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# Safety evaluation of certain mycotoxins in food

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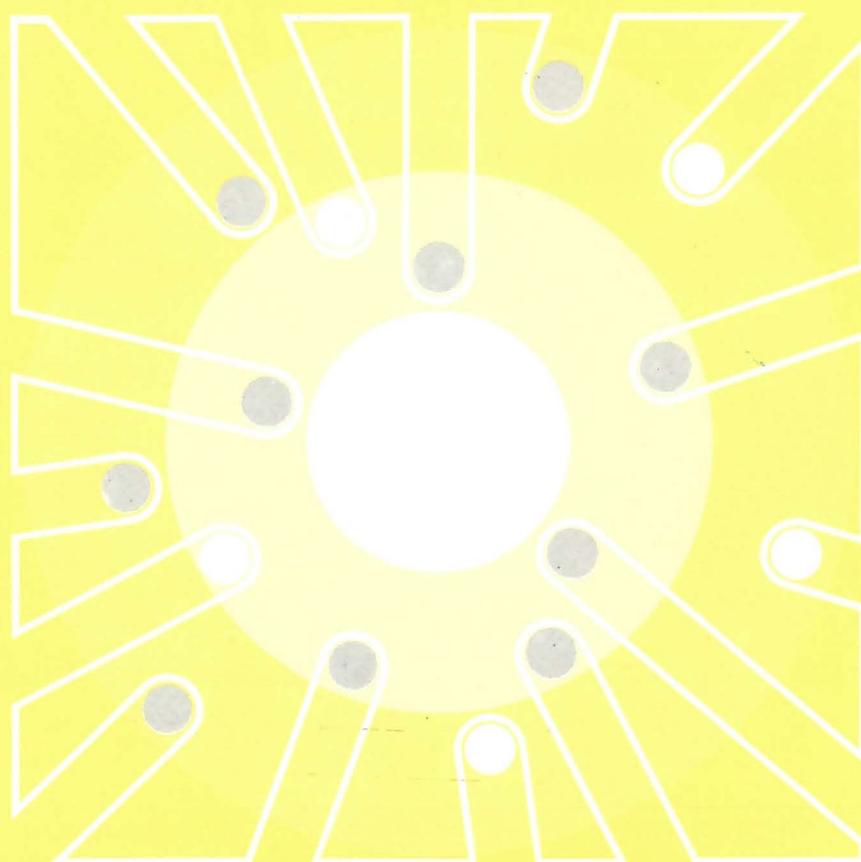
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**IPCS**

International Programme on Chemical Safety  
World Health Organization – Geneva



**WHO FOOD  
ADDITIVES  
SERIES: 47**

**Safety evaluation  
of certain  
mycotoxins in food**

Prepared by the Fifty-sixth meeting  
of the Joint FAO/WHO Expert  
Committee on Food Additives  
(JECFA)

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FOOD AND  
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PAPER**

**74**

**World Health Organization, Geneva, 2001**

**IPCS—International Programme on Chemical Safety**

**Food and  
Agriculture  
Organization  
of the  
United Nations  
2001**

This publication is a contribution to the International Programme on Chemical Safety.

The **International Programme on Chemical Safety (IPCS)**, established in 1980, is a joint venture of the United Nations Environment Programme (UNEP), the International Labour Organisation (ILO), and the World Health Organization (WHO). The overall objectives of the IPCS are to establish the scientific basis for assessing the risk to human health and the environment from exposure to chemicals, through international peer-review processes, as a prerequisite for the promotion of chemical safety, and to provide technical assistance in strengthening national capacities for the sound management of chemicals.

The **Inter-Organization Programme for the Sound Management of Chemicals (IOMC)** was established in 1995 by UNEP, ILO, the Food and Agriculture Organization of the United Nations, WHO, the United Nations Industrial Development Organization, and the Organisation for Economic Co-operation and Development (Participating Organizations), following recommendations made by the 1992 United Nations Conference on Environment and Development to strengthen cooperation and increase coordination in the field of chemical safety. The purpose of the IOMC is to promote coordination of the policies and activities pursued by the Participating Organizations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

Health & Environment International, Ltd, by agreement with WHO, performed independent literature searches on some of the substances on which data are summarized in this document in order to ensure that all relevant toxicological and related information was reviewed.

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## PREFACE

The monographs contained in this volume were prepared at the fifty-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met at WHO Headquarters in Geneva, Switzerland, 6–15 February 2001. These monographs summarize the data on selected mycotoxins reviewed by the Committee.

The fifty-sixth report of JECFA will be published by the World Health Organization in the WHO Technical Report Series. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1. Abbreviations used in the monographs are listed in Annex 2. The participants in the meeting are listed in Annex 3 of the present publication.

JECFA serves as a scientific advisory body to FAO, WHO, their Member States, and the Codex Alimentarius Commission, primarily through the Codex Committee on Food Additives and Contaminants and the Codex Committee on Residues of Veterinary Drugs in Foods, regarding the safety of food additives, residues of veterinary drugs, naturally occurring toxicants, and contaminants in food. Committees accomplish this task by preparing reports of their meetings and publishing specifications or residue monographs and toxicological monographs on substances that they have considered.

The monographs contained in this volume are based on working papers that were prepared by working groups before the meeting. A special acknowledgement is given at the beginning of each monograph to those who prepared these working papers. The monographs were edited by E. Heseltine, Lajarthe, 24290 St Léon-sur-Vézère, France.

The preparation and editing of the monographs included in this volume were made possible through the technical and financial contributions of the Participating Organizations of the International Programme on Chemical Safety (IPCS), which supports the activities of JECFA.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the organizations participating in the IPCS concerning the legal status of any country, territory, city, or area or its authorities, or concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by those organizations in preference to others of a similar nature that are not mentioned.

Any comments or new information on the biological or toxicological properties of the compounds evaluated in this publication should be addressed to: Joint WHO Secretary of the Joint FAO/WHO Expert Committee on Food Additives, International Programme on Chemical Safety, World Health Organization, Avenue Appia, 1211 Geneva 27, Switzerland.



## AFLATOXIN M<sub>1</sub>

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## 1. EXPLANATION

The Expert Committee was requested by the Codex Committee on Food Additives and Contaminants at its Thirty-second Session (Codex Alimentarius, 2000) to 'examine exposure to aflatoxin M<sub>1</sub> and to conduct a quantitative risk assessment' to compare the consequences of setting the maximum level in milk at 0.05 and 0.5 µg/kg.

Aflatoxins can be produced by three species of *Aspergillus*—*A. flavus*, *A. parasiticus*, and the rare *A. nomius*—which contaminate plants and plant products. *A. flavus* produces only B aflatoxins, while the other two species produce both B and G aflatoxins. Aflatoxins M<sub>1</sub> and M<sub>2</sub> are the hydroxylated metabolites of aflatoxins B<sub>1</sub> and B<sub>2</sub> and can be found in milk or milk products obtained from livestock that have ingested contaminated feed. The main sources of aflatoxins in feeds are peanut meal, maize and cottonseed meal.

Aflatoxins were evaluated by the Committee at its thirty-first, forty-sixth, and forty-ninth meetings (Annex 1, references 77, 122, and 131). At its forty-ninth meeting, the Committee considered estimates of the carcinogenic potency of aflatoxins and the potential risks associated with their intake. At that meeting, the Committee reviewed a wide range of studies conducted in animals and humans that provided qualitative and quantitative information on the hepatocarcinogenicity of aflatoxins. The Committee evaluated the potency of these contaminants, linked those potencies to estimates of intake, and discussed the potential impact of hypothetical standards on sample populations and their overall risk. In its evaluation, the Committee stated that the carcinogenic potency of aflatoxin M<sub>1</sub> in sensitive species is about one order of magnitude less than that of aflatoxin B<sub>1</sub>. In particular, the Committee noted that the carcinogenic potency of aflatoxin B<sub>1</sub> is substantially higher in carriers of hepatitis B virus (about 0.3 cancers per year per 100 000 persons per ng/kg bw per day), as determined by the presence in serum of the hepatitis B virus surface antigen (HBsAg<sup>+</sup> individuals), than in HBsAg<sup>-</sup> individuals (about 0.01 cancers per year per 100 000 persons per ng/kg bw per day). Populations with both a high prevalence of HBsAg<sup>+</sup> and a high aflatoxin intake might benefit from reductions in aflatoxin intake. The Committee also noted that vaccination against hepatitis B virus would reduce the

number of carriers of the virus, and thus reduce the potency of the aflatoxins in vaccinated populations, leading to a reduction in the risk for liver cancer.

The Committee at its forty-ninth meeting concluded that changing the hypothetical standard for aflatoxin B<sub>1</sub> from 20 µg/kg to 10 µg/kg would not result in any observable difference in rates of liver cancer.

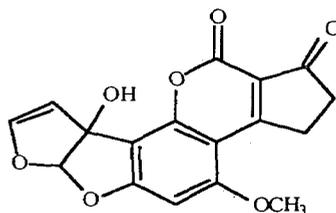
At its present meeting, the Committee reviewed studies published since its forty-ninth meeting, as well as other information, to elucidate further the carcinogenic potencies of aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> and the differences between animal species in their sensitivity to aflatoxins.

## 2. BIOLOGICAL DATA

### 2.1 Chemical structure

The chemical structure of aflatoxin M<sub>1</sub> is shown in Figure 1. Aflatoxin M<sub>1</sub> is the 4-hydroxy derivative of aflatoxin B<sub>1</sub> and is secreted in the milk of mammals that consume aflatoxin B<sub>1</sub>. Aflatoxin M<sub>1</sub> (CAS No. 6795-23-9) has a relative molecular mass of 328 Da and has the molecular formula C<sub>17</sub>H<sub>12</sub>O<sub>7</sub>.

Figure 1. Chemical structure of aflatoxin M<sub>1</sub>



### 2.2 Biochemical aspects

#### 2.2.1 Biotransformation

A complete review of studies of the metabolism of aflatoxins conducted up to 1997 can be found in the report of the monograph on aflatoxins published after the forty-ninth meeting of the Committee (Annex 1, reference 132) and in a book by Eaton and Groopman (1994). The papers highlighted in this section address studies of metabolic differences among species in their sensitivity to aflatoxins, comparisons of the toxicity of aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub>, studies of the differences in the carcinogenic potency of aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub>, and papers published since the last report of the Committee.

The metabolism of aflatoxin B<sub>1</sub> and the extent to which it binds to cell macromolecules were compared in liver slices from humans and rats, as rats are more sensitive to the carcinogenicity of aflatoxin B<sub>1</sub>. Liver slices were prepared from three human liver samples and incubated with [<sup>3</sup>H]aflatoxin B<sub>1</sub> at 0.5 µmol/L for 2 h. The rates of formation of oxidative metabolites and of specific binding to cell macromolecules showed significant interindividual variation. The rates of oxidative metabolism of aflatoxin B<sub>1</sub> to aflatoxin Q<sub>1</sub>, aflatoxin P<sub>1</sub>, and aflatoxin M<sub>1</sub> in the human liver samples were similar to those previously observed in rat liver slices. No aflatoxin B<sub>1</sub>-glutathione conjugate formation was detected in the human liver samples, and

there was much less specific binding of aflatoxin B<sub>1</sub> to cell macromolecules in the human than in the rat liver slices. For example, the level of binding between aflatoxin B<sub>1</sub> and DNA ranged from 3 to 26% of that in control rats. These results suggest that humans do not form as much aflatoxin B<sub>1</sub> 8,9-epoxide as rats, but they also suggest that humans do not have glutathione-S-transferase (GST) isozymes with high specific activity towards this epoxide. Significant individual differences in aflatoxin B<sub>1</sub> metabolism and binding suggest the presence of genetic and/or environmental factors that may result in large differences in susceptibility.

Aflatoxin M<sub>1</sub> is usually considered to be a detoxication by-product of aflatoxin B<sub>1</sub>; it is also the metabolite present in the milk of nursing women who eat foods containing the toxin. Aflatoxin B<sub>1</sub> epoxide has been shown to exist as two stereoisomers—endo- and exo-epoxides—the latter being the DNA-reactive form, and a similar situation may apply to aflatoxin M<sub>1</sub> epoxide. In a study of the metabolism of aflatoxins M<sub>1</sub> and B<sub>1</sub> in vitro in human liver microsomes, they had a very limited capacity to catalyse epoxidation of aflatoxin M<sub>1</sub>. The small amount of aflatoxin M<sub>1</sub> dihydrodiol formed from the epoxide also appeared to have a lower capacity to induce microsomal protein than did aflatoxin B<sub>1</sub> dihydrodiol. GST catalysed conjugation of the epoxides of both aflatoxins with glutathione; GST activity was present in mouse cytosol but not in the human liver fraction. The authors concluded that the difference between the genotoxic potency of the two toxins in vivo correlates with their mutagenicity in vitro, metabolic activation and DNA binding (Neal et al., 1998). In rats, however, activation of aflatoxin M<sub>1</sub> to the epoxide does not appear to be essential for its acute toxicity. Experiments in human cell lines indicated that cytochrome P450 (CYP) enzymes are involved in the cytotoxicity of aflatoxin B<sub>1</sub> but not of aflatoxin M<sub>1</sub>. Studies of the toxicity of aflatoxin M<sub>1</sub> in human lymphoblastoid cell lines expressing or not expressing human CYP enzymes showed a direct effect in the absence of metabolic activation, in contrast to aflatoxin B<sub>1</sub>. Aflatoxin M<sub>1</sub> is therefore not strictly a detoxication product of aflatoxin B<sub>1</sub> in biological responses in which cytotoxicity plays a significant role, such as immunotoxicity (Heinonen et al., 1996).

In studies of species sensitivity to the carcinogenicity of aflatoxin B<sub>1</sub>, mice were resistant because they constitutively express an  $\alpha$ -GST, which is strongly active against aflatoxin B<sub>1</sub> 8,9-epoxide, whereas rats, which do not express such a GST, were sensitive. Human hepatic  $\alpha$ -class GSTs have little capacity to detoxify aflatoxin B<sub>1</sub> 8,9-epoxide. The nonhuman primate *Macaca fascicularis* showed significant constitutive hepatic GST activity towards aflatoxin B<sub>1</sub> 8,9-epoxide. GSTs were purified from liver tissue from this species and characterized, and GST cDNAs were cloned by reverse transcriptase-coupled polymerase chain reaction (PCR). A protein, GSHA-GST, was purified by glutathione agarose affinity chromatography, which had stronger aflatoxin B<sub>1</sub> 8,9-epoxide-conjugating activity than other GST-containing peaks. The GSHA-GST was shown to belong to the  $\mu$  class. The authors then showed that two distinct  $\mu$ -class GST cDNAs have 97% and 98% homology with the human  $\mu$ -class GSTs hGSTM4 and hGSTM2, respectively.  $\mu$ -Class GSTs appear to be responsible for most of the conjugating activity of aflatoxin B<sub>1</sub> 8,9-epoxide in the liver of *M. fascicularis*. None of the known human  $\mu$ -class GSTs acts preferentially on the ultimate genotoxic aflatoxin B<sub>1</sub> metabolite exo-aflatoxin B<sub>1</sub> 8,9-epoxide, but large interindividual differences in the expression of GST isoforms have been shown in various tissues, and few human livers have been evaluated. The authors concluded

that identification of a potential human homologue of GSHA-GST would be relevant to the design of chemoprevention strategies to reduce aflatoxin B<sub>1</sub>-induced liver cancer in highly exposed populations. Nevertheless, induction of known human GSTs with little or no activity towards the epoxide of aflatoxin B<sub>1</sub> might be ineffective in reducing the genotoxicity of aflatoxin B<sub>1</sub> (Wang et al., 2000).

The extreme sensitivity of turkeys to aflatoxin B<sub>1</sub> was studied by measuring microsomal activation of aflatoxin B<sub>1</sub> to the 8,9-epoxide, the putative toxic intermediate, cytosolic GST-mediated detoxication of aflatoxin B<sub>1</sub> 8,9-epoxide, and hepatic phase I and phase II enzyme activities in 3-week-old male Oorlop turkeys. Liver microsomes prepared from these turkeys activated aflatoxin B<sub>1</sub> in vitro with an apparent  $K_m$  of 110  $\mu\text{mol/L}$  and a  $V_{max}$  of 1.25 nmol/mg per min. The involvement of CYP 1A2 and, to a lesser extent, 3A4 in the activation of aflatoxin B<sub>1</sub> was assessed with specific mammalian CYP inhibitors. The possible presence of avian orthologues of these CYPs was indicated by activity towards ethoxyresorufin and nifedipine, as well as by western immunoblotting with antibodies to human CYPs. GST-mediated conjugation of 1-chloro-2,4-dinitrobenzene and 3,4-dichloronitrobenzene was demonstrated in cytosol prepared from the turkey livers, but the rate was much lower than that observed in other species. The presence of  $\alpha$ - and  $\mu$ -class GSTs and another aflatoxin B<sub>1</sub> detoxifying enzyme, aflatoxin B<sub>1</sub> aldehyde reductase, was shown by western immunoblotting. Quinone reductase activity was also present in the cytosol. Furthermore, the cytosol showed no measurable GST-mediated detoxication of microsomally activated aflatoxin B<sub>1</sub>. Thus, turkeys are deficient in the most crucial aflatoxin B<sub>1</sub> detoxication pathway. The authors concluded that the extreme sensitivity of this species to aflatoxin B<sub>1</sub> is due to a combination of efficient aflatoxin B<sub>1</sub> activation and deficient detoxication by phase II enzymes such as GSTs (Klein et al., 2000).

The carcinogenicity of aflatoxin B<sub>1</sub>, aflatoxinol (aflatoxin L), aflatoxin M<sub>1</sub>, and aflatoxinol M<sub>1</sub> (aflatoxin LM<sub>1</sub>) was compared in terms of their binding to target organ DNA in rainbow trout. Tritiated compounds were synthesized, dose–response curves for DNA binding were established, and liver DNA binding indices were calculated for the four aflatoxins after a 2-week dietary intake by trout fry. The adduct levels increased linearly with dietary concentration, with relative DNA binding indices of 21, 20, 2.4, and  $2.2 \times 10^3$  (pmol/mg of DNA)/(pmol/g of diet) for aflatoxin M<sub>1</sub>, aflatoxin L, aflatoxin M<sub>1</sub>, and aflatoxin LM<sub>1</sub>, respectively.

In a similar protocol, over 7200 trout fry with an average initial body weight of 1.2 g were used to establish full carcinogen dose–response curves for each aflatoxin and an estimate of the DNA binding index after a single dose. Since trout are very sensitive,  $\leq 180 \mu\text{g}$  of each aflatoxin were required. Data analysed on logit incidence versus  $L_n$  dose coordinates generated four curves, which were modelled as parallel in slope over most or all the doses studied. In this analysis, the relative tumorigenic potencies were 1.0 for aflatoxin B<sub>1</sub>, 0.94 for aflatoxin L, 0.086 for aflatoxin M<sub>1</sub>, and 0.041 for aflatoxin LM<sub>1</sub>. When the data were plotted as logit incidence versus  $L_n$  adducts (effective dose received), dose–response relationships were found for all aflatoxin adducts, indicating that they are equally tumorigenic, except for aflatoxin LM<sub>1</sub>, which was two to three times less potent. Differences in the tumorigenicity of the four aflatoxins are largely or entirely accounted for by differences in uptake and

metabolism leading to DNA adduction, rather than to any inherent difference in tumour initiating potency per DNA adduct (Bailey et al., 1998).

Since most people are exposed to carcinogens in food intermittently, the effects of intermittent intake of aflatoxin B<sub>1</sub> on hepatic and testicular glutathione was studied in male Fischer 344 rats fed diets containing aflatoxin B<sub>1</sub> at a concentration of 0, 0.01, 0.04, 0.4, or 1.6 ppm at 4-week intervals up to 20 weeks. The control animals were fed an aflatoxin B<sub>1</sub>-free NIH-31 diet. Rats eating diets containing 0.01 ppm aflatoxin B<sub>1</sub> did not show induction of hepatic or testicular GST activity, but intermittent intake of concentrations of 0.04–1.6 ppm significantly increased GST activity. The increase in enzyme activity was proportional to the dose and the length of intake of aflatoxin B<sub>1</sub> (Sahu et al., 2000).

### 2.2.2 Effects of oltipraz and ethoxyquin

Oltipraz is a competitive and perhaps irreversible inhibitor of CYP 1A2 and 3A4, and addition of oltipraz to rat liver microsomes or to cultured human hepatocytes blocks the oxidative metabolism of aflatoxin B<sub>1</sub> to its 8,9-oxide and to the hydroxylated derivative aflatoxin M<sub>1</sub>. Inhibition of aflatoxin M<sub>1</sub> excretion in urine during dietary intervention with oltipraz was examined in male Fischer 344 rats before, during, and after transient intervention. The animals were housed individually in glass metabolism cages and given 25 µg of [<sup>3</sup>H]aflatoxin B<sub>1</sub> by gavage daily for 28 consecutive days. On days 6–16, half the rats were fed a diet supplemented with 0.075% oltipraz. Sequential 24-h urine samples were collected, and a subset was analysed for aflatoxin B<sub>1</sub> metabolites. Aflatoxin M<sub>1</sub> was the main metabolite in all samples, accounting for 2–6% of the administered dose. Its excretion was greatly reduced (by 77%) when oltipraz was added to the diet, but rapidly returned to control levels after cessation of the intervention. No such inhibition of aflatoxin M<sub>1</sub> excretion was seen in animals given oltipraz by gavage 24 h before dosing with aflatoxin B<sub>1</sub>. These findings are consistent with the view that oltipraz or a short-lived metabolite inhibits CYP 1A2 in vivo (Scholl et al., 1996).

The effects of cancer preventive agents on the metabolism of aflatoxin B was examined in non-human primates in a study designed to complement a human chemointervention trial, in which oltipraz, an antischistosomal drug approved by the Food and Drug Administration of the USA, was used to modulate the metabolism of aflatoxin B in a human population naturally exposed to this toxin in the diet. This study is discussed in section 5.3. The hepatic metabolism of aflatoxin B<sub>1</sub> was studied in macaque (*M. nemestrina*) and marmoset (*Callithrix jacchus*) monkeys and compared with that in humans. Thus, four adult male marmosets were used as controls, four were given oltipraz, and three received ethoxyquin. At time 0, each animal received a single dose of [<sup>3</sup>H]aflatoxin B at 100 µg/kg bw (0.5 mCi/kg) by gavage, and blood samples were drawn at 0, 2, 24, and 48 h. On days 16–28, the treated animals received the synthetic dithiolthione oltipraz at 18 mg/kg bw per day or the antioxidant ethoxyquin at 30 mg/kg bw per day in their diet, whereas the control animals received the vehicles only. On day 26, each animal received a second dose of [<sup>3</sup>H]aflatoxin B by gavage, and blood samples were drawn at 0, 2, 24, and 48 h. On day 28, the animals were killed, their livers were excised, and microsomal and cytosolic fractions were prepared. For comparison, livers from adult male

macaques were obtained from the University of Washington Primate Center (USA). Twelve human liver samples were also obtained from the University of Washington, and the microsomal fractions were pooled. Microsomal oxidation of aflatoxin B<sub>1</sub> and GST activity were measured, DNA adducts were isolated, and [<sup>3</sup>H]aflatoxin B-derived radioactivity in DNA fractions was quantified. [<sup>3</sup>H]Aflatoxin B<sub>1</sub>-albumin adducts in serum were determined.

The oxidative metabolism of aflatoxin B was quantitatively similar in the two monkey species and in humans. In contrast to macaques, humans and marmosets lacked aflatoxin B glutathione conjugating activity. As the metabolism of aflatoxin B in marmosets resembled that in humans more closely than that in macaques, the focus of the study was on marmosets. Both oltipraz and ethoxyquin induced aflatoxin B<sub>1</sub>-glutathione conjugating activity in the livers of some but not all marmosets. Oltipraz inhibited CYP-mediated activation of aflatoxin B to the ultimate carcinogenic metabolite, aflatoxin B<sub>1</sub> 8,9-epoxide, in vitro by up to 51%, and animals that received oltipraz in vivo showed a significant reduction (average, 53%) in aflatoxin B-DNA adduct formation in comparison with control animals.

The authors interpreted these findings as indicating that oltipraz and ethoxyquin induce modest aflatoxin B glutathione conjugating activity in the livers of some marmosets, most of the activity (about 70%) being directed against the exo isomer of aflatoxin B<sub>1</sub> 8,9-epoxide, which is by far the most potent DNA-reactive metabolite. Other workers have demonstrated aflatoxin B-mercapturic acid in the urine of marmosets exposed to aflatoxin B<sub>1</sub>. The hepatic GST activity towards aflatoxin B<sub>1</sub> 8,9-epoxide shown in this study in non-human primates was two orders of magnitude lower than that in mice, which are resistant to the carcinogenic effects of aflatoxin B<sub>1</sub>. The authors offered two explanations for the presence of DNA adducts and the decrease in steady state of exo-aflatoxin B<sub>1</sub> 8,9-epoxide shown in both treated groups: GST-mediated detoxication of exo-aflatoxin B<sub>1</sub> 8,9-epoxide and inhibition of the CYP(s) that form it. Consistent with the results of the chemoprevention trial in a human population (section 5.3), administration of oltipraz and ethoxyquin would be likely to attenuate the adverse effects of aflatoxin B in primates (Bammler et al., 2000).

In a study of the inhibition of aflatoxin M<sub>1</sub> production by bovine hepatocytes after intervention with oltipraz and another dithiolthione, 4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione, oltipraz inhibited the metabolism of aflatoxin B<sub>1</sub>, as neither aflatoxin M<sub>1</sub> nor aflatoxin B dihydrodiol (the second metabolite found in bovine hepatocytes) was formed. The second dithiolthione did not significantly inhibit aflatoxin B<sub>1</sub> metabolism. The authors suggested that the inhibition of aflatoxin B<sub>1</sub> metabolism by oltipraz was due to inhibition of the activity of several CYP enzymes. Although the authors proposed that oltipraz could be administered to dairy cows that had accidentally received aflatoxin B<sub>1</sub> in their feed, unmetabolized aflatoxin B<sub>1</sub> would still reach the systemic circulation. These results obtained in vitro should be confirmed in vivo (Kuilman et al., 2000).

The roles of coumarin, benzyl isothiocyanate, and indole-3-carbinol, which are present in vegetable-enriched diets and are believed to protect against malignant disease, in regulating GST and aldo-keto reductase activity were examined in rat liver. The drugs butylated hydroxyanisole, diethyl maleate, ethoxyquin, β-naphthoflavone, oltipraz, phenobarbital, and *trans*-stilbene oxide were also investigated. In

a complicated protocol, summarized briefly here, groups of three male and three female 10-week-old male and female Fischer 344 rats were given diets containing 0.75% butylated hydroxyanisole, 0.5% benzyl isothiocyanate, 0.5% coumarin, 0.5% ethoxyquin, 0.5% indole-3-carbinol, or 0.075% oltipraz for 2 weeks. Diethyl maleate at 0.5% was administered for 5 days in the food. *trans*-Stilbene oxide at 400 mg/kg bw was dissolved in 0.5 ml of peanut oil before daily intraperitoneal administration on 3 consecutive days, and  $\beta$ -naphthoflavone at 200 mg/kg bw was dissolved in phosphate-buffered saline before daily intraperitoneal administration for 7 consecutive days. Phenobarbital was added to the drinking-water at a concentration of 0.1% for 7 days.

For the short-term intervention study of the effect of coumarin on the development of preneoplastic foci, six groups of eight 12-week-old male Fischer 344 rats were given one of the following experimental diets for 13 weeks: RM1 control maintenance diet throughout, 0.05% coumarin in RM1 diet throughout, 2 mg/kg of diet aflatoxin B<sub>1</sub> in RM1 diet for 6 weeks followed by RM1 control diet for 7 weeks, 2 mg/kg of diet aflatoxin B<sub>1</sub> in RM1 diet throughout, 0.05% coumarin in RM<sub>1</sub> diet for 2 weeks followed by aflatoxin B<sub>1</sub> at 2 mg/kg of RM1 diet containing 0.05% coumarin for 11 weeks, or 2 mg/kg of diet aflatoxin B<sub>1</sub> in RM1 diet for 6 weeks followed by aflatoxin B<sub>1</sub> in RM1 diet containing 0.05% coumarin for 7 weeks.

In a long-term intervention study with coumarin to study tumour formation, six groups of eight 12-week-old male Fischer 344 rats were given the same diets described above but were placed on diets containing coumarin and aflatoxin B<sub>1</sub> for 24 weeks before being transferred to a control diet from week 25 until termination of the experiment at week 50. The animals were killed with CO<sub>2</sub>, and tissues were removed immediately. Microsomal and cytosolic fractions were prepared from fresh liver or from samples snap-frozen in liquid nitrogen.

Under these conditions, coumarin was the main inducer of aflatoxin B<sub>1</sub> aldehyde reductase and the aflatoxin-conjugating  $\mu$ -class GST A5 subunit in rat liver, increasing the concentrations of these proteins by 25–35 times. Coumarin caused similar increases in the concentration of  $\pi$ -class GST P1 subunit and NAD(P)H:quinone oxidoreductase in rat liver.

To assess the biological significance of enzyme induction by dietary coumarin, two intervention studies were performed, in which the ability of benzopyrone to inhibit aflatoxin B<sub>1</sub>-initiated preneoplastic nodules (at 13 weeks) or aflatoxin B<sub>1</sub>-initiated liver tumours (at 50 weeks) was investigated. Pretreatment with coumarin for 2 weeks before administration of aflatoxin B<sub>1</sub> and continued treatment during exposure to the carcinogen for a further 11 weeks protected the animals completely from development of hepatic preneoplastic lesions by 13 weeks. Treatment with coumarin in a longer-term dietary intervention, before and during exposure to aflatoxin B<sub>1</sub> for 24 weeks, resulted in significant inhibition of the number and size of tumours that developed by 50 weeks. The authors concluded that consumption of a coumarin-containing diet provides substantial protection against the initiation of hepatocarcinogenesis by aflatoxin B<sub>1</sub> in rats. The other phytochemicals and synthetic drugs tested in this study induced different zone- and sex-specific enzymes in the liver. The complexity of gene–environment interactions is emphasized by the fact that certain inducing agents can cause nuclear translocation of drug-metabolizing enzymes (Kelly et al., 2000).

## **2.3 Toxicological studies**

### **2.3.1 Acute toxicity**

The acute toxicity of aflatoxin M<sub>1</sub> was reviewed by van Egmond (1994) and is summarized only briefly here. In the 1960s, newly hatched ducklings were shown by several investigators to be extremely sensitive to both aflatoxin B<sub>1</sub> and aflatoxin M<sub>1</sub>, with LD<sub>50</sub> values of 12–16 µg per bird. Histopathological examination showed liver lesions similar to those caused by aflatoxin B<sub>1</sub> and necrosis of the renal tubules. Milk naturally contaminated with aflatoxin M<sub>1</sub> produced fewer lesions than artificially contaminated milk, however, suggesting differences in the bioavailability of naturally and artificially occurring aflatoxin M<sub>1</sub>. Studies on the acute toxicity of aflatoxins in 1-day-old ducklings suggest that aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> act by a similar mechanism in causing acute toxicity and subcellular alterations, such as changes in liver parenchymal cells, dissociation of ribosomes from the rough endoplasmic reticulum, and proliferation of the smooth endoplasmic reticulum, and that only the naturally occurring isomer of each aflatoxin is biologically active.

### **2.3.2 Long-term studies of toxicity and carcinogenicity**

van Egmond (1994) also summarized the results of long-term studies of toxicity. In a study by Sinnhuber in 1974, rainbow trout received diets containing aflatoxin B<sub>1</sub> at 4 µg/kg or aflatoxin M<sub>1</sub> at 0, 4, 16, 32, or 64 µg/kg for 12 months and then received a control diet. Selected groups were held for 20 months to determine the effect of maturation on tumour development, and some were fed aflatoxin M<sub>1</sub> at 20 µg/kg of diet for 5–30 days to determine the effect of limited oral intake of this toxin. Female trout with aflatoxin M<sub>1</sub>-induced hepatomas had a significantly higher mortality rate at maturation (16–20 months) than males. The trout receiving aflatoxin M<sub>1</sub> at 20 µg/kg of diet had a 3–12% incidence of hepatoma within 12 months. The author concluded that aflatoxin M<sub>1</sub> is a potent liver carcinogen but less potent than aflatoxin B<sub>1</sub>.

Canton et al. in 1975 fed rainbow trout diets containing aflatoxin M<sub>1</sub> at 0, 5.9, or 27 µg/kg and aflatoxin B<sub>1</sub> at 5.8 µg/kg for 16 months. The fish were killed after 5, 9, and 12 months. Degeneration of the liver was seen in all three groups and in the control group, but no tumours or preneoplastic changes were found. At 15 months, however, the survivors fed the diet containing 5.8% aflatoxin B<sub>1</sub> had a 13% incidence of hepatocellular carcinoma and a 23% incidence of hyperplastic nodules, and those fed the diet with 27.3 µg/kg aflatoxin M<sub>1</sub> had a 2% incidence of hepatocellular carcinoma and a 6% incidence of hyperplastic nodules. The investigators concluded that differences in trout strain could have contributed to the differences between their results and those of Sinnhuber, but that aflatoxin M<sub>1</sub> is less carcinogenic in trout than aflatoxin B<sub>1</sub>.

van Egmond (1994) summarized two further studies in rats. In a study in 1974, weanling Fischer rats were given 25 µg/day of synthetic aflatoxin M<sub>1</sub> by intubation on 5 days/week for 8 consecutive weeks. A second group of rats was given natural aflatoxin B<sub>1</sub> at the same concentration and under similar conditions. A control group was included. Only one rat (3%) given aflatoxin M<sub>1</sub> developed a hepatocellular carcinoma, whereas 28% had liver lesions (preneoplastic lesions). All rats receiving

aflatoxin B<sub>1</sub> developed tumours, whereas controls showed no significant liver lesions. The carcinogenic potency of aflatoxin M<sub>1</sub> was concluded to be much lower than that of aflatoxin B<sub>1</sub>.

In a second study, groups of Fischer rats were maintained on diets containing natural aflatoxin M<sub>1</sub> at 0, 0.5, 5, or 50 µg/kg and were killed between 18 and 22 months. Hepatocellular carcinomas were detected in 5% and neoplastic nodules in 15% of rats fed diets containing aflatoxin M<sub>1</sub> at 50 µg/kg between 19 and 20 months. No nodules or carcinomas were observed at the lower dose. Of rats fed the diet containing aflatoxin B<sub>1</sub> at 50 µg/kg, 95% developed hepatocellular carcinomas. Only a few rats at 50 µg/kg of aflatoxin M<sub>1</sub> developed intestinal carcinomas. The authors suggested that the greater polarity of aflatoxin M<sub>1</sub> than aflatoxin B<sub>1</sub> might be associated with the higher incidence of intestinal tumours. It was concluded that aflatoxin M<sub>1</sub> was a hepatic carcinogen, but with a potency 2–10% that of aflatoxin B<sub>1</sub> (Cullen et al., 1987, as described by van Egmond, 1994). This study is the one usually cited in comparisons of the carcinogenicity of aflatoxin B<sub>1</sub> and aflatoxin M<sub>1</sub>. van Egmond (1994) concluded that the toxicity of aflatoxin M<sub>1</sub> is similar to or slightly lower than that of aflatoxin B<sub>1</sub> in rats and ducklings, and the carcinogenicity of aflatoxin M<sub>1</sub> is probably one to two orders of magnitude lower than that of aflatoxin B<sub>1</sub> (see Table 1).

### 2.3.3 Genotoxicity

The potency of aflatoxin B<sub>1</sub> and aflatoxin M<sub>1</sub> in inducing DNA damage and genotoxicity was tested in *Drosophila melanogaster* in vivo in the mei-9a mei-41D5 DNA repair test and the mwh/flr3 wing spot test, respectively. In the repair test, larval stock consisting of meiotic recombination-deficient double-mutant mei-9a mei-41D5 males and repair-proficient females was exposed to the test agent, and preferential killing of the mutant larvae was taken as evidence of DNA damage. Aflatoxin M<sub>1</sub> was found to be a DNA-damaging agent, with an activity about one-

**Table 1. Comparative toxicity of aflatoxin M<sub>1</sub> and B<sub>1</sub>**

Species, strain	Sex	Route	Aflatoxin	LD <sub>50</sub>	Reference
Mouse, C57BL/6J, newborn	M,F	Intraperitoneal	AFP <sub>1</sub>	< 5% that of AFB <sub>1</sub>	Buchi et al. (1973)
Duck, Peking 40–50 g	NR	Oral	AFM <sub>1</sub> , AFM <sub>2</sub>	Similar to AFB <sub>1</sub> , < 4 x	Purchase (1967)
Peking duckling, 1-day-old	M,F	Oral	AFB <sub>1</sub>	0.34 mg/kg bw	Lijinsky & Butler (1966)
Rainbow trout, 270 days	M,F	Intraperitoneal	AFB <sub>1</sub>	0.81 mg/kg bw	Bauer et al. (1969)
Fischer rat, 70 days	M	Intraperitoneal	AFB <sub>1</sub>	0.75 mg/kg bw	McGuire (1969)
CFW Swiss mouse, 30 days	M	Intraperitoneal	AFB <sub>1</sub>	> 150 mg/kg bw	McGuire (1969)
Syrian hamster, 30 days	M	Oral	AFB <sub>1</sub>	10 mg/kg bw	Wogan (1966)

Adapted from Roebuck & Maxuitenko (1994)

third that of aflatoxin B<sub>1</sub>. In the wing spot test, in which larval flies trans-heterozygous for the somatic cell markers *mwh* and *flr3* were treated and the wings were inspected at adulthood for spots manifesting the phenotypes of the marker, the genotoxicity of aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> was similar. The authors concluded that aflatoxin M<sub>1</sub> is genotoxic in mammalian systems *in vivo* (Shibahara et al., 1995).

#### 2.3.4 Special studies

The tree shrew (*Tupaia belangeri chinensis*) is unique in that it can be infected with human hepatitis B virus (HBV) and is susceptible to aflatoxin B<sub>1</sub>-induced liver cancer; a synergistic interaction between HBV and aflatoxin B<sub>1</sub> for liver cancer has been observed. In studies in which the tree shrew model was used to evaluate experimental chemoprevention strategies for populations at high risk for liver cancer, two groups of tree shrews were fed milk containing aflatoxin B<sub>1</sub> at a concentration providing a dose of 400 µg/kg bw per day for 4 weeks. One week before administration of aflatoxin B<sub>1</sub>, one group also received oltipraz at 0.5 mmol/kg bw per day orally for 5 weeks. Samples of 1 ml of blood and 24-h urine were obtained from each animal at weekly intervals. Aflatoxin–albumin adducts in serum were identified by radioimmunological assay, and aflatoxin–N7-guanine adducts in urine were measured by high-performance liquid chromatography (HPLC). The concentration of aflatoxin–albumin adducts increased rapidly over 2 weeks, to reach a plateau at 20 pmol/mg of protein, and decreased after cessation of exposure to aflatoxin B<sub>1</sub>. Oltipraz significantly attenuated the overall burden of aflatoxin–albumin adducts throughout exposure, with a median reduction of 80%. As measured in a single cross-sectional analysis at the end of treatment with aflatoxin B<sub>1</sub>, oltipraz decreased the urinary aflatoxin–N7-guanine content by 93%. The authors concluded that oltipraz reduces risk biomarkers for aflatoxin B<sub>1</sub> in the tree shrew, as it does in rodents and humans, and established a rationale to evaluate cancer chemoprevention by oltipraz in tree shrews infected with human HBV and exposed to aflatoxin B<sub>1</sub>.

The authors recalled that reductions of comparable magnitude in both aflatoxin–albumin adducts in serum and aflatoxin–N7-guanine adducts in urine were found in rats pretreated with oltipraz and exposed to aflatoxin B<sub>1</sub>. Tree shrews appear to be less susceptible to hepatocarcinogenesis than rats. The tree shrew model is useful and may allow determination of whether agents such as oltipraz sustain their chemopreventive effect against aflatoxin in the presence of chronic infection with HBV (Li et al., 2000).

The effects of methyl deficiency and dietary restriction on hepatic-cell proliferation and telomerase activity were studied in 5-week-old male Fischer 344 rats pretreated with aflatoxin B<sub>1</sub> at 25 µg/rat per day by gavage on 5 days/week for 3 weeks or given solvent (100 µg of 75% dimethyl sulfoxide). The rats were then separated into groups fed a methyl-sufficient or -deficient diet *ad libitum* or with dietary restriction. When the rats were 15, 20, and 32 weeks of age, hepatic-cell proliferation, telomerase activity, and the number of GST-placental form (P)-positive foci were determined. Dietary restriction reduced hepatic-cell proliferation, while the methyl-deficient diet and aflatoxin B<sub>1</sub> pretreatment increased cell proliferation. Telomerase activity was decreased by dietary restriction and increased by the methyl-deficient diet and aflatoxin B<sub>1</sub> pretreatment. The same trend was observed for GST-P<sup>+</sup> foci in aflatoxin B<sub>1</sub>-pretreated rats: methyl deficiency increased the number of foci, and dietary

restriction decreased the number. These results are consistent with a role of telomerase in hepatocarcinogenesis, although the origin of the cells giving rise to the increase in telomerase activity was not determined (Chou et al., 2000).

In a study of the effect of ascorbic acid on the toxicity of aflatoxin B<sub>1</sub>, young guinea-pigs were either fed diets containing 0 or 25 mg/day of ascorbic acid or were given 300 mg/day by gavage for 21 days and the LD<sub>50</sub> dose of aflatoxin B<sub>1</sub> on day 22. Seven of 10 animals fed no ascorbic acid died within 73 h of administration of aflatoxin B<sub>1</sub>, and their livers showed massive regional necrosis and multilobular degeneration. None of the animals given 25 mg/day ascorbic acid died, but their livers showed changes similar to those seen in the group that received no ascorbic acid. The activities of serum alanine and aspartate aminotransferases were elevated.

None of the animals given 300 mg/day of ascorbic acid died or had pathological changes in the liver, and their alanine and aspartate aminotransferase activities were unaffected. Production of aflatoxin M<sub>1</sub> by liver microsomes tended to be higher than that in the other two groups. Three animals receiving 300 mg/day of ascorbic acid were given a second intraperitoneal LD<sub>50</sub> dose of aflatoxin B<sub>1</sub> 1 month after the first. One animal died, and the livers of all animals showed centrilobular degeneration and moderate necrosis in scattered hepatocytes. Hepatic microsomal CYP and cytosolic GST activities and aflatoxin M<sub>1</sub> production were drastically reduced, and the activities of alanine and aspartate aminotransferase were increased. The results indicate that intake of 300 mg of ascorbic acid virtually protected the animals from the acute toxicity of aflatoxin B<sub>1</sub> given by gavage but not when administered as a second dose intraperitoneally (Netke et al., 1997).

In a study of the effects of carotenoids on the initiation of liver carcinogenesis by aflatoxin B<sub>1</sub>, male weanling rats were fed  $\beta$ -carotene,  $\beta$ -apo-8'-carotenal, canthaxanthin, astaxanthin, or lycopene at 300 mg/kg of diet; an excess of vitamin A (21 000 retinol equivalents per kg of diet); or 3-methylcholanthrene at  $6 \times 20$  mg/kg bw intraperitoneally before and during treatment with aflatoxin B<sub>1</sub> at  $2 \times 1$  mg/kg bw. The rats were then treated with 2-acetylaminofluorene and partial hepatectomy, and GST-P<sup>+</sup> liver foci were detected and quantified. Aflatoxin B<sub>1</sub>-induced hepatic DNA damage was evaluated as single-strand breaks and binding of [<sup>3</sup>H]aflatoxin B<sub>1</sub> to liver DNA and plasma albumin *in vivo*. Modulation of aflatoxin B<sub>1</sub> metabolism by carotenoids or by 3-methylcholanthrene was investigated by incubation *in vitro* of [<sup>14</sup>C]aflatoxin B<sub>1</sub> with liver microsomes from rats that had been fed carotenoids or treated with 3-methylcholanthrene; the metabolites formed were analysed by HPLC.

Neither lycopene nor an excess of vitamin A had any effect, but  $\beta$ -carotene,  $\beta$ -apo-8'-carotenal, astaxanthin, and canthaxanthin decreased the metabolism of aflatoxin B<sub>1</sub> to aflatoxin M<sub>1</sub>, a less genotoxic metabolite. The authors concluded that these carotenoids exert their protective effect by deviating aflatoxin B<sub>1</sub> metabolism towards detoxication pathways. In contrast,  $\beta$ -carotene did not protect hepatic DNA from aflatoxin B<sub>1</sub>-induced alterations and affected the metabolism of aflatoxin B<sub>1</sub> to only a minor degree. Its protective effect against the initiation of liver preneoplastic foci by aflatoxin B<sub>1</sub> appears to be mediated by other mechanisms (Gradelet et al., 1998).

The hepatotoxicity of aflatoxin B<sub>1</sub> is augmented by bacterial endotoxin lipopolysaccharide in rats. At intraperitoneal doses > 1 mg/kg bw, aflatoxin B<sub>1</sub> caused

pronounced injury to the periportal regions of the liver. Male Sprague-Dawley rats were given aflatoxin B<sub>1</sub> at 1 mg/kg bw or the vehicle, 0.5% dimethyl sulfoxide and saline, and then *Escherichia coli* lipopolysaccharide ( $7.4 \times 10^6$  enzyme units per kg) or its saline vehicle 4 h later. Liver injury was assessed 6, 12, 24, 48, 72, or 96 h after administration of aflatoxin B<sub>1</sub>. Histological examination of liver sections and measurements of alanine and aspartate aminotransferase activity in serum were used to evaluate hepatic parenchymal-cell injury. Biliary-tract alterations were evaluated as increased concentration of serum bile acids and activities of  $\gamma$ -glutamyl-transferase, alkaline phosphatase, and 5'-nucleotidase in serum.

No or little injury was seen in rats treated with aflatoxin B<sub>1</sub> or lipopolysaccharide alone, but hepatic parenchymal-cell injury was pronounced by 24 h in the group treated with aflatoxin B<sub>1</sub> and lipopolysaccharide, returning to control values by 72 h. The injury began in the periportal region and spread mid-zonally with time. Changes in serum markers indicative of biliary-tract alterations were evident by 12 h, but the values had returned to control levels by 72 h. The nature of the hepatic lesions suggested that lipopolysaccharide potentiated the effects of aflatoxin B<sub>1</sub> on both parenchymal and bile-duct epithelial cells.

The authors suggested that the results of this study might partly explain the severity of human cases of acute aflatoxicosis. In addition, persons with hepatitis who have an inflammatory response may be predisposed to the carcinogenic effects of aflatoxin B<sub>1</sub>, as the results of this study suggest that inflammation accompanied by hepatic parenchymal-cell hyperplasia might contribute epigenetically to aflatoxin B-induced carcinogenesis by promoting tumour formation (Barton et al., 2000).

#### **2.4 Observations in domestic animals and veterinary toxicology**

Aflatoxin M<sub>1</sub>, like other aflatoxins, is produced by fungi that grow naturally on plants in the field or on stored feeds. Aflatoxins are among the most toxic of the known mycotoxins and have been implicated in the deaths of humans and animals that have consumed mouldy food. While the liver is the target organ for aflatoxicosis, aflatoxins are also found in other animal tissues and products, such as meat, milk, and eggs. As mature animals modify and eliminate toxins effectively, however, the main concern is long-term intake of low concentrations of these toxins, which can lead to cancer and immunosuppression. Although intake of low doses of aflatoxins may not cause death or tissue damage, it may severely affect the cost-effectiveness of animal production.

Sensitivity to aflatoxins varies from one species to another, and, within the same species, the severity of toxicity depends on dose, duration of intake, age, and breed of the animals, and their dietary protein content. The results of toxicological studies in domestic animals are given in Table 2.

In general, ingestion of aflatoxin results in a variety of clinical signs which depend on the amount consumed and the species and age of the animal. Aflatoxin may make an animal more susceptible to infectious diseases by impairing its immune system or potentiating a bacterial infection. Symptoms of secondary infection may obscure the symptoms of aflatoxicosis. Intake of aflatoxins during gestation may affect offspring as well as adults (Miller & Wilson, 1994).

The Food and Drug Administration (USA) set a tolerance limit of 20  $\mu\text{g}/\text{kg}$  for aflatoxins in maize and 0.5  $\mu\text{g}/\text{kg}$  of aflatoxin M<sub>1</sub> in milk. The latter can be achieved

**Table 2. Results of studies of the toxicity of aflatoxins in domestic animals**

Species	Sex	Aflatoxin	Dose	Toxicological end-point	Findings
Mink	Pregnant	Mixed	Diet, 10 µg/kg	Kit body weight at 3 weeks Kit mortality (birth to 3 weeks)	Decreased Increased
Hamster	Pregnant	B <sub>1</sub>	4–6 mg/kg bw	Hepatic, renal, and fetal lesions	Increased
Duck	Male and female	Mixed	Diet, 33 µg/kg	Thymus: Viable cells Cells/g tissue Spleen: Viable cells Cells/g tissue Bursa of Fabricius: Viable cells Cells/g tissue	Decreased Decreased Decreased Decreased Decreased Decreased
Chicken	Male	B <sub>1</sub>	Diet, 0.5–5.0 mg/kg, 5 weeks	Weight loss, decreased weight gain, impaired blood coagulation, poor pigmentation, decreased bone strength, and hepatic lesions	Positive
Calves	NR	B <sub>1</sub>	Single s.c. dose  0.8 mg/kg bw 1.8 mg/kg bw Daily dose: 42 mg over 3 months	Presence of aflatoxin B <sub>1</sub> , aflatoxin M <sub>1</sub> , aflatoxin L in tissues and urine	Positive Positive Negative
Pig	NR	Mixed	Diet: 1–4 mg/kg, 4 weeks; 0.4–0.8 mg/kg, 10 weeks	Weight loss, anorexia, haemorrhage, liver damage, and death	Positive
		B <sub>1</sub> , B <sub>2</sub> , G <sub>1</sub>	2.3–4.5 mg/kg	As above plus renal damage	Positive
Caprine	NR	B <sub>1</sub> , G <sub>1</sub> , M <sub>1</sub>	1.3–1.5 mg/day until death	Anorexia, depression, jaundice, liver and kidney damage, dark urine, and nasal discharge	Positive
Rabbit	NR	B <sub>1</sub>	25–626 µg/kg bw for 24 days	Hepatic lesions	Positive
Rat	NR	B <sub>1</sub>	7 mg/kg bw, once 0.5 mg/kg bw 4 days postnatally	Hepatic carcinoma Gastrointestinal, urogenital and hepatic carcinoma	Positive Positive

Table 2 (contd)

Species	Sex	Aflatoxin	Dose	Toxicological end-point	Findings
Guinea-pig	NR	B <sub>1</sub>	630 µg/kg bw once	Hepatic lesions	Positive
Monkey	NR	B <sub>1</sub>	0.01–1.0 mg/day until death	Hepatic lesions	Positive

Compiled from data reported by Aulerich et al. (1993), Miller & Wilson (1994), Shane (1994), Sabino et al. (1995), and Hurley et al. (1999)  
NR, not reported; s.c., subcutaneous

by consuming a diet contaminated with  $\leq 30$  µg/kg (Shane, 1994). The carry-over of aflatoxin from animal feed to milk and tissue is discussed in section 8.1.

## 2.5 Observations in humans

Liver cancer has been related to dietary intake of aflatoxins. The most recent epidemiological studies tend to indicate that individuals who are carriers of persistent viral infection with HBV and who are exposed to aflatoxin in their diets are at increased risk for progression to liver cancer as compared with HBV carriers who are not exposed to aflatoxins. No similar interaction has been reported with chronic infection with hepatitis C virus (HCV). The epidemiological studies to date have focused on aflatoxin B<sub>1</sub>; ingestion of aflatoxin M<sub>1</sub> with milk and milk products has not been directly related to liver cancer. Some of the epidemiological observations that implicate aflatoxins in the etiology of liver cancer derive from observations of unusual clusters of disease or unexplained trends in incidence. Additional indications are provided by the results of case-control and cohort studies based on adequate techniques and comprehensive evaluation of the risk factors in the etiology of liver cancer.

Increasing trends have been reported in the rates of hospitalization, incidence, and mortality attributable to liver cancer in the black and white populations of both sexes in the USA. The age-specific curves showed a shift towards liver cancer among persons aged 40–60. The authors discuss in detail and quite convincingly the factors that interfere in analyses of time trends for liver cancer, including the widespread introduction of new diagnostic means (ultrasound,  $\alpha$ -fetoprotein analysis), improved registration practices (histological confirmation and coding), and the quality of analysis (trend and birth-cohort analyses). The authors attribute the trend to intravenous drug abuse in the relevant generations, HCV being the predominant causative factor (El Serag & Mason, 1999).

HCV was also shown to be related to the increased rate of death from liver cancer in Japan after a vaccination campaign against tuberculosis under non-sterile conditions (Okuda, 1991).

Also in Japan, a report on trends in death from liver cancer showed that alcohol was the agent primarily responsible. Birth cohort analyses showed small effects. The effect of alcohol may have been overestimated because the incidence of liver

cancer was used as a surrogate measure of alcohol consumption by women, who are assumed to have low consumption of alcohol. Other surrogate measures used were the incidence of oesophageal cancer and the mortality rate from cirrhosis (Makimoto & Higuchi, 1999).

The role of chronic infection with HBV and HCV in the etiology of liver cancer is well established. Several epidemiological studies have examined the association between seropositivity for HBsAg and the risk for liver cancer. The risk estimates ranged from 3 to 30 in case-control studies and from 5.3 to 148 in cohort studies (IARC, 1994). A meta-analysis of studies published before 1998 gave an estimated relative risk of 17 for persons with antibodies to HCV who are HBsAg<sup>-</sup> (Donato et al., 1998). Table 3 shows current estimates of the attributable fractions for the main risk factors associated with liver cancer. Worldwide, 52% of liver cancer cases (230 000) have been attributed to chronic HBV infection, with 19 000 in developed countries and 210 000 in developing countries. The fraction of liver cancer cases attributable to HCV infection is 110 000 (25% of the world total), with 17 000 cases in developed countries and 93 000 in developing countries.

The presence of HBV DNA or HCV RNA serum and liver tumour tissue from patients with liver cancer, mostly in European countries, who were seronegative for antibodies to both viruses, was investigated in a collaborative multicentre study. Of

**Table 3. Risk factors for liver cancer and estimates of attributable fractions (%)**

Risk factor	Low-risk countries in Europe and the USA		Japan		High-risk countries in Africa and Asia	
	Estimate	Range	Estimate	Range	Estimate	Range
Hepatitis B virus	< 22	4–58	20	18–44	60	40–90
Hepatitis C virus <sup>a</sup>	60	12–72	63	48–94	< 10	Not evaluated
Aflatoxin	Little exposure		Little exposure		Heavy exposure <sup>b</sup>	
Alcohol		15–45	< 20			11–30
Tobacco	< 12		40	38–51	22 <sup>c</sup>	Not evaluated
Oral contraceptives		10–50 <sup>d</sup>	Not evaluated		8 <sup>e</sup>	Not evaluated
Other	< 5				< 5	

From Bosch et al. (1999). The attributable fractions do not necessarily add up to 100% because of multiple exposures and possible interactions between risk factors.

<sup>a</sup> Not including infection with both HBV and HCV; second-generation assays were used in few studies

<sup>b</sup> Attributable risk not quantified

<sup>c</sup> Estimates for HBsAg<sup>-</sup> black men > 50 years old (one study)

<sup>d</sup> Only in women

<sup>e</sup> Only in black women (one study)

the specimens, 33% contained HBV DNA and 7% contained HCV RNA (Brecht et al., 1998). The results have been confirmed. The trend suggests that, in countries where HBV is common, the presence of HBV DNA among HBsAg<sup>-</sup> patients with liver cancer is higher than the 33% found in Europe. These findings reinforce the strong relationship between HBV and HCV viral infections and liver cancer and suggest that the attributable fractions shown in Table 3 may be underestimates.

### 2.5.1 Correlation studies

In a review of several studies in China (some of which were evaluated by the Committee at its forty-ninth meeting), in which aflatoxin–albumin adducts were measured, a correlation was found between death from primary liver cancer and aflatoxin B<sub>1</sub>–albumin in serum from persons in Fusui but not in those from Shanghai. In Fusui County, primary liver cancer was correlated to intake of aflatoxin B<sub>1</sub> but not aflatoxin, M<sub>1</sub> and the decreasing trend in the aflatoxin–albumin adducts over time in Fusui were attributed to improved agricultural practices (Yu et al., 1998).

The mutation induced by aflatoxin B<sub>1</sub> in exon 3 of the human *HPRT* gene in B lymphoblasts is a GC to TA transversion at base 209, occurring in 17% of aflatoxin B<sub>1</sub>-induced mutants. In an analysis of the *HPRT* mutation frequency in an area with heavy intake of aflatoxin B<sub>1</sub>, the residents of Qidong County, China, were studied to determine the combined contributions of aflatoxins and other risk factors to the high incidence rate of liver cancer in the region. The study cohort comprised 42 men and 65 women aged 40–65. Blood samples were analysed for mean aflatoxin B<sub>1</sub> in albumin, *HPRT* mutations by a T-cell clonal assay, HBsAg status, serum alanine aminotransferase activity, leukocyte count, haemoglobin concentration, and platelet count. Subjects were categorized as having a low or a high intake of aflatoxin B<sub>1</sub> and were dichotomized around the population mean of aflatoxin–albumin adducts.

A major assumption in this study was that an individual's aflatoxin B<sub>1</sub> content was representative of his or her intake of aflatoxin throughout life, even though aflatoxin B<sub>1</sub>–albumin adducts indicate recent intake. The amounts of aflatoxin B<sub>1</sub> measured were comparable with those in previous year-long studies in this population. The typical mutation frequency in the *HPRT* gene in normal, healthy adults is 5–8 × 10<sup>-6</sup> per cell, whereas the frequency in this population was 26 × 10<sup>-6</sup>. Thus, the population was exposed to environmental agents that damage DNA. The authors concluded that the aflatoxin-induced DNA damage in T lymphocytes, assessed as the validated marker, albumin adducts, led to an increased mutation frequency, reflected as the increase in *HPRT* gene mutations (Wang et al., 1999a).

The limitation of the study is that HBsAg seropositivity was presumed to indicate the presence of HBV; however, epidemiological studies that rely on HBsAg status instead of detection of HBV DNA (Brecht et al., 1998; Bosch et al., 1999) systematically underestimate the risk due to HBV. Furthermore, HCV status was not measured.

### 2.5.2 Case–control and cohort studies

Studies on intake of aflatoxins and liver cancer published in 1997–2000 incorporated biomarkers of intake of aflatoxin in order to compare series of cases and controls. Some of these studies had the advantage of being nested in cohort

studies, thus including data from biological specimens collected some time before the occurrence of liver cancer.

A small case-control study in the Sudan showed a relationship between grain storage conditions and the aflatoxin contamination of peanuts, and some association between storage conditions and the occurrence of liver cancer (Omer et al., 1998).

A 10-year follow-up study for hepatocellular carcinoma in Qidong, China, was reported in which 145 carriers of HBV provided eight monthly urine samples which were tested for aflatoxin M<sub>1</sub> by a sensitive assay (3.6 ng/L). At the beginning of the study, 54% of the subjects had aflatoxin M<sub>1</sub> in their urine; 22 subsequently developed hepatocellular carcinoma. The predictors of liver cancer among HBV carriers were found to be the presence of aflatoxin M<sub>1</sub> in urine, antibodies to HCV, and a family history of hepatocellular carcinoma. The estimated relative risk associated with aflatoxin M<sub>1</sub> was 3.6 (95% confidence interval, 1.3–9.9). The authors compared this estimates with that for a cohort in Shanghai with exposure to both HBV and aflatoxins (relative risk, 59) and found no statistically significant difference, mainly because of the small number of cases in both studies. All four patients with hepatocellular carcinoma who had aflatoxin M<sub>1</sub> in their urine and who were tested for mutations in the *P53* oncogene showed the missense mutation in codon 249. The authors concluded that aflatoxin is a substantial risk factor for progression to hepatocellular carcinoma among carriers of HBV. The (unstable) estimated attributable fraction was 0.55 (0.09–0.94) (Sun et al., 1999).

In a prospective follow-up study of 737 HBV carriers and 699 with no HBV, aflatoxin-albumin adducts were measured in 30 HBsAg<sup>+</sup> patients with liver cancer and 150 controls (HBV status unclear). A significantly larger proportion of patients had adducts (odds ratio [OR], 3.5), and they had a significantly higher overall level of adducts than controls. The authors concluded that aflatoxins are a significant co-factor with HBV in the induction of primary liver cancer (Lu et al., 1998).

Forty-three patients with hepatocellular carcinoma in Taiwan were compared with 86 matched controls for the urinary concentration of aflatoxin metabolites and aflatoxin B<sub>1</sub>-albumin adducts in specimens taken in 1988–92 and for GST activity. All but one of the patients were HBsAg<sup>+</sup> and the other had antibodies to HCV. The levels of biomarkers of aflatoxin in urine differed somewhat between cases and controls. A trend to increased risk with urinary aflatoxin M<sub>1</sub> was reported, but the numbers were too small to ensure proper power. A high risk was found for persons with detectable levels of aflatoxin-albumin adducts and aflatoxin B<sub>1</sub>-N7 guanine adducts (OR, 10; 1.6–61). The authors concluded that aflatoxin is a significant factor for primary liver cancer in HBV carriers and that there may be an interaction between the GSTM1 genotype and intake of aflatoxin B<sub>1</sub> (Yu et al., 1997a).

A small case-control study was conducted among black Africans to determine the effect of iron overload and other environmental factors on the risk for hepatocellular carcinoma. The OR associated with HBV seropositivity in 24 cases and 48 hospital controls was 33 (7.2–150) for HCV infection, 6.4 (0.30–130) for alcohol consumption, 2.0 (0.50–8.2) for iron overload, and 11 (1.5–77) for aflatoxin-albumin adducts. There was no association with hepatocellular carcinoma (median prevalence

of adducts, 7.3 in cases and 22 in controls; OR not reported) (Mandishona et al., 1998).

Thus, three studies from Asia reported increased risks for persons infected with HBV and with either aflatoxin M<sub>1</sub> metabolites in urine or aflatoxin–albumin adducts in serum. The ORs reported ranged from 3 to 10. The evidence is not entirely consistent, and the study from southern Africa found no significant association between the presence of aflatoxin–albumin adducts in serum and liver cancer.

Other cohort studies gave conflicting results with regard to the role of aflatoxin in the etiology of liver cancer. A follow-up study was conducted to estimate the risk for primary liver cancer among male HBV carriers in areas with different intakes of aflatoxin: in Senegal, China, and persons of Asian origin resident in the USA. The cohorts were selected to examine why the estimated risk for liver cancer in some areas of China was significantly higher (two- to threefold) than that on the west coast of Africa. The prevalence of HBsAg was only moderately higher in Senegal (20% versus 16%), and the expected intake of aflatoxin was higher in the African setting. In an analysis of differences in host response to the viral infection in these populations, viral replication (HBV DNA detected by Southern blotting) was 25–30% in HBsAg carriers of all age groups in China, whereas in Senegal a strong decay in HBV DNA rates was seen with age, from 14% in persons aged 20–29 to 3% in those aged 30–49 and undetectable in persons > 50. Asian–American HBsAg carriers also showed a strong decline in HBV DNA with age, from 37% in the 20–29 age group to 5% in those over 50. Prolonged viral replication at a high titre correlated with several parameters of liver damage and may be a determinant in the high rate of mother-to-child transmission of HBV and in the incidence of liver cancer in Chinese populations .

In the first report of this study, the authors showed that the risk of an HBV carrier for progression to hepatocellular carcinoma was lower in Senegal (high risk for exposure to both HBV and aflatoxin) than in China (high risk for exposure to HBV but lower risk for intake of aflatoxin). This was contrary to expectations if a strong interaction between HBV and aflatoxins is the central determinant of liver cancer in these areas. In fact, active DNA replication throughout life seems to explain the higher progression rate in China and may be a consequence of the high rate of mother-to-child transmission of HBV and the high incidence of liver cancer in Chinese populations (Evans et al., 1998).

### **2.5.3 Metabolic epidemiological studies**

The metabolism of aflatoxins is not yet fully understood. It has been hypothesized that metabolic polymorphisms of the genes that regulate the metabolism of aflatoxins could explain the established interspecies differences in susceptibility to aflatoxin-induced carcinogenicity and the largely hypothetical differences in susceptibility among human groups. Some epidemiological studies have addressed the environmental factors that may modulate the natural history of aflatoxins and biomarkers of aflatoxins under various conditions of exposure.

The determinants of aflatoxin–albumin adducts in blood were investigated in 357 persons, including 181 chronic carriers of HBV, in The Gambia. Several environmental factors (season, place of residence, HBV status) and aspects of the

metabolism of aflatoxin (the GST genotypes M1, T1, P1 and epoxide hydrolase) were recorded, and the ratio of 6 $\beta$ -hydroxycortisol:cortisol as a marker of CYP 3A4 activity was measured in urine. The major determinants of the amounts of aflatoxin in blood were place of residence and season. The mean adduct levels were higher in persons without HBV infection and the GSTM1 null genotype. The authors concluded that environmental factors leading to food contamination are better determinants of intake than metabolic measures and are more amenable to intervention (Wild et al., 2000).

Understanding the natural history of the biomarkers used in epidemiological studies is an absolute requirement for proper interpretation of much of the available literature. In a study in Taiwan, aflatoxin B<sub>1</sub>-N<sup>7</sup>-guanine adducts were measured in urine as a function of the hormonal and nutritional parameters that may affect aflatoxin adduct formation. In a cross-sectional study of 42 male HBV carriers and 43 HBV-free men, adduct formation was detected in 42%. Significant determinants of adduct formation were HBV status, with higher levels in HBsAg carriers, and plasma concentrations of cholesterol,  $\alpha$ -tocopherol and  $\alpha$ - and  $\beta$ -carotene. The associations were significant and dose-dependent. Lycopene concentrations were inversely related to adduct formation. This study is significant in that it indicates some of the environmental and host determinants of adduct formation for use in etiological studies (Yu et al., 1997b).

If HBV status is directly related to adduct formation, case-control studies will systematically show that the presence of adducts in urine is a risk factor for hepatocellular carcinoma (Sohn et al., 2000). Nutritional determinants of adduct formation, if validated, should be treated as confounders in epidemiological studies in which urinary adducts are used as a biomarker. The roles of folate and other nutrients in the natural history of HBV infection and liver cancer have been confirmed in a number of studies (e.g. McGlynn et al., 1999), and the role of nutrients in the mutagenicity and carcinogenicity of aflatoxins is corroborated by evidence from experiments in rats (Soni et al., 1997) and in studies summarized in section 2.3.4.

GST expression was found to be inversely related to the HBV status of patients with normal livers, suggesting that viral replication decreases the ability of liver cells to detoxify liver carcinogens such as aflatoxin efficiently. Tissue from liver tumours had less GST activity, and subjects with the null GSTM1 genotype had less GST  $\alpha$ - and  $\mu$ -isoenzymes, with some overexpression of  $\pi$ . These results suggest that GST expression should be treated as a confounder in epidemiological studies in which urinary adducts are used as a biomarker (Zhou et al., 1997).

In a comparison of the sensitivity to mutagens of 28 cases of hepatocellular carcinoma and 110 controls, on the basis of the count of chromatid breaks induced by bleomycin or benzo[*a*]pyrene diol epoxide, the OR was 36. The tests show defects in predisposition to chromosome breakage or the capacity to repair chromatid breaks or both. The results suggest that individual susceptibility can be important in determining the outcome in exposed individuals. No epidemiological studies have been reported in which host factors determined by these methods were adjusted for (Wu et al., 1998).

#### **2.5.4 Intervention studies with vaccination against hepatitis B virus**

A study in Taiwan evaluated by the Committee at its forty-ninth meeting showed that, in an area hyperendemic for infection and with moderate-to-high intake of aflatoxins, immunization against HBV had reduced the rate of HBV carriage in 6-year-old children from about 10% in 1981–86 to 0.8–0.9% in the period 1990–94. A more recent report noted that 15–20% of the population of Taiwan were estimated to be HBV carriers in the early 1980s. A programme of mass vaccination against HBV was launched in 1982, and, since 1986, all newborns and, progressively, preschool children, primary-school children, adolescents, young adults, and others have also been vaccinated. The coverage of newborns is over 90%, and 79% of pregnant women are screened for HBsAg. The proportion of babies born to highly infectious mothers who also became carriers decreased from 86–96% to 12–14%. The average annual incidence of hepatocellular carcinoma in children aged 6–14 decreased significantly from 0.7 per 100 000 in 1981–86 to 0.36 per 100 000 in 1990–94; and the annual incidence of hepatocellular carcinoma in children aged 6–9 declined from 0.52 per 100 000 for those born in 1974–84 to 0.13 per 100 000 in those born in 1986–88. Thus, the mass vaccination programme has been highly effective in controlling chronic HBV infection and in preventing liver cancer in Taiwan. If a vaccination coverage rate of 90% of all newborns against HBV can be maintained, the carriage rate in Taiwan can be expected to decline to 0.1% by 2010. The cost of the programme has been about US\$ 100 million (Huang & Lin, 2000).

Recent reports from Taiwan and from other areas where massive HBV vaccination campaigns have been conducted have shown the presence of HBV mutants in the surface gene which induces chronic carriage among immunized children. The prognosis of these infected children is uncertain, but the observation should be considered in evaluating the occurrence of liver cancer in HBV-vaccinated populations (Hsu et al., 1999).

A study of vaccination against HBV was reported from the Republic of Korea, where the prevalence of HBV infection is among the highest in the world. In this prospective cohort study, 370 285 men over the age of 30 who were clinically free of liver disease and had not been vaccinated against HBV at the time of enrolment were included. About 5% of the cohort were HBsAg<sup>+</sup>, 78 094 had antibodies to the HBV surface marker, and 273 277 were negative for both. About 13% of the men had been vaccinated against HBV in 1985. Cases of liver cancer were ascertained by record linkage and from medical records covering 1986–89. A multivariate log-linear model was used to test for statistical significance and to estimate relative risks. The follow-up period represented 1 404 566 person-years (average, 3 years and 10 months), and 302 cases were ascertained, to give an overall incidence rate of liver cancer of 22 per 100 000 person-years. The relative risk for primary liver cancer was 18% (95% confidence interval, 14–23) among chronically infected men, 0.34 (0.19–0.60) among unvaccinated infected men, and 0.58 (0.31–1.1) in the vaccinated group. The study suggests that vaccination against HBV, even in adulthood, reduces the risk for liver cancer (Lee et al., 1998).

A report on the results of vaccination programmes in China and The Gambia showed that the vaccine must be given as early as possible in life: vaccination

within 48 h of birth reduced carriage by at least 70%. In China, some 40% of carriers of HBV were infected by perinatal transmission from their mothers. The effectiveness of vaccination has been reported to be 70% in some areas of China and as much as 90% in others. In Africa, introduction of the vaccine into the routine programme for infant vaccination reduced the carriage rate by 94%. In The Gambia, protection was shown to be maintained up to the age of 9 years, which is well past the age at which the risk of becoming a carrier is high; thus, these children effectively have lifelong protection against HBV-associated liver cancer (Wild & Hall, 2000).

Programmes to reduce the burden of liver cancer in developing countries should therefore give priority to HBV vaccination and to the prevention of HCV contamination. This implies reinforcing the control of blood and blood products and the use of sterile medical equipment. HBV carriers may benefit from reductions in intake of aflatoxins in their diets, and this may also offer some protection to HCV carriers. However, a reduction in intake of aflatoxin B<sub>1</sub> or a reduction in the concentration of aflatoxin M<sub>1</sub> in milk or milk products is unlikely to result in an observable reduction in the rate of liver cancer in most developed countries. In these populations, alcohol consumption may account for most cases of liver cancer without viral markers.

### **2.5.5 Intervention studies with oltipraz**

In spite of the effectiveness of hepatitis B vaccination in preventing chronic HBsAg carrier status in unexposed newborns, infants, and adults, a substantial number of persons (some 300 million worldwide) are HBsAg carriers. No effective treatment has been developed for these persons (Torresi & Locarnini, 2000).

Epidemiological studies suggest that dietary intake by chronic HBV carriers of aflatoxins may increase the rate of progression to hepatocellular carcinoma (Qian et al., 1994). Thus, it has been suggested that oltipraz, a drug that modifies the metabolism of aflatoxin and has a number of other biological properties (reviewed by Kensler et al., 1999) might be used as a chemopreventive agent. The experimental basis for this proposal is the demonstration of remarkable anticancer activity against aflatoxin B<sub>1</sub>-induced hepatocarcinogenesis in rats (see section 2.3.4). Oltipraz has also been evaluated as a chemopreventive agent for cancers of the colon, liver, bladder, and skin (reviewed by Kensler & Helzlsouer, 1985; Kensler et al., 1999).

In rats, continuous administration of oltipraz significantly reduced the formation of aflatoxin–albumin adducts and the occurrence of liver neoplasms (Kensler et al., 1997). Oltipraz affected the life cycle of HBV in vitro by blocking transcription in 2.2.15 cells, resulting in dose-related inhibition of HBV replication, perhaps mediated through induction of wild-type *p53* (Chi et al., 1998).

Oltipraz has been tested in phase I/II trials in China. The results of pilot studies for these trials showed reasonably good compliance with the regimen and mild toxicity, with no observed interaction with the HBV status of the individual (Jacobson et al., 1997). The results of another pilot trial showed that low daily doses of oltipraz induced phase-2 conjugation of aflatoxin, as measured by an increase in the urinary excretion of aflatoxin mercapturic acid, with no reduction in the concentration of aflatoxin M<sub>1</sub>.

Intermittent high doses of oltipraz decreased the phase-1 metabolism of aflatoxin, leading to a significant reduction in excretion of aflatoxin M<sub>1</sub>, indicating that the metabolic pathways of aflatoxins in humans can be strongly modified by oltipraz (Wang et al., 1999b). The long-term effects of this chemopreventive treatment remain to be established.

### 2.5.6 Other studies

Various biomarkers were studied in 23 cases of liver cancer in the USA. HBV markers including HBV DNA were found in 13 cases, HCV antibodies in sera in 5/22, overexpression of P53 in tissue in 5/23, and mutations in codon 249 in 0/5. Surprisingly, aflatoxin B<sub>1</sub>-DNA adducts were found in liver tumour tissue in 3/19 cases and aflatoxin B<sub>1</sub>-lysine adducts in sera in 5/5, none of which had concurrent overexpression of P53 or mutations at codon 249. Few cases were available for each test, and only the abstract has been published; however, the presence of markers of aflatoxin in cases of liver cancer in the USA is interesting because intake of aflatoxin in that country is expected to be low. HBV or HCV infection would also be expected to be infrequent, depending on the subpopulation sampled (Hoque et al., 1999).

Aflatoxin-albumin adducts were also identified in serum from 104 volunteers in the United Kingdom. There was no direct correlation with a particular food (Turner et al., 1998).

### 2.5.7 Studies of P53 as a marker of intake of aflatoxin

Mutations of *P53* are a relevant marker in the molecular epidemiology of liver cancer, as some 20% of cases show mutations of this oncogene. Moreover, a mutation at the third base of codon 249 (a GC to TA transversion leading to a change from arginine to serine) has been described in geographical correlation studies of intake of aflatoxin B<sub>1</sub>.

The IARC and other databases on p53 clearly describe the 'hot spot' at 249 as the predominant mutation in liver cancer. The IARC database is heavily biased by publication and reporting selection, but 35–40% of cases of liver cancer in areas where there is high intake of aflatoxins show the presence of the 249 mutation, whereas there is a much lower prevalence (0–2%) in areas where there is low intake (Soussi et al., 2000).

The most recent reviews of 'molecular fingerprints' of carcinogens appear to converge in accepting a few for which some specificity can be claimed: GC to TA transversions in lung cancer associated with smoking; GC to TA transversions in codon 249 in liver cancer associated with aflatoxin B<sub>1</sub>; and CC:GG to TT:AA transversions in skin cancer associated with exposure to ultra-violet light (Hainaut & Vahakangas, 1997; Wang & Groopman, 1999).

Since the last evaluation by the Committee, additional studies have shown that a mutation in codon 249 of *P53* is found regularly in a proportion of cases of liver cancer in certain countries and not in others. Several studies showed that these mutations are poorly correlated with another marker of aflatoxin intake, the presence of aflatoxin B<sub>1</sub> adducts, in hepatocellular carcinoma tissue (Hsie et al., 1995; Soini et al., 1996; Lunn et al., 1997). Experimental data have also shown that the mutation

can be induced in hepatocytes exposed to aflatoxin B<sub>1</sub>; aflatoxin metabolites bind to the third base in codon 249; and 249 ser p53 expression inhibits apoptosis and p53-mediated transcription and enhances liver cell growth in vitro (reviewed by Hussain & Harris, 2000).

Most of the studies reported below involved few cases, and even fewer cases with *P53* mutations, and many of the comparisons and ORs calculated from them are therefore quite unstable. Many of the reports focus on mutations and not on the full spectrum of genetic alterations in *P53* that characterizes hepatocellular carcinoma. Most of these mutations have been identified in individuals who are also HBV carriers. Of 21 cases of hepatocellular carcinoma in India, three had mutations in *P53* (two in 249 GT and one in a 250 CT transition). In investigations for HBV status, 59% of the cases were shown to have HBV DNA by dot blotting, 90% to have HBV DNA by polymer chain reaction, and 71% to be HBsAg<sup>+</sup> by enzyme-linked immunosorbent assay (ELISA). The report indicated that intake of aflatoxin was common in that part of India (Katiyar et al., 2000).

In a report on 24 cases of liver cancer in Shanghai and Qidong, China, all specimens had integrated HBV DNA, and 63% had the null GSTM1 genotype; 95% had alterations in *P53*: 12 had mutations in *P53*, and 13 had overexpression. Loss of heterozygosity at 4q was found in 50%, at 1p in 46%, at 16q in 42%, and at 13q in 38%. Mutation at codon 249 was found in seven cases from Qidong (all those with a *P53* mutation) and in three of five from Shanghai (Rashid et al., 1999).

Of 30 cases of hepatocellular carcinoma from Guangxi, China, an area of high risk for HBV and exposure to aflatoxin, 90% were HBsAg<sup>+</sup>, and 43% showed *P53* expression and a linear response with the stage of tumour (Qin et al., 1997).

Seven of 21 samples of tissue from patients with hepatocellular carcinoma in Tongan, China, had point mutations at codon 249 resulting in a G to T transversion. Only one of the patients was HBV-negative (method not stated). The authors also reported another case of HBV-negative hepatocellular carcinoma with a mutation at codon 249 of *P53* (Yang et al.; 1997).

In a study in Taiwan of 110 cases of liver cancer and 37 controls, HBV status was assessed by assay for HBsAg, intake of aflatoxin by aflatoxin B<sub>1</sub> adducts in liver tissue, *P53* status by immunohistochemistry, and DNA mutations by single-stranded conformation polymorphism and sequencing. The main findings were elevated risks associated with HBsAg seropositivity (OR, 8.4) and aflatoxin B<sub>1</sub> adducts (OR, 3.9), with an OR of 68 for both. *P53* mutations were found in 29% of cases, and mutations at codon 249 in 13%. The presence of aflatoxin B<sub>1</sub> adducts in liver tissue was related to the presence of *P53* protein and DNA mutations (borderline significance). Mutations in codon 249 were found only in HBsAg<sup>+</sup> subjects, suggesting that HBV is involved in the selection of these mutations. Because liver tissue was required for these comparisons, the controls were patients with liver or biliary-tract conditions, including hepatic metastases from other primary cancers (Lunn et al., 1997).

In a small correlation study, the *P53* mutation patterns in 31 cases of hepatocellular carcinoma from northern and southern Jiang-Su Province in China were compared. Mutations in codon 249 were found in 9/16 cases in the area with high intake of aflatoxin and 1/15 in the area with lower intake (Shimizu et al., 1999).

In a study in Spain, 120 paraffin blocks from hepatocellular carcinoma cases were studied by single-stranded conformation polymorphism and sequencing

techniques. No mutation was found in *P53*, although *P53* overexpression was found in 14 cases. The authors concluded that *P53* mutations are not common in hepatocellular carcinoma induction or promotion in Spain (Boix-Ferrero et al., 1999).

An investigation was conducted of circulating DNA for *P53* codon 249 mutations in a series of 53 hepatocellular carcinoma cases in The Gambia and in 13 patients with liver cirrhosis. There were 53 controls and a second control group of 60 French patients with a variety of liver conditions. The relevant mutation was found in 19 cases of hepatocellular carcinoma, two patients with cirrhosis and three controls. The OR for hepatocellular carcinoma was 16 (3–90). None of the patients in France had the 249 mutation (Kirk et al., 2000).

In a series of 62 hepatocellular carcinoma cases in Taiwan, *P53* mutations were investigated by single-stranded conformation polymorphism and sequencing; 37 of the patients were HBsAg<sup>+</sup> and 25 HBsAg<sup>-</sup>. Twenty patients had mutations, which were widely distributed along exons 5–8. Four patients, all HBsAg carriers, had a G to T mutation at codon 249 (Sheu, 1997).

A cluster of mutations was found at position 220 in *P53* in patients with genetic haemochromatosis and liver cancer. Mutations in codon 249 exon 7 A/T were observed in one case. Although anecdotal, these observations suggest that a mutation at this locus may also be acquired in other contexts (Vautier et al., 1999).

A recent, unpublished meta-analysis on the relationship between HBV, aflatoxin, and mutation at codon 249 of *P53* showed that the geographical relationship between intake of aflatoxins (broadly classified into three levels) and *P53* mutations (any spot) was strongly correlated. The correlation was due almost entirely to the G to T mutation, but a significant (albeit unstable) trend with mutations at other codons remained. There was no indication that the presence of the 249 mutation varied with HBV status, although one study showed a significant interaction (Stern et al., 2001, personal communication).

Table 4 summarizes the results of studies on *P53* mutations in cases of liver cancer. Intake of aflatoxins is expressed crudely in relation to the geographical source of the specimens, thus ignoring local and individual variation. Although *P53* mutations

**Table 4. *P53* mutations in cases of liver cancer according to exposure to aflatoxin**

Extent of exposure to aflatoxins	No. of cases of liver cancer	G–T mutations at <i>P53</i> codon 249		Total <i>P53</i> mutations		Mutations at codon 249/ total <i>P53</i> mutations (%)
		%	Range	%	Range	
High <sup>a</sup>	259	49	30–83	56	45–69	92
Intermediate <sup>b</sup>	495	10	0–30	28	13–50	27
Low <sup>c</sup>	651	2	0–11	24	0–35	2

From Lunn et al. (1997); Qin et al. (1997); Sheu (1997); Yang et al. (1997); Boix-Ferrero et al. (1999); Rashid et al. (1999); Shimizu et al. (1999); Katiyar et al. (2000)

<sup>a</sup> China, rural sub-Saharan Africa

<sup>b</sup> Urban China, urban sub-Saharan Africa, Thailand

<sup>c</sup> Europe, Japan, northern China, Singapore, USA

occur at both the hot spot (249 G to T) and other spots, mutations at codon 249 predominated (92% versus 2%) in relation to the geographical classification. The presence of the mutation was affected only moderately by the concurrent presence of HBV.

There is growing consensus that the mutational spectra of *P53*, and in particular codon 249, is a relevant biomarker of intake of aflatoxin B<sub>1</sub> in relation to hepatocellular carcinoma. In order to make full use of this biomarker, the natural history of the mutation should be characterized, and its relationship to the dose of aflatoxin B<sub>1</sub> and to better validated biomarkers, such as aflatoxin B<sub>1</sub>-albumin adducts in urine, should be established.

In epidemiological studies, the finding of this mutation in specimens of hepatocellular carcinoma can be interpreted as reflecting the involvement of aflatoxin B<sub>1</sub>, but its absence, particularly in cancers in people living in areas of heavy intake of aflatoxin B<sub>1</sub> should be interpreted with caution, as aflatoxin B<sub>1</sub> might induce hepatocellular carcinoma by mechanisms other than DNA damage.

### **3. ANALYTICAL METHODS**

#### **3.1 Screening tests**

Early detection of aflatoxin M<sub>1</sub> and removal of small lots of contaminated milk can prevent contamination of much larger volumes. Screening methods are particularly useful if they can be carried out quickly, easily, and economically. They should allow detection of concentrations of aflatoxin M<sub>1</sub> in milk as low as those detected by the ultimate quantitative methods (see section 3.2). Theoretically, screening methods should never give false-negative results.

The screening tests used for aflatoxin M<sub>1</sub> in milk and milk products are usually immunochemical. For aflatoxin M<sub>1</sub>, both radioimmunoassays and enzyme immunoassays have been developed (Frémy & Chu, 1989), although enzyme immunoassays are used more often.

The protocol for radioimmunoassay of aflatoxin M<sub>1</sub> usually includes simultaneous incubation of a test solution containing an unknown amount of aflatoxin M<sub>1</sub> (or a standard solution of a known amount of aflatoxin M<sub>1</sub> in phosphate buffer) with a constant amount of labelled aflatoxin and its specific antibody. Free and bound labelled aflatoxin are then separated, and the radiolabel on aflatoxin is determined. The aflatoxin M<sub>1</sub> concentration of the test solution is determined by comparing the results to a standard curve, which is established by plotting the ratio of bound and the initial (total) amount of labelled aflatoxin multiplied by 100 (% binding) versus the concentration of aflatoxin M<sub>1</sub> standard (Chu, 1984). Radioimmunoassay has found little use in routine investigations of aflatoxin M<sub>1</sub> in milk. A recent exception is an investigation of samples of milk in Thailand (Saitanu, 1997). There have been no collaborative studies on radioimmunoassays for aflatoxin M<sub>1</sub>.

Most enzyme immunoassays for aflatoxin M<sub>1</sub> are heterogeneous, with separation of the immunocomplex and the unreacted material. One of the commonest heterogeneous enzyme immunoassays, the ELISA, is generally used to determine aflatoxin M<sub>1</sub>. Several direct competitive ELISAs for aflatoxin M<sub>1</sub> are available commercially. The 96-well microtitre plate assay is most commonly used for quantitative measurements. ELISAs for aflatoxin M<sub>1</sub> are usually designed for rapid

screening. For legal purposes, positive results in an ELISA require confirmation by an accepted reference method.

One successful use of a direct competitive ELISA, in which the results were confirmed by a validated HPLC method, involved ELISA for the determination of aflatoxin M<sub>1</sub> in pasteurized milk, infant formula, powdered milk, and yoghurt (Kim et al., 2000). A valuable addition was comparison of the ELISA with the validated HPLC method of Ferguson-Foos & Warren (1984; see also section 3.2). Kim et al. concluded that the results for aflatoxin M<sub>1</sub> obtained with ELISA were similar to those obtained by HPLC. In a study in which ELISA and HPLC with immunoaffinity purification were compared, the latter technique was found to be superior (Biancardi, 1997). An interesting development is a portable field test involving a patented, membrane-based flow-through enzyme immunoassay (Sibanda et al., 1999), which can be carried out on farms. The kit comprises a nylon membrane spotted with anti-mouse antibodies, a plastic snap-fit device, absorbent cotton wool, mouse monoclonal antibodies against aflatoxin M<sub>1</sub>, and aflatoxin B<sub>1</sub>-horse radish peroxidase conjugate. Clean-up is done on an immunoaffinity column (see section 3.2). No collaborative studies have been carried out, at least not under the auspices of international organizations, of ELISAs for aflatoxin M<sub>1</sub>.

Techniques other than immunochemical procedures can, in principle, be used for rapid analysis of milk and milk products for aflatoxin M<sub>1</sub>. One such technique involves electrochemical flow injection monitoring on filter-supported bilayer lipid membranes (Andreou & Nikolelis, 1998). The method has not been formally validated.

It has been recommended that extensive collaborative studies be conducted to validate the performance characteristics of immunoassays, including their reproducibility and repeatability, accuracy, sensitivity, limits of detection, specificity, and selectivity (Frémy & Chu, 1989). This recommendation was echoed in 2000 (van Egmond, 2000), as no such studies have been carried out by AOAC International, the organization responsible for collaborative studies of methods of analysis (International Union of Pure and Applied Chemistry, 1989). The International Dairy Federation (1999) has produced a guideline document that outlines the parameters necessary for the evaluation and validation of competitive enzyme immunoassays for quantitative determination of aflatoxin M<sub>1</sub> in milk and milk products.

### **3.2 Quantitative methods**

Quantitative analytical methods for aflatoxin M<sub>1</sub> usually follow the general pattern for mycotoxin assays, i.e. extraction, clean-up, concentration, separation, detection, and quantification. A homogeneous sample of aflatoxin M<sub>1</sub> in milk is easily obtained, because the toxin is distributed evenly in fluid milk. The initial problem encountered in analysing milk is the extraction step. Because milk is a complex natural product, aflatoxin M<sub>1</sub> is not easily extracted and purified for final assay, owing (partly) to adsorption of aflatoxin M<sub>1</sub> to casein proteins. A process is required to separate aflatoxin M<sub>1</sub> from milk easily, efficiently, and economically. Once purified extracts are obtained, the concentration of aflatoxin M<sub>1</sub> can be determined in one of several ways. Most quantitative methods involve thin-layer chromatography (TLC) or HPLC. Aflatoxin M<sub>1</sub> is a weakly polar component and is extractable with solvents such as methanol, acetone, chloroform, or combinations of these solvents with water. In practice, the choice of solvent depends on the clean-up and the separation procedure.

The quantitative methods that have been developed and validated for aflatoxin M<sub>1</sub> in milk and milk products were originally designed to analyse milk powder. Milk was spray-dried or lyophilized to preserve its shelf life and to reduce sample bulk. Various mixtures of methanol and water (Masri et al., 1968, 1969a; Fehr et al., 1971), acetone and water and acetone, chloroform, and water (Purchase & Steyn, 1967) were used to extract aflatoxin M<sub>1</sub> from milk powder.

In the first effective method for the determination of aflatoxin M<sub>1</sub> in fluid milk, methanol and water were used as the extraction solvents (Jacobson et al., 1971). This method was modified by others (McKinney, 1972; Stubblefield & Shannon, 1974a), leading to a method suitable for collaborative studies. The method of Stubblefield & Shannon (1974a) involved extraction with acetone and water, precipitation with lead acetate solution to deproteinize the milk, and a defatting step with hexane. TLC with fluorescence detection was used for ultimate separation, detection, and quantification. The collaborative study was successful (Stubblefield & Shannon, 1974b), and the method became an official method for aflatoxin M<sub>1</sub> of the AOAC and IUPAC (AOAC official method 974.17, superseded in 1993; see Table 5 for performance characteristics).

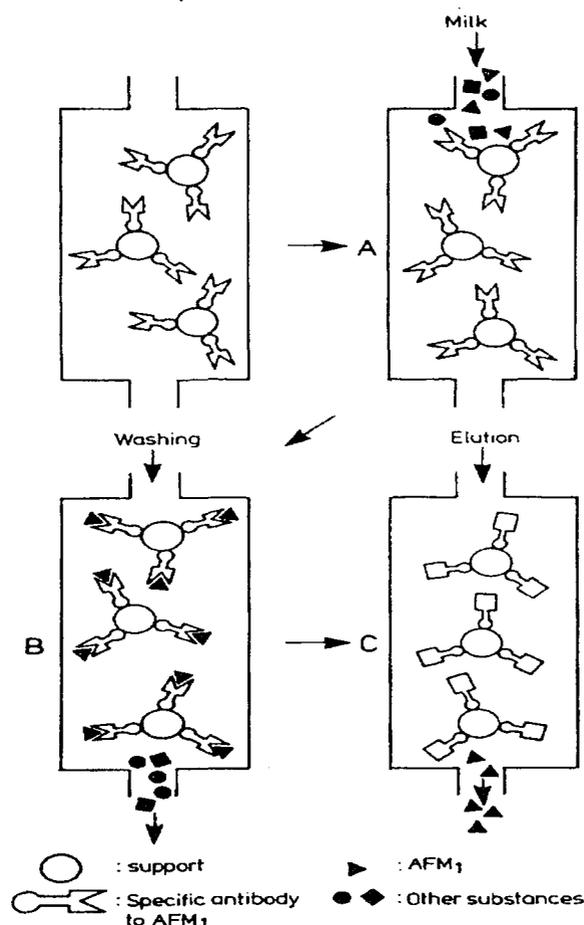
In another method, liquid milk was partitioned with chloroform in a separating funnel and cleaned-up over a small silica gel column. Final separation was by TLC with fluorescence detection, and aflatoxin M<sub>1</sub> spots were quantified by visual or densitometric estimation (Stubblefield, 1979). This method was further modified to allow determination of aflatoxin M<sub>1</sub> in cheese, in which two-dimensional TLC was used to improve separation of the aflatoxin M<sub>1</sub> spots from the background. The method was evaluated in an AOAC/IUPAC collaborative study (Stubblefield et al., 1980) and became an official AOAC method for aflatoxin M<sub>1</sub> in milk and cheese (AOAC official method 980.21; see Table 5 for performance characteristics).

With advances in HPLC methods in the 1980s, laboratories moved away from TLC to HPLC determination. In addition, factory-prepared solid-phase extraction columns were introduced for the purification of milk extracts. A method that successfully combined these two developments was that of Ferguson-Foos & Warren (1984), originally developed for normal-phase HPLC. The method was modified for reversed-phase HPLC, with the preparation of trifluoroacetic acid derivatives of aflatoxins M<sub>1</sub> and M<sub>2</sub>, and evaluated in an AOAC collaborative study (Stubblefield & Kwolek, 1986). The method became AOAC official method 986.16 for aflatoxin M<sub>1</sub> in fluid milk (see Table 5 for performance characteristics).

A more recent advance in quantitative extraction of aflatoxin M<sub>1</sub> and subsequent clean-up is use of immunoaffinity cartridges. These columns are composed of monoclonal antibodies specific for aflatoxin M<sub>1</sub>, which are immobilized on Sepharose® and packed into small cartridges (see Figure 2). The first published method for aflatoxin M<sub>1</sub> with immunoaffinity columns was that of Mortimer et al. (1987).

A milk sample containing aflatoxin M<sub>1</sub> is loaded onto the affinity gel column, and the antigen aflatoxin M<sub>1</sub> is selectively complexed by the specific antibodies on the solid support into an antibody–antigen complex. The column is then washed with water to remove all other matrix components of the sample. Aflatoxin M<sub>1</sub> is eluted from the column with a small volume of pure acetonitrile, and the eluate is concentrated and analysed by HPLC coupled with fluorescence detection. The method can be applied to whole milk, skimmed milk, and low-fat milk.

**Figure 2. Schematic diagram of immunoaffinity chromatography for concentration and purification of aflatoxin M<sub>1</sub>**



A, introduction of milk onto affinity gel column; B, removal of impurities; C, elution of aflatoxin M<sub>1</sub>

Modifications of the immunoaffinity-based methods for aflatoxin M<sub>1</sub> were subsequently published and studied collaboratively under the auspices of the International Dairy Federation (Tuinstra et al., 1993) and AOAC International (Dragacci et al., 2001) by groups of mainly European laboratories that could determine aflatoxin M<sub>1</sub> in milk at concentrations  $\leq 0.05 \mu\text{g/L}$ . The collaborative study of Tuinstra et al. led to International Dairy Federation Standard 171 (see Table 5 for performance characteristics). Another collaborative study resulted in approval as AOAC method 2000.08 (Dragacci et al., 2001; see Table 5 for performance characteristics).

The combination of immunoaffinity clean-up and liquid chromatography offers the best means for efficient purification and precise determination of low concentrations of aflatoxin M<sub>1</sub>. The approach is widely used in some parts of the world where low limits for aflatoxin M<sub>1</sub> in milk are in force, e.g. the European Union, where the method is successfully practised by the National Reference Laboratories for Milk and Milk Products (Dragacci et al., 2001). The method may, however, be too expensive for routine use in developing countries. An interesting lower-cost alternative is a method that combines immunoaffinity clean-up with TLC and a computer-based, low-cost densitometer. This method is being validated for detection of low concentrations of aflatoxin M<sub>1</sub> in a formal collaborative study.

**Table 5. Performance characteristics of methods of analysis for aflatoxin M<sub>1</sub> in milk and milk products that have been tested in laboratories providing acceptable results in formal collaborative studies by international organizations**

Sample	No. of laboratories	Mean (ng/kg)	Recovery (%)	Relative SD (%)			
				Repeatability	Reproducibility		
<i>AOAC method 974.17 (Stubblefield &amp; Shannon, 1974b), in accordance with Scott (1989)</i>							
Naturally contaminated:							
Powdered skimmed milk	14	1200	—	—	73		
Powdered whole milk <sup>a</sup>	30	2800	—	—	51		
Cheese (ricotta)	12	850	—	—	48		
Butter	8	480	—	—	45		
Spiked:							
Milk with 100 ng/kg	14	140	136	—	75		
Cheddar cheese with 500 ng/kg	11	270	54	—	65		
Uncontaminated:							
Milk	14	0	—	—	—		
Blue cheese	14	450 <sup>b</sup>	—	—	—		
Sample	No. of laboratories	Mean (ng/kg)	Recovery (%)	Relative SD (%); visual densitometry			
				Repeatability	Reproducibility		
<i>AOAC method 980.21 (Stubblefield et al., 1980), in accordance with Scott (1989)</i>							
Naturally contaminated:							
Powdered milk <sup>c</sup>	32	3000	—	36	23	36	38
Powdered milk <sup>c</sup>	38	4800	—	28	19	32	29
Cheese <sup>d</sup>	43	340	—	40	33	58	47
Cheese <sup>c</sup>	37	980	—	27	30	23	40
Cheese <sup>d</sup>	53	1400	—	19	20	33	42
Spiked:							
Powdered milk with 1120 ng/kg <sup>c</sup>	36	1000	91	32	21	71	34
Butter with 1000 ng/kg	12	450	45	8.0 <sup>e</sup>	68	24 <sup>e</sup>	82
Uncontaminated:							
Powdered milk <sup>c</sup>	42	—	—	—	—	—	—
Cheese <sup>c</sup>	38	—	—	—	—	—	—
Sample <sup>f</sup>	No. of values <sup>g</sup>	Mean (ng/L)	Recovery (%)	Relative SD (%)			
				Repeatability <sup>h</sup>	Reproducibility <sup>i</sup>		
<i>AOAC method 986.16 (Stubblefield &amp; Kwolek, 1986), in accordance with Scott (1989)</i>							
Spiked fluid milk							
At 770 ng/L	20 (25)	600	78	—	—		
At 155 ng/L	20 (26)	140	92	—	—		
At 67 ng/L	18 (22)	67	86	—	—		
At 415 ng/L	18 (23)	420	88	—	—		
At 1300 ng/L	20 (26)	1300	99	—	—		
At 116 ng/L	20 (26)	120	97	—	—		

Table 5. (contd)

Sample	No. of laboratories	Mean (ng/kg)	Recovery (%)	Relative SD (%)		
				Repeatability	Reproducibility	
<i>Dairy Federation standard 171 (Tuinstra et al., 1993)</i>						
Naturally contaminated milk powder	12	81	–	9.9	23	
	14	150	–	14	23	
	13	80	–	6.8	18	
	11	200	–	4.7	11	
	14	580	–	12	19	
Sample	No. of laboratories	Mean (ng/mL)	Recovery (%)	Relative SD (%)		HORRAT
				Repeatability	Reproducibility	
<i>AOAC method 2000.08 (Dragacci et al., 2001)</i>						
Naturally contaminated milk powder	12	0.023	–	17	27	0.33
	12	0.046	–	12	23	0.31
	12	0.100	–	8	21	0.33
Spiked milk powder with 0.05 ng/mL	10	0.037	74	18	31	0.44
Uncontaminated milk powder	12	–	–	–	–	–

SD, standard deviation; Recovery, mean recovery from sample spiked with known amount of aflatoxin M<sub>1</sub>; HORRAT, ratio of relative SD for reproducibility in the trial to that predicted. A HORRAT of 1 indicates a relative SD for reproducibility corresponding exactly to the Horwitz equation (Horwitz, 1989); a HORRAT  $\leq 1.0 \pm 0.5$  indicates normal reproducibility; a HORRAT  $> 1.5$  indicates that reproducibility is higher than expected, whereas a HORRAT  $> 2.0$  indicates problematic reproducibility (AOAC International, 2000).

- <sup>a</sup> Duplicate series of samples: no significant difference in means of two sets found by *t* test.
- <sup>b</sup> Fluorescent contaminant
- <sup>c</sup> Duplicate series of samples
- <sup>d</sup> Triplicate series of samples
- <sup>e</sup> Four samples instead of 12
- <sup>f</sup> Duplicate samples
- <sup>g</sup> In parentheses, total number of values including normal-phase data used for estimates of precision
- <sup>h</sup> Individual values were reported, but it was not clear from the original publication whether they related to repeatability or reproducibility. Relative SD for repeatability for all samples combined, 28%
- <sup>i</sup> Individual values were reported, but it was not clear from the original publication whether they related to repeatability or reproducibility. Relative SD for reproducibility for all samples combined, 44%
- <sup>j</sup> Values based on reconstituted milk prepared from the milk powder

Ensuring that regulations on limits for aflatoxin M<sub>1</sub> in milk and milk products are met requires validated methods of analysis. Several organizations (AOAC International, IUPAC, the International Dairy Federation, and the European Standardization Committee [Comité Européen de Normalisation]) have tested methods of analysis for aflatoxin M<sub>1</sub> collaboratively in order to establish their performance characteristics. The practical characteristics include: cost of performance, time required, and level of training needed. The scientific characteristics include: accuracy, precision, specificity, and lower limit of detection. Although all these characteristics are important, the most important from the regulatory point of view are accuracy and interlaboratory variation (reproducibility).

Few collaboratively studied methods of analysis for aflatoxin M<sub>1</sub> have been published in the scientific literature. Nearly all are based on TLC and HPLC. The performance characteristics of the methods described above, derived from collaborative studies, are summarized in Table 5.

Criteria for acceptance of collaboratively derived performance characteristics of methods for the determination of aflatoxin M<sub>1</sub> were not formalized until 1999 in countries that had regulations with respect to aflatoxin M<sub>1</sub>. In 1999, European Union legislation for aflatoxin M<sub>1</sub> came into force. The Directive (98/53/EC; Commission of the European Union, 1998) includes a section stating specific requirements for the methods of analysis used to determine concentrations of aflatoxins in certain foodstuffs, including aflatoxin M<sub>1</sub>. These requirements are based on report 13505 (Comité Européen de Normalisation, 1999). The recommended recovery of aflatoxin M<sub>1</sub> present in milk is 60–120% at a concentration of 0.01–0.05 µg/L and 70–110% at a concentration > 0.05 µg/L. The recommended precision of the relative standard deviation for reproducibility for all concentrations of aflatoxin M<sub>1</sub> in milk is that derived from the Horwitz (1989) equation, i.e.  $RSD_R = 2 (1 - 0.5 \log C)$ , where  $RSD_R$  is the relative standard deviation calculated from results generated under reproducible conditions [ $S_R/x$ ], and  $C$  is the concentration expressed as powers of 10 (e.g. 1 mg/kg (ppm) =  $10^{-6}$ ). The maximum permitted value is twice the RSDR. The detection limits of the methods used are not stated, as the precision values are given at the concentrations of interest.

Most collaborative studies on aflatoxin M<sub>1</sub> were carried out under the auspices of AOAC International, which did not require the reporting of recovery values or the limit of detection or determination in collaborative studies until 2000. In addition, reporting of HORRAT values was not mandatory, and no criteria were established for HORRAT values until 2000 (AOAC International, 2000). Thus, except for the collaborative study of Dragacci et al. (2001), there were no criteria to verify the performance of a method for aflatoxin M<sub>1</sub> in collaborative studies.

### **3.3 Analytical quality assurance**

The availability of collaboratively studied 'official' methods of analysis for aflatoxin M<sub>1</sub>, with acceptable performance characteristics, is no guarantee of accurate results. Check sample programmes for aflatoxins, including aflatoxin M<sub>1</sub> in milk, organized by IARC (Friesen & Garren, 1982) have shown that there can be wide variation in results. In compliance with the principles of analytical quality assurance, measurements of the mycotoxin by different laboratories should be reliable and comparable.

A quality assurance programme includes, when possible, use of (certified) reference materials and many other elements. Certified reference materials are stable, homogeneous products containing certified amounts of the analyte(s) of interest. Such materials for aflatoxin M<sub>1</sub> have been developed in the past with the coordination of the European Union's Community Bureau of Reference, now known as the Standards, Measurements and Testing Programme. Several full-cream milk powders certified for their aflatoxin M<sub>1</sub> content and an aflatoxin M<sub>1</sub> calibrating solution are available worldwide from the European Union Joint Research Centre, Institute for Reference Materials and Measurements in Geel, Belgium. Their characteristics are shown in Table 6 (Boenke, 1997). The supplies of certified milk powder reference materials are nearly exhausted, and the Institute is planning to produce and certify new batches in 2001.

Another increasingly important quality assurance component is proficiency testing. The European Union's Community Reference Laboratory for Milk and Milk Products, in Paris, France, conducted proficiency tests for national reference laboratories in 1996 (Dragacci et al., 1996) and 1998 (Grosso et al., 1999). The organizers concluded that, considering the very low concentrations of aflatoxin M<sub>1</sub> in the distributed samples, the network of national reference laboratories had shown good analytical competency for the determination of aflatoxin M<sub>1</sub> in milk at the permitted level in the European Union (0.05 µg/kg). It is foreseen that proficiency tests for aflatoxin M<sub>1</sub> will be expanded to include national reference laboratories that are charged with detecting mycotoxins. Laboratories that do not belong to the network can take part in the food analysis performance assessment scheme based in the United Kingdom but with worldwide participation. The scheme has organized rounds of testing for aflatoxin M<sub>1</sub> since 1999, and samples are sent out every 3–4 months (Ministry of Agriculture, Fisheries and Food, 1999, 2000a,b). Another possibility is participation in the laboratory proficiency programme of the American Oil Chemists (2000).

### 3.4 Conclusions

Many methods of analysis have become available for the determination of aflatoxin M<sub>1</sub> in milk and milk products, both for screening and for quantitative estimates. Most were developed for the analysis of milk and milk products, but they can be used for other dairy products, with minor modifications. The limits of determination have decreased over the years, while the precision of the methods has improved, as demonstrated in formal collaborative studies of performance characteristics. With modern methods of analysis, aflatoxin M<sub>1</sub> can be determined at concentrations well below 0.05 µg/kg of milk. The combination of immunoaffinity columns and liquid chromatography offers the best means for efficient clean-up and

**Table 6. Certified reference materials for aflatoxin M<sub>1</sub>**

Number	Matrix	Certified value (µg/kg)	Uncertainty (µg/kg)
CRM 282	Full-cream milk powder	< 0.05	Not reported
CRM 283	Full-cream milk powder	0.09	+ 0.04; - 0.02
CRM 285	Full-cream milk powder	0.76	± 0.05
RM 423	Chloroform	Information value: 9.93	Not reported

precise determination of low concentrations of aflatoxin M<sub>1</sub>. Cheaper alternatives based on immunoaffinity clean-up with TLC and computer-based densitometry have been developed and are being validated.

A value of 0.05 µg/kg of milk is currently the legal limit in those countries that have the most stringent regulations for aflatoxin M<sub>1</sub>. This value was proposed by the Codex Committee on Food Additives and Contaminants in 1992 (Codex Alimentarius, 1992), but it was still at the proposal stage in 2000, as several countries had reserved their positions (Codex Alimentarius, 2000). The availability of collaboratively studied methods (which usually involve TLC and HPLC) is, however, no guarantee of accurate results. The reliability and comparability of analytical results can be significantly improved by making use of reference materials and by taking part in proficiency studies. Certified milk powder reference materials for determination of aflatoxin M<sub>1</sub> are available, and proficiency studies with worldwide participation are organized.

#### **4. SAMPLING PROTOCOLS**

Two effective methods for controlling aflatoxin M<sub>1</sub> in the food supply are to sample dairy feed for aflatoxin B<sub>1</sub> or to sample the milk directly for aflatoxin M<sub>1</sub>. The performance of sampling plans for aflatoxin in granular feed products such as shelled maize (Park et al., 2000; Johansson et al., 2000a,b,c) and cottonseed (Whitaker et al., 1976) has been evaluated, but there has been little evaluation of sampling plans to detect aflatoxin M<sub>1</sub> in milk. It might be difficult to design an effective programme to control aflatoxin in granular feed, particularly at low concentrations, because of its heterogeneous distribution in these commodities, which results in wide sampling variation.

As the distribution of aflatoxin M<sub>1</sub> in liquid milk can be expected to be reasonably homogeneous, sampling of liquid milk for aflatoxin M<sub>1</sub> will be more accurate than sampling of granular feed products. Most of the uncertainty in estimates of aflatoxin M<sub>1</sub> in milk is probably associated with the analytical procedure.

The European Union, the South American Common Market (MERCOSUR), and the USA have designed plans for sampling aflatoxin M<sub>1</sub>. A Directive of the Commission of the European Union (1998) and a Decision (Commission of the European Union, 1991) specify that a minimum of 9.5 kg (or L) should be collected from a batch of milk mixed by manual or mechanical means and should be composed of at least five increments. The batch is accepted if the concentration of aflatoxin M<sub>1</sub> does not exceed the permitted limit. In the USA, the Food & Drug Administration (1996) stipulates that samples should consist of at least 10 lbs (4.5 kg) of milk, composed of no fewer than 10 randomly selected portions.

In the absence of information on the efficacy of sampling plans for the determination of aflatoxin M<sub>1</sub> in milk, it is recommended that the European model, in which a 500-g sample composed of five 100-g portions of milk is taken from a batch, be used for the minimum sample size and sample selection method.

#### **5. EFFECTS OF PROCESSING**

As aflatoxin M<sub>1</sub> occurs frequently in milk, two questions can be raised. What happens to the aflatoxin M<sub>1</sub> when contaminated milk is processed normally in the

dairy industry? What can be done to reduce or destroy aflatoxin M<sub>1</sub> in contaminated milk and milk products? The many investigations performed to resolve these questions were reviewed in detail by Yousef & Marth (1989). The main studies and conclusions are summarized below.

### **5.1 Fate of aflatoxin M<sub>1</sub> during processing of milk**

Treatments that are common in the dairy industry can be separated into two distinct processes: those that do not involve separation of milk components, such as heat treatment, low-temperature storage, and yoghurt preparation; and processes that involve separation of milk components, such as concentration, drying, and cheese and butter production.

The stability of aflatoxin M<sub>1</sub> during heat processing, such as pasteurization (Alcroft & Carnaghan, 1962) and heating milk directly on a fire for 3–4 h (Patel et al., 1981), has been studied. Although the results of the studies are not consistent, most indicate that such heat treatments do not change the amount of aflatoxin M<sub>1</sub> in these products appreciably. Studies of the stability of aflatoxin M<sub>1</sub> in milk during cool or frozen storage gave variable results (Yousef & Marth, 1989), but storage of frozen contaminated milk and other dairy products for a few months did not appear to affect the aflatoxin M<sub>1</sub> content. The manufacture of cultured dairy products, such as kefir and yoghurt, also did not significantly decrease the aflatoxin M<sub>1</sub> content (Wiseman & Marth, 1983).

The results of several investigations have been published in which the effects of removal of water on aflatoxin M<sub>1</sub> content were studied. The processes involved both heat- (spray or roller drying) and freeze-drying. The studies were reviewed by Yousef & Marth (1989). Large losses of aflatoxin M<sub>1</sub> were reported in some studies, whereas in others concentrating milk did not affect its aflatoxin M<sub>1</sub> content substantially. The few studies that addressed partitioning of aflatoxin M<sub>1</sub> during cream and butter processing confirmed that a small proportion of aflatoxin M<sub>1</sub> is carried over to cream and a yet smaller proportion to butter. No loss of aflatoxin M<sub>1</sub> occurred as the remainder was found in skim milk and buttermilk, respectively.

The manufacture of cheese involves several processes. Aflatoxin M<sub>1</sub> does not appear to be degraded in the first phase, conversion of milk into pressed curd, as the amount in whey and curd is approximately the same as in the original milk (Yousef & Marth, 1989). Aflatoxin M<sub>1</sub> seemed rather to occur predominantly with casein, so that cheese curd contained a higher concentration than whey. The association of aflatoxin M<sub>1</sub> with casein is also manifested in a higher concentration in cheese than in the milk from which the cheese is made. Yousef & Marth (1989) expressed the concentration of aflatoxin M<sub>1</sub> in milk divided by the concentration of aflatoxin M<sub>1</sub> in cheese as the enrichment factor. On the basis of several studies, these researchers concluded that the enrichment factor is 2.5–3.3 in many soft cheeses and 3.9–5.8 in hard cheeses. During the second phase of cheese manufacture, ripening, some discrepancies were found in the stability of aflatoxin M<sub>1</sub>, but, in general, it did not appear to be degraded during ripening.

### **5.2 Degradation of aflatoxin M<sub>1</sub> in milk**

The observation that the processes described above do not generally lead to loss of aflatoxin M<sub>1</sub> is of considerable practical importance. Several possibilities for

eliminating or inactivating aflatoxin M<sub>1</sub> in milk, involving chemical and physical treatment, have been investigated. The chemicals that have been studied for their ability to degrade aflatoxin M<sub>1</sub> are limited to those that are permitted as food additives: sulfites, bisulfites, and hydrogen peroxide (Applebaum & Marth, 1982a,b). When raw milk naturally contaminated with aflatoxin M<sub>1</sub> was treated with 0.4% potassium bisulfite at 25 °C for 5 h, the concentration decreased by 45%. A higher concentration of bisulfite was less effective. Aflatoxin M<sub>1</sub> in naturally contaminated milk was not affected by the presence of 1% hydrogen peroxide at 30 °C for 30 min, but addition of hydrogen peroxide at a concentration of 0.05–0.1% with lactoperoxidase reduced the amount by 50%.

Physical processes that have been explored to remove aflatoxin M<sub>1</sub> from milk include adsorption and radiation. Five per cent bentonite in milk adsorbed 89% of aflatoxin M<sub>1</sub> (Applebaum & Marth, 1982a). In a study of the effects of ultra-violet radiation with and without hydrogen peroxide, the concentration of aflatoxin M<sub>1</sub> was reduced by 3.6–100%, depending on the length of time the milk was exposed to radiation, the volume of treated milk, the presence of hydrogen peroxide, and other aspects of the experimental design (Yousef & Marth, 1985).

The chemical and physical treatments described are not readily applicable in the dairy industry, at least at present, as little is known about the biological safety, or the nutritional value of the treated products. Moreover, the costs of the processes may be considerable and prohibitive for large-scale application. If aflatoxin M<sub>1</sub> cannot be destroyed or removed readily, it can be excluded from milk only by eliminating aflatoxin B<sub>1</sub> from the diet of animals.

## **6. LEVELS AND PATTERNS OF CONTAMINATION OF FOOD COMMODITIES**

### **6.1 Results of surveys**

Data on contamination of milk and various milk products with aflatoxin M<sub>1</sub> were submitted to FAO by Argentina, Canada, the European Union, Norway, the Dubai Municipality of the United Arab Emirates, and the USA. Although not formally submitted, unpublished data were also available from an Indonesian research institute. Other data were taken from literature published between 1985 and early 2000 and from a review by Galvano et al. (1996a). In addition to published data, information was presented by the Philippines, the Republic of Korea, and Thailand. Data on natural occurrence before the mid-1980s and mid-1990s were surveyed by van Egmond (1989) and Galvano et al. (1996a), respectively. Although the available data are incomplete, they are presented in Appendix A.

None of the samples examined in Argentina in 1999 (Centro de Referencia de Micología, 1999) and Uruguay in 1993–95 (Pineiro et al., 1996) contained aflatoxins at concentrations > 0.05 µg/kg, and the maximum concentration was 0.03 µg/kg. Data for Brazil were taken from Martins & Martins (1986), de Sylos et al. (1996), Correa et al. (1997), Oliveira et al. (1997), Souza et al. (1999), and Prado et al. (1999, 2000). None of the milk samples was contaminated with aflatoxin M<sub>1</sub> at > 0.5 µg/kg. Four of 52 milk samples in 1992 contained > 0.05 µg/kg, with a maximum of 0.37 µg/kg (de Sylos et al., 1996), and 35 of 54 samples taken in 1992–93

contained 0.006–0.077 µg/kg (Prado et al., 1999, 2000). Of the samples of milk powder, 2% (6/300) of those taken in 1992–93 contained > 0.5 µg/kg, with a maximum of 1.0 µg/kg (Oliveira et al., 1997).

The data submitted by Health Canada for 1994, although limited, indicated that none of the processed milk samples contained > 0.5 µg/kg, and the mean was < 0.063 µg/kg. Data for 1997–98 (not shown in Appendix A), in which the number of samples was not stated, showed no aflatoxin M<sub>1</sub> in any of six types of milk at the limit of detection (LOD; 0.015 µg/kg).

Extensive data collected between 1995 and early 2000 were submitted by the Center for Food Safety and Applied Nutrition (Food & Drug Administration, 2000) in Washington DC, USA. This submission contained some industry-generated data, for 1998–2000 from southwestern USA ( $n = 5801$ ), the midwest ( $n = 438$ ), and southeastern USA ( $n = 13\ 093$ ), and data from three Food & Drug Administration (FDA) regional laboratories: laboratory A ( $n = 277$ ), laboratory B ( $n = 380$ ), and laboratory 27 ( $n = 4225$ ). No data were provided from northern USA because aflatoxin M<sub>1</sub> is not a problem under the climatic conditions that prevail.

In the surveys by FDA laboratory 27 for 1995–2000, 185 (4.6%) of 4000 raw milk samples were contaminated with aflatoxin M<sub>1</sub> at a concentration > 0.05 µg/kg and five (0.13%) at > 0.5 µg/kg, only in 1996. Of the finished milk samples during the same period, 10 (4.4%) of 225 samples contained 0.05–< 0.5 µg/kg. In midwestern and southwestern USA after 1998, 10 (2.3%) of 438 and 14 (0.11%) of 13 093 samples, respectively, contained > 0.5 µg/kg. The incidence of aflatoxin M<sub>1</sub> contamination was higher in southwestern (industry data) and southern states (FDA laboratory B) than in other areas. In 1998–2000, milk samples contaminated with > 0.05 and > 0.5 µg/kg were found in 21% (1239/5801) and 0.78% (45/5801) of southwestern states and in 40% (153/380) and 18% (68/380) of southern states, respectively. The numbers of samples for southwestern and southern states were provided within two distributions (0.00–0.5 µg/kg and > 0.5 µg/kg), with no information on mean or maximum values.

In Asia, high incidences and levels of aflatoxin M<sub>1</sub> contamination were found in Indonesia, the Philippines, and Thailand. Unpublished data for Indonesia in 1990–93 and 1999 were obtained from the Research Institute for Veterinary Science (2000) in Bogor. Of 342 milk samples, 199 samples (58%) contained aflatoxin M<sub>1</sub> (limit of quantification [LOQ], 0.1 µg/kg), and 73 (21%) contained > 0.5 µg/kg, with mean values of 0.31–5.4 µg/kg and maximum values of 2.0–23 µg/kg.

Data for Thailand in 1990–93 and 1995–96 were obtained from the Department of Medical Sciences, Ministry of Public Health (Boriboon & Suprasert, 1994) and the literature (Saitanu, 1997). Of 310 liquid milk samples, more than 261 (> 84%) were contaminated with aflatoxin M<sub>1</sub> at concentrations > 0.05 µg/kg, and 58 samples (19%) contained > 0.5 µg/kg, with a maximum of 6.6 µg/kg. In the Philippines, data from the Bureau of Animal Industry, Department of Agriculture, for 1997 (Begno, 1998) indicated that 88% and 18% of 91 milk samples were contaminated with aflatoxin M<sub>1</sub> at > 0.05 µg/kg and > 0.5 µg/kg, respectively.

The data for the Republic of Korea in 1995 and 1997 were taken from the literature (Shon et al., 1996; Kim et al., 2000). Of 134 liquid milk samples, 50 (37%) contained aflatoxin M<sub>1</sub> at a concentration > 0.05 µg/kg, with a maximum of 0.28 µg/kg. In the United Arab Emirates, data for 1998–99 from the Dubai Central Laboratory, Dubai

Municipality, showed that 33 (56%) of 59 liquid milk samples (including 15 imported products) were contaminated with > 0.05 µg/kg, with a maximum of 0.31 µg/kg.

The report from the Commission of the European Union (1999) provided data on aflatoxin M<sub>1</sub> concentrations in milk samples (total, 7573) in 1999 from 10 Member States: Austria (20 samples), Belgium (192), Finland (296), France (234), Germany (6537), Ireland (62), the Netherlands (30), Portugal (96), Sweden (11) and the United Kingdom (95). Of these, 7259 samples (96%) contained aflatoxin M<sub>1</sub> at concentrations below the LOQ or LOD of the method (0.001–0.03 µg/kg); 314 samples (4.2%) contained up to 0.05 µg/kg, and none of samples contained > 0.05 µg/kg.

The Commission of the European Union (1989–95) also provided the SCOOP report, which gives the concentrations of aflatoxin M<sub>1</sub> in milk analysed between 1984 and 1995 in eight Member States (Austria, Belgium, Denmark, Finland, France, Italy, Spain, and the United Kingdom). Of the 8791 milk samples (including crude and heat-treated milk), 34% contained concentrations below the LOQ (0.01 µg/kg)/LOD (0.001–0.01 µg/kg), 44% had concentrations up to 0.05 µg/kg, 0.33% (23 French and six British samples) had 0.051–0.1 µg/kg, and three samples from the United Kingdom had > 0.1 µg/kg, with a maximum of 0.22 µg/kg.

Additional information on a total of 18 945 samples taken in 1994–99 and 2000 was provided by the Commission of the European Union. Of 1048 samples taken in Finland in 1995–98, only one contained < 0.05 µg/kg and the others contained < 0.005 µg/kg. All 251 samples taken in France in 1998 contained < 0.05 µg/kg, and four had > 0.03 µg/kg). In 17 181 samples taken in Germany in 1996–98 and 2000, the maximum concentration was 0.033 µg/kg. All 168 samples of reconstituted milk taken in the Netherlands in 1998 contained < 0.01 µg/kg. The maximum concentration in 42 samples taken in Spain in 1998 was 0.027 µg/kg, and all 255 samples taken in the United Kingdom in 1994–95 had < 0.05 µg/kg.

Data submitted by the Norwegian Food Control Authority showed that 51 (94%) of 54 samples contained aflatoxin M<sub>1</sub> at a mean value of 0.0014 µg/kg and a maximum of 0.009 µg/kg. Data for Cyprus were derived from the literature (Ioannou-Kakouri et al., 1999). Of 112 samples, including 10 imported products, 11 were contaminated with aflatoxin M<sub>1</sub> at a level of 0.01–0.04 µg/kg.

## **6.2 Distribution curves**

Appendix B shows the distribution of aflatoxin M<sub>1</sub> contamination in various types of milk and milk products. Table 7 gives data calculated from the surveys of FDA laboratory B (1998–2000, 380 milk samples from southern USA), FDA laboratory 27 (1995–2000, 4225 raw and finished milk samples), and the Ministry of Public Health, Thailand (1990–93, 60 raw and pasteurized milk samples; Boriboon & Suprasert, 1994).

## **6.3 Annual variation**

Limited information was available on annual variations in the concentration of aflatoxin M<sub>1</sub> in milk. The most recent data are derived from the survey submitted by FDA Laboratory 27 for 1995–2000 (Table 8). The incidence of contamination with aflatoxin M<sub>1</sub> at a concentration > 0.05 µg/kg varied from 1.9% to 3.4% in raw milk and from 0.0% to 3.8% in finished milk in a 6-year survey, except in 1996, when these concentrations were found in 25% of raw milk samples and 19% of finished

**Table 7. Distribution of aflatoxin M<sub>1</sub> in milk in samples from Thailand and the USA**

Concentration of aflatoxin M <sub>1</sub> (µg/kg)	Southern USA (Laboratory B)		USA (Laboratory 27)		Thailand	
	No. of samples	% positive	No. of samples	% positive	No. of samples	% positive
≤ 0.05	20	12	1	0.5		
0.05–< 0.10	18	10	144	74	0	0.0
0.10 – < 0.20	32	18	29	15	2	9.5
0.20–< 0.30	18	10	8	4.1	3	14
0.30–< 0.40	14	8.0	5	2.6	1	4.8
0.40–< 0.50	15	8.6	3	1.5	4	19
0.50–< 1.00	40	23	2	1.0	9	43
1.00–< 2.00	11	6.3	3	1.5	1	4.8
2.00–< 3.00	5	2.9			0	0.0
3.00–< 4.00	0	0.0			0	0.0
4.00–< 5.00	4	2.3			0	0.0
5.00–< 6.00	1	0.6			0	0.0
6.00–< 7.00	2	1.1			1	4.8
7.00–< 8.00	0	0.0				
8.00–< 9.00	1	0.6				
9.00–< 10.00	1	0.6				
≥ 10.00	2	1.1				
Total no. positive	174		195		21	
Total no. analysed	380		4225		60	

milk samples. A concentration > 0.5 µg/kg was found in 1.3% of raw milk samples only in 1996.

In data for Germany in 1997–2000 submitted by the Commission of the European Union, 0.11–0.27% of samples were contaminated at > 0.005 µg/kg and 0.06–0.14% at > 0.01 µg/kg in 1997–98 and 2000, and 4.3% at 0.005–0.05 µg/kg and 0.45% at 0.01–0.05 µg/kg in 1999 (Appendix A).

Extensive data on annual variation is contained in the report of a 15-year survey (1978–92) of aflatoxin M<sub>1</sub> in milk and bulk raw milk in France (Table 9; Dragacci & Frémy, 1993). For 1978, 6246 milk samples were classified as containing < 0.05 µg/kg, 0.05–0.5 µg/kg, and > 0.5 µg/kg. According to this classification, two periods of contamination occurred in France, 1978–84 and the winters of 1988–91; highly contaminated samples, some containing up to 5 µg/kg, were found during the winters of 1978–83. The seasonal trend in milk contamination was attributed to the fact that cows receive less concentrated feed in summer, when they are grazing. Few contaminated samples were found between 1984 and 1988 and after 1991.

## 7. FOOD CONSUMPTION AND DIETARY INTAKE ESTIMATES

Dietary intake of aflatoxin M<sub>1</sub> was estimated from data on the concentrations of aflatoxin M<sub>1</sub> in milk submitted to FAO/WHO, from selected reports in the literature, and from data on milk consumption in the GEMS/Food Regional Diets (WHO, 1998).

**Table 8. Annual variations in aflatoxin M<sub>1</sub> contamination of milk in the USA, 1995–2000**

Year	Raw milk					Finished milk				
	No. of samples	> 0.05 µg/kg		> 0.5 µg/kg		No. of samples	> 0.05 µg/kg		> 0.5 µg/kg	
		No.	(%)	No.	(%)		No.	(%)	No.	(%)
1995	695	13	1.9	0	0	79	3	3.8	0	0
1996	381	94	25	5	1.3	21	4	19	0	0
1997	717	20	2.8	0	0	53	1	1.9	0	0
1998	855	16	1.9	0	0					
1999	877	26	3.0	0	0	60	2	3.3	0	0
2000	475	16	3.4	0	0	12	0	0	0	0

From US Food and Drug Administration Laboratory 27

**Table 9. Annual variation in aflatoxin M<sub>1</sub> contamination of milk in a 15-year survey (1978–92) in France**

Year: month	Milk					Bulk raw milk				
	No. of samples	> 0.05 µg/kg		> 0.5 µg/kg		No. of samples	> 0.05 µg/kg		> 0.5 µg/kg	
		No.	(%)	No.	(%)		No.	(%)	No.	(%)
1978:11–1979:5	49	29	59	1	2.0					
1979:6–10	37	6	16	0	0					
1979:11–1980:5	44	20	45	3	6.8	10	10	100	4	40
1980:6–10	58	12	21	0	0	26	23	89	5	19
1980:11–1981:5	104	62	60	1	0.96	67	50	75	5	7.5
1981:6–10	114	4	3.5	0	0	42	5	12	0	0
1981:11–1982:5	310	34	11	0	0	57	0	0	0	0
1982:6–10						33	1	3.0	0	0
1982:11–1983:5	238	10	4.2	0	0	54	1	1.8	0	0
1983:6–10	209	1	0.48	0	0	41	0	0	0	0
1983:11–1984:5	447	0	0	0	0	38	2	5.3	0	0
1984:6–10						18	0	0	0	0
1984:11–1985:5	494	7	1.4	0	0	37	5	14	0	0
1985:6–10						30	0	0	0	0
1985:11–1986:5	466	0	0	0	0	42	1	2.4	0	0
1986:6–10	265 <sup>a</sup>	0	0	0	0	26	0	0	0	0
1986:11–1987:5						24	0	0	0	0
1987:6–10	449 <sup>b</sup>	2	0.45	0	0	18	0	0	0	0
1987:11–1988:5	277 <sup>c</sup>	0	0	0	0	29	0	0	0	0
1988:6–10						27	0	0	0	0
1988:11–1989:5	549 <sup>d</sup>	2	0.36	0	0	8	0	0	0	0
1989:6–10						9	0	0	0	0
1989:11–1990:5	526 <sup>d</sup>	13	2.5	0	0	18	0	0	0	0
1990:6–10						19	0	0	0	0
1990:11–1991:5	550 <sup>d</sup>	3	0.55	0	0	35	0	0	0	0
1991:6–10	303 <sup>e</sup>	0	0	0	0	15	0	0	0	0
1991:11–1992:5						34	0	0	0	0

From Dragacci & Fremy (1993)

<sup>a</sup> 1986:9–11; <sup>b</sup> 1987:1–3, 9–11; <sup>c</sup> 1988:1–3; <sup>d</sup> 11–3; <sup>e</sup> 1991:9–11

**Table 10. Concentrations of aflatoxin M<sub>1</sub> in milk in Canada**

Years	Type of sample	No. of samples	Concentration (µg/kg)	
			Mean	Range
1993–94	Whole milk	15	< 0.069	< 0.015–0.21
	2% fat milk	7	< 0.043	< 0.015–0.16
	1% fat milk	1	< 0.015	
	Non-fat milk powder	2	< 0.037	< 0.015–0.059
	2% fat lactose-reduced milk	3	< 0.047	< 0.015–0.090
	Non-fat lactose-reduced milk	1	< 0.015	
	Evaporated milk	1	0.060	
1994–95	Whole milk	23	< 0.013	< 0.01–< 0.015
	2% fat milk	24	< 0.012	< 0.01–< 0.015
	Non-fat milk	8	< 0.013	< 0.01–< 0.015
	Whole-milk powder	1	< 0.1	
	Non-fat milk powder	6	< 0.032	< 0.01–< 0.015
	Low-fat flavoured milk	1	< 0.015	
	2% fat lactose-reduced milk	1	< 0.015	
1995–96	Whole milk	43	< 0.012	< 0.01–< 0.015
	2% fat milk	37	< 0.013	< 0.01–< 0.015
	1% fat milk	5	< 0.012	< 0.01–< 0.015
	Non-fat milk	5	< 0.01	
	Whole-milk powder	2	< 0.1	
	Non-fat milk powder	9	< 0.041	
	2% fat lactose-reduced milk	1	< 0.015	
	Whole goat milk	1	< 0.015	
1997–98	Whole milk	23	< 0.015	
	2% fat milk	35	< 0.015	
	1% fat milk	3	< 0.015	
	Non-fat milk	15	< 0.015	
	Whole-milk powder	1	< 0.015	
	Non-fat milk powder	16	< 0.015	
	Whole goat milk	2	< 0.015	
	Non-fat lactose-reduced milk	1	< 0.015	
	Chocolate milk	2	< 0.015	

### 7.1 National and regional assessments of intake

Eight data sets providing information on aflatoxin M<sub>1</sub> concentrations in milk were submitted for consideration by the Committee. Six of these were in the submissions from Argentina, Canada, the European Union, Norway, the United Arab Emirates, and the USA, and these provided the results of analyses for aflatoxin M<sub>1</sub> in milk from government or university institutions. Experimental data were available from an Indonesian research institute. The other two submissions were summaries of published papers from laboratories in Brazil and Uruguay. The eighth data set was in the form of summaries of published papers on the concentrations of aflatoxin M<sub>1</sub> in milk.

The data are discussed below and summarized in Appendix B, with separate entries for aflatoxin M<sub>1</sub> in different samples of milk. The data in Appendix B for which

the number of samples and the mean value were available are summarized in Appendix C by GEMS/Food regional diet.

### 7.1.1 Canada

Data for 1993–94, 1994–95, 1995–96, and 1997–98 were submitted by Health Canada (2000). The detection limit was 0.015 µg/kg, and the analytical method included immunoaffinity clean-up columns followed by liquid chromatography coupled with fluorimetric detection. In data for 1997–98, the mean concentrations and ranges of aflatoxin M<sub>1</sub> in nine types of milk were all < 0.015 µg/kg (Table 10).

### 7.1.2 Argentina

Data on the aflatoxin M<sub>1</sub> content of milk in Argentina were submitted by the Universidad Nacional de Lujan (2000). Concentrations of 0.0, 0.4, 0.6, 0.6, 0.6, and 0.9 µg/L (roughly equal to µg/kg) were found in six samples of fluid milk from Buenos Aires that were analysed in 1998. In response to a request about the analytical method and its LOQ/LOD, the method was identified as 'interchemistry', which is similar to the AOAC method but with a different clean-up procedure, allowing for an LOQ of 0.026 µg/L. It was also indicated that the milk had come from cows with problematic milk production which had been given a feed not typically used in Argentina (< 12% of milk production obtained from cows given this type of feed). Hence, the aflatoxin M<sub>1</sub> concentrations in the six milk samples may not be typical of those in milk in this country.

### 7.1.3 USA

Data on aflatoxin M<sub>1</sub> in milk in the USA were submitted by the FDA (2000), including some data provided by industry for 1998–2000 from the southwest ( $n = 5801$ ), midwest ( $n = 438$ ), and southeast ( $n = 13\,093$ ). The data from the southeast were provided as the number of samples within two distributions (0.00–0.5 ng/kg and > 0.5 ng/kg), with no information on mean or maximum values. The submission also included data from three FDA regional laboratories: Laboratory A provided data from southeastern states for 1999–2000 ( $n = 199$ ) and for 1997–2000 ( $n = 78$ ) within distributions of 0.00–0.5 ng/kg and > 0.5 ng/kg with no mean or maximum values. Laboratory B provided data from southern states for 1998 ( $n = 163$ ), 1999 ( $n = 167$ ), and 2000 ( $n = 49$ ). Laboratory 27 provided two sets of data for 1995–2000, one set for raw milk ( $n = 3942$ ) and one for finished milk ( $n = 273$ ). As the data from laboratories B and 27 were provided as individual numbers, the mean values could be calculated, and values < 0.05 µg/kg and < 0.5 µg/kg, maximum values, and values at the 90th percentile of consumption were determined.

Most of the values (72–100%) were 0–0.5 µg/kg (see Appendix B). The values for the southern states were higher than those for other regions.

### 7.1.4 Norway

Data were submitted by the Norwegian Food Control Authority, with clarification of the mean values for all samples and for positive samples, the analytical method, and individual values for all samples. Aflatoxin M<sub>1</sub> was determined in 54 samples of milk from 49 dairies in four areas of Norway in 1998. The LOD of the analytical

method was 0.0001 µg/kg. Aflatoxin M<sub>1</sub> was found in 51 (94%) of the samples, giving a mean value of 0.0014 µg/kg for all samples, a mean of 0.0014 µg/kg for positive samples, and a median value of 0.0009 µg/kg. The highest value was 0.009 µg/kg.

### 7.1.5 United Arab Emirates

Data on the concentrations of aflatoxin M<sub>1</sub> in eight types of milk product in 1998 and 1999 were submitted by the Dubai Municipality, United Arab Emirates (Dubai Central Laboratory, 2000). It was assumed that the milk described as from 'local dairies' was either raw or whole. The data are summarized in Table 11.

Although the original report indicated that the LOD was 0.005 ng/kg and the LOQ was 0.01 ng/kg, the Committee assumed that this was an error and that the values should be in µg/kg. '< LOQ' in Table 11 refers to the total number of samples less the number of samples designated as 'positive' in the report, as it was assumed that 'positive' values were those above the LOQ. These data are presented in Appendix B, but are not included in Appendix C because an overall mean for the samples was not provided and it could not be calculated because individual values were lacking.

### 7.1.6 European Union

The Commission of the European Union (1999) provided a summary of the concentrations of aflatoxin M<sub>1</sub> detected in 7573 milk samples in 1999 from 10 Member States: Austria, Belgium, Finland, France, Germany, Ireland, the Netherlands, Portugal, Sweden and the United Kingdom. Concentrations of aflatoxin M<sub>1</sub> below the LOD/LOQ (which differed among the countries) were found in 7259 (96%) of the samples. The concentrations in all 314 samples in which aflatoxin M<sub>1</sub> was detected (4.2%) were ≤ 0.05 µg/kg. Mean values were not reported.

The Commission of the European Union (1989–95) also provided a Scientific Cooperation (SCOOP) report, containing data for milk analysed between 1989 and 1995 in nine Member States. Of the 8791 samples, 3338 (38%) contained concentrations of aflatoxin M<sub>1</sub> below the LOQ/LOD; 1017 samples (12%) contained

**Table 11. Concentrations of aflatoxin M<sub>1</sub> in milk and milk products in Dubai Municipality, United Arab Emirates**

Year	Type of sample	No. of samples	< LOQ <sup>a</sup>		Maximum (µg/kg)	Positive	
			No.	%		No.	%
1998	Whole, raw milk	22	1	4.5	0.31	21	95
1999	Whole, raw milk	22	14	64	0.35	8	36
1998	UHT milk	11	11	100	0	0	0
1999	Imported milk	15	12	80	0.08	3	20
1999	Low-fat milk	11	2	18	0.24	9	82
1999	Non-fat milk	9	2	22	0.14	7	78
Total		90	42	65	0.35	48	53

<sup>a</sup> LOD, 0.005 µg/kg; LOQ, 0.01 µg/kg

concentrations < 0.05 µg/kg; six samples (0.07%) contained 0.05–0.1 µg/kg; and three samples (0.03%) contained > 0.1 µg/kg. The nine samples containing ≥ 0.05 µg/kg were from the United Kingdom.

### **7.1.7 Indonesia**

Appendix A gives unpublished data from the Research Institute for Veterinary Science in Bogor, Indonesia, on concentrations of aflatoxin M<sub>1</sub> in milk. The data include the mean and maximum concentrations of aflatoxin M<sub>1</sub> in 342 samples of milk collected during 1990–93 and 1999. The LOQ of the analytical method was 0.1 µg/kg. The concentration was below the LOQ in 143 samples, > 0.05 µg/kg in 199, and > 0.5 µg/kg in 73.

### **7.1.8 Summary of published data from Brazil and Uruguay**

A submission from Brazil (Ministerio da Saude, 2000) provided a summary of data on the concentrations of aflatoxin M<sub>1</sub> in samples of domestic cow milk from five references (Sabino et al., 1989; de Sylos et al., 1996; Souza et al., 1999; Prado et al., 1999, 2000). A second submission from Brazil (Rodriguez-Amaya, 2000) was a paper prepared for the Tenth International IUPAC Symposium on Mycotoxins and Phycotoxins, which summarized data on concentrations of aflatoxin M<sub>1</sub> in milk and milk products in Brazil (de Sylos et al., 1996; Correa et al., 1997; Oliveira et al., 1997) and Uruguay (Pineiro et al., 1996). The information from the eight papers (converted to µg/kg) is summarized in Table 12.

### **7.1.9 Additional data from the literature**

Additional information about concentrations of aflatoxin M<sub>1</sub> in milk was sought in the literature for guidance on appropriate levels for estimating daily intake and for information for geographic areas not covered by the submitted data. In particular, there were no submissions from Africa and few from the Far East or Middle East. Pertinent data (converted to µg/kg, assuming that 1 L of milk weighs 1.02 kg) are shown in Table 13. Other data were evaluated but not included in Table 13, for several reasons. Data reported by Lemieszek-Chodorowska (1974) for Poland and by Brewington & Weihrauch (1970) for the USA were considered to be too old; Fukal & Brezina (1991) did not provide mean values for their data from the former Czechoslovakia; the data from Cyprus (Ioannou-Kakouri et al., 1999) were of doubtful quality because of a low per cent recovery; Smith et al. (1994) in the USA reported data for only nine samples of goat milk, which is not commonly consumed in the USA; and the data of Galvano et al. (1998) for Italy probably duplicated data submitted by the Commission of the European Union. Other reports that did not contain pertinent data were those of Masri et al. (1969b), Grant & Carlson (1971), Purchase et al. (1972), Jung & Hanssen (1974), Stoloff et al. (1975), Kiermeier & Mashaley (1977), Nikov et al. (1991), el-Nezami et al. (1995), Saad et al. (1995), and Navas et al. (2000).

Additional data from the literature, summarized in Appendix A, were considered. There is already considerable overlap in the data in Appendices A and B, and there may be some duplication, because the Commission of the European Union provided summaries of data from individual Member States without identifying the sources of the data within each country. The criteria for selecting data from Appendix A for

**Table 12. Concentrations of aflatoxin M<sub>1</sub> in milk from Brazil and Uruguay in published reports**

Food	No. of samples	Positive samples		Concentration (µg/kg)		
		No.	%	All samples (mean)	Positive samples	
					Mean	Range
<i>de Sylos et al. (1996), Campinas, Brazil</i>						
Milk, pasteurized, 1989	51	0	0	0		
Milk, whole dry, 1989	20	0	0	0		
Milk, non-fat dry, 1989	15	0	0	0		
Yoghurt, 1990	30	0	0	0		
Milk, pasteurized, 1992	52	4	8	0.012	0.16	0.073–0.37
<i>Oliveira et al. (1997), Brazil</i>						
Milk, whole, from powder	300	33	11	0.030	0.27	0.10–1.0
Distribution:	267			89	0.0	
		17	6			0.10–0.20
		10	3			0.20–0.30
		6	2		> 0.50	
<i>Sabino et al. (1989), Brazil</i>						
Milk, market, 1979–81	100	1	1	0.2	0.2	
Milk, farm	50	9	18	0.086	0.48	0.10–1.7
<i>Souza et al. (1999), Brazil</i>						
Milk	110	27	24			
		5			0.05	0.038–0.071
<i>Prado et al. (1999), Brazil</i>						
Milk, all samples	61	50	82	0.020	0.025	0.006–0.077
Pasteurized	18	15	83	0.027	0.032	0.008–0.077
Long-life	21	16	76	0.018	0.023	0.006–0.070
Long-life, infant	11	9	82	0.025	0.030	0.007–0.050
Powder	6	6	100	0.012	0.012	0.006–0.038
Powder, infant	5	4	80	0.010	0.013	0.007–0.021
<i>Correa et al. (1997), Brazil</i>						
Milk, raw	144	0	0	0.0		
<i>Pineiro et al. (1996), Uruguay</i>						
Milk	22	7	32			< 0.2–> 0.5
Distribution:		15	68		< 0.2	
		6	27			0.2–0.5
		1	5		> 0.5	

addition to Appendix B were that the data were not already in Appendix B; they were relatively recent (1990 or later); they related to fluid milk or yoghurt, rather than dry milk or other dairy products; and the number of samples analysed and the mean concentration of aflatoxin M<sub>1</sub> in the samples was given. The data from Appendix A added to Appendix B were those from Africa (El-Gohary, 1996), France (Dragacci &

**Table 13. Concentrations of aflatoxin M<sub>1</sub> in milk, three countries (published reports)**

Food	No. of samples	Positive samples		Concentration (µg/kg)		
		No.	%	All samples (mean)	Positive samples	
					Mean	Range
<i>Karaioannoglou et al. (1989), Greece</i>						
Milk, raw	99	4	4.0	0.005	0.12	0.10–0.13
Milk, pasteurized	51	0	0.0	0.0		
<i>Markaki &amp; Melissari (1997), Athens, Greece</i>						
Milk, pasteurized	81	72	89	0.008	0.009	0.0005–0.18
Distribution:		9	11		0.0	
		31	38			0.0005–0.001
		32	40			0.0025–0.005
		9	11			0.010–0.18
<i>Rajan et al. (1995), India</i>						
Milk	504	89	18	0.20	1.2	0.10–3.5
<i>Kim et al. (2000), Seoul, Republic of Korea</i>						
Milk, pasteurized	70	53	76	0.014	0.018	0.002–0.037
Yoghurt	60	50	83	0.023	0.029	0.017–0.12
Infant formula	26	22	85	0.039	0.046	0.003–0.093
Milk, powdered	24	18	75	0.15	0.200	0.026–0.33

Frémy, 1993), the Philippines (Begino, 1998), Poland (Domagala et al., 1997), the Republic of Korea (Shon et al., 1996), Spain (Macho et al., 1992; Jalon et al., 1994), and Thailand (Boriboon & Suprasert, 1994; Saitanu, 1997). The data from France (Dragacci & Frémy, 1993) and Spain (Macho et al., 1992; Jalon et al., 1994) were assumed to be included in the summary from the Commission of the European Union and are therefore included in Appendix B but are not summarized in Appendix C, to avoid duplication.

#### **7.1.10 Summary of data on aflatoxin M<sub>1</sub> in milk**

The data in Appendix B are summarized in Appendix C for the five regional diets by aggregating milk types and/or periods from the same source or reference. The European regional diets are derived from data submitted from Canada, the Commission of the European Union and nine of its Member States, Norway, Poland, and the USA. Latin American diets are derived from data from Argentina, Brazil, and Uruguay. Far Eastern diets are represented by data from India, Indonesia, the Philippines, the Republic of Korea, and Thailand. Middle Eastern diets are represented by data from Greece and the United Arab Emirates, and African diets are represented by data from Egypt.

For the European regional diet, the mean value of aflatoxin M<sub>1</sub> in milk ranged from about 0.004 to 0.40 µg/kg on the basis of data from the Commission of the European Union, Norway, and the USA. The calculated weighted mean was 0.023 µg/kg.

For the Latin American regional diet, the mean value ranged from 0 to 0.52 µg/kg. The sources were six published papers (Sabino et al., 1989; de Sylos et al., 1996; Correa et al., 1997; Oliveira et al., 1997; Prado et al., 1999; Souza et al., 1999) and a submission of laboratory data from Argentina. The weighted mean was calculated to be 0.022 µg/kg.

For the Far Eastern regional diet, the sources were five published papers (Boriboon & Suprasert, 1994; Rajan et al., 1995; Shon et al., 1996; Begino, 1998; Kim et al., 2000) and a submission from a laboratory in Indonesia. The weighted mean was calculated to be 0.36 µg/kg. The values from Indonesia were higher than those from other countries in both the Far Eastern region and other regions.

For the Middle Eastern regional diet, the sources were two published papers (Karaioannoglou et al., 1989; Markaki & Melissari, 1997). The weighted mean was 0.005 µg/kg.

Only one source, a published paper from Egypt (El-Gohary, 1996), was available for the African regional diet. This source provided an average value for aflatoxin M<sub>1</sub> in powdered milk of 0.015 µg/kg, which would be equivalent to 0.0018 µg/kg in fluid milk, as 30 g of dried milk yields 244 g of fluid milk (the ratio of dried to fluid milk is 1:8.2, and  $0.015/8.2 = 0.0018$  µg/kg).

In the European, Latin American, Middle Eastern, and African diets, the weighted mean value for aflatoxin M<sub>1</sub> in milk was below the proposed maximum levels of 0.05 µg/kg and 0.5 µg/kg. In the Far Eastern regional diet, the weighted mean value for aflatoxin M<sub>1</sub> in milk (0.36 µg/kg) was greater than the proposed maximum level of 0.05 µg/kg but below 0.5 µg/kg.

## 7.2 Estimates of intake of aflatoxin M<sub>1</sub> from milk

### 7.2.1 Milk consumption

The GEMS/Food regional diets (WHO, 1998) provide the dietary intakes of food commodities in five geographical areas. Table 14 shows the intake of all milk and milk products for the five regions. The major food class in which aflatoxin M<sub>1</sub> was identified was milk, the term 'milk' being assumed to include the mammalian milks (buffalo, camel, cow, goat, and sheep) listed in the GEMS/Food regional diets and not cheese, butter, or other dairy products derived from milk. Because the GEMS/Food database does not include separate data on the consumption of fermented milks, it was assumed that consumption of yoghurt and other fermented milks was subsumed within the figures for milk.

**Table 14. Regional consumption of milk and milk products (g/person per day)**

Regional diet	Milk and milk products	All milk	Cows' milk
Far Eastern	33	32	23
African	42	42	36
Middle Eastern	130	120	80
Latin American	170	160	160
European	340	290	290

The figures for 'all milks' were used to estimate dietary intake, for two reasons. First, the group 'milk and milk products' includes butter, cheese, cream, and ghee, which were not specified to be of interest for this assignment. Second, aflatoxin M<sub>1</sub> occurs in milks other than cows' milk, and use of data only on consumption of cow milk might result in an underestimate of intake of aflatoxin M<sub>1</sub>. 'All milks' include milk (and presumably fermented milk) from buffalo, camels, cows, goats, and sheep. For all five geographical areas, cow milk was the major type of milk consumed.

### 7.2.2 Intake of aflatoxin M<sub>1</sub>

On the basis of the weighted mean concentrations of aflatoxin M<sub>1</sub> in milk in the five regions, the intake was 0.1 ng/day in the African diet, 0.6 ng/day in the Middle Eastern diet, 3.5 ng/day in the Latin American diet, 6.8 ng/day in the European diet, and 12 ng/day in the Far Eastern diet (Table 15). When these intakes are expressed as nanograms of aflatoxin M<sub>1</sub> per kg of body weight per day and assuming a body weight of 60 kg, the intakes are 0.002 for the African diet, 0.10 for the Middle Eastern diet, 0.058 for the Latin American diet, 0.11 for the European diet, and 0.20 for the Far Eastern diet.

One of the papers (Oliveira et al., 1997) provided an estimate of the aflatoxin M<sub>1</sub> intake of a 4-month-old child in Brazil on the basis of a rather high value for this compound in milk prepared from powder (0.27 µg/kg; range, 0.10–1.0 µg/kg). The estimated daily intake was 0.0037 µg/kg bw per day. If it is assumed that a 4-month-old child weighs about 6 kg, this would represent a daily intake of 0.022 µg (22 ng) of aflatoxin M<sub>1</sub> per day. No other literature or submitted national estimates were available on the intake of aflatoxin M<sub>1</sub>.

### 7.2.3 Effect of proposed maximum levels in foods on dietary intake of aflatoxin M<sub>1</sub>

One approach to determining the effect of applying the proposed maximum levels of 0.5 and 0.05 µg/kg for aflatoxin M<sub>1</sub> in milk on dietary intake is based on assessment of the mean aflatoxin M<sub>1</sub> concentration in all samples of milk, in all samples containing 0.5 µg/kg, and in all samples containing 0.05 µg/kg for a given population. These three concentrations can then be multiplied by the milk consumption of the population to determine the intakes. The most recent data (1999) from some Member States of the European Union, comprising 7573 samples, showed concentrations ≤ 0.05 µg/kg in all samples. The intake of aflatoxin M<sub>1</sub> would not differ if the maximum level was

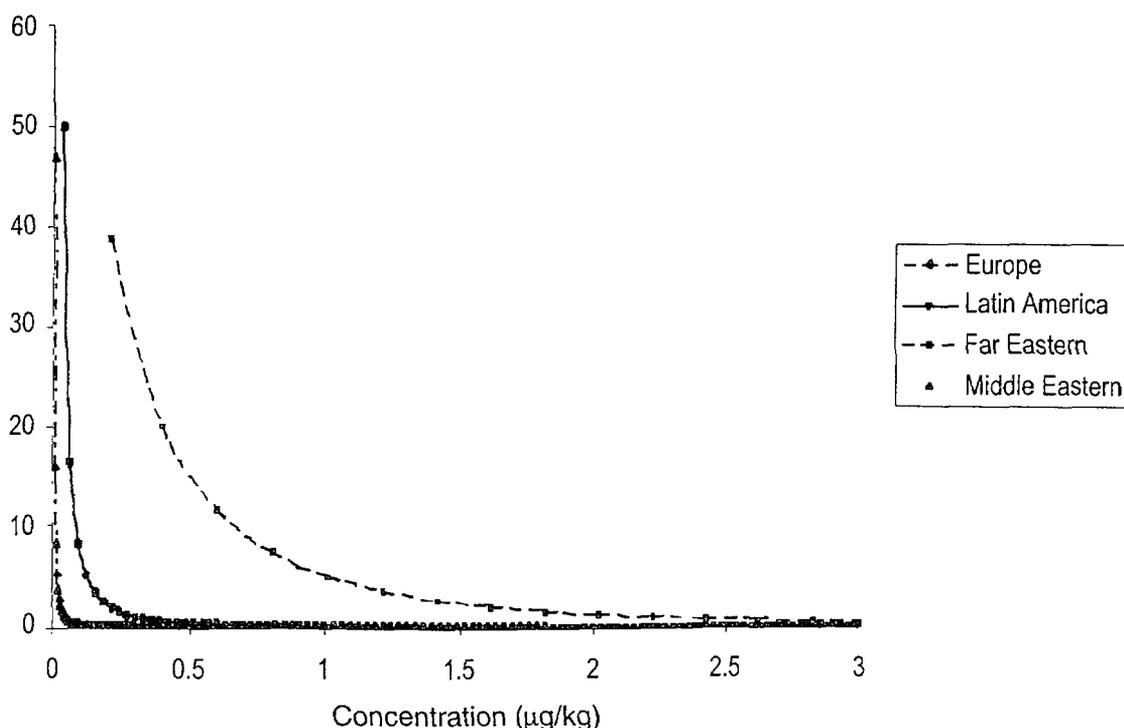
**Table 15. Daily intake of aflatoxin M<sub>1</sub> in all milk in the five regional diets**

Diet	Milk intake (kg/day)	Weighted mean		
		Aflatoxin in milk (µg/kg)	Aflatoxin intake	
			ng/person per day	ng/kg bw per day
European	0.29	0.023	6.8	0.11
Latin American	0.16	0.022	3.5	0.058
Far Eastern	0.032	0.36	12	0.20
Middle Eastern	0.12	0.005	0.6	0.10
African	0.042	0.002	0.1	0.002

0.5 or 0.05  $\mu\text{g}/\text{kg}$ , as none of the concentrations exceeded 0.05  $\mu\text{g}/\text{g}$ . A similar situation is seen with the most recent data from Canada, comprising 81 samples reported in 1997–98, as all milk samples analysed contained  $< 0.015 \mu\text{g}/\text{kg}$  (the LOD). Data on 3620 samples taken in the USA between 1995 and 2000 show that the mean intake of aflatoxin M<sub>1</sub> from all samples of milk was 0.0062  $\mu\text{g}/\text{kg}$ , resulting in an intake of 1.8 ng/person per day. If all samples containing  $\geq 0.5 \mu\text{g}/\text{kg}$  (3615; 99%) were omitted, the mean concentration would be 0.0046  $\mu\text{g}/\text{kg}$ , and the mean intake would be 1.3 ng/person per day. If all samples containing  $\geq 0.05 \mu\text{g}/\text{kg}$  (3491; 96%) were omitted, the mean concentration would be 0.00070  $\mu\text{g}/\text{kg}$ , and the intake would be 0.20 ng/person per day. The estimates are based on milk consumption in the GEMS/Foods European diet of 0.29 kg/person per day. The intakes of aflatoxin M<sub>1</sub> from milk are 1.8 ng/person per day for all samples, 1.4 ng/person per day when the maximum level is 0.5  $\mu\text{g}/\text{kg}$ , and 0.21 ng/person per day when the maximum level is 0.05  $\mu\text{g}/\text{kg}$ .

Another approach to determining the effect of the proposed maximum limits on dietary intake is to generate distribution curves for the concentrations of aflatoxin M<sub>1</sub> in milk in the regional data (Figure 3). Log normality of the distribution was assumed, the weighted mean concentrations of aflatoxin M<sub>1</sub> in the GEMS/Food regions were used, and the maximum values were reported. The distribution curve for the European regional diet shows that setting the maximum level at 0.05 or 0.5  $\mu\text{g}/\text{kg}$  would have no effect on intake. The distribution curve for milk samples in the Middle Eastern region, for which there were relatively few data, is similar to that for the European region. For the Latin American diet, selection of a maximum level of 0.5  $\mu\text{g}/\text{kg}$  would also have no effect; however, use of a maximum level of 0.05  $\mu\text{g}/\text{kg}$  would probably reduce intake. In the Far Eastern region, where milk is more heavily contaminated, intake of aflatoxin M<sub>1</sub> would be decreased at both proposed levels. It should be noted, however, that milk consumption is low in the Far East.

**Figure 3. Distribution curves for the concentrations of aflatoxin M<sub>1</sub> in milk according to the four GEMS/Foods regional diets**



The weighted mean concentrations of aflatoxin M<sub>1</sub> in milk in the regional diets are not the same in Appendix C and Figure 3 because generation of the distribution curves based on log normality required use of non-zero minimum values. Thus, the minimum values for aflatoxin M<sub>1</sub> in milk were assumed to be one-half of the LOD/LOQ, resulting in slightly different weighted mean values than those shown in Appendix B, which were generated from the number of samples and the mean value from each submission within each geographical region.

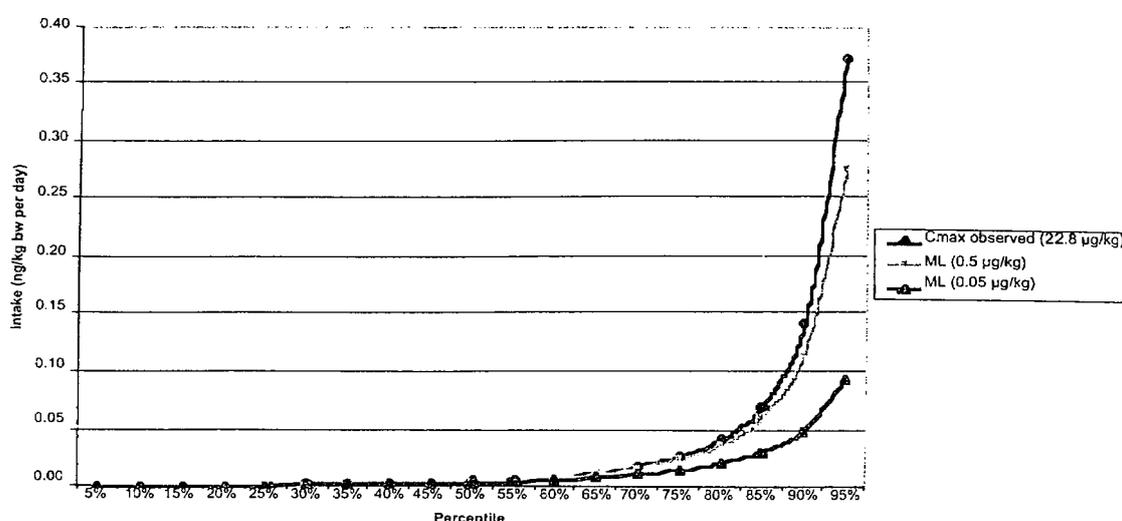
#### 7.2.4 Simulation of intake of aflatoxin M<sub>1</sub> by the French population

An example of intake of aflatoxin M<sub>1</sub> in milk was prepared from simulated distributions of contamination and of milk consumption by the French population (Figure 4). A probabilistic Monte-Carlo simulation was used to assess the intake of aflatoxin M<sub>1</sub> in milk. The software used was @risk, and the formula used to derive the contamination curves is:

$$\text{risk T lognormal (mean, SD, min, max).}$$

The data on food intake were taken from a French survey of individual food consumption conducted by the 'Association Sucre-Produits sucrés, Communication, Consommation' in 1994, and the results are those for participants who consumed milk. Of the 1161 persons surveyed, 79% consumed milk on the day of the survey. The mean consumption of milk was 27 g/week per kg bw, and the 95th percentile of consumption was 103 g/week per kg bw. The model is based on an assumption of log normality of the distribution and the mean, standard deviation, and minimum and maximum values for aflatoxin M<sub>1</sub> in milk. The minimum value is assumed to be zero, and the maximum observed value was 23 µg/kg. Three simulations were made, on the basis of the maximum observed concentration of aflatoxin M<sub>1</sub> in milk, the

**Figure 4. Simulated intake of aflatoxin M<sub>1</sub> from milk in France as a function of no maximum level, a maximum level of 0.5 µg/kg, and a maximum level of 0.05 µg/kg**



C<sub>max</sub>, maximum plasma concentration; ML, maximum level

upper proposed maximum level (0.5 µg/kg), and the lower proposed maximum level (0.05 µg/kg).

The results show no difference between the curves when there is no maximum level and the upper proposed maximum level is up to the 90th percentile of consumption. The curves for the upper and the lower maximum levels begin to separate at the 70th percentile. The intakes at the 90th percentiles are 0.05, 0.12, and 0.14 ng/kg bw per day for a maximum level of 0.05 µg/kg or 0.5 µg/kg and no maximum level, respectively. The intake at the 95th percentile, is 0.09, 0.27, and 0.37 ng/kg bw per day for a maximum level of 0.05 µg/kg, a maximum level of 0.05 µg/kg, and no maximum level, respectively.

#### **7.4 Limitations of intake estimates**

The validity and reliability of these estimates of intake of aflatoxin M<sub>1</sub> from milk are limited by a number of factors. Insufficient data on the concentrations of aflatoxin M<sub>1</sub> in milk were submitted for all five geographic regions and especially for the Middle East and Africa. As most of the submitted data were not accompanied by information on quality assurance, the validity or reliability of the resulting intake estimates cannot be assessed. Furthermore, little information was given about the sampling design or procedure used, and it is unlikely that the submitted data were representative of the area from which the samples were taken. Not all countries within a region submitted data, and the number of samples varied considerably. Very limited data were available from African and Middle Eastern countries. In addition, the concentration of aflatoxin M<sub>1</sub> in milk could vary considerably within each geographic region.

The use of different analytical methods by various investigators probably affected the reported concentrations of aflatoxin M<sub>1</sub> in milk, as some methods are more sensitive than others. The LOD/LOQ of the analytical method was less than the proposed maximum level of 0.05 µg/kg for the data from Indonesia, Thailand (Boriboon & Suprasert, 1994), and Uruguay. The lower values reported in these studies would not be accurate enough to determine the number of samples containing < 0.05 µg/kg. Comparison and aggregation of data from different laboratories is difficult not only because different analytical methods are used but also because the results are presented in different ways (distributions, means of positive values, values < LOD/LOQ, values below the maximum level) and in different units.

For studies in which a mean for all samples was not provided, the mean was calculated by assuming that values < LOQ/LOD were zero. In most cases, the number of samples at 0 and > 0 but < LOQ/LOD could not be determined. The assumption may have resulted in underestimation of the weighted mean concentrations of aflatoxin M<sub>1</sub> in milk.

The effects of processing, season, climate, and other environmental variables on the aflatoxin M<sub>1</sub> content of milk could not be ascertained, partly because these effects were not addressed in the various studies and reports, and also because various terms were used to describe milk and other dairy products. In most cases, the type of milk analysed was not specified.

For monitoring and evaluation of aflatoxin M<sub>1</sub> in milk in the future, it would be useful to obtain more data, especially from African, Far Eastern, Middle Eastern, and Latin American countries, and detailed information on sampling plans and quality

control of the analytical method in all submissions. In addition, individual data points should be provided for the construction of distribution curves and other statistical evaluations. The LOD and LOQ of the analytical methods should be provided. The samples that are analysed should be clearly described with regard to the type of milk, the extent of processing, geographical location, and whether or not the samples are representative of the entire country or region. Submissions should indicate whether values < LOD/LOQ are assigned a value of 0. They should provide information on the number of samples, the number of samples < LOD/LOQ, the number of samples containing aflatoxin M<sub>1</sub> at < 0.05 µg/kg and < 0.5 µg/kg, the mean concentration in each type of milk (by time, if appropriate), the maximum value, and the 90th percentile value.

## **8. PREVENTION AND CONTROL**

### **8.1 Carry-over from feed to cows' milk**

Since the observation that dairy cows consuming rations contaminated with aflatoxin B<sub>1</sub> excrete aflatoxin M<sub>1</sub> in their milk, various studies have been undertaken to establish the carry-over rates with consumption of high and low concentrations in feed. In studies conducted in the 1960s and 1970s, the aflatoxin B<sub>1</sub> intake was relatively high, the milk yields relatively low, and the analytical methods not well developed. Varied results were obtained, the concentration of aflatoxin M<sub>1</sub> in milk ranging from 0.2 to 4% of the concentration of aflatoxin B<sub>1</sub> ingested with the feed (van Egmond, 1989; Pettersson, 1997). Kiermeier et al. (1975, 1977) indicated that concentrations of aflatoxin M<sub>1</sub> in milk varied widely from animal to animal (even among animals in the same herd), from day to day, and from one milking to the next. The results of several researchers (van der Linde et al., 1964; Masri et al., 1969b; Kiermeier, 1973; McKinney et al., 1973; Polan et al., 1974) suggest that it takes 3–6 days of constant daily ingestion of aflatoxin B<sub>1</sub> before steady-state excretion of aflatoxin M<sub>1</sub> in milk is achieved, whereas aflatoxin M<sub>1</sub> becomes undetectable 2–4 days after withdrawal of animals from the contaminated diet.

In the 1980s and 1990s, more information became available on the carry-over of aflatoxin B<sub>1</sub> at concentrations in the range of the official limits. At the same time, the milk yields of cows increased considerably (up to 40 L/day), and analytical methods improved, became more reliable, and provided more precise results. The more recent studies widened the estimated range of concentrations of aflatoxin M<sub>1</sub> in milk from 0.3 to 6.2%. The highest percentage was found in a carry-over experiment in Dutch dairy cows in early and late lactation stages that were fed diets naturally contaminated with low concentrations of aflatoxin B<sub>1</sub> (Veldman et al., 1992). The authors assumed that the higher excretion ratios of cows with high milk production was a result of greater permeability of the cell membranes of the alveoli. The higher concentrations of aflatoxin M<sub>1</sub> excreted by cows with mastitis is also believed to be due to increased permeability of the membranes. A linear relation between aflatoxin B<sub>1</sub> intake and aflatoxin M<sub>1</sub> content was found for aflatoxin B<sub>1</sub> intakes of 5–80 µg:

$$\text{aflatoxin M}_1 \text{ (ng/kg of milk)} = 1.2 \times \text{aflatoxin B}_1 \text{ intake (}\mu\text{g/cow per day)} + 1.9.$$

Thus, production of milk containing aflatoxin M<sub>1</sub> at 0.05 µg/kg would require that the average intake of aflatoxin B<sub>1</sub> by dairy cows be limited to approximately 40 µg/day

per cow. On the basis of a daily consumption of 12 kg/cow of compound feed, the content of aflatoxin B<sub>1</sub> in the feed would have to be 3.4 µg/kg in order to meet the proposed Codex limit. Carry-over studies conducted in the 1990s of cows producing 10–20 L of milk per day (Harvey et al., 1991; Galvano et al., 1996b) showed much less carry-over (0.5–0.6%).

The variations in carry-over are significant at both high and low levels of contamination of feed with aflatoxin B<sub>1</sub>. Pettersson (1997) gave a number of reasons for the considerable variation. Apart from problems of analysis and experimental technique, there are large individual differences in excretion; however, this is outweighed by the fact that the studies were performed on groups of cows. Any differences by breed that may exist are not clearly discernible in the available studies. Differences in milk production are important. The studies of Pettersson et al. (1990), Veldman et al. (1992), and Veldman (1992) of cows with high milk production showed the greatest carry-over, ranging from 2.6 to 6.2%. Tables 16 and 17 present data from Park & Pohland (1986) on the carry-over of aflatoxin from feed to edible tissue.

## 8.2 Prevention and control of carry-over

The most effective way of controlling aflatoxin M<sub>1</sub> in the food supply is to reduce contamination with aflatoxin B<sub>1</sub> of raw materials and supplementary feedstuffs for dairy cattle. Specific regulations exist in many countries (FAO, 1997), and practical

**Table 16. Ratios of concentrations of aflatoxin in feed and in edible tissues**

Animal	Tissue	Aflatoxin	Feed:tissue ratio <sup>a</sup>
Beef cattle	Liver	B <sub>1</sub>	14 000
Dairy cattle	Milk	M <sub>1</sub>	75
		Aflatoxicol	195 000
Pigs	Liver	B <sub>1</sub>	800
Laying hens	Eggs	B <sub>1</sub>	2 200
Broiler chickens	Liver	B <sub>1</sub>	1 200

From Park & Pohland (1986)

<sup>a</sup> Concentration of aflatoxin B<sub>1</sub> in feed divided by the concentration of the specified aflatoxin in the specified tissue

**Table 17. Concentrations of aflatoxin B<sub>1</sub> required in feed to result in 0.1 ng/g of residues of aflatoxins in edible tissues**

Animal	Level of contamination with aflatoxin B <sub>1</sub> (ng/g)			
	Corn	Peanut meal	Cottonseed meal	Cottonseed
Beef cattle	1800	14 000	12 725	14 000
Dairy cattle	14	54	54	38
Pigs	105	730	1 600	
Laying hens	325	1 835	2 445	
Broiler chickens	180	925	1 200	

From Park & Pohland (1986)

programmes are being developed; e.g. the Codex Committee on Food Additives and Contaminants has developed a code of practice for reducing aflatoxin B<sub>1</sub> in raw materials (van Egmond et al., 1997). Reduction can be achieved by good manufacturing practices and good storage practices. If preventive measures fail to reduce fungal growth and aflatoxin B<sub>1</sub> formation in agricultural commodities intended for use as animal feeds, the last means for avoiding or reducing the occurrence of aflatoxins in feed is to eliminate (part of) the toxins. Feeds that have higher concentrations of aflatoxin B<sub>1</sub> may be acceptable for feeding to dairy animals if they are blended with feed that has lower concentrations, provided that the resultant aflatoxin M<sub>1</sub> concentration in milk does not exceed levels considered to be safe. In principle, aflatoxin-contaminated consignments of feeds can be decontaminated by removing the toxin (segregation) or by converting it to a non-toxic form (degradation). Degradation may be achieved by physical, chemical, or biological means.

Attempts have been made to degrade aflatoxins in feed by applying physical treatments such as heat, microwaves,  $\gamma$ -rays, X-rays, ultra-violet light and adsorption (van Egmond & Speijers, 1999). Degradation of aflatoxin M<sub>1</sub> has also been attempted by combined treatments, such as ultra-violet radiation followed by ultrafiltration. In most cases, neither heat treatment nor irradiation is effective. Adsorption of aflatoxins from animal feed onto bentonite and hydrated sodium calcium aluminosilicate (Veldman, 1992; Galvano et al., 1996a) has been used in the feed industry to reduce the aflatoxin M<sub>1</sub> content of milk (Harvey et al., 1991; Phillips et al., 1995).

A newer approach is use of oltipraz, a substituted dithiolthione that inhibits aflatoxin B<sub>1</sub> metabolism by inhibiting the activity of several cytochrome P450 enzymes (Kuilman et al., 2000). No aflatoxin M<sub>1</sub> formation was found in bovine hepatocytes incubated with aflatoxin B<sub>1</sub> and oltipraz. The findings suggest that oltipraz is highly effective in inhibiting aflatoxin M<sub>1</sub> contamination of milk from dairy cows exposed to aflatoxin B<sub>1</sub>-contaminated feeds.

Some chemical procedures have been developed to degrade aflatoxins in animal feed, usually based on addition of oxidizing agents, aldehydes, acids, and bases. The most widely used chemical detoxication reagent is ammonia, as an anhydrous vapour or as an aqueous solution. Treatment of aflatoxin B<sub>1</sub> with ammonia opens the lactone ring of the molecule. Ammoniation of agricultural commodities leads to decomposition of 95–98% of the aflatoxin B<sub>1</sub> present. This process is used in various countries for the decomposition of animal feedstuffs; however, it has not been formally approved by the European Union or the Food and Drug Administration of the USA because of controversy about the safety of products from animals that have been fed diets that have undergone chemical decontamination.

Park and Price (2001) reviewed studies conducted over more than three decades on the development and safety evaluation of procedures for reducing risks associated with contamination of agricultural commodities by aflatoxins. Use of ammonia to alter the chemical structure of aflatoxins and thus reduce their toxic and mutagenic potential has gained acceptance in many countries, including Brazil, France, Mexico, Senegal, South Africa, the Sudan, and some states of the USA. Use of ammonia to treat cottonseed feed for lactating cows in Arizona, USA, and to treat peanut meal in France has kept the milk supply free from aflatoxin contamination for almost 20 years. In the aflatoxin decontamination programme, in which the products were tested for aflatoxin concentrations both before and after ammoniation, the process was shown to result usually in no detectable aflatoxin M<sub>1</sub> residues in milk. The process and the treated products are accepted by the dairy industry.

Although several ammonia-based processes have been developed and studied, that in which ammonia (0.5–2.0%) is used under controlled conditions of moisture (12–16%), pressure (45–55 psi [3.2–3.9 kg/cm<sup>2</sup>]), and temperature (80–1000 °C) for 20–60 min, commonly called the ‘high-pressure, high-temperature method’, is the most efficient and produces the most reliably safe product. The procedure modifies the aflatoxin molecule chemically to compounds that are many orders of magnitude less toxic or mutagenic than the parent aflatoxin B<sub>1</sub> or undetectable after exhaustive extraction, isolation, and purification. The aflatoxin–ammonia reaction products in cottonseed and maize consist of 12–14% volatile compounds, 20–24% compounds extractable with methylene chloride, and 6–13% compounds extractable with methanol. Treatment with acid, base, and proteolytic enzymes released an additional 19–22% of the compounds. After solvent extraction and enzymatic digestion, the remaining cottonseed or maize matrix contained ammonia reaction products representing only 37% of the original aflatoxin concentration.

Studies of metabolism and excretion showed that the feed-bound ammonia–aflatoxin products are excreted primarily in the faeces. Exhaustive studies showed that the reaction products have minimal if any effect on the health of animals receiving rations containing ammonia-treated aflatoxin-contaminated maize, peanut, or cottonseed meal. The ambient temperature method, which usually requires a 3–6-week treatment period, effectively reduces aflatoxin concentrations, but it requires close monitoring, and the safety of the ammoniated product has not been demonstrated unequivocally.

The effect of ammonia on feed composition and by-products is usually increased concentrations of total and non-protein nitrogen, crude protein, ash, and soluble solids, with reduced concentrations of sulfur-containing amino acids, available lysine, and reducing sugars. Production parameters such as milk and egg quality have been shown to be significantly improved or not adversely affected by the treatment. Long-term, short-term, relay, and multi-generation feeding studies showed no toxic effects or lesions related to the ammoniation procedure. It has been approved for use in the USA for increasing non-protein nitrogen in animal feeds.

Isolated aflatoxin–ammonia reaction products in maize and cottonseed or human foods derived from animals fed ammonia-treated aflatoxin-contaminated feeds had significantly reduced toxic and mutagenic potential. No tumours or neoplastic lesions were observed in trout fed rations containing milk obtained from lactating dairy cows fed ammonia-treated aflatoxin-contaminated feed. Trout are highly susceptible to tumorigenesis by aflatoxins. High mutagenic activity in milk from cows exposed to aflatoxin B<sub>1</sub> was eliminated or reduced significantly by treating the feed with ammonia. Thus, some decontamination reaction products are somewhat toxic, mutagenic, or have DNA or protein covalent binding potential. The effects are, however, many orders of magnitude less than those of the parent aflatoxin B<sub>1</sub>. These compounds usually represent < 1% of the original aflatoxin concentration in the feed matrix. A comparison of the equivalent toxicity of aflatoxin after ammonia treatment showed that the aflatoxin–ammonia reaction products do not contribute significant toxic or mutagenic potential to the residual aflatoxin B<sub>1</sub> in ammoniated aflatoxin-contaminated feeds. Hence, the maximum level of aflatoxin in ammoniated aflatoxin-contaminated feeds would not have to be adjusted for the reaction products.

Alkaline heat treatment, or nixtamalization, which is used traditionally in the treatment of maize for the manufacture of tortillas, significantly reduced the concentration of aflatoxin. Subsequent studies showed, however, that much of the

original aflatoxin was re-formed when the products were acidified (Lopez-Garcia & Park, 1998).

In principle, biological methods could also be used to eliminate aflatoxins. For instance, procedures have been developed to degrade aflatoxins in feedstuffs by exposing them to the bacterium *Flavobacterium aurantiacum*. These studies have not yet led to application.

In conclusion, there are several possibilities for preventing the presence of aflatoxin M<sub>1</sub> in dairy products; each has its advantages and disadvantages. Although prevention of contamination of dairy cattle feed is the ideal, it may not be possible in practice. Various decontamination methods have a role to play in preventing and reducing the concentrations of aflatoxin M<sub>1</sub> in dairy products.

## **9. DOSE-RESPONSE RELATIONSHIP AND ESTIMATION OF CARCINOGENIC RISK**

### **9.1 Contribution of biochemical data to assessment of risk**

At its forty-ninth meeting, the Committee concluded that there was 'insufficient quantitative information available about competing aspects of metabolic activation and detoxication of aflatoxin B<sub>1</sub> in vivo in various species to describe quantitatively a species-dependent effect of metabolism on aflatoxin B<sub>1</sub> on carcinogenicity'. Further data have become available, however, to elucidate the effects of aflatoxin B<sub>1</sub> and aflatoxin M<sub>1</sub>.

Studies of slices of human liver showed wide variation in the metabolism and activation of aflatoxins among individuals, and genetic and environmental factors may affect this process in vitro. For example, ascorbate and coumarin seem to protect against the toxic effects of aflatoxins, and epidemiological studies have also shown the importance of genetic and environmental factors in liver cancer associated with intake of aflatoxins.

Human hepatocytes appear to form less aflatoxin B-oxide than rat cells, and human cells appear not to have the important detoxifying GST enzymes with high specific activity against aflatoxin B<sub>1</sub>. Some workers have shown no GST activity in human liver cytosol fractions. Human liver enzymes have a limited capacity to form the toxic epoxide form from aflatoxin M<sub>1</sub>. Aflatoxin M<sub>1</sub> is definitely cytotoxic, as is aflatoxin B<sub>1</sub>, in human hepatocytes, and this finding may have important implications for the effects of aflatoxin M<sub>1</sub> on immunocompetence and growth.

Aflatoxins may potentiate liver damage caused by bacterial lipopolysaccharide, which may affect acute toxicity in humans and domestic animals. This potentiation of liver damage by aflatoxins may also play a role in the interaction between hepatitis viruses and liver carcinogenesis.

Sensitivity to aflatoxins varies among species. In a highly sensitive species like the turkey, the crucial aflatoxin B<sub>1</sub>-detoxifying enzyme pathway (GST-mediated) is deficient, while there is efficient oxidation of aflatoxin B<sub>1</sub> to the epoxide. Hepatocytes from macaques, marmosets, and humans show a quantitatively similar ability to oxidatively metabolize aflatoxin B<sub>1</sub>. In one primate species (*M. fascicularis*), the  $\mu$  class of GSTs is the most important in detoxifying aflatoxin B<sub>1</sub>, but human cells have not been shown to duplicate this pattern of detoxication.

Oltipraz inhibits cytochrome P450 1A2 activity in rats and humans in vivo and in tree shrew hepatocytes. Thus, oxidation of aflatoxin B<sub>1</sub> to the 8,9-oxide and aflatoxin

M<sub>1</sub> is blocked. Oltipraz and ethoxyquin may also induce a modest increase in aflatoxin B glutathione conjugation activity.

The short-term toxicity of aflatoxin M<sub>1</sub> is similar to that of aflatoxin B<sub>1</sub>, and it appears to act by the same mechanism. In ducklings and rats, the short-term toxicity of aflatoxin M<sub>1</sub> was similar to or slightly less than that of aflatoxin B<sub>1</sub>. Aflatoxin M<sub>1</sub> is a less potent carcinogen than aflatoxin B<sub>1</sub>, even in Fischer rats and rainbow trout, which are very sensitive. Aflatoxin M<sub>1</sub> shows 2–10% of the carcinogenic activity of aflatoxin B<sub>1</sub>. In studies of genotoxicity, such as in *Drosophila melanogaster*, aflatoxin M<sub>1</sub> was about 10-fold less mutagenic than aflatoxin B<sub>1</sub>.

The relationship between dose or exposure to aflatoxin M<sub>1</sub>, HBV, and/or HCV and the incidence of hepatocellular carcinoma and other risk factors has not been studied epidemiologically in studies suitable for modelling a dose–response relationship or the carcinogenic potency of aflatoxin M<sub>1</sub>. However, a number of conclusions can be drawn from the existing studies on the potency of aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> and risk factors for human liver cancer.

There is consistent, growing evidence that hepatitis viruses play an important role in the etiology of liver cancer. Progress has been made in the use of markers (based on the PCR), which show an increased fraction of cases with virus and, in some examples, an increased fraction of controls with virus. The risk estimates therefore differ little from previous ones, but the attributable fractions may be increased in relation to those shown in the table in the report of the Committee at its forty-ninth meeting (modified as Table 3 above) and in other reviews. That is, the attributable fractions due to hepatitis viruses might be increased and that due to aflatoxin thereby decreased.

Many epidemiological studies have involved use of biomarkers of aflatoxins. Many included biomarkers in the metabolic pathways in which aflatoxin is detoxified and eliminated. Evidence is available to show that aflatoxin–albumin adducts reflect both aflatoxin intake in the diet and aflatoxin–DNA adducts in liver cells. However, biomarkers of aflatoxins do not allow a quantitative measure of aflatoxin intake over the long term, as they are limited by the half-time of the urinary metabolites or serum protein adducts.

The risk for hepatocellular carcinoma in relation to urinary excretion of aflatoxin M<sub>1</sub>, aflatoxin adducts, or aflatoxin–albumin adducts has been estimated in many studies. The most consistent finding is that HBV carriers have an increased risk when some of these biomarkers of intake are present. The increase is usually modest (two- to threefold, with some exceptions), but many of the studies are based on small numbers of cases and wide confidence intervals. Some of these studies are particularly valuable because urine or serum samples were collected before liver cancer occurred. However, HBV carriers may have some degree of liver disease at any time. Either of these two factors can alter the natural history of the biomarker itself. In addition, as can be seen in Table 3, HCV appears to be an increasingly important risk factor for hepatocellular carcinoma, especially in developed countries.

P53 mutations may be of considerable value for identifying cases of liver cancer that can be related to aflatoxin intake. In areas of high risk, however, only 30–50% of people can be shown to harbour the relevant mutation (codon 249), even though the entire population would have been exposed to aflatoxins. The specificity of the marker is thus low, and the negative results are difficult to interpret. In order to make full use of this important biomarker, its natural history should be characterized, and the dose–response relationship between aflatoxin B<sub>1</sub> and the mutation and the

relationship between better validated biomarkers (aflatoxin B<sub>1</sub>–albumin adducts in urine) and the occurrence of *P53* codon 249 mutations should be clarified. In epidemiological studies, the presence of mutations in specimens of hepatocellular carcinoma might be interpreted as reflecting the involvement of aflatoxin B<sub>1</sub> or aflatoxin M<sub>1</sub>, but the absence of mutations, particularly in cancers from areas where there is high aflatoxin B<sub>1</sub> intake (> 50% in most series), should be interpreted with caution, as aflatoxin B<sub>1</sub> and aflatoxin M<sub>1</sub> may induce hepatocellular carcinoma by other DNA damaging mechanisms.

Studies in which oltipraz is administered are likely to be informative if the proposed phase III trials are organized. In animals, olitpraz prevents aflatoxin-induced liver cancer. The results of studies in humans in China are awaited.

## 9.2 *General modelling issues*

The Committee at its forty-ninth meeting had an extensive discussion of general modelling issues. That discussion is relevant and will not be repeated here; a few general points will be emphasized.

The main weakness of the risk assessment performed by the previous Committee was the lack of an ideal data set. "...the best data set to use for dose–response analysis would be a human study in which dose is accurately measured, response is determined without error and there are no confounding factors which are unexplained." In the case of aflatoxin M<sub>1</sub>, there is no epidemiological study that addresses these factors adequately. The dose of aflatoxin M<sub>1</sub> or aflatoxin B<sub>1</sub> has not been measured accurately over the long period generally associated with cancer induction. The additional risk factors for hepatocellular carcinoma that interact with aflatoxins, such as HBV and HCV infection, cannot be quantified completely. Species differences in sensitivity to aflatoxins are beginning to be elucidated, but they cannot be quantified precisely.

As stated by the Committee at its forty-ninth meeting, quantitative risk assessment involves four basic issues: (1) choice of data; (2) measure of exposure; (3) measure of response; and (4) choice of a mathematical relationship between dose and response for a given data set.

### 9.2.1 *Choice of data*

The carcinogenic potency of aflatoxins was estimated in studies in several animal species and in several epidemiological studies. Data were available generally only for aflatoxin B<sub>1</sub>, sometimes for mixtures of aflatoxins, and rarely for aflatoxin M<sub>1</sub>. A study by Yeh et al. (1989) was the main source used by the Committee at its forty-ninth meeting and by most other groups for estimating the carcinogenic potency of aflatoxin B<sub>1</sub>. This study addressed the roles of HBV and aflatoxin B<sub>1</sub> in the development of primary hepatocellular carcinoma in a cohort of 7917 men aged 25–64 in southern Guangxi Province, China, where the incidence of this cancer is among the highest in the world. With 30 188 person-years accumulated, 149 deaths were observed, 76 (51%) of which were due to primary hepatocellular carcinoma. Of these patients, 69 (91%) were HBsAg<sup>+</sup> at the time of enrolment into the study, in contrast to 23% of all members of the cohort. Aflatoxin intake was estimated for the population as a whole and not for individuals. When the estimated aflatoxin B<sub>1</sub> intakes of subpopulations were plotted against the corresponding rates of death from primary hepatocellular carcinoma, a linear relationship was observed.

This study showed that HBV carriers in regions where HBV is highly prevalent and primary hepatocellular carcinoma is common are at high risk for this tumour. Further, the study indicated that the rate of death from this cancer is higher in an area of high aflatoxin B<sub>1</sub> intake than in areas of lower intake. The limitations of the study, first, that intake of aflatoxin B<sub>1</sub> was estimated from the availability of raw foodstuffs to the population and then attributed to individuals. Secondly, the correlation between the incidence of primary liver cancer and aflatoxin B<sub>1</sub> intake was not adjusted for any of possible confounders, such as HCV infection, alcohol drinking, tobacco use, or nutritional status. Thirdly, exposure to HBV may have been underestimated since PCR was not used. The potency of aflatoxin in patients with primary liver cancer who were HBsAg<sup>-</sup> was estimated on the basis of only seven cases, some of which would have been shown to be HBsAg<sup>+</sup> with PCR. Lastly, the prevalence of HBsAg was measured in a 25% sample of the cohort and attributed to the region.

The Committee at its forty-ninth meeting did not use the results of Campbell et al. (1990) in making quantitative estimates. This study, which showed no association between aflatoxin intake and the incidence of primary liver cancer in China, is more directly pertinent to determining the strength of the association between aflatoxin intake and the incidence of primary liver cancer (hazard assessment). A dose–response assessment, which is conducted after an assessment of the probability of an association on the basis of all the data, is guided by the selection of appropriate data, assuming that the association exists. A study with negative results is generally not suitable for this purpose.

In a cohort study in Shanghai, China (Ross et al., 1992; Qian et al., 1994), information from both measurements of biomarkers and dietary questionnaires was used to determine aflatoxin intake. The information on intake was, however, reported only as positive or negative, and the detailed data necessary for construction of a dose–response curve were not available. The study conducted by Wang et al. (1996) was the only one available to the Committee at its forty-ninth meeting in which HCV was considered, but the results were inconclusive.

In order to estimate the carcinogenic potency of aflatoxin M<sub>1</sub> the Committee at its present meeting used a comparative approach, based on the results of studies in laboratory animals and in vitro, as shown in Figure 5. Thus, the relative carcinogenic potency of aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> in Fischer rats was estimated from the study of Cullen et al. (1987), which was considered the most relevant study for risk assessment, as it is the only 2-year study available, and, as noted above, there are no epidemiological studies in which the intake of both aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> was determined. Additional studies were considered in order to determine the robustness of this estimate of relative potency across species, by extrapolation. None of the studies considered here directly took into account HBV or HCV infection or other factors that might interact with and modify the carcinogenicity of aflatoxin M<sub>1</sub>.

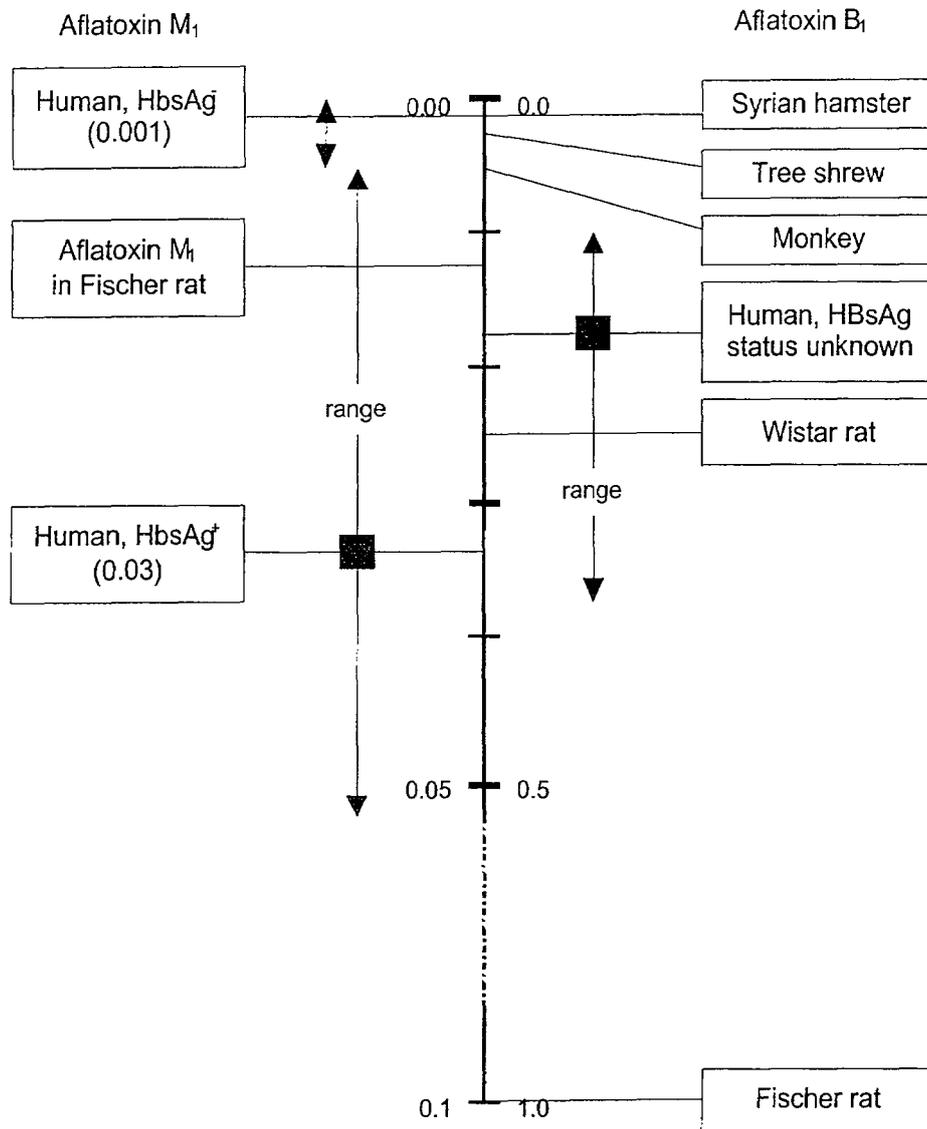
### **9.2.2 Measure of intake**

In all of the risk assessments, intake was expressed as the lifetime average intake in nanograms per kilogram of body weight per day.

### **9.2.3 Measure of response**

Although aflatoxin M<sub>1</sub> has been shown to be cytotoxic and to affect growth and the immune system, the focus of this risk assessment is its carcinogenicity.

Figure 5. Estimated potency (cancers/year per 100 000 people per ng/kg bw per day) of aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> for inducing liver cancer



Derived from epidemiological and toxicological studies. HBsAg<sup>±</sup> refers to presence or absence of the hepatitis B surface antigen in serum.

#### 9.2.4 Choice of mathematical model

The report of the forty-ninth meeting includes an abbreviated discussion of the mathematical models used in risk assessment. Since the data for aflatoxin M<sub>1</sub> are so limited, a simple linear extrapolation was used here.

#### 9.3 Potency estimates

Figure 5 was modified from the report of the forty-ninth meeting of the Committee. It presents potencies estimated from the results of experimental and epidemiological studies. The carcinogenic potency of aflatoxin B<sub>1</sub> in Fischer rats, a sensitive species, was arbitrarily set at 1; and the carcinogenic potencies of aflatoxin B<sub>1</sub> in other species are represented on the line as a fraction of 1, i.e. relative to that in Fischer rats. The

results of epidemiological studies in which the HBV infection status of the participants was unknown gave estimated potencies between those of HBV infected and uninfected persons. The potencies shown in Figure 5 do not apply directly to aflatoxin M<sub>1</sub>, since the estimates of intake given in many of the epidemiological studies did not include the contributions to total aflatoxin intake from milk and milk products. However, the potency of aflatoxin B<sub>1</sub> provides some information about that of aflatoxin M<sub>1</sub>.

The significance of estimates of the potency of aflatoxin B<sub>1</sub> relative to that of aflatoxin M<sub>1</sub> derives from the fact that aflatoxin M<sub>1</sub> is a metabolite of aflatoxin B<sub>1</sub>. At low intake, aflatoxin M<sub>1</sub> is a potentially significant (~10–25%) component of the metabolic pathway of aflatoxin B<sub>1</sub> in humans, as judged from studies of human liver slices and microsomes (Ramsdell & Eaton, 1990; Gallagher et al., 1996; Heinonen et al., 1996). Approximately 2–7% of ingested aflatoxin B<sub>1</sub> has been estimated to be excreted in the form of urinary aflatoxin M<sub>1</sub> (Groopman et al., 1992; Cheng et al., 1997). The difference in the percentage converted to aflatoxin M<sub>1</sub> and that excreted suggests that at least some of the aflatoxin M<sub>1</sub> derived from aflatoxin B<sub>1</sub> is further metabolized to the DNA-reactive epoxide. Consequently, estimates of the potency of aflatoxin B<sub>1</sub> provide some information about the potential risk posed by aflatoxin M<sub>1</sub>. Previous estimates of the carcinogenic potency of aflatoxin B<sub>1</sub> based on the correlation with rates of primary liver cancer implicitly include the effects of aflatoxin M<sub>1</sub> derived from metabolism of ingested aflatoxin B<sub>1</sub>. If exposure to aflatoxin M<sub>1</sub> derived from metabolism of aflatoxin B<sub>1</sub> is comparable in magnitude to the intake of aflatoxin M<sub>1</sub> by ingestion, the potency of aflatoxin M<sub>1</sub> can be no greater than that which has been estimated for aflatoxin B<sub>1</sub>, and is probably much less.

No epidemiological study equivalent to that of Yeh et al. (1989) for aflatoxin B<sub>1</sub> has been reported that addressed the carcinogenicity of aflatoxin M<sub>1</sub> in the presence and absence of HBV (and/or HCV). It was assumed that HBV and aflatoxin M<sub>1</sub> interact in increasing the risk for liver cancer in a manner comparable to that seen in the study of Yeh et al. (1989).

As no epidemiological studies have been reported that bear on the relationship between risk for primary liver cancer and intake of aflatoxin M<sub>1</sub> *per se*, the potency of aflatoxin M<sub>1</sub> was estimated from a comparative perspective. For the purposes of risk assessment, the 2-year study in male Fischer rats (Cullen et al., 1987) was used. In this study, groups of 62 male Fischer rats received diets containing 0.5, 5.0, or 50 µg/kg of aflatoxin M<sub>1</sub>. An additional 63 animals received agar diet without aflatoxin M. A positive control received a diet containing 50 µg/kg of aflatoxin B<sub>1</sub>, and an additional 42 rats were fed rodent chow. All animals were offered food and water *ad libitum*. The total intake of aflatoxin was estimated on the basis of food consumption: rats fed diets containing aflatoxin M<sub>1</sub> at 50 µg/kg ingested approximately 1 mg of aflatoxin M<sub>1</sub>, while those at 5.0 and 0.5 µg/kg groups ingested 0.1 and 0.01 mg, respectively. The group fed a diet containing aflatoxin B<sub>1</sub> had received a total of 0.8 mg by the time they were killed. Rats were scheduled for killing at 3, 6, 10, 16, 17, 19, and 21 months, but all surviving rats receiving aflatoxin B<sub>1</sub> were killed at 17 months.

In rats fed the diet containing aflatoxin M<sub>1</sub> at 50 µg/kg, hepatocellular carcinomas were detected in two of 18 rats killed at 21 months, and neoplastic nodules were found in six of 37 killed between 19 and 21 months. No nodules or carcinomas were observed in groups receiving the lower doses. Nineteen of 20 rats fed the diet

containing aflatoxin B<sub>1</sub> had developed hepatocellular carcinomas by 17 months. From these results, it was estimated that the potency of aflatoxin M<sub>1</sub> is 2–10% that of aflatoxin B<sub>1</sub> (Hsieh et al., 1984). A conservative view of this result is that the potency of aflatoxin M<sub>1</sub> is approximately one order of magnitude less than that of aflatoxin B<sub>1</sub> in that species. Therefore, the carcinogenic potency of aflatoxin M<sub>1</sub> in Fischer rats was placed at 0.1 the carcinogenic potency of aflatoxin B<sub>1</sub> in Figure 5.

The potency of aflatoxin M<sub>1</sub> in Fischer rats was calculated as follows:

$$2/18 \text{ risk} / (1 \text{ mg/lifetime} \times 21 \text{ months/lifetime} \times 31 \text{ days/month}) / 0.3 \text{ kg bw} = 0.00057 \text{ mg/kg bw per day.}$$

The corresponding calculation for aflatoxin B<sub>1</sub> in Fischer rats is:

$$19/20 \text{ risk} / (1 \text{ mg/lifetime} \times 17 \text{ months/lifetime} \times 31 \text{ days/month}) / 0.3 \text{ kg bw} = 0.006 \text{ mg/kg bw per day.}$$

Thus, the potency of aflatoxin B<sub>1</sub> is approximately 10 times that of aflatoxin M<sub>1</sub>. This is a conservative assessment of the carcinogenic potential of aflatoxin M<sub>1</sub> relative to aflatoxin B<sub>1</sub> because the tumour rate induced by aflatoxin B<sub>1</sub> at 17 months was compared directly with that induced by aflatoxin M<sub>1</sub> at 21 months. Although no hepatocellular carcinomas were observed in rats fed aflatoxin M<sub>1</sub> and killed at 17 months, this may have been a consequence of the sample size. In any event, the true, as opposed to the observed, tumour rate induced by aflatoxin M<sub>1</sub> at 17 months is certainly greater than 0 but lower than the rate at 21 months. This would imply a corresponding ratio greater than 10-fold.

Although the study of Cullen et al. (1987) was conducted with Fischer rats and did not include interaction with HBV or HCV, it is useful for estimating potency because the carcinogenicity of aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> is compared in the same experiment with the same control group. A limitation of this study for comparing the carcinogenic potency of aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> is that only one group received aflatoxin B<sub>1</sub>, and therefore a dose–response curve could not be constructed for aflatoxin B<sub>1</sub> under these experimental conditions. Hence, the potency of aflatoxin B<sub>1</sub> in this study was estimated from only one dose by linear extrapolation.

In the absence of epidemiological studies of aflatoxin M<sub>1</sub>, the relative risk associated with exposure to aflatoxin M<sub>1</sub> versus aflatoxin B<sub>1</sub> for human populations is assumed to be the same as that observed for Fischer rats. Use of this assumption is supported to some extent by studies of the relative potencies of the two toxins *in vitro*, as discussed below. The carcinogenic potency of aflatoxin M<sub>1</sub> in HBsAg<sup>-</sup> individuals is placed in Figure 5 at 0.001 or 1/10 of the previously estimated carcinogenic potency of aflatoxin B<sub>1</sub> in such persons. Similarly, the carcinogenic potency of aflatoxin M<sub>1</sub> in HBsAg<sup>+</sup> individuals is placed at 0.03, 1/10th of the previously estimated carcinogenic potency of aflatoxin B<sub>1</sub>. This approach, extrapolating potency, was adopted because of the apparent sensitivity of Fischer rats to aflatoxin B<sub>1</sub>.

As discussed in the report of the forty-ninth meeting, the customary three-quarters body weight scaling extrapolation of the potency of aflatoxin B<sub>1</sub> in Fischer rats to humans results in an estimate of 37 cases per 100 000/year per ng/kg bw per day, which is orders of magnitude greater than the range of potency of aflatoxin B<sub>1</sub> (0.002–0.036 per 100 000/year per ng of aflatoxin B<sub>1</sub> per kg bw/day) determined from epidemiological studies of aflatoxin B<sub>1</sub>. Consequently, aflatoxin B<sub>1</sub> is an extreme

exception to the general pattern of three-quarters body weight and/or surface area scaling that has been observed to hold for many carcinogens. Aflatoxin M<sub>1</sub> is also expected to be an exception to the general pattern.

A comparative study of aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> by Neal et al. (1998) suggested that cytotoxic effects in the MCL-5 lymphoblastoid cell line are generally absent at concentrations of aflatoxin B<sub>1</sub> < 0.05 µg/ml and of aflatoxin M<sub>1</sub> < 1 µg/ml. Although the susceptibility of liver cells to the cytotoxic effects of aflatoxins may differ from those of MCL-5 cells, the findings appear to be consistent with the conclusion that tumour induction in Fischer rats at the dietary concentration tested is likely to be a consequence of mutagenic effects of aflatoxin B<sub>1</sub> and aflatoxin M<sub>1</sub> rather than of increased liver cell proliferation due to cytotoxicity.

The relative potency of aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> in Fischer rats has been estimated in an animal model that is not infected with HBV, and no such studies in animals co-infected with HBV or any other virus (e.g. HCV) that causes significant toxicity, liver damage, and compensatory liver cell regeneration exist. Nevertheless, to the extent that the predominant mechanism of the carcinogenicity of aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> is likely to be genotoxic, at least at low levels of intake, it would appear reasonable to assume that the relative potency would be the same in populations with different background rates of liver cancer attributable to differences in general susceptibility to genotoxic carcinogens. Differences in the prevalence of HBV, which by itself is likely to cause liver cancer by a non-genotoxic process leading to enhanced cell proliferation, would appear to be represent an alteration in susceptibility to the effects of genotoxic agents.

The assumption that HBV affects the rates of primary liver cancer primarily by increasing and sustaining cell proliferation is supported by the study of Evans et al. (1998), discussed in section 2. Studies in woodchucks also seem to support the contention that the most significant mechanism of interaction between aflatoxins and hepatitis is increased cell proliferation, which fixes the adducts formed from the metabolism of aflatoxin B<sub>1</sub> (Izzotti et al., 1995). This may reasonably be expected to be the case for aflatoxin M<sub>1</sub> and HBV as well. Thus, in the absence of altered cell proliferation due to cytotoxic effects of either aflatoxin M<sub>1</sub> or aflatoxin B<sub>1</sub> *per se*, the induction of liver tumours by a genotoxic mechanism is generally correlated with adduct levels or other measures of genotoxic damage, irrespective of the background tumour rate (Poirier & Beland, 1994; Otteneder & Lutz, 1999; Wang & Groopman, 1999). This situation would appear to be likely for aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> at current levels of human intake.

Hepatitis may also significantly alter the metabolism of both aflatoxin B<sub>1</sub> and aflatoxin M<sub>1</sub>, although there is little evidence to suggest that the metabolism of one would be altered preferentially. Consequently, for the purposes of this assessment and in the absence of specific data to the contrary, the potency of aflatoxin M<sub>1</sub> and aflatoxin B<sub>1</sub> is assumed to be the same in HBsAg<sup>+</sup> and HBsAg<sup>-</sup> individuals.

The estimates of the potency of aflatoxin M<sub>1</sub> were combined with estimates of intake for the European regional diet. The potency of aflatoxin M<sub>1</sub> in a human population with an HBV prevalence rate *P* was projected to be the weighted combination of the potency estimates for HBsAg<sup>-</sup> individuals (0.001 cancers/100 000 per year per ng/kg bw per day) and HBsAg<sup>+</sup> individuals (0.03 cancers/100 000 per year per ng/kg bw per day). Specifically, the average potency of aflatoxin M<sub>1</sub> in a population of individuals with an HBV prevalence rate *P* was taken to be:

$$\text{Potency} = 0.001 \times (1-P) + 0.03 \times P.$$

Three rates of HBV prevalence were considered (1, 5, and 25%) in order to span the range of infection rates actually observed in various western and South-East Asian countries. The projected cancer risks were calculated as the product of the average potency and estimates of intake of aflatoxin M<sub>1</sub> in a European diet, corresponding to relatively high intake of milk and milk products. The projected risks were calculated by assuming various standards of contamination, including the current standard in the USA of 0.5 µg/kg and the proposed standard of 0.05 µg/kg. Assuming that all products are contaminated at the standard results in a worst-case projection of the population risk. For a given standard, the average level of contamination is likely to be less than the standard, since producers seek to ensure that their product meets the standard. The calculations are shown in Table 18.

The European diet was chosen from among the five regional diets because milk consumption is higher in that than in the other diets. It is obvious from the calculations that the additional risks for liver cancer predicted with the proposed maximum limit on aflatoxin M<sub>1</sub> of 0.05 µg/kg or with the current limit of 0.5 µg/kg are very small. For example, in a population with an HBV prevalence rate of 1%, the additional number of cases of liver cancer associated with aflatoxin M<sub>1</sub> contamination of milk at 0.5 µg/kg versus 0.05 µg/kg would be 0.0029 per 100 000 population/year (i.e. 0.0032–0.00032 cancers per 100 000 per year). Thus, in a population of 250 million with an HBV prevalence rate of 1%, the projected increase in risk would correspond to about seven additional cases per year at 0.5 µg/kg in comparison with the 0.05 µg/kg standard. This projected difference would not constitute a discernible reduction in the overall burden of liver cancer as measured in national cancer registries. Furthermore, the potency of aflatoxin M<sub>1</sub> would appear to be so low in HBsAg<sup>-</sup> individuals that no carcinogenic effect of aflatoxin M<sub>1</sub> intake in heavy consumers of milk and milk products could be demonstrated. The projected potency of aflatoxin M<sub>1</sub> in HBsAg<sup>+</sup> individuals is about 30-fold higher than that in HBsAg<sup>-</sup> individuals and comparable in magnitude to the estimated potency of aflatoxin B<sub>1</sub>.

**Table 18. Projected risk for liver cancer attributed to intake of aflatoxin M<sub>1</sub> in milk and comparison of effect of application of the proposed maximum levels**

Concentration of aflatoxin M <sub>1</sub> in milk (µg/kg)	HBsAg <sup>+</sup> (%)	Average potency <sup>a</sup>	Aflatoxin M <sub>1</sub> intake <sup>b</sup>		Prevalence of liver cancer attributable to aflatoxin M <sub>1</sub> (cancer cases/year per 10 <sup>6</sup> )
			ng/person per day	ng/kg bw per day <sup>c</sup>	
Weighted mean of 0.023	1	0.0013	6.8	0.11	1.5
	5	0.0025	6.8	0.11	2.8
	25	0.0083	6.8	0.11	9.4
0.05	1	0.0013	15	0.25	3.2
	5	0.0025	15	0.25	6
	25	0.0083	15	0.25	20
0.5	1	0.0013	150	2.5	32
	5	0.0025	150	2.5	60
	25	0.0083	150	2.5	200

<sup>a</sup> Potency in cases of cancer/year per 100 000 per ng/kg bw per day

<sup>b</sup> Based on a European-type diet

<sup>c</sup> Assuming a body weight of 60 kg

## 10. COMMENTS

### *Metabolism*

In all species and tissues tested to date, the mutagenicity, carcinogenicity and DNA binding activity of aflatoxin B<sub>1</sub> appear to result from its activation by cytochrome P450 enzymes to produce aflatoxin B<sub>1</sub>-8,9-epoxide. The metabolism of aflatoxin B<sub>1</sub> to the epoxide and to aflatoxin M<sub>1</sub> can be blocked in vitro (human hepatocytes) and in vivo (rats) by treatment with oltipraz, an antischistosomal drug, which blocks the formation of the epoxide and induces the major aflatoxin detoxication enzyme, glutathione-S-transferase (GST). Oltipraz is being tested in phase I and II clinical trials in China in the prevention of liver cancer; the results of these studies will be useful for clarifying the metabolism and mode of action of aflatoxins in humans.

Studies in human hepatocytes show wide variation among individuals in the metabolism and activation of aflatoxins. Human hepatocytes appear to form less of the epoxides of both aflatoxin B<sub>1</sub> and M<sub>1</sub> than rat hepatocytes. Conjugation of both epoxides with GST appears to occur more rapidly in mouse than in human hepatocytes. The details of the relationship between aflatoxin metabolism, activation, and detoxication in humans remains unclear.

### *Toxicological studies*

Aflatoxin M<sub>1</sub> is cytotoxic, as demonstrated in human hepatocytes in vitro and its acute toxicity in several species is similar to that of aflatoxin B<sub>1</sub>. In ducklings and rats, the acute and short-term toxicity of aflatoxin M<sub>1</sub> was similar to or slightly less than that of aflatoxin B<sub>1</sub>. In studies of carcinogenicity, aflatoxin M<sub>1</sub> was about one order of magnitude less potent than aflatoxin B<sub>1</sub>, even in sensitive species like the rainbow trout and the Fischer rat. The in vitro genotoxic potency of aflatoxin M<sub>1</sub> was similar to that of aflatoxin B<sub>1</sub> in some test systems and between one-half and one-sixth that of aflatoxin B<sub>1</sub> in other test systems.

### *Observations in humans*

No studies were available on the association between the dietary intake of aflatoxin M<sub>1</sub> and the risk for liver cancer. The Committee reviewed the literature on this topic published since the previous evaluation to determine if the additional studies provided more accurate estimates of the dose–response relationships than those used in 1997. Studies in which the recently developed biomarkers of exposure (aflatoxin-albumin adducts in serum, aflatoxin–N<sup>7</sup>-guanine adducts in urine, aflatoxin M<sub>1</sub> metabolites in urine or patterns of *P53* mutations) were used did not provide additional evidence that would allow more accurate risk assessments. Studies with more sensitive markers of exposure to hepatitis B and/or C viruses in patients with liver cancer strongly suggested that the estimated fraction of cases of human liver cancer attributable to these viral infections is increasing. As a consequence, estimates of the potency of aflatoxin B<sub>1</sub> estimated at the forty-ninth meeting in 1997 are likely to be overestimates. At its present meeting, the Committee made a conservative estimate of the potency of aflatoxin M<sub>1</sub> on the basis of the estimates for aflatoxin B<sub>1</sub>.

### *Analytical methods*

Screening tests for aflatoxin M<sub>1</sub> in milk and milk products include radioimmunoassay and enzyme-linked immunosorbent assay methods. The former have found

little application in routine investigations of aflatoxin M<sub>1</sub> in milk, whereas the latter are more often used. For regulatory purposes, positive results in enzyme-linked immunosorbent assays must be confirmed by an accepted reference method.

The quantitative analytical methods for aflatoxin M<sub>1</sub> include thin-layer and liquid chromatography. Many of these methods were developed for the analysis of milk and milk powder but can often be used for other dairy products. Five such methods have been studied in formal collaborative studies, and their performance characteristics have been published. With the development of liquid chromatography in the 1980s, most laboratories abandoned use of thin-layer chromatography for the analysis of aflatoxin M<sub>1</sub>. Use of immunoaffinity cartridges for clean-up of milk extracts was introduced subsequently, and the combination of immunoaffinity and liquid chromatography now offers the best means for efficient clean-up and precise determination of low concentrations of aflatoxin M<sub>1</sub>. A method involving a combination of immunoaffinity column clean-up with thin-layer chromatography and a computer-based low-cost densitometer is being validated for low concentrations of aflatoxins in a formal collaborative study.

#### *Sampling protocols*

Sampling plans for aflatoxins in granular feed products have been evaluated, but there has been little work on the evaluation of sampling plans to detect aflatoxin M<sub>1</sub> in milk. However, the European Union, the USA, and the Southern Common Market (Mercado Comun del Sur – MERCOSUR) have designed sampling plans for aflatoxin M<sub>1</sub>. A Directive of the Commission of the European Union specifies that from a batch of milk mixed by manual or mechanical means a minimum sample of 0.5 kg (or L) composed of at least five increments should be collected. The batch is accepted if the concentration of aflatoxin M<sub>1</sub> does not exceed the permitted limit. In the USA, the Food and Drug Administration stipulates that samples should consist of at least 4.5 kg of milk, composed of no fewer than 10 randomly selected portions.

As the distribution of aflatoxin M<sub>1</sub> in liquid milk can be expected to be reasonably homogeneous, sampling of liquid milk for aflatoxin M<sub>1</sub> will involve less uncertainty than sampling of granular feed products for aflatoxins. Most of the uncertainty in estimating aflatoxin M<sub>1</sub> in milk is probably associated with the analytical procedure.

#### *Effects of processing*

The results of the numerous studies on the effect of milk processing on the concentration of aflatoxin M<sub>1</sub> are variable. The concentration is not appreciably reduced by heat treatment. Production of yoghurt, cheese, cream, milk powder, or butter does not lead to loss of aflatoxin M<sub>1</sub>, although it is redistributed differentially into the products resulting from these processes.

Aflatoxin M<sub>1</sub> can be partially eliminated from milk by physical or chemical procedures, which include use of adsorbents, hydrogen peroxide, and ultra-violet radiation. These treatments are not readily applicable for the dairy industry, however, and their safety has not been tested; moreover, the costs may be prohibitive for large-scale application.

#### *Food consumption/dietary intake assessment*

Data on the concentrations of aflatoxin M<sub>1</sub> in milk were submitted by Argentina, Brazil, Canada, Indonesia, Norway, the United Arab Emirates, the USA, and several

Member States of the European Union; some data were also obtained from the literature. Some data may reflect biased or limited sampling designs and may not be representative of the area or nation in which the samples were obtained. Important information (number of samples, analytical quality assurance, mean value, individual sample values) was not available for some data sets. This does not imply that the data were not of good quality, but that they should be used with caution. All the data on aflatoxin M<sub>1</sub> that were submitted were used in the evaluation.

The dietary intake of aflatoxin M<sub>1</sub> was estimated from data on concentrations of aflatoxin M<sub>1</sub> in milk and from data on milk consumption in the GEMS/Food regional diets. The weighted mean concentration of aflatoxin M<sub>1</sub> in milk was 0.023 µg/kg in the European-type diet, 0.022 µg/kg in the Latin American diet, 0.36 µg/kg in the Far Eastern diet, 0.005 µg/kg in the Middle Eastern diet, and 0.002 µg/kg in the African diet. These mean concentrations were based on 10 778 milk samples for the European-type diet, 893 for the Latin American diet, 1191 for the Far Eastern diet, 231 for the Middle Eastern diet, and 15 for the African diet. The intake of aflatoxin M<sub>1</sub> from milk was calculated to be 6.8 ng/person per day for the European-type diet, 3.5 ng/person per day for the Latin American diet, 12 ng/person per day for the Far Eastern diet, 0.7 ng/person per day for the Middle Eastern diet, and 0.1 ng/person per day for the African diet. Intake calculated from the European regional diet was used for the assessment of cancer risk because this diet had the highest milk consumption. If all milk consumed were contaminated with aflatoxin M<sub>1</sub> at the proposed maximum levels of 0.05 µg/kg or 0.5 µg/kg, the intake of aflatoxin M<sub>1</sub> from milk in the European regional diet would be 15 ng/person per day or 150 ng/person per day, respectively.

One approach for determining the potential effects on dietary intake of the two proposed maximum levels for aflatoxin M<sub>1</sub> in milk involves estimating intake on the basis of mean aflatoxin M<sub>1</sub> concentration in milk for all samples, for all samples containing less than 0.5 µg/kg, and for all samples containing less than 0.05 µg/kg for a given population. The three calculated concentrations are multiplied by the milk consumption of the population of interest to determine the intake of aflatoxin M<sub>1</sub>. The most recent data on aflatoxin M<sub>1</sub> in milk in some European Union Member States (analyses of 7573 samples reported in 1999) indicated that all samples contained < 0.05 µg/kg, so the choice of either maximum level would not affect intake. Similarly, the data from Canada (81 samples reported in 1997-98) showed that all milk samples analysed contained < 0.015 µg/kg (the limit of detection) of aflatoxin M<sub>1</sub> so intake would not be affected at either level. The USA submitted individual data for 3620 samples collected between 1995 and 2000. On the basis of these data, the intakes of aflatoxin M<sub>1</sub> from milk were estimated to be 0.030 ng/kg of body weight per day for all samples, 0.023 ng/kg of body weight per day when a maximum level of 0.5 µg/kg was used, and 0.0035 ng/kg of body weight per day for a maximum level of 0.05 µg/kg. As other submissions and data from the literature did not include individual values for samples containing more than 0.5 µg/kg or more than 0.05 µg/kg aflatoxin M<sub>1</sub> in milk, similar calculations could not be performed on any of the other data.

Another approach for determining the effect of the proposed maximum levels on dietary intake is to generate distribution curves for the regional data for the concentrations of aflatoxin M<sub>1</sub> in milk. The distribution was constructed assuming log normality and with mean aflatoxin M<sub>1</sub> concentrations in the GEMS/Food regions and the maximum values reported. The distribution curve for the European regional

diet showed that if 0.05 and 0.5 µg/kg were set as the maximum levels, they would be at the extreme end of the distribution and consequently would have no effect on intake. The distribution curve for milk samples in the Middle Eastern diet, for which there were relatively few data, was similar to that for the European-type diet. For Latin American diets, selection of a maximum level of 0.5 µg/kg would also have no effect; however, use of a maximum level of 0.05 µg/kg would probably reduce intake. In the Far Eastern diet, in which the contamination of milk is higher, intake of aflatoxin M<sub>1</sub> would be decreased at both proposed levels, but it should be noted that milk consumption is low in the Far Eastern diet.

#### *Prevention and control*

About 0.3–6.2 % of aflatoxin B<sub>1</sub> in animal feed is transformed to aflatoxin M<sub>1</sub> in milk. For contaminated feed, a linear relationship has been found between intakes of aflatoxin B<sub>1</sub> in feed ranging from 5 to 80 µg/kg and the aflatoxin M<sub>1</sub> content of milk, as follows: aflatoxin M<sub>1</sub> (ng/kg milk) = [1.19 x aflatoxin B<sub>1</sub> intake (µg/cow/day)] + 1.9. Thus, production of milk containing aflatoxin M<sub>1</sub> at 0.05 µg/kg (the proposed Codex limit) would require that the average intake of aflatoxin B<sub>1</sub> by dairy cows be limited to approximately 40 µg/day/cow. On the basis of a daily feed consumption of 12 kg/cow of compound feeds, the use of the limit of 40 µg aflatoxin B<sub>1</sub> would mean that the content of aflatoxin B<sub>1</sub> in the feed would have to be 3.4 µg/kg in order to meet the limit of 0.05 µg/kg for aflatoxin M<sub>1</sub>.

The most effective means for controlling aflatoxin M<sub>1</sub> in the food supply is to control the amount of aflatoxin B<sub>1</sub> in feed for dairy cows. Specific regulations exist in many countries to control aflatoxin B<sub>1</sub> in the feed supply, but in parts of the world where cottonseed and maize are incorporated into animal feed, an effective aflatoxin control programme for feed may be difficult to design, particularly at low aflatoxin levels. The difficulty is related to the heterogeneous distribution of aflatoxin in these commodities, which results in a high degree of sampling variability.

The concentration of aflatoxin B<sub>1</sub> in feed can be reduced by good manufacturing practice and good storage practices. If preventive measures fail, however, aflatoxin B<sub>1</sub> can be reduced in feed by blending or by physical or chemical treatment. The physical treatments include heat, microwaves, gamma-rays, X-rays, ultra-violet light, and adsorption. Adsorption of aflatoxins onto hydrated sodium calcium aluminosilicate and other inert materials has been used in the animal feed industry in an attempt to reduce the aflatoxin M<sub>1</sub> content of milk. The most successful chemical procedure for degrading aflatoxins in animal feed is treatment with ammonia. Ammoniation of agricultural commodities leads to decomposition of 95–98 % of the aflatoxin B<sub>1</sub> present, and this procedure is used in various countries.

## **11. EVALUATION**

Since aflatoxin M<sub>1</sub> is a metabolite of aflatoxin B<sub>1</sub> and is presumed to induce liver cancer in rodents by a similar mechanism, estimates of the potency of aflatoxin B<sub>1</sub> can be used for determining the risk due to intake of aflatoxin M<sub>1</sub>. No adequate epidemiological studies exist on the dose–response relationships between the intake of aflatoxin M<sub>1</sub>, exposure to hepatitis B or C virus, and liver cancer. The Committee therefore assumed that aflatoxin M<sub>1</sub> acts similarly to aflatoxin B<sub>1</sub> with hepatitis B (and possibly) C virus. The Committee used the comparative figure for carcinogenic

potency derived at its forty-ninth meeting and assumed that the potency of aflatoxin M<sub>1</sub> was one-tenth that of aflatoxin B<sub>1</sub> in the Fischer rat. The carcinogenic potency of aflatoxin M<sub>1</sub> was estimated to be 0.001/100 000 per year per ng/kg of body weight per day in HBsAg<sup>-</sup> individuals and 0.03/100 000 per year per ng/kg of body weight per day in HBsAg<sup>+</sup> individuals.

The estimates of the potency of aflatoxin M<sub>1</sub> were combined with estimates of intake from the GEMS/Food European regional diet. The potency of aflatoxin M<sub>1</sub> in a human population with an HBsAg prevalence, *P*, was projected to be the weighted combination of the potency estimates for HBsAg<sup>-</sup> and HBsAg<sup>+</sup> individuals. Specifically, the average potency of aflatoxin M<sub>1</sub> in a population of individuals with a hepatitis B virus prevalence rate *P* was taken to be: Potency = 0.001 × (1−*P*) + 0.03 × *P*.

Three rates of HBsAg<sup>+</sup> prevalence (1, 5, and 25%) were considered to span the range of rates of infection with hepatitis B virus observed in various populations in western and South-East Asian countries. The risks for liver cancer were projected to be the product of the average potency values and estimates of intake of aflatoxin M<sub>1</sub> from a European-type diet, corresponding to a relatively high intake of milk and milk products. The projected risks due to aflatoxin M<sub>1</sub> were calculated for the two proposed maximum levels, 0.5 and 0.05 µg/kg. It was assumed that all products were contaminated at the two proposed maximum levels, giving worst-case projections of the population risk. Projected risks were also calculated for the weighted mean of 0.023 µg/kg of milk for the European regional diet (see Table 18).

The calculations show that, with worst-case assumptions, the projected risks for liver cancer at the proposed maximum levels of aflatoxin M<sub>1</sub> of 0.05 and 0.5 µg/kg are very small. For example, in a population with a prevalence of hepatitis B virus infection of 1%, which is typical for the USA and western Europe, the additional numbers of liver cancer cases associated with contamination of all milk with aflatoxin M<sub>1</sub> at 0.5 µg/kg versus 0.05 µg/kg would be 29 (i.e. 32 minus 3.2) cancers/1000 million persons per year. The potency of aflatoxin M<sub>1</sub> appears to be so low in HBsAg<sup>-</sup> individuals that a carcinogenic effect of aflatoxin M<sub>1</sub> intake in those who consume large quantities of milk and milk products in comparison with non-consumers of these products would be impossible to demonstrate.

Hepatitis B virus carriers might benefit from a reduction in the aflatoxin concentration in their diet, and the reduction might also offer some protection to hepatitis C virus carriers. Reduction of the current concentrations of aflatoxins in the diet in most developed countries is unlikely to produce an observable reduction in the rates of liver cancer.

At its present meeting, the Committee reiterated its previous conclusion that the liver cancer burden could best be reduced by giving priority to hepatitis B virus vaccination campaigns and to prevention of hepatitis C virus infection, which implies reinforcement of the control of blood and blood products and the use of sterile medical equipment.

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**Appendix A**

**Results of surveys for aflatoxin M<sub>1</sub>, showing concentrations and distribution of contamination in food commodities**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 0.05 (µg/kg)	n > 0.5 (µg/kg)	References	Sampling procedure
<b>Americas</b>											
Argentina	Farm milk	Mar–Sept 1999	56	0.01*	50	0.0017/0.030	0	0		CEREMIC; A, ELISA	Whole and defatted Commercial samples
	Powdered milk	Mar–Sept 1999	5	0.01*	1	0.010/0.014	0	0		(Ridascreen) (Ridascreen)	
	Pasteurized fluid milk	Mar–Sept 1999	16	0.01*	8	0.0065/0.017	0	0			
Brazil	Milk	NR	224	NR	220	Trace/0.002	0	0		P, Martins & Martins (1986)	
Brazil	Pasteurized milk	1992	52	0.025	48	0.012/0.37	4	0		P,S, de Sylos et al. (1996); A (HPLC), AOAC 986.16 (1990)	
Brazil	Raw milk	1992–93	144	NR	144	0	0	0	0	P, Correa et al. (1997)	
Brazil	Powdered milk, pasteurized	1989	86	0.15	86	0	0	NR	0	P,S, de Sylos et al. (1996); A (TLC), AOAC 980.21 (1990)	
Brazil	Milk powder, reconstituted for infants	Oct. 1992–Jan. 1993	300	0.1	267	0.030/1.0		> 33	6	P,S, A (ELISA), Oliveira et al. (1997)	
Brazil	Yoghurt	1990	30	0.15	30	0	0	0	0	P,S, de Sylos et al. (1996); A (TLC), AOAC 980.21 (1990)	
Brazil	Milk	1996–97	110	0.0063	83	NR/0.0071		0	0	P,S, Souza et al. (1999); A, ELISA	

Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 0.05 (µg/kg)	<i>n</i> > 0.5 (µg/kg)	References	Sampling procedure
<b>Americas (contd)</b>											
Brazil	Milk and milk products	1998–99	61	0.006	11	0.020/0.077		0.006–0.077	0	P,S, Prado et al. (2000); A, HPLC	
	Pasteurized milk		18	0.006	3	0.027/0.077		0.008–0.077	0		
	Long-life milk	1998–99	21	0.006	5	0.018/0.070		0.006–0.070	0		
	Long-life milk, infant		5	0.006	1	0.010/0.021		0	0		
	Milk powder		6	0.006	0	0.012/0.038		0	0		
	Milk powder, infant		11	0.006	2	0.025/0.050		0.007–0.050	0		
Canada	Homogenized milk	1994	15	0.015		< 0.069/0.21		NR	0	USA (FDA); industry Health Canada	
	2% partly skimmed milk	1994	7	0.015		< 0.034/0.16		NR	0		
	Skimmed milk powder	1994	2	0.015	1	< 0.030/0.059		1	0		
	Partly skimmed milk, lactose reduced	1994	2	0.015	1	< 0.053/0.090		1	0		
	1% partly skimmed milk	1994	1	0.015	1	0	0	0	0		
	Evaporated milk	1994	1	0.015	0	0.06		1	0		
	Partly skimmed milk, lactaid	1994	1	0.015	0	0.038		0	0		
	Skimmed milk, lacteeze	1994	1	0.015	1	0	0	0	0		
Uruguay	Milk	1993–95	22		15	0.002/ > 0.02		0	0	P, Pineiro et al. (1996)	
USA	Raw milk	1995	695		682	0.0017/0.33	0.000	13	0	FDA laboratory 27	
	Raw milk	1996	381		287	0.035/1.8	0.070	94	5		

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 0.05 (µg/kg)	n > 0.5 (µg/kg)	References	Sampling procedure
<b>Americas (contd)</b>											
USA	Raw milk	1997	717		697	0.0023/0.41	0.000	20	0	FDA laboratory 27	
	Raw milk	1998	855		839	0.0025/0.38	0.000	16	0		
	Raw milk	1999	877		851	0.0024/0.24	0.000	26	0		
	Raw milk	2000	475		459	0.0044/0.37	0.000	16	0		
	Finished milk	1995	79		76	0.0032/0.11	0.000	3	0		
	Finished milk	1996	21		17	0.0110/0.08	0.060	4	0		
	Finished milk	1997	53		52	0.0011/0.06	0.000	1	0		
	Finished milk	1999	60		58	0.0038/0.17	0.000	2	0		
	Finished milk	2000	12		12	0	0	0	0		
	Southwest	Milk	1998–2000	5 801	0.02*/ 0.05	4 562	NR/> 0.5		1239 639 (0.05–0.1) 399 (0.1–0.2) 134 (0.2–0.3) 11 (0.3–0.4) 11 (0.4–0.5)		45
Midwest	Milk	1998–2000	438	0.5	428	NR/> 0.5		NR	10	FDA; industry	
Southeast	Milk	1998–2000	13 093	0.5	13 079	NR/> 0.5		NR	14	FDA; industry	
Southern	Milk	1998	163	0.05	78	0.73/14.0	1.4	82	47	FDA laboratory B	
Southern	Milk	1999	168	0.05	96	0.17/5.2	0.56	59	20	FDA laboratory B	
Southern	Milk	2000	49	0.05	32	0.069/0.50	0.28	13	1	FDA laboratory B	
Southeast	Milk	Jun 1999–May 2000	199	0.1*	196	NR/< 0.25		3 (0.1–< 0.25)	0	FDA laboratory A	
Southeast	Milk	Jul 1997–May 2000	78	0.1*	49	NR/> 0.5		29 (< 0.1– > 0.5)		FDA; industry	

Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 0.05 (µg/kg)	n > 0.5 (µg/kg)	References	Sampling procedure
<b>Asia</b>											
China, north	Powdered milk	NR	NR	0.08*	2	NR		> 17 ( < 0.5)	3	P,S, Liu et al. (1992); A, ELISA	From factories and markets in 12 provinces
China, south	Powdered milk	NR	NR	0.08*	7	NR/4.2		>19 ( < 0.5)	33		
China	Powdered milk	1992–93	27	0.015	1	0.10/0.46		16	0	P,S, Kawamura et al. (1994);	
Taiwan	Fresh milk	Mar–Sept 1986	56	0.1	56	0		NR	0	P, van Egmond (1989)	
	Powdered milk	Mar–Sept 1986	161	0.1	161	0		NR	0		
India	Raw milk	1992–93	504		415	0.20/3.50		89	> 65	P,S, Rajan et al. (1995); A, AOAC (1990), HPLC	
	Raw milk	1993	325		289	0.12–1.0		36	3		
Indonesia	Milk	Jan 1990	55	0.1	15	1.3/21		> 40	16	P,S, RIVS (2000); A, AOAC (1984)	
	Milk	Sept 1990	30	0.1	0	5.4/23		> 30	8		
	Milk	July 1991	53	0.1	10	0.48/2.0		> 43	5		
	Milk	Dec 1991	50	0.1	32	0.31/3.4		> 18	4		
	Milk	Jan 1992	80	0.1	51	0.78/9.7		> 29	11		
Java Indonesia	Milk	Jan 1993	64	0.1	34	0.85/6.7		> 30	23	P,S, DL/RIVS (2000); A, AOAC (1984)	
	Milk/factory	Sept 1999	10	0.1	1	0.92/2.1		> 9	6		
	Ice cream/factory	Aug 1999	5	0.1	0	0.31/0.48		> 5	0		
	Ice cream/factory	Oct 1999	11	0.1	11	0		NR	0		
Korea, Republic of	Raw milk	Winter 1995	49	0.003*	0	0.065/0.16	0.120	29	0	P,S, and A (ELISA), Shon et al. (1996)	
	Market milk	Winter 1995	15	0.003*	0	0.13/0.28		18	0		
	Market milk	Summer, winter 1997	70	0.01 0.002	31 17	0.014/0.052 0.014/0.037		3 0	0 0		P,S,A (HPLC), Kim et al. (2000)

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 0.05 (µg/kg)	<i>n</i> > 0.5 (µg/kg)	References	Sampling procedure
<b>Asia</b> (contd)											
Korea, Republic of	Infant formula	Autumn 1997	26	0.01	8	0.043/0.13		12	0	P,S,A (HPLC), Kim et al. (2000)	
				0.002	4	0.039/0.93		9	0		
	Powdered milk	Autumn 1997	24	0.01	7	0.16/0.34		17	0		
				0.002	6	0.15/0.33		17	0		
	Yoghurt	Autumn, winter, 1997	60	0.01	29	0.023/0.12		9	0		
				0.002	10	0.024/0.17		10	0		
Philippines	Milk	1997	91	0.05	11	0.13/NR		80	16	P, Begino (1998)	
Thailand	Raw milk	1990–93	45	0.1	33	0.11/0.80	0.42	> 12	4	P,S, Boriboon & Suprasert (1994); A, IDF (1981) P,S, Saitanu (1997); A, RIA	~ 50 ml collected from 50-L farm milk can and tested within 3–4 days or stored at –15 °C until testing within 1 month
	Raw milk	1995–96	67	0.025	1	NR		57	17		
	Pasteurized milk	1990–93	15	0.1	6	0.80/6.6	1.1	> 9	7		
	Pasteurized milk	1995–96	63	0.025	0	NR		63	20		
	UHT milk		60	0.025	0	NR		60	7	P,S, Saitanu (1997); A, RIA	One sample from each lot of factory- treated products tested on arrival or kept at 4 °C and tested within 2 days
	Sterilized milk		60	0.025	0	NR		60	3		

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 0.05 (µg/kg)	<i>n</i> > 0.5 (µg/kg)	References	Sampling procedure
<b>Asia</b> (contd)											
Thailand	Powdered milk	1995–96	31	0.1	30	1.4		> 1	1	P,S, Boriboon & Suprasert (1994); A, IDF (1981)	Tested within 2 weeks Reconstituted with distilled water (1:7) to obtain 12.5% (w/v) immediately before testing
	Powdered milk, imported	1995–96	13	0.025	11	NR		2	0		
	Pelleted milk	1995–96	7	0.025	0	NR		7	1		
United Arab Emirates	Fresh milk	1998	22	0.01/0.005*	1	NR/0.31		21	0	Dubai Central Laboratory	immunoaffinity column–HPLC/fluorescence. Samples from local dairy farms. Three units of liquid milk mixed, and representative samples stored in a refrigerator
	UHT milk	1998	11	0.01/0.005*	11	0	0	0	0		
	Milk/imported	1999	15	0.01/0.005*	12	NR/0.08		NR	0		
	Low fat milk	1999	11	0.01/0.005*	2	NR/0.24		NR	0		
	Milk powder	1998	8	0.01/0.005*	8	0	0	0	0		
	Milk powder	1999	8	0.01/0.005*	8	0	0	0	0		
	Full cream milk	1999	22	0.01/0.005*	14	NR/0.35		NR	0		
	Skimmed milk	1999	9	0.01/0.005*	2	NR/0.14		NR	0		
	Milk-based infant formula	1998	11	0.01/0.005*	11	0	0	0	0		
	Milk-based infant formula	1999	3	0.01/0.005*	3	0	0	0	0		
<b>Europe</b>											
Austria	Milk powder	Feb 1983 Apr 1986	65	0.03	65	0	0	0	0	P, van Egmond (1989)	
Austria	Milk	1990–93	479	0.01	479	0.005/ < 0.01	0	0	0	SCOOP Report (EU)	
	Milk	1999	20	0.005	20	0	0	0	0		

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 0.05 (µg/kg)	n > 0.5 (µg/kg)	References	Sampling procedure
<b>Europe</b> (contd)											
Belgium	Commercial blended milk	Winter 1984– 85	233		204	0.02–0.15		NR	0	P, van Egmond (1989)	
		Winter 1985– 86	89	0.03	89	0	0	0	0		
	Milk	1993–94	153	0.01*	98	0.01/0.037		0	0	SCOOP Report (EU) EU	
	Milk	1999	192	0.005	168	NR/< 0.05		24 (< 0.05)	0		
Denmark	Milk	1990–94	1664	0.01*	1240	< 0.01/0.069		424 (< 0.05)	0	SCOOP Report (EU)	
Cyprus	Raw milk	1993,1995– 96	71	0.005	68	0.0015/0.04		3	0	P,S, Ioannou-Kakouri et al. (1999); A, Ioannou-Kakouri et al. (1995)	
	Pasteurized milk, full		19	0.01*	14	0.0040/0.02		0	0		
	Pasteurized milk, light		4	0.01*	3	0.01		0	0		
	Pasteurized milk, skimmed		8	0.01*	5	0.0075/0.04		0	0		
	Baby milk, imported		6	0.005	6	0		0	0		
	Evaporated milk, imported		4	0.005	4	0	0	0	0		
Czech Republic	Raw milk	1987–88	376	NR	330	0.002–< 0.50			0	P, Fukal et al. (1990)	
	Raw milk	1987–88	89	NR	62	0.020–< 0.50			0		
	Milk	NR	191	NR	166	0.050–0.10		25	0		P, Fukal (1988)
Finland	Milk powder	1986	14	0.1	14	0	0	0	0	P, van Egmond (1989)	

Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 0.05 (µg/kg)	n > 0.5 (µg/kg)	References	Sampling procedure
<b>Europe (contd)</b>											
Finland	Milk	1992–15	122	0.01*	122	0.005/< 0.01	0	0	0	SCOOP Report (EU)	
	Milk	1995	59	0.005	59	0	0	0	0		
	Milk	1996	112	0.005	112	0	0	0	0		
	Milk	1997	378	0.005	377	< 0.0001/< 0.05		1(< 0.05)	0		
	Milk	1998	499	0.005	499	0	0	0	0		
	Milk	1999	296	0.005	295	< 0.0002/< 0.05		1(< 0.05)	0		
France	Milk	Nov 1984–	494	0.05	487	NR		7	0	P,S, Dragacci & Frémy (1993); A, Frémy & Boursier (1981)	
		May 1985	466	0.05	466	0	0	0	0		
		Sept–Nov 1986	265	0.05	265	0	0	0	0		
		Jan–Mar, Sept–Nov 1987	449	0.05	447	NR		2	0		
		Jan–Mar 1988	277	0.05	277	0	0	0	0		
		Nov 1988– Mar 1989	549	0.05	547	NR		2	0		
	Milk	Nov 1989– Mar 1990	526	0.03	511	NR		13	0	P,S, Dragacci & Frémy (1993); A, Tuinstra et al. (1974)	
		Nov 1990– Mar 1991	550	0.03	544	NR		3	0		
		Sept–Nov 1991	303	0.03	300	NR		0	0		
	Bulk raw milk	Nov 1984– May 1985	37	0.05	32	NR		5	0	P,S, Dragacci & Frémy (1993); A, Frémy & Boursier (1981)	
		June–Oct 1985	30	0.05	30	0	0	0	0		

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 0.05 (µg/kg)	<i>n</i> > 0.5 (µg/kg)	References	Sampling procedure	
<b>Europe (contd)</b>												
France (contd)	Bulk raw milk	Nov 1985– May 1986	42	0.05	41	NR		1	0	P,S, Dragacci & Frémy (1993); A, Frémy & Boursier (1981)		
		June–Oct 1986	26	0.05	26	0	0	0	0			
		Nov 1986– May 1987	24	0.05	24	0	0	0	0			
		June–Oct 1987	18	0.05	18	0	0	0	0			
		Nov 1987– May 1988	29	0.05	29	0	0	0	0			
		June–Oct 1988	27	0.05	27	0	0	0	0			
		Nov 1988– May 1989	8	0.05	8	0	0	0	0			
		June–Oct 1989	9	0.05	9	0	0	0	0		P,S, Dragacci & Frémy (1993); A, Tuinstra et al. (1974)	
		Nov 1989– May 1990	18	0.05	18	0	0	0	0			
	June–Oct 1990	19	0.05	19	0	0	0	0				
	Nov 1990– May 1991	35	0.05	35	0	0	0	0				
	June–Oct 1991	15	0.05	15	0	0	0	0				
	Nov 1991– May 1992	34	0.05	34	0	0	0	0				
			LOQ: 0.05, ND – < 0.05; 0.03, ND – < 0.03									
		Cheese, milk	1989–94	2670	0.01	2600 (< 0.05)	0.016/0.37		21	0	SCOOP Report (EU)	
		Heat-treated milk	1990–95	165	0.01	160 (< 0.05)	0.015/NR		2	0		
		Powdered milk	1989–94	134	0.1	130 (< 0.05)	0.15/< 0.3	0	0	0		
	Milk	1998	251	0.03	247	NR/< 0.05		3 (0.03–0.05)				
	Milk	1999	234	0.03	234	NR	0	0	0			

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 0.05 (µg/kg)	<i>n</i> > 0.5 (µg/kg)	References	Sampling procedure
<b>Europe</b> (contd)											
Germany	Herd's bulk milk	Nov 1984–Apr 1985	135		NR	0.003–0.080		NR	0	P, van Egmond (1989)	
		Nov 1985–Apr 1986	242		NR	0.003–0.100		NR	0		
		May–June 1986	74		NR	0.003–0.020		0	0		
	Pasteurized commercial milk	Nov 1984–Apr 1985	132		NR	0.003–0.060		NR	0		
		Nov 1985–Apr 1986	16		NR	0.007–0.013		0	0		
		Nov 1985–Apr 1986	473		NR	0.004–0.010		0	0		
	Milk, butter, cream, cheese, ice cream	1991–93	1853	0.01*	1692	0.006/0.07		159 (< 0.05), 2(0.051–0.10)	0	SCOOP Report (EU)	
	Baby food		206	0.1*	171	0.007/0.04		35 (< 0.05)	0		
	Cheese		110	0.1*	104	0.055/0.15		1 (< 0.05), 5 (0.051–0.10)	0		
	Milk	1996	2822		NR	NR/(0.033)*		0	0		
		1997	4902		NR	NR/(0.014)*		11 (> 0.005), 7 (> 0.01)			
		1998	6150		NR	NR/(0.036)*		7 (> 0.005), 8 (> 0.01)			
1998–2000 (45)		6537	0.005	4775	NR		205 (< 0.05)	0			
	2000	3307	0.01	1550	NR		7 (< 0.05)	0			
				NR	NR (0.007, max stated value)		9 (> 0.005), 2 (> 0.01)	NR			

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 0.05 (µg/kg)	<i>n</i> > 0.5 (µg/kg)	References	Sampling procedure
<b>Europe</b> (contd)											
Greece	Raw milk	Nov 1986–Mar 1987	99	0.1*	95	0.10–0.13		> 4	0	P,S, Karaioannoglou et al. (1989); A, IDF (1982)	
	Pasteurized milk		51	0.1*	51	0	0	NR	0		
	Pasteurized milk	1995–96	81	0.005*	72	0.0072/0.18		3	0	P,S, and A, Markaki & Melissari (1997); A, Qian et al. (1994)	
Ireland	Milk	1999	62	0.02	60	NR/< 0.05		2 (< 0.05)		EU	
Italy	Milk	NR	59		13	Trace–0.38		NR	0	P, Davoli et al. (1986)	
	Milk	NR	27		3	0.005–0.065		NR	0	P, Gelosa (1986)	
	Milk	NR	107		102	0.024–0.094		NR	0	P, Gilli et al. (1987)	
	Milk	NR	104		104	0	0	0	0	P, Oliviero et al. (1987)	
	Milk, imported	1984	313	0.001*	271	0.001–0.050		0	0	P,S, Piva et al. (1987);	
	Milk, dairy farm	1985	276	0.001*	206	0.001–0.20		7	0	A (HPLC), AOAC (1984)	
	Milk, dairy farm	NR	176		176	0		0	0	P, Mosso et al. (1992)	
	Milk, dairy farm	NR	107		41	0.006–0.10		0	0	P, Bagni et al. (1992)	
	Milk, dairy farm	NR	107		51	0.003–0.060		NR	0	P, Bagni et al. (1992)	
	Milk, dairy farm	1995	159	0.001	23	0.009/0.11		2	0	P,S, Galvano et al. (1998); A, Mortimer et al. (1987)	
	Dry milk	NR	10		0	0.015–0.10		NR	0	P, Vittani (1987)	
	Dry milk	1995	97	0.001	16	0.018/0.10		10	0	P,S, Galvano et al. (1998); A, Mortimer et al. (1987)	
	Raw milk	NR	57		33	0.10–0.93		NR	NR	P, De Natale et al. (1989)	
	Raw milk	NR	60		57	0.10–0.28		NR	0		
	Pasteurized milk	NR	68		7	0.005–0.050		NR	0	P, Gelosa (1986)	
	Pasteurized milk	NR	30		3	0.003–0.022		0	0	P, Vittani (1987)	
	Yoghurt	1995	114	0.001	23	0.014/0.50		2	0	P,S, Galvano et al. (1998); A, Richard et al. (1993)	

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 0.05 (µg/kg)	<i>n</i> > 0.5 (µg/kg)	References	Sampling procedure
<b>Europe (contd)</b>											
Italy	Milk	1984–95	997	0.001*– 0.5*	579	NR/< 0.05	398 (< 0.05)	0		SCOOP Report (EU); A, Immunoaffinity– HPLC/fluorescence	
	Cheese	1984–95	1593	0.001*– 0.5*	1270	NR/< 0.05	323 (< 0.05)	0			
Netherlands	Bulk milk and milk powder	Jan–Apr 1985	209	0.010	40	0.010–0.070	NR	0		P, van Egmond (1989)	
		May–Aug 1985	207	0.010	54	0.010–0.050	NR	0			
		Sept–Dec 1985	207	0.010	19	0.010–0.090	NR	0			
		Jan–Apr 1986	212	0.010	38	0.010–0.090	NR	0			
		May–Aug 1986	204	0.010	89	0.010–0.070	NR	0			
		Sept–Dec 1986	202	0.010	37	0.010–0.050	NR	0			
	Milk	1990–93	1903	NR	331	0.009/0.02	1572 (< 0.05*, legal limit)	0		SCOOP Report (EU); A, HPLC/fluorescence EU	
Milk powder, home- produced	1998	168	0.01	168	0	0	0	0	EU		
Milk powder, imported	1998	39	0.01	NR	NR	NR	NR	NR	EU		
Milk	1999	30	0.005	25	NR/< 0.05	5 (< 0.05)	0	0	EU		
Norway	Milk	1998	54	0.0001	3	0.0014/0.009	0	0		Norwegian Food Control Authority	
Poland	Raw milk	1993–94	30	0.003*	24	0.0036–0.0106	0	0		P,S, Domagala et al. (1997)	
		1993–94	157	0.003*	120	< 0.010–0.025	0	0			
Portugal	Milk	1999	96	NR	28	NR/< 0.05	68 (< 0.05)	0		EU	

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 0.05 (µg/kg)	<i>n</i> > 0.5 (µg/kg)	References	Sampling procedure
<b>Europe (contd)</b>											
Spain	Raw milk	May–Nov 1990	61	0.01	52	0.010–< 0.050		0	0	P,S, Macho et al. (1992); A (HPLC), Mortimer et al. (1987) P, Jalon et al. (1994)	
	UHT milk	May–Nov 1990	33	0.01	28	0.010–0.025		0	0		
	Raw milk	May–Nov 1990	61	0.01	49	0.010–0.040		0	0		
			29	0.01	28	0.020–0.040		0	0		
			32	0.01	21	0.010–0.040		0	0		
	Milk	1989–92	307	0.005*–0.5*	234	0.006/0.046		73 (< 0.05)	0	SCOOP Report (EU); A, HPLC	**Excluding rejected data
	Butter, cream, cheese, and ice cream	1989–92	221**	0.005*–0.5*	217	0.022/0.29		5** (< 0.05)	0		
	Baby food, powdered milk	1989–92	4	0.005*–0.5*	0	0.06/0.09		4 (< 0.05)	0		
Confectionary	1989–92	2	0.005*–0.5*	0	0.033/0.05		2 (< 0.05)	0			
Milk (34 UHT & 8 pastuerized)	1998	42	NR	NR	NR/0.027		0	NR	EU		
Sweden	Milk	Jan–Mar 1986	268	0.005	239	0.005–0.31		NR	0	P, van Egmond (1989)	
	Milk	Apr–June 1986	271	0.005	118	0.005–0.050		NR	0		
	Milk powder	Dec 1985	14	0.005	0	0.006–0.057		NR	0		
	Milk	1999	11	0.005	11	0	0	0	0	EU	
United Kingdom	Milk, farm–gate	1988	118	0.01	107	0.010–0.18		2	0	P,S, MAFF (1993)	
	Milk, farm–gate	1989	127	0.01	101	0.010–0.16		3	0		
	Milk	1988–89, 1995	331	0.01	255	0.01/0.22		67 (< 0.05), 6 (0.051–0.10), 3 (> 0.10)	0	SCOOP Report (EU); A, HPLC/fluorescence	

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 0.05 (µg/kg)	<i>n</i> > 0.5 (µg/kg)	References	Sampling procedure
<b>Europe (contd)</b>											
United Kingdom	Powdered milk	1988–89, 1995	93	0.02	89	0.01/0.05	4 (< 0.05)		0	SCOOP Report (EU)	
	Yoghurt	1988–89, 1995	30	0.02	24	0.01/0.04		6 (< 0.05)	0		
	Cheese	1988–89, 1995	73	0.02	0	0.08/0.22		24 (< 0.05), 34 (0.051–0.10), 15 (> 0.10)	0		
	Retail milk	1994–95	162	0.01	74	NR		88 (< 0.05)	0	EU	
	Dried milk	1995	93	0.02	89	NR/0.05		4 (0.05)	0	EU	31 dried milk and 62 infant formula dried milk; 4 infant formula samples had max of 0.05, i.e. < 0.05 when reconstituted
	Milk	1999	95	0.001	93	NR/< 0.05		2 (< 0.05)	0	EU	

When the mean value for a positive samples was quoted and used (or its use implied), the data were recalculated, so all data represent the true mean for all samples, those below the limit of detection being considered 0. When possible, the numbers of samples with values > 0.05 and 0.5 µg/kg are included, as these values represent the legislative limits in many countries.

LOQ, limit of quantification; mean, true mean (for *n* analytical values; the true mean is the sum  $X_i / n$ , where  $X_i$  is the value of each analytical result); Max, maximum level

References: P, parent reference; S, sampling method; A, analytical method

\*, limit of detection

ND, not determined; NR, not reported; TLC, thin-layer chromatography; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; RIA, radioimmunoassay

CEREMIC, Centro de Referencia de Micología (Argentina); EU, European Union; FDA, Food and Drug Administration (USA); IDF, International Dairy Federation; MAFF, Ministry of Agriculture, Fisheries and Food (United Kingdom); RIVS, Research Institute for Veterinary Science (Indonesia)

**Appendix B**

**Concentrations of aflatoxin M<sub>1</sub> in milk**

Country	Commodity	Date	No. of samples	LOQ/LOQ µg/kg	n (%)				Mean (µg/kg)	Maximum (µg/kg)	90th %ile (µg/kg)	References
					< LOQ/LOQ	< 0.05 µg/kg	0.5 µg/kg	1 µg/kg				
<b>Submitted laboratory data</b>												
Argentina	Milk	1998	6	NR	1 (17) <sup>a</sup>	2 (33)	2 (33)	0 (0.0)	0.52	0.9	NR	Universidad de Lujan
Austria	Milk	1990–93	479	NR/0.01	479 (100)	479 (100)	479 (100)	0 (0.0)	0.005	< 0.01	NR	SCOOP Report
Belgium	Milk	1993–94	153	NR/0.01	98 (64)	153 (100)	153 (100)	0 (0.0)	0.01	0.037	NR	SCOOP Report
Canada	Milk, 7 types	1997–98	81	0.015/NR	81 (100.0)	81 (100)	81 (100)	0 (0.0)	< 0.015	< 0.015	NR	Health Canada (2000)
	Milk, 6 types	1995-96	92	0.015/NR	92 (100)	92 (100)	92 (100)	0 (0.0)	< 0.015	< 0.015	NR	
	Milk, 5 types	1994–95	57	0.015/NR	57 (100)	57 (100)	57 (100)	0 (0.0)	< 0.015	< 0.015	NR	
	Milk, 5 types	1993–94	27	0.015/NR	13 (48)	17 (63)	27 (100)	0 (0.0)	< 0.056	0.21	NR	
Denmark	Milk	1990–94	1664	0.01/NR	1240 (74)	1664 (100)	1664 (100)	0 (0.0)	< 0.01	0.069	NR	SCOOP Report
European Union	Milk	1999	7573	Variable	7259 (96)	7573(100)	7573(100)	0 (0.0)	NR	NR	NR	European Union
Finland	Milk	1992–95	122	0.01/NR	122 (100)	122 (100)	122 (100)	0 (0.0)	0.005	< 0.01	NR	SCOOP Report
France	Milk, raw	1989–94	2670	NR/0.01	0 (0.0)	2649 (99)	2670 (100)	0 (0.0)	0.016	0.37	NR	SCOOP Report
	Milk, pasteurized	1990–95	165	NR/0.01	0 (0.0)	163 (99)	165 (100)	0 (0.0)	0.015	NR	NR	SCOOP Report

Appendix B (contd)

Country	Commodity	Date	No. of samples	LOQ/LOQ µg/kg	n (%)				Mean (µg/kg)	Maximum (µg/kg)	90th %ile (µg/kg)	References
					< LOQ/LOQ	< 0.05 µg/kg	0.5 µg/kg	1 µg/kg				
Indonesia	Milk	1990-93, 1999	342	NR/0.1	143 (42)	NA	269 (79)	NR	0.86	23	NR	Research Institute for Veterinary Science
Italy	Milk	1984-95	997	0.001/NR	579 (58)	977 (98)	997 (100)	0 (0.0)	NR	NR	NR	SCOOP Report
Netherlands	Milk	1990-93	1903	NR	331 (17)	1572 (83)	1903 (100)	0 (0.0)	0.009	0.02	NR	SCOOP Report
Norway	Milk, dairy	1998	54	0.0001/NR	3 (5.6)	54 (100)	54 (100)	0 (0.0)	0.0014	0.009	NR	Food Control Authority
Spain	Milk	1989-92	307	0.005/NR	234 (76)	307 (100)	307 (100)	0 (0.0)	0.006	0.046	NR	SCOOP Report
United Arab Emirates	Milk, raw	1998	22	0.005/0.01	1 (4.5)	1 (4.5)	22 (100)	0 (0.0)	NR	0.31	NR	Dubai Central Laboratory
	Milk, whole	1999	22	0.005/0.01	14 (64)	NR	22 (100)	0 (0.0)	NR	0.35	NR	
	Milk, UHT	1998	11	0.005/0.01	11 (100)	11 (100)	11 (100)	0 (0.0)	0	0	0	
	Milk, imported	1999	15	0.005/0.01	12 (80)	NR	15 (100)	0 (0.0)	NR	0.08	NR	
	Milk, low-fat	1999	11	0.005/0.01	2 (18)	NR	11 (100)	0 (0.0)	NR	0.24	NR	
	Milk, non-fat	1999	9	0.005/0.01	2 (22)	NR	9 (100)	0 (0.0)	NR	0.14	NR	
United Kingdom	Milk	1988-89	331	NR/0.01	255 (77)	322 (97)	331 (100)	0 (0.0)	0.01	0.22	NR	SCOOP Report
USA												
Southwest	Milk	1998-2000	5801	0.02/0.05	4562 (79)	4562 (79)	5756 (99)	NR	NR	> 0.5	NR	Industry
Midwest	Milk	1998-2000	438	0.5/NQ	10 (2.3)	10 (2.3)	428 (98)	NR	NR	> 0.5	NR	Industry

**Appendix B** (contd)

Country	Commodity	Date	No. of samples	LOQ/LOQ µg/kg	n (%)				Mean (µg/kg)	Maximum (µg/kg)	90th %ile (µg/kg)	References
					< LOQ/LOQ	< 0.05 µg/kg	0.5 µg/kg	1 µg/kg				
<b>USA (contd)</b>												
	Southeast Milk	1998–2000	13 093	0.5/NQ	NR	NR	13 079 (100)	NR	NR	> 0.5	NR	Industry
	Southeast Milk	1999–2000	199	0.02/0.05	196 (98) <sup>a</sup>	NR	199 (100)	0 (0.0)	NR	< 0.5	NR	FDA Laboratory A
	Southeast Milk	1997–2000	78	0.02/0.05	49 (63) <sup>a</sup>	NR	71 (91)	NR	NR	> 0.5	NR	FDA Laboratory A
	Southern Milk	1998	163	0.02/0.05	81 (50)	81 (50)	117 (72)	21 (13)	0.73	14	1.5	FDA Laboratory B
	Southern Milk	1999	167	0.02/0.05	108 (65)	108 (65)	147 (88)	2 (1.2)	0.17	5.2	0.58	FDA Laboratory B
	Southern Milk	2000	49	0.02/0.05	36 (74)	36 (74)	49 (100)	0 (0.0)	0.07	0.5	0.28	FDA Laboratory B
USA	Milk, raw	1995	755	0.02/0.05	742 (98)	742 (98)	755 (100)	0 (0.0)	0.002	0.33	0	FDA Laboratory 27
	Milk, raw	1996	381	0.02/0.05	289 (76)	289 (76)	376 (99)	3 (0.8)	0.03	1.8	0.07	
	Milk, raw	1997	597	0.02/0.05	589 (99)	589 (99)	597 (100)	0 (0.0)	0.001	0.41	0	
	Milk, raw	1998	855	0.02/0.05	843 (99)	843 (99)	855 (100)	0 (0.0)	0.002	0.38	0	
	Milk, raw	1999	877	0.02/0.05	853 (97)	853 (97)	877 (100)	0 (0.0)	0.002	0.24	0	
	Milk, raw	2000	477	0.02/0.05	462 (97)	462 (97)	477 (100)	0 (0.0)	0.004	0.37	0	
	Milk, finished	1995	79	0.02/0.05	76 (96)	76 (96)	79 (100)	0 (0.0)	0.003	0.11	0	
	Milk, finished	1996	21	0.02/0.05	17 (81)	17 (81)	21 (100)	0 (0.0)	0.011	0.06	0.06	
	Milk, finished	1997	53	0.02/0.05	52 (98)	52 (98)	53 (100)	0 (0.0)	0.001	0.06	0	
	Milk, finished	1998	48	0.02/0.05	44 (92)	44 (92)	48 (100)	0 (0.0)	0.005	0.06	0	
	Milk, finished	1999	60	0.02/0.05	58 (97)	58 (97)	60 (100)	0 (0.0)	0.004	0.17	0	
	Milk, finished	2000	12	0.02/0.05	12 (100)	12 (100)	12 (100)	0 (0.0)	0	0	0	
<b>Literature data</b>												
Brazil	Milk, market	1979–81	100	NR	99 (99) <sup>a</sup>	99 (99)	100 (100)	0 (0.0)	0.002	0.2	NR	Sabino et al. (1989)
	Milk, farm	1979–81	50	NR	41 (82)	41 (82)	46 (92)	1 (2.0)	0.087	1.7	NR	
	Milk	1996–97	110	NR/0.0063	83 (76)	110 (100)	110 (100)	0 (0.0)	0.012	0.071	NR	Souza et al. (1999)
	Milk, fluid	1998–99	50	NR/0.006	10 (20) <sup>a</sup>	46 (92)	50 (100)	0 (0.0)	0.028	0.077	NR	Prado et al. (1999)

**Appendix B** (contd)

Country	Commodity	Date	No. of samples	LOQ/LOQ µg/kg	n (%)				Mean (µg/kg)	Maximum (µg/kg)	90th %ile (µg/kg)	References
					< LOQ/LOQ	< 0.05 µg/kg	0.5 µg/kg	1 µg/kg				
Brazil (contd)	Milk, reconstituted	1997	300	NR	267 (89)	267 (89)	294 (98)	0 (0.0)	0.03	1	NR	Oliveira et al. (1997)
	Milk, raw	1992–93	144	0.5/NR	144 (100)	144 (0.0)	144 (100)	0 (0.0)	0	0	0	Correa et al. (1997)
	Milk, powder	1989	35	NR	35 (100) <sup>a</sup>	35 (100)	35 (100)	0 (0.0)	0	0	0	de Sylos et al. (1996)
	Milk, past	1989	51	NR	51 (100) <sup>a</sup>	51 (100)	51 (100)	0 (0.0)	0	0	0	
	Milk, past	1992	52	NR	48 (92) <sup>a</sup>	48 (92)	52 (100)	0 (0.0)	0.012	0.37	NR	
	Yoghurt	1990	30	NR	30 (100)	30 (100)	30 (100)	0 (0.0)	0	0	0	
Egypt	Milk, reconstituted	1995	15	NR	14 (93)	15 (100)	15 (100)	0 (0.0)	0.0018 <sup>b</sup>	NR	NR	El-Gohary (1995)
France	Milk	1990–91	853	NR/0.05	844 (99)	850 (100)	853 (100)	0 (0.0)	NR	NR	NR	Dragacci & Fremy (1993)
	Milk, raw	1990–92	103	NR/0.05	103 (100)	103 (100)	103 (100)	0 (0.0)	0	0	0	
Greece	Milk, past	1997	81	NR/0.005	72 (89)	78 (96)	81 (100)	0 (0.0)	0.008	0.18	NR	Markaki & Melissari (1997)
	Milk, raw	1986–87	99	NR/0.1	95 (96)	NA	99 (100)	0 (0.0)	0.005	0.13	NR	Karaioannoglou et al. (1989)
	Milk, past	1986–87	51	NR/0.1	51 (100)	NA	51 (100)	0 (0.0)	0	0	0	
India	Milk, raw	1992–93	504	NR/0.1	415 (82)	415 (82)	NR	NR	0.20	3.5	NR	Rajan et al. (1995)
Italy	Milk	1995	159	0.001	23 (14)	NR	136 (100)	0 (0.0)	0.0086	0.11	NR	Galvano et al. (1998)
	Yoghurt	1995	114	0.001	23 (20)	NR	114 (100)	0 (0.0)	0.014	0.50	NR	
Philippines	Milk	1997	91	NR/0.05	11 (12)	11 (12)	75 (82)	NR	0.13	NR	NR	Begino (1998)

**Appendix B** (contd)

Country	Commodity	Date	No. of samples	LOQ/LOQ µg/kg	n (%)				Mean (µg/kg)	Maximum (µg/kg)	90th %ile (µg/kg)	References
					< LOQ/LOQ	< 0.05 µg/kg	0.5 µg/kg	1 µg/kg				
Poland	Milk, raw	1993–94	187	NR/0.003	144 (77)	187 (100)	187 (100)	0 (0.0)	NR	0.025	NR	Domagala et al. (1997)
Republic of Korea	Yoghurt	2000	60	NR/0.01	29 (48)	51 (85)	60 (100)	0 (0.0)	0.024	0.12	NR	Kim et al. (2000)
	Milk, past	2000	70	NR/0.01	31 (44)	67 (96)	70 (100)	0 (0.0)	0.014	0.052	NR	
	Milk, raw	1995	49	NR/0.003	0 (0.0)	20 (41)	49 (100)	0 (0.0)	0.065	0.16	0.12	Shon et al. (1996)
	Milk, market	1995	15	NR/0.003	0 (0.0)	0 (0.0)	15 (100)	0 (0.0)	0.13	0.28	NR	
Spain	Milk, raw	1990	61	NR/0.01	52 (85)	61 (100)	61 (100)	0 (0.0)	NR	< 0.050	NR	Macho et al. (1992)
	Milk, UHT	1990	33	NR/0.01	28 (85)	33 (100)	33 (100)	0 (0.0)	NR	0.025	NR	
	Milk, raw	1990	122	NR/0.01	98 (80)	122 (100)	122 (100)	0 (0.0)	NR	0.04	NR	Jalon et al. (1994)
Thailand	Milk, raw	1990–93	45	NR/0.1	33 (73)	NA	41 (91)	0 (0.0)	0.112	0.8	NR	Boriboon & Suprasert (1994)
	Milk, market	1995–96	183	NR/0.025	0 (0.0)	0 (0.0)	153 (84)	NR	NR	NR	NR	Saitanu (1997)
Uruguay	Milk	1993–95	22	0.2/NR	15 (68.2)	NA	21 (96)	NR	NR	20	NR	Pineiro et al. (1996)

NA, not applicable; NR, not reported; NQ, not quantified; past, pasteurized

<sup>a</sup> No samples; number of samples < LOD/LOQ may be higher

<sup>b</sup> Converted from 0.018 µg/kg of dry milk

## Appendix C

Concentrations of aflatoxin M<sub>1</sub> in milk (µg/kg) by regional diet

Diet	Commodity	Date	No. of samples	No. (%)			Mean (µg/kg)	Maximum (µg/kg)	Reference
				< 0.05 µg/kg	< 0.5 µg/kg	> 0.5 µg/kg			
<b>European</b>									
Austria	Milk	1990–93	479	479 (100)	479 (100)	0 (0.0)	0.005	< 0.01	SCOOP Report
Belgium	Milk	1993–94	153	153 (100)	153 (100)	0 (0.0)	0.01	0.037	SCOOP Report
Finland	Milk	1992–95	122	122 (100)	122 (100)	0 (0.0)	0.005	< 0.01	SCOOP Report
France	Milk, 2 types	1990–95	2 835	2812 (99)	2835 (100)	0 (0.0)	0.016	0.37	SCOOP Report
Netherlands	Milk	1990–93	1 903	1572 (83)	1903 (100)	0 (0.0)	0.009	0.02	SCOOP Report
Norway	Milk, dairy	1998	54	54 (100)	54 (100)	0 (0.0)	0.0014	0.009	Food Control Authority
Spain	Milk	1989–92	307	307 (100)	307 (100)	0 (0.0)	0.006	0.046	SCOOP Report
United Kingdom	Milk	1988–89	331	322 (97)	331 (100)	0 (0.0)	0.01	0.22	SCOOP Report
Southern USA	Milk	1998–2000	379	225 (59)	313 (83)	66 (17)	0.40	14	FDA Laboratory B
USA	Milk, raw	1995–2000	3 942	3778 (96)	3937 (100)	5 (0.1)	0.0048	1.8	FDA Laboratory 27
USA	Milk, finished	1995–2000	273	259 (95)	273 (100)	0 (0.0)	0.0037	0.17	FDA Laboratory 27
			10 778				Weighted mean =	0.023	

## Appendix C (contd)

Diet	Commodity	Date	No. of samples	No. (%)			Mean (µg/kg)	Maximum (µg/kg)	Reference
				< 0.05 µg/kg	< 0.5 µg/kg	> 0.5 µg/kg			
<b>Latin American</b>									
Argentina	Milk	1998	6	2 (33)	2 (33)	4 (67)	0.52	0.9	Universidad Nacional de Lujan (2000)
Brazil	Milk, 2 types	1979–81	150	140 (93)	146 (97)	4 (2.7)	0.03	1.7	Sabino et al. (1989)
Brazil	Milk, reconst.	1997	300	267 (89)	294 (98)	6 (2.0)	0.03	1	Oliveira et al. (1997)
Brazil	Milk and yoghurt	1989, 1990, 1992	133	129 (97)	133 (100)	0 (0.0)	0.0047	0.37	de Sylos et al. (1996)
Brazil	Milk	1996–97	110	110 (100)	110 (100)	0 (0.0)	0.012	0.071	Souza et al. (1999)
Brazil	Milk, various	1998–99	50	46 (92)	50 (100)	0 (0.0)	0.028	0.077	Prado et al. (1999)
Brazil	Milk, raw	1992–93	144	144 (100)	144 (100)	0 (0.0)	0	0	Correa et al. (1997)
			893		Weighted mean =		0.022		
<b>Far Eastern</b>									
India	Milk, raw	1992–93	504	415 (82)	NR	> 65 (> 13)	0.20	3.5	Rajan et al. (1995)
Indonesia	Milk	1990–93, 1999	342	> 199 (> 58)	269 (79)	73 (21)	0.86	23	Research Institute for Veterinary Science (2000)
Philippines	Milk	1997	91		75 (82)	16 (18)	0.13	NR	Begino (1998)
Republic of Korea	Milk and yoghurt	2000	130		130 (100)	0 (0.0)	0.019	0.124	Kim et al. (2000)
Republic of Korea	Milk, 2 types	1995	64		64 (100)	0 (0.0)	0.080	0.28	Shon et al. (1996)
Thailand	Milk, 2 types	1990–93	60		49 (82)	11 (18)	0.28	6.6	Boriboon & Suprasert (1994)
			1191		Weighted mean =		0.36		

**Appendix C** (contd)

Diet	Commodity	Date	No. of samples	No. (%)			Mean ( $\mu\text{g}/\text{kg}$ )	Maximum ( $\mu\text{g}/\text{kg}$ )	Reference
				< 0.05 $\mu\text{g}/\text{kg}$	< 0.5 $\mu\text{g}/\text{kg}$	> 0.5 $\mu\text{g}/\text{kg}$			
<b>Middle Eastern</b>									
Greece	Milk, past	1997	81	78 (89)	81 (100)	0 (0.0)	0.008	0.18	Markaki & Melissari (1997)
Greece	Milk, 2 types	1986–87	150	146 (97)	150 (100)	0 (0.0)	0.003	0.13	Karaioannoglou et al. (1989)
			231		Weighted mean =		0.005		
<b>African</b>									
Egypt	Milk, reconstituted	1995	15	15 (100)	15 (100)	0 (0.0)	0.0018**	NR	El-Gohary (1995)

\* Converted from 0.015  $\mu\text{g}/\text{kg}$  of dried milk

## FUMONISINS

First draft prepared by

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## 1. EXPLANATION

The Committee evaluated fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> at the request of the Codex Committee on Food Additives and Contaminants; these toxins had not been evaluated previously by the Committee. In 2000, a monograph on fumonisin B<sub>1</sub> was published (WHO, 2000a), which provided much of the information used in this evaluation.

Fumonisins are fungi produced by fungi of the genus *Fusarium*. The only species that produce significant quantities of fumonisins are *Fusarium verticillioides* (Sacc.) Nirenberg (= *F. moniliforme* (Sheldon)) and the related *F. proliferatum* (Matsushima) Nirenberg. At least 10 other *Fusarium* species also produce these toxins. *F. verticillioides* and *F. proliferatum* are among the most common fungi associated with maize, the most frequently contaminated food, and can be recovered from both damaged and undamaged maize kernels. These species cause *Fusarium* kernel rot of maize, an important disease in hot climates. A strong relationship also exists between insect damage and *Fusarium* kernel rot due to other *Fusarium* species, such as *F. graminearum*. Temperature stress may also play a role, especially in cultivars grown outside their area of adaptation. As *F. verticillioides* and *F. proliferatum* grow over a wide range of temperatures but only at relatively high water activities (above about 0.9), fumonisins are formed in maize only before harvest or during the early stage of drying. Except under extreme conditions, the concentrations of fumonisins do not increase during grain storage. Formation of fumonisins in the field correlates with the occurrence of *F. verticillioides* and *F. proliferatum*, which predominate during late maturity. Fumonisins are widely distributed geographically, and their natural occurrence in maize has been reported in many areas of the world. Of particular concern are the high concentrations found in maize produced and consumed by particular subpopulations, such as subsistence farmers. Considerable annual variations in contamination have been noted. Fumonisins occur infrequently in other foods, such as sorghum, asparagus, rice, beer, and mung beans.

Fumonisins are a group of structurally related compounds. Fumonisin B<sub>1</sub> is the diester of propane-1,2,3-tricarboxylic acid and 2*S*-amino-12*S*,16*R*-dimethyl-

3*S*,5*R*,10*R*,14*S*,15*R*-pentahydroxyeicosane in which the C-14 and C-15 hydroxy groups are esterified with the terminal carboxy group of propane-1,2,3-tricarboxylic acid. Fumonisin B<sub>2</sub> is the C-10 deoxy analogue of fumonisin B<sub>1</sub> in which the corresponding stereogenic units on the eicosane backbone have the same configuration. The full stereochemistry of fumonisin B<sub>3</sub> and B<sub>4</sub> is unknown, but the amino terminal of fumonisin B<sub>3</sub> has the same absolute configuration as that of fumonisin B<sub>1</sub>.

As most biological data were available on fumonisin B<sub>1</sub>, and maize is the major source of intake, the Committee focused its evaluation on toxicological studies of fumonisin B<sub>1</sub> and on studies of intake of contaminated maize and maize products. In many studies, culture materials and naturally contaminated maize were used, which can contain several other fumonisins, primarily fumonisins B<sub>2</sub> and B<sub>3</sub>. The toxicological profiles of these toxins are very similar to that of fumonisin B<sub>1</sub>. Various chemical derivatives of fumonisins have been tested in a number of biological systems to gain insight into structure–activity relationships. Briefly, the fumonisins of the B series that have been investigated are more toxic *in vivo* than their hydrolysed or *N*-acetylated counterparts. The free amino group appears to play a specific role in the biological activity of fumonisin B<sub>1</sub>.

## 2. BIOLOGICAL DATA

### 2.1 Biochemical aspects

A thorough review of the biochemical aspects of fumonisin B<sub>1</sub> is contained in *Fumonisin B<sub>1</sub>* (Environmental Health Criteria 219), published by WHO (2000a), and much of what follows is derived from that review, with relevant new publications. With a few exceptions, only references that were not included in that monograph, recent or critical reviews, or studies with stated doses are cited here.

In many studies on fumonisins in animals *in vivo*, culture materials or naturally contaminated maize were used in which not only fumonisin B<sub>1</sub> but also other fumonisins (primarily fumonisin B<sub>2</sub> and B<sub>3</sub>) can be found. Various chemical derivatives of fumonisins have been studied in several biological systems to gain insight into the structural requirements of fumonisin-induced toxicity and biochemical mechanisms *in vivo* (see WHO, 2000a, pp. 79–80 for additional details). Briefly, the fumonisins of the B series that have been investigated are more toxic *in vivo* than their hydrolysed or *N*-acetylated counterparts. The free amino group has an important role, as the *N*-acetyl derivatives are less toxic in primary hepatocytes and hydrolysed fumonisin B<sub>1</sub> is more toxic. The lesser toxicity of hydrolysed fumonisin B<sub>1</sub> in rat liver is not due to reduced absorption. The fungus previously known as *F. moniliforme* Sheldon is referred to in this monograph as *F. verticillioides* (Sacc.) Nirenberg.

#### 2.1.1 Absorption, distribution, and excretion

In rats and most other animals, the kinetics of absorption of fumonisin B<sub>1</sub> indicates rapid distribution and elimination that is adequately described by a two- or three-compartment model (most recently, Martinez-Larranaga et al., 1999). Little fumonisin B<sub>1</sub> was detected in plasma and tissues after oral administration, indicating that absorption is negligible ( $\leq 4\%$  of the dose). Fumonisin B<sub>2</sub> may be less bioavailable than fumonisin B<sub>1</sub>, and proportionally less fumonisin B<sub>2</sub> is excreted in bile (Shephard

& Snijman, 1999). In rats treated by gavage, more hydrolysed [ $^{14}\text{C}$ ]fumonisin  $\text{B}_1$  was excreted in urine than [ $^{14}\text{C}$ ]fumonisin  $\text{B}_1$  or the [ $^{14}\text{C}$ ]fumonisin  $\text{B}_1$ -fructose adduct. For example, in female Fischer 344NHsd rats, 17% of a dose of hydrolysed [ $^{14}\text{C}$ ]fumonisin  $\text{B}_1$  and 0.7% of a dose of [ $^{14}\text{C}$ ]fumonisin  $\text{B}_1$  was recovered in urine after administration by gavage. The authors concluded that hydrolysed fumonisin  $\text{B}_1$  was better absorbed than fumonisin  $\text{B}_1$ , although the biliary excretion of the two fumonisins was similar (Dantzer et al., 1999). While fumonisin  $\text{B}_1$  was distributed to most tissues, the liver and kidney retained most of the absorbed material, the liver retaining more toxin than the kidney in some studies and the inverse in others. In a study in male Wistar rats given pure fumonisin  $\text{B}_1$  by gavage, kidney contained > 10 times as much fumonisin  $\text{B}_1$  as liver (Martinez-Larranaga et al., 1999). This study confirmed previous reports that fumonisin  $\text{B}_1$  persists in rat liver and kidney much longer than in plasma. It was estimated that, after administration to pigs of fumonisin  $\text{B}_1$  in the diet at 2–3 mg/kg bw, a withdrawal period of at least 2 weeks would be required to eliminate the toxin from liver and kidney. The material retained in liver and kidney is primarily unmetabolized fumonisin  $\text{B}_1$ , as shown in several studies of the persistence in kidney of free sphinganine (a biomarker for fumonisin). Thus, while fumonisin  $\text{B}_1$  is eliminated rapidly, the concentration of the biomarker in rat kidney (but not liver) is more persistent (most recently, Enongene et al., 2000; Garren et al., 2000). Similar kinetics was seen in the livers of non-human primates given a single dose of pure fumonisin  $\text{B}_1$  at 10 mg/kg bw by gavage, which increased the serum concentrations of sphingoid bases, cholesterol, and enzymes indicative of liver function, and these remained elevated for several weeks after dosing (van der Westhuizen et al., 2001).

In pregnant rats, low concentrations of fumonisin  $\text{B}_1$  were recovered in uteri (0.24–0.44%), individual placentae (0–0.4%), and fetuses ( $\leq 0.015\%$ ), indicating the absence of placental transfer of fumonisin  $\text{B}_1$ . Similar results were reported in rabbits. There is also little evidence of significant transfer during lactation. For example, no fumonisin  $\text{B}_1$  was detected in the milk of lactating sows fed diets containing non-lethal concentrations of fumonisin  $\text{B}_1$ , and there was no evidence of toxicosis in their suckling piglets. In a study in which lactating cows were given fumonisin  $\text{B}_1$  intravenously, the carry-over rate into the milk reached a maximum of 0.11%. In other studies, no fumonisins were detected in cows' milk, and fumonisin  $\text{B}_1$  was found in only 1 of 165 samples of milk in the USA at a concentration of 5 ng/ml. The finding that little fumonisin  $\text{B}_1$  is retained in tissues, milk, or eggs has led to the conclusion that fumonisin residues in food products derived from animals are insufficient to make them injurious to consumers.

After intraperitoneally or intravenously administered fumonisin  $\text{B}_1$  has been distributed, its initial elimination is rapid, with no evidence of metabolism. In vervet monkeys (*Cercopithecus aethiops*) treated intraperitoneally with fumonisin  $\text{B}_2$ , elimination was rapid and followed a bi-exponential pattern (half-time, 18 min), similar to that of the elimination of fumonisin  $\text{B}_1$  (half-time, 40 min). The elimination kinetics in non-human primates after oral dosing has not been determined, but peak plasma concentrations of fumonisin  $\text{B}_1$  and  $\text{B}_2$  occurred 1 to several hours after a dose of 7.5 mg/kg bw by gavage, and the plasma fumonisin concentrations ranged from < 20 ng/ml to nearly 210 ng/ml. Thus, the elimination kinetics after oral dosing is not easily described, unlike that of intraperitoneal or intravenous dosing. Furthermore, an oral dose of fumonisin cannot be fully accounted for (Shephard & Snijman, 1999).

As the rate of elimination of fumonisin B<sub>1</sub> is a function of body weight, elimination is rapid in mice but would be much longer in humans (DeLongchamp & Young, 2001).

### 2.1.2 Biotransformation

There is little or no evidence that fumonisins are metabolized *in vitro* or *in vivo* in animals, even though they are clearly excreted in bile. A study in which primary hepatocytes were exposed to [<sup>14</sup>C]fumonisin B<sub>1</sub> showed that the toxin is associated with both the soluble and the insoluble membrane compartments of the cell, and no metabolites were detected after a 44-h incubation. Incubation with rat liver microsomal preparations also showed no metabolism by cytochrome P450 (CYP), microsomal esterase, or any other microsomal enzyme. Incubation with a triglyceride hepatic endothelial lipase or a porcine pancreatic lipase also did not result in hydrolysis of the tricarboxylic acid moieties of fumonisin B<sub>1</sub>.

Several studies in which different routes of exposure and different animal species were used showed that fumonisins are excreted primarily in the faeces, either unchanged or with loss of one or both of the tricarboxylic acid side-chains. The material excreted in bile is still biologically active, since fumonisin B<sub>1</sub> given subcutaneously to mice rapidly entered the small intestine, where it inhibited ceramide synthase (Enongene et al., 2000). Loss of the tricarboxylic acid side-chains probably occurs in the gut, since after partial hydrolysis resulting in removal of only one of the two side-chains and full hydrolysis fumonisin B<sub>1</sub> is recovered in the faeces but not in the bile. This finding was confirmed in a study in vervet monkeys (Shephard & Snijman, 1999). Most of the hydrolysed fumonisin B<sub>2</sub> was present as a mixture of the two possible partially hydrolysed forms, while fully hydrolysed fumonisin B<sub>2</sub> was a minor constituent. No hydrolysis products were found in urine, confirming that fumonisin was hydrolysed in the gut, probably by microbial degradation. While there is no evidence that fumonisin is metabolized by CYP enzymes, some studies have shown that fumonisins can alter their activity, and this observation was confirmed *in vitro* and *in vivo* (Merrill et al., 1999a; Spotti et al., 2000). In some studies, the effects on CYP activity have been shown to be the result of fumonisin-induced alterations in sphingolipid metabolism (Merrill et al., 1999b). For example, in HepG2 cells, fumonisin B<sub>1</sub> inhibited the induction of CYP 1A1 (which metabolizes aryl hydrocarbons such as methylcholanthrene) (Merrill et al., 1999a). In rats given fumonisin B<sub>1</sub> by gavage at 2 mg/kg bw, there was inhibition of CYP 2C11 and to a lesser extent of CYP 1A2 (Spotti et al., 2000). The inhibition of CYP 2C11 was attributed to suppression of protein kinase activity due to inhibition of sphingolipid biosynthesis. Sphingosine, a sphingolipid that accumulates in animals exposed to fumonisin B<sub>1</sub>, was also shown to inhibit CYP 2C11 in rat hepatocytes (Merrill et al., 1999a). Feeding rainbow trout a diet containing fumonisin B<sub>1</sub> at a concentration of 104 mg/kg had no effect on acetylated fumonisin B<sub>1</sub>-DNA adduct formation (Carlson et al., 2001).

While there is little evidence that absorbed fumonisins are metabolized in animals, removal of the tricarboxylic acid side-chains (producing hydrolysed fumonisin B<sub>1</sub>) converts this inhibitor of ceramide synthase into a substrate for the enzyme. The product of this reaction, *N*-palmitoyl-hydrolysed fumonisin B<sub>1</sub>, also inhibits ceramide synthase *in vitro*. It is not known whether this product is formed *in vivo*, but it is more toxic than fumonisin B<sub>1</sub> or hydrolysed fumonisin B<sub>1</sub> for HT-29 cells (Merrill et al.,

2001). Since hydrolysed fumonisin B<sub>1</sub> and hydrolysed fumonisin B<sub>2</sub> are major breakdown products in nixtamalized maize products and are also produced in the gut from fumonisin B<sub>1</sub> and B<sub>2</sub>, the toxicity of the hydrolysed toxins should be addressed.

### **2.1.3 Effects on enzymes, biochemical parameters, and cellular regulation**

#### *(a) Biochemical modes of action*

Several biochemical reactions have been proposed to explain all or some of the toxic effects of fumonisins in animals. Two of them invoke disruption of lipid metabolism as the initial site of action, and they are similar in other respects (Gelderblom et al., 2001a; Merrill et al., 2001; Riley et al., 2001). Both of these hypothesized mechanisms are supported by data obtained in *in vivo* (Table 1) and *in vitro* (Table 2), in short-term and long-term studies in rodents (National Toxicology Program, 1999; Delongchamp & Young, 2001; Gelderblom et al., 2001a; Riley et al., 2001; Voss et al., 2001), long-term studies of carcinogenicity in trout (Carlson et al., 2001), and short-term studies of toxicity in other animals (WHO, 2000a). The first proposed lipid-based mechanism involves inhibition of ceramide synthase, a key enzyme in the biosynthesis of sphingolipids (reviewed extensively in WHO, 2000a). The second mechanism involves changes in the polyunsaturated fatty acid and phospholipid pools. Both lead ultimately to lipid-mediated alterations in signalling and metabolic pathways crucial to cell growth, death, and differentiation. Several studies *in vitro* indicate that fumonisin-induced changes in key enzymes involved in cell cycle regulation, differentiation, and/or apoptosis are initial or secondary triggers (most recently, Pinelli et al., 1999; Mobio et al., 2000a; Table 2).

#### *(i) Inhibition of ceramide synthase*

The structural similarity between sphinganine and fumonisin B<sub>1</sub> led to the hypothesis that this mycotoxin acts by disrupting the metabolism or a function of sphingolipids. There is considerable support for the hypothesis that fumonisin-induced disruption of sphingolipid metabolism is an important event in the cascade of events leading to altered cell growth, differentiation, and cell injury observed both *in vitro* and *in vivo* (Tables 1–3). A complete review of the literature on this subject is beyond the scope of this monograph, and the interested reader is referred to WHO (2000a) and reviews by Merrill et al. (2001) and Riley et al. (2001)

Fumonisin B<sub>1</sub> strongly inhibited the acylation of sphinganine and sphingosine in all cell lines and all animals, plants, and fungi in which it has been tested. Ceramide synthase recognizes both the amino group (sphingoid-binding domain) and the tricarboxylic acid side-chains (fatty acyl-coenzyme A domain) of fumonisin B<sub>1</sub>. While removal of the tricarboxylic acid side-chains reduces the ability of fumonisin B<sub>1</sub> to inhibit ceramide synthase, *N*-acetylation completely abolishes the inhibitory activity (Norred et al., 2001). Complete inhibition of ceramide synthase by fumonisins causes a rapid increase in the intracellular concentration of sphinganine and sometimes of sphingosine, both *in vivo* and *in vitro*. *In vivo* there is a close relationship between sphinganine accumulation and the expression of toxicity in liver and kidney (DeLongchamp & Young, 2001). Once accumulated, free sphingoid bases can persist in tissues (especially kidney) much longer than fumonisin B<sub>1</sub> (Shephard & Snijman,

**Table 1. Selected biochemical mechanisms for the toxicity of fumonisin B<sub>1</sub> in animal models in which the proposed biochemical action has been shown to be related to specific effects**

Description	Model (Species, tissues)	Action	Biochemical effects [lowest oral dose that caused an effector to change]	Correlated adverse effects and associated molecular events
<b>Altered lipid metabolism</b> Disruption of sphingolipid metabolism	Rat/L,K, H,S,U	Ceramide synthase inhibition	Increased sphingoid bases and decreased complex sphingolipids [kidney, 1 mg/kg FB <sub>1</sub> , equivalent to 0.1 mg/kg bw per day (Wang et al., 1999)]	Increased apoptotic and oncotoc necrosis in liver and kidney, mitogenesis, decreased heart weight, kidney tumours, liver tumour promotion
	Mouse/L,K,GI,S		Increased sphingoid bases and decreased complex sphingolipids [kidney, 0.3 mg/kg bw per day (Enongene et al., 2001)]	Increased apoptotic and oncotoc necrosis in liver and kidney, altered TNF $\alpha$ expression, liver tumours
	Rabbit/L,K, S,U		Increased sphingoid bases [kidney, 0.1 mg/kg bw per day (LaBorde et al., 1997)]	Nephrotoxicity
	Pig/L, K, Ln, H, S		Increased sphingoid bases and decreased complex sphingolipids [serum, 5 mg/kg FB <sub>1</sub> , equivalent to 0.2 mg/kg bw per day (Riley et al., 1993)]	Hepatotoxicity, cardiovascular toxicity, pulmonary oedema syndrome
	Monkey/L,K,S,U		Increased sphingoid bases [serum, 1.0 mg/kg bw per day (van der Westhuizen et al., 2001)]	Hepatotoxicity, nephrotoxicity

**Table 1** (contd)

Description	Model (Species, tissues)	Action	Biochemical effects [lowest oral dose that caused an effector to change]	Correlated adverse effects and associated molecular events
Disruption of sphingolipid metabolism (contd)	Horse/L, K, S	Ceramide synthase inhibition	Increased sphingoid bases and decreased complex sphingolipids [serum, 22 mg/kg total fumonisins, equivalent to 0.44 mg/kg bw per day (Wang et al, 1992)]	Hepatotoxicity, cardiovascular toxicity, leukoencephalomalacia
	Trout, L, K, S		Increased sphingoid bases and decreased complex sphingolipids [liver, 25 mg/kg FB <sub>1</sub> , equivalent to 3.75 mg/kg bw per day on basis of study in mice (Carlson et al., 2001)]	Promotion of liver tumours induced by direct or indirect carcinogens
Disruption of fatty acid and phospholipid metabolism	Rat/L, S	Impairment of N-6 fatty acid metabolism, phospholipid metabolism, and ceramide synthase inhibition	Alterations in absolute and relative amounts of phosphatidylcholine and ethanolamine and in degree of saturation of fatty acids in phosphatidylcholine and ethanolamine in microsomal, mitochondrial, plasma, nuclear cell membranes and membranes associated with hepatic nodules; in particular, relative and absolute amounts of fatty acid products of N-6 and N-3 pathway. Also, increased free sphingoid bases and decreased sphingomyelin. [Liver, 10 mg/kg FB <sub>1</sub> , equivalent to 0.5 mg/kg bw per day (Gelderblom et al., 1997)]	Increased lipid peroxidation, mitoinhibition, hepatotoxicity, growth of hepatocyte nodules, increased expression of hepatocyte growth factor and tumour growth factor- $\alpha$ , c-myc, alterations in retinoblastoma pathway, deregulation of cell cycle control by overexpression of cyclin D1, liver tumour promotion and hepatocarcinogenicity

**Table 1** (contd)

Description	Model (Species, tissues)	Action	Biochemical effects [lowest oral dose that caused an effector to change]	Correlated adverse effects and associated molecular events
<b>Increased oxidative stress</b>	Rat/L, SI	Lipid oxidation	Increases superoxide radicals, increased lipid radicals [Liver, 16 mg/kg bw per day Lemmer et al., 1999a]	Increased iron-induced lipid peroxidation, oxidative DNA damage, protection from toxicity by antioxidants

Abbreviations: FB<sub>1</sub>, fumonisin B<sub>1</sub>; L, liver; K, kidney; Ln, lung; H, heart; GI, digestive epithelia; M, muscle; SI, spleen; S, serum; U, urine; TNF, tumour necrosis factor

**Table 2. Selected biochemical mechanisms for the toxicity of fumonisin B<sub>1</sub> in vitro in models in which the proposed biochemical action has been shown to be related to specific molecular events or physiological or toxic effects**

Description	Model	Action	Biochemical effectors (lowest concentration)	Molecular targets	Correlated adverse effect
<b>Altered lipid metabolism</b>					
Disruption of sphingolipid metabolism	Microsomes: Rat liver and mouse cerebellar neurons	Ceramide synthase inhibition	Competitive inhibition with fatty acids or sphingoid bases as substrates [IC <sub>50</sub> ≤ 0.075 μmol/L FB <sub>1</sub> (Merrill et al., 1993)]	NA	NA
	Primary cultures: Rat hepatocytes, liver and kidney slices, hippocampal neurons, cerebellar Purkinje cells, fetal glial cells, sympathetic neurons; mouse neuronal cells and spinal cord cultures; chick embryos; pig endothelial cells; human keratinocytes		Decreased biosynthesis of <i>N</i> -acetylated sphingoid bases (ceramides), increased sphingoid bases, increased sphingoid base 1-phosphates, Increased fatty aldehydes, Increased phosphatidylcholine and phosphatidylethanolamine biosynthesis (in rat hepatocytes), decreased glycosphingolipids, sphingomyelin, and other complex sphingolipids [0.1 μmol/L FB <sub>1</sub> ] (Wang et al., 1991)	Altered amounts of sphingolipid and glycerophospholipid second messengers; decreased expression of glycosphingolipid receptors.	In hepatocytes, cytotoxicity and mitoinhibition reported but only at concentrations (≥ 75 μmol/L) far in excess of those that cause maximal inhibition of ceramide synthase (~1 μmol/L). In other primary cultures, sphingolipid-dependent growth inhibition, apoptosis, and functional effects have been found; e.g. sphinganine-dependent apoptosis, glycosphingolipid-dependent, growth factor-stimulated axonal growth, cytokine-induced adhesion molecule expression and bacterial toxin binding.

Table 2 (contd)

Description	Model	Action	Biochemical effectors (lowest concentration)	Molecular targets	Correlated adverse effect
Disruption of sphingolipid metabolism (contd)	Cell lines: Pig renal; mouse fibroblast, macrophage, and melanoma; hamster ovary; monkey kidney; human colon	Ceramide synthase inhibition	Increased sphingoid bases, decreased complex sphingolipids, and/or other specific glycosphingolipids, increased phosphatidylethanolamine, sphingoid-base-1-phosphates [1 $\mu\text{mol/L}$ FB <sub>1</sub> ; 10 $\mu\text{mol/L}$ hydrolysed FB <sub>1</sub> (Schmelz et al., 1998)].	Altered amounts of sphingolipid second messengers, activity of protein kinases, expression of cell cycle proteins, adhesion molecules (ICAM-1, integrins), and bacterial toxin and vitamin receptors	Increased sphinganine-dependent apoptotic and/or oncotic necrosis, and/or altered proliferation. Decreased glycosphingolipid-dependent cell adhesion, growth, altered cell morphology, or differentiation, and altered vitamin transport
Disruption of fatty acid and phospholipid metabolism	Primary cultures: Rat hepatocytes	Impaired N-6 fatty acid metabolism, phospholipid metabolism, and ceramide synthase inhibition	Decreased biosynthesis of neutral lipids, triglycerides, and cholesterol; increased phosphatidylcholine and ethanolamine, decreased sphingomyelin, increased free sphinganine. Altered fatty acid saturation profiles in various lipid pools, in particular accumulation of C18:2 $\omega$ 6 and C20:4 $\omega$ 6 [other than increase in free sphinganine: 150 $\mu\text{mol/L}$ FB <sub>1</sub> (Gelderblom et al., 1996a)]	Altered amounts of lipids required for maintaining membrane fluidity and as substrates for signalling pathways that regulate the epidermal growth factor-induced mitogenic response in hepatocytes. In particular, disruption of prostaglandin-mediated responses	Increased cytotoxicity and inhibition of epidermal growth factor-induced mitogenesis. Altered prostaglandin and arachidonic acid-induced cytotoxicity

**Table 2** (contd)

Description	Model	Action	Biochemical effectors (lowest concentration)	Molecular targets	Correlated adverse effect
<b>Oxidative stress</b>	Artificial membranes	Altered redox state	Increased membrane disorder, increased oxygen transport, increased free radicals [250 µmol/L FB <sub>1</sub> (Yin et al., 1998)]	Decreased membrane integrity	Increased membrane permeability
	Isolated organelles: Rat liver nuclei		Lipid radicals	Decreased membrane integrity	Increased lipid peroxidation
	Primary cultures: Rat hepatocytes		Superoxide radicals and hydrogen peroxide, lipid radicals [5 µmol/L FB <sub>1</sub> (Lee & Lee, submitted)]	Increased oxidative damage to macromolecules and membrane lipids	Increased DNA fragmentation, lipid peroxidation, cytotoxicity, and protection by antioxidants
	Cell lines: Rat brain glioma; pig renal; monkey kidney		Oxidants [0.14 µmol/L FB <sub>1</sub> (Abado-Becongnee et al., 1998)]	Increased oxidative damage to macromolecules, glutathione depletion	Increased DNA fragmentation, hypermethylation of DNA, lipid peroxidation, cytotoxicity, cell growth, protection by antioxidants
<b>Other mechanisms</b>	Isolated enzymes: bacterially expressed	Protein phosphatase inhibition	Direct inhibition [80 µmol/L FB <sub>1</sub> (Fukuda et al., 1996)]	Dephosphorylation of proteins	None

Table 2 (contd)

Description	Model	Action	Biochemical effectors (lowest concentration)	Molecular targets	Correlated adverse effect
<b>Other mechanisms (contd)</b>	Isolated organelles: Rat brain membranes; bovine retina rod segments	Altered GTP binding and GTPase activity	FB <sub>1</sub> , hydrolysed FB <sub>1</sub> , and sphingoid bases [IC <sub>50</sub> = 75 µmol/L FB <sub>1</sub> (Ho et al., 1996)]	GTP binding proteins	None
	Cell lines: Mouse fibroblasts, macrophages; monkey kidney; human epithelium	Activation or inhibition of specific protein activities	FB <sub>1</sub> or unidentified effector (hypothesized sphingolipid effect) [1 µmol/L FB <sub>1</sub> (Huang et al., 1995; Rotter & Oh, 1996)]	Repressed protein kinase C; stimulated nitric oxide synthase, mitogen-activated protein kinases, and cytoplasmic phospholipase A2; altered expression of cyclins and cytokine signalling pathways	None or altered cell cycle progression, altered growth, mitogenesis, or apoptosis
	Primary cultures: Rat cerebrocortical slices	Protein kinase C <i>trans</i> -location	Direct inhibition [1 nmol/L FB <sub>1</sub> (Yeung et al., 1996)]	Activated protein kinase C and increased membrane association	None
	Isolated tissues: Frog atrial muscle	Calcium blockade	Inhibition of calcium entry [100 µmol/L FB <sub>1</sub> (Sauviat et al., 1991)]	Calcium channels	Altered muscle contractility

Abbreviations: FB<sub>1</sub>, fumonisin B<sub>1</sub>; IC<sub>50</sub>, median inhibitory concentration; GTP, guanosine triphosphate; NA, not applicable

1999; Enongene et al., 2000). In the urine of rats fed fumonisin B<sub>1</sub>, > 95% of the free sphinganine was recovered in dead cells. An oral dose of fumonisin B<sub>1</sub> insufficient to increase the concentration of free sphinganine (1 mg/kg of diet, equivalent to 0.1 mg/kg bw per day) can prolong the half-life of free sphinganine in urine of rats after they have been taken off diets that contained a dose sufficient to cause free sphinganine (10 mg/kg of diet, equivalent to 1 mg/kg bw per day) to accumulate in urine (Wang et al., 1999). This observation that a sub-threshold dose can prolong the increase in free sphinganine caused by a higher dose has been confirmed in mice treated by oral gavage (Enongene et al., 2001). Fumonisin B<sub>1</sub>-induced increases in free sphingoid bases and toxicity are both reversible, although elimination of free sphinganine from the liver is faster than from the kidney (Enongene et al., 2000).

A portion of the accumulated sphinganine is metabolized to sphinganine 1-phosphate and then cleaved into a fatty aldehyde and ethanolamine phosphate, both of which can be redirected into other biosynthetic pathways, such as increased biosynthesis of phosphatidylethanolamine. Free sphinganine that is not degraded can be acylated to form C-2 dihydroceramide (Merrill et al., 2001). Disrupted sphingolipid metabolism leads to imbalances in phosphoglycerolipid, fatty acid, and cholesterol metabolism by altering phosphatidic acid phosphatase and monoacylglycerol acyltransferase. Thus, inhibition of ceramide synthase by fumonisin B<sub>1</sub> can cause a wide spectrum of changes in lipid metabolism and associated lipid-dependent signalling pathways (reviewed by Merrill et al., 2001).

In short-term studies with mice, rats, and rabbits, disruption of sphingolipid metabolism, as shown by statistically significant increases in the free sphinganine concentration, occurred at doses at or below those that cause liver or kidney lesions (Table 1; for review, see Riley et al., 2001). In a long-term study in which Fischer 344/N Nctr BR rats were fed diets containing pure fumonisin B<sub>1</sub>, the increase in the ratio of sphinganine:sphingosine in kidney and urine correlated with increased incidences of non-neoplastic and neoplastic lesions in the kidney (National Toxicology Program, 1999). In the livers of female B6C3F<sub>1</sub>/Nctr BR mice, the concentrations of free sphinganine and the ratio of sphinganine:sphingosine were increased after 3 and 9 weeks on a diet containing fumonisin B<sub>1</sub> at 50 or 80 mg/kg, which doses induced liver adenoma and carcinoma (National Toxicology Program, 1999). In rainbow trout, fumonisin B<sub>1</sub> was not a complete carcinogen, but there was a close correlation between promotion of hepatocarcinogenicity caused by aflatoxin B<sub>1</sub> and the concentration of free sphinganine in liver (Carlson et al., 2001; Riley et al., 2001). A mathematical model based on the data from the study of the National Toxicology Program confirmed that the concentration of sphinganine in mouse liver and rat kidney was a dose-related biomarker of fumonisin B<sub>1</sub>-induced cell death (DeLongchamp & Young, 2001). In Sprague-Dawley rats fed AIN-76 diets containing pure fumonisin B<sub>1</sub>, the LOEL for an increased urinary concentration of free sphinganine was 5 mg/kg of diet (equivalent to 0.5 mg/kg bw per day) (Wang et al., 1999). In rats fed a diet containing a mixture of fumonisins from culture material for 13 days, the NOEL was 1–2 mg/kg of diet (equivalent to 0.1–0.2 mg/kg bw per day) (Solfrizzo et al., 1997).

The potential problems of using increased free sphinganine as a functional biomarker of human exposure to fumonisin B<sub>1</sub> have been reviewed (Turner et al.,

1999). In a study in men in China, the ratio of free sphinganine to free sphingosine in urine was significantly greater for men in households where the estimated daily intake of fumonisin B<sub>1</sub> was > 110 µg/kg bw per day (Qui et al., 2001). In several other studies, an increase in free sphinganine in human urine or blood did not appear to be associated with fumonisin intake (e.g. van der Westhuizen et al., 1999).

In cultured cells, the sphingolipid-dependent mechanisms for inducing apoptosis include accumulation of excess ceramide, glucosylceramide (Korkotian et al., 1999), or sphingoid bases and depletion of ceramide, or more complex sphingolipids (Table 2). Conversely, the balance between sphingosine 1-phosphate and ceramide is critical for signalling proliferation or cell survival (Spiegel, 1998). A diversity of alterations in cellular regulation resulting from disruption of sphingolipid metabolism by fumonisin B can also be expected. This is demonstrated in numerous studies of the identify of cell processes mediated by ceramides (Table 3). Many of these processes are relevant to understanding the toxicity and carcinogenicity of fumonisin B<sub>1</sub>, in particular, the ability of this toxin to protect oxidant-damaged cells from apoptosis and to alter the proliferative response (Table 3).

There is no doubt that loss of complex sphingolipids also plays a role in the abnormal behaviour, altered morphology, and altered proliferation of fumonisin-treated cells (Tables 1 and 2), in particular the ability of fumonisin B<sub>1</sub> to alter the function of specific glycosphingolipids and lipid rafts (membrane associations of sphingolipids, ceramide-anchored proteins, and other lipids). Examples are functions such as vitamin and toxin transport and cell–cell and cell–substratum contact (Table 3).

Inhibition of ceramide biosynthesis by fumonisins also protects cells from oxidant-induced cell death (Table 3). As the metabolism of ceramides is sensitive to the redox state of the cell, it is of particular interest that mice, pigs, and horses treated with fumonisin have increased amounts of complex sphingolipids containing sphinganine as the long-chain sphingoid-base backbone. The ceramide generated from these complex sphingolipids is dihydroceramide, the form of ceramide that is inactive in ceramide signalling and does not induce apoptosis of oxidant-damaged hepatocytes (Arora et al., 1997). Dihydroceramide also occurs in greater amounts in mouse hepatoma cells, in which 37% of the ceramides contain sphinganine, as compared with 5% in normal rat liver (Rylova et al., 1999).

#### *(ii) Altered fatty acid metabolism in liver*

Essential fatty acids are major constituents of all cell membrane glycerophospholipids, sphingolipids, and triglycerides. Apart from being structural components of all membranes, they are precursors of eicosanoids, prostaglandins, leukotrienes, and other oxygenated derivatives. In addition, the regulated turnover of membrane phospholipids is important in many intracellular signalling systems known to regulate cell growth, death, and differentiation. In rat liver, fumonisin B<sub>1</sub> induced changes in phospholipids and their fatty acid composition that markedly affected the many cell functions that may contribute to its toxicity and carcinogenicity. The following summary is from the review of Gelderblom et al. (2001a).

In primary hepatocytes treated with fumonisin B<sub>1</sub>, incorporation of [<sup>14</sup>C]palmitic acid into total lipids, neutral lipids, triacylglycerol, and cholesterol esters were

**Table 3. Use of fumonisin B<sub>1</sub> (FB<sub>1</sub>) in research: sphingosine-, ceramide-, and glycosylceramide-mediated processes in vitro are sensitive to fumonisin-inhibited ceramide synthase. Models or processes not summarized previously (WHO, 2000a) are shown in bold.**

Description	Model	Process affected [lowest fumonisin concentration]	Correlated effects and associated molecular events
Inhibition of ceramide- or glucosylceramide-induced apoptotic or oncotic cell death	Primary cultures: Hen granulosa; rat pancreas, <b>hippocampal neurons; bovine cerebral endothelial, aortic endothelial</b>	Inhibition of free fatty acid-induced DNA fragmentation, TNF- $\alpha$ -cycloheximide-induced cell death, <b>direct DNA damage-induced apoptosis, glucocerebrosidase-induced apoptosis</b> [0.01 $\mu$ mol/L FB <sub>1</sub> (Xu et al., 1998)]	Inhibition of apoptotic or oncotic necrosis induced by antineoplastic agents or other therapeutic agents designed to kill cancer cells selectively, <b>treatments designed to introduce lethal double-strand breaks in DNA, glucocerebrosidase accumulation (Gaucher disease)</b>
	Cell lines: <b>Human breast cancer, prostate cancer, monocytic leukaemia, promyelocytic, immortalized B cells</b> ; mouse fibroblast, haematopoietic, <b>oligodendrocyte</b> ; pig renal; <b>HaCaT; PC12W cells</b>	Inhibition of poly(ADP-ribose) polymerase processing, <b>modulation of multidrug resistance</b> , inhibition of interleukin converting enzyme-like proteinase activity, inhibition of cell death induced by tetraphorbolacetate, mitochondrial-derived palmitate, chemical hypoxia, hexadecylphosphocholine, <b>taxol, TNF<math>\alpha</math>/PKC-<math>\beta</math>, lymphotoxin, angiotensin II</b> [10 $\mu$ mol/L FB <sub>1</sub> (Charles et al., 2000)]	
Altered cell cycle progression	Primary cultures: Human peripheral T cells; frog oocytes	Inhibition of Fas (CD95)-induced proliferation, induction of oocyte maturation [0.1 $\mu$ mol/L FB <sub>1</sub> (Sakata et al., 1998)]	Altered cell growth or differentiation
	Cell lines: Human diploid fibroblasts	Inhibition of Rb protein dephosphorylation [25 $\mu$ mol/L FB <sub>1</sub> (Lee et al., 1998)]	

Table 3 (contd)

Description	Model	Process affected [lowest fumonisin concentration]	Correlated effects and associated molecular events
Arachidonic acid release	Cell lines: Mouse macrophage	Inhibition of arachadonic acid release [0.2 µmol/L FB <sub>1</sub> (Balsinde et al., 1997)]	Inhibition of endotoxin/platelet activating factor-induced arachidonate mobilization
Lipid raft function or processes involving uptake or release of toxins or other chemicals	Primary cultures: <b>Mouse spinal cord</b>	<b>Reduced expression of tetanus and cholera toxin receptors [20 µmol/L FB<sub>1</sub> (Williamson et al., 1999)]</b>	Prevention of toxin-induced neurotransmitter release and cell death, folate, Shiga toxin, and saposin transport, toxin-induced cell death, altered protein processing
	Cell lines: Human colon, epidermoid carcinoma; <b>rat kidney; ScN2a; hamster ovary</b>	Reduced receptor function or expression (vitamin receptors, lipopolysaccharide (CD14), Shiga toxin), <b>increased formation of scrapie prion protein, altered sorting</b> [10 µmol/L FB <sub>1</sub> (Sandvig et al., 1996)]	
Cell matrix and cell–cell adhesion	Primary cultures: Human keratinocytes	Modulation of adhesion molecule and antigen expression	Disruption of cell–substrate and cell–cell contact
	Cell line: <b>Mouse melanoma;</b> pig kidney	<b>Inhibition of fibronectin binding;</b> reduced junctional integrity [1 µmol/L FB <sub>1</sub> (Pelagalli et al., 1999)]	
Other mechanisms	Primary culture: <b>Rat cortical neurons</b>	Attenuation of ischaemic tolerance	Prevention of protective effects of hypoxic preconditioning
	Cell line: <b>Monkey kidney SV40, transformed</b>	Increased p21-activated serine/ threonine kinases activity [5 µmol/L FB <sub>1</sub> (Bokoch et al., 1998)]	Unknown

PKC, phosphatase kinase C; TNF, tumour necrosis factor

decreased, whereas it was increased in phospholipids (Gelderblom et al., 1996a). There was a concomitant increase in the cellular concentrations of phosphatidylcholine and phosphatidylethanolamine, while the total cholesterol concentration decreased. The concentration and labelling of sphingomyelin was also decreased. Changes in the concentrations of specific polyunsaturated fatty acids were attributed to disruption of the  $\Delta 6$  desaturase and cyclo-oxygenase metabolic pathways. These could be important in fumonisin B<sub>1</sub>-induced toxicity in primary hepatocytes.

Fumonisin B<sub>1</sub> disrupted fatty acid and phospholipid biosynthesis in rat liver in vivo. In contrast to the situation in vitro, the main changes were associated with the phosphatidylethanolamine and phosphatidylcholine fractions, while the concentration of cholesterol was increased in both serum and liver (Tables 1 and 2). A characteristic fatty acid pattern was seen in the livers of rats given diets containing purified fumonisin B<sub>1</sub> at concentrations that caused preneoplastic hepatic lesions in rats treated only with fumonisin B<sub>1</sub> ( $\geq 1.6$  mg/kg bw per day) or in rats fed fumonisin B<sub>1</sub> after treatment with known liver cancer initiators such as *N*-nitrosodiethylamine ( $\geq 0.8$  mg/kg bw per day). The pattern includes:

- increased amounts of saturated and monounsaturated fatty acids (C18:1 $\omega$ 9) in phosphatidylcholine and phosphatidylethanolamine;
- increased relative amount of C18:2 $\omega$ 6 in phosphatidylcholine and increased absolute amount in phosphatidylcholine and phosphatidylethanolamine;
- decreased relative and absolute amounts of C20:4 $\omega$ 6 in phosphatidylcholine and increases in phosphatidylethanolamine;
- decreased relative and absolute amounts of C22:4 $\omega$ 6 and C22:5 $\omega$ 6 in phosphatidylcholine, increased relative amount of C22:5 $\omega$ 6 in phosphatidylethanolamine; decreased amount of C22:6 $\omega$ 3 in phosphatidylcholine and increase in phosphatidylethanolamine;
- decreased relative and absolute amounts of total polyunsaturated fatty acids in phosphatidylcholine and decreased relative amounts in phosphatidylethanolamine;
- decreased ratio of polyunsaturated to saturated fatty acids in phosphatidylcholine and phosphatidylethanolamine.

As glycerophospholipids are important components of many cellular signalling pathways, perturbation of the phospholipid and fatty acid composition of cellular membranes could have a strong effect on processes that control cell growth and cell death (Tables 1 and 2). For example, increased expression of hepatocyte growth factor, transforming growth factor (TGF)- $\alpha$  and especially TGF- $\beta$ 1 and *c-myc* was seen in rat liver during short-term feeding with fumonisin B<sub>1</sub> (Lemmer et al., 1999b). Overexpression of TGF- $\beta$ 1 may contribute to the increased apoptosis seen in the livers of rodents fed fumonisin B<sub>1</sub>. The proto-oncogene *c-myc* is a positive regulator of cell proliferation that is involved in tumour progression (Nagy et al., 1988); it has also been implicated in TGF- $\beta$ 1 signalling (Alexandrow & Moses, 1995). Increased expression of *c-myc* and TGF- $\beta$ 1 may cooperate in the promotion of liver tumours during feeding of fumonisin B<sub>1</sub>, possibly by providing an environment that selects for the growth of TGF- $\beta$ 1-resistant transformed liver cells. Overexpression of *c-myc*, depletion of growth factors and/or disruption of growth signalling pathways could result in imbalances in cell cycle progression and hence the induction of apoptosis (Steiner et al., 1996). In this regard, fumonisin B<sub>1</sub> overexpressed *c-myc* in rat liver (Lemmer et al., 1999b), while it disrupted growth-related responses in cell types such as primary hepatocytes and in the liver in vivo (reviewed by Gelderblom et al., 2001a).

In male BDIX rats fed diets containing purified fumonisin B<sub>1</sub> for 2 years, cyclin D1 accumulated in the nuclei of altered hepatocytes in foci, nodules, adenomas, and carcinomas (Ramljak et al., 2000). In male Fischer rats fed diets containing fumonisin B<sub>1</sub> for 21 days, the concentration of cyclin D1 protein in liver was increased up to fivefold in a dose-dependent manner, with no simultaneous increase in mRNA. A fumonisin B<sub>1</sub>-induced increase in cyclin-dependent kinase 4 was confirmed by increased phosphorylation of the retinoblastoma protein; the accumulation of cyclin D1 appeared to result from stabilization of the protein associated with activation of protein kinase B (Akt), followed by inhibition of glycogen synthase kinase 3 $\beta$  activity. Akt is part of the anti-apoptotic phosphatidylinositol 3 kinase cell survival pathway (Dudek et al., 1997) and can be activated by stimuli involving growth factors and cytokines and inhibited by the pro-apoptotic molecule, ceramide (Franke et al., 1997; Zhou et al., 1998). Therefore, the modulating effects of fumonisin B<sub>1</sub> on both sphingolipids and phospholipids could play a major role in molecular events involving the stability of cyclin D1 protein (Ramljak et al., 2000).

It has been proposed that glycerophospholipids and the sphingolipid cycle interact to control a variety of cellular processes, including apoptosis. For example, C20:4 $\omega$ 6 generated by cytosolic phospholipase A<sub>2</sub> initiated sphingomyelin hydrolysis, whereas ceramide generated *de novo* during ceramide synthase stimulated C20:4 $\omega$ 6 release via secretory phospholipase A<sub>2</sub> (Jayadev et al., 1994; Balsinde et al., 1997). Ceramide has also been shown to regulate transcription of cyclooxygenase-2 (Subbaramaiah et al., 1998). A similar interactive pathway is likely to exist for fumonisins in the liver to regulate processes related to cell proliferation and apoptosis. Fumonisin B<sub>1</sub> induces similar changes in phospholipids and in the profile of fatty acids in both the liver and hepatocyte nodules (Abel et al., 2001). However, subsequent effects on sphingolipid and/or prostaglandin production appear to inhibit the growth of normal hepatocytes, which, with the overexpression of TGF $\beta$ -1 and *c-myc*, could affect apoptosis. Oxidative damage and the resultant lipid peroxidation products may further enhance apoptosis in the liver (Chen et al., 1997). Conversely, the increased C18:1 $\omega$ 9, the decreased long-chain polyunsaturated fatty acid concentration, and a phosphatidylethanolamine-associated increase in C20:4 $\omega$ 6 fatty acids are critical for cell proliferation (Tang et al., 1993; Horribin, 1994), especially in initiated cell populations.

#### (b) Other biochemical changes

Several studies of fumonisins *in vitro* showed changes in cellular regulation and function that were attributed to actions independent of altered lipid metabolism (Tables 1 and 2). Many of these effects might be relevant to the toxicity of fumonisins.

Thus, fumonisin-induced disruption of lipid metabolism is observed both *in vitro* and *in vivo*. The biochemical consequences of the disruption of sphingolipid metabolism that are most likely to alter cell regulation are increased concentrations of free sphingoid bases and their 1-phosphates, alterations in complex sphingolipids, and decreased ceramide biosynthesis. Because free sphingoid bases and ceramide can induce cell death, inhibition of ceramide synthase can inhibit cell death induced by ceramide but can promote cell death induced by free sphingoid bases. The kinetics of the increases and decreases in the various bioactive sphingolipid pools in liver, kidney, lung, and heart is also important in the toxicity of these toxins. Fumonisin also induced changes in fatty acids and phospholipids in primary rat hepatocytes

and rat liver in vivo, which closely reflected those expected from disruption of the  $\Delta 6$  desaturase enzyme, the rate-limiting enzyme in fatty acid metabolism, and disruption of prostaglandin biosynthesis. The changes in fatty acid and phospholipid metabolism that probably alter cell regulation are changes in the degree of saturation of fatty acids in the phospholipid pools, increases in the ratio of phosphatidylcholine to phosphatidylethanolamine, changes in prostaglandin biosynthesis, and altered ceramide production.

Fumonisin also affect sites of cellular regulation that are apparently independent of the disruption of lipid metabolism. Nevertheless, disruption of lipid metabolism, membrane structure, and signal transduction pathways mediated by lipid second messengers appear to be important in all the proposed mechanisms of action.

#### **2.1.4 Hypothesized cellular mechanisms of toxicity and carcinogenicity**

Two cellular modes of action for the toxicity and carcinogenicity of fumonisin B<sub>1</sub> have been proposed that are well supported by results obtained in vivo. In both hypotheses, altered lipid metabolism is the initial biochemical mechanism. In one hypothesis, the initial biochemical lesion is presumed to be inhibition of ceramide synthase (Merrill et al., 2001; Riley et al., 2001), and in the other, the biochemical lesion is attributed to disruption of the  $\Delta 6$  desaturase and cyclooxygenase metabolic pathways (Gelderblom et al., 2001a). In both hypotheses, it is assumed that other initial sites of action could contribute to the observed cellular responses. The two invoke similar cellular mechanisms, to the extent that fumonisin B<sub>1</sub>-induced imbalances in the rates of cell death and proliferation in target tissues are considered to contribute to cancer development (Dragan et al., 2001; Howard et al., 2001a).

Fumonisin-induced disruption of sphingolipid metabolism in target tissues has been demonstrated in many independent studies. Nonetheless, the way in which disrupted sphingolipid metabolism contributes to toxicity in rodents is unclear. Current understanding of the sphingolipid signalling pathways (reviewed by Merrill et al., 2001; Riley et al., 2001) indicates that the balance between the intracellular concentrations of sphingolipid effectors that protect cells from apoptosis (decreased ceramide, increased sphingosine 1-phosphate) and the effectors that induce apoptosis (increased ceramide, increased free sphingoid bases, increased fatty acids) determines the cellular response. Cells sensitive to the proliferative effect of decreased ceramide and increased sphingosine 1-phosphate will be selected to survive and proliferate. Conversely, when the rate of increase in free sphingoid bases exceeds a cell's ability to convert sphinganine or sphingosine to dihydroceramide or ceramide or their sphingoid base 1-phosphate, then free sphingoid bases will accumulate to toxic levels. In this case, cells that are sensitive to sphingoid base-induced growth arrest will cease growing, and insensitive cells will survive. Thus, the kinetics of fumonisin elimination (rapid), the affinity of fumonisin B<sub>1</sub> for ceramide synthase (competitive and reversible), and the kinetics of sphinganine elimination (persistent but reversible) could affect the time course, amplitude, and frequency of peaks in the intracellular concentrations of ceramide, sphingoid base-1 phosphates, and free sphinganine in tissues of animals given diets containing fumonisins. This is important, because the balance between the rates of apoptosis and proliferation is a critical determinant in hepato- and nephrotoxicity and tumorigenesis in animal models (Dragan et al., 2001; Howard et al., 2001a; Voss et al., 2001). At the cellular level, apoptotic necrosis should be considered to be similar

to oncotic necrosis (as defined by Levin et al., 1999), in that both lead to a regenerative process involving sustained cell proliferation (Dragan et al., 2001; Hard et al., 2001). Numerous endogenous processes can cause DNA damage; while most are repaired, an unrepaired mutation in DNA can occur. Increased DNA replication can thus increase the risk that damaged DNA is also replicated, resulting in an increased cancer risk. With respect to fumonisin B<sub>1</sub>, this hypothesis is best supported by observations in rat kidney, as in liver apoptotic and oncotic necrosis occur concurrently (Dragan et al., 2001). Nonetheless, regeneration after either apoptotic or oncotic necrosis is observed at the same doses that cause cancer development.

Fumonisin B<sub>1</sub> alters normal cell proliferation in rat liver *in vivo* (Gelderblom et al., 1994, 1996a; Li et al., 2000) and in primary rat hepatocytes and many other cell lines *in vitro* (Gelderblom et al., 1994; Tolleson et al., 1996). Differential inhibition of cell proliferation is a possible mechanism: hepatocytes resistant to fumonisin B<sub>1</sub>-induced inhibition of cell growth are selectively stimulated to grow by creating an environment in which the growth of normal cells is impaired. Selective inhibition of normal cell growth could increase the chances of survival of cells initiated by processes such as free-radical damage, leading ultimately to manifestations of cancer initiation in liver, such as glutathione *S*-transferase, placental form (GST-P)<sup>+</sup> foci and hepatocyte nodules. Fumonisin B<sub>1</sub> may be not only a complete carcinogen but also promote cancer, and this should be considered in models for risk assessment.

Disruption of the  $\Delta 6$  desaturase and cyclooxygenase metabolic pathways in the livers of male rats has been well documented. Disruption of lipid-mediated growth stimulation in the liver could be important in establishing a growth differential that results in clonal expansion of certain cell types associated with neoplastic development. For example, disruption of C20:4 $\omega$ 6 metabolism altered the mitogenic response to epidermal growth factor in primary rat hepatocytes, a known property of many hepatocarcinogens (Gelderblom et al., 1999a). Three lines of evidence support the hypothesis that fumonisin B<sub>1</sub>-induced alteration of lipid metabolism contributes to establishing a growth differential in rat liver. First, the lipid parameters associated with increased cell proliferation in hepatocyte nodules closely mimic those of normal regeneration in the liver; one major difference is that the changes in the nodules are persistent, whereas they are reversed in regenerating liver (Abel et al., 2001). The increased concentrations of phosphatidylethanolamine and C20:4 $\omega$ 6 are of special interest, as this fatty acid is known to regulate many processes related to cell growth, such as proliferation and apoptosis (Khan et al., 1995). Several studies indicated that fumonisin B<sub>1</sub> interacts with C20:4 $\omega$ 6 metabolism in normal and cancer cell lines *in vitro* (Gelderblom et al., 1999a; Pinelli et al., 1999; Seegers et al., 2000). Second, alterations in the N-6 fatty acid desaturase pathway and the subsequent decrease in long-chain polyunsaturated fatty acids would result in a more rigid membrane structure. This could provide a survival advantage for hepatocytes under stress, since the membranes will be resistant to oxidative damage. Such membrane changes occur preferentially in hepatocyte nodules, creating an environment for differential growth relative to the surrounding normal tissue. Changes in membrane fluidity could also alter the response of membrane receptors and enzymes by affecting their mobility in the bilayer. Third, lipid metabolites, and in particular glycerophospholipids, are important components of many cellular signalling systems that control the balance between cell growth and cell death.

In the classical model of cancer initiation by genotoxic carcinogens, fumonisins did not increase the incidence of hepatocellular foci after single or multiple doses

(Gelderblom et al., 1992). Subsequent studies indicated that the 'effective dose' for induction of preneoplastic lesions in liver, such as GST-P<sup>+</sup> foci and hepatocyte nodules, in male Fischer rats depends on the dose and duration of exposure (Gelderblom et al., 1994). The toxicity of fumonisin B<sub>1</sub> in rat liver appears to play an important role in cancer development (Abel & Gelderblom, 1998). Induction of oxidative damage and lipid peroxidation as a consequence of toxicity (Tables 1 and 2) could lead to DNA damage. Changes in the balance of the various cell regulatory molecules, such as those found in the livers of rats fed diets containing fumonisin B<sub>1</sub>, are likely to be involved in induction of a growth differential in which the growth of initiated cells is selectively stimulated and cancer develops.

## 2.2 Toxicological studies

Many of the studies summarized in this section are also described in the IPCS monograph (WHO, 2000a). The pathological findings and doses in pertinent studies are given in Tables 4–6. The diets used in most of the studies summarized in this section differed markedly in nutritional composition. As the proposed mechanisms of action involve alterations in *de novo* biosynthesis, nutritional factors might be important in toxic end-points. The liver was the target for fumonisin B<sub>1</sub> in all animals in which toxicity was observed, and the kidney was also a target in many animals. In both liver and kidney, fumonisin B-induced toxicity is often characterized initially by increased apoptotic and oncotoc necrosis, regeneration, and, in the case of liver, bile-duct hyperplasia (Tables 4 and 5). In rodents, the toxicity of fumonisin B<sub>1</sub> depends on the strain and sex. For example, male BD IX rats appeared to be more resistant to the nephrotoxic effects of fumonisin B<sub>1</sub> than male Fischer 344N, male Sprague-Dawley, and male RIVM:WU rats.

*F. verticillioides* culture material and naturally contaminated maize can contain various fumonisins and other mycotoxins. However, naturally contaminated maize and culture material of the *F. verticillioides* isolate known as MRC 826 contain predominantly fumonisins of the B series. Therefore, the results of studies with these materials corroborate those of studies in which pure fumonisin B<sub>1</sub> was used. Although the results were not used for hazard characterization or risk assessment, when possible, the NOEL or LOEL was calculated for comparison with the results of studies with pure fumonisin B<sub>1</sub>.

### 2.2.1 Acute toxicity

No studies have been published on the lethality of single doses of pure fumonisin B<sub>1</sub> in laboratory animals. The few available studies indicate that fumonisins are not acutely toxic. For example, mice given a single dose of 25 mg/kg bw by gavage or subcutaneous injection showed reversible alterations in cytokine expression, serum enzymes activity, and blood cell counts (Bhandari et al., 2001).

### 2.2.2 Short-term studies of toxicity

#### (a) BALB/c mice

Male mice were given a subcutaneous dose of fumonisin B<sub>1</sub> at 0.3, 0.8, 2.3, or 6.8 mg/kg bw per day for 5 days. Apoptosis was detected in the livers of mice at doses  $\geq$  0.8 mg/kg bw per day and in the kidney at all doses (Sharma et al., 1997; Tsunoda et al., 1998). If it is assumed that 10% of an oral dose would be absorbed

in mice, the calculated LOEL for oral administration would be 8 mg/kg bw per day in liver and 3 mg/kg bw per day in kidney. The relative weight of the kidney was decreased at all doses except 0.8 mg/kg bw per day; no effect was observed on the relative weight of the liver. Increased apoptosis in liver was also seen in subsequent studies with various strains of mice and transgenic mouse models. The response to fumonisin differed in some transgenic mouse models in comparison with the wild type (Sharma et al., 2000a,b,c). The results of studies with mice that overexpress or lack tumour necrosis factor (TNF)- $\alpha$  suggest that this pathway plays a role in the hepatic toxicity of fumonisin B<sub>1</sub> in mice.

(b) *B6C3F<sub>1</sub> mice*

Male and female mice were fed diets containing fumonisin B<sub>1</sub> at a concentration of 1, 3, 9, 27, or 81 mg/kg for 90 days, providing mean intakes of 0.3, 0.9, 2.5, 7.4, and 23 mg/kg bw per day for males and 0.3, 1, 3, 9.7, and 29 mg/kg bw per day for females. The serum concentrations of cholesterol and total bilirubin and the activities of alanine and aspartate aminotransferases, alkaline phosphatase, and lactate dehydrogenase were significantly increased in female mice at the high dose, with no effect in males. The changes were paralleled by histological alterations in the livers of the female mice, mainly restricted to the centrilobular zone. No lesions were reported in the kidney, but some were detected in the adrenal cortex (presumably a normal physiological reaction to stress induced by the treatment) in all females given the highest dose and two females given the next lowest dose (Table 4; Voss et al., 1995a).

Adult male and female mice were given fumonisin B<sub>1</sub> at a daily dose of 1, 5, 15, 35, or 75 mg/kg bw per day by gavage for 14 days. Hepatotoxicity was observed in animals of each sex, but renal toxicity was seen only in females. Females were more sensitive than males to these effects. Single-cell necrosis was found in the liver at a dose of 35 mg/kg bw per day in males and 15 mg/kg bw per day in females. Increased hepatocyte mitosis was seen at 75 mg/kg bw per day in males and  $\geq 5$  mg/kg bw per day in females. Mild single-cell necrosis was detected in the cortical and medullary tubules only in female mice at 15–75 mg/kg bw per day. Males (at doses  $\geq 35$  mg/kg bw per day) and females (at doses  $\geq 15$  mg/kg bw per day) had moderate diffuse vacuolization of adrenal cortical-cell cytoplasm. Mild thymic cortical lymphocytolysis was noted in a few female mice that received doses  $\geq 35$  mg/kg bw per day (Table 4; Bondy et al., 1997).

Male and female mice were fed diets containing fumonisin B<sub>1</sub> at a concentration of 99, 160, 230, or 480 mg/kg for 28 days in order to establish the doses for a 2-year bioassay. Males at the highest concentration developed liver lesions. Changes in clinical chemical end-points and cell cycle progression indicative of increased proliferation paralleled the pathological changes. Similar changes were seen in females but at all doses. Thus, female mice were more sensitive than males to the hepatic toxicity of fumonisin B<sub>1</sub>. No NOEL could be identified, as liver lesions were seen in females at all doses (Table 4; National Toxicology Program, 1999).

(c) *BD IX rats*

Male rats were fed diets containing fumonisin B<sub>1</sub> at a concentration of 0.1% for 33 days. Major changes were reported in the liver and mild changes in kidney

**Table 4. Results of studies in male and female B6C3F<sub>1</sub> mice given diets containing purified fumonisin B<sub>1</sub>**

Sex	Treatment (mg/kg)	Fumonisin B <sub>1</sub> intake (mg/kg bw per day)	Main pathological lesions or effects		Reference
			Liver	Kidney	
Males and females	NIH open formula 07 diet, 90 days				Voss et al. (1995a)
Males	1	0.3	No lesions	No lesions	
	3	0.8			
	9	2.4			
	27	7.4			
	81	23			
Females	1	0.3	Highest dose only: Necrosis, mitotic figures, hepatocyte collapse of centrilobular zone; infiltration of inflammatory cells		
	3	1			
	9	3			
	27	9.7			
	81	29			
Males and females al. single-cell	Gavage, 14 days	1 (1997)	Males: Increased cholesterol, alanine 5 necrosis ( $\geq 35$ mg/kg bw per day). Increased mitosis at 75 mg/kg bw per day	No lesions aminotransferase activity,	Bondy et al.
		15	Females: Increased cholesterol, alanine aminotransferase activity, single-cell necrosis ( $\geq 15$ mg/kg bw per day). Increased mitosis at 5 mg/kg bw per day	Females: single-cell necrosis and vacuolization of cytoplasm in the cortical and medullary tubules at $\geq 15$ mg/kg bw per day	
		35			
		75			

Table 4 (contd)

Sex	Treatment (mg/kg)	Fumonisin B <sub>1</sub> intake (mg/kg bw per day)	Main pathological lesions or effects		Reference
			Liver	Kidney	
Males and females	NIH 31 diet, 28 days				National Toxicology Program (1999)
Males	99	19	Highest dose only: Hepatocellular necrosis, periportal hypertrophy, diffuse centrilobular hyperplasia, Kupffer cell hyperplasia	No lesions in any group	
	160	31			
	230	44			
	480	93			
Females	99	24	All treated animals: Hepatocellular necroses, periportal hypertrophy, diffuse centrilobular hyperplasia, Kupffer cell hyperplasia > 160 mg/kg: Bile canaliculi hyperplasia	No lesions in any group	
	160	41			
	230	62			
	480	100			
Males and females	NIH 31 diet, 728 days				National Toxicology Program (1999); Howard et al. (2001b)
Males	5	0.5	Hepatocyte hypertrophy (10/47, 9/47, 24/48, 25/48, 30/48), Multifocal necrosis (1/47, 1/47, 0/48, 4/48, 14/48) Hepato- cellular adenomas (9/47, 7/47, 7/48, 6/48, 8/48), Hepatocellular carcinomas (4/47, 3/47, 4/48, 3/48, 2/48)	No lesions in any group	
	15	1.6			
	50	9.0			
	150	15			
Females	5	0.7	Hepatocyte hypertrophy (0/47, 0/48, 0/48, 7/47, 31/45), apoptosis (0/47, 0/48, 0/48, 7/47, 14/45), multifocal necrosis (1/47, 1/48, 1/48, 29/47, 26/45). Hepatocellular adenomas (5/47, 3/48, 1/48, 16/47, 31/45), hepatocellular carcinomas (0/47, 0/48, 0/48, 10/47, 9/45)	No lesions in any group	
	15	1.9			
	50	6.6			
	80	13			

(Table 5). After 3 days of treatment of male rats with fumonisin B<sub>1</sub> at 240 mg/kg bw per day by gavage, major pathological lesions were observed in the liver and only minor changes in kidney. Severe disseminated acute myocardial necrosis and severe pulmonary oedema were observed in two rats. At lower doses but longer treatment (9–12 days), pathological changes were observed only in liver. Early signs of bile-duct proliferation and fibrosis radiating from the portal areas were noted, and the nuclei of a few hepatocytes were enlarged (Gelderblom et al., 1988).

Culture material from *F. verticillioides*, strain MRC 826, was fed to male rats for 77 days. All developed cirrhosis, intraventricular cardiac thrombosis and nephrosis (Kriek et al., 1981a).

(d) *Fischer rats*

When male rats were fed diets containing fumonisin B<sub>1</sub> at a concentration of 0.1% for 55 days, the pathological lesions reported in the livers were similar to those described in male BD IX rats. Hepatocyte nodules were induced when the fumonisin B<sub>1</sub>-fed rats were subjected to promotion by 2-acetylaminofluorene and partial hepatectomy (Gelderblom et al., 1992). In subsequent studies, in which rats were given diets containing 25–750 mg/kg, histopathological lesions were observed in the livers of animals at 500 and 750 mg/kg of diet after 21 days. Similar changes, although less pronounced, were observed in the rats that received 100 and 250 mg/kg of diet. A few necrotic cells were detected in the livers of rats at 50 mg/kg of diet, but none were observed at 25 mg/kg of diet. Measurements indicated that the mean daily intake of fumonisin B<sub>1</sub> at these six doses was 1.7, 3.5, 7.2, 15, 25, and 31 mg/kg bw per day. Twenty-one days after the promoting treatment, GST-P<sup>+</sup> foci and nodules were induced at 250 mg/kg of diet or a daily intake  $\geq$  15 mg/kg bw. The dose-dependency for inducing the putative preneoplastic lesions in liver was also seen after daily treatment by gavage for 14 days. After the promoting treatment, hepatocyte nodules and toxic effects in the liver were seen only in rats that received a daily dose  $\geq$  8.5 mg/kg bw. These studies showed that fumonisin B<sub>1</sub>-induced hepatotoxicity was a prerequisite for development of the preneoplastic lesions in rat liver (Table 5; Gelderblom et al., 1994).

In a study of the cancer promoting potential of fumonisin B<sub>1</sub>, similar dietary concentrations were fed to male rats over 21 days. Pathological changes were reported in the livers of rats receiving 50 mg/kg of diet (3.5 mg/kg bw per day) but not in those that received 10 mg/kg of diet (0.7 mg/kg bw per day), and cancer promotion was seen in *N*-nitrosodiethylamine-initiated rats at the higher concentration (Table 5; Gelderblom et al., 1996b). Microscopic lesions were seen in the kidneys of rats that received the low dose (W.C.A. Gelderblom, unpublished data) and in the inner cortex and outer medulla of rats at the high dose. The changes were common in animals at the high dose and only mild at 10 mg/kg of diet (0.7 mg/kg bw per day). These results indicate that similar lesions are induced by comparable doses of fumonisin B<sub>1</sub> in the livers of male BD IX and Fischer rats, but in Fischer rats lesions were detected in the livers at a dietary concentration as low as 50 mg/kg and in the kidneys at a concentration as low as 10 mg/kg.

**Table 5. Results of studies in rats given diets containing purified fumonisin B<sub>1</sub>**

Strain and sex	Treatment (mg/kg)	Fumonisin B <sub>1</sub> intake (mg/kg bw per day)	Main pathological lesions or effects		Reference
			Liver	Kidney	
BD IX, male	Gavage	240, 3 days	75% of rats died; toxic hepatitis, myocardial necrosis, pulmonary oedema, bile-duct proliferation	ND	Gelderblom et al. (1988)
		70, 9 days	Single-cell necrosis; hydrophic degeneration	ND	
		48, 12 days 70, 33 days	Fibrosis Bile-duct proliferation; fibrosis; hepatocyte nodules	Proximal convoluted tubule: fatty changes; scant necrosis	
BD IX, male	Semi-purified diet, 780 days 50	1.6	Cirrhosis (15/15); regenerative nodules (15/15); cholangio-fibrosis (15/15); hepatocellular carcinoma (10/15)	Mild changes in proximal convoluted tubule towards end of experiment	Gelderblom et al. (1991)
BD IX, male	Semi-purified diet, 690 days	1	No lesions	No lesions	Gelderblom et al. (2001a)
		10	Mild changes	At 10 and 25 mg/kg:	
		25	Anisokaryosis (13/17); hepatocyte nodules (9/17); oval-cell proliferation (2/17); bile-duct hyperplasia (3/17); portal fibrosis (5/17); ground-glass foci (5/17); GST-P <sup>+</sup> foci (11/11)	Chronic, low-grade renal toxicity limited to proximal and distal tubules	

**Table 5** (contd)

Strain and sex	Treatment (mg/kg)	Fumonisin B <sub>1</sub> intake (mg/kg bw per day)	Main pathological lesions or effects		Reference
			Liver	Kidney	
Fischer 344, male	101, Dyetts Inc. diet, 55 days, 1000	60	Bile -duct proliferation, hepatocyte nodules, hepatocyte necrosis	ND	Gelderblom et al. (1992)
Fischer 344, male	AIN-76 diet, 21 days				Gelderblom et al. (1994)
	25	1.7	No lesions		
	50	3.5	No lesions		
	100	7.2	A few necrotic cells		
	250	15	Bile-duct proliferation		
	500	25	Apoptosis		
	750	31	Degenerative changes		
	Gavage, 14 days				
	59	4	As above; hepatocyte nodules	ND	
	120	8.5	after promotion with acetylami- nofluorene and partial hepatectomy		
	230	16			
	320	23			
Fischer 344, male	AIN-76 diet, 21 days				Gelderblom et al. (1996c)
	10	0.7	No lesions	All treated groups:	
	50 <sup>a</sup>	3.5	Scattered necrotic cells	Nephrosis, necrotic	
	100	6.8	Apoptosis, ductile endothelial cell proliferation, mitotic figures	epithelial cells, apoptosis, hypereosinophilia, slough- ing of epithelial cells	
	250	15	Nodular regeneration, fibrosis, ductile endothelial cell proliferation		
	500	25	Distortion of lobular structure		

**Table 5** (contd)

Strain and sex	Treatment (mg/kg)	Fumonisin B <sub>1</sub> intake (mg/kg bw per day)	Main pathological lesions or effects		Reference
			Liver	Kidney	
Fischer 344, male	AIN-76 diet, 35 days, 250	15	Hepatocyte necrosis, apoptosis, stellate-cell proliferation, fibrosis, regenerative nodules, foci and nodules, oval-cell proliferation	ND	Lemmer et al. (1999a,b)
Fischer 344, male	35 days at 15 mg/kg bw, 150 days at 100 mg/kg bw, 185 days on AIN-76 diet without FB <sub>1</sub>	15	GST-P <sup>+</sup> foci and nodules, fibrosis, oval-cell proliferation, cholangiofibrosis Dysplastic liver nodules (6/6), cholangiofibrosis (1/6), hepatocellular carcinoma (1/6)	ND	Lemmer (2000)
	250 100 "Stop model"	7			
Fischer 344 Males <sup>b</sup>	NIH 31 diet, 28 days				
	99	12	No lesions	All treated groups: Increased apoptosis, degeneration, and mitosis; decreased relative kidney weights	Tolleson et al. (1996); National Toxicology Program (1999); Howard et al. (2001b)
	160	20	No lesions		
	230	28	Increased apoptosis and degeneration at doses > 20 mg/kg bw per day. Increased, mitosis <sup>c</sup> , bile-duct hyperplasia, decreased relative liver weight at doses > 28 mg/kg bw per day		
	480	56			

**Table 5** (contd)

Strain and sex	Treatment (mg/kg)	Fumonisin B <sub>1</sub> intake (mg/kg bw per day)	Main pathological lesions or effects		Reference	
			Liver	Kidney		
Fischer 344 Females <sup>b</sup>	NIH 31 diet, 28 days				Tolleson et al. (1996); National Toxicology Program (1999); Howard et al. (2001b)	
	99	12	Increased apoptosis in all groups; increased mitosis and degeneration at doses > 12 mg/kg bw per day; bile- duct hyperplasia and decreased relative liver weights at doses > 20 mg/kg bw per day	Decreased relative kidney weights and increased mitosis in all groups; increased apop- tosis, degeneration at doses > 12 mg/kg bw per day		
	160	20				
	230	28				
	480	56				
Fischer 344	NIH open formula 07, 90 days				Voss et al. (1995a)	
	Males		No lesions	No lesions		
	1	0.1				
	3	0.2				
	9	0.6				
	27	1.9				
	81	5.7	Single-cell necrosis, nec- rotic tubule epithelial cells, eosinophilic cyto- plasm, sloughing of tu- bule epithelia			
	Females			No lesions		No lesions
	1	0.1				
	3	0.3				
9	0.7					
27	2.2					
91	6.4	Lesions				

Table 5 (contd)

Strain and sex	Treatment (mg/kg)	Fumonisin B <sub>1</sub> intake (mg/kg bw per day)	Main pathological lesions or effects		Reference	
			Liver	Kidney		
Fischer 344 Males	Diet, 728 days	Weeks 51–104			National Toxicology Program (1999); Hard et al. (2001); Howard et al. (2001b)	
	5	0.22	No lesions	(Data from Hard et al., 2001) Adenoma (0/48, 0/40, 0/48, 4/48, 6/48); carcinoma (0/48, 0/40, 0/48, 8/48, 10/48); atypical tubule (0/48, 0/40, 0/48, 4/48, 9/48); apoptosis and regeneration sustained throughout study at doses ≥ 0.67 mg/kg bw		
	15	0.67				
	50	2.2				
	150	6.6				
Females	5	0.27	No lesions	No lesions		
	15	0.78				
	50	2.6				
	100	5.2				
Sprague-Dawley, male and female	RMH 3000 diet, 28 days				Voss et al. (1993, 1995b)	
	15	1.4	No lesions	Single-cell necrosis, sloughing of tubular epithelia, epithelial hyperplasia, cytoplasmic basophilia		
	50	4.1	Mild changes			
	150	13	Single-cell necrosis, cell and nuclear polymorphism, bile-duct proliferation (?); lesions more severe in females	Males, all doses: females, ≥ 4.7 mg/kg bw per day		

Table 5 (contd)

Strain and sex	Treatment (mg/kg)	Fumonisin B <sub>1</sub> intake (mg/kg bw per day)	Main pathological lesions or effects		Reference
			Liver	Kidney	
Sprague-Dawley, male and female	Gavage, 11 days	1	Males: single-cell necrosis, increased alanine and aspartate aminotransferases (15 mg/kg bw per day), mitosis (35 mg/kg bw per day) Females: increased cholesterol (5 mg/kg bw per day); single-cell necrosis, mitosis, increased alanine aminotransferase at 15 mg/kg bw per day	Necrosis of tubular epithelia, anisokaryosis, cytoplasmic basophilia, atrophy of tubular epithelia Males, all doses; females, ≥ 5 mg/kg bw per day	Bondy et al. (1996, 1998)
		5			
		15			
		35			
		75			
Sprague-Dawley, male and female	Gavage, 11 days	1	Males: GST-P <sup>+</sup> foci and PCNA <sup>+</sup> nuclei at doses ≥ 35 mg/kg bw per day. Females: GST-P <sup>+</sup> foci at 75 mg/kg bw per day. PCNA <sup>+</sup> nuclei at 35 and 75 mg/kg bw per day	Not reported, but experimental design similar to Bondy et al. (1996)	Mehta et al. (1998)
		5			
		15			
		35			
		75			
RIVM:WU, male	Gavage, 28 days	0.2	No lesions at any dose Increased γ-glutamyl transferase activity at 0.75 and 3 mg/kg bw per day	Dose-dependent increase in tubular cell death at all doses; increased apoptosis and mitosis at 0.75 and 3 mg/kg bw per day	de Nijs (1997)
		0.8			
		3.0			

ND, not determined; GST-P<sup>+</sup>, positive for glutathione *S*-transferase, placental form; PCNA<sup>+</sup>, positive for proliferating cell nuclear antigen

<sup>a</sup> 50 mg/kg of diet; promotion in rats initiated with *N*-nitrosodiethylamine

<sup>b</sup> Final body weight significantly lower after 28 days in males and females at 484 mg/kg of diet

<sup>c</sup> Mitosis based on morphological evaluation was less than that measured in the PCNA assay.

The kinetics of hepatocyte injury in liver was investigated in male rats fed a diet containing fumonisin B<sub>1</sub> at 250 mg/kg for 5 weeks. Hepatocyte necrosis and apoptosis were found mainly in zone 3 of the liver lobule. Hepatocyte injury and death were mirrored by desmin-positive hepatic stellate-cell proliferation and marked fibrosis, with progressive disturbance of the architecture and formation of regenerative nodules. Oval-cell proliferation was seen in the presence of hepatocyte mitotic activity. Oval-cell (OV-6 positive) proliferation was noted from week 2; glutathione S-transferase-positive hepatic foci and nodules developed; and, later, oval cells were seen inside some of the 'atypical' nodules (Table 5; Lemmer et al., 1999b).

In a subsequent experiment, groups of 12 male Fischer rats were fed diets containing fumonisin B<sub>1</sub> at a concentration of 250 mg/kg for 5 weeks followed by 100 mg/kg of diet for the remainder of the experiment, up to 6 months, when exposure was discontinued ('stop model'). Liver biopsy samples were collected from a subpopulation of the rats for another 6 months. The body-weight gain of treated animals was significantly lower after 6 months. A variety of preneoplastic and neoplastic hepatic lesions were observed in the samples collected. The intake of fumonisin B<sub>1</sub> was estimated to be 15 mg/kg bw per day (Table 5; Vessey et al., 1999; Lemmer, 2000).

Male and female rats were fed diets containing fumonisin B<sub>1</sub> at a concentration of 99, 160, 230, or 480 mg/kg for 28 days, in order to set the doses for a 2-year bioassay, providing average daily doses of the toxin of 12, 20, 28, and 56 mg/kg bw per day. The body weights of both males and females were at the highest dose were decreased. Similar pathological lesions and effects were seen in the livers and kidneys of males and females. The male kidney was the most sensitive target for fumonisin B<sub>1</sub>, and the liver was more severely affected in females than in males. The earliest cellular response in both liver and kidney was increased apoptosis, which was accompanied by increased cell proliferation. Structural degeneration as a result of apoptosis was seen in both liver and kidney. In females, the NOEL for bile-duct hyperplasia and decreased liver weight was 28 mg/kg bw per day, and that for liver degeneration and increased hepatocellular mitosis was 20 mg/kg bw per day. The NOEL for increased hepatocellular apoptosis and for decreased kidney weight, increased mitosis, increased structural degeneration, and increased mitosis in males was < 12 mg/kg bw per day (Table 5; Tolleson et al., 1996; National Toxicology Program, 1999; Howard et al., 2001b).

Male and female rats were fed diets containing fumonisin B<sub>1</sub> at a concentration of 1, 3, 9, 27, or 81 mg/kg for 90 days. No hepatotoxic effects were seen in male or female rats, whereas renal toxicity was found in male rats fed diets containing  $\geq 9$  mg/kg. Female rats were more resistant, as renal changes were observed only at 81 mg/kg of diet (5.7 mg/kg bw per day); however, when kidney weight was used as a marker, a significant difference was found between control females and females fed 9 mg/kg of diet (0.7 mg/kg bw per day). The NOEL for renal toxicity in male rats was 0.2 mg/kg bw per day (Table 5; Voss et al., 1995a).

A maize sample naturally contaminated with *F. verticillioides* that had been involved in an epizootic of equine leukoencephalomalacia in the USA was fed to male rats for 5–6 months. The liver was the major organ affected (Table 6; Wilson et

al., 1985). Retrospective analyses indicated the the total concentration of fumonisin B<sub>1</sub> and B<sub>2</sub> was 33 mg/kg, and only trace amounts of aflatoxin B<sub>1</sub> and B<sub>2</sub> were present. Dietary deficiencies, especially those related to methionine and choline, were suggested to have accelerated and/or promoted the liver lesions. The mean fumonisin B intake was calculated to be equivalent to 2.3–3.2 mg/kg bw per day (Gelderblom et al., 2001a).

(e) *Sprague-Dawley rats*

Male and female rats were fed diets containing fumonisin B<sub>1</sub> at a concentration of 15, 50, or 150 mg/kg for 4 weeks, providing estimated daily intakes of 1.4, 4.7, and 14 mg/kg bw per day for males and 1.4, 4.1, and 13 mg/kg bw per day for females. Only mild changes were observed by light microscopy in the livers of rats fed the high dose. The NOEL for effects on the liver was thus 4.1–13 mg/kg bw per day. The nephrotoxic changes were localized in the proximal convoluted tubules of males fed diets containing  $\geq 15$  mg/kg and of females at  $\geq 50$  mg/kg. The NOEL for effects on the kidney was  $< 1.4$  mg/kg bw per day in males and 1.4 mg/kg bw per day in females (Table 5; Voss et al., 1993, 1995b). Ultrastructural changes were observed in the livers in males and females at doses  $\geq 15$  and  $\geq 50$  mg/kg of diet, respectively. The activity of serum enzymes and the concentrations of cholesterol and triglycerides were increased at 150 mg/kg of diet (Riley et al., 1994). In a subsequent study in male rats dosed intraperitoneally with fumonisin B<sub>1</sub> at 2 mg/kg bw per day for 4 days, alanine aminotransferase activity, serum cholesterol concentration, and apoptosis (on the basis of morphology and the terminal UTP end-labelling assay) were increased in liver on day 3. Although increased proliferation was also seen on day 3, it was not statistically significant until day 5 (only liver was examined on days 1, 3, and 5) (Li et al., 2000).

In male and female rats given fumonisin B<sub>1</sub> at 1, 5, 15, 35, or 75 mg/kg bw for 11 days by gavage, the histopathological changes in the kidneys were similar to those described in other studies, and males were more sensitive to the renal effects ( $< 1.0$  mg/kg bw per day for males and 5 mg/kg bw per day for females). Primary changes associated with bone-marrow toxicity were also found in both sexes. Hepatotoxicity, reduced liver weight, and increased vacuolization of adrenal cortical cells were found in female rats treated with doses  $\geq 15$  mg/kg bw per day. Elevated cholesterol concentrations were observed in female rats from 5 mg/kg bw per day. Changes in serum enzyme activity and cholesterol concentration in males were markedly increased from 15 mg/kg bw per day, while single-cell necrosis and mitosis were seen at 15–75 mg/kg bw per day. Mild lymphocytosis in the thymic cortex was seen in rats treated at 5 mg/kg bw per day and persisted in rats at the highest dose. It was concluded that male rats are marginally more sensitive than female rats to fumonisin B<sub>1</sub> (Table 5; Bondy et al., 1996, 1998).

The number of GST-P<sup>+</sup> hepatocytes was increased in males and females given 35 and 75 mg/kg bw per day by gavage, respectively, for 11 days. In the same study, the area occupied by GST-P<sup>+</sup> minifoci was increased significantly in both sexes at 75 mg/kg bw per day. The hepatocyte proliferation rate, measured as the expression of proliferating cell nuclear antigen (PCNA), was significantly enhanced in the livers of rats in which the number of GST-P<sup>+</sup> cells was increased ((Mehta et

al., 1998). Pathological changes occurred at the same doses in males and females, although they appeared to be more severe in males (Bondy et al., 1996, 1998). After intraperitoneal administration of a single dose of 7.5 or 10 mg/kg bw to male rats on four consecutive days, only the higher dose significantly enhanced the induction of GST-P<sup>+</sup> minifoci. The authors suggested that, like known genotoxic carcinogens, fumonisin B<sub>1</sub> can induce GST-P<sup>+</sup> hepatocytes and their subsequent development into minifoci in the presence of enhanced hepatocyte proliferation, presumably in response to toxicity.

Maize samples associated with outbreaks of equine leukoencephalomalacia fed to male rats for 28 days caused degenerative changes in the liver and kidney similar to those seen with purified fumonisin B<sub>1</sub> (Table 6; Voss et al., 1989). The toxicity was probably exacerbated by the nutritionally imbalanced diet. On the basis of the estimated feed intake and the estimated fumonisin B content of the two samples (150 and 20 mg/kg of feed), the mean daily intake of fumonisin B<sub>1</sub> and B<sub>2</sub> was equivalent to 13 and 1.7 mg/kg bw, respectively (Plattner et al., 1990).

Male rats were fed diets containing *F. verticillioides* MRC 826 culture material at a concentration of 270 mg/kg for 28 days, providing an intake of fumonisin B equivalent to 18 mg/kg bw per day. Pathological lesions were found in the liver (Table 5; Voss et al., 1990). Retrospective examination (K. Voss, personal communication) revealed renal lesions similar to those described by Voss et al. (1989), who reported on the effects of three batches of *F. verticillioides* that produced mainly fumonisin B<sub>1</sub>, B<sub>2</sub>, or B<sub>3</sub>. Concentrations of fumonisin B of 4.6–6.9, 32–53, and 220–300 mg/kg were incorporated into the diets, resulting in estimated intakes of 0.4–0.6, 2.7–4.5, and 14–20 mg/kg bw per day. Liver lesions were reported at the two higher doses, whereas renal lesions were observed at all doses (Table 6; Voss et al., 1998). The toxicological effects of fumonisin B<sub>2</sub>- and fumonisin B<sub>3</sub>-containing cultures, fed at similar doses, mimicked those of the fumonisin B<sub>1</sub> culture material. The toxicity was reversed after 3 weeks on fumonisin B-free diets.

(f) *RIVM rats*

Male rats were given pure fumonisin B<sub>1</sub> at a dose of 0.19, 0.75, or 3 mg/kg bw per day by gavage for 28 days. Treatment had no effect on body weight, but the kidney weight was significantly reduced in animals given the highest dose, and increased apoptosis and renal tubular-cell death were seen at the two higher doses. The serum concentrations of urea and creatinine were not altered. There was no histological indication of liver toxicity, but serum  $\gamma$ -glutamyl transferase activity was significantly increased at 0.75 and 3 mg/kg bw per day; no change was observed in the activity of alanine and aspartate transaminases. The NOEL for increased renal tubular-cell death was 0.19 mg/kg bw per day (Table 5; de Nijs, 1997).

(g) *Other species*

As maize is an important component of many animal feeds, numerous studies have been conducted with agriculturally important species and pure fumonisins, contaminated maize screenings, or maize culture material of *F. verticillioides*. Examples of these studies include those in catfish, cattle (Mathur et al., 2000),

**Table 6. Results of studies in rats given diets containing samples naturally contaminated with fumonisin B<sub>1</sub> or fungal cultures**

Strain and sex	Dietary addition, duration	Fumonisin B intake (mg/kg bw per day)	Main pathological lesions or effects		Reference
			Liver	Kidney	
Sprague-Dawley, male	Maize screenings <sup>a</sup> , 28 days	13	Single-cell necrosis, fibrosis (mild), bile-duct hyperplasia, adenofibrosis, mitotic figures, disruption of lobular structure	Tubular basophilia, cytoplasmic vacuolization, sloughing of tubular epithelia, single-cell necrosis, epithelial hyperplasia	Voss et al. (1989)
		1.7	Mild changes		
Sprague-Dawley, male	MRC 826 diet, 28 days	18	Bile-duct proliferation, single-cell necrosis, minimal fibrosis, mitotic figures, inflammatory cells	Retrospective examination revealed lesions similar to those described above.	Voss et al. (1990)
Sprague-Dawley, male	Fungal culture, 21 days)	0.4–0.6 2.7–4.5 14–20	No lesions Mild changes Single-cell necrosis and bile-duct hyperplasia	All groups: basophilic epithelial cells, apoptosis, sloughing of tubular epithelia	Voss et al. (1998)
Fischer 344, male	Contaminated maize, 123–176 days	2.3–3.2	Neoplastic nodules (12/12); adenofibrosis (12/12); cholangiocarcinoma (12/12)	No lesions	Wilson et al. (1985)

Table 6 (contd)

Strain and sex	Dietary addition, duration	Fumonisin B intake (mg/kg bw per day)	Main pathological lesions or effects		Reference
			Liver	Kidney	
BD IX	Mouldy maize in Transkei diet, 610–691 days	0.65–1.4	Proliferating bile-duct oval cells (grade 3; 17/23), hyperplastic nodules (grade 3; 8/23)	Not reported	Purchase & Joubert (1970); Purchase et al. (1975)
BD IX, male	MRC 826 in Epol diet; 288 days on 4%, 606 days on 2%; total, 894 days	6.9 (4%) 3.2 (2%)	Cirrhosis (20/20); adenofibrosis (19/20); ductular carcinoma (10/20); hepatocellular carcinoma (12/20); basal-cell hyperplasia (11/15)	No lesions	Marasas et al. (1984)
BD IX, male	MRC 826 in semi-purified diet; 211 days on 0.25%, 311 days on 0.5%, 81 days on 0.75%, 266 days on 0.5%; total, 869 days	0.4 (0.25%) 0.9 (0.5%) 1.3 (0.75%)	Neoplastic nodules (18/21); GGT <sup>+</sup> foci (18/21); fatty change (21/21); hepatocellular carcinoma (2/21); ductular hyperplasia (21/21); adenofibrosis (19/21); cholangiocarcinoma (8/21)	No lesions reported	Jaskiewicz et al. (1987a)

GGT<sup>+</sup>, positive for  $\gamma$ -glutamyl transpeptidase

<sup>a</sup> Naturally contaminated maize involved in field outbreaks of equine leukoencephalomalacia in the USA

goats (Gurung et al., 1998), lambs, mink, poultry, and rabbits. In all cases in which toxicity was seen, it involved the liver and/or kidney, heart, or homologous organs, and, when measured, increased concentrations of free sphinganine in tissues, serum, or urine. Of all the agriculturally important species studied, fetal male rabbit appeared to be the most sensitive to fumonisin-induced toxicity, on the basis of decreased absolute kidney weight (LOEL, 0.1 mg/kg bw per day by gavage); however, when the kidney weight was normalized to the fetal body weight, the difference was not significant (LaBorde et al., 1997).

### **2.2.3 Long-term studies of toxicity and carcinogenicity**

#### *(a) B6C3F<sub>1</sub> mice*

Male and female mice were fed diets containing pure fumonisin B<sub>1</sub> at a concentration of 0, 5, 15, 50, or 80 mg/kg for 2 years, providing doses of 0, 0.65, 1.9, 6.6, and 13 mg/kg bw per day for females and 0, 0.53, 1.6, 9.0, and 15 mg/kg bw per day for males during weeks 51–104 of the study. No differences were found in the body weights of treated and control animals; however, the body weights of females were 30% lower and those of males about 15% lower than those of B6C3F<sub>1</sub>/NCTR BR mice in other studies at the laboratory. Analysis of feed consumption rates showed that the mice were consuming about 30% less feed than mice in other studies at the laboratory, and this was found to be due to reduced availability of feed through the screen feeders, although the particle size of the powdered feed was not altered by the addition of fumonisin B<sub>1</sub>. As a result, the tumour incidences were about 12% lower than expected (Howard et al., 2001b). The survival of female mice at the highest dose was shorter than that of mice on control diets or diets containing 50 mg/kg. The decrease in survival began at about 1 year of age and continued until the end of the study. The survival rates of females at other doses were similar to those of female controls. Treatment had no effect on the survival of male mice.

Hepatocellular adenomas were found in 12% of female mice given control diet and in 6.5% at 5 mg/kg, 2.1% at 15 mg/kg (not statistically significant), 36% at 50 mg/kg, and 74% at 80 mg/kg of diet. Hepatocellular carcinomas were not found in females receiving 0, 5, or 15 mg/kg of diet but occurred in 22% of those at 50 mg/kg of diet and 23% of those at 80 mg/kg of diet. The combined incidence of hepatocellular adenomas and carcinomas was thus 12% in female controls, 43% at 50 mg/kg, and 88% at 80 mg/kg of diet. The increases in the incidence rates of adenomas and carcinomas at 50 and 80 mg/kg of diet were statistically significant. The hepatocellular adenomas were characterized by distinct foci of eosinophilic or basophilic cells and routinely compressed the adjacent normal parenchymal cells. The carcinomas were characterized by poorly differentiated anaplastic cells. The increased incidences were accompanied by an increased prevalence of hepatocellular hypertrophy and multifocal necrosis. Male mice showed no increase in the incidence of neoplasia of any type, including the liver: about 25% of the mice had hepatocellular adenomas or carcinomas. Although hypertrophy was correlated with tumour incidence in the female mice, it was also present in the livers of the male mice at 80 and 150 mg/kg of diet in the absence of an increased tumour incidence. The NOEL for carcinogenicity in these feed-restricted female mice was 15 mg/kg of diet, equal to 1.9 mg/kg bw per day (Table 4; National Toxicology Program, 1999).

(b) *BD IX rats*

Male rats received a semi-purified diet marginally deficient in vitamins, lipotropes, and some minerals containing fumonisin B<sub>1</sub> at a concentration of 50 mg/kg, equivalent to 1.6 mg/kg bw per day. From 18 months onwards, some hepatocyte nodules showed dysplasia and nuclear atypia characteristic of preneoplastic changes, a few of which were transformed into hepatocellular carcinoma. Of the 15 rats that were killed between 18 and 26 months, 10 developed hepatocellular carcinoma, of which two metastasized to the lung and heart and one to the kidneys. Another lesion that was present consistently from 6 months onwards was cholangiofibrosis, which developed into cholangiocarcinoma in some rats towards the end of the experiment. Histopathological changes in the kidney were observed rarely, although fumonisin B<sub>1</sub> tended to cause some lesions towards the end of the experiment. These lesions were not specific, although scant necrosis and other degenerative changes were found in the proximal convoluted tubules (Table 5; Gelderblom et al., 1991).

The dose–response relationship between fumonisin B<sub>1</sub> and hepatocarcinogenesis was studied in BD IX rats given the same diet as used in the previous study but containing fumonisin B<sub>1</sub> at a concentration of 1, 10, or 25 mg/kg. Minor changes were found in some rats given 10 mg/kg of diet and only minimal changes in those given 1 mg/kg of diet. The rats that received 25 mg/kg of diet had apoptosis, proliferation of duct epithelial cells, and mild fibrosis, which in some cases caused bridging between the portal tracts, resulting in a slight distortion of the architecture of the liver in some rats. One rat in this group showed a large focal area of adenofibrosis. In rats killed at 24 months, hepatocyte nodules were found in 9/17 rats given 25 mg/kg of diet. Other pathological changes in the liver included mild periacinar fatty changes, mild-to-prominent anisonucleosis, ground-glass foci, and a few apoptotic bodies, mainly in rats given 10 or 25 mg/kg of diet. Lesions in the kidneys were restricted to the tubular epithelium and included the presence of granular casts, necrosis, apoptosis, calcification, and regenerative foci (which may also be interpreted as hyperplastic foci) of the tubular epithelium in the proximal convoluted tubules. These lesions were found mainly at 25 mg/kg of diet and to a lesser extent at 10 mg/kg of diet, representing mean daily intakes of 0.8 and 0.3 mg/kg bw, respectively (Table 5; Gelderblom et al., 2001b).

Lesions were induced in the livers of rats fed food samples intended for human consumption for 610–691 days. The food samples were obtained from an area with a high incidence of oesophageal cancer in the Kentani district of the Transkei, South Africa. Much of the maize used was damaged by mould growth (Purchase & Joubert, 1970). The diet that caused the most pronounced liver lesions consisted of maize, beans, a salt mixture, and *imifino*. Several rats also showed myocardial fibrosis, while some treatments induced epithelial-cell dysplasia of the oesophagus. As no aflatoxin was detected in the samples, it was suggested that the food contained other toxic principles that were responsible for the lesions. No detailed mycological analyses were performed on the samples, but a retrospective analysis of maize collected in the same region revealed relatively high concentrations of fumonisins (Rheeder et al., 1992). The mean intake of fumonisin B was calculated to be equivalent to 0.65–1.4 mg/kg bw per day (Table 6; Purchase et al., 1975).

Commercial rat feed containing freeze-dried maize cultures of *F. verticillioides* MRC 826 was fed to male rats for 763 days. None of the rats survived the highest dietary concentration, calculated to provide 8% fumonisin B. Of rats fed diets containing 2% and 4% (equivalent to 3.2 and 6.9 mg/kg bw per day), 80% developed hepatocellular carcinoma and 63% developed ductular carcinoma in the liver. These tumours invariably developed in severely cirrhotic livers showing nodular hyperplasia and other changes. Pulmonary metastases developed in three rats at each dietary concentration. Adenofibrosis developed in all the animals and progressed to neoplastic lesions, referred to as 'cholangiocarcinoma', that were seen macroscopically to extend above the surface of the liver. Other pathological changes included endothelial hyperplasia of the ventricular endocardium and/or intraventricular thrombosis and oesophageal basal-cell hyperplasia in about 50% of rats that received the culture material (Table 6; Marasas et al., 1984). The mild basal-cell hyperplasia in the oesophagus and the cardiac changes were not reproduced with fumonisin B<sub>1</sub> (Gelderblom et al., 2001a).

The same freeze-dried culture material at concentrations of 0.25–0.75% was given to male rats in a semi-synthetic diet marginally deficient in certain vitamins and minerals. After a feeding period of 23–27 months, two of 21 rats developed hepatocellular carcinoma with lung metastases and eight of 21 animals developed cholangiocarcinoma. Other changes in the livers are summarized in Table 6. No lesions were found in the kidney. Basal-cell hyperplasia was prominent in the oesophageal epithelium; myocardial disseminated fibrosis occurred frequently; and endocardial and subcardial fibrosis were seen in three rats. The low incidence of liver tumours was due to the minimal hepatotoxicity induced by the relatively low dietary concentrations of culture material. The estimated intake of fumonisin B was 0.4–1.3 mg/kg bw per day (Table 6; Jaskiewicz et al., 1987a).

(c) *Fischer 344N rats*

Male and female rats were fed diets containing pure fumonisin B<sub>1</sub> at a concentration of 0, 5, 15, 50, or 100 mg/kg for 2 years, equal to mean doses of 0, 0.27, 0.78, 2.6, and 5.2 mg/kg bw per day for females and 0, 0.22, 0.67, 2.2, and 6.6 mg/kg bw per day for males between weeks 51 and 104 (Howard et al., 2001b). There was no dose-related difference in the survival of rats at 104 weeks. Females had decreased weight only at the highest dose, and males showed no treatment-related changes in body weight. Serum from male rats killed at 6, 10, 14, or 26 weeks showed no treatment-related changes in cholesterol or triglyceride concentration or alanine aminotransferase activity. The relative weight of the liver in males, but not females, was significantly decreased at all doses, and the relative weight of the kidney was decreased in males and females at concentrations  $\geq 15$  mg/kg of diet.

Necropsy and microscopic evaluation showed an increase in the number of basophilic foci in the livers of males at 150 mg/kg of diet. The incidence of renal tubular adenomas and carcinomas was increased in males at 50 and 150 mg/kg of diet, but no tumours were found in the kidneys of males at lower doses. A re-evaluation of the renal tumours (Hard et al., 2001) showed that tubular adenomas were present in four and renal tubular carcinomas in eight of 48 male rats at 50 mg/kg of diet; a total of 10 rats bore tumours. At the highest dose, six of 48 rats developed adenomas

and 10 of 48 developed carcinomas. These increases were statistically significant (Howard et al., 2001a). The increased incidence of renal tumours was accompanied by an increased incidence of foci of atypical tubular hyperplasia, in 4/48 and 9/48 rats at 50 and 150 mg/kg of diet, respectively (Hard et al., 2001). Howard et al. (2001b) also reported renal tubular epithelial-cell hyperplasia at these doses at 2 years, in 2/48, 1/40, 4/48, 14/48, and 8/48 of male rats receiving 0, 5, 15, 50, and 150 mg/kg of diet, respectively. Similarly, increased renal tubular epithelial-cell apoptosis and proliferation were detected at the two higher doses in male rats killed after 6, 10, 14, and 26 weeks on fumonisin B<sub>1</sub>-containing diets.

In a detailed review of the renal histopathology, nephrotoxicity, including apoptosis and regeneration, was found in male rats throughout treatment at concentrations  $\geq 15$  mg/kg of diet. The single-cell death observed was usually preceded by loss of cell anchorage to the basement membrane and detachment into the lumen. The renal tubular adenomas were characterized by a defined focus of expansive tubular cells, and the nuclear and cell volumes were greater in the adenoma cells than in normal adjacent cells. The cytoplasm of the adenoma cells stained clear to basophilic. The renal tubular carcinomas were a rare, highly malignant variant characterized as growths of abnormal and atypical cells (anaplastic) that compressed and invaded neighbouring normal tissue. The cells within the growing boundary of the carcinoma contained basophilic cytoplasm with typically increased volume and hyperchromatic nuclei. Necrosis was evident within the larger carcinomas. In many of the carcinomas, small renal tubule-like structures were evident, and these carcinomas metastasized to the lung and lymphatic tissues. Female rats showed no significant increase in the incidence of fumonisin B<sub>1</sub>-induced tumours. One renal adenoma was detected in a female rat at 50 mg/kg of diet, and one renal tubular carcinoma was found in a female that consumed 100 mg/kg of diet. The NOEL for induction of renal tumours in male rats was 0.67 mg/kg bw per day, whereas the NOEL for renal toxicity was 0.22 mg/kg bw per day (Hard et al., 2001).

#### (d) Trout

Rainbow trout were fed Oregon test diet containing pure fumonisin B<sub>1</sub> at a concentration of 0, 3.2, 23, or 100 mg/kg for 34 weeks, in the absence or presence of an initiator, equivalent to doses of 0.2, 1.3, and 3.5 mg/kg bw per day on the basis of the consumption of this diet by trout of 5.6% of body weight per day. In trout fed the treated diet, in the absence of initiation, no tumours or lesions were found after 60 weeks in any tissue examined (liver, kidney, stomach, swim bladder). Feeding of diets containing fumonisin B<sub>1</sub> for 42 weeks increased the incidence of liver tumours in trout fry initiated by immersion for 30 min in a bath containing either aflatoxin B<sub>1</sub> (100 ng/ml) or *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (35  $\mu$ g/ml). The calculated NOEL for promotion of liver tumours was 0.2 mg/kg bw per day (Carlson et al., 2001).

#### (e) Baboons

The major pathological effects in baboons fed diets containing culture material of *F. verticillioides* included acute congestive heart failure in two baboons after 143 and 248 days. One baboon developed cirrhosis after being fed the diet for 720 days (Kriek et al., 1981b).

(f) *Vervet monkeys*

Culture material of *F. verticillioides* strain MRC 826 was fed to male and female vervet monkeys (*Cercopithecus aethiops*) for 13.5 years (WHO, 2000a; Gelderblom et al., 2001c). Analysis of feed intake and degree of fungal contamination indicated a fumonisin B content of 8.2–13 mg/kg of diet. Toxicity was monitored by bimonthly clinical chemical analyses throughout treatment, and liver biopsy samples were taken at regular intervals up to 4.5 years. The threshold dose of fumonisin B for renal and hepatic damage was calculated to be 0.11–0.18 mg/kg bw per day. Typical lesions observed in the liver of animals at the high dose included portal-to-portal fibrosis, hepatocyte nodules, bile-duct proliferation, apoptosis, and an increased sphinganine:sphingosine ratio (Fincham et al., 1992; Shephard et al., 1996a). The kidneys of these monkeys have not yet been examined for histopathological changes. The LOEL for sphingolipid changes in the serum was 22–48 mg/kg of diet, equivalent to 0.29–0.64 mg/kg bw per day. Other parameters that were also affected throughout the study as a result of the treatment included lipid parameters associated with hypercholesterolaemia and significantly decreased white and red blood cell and platelet counts.

A working group convened by IARC (IARC, 1993) reached the following conclusions about the carcinogenicity of toxins derived from *F. verticillioides*, including fumonisin B<sub>1</sub>:

- There is *inadequate evidence* in humans for the carcinogenicity of toxins derived from *F. verticillioides*.
- There is *sufficient evidence* in experimental animals for the carcinogenicity of cultures of *F. verticillioides* that contain significant amounts of fumonisins.
- There is *limited evidence* in experimental animals for the carcinogenicity of fumonisin B<sub>1</sub>.

Overall evaluation: Toxins derived from *Fusarium verticillioides* are *possibly carcinogenic to humans* (Group 2B).

#### 2.2.4 *Genotoxicity*

The possible genotoxicity of fumonisins has been assessed *in vivo* and *in vitro* (reviewed in WHO, 2000a; Table 7). The results of several independent studies showed that fumonisin B<sub>1</sub> and other fumonisins were not mutagenic in several strains of *Salmonella typhimurium*, with and without addition of microsomal activation from rat liver. Tests for gene mutation in *Escherichia coli* PQ37, differential DNA repair in *E. coli* K12, and DNA repair *in vivo* and *in vitro* in two laboratories also gave negative results.

In one unconfirmed report, fumonisin B<sub>1</sub> was active in a commercial bioluminescent bacterial assay. It increased the micronucleus frequency in primary rat hepatocytes in a non-concentration-dependent manner and caused a significant, concentration-dependent increase in the frequency of chromosomal aberrations. The Commission of the European Union (2000) considered that this report was unreliable owing to methodological limitations. Fumonisin B<sub>1</sub> transformed mouse embryo cells only at a concentration of 500 µg/ml and not at lower or higher concentrations.

Evidence *in vitro* and *in vivo* indicates that fumonisin B<sub>1</sub> can damage DNA indirectly by increasing oxidative stress (Atroshi et al., 1999; Mobio et al., 2000b).

Table 7. Results of assays for the genotoxicity of fumonisins B<sub>1</sub>(FB)

End-point	Test object	Concentration	Results	Reference
<i>in vitro</i>				
Reverse mutation	<i>S typhimurium</i> TA97a, TA98, TA100, TA102	FB <sub>1</sub> , FB <sub>2</sub> (1–10 mg/plate), and FB <sub>3</sub> (5 mg/plate)	Negative <sup>a</sup>	Gelderblom & Snyman (1991)
Reverse mutation	<i>S typhimurium</i> TA98, TA100	FB <sub>1</sub> (0.7–500 µg/plate)	Negative <sup>a</sup>	Knasmüller et al. (1997)
Unscheduled DNA synthesis	Primary rat hepatocytes	FB <sub>1</sub> (80 µmol/ plate); FB <sub>2</sub> (40 µmol/plate)	Negative	Gelderblom et al. (1992)
Unscheduled DNA synthesis	Primary rat hepatocytes	FB <sub>1</sub> (0.5–250 µmol/plate)	Negative	Norred et al. (1992)
Chromosomal aberrations	Primary rat hepatocytes	FB <sub>1</sub> (0.01–100 µg/ml)	Positive <sup>b</sup>	Knasmüller et al. (1997)
Micronucleus formation			Negative	
Gene mutation	<i>E. coli</i>	FB <sub>1</sub> (5–500 µg/ plate)	Negative <sup>a</sup>	
DNA repair	<i>E. coli</i>	0.7–500 µg/plate	Negative <sup>c</sup>	
DNA binding	Oligonucleotides	2.5 nmol/µl	Negative	Pocsfalvi et al. (2000)
Transformation	Mouse embryo cells	500 µg/ml	Positive <sup>c</sup>	Sheu et al. (1996)
Hypermethylation of DNA	C6 glioma cells	9–18 µmol/L	Positive	Mobio et al. (2000b)
Lipid peroxidation	Rat liver nuclei	FB <sub>1</sub> (40–300 µmol/L)	Positive	Sahu et al. (1998)
Lipid peroxidation	Phosphatidyl- choline bilayers	FB <sub>1</sub> (10 mmol/L)	Positive	Yin et al. (1998)
Lipid peroxidation	Vero cells	0.14–70 µmol/L	Positive	Abado-Becongnee et al. (1998)
Lipid peroxidation	C6 glioma cells	3–27 µmol/L	Positive	Mobio et al. (2000a)
Lipid peroxidation	Primary hepatocytes	75–500 µmol/L	Positive	Abel & Gelderblom (1998)
Adduct formation	<sup>32</sup> P-Postlabelling	Not reported	Negative	P. Howard (personal communication)

Table 7 (contd)

End-point	Test object	Concentration	Results	Reference
<i>In vivo</i>				
Unscheduled DNA synthesis	Rat liver (single dose by gavage)	FB <sub>1</sub> (100 mg/kg bw) FB <sub>2</sub> (100 mg/kg bw)	Negative	Gelderblom et al. (1992)
Lipid peroxidation	Rat liver Plasma membranes, microsomes, nuclei, mitochondria	250 mg/kg of diet (21 days)	Positive <sup>d</sup> Mild to significant effect	Abel & Gelderblom (1998)
Lipid peroxidation	Rat liver	250 mg/kg of diet plus 1–2% carbonyl iron (35 days)	Positive <sup>d</sup>	Lemmer et al. (1999a)
Lipid peroxidation	Mouse liver	2.25 mg/kg bw per day (subcutaneous, 5 days)	Negative <sup>e</sup>	Riley et al. (2001)

<sup>a</sup> With and without added rat liver microsomal fraction

<sup>b</sup> The European Commission (2000) considered this report unreliable owing to methodological limitations.

<sup>c</sup> No activity at lower or higher concentration

<sup>d</sup> Dose shown to initiate cancer in rat liver

<sup>e</sup> Hepatotoxic dose

Oxidative damage was closely associated with fumonisin B<sub>1</sub>-induced hepatotoxicity and induction of putative preneoplastic lesions *in vivo*, while the plasma and microsomal membranes, and to some extent the mitochondria and nuclei, appeared to be significantly affected by lipid peroxidation (Abel & Gelderblom, 1998).

Studies in primary hepatocytes showed a similar relationship between cytotoxicity and lipid peroxidation. Although lipid peroxidation was prevented by the addition of  $\alpha$ -tocopherol, cytotoxicity was reduced, but not to baseline levels, suggesting that the cytotoxicity was due not only to oxidative damage but also to fumonisin B<sub>1</sub>-induced toxic effects. Fumonisin B<sub>1</sub> potentiated the effect of iron on lipid peroxidation and was toxic to the liver, independently of its effect on lipid peroxidation (Lemmer et al., 1999a). The toxin induced lipid peroxidation in cell membrane preparations (Yin et al., 1998) and isolated rat liver nuclei. Nuclear membrane lipid peroxidation with concomitant DNA strand breaks was found in isolated rat liver nuclei treated with fumonisin B<sub>1</sub> *in vitro* at concentrations of 40–300  $\mu$ mol/L (Sahu et al., 1998). It was suggested that the formation of hydroxy and peroxy radicals in the close vicinity of nuclear material could induce DNA strand breaks. It is not known whether the induction of chromosomal aberrations in primary hepatocytes (Knasmüller et al., 1997) is related to oxidative damage. Oxidative damage has been reported in Vero cells (Abado-Becongnee et al., 1998) treated with fumonisin B<sub>1</sub> at a concentration

(0.14  $\mu\text{mol/L}$ ) below that which inhibits protein and DNA synthesis ( $> 14 \mu\text{mol/L}$ ). However, in mice, fumonisin B<sub>1</sub>-induced liver toxicity occurred in the absence of evidence of increased lipid peroxidation (Riley et al., 2001). In numerous studies in vitro, fumonisin was used to protect cells from oxidant-induced cell death (for examples, see Table 3). Whether this occurs in vivo is unknown.

Although fumonisin B<sub>1</sub> caused oxidative damage to DNA in vitro, there is no compelling evidence to suggest that it binds covalently to DNA. Extracts of *Fusarium* fungi have been shown to form DNA adducts (Lu et al., 1988; Bever et al., 2001), as detected by the sensitive <sup>32</sup>P-postlabelling method. However, DNA adducts did not form after incubation of fumonisin B<sub>1</sub> with DNA in the presence or absence of microsomal protein (rat liver microsomes), and are probably formed by other mycotoxins produced by the fungus (P. Howard, personal communication). Using mild electrospray ionization mass spectrometry, Pocsfalvi et al. (2000) found no evidence of a specific interaction between fumonisin B<sub>1</sub> and single- or double-stranded oligonucleotides. Specific non-covalent interactions were noted with fusaproliferin and beauvercin, two mycotoxins produced by *F. proliferatum* and *F. subglutinans*.

### 2.2.5 Reproductive toxicity

While fumonisins are embryotoxic in vitro, there were no published data to support the conclusion that they are developmental or reproductive toxicants in farm animals or humans (WHO, 2000a). Fumonisin B<sub>1</sub> inhibits the biosynthesis of the glycosylphosphatidylinositol-anchored folate transporter in vitro, and folate deficiency is associated with an increased risk for neural tube defects. Inhibition of the folate transporter in vivo has not been confirmed in feeding studies. There is no evidence of neonatal toxicity in laboratory animals. Except in one study in Syrian hamsters, embryotoxicity occurred secondary to maternal toxicity. In Syrian hamsters, administration of fumonisin B<sub>1</sub> by gavage at a dose of 18 mg/kg bw per day on days 8 to 10 or 12 of gestation resulted in a statistically significant increase in the number of fetal deaths. The fact that there was no maternal toxicity at this dose indicates that the Syrian hamster is extremely resistant to the effects of fumonisin. In rats and mice, reproductive and developmental effects occurred at doses that were also maternally toxic, suggesting that the reproductive and developmental toxicity of fumonisin is mediated through maternal toxicity. However, in rabbits, maternal toxicity was observed at a daily dose (given by gavage in water) as low as 0.25 mg/kg bw on days 3–19 of gestation, but there was no increase in the frequency of fetal loss or of gross visceral or skeletal abnormalities at any maternal dose of fumonisin B<sub>1</sub> (0–1.75 mg/kg bw per day); however, slight decreases in fetal weight did occur at maternally toxic doses.

### 2.3 Observations in domestic animals/veterinary toxicology

Because fumonisins are known to cause field outbreaks of equine leukoencephalomalacia and porcine pulmonary oedema, many studies have been undertaken to better understand the physiological and biochemical mechanisms responsible for these brain and lung diseases. The cardiovascular changes in equids are similar to those seen in pigs during onset of the syndrome (Smith et al., 1999; Constable et al., 2000a; Smith et al., 2000), and the results of studies suggest a

common underlying mechanism for the two diseases involving sphingosine-mediated L-type calcium channel blockade (Constable et al., 2000b).

A thorough review of studies on these two diseases is contained in the IPCS monograph (WHO, 2000a). The following is a brief summary of that review.

### **2.3.1 Equine leukoencephalomalacia**

Equine leukoencephalomalacia syndrome is a sporadic condition characterized by the presence of liquefactive necrotic lesions in the cerebrum. The disease appears to be unique to equids, although brain lesions have also been reported in rabbits (Bucci et al., 1996), pigs (Fazekas et al., 1998), and carp fed fumonisins (Pepeljnjak et al., 2000). The brain lesions in rabbits and pigs could not be reproduced in subsequent studies (T. Bucci, personal communication; Zomborszky et al., 2000), and the study in carp has not been repeated.

Analysis of feeds from confirmed cases of equine leukoencephalomalacia in the USA indicated that consumption of feed with a fumonisin B<sub>1</sub> concentration > 10 mg/kg of diet (equivalent to 0.2 mg/kg bw per day) was associated with an increased risk for developing the disease, whereas a concentration < 6 mg/kg of diet (equivalent to 0.12 mg/kg bw per day) was not. The minimum oral dose sufficient to induce equine leukoencephalomalacia appeared to be 15–22 mg/kg of diet (equivalent to 0.30 mg/kg bw per day for 150 days to 0.44 mg/kg bw per day for 241 days) in studies of naturally contaminated maize screenings or culture material (*F. proliferatum*) containing fumonisins. The minimum oral dose of pure fumonisin B<sub>1</sub> that will induce equine leukoencephalomalacia is unknown. The minimum intravenous dose of pure fumonisin B<sub>1</sub> that induced neurological abnormalities was 0.01–0.05 mg/kg bw per day. If the intravenous dose is assumed to represent 5% of the oral dose, the equivalent oral dose would be 0.2–1.0 mg/kg bw per day. The NOEL for cardiovascular abnormalities was 0.2 mg/kg bw per day, but the NOEL for serum biochemical abnormalities was equivalent to < 0.2 mg/kg bw per day (Constable et al., 2000b).

Equine leukoencephalomalacia has been reproduced by giving fumonisin B<sub>1</sub> orally or intravenously, giving naturally contaminated maize screenings, or giving diets containing *F. proliferatum* maize culture material with predominantly fumonisin B<sub>1</sub> or fumonisin B<sub>2</sub>. In addition to the brain lesions, histopathological abnormalities are usually found in the liver, and recent studies suggest that fumonisin B<sub>1</sub> is also nephrotoxic in horses. Changes in serum enzymes indicative of liver damage and behavioural changes are usually preceded by increased concentrations of free sphingoid bases and cholesterol in serum or plasma. It has been hypothesized that equine leukoencephalomalacia is a result of cerebral oedema due to an inability to shut down the blood flow to the brain when the horse lowers its head to eat and drink (Constable et al., 2000b).

Evidence of disruption of sphingolipid metabolism is an early indicator of exposure of horses to fumonisins. For example, all ponies fed diets containing maize screenings naturally contaminated with fumonisins at ≥ 22 mg/kg (primarily fumonisin B<sub>1</sub>) had large increases in the serum concentration of free sphinganine. The increase was reversible. This and the increased sphinganine:sphingosine ratio occur before increases in serum transaminase activity and clinical signs of equine leukoencephalomalacia.

### 2.3.2 Porcine pulmonary oedema

Porcine pulmonary oedema is also believed to be induced by cardiovascular dysfunction. Significant changes in oxygen consumption and in several haemodynamic parameters are seen before the onset of clinical signs in pigs fed diets containing fumonisins, suggesting that pulmonary hypertension caused by hypoxic vasoconstriction might contribute to the syndrome. Fumonisin B<sub>1</sub> has been shown to reduce the mechanical efficiency of the left ventricle (Constable et al., 2000a), suggesting that pulmonary oedema in pigs is due primarily to acute left-sided heart failure (Smith et al., 1999; Constable et al., 2000a; Smith et al., 2000). Hepatotoxicity is usually observed at doses lower than those that induce the pulmonary oedema. Liver lesions were induced by maize screenings providing a dose of fumonisin of 1.1 mg/kg bw per day (17 mg/kg of diet). In a study in weaned piglets, mild pulmonary oedema was induced in three of four animals fed diets that contained 10 mg/kg (equivalent to 0.4 mg/kg bw per day) of fumonisin B<sub>1</sub> from *F. verticillioides* culture material for 4 weeks (Zomborszky et al., 2000). Subtle changes in performance were reported at a concentration of fumonisin B<sub>1</sub> (1 mg/kg), which does not cause overt toxicity. Erratic growth was reported at 0.1 mg/kg (Rotter et al., 1996).

In 1989–90, outbreaks of this disease were reported in various parts of the USA. Maize screenings obtained from farms where pigs had died of porcine pulmonary oedema were contaminated predominantly with *F. verticillioides*. The concentrations of fumonisin B<sub>1</sub> in the suspected feeds ranged from 20 to 360 mg/kg, while those in other feeds was < 8 mg/kg (Ross et al., 1991). The clinical signs of porcine pulmonary oedema include dyspnoea, weakness, and cyanosis. At necropsy, the animals have varying degrees of interstitial and interlobular oedema, with pulmonary oedema and hydrothorax. Toxic hepatitis occurs concurrently. The concentrations of fumonisin B<sub>1</sub> in feed associated with this disease are usually much greater than those associated with outbreaks of equine leukoencephalomalacia. Purified fumonisin B<sub>1</sub> has been shown to reproduce the disease when administered intravenously. Porcine pulmonary oedema has not yet been reproduced by oral administration of pure fumonisins, although it has been induced many times with culture material containing fumonisin B<sub>1</sub>.

In pigs fed diets prepared from naturally contaminated maize screenings, there was a dose–response relationship between the dose of fumonisin, pathological lesions in the liver, and the ratio of free sphinganine to free sphingosine in serum or liver. After 14 days, statistically significant increases in the serum ratio of free sphinganine to free sphingosine were observed at a concentration of total fumonisins in feed as low as 5 mg/kg (equivalent to 0.2 mg/kg bw per day). The concentrations of free sphingoid bases were also significantly elevated in kidney and lung at doses that induced no signs of toxicity in these organs. In pigs fed fumonisin in maize culture materials, significant effects on cardiovascular function were associated with significant increases in free sphingoid base concentrations in heart tissue. Subsequent studies showed that damage to pig alveolar endothelial cells in vivo was preceded by accumulation of free sphingoid bases in lung tissue. Increased activity of serum enzymes and increased concentrations of free sphingoid bases in serum were seen at all doses in the study of Zomborszky et al. (2000). It has been hypothesized that the cardiovascular alterations leading to acute left-sided heart failure are a consequence of sphingoid-base-induced inhibition of L-type calcium

channels (Smith et al., 1999; Constable et al., 2000a; Smith et al., 2000). The minimum oral dose needed to induce porcine pulmonary oedema has not been clearly established; however, Smith et al. (1999) predicted that when the concentrations of free sphinganine and free sphingosine in plasma are  $\geq 2.2$  and  $1 \mu\text{mol/L}$ , respectively, haemodynamic changes will occur.

## 2.4 Observations in humans

The association between fumonisins and other fungal toxins and risk factors for various human diseases are summarized in Table 8.

### 2.4.1 Oesophageal cancer

An association has been established between the occurrence of the fungus, *Fusarium verticillioides* (Sacc.) Nirenberg (= *Fusarium moniliforme* Sheldon) on maize and the incidence of oesophageal cancer in various regions of the world. Geographical differences in demography, ethnic groups, genetic susceptibility, culture, economy and nutritional status all affect the rates of disease; however, some common risk factors are emerging, such as having maize as the main dietary staple and, to some extent, a low socioeconomic status. Thus, high incidences of oesophageal cancer have been associated with limited diets consisting mainly of wheat or maize and low contents of certain minerals and vitamins (Blot, 1994). Fungal contamination of maize and wheat attracted the interest of many investigators, who have characterized the toxic, mutagenic, and carcinogenic metabolites of the major fungal contaminants of these grains. The possible involvement of *F. verticillioides* and fumonisins in the development of oesophageal cancer in various regions of the world is evaluated on the basis of incidence rates, the presence of *Fusarium* spp. in maize, the presence of fumonisins and other *Fusarium* mycotoxins, and nutritional deficiencies and other dietary risk factors. Those aspects that were addressed by WHO (2000) are not discussed in detail.

#### (a) South Africa

A dramatic increase in the incidence of oesophageal cancer in the Transkei region of Eastern Cape Province, South Africa, was first described by Burrell (1957, 1962; Burrell et al., 1966). Subsequent reports were published for the periods 1955–57 and 1965–69 covering all districts of the Transkei (Rose, 1965, 1973; Rose & Fellingham, 1981) and for the periods 1981–84 (Jaskiewicz et al., 1987b) and 1985–90 (Makaula et al., 1996) focusing on cancer incidence in four selected districts: two areas of high incidence (Centane or Kentani and Butterworth in the south-west) and two areas of low incidence (Lisikisiki and Bizana in the north-east). Data for 1991–95 indicate that the age-standardized incidence rate has decreased in Butterworth to 51 per 100 000 but increased in Lusikisiki and Bizana to 37 per 100 000. Conversely, the rate in Centane has remained consistently high in males, at 56 per 100 000.

A comparative study of the incidence of *Fusarium* spp. in maize in regions of low (Bizana and Lusikisiki) and high (Butterworth and Centane) incidence of oesophageal cancer in the Transkei indicated that three *Fusarium* species (*F. graminearum*, *F. verticillioides*, and *F. subglutinans*) were the predominant fungal contaminants of home-grown maize during the 1976–77 season (Marasas et al., 1979). The

**Table 8. Interaction of fumonisins with other fungal toxins and risk factors in the development of disease in humans**

Geographic region/ country	Age-standardized incidence (No. of cases/100 000)	Fungal infection of major dietary staples	Mycotoxin contamination	Dietary and other risk factors
<i>Oesophageal cancer</i>				
Transkei, Southern Africa (rural)	Males: Lusikisiki, 51; Bizana, 37; Butterworth, 43; Centane, 56 (Makaula et al., 1996)	Maize: <i>F. verticillioides</i> and <i>F. graminearum</i> (Marasas et al., 1981; Gelderblom et al., 1984; Marasas et al., 1988; Sydenham et al., 1990; Van Rensburg et al., 1990; Rheeder et al., 1992; Bever et al., 2001)	Healthy maize: fumonisin B (2.0–2.1 mg/kg) Mouldy maize: fumonisin B (32–67 mg/kg) Deoxynivalenol (2.9 mg/kg), nivalenol (4.6 mg/kg), zearalenone (1.3 mg/kg), aflatoxin B <sub>1</sub> (0.66 µg/kg), moniliformin (3.5 mg/kg)	Vitamin A, E, and B <sub>12</sub> , folate, selenium deficiencies (Van Helden et al., 1987; Jaskiewicz et al., 1987c, 1988a,b) Smoking: some relationship Alcohol: some relationship (Rose, 1973; Van Rensburg, 1985; Sammon, 1992)
Henan, Hebei, Linxian, and Shanxi provinces, northern China	Yancheng, 135; Hebei, 140; Linxian, 108 (Yang, 1980)	Wheat, maize, dried sweet potato, rice, soya bean; <i>Penicillium</i> spp., <i>Aspergillus</i> spp., <i>F. verticillioides</i> predominant fungi (Zhen, 1984; Luo et al., 1990; Chu & Li, 1994; Yoshizawa et al., 1994; Gao & Yoshizawa, 1997; Zhang et al., 1997)	Healthy maize: fumonisin B <sub>1</sub> (0.7–3.5 mg/kg). One study: fumonisin B <sub>1</sub> (35 mg/kg) Mouldy maize: fumonisin B <sub>1</sub> (74 mg/kg) Aflatoxin B <sub>1</sub> (8.6–10 µg/kg), type A (630 µg/kg) and B (2400 µg/kg); trichothecenes, deoxynivalenol (0.02–3.5 mg/kg), nivalenol (0.05–0.9 mg/kg), zearalenone (0.06 mg/kg)	Low intake of vitamins A and C. Inverse relationship with molybdenum, manganese, zinc; no relationship with pickled vegetables Alcohol intake: no association; smoking: mild risk factor Nitrosamines: Bread inoculated with fungi and tumour induction in rat oesophagus (Li et al., 1979, 1980; Yang, 1980; Li et al., 1982, 1989)

Table 8 (contd)

Geographic region/ country	Age-standardized incidence (No. of cases/100 000)	Fungal infection of major dietary staples	Mycotoxin contamination	Dietary and other risk factors
<i>Oesophageal cancer</i> (contd)				
Mazandaran, Province, Gonbad region, Caspian littoral of Iran	Females: 262 Males: 206 (Kmet & Mahboubi, 1972; Hormozdiari et al., 1975)	<i>Aspergillus</i> , <i>Fusarium</i> , <i>Penicillium</i> spp. on maize. <i>F. verticillioides</i> and <i>F. proliferatum</i> important spp. <i>Alternaria alternata</i> (Kmet & Mah- boubi, 1972; Hormozdiari et al., 1975; Chen et al., 1992; Bujari & Ershad, 1993; Shephard et al., 2000)	Healthy maize: fumonisin B (1.6–6.1 mg/kg) Aflatoxins, polycyclic aromatic hydrocarbons, nitrosamines	Micronutrient deficiencies: iron, manganese, copper, zinc, vita- mins A, C, riboflavin. Nonsignificant roles for alcohol and tobacco smoking (women). <i>Nass</i> , mixture of opium, lime, and ash, risk factor in men. Thermal irritation with hot tea, bread contamination with silica, fibre, consumption of sour pomegranate seeds, black pepper, and garlic (Kmet & Mahboubi, 1972; Joint Iran/IARC Study Group, 1977; Cook-Mozaffari et al., 1979; O'Neil et al., 1980; Ghadirian, 1987)
Friuli– Venezia Giullia, northeast Italy	Pordenone Province Males: 17 (Franceschi et al., 1990)	Fumonisin-producing <i>Fusarium</i> species (Logrieco et al., 1995)	Fumonisins: fumonisin B <sub>1</sub> (0.15–0.38 mg/kg), fumonisin B <sub>2</sub> (0.06–0.91 mg/kg)	Consumption of polenta. Low intake of micronutrients such as riboflavin and niacin; interactive role of alcohol (Rossi et al., 1982; Franceschi et al., 1990)

Table 8 (contd)

Geographic region/ country	Age-standardized incidence (No. of cases/100 000)	Fungal infection of major dietary staples	Mycotoxin contamination	Dietary and other risk factors
<i>Oesophageal cancer</i> (contd)				
Western and central Kenya	45% of cases (Gatei et al., 1978)	Maize: <i>F. verticillioides</i> (Macdonald & Chapman, 1996; Kedera et al. 1999; van der Westhuizen et al., 1999)	Healthy maize: fumonisin B (0.06–1.0 mg/kg) Poor quality maize: fumonisin B <sub>1</sub> (3.6–12 mg/kg) Breakfast cereals: total fumonisin B (4.9 mg/kg) (Sydenham et al., 1993)	Dietary patterns and tribal cus- toms vary; alcohol consump- tion; geographical and ethnic variations (Gatei et al., 1978) No studies
Zimbabwe	Males: Harare: 30 Bulawayo: 59 (Bassett et al., 1995)	No studies		
Charleston County, South Carolina, USA	Black males, 170 (death rate) (Fraumeni & Blot, 1977; O'Brien et al., 1982; Brown et al., 1988)	No studies	Maize-based human foods Fumonisin B <sub>1</sub> (0.1-1.9 mg/kg) Fumonisin B <sub>2</sub> (0.07-0.46 mg/kg) (Sydenham et al., 1991)	Low socioeconomic status, tobacco and alcohol ('moon- shine' distilled from fermented maize meal); low intake of fresh fruits (Fraumeni & Blot, 1977; Brown et al., 1988)
Southern Brazil	Santa Catarina, Paranà, Rio Grande do Sul Males: 18 (Instituto Nacional do Cancer, 1989)	Maize: <i>F. verticillioides</i> , <i>A. flavus</i> (Sydenham et al., 1992a; Hirooka et al., 1996; Scaff & Scussel, 1999a,b; Camargos et al., 2000; Hermans et al., 2000; Machinski & Valente Soares, 2000)	Animal mycotoxicosis: Fumonisin B <sub>1</sub> (38 mg/kg); fumonisin B <sub>2</sub> (12 mg/kg) Maize samples: Fumonisin B <sub>1</sub> (2.7–11 mg/kg) Fumonisin B <sub>2</sub> (2.3–10 mg/kg) Maize-based food: Fumonisin B <sub>1</sub> (0.04–12 mg/kg) Fumonisin B <sub>2</sub> (0.01–10 mg/kg) Markets and supermarkets: Fumonisin B <sub>1</sub> ( $\leq$ 32 mg/kg)	Farm workers, smoking and drinking: regional variation; hot beverages (maté and chimarrao) (Victoria et al., 1987; Dietz et al., 1998; Scaff & Scussel, 1999a,b)

Table 8 (contd)

Geographic region/country	Age-standardized incidence (No. of cases/100 000)	Fungal infection of major dietary staples	Mycotoxin contamination	Dietary and other risk factors
<i>Liver cancer</i>				
Jiangsu County, China	Haimen: 52–65 (mortality rate) (Ueno et al., 1996, 1997)	No studies	Maize: Fumonisin B (0.16–26 mg/kg) Aflatoxin B <sub>1</sub> (≤ 31 µg/kg) Deoxynivalenol (0.89 mg/kg) (Ueno et al., 1997)	Microcystins (Ueno et al., 1996)
Transkei, South Africa	Kentani: 2.4–7.7 Lusikisiki: 13 (Jaskiewicz et al., 1987b; Makaula et al., 1996)	Maize-based food and home-grown maize (Sydenham et al., 1990; Van Rensburg et al., 1990; Rheeder et al., 1992)	Aflatoxin B <sub>1</sub> (16 ng/kg bw) Healthy maize: fumonisin B (2.0–2.1 mg/kg) Mouldy maize: fumonisin B (32–67 mg/kg) Deoxynivalenol (2.9 mg/kg), nivalenol (4.6 mg/kg), zearalenone (1.3 mg/kg), aflatoxin B <sub>1</sub> (0.66 µg/kg), moniliformin (3.5 mg/kg)	Low socioeconomic status; nutritional deficiencies (Van Rensburg, 1985; Van Helden et al., 1987; Jaskiewicz et al., 1987c, 1988a,b)
<i>Neural tube defects</i>				
Southern Texas, USA	Lower Rio Grande valley: 27/10 000 (Hendricks, 1999; Missmer et al., 2000)	Case-control study, 1995–99, Texas Health Department No data on fungal contamination	Maize-based foods: 1.2 mg/kg (Sydenham et al., 1991) 1997–98 study of maize meal: degermed, 0.15 mg/kg; whole 1.2 mg/kg	Tortillas; sphingonine: sphinganine ratio in serum; protective effect of folate supplementation (Missmer et al., 2000)

Table 8 (contd)

Geographic region/ country	Age-standardized incidence (No. of cases/100 000)	Fungal infection of major dietary staples	Mycotoxin contamination	Dietary and other risk factors
<i>Neural tube defects (contd)</i>				
China	Hebei and Shanxi Provinces: 60/10 000 (Moore et al., 1997)	<i>Penicillium</i> , <i>Aspergillus</i> spp., <i>F. verticillioides</i> in wheat, maize (Zhen, 1984; Chu & Li, 1994; Yoshizawa et al., 1994)	Healthy maize: fumonisin B <sub>1</sub> (0.7–3.5 mg/kg); one study, 35 mg/kg Mouldy maize: fumonisin B <sub>1</sub> (74 mg/kg)	Low socioeconomic status; nutritional deficiencies (Yang, 1980)
Transkei and Mpumalanga South Africa	Umzimkulu: 38/10 000; Mpumalanga: 3.6/10 000 (Ncayiyana, 1986; Venter et al., 1995)	<i>F. verticillioides</i> , <i>F. proliferatum</i> , <i>F. graminearum</i> , <i>F. subglutinans</i> (Rheeder et al., 1992)	Healthy maize: fumonisin B <sub>1</sub> (0.3–0.6 mg/kg) Mouldy maize: fumonisin B <sub>1</sub> (5–9 mg/kg)	Low socioeconomic status; nutritional deficiencies (Van Rensburg et al., 1985; Van Helden et al., 1987; Jaskiewicz et al., 1987b, 1988a,b)
<i>Foodborne disease outbreak</i>				
Southern India	Deccan Plateau; gastrointestinal disease (Bhat et al., 1997; Shetty & Bhat, 1997; Vasanthi & Bhat, 1998)	Mouldy sorghum and maize <i>Fusarium</i> , <i>Aspergillus</i> , <i>Alternaria</i> spp. (Bhat et al., 1997; Prathap- kumar et al., 1997)	Sorghum: fumonisin B <sub>1</sub> (0.14– 7.8 mg/kg); aflatoxin B <sub>1</sub> (trace– 0.08 mg/kg) Maize: fumonisin B <sub>1</sub> (0.25–65 mg/kg); aflatoxin B <sub>1</sub> (0.05–0.93 mg/kg)	Low socioeconomic status; lack of access to other foods such as rice (Bhat et al., 1997)

association between the occurrence of *F. verticillioides* in maize and the incidence of oesophageal cancer was established in a detailed mycological study of home-grown maize in the four districts, carried out for six seasons in 1976–79, 1985–86, and 1989 (Marasas et al., 1981, 1988; Sydenham et al., 1990; Rheeder et al., 1992). The incidences of *F. graminearum* and *F. subglutinans* and the concentrations of toxins produced by these *Fusarium* species, i.e. deoxynivalenol, nivalenol, and zearalenone and moniliformin, respectively, were significantly higher in home-grown maize from the area of low incidence of oesophageal cancer than that of high incidence (Sydenham et al., 1990; Rheeder et al., 1992). Significantly higher concentrations of fumonisin B<sub>1</sub> and fumonisin B<sub>2</sub> were detected in visibly uncontaminated ('healthy') and mouldy samples of maize from the high-incidence areas than the low-incidence areas. The environmental conditions in the high-incidence area favour the colonization of maize ears and toxin production by *F. verticillioides*, whereas the conditions in the low-incidence area favour *F. graminearum* and *F. subglutinans*. This interaction was clear from a mycological analysis of maize harvested during 1985 (Sydenham et al., 1991), while variable results were obtained in maize samples collected during the 1976 and 1977 seasons (Marasas et al., 1979). The population in these regions is exposed not only to the known *Fusarium* toxins but to various other mycotoxins that may play a role in the development of cancer in humans, and specifically cancers of the oesophagus and the liver. These toxins include aflatoxin B<sub>1</sub>, present in food and traditional beer (Van Rensburg et al., 1990), the mutagen fusarin C (Gelderblom et al., 1984), and other compounds produced by *F. verticillioides* (Bever et al., 2001).

People in the high-incidence area for oesophageal cancer in the Transkei were exposed not only to mycotoxin contamination of maize but also had nutritional deficiencies, such as of vitamins A, E, and B<sub>12</sub>, selenium, and folate, when compared with people in the low-incidence areas (Van Helden et al., 1987; Jaskiewicz et al., 1987b, 1988a,b). In contrast to the study of Van Rensburg et al. (1983), a study by Jaskiewicz et al. (1988b) showed no difference in the plasma concentrations of zinc, copper, and magnesium in people in the low- and high-incidence areas. Deficiencies in these micronutrients and in manganese and molybdenum have been implicated as risk factors for oesophageal cancer in populations that subsist on either maize or wheat (Van Rensburg, 1985). A case–control study in Zulu men with oesophageal cancer carried out in Durban, South Africa, indicated that consumption of purchased maize meal is one of the major risk factors for development of the disease (Van Rensburg et al., 1985). Mineral deficiencies in the soil were implicated as another possible risk factor in the high-risk areas in the Transkei (Burrell et al., 1966); however, a study of the elemental content of soil and maize leaves (Rheeder et al., 1994) showed no deficiency in mineral elements and no association with the risk for oesophageal cancer.

Alcohol consumption and tobacco smoking are widely regarded as major factors in the development of oesophageal cancer in developed countries (Blot, 1994). In the endemic areas in the Transkei, a number of the patients did not use tobacco or consume alcohol, hence ruling out these factors as the sole causative agents (Rose, 1973; Sammon, 1992). In Zulu men, however, smoking was identified as a risk factor, while alcohol consumption had no appreciable effect (Van Rensburg et al., 1985).

(b) China

A survey in Henan, Hebei, and Shanxi, three provinces in northern China, showed that the highest mortality rates from oesophageal cancer were those in two counties, Yancheng and Hebei, with crude rates of 135 and 140 per 100 000, respectively (Yang, 1980). Data for 12 years (1959–70) in a cancer registry in Linxian County showed an average incidence rate of 108 per 100 000, while the lowest rates were recorded in Hunyan County (1.4 per 100 000) and Tatong County (2.8 per 100 000). The male:female ratio ranged from 1.44:1 to 2.63:1.

The fungal contamination of 1121 food samples of wheat, maize, dried sweet potato, rice, and soya bean obtained from five counties of high incidence and three of low incidence for oesophageal cancer in Henan Province indicated that, in addition to *Penicillium* and *Aspergillus* species, the incidence of *F. verticillioides*, was significantly higher in the high-incidence area (Zhen, 1984). An increased risk was associated with a high intake of wheat and maize, further emphasizing the importance of performing detailed studies on the fungal contamination of these food commodities (Li et al., 1989).

Analysis of 31 maize samples collected from households in Linxian and Cixian counties showed high concentrations of fumonisin B<sub>1</sub> in both mouldy and healthy samples; low concentrations of aflatoxin B<sub>1</sub> and various type A and B trichothecenes were also found in the mouldy samples (Chu & Li, 1994). In studies on maize (Yoshizawa et al., 1994) and on wheat and maize (Gao & Yoshizawa, 1997), the frequency of fumonisin contamination was higher in Linxian County, while the mean concentrations of fumonisin B mycotoxins were similar to those in Shangqiu County, a low-incidence area of oesophageal cancer in Henan Province. The samples were also frequently co-contaminated with trichothecenes, consisting mainly of deoxynivalenol and to some extent nivalenol. The concentrations of these mycotoxins were significantly higher in Linxian, and zearalenone was detected only in the maize samples from this County.

Aflatoxin B<sub>1</sub> and fumonisin B contamination was measured in 246 samples of healthy maize kernels collected from villages in the high-risk counties of Cixian, Linxian, and Anyang and the low-risk counties of Fanxian and Yanqui, during 1995–96. Once again, the frequency of fumonisin contamination was higher in the high-incidence areas (65%) than in the low-incidence areas (28%). Although no clear relationship could be detected between fumonisin contamination and oesophageal cancer incidence, people in the high-incidence area were exposed to higher mean concentrations of fumonisin B in maize than those in the low-incidence areas (Zhang et al., 1997). Significantly greater contamination with deoxynivalenol, nivalenol, and zearalenone was found in the main staple foods (maize and wheat) in the high-incidence county of Linxian than in the low-incidence area, Shangui (Luo et al., 1990).

A nutritionally inadequate diet appears to play an important role in the incidence of oesophageal cancer and the development of the precancerous dysplastic state. The diet in Linxian consisted mainly of maize, wheat, millet, rice, and some seasonal vegetables. Up to 80% of the calorie intake was obtained from grains, while consumption of fruit and vegetables was low, resulting in deficiencies in vitamins A and C during certain times of the year. Detailed analyses of food and drinking-water indicated an inverse correlation between the rate of mortality from oesophageal cancer and the mineral content of the diet, including molybdenum, manganese, and

zinc. Analyses of hair, serum, and urine indicated that the molybdenum content was significantly lower in men from Linxian than from the low-incidence areas of Yuxian and Xinyangxian. Similar studies carried out in oesophageal cancer patients in Henan indicated lower zinc concentrations than in normal subjects. Analysis of resected oesophagi from patients in Linxian showed significantly lower molybdenum concentrations in cancerous tissue. The consumption of pickled vegetables heavily infested with various *Aspergillus* and *Fusarium* spp. and mouldy food is common in the high-incidence areas, whereas the intake in the areas of lower incidence is less common (Yang, 1980).

Li et al. (1989) found no association with the intake of pickled vegetables, however, while the intake of wheat and maize was associated with an increased risk and that of fresh vegetables and fruit with a decreased risk for oesophageal cancer. No association was found with alcohol intake, while smoking was a mild risk factor. The presence of nitrosamine precursors in drinking-water and food and the formation of nitrosamines in the stomach have been associated with the development of oesophageal cancer (Yang, 1980). Various nitrosamines (*N*-nitrosodimethylamine, *N*-nitrosodiethylamine, and *N*-nitrosomethylbenzylamine) were formed in maize bread inoculated with *F. verticillioides* in the presence of low concentrations of sodium nitrate, and secondary nitrosamines were detected with some *Aspergillus* and *Penicillium* species (Li et al., 1979). These findings have not, however, been confirmed. Maize meal infested with *F. verticillioides* induced tumours in the oesophagus and stomach of rats and mice (Li et al., 1980, 1982).

(c) *Islamic Republic of Iran*

The incidence of oesophageal cancer varied by up to 30-fold in men and sixfold in women along a 300-mile stretch of the Caspian littoral of Iran. Gonbad, an eastern region of Mazandaran Province, was regarded as one of the world's 'hotspots' for oesophageal cancer in 1960–61, with truncated age-standardized rates of 206 per 100 000 for men and 262 per 100 000 for women (Kmet & Mahboubi, 1972; Hormozdiari et al., 1975). The incidence was higher among women than men in the high-incidence area. Lower incidences were recorded in the western part of the Caspian littoral.

A survey of the mycoflora of maize kernels collected from seed production centres in Sari, Moghan, and Karaj indicated that isolates of *Aspergillus*, *Fusarium*, and *Penicillium* were dominant (Bujari & Ershad, 1993). These include the fumonisin-producing species *F. verticillioides* and *F. proliferatum*. Shephard et al. (2000) reported that maize in Mazandaran Province contained higher concentrations of fumonisins than maize in Isfahan Province to the south of Mazandaran, a low-incidence area for oesophageal cancer. This report of the occurrence of fumonisins in maize in a high-risk area for oesophageal cancer in Iran must be followed up by studies of the maize consumption pattern in this region, the fungal contamination, and the presence of other *Fusarium* mycotoxins in maize. Furthermore, the samples were intended for animal consumption.

Heavy fungal contamination was also found in wheat stored in underground pits in Iran, the most prevalent fungus being *Alternaria alternata* (Kmet & Mahboubi, 1972). The toxins associated with this fungus have a similar structure to fumonisins and have similar biological effects in plants and cultured mammalian cells (van der Westhuizen et al., 1998). It is not known whether they also mimic the characteristic

biological effects of fumonisins in animals. It has been reported that *A. alternata* can produce fumonisins (Chen et al., 1992).

In a detailed study of the distribution of exogenous factors related to the differences in the incidence of oesophageal cancer in the Caspian littoral, no single causative agent could be identified. Bread and sheep and goat milk or yoghurt were the main dietary staples in high-incidence areas, while rice was the mainly staple in low-incidence regions (Hormozdiari et al., 1975). A low intake of vitamins A and C, riboflavin, animal protein, fresh vegetables and fruit and a high intake of bread and tea were common in the high-incidence areas. The micronutrient deficiencies are typical of those among persons of low socioeconomic status (Joint Iran-ARC Study Group, 1977; Cook-Mozaffari et al., 1979). Food samples from both the low- and the high-incidence areas contained low concentrations of aflatoxins, polycyclic aromatic hydrocarbons, and nitrosamines (Hormozdiari et al., 1975). Alcohol and tobacco smoking were reported to have no role, as intake was negligible and the women abstained from both alcohol and *nass*, a mixture of opium, lime, and ash favoured by some male inhabitants. Studies performed by the Joint Iran-IARC Study Group (1977) and Ghadirian et al. (1985) provided some support for the hypothesis that pyrolysate products of opium are involved in the etiology of oesophageal cancer. Subsequent studies by Kmet & Mahboubi (1972), Ghadirian (1987), and O'Neil et al. (1980) implicated deficiencies of micronutrients such as iron, manganese, copper, and zinc, thermal irritation from hot tea, contamination of bread with silica fibre, and consumption by women of a diet consisting of sour pomegranate seeds, black pepper, and garlic.

#### (d) Northern Italy

The standardized mortality rate for oesophageal cancer among men in Pordenone Province in the Friuli-Venezia Giulia region of northeastern Italy was 17 per 100 000 (Franceschi et al., 1990). Fumonsin-producing *Fusarium* species were shown to be present in maize produced in northern Italy (Logrieco et al., 1995). One study showed the presence of fumonisin B in 20 samples of polenta at concentrations of 0.15–3.8 mg/kg (Pascale et al., 1995).

Two studies showed a correlation between consumption of maize, particularly polenta, and the incidence of cancers of the upper digestive track and oesophagus in the Friuli-Venezia Giulia region (Rossi et al., 1982; Franceschi et al., 1990). In both studies, deficiencies of several micronutrients in refined maize, including riboflavin and niacin, were implicated, especially in conjunction with alcohol consumption, which may aggravate the nutritional deficiency induced by maize-based diets.

#### (e) Kenya

A retrospective study in Kenya of 667 cases of oesophageal cancer from the major hospitals and the Kenya Cancer Registry during the period 1968–75 showed a male:female ratio of 8:1. About 45% of the cases were from western Kenya and 45% from the centre, which are considered to be high-incidence areas as compared with the coastal and northern regions and the Rift Valley (Gatei et al., 1978).

A survey of fungal contamination of maize showed that *F. verticillioides* was the most frequent contaminant in maize kernels from western and central Kenya (Macdonald & Chapman, 1996), and the finding was confirmed on screening the

fungal contamination of 150 maize kernel samples collected in the tropical highlands of western Kenya. Chemical analyses of 197 samples collected in this region showed little fumonisin contamination: 46% of the samples contained concentrations above the limit of detection (100 µg/kg), 5% contained > 1 mg/kg, and a few samples (of poor quality) contained fumonisin B<sub>1</sub> at 3.6–12 mg/kg (Kedera et al., 1999). A low concentration (0.06 mg/kg) of total fumonisins was also detected in only one of seven samples collected in western Kenya (Van der Westhuizen et al., 1999).

Consumption of alcohol does not explain the geographical and ethnic variations, as dietary patterns and tribal customs in Kenya vary considerably (Gatei et al., 1978). No significant correlation was observed between the presence of nitrosamine-like compounds and the incidence of oesophageal cancer. The spatial distribution corresponds to the annual rainfall patterns, with high-incidence areas in regions of heavy rainfall.

(f) *Zimbabwe*

The population-based cancer registry in Zimbabwe showed cancer incidence rates similar to those in other countries of sub-Saharan Africa, with high rates of cancers of the liver, prostate, cervix, and oesophagus (Bassett et al., 1995). The age-standardized incidence rates of oesophageal cancer among men were 30 per 100 000 in Harare in 1990–92 and 59 per 100 000 Bulawayo, further to the south, in 1963–72, and the rates in females were about 8 per 100 000 in both locations. Only three maize-based samples were analysed for fumonisins; one sample, a breakfast cereal intended for human consumption, contained fumonisin B at up to 4.9 mg/kg (Sydenham et al., 1993).

(g) *USA*

A survey of the mortality rates from oesophageal cancer in the USA between 1950 and 1969 indicated a clustering of cases among African-Americans in a narrow region along the southeastern coast of the Atlantic (Fraumeni & Blot, 1977; O'Brien et al., 1982; Brown et al., 1988). A small area of the Sea Islands and the coastal mainland in the vicinity of Charleston, South Carolina, was found to have a particularly high rate of death from this cancer. In Charleston County, the death rate among black males was about 170 per 100 000, and that among black females was 12 per 100 000, which is significantly higher than the State rate of 6.2 per 100 000.

Seven maize-based human foods purchased in retail outlets in Charleston in 1989 contained mean concentrations of fumonisin B<sub>1</sub> and fumonisin B<sub>2</sub> of 0.64 and 0.18 mg/kg, respectively (Sydenham et al., 1991).

Mortality rates in the USA tended to be inversely proportional to the socioeconomic indices of income and education (Fraumeni & Blot, 1977). The increased risk among black men in coastal South Carolina was associated with use of tobacco and alcohol, including 'moonshine' distilled from fermented maize meal and a low intake of fresh fruits (Brown et al., 1988).

(h) *Brazil*

Cancer of the oesophagus is the seventh most important cause of death from cancer among males in Brazil. The southern regions of Santa Catarina, Paraná, and Rio Grande do Sul have the highest incidence rate for oesophageal cancer in

the country, 18 per 100 000 (Instituto Nacional do Cancer, 1989). The incidence is three to four times higher in men than in women, and marked differences in incidence are found in small geographical areas and as a function of time.

The south and western regions of Santa Catarina State, where there is a high volume of maize production and heavy consumption of maize by-products (especially polenta in rural areas), also have the highest incidence of oesophageal cancer. *F. verticillioides* was the predominant fungal contaminant in feed samples associated with mycotoxicoses in animals in Paraná (Sydenham et al., 1992a), and *F. verticillioides* and *A. flavus* were the most prevalent fungal contaminants in maize samples during the 1990–91 crop year in various regions of Paraná and the tropical regions, including Mate Grosso do Sul and Goiás (Hirooka et al., 1996). A survey of the mycoflora in postharvested and stored maize in São Paulo indicated that *Fusarium*, *Penicillium*, and *Aspergillus* spp. were most common (Pozzi et al., 1995).

Fumonisin B<sub>1</sub> and B<sub>2</sub> concentrations  $\leq 38$  and 12 mg/kg, respectively, were found in feed samples associated with outbreaks of mycotoxicoses in the State of Paraná (Sydenham et al., 1992a). Concentrations of up to 10 mg/kg of the two toxins were found in samples collected in the tropical regions of Mate Grosso do Sul, with lower concentrations in Paraná: fumonisin B<sub>1</sub> at 4 mg/kg and fumonisin B<sub>2</sub> at 3 mg/kg (Hirooka et al., 1996). Maize and maize-based food samples from Paraná and the southeast (São Paulo) contained fumonisin B<sub>1</sub> and fumonisin B<sub>2</sub> at concentrations up to 12 mg/kg and 10 mg/kg, respectively (Scaff & Scussel, 1999a). In São Paulo, maize cultivars grown in various regions contained up to 7 mg/kg of fumonisin B<sub>1</sub> and 2 mg/kg of fumonisin B<sub>2</sub> (Camargos et al., 2000). A high concentration of fumonisin B<sub>1</sub> (5 mg/kg) was found in maize meal samples from markets and supermarkets in Campiñas, São Paulo (Machinski & Valente Soares, 2000). Fumonisin B<sub>1</sub> was detected at up to 32 mg/kg in maize intended for human consumption collected in the western part of Santa Catarina (Hermans et al., 2000).

No information is available about the nutritional status of the populations of the regions of high incidence of oesophageal cancer in Brazil. Smoking and alcohol drinking have been implicated, although the prevalence of these habits does not differ from those in regions with lower incidence rates (Scaff & Scussel, 1999b). The consumption of hot beverages such as maté and chimarrao has also been implicated as a possible risk factor for oesophageal cancer (Victoria et al., 1987). An epidemiological study in Rio Grande do Sul indicated that smoking, alcohol and maté drinking, farm work, and having a father with cancer were more frequent in cases of oesophageal cancer (Dietz et al. (1998). Other studies suggested that consumption of maize, especially polenta, is a risk factor for this cancer in rural areas in southern and western Santa Catarina (Scaff & Scussel, 1999b).

#### 2.4.2 Liver cancer

The role of fumonisins in the causation of liver cancer was evaluated in Haimen (Jiangsu County) and Penlai (Shandong Province), China, the mortality rate in Haimen (52–65 per 100 000) being about fourfold higher than that in Penlai. In a 3-year survey of 240 maize samples, a 10–50-fold higher fumonisin B content was found in Haimen. Most of the samples also contained aflatoxin B<sub>1</sub>, but there was no significant difference between the two regions. However, deoxynivalenol was detected mainly in maize samples in Haimen. The authors suggested that a synergistic interaction between fumonisins, aflatoxins, and trichothecenes and the algal toxins,

microcystins (Ueno et al., 1996), might contribute to the development of liver cancer (Ueno et al., 1997).

In Transkei, South Africa, the intake of aflatoxin B<sub>1</sub> correlated with the incidence of liver cancer (Van Rensburg et al., 1990), the rates varying between 3.8 per 100 000 in Butterworth and 7.7 per 100 000 in Kentani (Jaskiewicz et al., 1987b) and 2.4 per 100 000 in Kentani and 13 per 100 000 in Lusikisiki (Makaula et al., 1996), with no apparent association with the concentration of fumonisins in maize.

Experimental evidence for synergistic interactions between aflatoxin B<sub>1</sub> and fumonisin B<sub>1</sub> (Gelderblom et al., 1999a; Carlson et al., 2001) and between aflatoxin B<sub>1</sub> and nivalenol (Ueno et al., 1992) in inducing hepatic cancer in rats was reported.

#### **2.4.3 Neural tube defects**

In South Africa, high incidence rates of neural tube defects were recorded in Mpumalanga Province (3.6 per 1000) and Umzimkulu District (3.8 per 1000) in the Transkei (Venter et al., 1995; Ncayiyana, 1986). The rate in rural Transkei is approximately 5–10 times higher than that for blacks in Cape Town (Cornell et al., 1983). High rates of neural tube defects (5.7 per 1000) were also recorded in Hebei Province, China (Moore et al., 1997), and in northeastern USA between 1920 and 1949, peaking between 1929 and 1932 (2.3–4.3 per 1000). The latter epidemic seemed to correspond to two major socioeconomic events: the great depression and alcohol prohibition, although there are some discrepancies (Machon & Yen, 1971). A high rate of neural tube defects (2.7 per 1000) was also recorded in the lower Rio Grande valley in southern Texas, USA, among the offspring of women who had conceived during 1990–91 (Hendricks, 1999). An association between the epizootics of equine leukoencephalomalacia and porcine pulmonary oedema that occurred late in 1989 in the USA has been postulated (Ross et al., 1991). The concentrations of fumonisins in maize-based foods were high in both the Transkei and Hebei Province (Sydenham et al., 1990; Chu & Li, 1994), and maize-meal foods obtained in the USA during 1990–91 also had a relatively high concentration (1.2 mg/kg) of fumonisin B mycotoxins (Sydenham et al., 1991).

Interference with folate metabolism has been related to the development of neural tube defects, as supplementation with folate decreased the risk (Missmer et al., 2000). The blockage of folate uptake, a critical requirement during organogenesis (Lucock et al., 1998), by fumonisins was also implicated in the induction of neural tube defects (Stevens & Tang, 1997; Hendricks, 1999).

#### **2.4.4 Acute mycotoxicosis**

Consumption of rain-damaged, mouldy sorghum and maize by the inhabitants of 27 villages in the Deccan Plateau in southern India resulted in an episode of human mycotoxicosis in 1995. Diarrhoea was reproduced in 1-day-old cockerels fed contaminated grain from the affected households. An epidemiological survey in India indicated that consumption of unleavened bread prepared from mouldy sorghum or maize resulted in a gastrointestinal disease characterized by abdominal pain, borborymi, and diarrhoea. The victims were of low socioeconomic status and ate the mouldy grains mainly because of lack of access to other foods, such as rice.

The dominant mycoflora in the sorghum were *Aspergillus*, *Fusarium*, and *Alternaria* spp., while the first two fungal species were dominant in maize. Fumonisin

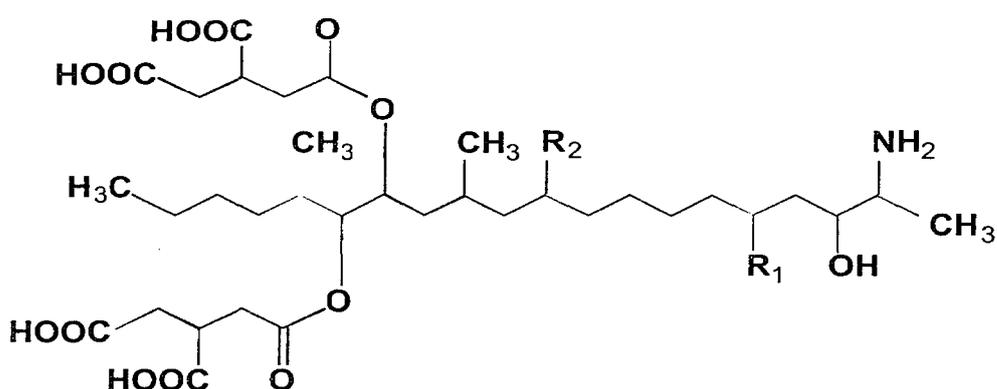
B<sub>1</sub> was the most common mycotoxin in both sorghum and maize samples, and a relatively high concentration of aflatoxin B<sub>1</sub> was also detected in the maize. Fumonisin B<sub>1</sub> concentrations up to 8.5 mg/kg were associated with diarrhoea in laying hens and 1-day-old cockerels, and addition of fumonisin B<sub>1</sub> at 8 or 16 mg/kg of normal diet induced a similar response (Shetty & Bhat, 1997). The control of mycotoxins in human foods in India, especially as related to intake of aflatoxins and fumonisins, has been reviewed (Vasanthi & Bhat, 1998).

### 3. ANALYTICAL METHODS

#### 3.1 Chemistry

The chemical structures of fumonisin B<sub>1</sub>, fumonisin B<sub>2</sub>, fumonisin B<sub>3</sub>, and fumonisin B<sub>4</sub> are given in Figure 1. Fumonisin B<sub>1</sub> is the diester of propane-1,2,3-tricarboxylic acid and 2*S*-amino-12*S*,16*R*-dimethyl-3*S*,5*R*,10*R*,14*S*,15*R*-pentahydroxyeicosane in which the C-14 and C-15 hydroxy groups are esterified with the terminal carboxy group of propane-1,2,3-tricarboxylic acid (Bezuidenhout et al., 1988; Hoye et al., 1994). Fumonisin B<sub>2</sub> is the C-10 deoxy analogue of fumonisin B<sub>1</sub> in which the corresponding stereogenic centres on the eicosane backbone have the same configuration (Bezuidenhout et al., 1988; Harmange et al., 1994). The full stereochemical configurations of fumonisin B<sub>3</sub> and fumonisin B<sub>4</sub> are unknown, although the amino terminal of fumonisin B<sub>3</sub> has the same absolute configuration as that of fumonisin B<sub>1</sub> (i.e. 2*S*, 3*S*) (Hartl & Humpf, 1998). The configuration of the chiral centre on the tricarboxylic acid moieties has been determined by three groups, but with conflicting results. In the initial publication of the stereochemistry of fumonisin B<sub>1</sub>, the *S* configuration was assigned to this centre (Shier et al., 1995), but subsequently both fumonisin B<sub>1</sub> and fumonisin B<sub>2</sub> were reported to have an *R*

Figure 1. Chemical structures of fumonisins



	R <sub>1</sub>	R <sub>2</sub>	Formula	CAS Number	Molecular mass
Fumonisin B <sub>1</sub>	OH	OH	C <sub>34</sub> H <sub>59</sub> NO <sub>15</sub>	116355-83-0	721.838
Fumonisin B <sub>2</sub>	OH	H	C <sub>34</sub> H <sub>59</sub> NO <sub>14</sub>	116355-84-1	705.839
Fumonisin B <sub>3</sub>	H	OH	C <sub>34</sub> H <sub>59</sub> NO <sub>14</sub>	136379-59-4	705.839
Fumonisin B <sub>4</sub>	H	H	C <sub>34</sub> H <sub>59</sub> NO <sub>13</sub>	136379-60-7	689.840

configuration (Boyle & Kishi, 1995a,b). A later study with a synthetic, optically active  $\gamma$ -lactone related to the tricarboxylic acid correlated this with the same lactone in fumonisin B<sub>1</sub> and confirmed the *R* configuration for the chiral site on the side-chains (Edwards et al., 1999).

The most abundant fumonisins in naturally contaminated maize and maize-based products are fumonisins B<sub>1</sub> and B<sub>2</sub>. Hence, analytical methods (and surveys) have been developed mainly for these two toxins. In general, methods developed for fumonisins B<sub>1</sub> and B<sub>2</sub> have been found to be valid for fumonisin B<sub>3</sub> as well (Sydenham et al., 1992b), although use of these methods is limited by problems in the supply of analytical standards for fumonisin B<sub>3</sub>. No specific analytical methods have been developed for fumonisin B<sub>4</sub>, and little is known about its natural occurrence.

### 3.2 Screening tests

Screening tests for fumonisins are based either on thin-layer chromatography (TLC) separation after appropriate clean-up of maize extracts or on commercially available enzyme-linked immunosorbent assays (ELISAs). Other immunologically based methods, such as dipstick (Schneider et al., 1995) and biosensor methods (Thompson & Maragos, 1996; Maragos, 1997; Mullett et al., 1998), have been described but have not found general use. Immunoaffinity columns have been designed to purify extracts before high-performance liquid chromatography (HPLC) separation and quantification of fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> analogues, and have also been used in a direct fluorimetric method for rapid determination of 'total fumonisin' (Duncan et al., 1998).

The TLC and other chromatographic methods for fumonisins have been reviewed (Shephard, 1998). The reversed-phase technique developed by Rottinghaus et al. (1992) has been used in surveys of contamination of maize with fumonisins (Shelby et al., 1994a). When combined with an efficient extract clean-up procedure based on use of immunoaffinity columns and detection by densitometry, TLC can be considered quantitative (Preis & Vargas, 2000).

The performance characteristics of screening tests for fumonisins in maize, based on interlaboratory collaborative studies, have not been reported in the literature. However, in-house comparisons between HPLC methods and the various screening tests have been described. The TLC method of Rottinghaus et al. (1992) has been compared with HPLC over a contamination range of fumonisin B<sub>1</sub> of 1–250 mg/kg (correlation coefficient,  $r = 0.953$ ;  $p < 0.0005$ ; Schaafsma et al., 1998). The results obtained with a fibre-optic immunosensor in a direct competitive monoclonal antibody format with a fumonisin B<sub>1</sub>–fluorescein isothiocyanate conjugate compare favourably with those obtained with HPLC (Maragos, 1997).

The commercial availability of ELISA methods has made them popular for screening for fumonisin contamination. Although the antibodies used in ELISAs are raised against fumonisin B<sub>1</sub>, they generally have significant (but lower) cross-reactivity with fumonisins B<sub>2</sub> and B<sub>3</sub>. The performance of ELISAs is generally assessed by comparison with HPLC determination of fumonisins and has been found to depend on the antibody used (Pestka et al., 1994; Usleber et al., 1994; Sydenham et al., 1996a,b; Kulisek & Hazebroek, 2000). The correlation between the results of HPLC and ELISA for naturally contaminated samples has been reported to vary from 0.51 ( $p < 0.05$ ; Pestka et al., 1994) to 0.97 ( $p < 0.001$ ; Sydenham et al., 1996a). However,

such comparisons have generally shown an overall trend for the concentrations of 'total fumonisins' with ELISA to be greater than those determined in the same samples by HPLC.

### 3.3 Quantitative methods

Quantitative analytical methods for fumonisins have been reviewed (Sydenham & Shephard, 1996; Wilson et al., 1998; Shephard, 1998). Almost all these methods involve HPLC separation of fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> after solvent extraction from maize or maize-based food matrices and purification by solid-phase extraction. In most surveys and studies on the natural occurrence of fumonisins involved use of variations or minor modifications of a few basic analytical methods.

The first quantitative HPLC method for determining fumonisins B<sub>1</sub> and B<sub>2</sub> in naturally contaminated maize involved extraction with methanol:water (3:1), clean-up on strong anion exchange solid-phase extraction cartridges, and quantification by reversed-phase HPLC after precolumn derivatization with the fluorogenic reagent, *ortho*-phthaldialdehyde (Shephard et al., 1990). The reproducibility of this method when used for naturally contaminated maize was studied collaboratively under the sponsorship of the Commission on Food Chemistry of IUPAC (Thiel et al., 1993). The method was subsequently improved and extended to include the determination of fumonisin B<sub>3</sub> (Sydenham et al., 1992b). A further collaborative study of its accuracy and reproducibility was conducted under the auspices of both the Commission on Food Chemistry of IUPAC and AOAC International and resulted in adoption of the method by the latter (Sydenham et al., 1996c). It has now been accepted as official AOAC method 995.15 for the determination of fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> in maize (Trucksess, 2000). The performance characteristics in the international collaborative trials are given in Tables 9–12.

Modifications to the above method led to the development of other analytical methods. Acetonitrile:water (1:1) was formulated as an alternative extraction solvent, while reversed-phase C<sub>18</sub> solid-phase extraction cartridges have been used to purify extracts (Bennett & Richard, 1994; Rice et al., 1995). Although these cartridges yield less pure extracts, they are necessary for determination of the hydrolysis products of fumonisins and for cases in which strong anion exchange columns give poor recovery (Shephard, 1998). The introduction of immunoaffinity columns containing antibodies reactive towards fumonisins has greatly improved the clean-up step in analytical methods (Ware et al., 1994; Duncan et al., 1998). Most researchers report ed quantification by means of precolumn derivatization with *ortho*-phthaldialdehyde, despite its limited stability. Use of naphthalene-2,3-dicarboxaldehyde has been proposed as an alternative (Bennet & Richard, 1994). Recent advances in analytical instrumentation have resulted in the introduction of bench-top liquid chromatography–mass spectrometers, which are more sensitive and specific for the detection and quantification of fumonisins (Shephard, 1998).

The official AOAC International method for fumonisins in maize has also been used for maize-based foods (Stack & Eppley, 1992), although problems have been reported in the recovery of fumonisins from certain matrices (Scott & Lawrence, 1994). In order to improve the recovery from these matrices, other extraction solvents have been investigated, while retaining the solid-phase extraction clean-up steps and *ortho*-phthaldialdehyde derivatization for HPLC quantification. The alternative solvents include methanol:borate buffer (pH 9.2; 3:1), methanol:water:hydrochloric

**Table 9. Results of a collaborative study by 11 laboratories of the repeatability and reproducibility of the method of Shephard et al. (1990) for the determination of fumonisins B<sub>1</sub> and B<sub>2</sub> in naturally contaminated maize**

Mean (µg/kg)	S <sub>r</sub> (µg/kg)	RSD <sub>r</sub> (%)	S <sub>R</sub> (µg/kg)	RSD <sub>R</sub> (%)	No. of outliers	HORRAT value
<i>Fumonisin B<sub>1</sub></i>						
< 50	–	–	–	–		
226	33	15	56	25	1	1.2
340	84	26	86	26	1	1.4
560	44	7.7	100	18	1	1.0
1200	170	15	310	27	1	1.7
1983	260	13	460	23	1	1.6
<i>Fumonisin B<sub>2</sub></i>						
< 50	–	–	–	–		
74	13	17	28	38	1	1.6
100	37	37	46	46	1	2.0
260	32	12	71	28	1	1.4
420	66	16	140	34	1	1.9
740	130	18	270	36	1	2.2

From Thiel et al. (1993)

S<sub>r</sub>, standard deviation for repeatability; S<sub>R</sub>, standard deviation for reproducibility; RSD<sub>r</sub>, relative S<sub>r</sub>; RSD<sub>R</sub>, relative S<sub>R</sub>; HORRAT, ratio of relative SD for reproducibility in the trial to that predicted. A HORRAT of 1 indicates a relative SD for reproducibility corresponding exactly to the Horwitz equation (Horwitz, 1989); a HORRAT ≤ 1.0 ± 0.5 indicates normal reproducibility; a HORRAT > 1.5 indicates that reproducibility is higher than expected, whereas a HORRAT > 2.0 indicates problematic reproducibility (AOAC International, 2000).

acid (2 or 5 N; 3:1:0.3), methanol:0.1 mol/L HCl (3:1); acetonitrile:methanol:water (1:1:2), and acetonitrile:phosphate buffer (0.1 mol/L; pH 3.0; 1:1) (Scott & Lawrence, 1994; Zoller et al., 1994; Scott & Lawrence, 1996; Scott et al., 1999; Solfrizzo et al., 2000; De Girolamo et al., 2001).

As part of a programme to develop analytical methods for mycotoxins in foods at concentrations of interest for future legislation for the European Union, methods for fumonisins have been investigated within the Measurements and Testing Programme and the Community Bureau of Reference. A comparative study was conducted by 24 European laboratories on a contaminated maize sample containing fumonisin B<sub>1</sub> at approximately 2000 µg/kg and fumonisin B<sub>2</sub> at 1000 µg/kg (Visconti & Boenke, 1995; Visconti et al., 1996a). The results are summarized in Table 11.

Further improvements have been made in the analytical methods for fumonisins in maize, cornflakes, maize muffins, extruded maize, and infant formula, resulting in adoption of acetonitrile:methanol:water (1:1:2) as the extraction solvent and use of immunoaffinity columns for clean-up (Visconti et al., 1999; Solfrizzo et al., 2000). These extraction and clean-up techniques and HPLC quantification of preformed *ortho*-phthaldialdehyde derivatives of fumonisins B<sub>1</sub> and B<sub>2</sub> were used in a collaborative study of maize and cornflakes, which resulted in the adoption of the

**Table 10. Results of collaborative study by 12 laboratories of the accuracy and reproducibility of the method of Sydenham et al. (1992a) for the determination of fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> in spiked and naturally contaminated maize**

Spiking concentration (µg/kg)	Mean (µg/kg)	Mean recovery (%)	S <sub>r</sub> (µg/kg)	RSD <sub>r</sub> (%)	S <sub>R</sub> (µg/kg)	RSD <sub>R</sub> (%)	No. of outliers	HORRAT value
<i>Fumonisin B<sub>1</sub></i>								
< 50	—	—	—	—	—	—	—	—
500	400	81	29	7.1	56	14	1	0.75
1000	810	81	47	5.8	130	16	—	0.95
2000	1600	81	120	7.7	260	16	—	1.1
4000	3200	81	200	6.2	490	15	—	1.1
8000	6700	84	740	11	1100	16	—	1.4
Naturally contaminated	4200	—	560	13	940	22	—	1.7
<i>Fumonisin B<sub>2</sub></i>								
< 50	—	—	—	—	—	—	—	—
200	150	76	13	8.4	25	16	1	0.77
400	310	78	27	8.5	49	16	—	0.83
800	620	77	73	12	120	19	—	1.1
1600	1300	81	93	7.2	220	17	—	1.1
3200	2600	82	320	12	470	18	—	1.3
Naturally contaminated	1200	—	220	18	330	27	—	1.7
<i>Fumonisin B<sub>3</sub></i>								
< 50	—	—	—	—	—	—	—	—
100	76	76	7.7	10	15	20	1	0.83
200	160	81	28	17	35	22	—	1.0
400	320	80	49	15	71	22	—	1.2
800	660	82	53	8.0	140	21	—	1.2
1600	1400	87	200	14	270	20	—	1.3
Naturally contaminated	370	—	63	17	93	25	—	1.3

From Sydenham et al. (1996c). For abbreviations, see footnote to Table 9.

method by AOAC International (Visconti et al., 2001). The performance characteristics achieved in the collaborative study are shown in Table 12.

A collaborative study for determination of total fumonisins with a commercially available ELISA kit is under consideration by the methods programme of AOAC International (2000).

**Table 11. Results of comparative study in 24 European laboratories of a maize sample containing fumonisin B<sub>1</sub> at approximately 2000 µg/kg and fumonisin B<sub>2</sub> at 1000 µg/kg prepared by spiking a naturally contaminated, ground maize sample**

Result	Fumonisin B <sub>1</sub>	Fumonisin B <sub>2</sub>
No. acceptable data sets	25	25
No. outliers	1	1
Overall mean (corrected for recovery)	2300 µg/kg	1200 µg/kg
RSD <sub>r</sub>	10%	11%
RSD <sub>R</sub>	11%	13%
Overall mean recovery	70 ± 14%	69 ± 16%
Recovery SD (within laboratories)	6%	7%
Recovery SD (between laboratories)	16%	15%
Recovery: Extraction by shaking ( <i>n</i> = 9)	85 ± 12%	86 ± 14%
Recovery: Extraction by blending ( <i>n</i> = 16)	62 ± 6%	60 ± 6%

From Visconti & Boenke (1995); Visconti et al., 1996a). All laboratories used methods derived from that of Shephard et al. (1990), with modifications. For abbreviations, see footnote to Table 9.

#### 4. SAMPLING PROTOCOLS

Whitaker and co-workers have studied the sampling variance associated with the testing of shelled maize for fumonisin (Whitaker et al., 1998). A bulk sample of about 45 kg (100 lbs) was taken from each of 24 batches of shelled maize which had been harvested from 24 fields in North Carolina, USA. Each bulk sample was riffle-divided into 32 1.1-kg test samples, and these were comminuted in a Romer mill. A nested design was used to determine the variation associated with sampling, sample preparation, and analysis. Briefly, 10 batches with a wide range of fumonisin concentrations were selected. From each batch, 10 comminuted test samples were taken randomly, and two 25-g portions were taken from each by riffle division. Finally, the concentrations of fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> were determined by AOAC official method 995.15 (Sydenham et al., 1996c). At a batch contamination concentration of 2 mg/kg, the coefficient of variation associated with sampling (1.1-kg sample) was 17%, that associated with sample preparation (Romer mill and 25-g analytical portion) was 9.1%, and that for analysis was 9.7%. These values were independent of the fumonisin type (B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, or total). The coefficient of variation associated with the total test procedure (sampling, sample preparation, and analysis) was 45%, which was of the same order of magnitude as that for measuring aflatoxin in shelled maize by a similar test procedure.

The proposed sampling plans are shown in Table 13. Some general precepts are:

**Table 12. Results of collaborative study by 23 laboratories of the reproducibility of high-performance liquid chromatography with immunoaffinity column clean-up for determining fumonisins B<sub>1</sub> and B<sub>2</sub> in maize and cornflakes**

	Mean (µg/kg)	Mean recovery (%)	S <sub>r</sub> (µg/kg)	RSD <sub>r</sub> (%)	S <sub>R</sub> (µg/kg)	RSD <sub>R</sub> (%)	No. of outliers	HORRAT value
<b>Analysis of spiked maize and naturally contaminated maize</b>								
<i>Fumonisin B<sub>1</sub></i>								
< 0.05 µg/kg	0.04	—	—	—	—	—	—	—
0.80 µg/kg	0.65	76	0.14	21	0.16	26	—	1.5
Naturally contaminated	0.37	—	0.09	24	0.10	28	—	1.5
Naturally contaminated	0.78	—	0.15	19	0.20	26	1	1.5
Naturally contaminated	1.4	—	0.28	20	0.31	22	—	1.4
<i>Fumonisin B<sub>2</sub></i>								
< 0.05 µg/kg	0.01	—	—	—	—	—	—	—
0.40 µg/kg	0.30	72	0.06	18	0.07	23	—	1.2
Naturally contaminated	0.09	—	0.02	22	0.02	22	4	0.96
Naturally contaminated	0.20	—	0.05	27	0.06	30	—	1.5
Naturally contaminated	0.56	—	0.13	22	0.14	26	—	1.5
<b>Analysis of spiked cornflakes and cornflakes with added naturally contaminated maize</b>								
<i>Fumonisin B<sub>1</sub></i>								
< 0.05 µg/kg	0.04	—	—	—	—	—	—	—
0.80 µg/kg	0.92	110	0.09	9.2	0.27	30	1	1.8
Naturally contaminated <sup>a</sup>	0.32	—	0.07	21	0.10	32	1	1.7
Naturally contaminated <sup>a</sup>	0.57	—	0.09	15	0.16	28	—	1.6
Naturally contaminated <sup>a</sup>	1.0	—	0.12	11	0.29	27	—	1.7
<i>Fumonisin B<sub>2</sub></i>								
< 0.05 µg/kg	0.00	—	—	—	—	—	—	—
0.40 µg/kg	0.39	97	0.03	7.7	0.12	31	1	1.7
Naturally contaminated <sup>a</sup>	0.13	—	0.03	22	0.04	35	—	1.6
Naturally contaminated <sup>a</sup>	0.24	—	0.04	15	0.07	28	—	1.4
Naturally contaminated <sup>a</sup>	0.46	—	0.05	10	0.12	26	—	1.4

From Visconti et al. (2001). For abbreviations, see footnote to Table 9.

<sup>a</sup> Spiked with minimal amounts (0.8–3.0%) of ground, naturally contaminated maize

- A minimum of 30 batches of food should be sampled from each region (i.e. country, agroclimatic region within a country).
- 'Samples' should refer to initial samples collected from a batch, which may be large bulk samples or smaller composite samples.
- 'Sub-samples' should refer to samples produced by riffle division of unground bulk samples or samples produced by comminuting and subdividing smaller composite samples.
- 'Analytical samples' should refer to samples subjected to analysis, which are usually small portions drawn from subsamples.
- The coefficient of variation of the sampling plan should be no more than 30%.
- The coefficient of variation of the complete analytical method (extraction, clean-up, quantification) should be no more than 10%.

## 5. LEVELS AND PATTERNS OF CONTAMINATION OF FOOD COMMODITIES

### 5.1 Results of surveys

Fumonisins are primarily produced by the fungi *F. verticillioides* (Sacc.) Nirenberg and *F. proliferatum* (Matsushima) Nirenberg, which are major pathogens of maize (*Zea mays* L.) around the world. The commodities most often contaminated with fumonisins are therefore maize and maize-based foods. Data on contamination of maize by fumonisins was submitted to the Committee by Argentina, Brazil, Canada, China, Denmark, Sweden, the United Kingdom, and the USA. Further data were derived from the literature published between 1995 and early 2000. Studies of the natural occurrence of fumonisins conducted before 1995 were reviewed by Shephard et al. (1996b).

Data on the occurrence of fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> are presented in Appendices A, B, and C, respectively. The information is often incomplete: most authors reported the limit of detection (LOD), rather than the limit of quantification (LOQ), of the analytical method; the mean value in samples is frequently given, and in these cases (or when its use implied), the data were recalculated so that all data represent the means of samples, those below the limit of detection being taken as zero. When possible, the numbers of samples containing > 1000 and > 2000 µg/kg are also given. The former level represents the legislative limit for fumonisins B<sub>1</sub> and B<sub>2</sub> in Switzerland (FAO, 1997). The references in the appendices show the primary reference, which usually includes details on sampling and some information on analytical method; further references to methods are recorded as 'A ='. Few references were given for sampling methods.

Little is known about the natural occurrence of fumonisin B<sub>4</sub>. It is produced by strains of *F. verticillioides*, generally at lower concentrations than fumonisin B<sub>1</sub>, B<sub>2</sub>, or B<sub>3</sub> (Abbas et al., 1992; Plattner et al., 1996). Fumonisin B<sub>4</sub> was identified in 23 of 44 mouldy maize samples in the Republic of Korea (mean, 1600 µg/kg; range, 80–11 000 µg/kg) at concentrations lower than those of fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> in these samples (Seo & Lee, 1999).

Some surveys have been undertaken of contamination of food commodities other than maize products. Analysis of 41 local and imported beers in Canada by HPLC showed a low incidence of contamination, with only four samples containing

**Table 13. Proposed sampling plans**

Commodity	Increments (n x y grams)	Subsample size (kg)	Notes
<i>Fumonisin</i>			
Maize			
Whole corn	50 x 100	5.0	Whitaker et al. (1998): Sampling variation for fumonisins in maize similar to that reported for aflatoxins
Corn-on-the-cob	50 cobs	7.5	Assuming that core of cob contributes about 30% of total weight of cob and that a cob yields about 100 g of kernels Coker et al. (in press).
Cornflour			Assumed that sampling variance for these commodities was similar to that associated with aflatoxin in comminuted feeds; suggested sampling plan associated with sampling precision of 12.5% for aflatoxin in comminuted feeds
Maize meal			
Maize grits			
Bran			
Processed maize foods, e.g. cornflakes, tortilla chips, popcorn, muffin mix, starch	10 x 100	1.0	
<i>Deoxynivalenol and T-2 and HT-2 toxins</i>			No data on sampling variance for T-2 or HT-2 toxins; assumed to be similar to that for deoxynivalenol
Maize			
Whole corn	20 x 100	2.0	Sample size for maize set at double the sample weight required for estimating deoxynivalenol in wheat and barley
Corn-on-the-cob	20 cobs	3.0	Assuming that core of cob contributes about 30% of total weight of cob and that a cob yields about 100 g of kernels
Maize grits			Sample size for deoxynivalenol in cereal products arbitrarily set at half that required for fumonisins and ochratoxin A
Processed maize foods, e.g. cornflakes, tortilla chips, popcorn, muffin mix, starch	10 x 50	0.5	
Wheat			Whitaker et al. (2000)
Barley	20 x 50	1.0	Freese et al. (2000)
Oats			
Rye			

**Table 13** (contd)

Commodity	Increments (n x y grams)	Subsample size (kg)	Notes
<i>Deoxynivalenol and T-2 and HT-2 toxins (contd)</i>			
Flour, meal, and bran of all origins	10 x 50	0.5	Sample size for deoxynivalenol in cereal products arbitrarily set at half that required for fumonisins and ochratoxin A
Bread			
<i>Ochratoxin A</i>			
Maize			
Whole maize	50 x 100	5.0	Sampling variability for ochratoxin A assumed to be similar to that for fumonisins (and aflatoxins; see above) Assuming that core of cob contributes about 30% of total weight of cob and that a cob yields about 100 g of kernels
Corn-on-the-cob	50 cobs	7.5	
Maize grits			
Processed maize foods, e.g. cornflakes, tortilla chips, popcorn, muffin mix, starch	10 x 100	1.0	Coker et al. (in press): Assumed that sampling variance for these commodities was similar to that associated with aflatoxin n comminuted feeds; suggested sampling plan associated with sampling precision of 12.5% for aflatoxin in comminuted feeds
Wheat			
Barley			
Rice (including de-husked and polished)			
Peas and beans (including coffee beans)	50 x 100	5.0	Sampling variability for ochratoxin A assumed to be similar to that of fumonisins (and aflatoxins; see above)
Dried fruit, (e.g. raisins, currants, sultanas, figs, dates, apricots)			
Flour, meal, and bran of all origins			
Bread	10 x 100	1.0	Coker et al. (in press): Assumed that sampling variance for these commodities was similar to that associated with aflatoxin n comminuted feeds; suggested sampling plan associated with sampling precision of 12.5% for aflatoxin in comminuted feeds
Ground and instant coffee			
Cocoa powder			

**Table 13** (contd)

Commodity	Increments (n x y grams)	Subsample size (kg)	Notes
<i>Ochratoxin</i> (contd)			
Beverages (e.g. coffee, wine, grape juice)	5 x 100	0.5	Commission of the European Union (1998a): Assumed that sampling variance of mycotoxins in beverages was similar to that for aflatoxin M <sub>1</sub> in milk
<i>Aflatoxin M<sub>1</sub></i>			
Milk (liquid and dry), e.g. raw, pasteurized, homogenized, UHT, skimmed, semi-skimmed, evaporated, infant formula Milk products	5 x 100	0.5	Commission of the European Union (1998a): Assumed that sampling variance of mycotoxins in beverages was similar to that for aflatoxin M <sub>1</sub> in milk

> 2 ng/ml fumonisin B<sub>1</sub> (maximum, 59 ng/ml) (Scott & Lawrence, 1995). Of these, three contained fumonisin B<sub>2</sub> at a concentration > 2 ng/ml (maximum, 12 ng/ml). Of the rest, seven contained 1 ng/ml or traces of fumonisin B<sub>1</sub>. A survey of 46 imported beer samples in Canada by ELISA showed that 11 samples contained > 1 ng/ml (maximum, 25 ng/ml), and a further 11 contained 1 ng/ml or traces > 0.15 ng/ml (Scott et al., 1997). A similar survey of 32 Spanish beer samples by ELISA showed 14 positive samples (LOD, 3 ng/ml) with a range of 4.8–86 ng/ml (Torres et al., 1998). Another survey by HPLC of 29 domestic and imported beers in the USA showed 25 positive samples (> 0.3 ng/ml), with 17 samples containing > 1 ng/ml (maximum, 13 ng/ml) for fumonisins B<sub>1</sub> plus B<sub>2</sub> (Hlywka & Bullerman, 1999).

Investigation of milk and beef for contamination by fumonisins failed to raise any concern. Fumonisins were detected in beef muscle only after continuous exposure to highly contaminated feed (Smith & Thakur, 1996). In a survey of 165 milk samples in the USA, only one was found to be contaminated with fumonisin B<sub>1</sub> at a concentration of 1.3 ng/ml by gas chromatography–mass spectrometry and > 5 ng/ml by HPLC (Maragos & Richard, 1994). Studies in which cows were given feed contaminated with culture material equivalent to 75 mg/kg of feed or pure fumonisin B<sub>1</sub> at a dose equivalent to contamination of feed with 125 mg/kg showed no detectable carry-over of fumonisins into milk (LOD, approximately 5 ng/ml) (Scott et al., 1994; Richard et al., 1996). Re-analysis of these milk samples by ELISA (LOD, 0.5 ng/ml) also showed no carry-over into the milk of treated cows (Prelusky et al., 1996a). Similar studies in lactating sows given a diet containing fumonisin B<sub>1</sub> at 100 mg/kg for 14 days showed no toxin in the milk at a LOD of 30 µg/kg (Becker et al., 1995). Pigs fed diets containing the toxin at 2–3 mg/kg also did not show accumulation of fumonisin residues in muscle, although residues did accumulate in kidneys and liver while the contaminated feed was being consumed (Prelusky et al., 1994, 1996a,b). Replacement of the contaminated feed by clean feed resulted in a rapid decline in the concentrations. Studies in laying hens also showed no fumonisin residues in most tissue samples or in eggs (Vudathala et al., 1994; Prelusky et al., 1996a).

Contamination of rice with fumonisins was reported in the USA in samples collected from fields known to have plants with symptoms of *Fusarium* sheath rot (Abbas et al., 1998). Fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> were detected (LOD, 500 µg/kg by HPLC) in 8 of 20 samples at concentrations ≤ 5600 µg/kg. The appendices give the results of determinations in rice samples in Argentina, in which four of five imported samples showed contamination at maximum concentrations of 229 µg/kg fumonisin B<sub>1</sub>, 126 µg/kg fumonisin B<sub>2</sub>, and 16 µg/kg fumonisin B<sub>3</sub>. A further 11 samples of locally produced rice showed no fumonisin contamination (LOQ, 8 µg/kg), and five rice samples included in a survey of foods in the United Kingdom also contained no detectable fumonisin (LOD, 10 µg/kg) (Patel et al., 1997). Fumonisin B<sub>1</sub> has been reported in rice in China (Appendix A). It has also been found on *Fusarium*-infected mouldy navy, adzuki, and mung beans (Tseng et al., 1995; Tseng & Tu, 1997) and in *F. proliferatum*-infected asparagus plants (Logrieco et al., 1998). In a survey of 35 sorghum syrup samples in the USA, one contained fumonisin B<sub>1</sub> (at 120 µg/kg; LOQ, 100 µg/kg; LOD, 10 µg/kg) (Trucksess et al., 2000). A single analysis of a sorghum meal sample in Burundi showed a very high concentration (28 200 µg/kg) of fumonisin B<sub>1</sub>, but five sorghum samples were not contaminated (LOD not given) (Munimbazi & Bullerman, 1996). In an analysis of 20 sorghum and sorghum meal

samples in Botswana, three contained fumonisin B<sub>1</sub> at a concentration > 20 µg/kg (mean, 43 µg/kg; maximum, 60 µg/kg; LOD, 20 µg/kg) (Siame et al., 1998). A further two samples of sorghum meal in Botswana contained traces of fumonisin B<sub>1</sub> (20 µg/kg) (LOD, < 20 µg/kg) (Doko et al., 1996), and five samples collected from control households during an epidemiological study in India contained fumonisin B<sub>1</sub> at a concentration of 70–360 µg/kg (Bhat et al., 1997). In an extensive study of 43 normal sorghum samples in India, only two had concentrations above the LOD of 25 µg/kg (range, 150–510 µg/kg) (Shetty & Bhat, 1997). A survey of the effects of storage on sorghum in Brazil over 1 year showed mean concentrations of 110–150 µg/kg (Da Silva et al., 2000).

## 5.2 Distribution

When numerical data on the distribution of fumonisins in harvested maize and maize-based products were available in the surveyed literature, the numbers are given in Appendix A. These data include the combined results from surveys in the USA on a wide range of maize-based products sampled between 1990 and 1994 (Pohland, 1996). A number of authors presented distributions in the form of histograms, from which it was not always possible to extract unambiguous, relevant numerical information.

## 5.3 Annual variation

Limited data were available on annual variation in fumonisin concentrations in maize harvested in consecutive years, although it is clear that considerable variation can occur. The most extensive data on annual variation was collected for maize sampled in Iowa, USA, between 1988 and 1996 (Appendices A–C; Murphy et al., 1993; Rice & Ross, 1994; P.A. Murphy, personal communication). During the initial years of the survey (1988–91), the average concentration of fumonisin B<sub>1</sub> was > 2000 µg/kg each year, whereas in 1992–96 the concentration was significantly lower, with a mean < 450 µg/kg. A 5-year survey (1989–93) was conducted in South Africa on fumonisin concentrations in white and yellow maize. The concentrations of fumonisin B<sub>1</sub> in white maize over the 5 years ranged from 320 to 570 µg/kg; those in yellow maize ranged from 170 to 190 µg/kg during the first 4 years and rose to 680 µg/kg in the last year (Shephard et al., 1996b). Contamination of maize in Croatia with fumonisins B<sub>1</sub> and B<sub>2</sub> over two consecutive seasons (1996 and 1997) has also been reported (Appendix A; Jurjevic et al., 1999). The data for Brazil drawn from the GEMS/Food programme is poor (Appendices A and B). Similar data are available for Argentina over a number of years (Appendices A–C), although the geographical origin of the samples included in the survey varied slightly.

The annual variation in total fumonisin concentrations over the 5-year period 1994–98 in various maize-based food products in the USA is shown in Appendix A, with data on contamination of maize meal (degerminated, partially degerminated, and whole grain) from the 1997 and 1998 US crops.

## 6. EFFECTS OF PROCESSING

The stability of mycotoxins during food processing is affected by many factors, including: the moisture of the product, the toxin concentration and its location, the

presence of additives, and the type of food matrix (Scott, 1984). These factors should therefore be considered with respect to each type of processing (e.g. milling, roasting, canning, oil extraction) when estimating the fate of the mycotoxin. After decomposition or destruction of mycotoxins, the resulting compound(s) should be identified to ensure that undesirable materials (with harmful biological effects) do not contaminate the food.

### **6.1 Dry and wet milling**

Dry milling of maize is a physical process by which the components of the grain are separated. The primary products derived from the process are grits, bran, germ, meal, and flour (Alexander, 1987). Since fumonisins are not expected to be destroyed during this process, their distribution has been evaluated in the fractions obtained from commercially and experimentally dry-milled maize. After commercial dry milling, fumonisins were found in flaking grits, flour, germ, and bran. The pattern of distribution after experimental dry milling varied slightly for different types of maize, but in general the concentrations were lower in grits and higher in germ, bran, and fines. As all the fractions found to contain fumonisins are used in the production of animal or human food, maize-based ingredients should be monitored for the presence of fumonisins (Katta et al., 1997).

Wet milling is used to obtain products such as germ (for oil and feed preparation), fibres and gluten (used in animal feed), and starch (for e.g. syrup). Analysis of the fractions obtained from laboratory-scale wet milling of maize contaminated with fumonisins revealed that the starch fractions did not contain measurable amounts of fumonisin B<sub>1</sub>; 22% of the toxin was found in the steeping and process water, while the fumonisin content of the other fractions diminished in the order gluten > fibre > germ and represented 10–40% of the concentration found in the original maize. The presence of fumonisin in gluten, fibres, and germ could be a hazard to animal health, since these products are used as feed ingredients (Bennett et al., 1996).

### **6.2 Removal of fumonisins**

As maize contaminated with fumonisin B<sub>1</sub> has a low density, 86% of the toxin can be removed in the buoyant fraction after treatment with saturated sodium chloride solution (Shetty & Bhat, 1999). Steeping maize in water, which is a step in wet milling, was also effective in reducing the concentrations of fumonisins B<sub>1</sub> and B<sub>2</sub> in maize, but sulfur dioxide delayed toxin extraction from the contaminated grain (Canela et al., 1996). Nevertheless, steeping maize in 0.2% sulfur dioxide solution containing fumonisin B<sub>1</sub> significantly decreased the amount of toxin in the solution, indicating that maize may contain fumonisin-binding constituents (Pujol et al., 1999).

### **6.3 Heating**

The effects of heating on the stability of fumonisins during various food processing procedures have been studied. The temperatures required to achieve reductions > 50% in the concentration of fumonisin B<sub>1</sub> and/or fumonisin B<sub>2</sub> varied according to the process: for example, 190 °C for dry or moist maize meal (Scott & Lawrence, 1994) or frying maize chips (Jackson et al., 1997); extrusion temperatures for batter

made from maize flour (Pineiro et al., 1999); 218 °C in roasting maize meal (Castelo et al., 1998a), and 160 °C for extrusion cooking of maize grits (Katta et al., 1999). The results indicate that fumonisins are fairly stable to heat and that significant removal occurs only during processes that reach temperatures > 150 °C. At each temperature tested in a specific food-processing system, the stability of the fumonisin was time-dependent (Jackson et al., 1996) and was strongly affected by other factors, some of which are mentioned above. The efficiency with which fumonisins are extracted from heated foods may decrease with time (Bordson et al., 1993), probably because of binding to the food matrix (Scott & Lawrence, 1994). As a consequence, controlled recovery experiments must be conducted for each type of processing system to ensure that the most appropriate methods of detection are used (Scott & Lawrence, 1994). Methods are needed to differentiate between binding and removal of fumonisins (Bullerman & Tsai, 1994).

#### 6.4 Nixtamalization

Populations who consume large amounts of maize and maize products (e.g. in countries in South and Central America) are at high risk of exposure to fumonisins. The technique for preparing staple foods in these countries, such as tortillas, involves alkaline cooking and heating (nixtamalization), during which hydrolysed fumonisins are formed. Hydrolysed fumonisin B has been detected in commercial *masa* and tortilla chips, probably due to formation during nixtamalization (Murphy et al., 1996). In studies of the fate of fumonisins during processing and toxicological studies of the products, nixtamalized *F. moniliforme* culture (Voss et al., 1996a) and a maize-based diet containing *F. proliferatum* remained toxic after processing (Hendrich et al., 1993). Although the main toxic product formed from fumonisin during nixtamalization is hydrolysed fumonisin, other products may be formed (Hendrich et al., 1993).

Treatment of fumonisin-contaminated, ground maize with calcium hydroxide resulted in transfer of the aminopentol moiety and, possibly, also the tricarballic acid moiety to the aqueous fraction; these could easily be separated. Maize kernels from which the pericarp had been partially removed contained significantly greater amounts of fumonisin B<sub>1</sub> and its aminopentol moiety, indicating that this treatment would reduce the concentration of fumonisins in maize (Sydenham et al., 1995). Use of a modified nixtamalization procedure on maize contaminated with fumonisin B<sub>1</sub> resulted in a 100% reduction in the fumonisin content and removed the mutagenic potential of the maize extracts (Park et al., 1996).

Although it was expected that the fumonisin in nixtamalized maize products would be in the hydrolysed form, the content of that form in *masa* and tortillas in Mexico was lower than the content of fumonisins B<sub>1</sub> and B<sub>2</sub>. Several explanations were proposed, including incomplete nixtamalization, a high initial concentration of fumonisin, and only partial removal of the pericarp, in which most of the fumonisin is located (Dombrink-Kurtzman & Dvorak, 1999).

Better control of fumonisins can thus be achieved by monitoring the degree of pericarp removal and analysing samples from each stage of production. Nixtamalization alone does not ensure complete detoxication of fumonisin-contaminated maize, and the process should be carefully controlled to ensure at least a significant reduction in fumonisins and other toxic products in the processed material.

### 6.5 Fermentation

The fermentation of naturally contaminated maize to produce ethanol led to only limited degradation of fumonisin B<sub>1</sub>, most of which was recovered in distillers' dried grain, thin stillage, and distillers' soluble fractions (Bothast et al., 1992). Fumonisin B<sub>1</sub> has also been found in beer, indicating that the toxins persist under the conditions (temperature, pH) prevailing during the brewing process (Scott & Lawrence, 1995; Scott et al., 1997; Hlywka & Bullerman, 1999).

## 7. FOOD CONSUMPTION AND DIETARY INTAKE ESTIMATES

Surveys of the frequency of occurrence and concentrations in maize and maize products throughout the world are summarized in Appendices A–C. Over 60% of the surveyed products contained detectable amounts of fumonisins. Unfortunately, most of the reports did not contain sufficient information to allow determination of the representativeness of the data, i.e. they lacked information on sampling methods and involved insufficient numbers of samples to allow statistical evaluation of the fumonisin contamination of the diet.

The highest concentrations of fumonisins are found in visibly damaged or mouldy maize. The data show that the concentrations and incidence of contamination vary considerably in relation to the commodity tested and the source. The highest frequency was recorded in maize feeds, followed by ground maize products such as flour, grits, polenta, semolina, and gluten, maize kernels, and miscellaneous maize foods. The list of commercial retail foods that may be contaminated with fumonisins includes maize flour, grits, polenta, semolina, snacks, cornflakes, sweet, canned, and frozen maize, extruded maize, maize bread, maize-extruded bread, biscuits, cereals, chips, pastes, starch, infant foods, gruel, purée, noodles, popcorn, porridge, tortillas, tortilla chips, *masa*, popped maize, soup, tacos, and tostadas. Generally, processed maize foods have lower concentrations and a lower frequency of contamination than untreated foods. These differences may be the result of dilution of maize in food commodities or may depend on differences in maize cultivars or in the quality requirements for different destinations. Additionally, fumonisins are water-soluble, and processes that involve washing or water treatment may result in their partial or complete removal from the final food product.

In this evaluation, only the consumption of contaminated maize or maize-containing food products was considered, as the contributions of other commodities to the intake of fumonisins are too low and too variable to affect overall long-term exposure significantly.

Dietary intake of fumonisins was assessed in accordance with the recommendations of an FAO/WHO workshop on methods for assessing exposure to contaminants and toxins (WHO, 2000b). That workshop recommended that the best available data on concentrations in foods be used to estimate intake. For commodities that contribute significantly to intake, distribution curves should be generated to provide options to governments and national and international regulatory agencies for use in risk management. The Workshop further recommended that international estimates of dietary intake be generated by multiplying mean or median concentrations by the values for consumption of a commodity in the five GEMS/Food regional diets (WHO, 1998), which were derived from food balance sheets

compiled by FAO. Since these sheets are available for most countries, they provide a set of data that are comparable across countries and regions of the world (WHO, 2000b). The five regions represented by the diets are Africa, Europe (which includes Australia, Canada, New Zealand, and the USA), the Far East, Latin America, and the Middle East. The regional diets represent the average per-capita availability of food commodities rather than actual food consumption, and data on availability generally result in an overestimate of consumption by about 15% (WHO, 1998). The workshop noted that, if available, national intake estimates should also be reported, as they may provide information about the intake of specific population subgroups or consumers of large amounts, which cannot be derived from GEMS/Food regional diets.

As the toxic effects in humans attributed to consumption of fumonisins in food are primarily of a long-term nature, this assessment is concerned solely with long-term intake.

### **7.1 *Estimated concentrations of fumonisins in foods***

Nine countries, Argentina, Brazil, Canada, China, Denmark, Sweden, the United Kingdom, the United States, and Uruguay, submitted information on the concentrations of fumonisins in maize and maize-derived foods. Fumonisins were detected in over 60% of all food products tested. The rate of detection was much lower in sound maize than in mouldy maize, and processed maize-containing foods generally contained lower concentrations of fumonisins than maize grain, flour, or grits.

A frequency distribution of the concentrations of fumonisins in maize was derived from available data in 1997 and published as part of an assessment of human intake of fumonisins in the Netherlands (de Nijs et al., 1998a). All maize consumed in the Netherlands is imported, and most was from Europe, South America, and the USA, although some was imported from Asia and Africa. As the concentrations of fumonisins and the incidences of fumonisin contamination reflected those found in the submitted data, for the purposes of this analysis, they were taken as representative of the maize available in trade throughout the world. Analysis of data available since 1997 showed little change in the patterns of incidence and concentration of fumonisins in maize and maize-based foods.

The concentrations of fumonisins were shown by the least-squares method to be distributed log-normally. The arithmetic mean concentration in the 349 samples used in the distribution was 1.36 mg/kg, and this distribution was combined with appropriate food intakes for assessing intake.

### **7.2 *Estimates of intake at the international level***

Data on food intake from the GEMS/Food regional diets (Table 14; WHO, 1998) were combined with the distribution of fumonisin concentrations in maize in commerce described above to yield estimates of fumonisin intake. A commercially available software product, @Risk (Palisade Corp.) was used with Microsoft Excel 97 to derive the distributions. Each simulation was run with Latin Hypercube sampling and 25 000 iterations.

Three scenarios were examined. In the first scenario, the per-capita consumption of maize in the GEMS/Food diets was combined with the distribution of concentrations of fumonisins to yield a distribution of fumonisin intake (Table 15). In the second

**Table 14. Intake of foods containing fumonisins in the GEMS/Food regional diets**

Commodity	GEMS code	Diet (g of food per person per day)				
		Middle Eastern	Far Eastern	African	Latin American	European
Maize, all	GC 645	48	31	110	42	8.8
Maize		16	0	0	1.5	0
Maize, flour	CF 1255	32	31	110	40	8.8
Sweet maize (kernels)	VO 1275	0	0	3.3	0	6.2
Sweet maize (cob)	VO 447	0	0	4.4	0	8.3
Popcorn	GC 656	0.2	0.2	0.2	0.2	0.2
All cereals	GC 80	430	450	330	250	230

scenario, a hypothetical distribution of maize consumption was estimated by assuming that it is log-normally distributed in each diet, with a standard deviation equal to 66% of the mean consumption. This standard deviation was based on empirical observations of various distributions of commonly eaten foods in the USA (Table 16). This scenario provides a better evaluation of the high end of the intake

**Table 15. Scenario 1: Distribution of intake of fumonisins**

Intake of fumonisins (%)	Intake of fumonisins ( $\mu\text{g/day}$ per person)				
	Middle Eastern	Far Eastern	African	Latin American	European
Minimum	0.2	0.1	0.4	0.2	0.0
Maximum	1100	740	2500	990	210
Mean	66	43	140	57	12
Standard deviation	120	76	260	100	21
5	0.3	0.2	0.7	0.3	0.1
10	0.6	0.4	1.4	0.6	0.1
15	1.4	0.9	3.1	1.2	0.3
20	2.0	1.3	4.4	1.7	0.4
25	3.0	1.9	6.6	2.6	0.5
30	4.4	2.9	9.7	3.8	0.8
35	6.3	4.1	14	5.5	1.2
40	9.6	6.2	21	8.3	1.7
45	14	9.1	31	12	2.6
50	20	13	44	17	3.7
55	26	17	57	22	4.7
60	33	22	74	29	6.1
65	43	28	94	37	7.8
70	54	35	120	47	9.9
75	70	45	150	61	13
80	97	63	210	84	18
85	140	89	300	120	25
90	200	130	440	170	36
95	300	190	660	260	54
97.5	370	240	810	320	67

**Table 16. Scenario 2: Distribution of intake of fumonisins**

Intake of fumonisins (%)	Intake of fumonisins ( $\mu\text{g}/\text{day}$ per person)				
	Middle Eastern	Far Eastern	African	Latin American	European
Minimum	0.0	0.0	0.1	0.0	0.0
Maximum	4300	2200	9000	3000	490
Mean	65	42	140	57	12
Standard deviation	140	93	340	130	26
5	0.3	0.2	0.6	0.2	0.0
10	0.6	0.3	1.2	0.5	0.1
15	1.0	0.6	2.1	0.8	0.2
20	1.7	1.0	3.4	1.4	0.3
25	2.5	1.6	5.2	2.1	0.4
30	3.7	2.3	7.8	3.0	0.7
35	5.4	3.4	11	4.4	1.0
40	7.9	4.9	17	6.5	1.4
45	11	7.0	24	9.1	1.9
50	15	9.7	33	13	2.7
55	21	13	45	17	3.6
60	28	17	59	23	4.9
65	36	23	77	30	6.5
70	47	30	100	40	8.6
75	63	40	130	53	12
80	84	55	180	72	16
85	120	77	260	100	22
90	180	110	400	1530	33
95	300	190	670	260	55
97.5	440	300	980	390	82

distribution of fumonisins; however, the mean intake in this scenario is the same as that in the first. The third scenario was intended to mimic the worst case, in which the only grain that a person consumes is maize (Table 17). This extremely conservative scenario could describe the intake of fumonisins by individuals who either have no access to grains other than maize (e.g. subsistence farmers) or are allergic to other grains, such as wheat and rice. In this scenario, the per-capita consumption of all grains was combined with the distribution of fumonisin intakes.

The mean intake of fumonisins in scenarios 1 and 2 ranged from 12  $\mu\text{g}/\text{day}$  per person in the European diet to 140  $\mu\text{g}/\text{day}$  per person in the African diet. These estimates were based on the assumption that an individual consumes randomly contaminated maize over a lifetime and will consume maize at a daily rate equal to the per-capita disappearance of maize. Use of the presumed distribution of maize consumption, as in the second scenario, allows examination of fumonisin intake by people who consume larger amounts of maize over a lifetime as well as those who consume maize with higher concentrations of fumonisins in the long term. Over a lifetime, a person would be expected to consume maize with concentrations of fumonisins at approaching the mean of the distribution. Only those individuals in areas where maize is regularly and heavily contaminated would fall into the high end of the distribution of intake of fumonisins in this scenario. The intake of fumonisins

**Table 17. Scenario 3: Distribution of intake of fumonisins**

Intake of fumonisins (%)	Intake of fumonisins ( $\mu\text{g/day}$ per person)				
	Middle Eastern	Far Eastern	African	Latin American	European
Minimum	0.4	0.3	0.2	0.2	0.2
Maximum	30 000	33 000	24 000	17 000	13 000
Mean	590	610	430	350	310
Standard deviation	1 200	1 300	920	770	660
5	2.5	2.6	1.8	1.4	1.3
10	5.0	5.3	3.7	2.9	2.7
15	9.7	9.9	7.0	5.4	5.0
20	15	16	11	8.7	8.0
25	23	24	17	13	12
30	34	35	25	20	18
35	50	51	36	29	26
40	72	75	53	41	37
45	100	110	76	59	54
50	140	150	100	82	75
55	190	200	140	110	100
60	250	260	190	140	130
65	330	340	240	190	170
70	440	450	320	250	220
75	580	600	420	330	300
80	780	830	580	440	410
85	1 100	1 200	800	630	580
90	1 600	1 700	1 200	940	860
95	2 700	2 800	2 000	1 600	1 400
97.5	4 000	4 000	2 900	2 300	2 100

at the 97.5th percentile in this scenario ranges from 82  $\mu\text{g/day}$  per person in the European diet to 980  $\mu\text{g/day}$  per person in the African diet. Below this percentile, the predicted intakes in the two scenarios are not appreciably different.

The predicted intake of fumonisins in the third scenario, which describes the potential intake of fumonisins by persons who eat maize in place of all other grains, is appreciably higher than those in the first two scenarios. It must be emphasized that the number of individuals covered by this scenario is extremely small on a global basis and consists primarily of rural subsistence farmers, who are not representative of national or GEMS/Food regional populations. The mean intake in this scenario ranged from 310  $\mu\text{g/day}$  per person in the European diet to 610  $\mu\text{g/day}$  per person in the Far Eastern diet (in which the diet would typically be dominated by rice). The 95th percentile intake ranged from 1400  $\mu\text{g/day}$  per person in the European diet to 2800  $\mu\text{g/day}$  per person in the Far Eastern diet.

### 7.3 National estimates of intake

Although nine countries submitted information on the concentrations of fumonisins in maize and maize-based foods, only the United Kingdom presented information that allowed estimation of national intake of fumonisins. Nevertheless, several national

estimates have been published, from Argentina, Canada, the Netherlands, South Africa, Switzerland, and the USA (Kuiper-Goodman et al., 1996; Humphreys et al., 2000; Marasas, 1997; de Nijs, 1997; Solovey et al., 1999). The mean daily intake of persons aged 15–55 in Argentina was estimated to be 0.2 µg/kg bw per day. Intake in Canada between 1991 and early 1995 was estimated to be 0.017–0.089 µg/kg bw per day (Kuiper-Goodman et al., 1996).

On the basis of daily average consumption of maize and maize products of 3 g for the general population, 42 g for regular eaters of maize products, and 160 g for persons intolerant to gluten in the Netherlands, the estimated daily intakes of fumonisin B<sub>1</sub> were 0.06, 1.0, and 3.7 µg/kg bw per day, respectively, assuming a mean fumonisin B<sub>1</sub> content of 1.36 mg/kg of maize produce. It was estimated conservatively that 97% of individuals with gluten intolerance had a daily intake of fumonisin B<sub>1</sub> of at least 1 µg, and 37% had an intake of at least 100 µg, while the proportions of the general population exposed to these concentrations of fumonisin B<sub>1</sub> were 49% and 1%, respectively (de Nijs, 1997; de Nijs et al., 1998b).

The intakes of fumonisin B<sub>1</sub> in the Transkei, South Africa, were estimated to be 14 and 440 µg/kg bw per day from healthy and mouldy maize, respectively (Thiel et al., 1992). The probable daily intake of rural blacks in the Transkei consuming home-grown mouldy maize was estimated to vary from 1.2 to 355 µg/kg bw per day (Marasas, 1997).

The mean daily intake of fumonisins in Switzerland was estimated to be 0.030 µg/kg bw per day (Zoller et al., 1994).

In the United Kingdom, various maize-based foods are consumed at levels of 0.6–12 g/day (Gregory et al., 1990). Polenta was found to contain the highest concentration of fumonisins, with a mean of 530 µg/kg and a maximum of 2100 µg/kg. All other retail foods contained < 100 µg/kg. On the basis of the mean consumption of polenta (maize meal), breakfast cereals, popcorn, and maize-based snacks and the mean fumonisin concentrations in these foods, the intake of fumonisins would be 1.8 mg/day per person, or approximately 0.03 µg/kg bw per day. Intake of fumonisin B<sub>1</sub> at the 90th percentile of intake can be approximated by tripling the mean.

A preliminary estimate of the intake of fumonisins by maize eaters in the USA was 0.08 µg/kg bw per day (Humphreys et al., 2000).

National estimates of intake vary considerably according to the source and amount of maize in the diet as well as the prevalence of *Fusarium* kernel rot in the harvested crop. The national estimates are summarized in Table 18, in which intakes on a body-weight basis have been converted to micrograms per day by assuming an average body weight of 60 kg for an adult over a lifetime.

All the national estimates of fumonisin intake are appreciably lower than the international estimates prepared from the GEMS/Food diets. A number of factors may contribute to these differences. First, many of the national estimates were based on intakes of maize-containing food products, which are generally less heavily contaminated with fumonisins than the maize or maize flour used to make them. Secondly, the intakes of individual food products used in the national estimates were typically lower than the overall consumption of maize in the GEMS/Food diets. Finally, exported maize appears to be more heavily contaminated with fumonisins than maize used domestically in food products (Rheeder et al., 1994), due perhaps to lengthy storage before transport or a lack of strict regulation of exported maize consignments.

**Table 18. National estimates of fumonisin intake**

Country	Intake ( $\mu\text{g}/\text{kg}$ bw per day)	
	Mean or median	High
Argentina	0.2	
Canada	0.02	0.08
Netherlands	0.06, 1.0	
Switzerland	0.03	
United Kingdom <sup>a</sup>	0.03	0.1
USA	0.08	

<sup>a</sup> Calculated for this risk assessment from data submitted to WHO

#### 7.4 Potential effects on intake of limits and their enforcement

Imposition of limits on the concentration of fumonisins in maize in international trade would probably affect the intake of fumonisins, especially at the high end of the distribution. Table 19 shows the potential intakes of fumonisins in the GEMS/ Food African diet if a limit of 1, 2, 5, or 10 mg/kg was imposed and enforced or with the default assumption of no limit. The model and data used to produce the estimates in Table 15 were used. The main assumption in this model is that maize would not be consumed at a concentration above the maximum limit. Additionally, it was assumed that the existence of a limit would have no effect on the distribution of fumonisins in maize below that limit, i.e. that no producer would seek to improve the overall distribution of fumonisins in maize to ensure that the maximal number of samples was acceptable. An approximation of the percentage of maize samples that would be rejected at each limit, assuming no change in production practices, is included.

## 8. PREVENTION AND CONTROL

### 8.1 Fumonisin-producing fungi

At least 12 fumonisins and structurally related analogues have been recognized and are categorized into five groups, A, B, C, P, and H ( Musser & Plattner, 1997). Of these, the fumonisins of the B series ( $B_1$ ,  $B_2$ ,  $B_3$ ) are the most widespread in nature

**Table 19. Potential intake of fumonisins from maize in the African diet when various limits are imposed and enforced**

Limit (mg/kg)	Intake of fumonisins ( $\mu\text{g}/\text{day}$ per person)						
	Mean	Minimum	Maximum	50th %ile	90th %ile	95th %ile	% excluded
None	140	0.4	2500	44	440	660	0
1	27	0.4	110	13	77	90	32
2	46	0.4	210	21	130	160	20
5	86	0.4	530	34	260	370	7.6
10	120	0.4	1100	42	400	580	1.6

(Sydenham et al., 1991; Nelson et al., 1993). The fumonisins are produced only by *Fusarium* spp. In a survey of the production of fumonisins B<sub>1</sub> and B<sub>2</sub> by 40 toxic *Fusarium* isolates, the toxins were produced only by *F. moniliforme*, *F. proliferatum* (both belong to the section *Liseola*), and *F. nygami* (Thiel et al., 1991). Strains of *F. anthropilum*, *F. dlamini*, and *F. napiforme* also produced fumonisins, but *F. subglutinans* and *F. beomiforme* did not (Nelson et al., 1992).

The fumonisin-producing fungi are common in grain. *F. moniliforme* is considered to be the main cause of *Fusarium* kernel rot, which occurs especially during warm, dry weather. *F. moniliforme*, *F. proliferatum*, *F. nygamai*, and *F. napiforme* are the most important producers of fumonisin B<sub>1</sub> because of their association with food grains such as maize, millet, and sorghum (Nelson et al., 1993). Strains of *F. moniliforme* collected from various substrates and geographical areas were all found to produce fumonisins (Nelson et al., 1991), and the natural occurrence of fumonisins in foods and grains, especially maize, throughout the world is well documented (reviewed by Marasas, 1996; Shephard et al., 1996b; Munkvold & Desjardins, 1997). The potential presence of fumonisins in agricultural produce and processed foods is therefore a serious threat to public health, and efforts have been made to find ways of preventing or reducing the accumulation of fumonisins.

## **8.2 Pre-harvest control**

### **8.2.1 Cultural practices**

The prevalence of *Fusarium* and the subsequent production of fumonisins are enhanced in warm climates and under drought conditions, factors that cannot be controlled, although growers and consumers should be made aware that high concentrations of fumonisins are to be expected under such conditions. Insect damage also affects the accumulation of fumonisins, and both the prevalence and degree of insect damage are significantly correlated with the concentrations of the toxins. Maintenance of rigorous insect control may assist in reducing fumonisin contamination.

### **8.2.2 Genetic improvement**

Maize hybrids that differ with respect to fumonisin accumulation have been identified, some of which contained only low concentrations of fumonisins (Shetty & Bhat, 1977; King & Scott, 1981; Shelby et al., 1994b; Doko et al., 1995; Visconti, 1996). However, hybrids grown in areas outside those to which they were adapted had enhanced fumonisins contents (Shelby et al., 1994b) as a result of the different environmental conditions. Hybrids that accumulate lower concentrations of fumonisins should therefore be screened in each growing area as part of a selection programme. Several genetic mechanisms may be responsible for the low incidence of toxins in some plants. These include factors related to inhibition of fungal invasion (e.g. hardness and composition of kernels; silk composition and viability; inhibitory compounds such as phytoalexins), the presence of enzymes that can degrade fumonisins (Duvick, 1999), and mechanical barriers (e.g. tight husks) or induced resistance to insect penetration. In maize hybrids genetically engineered for insect resistance (by insertion of *Bacillus thuringiensis* genes encoding the  $\delta$ -endotoxin CryIA(b) expressed in kernels), the kernels consistently had less *Fusarium* ear rot and *Fusarium* infection than kernels from normal plants (Munkvold et al., 1997).

Furthermore, the concentrations of fumonisins in the transgenic hybrids were lower than those in their normal counterparts (Munkvold et al., 1999). Thus, engineering of maize for insect resistance may reduce *Fusarium* infection in the kernels and the subsequent accumulation of fumonisin. Several mechanisms might be involved in the reduced concentrations of fumonisins in certain varieties, however. Their elucidation would provide a basis for planning breeding or genetic engineering strategies designed to develop host resistance.

### **8.3 Post-harvest control**

The strategies for coping with mycotoxins after harvest include inhibition of fungal growth by methods including chemical and physical means, natural products, and biological control; removal of grains or particles suspected to contain toxins (segregation); and removal or destruction of existing mycotoxins by e.g. physical means, chemical treatment, adsorption, or biological degradation. Different methods can be combined to achieve a synergistic effect, expressed as enhanced activity.

As fungi will not grow if the water activity ( $a_w$ ) is lower than 0.65–0.70, drying of freshly harvested kernels is an elementary step towards reducing the accumulation of mycotoxins after harvest. The safe moisture content of maize is 14–15%.

#### **8.3.1 Fumonisin production in maize treated with grain preservatives**

The effect of propionate preservatives on the growth of *F. verticillioides* and *F. proliferatum* on irradiated maize and their production of fumonisin B<sub>1</sub> was affected by the  $a_w$ , temperature, and the concentration and source of propionate. Fumonisin production by *F. verticillioides* was not affected by this treatment, but increasing the dose of propionate decreased fumonisin production by *F. proliferatum* at 15 °C, regardless of the  $a_w$ . It was concluded that environmental factors, fungal colonization, and preservatives interact in the grain ecosystem. Fumonisin production did not correlate with the fungal growth rate but was affected by interactions between growth conditions and propionate concentration (Marin et al., 1999).

#### **8.3.2 Separation of grain screenings**

Maize screenings can contain significantly higher concentrations of fumonisin than whole grain (Ross et al., 1991; Murphy et al., 1993), but no major size-associated segregation of fumonisin in screenings was found (Murphy et al., 1993). Feeding animals maize screenings has been correlated directly with diseases caused by fumonisins (equine leukoencephalomalacia and porcine pulmonary oedema) (Harrison et al., 1990; Ross et al., 1990; Wilson et al., 1990). Thus, removal of all dockage constituents and bulk cleaning might reduce the concentrations of fumonisins (Sydenham et al., 1994; Malone et al., 1998) and, in turn, the incidence of animal and human intoxications. However, since fumonisins can occur in whole, undamaged grain, methods for removing fumonisin-containing materials must be improved before the grain enters food-processing operations (Bullerman, 1996; FAO/WHO, 2000).

### **8.4 Chemical and physical decontamination**

As ammoniation is known to detoxify aflatoxins, its effect on fumonisins was also studied. Under atmospheric pressure and ambient temperature, ammoniation

did not significantly reduce the concentration of fumonisins in maize (Norred et al., 1991; Sydenham et al., 1995), but ammoniation under high temperature or pressure reduced the concentration by 79%, leaving no mutagenic effect, indicating that ammoniation under the conditions described can be regarded as safe (Park et al., 1992). Several parameters, such as temperature, length of application, moisture content, ammonia concentration, and toxin concentration, could affect the efficacy of ammoniation, and the roles of these parameters in the process should be studied. Furthermore, the toxicity of the resulting compounds should be further analysed.

Another approach to the detoxication of fumonisin B<sub>1</sub> involves a reaction with fructose to block the amine group, which is critical for its toxicity. The toxicity of fumonisin B<sub>1</sub> to rats was indeed eliminated by such treatment, suggesting a new mechanism for fumonisin detoxication (Lu et al., 1997).

Exposure of maize to 15 kGy of  $\gamma$ -irradiation, which is known to destroy mycotoxins, sterilized the maize but reduced the concentrations of fumonisins B<sub>1</sub> and B<sub>2</sub> by only 20% (Visconti et al., 1996b).

## **9. DOSE-RESPONSE ANALYSIS AND ESTIMATION OF CARCINOGENIC AND TOXIC RISK**

### **9.1 Safety assessment**

The extent to which a food contaminant is judged to represent a potential public health risk is usually assessed by comparing likely intake with a measure such as the tolerable daily intake (TDI). The TDI is derived by applying safety factors to the NOEL in the study considered to be critical, whether it was conducted in laboratory animals or humans. These factors account for extrapolation from animals to humans, variation in the human response, and, sometimes, for less-than-adequate data. This approach was followed by Kuiper-Goodman et al. (1996) in an assessment of fumonisin in maize products, noting that the point estimate of the intake of fumonisin by the group with the highest intake, 0.089  $\mu\text{g}/\text{day}$ , was more than three orders of magnitude lower than the NOEL in studies in animals. A similar assessment was made with similar conclusions by the Nordic Council (TemaNord, 1998). However, de Nijs (1997) found no margin of safety for a sensitive sub-population, patients with coeliac disease, and the approach could not be used to derive an estimate of the probability of harm. These assessments are summarized in Table 20.

Tables 21–24 show the LOELs and NOELs for renal and hepatic effects in studies in which experimental animals were fed fumonisin B-contaminated maize or culture material.

### **9.2 Quantitative risk assessment**

#### **9.2.1 Adverse effects other than cancer**

Quantitative assessments were made of the magnitude of the adverse effects, variations in intake of fumonisins among populations, and the uncertainties of correlations, expressed as a range of possible outcomes. The most sensitive adverse response in rats, nephrotoxicity, was used for the dose–response analysis (Voss et al., 1995a). Dietary intake of fumonisin in maize products was estimated from a 3-

**Table 20. Results of previous safety assessments**

End-point	NOEL or LOEL (µg/kg bw per day)	Safety factor	ADI or TDI (µg/kg bw)	Reference
Renal apoptosis	LOEL: 190	100	0.50	de Nijs (1997)
Rat; cancer		100 1000 5000	8 0.8 0.16	Marasas (1997)
Rat: kidney	NOEL: 0.2	100	2	Commission of the European Union (1998b)

**Table 21. NOEL or LOEL for hepatic effects in studies of rodents given purified fumonisin B<sub>1</sub> by oral administration**

Species and strain	Duration (days)	Route	NOEL/LOEL <sup>a</sup> (mg/kg bw per day)	Reference
B6C3F <sub>1</sub> mouse	90	Diet	9.7/29	Voss et al. (1995a)
B6C3F <sub>1</sub> mouse	14	Gavage	5/15 <sup>c</sup>	Bondy et al. (1997)
B6C3F <sub>1</sub> mouse	28	Diet	< 24	National Toxicology Program (1999)
B6C3F <sub>1</sub> mouse	728	Diet	0.7/1.9 <sup>d</sup> 1.9/6.6 (tumours)	National Toxicology Program (1999)
BD IX rat	780/690	Diet	0.03/0.3 0.8/1.6 (tumours)	Gelderblom et al. (1991, 2001b)
Fischer 344 rat	21	Diet	1.7/3.5	Gelderblom et al. (1994)
Fischer 344 rat	14	Gavage	4/8.5	Gelderblom et al. (1994)
Fischer 344 rat	21	Diet	0.7/3.5	Gelderblom et al. (1996c and unpublished)
Fischer 344 rat	28	Diet	< 12	National Toxicology Program (1999)
Fischer 344 rat	90	Diet	> 6.4	Voss et al. (1995a)
Fischer 344 rat	728	Diet	> 6.6	National Toxicology Program (1999)
Sprague-Dawley rat	28	Diet	1.4/4.1	Voss et al. (1993)
Sprague-Dawley rat	11	Gavage	5/15	Bondy et al. (1996, 1998)
Sprague-Dawley rat	11	Gavage	15/35 <sup>b</sup>	Mehta et al. (1998)
RIVM:WU rat	28	Gavage	> 3.0	de Nijs (1997)

<sup>a</sup> Values preceded by '<' indicates the NOEL was less than the value shown; '>' indicates that the LOEL would be greater than the value shown; '/' indicates that the first number is the NOEL and the second is the LOEL.

<sup>b</sup> Based on foci positive for glutathione-S-transferase, placental form

<sup>c</sup> Increased mitosis observed in females at 5 mg/kg bw per day

<sup>d</sup> Based on hepatocellular hypertrophy

**Table 22. NOEL or LOEL for renal effects in studies of rodents given purified fumonisin B<sub>1</sub> by oral administration**

Species and strain	Duration (days)	Route	NOEL/LOEL <sup>a</sup> (mg/kg bw per day)	Reference
B6C3F <sub>1</sub> mouse	90	Diet	> 29	Voss et al. (1995a)
B6C3F <sub>1</sub> mouse	14	Gavage	5/15	Bondy et al. (1997)
B6C3F <sub>1</sub> mouse	28	Diet	> 105	National Toxicology Program (1999)
B6C3F <sub>1</sub> mouse	728	Diet	> 15	National Toxicology Program (1999)
BD IX rat	780/690	Diet	0.03/0.3	Gelderblom et al. 1991, 2001b)
Fischer 344 rat	21	Diet	< 0.7	Gelderblom et al. (1996c and unpublished)
Fischer 344 rat	28	Diet	< 12	National Toxicology Program (1999)
Fischer 344 rat	90	Diet	0.2/0.6	Voss et al. (1995a)
Fischer 344 rat	728	Diet	0.22/0.67 0.67/2.2 (tumours)	National Toxicology Program (1999)
Sprague-Dawley rat	28	Diet	< 1.4	Voss et al. (1993)
Sprague-Dawley rat	11	Gavage	< 1	Bondy et al. (1996, 1998)
RIVM:WU rat	28	Gavage	< 0.2	de Nijs (1997)

<sup>a</sup> Values preceded by '<' indicates the NOEL was less than the value shown; '>' indicates that the LOEL would be greater than the value shown; '/' indicates that the first number is the NOEL and the second is the LOEL.

day survey of 15 368 persons (US Department of Agriculture, 1989–90). The proportion of maize in each product consumed was estimated from standard recipes. The concentrations of fumonisins in maize products were estimated from surveys by the US Department of Agriculture, and the distribution of fumonisin consumption was modelled for each individual in the surveyed population. The uncertainties associated with the predictions made from each model and with model selection are described. The results of the analyses of the dose–response relationship and intake were assimilated in a two-dimensional Monte-Carlo simulation. The distributions representing variation and uncertainty were selected iteratively to form a two-dimensional array of estimates of the magnitude of harm to individuals and to the population as a whole.

The level of risk associated with fumonisin intake is described first, and then the reductions in risk achieved with two alternative risk management options (reduced concentration and reduced consumption) to gauge their relative effectiveness are described. Nephrotoxicity was used as the end-point because data on the dose–response relationship in individual animals were available (Voss et al., 1995a). The scenarios were designed to show how risk changes when exposure was altered by (1) limiting the intake of fumonisins in maize products and (2) decreasing the consumption of maize by frequent consumers.

**Table 23. NOEL or LOEL for renal and hepatic effects in studies of rats given diets containing naturally contaminated maize or fungal culture material containing fumonisins**

Strain	Duration (days)	Site (end-point)	NOEL/LOEL <sup>a</sup> (mg/kg bw per day)	Reference
Sprague-Dawley	21	Kidney Liver	< 0.6 0.6/4.5	Voss et al. (1998)
Fischer 344	176	Kidney Liver (tumours)	> 3.2 < 2.3	Wilson et al. (1985)
BDIX	610–691	Liver	< 0.65	Purchase & Joubert (1970); Purchase et al. (1975)
BDIX	894	Kidney Liver (tumours)	> 6.9 < 3.2	Marasas et al. (1984)
BDIX	869	Kidney Liver (tumours)	> 1.3 < 0.4	Jaskiewicz et al. (1987a)

<sup>a</sup> Values preceded by '<' indicates the NOEL was less than the value shown; '>' indicates that the LOEL would be greater than the value shown; '/' indicates that the first number is the NOEL and the second is the LOEL.

(a) *Analytical method*

Three software routines were written in Microsoft Excel macro language (Visual Basic for Applications) for fitting curves and for Monte-Carlo simulations.

- (1) ParamFit generates a two-dimensional description of the data from a number of alternative frequency (variability) models to represent uncertainty. Uncertainty associated with model selection is represented by distributing the frequency of use of each of the alternative models according to a specified weighting criterion (i.e. a balance between goodness-of-fit and the number of parameters).
- (2) The Quantitative Risk and Response program models the relationship between dose and response. It models both the magnitude of individual subject response and population variability, and it accounts for model uncertainty associated with the predictions. The program requires a data set of toxicological observations for individual subjects in which the dose and magnitude of response are specified. These data are used to derive a cumulative response distribution for each dose. The program generates two Excel functions: one that predicts response as a function of dose and another that predicts frequency as a function of response. These functions can be used as part of a Monte-Carlo simulation. Two distributions indicate uncertainty, in addition to the distributions of population variation: One is a frequency-based reflection of deviation of the data set from the individual models used to describe the data, while the other is a model probability distribution representing the uncertainty due to model selection.

**Table 24. NOEL or LOEL for various end-points in studies of animals other than rodents given diets containing purified fumonisin B<sub>1</sub>, MRC 826, other fungal cultures, or naturally contaminated maize**

Species	Treatment	Duration	Site (end-point)	NOEL/LOEL <sup>a</sup> (mg/kg bw per day)	Reference
Vervet monkey	MRC 826	13.5 years	Kidney Liver	0.11/0.18 0.11/0.18	Gelderblom et al. (2001c)
Equid	Contaminated maize and fungal culture	150 and 241 days	Equine leukoencephalomalacia	0.30/0.44	Wang et al. (1992)
Pig	Fungal culture	28 days	Liver and lung	< 0.4	Zomborszky et al. (2000)
Pig	Contaminated maize	14 days	Liver Lung	0.2/.92 4/7	Riley et al. (1993)
Rabbit	Purified fumonisin B <sub>1</sub> by gavage	17 days	Kidney	< 0.1	LaBorde et al. (1997)

<sup>a</sup> Values preceded by '<' indicates the NOEL was less than the value shown; '>' indicates that the LOEL would be greater than the value shown; '/' indicates that the first number is the NOEL and the second is the LOEL.

(3) MC2D runs two-dimensional Monte-Carlo simulations. The distribution of the model output is estimated by sampling the input distributions repeatedly. MC2D integrates the sources of variability and the sources of uncertainty into separate distributional dimensions. A Boolean variable on the worksheet controls the behaviour of random number generators in the model by regulating whether a given distribution is re-sampled for every calculation (for variability distributions) or re-sampled only at the start of a new uncertainty iteration (for uncertainty statements). Results from up to 10 output cells are collected. In the present analysis, one output cell was used for each of the four risk management scenarios. After the simulation, MC2D can generate a standard table of specific percentiles for each of the output cells chosen. The net difference across the entire simulation for two output cells can also be calculated.

In both the Paramfit and Quantitative Risk and Response programs, the goodness of fit of each model was optimized by nonlinear regression (Excel Solver). Model weights were calculated from an algorithm that rewards models for goodness-of-fit and penalizes them for use of extra parameters.

#### (b) Data and model

Data from surveys of the US Department of Agriculture conducted in 1994 and 1995 were used to estimate the amount of fumonisin in maize, and these are summarized in Table 25. These data did not distinguish between sweet and field

maize which are reported to have different concentrations of fumonisins (Trucksess et al., 1995); these differences may be real or be artefacts, since the sugars of sweet maize may hinder fumonisin extraction. Sweet maize constitutes only 5% of the maize produced in the USA. Both fumonisin B<sub>1</sub> and fumonisin B<sub>2</sub> were assayed. Because the concentration of fumonisin B<sub>2</sub> was often below the LOD, the concentrations of fumonisin B<sub>1</sub> were modelled, and those of total fumonisins were estimated to be 1.25 times the fumonisin B<sub>1</sub> concentration. The distributions for shelled maize, maize meal, maize flour, and popcorn were modelled with ParamFit, and the distributions in corn flakes, maize grits, maize chips, and maize tortillas were described as a series of percentiles corresponding to actual values; values below the LOD were considered to be uniformly distributed between 0 and the LOD of 20 mg/kg.

The consumption of maize products was evaluated from a US Department of Agriculture survey conducted in 1989–91, in which the 3-day eating habits of 15 368 people were recorded. The amount and type of maize product in each food consumed was calculated from a standard recipe. Maize starch and maize syrup were not included in the analysis as they are minor components in many recipes and because most fumonisin is removed from these products during processing.

Some form of maize product was consumed by 2655 persons in this population during the 3-day survey period. As a 90-day experiment would correspond to about 1/16th of the rats' lifespan, a human exposure period of 360 days was simulated by randomly re-sampling (bootstrapping) each of the 3-day exposure records 360 times. The simulation was repeated 10 times to represent the uncertainty resulting from this sampling exercise.

As the US Department of Agriculture survey contains information about the age of each subject but not their body weights, intakes were calculated on the basis of the mean weights (National Academy of Sciences, 1989) for the age group corresponding to each individual in the survey.

**Table 25. Concentrations of fumonisin B<sub>1</sub> in maize products (µg/kg)**

Maize product	No. of samples	Minimum	Maximum	Average
Shelled maize	78	ND	3100	310
Maize meal	64	ND	1900	160
Maize flour	15	ND	150	37
Popcorn	15	ND	72	22
Maize grits	15	ND	180	30
Tortillas	12	ND	24	12
Maize chips	6	ND	ND	ND
Cereal	5	ND	ND	ND

ND, below the LOD of about 20 µg/kg

The concentrations of fumonisin B<sub>1</sub> in maize products were fitted with 10 alternative distributions: exponential, normal, gamma, log-normal, logistic, Cauchy, beta, triangular, rectangular, and Weibull. The best model for each product, as judged by the same criteria used for weighting models (goodness-of-fit and number of parameters), were used to describe the distribution of concentrations of fumonisin B<sub>1</sub> in each commodity.

(c) *Dose–response relationship for fumonisin and nephrotoxicity*

A dose–response relationship for fumonisin and nephrotoxicity was based on data from the study of Voss et al. (1995a) in which rats were given feed containing fumonisin B<sub>1</sub> at a concentration of 0, 1, 3, 9, 27, or 81 mg/kg. The end-point was lesions on a rating scale of 0–3, the smallest observable lesion being given a score of 1. Because the females had lesions only at the highest dose, no dose–response relationship could be derived. This dose was about 10 times that which caused adverse effects in males, and the females were estimated to be about 10 times less sensitive than males. A rectangular distribution with a range of 5–15 was used to represent the uncertainty of the relative dose in females. Thus, only the dose–response relationship in males was derived directly from the data.

The data were fitted into 273 models that differed in the dose–effect function (e.g. linear, sigmoidal, exponential), the population model (e.g. normal, log-normal), and the presence of dose-independent (background) factors. The uncertainty of the model was represented by weighting; no one model was chosen, and the validity of each model was given a relative probability (see discussion of quantitative risk and response).

Differences between rats and humans in fumonisin-induced nephrotoxicity would best be approximated by using data specific for toxicity induced by fumonisin-like compounds in the two species; however, such information was not available. In this analysis, the interspecies comparison was based on the ratio of the maximal tolerated dose in humans to the LD<sub>10</sub> value in rats in their responses to alkylating agents, a class of compounds for which data on both rats and humans are available (Travis & White, 1988). The resulting frequency distribution of the toxicity ratios was used to extrapolate and represent the uncertainty associated with this inference. Human variation, based on a compilation of pharmacokinetics (Hattis et al., 1987), was accounted for by a log<sub>10</sub> geometric standard deviation.

(d) *Simulations*

Simulations were conducted in two steps. The first was an analysis of intake with a minimal number of uncertainty iterations (10) but which simulated a 360-day intake for each of the 2655 maize-eating individuals. The second step was a human response simulation which contained 500 uncertainty iterations (outer loop) and 2655 variability iterations (inner loop). The second step involved four procedures: One of the 10 intake runs was selected randomly at the start of each uncertainty iteration. The human dose was adjusted to an equivalent rodent dose from the species extrapolation ratio, which was re-sampled at the start of each uncertainty iteration. The dose was adjusted to reflect human variation (the distribution of variation was re-sampled at each iteration). The response was calculated from the male dose–response mode. For females in the consumption survey, the dose was adjusted downwards by a factor of 10 to reflect the lower sensitivity of female rats.

*Intake simulation:* The scenarios designed to simulate concentration limits were generated by truncating the distributions of fumonisins in maize products (e.g. limit of 1 mg/kg). The effects on the intake of fumonisin B of varying the concentration limit of fumonisin in maize are summarized in Table 26. The reduction in intake of fumonisin at any percentile is not proportional to the reduction in the limit. Eaters in

the lower percentile of consumption are not affected. Even at the restrictive limit of 0.5 mg/kg, which would eliminate much of the maize supply, the fumonisin intake of 50th percentile eaters would vary by less than 1.5  $\mu\text{g}/\text{day}$  with the six concentration limits and no limit (the concentration of fumonisins at the time of the survey). The 0.5 mg/kg limit resulted in a predicted reduction of intake of about 40% for people in the highest percentiles.

The scenarios that simulate consumption limits were generated by truncating the daily consumption (e.g. a limit of 100 g/day). Table 27 summarizes the effect of varying the consumption limit on intake. Again, eaters in the lower percentile are not affected. Eaters in the 50th percentile are affected only by about 1  $\mu\text{g}/\text{day}$  at the 25 g/day limit. Eaters in the upper percentile are greatly affected; the fumonisin intake of eaters in the 95th percentile is reduced by about two-thirds, or 20  $\mu\text{g}/\text{day}$ , and that of eaters in the 99th percentile is reduced by over sevenfold, or > 37  $\mu\text{g}/\text{day}$ . In this assessment, reducing intake of fumonisin by lowering maize consumption was more effective than lowering the concentration of fumonisin in maize.

*Response simulation for fumonisin-induced nephrotoxicity:* The simulation of fumonisin B-induced human nephrotoxicity consisted of integration of the intake assessment, the dose–response function, and the functions accounting for human variation and species extrapolation. Table 28 shows the predicted severity of human nephrotoxicity when maize is consumed as described in the survey and for each of the scenarios (concentration and consumption). The units in the table represent a rating scale for nephrotoxicity of 0–3, 1 representing the lowest observable effect. As all the predicted values are below 1, no observable effect is anticipated. The predicted nephrotoxicity represents the background rate and is largely independent of maize consumption, and therefore of the concentration of fumonisin. This is shown more clearly in Tables 29 and 30, which show little difference in the predicted rate of nephrotoxicity with different scenerios. The tables give a comparison of the

**Table 26. Effects of different concentration limits on intake of fumonisin B**

Consumption	Intake of fumonisin B ( $\mu\text{g}/\text{day}$ per person) at limits of:						
	0.5 mg/kg	1 mg/kg	1.5 mg/kg	2 mg/kg	2.5 mg/kg	3 mg/kg	None
Minimum	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.01	0.04	0.04	0.04	0.04	0.04	0.04	0.04
0.05	0.09	0.09	0.09	0.09	0.09	0.09	0.09
0.10	0.12	0.12	0.12	0.12	0.12	0.12	0.12
0.25	0.30	0.30	0.31	0.31	0.31	0.31	0.31
0.50	2.2	2.6	2.8	3.0	3.0	3.1	3.6
0.75	6.6	9.0	10	11	11	11	11
0.90	12	17	19	20	20	21	21
0.95	17	24	27	29	29	30	31
0.99	28	38	43	46	47	48	48
Maximum	59	83	95	100	100	100	100

Each value is the mean of 10 uncertainty iterations. The reduction in exposure at any percentile is not proportional to the reduction in the limit; even the draconian limit of 0.5 mg/kg would result in a predicted reduction in exposure of only 50% at the upper percentiles.

**Table 27. Effects of different consumption limits of maize on intake of fumonisin B**

Consumption	Intake of fumonisin B ( $\mu\text{g/day}$ per person) at maize consumption limit of:				
	25 g/day	50 g/day	100 g/day	300 g/day	No limit
Minimum	0.00	0.00	0.00	0.00	0.00
0.01	0.04	0.04	0.04	0.04	0.04
0.05	0.09	0.09	0.09	0.09	0.09
0.10	0.12	0.12	0.12	0.12	0.12
0.25	0.30	0.31	0.31	0.31	0.31
0.50	2.6	3.0	3.2	3.6	3.6
0.75	8.0	11	11	11	11
0.90	9.5	18	21	21	21
0.95	9.7	19	28	30	30
0.99	10	20	38	47	47
Maximum	15	29	41	100	100

Each value is the mean of 10 uncertainty iterations. Limiting maize intake alters the exposure of persons with a high intake. For instance, in the consumption survey only two individuals ate more than 300 g/day. This limit therefore changes the position only of those two individuals in the distribution relative to the other consumers.

concentration and consumption limit scenarios by subtracting one array (simulation) of variability-by-uncertainty from the other. Table 29 shows the result of subtracting the background (no maize) rate from the concentration limit scenario, and Table 30 shows the result of subtracting the background (no maize) rate from the consumption limit scenario.

The assessment of dietary intake of fumonisins presented here provides a quantitative estimate of the risk at concentrations above the ADI or TDI. In particular, it provides a quantitative description of the level of risk reduction achieved by two alternative risk management options. It shows incremental risk with consumption of a toxin. Dietary intake of fumonisins is a useful case study for this type of assessment, since, like many environmental contaminants, whether of natural or anthropogenic origin, it is widespread. Efforts to avoid or reduce intake and the resulting risk become major considerations in assessing the public health significance of an environmental contaminant like fumonisin. Safety assessments serve to screen out contaminants that present little risk. They may not be adequate when the intake of a contaminant, such as fumonisin, already exceeds a safe level and when reasonable, achievable measures for reducing intake remain to be identified.

The model presented here suggests that renal toxicity would not be expected to be detected at current levels of intake. Table 28 shows that the predicted values for renal lesions are lower than those that could be observed (i.e.  $\geq 1$ ). The results presented in Tables 29 and 30 show that reducing intake by reducing consumption of fumonisin-containing products would be more effective in reducing human risk of kidney damage than would lowering the concentration of fumonisin permitted in maize by a similar factor. Limiting maize consumption alters only the intake of fumonisin by persons with a high maize intake.

**Table 28. Distributions of pathologist's rating of nephrotoxicity with limits on concentration of fumonisin B and consumption of maize**

Simulation	Uncertainty/variability					
	Average/median	Average/0.95	Median/median	Median/0.95	0.95/median	0.95/0.95
Null limit, fumonisin B	0.264535	0.264978	0.311075	0.311078	0.397879	0.397879
Null limit, maize	0.264536	0.264978	0.311075	0.311078	0.397879	0.397879
1 mg/kg limit, fumonisin B	0.264533	0.264886	0.311075	0.311078	0.397879	0.397879
3 mg/kg limit, fumonisin B	0.264535	0.264960	0.311075	0.311078	0.397879	0.397879
20 g/day limit, maize consumption	0.264529	0.264712	0.311075	0.311078	0.397879	0.397879
100 g/day limit, maize consumption	0.264535	0.264950	0.311075	0.311078	0.397879	0.397879
No maize	0.264522	0.264522	0.311075	0.311075	0.397879	0.397879

Pathologist's rating of nephrotoxicity, from 0 to 3, with 1 representing the lowest observable effect. As all the predicted values are < 1, no observable effect is anticipated. The first two simulations are identical; the slight difference in the average–median value reflects random variation. The predicted response values are largely independent of dose and therefore of maize consumption.

**Table 29. Effects of limiting the concentration of fumonisin B on a pathologist's rating of nephrotoxicity**

Simulation	Uncertainty/variability					
	Average/median	Average/0.95	Median/median	Median/0.95	0.95/median	0.95/0.95
Null limit, fumonisin B	0.000013	0.000456	0.000000	0.000156	0.000036	0.001542
1 mg/kg limit, fumonisin B	0.000012	0.264960	0.000000	0.000150	0.000034	0.001492
3 mg/kg limit, fumonisin B	0.000011	0.000364	0.000000	0.000121	0.000028	0.001204

Pathologist's rating of nephrotoxicity, from 0 to 3, with 1 representing the lowest observable effect. The background level of nephrotoxicity associated with each fumonisin concentration limit has been subtracted.

**Table 30. Effects of limiting the consumption of maize on a pathologist's rating of nephrotoxicity**

Simulation	Uncertainty/variability					
	Average/median	Average/0.95	Median/median	Median/0.95	0.95/median	0.95/0.95
Null limit, maize	0.000013	0.000455	0.000000	0.000154	0.000036	0.001528
20 g/day limit, maize consumption	0.000013	0.000428	0.000000	0.000144	0.000035	0.001446
100 g/day limit, maize consumption	0.000007	0.000190	0.000000	0.000056	0.000017	0.000602

Pathologist's rating of nephrotoxicity, from 0 to 3, with 1 representing the lowest observable effect. The background level of nephrotoxicity at each limit of maize intake has been subtracted. As all the predicted values are < 1, no observable effect is anticipated. The percentiles do not reflect the same individual values for uncertainty or variability as in Table 28, as the values were sorted after subtraction of the background nephrotoxicity.

The risks and the uncertainties of the models associated with human intake of fumonisin have thus been described. For each input, a range of values could be derived, depending on which model is used. Use of multiple models yields the model uncertainty, which is measured by the range of values for a given input from a series of weighted models. Some uncertainties were not represented in the analysis, such as the uncertainty due to unknown relative proportions of field maize to sweet maize eaten and whether maize consumption would vary over a longer period than represented in the 3-day survey. Other uncertainties include whether components of maize other than fumonisin are also toxic and the appropriateness of extrapolating from animals to humans. The kidney, the most sensitive target organ in rats, a species known to have renal problems, may not be the most sensitive target for fumonisins in humans. Knowledge of the mechanisms of toxicity, perhaps through L-calcium channels (Smith et al., 1999, 2000) or disruption of sphingolipid metabolism (Riley et al., 1994, 1996, 1997), could be used quantitatively in species extrapolation and to describe variation. Either of these mechanisms, which are not exclusive, could allow a signalling event in a variety of responses. The data used here are, however, unique in that they provide dose–response relationships for individual animals, a feature necessary for modelling with Monte Carlo analysis. Data of this type for other end-points would make other analyses possible.

On the basis of this analysis, the current intake of fumonisin in the USA would not be expected to result in renal lesions, even for eaters at upper percentiles of consumption. Even if dietary intake doubled, no measurable toxicity would be expected in the population of the USA. Limiting maize consumption would be more effective, would disrupt the eating habits of fewer people, and would eliminate less of the maize crop than would limiting the concentration of fumonisins in maize.

### 9.2.2 Dose–response relationship for cancer

#### (a) Liver

Data from the National Toxicology Program study of the carcinogenicity of fumonisin B<sub>1</sub> in B6C3F<sub>1</sub> mice were used to fit the Moolgavkar–Venzon–Knudson (MVK) two-stage, clonal expansion model of carcinogenesis (Moolgavkar & Venzon, 1979; Moolgavkar & Knudson, 1981; Kodell et al., 2001). The data included tissue weight, cell proliferation, cell death, and sphingolipid metabolism in the primary target organ. This model was used to predict 2-year liver tumour rates in female and male mice on the basis of the effects of different doses of fumonisin B<sub>1</sub> on the growth of normal cells and on the proliferation of preneoplastic cells as a compensatory response to sphinganine-induced cell death. The model reproduced the observed tumour rates reasonably well. Increased rates above background in females were predicted only at the highest dose.

The MVK model allows for birth and death of preneoplastic cells. It includes the assumption that a normal cell can undergo an initial mutation to become a preneoplastic cell at a certain rate ( $\mu_1$ ) and that this cell can in turn undergo a second mutation to generate a malignant cell at another specified rate ( $\mu_2$ ).

Preneoplastic cells can divide to produce two daughter cells at a rate  $\beta(t)$ , or they can die at death rate  $\delta(t)$ . In this implementation of the MVK model,  $\mu_1$  and  $\mu_2$  were assumed to be constant with respect to time, while  $\beta(t)$  and  $\delta(t)$  were assumed to be time-dependent. Data on liver weights were supplemented with data on body

weights to estimate the net growth rate of the liver over time. Data on PCNA were used to estimate the cell birth rate,  $\beta(t)$ , in the liver over time, while the cell death rate,  $\delta(t)$ , was estimated as the difference between the cell birth rate and the net growth rate of the liver. The differential effect of fumonisin B<sub>1</sub> on  $\delta(t)$  and, consequently, on  $\beta(t)$  is proportional to the concentration of sphinganine in the liver. The mutation rates,  $\mu_1$ , are assumed to be unaffected by fumonisin B<sub>1</sub>, consistent with evidence indicating that fumonisin B<sub>1</sub> is not genotoxic (Cohen et al., 2000). The model describes the probability of such an occurrence at any time (Zheng, 1997), according to the following formula. The programming code for implementation of the model was provided by Zheng (1998).

The liver weights of four animals at each interim sacrifice (3, 7, 9, and 24 weeks) and of all animals killed at 104 weeks were recorded. The body weight of each animal was recorded each week. As the average liver weights throughout the 104-week study were needed to implement the MVK model, liver weights for the weeks

$$P(t_0) = 1 - \exp\left\{-\int_0^{t_0} \mu_1 N(t) [z(t) - 1] dt\right\}$$

where  $z'(t) = \beta(t)[z(t)]^2 + [\beta(t) + \delta(t) + \mu_2]z(t) - \delta(t)$  and  $z(t_0) = 1$ .  $t_0 > 0$ .

with no observations were estimated from an allometric relationship with body weight, according to the equation:

$$LW(t) = a[BW(t)]^b$$

where  $BW(t)$  and  $LW(t)$  represent, respectively, the body weight and liver weight of a female mouse at time  $t$ . This equation may be expressed as:

$$\log_e[LW(t)] = \log_e(a) + b \times \log_e[BW(t)].$$

A similar fit was performed for each dose group, and the liver weights for intermediate times were inferred from each dose-specific allometric equation, on the basis of observed weekly body weights. The curves for 0, 5, and 15 mg/kg of diet are similar, while those for 50 mg/kg of diet were about 25% higher and those for 80 mg/kg of diet were 2–2.5 times higher. For each dose group, a locally linear smoothing technique (Fan & Gijbels, 1996) was used to approximate the derivative of the  $\log_e$ -transformed liver weight:

$$d[\log_e LW(t)]/dt$$

over the range 0–104 weeks. This derivative was used as an estimate of the net growth rate of the liver over time. During the first 12 months, the growth rate at 5 mg/kg of diet was roughly 10% more than that of the controls, 20% higher at 15 mg/kg of diet, 50% higher at 50 mg/kg of diet, and 2.5–3 times higher at 80 mg/kg of diet. The liver growth rates were somewhat erratic after 12 months. The number of cells in the liver each week,  $N(t)$ , was estimated from:

$$N(t) = LW(t)/CW$$

where  $CW$  represents the average weight of a mouse liver cell, which has been reported to be approximately  $6.6 \times 10^{-6}$  (Altman & Dittmer, 1962).

The percentages of PCNA-labelled cells in actively replicating cells were used to estimate the cell birth rates.

As the PCNA values for individual animals were somewhat erratic, the raw data were considered unreliable for regression modelling. Although the regression fit to the weekly data was quite satisfactory, the somewhat erratic, non-monotone nature of the data for dose groups made these results difficult to fit. It was decided that a simple cubic (monotone) curve was a reasonable compromise between the increasing PCNA responses at 5 and 15 mg/kg of diet and the inverted response at 50 mg/kg of diet. The birth rate,  $\beta(t)$ , for dose  $d$  and time  $t$  was estimated from:

$$\beta(t) = (1/\text{cell cycle}) \times [0.31154 + 3.35 \times 10^{-7}d^3 - 0.05562 \times \log_e(t)],$$

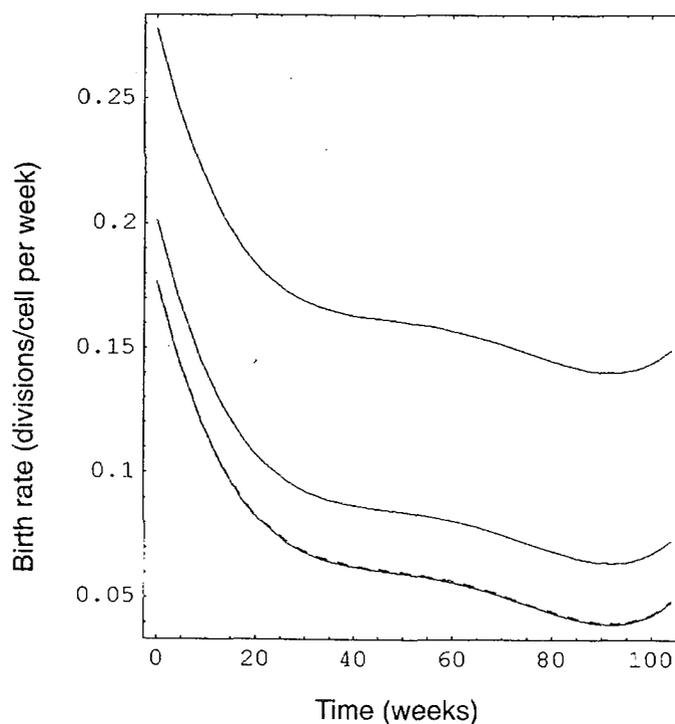
where the cell cycle was taken to be 33.6 h. The plot of  $\beta(t)$  for the dose groups is shown in Figure 2. The values for 0, 5, and 15 mg/kg of diet were almost indistinguishable.

The study included data on apoptosis in the liver at the 3-, 7-, 9-, and 24-week interim kills and at terminal sacrifice. However, these data reflect only incidence and severity, not rates of cell death. Hence, an indirect approach had to be devised to estimate the cell death rate,  $\delta(t)$ . This was done by using the estimated net growth rate of the liver with the estimated cell birth rate. This approach proved to be a key factor in using the model. Specifically, the relationship

$$\delta(t) = \beta(t) - d[\log_e LW(t)]/dt$$

was used to estimate the cell death rate for each dose group. The shapes of the death-rate curves are similar to the birth-rate curves, but slightly less steep (Figure 3).

**Figure 2. Birth rates of liver cells in mice at different concentrations of fumonisin  $B_1$  in the diet**



The average sphinganine concentrations in the livers of female mice were 2.3, 1.8, 3.1, 5.1, and 5.3 nmol/g at 0, 5, 15, 50, and 80 mg/kg of diet, respectively. These concentrations were used to model the effect of fumonisin B<sub>1</sub> on the cell death rate according to the relationship

$$\begin{aligned} \delta_P(t) &= (1 + \emptyset \times S) \delta_n(t) \\ &= \delta_n(t) + (\emptyset \times S \times \delta_N(t)) \end{aligned}$$

where the subscripts *P* and *N* denote, respectively, preneoplastic and normal cells, *S* denotes the average concentration of sphinganine (mmol/g) in the liver, and  $\emptyset$  is a constant. Thus, fumonisin B<sub>1</sub> was assumed to increase the rate of apoptosis of preneoplastic liver cells over that of normal cells by an amount  $\emptyset \cdot S \cdot \delta_N(t)$ . The compensatory proliferation of preneoplastic cells was modelled according to the relationship:

$$\beta_P(t) = \beta_N(t) + \gamma - \emptyset - S - \beta_N(t),$$

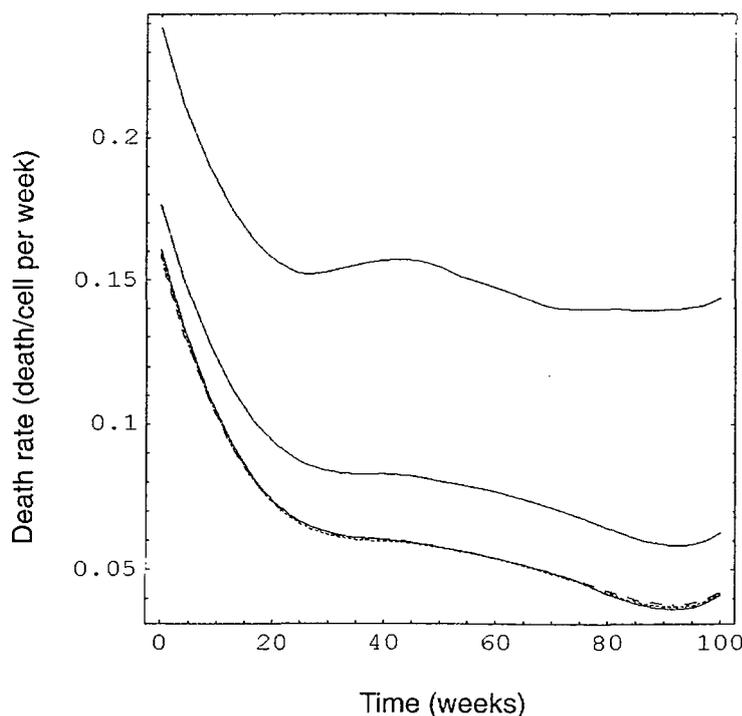
which indicates that the birth rate of preneoplastic cells is increased above that of normal cells by a constant  $\gamma$  times the increase in the cell death rate. The constants  $\emptyset$  and  $\gamma$  were assigned values of 0.2 and 1.38, respectively, to obtain representative predictions of the tumour incidences at 104 weeks.

Although not a key assumption for model fitting, it was considered plausible that the second mutation rate,  $\mu_2$ , might be higher than the first mutation rate,  $\mu_1$ . Hence, the relationship

$$\mu_1 = k \mu_2$$

was adopted, where *k* is a constant ( $0 < k < 1$ ). A reasonably good fit to the data on tumours at 104 weeks was obtained with  $\mu_1 = 1.0 \times 10^{-7}$  and  $k = 0.715$ .

**Figure 3. Death rates of liver cells in mice at different concentrations of fumonisin B<sub>1</sub> in the diet**

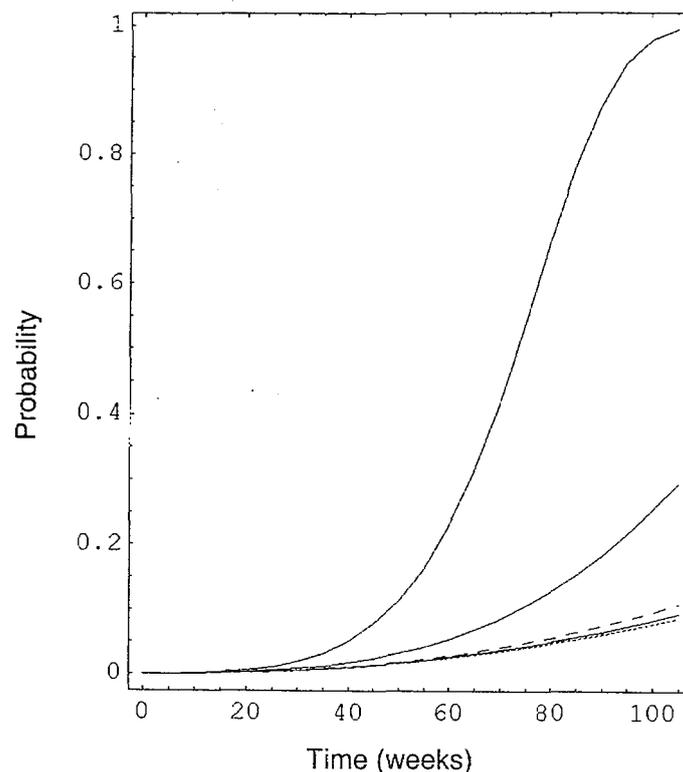


Putting all the model components together, i.e. cell growth rate, cell number, cell birth and death rates, liver sphinganine concentration, and rates of mutation, gave the predicted tumour probabilities shown in Figure 4. The plots reflect the fact that the MVK model is used to predict the tumour incidence over the entire interval between 0 and 104 weeks, even though the tumour incidences at 104 weeks were the only ones used to infer representative mutation rates.

The predicted incidences were 0.091, 0.084, 0.105, 0.284, and 0.992 at 0, 5, 15, 50, and 80 mg/kg of diet, respectively whereas the observed incidences (corrected for survival) were 0.117, 0.065, 0.021, 0.427, and 0.883 at 104 weeks. The predicted incidence at 5 mg/kg of diet is slightly lower than that at 0 mg/kg of diet, in keeping with the observed data, but the pronounced decrease at 15 mg/kg of diet in the observed data is not reproduced. Although there is both over- and under-prediction of dose-specific incidences, the predictions are reasonably representative of the observed values. It should be remembered that predictions were made for each dose group separately, i.e. there was no mathematical dose-response relationship. Clearly, the model predicts little or no risk for liver tumour development at low doses of fumonisin B<sub>1</sub> but a marked increase in risk at the higher doses.

In order to validate the predictions of tumour risk in females, the model was implemented a second time with data on the mechanism of action in males. Thus, liver and body weights, cell birth rates (PCNA), and sphinganine concentrations in the liver were used in the same way for males as was done for females. The predictions for males were 0.199, 0.201, 0.198, 0.233, and 0.237 at 0, 5, 15, 80, and 150 mg/kg of diet, respectively, whereas the observed values were 0.268, 0.211, 0.190, 0.213, and 0.213. The predictions are slightly higher at 80 and 150 mg/kg of diet than at 0, 5, and 15 mg/kg of diet, but they are all within a 4% range of the

**Figure 4. Probability of liver tumour occurrence over time in mice fed diets containing fumonisin B at various concentrations**



observed values and are consistent with a lack of a dose–response relationship. The value at 15 mg/kg of diet was actually slightly below that of controls. The prediction of little or no risk for males at low doses of fumonisin B<sub>1</sub> is the same as the prediction for females. The hypothesis of cell death and compensatory proliferation on which the model for females was based appears to be validated by the excellent agreement of the fitted and observed values for males.

Although the range of measured concentrations of sphinganine in liver was not markedly different in males and females, the cell birth and death rates were estimated to be much higher in females than in males on the basis of the observed PCNA and liver-weight data. Hence, elevated sphinganine levels at higher doses had a much greater effect on the tumour incidence in females than in males because of the magnitude of the difference in estimated cell birth and death rates that sphinganine was assumed to affect. Thus, the presence of a dose–response relationship for tumours in females and the absence of such a relationship in males appears to be due primarily to a difference in estimated cell turnover in the liver.

How fumonisin B<sub>1</sub> increases apoptosis by interfering with sphingolipid metabolism is not well understood (Riley et al., 1996; Merrill et al., 1997; Tsunoda et al., 1998). The MVK model assumes that sphinganine concentrations in target organs provide the best biomarker for this mechanism (DeLongchamp & Young, 2001), although other assumptions are equally plausible. The model is an oversimplification of the biological mechanism by which fumonisin B<sub>1</sub> causes liver tumours in mice. In fact, the assumption of a direct effect of sphinganine on the death rates only of preneoplastic cells is an inaccurate reflection of the apoptosis hypothesis. Fumonisin B<sub>1</sub> is believed to affect the death rates of normal cells also because of sphinganine build-up. The MVK model does not account for this directly. A more complex model could be considered, but increased complexity would be accompanied by increasing difficulty in resolving the model with the data. Direct measurements of the rates of apoptosis in both normal and preneoplastic cells of mice exposed to fumonisin B<sub>1</sub> would help resolve these issues. The model used here is a reasonable representation of the tumour incidence in mice of each sex. It is based on insertion of mechanistic data into a mathematical model of cancer development, which embodies a plausible hypothesis for how fumonisin B<sub>1</sub> increases the incidence of liver tumours.

#### *(b) Kidney cancer*

Dose–response models for tumour incidence can provide estimates of risk at the PMTDI which is based on effects in the kidney. These models can also provide a PMTDI for cancer for comparison with the PMTDI for other effects. A dose–response curve represents the cumulative probability of a tumour occurring on the basis of an underlying distribution of interindividual differences in susceptibility to a chemical.

For example, many biological effects are approximately described by a log-normal distribution (Mantel & Bryan, 1961). That is, the logarithm of doses that produce a biological effect is approximately normally distributed. The log-normal distribution is described by the mean (median) and standard deviation. Hence, estimates of the mean and standard deviation in bioassays provide the data necessary for estimating by how much the dose must be reduced below the NOEL to achieve a low level of cancer risk. Typically, a default safety factor of 10 is used to account for interindividual differences in susceptibility. Alternatively, the distribution of doses that cause tumours (or, equivalently, the resulting log-probit dose–response curve) can provide a safety factor for interindividual variation to replace the default factor of 10.

A log-normal distribution was estimated for renal tumours (adenomas and carcinomas) in male Fischer 344 rats observed in the study conducted for the National Toxicology Program (1999) in the USA. Two other commonly used models, the log-logistic and Weibull, were also fit to these data to indicate the effect of the choice of dose-response model (or equivalent underlying distribution of interindividual variation). All three dose-response models have shallow slopes at low doses, i.e. little increase in tumour incidence as the dose increases. At higher doses, these dose-response models curve upwards sharply as detoxication processes become saturated. Further, the log-logistic model is based on how a chemical affects the toxicokinetics of the formation and reduction of a chemically active agent, and the Weibull distribution is based on the assumption that multiple hits at a target site (cell) are required to affect the carcinogenic process.

As it is unlikely that a low tumour incidence would be observed in a sample of 48 animals, the zero incidence at the two lowest doses was replaced by a conservative Bayesian estimated incidence of  $1/2n$ , where  $n$  is the number of animals. Hence, an incidence of  $1/(2 \times 48) = 0.01$  was assigned to the group receiving 0.67 mg/kg bw per day. Since no renal tumours were induced at that dose, it was assumed that the animals would not have had tumours at 0.22 mg/kg bw per day. The estimated incidence for the 48 animals without tumours at this dose was  $1/(2 \times 96) = 0.005$ .

The estimated dose-response relationship for the log-probit model is:

$$P = \Phi(\ln \text{ median} = 2.114, \text{ standard deviation of } \ln d = 1.26)$$

where  $P$  is the estimated probability of tumour occurrence,  $d$  is dose expressed as mg/kg bw per day, and  $\Phi$  denotes the cumulative normal distribution.

The estimated dose-response curves for the other models are:

#### Log-logistic

$$P = 1/[1 + e^{-(3.258 + 1.686 \ln d)}]$$

#### Weibull

$$P = 1 - e^{-(0.0283 d(E)^{1.62})}$$

where  $d(E)^{1.62}$  is  $d$  raised to the 1.62 power.

The slope of the dose-response curve accounts for intraspecies variation in the sensitivity of rats to fumonisin. In addition, allowing for a safety factor of 10 for animal to human extrapolation, the cancer risks of individuals exposed for a lifetime at the PMTDI/10, PMTDI/2, PMTDI, and 2 x PMTDI, where the PMTDI = 0.002 mg/kg bw per day, for each of the models are presented in Table 31. Since the Bayesian procedure increased the tumour incidence at the two lower doses, the cancer risks at low doses are likely to be overestimated.

**Table 31. Conservative (probably over-) estimates of the cancer risk associated with lifetime exposure to fumonisin B<sub>1</sub> relative to the PMTDI of 0.002 mg/kg bw**

Model	PMTDI/10	PMTDI/2	PMTDI	2 X PMTDI
Log-probit	0	$4.8 \times 10^{-8}$	$0.9 \times 10^{-6}$	$1.2 \times 10^{-5}$
Log-logistic	$1.1 \times 10^{-6}$	$1.6 \times 10^{-5}$	$5.3 \times 10^{-5}$	$1.7 \times 10^{-4}$
Weibull	$1.2 \times 10^{-6}$	$1.6 \times 10^{-5}$	$5.0 \times 10^{-5}$	$1.5 \times 10^{-4}$

## 10. COMMENTS

### *Absorption, distribution, metabolism and excretion*

In all animal species studied, fumonisins are poorly absorbed from the digestive tract and are rapidly distributed and eliminated. The liver and kidney retain most of the absorbed material, and fumonisin B<sub>1</sub> persists longer in rat liver and kidney than in plasma. In pregnant rats and rabbits, very low concentrations of fumonisin B<sub>1</sub> were recovered in the uterus and placenta. No fumonisin B<sub>1</sub> was found in fetuses, indicating the absence of placental transfer. There was little evidence of significant transfer during lactation, and fumonisins do not appear to be metabolized *in vitro* or *in vivo*. Although fumonisins are not metabolized by cytochrome P450 enzymes, fumonisin B<sub>1</sub> can alter the activity of these enzymes through mechanisms that alter sphingolipid biosynthesis. Fumonisins are structurally related to sphingoid bases. Removal of the tricarballylic acid side-chains, presumably by the microbial flora of the gut, converts fumonisin B<sub>1</sub> into a substrate for ceramide synthase. The product of the enzyme reaction, like fumonisin B<sub>1</sub>, is an inhibitor of the enzyme *in vitro*.

### *Toxicological studies*

In all animal species studied, the liver was a target for fumonisin B<sub>1</sub>; the kidney was also a target in many species. In kidney, the early effects are often increases in free sphingoid bases, renal tubule-cell apoptosis, and cell regeneration. In the liver, apoptotic and oncotic necrosis, oval-cell proliferation, bile-duct hyperplasia, and regeneration are early signs of toxicity. In studies in rats and trout fed known cancer initiators and with various initiation and promotion protocols, purified fumonisin B<sub>1</sub> enhanced liver cancer development. Brief administration of high doses or longer administration of lower doses that cause significant hepatotoxicity resulted in the appearance of foci positive for glutathione-S-transferase (placental form), hepatocellular nodules, and other precursors of liver tumour development. In rodents, the toxicity of fumonisin B<sub>1</sub> was strain- and sex-dependent. For example, male BDIX rats appeared to be more sensitive to the hepatotoxic effects of fumonisin B<sub>1</sub> than male Fischer 344N, male Sprague-Dawley, and male RIVM:WU rats, in which nephrotoxicity was observed at lower doses than hepatotoxicity. In mice, the liver is more sensitive than the kidney to the toxicity of fumonisin B<sub>1</sub>. Female mice were more sensitive than males. In long-term feeding studies, purified fumonisin B<sub>1</sub> caused both liver and kidney tumours in rodents. The kidney carcinomas induced in male Fischer 344N rats by fumonisin B<sub>1</sub> were a highly malignant variant of renal tubule tumour, but the significance of their aggressive nature was unclear. The NOEL for renal cancer in Fischer 344N rats was 0.67 mg/kg bw per day (Table 32), and the NOEL for renal toxicity was 0.2 mg/kg bw per day (Table 33). The NOEL for liver cancer in male BD IX rats was 0.8 mg/kg bw per day, and the NOEL in feed-restricted female B6C3F<sub>1</sub> mice was 1.9 mg/kg bw per day.

Studies in rodents, non-human primates, and other animal species given *F. verticillioides* culture material from an isolate that produces predominantly fumonisin B<sub>1</sub> (isolate MRC 826) or maize naturally contaminated with fumonisins showed toxic effects in the liver and kidney that were similar to those in studies with purified fumonisin B<sub>1</sub>. Both MRC 826 and naturally contaminated maize caused liver tumours in rats at doses similar to those that caused liver tumours in rodents fed purified fumonisin B<sub>1</sub>. The NOEL for the renal and hepatic toxicity of all fumonisins in vervet

**Table 32. Dose-response relationship for renal toxicity and tumours in male Fischer 344N rats fed diets containing purified fumonisin B<sub>1</sub> for 2 years**

Dose of fumonisin B <sub>1</sub> (mg/kg bw per day)	No. of animals showing signs of renal toxicity and tumours		
	Cytotoxic or regenerative lesions	Atypical tubule hyperplasia	Renal tumours
Untreated controls	0/42	0/48	0/48
0.22	0/40	0/40	0/40
0.67	23/33	0/48	0/48
2.2	42/42	4/48	10/48
6.6	43/43	9/48	16/48

**Table 33. Dose-response relationship for renal toxicity in male Fischer 344N rats fed diets containing purified fumonisin B<sub>1</sub> for 90 days**

Dose of fumonisin B <sub>1</sub> (mg/kg bw per day)	No. of animals showing signs of renal toxicity
Untreated controls	0/10
0.1	0/10
0.2	0/10
0.6	9/10
1.9	10/10
5.7	10/10

monkeys fed a diet containing MRC 826 culture material was 0.11 mg/kg bw per day. Purified fumonisin B<sub>1</sub>, *F. verticillioides* culture material, and naturally contaminated maize all induced not only hepatic toxicity but also leukoencephalomalacia in equids and pulmonary oedema and hydrothorax in pigs. Both diseases appeared to occur secondarily to cardiovascular dysfunction. Cardiovascular effects have also been seen in other species. Field outbreaks of equine leukoencephalomalacia and porcine pulmonary oedema associated with consumption of fumonisin-contaminated maize have been reported in the USA and elsewhere. The NOEL for fumonisin B<sub>1</sub> in equine leukoencephalomalacia was equivalent to 0.3 mg/kg bw per day for animals fed diets containing *Fusarium* culture material. In pigs fed *Fusarium* culture material, evidence of pulmonary oedema was detected at a concentration of fumonisin B<sub>1</sub> equivalent to 0.4 mg/kg bw per day. For pigs fed naturally contaminated maize, the concentration of fumonisin B<sub>1</sub> required to induce pulmonary oedema was much higher, although the NOEL for liver toxicity was similar (equivalent to 0.2 mg/kg bw per day).

Several biochemical modes of action have been postulated to explain the induction by fumonisins of disease in animals. Two hypotheses involve disruption of lipid metabolism as the initial step. The first proposed mechanism involves disruption of sphingolipid metabolism through inhibition of ceramide synthase. The demon-

strated consequences of inhibition of this enzyme in liver and kidney are changes in all the major pools of sphingolipids, including increased concentrations of free sphingoid bases and free sphingoid-base metabolites and decreased biosynthesis of ceramide and other sphingolipids containing ceramide. Glycerophospholipid metabolism is also affected. Clear evidence of fumonisin-induced disruption of sphingolipid metabolism has been obtained in all target tissues except brain and in all species tested. The second proposed mechanism involves disruption of fatty acid and glycerophospholipid metabolism. Fumonisin-induced changes in fatty acid profiles and prostaglandins have been demonstrated *in vivo* in rat liver. These two proposed lipid-based mechanisms of action are similar in many respects with regard to their ultimate effects on cell physiology and are consistent with data obtained *in vitro*, in short-term studies of toxicity, and in long-term studies of carcinogenicity in rodents. Fumonisin also affect sites of cellular regulation that are apparently independent of the disruption of lipid metabolism, but cancer and the other toxic effects observed in animals appear to depend on disruption of various aspects of lipid metabolism, membrane structure, and signal transduction pathways mediated by lipid second messengers. The demonstrated cellular effects include altered cell proliferation, altered rates of apoptosis, altered intracellular communication and cell adhesion, induction of oxidative stress, and modulation of gene expression. Since the proposed biochemical mechanisms of action involve alterations in *de novo* biosynthetic pathways, nutritional factors could play an important role in determining the potency of fumonisin B<sub>1</sub> and the observed toxicological effects in rodents.

The available *in-vivo* observations are consistent with a proposed mode of action for fumonisin B<sub>1</sub>-induced toxicity that is dependent on perturbed lipid metabolism. The resulting increase in cell death coupled with regenerative cell proliferation, possibly by generation of oxidative damage, could in turn lead to increased incidences of tumours in target tissues. The primary evidence for sustained cell loss and regeneration is the observations of such effects in rat kidneys.

In a small number of studies *in vitro* and a single study *in vivo*, neither fumonisin B<sub>1</sub> nor any other fumonisin was shown unequivocally to be genotoxic. Similarly, no adducts of fumonisin with DNA have been found.

While there was evidence that fumonisins are embryotoxic *in vitro*, no published data exist to support the conclusion that fumonisins cause developmental or reproductive toxicity in farm animals. Except in one study in hamsters, embryotoxicity occurred in laboratory animals (rats, mice, and rabbits) secondarily to maternal toxicity.

Consumption of mouldy sorghum or maize containing fumonisin B<sub>1</sub> at up to 64 mg/kg was associated with an outbreak of human disease in India involving gastrointestinal symptoms. The grain was also reported to be contaminated with other toxigenic fungi.

The available evidence for an association between the intake of fumonisins and human cancer was limited to a few correlation studies. Typically, these involved a few regions in which populations were broadly classified with regard to their risk for oesophageal or liver cancer. The regions were then compared with respect to the proportions of contaminated samples and the level of contamination. In some studies, the measures of intake of fumonisins were indirect, and the incidence of disease was related to consumption of certain foods, notably maize. Taken together, the results of these studies could be interpreted as indicating an association between

fungal contamination of foodstuffs and oesophageal cancer or liver cancer. However, bias, chance, or confounding could not be excluded, and hence there was only limited evidence of an independent carcinogenic effect of fumonisins.

A specific role for fumonisins in the development of neural tube defects has been proposed. The hypothesis includes a critical role of fumonisins in disruption of folate membrane transport, but no specific studies have been designed or performed to confirm this mechanism.

#### *Analytical methods*

Two validated analytical methods based on liquid chromatography have been developed for fumonisins. The first method, based on strong anion exchange clean-up of a solvent extract, was validated for fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> in maize. Although this method has been used to determine fumonisins in maize-based foods, recovery from certain food matrices can be problematic. A second method with improved extraction efficiency and immunoaffinity column clean-up has been validated for fumonisins B<sub>1</sub> and B<sub>2</sub> in maize and cornflakes. Although methods for unequivocal detection based on liquid chromatography with mass spectrometric detection are also available, their high cost prohibits their routine use. Screening tests based on thin-layer chromatography and, for the combined B-series fumonisins, enzyme-linked immunosorbent assays, have also been developed. No methods specific for fumonisin B<sub>4</sub> have been described, and little is known about its occurrence, although the limited evidence suggests that it occurs at lower concentrations than fumonisin B<sub>1</sub>, B<sub>2</sub>, or B<sub>3</sub>, which were the subject of the present evaluation. The absence of a method to determine the concentration of fumonisin calibrant solutions remains a problem. In the laboratories that provided data on the natural occurrence of fumonisins in maize and maize-based foods for the current assessment, liquid chromatography was used predominantly, with solvent extraction, solid phase extraction clean-up, and quantification by pre-column formation of fluorescent *ortho*-phthaldialdehyde derivatives. The limits of detection were generally equal to or below 50 µg/kg and the analytical recovery greater than 70%.

#### *Sampling protocols*

The sampling variance in the testing of shelled maize for fumonisins was studied after collection of a large bulk sample and riffle-division into 1.1-kg test samples. At a batch contamination concentration of 2 mg/kg, the coefficients of variation were 17% associated with sampling (1.1-kg sample), 9.1% with sample preparation (Romer® mill and 25-g analytical portion), and 9.7% with analysis; they were independent of the fumonisin(s) tested (fumonisin B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, or all fumonisins). The coefficient of variation associated with the whole test procedure (sampling, sample preparation, and analysis) was 21%, which was of the same order of magnitude as that for the measurement of aflatoxin in shelled maize with a similar test procedure.

#### *Effects of processing*

The effects of various food processing procedures on the levels of fumonisin contamination have been studied. For example, maize screenings contain higher concentrations of fumonisins than whole grain. Separation and removal of screenings is a useful method for reducing the amount of fumonisins entering storage. Steeping

maize in aqueous solutions during wet milling results in extraction of fumonisins and is thus effective in reducing the concentration in maize products. Fumonisins are fairly heat-stable, and the toxin content is significantly reduced only during processes in which the temperature exceeds 150 °C. Dry milling of maize results in distribution of fumonisin into the various maize constituents. In wet milling, some fumonisin is extracted into the steeping water. There is little degradation of fumonisins during fermentation. Alkaline cooking and heating (nixtamalization), which result in the production of hydrolysis products, does not completely detoxify fumonisin-contaminated maize. In each process, many parameters affect the fate of the fumonisins. In addition, toxic compounds resulting from the conversion of fumonisins may appear during processing.

#### *Food consumption and dietary intake assessment*

Distributions of estimates of the intake of fumonisin B<sub>1</sub> around the world were based on the GEMS/Food regional diets and a published distribution of the concentrations of fumonisin B<sub>1</sub> in maize. Data that supported use of the published distribution were submitted by Argentina, Brazil, Canada, China, Denmark, Sweden, the United Kingdom, Uruguay, and the USA. The international estimates were made on the assumption that all maize consumed contains fumonisin B<sub>1</sub> at the concentration found in the unprocessed maize samples that were used to construct the distribution curve. While the mean concentration of fumonisin B<sub>1</sub> used was 1.4 mg/kg of unprocessed maize (median, 0.42 mg/kg), surveys of fumonisin B<sub>1</sub> over several years have shown that the median or mean concentration in maize varies greatly. The mean in sound maize in international trade in any given year could be expected to be between 0.2 and 2.5 mg/kg. Use of these concentrations of fumonisin B<sub>1</sub> with the maize intakes in the GEMS/Food regional diets would alter the mean of the expected distribution of intake of fumonisin B<sub>1</sub> by one-seventh to twice that reported here.

The estimated mean intake of fumonisin B<sub>1</sub> ranged from 0.2 µg/kg bw per day in the European-type diet to 2.4 µg/kg bw per day in the African diet (Table 34).

The Committee also considered published national estimates of fumonisin B<sub>1</sub> intake from Argentina, Canada, the Netherlands, Switzerland, and the USA. Additionally, fumonisin B<sub>1</sub> intake was estimated on the basis of consumption of food containing maize and associated fumonisin B<sub>1</sub> concentrations submitted by the United Kingdom. The estimates of national intake of fumonisin B<sub>1</sub> were lower than the international estimates presented here because they took into account the effects of processing, and because the national estimates were prepared from more specific

**Table 34. Estimated intake of fumonisin B<sub>1</sub> based on GEMS/Food regional diets**

	Intake (µg/kg bw per day)				
	Middle Eastern	Far Eastern	African	Latin American	European
Mean	1.1	0.7	2.4	1.0	0.2
90th percentile	3.3	2.1	7.3	2.9	0.6

data, i.e. the intakes of foods as consumed were considered rather than raw agricultural commodities. The mean estimates of national intake of fumonisin B<sub>1</sub> ranged from 0.02 µg/kg bw per day to 1.0 µg/kg bw per day (Table 35). These estimates also included certain assumptions that ensure conservatism, such as the assumption that all persons consume food containing fumonisins B<sub>1</sub> at the default concentration. Finally, the Committee noted that subsistence farmers, who grow and eat their own maize, might consume larger amounts of fumonisin B<sub>1</sub> than those reported here.

When they were quantified in the same sample, the ratio of fumonisin B<sub>1</sub>:B<sub>2</sub>:B<sub>3</sub> was approximately 10:3:1. To approximate the intake of all three fumonisins, therefore, the intake figures for fumonisin B<sub>1</sub> in this evaluation should be increased by 40%.

#### *Prevention and control*

The strategies for pre-harvest reduction of fumonisin contamination include agricultural practices, plant breeding, and genetic engineering. However, any breeding programme should take into account the growth conditions in specific regions to ensure full adaptation of the variety(ies) developed. The main means for preventing fumonisin contamination after harvest is immediate drying of the grain. Treatments with chemical preservatives before storage or by physical means, such as temperature reduction or modified atmospheres, during storage can also prevent fungal growth and subsequent mycotoxin formation.

## 11. EVALUATION

Nephrotoxicity, which was observed in several strains of rat, was the most sensitive toxic effect of pure fumonisin B<sub>1</sub>. Since the available studies clearly indicated that long-term renal toxicity is a prerequisite for renal carcinogenesis, the potential for the latter is subsumed by the dose–response relationship for renal toxicity. Therefore, the pivotal studies that could serve as the basis for a tolerable intake of fumonisin B<sub>1</sub> were the short-term and long-term studies of toxicity in rodents (see Tables 32 and 33). On the basis of these studies, the overall NOEL for renal toxicity was 0.2 mg/kg bw per day.

**Table 35. National estimates of intake of fumonisin B<sub>1</sub>**

Country	Intake (µg/kg bw per day)	
	Mean or median	90th percentile
Argentina	0.2	NR
Canada	0.02	0.08
Netherlands	0.06, 1.0 <sup>a</sup>	NR
Switzerland	0.03	NR
United Kingdom	0.03	0.1
USA	0.08	NR

NR, not reported or calculated

<sup>a</sup> The first value is for the whole population, the second for regular maize eaters.

The Committee allocated a group provisional maximum tolerable daily intake (PMTDI) for fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub>, alone or in combination, of 2 µg/kg bw per day on the basis of the NOEL of 0.2 mg/kg bw per day and a safety factor of 100. All of the estimates of intake of fumonisin B<sub>1</sub> based on the available data on national consumption were well below the group PMTDI. This remains true even when intake estimates for fumonisin B<sub>1</sub> are increased by 40% to account for the presence of fumonisins B<sub>2</sub> and B<sub>3</sub>.

The Committee was aware of an unpublished risk assessment in which the data on renal tumours had been used, and noted that the estimated risk was negligible at intakes below the group PMTDI established at the present meeting.

#### *Recommendations*

The Committee acknowledged the need for research in areas recommended in WHO Environmental Health Criteria (WHO, 1999). The Committee identified the following additional recommendations:

- As renal toxicity is the critical effect required for fumonisin-induced renal carcinogenesis in male rats, the tumour incidence in the male rat kidney should be modelled with biologically-based procedures.
- The biochemical and physiological mechanism(s) underlying the highly aggressive behaviour of fumonisin-induced renal tubular carcinomas in Fischer 344N rats should be investigated, including studies on the effects of fumonisin B<sub>1</sub> on the expression of cell adhesion molecules.
- The biochemical and physiological mechanism for the apparently different sensitivities of Fischer 344N and BDIX rats to fumonisin-induced liver toxicity should be investigated.
- Investigations should be conducted to determine whether dietary factors such as folate, vitamin E, and choline modify renal or hepatic toxicity induced by fumonisin B<sub>1</sub> in laboratory animals.
- The ability of fumonisin B<sub>1</sub> to alter the transport of folate at the cellular level and placental transport to the fetus in vivo should be investigated.
- Investigations should be conducted to determine the role of inhibition by fumonisins of ceramide biosynthesis in protection of cells from ceramide-mediated apoptosis induced by mitochondrial dysfunction.
- The relationship between the intake of fumonisin and human disease in areas where nixtamalized maize-products comprise a large portion of the diet should be investigated. Particular emphasis should be placed on diseases of the liver and kidney and other diseases suspected of being associated with the intake of fumonisin B<sub>1</sub>, such as nasopharyngeal and oesophageal cancers and neural tube defects.
- The ability of fumonisins to modify the expression of receptors for microbial pathogens and toxins that are associated with renal and hepatic disease in humans should be investigated.

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## Appendix A

Results of surveys for fumonisin B<sub>1</sub>, showing concentrations and distribution of contamination in food commodities

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b>											
<b>North America</b>											
Canada	Domestic	1993	98	No data	89	69/1800	< 200	3	0	Miller et al., 1995	Frequent small samples from grain handling equipment. Samples Pooled, mixed and 2.2-kg subsample ground
USA	Iowa State	1988	22	250	No data	2500/14 900	No data	No data	No data	Murphy et al., 1993; A = Ross et al., 1991	Random samples from trucks at grain elevators; 400-g subsample ground before analysis
		1989	44	250	No data	2900/37 900	No data	No data	No data		
		1990	59	250	No data	3300/19 100	No data	No data	No data		
		1991	50	250	No data	2900/15 800	No data	No data	No data		
		1992	80	250	No data	50/1600	No data	No data	0	Rice & Ross, 1994; L.G. Rice, P.F. Ross, USDA Veterinary Services Laboratory; P.A. Murphy, Iowa State University	
		1993	43	250	No data	340/1900	No data	No data	0		
		1994	37	250	No data	240/2200	No data	No data	No data		
		1995	85	250	No data	440/6500	No data	No data	No data		
		1996	93	250	No data	130/3300	No data	No data	No data		
<b>South and Central America</b>											
Argentina	Domestic	Apr–Nov 1998	34	8	20	104/744	499	0	0	GEMS/Food programme	Statistically based and representative for part of country
	Domestic	Jan–Oct 1999	186	8	2	3299/15 560	8558	132	89		
	Domestic	May–Jun 1999	66	8	17	840/9377	1940	10	6		
	Domestic	Jan–Aug 2000	56	8	10	545/4982	1647	9	3		

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b> (contd)											
Argentina (contd)	Rice, imported	Nov–Dec 1999	5	8	1	60/229	No data	0	0	GEMS/Food programme	Not statistically based, samples from whole country
	Rice, husked, domestic	Apr–Jun 1999	6	8	6	—/—	—	—	—	GEMS/Food programme	Not statistically based, samples from part of country
	Rice, polished, domestic	Apr–Jun 1999	5	8	5	—/—	—	—	—		
Brazil	Domestic	1997–98	110	20*	0	No data/44	No data			GEMS/Food programme	Statistically based, repre- sentative for part of country
	Domestic	1994-95	105	20*	0	No data/6.6	No data				
	Domestic	No data	150	No data	No data	4.6/13(?)	No data				
	Domestic	4/1995– 4/1996	150	0.05(?)	1	580/13 460	No data				
	Domestic	4/1995– 4/1996	150	0.093(?)	3	1140/22600	No data				
	Domestic	1998	214	100*	2	No data/6000	No data	172	48>3000	Preis & Vargas, 2000; A = Trucksess et al., 1995; Thiel et al., 1993	Maize collected in vari- ous regions
	Domestic	1990–91	48	No data	1	5380/18 520	8750	46	42	Hirooka et al., 1996; A = Shephard et al., 1990; Ueno et al., 1993; mixed, reduced to 2 kg, S = Schmitt & Hurburgh, and ground 1989	Samples collected from silos and warehouses,

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b> (contd)											
Colombia	Domestic 1999	Feb–Aug	15	20*	7	259/2170	No data	No data	No data	Perilla & Diaz, 1998; A = Sydenham et al., 1996c; Patel et al., 1997; S = Campbell et al., 1986; Park & Pohland, 1989	Samples (minimum, 500 g) purchased at random from most popular retail outlets, ground and sub- sampled in grinding–sub- sampling mill (Romer series II)
Costa Rica	Domestic	1992–93	64	No data	4	2650/6320	5350	58	39	Viquez et al., 1996; A = Shephard et al., 1990	Sampling by agricultural agency: representative daily samples (5 kg) reduced to 8-kg sub- sample/week, which was milled and divided to yield 2 kg/week; over 3 weeks, 6-kg sample reduced to 1 kg
Honduras	Domestic 11/1993	10/1992, 11/1993	23	No data	0	1357/6555	2248	11	6	Julian et al., 1995; A = Sydenham et al., 1992b	Samples collected by systematic sampling of ears in field (2 transects/ field, ears taken at 10-m intervals (total, 25 ears per sample) or from farm stores (25 ears from different areas of store); samples ground, mixed in vane mixer, and test portions taken

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize (contd)</b>											
Uruguay	Maize kernels	1995–96	22	50*	11	938/5787	3025	7	3	Pineiro et al., 1997; A = Scott & Lawrence, 1996; Sydenham et al., 1996c	Samples purchased from local shops; 1-kg sub- samples ground and mixed well
Venezuela	Domestic	Oct 1993	37	10*	6	1459/15 050	2626	11	5	Medina-Martinez & Martinez, 2000; A = Stack & Eppley, 1992; S = COVENIN, 1982	Yellow maize collected as single random pur- chases from retail outlets in Caracas. White maize withdrawn from trucks at grain elevators; sub- samples prepared by grinding in a disk mill (Quaker model 4E)
<b>Europe</b>											
Croatia	(Fumonisin B <sub>1</sub> and B <sub>2</sub> )										
	Domestic	1996	105	10*	3	627/11661	No data	7	5>5000	Jurjevic et al., 1999	Samples (whole ear, 1.5– 2 kg) collected randomly from several farms in 14 counties. 270–320 g stored and ground before analysis
	Domestic	1997	104	10*	7	125/2524	No data	1	0>5000		
Hungary	Non-mouldy		28	50*	26	7/151	No data	0	0	Fazekas et al., 1996;	Storage samples from private producers and feed mixing plants: har- vested autumn 1993; collected 12/93–2/94
	Mouldy		24	50*	7	1842/19 800	No data	No data	No data	A = Shephard et al., 1990	

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b> (contd)											
Hungary (contd)	Random	1994	23	50*	16	463/5100	No data	No data	No data	Fazekas et al., 1996; A = Shephard et al., 1990	Harvest samples: ran- dom samples of 50 ears taken on each maize field
	Mouldy	1994	23	50*	7	4619/52 400	No data	No data	No data		
Italy	Visibly mouldy	1994	22	5000*	2	67 000/ 300 000	180 000	No data	No data	Ritieni et al., 1997; A = Shephard et al., 1990	Ears collected before harvest from different fields
Netherlands	Imported	1994–96	62	25	1	620/3350	1218	11	3	de Nijs et al., 1998b; A = Shephard et al., 1990	Kernels obtained from bulk ship loads or trailers; samples ground before analysis
Spain	Domestic	1994–96	55	60*	7	4200/19 200	No data	No data	No data	Castella et al., 1999; A = Sydenham et al., 1992a	Samples (1 kg represen- tative) obtained from agricultural cooperatives and factories; sub- sampled into 200-g ali- quot for analysis
Sweden	Imported	Jan–Dec 1996	42	5	23	45/393	159	No data	No data	GEMS/Food programme	Statistically based, repre- sentative for part of country

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize (contd)</b>											
United Kingdom	(Data corrected for analytical recovery) Imported	6/1998– 4/1999	139	20	5	795/3406	No data	39	0>5000	Scudamore & Patel, 2000; A=Shephard et al., 1990 Food Standards Agency, 2000	Manchester: 20 incremental samples (0.5 kg) taken from conveyer going to mill over 2 h and bulked. Merseyside: 100 incremental samples (0.3 kg) taken manually from 25 points in selected holds during discharge, bulked, mixed, and 10-kg subsample analysed
	Imported	6/1998– 4/1999	All fumonisins 139	20	5	1160/5007	No data	48	1>5000	Scudamore & Patel, 2000; A=Shephard et al., 1990 Food Standards Agency, 2000	Mill 1: On each occasion, 20 (14–27) consecutive lorries (0.5 kg) sampled with probe, combined to 10-kg analytical sample Mill 2: Every third lorry sampled (1.5-kg tailgate sample, of which 0.5 kg supplied); 30 samples bulked to give 15-kg analytical sample Mill 3: 1–2 kg snatch samples from conveyer 4 times/day for 5 days; 0.5 kg from each to give 10 kg for analysis All samples ground and mixed in horizontal mixer
			Distribution: 30–100: 25; 101–500: 42; 501–1000: 28; 1001–5000: 39								
			Distribution: 30–100: 14; 101–500: 41; 501–1000: 30; 1001–5000: 48; > 5000: 1								

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b> (contd)											
<b>Africa</b>											
Botswana	Domestic	1/1996– 12/1997	33	20*	5	210/1270	No data	No data	0	Siame et al., 1998; A = Doko et al., 1995	Samples (2–5 kg) collected from storage depots or purchased from retail outlets; ground and mixed before aliquots taken for analysis
Burundi	Domestic	No data	6	No data	0	12 200-75 200	No data	No data	No data	Munimbazi & Bullerman, 1996; A = Rice & Ross, 1994	Samples collected from markets
Kenya	Smallholder farms	1996	197	100*	104	316/12 000	No data	10	No data	Kedera et al., 1999; Sydenham et al., 1992b	Shelled maize kernels (0.5–1.0 kg) collected from randomly selected farms. 50-g sample ground, 5 g extracted
Kenya	Kernels	1994	1	20*	0	780/780	No data	0	0	Doko et al., 1996; A = Sydenham et al., 1992b; Doko et al., 1995	Samples (1–5 kg) drawn from sample lots, ground, and mixed before aliquots taken for analysis
Malawi			8	20*	1	59/115	No data	0	0		
Mozambique			3	20*	0	260/295	No data	0	0		
South Africa	White maize	1994–95	143	20*	No data	637/12 963	No data	No data	No data	Rava, 1996; A = Sydenham et al., 1992b	Samples collected from mills throughout country and ground before analysis
	Yellow maize	1994–95	148	20*	No data	664/5062	No data	No data	No data		

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b> (contd)											
South Africa	White grade 1	1993	No data	20*	No data	329/5637	No data	No data	No data	Rava et al., 1996; A = Sydenham et al., 1992b	Representative samples (3 kg) collected at harvest from silos in main production zones; 500-g sample obtained from riffle splitter and ground
	White grade 2	1993	No data	20*	No data	311/2078	No data	No data	No data		
	White grade 3	1993	No data	20*	No data	161/1128	No data	No data	0		
	Yellow grade 1	1993	No data	20*	No data	589/11773	No data	No data	No data		
	Yellow grade 2	1993	No data	20*	No data	767/4991	No data	No data	No data		
	Yellow grade 3	1993	No data	20*	No data	849/5629	No data	No data	No data		
Tanzania	Kernels	1994	9	20*	1	71/165	No data	0	0	Doko et al., 1996; A = Sydenham et al., 1992b; Doko et al., 1995	Samples (1–5 kg) drawn from sample lots, ground, and mixed before aliquots taken for analysis
Uganda			1	20*	0	605/605	No data	0	0		
Zimbabwe			2	20*	1	63/125	No data	0	0		
<b>Asia</b>											
China	Maize	Spring 1996	177	10	83	8880/78 370	25 260	91	77>5000	China	No data
	Rice		155	10	130	1410/31830	5990	25	17>5000		
China Linq County, Shandong Province		1996	16	500*	14	288/2400	1100	2	2	Groves et al., 1999; A = Rice et al., 1995	Random selection of households (3) in random selection of villages (7); samples frozen and 5-g portions cut for analysis

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b> (contd)											
China	(Total fumonisins by ELISA)										
Linxian & Anyang counties, Henan Province & Cixian County, Hebei Province		Autumn 1995	164	500*	58	700/16000	No data	No data	No data	Zhang et al., 1997	Samples collected from farmers 10/1995–3/1996 and milled
Fanxian & Yanqing counties		Autumn 1995	82	500*	59	200/1500	No data	No data	0		
Linxian County, Henan Province		1994	34	No data	7	2168/21000	No data	No data	No data	Gao & Yoshizawa, 1997; A = Yoshizawa et al., 1994	Samples from 1994 harvest collected from peasant families during Jan–Feb 1995
Shangqiu County, Henan Province		1994	20	No data	10	1351/8470	No data	No data	No data		
Haimen, Jiangsu County		Apr–Jul 1993	40	50*	3	4727/25 970	11 074	26	20	Ueno et al., 1997; A = Shephard et al., 1990; Ueno et al., 1993	Kernels collected rand- omly from agricultural stocks. 25-g sample milled and 5 g extracted
Penlai, Shandong Province		Apr–Jul 1993	40	50*	24	260/3190	704	4	1		

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b> (contd)											
India	Domestic	1995	6	No data	No data	Range, 50–240	No data	0	0	Bhat et al., 1997; A = Stack & Eppley, 1992	Samples (0.25–5 kg) collected from house- holds
	Domestic	No data	35	10*	9	Geometric mean of posi- tives, 620/4740	No data	9	7	Shetty & Bhat, 1997; A = Stack & Eppley, 1992	Samples collected from households and retail shops
				Distribution: < 500: 21; 500–1000: 5; 1000–1500: 1; 1500–2000: 1; > 2000: 7							
Indonesia	Domestic	Nov 1995	16	50*	0	788/2440	1450	6	1	Ali et al., 1998; A = Yoshizawa et al., 1994, 1996	Samples ground and aliquots taken from 200 g
	Domestic	1992–94	12	50*	5	492/1780	No data	No data	No data	Yamashita et al., 1995; A = Yoshizawa et al., 1994	Samples collected at random from stores of wholesalers, retailers, university farms, and local farmers; ground and stored
Iran	Mazandaran	Sept 1998	11	10*	0	2269/3980	3360	11	4	Shephard et al., 2000; A = Sydenham et al., 1996c	Farmers' maize lots col- lected at random from consignments sold to Iranian Agriculture Office; total sample ground

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b> (contd)											
Iran	Isfahan	Oct 1998	8	10*	0	169/590	559	0	0	Shephard et al., 2000; A = Sydenham et al., 1996c	Maize ears bought at different periods from local retail market; total sample ground
Korea, Republic of	Mouldy	Nov 1997	36	50*	3	21 300/ 168 800	No data	No data	No data	Sohn et al., 1999; A = Xie et al., 1997	Samples collected from households and milled
	Healthy	Nov 1997	35	50*	25	910/12 500	No data	No data	No data		
Nepal	(Total fumonisins by HPLC) Domestic Feb–Jul 1997		58	100*	6	493/8400	No data	No data	No data	Desjardin et al., 2000	Samples (0.25–0.5 kg) collected from farms and markets in 10 districts; 100 g ground and 10 g extracted
Philippines	(Total fumonisins by ELISA) Domestic Jun–Nov 1995		10	200*	1	2000/10 000	No data	No data	No data	Bryden et al., 1998	Samples (1 kg) collected from major storage sites, subsampling, and ground
	Domestic	1992–94	50	50*	24	218/1820	No data	No data	0	Yamashita et al., 1995; A = Yoshizawa et al., 1994	Samples collected at random from stores of wholesalers, retailers, university farms, and local farmers; ground and stored

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b> (contd)											
Philippines	Seed maize	1992–94	24	50*	14	148/970	No data	0	0	Yoshizawa & Yamashita, 1995; A = Shephard et al., 1990	At least 100 g collected, 50 g ground, and 20 g extracted
	Feed maize	1992-1994	22	50*	7	320/1820	No data	No data	0		
	Food maize	1992-1994	23	50*	16	41/268	No data	0	0		
Taiwan	Domestic	1996–97	110	40*	61	79/1148	No data	No data	0	Tseng & Liu, 1999; A = Shephard et al., 1990	Samples (1 kg) collected from 8 districts; sub-samples (200 g) ground and 25 g extracted
				Distribution: < 100: 87; 101–200: 16; 201–300: 4; > 300: 3							
Thailand	Visibly mouldy human food	1992	5	50*	1	4277/18800	No data	3	1	Yoshizawa et al., 1996; A = Shephard et al., 1990	Samples collected before harvest; random 100-g aliquots analysed; 50 g milled and 20-g sub-sample extracted
	Domestic	1992–94	27	50*	8	1112/18 800	No data	No data	No data	Yamashita et al., 1995; A = Yoshizawa et al., 1994	Samples collected at random from stores of wholesalers, retailers, university farms, and local farmers; ground and stored
Viet Nam	(Total fumonisins by ELISA) Domestic	Jun–Nov 1995	12	200*	0	6600/9100	No data	No data	No data	Bryden et al., 1998	Samples (1 kg) collected from major storage sites, subsampled, and ground

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b> (contd)											
Viet Nam	Maize, feed	1993	15	50*	7	587/3447	1506	4	1	Wang et al., 1995; A =	Random samples from various locations. 25 g milled, and 5 g extracted
	Maize powder, feed	1993	17	50*	2	688/1516	1179	4	0	Ueno et al., 1993	
<b>Australia</b>											
Australia	(Total fumonisins by ELISA) Domestic	Jun–Nov 1995	70	200*	3	6700/40 600	No data	No data	No data	Bryden et al., 1998	Samples (1 kg) collected from major storage sites, subsampled, and ground
<b>Processed maize-based human food</b>											
<b>North America</b>											
Canada	(* number and mean of positive samples)										
	Maize kernel, 1996–97 meal, flour		21*	No data	No data	190*/850	No data	0	0	Canada	No data
	Maize snacks (alkali-processed)		20*	No data	No data	203*/970	No data	0	0		No data
	Maize snacks (non-alkali-processed)		6*	No data	No data	65*/100	No data	0	0		No data
	Canned		9*	No data	No data	48*/90	No data	0	0		No data
	Frozen		2*	No data	No data	35*/40	No data	0	0		No data
	Maize bread		1*	No data	No data	30*/30	No data	0	0		No data
	Maize-based breakfast cereals		8*	No data	No data	80*/170	No data	0	0		No data
	Fresh tortillas		1*	No data	No data	750*/750	No data	0	0		No data

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food</b> (contd)											
Canada (contd)	Maize-based infant foods:	1997–98								Canada	
	Mixed cereals		16*	No data	No data	26*/110	No data	0	0		No data
	Cereal with formula		6*	No data	No data	<10*/<10	No data	0	0		No data
	Creamed maize or maize / vegetables		6*	No data	No data	<10*/<10	No data	0	0		No data
	Infant cereals:	1998–99								Canada	
	Mixed cereals with fruit / formula		13*	No data	No data	<10*/<10	No data	0	0		No data
	Rice-based		16*	No data	No data	<10*/<10	No data	0	0		No data
	Soya-based		14*	No data	No data	20*/60	No data	0	0		No data
	Mixed cereals		20*	No data	No data	20*/70	No data	0	0		No data
	Tortilla, nacho, maize chips, taco shells	1996	17	20*	12	23/216	65	0	0	Scott & Lawrence, 1996	Samples purchased from retail outlets, mostly in Ottawa; sample sizes 125–900 g; ground
	Hydrolysed fumonisin B <sub>1</sub>		17	10*	16	2/40	<10	0	0		
	Maize tortilla, dried		10	20*	2	202/612	425	0	0		
	Hydrolysed fumonisin B <sub>1</sub>		10	10*	5	20/60	51	0	0		
USA	Maize meal, degermed	1997	602	100*	278	130/1080	300	1	0	USA	Sampled 1998
	Maize meal, partly degermed		20	100*	6	230/1130	550	1	0		
	Maize meal, whole grain		50	100*	5	910/4820	2852	10	6		
	Maize meal, degermed	1998	561	100*	439	130/7010	260	21	8	USA	Sampled 1999

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
USA (contd)	Maize meal, 1998 partly degermed		20	100*	6	730/3030	2050	5	2	USA	Sampled 1999
	Maize meal, whole grain		39	100*	2	950/5500	2414	11	5		
	Canned maize	Spring 1993	70	25	42	11/235	No data	0	0	Trucksess et al., 1995	Samples collected by 10 FDA district offices from retail establishments representing, where possible, different manu- facturers and distributors
	Frozen maize		27	25	18	18/350	No data	0	0		
	Maize meal	No data	10	75*	<75–5916	No data	No data	No data	No data	Castelo et al., 1998b; A = Rice et al., 1995	Samples purchased in Maryland, Nebraska, and Arizona; ground before analysis
	Muffin mix	No data	6	75*	<75–417	No data	0	0	0		
	Maize bread	No data	4	75*	<75–1020	No data	No data	0	0		
	Tortilla chips	No data	19	75*	<75–1565	No data	No data	0	0		
	Maize tortilla	No data	5	75*	<75–330	No data	0	0	0		
	Cornflakes	No data	6	75*	<75–88	No data	0	0	0		
	Maize starch	No data	1	75*	<75	No data	0	0	0		
	Popcorn	No data	1	75*	<75	No data	0	0	0		
	Infant foods	No data	2	75*	<75	No data	0	0	0		
	(Total fumonisins by HPLC)										
	Maize, shelled	1994	41	20*	6	179/1100	No data	No data	0	USA	No data
		1995	78	20*	12	406/4400	No data	No data	No data		No data
		1996	76	20*	32	562/14900	No data	No data	No data		No data
		1997	43	20*	6	516/5100	No data	No data	No data		No data
		1998	134	20*	85	497/7300	No data	No data	No data		No data
	Maize meal	1994	39	20*	2	266/1300	No data	No data	0		No data
		1995	64	20*	14	188/2500	No data	No data	No data		No data

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
USA (contd)	Maize meal	1996	35	20*	28	142/2700	No data	No data	No data	USA	No data
		1997	40	20*	9	295/1800	No data	No data	0		No data
		1998	69	20*	45	177/1600	No data	No data	0		No data
	Maize flour	1994	18	20*	2	178/630	No data	0	0	USA	No data
		1995	15	20*	7	37/190	No data	0	0		No data
		1996	17	20*	13	80/860	No data	0	0		No data
		1997	19	20*	1	332/1300	No data	No data	0		No data
	Maize grits	1998	15	20*	13	113/1300	No data	No data	0		No data
		1994	8	20*	4	60/231	No data	0	0	USA	No data
		1995	6	20*	2	60/215	No data	0	0		No data
		1996	6	20*	5	17/100	No data	0	0		No data
		1997	9	20*	3	127/300	No data	0	0		No data
	Hominy grits	1998	5	20*	5	—/—	No data	—	—		No data
		1994	0	20*	—	—/—	No data	—	—	USA	No data
		1995	7	20*	6	9/60	No data	0	0		No data
		1996	2	20*	1	110/220	No data	0	0		No data
		1997	3	20*	1	333/600	No data	0	0		No data
	Maize chips, tortilla	1998	10	20*	10	—/—	No data	—	—		No data
		1994	17	20*	13	19/164	No data	0	0	USA	No data
		1995	6	20*	5	8/50	No data	0	0		No data
		1996	5	20*	5	—/—	No data	—	—		No data
		1997	27	20*	19	83/740	No data	0	0		No data
	Maize muffin mix	1998	32	20*	30	33/1000	No data	No data	0		No data
		1994	5	20*	5	—/—	No data	—	—	USA	No data
1995		8	20*	6	15/93	No data	0	0		No data	
1996		3	20*	3	—/—	No data	—	—		No data	
1997		6	20*	4	33/100	No data	0	0		No data	
		1998	16	20*	15	25/400	No data	0	0	USA	No data

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
USA (contd)											
	Cornflakes, dry cereals	1994	9	20*	9	—/—	No data	--	—	USA	No data
		1995	4	20*	4	—/—	No data	—	—		No data
		1996	12	20*	12	—/—	No data	—	—		No data
		1997	15	20*	14	2/30	No data	0	0		No data
		1998	27	20*	27	—/—	No data	—	—		No data
	Starch	1994	5	20*	5	—/—	No data	—	—	USA	No data
		1995	5	20*	4	3/13	No data	0	0		No data
		1996	3	20*	3	—/—	No data	—	—		No data
		1997	9	20*	9	—/—	No data	—	—		No data
		1998	9	20*	9	—/—	No data	—	—		No data
	Popcorn	1994	17	20*	6	71/280	No data	0	0	USA	No data
		1995	15	20*	10	17/90	No data	0	0		No data
		1996	14	20*	12	49/570	No data	0	0		No data
		1997	11	20*	4	235/1800	No data	No data	0		No data
		1998	25	20*	22	47/960	No data	0	0		No data
	Tortillas	No data	52	10*	2	187/750	326	0	0	Stack, 1998; A = Rice et al., 1995	Samples from Texas— Mexico border
		Hydrolysed fumonisin B <sub>1</sub>		10*	4	82/204	104	0	0		
	Masa	No data	8	10*	0	262/689	418	0	0		
		Hydrolysed fumonisin B <sub>1</sub>		10*	2	64/178	127	0	0		
	Starch	No data	4	10*	2	158/335	No data	0	0	Maragos et al., 1997;	No data
	Grits	No data	4	10*	1	4889/10110	No data	No data	No data	A = Maragos & Richard, 1994	
	Masa	No data	2	10*	1	55/110	No data	0	0		
	Popcorn	Feb-May 1993/	18	40*	17	4/69	<40	0	0	Rumbeiha & Oehme, 1997; A = Sydenham et al., 1992b	Purchased in Manhattan, Kansas; 500 g blended and 25 g extracted

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
USA (contd)	Breakfast cereals	Feb–May 1993	16	40*	16	—/—	—	—	—	Rumbeiha & Oehme, 1997; A = Sydenham et al., 1992b	Purchased in Manhattan, Kansas; 500 g blended and 25 g extracted
	Snacks		40	40*	40	—/—	—	—	—		
	Maize flour		25	40*	12	82/349	168	—	—		
	Tortilla shells		5	40*	5	—/—	—	—	—		
	Sweet maize		8	40*	8	—/—	—	—	—		
	Maize oil		4	40*	4	—/—	—	—	—		
	Total fumonisins	1990–94				0–250	251-500	501-1000	>1000	Pohland, 1996	No data
	Canned maize		70	No data	42	28					
	Frozen maize		27	No data	18	8	1				
	Maize bran cereal		7	No data	0	4	3				
	Maize bread mix		13	No data	2	3	1	3	4		
	Maize cereal		3	No data	3						
	Maize chips		8	No data	7	1					
	Cornflakes		13	No data	11	2					
	Maize grits		21	No data	4	4	9	4			
	Maize hominy		1	No data	0	1					
	Maize meal		98	No data	2	34	15	19	28		
	Maize muffin mix		9	No data	5	2	1	1			
	Maize pops cereal		5	No data	5						
	Maize tortillas			15	No data	9	6				

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
USA (contd)	Maize tortilla chips	1990-94	2	No data	1	1				Pohland, 1996	No data
	Shelled maize			41	No data	6	19	8	7	1	
	Maize starch		5	No data	5						
	Fibre cereal		3	No data	3						
	Popcorn		20	No data	6	13	1				
	Spoon bread mix		1	No data	0			1			
<b>South and Central America</b>											
Argentina	Maize flour	Apr-Dec 1999	11	8	0	188/540	540	0	0	GEMS/Food programme	Statistically based and representative for part of country
	Popcorn	May-Jun 1999/	42	8	1	1084/14241	4311	6	4		
	Maize flour	10/1996-1/1997	15	11*	1	341/1860	638	1	0	Hennigen et al., 2000; A = Sydenham et al., 1996c	Samples (1 kg) purchased from food shops and supermarkets in Buenos Aires, ground and well mixed by hand before analysis
	Maize grits	10/1996-1/1997	4	11*	2	147/494	373	0	0		
	Maize flour	Jan 1998	14	11*	0	882/4987	1435	5	1		
	Maize grits	Jan 1998	2	11*	0	577/832	No data	0	0		
	Maize meal	1997	21	1.5*	2	503/2860	1357	2	0	Solovey et al., 1999; A = Sydenham et al., 1996c	Randomly purchased from commercial outlets; total sample ground
	Cornflakes	1997	17	1.5*	1	11/38	32	0	0		
	Maize meal:	Distribution of total fumonisins: 0-500: 14; 501-1000: 2; 1001-1500: 3; >1500: 2									

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure	
<b>Processed maize-based human food (contd)</b>												
Brazil	Canned sweet maize	Mar–May 1999	11	20*	9	10/80	No data	0	0	Machinski & Valente Soares, 2000; A = Shephard et al., 1990	Samples (minimum 500 g) purchased from retail stores in Campinas and ground, homogenized, or both	
	Cornflakes		4	20*	3	170/660	No data	0	0			
	Maize flour		11	20*	2	610/1460	No data	No data	0			
	Maize flour, baby cereal		2	20*	1	220/440	No data	0	0			
	Maize grits		2	20*	0	700/1230	No data	No data	0			
	Maize meal		9	20*	0	2290/4930	No data	No data	No data			
	Corn-on-the-cob			7	20*	7	—/—	—	—			—
	Curau		2	20*	2	—/—	—	—	—			—
	Maize, degermed		11	20*	3	840/4520	No data	No data	No data			
	Pamonha		7	20*	7	—/—	—	—	—			—
	Popcorn		1	9	20*	5	330/1720	No data	No data			0
	Pre-cooked maize meal		6	20*	2	840/1790	No data	No data	0			—
	Colombia	Pre-cooked maize meal	Feb–Aug 1998	15	20*	2	102/230	No data	0			0
Popcorn			8	20*	4	84/246	No data	0	0			
Maize meal			7	20*	3	123/408	No data	0	0			
“Arepas”			6	20*	4	18/61	No data	0	0			
Maize snacks			6	20*	4	25/127	No data	0	0			
Maize starch		3	20*	3	—/—	—	—	—	—			
Guatemala	Tortillas	Aug–Sep 1995								Meredith et al., 1999	Samples collected from households, frozen, freeze-dried, and ground; 5–10 g extracted	
	Santa Maria de Jesus	FumonisinB <sub>1</sub> 38 Hydrolysed fumonisinB <sub>1</sub>	38	400*	31	850/6500	No data	No data	No data			
			38	400*	12	26 100/185 100	No data	No data	No data			

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
Guatemala (contd)	Tortillas	Aug–Sep 1995								Meredith et al., 1999	Samples collected from households, frozen, freeze-dried, and ground; 5–10 g extracted
	Patzicia	FumonisinB <sub>1</sub> , Hydrolysed	35 35	400* 400*	24 24	2200/11 600 5700/31 700	No data No data	No data No data	No data No data		
	Nixtamal										
	Santa Maria de Jesus	FumonisinB <sub>1</sub> , Hydrolysed	46 46	400* 400*	0 46	13600/77200 —/—	No data —	No data —	No data —		
	Patzicia	FumonisinB <sub>1</sub> , Hydrolysed	50 50	400* 400*	33 50	4400(?) / 5000 —/—	No data —	No data —	No data —		
Mexico	Tortillas	No data Hydrolysed	7 7	10* 10*	0 2	601/1070 16/50	No data No data	1 0	0 0	Dombrink-Kurtzman & Dvorak, 1999; A = Sydenham et al., 1995; Bennett & Richard, 1994	No data
	Masa	No data Hydrolysed	2 2	10* 10*	0 1	1195/1800 50/100	No data No data	1 0	0 0		
Uruguay	Polenta	1/1995– 4/1996	8	50*	5	101/427	322	0	0	Pineiro et al., 1997; A = Scott & Lawrence, 1996; Sydenham et al., 1996c	Samples purchased from local shops; 1-kg subsamples ground and mixed well
	Maize starch		4	50*	4	—/—	—	—	—		
	Popcorn		2	50*	1	100/199	No data	0	0		
	Snacks		5	50*	3	93/314	249	0	0		
	Breakfast cereal		3	50*	2	73/218	No data	0	0		
	Canned maize		4	50*	4	—/—	—	—	—		
	Frozen maize		2	50*	50*	1	78/155	No data	0		
	Maize grits		1	50*	1	—/—	—	—	—		

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
<b>Europe</b>											
(Total fumonisins by ELISA; * mean calculated with samples <LOQ taken as 4.5 µg/kg)											
Czech Republic	Maize-extruded bread	Autumn 1995–	35	9	5	270*/1808	910	No data	0	Ostry & Ruprich, 1998	Samples purchased in shopping network in 13 areas
	Maize-extruded products	Spring 1996	26	9	0	301*/1178	1062	No data	0		
	Maize flour		22	9	0	187*/487	355	0	0		
	Maize instant porridge	19	9	1	124*/788	273	0	0	0		
	Maize paste		11	9	5	75*/511	111	0	0		
	Polenta		7	9	1	559*/1243	1221	No data	0		
Denmark	Cornflakes	1996	10	1*	4	110/1030	No data	No data	No data	Petersen, 2000; A =	Samples purchased in retail shops, homogenised in a mixer before analysis
	Maize flour		8	1*	2	33/86	No data	No data	No data	Sydenham et al.,	
	Maize grits		4	1*	3	2/7	No data	0	0	1992b	
	Maize starch		6	1*	6	0/0	No data	0	0		
	Corn-on-the-cob		4	1*	4	0/0	No data	0	0		
	Maize snacks	10	1*	4	16/65	No data	0	0	0		
	Polenta		1	1*	0	84/84	No data	0	0		
	Popcorn		9	1*	5	54/474	No data	0	0		
	Sweet maize	16	1*	16	0/0	—	0	0	0		
Germany	Maize grits	No data	6	0.8	0	92/202	No data	0	0	Lukacs et al., 1996;	Samples from market
	Maize meal		2	0.8	0	86/138	No data	0	0	A = Zoller et al., 1994	
	Sweet maize	6	0.8	6	—/—	—	—	—	—		
	Popcorn		2	0.8	0	<1/—	No data	0	0		
	Cornflakes		2	0.8	0	<2/—	No data	0	0		
	Infant foods		4	0.8	2	9/19	No data	0	0		

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 1000 µg/kg	n > 2000 µg/kg	References	Sampling procedure		
<b>Processed maize-based human food (contd)</b>													
Germany (contd)	Maize grits	No data	11	20	1	208/712	519	0	0	Hartl et al., 1999	Commercial samples blended; 5 g extracted		
	Maize meal	No data	6	20	0	2272/7647	5343	4	2				
Italy	Polenta	No data	20	20*	0	1380/3760	3595	9	4	Pascale et al., 1995; A = Doko & Visconti, 1994	Samples purchased in local retail market in Apulia		
Netherlands	(Corrected for analytical recovery)										de Nijs et al., 1998b; Shephard et al., 1990	Samples purchased in local retail stores and ground when necessary	
	Minimally treated maize:												
	For bread	1996		19	25	25	11	98/380	246	0			0
	For popcorn	1995		10	25	25	9	11/110	11	0			0
	For flour	1995	7	25	2	41/90	72	0	0	0			
	Polenta	1995	3	25	1	No data/40	No data	0	0	0			
	Processed maize:												
	Tostada	1995	1	25	1	—/—	—	—	—	—			—
	Canned	1995	6	25	6	—/—	—	—	—	—			—
	Starch	1995	5	25	5	—/—	—	—	—	—			—
	Bread	1995	2	25	1	40/80	No data	0	0	0			
	Popped	1995	5	25	2	no data/300	No data	0	0	0			
	Flour mixes	1995	6	25	6	—/—	—	—	—	—			—
Maize chips	1995	9	25	6	no data/160	No data	0	0	0				
Cornflakes	1995	5	25	4	286/1430	No data	0	0	0				
Sweden	Cornflakes	No data	6	1*	3	14/34	No data	0	0	Moller & Gustavsson, 2000	Samples taken randomly		
	Fresh, frozen, canned maize		13	1*	13	—/—	—	—	—				
	Maize chips		7	1*	0	143/252	No data	0	0				

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 1000 µg/kg	n > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
Sweden (contd)	Maize grits, flour, gruel	No data	8	1*	4	16/52	No data	0	0	Moller & Gustavsson, 2000	Samples taken randomly
	Popcorn	No data	8	1*	2	70/393	No data	0	0		
United Kingdom	(Total fumonisins by HPLC; corrected for recovery)										
	Polenta	1994–95	20	10*	4	529/2124	No data	4	No data	Patel et al., 1997; A = Shephard et al., 1990; Food Standards Agency, 2000	Samples (minimum 0.5 kg) purchased from retail outlets, ground or homogenized, and sub-sampled before analysis
	Breakfast cereals		50	10*	38	22/194	No data	0	0		
	Popcorn, ready-made		9	10*	9	—/—	—	—	—		
	Popcorn		13	10*	7	76/784	No data	0	0		
	Maize snacks		40	10*	9	42/220	No data	0	0		
	Tortilla, taco, enchilada		20	10*	14	6/31	No data	0	0		
	Maize thickener		21	10*	17	15/110	No data	0	0		
	Maize syrup		11	10*	11	—/—	—	—	—		
	Maize oil		20	10*	20	—/—	—	—	—		
	Corn-on-the-cob		20	10*	20	—/—	—	—	—		
Sweet maize		22	10*	21	<1/11	No data	0	0			
<b>Africa</b>											
Botswana	Maize meal	1994	4	20*	0	185/255	No data	0	0	Doko et al., 1996; A = Sydenham et al., 1992b; Doko et al., 1995	Samples (1–5 kg) purchased from retail outlets, ground, and mixed before aliquots taken for analysis

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
South Africa	Maize meal	1994	2	20*	0	65/70	No data	0	0	Doko et al., 1996; A = Sydenham et al., 1992b; Doko et al., 1995	Samples (1–5 kg) purchased from retail outlets, ground and mixed before aliquots taken for analysis
	Samp	1994–95	13	20*	No data	461/1994	No data	No data	0	Rava, 1996; A =	Samples collected from mills throughout country and ground before analysis
	Maize rice		11	20*	No data	295/991	No data	0	0	Sydenham et al., 1992b	
	Maize grits		5	20*	No data	554/1800	No data	No data	0		
	Maize flour		2	20*	No data	532/549	No data	0	0		
	Super meal		25	20*	No data	134/871	No data	0	0		
	Special meal		36	20*	No data	378/1400	No data	No data	0		
	Sifted meal		47	20*	No data	562/4482	No data	No data	No data		
	Unsifted meal		19	20*	No data	827/3929	No data	No data	No data		
	Germ meal		8	20*	No data	437/1288	No data	No data	0		
	Maize bran		32	20*	No data	1324/8180	No data	No data	No data		
	Screenings		7	20*	No data	6651/15716	No data	No data	No data		
Zambia	Maize meal	1994	1	20*	0	740/740	No data	0	0	Doko et al., 1996; A =	Samples (1–5 kg) drawn from sample lots, ground, and mixed before aliquots taken for analysis
Zimbabwe	Maize meal	1994	4	20*	0	625/1910	No data	1	0	Sydenham et al., 1992b; Doko et al., 1995	

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
<b>Asia</b>											
China (Linqu County, Shandong)	Maize meal	1996	14	500*	7	1064/8800	1840	3	2	Groves et al., 1999; A = Rice et al., 1995	Random selection of households (3) in random selection of villages (7); samples frozen and 5 g portion cut for analysis
	Batter	1996	32	500*	26	494/7200	780	2	2		
	Pancake	1996	16	500*	10	400/2200	1050	3	2		
Nepal	(Total fumonisins by HPLC)									Desjardins et al., 2000	Samples (0.25–0.5 kg) collected from farms and markets in 10 districts; 100 g ground and 10 g extracted
	Maize flour	Feb–Jul	8	100*	2	600/2400	No data	No data	No data		
	Cornflakes	1997	2	100*	2	—/—	—	—	—		
Taiwan	Maize snacks	8/1994– 12/1995	78	40*	52	152/2395	No data	No data	No data	Tseng & Liu, 1997; A = Shephard et al., 1990	Random purchases from retail markets in various districts; 150-g sub- sample ground and 25 g extracted
	Sweet canned maize		24	40*	12	200/1089	No data	No data	0		
	Popcorn		22	40*	15	111/1003	No data	No data	0		
	Cornflakes		17	40*	13	117/1281	No data	No data	0		
	Maize grits		4	40*	4	—/—	—	—	—		
	Maize flour		2	40*	1	304/608	No data	0	0		

LOQ, limit of quantification; \*, limit of detection; mean: true mean (for *n* analytical values, the true mean is the sum  $X_i/n$ , where  $X_i$  is the value of each analytical result; for not detected,  $X_i = 0$ ); max: maximum concentration; COVENIN, Comision Venezolana de Normas Industriales; FDA, Food and Drug Administration; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography  
References: P, parent reference, S, sampling method, A, analytical method

Appendix B

Results of surveys for fumonisin B<sub>2</sub>, showing concentrations and distribution of contamination in food commodities

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 1000 µg/kg	n > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b>											
<b>North America</b>											
Canada	Domestic	1993	98	No data	89	54/1000	<200	1	0	Miller et al., 1995	Frequent small samples taken from grain handling equipment; samples pooled, mixed, and 2.2-kg subsample ground
USA	Iowa State	1988	22	500	No data	700/5700	No data	No data	No data	Murphy et al., 1993; A = Ross et al., 1991	Random samples taken from trucks at grain elevators; 400-g subsample ground before analysis
		1989	44	500	No data	800/12300	No data	No data	No data		
		1990	59	500	No data	900/6100	No data	No data	No data		
		1991	50	500	No data	800/4400	No data	No data	No data		
		1992	80	500	No data	10/600	No data	0	0		
		1993	43	500	No data	200/1100	No data	No data	0		
		1994	37	500	No data	90/1100	No data	No data	0		
		1995	85	500	No data	60/1800	No data	No data	0		
1996	93	500	No data	30/—	No data	No data	0	Rice & Ross, 1994; L.G. Rice, P.F. Ross, USDA Veterinary Services Laboratory; P.A. Murphy, Iowa State University			
<b>South and Central America</b>											
Argentina	Domestic	Apr–Nov 1998	34	10	28	52/446	279	0	0	GEMS/Food programme	Statistically based, representative for part of country
	Domestic	Jan–Oct 1999	186	10	5	1451/6944	3847	81	47		
	Domestic	May–Jun 1999	66	10	34	208/4622	264	3	2		

**Appendix B** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b> (contd)											
Argentina (contd)	Domestic	Jan–Aug 2000	56	10	40	103/995	442	0	0		
	Rice, imported	Nov–Dec 1998	5	10	3	33/126	No data	0	0	GEMS/Food programme	Not statistically based, samples from whole country
	Rice, husked, domestic	Apr–Jun 1999	6	10	6	—/—	—	—	—		
Rice, polished domestic	Apr–Jun 1999	5	10	5	—/—	—	—	—			
Brazil	Domestic	4/1995– 4/1996	150	0.08	11	200/6920	No data	No data	No data	GEMS/Food programme	Statistically based, repre- sentative of part of coun- try
	Domestic	1990–91	48	No data	2	4620/19 130	9060	46	39	Hirooka et al., 1996; A = Shephard et al., 1990; Ueno et al., 1993; S = Schmitt & Hurburgh, 1989	Samples collected from silos and warehouses, mixed, reduced to 2 kg, and ground
Colombia	Domestic	Feb–Aug 1998	15	20*	10	102/833	No data	0	0	Perilla & Diaz, 1998; A = Sydenham et al., 1996c; Patel et al., 1997; S = Campbell et al., 1986; Park & Pohland, 1989	Samples (minimum 500 g) purchased at random from most popular retail shops; ground, sub- sampled in grinding– subsampling mill (Romer series II)

## Appendix B (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize (contd)</b>											
<b>Europe</b>											
Spain	Domestic	1994–96	55	100*	33	760/5900	No data	No data	No data	Castella et al., 1999; A = Sydenham et al., 1992a	Samples (1 kg represent- ative) obtained from agri- cultural cooperatives and factories; subsampled in- to 200-g aliquot
<b>Africa</b>											
Kenya	Kernels	1994	1	20*	0	275/275	No data	0	0	Doko et al., 1996; A =	Samples (1–5 kg) drawn from sample lots, ground and mixed before ali- quots taken for analysis
Malawi	Kernels	1994	8	20*	7	4/30	No data	0	0	Sydenham et al., 1992b;	
Mozambique	Kernels	1994	3	20*	0	90/110	No data	0	0	Doko et al., 1995	
South Africa	White maize	1994–95	143	20*	No data	118/4187	No data	No data	No data	Rava, 1996; A = Syden-	Samples collected from mills throughout country, ground before analysis Representative samples (3 kg) collected at har- vest from silos in main production zones; 500-g sample obtained from riffle splitter and ground
	Yellow maize	1994–95	148	20*	No data	148/2000	No data	No data	No data	ham et al., 1992b	
	White grade 1	1993	No data	20*	No data	54/1430	No data	No data	0	Rava et al., 1996; A = Sydenham et al., 1992b	
	White grade 2	1993	No data	20*	No data	70/842	No data	0	0		
	White grade 3	1993	No data	20*	No data	30/200	No data	0	0		
	Yellow grade 1	1993	No data	20*	No data	198/5690	No data	No data	No data		
	Yellow grade 2	1993	No data	20*	No data	257/2039	No data	No data	No data		
	Yellow grade 3	1993	No data	20*	No data	227/1800	No data	No data	0		

## Appendix B (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b> (contd)											
Tanzania	Kernels	1994	9	20*	8	7/60	No data	0	0	Doko et al., 1996; A =	Samples (1–5 kg) drawn from sample lots, ground and mixed before aliquots taken for analysis
Uganda	Kernels	1994	1	20*	0	155/155	No data	0	0	Sydenham et al.,	
Zimbabwe	Kernels	1994	2	20*	1	20/40	No data	0	0	1992b; Doko et al., 1995	
<b>Asia</b>											
China	Linqu County, Shandong Province	1996	16	500*	12	200/1000	800	1	0	Groves et al., 1999; A = Rice et al., 1995	Random selection of households (3) in random selection of villages (7); samples frozen and 5-g portion cut for analysis Samples from 1994 harvest collected from peasant families during Jan–Feb 1995
		1994	34	No data	14	409/4350	No data	No data	No data	Gao & Yoshizawa, 1997; A = Yoshizawa et al., 1994	
	Shangqiu County, Henan Province	1994	20	No data	12	236/1220	No data	No data	0		
	Haimen, Jiangsu counties	Apr–Jul 1993	40	100*	15	1263/6770	2748	17	11	Ueno et al., 1997; A = Shephard et al., 1990; Ueno et al., 1993	
	Penlai, Shandong Province	Apr–Jul 1993	40	100*	33	87/1190	280	1	0		

## Appendix B (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize (contd)</b>											
Indonesia	Domestic	Nov 1995	16	50*	9	80/376	266	0	0	Ali et al., 1998; A = Yoshizawa et al., 1994, 1996	Samples ground and aliquots taken from 200 g
	Domestic	1992–94	12	50*	9	111/556	No data	0	0	Yamashita et al., 1995; A = Yoshizawa et al., 1994	Samples collected at random from stores of wholesalers, retailers, university farms, and local farmers; ground and stored
Iran	Mazandaran	Sep 1998	11	10*	0	512/1175	940	1	0	Shephard et al., 2000; A = Sydenham et al., 1996c	Farmers' maize lots collected at random from consignments sold to Iranian Agriculture Office; total sample ground
	Isfahan	Oct 1998	8	10*	6	16/75	58	0	0		Maize ears bought at various periods from local retail market; total sample ground
Korea Republic of	Mouldy	Nov 1997	36	50*	5	6500/48400	No data	No data	No data	Sohn et al., 1999;	Samples collected from households and milled
	Healthy	1997/Nov	35	50*	27	250/5400	No data	No data	No data	A = Xie et al., 1997	
Philippines	Domestic	1992–94	50	50*	44	34/1210	No data	No data	0	Yamashita et al., 1995; A = Yoshizawa et al., 1994	Samples collected at random from stores of wholesalers, retailers, university farms, and local farmers; ground and stored

## Appendix B (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize (contd)</b>											
Philippines	Seed maize	1992–94	24	50*	22	7/118	No data	0	0	Yoshizawa & Yamashita, 1995; A = Shephard et al., 1990	At least 100-g sample collected, 50 g ground, and 20 g extracted
	Feed maize		22	50*	18	70/1210	No data	No data	0		
	Food maize		23	50*	23	—/—	—	—	—		
Taiwan	Domestic	1996–97	110	80*	108	4/255	No data	0	0	Tseng & Liu, 1999; A = Shephard et al., 1990	Samples (1 kg) collected from 8 districts; subsamples (200 g) ground and 25 g extracted
Thailand	Visibly mouldy human food	1992	5	50*	2	379/1400	No data	1	0	Yoshizawa et al., 1996; A = Shephard et al., 1990	Samples collected before harvest; random 100-g aliquots analysed; 50 g milled and 20-g subsample extracted
	Domestic	1992–94	27	50*	15	112/1400	No data	No data	0	Yamashita et al., 1995; A = Yoshizawa et al., 1994	Samples collected at random from stores of wholesalers, retailers, university farms, and local farmers; ground and stored
Viet Nam	Maize (feed)	1993	15	100*	11	74/560	184	0	0	Wang et al., 1995; A = Ueno et al., 1993	Random samples from various locations; 25 g milled and 5 g extracted
	Maize powder, feed	1993	17	100*	5	204/401	375	0	0		

Appendix B (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food</b>											
<b>North America</b>											
Canada	(* number and mean of samples positive for fumonisin B <sub>1</sub> (not fumonisin B <sub>2</sub> ))										
	<i>Maize products: 1996–97</i>										
	Maize kernel, meal, flour		21*	No data	No data	34*/60 (n=16) 77*/200 (n=5)	No data	0	0	Canada	No data
	Maize snacks (alkali -processed)		20*	No data	No data	57*/240 (n=13) 76*/270 (n=7)	No data	0	0	Canada	No data
	Maize snacks (not alkali-processed)		6*	No data	No data	29*/40 (n=4) <38*/<50 (n=2)	No data	0	0	Canada	No data
	Canned maize		9*	No data	No data	<25*/<25 (n=8) <30*/<30 (n=1)	No data	0	0	Canada	No data
	Frozen maize		2*	No data	No data	<25*/<25	No data	0	0	Canada	No data
	Maize bread		1*	No data	No data	<25*/<25	No data	0	0	Canada	No data
	Maize-based breakfast cereals		8*	No data	No data	33*/40 (n=2) <28*/<40 (n=6)	No data	0	0	Canada	No data
	Fresh tortillas		1*	No data	No data	190*/190	No data	0	0	Canada	No data
	<i>Maize-based infant foods: 1997–98</i>										
	Mixed cereals		16*	No data	No data	11*/20	No data	0	0	Canada	No data
	Cereal with formula		6*	No data	No data	<10*/<10	No data	0	0	Canada	No data
	Creamed maize or maize/vegetables		6*	No data	No data	<10*/<10	No data	0	0	Canada	No data
	<i>Infant cereals: 1998–99</i>										
	Mixed cereals with fruit/formula		13*	No data	No data	<10*/<10	No data	0	0	Canada	No data
	Rice-based		16*	No data	No data	<10*/<10	No data	0	0	Canada	No data
	Soya-based		14*	No data	No data	11*/20	No data	0	0	Canada	No data
	Mixed cereals		20*	No data	No data	11*/20	No data	0	0	Canada	No data

## Appendix B (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food</b>											
USA	Maize meal, 1997 degermed		602	100*	575	10/450	<10	0	0	USA	Sampled 1998
	Maize meal, partially de- germed		20	100*	17	40/360	193	0	0		
	Maize meal, whole grain		50	100*	25	250/1420	1176	6	0		
	Maize meal, 1998 degermed		561	100*	525	30/2480	<10	2	1	USA	Sampled 1999
	Maize meal, partially de- germed		20	100*	14	170/1130	569	1	0		
	Maize meal, whole grain		39	100*	21	240/1830	786	4	0		
	Starch	No data	4	10*	2	32/84	No data	0	0	Maragos et al., 1997;	No data
	Grits		4	10*	2	915/1910	No data	No data	0	A = Maragos & Richard, 1994	
<b>South and Central America</b>											
Argentina	Maize flour	Apr–Dec 1999	11	10	2	67/341	311	0	0	GEMS/Food programme	Statistically based, repre- sentative of part of country
	Popcorn	May–Jun 1999	42	10	3	575/8008	2045	5	4	GEMS/Food programme	Statistically based, repre- sentative for part of country

**Appendix B** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food</b> (contd)											
Argentina (contd)	Cornflour	10/1996– 1/1997	15	11*	4	113/768	190	0	0	Hennigen et al., 2000; A = Sydenham et al., 1996c	Samples (1 kg) pur- chased from food shops and supermarkets in
	Maize grits	10/1996– 1/1997	4	11*	2	30/100	76	0	0		
	Maize flour	Jan 1998	14	11*	1	308/1818	649	1	0	Solovey et al., 1999; A = Sydenham et al., 1996c	Buenos Aires; ground and well mixed by hand before analysis Randomly purchased from comercial outlets; total sample ground
	Maize grits	Jan 1998	2	11*	0	275/324	No data	0	0		
	Maize meal	1997	21	1.5*	9	133/1090	376	1	0		
	Maize flakes	1997	17	1.5*	17	—/—	—	—	—		
Brazil	Canned sweet maize	Mar–May 1999	11	20*	5	80/210	No data	0	0	Machinski & Valente Soares, 2000; A = Shephard et al., 1990	Samples (minimum 500 g) purchased from retail shops in Campinas and ground. homogenized, or both
	Cornflakes		4	20*	3	80/30 (?)	No data	0	0		
	Maize flour		11	20*	2	220/510	No data	0	0		
	Cornflour, baby cereal		2	20*	1	30/50	No data	0	0		
	Maize grits		2	20*	0	180/300	No data	0	0		
	Maize meal		9	20*	0	600/1380	No data	No data	0		
	Corn-on-the-cob		7	20*	7	—/—	—	—	—		
	Curau		2	20*	2	—/—	—	—	—		
	Dergermmed maize		11	20*	3	150/640	No data	0	0		
	Pamonha		7	20*	7	—/—	—	—	—		
	Popcorn		9	20*	5	80/300	No data	0	0		
	Pre-cooked maize meal		6	20*	2	200/420	No data	0	0		

## Appendix B (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
Colombia	Pre-cooked maize meal	Feb–Aug 1998	15	20*	8	20/81	No data	0	0	Perilla & Diaz, 1998; A = Sydenham et al., 1996c; Patel et al., 1997; S = Campbell et al., 1986; Park & Pohl- and, 1989	Samples (minimum 500 g) purchased at random from most popular retail shops; samples ground and subsampled in grind- ing–subsampling mill (Romer series II)
	Popcorn		8	20*	5	27/78	No data	0	0		
	Maize meal		7	20*	5	24/105	No data	0	0		
	“Arepas”		6	20*	3	30/93	No data	0	0		
	Maize snacks		6	20*	5	12/73	No data	0	0		
	Maize starch		3	20*	3	—/—	—	—	—		
Mexico	Tortillas	No data	7	10*	1	76/180	No data	0	0	Dombrink-Kurtzman & Dvorak, 1999 A = Sydenham et al., 1995; Bennett & Richard, 1994	No data
	Masa	No data	2	10*	0	745/1380	No data	1	0		
<b>Europe</b>											
Denmark	Cornflakes	1996	10	3*	8	25/243	No data	0	0	Petersen, 2000; A = Sydenham et al., 1992b	Samples purchased in retail stores, homog- enized in mixer before analysis
	Maize flour		8	3*	2	10/24	No data	0	0		
	Maize grits		4	3*	4	—/—	No data	0	0		
	Maize starch		6	3*	6	—/—	No data	0	0		
	Corn-on-the-cob		4	3*	4	—/—	No data	0	0		
	Maize snacks		10	3*	7	2/8	No data	0	0		
	Polenta		1	3*	0	22/22	No data	0	0		
	Popcorn		9	3*	8	7/59	No data	0	0		
Sweet maize		16	3*	16	—/—	No data	0	0			

**Appendix B** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food</b> (contd)											
Germany	Maize grits	No data	6	0.8	0	40/112	No data	0	0	Lukacs et al., 1996; A = Zoller et al., 1994	Samples from market
	Maize meal		2	0.8	0	39/62	No data	0	0		
	Sweet maize		6	0.8	0	—/—	—	—	—		
	Popcorn		2	0.8	0	<1/—	No data	0	0		
	Cornflakes		2	0.8	0	<2/—	No data	0	0		
	Infant foods		4	0.8	2	8/10	No data	0	0		
Italy	Polenta	No data	20	20*	0	366/910	840	0	0	Pascale et al., 1995; A = Doko & Visconti, 1994	Samples purchased in local retail market in Apulia
Sweden	Cornflakes	No data	6	1*	6	—/—	—	—	—	Moller & Gustavsson, 2000	Samples taken randomly
	Fresh, fro- zen, canned maize		13	1*	13	—/—	—	—	—		
	Maize chips		7	1*	2	27/56	No data	0	0		
	Maize grits, flour, gruel		8	1*	7	1/11	No data	0	0		
	Popcorn		8	1*	5	7/40	No data	0	0		
<b>Africa</b> Botswana	Maize meal	1994	4	20*	2	40/85	No data	0	0	Doko et al., 1996; A = Sydenham et al., 1992b; Doko et al., 1995	Samples (1–5 kg) pur- chased from retail out- lets, ground and mixed before aliquots taken for analysis

## Appendix B (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
South Africa	Maize meal	1994	2	20*	2	—/—	—	—	—	Doko et al., 1996; A = Sydenham et al., 1992b; Doko et al., 1995	Samples (1–5 kg) pur- chased from retail out- lets, ground and mixed before aliquots taken for analysis
	Samp	1994–95	13	20*	No data	3/41	No data	0	0	Rava, 1996; A =	Samples collected from
	Maize rice		11	20*	No data	—/—	—	—	—	Sydenham et al., 1992b	mills throughout country, ground before analysis
	Maize grits		5	20*	No data	13/63	No data	0	0		
	Maize flour		2	20*	No data	—/—	—	—	—		
	Super meal		25	20*	No data	—/—	—	—	—		
	Special meal		36	20*	No data	32/507	No data	0	0		
	Sifted meal		47	20*	No data	87/1223	No data	No data	0		
	Unsifted meal		19	20*	No data	148/1100	No data	No data	0		
	Germ meal		8	20*	No data	25/200	No data	0	0		
	Maize bran		32	20*	No data	338/2368	No data	No data	No data		
	Screenings		7	20*	No data	1628/3718	No data	No data	No data		
Zambia	Maize meal	1994	1	20*	0	380/380	No data	0	0	Doko et al., 1996; A =	Samples (1–5 kg) drawn
Zimbabwe	Maize meal	1994	4	20*	2	193/620	No data	0	0	Sydenham et al., 1992b; Doko et al., 1995	from sample lots, ground and mixed before ali- quots taken for analysis
<b>Asia</b>											
China	Maize meal	1996	14	500*	10	350/2800	880	2	1	Groves et al., 1999; A =	Random selection of
(Linq County, Shandong)	Batter	1996	32	500*	30	31/500	<500	0	0	Rice et al., 1995	households (3) in random selection of villages (7); samples frozen, 5-g portion cut for analysis
	Pancake	1996	16	500*	14	113/1100	<500	1	0		

**Appendix B** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food</b> (contd)											
Taiwan	Maize snacks	8/1994– 12/1995	78	80*	62	30/715	No data	0	0	Tseng & Liu, 1997; A = Shephard et al., 1990	Random purchases from retail markets in various districts; 150-g subsam- ple ground and 25 g extracted
	Sweet, canned maize		24	80*	18	16/658	No data	0	0		
	Popcorn		22	80*	15	37/273	No data	0	0		
	Cornflakes		17	80*	14	29/466	No data	0	0		
	Maize grits		4	80*	4	—/—	—	—	—		
	Maize flour		2	80*	2	—/—	—	—	—		

LOQ, limit of quantification; \*, limit of detection; mean: true mean (for *n* analytical values, the true mean is the sum  $X_i/n$ , where  $X_i$  is the value of each analytical result; for not detected,  $X_i = 0$ ); max: maximum concentration

References: P, parent reference, S, sampling method, A, analytical method

## Appendix C

**Results of surveys for fumonisin B<sub>3</sub>, showing concentrations and distribution of contamination in food commodities**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b>											
<b>North America</b>											
USA	Iowa State	1988	22	500	No data	200/2100	No data	No data	No data	Murphy et al., 1993; A = Ross et al., 1991	Random samples taken from trucks at grain elevators; 400-g sub- sample ground before analysis
		1989	44	500	No data	200/4000	No data	No data	No data		
		1990	59	500	No data	300/2800	No data	No data	No data		
		1991	50	500	No data	400/2300	No data	No data	No data		
		1992	80	500	No data	—/—	—	—	—	Rice & Ross, 1994; L.G. Rice, P.F. Ross, USDA Veterinary Ser- vices Laboratory; P.A. Murphy, Iowa State University	
		1993	43	500	No data	30/700	No data	0	0		
		1995	85	500	No data	20/600	No data	0	0		
		1994	37	500	No data	30/600	No data	0	0		
		1996	93	500	No data	—/—	No data	—	—		
<b>South and Central America</b>											
Argentina	Domestic	Apr–Nov 1998	34	8	29	8/61	44	0	0	GEMS/Food programme	Statistically based, repre- sentative of part of country
	Domestic	Jan–Oct 1999	186	8	8	341/1684	874	15	0		
	Domestic	May–Jun 1999	66	8	39	51/867	155	0	0		
	Domestic	Jan–Aug 2000	56	8	43	39/442	202	0	0		
	Rice, imported	Nov–Dec 1998	5	8	4	3/16	—	0	0	GEMS/Food programme	Not statistically based, samples from whole country

## Appendix C (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b>											
Argentina (contd)	Rice, husked, domestic	Apr–Jun 1999	6	8	6	—/—	—	—	—	GEMS/Food programme	Not statistically based, samples from part of country
	Rice, poli- shed domestic	Apr–Jun 1999	5	8	5	—/—	—	—	—	GEMS/Food programme	Not statistically based, samples from part of country
<b>Africa</b>											
Kenya	Kernels	1994	1	20*	0	130/130	No data	0	0	Doko et al., 1996; A =	Samples (1–5 kg) drawn from sample lots, ground, and mixed before ali- quots taken for analysis
Malawi	Kernels	1994	8	20*	8	—/—	—	—	—	Sydenham et al., 1992b; Doko et al., 1995	
Mozambique	Domestic	1994	3	20*	0	40/50	No data	0	0		
South Africa	White maize	1994–95	143	20*	No data	83/3110	No data	No data	No data	Rava, 1996; A = Syden-	Samples collected from mills throughout country, ground before analysis Representative samples (3 kg) collected at har- vest from silos in main production zones; 500-g sample obtained from riffle splitter and ground
	Yellow maize	1994–95	148	20*	No data	54/1431	No data	No data	No data	ham et al., 1992b	
	White grade 1	1993	No data	20*	No data	25/355	No data	0	0	Rava et al., 1996; A = Sydenham et al., 1992b	
	White grade 2	1993	No data	20*	No data	38/400	No data	0	0		
	White grade 3	1993	No data	20*	No data	—/—	—	—	—		
	Yellow grade 1	1993	No data	20*	No data	71/1570	No data	No data	0		
	Yellow grade 2	1993	No data	20*	No data	167/1963	No data	No data	0		
	Yellow grade 3	1993	No data	20*	No data	112/729	No data	0	0		

## Appendix C (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b>											
Tanzania	Kernels	1994	9	20*	9	—/—	—	—	—	Doko et al., 1996; A =	Samples (1–5 kg) drawn from sample lots, ground, and mixed before aliquots taken for analysis
Uganda	Kernels	1994	1	20*	0	85/85	No data	0	0	Sydenham et al.,	
Zimbabwe	Kernels	1994	2	20*	2	—/—	—	—	—	1996b; Doko et al., 1995	
<b>Asia</b>											
China	Linqi County, Shandong Province	1996	16	500*	15	31/500	<500	0	0	Groves et al., 1999; A = Rice et al., 1995	Random selection of households (3) in random selection of villages (7); samples frozen and 5-g portion cut for analysis Samples from 1994 harvest collected from peasant families during Jan–Feb 1995
	Linxian County, Henan Province	1994	34	No data	19	169/1660	No data	No data	0	Gao & Yoshizawa, 1997; A = Yoshizawa et al., 1994	
	Shangqiu County, Henan Province	1994	20	No data	13	109/576	No data	0	0		
	Haimen, Jiangsu counties	Apr–Jul 1993	40	100*	15	700/4130	1950	10	4	Ueno et al., 1997; A = Shephard et al., 1990; Ueno et al., 1993	
	Penlai, Shandong Province	Apr–Jul 1993	40	100*	36	17/270	14	0	0		Kernels collected randomly from agricultural stocks; 25-g sample milled and 5 g extracted

## Appendix C (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Unprocessed maize</b>											
Indonesia	Domestic	Nov 1995	16	50*	12	27/222	76	0	0	Ali et al., 1998; A = Yoshizawa et al., 1994, 1996	Samples ground and aliquots taken from 200 g
Iran	Mazandaran	Sep 1998	11	10*	0	361/960	550	0	0	Shephard et al., 2000; A = Sydenham et al., 1996c	Farmers' maize lots collected at random from consignments sold to Iranian Agriculture Office; total sample ground Maize ears bought at various periods from l local retail market; total sample ground
	Isfahan	Oct 1998	8	10*	6	16/75	58	0	0		
Korea, Republic of	Mouldy	Nov 1997	36	50*	5	5400/10600	No data	No data	No data	Sohn et al., 1999; A = Xie et al., 1997	Samples collected from households and milled
	Healthy	Nov 1997	35	50*	28	60/500	No data	0	0		
Viet Nam	Maize, feed	1993	15	100*	12	46/432	135	0	0	Wang et al., 1995; A = Ueno et al., 1993	Random samples from various locations; 25 g milled, 5 g extracted
	Maize powder, feed	1993	17	100*	7	104/268	203	0	0		
<b>Processed maize-based human food</b>											
<b>North America</b>											
USA	Maize meal, 1997 degermed		602	100*	602	—/—	—	—	—	USA	Sampled 1998
	Maize meal, partially degermed		20	100*	20	—/—	—	—	—		

## Appendix C (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
USA (contd)	Maize meal, 1997 whole grain		50	100*	40	40/290	180	0	0	USA	Sampled 1998
	Maize meal, 1998 degermed		561	100*	552	4/610	<10	0	0	USA	Sampled 1999
	Maize meal, partially degermed		20	100*	18	20/270	11	0	0		
	Maize meal, whole grain		39	100*	34	40/480	18	0	0		
<b>South and Central America</b>											
Argentina	Maize flour	Apr–Dec 1999	11	8	2	42/312	260	0	0	GEMS/Food programme	Statistically based; repre- sentative of part of country
	Popcorn	May–Jun 1999	42	8	6	160/2027	658	2	1		
	Maize meal	1997	21	1.5*	10	78/1015	132	1	0	Solovey et al., 1999;	Randomly purchased
	Cornflakes	1997	17	1.5*	17	—/—	—	—	—	A = Sydenham et al., 1996c	from commercial outlets; total sample ground
<b>Africa</b>											
Botswana	Maize meal	1994	4	20*	3	8/30	No data	0	0	Doko et al., 1996; A = Sydenham et al., 1992b; Doko et al., 1995	Samples (1–5 kg) pur- chased from retail out- lets, ground, and mixed before aliquots taken for analysis

## Appendix C (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food (contd)</b>											
South Africa	Maize meal	1994	2	20*	2	—/—	—	—	—	Doko et al., 1996; A = Sydenham et al., 1992b; Doko et al., 1995	
	Samp	1994–95	13	20*	No data	—/—	—	—	—	Rava, 1996; A = Sydenham et al., 1992b	Samples collected from mills throughout country and ground before analysis
	Maize rice		11	20*	No data	—/—	—	—	—		
	Maize grit		5	20*	No data	—/—	—	—	—		
	Maize flour		2	20*	No data	—/—	—	—	—		
	Super meal		25	20*	No data	—/—	—	—	—		
	Special meal		36	20*	No data	4/100	No data	0	0		
	Sifted meal		47	20*	No data	23/603	No data	0	0		
	Unsifted meal		19	20*	No data	64/522	No data	0	0		
	Germ meal		8	20*	No data	6/48	No data	0	0		
	Maize bran		32	20*	No data	126/2008	No data	No data	No data		
	Screenings		7	20*	No data	599/1604	No data	No data	0		
Zambia	Maize meal	1994	1	20*	0	85/85	No data	0	0	Doko et al., 1996; A =	Samples (1–5 kg) pur- chased from retail out- lets, ground, and mixed before aliquots taken for analysis
Zimbabwe	Maize meal	1994	4	20*	2	65/205	No data	0	0	Sydenham et al., 1992b; Doko et al., 1995	

**Appendix C** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 1000 µg/kg	<i>n</i> > 2000 µg/kg	References	Sampling procedure
<b>Processed maize-based human food</b> (contd)											
<b>Asia</b>											
China	Maize meal	1996	14	500*	12	107/900	< 500	0	0	Groves et al., 1999; A = Rice et al., 1995	Random selection of households (3) in random selection of villages (7); samples frozen and 5-g portion cut for analysis
(Linqi County, Shandong Province)	Batter	1996	32	500*	31	28/900	<500	0	0		
	Pancake	1996	16	500*	14	81/700	<500	0	0		

LOQ, limit of quantification; \*, limit of detection; mean: true mean (for *n* analytical values, the true mean is the sum  $X_i / n$ , where  $X_i$  is the value of each analytical result; for not detected,  $X_i = 0$ ); max: maximum concentration

References: P, parent reference, S, sampling method, A, analytical method



## OCHRATOXIN A

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## 1. EXPLANATION

Ochratoxin A was evaluated by the Committee at its thirty-seventh meeting (Annex 1, reference 94), when it established a provisional tolerable weekly intake (PTWI) of 112 ng/kg bw, on the basis of deterioration of renal function in pigs, for which the lowest-observed-effect level (LOEL) was 0.008 mg/kg bw per day, and a safety factor of 500. At that time, the Committee recommended that further studies be conducted to elucidate the role of ochratoxin A (and other mycotoxins) in causing nephropathy in pigs and humans, the mechanisms of induction of tumours, and the role of phenylalanine in antagonizing the adverse effects of ochratoxin A. (The present Committee noted that the adverse effects noted at the thirty-seventh meeting consisted of nephrotoxicity.) Ochratoxin A was re-evaluated by the Committee at its forty-fourth meeting (Annex 1, reference 116), when it considered toxicological data that had become available since the previous evaluation, including studies on the epidemiology of nephropathy, on genotoxicity and on experimental nephrotoxicity. At that meeting, the Committee reconfirmed the PTWI, rounding it to 100 ng/kg bw, and reiterated its request for further studies on ochratoxin A.

The Codex Committee on Food Additives and Contaminants at its Thirty-first Session requested the Expert Committee to perform a risk assessment of the consequences of establishing a maximum level of 5 or 20 µg/kg in cereals and cereal products.

Ochratoxin A is produced by a single *Penicillium* species, *P. verrucosum*, by *Aspergillus ochraceus* and several related *Aspergillus* species, and by *A. carbonarius*,

with a small percentage of isolates of the closely related *A. niger*. These three groups of species differ in their ecological niches, in the commodities affected, and in the frequency of their occurrence in different geographical regions. *P. verrucosum* grows only at temperatures below 30 °C and at water activity as low as 0.8. It is therefore found only in cool temperate regions; it is the source of ochratoxin A in cereals and cereal products in Canada and Europe. As cereals are widely used in animal feeds in Europe, and ochratoxin A is relatively stable in vivo, this mycotoxin is also found in some animal products in that region, especially in pig kidney and liver. As *P. verrucosum* does not occur in the tropics and subtropics, cereals from these regions are unlikely to contain ochratoxin A from this source. *A. ochraceus* grows at moderate temperatures and at a water activity above 0.8. It is found sporadically in a wide range of stored food commodities, including cereals, but is seldom the cause of substantial concentrations of ochratoxin A. It may also infect coffee beans during sun-drying and is a source of ochratoxin A in green coffee beans. *A. carbonarius* grows at high temperatures and is associated with maturing fruits, especially grapes. Because of its black spores, it is highly resistant to sunlight and survives sun-drying. It is the source of ochratoxin A in fresh grapes, dried vine fruits, and wine; it is also one source of ochratoxin A in coffee.

The Committee considered several new studies that had become available since the previous evaluation of ochratoxin A. These included further studies of absorption, distribution (including secretion into the milk of experimental animals), metabolism, and excretion; biochemical studies; toxicological studies on genotoxicity, immunotoxicity, neurotoxicity, embryotoxicity, and hepatotoxicity; and studies on the mechanisms of cytotoxicity and nephrotoxicity. The results of epidemiological studies were also reviewed. New data from surveys of food commodities for ochratoxin A and of food consumption were also considered, and intakes were estimated for various countries and regions of the world.

## **2. BIOLOGICAL DATA**

### **2.1 Biochemical aspects**

#### **2.1.1 Absorption, distribution, and excretion**

##### *(a) Absorption*

It has been suggested that, in most species, ochratoxin A is absorbed from the stomach as a result of its acidic properties ( $pK_a = 7.1$ ) (Galtier, 1978; Roth et al., 1988). In studies of animals with ligated gastrointestinal loops, however, the small intestine was found to be the major site of absorption, with maximal absorption from the proximal jejunum. Absorption from the jejunum can take place against a concentration gradient and depends on the pH at the mucosal surface of the jejunum. Ochratoxin A that is so transferred is lipid-soluble and non-ionized (Kumagai & Aibara, 1982; Kumagai, 1988).

The results of studies in which a low dose of [<sup>3</sup>H]ochratoxin A was given by intubation to mice were interpreted by the authors as indicating rapid absorption from the stomach, but they could also be interpreted as showing that intestinal absorption is the major route, with rapid transit from the stomach to the intestine.

Secondary peaks of ochratoxin A found in the intestinal contents and serum may have been a consequence of enterohepatic circulation, since the biliary excretion of this toxin is very efficient (Fuchs et al., 1988a; Roth et al., 1988).

The overall percentage of ochratoxin A absorbed was 66% in pigs, 56% in rats, 56% in rabbits, and 40% in chickens (Suzuki et al., 1977; Galtier et al., 1981).

In male Wistar rats that received a single intratracheal dose of crystalline ochratoxin A (purity unknown) at 50 ng/g bw, absorption from the lungs was found to be very efficient, the bioavailability being calculated as 98%. The biological half-life of ochratoxin A was estimated to be 127 h. The toxicokinetics of the toxin when given intratracheally, orally, or intravenously were comparable (Breitholtz-Emanuelsson et al., 1995).

Phenylalanine given to mice by gavage with ochratoxin A in a 10:1 molar ratio appeared to increase the absorption of ochratoxin A from the stomach and intestine and to increase gastrointestinal transit. This resulted in an eightfold higher concentration of ochratoxin A in serum and a fourfold higher concentration in liver during the next 12 h (Roth et al., 1988).

#### *(b) Distribution*

The bioavailability of ochratoxin A, estimated from a comparison of the maximal serum concentration after oral and intravenous administration, was very low in fish but 44 and 97% for two mammalian species investigated (Hagelberg et al., 1989). Once it reaches the blood, ochratoxin A bound readily to serum albumin (Galtier et al., 1980) and other macromolecules (Hult & Fuchs, 1986). Erythrocytes contained only traces (Galtier, 1978).

The association constant for the binding of ochratoxin A to serum albumin was  $7.1 \times 10^4$  per mol for pigs,  $5.1 \times 10^4$  per mol for chickens, and  $4.0 \times 10^4$  per mol for rats (Galtier et al., 1981). The fraction of ochratoxin A bound to serum albumin and other macromolecules constitutes a mobile reserve of mycotoxin that can be made available for release to the tissues for a long time (Galtier, 1978; Hult et al., 1982). Studies with albumin-deficient rats showed that the main effect of ochratoxin A binding to serum albumin is to retard its elimination by limiting its transfer from the bloodstream to hepatic and renal cells (Kumagai, 1985).

In studies of the stability of ochratoxin A bound to porcine albumin, it was displaced by the acidic drug phenylbutazone, so that more free toxin was available. In male rats, ochratoxin A was more toxic in the presence of phenylbutazone, with a significant decrease in the LD<sub>50</sub> value from 33 to 21 mg/kg bw (Galtier et al., 1980).

Ochratoxin A had strong affinity for an unidentified serum macromolecule (relative molecular mass, 20 000), with association constants of  $2.3 \times 10^{10}$  per mol in human serum and  $0.59 \times 10^{10}$  per mol in porcine serum. The specific binding of this macromolecule was saturated at concentrations of ochratoxin A of 10–20 ng/ml serum. Significant amounts of serum albumin were bound at higher concentrations

of ochratoxin A, with saturation above several hundred micrograms per millilitre of serum (Stojkovic et al., 1984; Hult & Fuchs, 1986).

The fraction of ochratoxin A that remained unbound to two identified plasma proteins was 0.02% in humans and rats, 0.08% in monkeys, 0.1% in mice and pigs, and 22% in fish (Hagelberg et al., 1989).

Once ochratoxin A has been absorbed, the concentrations of the toxin and its metabolites in tissues and plasma residues depend on the length of feeding, the dose, whether the ochratoxin A is naturally occurring or crystalline, the route, the degree of serum binding, the half-life of ochratoxin A, and the duration on an ochratoxin A-free diet before sacrifice. These factors are important in assessing the natural occurrence of residues in animal tissues (Kuiper-Goodman & Scott, 1989).

After a single oral dose, the maximum serum concentrations of ochratoxin A were found within 10–48 h in pigs and rats (Suzuki et al., 1977; Galtier, 1978; Galtier et al., 1981; Mortensen et al., 1983a), at 2–4 h in ruminant calves (Sreemannarayana et al., 1988), after 1 h in rabbits, and after 0.33 h in chickens (Galtier et al., 1981). Maximum concentrations in tissues were found within 48 h in rats.

Wide species differences have been reported in the serum half-life of ochratoxin A. The half-life after oral administration was found to be 510 h in *Macaca mulata* monkeys (Hagelberg et al., 1989), 72–120 h in pigs (Galtier et al., 1981; Mortensen et al., 1983a), 77 h in pre-ruminant calves (Sreemannarayana et al., 1988), 55–120 h in rats (Galtier et al., 1979; Ballinger et al., 1986; Hagelberg et al., 1989), 6.7 h in quail (Hagelberg et al., 1989), and 4.1 h in chickens (Galtier et al., 1981). In those species tested, the serum half-time was longer after intravenous administration (Hagelberg et al., 1989), perhaps due in part to differences in absorption (Galtier et al., 1981), differences in peak plasma concentrations (see above), and species differences in the degree of binding to serum macromolecules, including albumin.

The rate of disappearance of ochratoxin A was slower from blood than from kidney, liver, and other tissues in pigs (Hult et al., 1979).

Whole-body autoradiography of mice after a single intravenous dose of [<sup>14</sup>C]ochratoxin A at approximately 200 µg/kg bw showed that the toxin persisted for > 4 days in the blood, interpreted as showing that the toxin is present mainly in bound form at this dose (Fuchs et al., 1988a). In a similar experiment in rats, the distribution after 24 h was greatest in lung and, in decreasing order, in adrenal medulla, skin, liver, myocardium, kidney, salivary gland, adrenal cortex, muscle, gastric mucosa, and bone marrow (Breitholtz-Emanuelsson et al., 1992). The tissue distribution in pigs, rats, chickens, and goats generally followed the order kidney > liver > muscle > fat (Harwig et al., 1983) or kidney > muscle > liver > fat (Mortensen et al., 1983a; Madsen et al., 1982).

In hens fed ochratoxin A, none was found in eggs (Krogh et al., 1976). In another study, it was found in eggs when the birds were fed 10 mg/kg bw (Juszkiewicz et al., 1982). A study of the tissue distribution of [<sup>14</sup>C]ochratoxin A in laying Japanese quail

showed specific retention of unidentified radiolabel as a ring-shaped deposition in eggs, indicating that the toxin could be deposited over a short period (Fuchs et al., 1988b). Egg-laying Japanese quail were given a single oral dose of 0, 1, 5, or 20 mg/kg bw. The concentrations of ochratoxin A in abdominal yolk of birds 6 h later were 13 µg/kg in those given 5 mg/kg bw and 34 µg/kg in those given 20 mg/kg bw. The toxin was still present in abdominal yolks 4 days after administration, and the mean concentration was 10-fold higher than in whole eggs. No ochratoxin A was found in eggs of birds given 1 mg/kg bw (Piskorska-Pliszczynska & Juszkiwicz, 1990).

Lactating rats, treated orally with a single dose of ochratoxin A up to 250 µg/kg bw excreted the toxin in their milk. The milk: blood concentration ratio was 0.4 at 24 h and 0.7 at 72 h. A linear relationship was found between the concentration of ochratoxin A in the dam's milk and that in the blood and kidneys of pups at 72 h. The pup milk: blood concentration ratio was approximately 1.7. At 72 h, the suckling pups had higher concentrations of ochratoxin A than their dams in both blood and kidney (Breitholtz-Emanuelsson et al., 1993a).

Whole-body autoradiography after intravenous administration of high doses of [<sup>14</sup>C]ochratoxin A showed that it could cross the placenta more readily when given on days 8 and 9 than day 10 of gestation, with radiolabel appearing within 20 min in the uterine wall, placenta, and fetal tissues. Ochratoxin A given to mice on day 17 of gestation resulted in very little radiolabel in fetuses (Appelgren & Arora, 1983a,b).

Differences in fetal uptake of ochratoxin A at different times during gestation were suggested to be due to differences in the placenta, which was considered to be completely developed by day 9 of gestation. After intraperitoneal injection of ochratoxin A on day 11 or 13 of gestation, residues appeared more slowly and reached maximum values 30–48 h after dosing. The concentrations in the placenta were high 2–6 h after injection and then decreased more slowly than from other tissues. The serum half-life of ochratoxin A was 29 h at day 11 and 24 h at day 13 of gestation. The authors considered the embryo to be a 'deep compartment' (Fukui et al., 1987).

A group of 39 female Sprague-Dawley rats received ochratoxin A orally at 50 µg/kg bw five times a week for 2 weeks before mating, during gestation, and then 7 days a week during lactation. Pups from ochratoxin A-treated dams were cross-fostered at birth to control dams and *vice versa*. Treatment did not affect maternal body weight nor alter the birthweight or development of pups. The concentrations of ochratoxin A in the blood and kidney of exposed pups were three to four times higher than those in the dams. No differences in weight gain or in body or kidney weight were seen between pups exposed *in utero*, via lactation, or both. The transfer of ochratoxin A to milk was very efficient (60% of the blood concentration at 8 weeks). The highest blood and kidney concentrations were found in offspring exposed *in utero* and via milk, but the most significant exposure was via milk (Hallén et al., 1998).

After subcutaneous administration of [<sup>3</sup>H]ochratoxin A to rats on day 12 of gestation, fetal uptake was delayed, with maximum concentrations 48–72 h after dosing, representing about 0.1% of the dose administered (Ballinger et al., 1986).

Four lactating Blanc de Termonde rabbits received ochratoxin A from feed naturally contaminated at 190 ng/g, equivalent to 16 µg/kg bw, on days 3–19 of lactation. The toxin was effectively transported from blood to milk and subsequently to the offspring. Higher concentrations were found in maternal plasma than in milk, and a linear relationship was found between the concentrations in milk and plasma of offspring. The plasma:kidney concentrations were much higher in offspring than in adults, perhaps due to slower detoxication in the former (Ferrufino-Guardia et al., 2000).

Ochratoxin A given at 0.38 mg/kg bw to pregnant sows on days 21–28 of pregnancy did not cross the placenta (Patterson et al., 1976). Similarly, no residues were found in piglets of sows fed diets containing ochratoxin A at 7–16 µg/kg bw per day throughout gestation (Mortensen et al., 1983b). In a more recent study, however, ochratoxin A was transmitted to six piglets *in utero* when the sow was fed naturally contaminated feed; the blood concentrations in the newborn piglets were 0.075–0.12 ng/ml, whereas that in the sow was 0.20 ng/ml (Barnikol & Thalmann, 1988).

### (c) Excretion

Both biliary excretion and glomerular filtration play important roles in the plasma clearance of ochratoxin A in rats. This is related to its relative molecular mass of 403.8, since both pathways are used in this species for substances with relative molecular masses between 350 and 450. Thus, in rats, both the urinary and faecal excretory routes are important, the relative contribution of each depending on factors such as route of administration and dose (Kuiper-Goodman & Scott, 1989).

In all species, the relative contribution of each excretory route is also influenced by the degree of serum macromolecular binding and differences in the degree of enterohepatic recirculation of ochratoxin A (Hagelberg et al., 1989).

In rats, the major excretory products were ochratoxin  $\alpha$  (in urine and faeces), ochratoxin A, and the 4R-OH-ochratoxin A epimer. In urine, these represented 25–27%, 6%, and 1–1.5% of the administered dose, respectively (Storen et al., 1982a).

Up to 33% of the radiolabel on an orally administered dose of ochratoxin A was excreted into the bile of rats up to 6 h after dosing; only trace amounts of ochratoxin  $\alpha$  were detected in the bile (Suzuki et al., 1977).

Biliary excretion of ochratoxin A was increased and urinary excretion of ochratoxin A and ochratoxin  $\alpha$  was decreased in mice pretreated with phenobarbital (Moroi et al., 1985).

When ochratoxin A was administered to rats intraperitoneally, only traces of ochratoxin A and ochratoxin  $\alpha$  were identified in faeces, whereas after oral administration 12% ochratoxin A and 9% ochratoxin  $\alpha$  were found in faeces (Storen et al., 1982a).

In pre-ruminant and ruminant calves, 85–90% of orally administered ochratoxin A was excreted as ochratoxin  $\alpha$ , most of it in the urine (Sreemannarayana et al., 1988).

### 2.1.2 Biotransformation

Ochratoxin A is hydrolysed to the non-toxic ochratoxin  $\alpha$  at various sites. In rats, detoxication by hydrolysis to ochratoxin  $\alpha$  is a function of the bacterial microflora of the caecum (Galtier, 1978). The enzymes responsible for hydrolysis to ochratoxin  $\alpha$  in cows and rodents are carboxypeptidase A and chymotrypsin (Pitout, 1969a,b; Pitout & Nel, 1969). Other mycotoxins such as penicilloic acid inhibit this reaction (Parker et al., 1982). Inhibition of the flora of the lower gastrointestinal tract of rats by neomycin reduced hydrolysis of ochratoxin A to ochratoxin  $\alpha$  and increased the blood concentration of ochratoxin A (Madhyastha et al., 1992).

Studies with rat tissue homogenate showed that the duodenum, ileum, and pancreas also have a high capacity to carry out this reaction, whereas the activity in the liver and kidney was low (Suzuki et al., 1977). It was non-existent in rat hepatocytes (Hansen et al., 1982) and rabbit and rat liver (Kanisawa et al., 1979; Stormer et al., 1983).

In rats given [ $^{14}\text{C}$ ]ochratoxin A, most of the radiolabel was attached to ochratoxin A, indicating that efficient metabolism of this toxin is lacking in most tissues other than the intestine (Galtier et al., 1979).

Incubation of the contents of the four stomachs of cows indicated effective hydrolysis of ochratoxin A to ochratoxin  $\alpha$  by the ruminant protozoa. Assuming a similar reaction velocity *in vivo*, it was estimated that up to 12 mg/kg of feed could be degraded (Hult et al., 1976; Pettersson et al., 1982), so that this species is assumed to be relatively resistant to the effects of ochratoxin A in feed. Sheep also have a good capacity to detoxify ochratoxin A before it reaches the blood (Kiessling et al., 1984).

Studies in mice suggest that ochratoxin A circulates from the liver into the bile and into the intestine, where it is hydrolysed to ochratoxin  $\alpha$  (Moroi et al., 1985).

About 25–27% of ochratoxin A, given either intraperitoneally or orally to rats, was present as ochratoxin  $\alpha$  in the urine. Its presence in the urine can be explained by reabsorption from the intestine (Storen et al., 1982a). A similar mechanism of intestinal reabsorption of ochratoxin  $\alpha$  has been suggested to occur in ruminant calves (Sreemannarayana et al., 1988).

Other minor urinary metabolites of ochratoxin A are 4-OH (4R- and 4S) epimers produced in rat and rabbit liver (Størmer et al., 1981) and rat kidney (Stein et al., 1985) by the action of cytochromes P450 (CYPs; Størmer et al., 1981, 1983). The 4R-OH epimer, which is considered less toxic than ochratoxin A, is the main one formed in human and rat liver microsomal systems (Størmer et al., 1981), whereas the 4S-OH epimer is more prevalent in pig liver microsomes. No data were available on its toxicity (Moroi et al., 1985).

The biotransformation of ochratoxin A has also been studied in various microsomal preparations and in recombinant human and rat CYP preparations (Gautier et al., 2001; Zepnik et al., 2001). Incubation of ochratoxin A with liver microsomes from

rats and mice produced 4R- and 4S-hydroxyochratoxin A, but at very low rates, whereas oxidation of ochratoxin A was not observed in kidney microsomes from these species. 4R-Hydroxyochratoxin A was also formed at low rates by recombinant human CYP 3A4 (both studies), CYP 1A1 and CYP 2C9-1 (both in single studies), while conflicting results were obtained with CYP1A2. Oxidation was not observed with recombinant human CYP 2E1 or rat CYP 1A2 or the male rat-specific CYP 2C11 (all in one study). Prostaglandin H-synthase produced small amounts of a non-polar product.

The 10-OH derivative was formed from ochratoxin A in a rabbit liver microsomal system (Størmer et al., 1983). Ochratoxin C, a metabolite of ochratoxin A produced in rumenal fluid, is as toxic as ochratoxin A (cited by Galtier et al., 1981). Ochratoxin B, a dechloro derivative of ochratoxin A, may occur with ochratoxin A in cereal products. In rats, it is less toxic than ochratoxin A and is metabolized to 4-OH-ochratoxin B and ochratoxin  $\beta$  (Størmer et al., 1985).

Ochratoxin B was not antagonistic to ochratoxin A with respect to effects on the formation of phenylalanyl-tRNA and protein synthesis (Roth et al., 1989).

Many researchers consider that the toxicity of ochratoxin A is due to one of its metabolites. The studies cited above indicate, however, that, in rats, ochratoxin A itself, rather than one of its metabolites, is the active toxic agent, since the known metabolites are less toxic than or as toxic as ochratoxin A. This conclusion concurs with findings in mice, in which the LD<sub>50</sub> of ochratoxin A increased by 1.5- to 2-fold after the animals were fed phenobarbital at 500 mg/kg of diet for 1 week before oral or intraperitoneal administration (Moroi et al., 1985).

Similarly, pretreatment with sodium phenobarbital at 80 mg/kg bw per day by gavage for 5 days or 3-methylcholanthrene at 20 mg/kg bw per day by gavage for 2 days resulted in increased LD<sub>50</sub> values for ochratoxin A. With phenobarbital, the difference was smaller 144 h after dosing with ochratoxin A than at 48 h. Administration of piperonyl butoxide, an inhibitor of microsomal monooxygenases, decreased the 144-h LD<sub>50</sub> of ochratoxin A from 40 to 19 mg/kg bw (Chakor et al., 1988). In contrast, preliminary studies in mice showed that simultaneous feeding of phenobarbital slightly increased the incidence of liver tumours seen with ochratoxin A alone, and that the mice developed large, multiple hepatomas (Suzuki et al., 1986).

Few data are available on the metabolic disposition of ochratoxin A in humans. It has been suggested that it has a long serum half-life, on the basis of its strong binding to human serum macromolecules (Bauer & Gareis, 1987; Hagelberg et al., 1989).

### **2.1.3 Effects on enzymes and other biochemical parameters**

The activities of glycolytic enzymes were reduced, whereas those of gluconeogenic enzymes were increased. The diabetogenic effect of ochratoxin A was thought to be due to inhibited synthesis and/or release of insulin from pancreatic cells, thereby suppressing glycolysis and glycogenesis and enhancing gluconeogenesis and glycogenolysis (Subramanian et al., 1989).

Calcium homeostasis was studied in rats treated intraperitoneally with ochratoxin A at a single dose of 10 mg/kg bw or multiple doses of 0.5–2 mg/kg bw per day. An increase in renal endoplasmic reticulum calcium pump activity was observed, suggesting an association with ochratoxin A-induced renal cytotoxicity (Rahimtula & Chong, 1991).

Studies with pig renal cortical explants indicated that inhibition of the biosynthesis of macromolecules (protein, RNA and DNA) by ochratoxin A was not due to impairment of cellular respiration (Braunberg et al., 1992).

The biochemistry and molecular aspects of the action of ochratoxin A in both prokaryotes and eukaryotes have been reviewed (Röschenthaler et al., 1984). The findings are inconsistent, owing to differences and limitations in experimental models and procedures as well as interfering factors, especially in more complex organisms. In prokaryotes (Konrad & Röschenthaler, 1977), eukaryotic microorganisms (Creppy et al., 1979a), mammalian cells (Creppy et al., 1980a, 1983a), and experimental animals in vivo (Creppy et al., 1980b, 1984), the primary effect of ochratoxin A is inhibition of protein synthesis; secondarily, RNA and DNA synthesis may be inhibited.

The inhibition of protein synthesis is specific and occurs at the post-transcriptional level, ochratoxin A having a direct effect on the translation step in protein synthesis. This involves competitive inhibition of phenylalanine-tRNA<sup>Phe</sup> synthetase, so that amino-acylation and peptide elongation are stopped. This reaction is fundamental for all living organisms. In yeast, the first part of this reaction, phenylalanine-dependent pyrophosphate exchange, was inhibited five times more than transfer to tRNA, the second part. In this reaction ochratoxin A may be regarded as an analogue of phenylalanine, and in cell cultures the competitive inhibition could be reversed by an increase in phenylalanine concentration (Creppy et al., 1979a). Similarly, in mice, the lethality of a single dose of 0.8 mg of ochratoxin A injected intraperitoneally was completely prevented by simultaneous injection of 1 mg of phenylalanine (Creppy et al., 1980b).

In yeast, the effect on protein synthesis of the rR-OH-ochratoxin A epimer was similar to that of ochratoxin A, but ochratoxin  $\alpha$ , which lacks the phenylalanine moiety, had no effect (Creppy et al., 1983a). Analogues of ochratoxin A in which phenylalanine has been replaced by other amino acids, such as tyrosine, inhibit the respective amino acid-specific tRNA synthetases similarly (Creppy et al., 1983b).

The binding affinity of phenylalanine-tRNA<sup>Phe</sup> synthetase for ochratoxin A is weaker than for phenylalanine and ranges from 1/300 in yeast ( $K_M = 1.3$  mmol/L for ochratoxin A; 3.3  $\mu$ mol/L for phenylalanine) (Creppy et al., 1983a) to 1/20 in rat liver ( $K_m = 0.28$  mmol/L for ochratoxin A; 6  $\mu$ mol/L for phenylalanine) (Röschenthaler et al., 1984). Despite these differences in binding affinity, the inhibition of phenylalanine-tRNA<sup>Phe</sup> by ochratoxin A is very effective, since the toxin is more readily concentrated by cells than phenylalanine. The concentration of ochratoxin A inside hepatoma cells was 200- to 300-fold that in the medium (Creppy et al., 1983a).

A dose-related inhibition of protein synthesis was found in mice given ochratoxin A intraperitoneally at a dose  $\geq 1$  mg/kg bw. The degree of inhibition of protein synthesis

5 h after administration of ochratoxin A at 1 mg/kg bw was 26% in liver, 68% in kidney, and 75% in spleen as compared with controls (Creppy et al., 1984).

Ochratoxin A may also act on other enzymes that use phenylalanine as a substrate, although no direct effect on the activity of other isolated enzyme systems has been demonstrated (Röschenthaler et al., 1984). In kidney slices from rats 2 days after they had been fed ochratoxin A at 2 mg/kg bw, the activity of renal phosphoenolpyruvate carboxykinase, a key enzyme in the gluconeogenic pathway, was lowered by 50% (Meisner & Krogh, 1986). The inhibition was due indirectly to specific degradation of the mRNA coding for this enzyme. A similar effect was not seen in rat liver (Meisner et al., 1983).

The effect of ochratoxin A on phenylalanine metabolism was studied in isolated hepatocytes and in liver homogenates from male rats treated in vivo. Both the hydroxylation of phenylalanine to tyrosine and the subsequent metabolism of tyrosine, as measured by homogenate oxidation, were inhibited when ochratoxin A at a concentration of 0.12–1.4 mmol/L was incubated with isolated hepatocytes (Creppy et al., 1990).

Ochratoxin A enhanced NADPH- or ascorbate-dependent lipid peroxidation in rat liver microsomes and NADPH-dependent lipid peroxidation in kidney microsomes in vitro, as measured by malondialdehyde formation or oxygen uptake. It was suggested that ochratoxin A stimulates lipid peroxidation by complexing  $\text{Fe}^{3+}$  and facilitating its reduction. Subsequent to oxygen binding, an iron–oxygen complex initiates lipid peroxidation. Cytochrome P450, free active oxygen species, and free hydroxy radicals do not appear to be involved in  $\text{Fe}^{3+}$ –ochratoxin A-stimulated lipid peroxidation. Oral administration of ochratoxin A at 6 mg/kg bw to rats appeared to increase lipid peroxidation in vivo, causing a sevenfold increase in ethane exhalation (Rahimtula et al., 1988; Omar et al., 1990).

In pig renal cortical tissue, ochratoxin A and citrinin added singly or in combination at a concentration of  $10^{-6}$  or  $10^{-3}$  mol/L did not elicit consistent or strong synergistic effects, as measured by transport of tetraethylammonium and paraaminohippurate ions, or protein synthesis measured with [ $^3\text{H}$ ]leucine (Braunberg et al., 1994).

The effects of superoxide dismutase and catalase on ochratoxin A-induced nephrotoxicity were studied. Superoxide removes oxygen by converting it to hydrogen peroxide; this enzyme works in conjunction with catalase, which removes hydrogen peroxide within cells. Rats were given 20 mg/kg bw of each enzyme by subcutaneous injection every 48 h, 1 h before gavage with ochratoxin A at 290  $\mu\text{g}/\text{kg}$  bw every 48 h, for 3 weeks. Superoxide dismutase and catalase prevented most of the nephrotoxic effects induced by ochratoxin A, observed as enzymuria, proteinuria, and creatinaemia, and increased the urinary excretion of ochratoxin A. The results indicated that superoxide radicals and hydrogen peroxide are likely to be involved in the nephrotoxic effects of ochratoxin A in vivo (Baudrimont et al., 1994).

After short-term administration of ochratoxin A to rats, the renal proximal tubule did not appear to be the main target for nephrotoxicity, although decreased capacity

to eliminate the toxin may result in a self-enhancing effect (Gekle & Silbernagl, 1994). The main renal effect of ochratoxin A in rats was found in the 'postproximal' nephron, as measured by a reduced glomerular filtration rate, increased fractional water, Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup> excretion, and increased dependence of osmol clearance on urine flow. In addition, ochratoxin A blocked membrane anion conductance in canine kidney cells in vitro (Gekle et al., 1993).

## 2.2 Toxicological studies

### 2.2.1 Acute toxicity

The LD<sub>50</sub> values found in various species treated by various routes are shown in Table 1. Dogs and pigs were the most sensitive species and rats and mice the least sensitive. Simultaneous oral administration of phenylalanine at 100 mg/kg bw to mice increased the oral LD<sub>50</sub> from 46 mg/kg bw to 71 mg/kg bw (Moroi et al., 1985). As is the case with many xenobiotics, neonatal rats were considerably more susceptible than adults.

Histopathological and electron microscopic studies were conducted with groups of 10 male Long-Evans and Sprague-Dawley rats given benzene-free ochratoxin A at a single dose of 0, 17, or 22 mg/kg bw in 0.1 mol/L sodium bicarbonate by gavage and examined for up to 48 h afterwards. The earliest changes were multifocal haemorrhages in many organs and fibrin thrombi in the spleen, the choroid plexus of the brain, liver, kidney and heart, suggesting disseminated intravascular coagulation. The effect was postulated by the authors to be due to activation of extrinsic and intrinsic systems of coagulation. Other changes were hepatic and lymphoid necrosis, enteritis with villous atrophy, affecting the jejunum most severely, and nephrosis. The myocardial changes were considered to be related to shock and subsequent ischaemic injuries (Albassam et al., 1987).

### 2.2.2 Short-term studies of toxicity

Ochratoxin A had nephrotoxic effects in all monogastric mammalian species tested so far (Kuiper-Goodman & Scott, 1989). The results of short-term studies with this toxin are shown in Table 2.

**Table 1. LD<sub>50</sub> values for ochratoxin A in various species**

Species	LD <sub>50</sub> (mg/kg bw)		
	Oral	Intraperitoneal	Intravenous
Mouse	46–58	22–40	26–34
Rat	20–30	13	13
Rat neonate	3.9		
Dog	0.2		
Pig	1		
Chicken	3.3		

Based on a literature compilation by Harwig et al. (1983)

**Table 2. Results of short-term studies of the toxicity of ochratoxin A**

Species, strain, sex, age	No.	Route	Dose (mg/kg bw per day [mg/kg of diet])	Time (days)	NOEL (mg/kg bw per day)	Effects	Reference
Rat, Wistar, male, weanling	10	Diet	0.24–2.4 [2.4–24]	14	~0.48 ~0.48 ~0.96 < 0.24 < 0.24	Growth retardation Increased serum blood urea nitrogen Increased kidney weight Decreased urine volume Renal lesions	Munro et al. (1974)
Rat Wistar, male, female, weanling	15	Diet	0.015–0.37 [0.2–5]	90	~0.075 ~0.016  0.37	Reduced weight gain Reduced kidney weight; no change in blood urea nitrogen, urinary or haematological parameters Desquamation; increase in smooth endoplasmic reticulum, changes in rough endoplasmic reticulum, basement membrane thickening of proximal convoluted tubule cells; increased eosinophilia and karyomegaly in proximal convoluted tubule cells	Munro et al. (1974)
Rat, Wistar, male, adult	5	Gavage	5	3	< 5	Reduced <i>para</i> -amino hippuric acid clearance, basement membrane thickening	Suzuki et al. (1975)

**Table 2** (contd)

Species, strain, sex, age	No.	Route	Dose (mg/kg bw per day [mg/kg of diet])	Time (days)	NOEL (mg/kg bw per day)	Effects	Reference
Rat, Wistar, male, adult	10	Gavage	0.5–2	10	1 < 0.5	Increased blood urea nitrogen Increased urine volume	Hatey & Galtier (1977)
Rat, Sprague-Dawley and Wistar, male, female adult	4–6	intraperitoneal	0.75, 2	5–7	< 0.75	Decreased body weight; increased urine flow; decreased urine osmolality; increased urinary protein; increased urinary glucose; impaired urinary transport of organic substances; Sprague-Dawley more sensitive than Wistar, females less sensitive than males	Berndt & Hayes (1979)
Rat, Wistar, male, adult	14	Gavage	4	4–10	< 4	Decreased factors II, VI, X; decreased plasma fibrinogen; decreased thrombocyte, megakaryocyte counts	Galtier et al. (1979)
Rat, Wistar, male, adult	9	Gavage	4	10	< 4	Hypothermia, cachexia, tremors, diarrhoea	Galtier et al. (1980)
Rat, Wistar, male, adult	3	Gavage	0.14–2	56–84	< 0.14	Decreased kidney enzyme activity; increased urinary enzyme activity	Kane et al. (1986a)

**Table 2** (contd)

Species, strain, sex, age	No.	Route	Dose (mg/kg bw per day [mg/kg of diet])	Time (days)	NOEL (mg/kg bw per day)	Effects	Reference
Rat, Fischer 344/N, male, female, weanling	5	Gavage	1-6	16 (12 doses)	1	Increased relative kidney, heart, and brain weight; thymus atrophy; forestomach necrosis; adrenal haemorrhage	National Toxicology Program (1989)
					< 1	Bone-marrow hypoplasia	
					< 1	Renal nephropathy	
Rat, Fischer 344/N, male, female, weanling	10	Gavage	0.06-1	91	0.12, males 0.12, males 0.06 < 0.06	Growth retardation Reduced relative kidney weight Kidney tubular necrosis Karyomegaly	National Toxicology Program (1989)
Dog, beagle, male, young	3-6	Capsule	0.1-0.2	14	0.2 < 0.1 < 0.1	No change in kidney function Renal tubular necrosis Proximal tubule changes; thymus, lymphoid necrosis	Kitchen et al. (1977a,b,c)
Pig, female, 8-12 weeks	3-6	Diet	0.008, 0.04, 0.2 [0.2, 1, 5]	5-90	< 0.008	Renal enzyme changes; changes in renal function	Elling (1979a); Krogh et al. (1988)

*(a) Rats*

Groups of 10 male weanling Wistar rats were fed semi-purified diets containing ochratoxin A at a concentration of 0, 2.4, 4.8, 9.6, or 24 mg/kg, equivalent to 0, 0.24, 0.48, 0.96, and 2.4 mg/kg bw per day, for 14 days. At the two higher doses, growth retardation, reduced food consumption, and increased serum urea nitrogen were seen. At the highest dose, the relative kidney weight was increased. Renal lesions, involving degenerative changes in the entire tubular system, and a decrease in urine volume were seen at all doses. Increased eosinophilia and karyomegaly in cells of the proximal convoluted tubules were noted at all doses (Munro et al., 1974).

Semi-purified diets containing ochratoxin A at 0, 0.2, 1, or 5 mg/kg, equivalent to 0, 0.015, 0.075, or 0.37 mg/kg bw per day, were fed to groups of 15 weanling Wistar rats of each sex for 90 days. At that time, eight animals from each group were killed, and the remaining rats were fed control diet for an additional 90 days. No changes in blood urea nitrogen or urinary or haematological parameters were seen at any dose. After 90 days at the two higher dietary concentrations, the relative kidney weights were reduced in animals of each sex; these had returned to control values after the 90-day recovery period, except in males at the highest dose. Dose-related changes in morphological appearance were seen after 90 days of treatment at doses  $\geq 0.2$  mg/kg of diet and involved karyomegaly and increased eosinophilia in cells of the proximal convoluted tubules. The authors considered the latter change to be a phenomenon of ageing which had been accelerated by administration of ochratoxin A. Desquamation of proximal tubular cells, autolysis, changes in the rough and smooth endoplasmic reticulum, and tubular basement membrane thickening up to 4  $\mu\text{m}$  were noted at the highest dose. In animals at the highest dose that were subsequently given control diet for 90 days, the karyomegaly and tubular basement membrane thickening persisted, but otherwise the kidneys appeared normal (Munro et al., 1974).

Similar effects were seen when ochratoxin A was administered to groups of four to six adult Sprague-Dawley and Wistar rats by intraperitoneal injection for 5–7 days at a dose of 0, 0.75, or 2 mg/kg bw per day. Decreased body weight, increased urine flow, increased urinary protein, increased urinary glucose, and impaired urinary transport of organic substances were seen at all doses. Sprague-Dawley rats were more sensitive than Wistar rats, and males were more sensitive than females. It was suggested that the increased urinary protein indicated interference with protein reabsorption by cells of the convoluted tubules (Berndt & Hayes, 1979).

Ochratoxin A was administered by gavage in maize oil to groups of five weanling male and female Fischer 344/N rats at a dose of 0, 1, 4, or 16 mg/kg bw per day on 5 days per week for a total of 12 doses over 16 days. All rats that received the highest dose had diarrhoea and nasal discharge and died before the end of the study. Increased relative weights of kidneys, heart, and brain, thymus atrophy, forestomach necrosis and/or hyperplasia, and haemorrhage of adrenal glands were seen at the two higher doses. Bone-marrow hypoplasia and nephropathy were seen at all doses, involving renal tubular degenerative and regenerative changes (National Toxicology Program, 1989).

Ochratoxin A was administered by gavage in maize oil to groups of 10 male and female weanling Fischer 344/N rats at a dose of 0, 0.06, 0.12, 0.25, 0.5, or 1 mg/kg bw per day for 5 days/week for 91 days. Growth retardation and a reduced relative kidney weight were seen in males at the two higher doses. The NOEL for renal tubular necrosis was 0.062 mg/kg bw, but karyomegaly of dose-related severity was observed in the proximal tubules at all doses. Milder renal changes consisting of tubular atrophy were seen at lower doses (National Toxicology Program, 1989).

Groups of 15 weanling rats were given ochratoxin A in 0.1 mol/L sodium bicarbonate at a dose of 0 or 100 µg/rat (equivalent to 1.25 mg/kg bw per day) by gavage for 8 weeks. Blood samples from fasted treated rats contained about twice the amount of glucose as those of controls. In a glucose tolerance test, the insulin concentration did not reach that in control rats. Total carbohydrate and glycogen concentrations in liver of treated rats were reduced, as seen earlier (Suzuki et al., 1975; T. Kuiper-Goodman, personal observation).

*(b) Dogs*

Groups of three to six young beagle dogs were given ochratoxin A by capsule at a dose of 0, 0.1, or 0.2 mg/kg bw per day for 14 days. No changes were observed in renal function, but tubular necrosis and ultrastructural changes in the proximal tubules were observed at all doses. Necrosis of lymphoid tissues of the thymus and tonsils was also seen at all doses (Kitchen et al., 1977a,b,c).

*(c) Pigs*

In a series of experiments, groups of three to six sows were given feed containing ochratoxin A at a concentration of 0, 0.2, 1, or 5 mg/kg, equivalent to 0, 0.008, 0.04, and 0.2 mg/kg bw per day, for periods of 5 days, 8 or 12 weeks, or up to 2 years. Decreased renal function, nephropathy, and reduced renal enzyme activity were reported. Progressive nephropathy but no renal failure was seen in female pigs given feed containing 1 mg/kg for 2 years; no results were reported for male pigs (Krogh & Elling, 1977; Elling, 1979a,b, 1983; Elling et al., 1985; Krogh et al., 1988).

*(d) Chickens*

In groups of 10 broiler chicken given ochratoxin A at a dietary concentration of 4 mg/kg for 2 months, the mortality rate was 42%. When the feed was supplemented with 0.8 or 2.4% L-phenylalanine, the mortality rate decreased to 12 and 15%, respectively (Gibson et al., 1990).

### **2.2.3 Long-term studies of toxicity and carcinogenicity**

*Mice*

Diets containing ochratoxin A at 0 or 40 mg/kg, equivalent to 5.6 mg/kg bw per day, were fed to groups of adult male 10 ddY mice for 44 weeks, followed by 5 weeks of basal diet. Of the nine surviving treated mice, five had hepatic-cell tumours, nine had renal cystic adenomas, and two had solid renal-cell tumours (terms used by the authors). No hepatic or renal tumours were observed in control mice, and no data

on the incidence of these tumours in other control groups of this strain of mice were presented. It was not clear indicated whether the liver tumours were benign or malignant (Kanisawa & Suzuki, 1978).

In a second study from the same laboratory, diets containing ochratoxin A at 0 or 25 mg/kg, equivalent to 3.5 mg/kg bw per day, were fed to groups of 20 6-week-old male DDD mice for 70 weeks. All 20 surviving treated mice had renal cystic adenomas, six had solid renal tumours, and eight had hepatic-cell tumours. One of the 17 control mice had a hepatic-cell tumour (Kanisawa, 1984).

In a third study from the same laboratory, the mice were not exposed for life but for 70 weeks. Diets containing ochratoxin A at 0 or 50 mg/kg, equivalent to 7 mg/kg bw per day, were fed to groups of 16 adult male ddY mice for 5–30 weeks, followed by control diet for 40–65 weeks. No renal or liver tumours were observed in control mice or in mice fed ochratoxin A for  $\leq 10$  weeks. The incidences of renal-cell tumours were 3/15, 1/14, 2/15, and 4/17 after 15, 20, 25, and 30 weeks on the ochratoxin A-containing diet, respectively. The incidence of renal cystic adenomas was not indicated. A significant increase in the incidence of liver tumours was observed after mice had been fed ochratoxin A for 25 weeks (5/15) or 30 weeks (6/17). These results indicated that the renal and liver tumours persisted through subsequent feeding of control diet (Kanisawa, 1984).

In these studies, two types of renal tumour were distinguished by the authors: papillary cyst adenomas (benign) and solid renal-cell tumours, which contained atypical cells, displayed infiltrative growth, and were interpreted by the Committee as malignant. Preneoplastic renal lesions were frequent and multiple and consisted of distended tubules with atypical epithelial cells. No metastases attributable to the kidney or liver tumours were found.

Diets containing ochratoxin A at a concentration of 0, 1, or 40 mg/kg were fed to groups of 50 weanling B6C3F<sub>1</sub> mice of each sex for 24 months. The test compound contained about 84% ochratoxin A, 7% ochratoxin B, and 9% benzene. Dead and moribund mice were identified daily. The mice were examined and weighed, and their food consumption was recorded weekly for the first 4 weeks, then monthly. Animals at the high dose showed decreased body weights, by 25% for females and 33% for males, indicating that the maximum tolerated dose was exceeded, although no other signs of toxicity were observed. Nephropathy, characterized by cystic dilatation of renal tubules often with hyperplasia of the lining epithelium, was seen only in mice fed diets containing the highest concentration and was more severe in males than in females. No nephropathy was found in males or females given a control diet or the lower concentration of ochratoxin A. Benign and malignant renal tumours were seen only in male mice fed diets containing the high concentration, at incidences of 53% and 29%, respectively (combined incidence, 63%). No metastases from the renal tumours were found.

When the combined incidence of hepatocellular adenomas and carcinomas in treated mice was compared with that in concurrent controls, the increase was statistically significant in both male and female mice given the high dose; however, the 20% incidence in males was within the range of past controls of 0–22% for this strain of mouse, but the 14% incidence in females was greater than the incidence of

0–3.9% in previous controls (Ward et al., 1979). The authors noted that the ochratoxin A used in their study contained 9% benzene, a proven carcinogen, and thus the possibility of synergism must be considered. The presence of renal tumours in males did not decrease their survival rate. In fact, the survival rates of males at 18 months were 75% in the controls and 65% among those at 1 mg/kg of diet, compared with 98% for those at 40 mg/kg of diet, owing to a high incidence of fatal obstructive urinary-tract disease among the controls and low-dose mice, with onset as early as 4 months (Bendele et al., 1985a). It was suggested that the apparent protective effect of ochratoxin A at 40 mg/kg of diet was due to inhibition of the growth of gram-positive bacteria and to the induction of polyuria as a result of renal proximal tubule damage (Bendele & Carlton, 1986). Group caging and fighting-related lesions of the prepuce and penis may have contributed to the chronic uropathy (Rao, 1987).

### Rats

Groups of 80 male and female Fischer 344/N rats were given ochratoxin A by gavage in maize oil at a concentration of 0, 21, 70, or 210 µg/kg bw per day, 5 days/week for 9 months, 15 months, or 103 weeks. The rats were observed twice daily, and body weights and food consumption were recorded weekly for the first 13 weeks and then monthly. Feed and water were available *ad libitum*. Groups of 15 rats of each sex were killed after 9 and 15 months. The body weight of rats at the highest dose was decreased by 4–7% between 18 and 77 weeks for male rats and between 6 and 89 weeks for female rats. No compound-related clinical signs were seen, and the results of haematological and serum chemical analyses showed no effects of biological significance. Urinary analysis indicated a mild to moderate change in the ability to concentrate urine, with no other change in renal function.

The incidences of renal adenomas in males were 1/50, 1/51, 6/51, and 10/50 and those of renal carcinomas were 0/50, 0/51, 16/51, and 30/50, in the four groups, respectively. The combined incidences of renal tubule-cell adenomas and carcinomas were 20/51 and 36/50 at the two higher doses. At the highest dose, many of the renal adenomas and carcinomas were multiple or bilateral. There was a dose-related increase in the number of males found dead or moribund before the terminal sacrifice (7, 19, 23, and 26, respectively, at 0, 21, 70, and 210 µg/kg bw per day). The decreased survival rates among rats at the two higher doses were attributed by the authors to the presence of kidney tumours, since 15/23 and 18/26 rats that died at these two doses had kidney tumours. In addition, a larger proportion of animals that died before the terminal sacrifice had carcinomas that had become metastatic (3/8 and 11/15 at the intermediate and high doses, respectively) than of animals killed at terminal sacrifice (0/7 and 3/15 at the intermediate and high doses, respectively). In male rats given the low dose of ochratoxin A, only one kidney tumour was present, although the decrease in survival was similar to that of rats at the two higher doses. The reduced survival of this group must therefore be attributed to a non-neoplastic treatment-related effect. In females, the combined incidences of renal adenomas and carcinomas were 0/50, 0/51, 2/50, and 8/50 at 0, 21, 70, and 210 µg/kg bw per day, respectively. The significance of the ochratoxin A-induced renal carcinomas in rats is increased by the high frequency of metastases, attributed to renal-cell carcinomas, mainly in the lungs and lymph nodes. Females at the high dose also had a greater multiplicity of fibroadenomas in the mammary gland (14/50) than controls and rats at lower doses (4–5/50).

The non-neoplastic lesions involved mainly the kidney. Chronic diffuse nephropathy, common to old rats, was seen at about the same incidence in all groups, but the extent and grade were not reported. Karyomegaly or karyocytomegaly (large kidney epithelial cells with giant polyploid nuclei and prominent nucleoli) was seen in all males and females at the two higher doses, and it was the most consistent finding in these groups at the 9- and 15-month interim sacrifices as well as in a preliminary 13-week study (National Toxicology Program, 1989).

In reviewing these data at its forty-fourth meeting, the Committee noted that renal carcinomas were found in 16/51 male rats at 70  $\mu\text{g}/\text{kg}$  bw per day and in 30/50 at 210  $\mu\text{g}/\text{kg}$  bw per day; no carcinomas were found at the lower doses. In female rats, renal carcinomas were less common, with 0/50, 1/50, and 3/50 animals showing carcinomas at the low, intermediate, and high doses, respectively. Renal adenomas were found in all groups of male rats, increasing in frequency with increasing dose. In the female rats, renal adenomas were found only at the two higher doses. Fibroadenomas in the mammary gland were found in 45–56% of treated females, a significantly higher percentage than in the control group (Annex 1, reference 117).

The slides of the kidneys from the National Toxicology Program study were reviewed subsequently (Hard, 2000). The review confirmed that the site of injury was the straight segment of proximal tubule S3 in the outer stripe of the outer medulla. In the 2-year bioassay, the lesion consisted of contraction and disorganization of the normal linear pattern of the S3 tubules due to marked development of karyomegaly and cytomegaly. This change showed a clear dose–response relationship in both males and females. The 16-day and 13-week studies showed that this response was preceded by focal tubule basophilia involving mainly the outer stripe of the outer medulla, associated with single-cell death, increased mitotic activity, and some simple tubule hyperplasia. Other non-neoplastic lesions involving only the outer stripe of the outer medulla in the 2-year bioassay were dilated atypical tubules, chromophobic tubules, and cystic tubules, the latter being more prominent in females than in males. The review also confirmed that low (microgram) concentrations of ochratoxin A induced a high incidence of renal tubule tumours (74% in males at the high dose), with carcinomas predominating over adenomas. The carcinomas had a relatively rapid onset, progressing with malignant and aggressive behaviour, some tumours showing a tendency towards an uncommon anaplastic phenotype. There was a relatively high incidence of metastasis, and some tumours were undoubtedly the cause of death. These various features of the ochratoxin A-induced tumours distinguish them from the kidney tumours induced by model non-genotoxic renal carcinogens such as d-limonene and chloroform. However, the tendency towards anaplasia and their aggressive nature were reminiscent of renal tubule tumours induced by fumonisin B<sub>1</sub>. Renal tumour development was clearly related to the site of ochratoxin A-induced tubule damage, in that preneoplastic atypical tubule hyperplasia, adenomas, and very early carcinomas developed within the outer stripe of the outer medulla. However, a mode of action of sustained cytotoxicity and compensatory cell regeneration coupled with simple tubule hyperplasia, although a possibility, could not be established within the limits of conventional histology alone. Nevertheless, the very high incidence of renal neoplasms, their relatively rapid onset and highly malignant behaviour, coupled with a tendency towards an aggressive anaplastic phenotype and their contribution to

death all favour a conclusion that ochratoxin A-induced renal tumour development occurs via DNA reactivity.

The Committee noted that the long-term effects were preceded by evidence of renal toxicity in the 16-day and 13-week studies. It is unclear whether the malignancy and aggressive nature of the tumours is a secure indication that the mechanism of induction is via DNA reactivity. The analogy with tumours induced by fumonisin B<sub>1</sub> is not evidence of a genotoxic mechanism, since it has been postulated that the mechanism by which fumonisins induce tumours may be indirect, involving altered sphingolipid metabolism.

#### 2.2.4 Genotoxicity

The results of studies of genotoxicity with ochratoxin A are summarized in Table 3.

##### (a) DNA adducts

Almost all the available studies in which DNA adducts were detected by <sup>32</sup>P-postlabelling after exposure to ochratoxin A were from one laboratory (Pfohl-Leszkowicz et al., 1991, 1993; Grosse et al., 1995, 1997; Castegnaro et al., 1998; Pfohl-Leszkowicz et al., 1998). All showed positive results in rats and mice given 0.4–2.5 mg/kg bw for 1–16 days or even up to 2 years. The number of adducts ranged from 1 to 200/10<sup>9</sup> nucleotides in kidney DNA. However, the nonspecific postlabelling technique used may have resulted in adducts that did not contain an ochratoxin A or ochratoxin A metabolite moiety. At least some of the adducts might have been due to ochratoxin A-induced cytotoxic effects that generate reactive oxygen species. Thus, Grosse et al. (1997) found that prior treatment of rats with superoxide dismutase or catalase, ascorbic acid, or  $\alpha$ -tocopherol significantly decreased the number of adducts.

Indications that oxidative damage to DNA is not the only source of the presumed adducts are provided by the results of experiments *in vitro* with purified DNA and mononucleotides incubated with kidney or liver microsomes from mouse and rabbit, ochratoxin A, and either NADPH or arachidonic acid as cofactors (Obrecht-Pflumio & Dirheimer, 2000). Presumed adducts were obtained in all cases but particularly with mouse and rabbit kidney microsomes and arachidonic acid as the cofactor. Liver microsomes were much less active. With NADPH as the cofactor with mouse kidney microsomal enzymes, the adduct level was only 44% that obtained with arachidonic acid. When dAMP, dGMP, dTMP, and dCMP were used as substrates, three adducts were formed with dGMP, mouse kidney microsomes, and either cofactor. However, only one of these adducts was common to the two cofactors. Inhibition of lipid peroxidation and the generation of hydroxyl radicals with desferrioxamine B methanesulfonate did not change the adduct profile. The major adduct obtained with dGMP co-chromatographed with the major adduct obtained with purified DNA. No adducts were obtained with the other three mononucleotides.

In contrast to these results with <sup>32</sup>P-postlabelling methods, Schlatter et al. (1996) and Rasonyi (1995) reported that the level of covalent binding of [<sup>3</sup>H]ochratoxin A to DNA was below the limit of detection (LOD) of scintillation counting in kidney and liver (< 1.3/10<sup>10</sup> and 5.6/10<sup>11</sup> DNA bases, respectively). In addition, Gautier et al. (2001), using scintillation counting, did not find covalent binding of [<sup>3</sup>H]ochratoxin A to the DNA of the kidneys of male Fischer 344 rats dosed by gavage 24 h earlier.

Table 3. Results of assays for genotoxicity with ochratoxin A

Test system	Test object	Concentration	Results	Reference
<i>In vitro</i> Reverse mutation	<i>S. typhimurium</i> TA 98, TA100, TA1535, TA1537, TA1538	0.4–400 µg/plate	Negative (highly variable TA100 controls, not tested to cytotoxicity)	Wehner et al. (1978); Kuczuk et al. (1978)
Reverse mutation	<i>S. typhimurium</i> TA100, TA1538	~ 200 µg/plate	Negative with mouse and rat liver activation	Bartsch et al. (1980)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538	50–600 µg/plate	Negative	Bendele et al. (1985b)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538, G46, G3076, D3052	0.1–100 µg/ml	Negative	Bendele et al. (1985b)
Reverse mutation	<i>S. typhimurium</i> TA1538	0.1–500 µg/plate (mixture of ochratoxin A: ochratoxin B, 17)	Positive > 100 µg/plate	Kuczuk et al. (1978)
Reverse mutation	<i>S. typhimurium</i> TA97, TA98, TA100, TA1535	1–100 µg/plate	Negative with hamster or rat liver activation	National Toxicology Program (1989)
Reverse mutation	<i>S. typhimurium</i> TA98, TA1535, TA1538	0–1200 µg/plate	Positive only after activation by mouse kidney microsomes	Obrecht-Pflumio et al. (1999)
Reverse mutation	<i>S. typhimurium</i> TA100, TA2638	0–200 µg/plate	Negative in pre-incubation assay with mouse liver and kidney, and isolated enzyme activation systems	Zepnik et al. (2001)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538		Positive after activation by medium derived from hepatocytes exposed to ochratoxin A	Hennig et al. (1991)
Gene mutation	<i>S. cerevisiae</i> D3	75, 200 µg/plate		Kuczuk et al. (1978)

Table 3 (contd)

Test system	Test object	Concentration	Results	Reference
Gene mutation	<i>B. subtilis</i> rec	20–100 µg/disc	Negative	Ueno & Kubota (1976)
DNA repair	<i>E. coli</i> , SOS assay	1–2 mg/100 µl	Negative	Reiss (1986); Auffray & Boutibonnes (1986)
DNA repair	<i>E. coli</i> WP2	Gradient plate, not stated	Negative	Bendele et al. (1985b)
Forward gene mutation	Mouse lymphoma cells, <i>Tk</i> locus	0.1–13 µg/ml	Negative (>12 µg/ml cytotoxic)	Bendele et al. (1985b)
Gene mutation	C3H mouse mammary cells	5–10 µg/ml	Negative (10 µg/ml cytotoxic)	Umeda et al. (1977)
Gene mutation ( <i>lacZ</i> on shuttle vector)	NIH 3T3 cells transfected with human cytochrome P450	25 µg/ml	Positive	De Groene et al. (1996)
Unscheduled DNA synthesis	Fischer 344 rat primary hepatocytes	0.000025–500 µg/ml (2 lots tested at 15 doses)	Negative (> 0.05 µg/ml cytotoxic)	Bendele et al. (1985b)
Unscheduled DNA synthesis	ACI rat primary hepatocytes	0.4, 4 µg/ml	Weakly positive at 0.4, cytotoxic at 4.0 µg/ml	Mori et al. (1984)
Unscheduled DNA synthesis	C3H mouse primary hepatocytes	4, 40 µg/ml	Weakly positive at 4.0, cytotoxic at 40 µg/ml	Mori et al. (1984)
Unscheduled DNA synthesis	Rat hepatocytes; porcine urinary bladder epithelial cells	250 nmol/L–1 µmol/L	Positive	Dorrenhaus & Follmann (1997)
Unscheduled DNA synthesis	Cultured human urothelial cells	0.005–0.05 µmol/L	Positive	Flieger et al. (1998)
Unscheduled DNA synthesis	Primary human urothelial cells	10–2000 nmol/L	Positive	Dorrenhaus et al. (2000)
DNA strand break, alkaline elution	Chinese hamster ovary cells; rat fibroblasts	200 µg/ml	Positive (1.2 strand breaks/10 <sup>9</sup> Da)	Stetina & Votava (1986)
DNA damage	Mouse spleen, phytohaemagglutinin-stimulated	1–10 µg/ml	Positive (dose-related)	Creppy et al. (1985)

Table 3 (contd)

Test system	Test object	Concentration	Results	Reference
DNA damage, <sup>32</sup> P-postlabeling assay	Mouse kidney, liver, spleen	0.6, 1.2, 2.5 mg/kg bw	Positive ('adducts' not shown to contain bound ochratoxin A)	Pfohl-Leszkowicz et al. (1991)
DNA binding	Rat kidney, liver, seminal vesicle; mouse kidney	100 µmol/L incubated with S9 protein	Negative	Gautier et al. (2001)
Sister chromatid exchange	Human peripheral blood lymphocytes	5–10 µg/ml	Negative (mitotic inhibition at 10 µg/ml)	Cooray (1984)
Sister chromatid exchange	Chinese hamster ovary cells, 26 h with ochratoxin A	0.5–5 µg/ml	Negative	National Toxicology Program (1989)
Sister chromatid exchange	Chinese hamster ovary cells, 2 h with ochratoxin A	5–160 µg/ml	Positive (frequency ≤ 37% above control, weak dose–response relationship)	National Toxicology Program (1989)
Sister chromatid exchange	Human lymphocytes		Positive	Hennig et al. (1991)
Chromosomal aberration	Chinese hamster ovary cells, 8–10 h with ochratoxin A	30–160 µg/ml	Negative	National Toxicology Program (1989)
	2 h with ochratoxin	100–300 µg/ml	Negative	
Chromosomal aberration	Human lymphocytes, 48 h with ochratoxin A	4.5 µg/ml	Positive (4.5–5-fold increase)	Manolova et al. (1990)
Micronucleus formation	Ovine seminal vesicle cell cultures	12–30 µmol/L	Positive <sup>a</sup>	Degen et al. (1997)
Micronucleus formation	Syrian hamster embryo fibroblasts		Positive <sup>b</sup>	Dopp et al. (1999)
<i>In vivo</i> Chromosomal aberration	Mouse	1 µg/kg bw per day in diet, 45 days	Positive (ameliorated by 10 mg/kg bw ascorbic acid)	Bose & Sinha (1994)
Chromosomal aberration	Mouse	1 µg/kg bw per day in diet, 14 days	Positive (ameliorated by 130 IU vitamin A/kg bw)	Kumari & Sinha (1994)

Table 3 (contd)

Test system	Test object	Concentration	Results	Reference
Sister chromatid exchange	Chinese hamster bone marrow	25–400 mg/kg bw by gavage	Negative (> 100 mg/kg bw cytotoxic)	Bendele et al. (1985b)
DNA damage (single-strand breaks)	BALB/c mouse Spleen 4, 16, 24 h after treatment Kidney 24, 48 h after treatment Liver 24, 48, 72 h after treatment	2500 µg/kg bw intraperitoneally	Positive (max. response at 24 h) Positive (max. response at 24 h) Positive (max. response at 48 h; recovery at 72 h)	Creppy et al. (1985)
DNA damage	Wistar rat kidney, liver	290 µg/kg bw by gavage every 48 h for 6 or 12 weeks	Positive, no recovery between treatments	Kane et al. (1986b)

<sup>a</sup> No inhibition by indomethacin, suggesting absence of activation by prostaglandin H synthase

<sup>b</sup> Clastogenic effects due to changes in intracellular calcium

The LOD was 2.7 adducts/10<sup>9</sup> purified DNA bases. The authors also used a <sup>32</sup>P-postlabelling method with these rat kidney DNA samples and found adducts at levels ranging from 31 to 71/10<sup>9</sup> DNA bases 24 h after dosing, compared with 6–24/10<sup>9</sup> DNA bases in untreated controls. Since the adducts occurred at a level 3–17 times higher than the detection limit for scintillation counting and there was no evidence of tritium exchange, most, if not all, of the adducts observed by the <sup>32</sup>P-postlabelling method would not have contained an ochratoxin A moiety.

Furthermore, no adducts were found (detection limit, 20 adducts/10<sup>9</sup> DNA bases) by scintillation counting when DNA and [<sup>3</sup>H]ochratoxin A were incubated in the presence of male rat kidney microsomes with NADPH, mouse kidney microsomes with NADPH, rat seminal vesicle microsomes with arachidonic acid, or horseradish peroxidase with hydrogen peroxide.

#### (b) DNA damage and repair

There was no evidence of DNA repair as a result of possible DNA damage in bacteria, whereas DNA single-strand breaks were consistently induced in cultured mammalian cells. DNA single-strand breaks were also observed *in vivo* in spleen, liver, and kidney cells of mice after intraperitoneal injection of ochratoxin A. DNA repair, manifested as unscheduled DNA synthesis, was observed in most studies with primary cultures of rat and mouse hepatocytes, porcine epithelial cells from bladder, and human urothelial cells.

(c) *Gene mutation*

Most tests for gene mutation induction in bacteria showed no effect of exposure to ochratoxin A. Two studies showed positive results. One was in *S. typhimurium* strains TA1535 and TA1538 treated in the presence of mouse kidney microsomes (Obrecht-Pflumio et al., 1999), while the other was in *S. typhimurium* strains TA1535, TA1538, and TA100 treated with the culture medium of rat hepatocytes exposed to ochratoxin A (Hennig et al., 1991). Both papers described preliminary results that required further investigation before they could be readily accepted. It should be noted, however, that Hennig et al. (1991) obtained negative results with the same bacterial strains when rat liver microsomes were used as the exogenous metabolic activation system. This portion of the results has been confirmed in independent studies in other laboratories.

Gene mutations were not induced in the yeast *Saccharomyces cerevisiae* D3 (Kuczuk et al., 1978). In mammalian cells, gene mutations were not induced in two studies, while positive results were observed in a third. The last study was performed with NIH 3T3 cells transfected with a human CYP gene (De Groene et al., 1996) at a concentration of 25 µg/ml. In the studies with negative results, concentrations of 10 µg/ml (C3H mouse mammary cells) and > 12 µg/ml (mouse lymphoma L5178Y cells) were cytotoxic. The positive result therefore requires confirmation. No studies of mutation *in vivo* have been reported.

(d) *Chromosomal aberrations*

Sister chromatid exchange was induced in two of four studies *in vitro* but not in a single study *in vivo* after gavage of a range of doses that included cytotoxic doses.

Chromosomal aberrations were not induced in Chinese hamster ovary cells (National Toxicology Program, 1989) but were induced in cultured human lymphocytes (Manolova et al., 1990), and micronuclei were induced in ovine seminal vesicle cells and Syrian hamster embryo fibroblasts. *In vivo*, chromosomal aberrations were induced in mouse cells, an effect that was reduced by treatment of the mice with either ascorbic acid (by gavage) or vitamin A (in the diet). These protective effects are consistent with the observation that the formation of <sup>32</sup>P-postlabelling spots was prevented in some studies in which mice were treated with ochratoxin A (Grosse et al., 1997).

### 2.2.5 *Reproductive toxicity*

No adequate studies on the reproductive toxicity of ochratoxin A were available for review. Several studies of effects on developmental toxicity are summarized.

(a) *Mice*

Groups of 4–26 pregnant CBA mice were given a single dose of ochratoxin A in maize oil by gavage at 0, 1, 2, or 4 mg/kg bw on day 8 or 9 of gestation (day of vaginal plug considered to be day 1 after conception) or 4 mg/kg bw per day 2 days before mating and on days 2, 4, 6, 7, 10, and 14 of gestation, and observed until day 19. At this time, the numbers of viable and dead fetuses and the number of resorption sites were determined, and fetuses were weighed and examined for morphological

changes. No mention was made of maternal toxicity. Prenatal survival was decreased in groups that had received 4 mg/kg bw on days 7 (24% deaths), 8 (17% deaths), and 9 (22% deaths) of gestation. Overt craniofacial anomalies were seen only after treatment on day 8 or 9; their incidence, multiplicity, and severity increased with increasing dose, the peak effect being seen on day 9. The incidences of malformed pups among surviving pups were 0%, 0%, 8.1%, and 16% of mice given ochratoxin A at 0, 1, 2, or 4 mg/kg bw on day 8 of gestation, and 0%, 29%, 42%, and 91% of mice given the same doses on day 9 of gestation. The mean number of malformations per fetus was 0.3 and 2.3 on days 8 and 9 at 4 mg/kg bw, and 1.7 and 3.9 in animals given 8 mg/kg bw in a separate study. The central nervous system, the eye, and the axial skeleton were the main systems affected. The most important malformations were those affecting the craniofacial structures, including aplasia and dysplasia of the upper facial structures, such as exencephaly, microcephaly, blunt jaws, anophthalmia, microphthalmia, and median cleft face. In animals treated on day 9 of gestation at 4 mg/kg bw, the incidences of the various major anomalies were exencephaly, 89%; anophthalmia, 45%; microphthalmia, 27%; open eyelids, 16%; agenesis of external nares, 21%; cleft lip, 7.1%; median cleft face, 8.9%; and malformed jaws or short maxilla with protruding tongue, 41%. The craniofacial anomalies were considered by the authors to have arisen from failure of closure of the neurocranium, resulting in abnormal configuration, position, and size of the bones of the base and lateral walls of the skull (Arora & Frölen, 1981).

The effects of protein deprivation on the teratogenic effects of ochratoxin A were studied in groups of 10–13 CD-1 mice, maintained on diets providing 26% (control), 16%, 8%, and 4% purified protein (casein), after mating and throughout gestation. A single dose of ochratoxin A in 0.1 N sodium bicarbonate was administered by gavage at a dose of 0, 2, or 3 mg/kg bw on day 8 of gestation (vaginal plug considered to be day 1), and the mice were killed on day 18 of gestation for examination. The dams were monitored twice daily, and food consumption was monitored. Diets and water were available *ad libitum*.

Ochratoxin A treatment did not affect maternal food consumption, but maternal deaths were significantly more frequent in the group receiving ochratoxin A at 3 mg/kg bw and 26% protein (five deaths), in that given the same dose and 4% protein (four deaths), and in that given 2 mg/kg bw and 4% protein (nine deaths), with no deaths in the untreated groups. The percentages of litters with grossly malformed fetuses and the percentages of malformed fetuses (in brackets) for each of the four diets (26, 16, 8, and 4% protein, respectively) were 58 (25), 50 (17), 75 (45), and 100 (81) at 3 mg/kg bw; 25 (5), 50 (21), 30 (13), and 100 (78) at 2 mg/kg bw; and 0 (0), 0 (0), 18 (3), and 31 (9.8) without ochratoxin A. The fetal weights were reduced as a result of treatment and protein deprivation. Craniofacial malformations were the commonest abnormality, but at lower protein concentrations gross malformations affecting the limbs and tail were also seen (Singh & Hood, 1985).

In microcephalic mice derived from females treated intraperitoneally with ochratoxin A at 3 mg/kg bw on day 10 of gestation, a quantitative assessment of neurons and synapses at 6 weeks of age showed that the somatosensory cortices of treated mice had fewer synapses per neuron than those of controls, indicating reduced dendritic growth (Fukui et al., 1992).

*(b) Rats*

Five groups of 12–20 pregnant Wistar rats were given ochratoxin A at a total dose of 5 mg/kg bw in 0.16 mol/L sodium bicarbonate by gavage, as follows: a single dose of 2.5 mg/kg bw on each of days 8 and 9 of gestation (vaginal plug considered to be day 1), a dose of 1.2 mg/kg bw on each of days 8–11 of gestation, a dose of 0.83 mg/kg bw on each of days 8–13 of gestation, or a dose of 0.63 mg/kg bw on each of days 8–15 of gestation. A control group was given the vehicle only. In a similar way, three groups of 20 rats were given ochratoxin A at a single dose of 2.5 mg/kg bw on each of days 8 and 9 of gestation or a dose of 1.7 mg/kg bw on each of days 8–10 of gestation. The rats were killed on day 20 of gestation. No significant difference was seen in the number of implantations per female in the various groups. Females that had received the same total amount of ochratoxin A but divided into fewer single doses and early in gestation were most affected. There was a dose-related increase in the number of resorptions per female and decreases in the mean number of fetuses per female, mean fetal weight, and mean placental weight. A high dose-related incidence of fetal haemorrhages (seen at 2, 2.5, and 4 times the 1.2 mg/kg dose) and coelosome with or without oedema were considered to be teratogenic responses (Moré & Galtier, 1974).

In a study from the same laboratory, a similar protocol for administration of ochratoxin A was used, but the rats were observed until 82 days after birth. Dose-related decreases in the mean number of newborn rats, the mean number of rats alive at 4 days, and the viability index were seen, but not in the lactation index. In the group given 2.5 mg/kg bw twice, the mean body weights of male and female offspring at 82 days were reduced by 12 and 8%, respectively. Hydrocephalus was observed on day 15 after birth in 26% of the male offspring at that dose, and 40% of these animals died by 20 days after birth. A second generation was bred to examine residual maternal or paternal effects, without further administration of ochratoxin A. No differences in reproductive parameters were noted, and few details were given (Moré & Galtier, 1975).

A dose of 0.5 mg/kg bw given by gavage to rats on days 11–14 of gestation caused learning deficits in pups tested for 26 weeks (Kihara et al., 1984).

Oral administration of ochratoxin A to pregnant rats at 1 mg/kg bw per day on days 6–15 of gestation resulted in decreased fetal weight and increased numbers of resorptions but no overt adverse effects on the dams. Skeletal and/or lung malformations were reported in up to 20% of the fetuses; the incidence of renal malformations was 40%. Concurrent administration of methionine at 43 mg/kg bw protected against these adverse effects (Abdel-Wahhab et al., 1999).

Other studies on the teratogenicity of ochratoxin A in mice and rats treated intraperitoneally or subcutaneously were reviewed by Kuiper-Goodman & Scott (1989).

*(c) Chickens*

The embryotoxic potential of ochratoxin A was tested in chicks by injecting hens' eggs on day 3 and incubating them until day 13 or 18, when visible abnormalities,

weight, and length of chicks were recorded. A dose-related increase in the mortality rate was seen after injection of 1–2 µg of ochratoxin A. An increased frequency of abnormalities was seen in one of the two reported experiments (Edrington et al., 1995). The Committee noted that this is not a validated method, and the results could not be used in risk assessment.

*(d) In vitro*

Prechondrogenic mesenchymal cells from the limb buds of 4-day-old chick embryos were cultured with ochratoxin A for 6 days. Ochratoxin A inhibited the accumulation of cartilage proteoglycans and general protein synthesis in a dose-related manner (Wiger & Stormer, 1990).

Rat embryos explanted on day 10 of gestation were cultured in a medium containing ochratoxin A at concentrations up to 300 µg/ml. Dose-dependent reductions in the protein and DNA content of the embryos were seen. The malformations induced included hypoplasia of the telencephalon, stunted limb bud development, and decreased size of mandibular and maxillary bones. Cellular necrosis of mesodermal and neuroectodermal structures was observed (Mayura et al., 1989).

### **2.2.6 Special studies**

*(a) Covalent binding to nucleic acids and/or proteins*

Preliminary observations indicated no specific binding of ochratoxin A to macromolecules in porcine kidney cytosol (Stojković et al., 1984).

Subcellular fractions of a number of kidney-derived cell lines and rat intestine, liver, spleen, kidney, and plasma were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then incubated with ochratoxin A coupled with horseradish peroxidase in order to locate the ochratoxin A-binding proteins. The toxin was shown to bind to virtually all rat blood serum proteins and to some proteins in rat intestine, liver, spleen, and kidney, particularly at 60, 40, and 27 kDa. Binding of ochratoxin A to the 60- and 27-kDa proteins, but not the 40-kDa protein, was inhibited by phenylalanine and aspartame in liver but not in the other organs. The binding of ochratoxin A to cytosolic or organelle proteins was comparable in all the kidney cell lines, which were derived from various species and from various regions of the kidney. Phenylalanine and aspartame had no effect on the binding. The authors concluded that ochratoxin A can bind to several cellular proteins, and that this accounts for its accumulation in cells, but that the results do not explain the protective effects of phenylalanine and aspartame described previously (Schwerdt et al., 1999a).

*(b) Immunotoxicity*

*Mice*

The size of the mouse thymus was reduced to 33% that of controls after four intraperitoneal injections of ochratoxin A at 20 mg/kg bw on alternate days, a dose

which caused minimal nephrotoxicity. Bone marrow depression was shown as dose-related, significantly ( $p < 0.01$ ) decreased marrow cellularity, including a reduction in bone marrow macrophage–granulocyte progenitors, a decreased number of haematopoietic stem cells and a significant decrease in erythropoiesis as measured by  $^{59}\text{Fe}$  uptake; increased phagocytosis by macrophages was also observed (Boorman et al., 1984).

Residual damage was seen 3 weeks after exposure as increased sensitivity to radiation, even though bone-marrow cellularity and the peripheral blood count had returned to normal (Hong et al., 1988; National Toxicology Program, 1989).

Ochratoxin A administered to 8–10-week-old Swiss mice at 5 mg/kg bw per day by intraperitoneal injection for 50 days reduced the antibody response to *Brucella abortis*, a cell-mediated immune response. This was postulated to be due to suppression of immunoglobulin (Ig)M synthesis. The same treatment also reduced mitogen (concanavalin A)-induced blast formation in lymphocytes derived from mouse spleen (Prior & Sisodia, 1982).

Groups of eight female BALB/c mice were fed diets containing ochratoxin A at a concentration of 6, 250, or 2600  $\mu\text{g}/\text{kg}$  for 28 or 90 days, equivalent to 1, 40, and 400  $\mu\text{g}/\text{kg}$  bw per day. Treatment did not cause changes in body or lymphoid organ weights. Kidney weights were reduced at the two higher doses at 28 days and at the highest dose at 90 days. The concentrations of ochratoxin A in kidney were clearly dose-related. No differences in leukocyte count were observed, but a significant reduction in the number of spleen cells (by about 20%) was observed at the highest dose after 90 days. No changes were observed in blood or thymic T lymphocytes at 28 days, but a decreased proportion of mature  $\text{CD4}^+$  and  $\text{CD8}^+$  cells was seen with a corresponding increase in the immature double-positive sub-population at the two higher doses after 90 days. After 28 days, the primary (humoral) antibody response to sheep red blood cells was significantly suppressed in a dose-dependent manner at the two higher doses. The antibody response to another T-cell-dependent antigen (viral antigen PR8) was not affected, suggesting that exposure to ochratoxin A alters certain immune functions in mice, and, as previously demonstrated, the spleen may be the most sensitive immune tissue to ochratoxin A. Differences in the proportions of mature and immature  $\text{CD4}^+$  and  $\text{CD8}^+$  populations suggest that ochratoxin A may affect late-stage differentiation of T cells (Thuvander et al., 1995).

Female BALB/c mice were given diets containing ochratoxin A to provide a calculated average dietary intake of 5–30  $\mu\text{g}/\text{kg}$  bw per day for 2 weeks before mating. At birth, the pups were cross-fostered to unexposed dams. Exposed and control pups were killed at 14 and 28 days of age. Ochratoxin A did not effect the reproductive outcome or body weight of pups. No differences in spleen or thymus weight or cell numbers were observed on day 14, but significant increases were seen in both thymus weights (by 20%) and cell number (by 67%) in the offspring of dams at the high dose on day 28. Although the percentages of splenic  $\text{CD4}^+$  and  $\text{CD8}^+$  cells were decreased in pups at the high dose, there were no alterations in absolute numbers. No significant differences were observed in the proliferative responses of splenic or thymic lymphocytes to mitogens nor in the production of

interleukin-2 in concanavalin A-stimulated cell cultures. No significant differences in the humoral antibody response to sheep red blood cells or viral antigen PR8 were found. Natural killer cell activity on day 28 was not affected by prenatal exposure to ochratoxin A. Thus, the treatment did not suppress immune function but altered the absolute and relative numbers of lymphocyte subpopulations in lymphoid organs (Thuvander et al., 1996a).

Groups of 10 Han-NMRI mice (sex not specified) received commercial (Serva) or 'raw' ochratoxin A at a dose of 1, 3, or 6 mg/kg bw per day by intraperitoneal injection for 8–17 days and were then monitored for up to 20 days. Animals receiving 'raw' ochratoxin A at 3 mg/kg bw per day had a significantly lower body weight than controls on days 5–17; however, this correlated with a reduction in feed consumption. No significant change in body weight was noted in the groups receiving crystalline ochratoxin A. The total leukocyte count was unchanged in all groups; however, lymphopenia, neutrophilia, and eosinophilia were observed at 3 and 6 mg/kg bw per day. The blood IgM titre was suppressed at these doses in a dose-dependent manner. The authors concluded that ochratoxin A has a nonselective suppressive effect on various immune reactions, but the paper contains inadequate detail to verify their conclusion (Müller et al., 1995).

### *Rats*

Bone-marrow hypocellularity and a reduced thymic size were also seen in Fischer rats given ochratoxin A at 1 or 4 mg/kg bw per day by gavage for 16 days (National Toxicology Program, 1989).

Necrosis of germinal centres in the spleen and lymph nodes was seen in Wistar rats given a single dose of ochratoxin A at 5–50 mg/kg bw (Kanisaw et al., 1977) and in dogs given ochratoxin A by capsule at doses of 0.1–0.2 mg/kg bw per day for 14 days (Kitchen et al., 1977c).

The effects of ochratoxin A on the bone marrow and lymphatic cell population may reflect the sensitivity of these cells to the inhibition of protein synthesis induced by ochratoxin A. These effects on the structural components of the immune system indicated that ochratoxin A is likely to have an effect on immune function.

The immunotoxic effects of perinatal exposure to ochratoxin A were investigated in the offspring of Sprague-Dawley rats treated singly or repeatedly. In a short-term study, dams received a single oral dose of 10, 50, or 250 µg/kg bw on day 11 of lactation, and the pups were examined on day 14. Dose-dependent uptake of ochratoxin A was observed in both dams and pups. The toxin did not induce consistent changes in the weights of the lymphoid organs of pups. A small but significant increase in the number of thymocytes was observed in offspring of dams dosed at 50 µg/kg bw, but it was not dose-dependent. A small but significant decrease in the proliferative response of splenocytes to T-cell mitogen lipopolysaccharide was seen in pups of dams given 250 µg/kg bw. In contrast, exposure to 10–50 µg/kg bw per day resulted in significant increases in the proliferative responses of both splenocytes and thymocytes of pups to concanavalin A. This was not seen at the higher dose. The authors proposed that short-term exposure of suckling pups via the milk stimulates

the immune response, measured as proliferation of lymphocytes in response to concanavalin A and lipopolysaccharide (Thuvander et al., 1996b).

In a long-term study, dams received repeated oral doses of ochratoxin A at 50 µg/kg bw on 5 days/week for 2 weeks before mating, during gestation, and then 7 days/week until weaning. At parturition, the number of pups was reduced to eight per litter and they were cross-fostered to produce groups of prenatally, postnatally, and pre- and postnatally exposed pups. The highest blood concentrations of ochratoxin A were detected in pups exposed both pre- and postnatally, but exposure via the milk appeared to account for most of the content. Long-term exposure to 50 µg/kg bw per day did not induce any consistent changes in body or lymphoid organ weights of pups, but prenatal exposure suppressed the lymphocyte response to both B- and T-cell mitogens at 14 days of age. The background proliferation of unstimulated cells was significantly suppressed in cultures from prenatally exposed pups. These effects were not observed in pups exposed during lactation, although the blood concentrations were higher in pups exposed postnatally. Prenatally exposed pups showed a significantly lower primary antibody response to PR8 viral antigen ( $\pm 0.36$ ). No significant difference in the natural killer cell activity of splenocytes was measured in exposed pups at 13 weeks of age. The authors concluded that long-term prenatal exposure to ochratoxin A, but not postnatal exposure via milk, may cause immunosuppression; however, short-term postnatal exposure may stimulate proliferation of lymphocytes in response to mitogens (Thuvander et al., 1996b).

The Committee noted that no details were given about the ochratoxin A used. These authors previously used commercial ochratoxin A, but in this paper they quoted a 1984 reference for details of how the ochratoxin A was produced. The size of the groups was not given, but they seem to have consisted of four to five dams.

### *Pigs*

Groups of six weanling hybrid pigs received either pure or crude ochratoxin A at doses of 7–50 µg/kg bw per day by subcutaneous injection for 19–39 days. The animals were immunized 8 days after ochratoxin A challenge with *Pasteurella* by inhalation. The authors stated that ochratoxin A had no effect on body-weight gain and that the serum concentrations were dose-dependent. The concentrations were reported to be lower after administration of crude ochratoxin A than pure material. A reduction in relative lymphocyte count and increases in total leukocyte, relative neutrophil, and eosinophil counts were seen. Crude toxin had a greater effect than pure toxin. Ochratoxin A decreased the phagocytosis index of individual cells and decreased expression of SWC1 (a lymphocyte cell surface marker) but did not change lymphocyte proliferation (Müller et al., 1999).

The Committee noted that many of the results were conflicting and the study was inadequately reported.

### *Chickens*

In chickens fed diets containing ochratoxin A at a concentration of 2–4 mg/kg for 20 days, the lymphoid cell population of immune organs was decreased (Dwivedi & Burns, 1984a).

Several studies have shown that ochratoxin A affects both humoral and cell-mediated immunity. In chickens fed a diet containing ochratoxin A at 5 mg/kg for 56 days, the contents of  $\alpha$ 1-,  $\alpha$ 2-,  $\beta$ -, and  $\gamma$ -globulins in plasma were reduced (Rupić *et al.*, 1978).

In chickens fed diets containing ochratoxin A at a concentration of 2–4 mg/kg for 20 days, immunoglobulin (Ig)G, IgA, and IgM in lymphoid tissues and serum were depressed (Dwivedi & Burns, 1984b), and complement activity was slightly affected in birds fed at diets containing 2 mg/kg for 5–6 weeks (Campbell *et al.*, 1983).

Ochratoxin A also reduced IgG and increased IgM in the bursa of Fabricius in chick embryos that had been injected with 2.5  $\mu$ g of the toxin on day 13. This did not affect their immunocompetence, however, as seen after challenge of the hatched chickens with *E. coli* at 1, 2, and 4 weeks of age, indicating that the effect on immunoglobulins may have been transient (Harvey *et al.*, 1987).

Immunosuppression was observed in chickens fed diets containing ochratoxin A at 0.5 or 2 mg/kg for 21 days. When compared with controls, the treated animals had reduced total serum protein, lymphocyte counts, and weights of the thymus, bursa of Fabricius, and spleen (Singh *et al.*, 1990).

#### *In vitro*

The effects of ochratoxin A on T-cell activation were investigated in purified (> 95%) human lymphocytes cultured in medium containing 1% bovine serum albumin. Intracellular free  $\text{Ca}^{2+}$  and activation of protein kinase C were measured as indicators of the early stages of activation; the effect on phytohaemagglutinin-induced proliferation was measured as a late event mediated by expression of functional interleukin-2 receptors. The early-stage events were not inhibited by ochratoxin A at a concentration of 12  $\mu$ mol/L. In contrast, incubation of ochratoxin A with phytohaemagglutinin-stimulated lymphocytes resulted in inhibition of DNA synthesis at concentrations  $\geq$  6.4  $\mu$ mol/L. Protein synthesis in resting lymphocytes was markedly inhibited by 12  $\mu$ mol/L but to a lesser extent in phytohaemagglutinin-stimulated lymphocytes. The authors concluded that ochratoxin A can block DNA synthesis at a late stage in lymphocyte activation and that this effect may be partially mediated by inhibition of protein synthesis (Størmer & Lea, 1995).

Ochratoxin A also inhibited the proliferative response of bovine peripheral blood mononuclear cells cultured in 10% fetal calf serum. The  $\text{ID}_{50}$  value varied from 0.1 to 4  $\mu$ g/ml, depending on the mitogen used to stimulate the cells and the incubation time. The authors considered these results indicative of immunosuppressive potential (Charoenpornsook *et al.*, 1998).

#### (c) Neurotoxicity

##### *Rats*

Three male Wistar rats received 1 nmol (about 400 ng) of ochratoxin A by intracerebral administration and four received a diet containing 290  $\mu$ g/kg by oral

gavage for 8 days. The animals were killed 24 h after dosing. Although ochratoxin A was detected in areas of the central nervous system after intracerebral injection, it was not detected in the periphery or blood, kidney, or urine, indicating that it is transferred poorly or not at all from the spinal fluid to blood, kidney, or urine. After administration in the diet, the ventral mesencephalon, hippocampus, striatum, and cerebellum were the main targets of cytotoxicity in rat brain (Belmadani et al., 1998a)

Four male Wistar rats received ochratoxin A at 290  $\mu\text{g}/\text{kg}$  bw orally every 48 h for 1–6 weeks. The treated animals had a slight reduction in body weight after 4 weeks, but feed and water consumption were not significantly different from those of controls. Ochratoxin A accumulated in the brain in a linear time-dependent manner, to reach about 100 ng/g of brain after 6 weeks. The toxin was shown to change the concentrations of the amino acids tyrosine and phenanthrene and to damage tissues in the hippocampus (Belmadani et al., 1998b)

Ten adult female Fischer rats received ochratoxin A at 120  $\mu\text{g}/\text{kg}$  bw per day by oral gavage for 10, 20, or 35 days. Treatment altered the activity of all enzymes tested. Significant increases in  $\gamma$ -glutamyl transferase activity were observed in the three brain regions examined. The changes in the other enzyme activities were regionally selective, but most of the activities had returned to control levels by day 35 of dosing (Zanić-Grubisić et al., 1996).

(b) *In vitro*

The neurotoxicity of ochratoxin A has been investigated in nerve tissue cell cultures (embryonic chick neural retina and brain) and cultured meningeal fibroblasts. The cells were incubated with ochratoxin A in serum-free medium for 8 days. The median inhibitory concentration ( $\text{IC}_{50}$ ) for a number of parameters of cytotoxicity (cellular protein, 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide [MTT] reduction, neutral red uptake) were found to be about 170 nmol/L in all three culture systems, indicating that ochratoxin A did not have cell-specific effects. Ochratoxin B and the heat-induced 3S-epimer of ochratoxin A induced comparable effects at 19- and 10-fold higher concentrations, respectively (Bruinink et al., 1997).

In a study with a similar protocol, markers of neuritic outgrowth and differentiation (NF68 and 160 kDa, MAP2 and MAP5) were affected at significantly lower concentrations than the markers of cytotoxicity. Although the presentation of the data is unclear, the  $\text{IC}_{50}$  values for the most sensitive parameters appeared to be 20–50 nmol/L for the embryonic brain and neural retinal cultures. Binding of ochratoxin A to bovine serum albumin resulted in significantly decreased potency ( $\text{IC}_{50}$  values increased by 15–30-fold). Differences were noted between serum-free primary cultures and the cell lines. In these culture systems, phenylalanine did not decrease the effects of ochratoxin A and in contrast appeared to cause a concentration-related decrease in the  $\text{IC}_{50}$  [no statistical analysis presented]. The authors concluded that ochratoxin A specifically affected neurite formation and that its toxicity was decreased by protein binding but not by phenylalanine (Bruinink & Sidler, 1997).

These authors also investigated whether the effects of ochratoxin A could be attributed to its isocoumarin structure, by comparing the toxicity of ochratoxin A with that of ochratoxin  $\alpha$  and ochracin in serum-free embryonic chick brain cultures.

Ochratoxin A decreased the end-points at concentrations > 15 nmol/L, with a greater effect on neurite outgrowth (neurofilament 68 kD). Ochratoxin  $\alpha$  and ochracin had minimal effects at concentrations up to 1 mmol/L. The isocoumarin structure was therefore considered not to be responsible for the toxicity of ochratoxin A in this brain cell culture model (Bruinink et al., 1998).

The regional selectivity of ochratoxin A was investigated in primary cultures of neurons and astrocytes isolated from embryonic or newborn rat brain ventral mesencephalon and cerebellum. The cultures were exposed to ochratoxin A in a medium containing 10% fetal calf serum for 46 h, before measurement of DNA and protein synthesis, lactate dehydrogenase leakage, and lipid peroxidation. Ochratoxin A inhibited protein and DNA synthesis in all cell types, with  $IC_{50}$  values ranging from 14 to 69  $\mu$ mol/L. Neuronal cells were more sensitive than astrocytes, and the cells of the ventral mesencephalon were more sensitive than those of the cerebellum. Increases in lactate dehydrogenase leakage and lipid peroxidation were also seen, but the sensitivity of the cell types did not mirror that for DNA and protein synthesis. The authors concluded that ochratoxin A is neurotoxic and may affect particular structures of the brain (Bruinink et al., 1998).

(d) *Nephrotoxicity*

*In vivo*

Renal function and morphology are greatly affected at high doses of ochratoxin A, as indicated by increased kidney weight, urine volume, blood urea nitrogen (Hatey & Galtier, 1977), urinary glucose, and proteinuria (Berndt & Hayes, 1979). The last two findings indicate that the site of reabsorption, i.e. the proximal convoluted tubules, is damaged. The NOELs for changes in renal function depend on the species and on the parameter tested. At low doses of ochratoxin A, no increase in blood urea nitrogen, creatinine, or glucose was found in the urine of male or female rats given 210  $\mu$ g/kg bw per day by gavage for 6–12 months, but a mild to moderate decrease in the ability to concentrate urine was seen. The NOEL for this effect was 70  $\mu$ g/kg bw per day for male rats and 21  $\mu$ g/kg bw per day for female rats (National Toxicology Program, 1989).

Various groups of investigators have shown that this specific nephrotoxic effect is due to an ochratoxin A-induced defect of the organic anion transport mechanism located on the brush border of the proximal convoluted tubule cells and basolateral membranes (Endou et al., 1986; Sokol et al., 1988). The organic ion transport system is also the mechanism by which ochratoxin A enters proximal tubular cells (Friis et al., 1988; Sokol et al., 1988).

The middle (S2) and terminal (S3) segments of the proximal tubule of isolated nephron segments were found to be the most sensitive to the toxic effects of ochratoxin A (0.05 mmol/L), as shown by a significant decrease in cellular ATP and a dose-related decrease in mitochondrial ATP content (Jung & Endou, 1989).

Several investigators have measured the effect of ochratoxin A on the release of enzymes from the kidney into the urine. Changes in enzyme and protein patterns can be used to distinguish different types of renal injury (Stonard et al., 1987).

Subcutaneous doses of ochratoxin A at 10 mg/kg bw for 5 days decreased the activity of muramidase and then decreased the activities of lactate dehydrogenase, alkaline phosphatase, glutamate dehydrogenase, and acid phosphatase in the kidney (Ngaha, 1985). The activities of alanine peptidase, leucine amino peptidase, and alkaline phosphatase were decreased by 60%, 50%, and 35%, respectively in isolated kidney tubules in the presence of 0.1 mmol/L ochratoxin A (Endou et al., 1986).

In male rats given ochratoxin A at 0.1–2 mg/kg bw per day orally for 2–5 days, the phosphoenolpyruvate carboxykinase activity decreased by 50–70% at the highest dose (Meisner et al., 1983; Meisner & Krogh, 1986). The minimum effect level was 0.1 mg/kg bw per day (Meisner & Polsinelli, 1986); at 2 mg/kg bw per day, enzymes such as pyruvate carboxylase, malate dehydrogenase, hexokinase, and  $\gamma$ -glutamyl transpeptidase were not affected (Meisner & Selanik, 1979).

In rats given ochratoxin A by gavage at a dose of 0.14 mg/kg bw every 48 h (equivalent to about 2 mg/kg diet) for 8–12 weeks, the activities of lactate dehydrogenase, alkaline phosphatase, leucine aminopeptidase, and  $\gamma$ -glutamyl transferase decreased significantly. The last three enzymes are located in the brush border of the proximal convoluted tubules, indicating damage at that site. Concomitantly with the decrease of enzyme activity in the kidney, these enzymes appeared in the urine. A late event was a urinary increase in the activity of *N*-acetyl  $\beta$ -D-glucosidase, a lysosomal enzyme. The activity of this enzyme in the kidney was not affected (Kane et al., 1986a). The late appearance of this enzyme may indicate active regeneration and exfoliation of necrotic proximal convoluted tubular cells, releasing lysosomal enzymes (Stonard et al., 1987). In this study, *para*-aminohippurate clearance was reduced initially by 56% at 2 weeks and 8% at 12 weeks of dosing, indicating damage followed by regeneration.

Pigs are very sensitive to the effect of ochratoxin A on renal enzyme activity. In the kidneys of pigs fed diets containing ochratoxin A at 0.2–1 mg/kg, equivalent to 0.008–0.041 mg/kg bw per day, a dose-related decrease in the activity of phosphoenolpyruvate carboxykinase and  $\gamma$ -glutamyl transpeptidase was accompanied by a dose-related decrease in renal function, as indicated by a reduction in the maximal tubular excretion of *para*-aminohippurate per clearance of inulin and an increase in glucose excretion. Only cytosolic, and not mitochondrial, phosphoenolpyruvate carboxykinase activity was inhibited (Meisner & Krogh, 1986; Krogh et al., 1988).

Subcutaneous administration of superoxide dismutase and catalase together was found to offset the nephrotoxic effects of ochratoxin A, leading to the suggestion that superoxide radicals and hydrogen peroxide are likely to be involved in the nephrotoxic effects of ochratoxin A in vivo (Baudrimont et al., 1994).

Male Wistar rats weighing 70–150 g were given ochratoxin A in order to determine its effects on the pH in the vasa recta of the renal papilla after a single intravenous injection of 3  $\mu$ mol/kg bw or six intraperitoneal injections of 1.2  $\mu$ mol/kg bw per day. Both regimes increased the pH in the descending and ascending vasa recta, with no significant difference between the renal arterial and aortic pH values, in serum and urine osmolality, or in urinary flow rate. An increased pH over that before treatment

was detected in the collecting ducts of individual animals after an intravenous dose, but the differences between groups were not significant. Treated rats had a lower body-weight gain (19%) than controls (34%) during the 6-day intraperitoneal treatment. The authors suggested that ochratoxin A upsets pH homeostasis in the interstitium of the renal papilla, leading to alkalinization, in addition to impairment of urinary acidification (Kuramochi et al., 1997a).

In a subsequent study in which male Wistar rats were treated intravenously with ochratoxin A at 3  $\mu\text{mol/kg}$  bw, the pH in the proximal tubule, distal tubule, and collecting ducts and the descending and ascending vasa recta was increased. The concentration of bicarbonate ion increased significantly in the proximal tubule and collecting ducts of treated animals, but there were no significant differences between treated and control animals in any of the parameters in aortic blood. No significant differences were observed in  $\text{pCO}_2$  in any region, and there were no significant differences in serum or urine osmolality or urinary flow rate. The authors concluded that ochratoxin A increased the pH and bicarbonate ion concentration in the tubular fluid or vasa recta but did not alter  $\text{pCO}_2$ . They hypothesized that this disturbance in pH homeostasis could contribute to alterations in acid–base status and hence to the nephrotoxicity of ochratoxin A (Kuramochi et al., 1997b).

Groups of Wistar rats (sex not specified) were given ochratoxin A at 0, 0.4, or 0.8 mg/kg bw intraperitoneally every 72 h for 90 days in order to investigate the relationship between the pathogenesis of nephropathy and the genotoxic and carcinogenic effects of ochratoxin A. Treatment resulted in significant, dose-related decreases in relative kidney weight (80 and 77% of control), average kidney length (83 and 78% of control), and creatinine clearance (90 and 76% of control) at 0.4 and 0.8 mg/kg bw, respectively. Severe renal atrophy was reported in animals at both doses, but these were not dose-related. Dose-dependent concentrations of ochratoxin A were found in the blood (920 and 1900 ng/ml) and kidney (30 and 170 ng/g) at the two doses, respectively. The urinary concentration was similar at both doses, consistent with the low urinary elimination of ochratoxin A. Histological examination of the kidneys of rats given 0.8 mg/kg bw every 72 h for 30 days showed giant karyomegalic tubule cells with limited degeneration of interstitial tissue and fewer apoptotic bodies in the tubule epithelium than in the kidneys of control animals. The authors also found abnormal mitoses after 30 days of dosing and suggested that regeneration would have occurred if treatment had been stopped (Maaroufi et al., 1999).

Administration of ochratoxin A at 1 or 3 mg/kg of diet and cholestyramine at 1 or 5% of diet for up to 14 days decreased the plasma concentrations of ochratoxin A and the urinary and biliary excretion of this toxin and its metabolites. Increased concentrations of ochratoxin A were found in the faeces. The authors suggested that cholestyramine may have decreased the absorption of ochratoxin A, either by interfering with bile acid secretion or by direct binding (Kerkadi et al., 1998).

These authors subsequently confirmed that cholestyramine can bind ochratoxin A and bile salts *in vitro* and that depletion of bile salts by interruption of enterohepatic circulation in rats resulted in decreased plasma concentrations of ochratoxin A (Kerkadi et al., 1999).

*In vitro*

When ochratoxin A was added to isolated rat renal proximal tubules in suspension, mitochondrial dysfunction was seen as an early event in the process of nephrotoxicity. Mitochondrial impairment apparently occurred at sites I and II of the respiratory chain. Although lipid oxidation occurred before cell death, it did not seem to be responsible for the toxic effect (Aleo et al., 1991).

The effects of ochratoxin A on cell growth, cell viability and transepithelial transport were investigated in male Wistar rat proximal tubule cells in serum-free primary culture. Biphasic effects were reported, depending on the concentration of ochratoxin A (0.1–10  $\mu\text{mol/L}$ ) and the incubation time (24–72 h). The authors considered that these effects could be explained by accelerated cell growth, followed by decreased DNA synthesis due to increased cell density. Addition of albumin or acidification of the culture medium reduced the effects of ochratoxin A in a concentration-dependent manner, whereas alkalization to pH 7.7 had little effect. Transepithelial electrolyte transport was disrupted at 10  $\mu\text{mol/L}$  but not at 0.1  $\mu\text{mol/L}$ . The authors concluded that ochratoxin A at physiological (nanomolar) concentrations can stimulate proliferation of proximal tubule cells without exerting toxic effects or reducing cell viability, and that this effect may be mediated by ochratoxin A-induced changes of cellular pH homeostasis (Gekle et al., 1995).

Transport of ochratoxin A by the kidney-specific organic anion transporter 1 (ochratoxin AT1) was investigated in *Xenopus* oocytes, which transiently express ochratoxin AT1, and cultured S3 cells, with stable expression of ochratoxin AT1, in 5% fetal calf serum. Oocytes with ochratoxin AT1 took up significantly more ochratoxin A (4  $\mu\text{mol/L}$ ) than oocytes transfected with the vector alone. *para*-Aminohippurate, probenecid, prioxicam, octanoate, and citronin, which have been reported previously to inhibit the nephrotoxicity of ochratoxin A, inhibited uptake. Uptake of the compound was also greater in ochratoxin AT1-expressing S3 cells than in the mock-transfected parent cells. Cell proliferation was significantly decreased by ochratoxin A at 2 and 10  $\mu\text{mol/L}$ , and their viability was decreased by 10  $\mu\text{mol/L}$ , in ochratoxin AT1-expressing S3 cells but not in the mock-transfected cells. Incubation with *para*-aminohippurate suppressed the effects of ochratoxin A. The authors concluded that ochratoxin AT1 plays a pivotal role in the nephrotoxicity of mycotoxins (Tsuda et al., 1999).

The transport of ochratoxin A across the renal peritubular membrane was studied in suspensions of freshly isolated rabbit renal proximal tubules, in order to investigate whether the accumulation of ochratoxin A in proximal tubule cells is involved in its nephrotoxicity. The accumulation and kinetics were determined by fluorescence, which correlates linearly with the concentration of ochratoxin A over a range of 1–70 nmol/L. Accumulation of 10  $\mu\text{mol/L}$  was approximately linear for 60 s and approached steady state after 5 min. The uptake was almost completely blocked by 2 mmol/L probenecid, which inhibits the organic anion transport pathway. *para*-Aminohippurate, which is the prototypic substrate for the peritubular organic anion transporter, also inhibited ochratoxin A uptake, but only by 40–50% at a concentration of 2.5 mmol/L, indicating that uptake occurs by a mechanism in addition to the organic anion transporter. Use of other inhibitors indicated that phenylalanine was

not involved, but that a fatty acid transporter may also contribute to uptake of ochratoxin A. The overall results suggest that the peritubular membrane is a significant site for accumulation of ochratoxin A (Groves et al., 1998).

The potential of ochratoxin A to induce apoptosis was examined in human proximal tubule-derived cells (IHKE cells) and compared with that in renal-cell lines derived from opossum proximal tubule (OK cells) and from canine renal collecting duct (MDCK-C11 and MDCK-C7 cells), cultured in 19% fetal calf serum. Ochratoxin A induced a time- and concentration-dependent increase in caspase 3 activity in IHKE cells. Significant increases were seen at 5 nmol/L and a 7-day incubation and at 10 nmol/L with a 24 or 72-h incubation. DNA fragmentation and chromatin condensation confirmed the occurrence of apoptosis at concentrations of 30 and 100 nmol/L. The free-radical scavenger *N*-acetylcysteine and the intracellular calcium chelator BAPTA-AM, had no effect on ochratoxin A-induced caspase 3 activation, indicating that the mechanism did not involve free-radical production or disturbed calcium homeostasis. IHKE cells were more sensitive to low concentrations of ochratoxin A than MDCK-C11, MDCK-C7, or OK cells. The authors concluded that low concentrations of ochratoxin A led to caspase 3 activation and subsequently apoptosis in cultured human proximal tubule cells but that the mechanism was unclear (Schwerdt et al., 1999b).

The ability of ochratoxin A to activate c-Jun *N*-terminal kinase has also been investigated in two clones of the MDCK kidney-derived cell line (C7 resembling principal cells and C11 resembling intercalated cells), cultured in 10% fetal calf serum. Incubation with ochratoxin A for 8 h at 10 nmol/L, 100 nmol/L, or 1  $\mu$ mol/L resulted in stimulation of c-Jun *N*-terminal kinase 1 in MDCK-C7 cells but not in MDCK-C11 cells. Apoptosis, as measured by caspase 3 activity and DNA fragmentation, was observed in MDCK-C7 cells treated with ochratoxin A at 100 nmol/L, which did not cause necrosis as measured by leakage of the cytosolic enzyme lactate dehydrogenase. MDCK-C11 cells were less responsive than MDCK-C7 cells, with a smaller increase in caspase 3 activity at 300 nmol/L ochratoxin A. Lactate dehydrogenase leakage was proportional to DNA fragmentation, indicating that ochratoxin A primarily caused necrosis in MDCK-11 cells. Ochratoxin A at 0.1–0.5  $\mu$ mol/L also potentiated the pro-apoptotic action of tumour necrosis factor- $\alpha$  in a concentration-dependent manner, with a greater effect in MDCK-C7 cells than in MDCK-C11 cells. The cell specificity demonstrated in this study indicates that c-Jun *N*-terminal kinase signalling pathways may play a role in ochratoxin A-induced apoptosis. The authors suggested that this may explain some of the changes in renal function and teratogenicity induced by the toxin (Gekle et al., 2000).

Ochratoxin A inhibited protein synthesis and caused leakage of cytosolic enzymes in Vero monkey kidney cells cultured in the presence of 5% newborn calf serum. Incubation with ochratoxin A for 24 h in the presence of aspartame at 250  $\mu$ mol/L increased the  $IC_{50}$  for inhibition of protein synthesis from 14  $\mu$ mol/L to 22  $\mu$ mol/L. An increase to 34  $\mu$ mol/L was seen when the cells were incubated with the same concentration of aspartame for 24 h before addition of ochratoxin A. Aspartame was also shown to prevent binding of ochratoxin A to plasma proteins and to displace ochratoxin A already bound to plasma proteins. The authors concluded that aspartame could decrease the toxicity of ochratoxin A by affecting binding to plasma

proteins as well as by preventing inhibition of protein synthesis (Baudrimont et al., 1997). The Committee noted that these effects required high concentrations of aspartame.

(e) *Mechanism of tumorigenesis*

The mechanisms of tumour induction in rodent kidney by ochratoxin A have been addressed in many studies, including investigations of the role of biotransformation and bioactivation and the formation of ochratoxin A-derived nucleic acid derivatives in target and non-target organs for toxicity. The results diverge, as do those of the studies on mutagenicity. Although no definite mechanism for the carcinogenicity of ochratoxin A to rodent kidney has been described, non-genotoxic events make a major contribution to the induction and progression of ochratoxin A-derived renal tumours.

Several studies have addressed the biotransformation of ochratoxin A and its role in its toxicity. Biotransformation has been postulated to be involved in the DNA binding and renal tumorigenicity of ochratoxin A, and a variety of CYPs, peroxidases, and glutathione *S*-transferases have been suggested to catalyse the transformation of ochratoxin A to reactive intermediates (Hietanen et al., 1991; Hennig et al., 1991; Würigler et al., 1991; Malaveille et al., 1994; Fink-Gremmels et al., 1995; Obrecht-Pflumio et al., 1996; Grosse et al., 1997; Pfohl-Leszkowicz et al., 1998; Obrecht-Pflumio et al., 1999; El Adlouni et al., 2000). However, none of these studies assessed the capacity of the respective enzymes to transform ochratoxin A to metabolites or suggested the structure(s) of a reactive metabolite (Castegnaro et al., 1998). Most studies assessed potentially relevant end-points in the toxicity of ochratoxin A and their modulation by changes in xenobiotic-metabolizing enzyme activities. Because of these limitations, no conclusions can be drawn about the mechanisms of ochratoxin A-induced tumour formation in rat kidney.

The possible biotransformation reactions of ochratoxin A have been postulated on the basis of rigorous analytical chemistry. Formation of an ochratoxin A-derived reactive quinone was suggested (Gillman et al., 1999), but this metabolite was formed only by a chemical system that mimics the CYP system. The ochratoxin A-derived reactive quinone was not detected by the use of isolated enzymes and microsomes with high activity for specific CYPs, and only 4R- and 4S-hydroxy-ochratoxin A were formed at very low yields (Gautier et al., 2001; Zepnik et al., 2001). Subcellular fractions rich in prostaglandin synthase activity or purified CYP enzymes also did not catalyse the formation of reactive ochratoxin A metabolites (Gautier et al., 2001).

The known mechanisms of formation of ochratoxin A metabolites (insertion of an oxygen into a carbon–hydrogen bond) do not suggest formation of reactive and toxic intermediates. The lack of involvement of CYP-mediated oxidation in the toxicity of ochratoxin A is supported by the observation that increasing the rates of biotransformation of the toxin by induction of CYP decreases its renal toxicity (Omar et al., 1996), and the observation of typical toxic effects of ochratoxin A in cell systems with very low or no CYP activity (Seegers et al., 1994; Hoehler et al., 1996; Xiao et al., 1996; Dopp et al., 1999). The formation of ochratoxin A-derived radicals capable of interacting with macromolecules is also not indicated. In contrast, the electron spin resonance spectra suggest the formation of hydroxy radicals (Hoehler et al., 1996, 1997).

Formation of DNA adducts has also been postulated as an important event in the tumorigenicity of ochratoxin A. The formation of spots interpreted as ochratoxin A-derived DNA adducts was observed in target tissues in rodents by the very sensitive  $^{32}\text{P}$ -postlabelling assay. The nature of the DNA damage and/or mutations caused by ochratoxin A is unknown (Pfohl-Leskowicz et al., 1991; Würzler et al., 1991; Grosse et al., 1995, 1997; Obrecht-Pflumio & Dirheimer, 2000). The end-points in many of the studies on the mechanisms of tumorigenicity of ochratoxin A was the possible formation of DNA adducts (spots by  $^{32}\text{P}$ -postlabelling). However, a role of DNA binding of ochratoxin A is not supported by the results of studies of biotransformation cited above or of experiments to investigate the binding of radiolabelled ochratoxin A to nucleic acids (Gautier et al., 2001). Studies of DNA binding with [ $^3\text{H}$ ]ochratoxin A revealed no binding of 'metabolically activated' ochratoxin A to calf thymus DNA *in vitro* or to DNA from rat liver or kidney *in vivo*. The sensitivity of these experiments was similar to that of the postlabelling studies. Lack of DNA binding of ochratoxin A or its metabolites was observed *in vivo* after administration of a single dose of [ $^3\text{H}$ ]ochratoxin A (Rasonyi, 1995).

In summary, these data cast doubt on the hypothesis that ochratoxin A causes renal tumours by covalent binding of reactive intermediates to DNA. The hypothesis that DNA damage induced by ochratoxin A is due to oxidative stress represents an alternative explanation for the discrepant data and is more consistent with the observations. Several experimental observations support this hypothesis. An unusually large number of DNA adducts (up to 30 individual adducts) was formed from ochratoxin A in low yields in various experimental systems (Castegnaro et al., 1998; Pfohl-Leskowicz et al., 1998). Patterns of modifications similar to those observed with ochratoxin A by postlabelling were observed in kidney DNA of rodents exposed to iron(III) nitrilotriacetate (Randerath et al., 1995), a renal carcinogen that acts through oxidative stress, or in DNA exposed to hydrogen peroxide (Randerath et al., 1996). Some of these results are consistent with a major role of oxidative stress in the toxicity of ochratoxin A. For example, antioxidants prevent the induction of DNA damage by ochratoxin A in mice (Grosse et al., 1997).

Induction of renal toxicity, oxidative stress due to mitochondrial dysfunction, and persistent cell proliferation represent an alternative mechanism for the renal carcinogenicity of ochratoxin A. The toxin is known to induce oxidative stress (Aleo et al., 1991) and the formation of hydrogen peroxides (Omar et al., 1990). In addition, mechanisms linked to long-term renal toxicity and oxidative stress are known to play an important role in tumour induction in rat kidney (Swenberg & Maronpot, 1991; Dietrich & Swenberg, 1993; Hard, 1998). Several non-genotoxic chemicals that do not undergo bioactivation reactions induce renal tumours in rodents. For example, DNA damage and cellular toxicity mediated by oxidative stress seem to be involved in the renal carcinogenicity of iron(III) nitrilotriacetate and potassium bromate in rodents. These compounds are potent renal carcinogens and induce renal tumours in rodents in high yields after short exposure (Li et al., 1987; Wolf et al., 1998). Sex differences in tumour incidences are also seen with these compounds. For example, as seen with ochratoxin A, male rats are more susceptible to renal tumour induction by potassium bromate (Kurokawa et al., 1983, 1990; Umemura et al., 1998).

(f) *Mechanisms of cytotoxicity*

Ochratoxin A induced apoptosis in the HL-60 human promyelotic leukaemia cell line as seen by a DNA fragmentation technique and ultrastructural observation. Incubation for 24 h with ochratoxin A at a concentration of 3–4 µg/ml resulted in both apoptosis and cytotoxicity, as measured by MTT reduction (Ueno et al., 1995).

A brief report on the possible etiology of Balkan endemic nephropathy noted that apoptosis was not observed in kidneys of rats given ochratoxin A in the diet at 0.8 mg/rat per day for 5 days, which was sufficient to cause extensive necrosis (Mantle et al., 1998). No other details were available.

The toxicity of ochratoxin A, three natural analogues, and 10 synthetic analogues was compared in vitro and in vivo in order to identify the active moiety of the ochratoxin A structure. The studies in vivo involved intraperitoneal injection of mice and intravenous injection of rats, with lethality as the end-point. The hydroxyl, carboxyl, chlorine, and lactone groups of ochratoxin A affected its bactericidal activity (*Bacillus brevis*), its cytotoxicity to HeLa cells, and its toxicity to mice and rats. Its biological reactivity may be partly associated with the lactone carbonyl group of the isocoumarin moiety. There appeared to be no direct relationship between toxicity and the extent of iron chelation. In addition, formation of a previously undescribed ring-opened metabolite of ochratoxin A was detected in the bile but not in the blood or urine of rats after instillation of 100 µg of ochratoxin A into the carotid artery (Xiao et al., 1996).

The Committee noted that these studies are not helpful for risk assessment, because high doses were given by injection and lethality was the only end-point. Furthermore, the studies were inadequately reported.

(g) *Effects on the male reproductive system*

Ochratoxin A inhibited testosterone secretion in isolated testicular interstitial cells of gerbils (Fenske & Fink-Gremmels, 1990).

Male rats treated by gavage with ochratoxin A at 290 µg/kg bw every second day for up to 8 weeks showed a twofold increase in the testicular content of testosterone and accumulation of premeiotic germinal cells, as measured by increases in  $\alpha$ -amylase, alkaline phosphatase, and  $\gamma$ -glutamyl transpeptidase activities in testis homogenate. All of these effects were indicative of a disturbance of spermatogenesis (Gharbi et al., 1993).

## **2.3 Observations in domestic animals and veterinary toxicology**

### **2.3.1 Chickens**

Groups of 20 Peterson x Hubbard broiler chickens were fed diets containing ochratoxin A alone at 0 or 2.5 mg/kg of diet or in combination with cyclopiazonic acid for 3 weeks. A significant reduction in body-weight gain was seen by the second week of feeding and was still present at the third week (by 19%). The relative kidney weight was increased in the group given ochratoxin A, and significant increases in serum uric acid and triglycerides but decreased total protein, albumin, and cholesterol were seen (Gentles et al., 1999).

### **2.3.2 Pigs**

Commercial ochratoxin A was administered orally at a dose of at 20 or 40 µg/day for 5 weeks to groups of two sexually mature male Hungarian large white and Dutch Landrace boars weighing 250 kg. Ochratoxin A was detected in serum and seminal plasma of both groups (Solti et al., 1996).

## **2.4 Observations in humans**

### **2.4.1 Biomarkers of exposure**

Ochratoxin A has a half-life of about 35 days in humans (Bauer & Gareis, 1987; Hagelberg et al., 1989; Studer-Rohr et al., 1995), and the blood concentrations are considered to represent a convenient biomarker of exposure during recent weeks. This biomarker has been used extensively in epidemiological studies (see below). Similar estimates of exposure have been derived from dietary surveys and from blood analyses, suggesting that the latter is a reliable biomarker.

### **2.4.2 Biomarkers of effects**

The nephrotoxic effect of ochratoxin A is detectable by urinary analysis, but this is a relatively non-specific effect and late in onset. Anaemia is an early manifestation but is also non-specific, and early diagnosis is difficult.

### **2.4.3 Epidemiological studies**

Since ochratoxin A was suggested to be a possible determinant of endemic nephropathy, considerable efforts have been made to determine a correlation between human exposure to this toxin and the incidence of the disease. Endemic nephropathy is a fatal human renal disease, recognized as a specific entity and affecting predominantly rural populations in limited areas of the central Balkan peninsula. So far, the disease has been reported in Bosnia and Herzegovina, Bulgaria, Croatia, Romania, and Yugoslavia (Serbia). The disease was first recognized in the 1950s (Tancev et al., 1956), but there is evidence that it occurred even earlier (Belicza et al., 1979).

The disease starts without an acute episode. Onset is common between the ages of 30 and 50, although there have been reports of patients aged 10–19 (Stoyanov et al., 1978). Its progress is very slow, and after development of nonspecific signs and symptoms there is atypical manifestation of renal impairment (Radonić et al., 1966). The effect on the primary tubules is characterized by a decrease in tubular transport and becomes evident through proteinuria. As a rule, the proteinuria is very mild and is accompanied by the characteristic presence of low-relative-molecular-mass proteins (Hall & Vasiljević, 1973). Anaemia of the normochromic type is among the first signs of the disease and precedes clinical manifestation of renal impairment (Radonić et al., 1966). The ultrasonic appearance of the kidney is normal at the early stage of the disease, but it becomes smaller as the disease progresses (Boršo, 1996). Since there are neither characteristic clinical data nor pathognomonic laboratory indicators, the early diagnosis of endemic nephropathy is difficult and relies on repeated findings of proteinuria, creatininaemia, anaemia, and a family history of the disease.

The prevalence rate of the disease is reported to be 2–10%. In the endemic area of Croatia, a systematic field survey of cases between 1975 and 1990 revealed a prevalence of 0.5–4.4%. The average specific mortality (based on official statistics and documented cases) during the period 1957–84 was 1.5/1000 per year, although some studies have shown that the mortality rate is actually more than twice as high. The disease affects more women than men, and women die more frequently from endemic nephropathy (Ceović et al., 1992).

A remarkable reduction in the size of the kidney is seen *post mortem*: in one extreme case, one organ weighed only 20 g. In almost all advanced cases, a characteristic pale dirty yellow discolouration of the skin was common, with a peculiar yellowish colouration of the adipose tissue. The shrinking is progressive, and the organs can be normal or rather small in the early stages of the disease. The kidneys are pale grey and hard to cut (Vukelić et al., 1991). Pathomorphologically, the disease can be described as interstitial, bilateral, non-inflammatory, and non-obstructive nephropathy with heavy damage to the tubular epithelium and extensive interstitial fibrosis starting in the cortex (Vukelić et al., 1992).

The reported incidence of epithelial tumours of the upper urinary tract is much higher in endemic than in non-endemic areas (Chernozemsky et al., 1977; Nicolov et al., 1978; Ceović & Miletić-Medved, 1996). In the endemic region of Croatia, the prevalence of tumours of the pyelon and ureter is 11 times that in the non-endemic area (Vukelić et al., 1987). Of the malignant tumours, transitional-cell carcinomas were the most frequent (95%); squamous-cell carcinomas were seen in only 5% of cases. Generally, the differences in urothelial tumours between endemic and non-endemic regions include the following: the incidence of tumours is higher in the endemic region, and they affect younger people and women more frequently; the renal pelvis and urethra are the usual sites of tumours in the endemic region, whereas in non-endemic regions the most frequent site is the urinary bladder (Vukelić & Šostarić, 1991). A study of 766 patients treated at the Belgrade Department of Urology for upper urinary tract tumours in 1970–97 showed that the incidence of these tumours was 68% in patients from endemic and probably endemic regions and 32% in patients from non-endemic regions in Yugoslavia (Serbia). The tumours were more frequent in women. A much higher incidence of bilateral tumours was reported in patients from the endemic region (13%) than from non-endemic regions (2%) (Djokić et al., 1999; Table 4).

Striking similarities between the changes in the renal structure and function found in endemic nephropathy and in ochratoxin A-induced porcine nephropathy suggested a common causal relationship (Krogh, 1974). Epidemiological similarities, in particular the endemic occurrence (Krogh, 1976), support the hypothesis that ochratoxin A is a causative agent of endemic nephropathy in humans.

Although ochratoxin A has been found as a contaminant of food and feed all over the world (Krogh, 1992), food samples collected in the endemic areas showed higher contamination. In 1979 in the endemic region of Croatia, ochratoxin A was found in 9.4% of food samples. In a 5-year study in Bulgaria in which 524 food

**Table 4. Incidence, by anatomical location, of urothelial tumours among inhabitants of areas endemic and non-endemic for endemic nephropathy**

Anatomical location	Endemic area (10 094 inhabitants)		Non-endemic area (96 306 inhabitants)	
	No.	%	No:	%
Pyelon	29	0.286	20	0.021
Ureter	9	0.089	13	0.013
Urinary bladder	23	0.23	86	0.089
Combination (pyelon–ureter)	6	0.059	7	0.007
Total	67	0.66	126	0.13

From Vukelić et al. (1992)

samples from endemic and control villages were analysed, the frequency of positive samples from endemic villages was several times higher than that from non-endemic villages (Pavlović et al., 1979).

Ochratoxin A was first detected in humans in blood samples from inhabitants of endemic villages (Hult et al., 1982), at a much higher concentration than in non-endemic villages. The prevalence rate was 17% in endemic and 6.0% in non-endemic villages, and similar rates were found in blood samples from endemic (18%) and non-endemic (7.7%) areas in Bulgaria (Petkova-Bocharova et al., 1988).

Low blood concentrations of ochratoxin A have been found in countries where endemic nephropathy has not been detected, such as Canada, the Czech Republic, Egypt, France, Germany, Italy, Sweden, Switzerland, and Tunisia (Bauer & Gareis, 1987; Hadlok, 1993; Breitholtz-Emanuelsson et al., 1994; Zimmerli & Dick, 1995; Malir et al., 1998; Wafa et al., 1998). Some regional differences in exposure to ochratoxin A have been found (Breitholtz et al., 1991; Creppy et al., 1993; Maaroufi et al., 1995a; Scott et al., 1998), and in Croatia in a study of blood from donors in five major cities (Peraica et al., 1999). The mean concentration in the 250 samples was 0.39 ng/ml of plasma, and 59% of samples contained the toxin (detection limit, 0.2 ng/ml). The highest frequency of positive samples (100%), the highest mean ochratoxin A concentration (0.68 ng/ml), and the largest number of samples with a concentration > 1.0 ng/ml (18%) were found in a city relatively near the endemic region. The concentrations reported in blood from healthy persons are shown in Table 5.

Ochratoxin A has been found in human milk. Nine of 50 samples of milk from women in various regions of Italy contained the toxin, at concentrations of 1.2–6.6 ng/ml of milk (Micco et al., 1991).

**Table 5. Occurrence of ochratoxin A in blood samples from healthy persons**

Country	Period of collection	Positive/analysed		Concentration (ng/ml)		Reference
		No.	%	Mean	Range	
Bulgaria	1984–90	9/125	7		1.0–10	Petkova-Bocharova et al. (1991)
Canada	1994	144/144	100	0.88	0.29–2.4	Scott et al. (1998)
Czechoslovakia	1990	35/143	24	0.14	0.1–1.3	Fukal & Reisnerova (1990)
Czech Republic	1994	734/809	91	0.23	0.1–14	Malir et al. (1998)
	1995	404/413	98	0.24	0.1–1.9	
Croatia	1997	148/249	59	0.39	0.2–16	Peraica et al., (1999)
Denmark	1986			1.5	0.1–9.7	Hald (1991)
	1987			2.3	0.1–9.4	
	1988			1.6	0.1–13	
France	1991–92					Creppy et al. (1993)
Alsace		97/500	19		0.1–12	
Aquitaine		385/2055	19		0.1–160	
Rhone-Alpe		75/515	15		0.1–4.3	
Germany	1977	84/165	51	0.79	0.1–14	Bauer & Gareis (1987)
	1985	89/141/	63	0.42	0.1–1.8	Hadlok (1993)
	1988	142/208	68	0.75	0.1–8.4	Solti et al. (1997)
Hungary	1995	291/355	82		0.2–10	Tapai et al. (1997)
	1997	213/277	77		0.1–1.4	Breitholtz-Emanuelsson et al. (1994)
Italy	1992	65/65	100	0.53	0.1–2.0	Ueno et al. (1998)
Japan, Tokyo	1992–96	156/184	85	0.068	0.004–0.28	Golinski (1987)
Poland	1983–84	25/397	6	0.21	1.0–13	Jonsyn (1996)
	1984–85	52/668	8	0.31	1.0–40	Jimenez et al. (1998)
Sierra Leone	1996	12/36	33 <sup>a</sup>		1.5–18	Zimmerli & Dick (1995)
Spain	1996–98	40/75	53	0.71	0.5–4.0	Breitholtz et al. (1991)
Switzerland	1992–93					
North of Alps		251/252	100		0.06–2.1	
South of Alps		116/116	100		0.11–6.0	
Sweden	1989					Breitholtz-Emanuelsson et al. (1993b)
Visby		29/99	29	0.26	0.3–7.0	
Uppsala		3/99	3	0.02	0.3–0.8	
Ostersund		6/99	6	0.03	0.3–0.8	
	1990–91	39/39	100	0.17	0.09–0.94	Maaroufi et al. (1995a)
Tunisia	1993–95	73/140	52	1.2	0.1–8.8	

From Peraica et al. (1999). Mean concentration calculated for all samples, range given only for positive samples

<sup>a</sup> Non-breastfed infants up to 5 years of age

### 3. ANALYTICAL METHODS

Ochratoxin A has been found in many commodities, including cereals, cereal products, coffee, grapes, dried vine fruit, grape juice, wine, cocoa and chocolate, beer, meat, pork products, pulses, milk and milk products, and spices. Several published analytical methods for the determination of ochratoxin A in maize, barley, wheat, wheat bran, wheat wholemeal, rye, wine, beer, and roasted coffee have been formally validated in collaborative studies. The methods are based on liquid chromatography (LC) with fluorescence detection, include a solid-phase extraction clean-up step with reversed-phase C<sub>18</sub>, silica gel 60, or immunoaffinity columns, and can guarantee detection of < 0.5 µg/kg. These methods have also been used successfully to analyse a number of other cereals, cereal products, and dried fruit. The first LC method for determining ochratoxin A in maize and barley was validated in a collaborative study with materials spiked with ochratoxin A in the range of 10–50 ng/g. Ochratoxin A was extracted from grains with chloroform:aqueous phosphoric acid and isolated by liquid–liquid partitioning into aqueous bicarbonate solution that had been cleaned-up on a C<sub>18</sub> (solid-phase extraction) cartridge. Identification and quantification were performed by reversed-phase LC with fluorescence detection. The identity of ochratoxin A in samples that contained it was confirmed by methyl ester derivatization followed by LC analysis (Nesheim et al., 1992). The performance characteristics achieved in an international collaborative study involving 16 laboratories are shown in Table 6. The method, which is quantitative for ochratoxin A at concentrations ≥ 10 µg/kg in maize and barley, has been accepted as final-action AOAC International Official Method 991.44.

This method was successively validated for other cereals and at lower ochratoxin A concentrations (Larsson & Moeller, 1996). Spiked and naturally contaminated barley, wheat bran, and rye containing ochratoxin A at concentrations of 2–9 µg/kg were used. The performance characteristics achieved in the international collaborative study involving 12 laboratories are shown in Table 7. The European Committee for Standardisation (CEN, technical committee 275/WG5 'Biotoxins'), which uses specific criteria to select methods, has adopted this method as CEN standard (EN ISO 15141-2) for determination of ochratoxin A in barley, maize, and wheat bran.

The second LC method, adopted as CEN standard (EN ISO 15141-1) for determination of ochratoxin A in cereals and cereal products, was validated in a collaborative study on wheat wholemeal containing ochratoxin A at 0.4 or 1.2 µg/kg (Majerus et al., 1994). Ochratoxin A was extracted from grains with toluene after addition of hydrochloric acid and magnesium chloride solution. The filtered extract was cleaned-up on a mini-silica gel column, and ochratoxin A was determined by reversed-phase LC with fluorescence detection. The performance characteristics achieved in the collaborative study involving 13 laboratories according to ISO 5725: 1986 are shown in Table 8. Laboratory experience has shown that this method is also applicable to cereals, dried fruits, oilseeds, pulses, wine, beer, fruit juices, and raw coffee (Jiao et al., 1992; Majerus et al., 1993; Jiao et al., 1994).

During the past few years, the use of antibody-based immunoaffinity columns in the clean-up step has improved the analysis of ochratoxin A. Two methods based on immunoaffinity clean-up for determination of ochratoxin A in barley and roasted coffee have been developed and validated in collaborative studies under the auspices of the European Commission, Standard and Measurement Testing programme

**Table 6. Results of collaborative study for determination of ochratoxin A in maize and barley**

Matrix	Mean (ng/g)	No. acceptable results	Mean recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Maize	0.8	15	—	—	—
	8.2	15	82	—	21
	16	15	82	20	28
	40	14	77	—	32
Barley	0.8	15	—	—	—
	7.4	15	74	—	27
	14	14	72	7.9	26
	3	15	74	—	28

RSD<sub>r</sub>, relative standard deviation for repeatability; RSD<sub>R</sub>, relative standard deviation for reproducibility

**Table 7. Results of collaborative study for determination of ochratoxin A in rye, barley, and wheat bran**

Matrix	Mean (ng/g)	No. acceptable results	Mean recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Rye	2.8	12	64	22	29
	4.8	12	65	16	23
Barley	2.9 <sup>a</sup>	12	—	17	22
	3.0 <sup>a</sup>	12	—	15	23
Wheat bran	3.8	12	70	21	24
	4.5	12	68	17	26

For abbreviations, see Table 6.

<sup>a</sup> Naturally contaminated sample

**Table 8. Results of collaborative study for determination of ochratoxin A in wholemeal wheat**

Matrix	Mean (ng/g)	No. acceptable results	Mean recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Wheat, wholemeal	0.41	13	80	15	26
	1.2	13	80	20	32

For abbreviations, see Table 6.

(Entwisle et al., 2000a,b). Ochratoxin A was extracted from barley with acetonitrile: water solution, and the filtered sample extract was diluted with phosphate-buffered saline that had been cleaned-up by passage through an immunoaffinity column. For the determination of ochratoxin A in roasted coffee, phenyl silane solid-phase extraction clean-up before the immunoaffinity column stage was introduced to avoid any deleterious effects of caffeine on the immunoaffinity columns (Koch et al., 1996; Entwisle et al., 2000b). In both these methods, ochratoxin A was identified and

quantified by reversed-phase LC with fluorescence detection. Spiked and naturally contaminated samples containing ochratoxin A at 1.2–3.7  $\mu\text{g}/\text{kg}$  were used in the collaborative study. These two methods have been accepted by AOAC International as first-action methods and are being considered for adoption by the CEN as standards. The performance characteristics achieved in the collaborative studies involving 15 laboratories are shown in Tables 9 and 10 for barley and roasted coffee, respectively.

The occurrence of ochratoxin A in wine, especially red wine, was first reported by Zimmerli and Dick (1995). The analytical method they used involved extraction of ochratoxin A by liquid–liquid partitioning with chloroform, clean-up on an immunoaffinity column, and determination by reversed-phase LC with fluorescence detection. A more accurate and precise method has since been developed for determination of ochratoxin A in red, rosé, and white wine (Visconti et al., 1999) and beer (Visconti et al., 2000a). After simple dilution with water containing polyethylene glycol and  $\text{NaHCO}_3$ , wine or beer samples were cleaned-up on immunoaffinity columns and analysed by reversed-phase LC with fluorescence detection. The method was validated in a collaborative study. It has been adopted as a First Action Method by AOAC International and is being considered for adoption by the CEN as a standard (Visconti et al., 2000b). The performance characteristic obtained in the collaborative study are reported in Table 11.

The European Commission, Measurement and Testing Programme sponsored projects to improve the method and to prepare certified reference materials for determination of ochratoxin A in wheat and pig kidney (Hald et al., 1993; Wood et

**Table 9. Results of collaborative study for determination of ochratoxin A in barley**

Matrix	Mean (ng/g)	No. of acceptable results	Mean recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Barley	0.1 <sup>a</sup>	14	–	26	72
	1.3 <sup>b</sup>	15	–	24	33
	3.0 <sup>b</sup>	14	–	12	17
	4.5 <sup>b</sup>	12	–	14	15
	3.7	12	93	4	12

For abbreviations, see Table 6.

<sup>a</sup> Blank sample

**Table 10. Results of collaborative study for determination of ochratoxin A in roasted coffee**

Matrix	Mean (ng/g)	No. of acceptable results	Mean recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Roasted coffee	0.1 <sup>a</sup>	13	–	27	71
	1.2 <sup>a</sup>	14	–	22	26
	2.6 <sup>a</sup>	15	–	11	15
	5.4 <sup>a</sup>	12	–	2	14
	3.5	13	85	6	13

For abbreviations, see Table 6.

<sup>a</sup> Naturally contaminated sample

**Table 11. Results of collaborative study for determination of ochratoxin A in wine and beer**

Matrix	Mean (ng/g)	No. of acceptable results	Mean recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
White wine	< 0.01 <sup>a</sup>	—	—	—	—
	0.10	13	100	10	14
	1.0	14	91	6.6	14
	1.8	14	88	8.5	13
	0.28 <sup>b</sup>	15	—	11	15
Red wine	< 0.01 <sup>a</sup>	—	—	—	—
	0.19	12	93	5.5	9.9
	0.81	14	90	9.9	12
	2.5	15	85	8.9	13
	1.7 <sup>b</sup>	14	—	11	13
Beer	< 0.01 <sup>a</sup>	—	—	—	—
	0.19	13	95	10	18
	0.70	15	87	7.2	18
	1.4	13	94	4.6	16
	0.069 <sup>b</sup>	14	—	19	20

For abbreviations, see Table 6.

<sup>a</sup> Blank sample

<sup>b</sup> Naturally contaminated sample

al., 1996; Entwisle et al., 1996; Wood et al., 1997; Williams et al., 1998). The study in wheat involved 26 European laboratories and was conducted in three phases: a first comparison of procedures, a second comparison of procedures, and certification of two reference materials. Various procedures were compared in the first step, including chloroform, methanol, toluene, or ethyl acetate for extraction, and silica, reversed-phase, or immunoaffinity columns for clean-up. LC was used for determination in all laboratories except one, where thin-layer chromatography (TLC) was used (Hald et al., 1993). In the second comparison of procedures, all laboratories used high-performance liquid chromatography (HPLC) for quantification of ochratoxin A, whereas acetonitrile, chloroform, dichloromethane, ethyl acetate, methanol, and toluene were used as extraction solvents. Various clean-up procedures were used, including silica, reversed-phase, diatomaceous earth, and immunoaffinity columns and liquid-liquid partitioning. Fifteen of 26 participants had results that fulfilled the agreed acceptance criteria and therefore participated in the certification study (Wood et al., 1996). The results of nine of these 15 laboratories were accepted for certification of the two wheat reference materials. The methods used by these nine laboratories for setting the certification value of ochratoxin A in blank and naturally contaminated wheat flour are summarized in Table 12. The two certified reference materials are useful for ensuring the quality of analyses and can be used to prepare in-house reference materials easily and inexpensively. Certified reference materials for mycotoxins, including ochratoxin A, are available from the European Union Joint Research Centre, Institute for Reference Materials and Measurements, Geel, Belgium.

**Table 12. Methods used by laboratories accepted for setting the certification value for ochratoxin A in wheat flour**

Extraction	No. of laboratories	Clean-up
25-g test portion 125 ml CHCl <sub>3</sub> 12.5 ml 0.1 mol/L H <sub>3</sub> PO <sub>4</sub>	4	Silica column (1 laboratory) C <sub>18</sub> column (2 laboratories) Liquid-liquid defatting (1 laboratory)
25-g test portion 125 ml toluene 30 ml 2 mol/L HCl 25 ml 0.4 mol/L MgCl <sub>2</sub>	3	Silica column
25-g test portion 24 ml acetonitrile 16 ml phosphate-buffered saline	1	Immunoaffinity column
25-g test portion 80 ml ethyl acetate 8 ml 5% acetic acid	1	C <sub>18</sub> column

All laboratories used liquid chromatography with reversed-phase C<sub>18</sub> and fluorescence detection

Two comparative studies of methods for the analysis of ochratoxin A in freeze-dried pig kidney were performed in order to determine the feasibility of preparing certified reference materials. In the first study, almost all of the 20 European laboratories reported lower than usual recoveries for the freeze-dried material (Entwisle et al., 1996). The methods used were similar to those used in the comparative studies for ochratoxin A in wheat (see above). The results of the second comparative study of methods for the analysis of ochratoxin A in freeze-dried pig kidney indicated the following: the reconstitution of freeze-dried material is crucial, as small lumps of powdered material may be formed; in recovery experiments, the spiking solution must be added to reconstituted material and not to powdered material in order to avoid formation of small lumps; immunoaffinity clean-up resulted in clearer extracts and chromatograms than reversed-phase or silica gel columns or liquid-liquid partitioning; the use of 1 mol/L instead of 0.1 mol/L phosphoric acid in the extraction step did not improve the recovery of ochratoxin A (Williams et al., 1998).

### 3.1 Screening tests

Screening methods based on TLC are available, and one has been collaboratively validated (Nesheim et al., 1973). These methods are used in only a few laboratories since they do not provide an adequate limit of quantification (LOQ). Enzyme-linked immunoabsorbent assays (ELISAs) have been developed for the detection of ochratoxin A in pig kidney, animal and human sera, cereals, and mixed feed. The results obtained with these methods require confirmation since the antibodies produced often show cross-reactivity to compounds similar to ochratoxin A. These methods were not used in the surveys considered by the Committee.

### 3.2 Conclusions

Because of the large number of commodities contaminated with ochratoxin A, several LC analytical methods have been proposed. Validated analytical methods are available for accurate and precise determination of ochratoxin A in maize, barley, rye, wheat, wheat bran, wheat wholemeal, roasted coffee, wine, and beer. The introduction of immunoaffinity columns has improved analytical methods for ochratoxin A. Use of these columns reduces the need for dangerous solvents, drastically improves the clean-up of extracts, improves detection, and simplifies sample preparation and clean-up.

The analytical methods used for the determination of ochratoxin A in foods are summarized in Table 13.

## 4. EFFECTS OF PROCESSING

The main foods in which the effects of processing have been studied are cereals and coffee, although a little work has been carried out on other foods as well.

### 4.1 Cereals

In flour manufacture, some parts of the wheat grain are removed, possibly reducing the concentrations of ochratoxin A in flour and subsequent products. This was investigated by Osborne et al. (1996), who allowed growth of a toxin-producing strain of *P. verrucosum* in two samples of clean hard and soft wheat and then produced both white and wholemeal flour and bread from them. The process greatly influenced the final concentration of ochratoxin A in the bread (Table 14). The scouring process used by Osborne et al. (1996) resulted in a reduction of more than 50% in the concentration of ochratoxin A, but this process is not standard milling practice. Milling hard wheat to produce white flour resulted in an approximately 65% reduction, and a further 10% decrease occurred during baking. The reduction in soft wheat was much smaller; explanations were offered, but these were somewhat academic because bread is usually made from hard wheat. Wholemeal flour and bread showed much smaller reductions in the concentration of ochratoxin A during processing, as might be expected, because less of the grain is discarded. The loss during milling was only 10%, with a further 4% loss during baking (Table 14).

In a study of the stability of ochratoxin A in cereals during heating, a toxigenic strain of *A. ochraceus* on wheat, and dry and moistened samples were heated at several temperatures and times and the level of destruction recorded. From those figures, the times to 50% destruction of ochratoxin A under various heating conditions were calculated (Table 15; Boudra et al., 1995).

A reduction in ochratoxin A concentrations resulting from manufacture of retail products from contaminated raw materials was reported by the Ministry of Agriculture, Fisheries and Food (1996a). The processes involved in making breakfast cereals and biscuits caused substantial reductions in the ochratoxin A content; however manufacture of egg noodles and pasta caused little or no reduction (Table 16).

### 4.2 Coffee

The perception that coffee may be an important source of ochratoxin A led to much work on its formation, destruction during roasting, and presence in brewed

**Table 13. Analytical methods used to determine ochratoxin A in foods**

Reference	Commodity	Test portion (g)	Extraction	Clean-up	Quantification	LOD/LOQ (µg/kg)	Accuracy			
							Spiking (µg/kg)	Recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Wolff et al. (2000)	Wine, juices, oil, vinegar	5 (ml)	Diluted with 1 ml PBS	Immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.01/				
Wolff et al. (2000)	Meat and meat products	25	HCl-MgCl <sub>2</sub> solution + CHCl <sub>3</sub>	Liquid-liquid partitioning with NaHCO <sub>3</sub> + immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.01/	0.05 –5.0	34–104		
Wolff et al. (2000)	Cereals	40	30 ml HCl-MgCl <sub>2</sub> + 125 ml toluene	SiO <sub>2</sub> column Sep - pak	HPLC (RP18)/ fluorescence detection	0.01/				
Wolff et al. (2000)	Beer	5 (ml)	Diluted with 1 ml PBS	Immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.01/				
Wolff et al. (2000)	Coffee, tea	25	500 ml H <sub>2</sub> O-NaHCO <sub>3</sub>	Dilution with PBS and clean-up on immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.3/				
Wolff et al. (2000)	Milk products, sweets, oil-seeds	50	200 ml acetonitrile: water (6:4) 2-min blending	Dilution with PBS and clean-up on immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.01/				

Table 13 (contd)

Reference	Commodity	Test portion (g)	Extraction	Clean-up	Quantification	LOD/LOQ ( $\mu\text{g}/\text{kg}$ )	Accuracy			
							Spiking ( $\mu\text{g}/\text{kg}$ )	Recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Langseth et al. (1989); Langseth (1999)	Cereals	25	125 ml $\text{CHCl}_3$ + 12.5 ml 0.1 mol/L $\text{H}_3\text{PO}_4$ ; shaking 60 min	Silica Sep-pack cartridge or immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.01/	10	80–114		
Larsson & Moeller (1996)	Wheat, barley, rye	50	25 ml 0.1 mol/L $\text{H}_3\text{PO}_4$ + 250 ml $\text{CHCl}_3$ + 10 g diatomaceous earth; 3-min blending	Liquid-liquid partitioning with 3% $\text{NaHCO}_3$ + C18 (Sep-pack)	HPLC (RP18)/ fluorescence detection	0.1/	4.96 5.95 7.44	72 69 66	17–22	24–28
Soares et al. (1985)	Corn, peanuts, beans, rice, cassava	50	270 ml MeOH + 30 ml 4% KCl; 5 min blending	Clarification with $(\text{NH}_4)_2\text{SO}_4$ and Hyflo Super-Cel; dilution with water and liquid-liquid partitioning with $\text{CHCl}_3$	Visual TLC (20 x 20 cm silica gel 60)	10/	10–400	86–125	0–24	
Trucksess et al. (1999)	Wheat, barley, coffee	25	100 ml MeOH:1% $\text{NaHCO}_3$ (7+3); 3-min blending	Dilution with PBS and clean-up on Immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.03/	1–4	71–96	2–17	

Table 13 (contd)

Reference	Commodity	Test portion (g)	Extraction	Clean-up	Quantification	LOD/LOQ ( $\mu\text{g}/\text{kg}$ )	Accuracy		
							Spiking (%)	Recovery (%)	RSD <sub>r</sub> (%)
Solfrizzo et al. (1998)	Wheat, oats	10	60 ml $\text{CHCl}_3$ + 5 ml 0.1 mol/L $\text{H}_3\text{PO}_4$ 30 min shaking	Dried extract dissolved in HPLC mobile phase and defatted with <i>n</i> -hexane by liquid-liquid partitioning	HPLC (RP18)/fluorescence detection	/0.8	1-100	82-104	3-7
Jorgensen et al. (1996)	Wheat, rye, barley, oats, bran, pork, poultry meat and liver	50	250 ml $\text{CH}_2\text{Cl}_2$ :EtOH (4+1) + 25 ml 0.1 mol/L $\text{H}_3\text{PO}_4$ 30-min shaking	Liquid-liquid partitioning with 0.35 mol/L $\text{NaHCO}_3$ +EtOH (5+2) and $\text{CH}_2\text{Cl}_2$	HPLC (RP18)/fluorescence detection	0.05/ 0.5-4 (meat, liver)	5 60-121	80-90	<15
Jorgensen (1998)	Beer	150 (ml)	Beer degassed for 60 min	Degassed beer passed on immuno-affinity column	HPLC (RP18)/fluorescence detection		0.001/	0.3	66-113
Jorgensen (1998)	Roasted coffee	50	200 ml 1% m/m $\text{NaHCO}_3$ ; 2-min blending	Dilution with PBS and clean-up on immuno-affinity column	HPLC (RP18)/fluorescence detection		0.1/	5	59-83
Jorgensen (1998)	Pulses	50	200 ml acetonitrile: water (6:4); 2-min blending	Dilution with PBS and clean-up on immuno-affinity column	HPLC (RP18)/fluorescence detection		0.1/	5	78-103

Table 13 (contd)

Reference	Commodity	Test portion (g)	Extraction	Clean-up	Quantification	LOD/LOQ ( $\mu\text{g}/\text{kg}$ )	Accuracy			
							Spiking ( $\mu\text{g}/\text{kg}$ )	Recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Patel et al. (1996)	Cereals, oils, nuts, seeds, herbs, pickles, canned food	25	125 ml $\text{CHCl}_3$ + 12.5 ml 0.1 mol/L $\text{H}_3\text{PO}_4$ 30-min shaking	Silica Sep-pack cartridge	HPLC (RP18)/fluorescence detection		0.1/	10	90	
Maaroufi et al. (1995)	Olives, cereals and derived foods, vegetables	100	120 ml $\text{CHCl}_3$ +HCl (100+1); shaking overnight	Preparative TLC	HPLC (RP18)/fluorescence detection	0.1/	10–100	60–85		
Sharman et al. (1992)	Cereal products	10	40 ml PBS:MeOH (50:50); 3–5-min blending	Immunoaffinity column	HPLC (RP18)/fluorescence detection	0.2/	10	74	4	
Sharman et al. (1992)	Animal products	10	100 ml $\text{CHCl}_3$ + 0.6 g 85% $\text{H}_3\text{PO}_4$ 3–5-min blending	Immunoaffinity column	HPLC (RP18)/fluorescence detection	0.2/	10	74–79	3–6	
Rao (2000)	Foods	NR	Acetonitrile:water or MeOH:water	Immunoaffinity column	HPLC (RP18)/fluorescence detection	0.2/0.5	1–5	70–126	8–13	
MAFF (1999a)	Cereals	25	$\text{CHCl}_3$ + 0.1 mol/L $\text{H}_3\text{PO}_4$	Silica Sep-pack cartridge	HPLC (RP18)/fluorescence detection	0.1/0.2	2	71–104		

Table 13 (contd)

Reference	Commodity	Test portion (g)	Extraction	Clean-up	Quantification	LOD/LOQ ( $\mu\text{g}/\text{kg}$ )	Accuracy			
							Spiking ( $\mu\text{g}/\text{kg}$ )	Recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Howell & Taylor (1981; modified)	Maize	25	250 ml $\text{CHCl}_3$ + 25 ml 0.1 mol/L $\text{H}_3\text{PO}_4$ 30-min shaking	Silica Sep-Pak cartridge	HPLC (RP18)/ fluorescence detection	0.1/0.2	2	90–98	5	
Pineiro & Giribone (1994)	Various foods and feeds	NR	NR	NR	TLC	50/				(
MAFF (1999b)	Dried fruit, chocolate, cocoa, pulses	NR	NR	Immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.1/0.2		79–92	1.7–11.1	
MAFF (1999b)	Wine, grape juice	NR	NR	Immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.01/0.02		79–92	5.9–7.0	
MAFF (1999c)	Total diet	NR	$\text{CHCl}_3$ : $\text{H}_3\text{PO}_4$	Liquid–liquid partitioning with $\text{NaHCO}_3$ solution and clean-up on immunoaffinity column	HPLC (RP18)/ fluorescence detection		/0.002		89	16.8
MAFF (1996b)	Green coffee beans	50	25 ml 0.1 mol/L $\text{H}_3\text{PO}_4$ + 250 ml $\text{CHCl}_3$ + 10 g diatomaceous earth; 3-min blending	Liquid–liquid partitioning with 3% $\text{NaHCO}_3$ , immunoaffinity and C18 (Sep-pack)	HPLC (RP18)/ fluorescence detection		/0.26		73.3	18

Table 13 (contd)

Reference	Commodity	Test portion (g)	Extraction	Clean-up	Quantification	LOD/LOQ	Accuracy			
						( $\mu\text{g}/\text{kg}$ )	Spiking (%)	Recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Stegen et al. (1997)	Coffee products	Cooperative study with 9 laboratories using different methods			HPLC (RP18)/fluorescence	0.2–1/			20	42 (CV)
Nesheim et al. (1973)	Barley	50	25 ml 0.1 mol/L H <sub>3</sub> PO <sub>4</sub> + 250 ml CHCl <sub>3</sub> + 10 g	detection Liquid–liquid partitioning	TLC sprayed with ammonia and scanned with fluoridensitometer	2/				
Scott et al. (1991)	Meat, kidney, liver	25	100 ml CHCl <sub>3</sub> + 50 ml 2 nmol/L NaCl + 50 ml 0.5 mol/L H <sub>3</sub> PO <sub>4</sub> ; 60-min shaking	Silica gel column	HPLC (RP18)/fluorescence detection	0.5/	1–10	93–106	9–12	
Patel et al. (1997)	Roasted and soluble coffee	25	125 ml CHCl <sub>3</sub> + 12.5 ml 0.1 mol/L H <sub>3</sub> PO <sub>4</sub> ; 30-min shaking	Silica Sep-Pak cartridge + immunoaffinity column	HPLC (RP18)/fluorescence detection	0.1/	2	91	5	
Burdaspal & Legarda (2000)	Baby food	25	100 ml 0.5 mol/L H <sub>3</sub> PO <sub>4</sub> 2 mol/L NaCl + 50 ml <i>tert</i> -butyl methyl ether; 2-min blending	Liquid–liquid partitioning + immunoaffinity clean-up	HPLC (RP18)/enhanced fluorescence detection	/0.008	< 1	91–109	18	

Table 13 (contd)

Reference	Commodity	Test portion (g)	Extraction	Clean-up	Quantification	LOD/LOQ ( $\mu\text{g}/\text{kg}$ )	Accuracy			
							Spiking ( $\mu\text{g}/\text{kg}$ )	Recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Zimmerli & Dick (1995, 1996)	Wine, grape juice	5 (ml)	Dilution with $\text{H}_3\text{PO}_4$ : 2 mol/L NaCl (33.7:966.3), extraction with 5 ml $\text{CHCl}_3$ 1-min vortex	immunoaffinity column	HPLC (RP18)/ enhanced fluorescence detection	0.003/0.005	0.055	74–91	6	
Burdaspal & Legarda (1998a)	Beer	5	Dilution with 1 ml 1% $\text{NaHCO}_3$ 15% NaCl	Immunoaffinity column	HPLC (RP18)/ enhanced fluorescence detection	0.004/	0.114	100	2.4	
Ueno	Coffee Canned coffee Wine and beer	0.5 5 (ml) 5 (ml)	8 ml 1% $\text{Na}_2\text{CO}_3$ and diluted with PBS Filtration Mixed with 1 ml 2.5% $\text{Na}_2\text{CO}_3$ 1.5% NaCl	Immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.003–0.06/	NR	80–90	(1998)	
Visconti et al. (1999, 2000a)	Wine, beer	10 (ml) 10 ml	Dilution with 1% PEG and 5% $\text{NaHCO}_3$	Immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.01/	0.1–3.0	85–102	5–19	10–20
Ottender & Majerus (2000)	Wine	25 (ml)	Dilution with 25 ml PBS and pH 7.0–7.5	Immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.01/			8.4	

Table 13 (contd)

Reference	Commodity	Test portion (g)	Extraction	Clean-up	Quantification	LOD/LOQ ( $\mu\text{g}/\text{kg}$ )	Accuracy			
							Spiking ( $\mu\text{g}/\text{kg}$ )	Recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Koch et al. (1996)	Roasted coffee	15	150 ml MeOH +	Double clean-up: 3% NaHCO <sub>3</sub> (1+1, v/v) 30-min shaking column	HPLC (RP18)/ phenyl silane column and immunoaffinity	0.1/	6	87–94		
							fluorescence detection			
Majerus et al. (1994)	Wheat whole-meal, cereals, dried fruits, pulses, wine, beer, fruit juice, raw coffee	20	30 ml HCl+ 50 ml 0.4 mol/L MgCl <sub>2</sub> ; extracted with 100 ml toluene by shaking 60 min	Silica gel column	HPLC (RP18)/ fluorescence detection	0.1/ 1.2	0.41 80	80 20	15 32	26
Leoni et al. (2000)	Instant and roasted coffee	10	200 ml 1% NaHCO <sub>3</sub> 2-min blending	Immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.2/	10	77–100		
Sizoo & van Egmond	Wheat and wheat products	25	CH <sub>3</sub> CN/water	Immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.3/1	5	99	6	
Inspectorate of Health Protection	Wheat and wheat products	20	MeOH/water or CH <sub>3</sub> CN/water	Immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.1/0.25	0.51–54	86–106	8	NA

Table 13 (contd)

Reference	Commodity	Test portion (g)	Extraction	Clean-up	Quantification	LOD/LOQ (µg/kg)	Accuracy			
							Spiking (µg/kg)	Recovery (%)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Inspectorate for Health Protection	Wine	5 (ml)	Dilution with water	Immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.05/0.1	0.33	83–93	9	NA
Inspectorate for Health Protection	Roasted coffee	10	MeOH/3% NaHCO <sub>3</sub>	Phenyl silane column + immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.13/0.25	0.2–1.0	76–104	33	NA
Inspectorate for Health Protection	Paprika, pepper, nuts	20	MeOH/water or CH <sub>3</sub> CN/water	Immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.13/0.25	NA	> 70	NA	NA
Entwisle et al. (2000b)	Roasted coffee	15	MeOH/3% NaHCO <sub>3</sub>	Phenyl silane column + immunoaffinity column	HPLC (RP18)/ fluorescence detection	0.2/	4	65–97	2.0–22	14–26

CV, coefficient of variation; HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; MAFF, Ministry of Agriculture, Fisheries and Food (United Kingdom); RSD<sub>r</sub>, relative standard deviation for repeatability; RSD<sub>R</sub>, relative standard deviation for reproducibility; NR, not reported; PBS, phosphate-buffered saline; PEG, polyethylene glycol; TLC, thin-layer chromatography

**Table 14. Effect of flour and bread manufacture on the concentration of ochratoxin A (fg/kg) in wheat**

Wheat type	Initial concentration	Cleaning process	After cleaning	White flour	White bread	Wholemeal flour	Wholemeal bread
Hard	618	Clean	624 (0)	209 (66)	140 (77)	555 (10)	531 (14)
		Clean and scour	256 (59)	60 (90)	72 (88)	127 (80)	226 (63)
Soft	643	Clean	473 (27)	389 (40)	224 (65)	553 (9)	476 (26)
		Clean and scour	179 (72)	111 (83)	94 (85)	189 (71)	160 (75)

From Osborne et al. (1996). Percentage reductions are given in parentheses. All values are from duplicate experiments, with generally good agreement.

**Table 15. Time to reduce ochratoxin A content in wheat by 50% under various conditions**

Moisture content of wheat	Temperature of heating (°C)	Time to 50% destruction of ochratoxin A (min)
Dry wheat	100	700
	150	200
	200	12
	250	6
Wet wheat	100	140
	150	60
	200	19

From Boudra et al. (1995). The wheat samples had been ground; the dry wheat had been dried in a vacuum oven, the wet wheat was 50:50 dry wheat and water.

**Table 16. Reduction in concentrations of ochratoxin A during food manufacture**

Raw material	Ochratoxin A (fg/kg)	Rate of incorporation of raw material into food	Retail food	Ochratoxin A (fg/kg)	Reduction (%) (allowing for incorporation rate)
Breakfast cereal grain	0.8	43	Breakfast cereal	< 0.2	≥ 50
Breakfast cereal grain	0.8	90	Breakfast cereal	< 0.2	≥ 76
Biscuit flour	6.4	40	Biscuits	0.4	84
Durum wheat flour	1.4	82.5	Egg noodles	1.0	13
Durum wheat flour	0.9	82.5	Egg noodles	0.8	0
Durum wheat flour	1.6	82.5	Egg noodles	1.3	2
Semolina	0.8	100	Pasta (dried)	0.6	25
Semolina	0.8	100	Pasta (dried)	1.0	0

Modified from Ministry of Agriculture, Fisheries and Food (1996a)

coffee. It now seems clear, however, that coffee is not a major source of ochratoxin A in the normal diet (Ministry of Agriculture, Fisheries and Food, 1995).

Reductions in the concentration of ochratoxin A during cleaning and roasting of coffee have been studied, with variable results. Wilkens & Jörissen (1999) showed that cyclone cleaning of green coffee beans had little effect: although the ochratoxin A concentration in the discarded fraction was high, the dust comprised < 1% of the weight of the cleaned coffee. Sorting with colour sorters resulted in some reduction, and steaming caused a mean 25% reduction. Decaffeination is an effective process, resulting in 92% reduction (Heilmann et al., 1999).

Several reports on the effect of roasting on the concentration of ochratoxin A are summarized in Table 17. The reported reductions vary, but a major factor in such variation is that the natural concentration of ochratoxin A in coffee destined for retail markets, i.e. in coffee of sufficient quality for human consumption, seldom exceeds 10 µg/kg. Reports based on samples of such coffee usually showed high rates of reduction, e.g. 50–90% (Micco et al., 1989), 30–90% (Wilkens & Jörissen, 1999), and 81% (Blanc et al., 1998). Much less reduction (0–22%) was reported by Studer-Rohr et al. (1995); however, they studied green coffee beans that were naturally spoiled or on which a culture of *A. ochraceus* had been grown in the laboratory,

**Table 17. Reduction in concentrations of ochratoxin A during roasting of coffee**

Sample origin	Method of contamination	Ochratoxin A concentration (fg/kg)		Reduction (%)	Reference
		In green coffee	After roasting		
Zaire	Natural	8.6	0.2	98	Micco et al. (1989)
Conillon	Natural	4.0	0.3	92	Micco et al. (1989)
Santos	Spiked	46	6.1	87	Micco et al. (1989)
Costa Rica	Spiked	42	20	49	Micco et al. (1989)
Brazil/Ivory Coast	Spiked	47	11	80	Micco et al. (1989)
Thailand Robusta	Natural	7.3	1.4	81	Blanc et al. (1998)
Commercial	Spiked	780	890	0	Studer-Rohr et al. (1995)
	Spiked	1300	1200	11	Studer-Rohr et al. (1995)
	Natural (spoiled)	360	280	22	Studer-Rohr et al. (1995)
	Natural (spoiled)	140	121	16	Studer-Rohr et al. (1995)
Unknown	Natural	92	92	0	Studer-Rohr et al. (1995)
	Natural	0.90	0.63	30	Wilkens & Jörissen (1999)
	Natural	9.9	2.1	79	Wilkens & Jörissen (1999)
Ivory Coast Robusta	Natural	18	1.9	89	Wilkens & Jörissen (1999)
	Natural	4.9	1.5	69	Stegen et al. (2001)

creating artificially high concentrations (90–1300 µg/kg). It is unlikely that such high concentrations are relevant to the conditions found in commercial beans or that degradation of such high concentrations by natural chemical reactions during heating would resemble those that occur in the minute traces of ochratoxin A normally found in coffee beans.

## 5. LEVELS AND PATTERNS OF CONTAMINATION OF FOOD COMMODITIES

### 5.1 *Ochratoxin A*-producing fungi

Ochratoxin A was originally described as a metabolite of *Aspergillus ochraceus* in laboratory experiments (van der Merwe et al., 1965). It was subsequently reported in several related *Aspergillus* species (Ciegler, 1972; Hesselstine et al., 1972), but the first report of its natural occurrence, and its potential importance, was in a different source, a *Penicillium* species (Scott et al., 1970; Krogh et al., 1973). Recently, *A. carbonarius* was identified as a third major source, with a low percentage of isolates of the closely related species *A. niger* (Abarca et al., 1994; Téren et al., 1996). It is now clear that ochratoxin A is produced by a single *Penicillium* species, *P. verrucosum*, and a rather remarkable range of *Aspergillus* species. The following sections deal with these species in more detail.

#### 5.1.1 *Aspergillus* species that produce ochratoxin A

The latest information (Frisvad & Samson, 2000 and unpublished data) indicates that ochratoxin A is produced by only a few species related to *A. ochraceus*, all of which are classified in *Aspergillus* subgenus *Circumdati* section *Circumdati*. Apart from *A. ochraceus*, this group of ochratoxin A producers includes two ascosporic fungi, *Neopetromyces muricatus* (asexual state *A. muricatus*) and *Petromyces alliaceus* (asexual state *A. alliaceus*) plus *Aspergillus sclerotiorum* and *A. sulphureus*. *N. muricatus* is the correct name for isolates producing ochratoxin A that were previously identified as *A. melleus*. Both *N. muricatus* and *P. alliaceus* are uncommon species. *P. sclerotiorum* isolates make this toxin only rarely, and although isolates of *A. sulphureus* are usually producers, this is a rare species (J.C. Frisvad, personal communication). Apart from *A. ochraceus*, all of these species are very uncommon in foods and are not known to cause food spoilage. Hence, the only species of any importance for ochratoxin A production in the *Aspergillus* section *Circumdati* is *A. ochraceus*.

*A. carbonarius* has been recognized as a source of ochratoxin A only recently (Horie, 1995; Téren et al., 1996; Wicklow et al., 1996). It is now known that most if not all isolates of *A. carbonarius* are producers when grown in pure culture (Heenan et al., 1998; Taniwaki et al., 1999; J.I. Pitt and colleagues, unpublished), although the extent of production is variable. The closely related species *A. niger* has also been reported reliably as a producer (Ueno et al., 1991; Abarca et al., 1994; Heenan et al., 1998; Taniwaki et al., 1999; J.I. Pitt and colleagues, unpublished). All reports agree, however, that ochratoxin A production by *A. niger* is very uncommon, being formed under pure culture conditions by only 1–2% of isolates. *A. carbonarius* and *A. niger* are classified in *Aspergillus* subgenus *Circumdati* section *Nigri*.

A number of other species of *Aspergillus* have been reported to produce ochratoxin A, but in the opinion of Dr J.C. Frisvad, Department of Biotechnology, Technical University of Denmark, an authority on *Aspergillus* secondary metabolism, none of these has been substantiated (personal communication to J.I. Pitt, 2000).

### 5.1.2 *Penicillium* species that produce ochratoxin A

Soon after the isolation of ochratoxin A from *A. ochraceus*, the formation of ochratoxin A by a *Penicillium* species, *P. viridicatum*, was reported (van Walbeek et al., 1969), and its natural occurrence was confirmed (Krogh et al., 1973). The view that *P. viridicatum* was a major source of ochratoxin A in foods and feeds in some parts of the world was accepted for more than a decade. The species involved was later correctly identified as *P. verrucosum* (Pitt, 1987), as confirmed by others (Frisvad, 1989; Frisvad & Filtenborg, 1989). Although a number of more recent reports refer to ochratoxin A production by other, often unspecified, *Penicillium* species, this is known to be erroneous (Frisvad 1989; Frisvad & Filtenborg, 1989; Pitt & Hocking, 1997). It is now clear that *P. verrucosum* is the only *Penicillium* species that has been shown to produce ochratoxin A. It is classified in *Penicillium* subgenus *Penicillium* section *Penicillium*, along with many other mycotoxin-producing species.

### 5.1.3 *Physiology and ecology of fungi that produce ochratoxin A*

Each of the three major producers of ochratoxin A, *P. verrucosum*, *A. ochraceus*, and *A. carbonarius*, has a quite different physiology and consequently quite different ecological habitat. To understand the kinds of foods in which ochratoxin A occurs and to predict the potential for its formation, it is necessary to understand the physiology and ecology of these species and the differences between them.

#### (a) *Penicillium verrucosum*

*P. verrucosum* is a slowly growing species under any conditions but is capable of growth at low water activity ( $a_w$ ) (down to 0.80) and at low temperature (range, 0–31 °C; optimum, 20 °C) (Pitt & Hocking, 1997).

A notable feature of the ecology of *P. verrucosum* is that it grows only at lower temperatures. This results in a distribution which is apparently confined to cool temperate regions. Its major food habitat is cereal crops grown in cool temperate climates, ranging across northern and central Europe and Canada. It also occurs in European meat products and in cheese. It appears to be uncommon, indeed almost unknown, in warm climates or in other kinds of foods. The occurrence of this species in European cereals has two consequences: ochratoxin A is present in many kinds of European cereal products, especially bread and flour-based foods, and in animals that eat cereals as a major dietary component. Ochratoxin A was detected in Danish pig meat 25 years ago (Krogh et al., 1973), and its implications for human and animal health were recognized at the same time. As bread, other cereal products, and pig meats are major components of the European diet, the further consequence is that most Europeans who have been tested had appreciable concentrations of ochratoxin A in their blood (Hald, 1991; Petkova-Bocharova & Castegnaro, 1991; Breitholtz-Emanuelsson et al., 1993b; Zimmerli & Dick, 1995; Burdaspal & Legarda, 1998; Jiménez et al., 1998b; Scott et al., 1998). There is no doubt that this results from the growth of *P. verrucosum* in cereals.

(b) *Aspergillus ochraceus*

*A. ochraceus* can be described as a mesophilic xerophile. Growth occurs between 8 and 37 °C, with the optimum at 24–31 °C. Optimal conditions for growth are 0.95–0.99  $a_w$ , while the lower limit for growth is 0.79  $a_w$  on media containing sugars and down to 0.81  $a_w$  on media based on NaCl. *A. ochraceus* grows slowly at pH 2.2 and well between pH 3 and 10 (Pitt & Hocking, 1997).

*A. ochraceus* has been isolated from a wide range of food products but is more common in dried and stored foods than elsewhere. Stored foods from which it has been isolated include smoked and salted dried fish, dried beans, biltong, soya beans, chickpeas, rapeseed, pepper, dried fruit, and sesame seeds. Nuts are also a major source, especially pecans and pistachios, and also peanuts, hazelnuts, and walnuts. It has been reported infrequently in cereals and cereal products, including rice, barley, maize, wheat, flour, and bran. *A. ochraceus* has also been reported in cheese, spices, black olives, cassava, and processed meats. However, this species rarely causes spoilage and is often found in foods only at low concentrations; its presence is therefore not a good indicator of significant mycotoxin production (Pitt & Hocking, 1997).

Several workers have detected *A. ochraceus* in green coffee beans (Levi et al., 1974; Cantafora et al., 1983; Tsubouchi et al., 1984; Micco et al., 1989; Studer-Rohr et al., 1994), and this species appears to be one source of ochratoxin A in coffee (Taniwaki et al., 1999).

*A. ochraceus* has been isolated from a variety of South-East Asian commodities, including maize, peanuts, soya beans and other beans, cashews, and sorghum. Its presence or absence in any sample was probably related to the length of storage rather than to geographical location or other factors (Pitt et al., 1993, 1994, 1998).

As noted above, a few species closely related to *A. ochraceus* can produce ochratoxin A. Little is known about the physiology and ecology of any of these species, but what information there is suggests that their important features are similar to those of *A. ochraceus*. The occurrence of any of them in foods or food commodities is very rare, however.

(c) *Aspergillus carbonarius*

Relatively little is known about the third major ochratoxin A producer, *A. carbonarius*. The ability of this species to produce ochratoxin A was reported only recently (Horie, 1995; Téren et al., 1996; Varga et al., 1996; Heenan et al., 1998). It resembles *A. niger* in many features, and indeed the two species are very closely related. *A. carbonarius* differs from *A. niger* most notably in the production of larger spores, although other minor morphological differences exist. The available information on its physiology indicates a broad similarity to *A. niger*. However, preliminary studies indicate that *A. carbonarius* grows at rather lower temperatures than *A. niger*, with a maximum around 40 °C and optimal conditions about 32–35 °C. The ability to grow at reduced  $a_w$  is also more restricted: germination occurs down to 0.82  $a_w$  at 25 and 30 °C. Unlike *A. niger*, *A. carbonarius* failed to germinate at 0.82  $a_w$  and 37 °C (S.-L. Leong & J.I. Pitt, unpublished data).

*A. carbonarius* appears to be less common than *A. niger*, but many surveys of Aspergilli in foods have probably not differentiated the two species, calling all black Aspergilli *A. niger*.

As a result of the high resistance of the black Aspergilli, which include *A. niger*, *A. carbonarius*, and *A. japonicus*, to sunlight and ultra-violet light, a major habitat of these species is dried vine fruits, which in most producing countries are dried in the sun without preservatives. The incidence of the black Aspergilli in grapes at harvest and during drying has been studied in the major grape-growing region surrounding Mildura, Victoria, Australia, which is an irrigated area with a hot (35–42 °C) climate during the harvest season. The three major black species, *A. niger*, *A. carbonarius*, and *A. japonicus*, were all very common. The percentage of each species varied from season to season, presumably due to seasonal differences in climatic factors, especially average temperatures and rainfall patterns (S.-L. Leong & J.I. Pitt, unpublished data).

In that study, 470 *A. niger* isolates, 200 of *A. japonicus*, and 245 of *A. carbonarius* were isolated and identified. All were subsequently assayed for ochratoxin A production. The techniques included examination under ultra-violet light after growth on coconut cream agar (Heenan et al., 1998), TLC from agar plugs (Filténborg & Frisvad, 1980), and, on some isolates, growth in culture, extraction, and HPLC. *A. japonicus* did not make ochratoxin A, *A. niger* made it rarely and at low concentrations, but all isolates of *A. carbonarius* were capable of producing ochratoxin A. These findings indicate that it is extremely likely that *A. carbonarius* is the major source of ochratoxin A in grapes and grape products, including table grapes, wines, and dried vine fruits.

#### (d) *Aspergillus niger*

A detailed account of the ecology of *A. niger* is included here on the basis that *A. carbonarius* probably occurs in most habitats in which *A. niger* has been found. However, this must be regarded as a hypothesis, not a factual statement, at present.

Like many Aspergilli, *A. niger* grows optimally at relatively high temperatures, with minimal growth at 6–8 °C, maximal growth at 45–47 °C, and optimal conditions of 35–37 °C. *A. niger* is a xerophile with germination reported at 0.77  $a_w$  at 35 °C. The growth rates vary only slightly on media based on sugars, NaCl, or glycerol or at pH 4.0 and 6.5, and at various water activities. Thus, the growth of *A. niger* appears to be little affected by food type. *A. niger* can grow down to pH 2.0 at high  $a_w$  (Pitt & Hocking, 1997).

Among the fungi most commonly reported in foods, *A. niger* is prevalent in warmer climates, both in field situations and stored foods. It is by far the commonest *Aspergillus* species responsible for post-harvest decay of fresh fruit, including apples, pears, peaches, citrus, grapes, figs, strawberries, mangoes, tomatoes, and melons and some vegetables, especially onions, garlic, and yams (Snowdon, 1990, 1991). Most of these diseases are sporadic and of minor significance. *A. niger* sometimes causes kernel rot in cashews and can cause thread mould spoilage of cheese (Hocking & Faedo, 1992).

*A. niger* is among the commonest fungi isolated from nuts, especially peanuts, and has also been reported in pecans, pistachios, hazelnuts, walnuts, coconut, and copra. Cereals and oilseeds are also sources, especially maize and also barley, soya beans, canola, sorghum, stored and parboiled rice, and dried beans (Pitt & Hocking, 1997).

*A. niger* has commonly been isolated from South-East Asian foods (Pitt et al., 1993, 1994, 1998). The heaviest contamination was found in peanuts, maize,

cashews, copra, pepper, and spices from Indonesia, the Philippines, and Thailand and in kemiri nuts from Indonesia.

In terms of mycotoxin production, *A. nigeris* usually regarded as a benign fungus and has been widely used in food processing. It is categorized as 'generally regarded as safe' by the Government of the USA. However, 2 of 19 *A. niger* isolates were reported to produce ochratoxin A by Abarca et al. (1994) and 2 of 115 by Heenan et al. (1998). Ochratoxin A production by *A. niger* in commercially grown crops appears possible but is probably uncommon.

## 5.2 Results of surveys

The results of surveys for ochratoxin A are shown in Appendix A. As contamination with ochratoxin-producing fungi is widespread, numerous commodities have been analysed, including cereal and cereal products, green and roasted coffee, dried fruits, wine, grape juice, cocoa and chocolate, herbs and spices, canned foods, oils, olive, pulses, chickpeas, lentils, soya products, sweets, milk and milk products, meat, kidney, liver, beer, tea, vinegar, mustard, baby food, and house dust.

Most of the information for the past 5 years was taken from the literature and one Internet site. Other data were submitted to the Committee for the current meeting. The natural occurrence of ochratoxin A before 1995 was reviewed by WHO (1990) and the Commission of the European Union (1997).

The total number of samples for which data are shown in Appendix A was 23 167, with 85% from Europe (Croatia, Denmark, Finland, France, Germany, Italy, the Netherlands, Norway, Spain, Sweden, Switzerland, and the United Kingdom), 7% from South America (Brazil and Uruguay), 6% from North America (Canada and the USA), 1% from Africa (Sierra Leone and Tunisia), and 1% from Asia (Dubai and Japan).

When mean values and 90th percentiles were not available, they were calculated from a single datum, if available, assuming 0 for those samples containing no detectable toxin. The parent reference (P), analytical method (A) and sampling method used (S) are shown for each entry. When available, details of the sampling procedure are reported. The analytical methods cited in Appendix A are described in Table 13.

Adequate sampling procedures should be used for future surveys of ochratoxin A in cereals and cereal products. For example, 10 of 22 submitted papers giving data on cereals described the sampling procedure, whereas no description was reported in the remaining 12 papers. When the collected sample was small, the number of total samples was adequately reduced in order to limit the impact of occasionally high values on the weighted mean concentration (Jorgensen et al., 1996; Solfrizzo et al., 1998; Jurcevic et al., 1999). No sampling plans for the determination of ochratoxin A in foods have been published, and details of sampling variation have not been reported.

Most of the samples reported in Appendix A (95%) were analysed by LC with fluorescence detection, and the LOD or LOQ was usually < 0.5 µg/kg. The remaining 5% of samples were analysed by TLC with limits of detection or quantification > 5 µg/kg. These data were not used to estimate intake as the analytical method used did not provide an LOQ down to 5 µg/kg, the lower level that the Committee was requested to evaluate by the Codex Committee on Food Additives and Contaminants. In fact, only one of the 1225 samples analysed by TLC, a sample of

wheat, was found to be contaminated with 40 µg/kg of ochratoxin A (Pineiro & Giribone, 1994; Furlong et al., 1995a; Pineiro et al., 1996). When a more sensitive method was used (LOD = 0.2 µg/kg), most of the samples (78%) from the same geographical area were contaminated with ochratoxin A (Leoni et al., 2000).

The incidence of contaminated samples varied by commodity, and the incidence was higher in the same commodity when analytical methods with lower LOQs were used. For example, a survey of 300 samples of pig kidney in Denmark showed a high incidence of contamination (79%), and five samples were contaminated at > 5 µg/kg (Petersen, 2000). Similar results were obtained in an analysis of 61 samples of pig kidney collected in Germany (Wolff et al., 2000), whereas a low incidence of positive samples (6%) was found among 1010 samples collected in France (Dragacci et al., 1999). The different analytical methods used in these studies, with different LOQs (0.06 µg/kg and 0.5 µg/kg), could explain the discrepant results.

The concentrations of ochratoxin A in the various commodities were highly variable; 1.4% and 0.6% of all samples contained more than 5 µg/kg and 20 µg/kg, respectively. Within one type of cereal, 1.2% and 0.3% of samples contained more than 5 µg/kg and 20 µg/kg, respectively. Within cereal products, 0.3% and 0.05% of samples contained more than 5 µg/kg and 20 µg/kg, respectively. For this calculation, the cereal product samples from Tunisia were not considered, as they were selected samples from families of patients with nephropathy (Maaroufi et al., 1995b).

The weighted mean concentrations of ochratoxin A in cereals, cereal products, cocoa and chocolate, coffee, dried vine fruit, grape juice, pig kidney, other products of animal origin, and wine are shown in Table 18. Samples for which the mean concentration of ochratoxin A was lacking were not considered. High concentrations ( $\leq 50$  µg/kg) were also reported in herbs and spices (Patel et al., 1996; Rao, 2000) as well as in house dust ( $\leq 1600$  µg/kg), confirming the widespread presence of ochratoxin A (Richard et al., 1999). Low concentrations were found in all 50 samples of the total diet analysed in the United Kingdom by a very sensitive method capable of detecting down to 0.002 µg/kg (Ministry of Agriculture, Fisheries and Food, 1999b).

### 5.3 Distribution

As most of the samples (85%) represented the European diet, it is difficult to evaluate the geographical distribution of ochratoxin A. The data indicate that it occurs in coffee regardless of the geographical origin of the samples. For example, high percentages of contaminated samples were found in all countries and regions where coffee has been analysed, i.e. Brazil, Canada, Dubai, Europe, including eastern Europe, Japan, and the USA (Pittet et al., 1996; Stegen et al., 1997; Ueno, 1998; Trucksess et al., 1999; Government of Canada; Leoni et al., 2000; Rao, 2000; Wolff et al., 2000). There is clear evidence for the occurrence of ochratoxin A in cereals and cereal products in the European diet, but little information was available for other diets. The limited data indicate that ochratoxin A is also found in cereals produced elsewhere; in particular, contamination was found in 1 of 10 wheat samples and 3 of 28 mixed cereal samples in Dubai (Rao, 2000) and 56 of 383 wheat samples and 11 of 103 barley samples in the USA (Trucksess et al., 1999).

Ochratoxin A was found in 13 of 25 oat samples and 8 of 22 rye samples imported into Denmark (Jorgensen et al., 1996), 15 of 108 wheat samples and 12 of 41 rye samples imported into Norway (Langseth, 1999), and 14 of 139 maize samples imported into the United Kingdom (Scudamore & Patel, 2000). The data from Brazil

**Table 18. Weighted mean concentrations of ochratoxin A in commodities evaluated**

Commodity	No. of samples	Weighted mean concentration ( $\mu\text{g}/\text{kg}$ )
Beer	660	0.025
Cereals, all	2700	0.94
Barley	350	0.53
Maize	95	7.5
Oats	280	0.44
Rice	45	0.06
Rye	790	1.2
Wheat	1200	0.38
Cereal products	1500	0.19
Cocoa and chocolate	270	0.18
Coffee, green and roasted	1900	0.86
Green	130	1.0
Roasted	1700	0.76
Instant	290	1.4
Dried vine fruit	860	2.3
Grape juice	68	0.44
Pig kidney	380	0.12
Products of animal origin (liver, meat, sausages)	810	0.052
Wine, all	1800	0.32
Red	1300	0.4
White	260	0.1

and Uruguay could not be used as they had been obtained by analytical methods with high LODs, ranging from 5  $\mu\text{g}/\text{kg}$  to 50  $\mu\text{g}/\text{kg}$ , which are inadequate to detect and measure ochratoxin A at 0.94  $\mu\text{g}/\text{kg}$  and 0.19  $\mu\text{g}/\text{kg}$ , the weighted mean concentrations found in Europe and the USA in cereals and cereal products, respectively (Pineiro & Giribone, 1994; Furlong et al., 1995a,b; Soares & Furlani, 1996; Pineiro et al., 1996).

Indirect evidence for the occurrence of ochratoxin A in foodstuffs in Africa is provided by the high incidence of contaminated samples (35%) and the high mean ochratoxin A concentration (7.9  $\mu\text{g}/\text{kg}$ ) found in breast milk collected in Sierra Leone, where some infants were exposed to concentrations that far exceeded the permissible levels in animal feeds in developed countries (Jonsyn et al., 1995). These high concentrations indicate considerable human exposure to this toxin from food; however, no surveys have been reported from that area. More surveys are needed in regions of the world other than Europe in order that intake in those regions can be assessed.

An unexpectedly high mean concentration of ochratoxin A,  $\leq 33\,000$   $\mu\text{g}/\text{kg}$ , was reported in foods (wheat, barley, mixed cereals, dried vegetables, and olives) collected in Tunisia (Maaroufi et al., 1995b). One of the authors (E. Creppy) of the paper was contacted in order to check whether results had erroneously been reported as ng/kg instead of  $\mu\text{g}/\text{kg}$ . The response confirmed the concentrations reported in the paper, and the information was provided that the samples had been taken from members of families with one or more patient with nephropathy. These results should

be confirmed in further studies with validated methods for determination of ochratoxin A, and they were not considered for estimating intake.

#### **5.4 Annual variation**

Data on annual variations in contamination with ochratoxin A were available for wine (Pietri, 2000), wheat, barley (Scudamore, 1999), and maize (Jurcevic et al., 1999). Higher incidences of contaminated samples and higher mean concentrations of ochratoxin A were found in maize collected in Croatia in 1997 (35%, 57 µg/kg) than in 1996 (10%, 38 µg/kg) (Jurcevic et al., 1999). A survey of wheat and barley was carried out in the United Kingdom during the crop year 1993–94. In 1993, only two of 611 cereal samples (0.3%) were contaminated, each containing 15 µg/kg. In 1994, the incidence was much higher, with 22 of 450 samples (9%) containing ochratoxin A at concentrations ranging from 1 to 32 µg/kg (Scudamore, 1999). Limited data from Italy for red wine (169 samples) showed no substantial variation of incidence over the years 1996–99, ranging from 70% to 100%, whereas the mean concentration was 0.54–0.76 µg/kg in 1996–98 (54 samples) and 2.1 µg/kg in 1999 (115 samples) (Pietri, 2000).

## **6. FOOD CONSUMPTION AND DIETARY INTAKE ASSESSMENTS**

### **6.1 National and regional estimates of intake**

Ochratoxin A was first encountered as a natural contaminant in maize and to a lesser extent in some beans, including coffee and cocoa. Residues of ochratoxin A are not generally found in ruminants, because the toxin is cleaved in the rumen by protozoan and bacterial enzymes. Residues have been detected in a number of tissues of non-ruminant food animals, such as pigs, and in the muscle of hens and chickens but not in eggs.

Three types of data were submitted, the first on the occurrence of ochratoxin A in foods, the second on the intake of potentially contaminated foods, and the last on biomarkers of the exposure for use in epidemiological studies.

#### **6.1.1 Occurrence of ochratoxin A in foods**

Data were submitted by 13 countries: Argentina, Brazil, Canada, China, Denmark, France, Germany, Italy, Norway, Sweden, the United Kingdom, Uruguay, and the USA. Additional data were submitted by the Commission of the European Union and the Coffee Science Information Centre. Most of the data referred to cereals and cereal products, of which a total of 7877 samples were analysed. More than 2000 analyses were provided by Germany, pooled into six categories; 485 were from the USA, 212 from Sweden, 117 from Canada, and 75 from France. More than 2500 analyses were provided by the Commission of the European Union on the contamination of cereals by ochratoxin A in Denmark, Italy, The Netherlands, Spain, and the United Kingdom.

Brazil submitted information about ochratoxin A contamination in the electronic submission format of GEMS/Food. The data related to peanuts, grapes, beans, coffee, and maize, with a total number of 806 samples. The results for most of the

samples (624) were, however, expressed as a percentage of positive values or as a range.

Canada submitted the results of surveys conducted between 1997 and 1999 on ochratoxin A in coffee and cereals, in a total of 101 samples of coffee and 117 samples of cereals and cereal products.

China submitted analytical data for 1989–91. The LOQ was very high (10 µg/kg), and none of the samples had concentrations that exceeded this value.

A sample of kidney and a sample of muscle were taken from 300 pigs from all parts of Denmark in 1999 and were analysed for their content of ochratoxin A. The analyses were performed at the Division of Chemical Contaminants, Danish Veterinary and Food Administration.

France provided results on the occurrence of ochratoxin A in pork offal (1011 samples) and in various fruits and vegetables (333 samples). The mean, the median, and the distribution of the contamination in intervals were provided for each food category.

Germany provided information on a total of 6476 samples of various foods analysed between 1995 and 1998. The data were very detailed, giving the number of samples, the LOQ of the method of analysis, the number of samples with concentrations above the LOQ, the percentage of samples with amounts below the LOQ, and the range of ochratoxin A concentrations for each food category, and, in a majority of cases, the mean, median, and 90th percentile of the distribution of contamination.

Italy provided data on the occurrence of ochratoxin A in 280 samples of wine.

Norway provided results for more than 1000 samples of cereals and cereal products.

Sweden submitted information on ochratoxin A contamination in the GEMS/Food electronic submission format. The data were for wheat, oat, rye, beans, peas, and coffee (soluble, green, and roasted). Analytical results were also provided for each sample of wheat, pulses, and coffee, making it possible to calculate the standard deviation of the distribution.

More than 2000 analytical results for various commodities were submitted by the United Kingdom.

Uruguay submitted the abstracts of scientific publications presented during the last meeting of AOAC/IUPAC (Guaruja, May 2000). Only one publication was relevant to the occurrence of ochratoxin A and gave the percentage of positive values in 600 samples of maize collected between 1991 and 1998 in Argentina, Brazil, and Paraguay.

The USA submitted data from five laboratories, reflecting the concentrations of ochratoxin A in coffee, raisins, wheat, and barley. Some of the results for green coffee (180 samples) and soluble coffee (23 samples) were expressed as percentages of positive values.

The Commission of the European Union submitted an assessment of the dietary intake of ochratoxin A by the populations of Member States of the European Union, for which data on occurrence were collected from the 12 Member States and Norway. 'Best estimates' were made of the mean concentrations in foods and food groups, and these are included in this monograph except for countries that provided more recent and more accurate data directly to the Committee, i.e. France, Germany, and Sweden.

The Coffee Science Information Centre provided information on total exposure to ochratoxin A contained in the position paper of the Codex Alimentarius Commission (CX/FAC 99/14), the report of a survey by the Ministry of Agriculture, Fisheries and Food of the United Kingdom of human exposure to ochratoxin A, and several published papers on the occurrence of ochratoxin A in coffee in European Member States and the USA (Patel et al., 1997; Stegen et al., 1997; Burdaspal & Legarda, 1998b; Jorgensen, 1998; Trucksess et al., 1999).

### **6.1.2 Consumption of potentially contaminated foods**

National food consumption data were provided by France, Germany, Sweden, and the Commission of the European Union.

The distribution of consumption of foods in the relevant food categories was provided on the basis of individual data. A report in which data on food intake were compared with data on contamination by ochratoxin A indicated the distribution of intake and the probability of high intake.

The German approach consisted of combining the median and the 90th percentile of the distribution of contamination with various portion sizes (small, medium, and large) obtained in 3-day and 4-week studies of mean intake. The basis of the intake studies was not provided. Surprisingly, the intakes in the longer study were higher than those obtained in the shorter study.

A Swedish report provided an estimate of the intake of ochratoxin A from various foods and was based on data on food consumption from their study of dietary habits and nutrient intake in Sweden (Becher, 1992; Swedish National Food Administration, 1994).

Data on consumption were submitted in two reports from the European Union Scientific Cooperation (SCOOP) programme: from Task Group 3.2.2 (Commission of the European Union, 1997) and from Task Group 4.1 (Commission of the European Union, 1996). These reports provided a compilation of the mean intakes of various food categories across the European Union.

### **6.1.3 Biomarkers of exposure**

The results of intake assessments that included biomarkers were provided by Sweden and the Commission of the European Union. The results of a study in the United Kingdom, available on the Internet, were submitted by the Coffee Science Information Centre.

Sweden submitted a publication on the concentrations of ochratoxin A in blood from Norwegian and Swedish donors (Thuvander et al., 2000). The mean concentration of ochratoxin A in plasma ranged from 0.17 to 0.21 ng/ml, and the 95th percentile concentration was 0.38–0.57 ng/ml.

The Ministry of Agriculture, Fisheries and Food in the United Kingdom conducted a survey of intake of ochratoxin A in which samples of duplicate diets, plasma, and urine were collected each week from 50 volunteers living in one area of the United Kingdom. A statistical analysis of the results indicated a stronger correlation between the urinary concentration of ochratoxin A and the level of consumption than with the plasma concentration.

SCOOP report 3.2.2 contains data on the occurrence of ochratoxin A in plasma and milk collected from healthy persons between 1977 and 1994 in Denmark, France, Germany, Italy, and Sweden. These data showed a mean concentration of ochratoxin A in plasma of 1.8 ng/ml in Denmark (1986–88), 0.4 ng/ml in France (1993), 0.45 ng/ml in Germany (1977–94), 0.53 ng/ml in Italy (1992), and 0.18 ng/ml in Sweden (1994).

## **6.2 Assessment of intake at the international level**

Intake at the international level was assessed from data on mean consumption combined with the weighted mean of contamination. As ochratoxin A occurs mainly in Europe, data on food consumption in Europe obtained from the GEMS/Food programme were considered the most relevant for risk assessment. As no information was available on coffee, beer, wine, or fruit juices in the GEMS/Food database, the mean intakes of the food categories in Europe were obtained from a report of the Commission of the European Union (1997), and those for coffee from a published paper (Jorgensen, 1998).

The available data on levels of contamination were aggregated as per the recommendations of a FAO/WHO workshop (Geneva, June 2000). The national results were presented in an aggregated format, so that one figure could represent the mean of a large number of individual samples. The first step therefore consisted of weighting each result as a function of the number of samples it represented. Each result was then multiplied by the number of individual samples in the original survey. The sum was then divided by the total number of individual samples. The result of this operation provided a weighted mean level of contamination for the food category considered. Some data were not used because they were expressed only in terms of the presence or absence of ochratoxin A, with no quantification of the mean level of contamination. As it was not possible to identify analytical results for targeted samples, all the data were considered to be representative of the total contamination of foodstuffs.

Each weighted mean can therefore be multiplied by the mean consumption of the corresponding food category to derive the contribution of that food category to human intake. The results are presented in Table 19. With this approach, the mean total intake of ochratoxin A was about 45 ng/kg bw per week, assuming a body weight of 60 kg.

From these calculations, various food categories could be classified as a function of their potential impact in terms of public health. Cereals and wine contributed about 25 and 10 ng/kg bw per week, respectively, to average intake, whereas grape juice and coffee each contributed 2–3 ng/kg bw per week. Other food products (dried fruits, beer, tea, milk, cocoa, poultry, and pulses) contributed < 1 ng/kg bw per week. Most of the results submitted for pig meat and products were for pig liver and kidney, whereas the figure for food consumption was based on pig meat: the resulting estimate of 1.5 ng/kg bw per week can therefore be considered a gross overestimate of intake.

The data on 'cereal products' included several foods manufactured from cereals. In these products, the reported mean levels of contamination were about one-fifth those in the raw material. However, these data were not adequate for use in the intake assessment.

The second step, relating to contamination of the most relevant food categories for human intake (i.e. cereals, wine, grape juice, and coffee), consisted of simulating a worldwide distribution of ochratoxin A on the basis of several assumptions (WHO, 2000b). The first concerns the form of the distribution curve. It is generally considered that food contaminants follow a log-normal distribution. In order to construct a global distribution curve assuming log-normality, the mean and the standard deviation must be determined.

The results are presented in Table 20 and in Figures 1–4.

### 6.3 Impact of alternative maximum limits on intake

In order to assess intake from cereals, a probabilistic approach was used, in which a simulated distribution of contamination and the distribution of cereal consumption in France were used.

**Table 19. Classification of food categories as a function of their relative contribution to human exposure**

Food category	Contamination (µg/kg)	Intake		
		g	µg/person per week	ng/kg bw per week
Cereals <sup>a</sup>	0.94	230	1.5	25
Beer <sup>b</sup>	0.023	260	0.04	0.69
Wine <sup>c</sup>	0.32	240	0.54	8.9
Grape juice <sup>d</sup>	0.39	69	0.19	3.1
Tea	0.3	2.3	0.00	0.08
Cocoa	0.55	6.3	0.02	0.40
Pork	0.17	76	0.09	1.5
Poultry	0.041	53	0.02	0.25
Dried fruits	2.2	2.3	0.03	0.58
Pulses	0.19	25	0.03	0.55
Roasted coffee <sup>e</sup>	0.76	24	0.13	2.1

<sup>a</sup> From GEMS/Food database, data for the Far Eastern diet

<sup>b</sup> From SCOOP report 4.1, data for Norway

<sup>c</sup> From SCOOP report 4.1, data for the United Kingdom

<sup>d</sup> From SCOOP report 4.1, data for Portugal

<sup>e</sup> From Jorgensen (1998)

For cereal consumption, the results of a survey of food consumption by 1161 individuals were used, with individual body weights, assuming that 100% were consumers with a mean consumption of 28 g/week per kg bw and consumption at the 95th percentile of 61 g/week per kg bw.

For contamination of cereals, assuming that the distribution is log-normal, the model included the mean and the standard deviation and, for the specific task, the minimum and the maximum values. The maximum value was assumed to be 0 in all situations. Three simulations were made, the first with the maximum observed value (121  $\mu\text{g}/\text{kg}$ ), the second with the higher proposed maximum limit (20  $\mu\text{g}/\text{kg}$ ), and the third with the lower proposed maximum limit (5  $\mu\text{g}/\text{kg}$ ).

A probabilistic approach with a Monte-Carlo simulation made it possible to assess the intake of ochratoxin A by the population. The software used was @risk, and the equation for constructing the curves for contamination was riskTlognorm (mean, SD, min, max).

**Table 20. Distribution of contamination of major food categories with ochratoxin A**

Food category	No. of samples	Weighted mean	Standard deviation (ln)
Cereals	2714	0.94	1.23
Cereal products	1536	0.19	1.96
Wine	1834	0.32	1.26
Grape juice	68	0.44	0.48
Green coffee	127	1.02	6.12
Roasted coffee	1726	0.76	0.29
Grapes	857	2.29	0.79

**Figure 1. Simulated distribution of ochratoxin A in cereals**

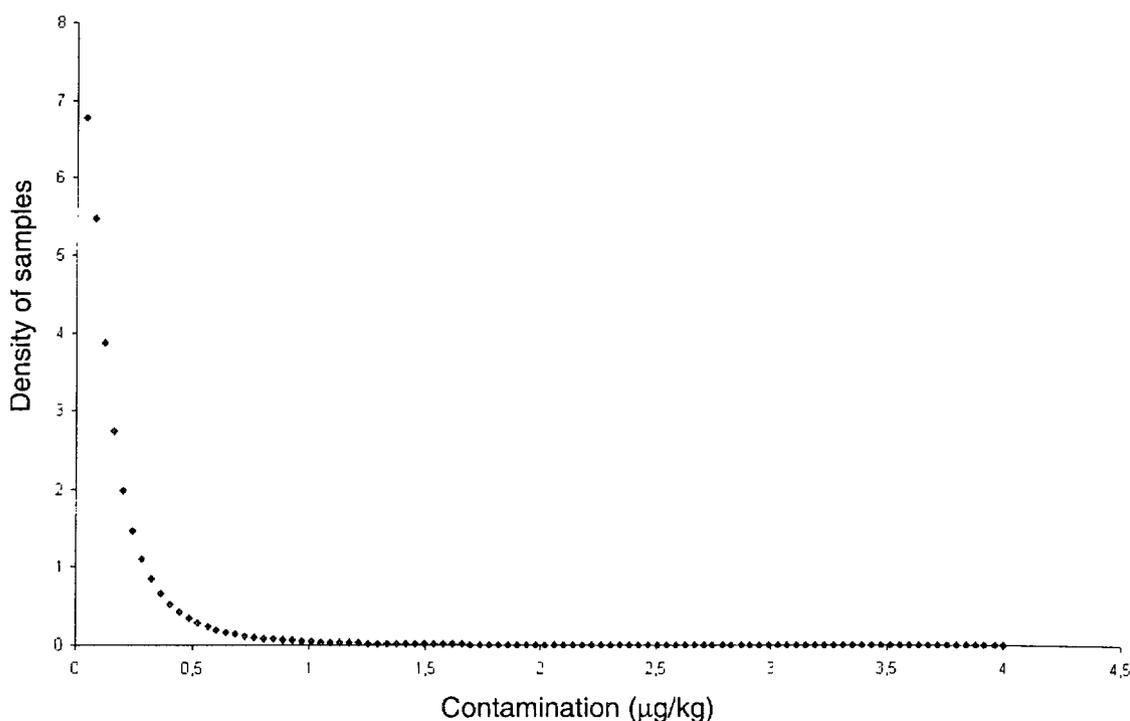


Figure 2. Simulated distribution of ochratoxin A in roasted coffee

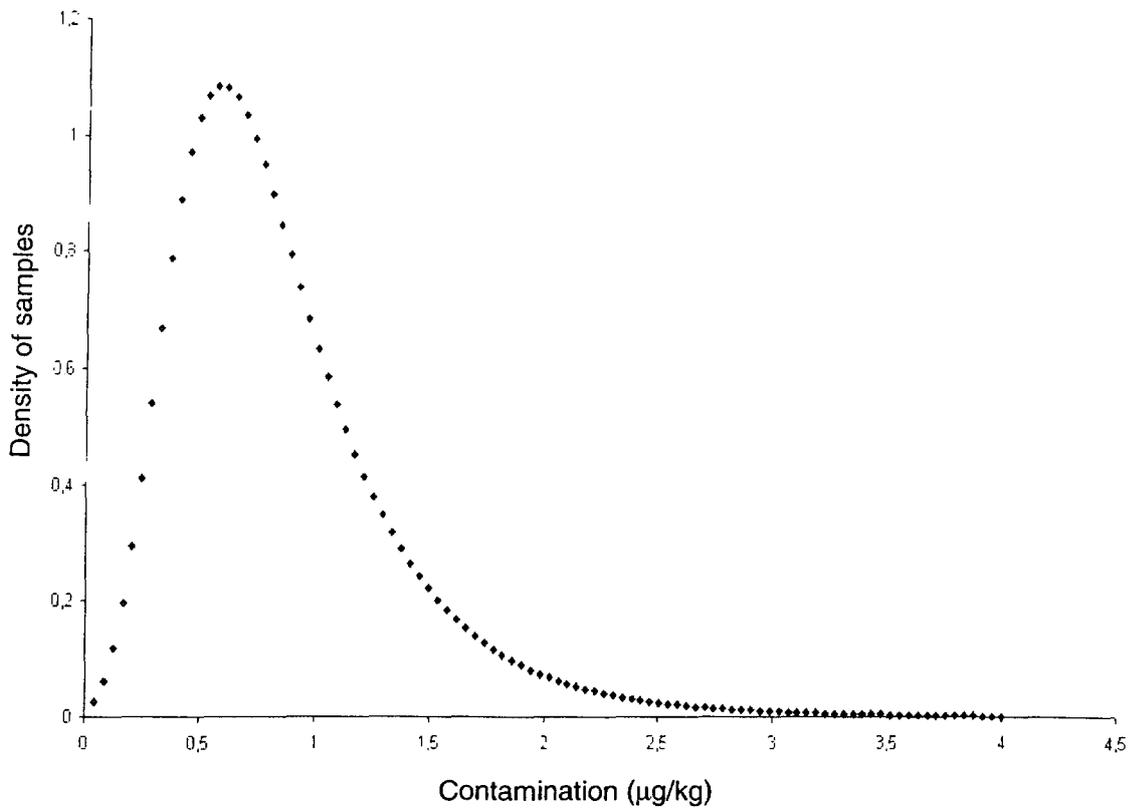
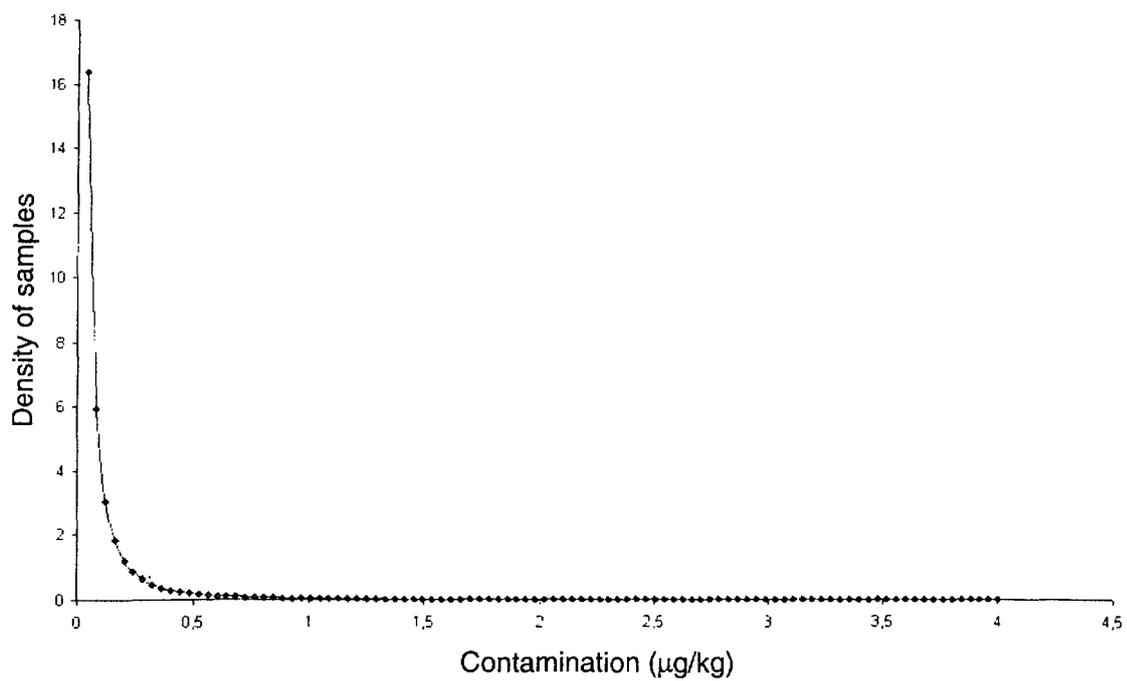
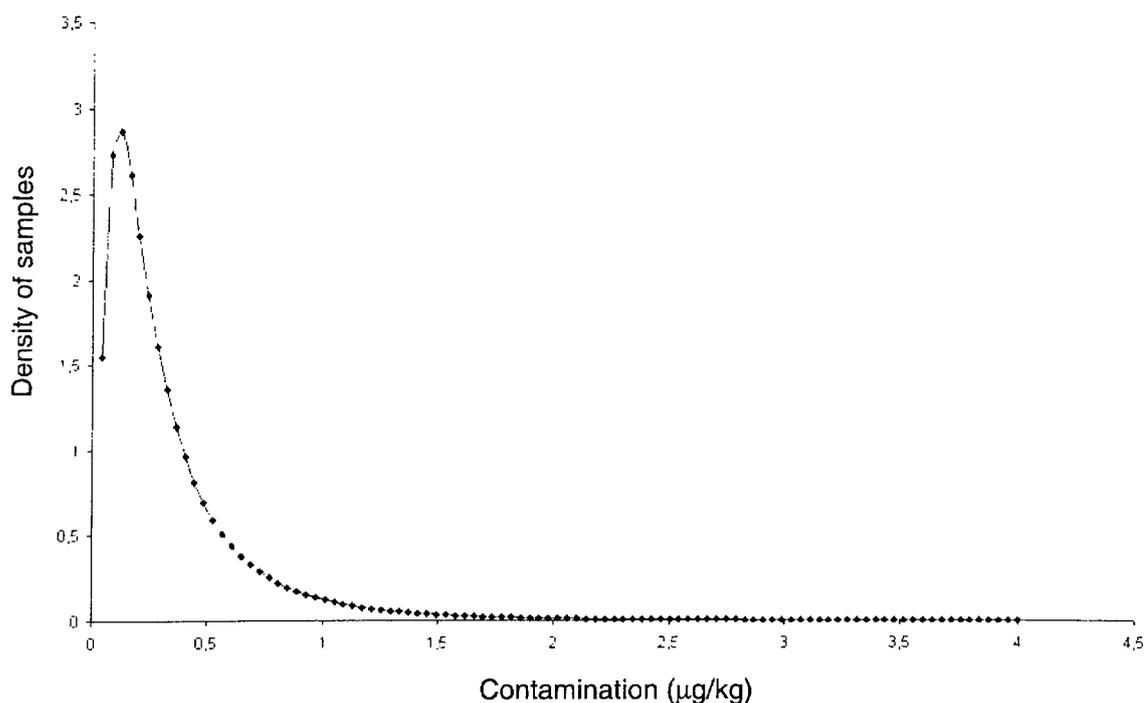


Figure 3. Simulated distribution of ochratoxin A in wine



**Figure 4. Simulated distribution of ochratoxin A in grape juice**



This simulation, which was considered to be realistic for European-type diets, showed that the intake of ochratoxin A by consumers of cereals at the 95th percentile would be 92 ng/kg bw per week. Use of the proposed maximum limit of 5 µg/kg, as opposed to 20 µg/kg, would have a statistically significant effect on intake only for consumers of cereals above the 95th percentile. However, the difference would be very small (84 vs 92 ng/kg of body weight per week) in view of the distribution of the level of contamination indicated by the available data.

## **7. PREVENTION AND CONTROL OF OCHRATOXIN A PRODUCTION**

Control of ochratoxin A in foods depends on the kind of crop and its geographical location, as those two factors are primarily responsible for determining which of the three major ochratoxin A-producing fungi is likely to grow and produce toxin. Moreover, as experience with other mycotoxins has shown, the likelihood and extent of toxin production by any particular species is greatly influenced by whether the fungus concerned has a particular affinity for a specific crop, i.e. can invade and grow in a crop before or during harvesting and drying. As these factors, and the physiology of the three species, differ, the control of ochratoxin A production by each species is considered separately.

### **7.1 By *Aspergillus ochraceus***

As stated above, *A. ochraceus* occurs primarily in stored foods, and no association with plants before harvest is known. Its control therefore consists mainly of the standard methods used for controlling the growth of any fungus in dried foods.

### 7.1.1 Grains

The main commodities in which *A. ochraceus* may produce ochratoxin A are stored grains. The traditional way of avoiding microbial growth in grains is to dry them thoroughly and to keep them dry. Adequate ventilation in storage bins will remove moisture, prevent condensation, lower and equilibrate temperatures, and prevent heating. Over the past 20 years, bag stacks and manual handling of grain have given way to bulk handling and storage, with great improvements in the control of insect and fungal damage, even in some tropical areas (Champ & Highley, 1988). Drying techniques have made great advances, with sophisticated computer control of drying rates and temperatures now in use, at least in developed countries. Control of insect and fungal damage in grain stores is of particular importance in the tropics, where most grains are stored in sacks in warehouses unsuitable for sealing and fumigation. Recent approaches to sealing stacks of bags in such stores, fumigating and then maintaining the sealed stacks under controlled atmospheres have shown the potential to greatly reduce grain losses (Annis, 1990a; Graver, 1990).

The moisture content of grains must be reduced to below 0.8  $a_w$  (17–19% moisture; Iglesias & Chirife, 1982) to prevent ochratoxin A formation by *A. ochraceus*.

Modern approaches to grain storage rely on fumigation and sealed storage under controlled atmospheres, especially in tropical and subtropical regions where insect damage is a major problem (Champ et al., 1990). Fumigants, highly toxic gases or vapours added to grain stores specifically to kill insects, in some cases also achieve fungal destruction. Fumigants are usually used as a rapid method for killing insects and are subsequently removed by ventilation. A variety of gases have been used as fumigants, either singly or in combination, including ethylene dichloride, carbon tetrachloride, carbon disulfide, ethylene dibromide, chloropicrin, hydrogen cyanide, ethylene oxide, methyl chloride, methyl bromide, and phosphine. For various reasons, only methyl bromide and phosphine are in widespread use (Annis, 1990b). The concentrations recommended for use are given in Table 21. Environmental considerations are resulting in the phasing out of methyl bromide, and the search for alternative fumigants continues.

Controlled atmospheres may be used for grain storage. This technique relies on continuous application of atmospheres low in  $O_2$  or with high  $CO_2$  concentrations. The recommended approach is to add such gas mixtures to sealed storage and maintain the grain in totally sealed systems. Where this is not practicable, continuous flow of such gas mixtures may be possible (Annis, 1990b). The recommended  $O_2$  and  $CO_2$  concentrations are given in Table 21.

Fumigation and controlled atmospheres help control mould growth on grains by directly destroying spores, by inhibiting growth, or by killing insects that damage kernels. Fumigants that merely destroy insects have no lasting effect on mould growth (Vandegrift et al., 1973); however, methyl bromide destroys fungi as well as insects (Majumder, 1974), and phosphine has some fungicidal properties (Hocking & Banks, 1993). Modified atmospheres that control insects may have a substantial effect in controlling fungi as well (Hocking, 1990).

Many investigators have suggested using heat or chlorine to destroy microorganisms in grains; however, this technique has had little use, with the recognition that such remedial processes do not destroy mycotoxins and are not a substitute for clean grain. In the same way, ionizing radiation at 2–3 kGy destroys moulds that

**Table 21. Suggested target doses for gaseous treatments of grain at 25 °C**

Gas	Time (days) <sup>a</sup>	Concentration <sup>b</sup>	Concentration x time
Carbon dioxide	15	> 35%	–
Oxygen	20	< 1%	–
Phosphine	7	100 mg/m <sup>3</sup>	–
Methyl bromide	1–2	–	150 g h/m <sup>3</sup>
Hydrogen cyanide	1	Not well defined	–

From Annis (1990b)

<sup>a</sup> In cases of slow gas introduction or poor gas distribution, longer exposure may be necessary.

<sup>b</sup> Minimum concentration achieved at end of exposure

ordinarily spoil rice (Iizuka & Ito, 1968; Ito et al., 1971), but this process remains illegal or unacceptable to consumers in many countries.

### 7.1.2 Coffee

Control of ochratoxin A production in coffee by either *A. ochraceus* or *A. carbonarius* is similar, but the control measures used are different from those for other commodities. Although some research is still required, it appears that ochratoxin A can be controlled in coffee by good manufacturing practice. Studies in Brazil by Taniwaki et al. (1999) and Taniwaki and Pitt (unpublished) have shown that mould growth and ochratoxin A production occur only during drying of green coffee beans, and that if drying is rapid and effective ochratoxin A will not be produced. Good sun-drying or a combination of sun-drying and mechanical dehydration provide effective control. No evidence has been found that either *A. ochraceus* or *A. carbonarius* invades coffee beans before harvest or has an association with the coffee tree.

The formation of ochratoxin A during drying of coffee was studied in Thailand by Bucheli et al. (2000), who showed that the toxin was normally produced during sun-drying of coffee in that country, that overripe cherries were more susceptible than green ones, and that defects, especially the inclusion of husks, were the most important source of ochratoxin A contamination. They agreed with Taniwaki et al. (1999), that better quality raw material, appropriate drying and dehulling procedures, and reduction of defects can substantially reduce the concentration of ochratoxin A in green coffee.

The formation of ochratoxin A did not increase during storage for 18 months in Thailand, even with bag storage at high humidity (Bucheli et al., 1998). However, as the initial  $a_w$  of the beans stored in bags was 0.72 and did not exceed 0.75 even in the rainy season, these results are not surprising.

The suggestion by Mantle (1998) that ochratoxin A in coffee beans may result from uptake of ochratoxin A in soil by the roots of the coffee tree and then translocation is conjectural at best.

## 7.2 By *Aspergillus carbonarius*

The available evidence indicates that *A. carbonarius* and *A. niger* are not pathogens on fruit, but saprophytes, and hence cannot gain entry to sound fruit.

However, damage to fruit by any means, mechanical, chemical, or by disease microorganisms, may allow entry into fruit tissue, where the low pH, high sugar, and often warm temperature provide an ideal habitat for these species. This is especially true of grapes, which have very tough skins. When the skins are intact, they are resistant to attacks by these fungi, but ideal growth conditions prevail once the skin is disrupted. Control of growth of these species in grapes before harvest therefore relies on:

- control of pathogenic fungi, especially *Rhizopus stolonifer*, *Botrytis cinerea*, and powdery mildews such as *Erysiphe* species;
- control of mechanical damage, from pruning, leaf reduction and, for dried fruit, harvesting equipment; and
- control of splitting due to rain just before harvest.

Growth of fungal pathogens is especially difficult to control on cultivars that have tight bunches or excessive leaf cover over bunches, or under rainy or misty conditions at harvest time. Control of *Rhizopus stolonifer*, *Botrytis cinerea*, and *Erysiphe* species relies primarily on vineyard hygiene, with removal of diseased plant tissue, thinning of tight bunches, and removal of excessively tight leaf clusters (Snowdon, 1990; Emmett et al., 1992). Fungicides are sometimes used against pathogenic fungi, but the effectiveness of such treatments is variable and depends on seasonal and geographic factors (Nair et al., 1987; Snowdon, 1990).

Control of mechanical damage relies on good farm management. In many countries, the importance of minimizing damage to grapes probably still needs emphasis. This is a serious problem when grapes are to be dried, as mechanical damage at harvest is difficult to avoid and the length of the drying process (2 weeks or more) provides ample time for growth of *A. carbonarius* and ochratoxin A production. This is much less of a problem with wine grapes, which are usually crushed within a few hours of picking, and it is a reasonable assumption that the rapid establishment of anaerobic conditions prevents further growth of *A. carbonarius* or ochratoxin A production.

Some cultivars, especially the sultana grapes favoured for drying, are susceptible to rain damage during the week preceding harvest, when turgor pressure inside the fruit is high and the skins often inflexible. Splitting around the neck of the grape below the stem provides an ideal environment for invasion by *A. carbonarius*. Control is very difficult. The only useful recommendation is to cut vines or bunches and commence drying as quickly as possible after rain, if damage is seen as a possibility.

Little has been published specifically about preharvest and postharvest control of ochratoxin A production in coffee by *A. carbonarius*, but see the section on *A. ochraceus*, above.

### 7.3 By *Penicillium verrucosum*

Little published information exists about the time of invasion of cereal crops by *P. verrucosum*. It is commonly stated that this species is a storage fungus, invading after harvest, but that does not explain the paradox that *P. verrucosum* is a slowly growing, not notably xerophilic species which should compete poorly against many other species known to spoil grains. There have been few reports on the incidence of *P. verrucosum* in Canadian or northern European grain. Frisvad & Vuif (1986) found *P. viridicatum* Group II (= *P. verrucosum*) in each of 70 samples of Danish

grain containing ochratoxin A. Holmberg et al. (1991) found that the incidence of storage fungi, and particularly *P. verrucosum*, was significantly higher in feed samples (barley and oats or cereal mash feed) of pig herds infected with ochratoxin A: Ochratoxin A-producing *P. verrucosum* was found in 60% of feed samples of infected herds and in only 5% of feed of uninfected herds. Moreover, as ochratoxin A is known to occur in grain in cold climates and no *Aspergillus* species is likely to occur in such grain, and as *P. verrucosum* is the only *Penicillium* species that produces ochratoxin A, it is likely that *P. verrucosum* is the source of ochratoxin A in Canadian and northern European grains.

The occurrence of ochratoxin A in such grains is attributed to insufficient drying or over-long storage before drying. Jonsson et al. (1997) studied the effect of moisture content, temperature, and time on the growth of moulds and the production of ochratoxin A in winter wheat. The maximum storage time without mould growth appeared to be halved when the moisture content at harvest was increased by 2–3% or if the storage temperature was increased by 5 °C. Ochratoxin A could generally be detected quite soon after microbial growth had begun.

The occurrence of ochratoxin A in dried grain used for human food can be controlled by analysis and segregation of defective lots. Little or no information exists about whether this procedure is practised anywhere in the world.

## 8. COMMENTS

### *Absorption, distribution, metabolism and excretion*

Ochratoxin A is slowly absorbed from the gastrointestinal tract. It is distributed in a number of species via the blood, mainly to the kidneys, lower concentrations being found in liver, muscle, and fat. Transfer to milk has been demonstrated in rats, rabbits, and humans, but little is transferred to the milk of ruminants owing to metabolism of ochratoxin A by the ruminal microflora. The major metabolite of ochratoxin A in all species examined is ochratoxin  $\alpha$ . This and minor metabolites that have been identified are all reported to be less toxic than ochratoxin A itself. Ochratoxin A is excreted in urine and faeces, and the relative contribution of each of these routes in different species is influenced by the extent of the enterohepatic recirculation of ochratoxin A and its binding to serum macromolecules. These factors are also important in the determination of the serum half-life of ochratoxin A, which varies widely among species. It has a long half-life in non-ruminant mammals, e.g. 24–39 h in mice, 55–120 h in rats, 72–120 h in pigs, 510 h in one macaque, and 840 h in a volunteer.

### *Toxicological studies*

Ochratoxin A has been shown to be nephrotoxic in all mammalian species tested. Its main target is the renal proximal tubule, where it exerts cytotoxic and carcinogenic effects. Significant sex and species differences in sensitivity to nephrotoxicity were evident, in the order pig > rat > mouse. The doses at which carcinogenicity was observed in rodents were higher than those that caused nephrotoxicity. The Committee reconsidered the report of the study of carcinogenicity conducted by the National Toxicology Program (USA) in 1989 and noted the consistent presence and severity of karyomegaly in male and female rats and the aggressive nature of the

renal tumours in this study. However, the biological and mechanistic significance of these observations was unclear.

Gene mutations were induced in bacteria and mammalian cells in a few studies of genotoxicity, but not in most. Ochratoxin A did, however, induce DNA damage, DNA repair, and chromosomal aberrations in mammalian cells in vitro and DNA damage and chromosomal aberrations in mice treated in vivo. Putative DNA adducts were found consistently with a  $^{32}\text{P}$ -postlabelling method in the kidneys of mice and rats dosed with ochratoxin A, but none of these adducts has been demonstrated to contain fragments of ochratoxin A. It was therefore uncertain whether ochratoxin A interacts directly with DNA or whether it acts by generating reactive oxygen species. There was no indication that a reactive metabolite of ochratoxin A is generated in vivo. Ochratoxin A is thus genotoxic both in vitro and in vivo, but the mechanism of genotoxicity is unclear and there was no evidence that it is mediated by direct interaction with DNA. The doses used in the studies of genetic toxicity were in the same range as those at which the incidence of renal tumours was increased in mice. In rats, however, the incidences of nephrotoxicity and renal tumours were increased at much lower doses; therefore the contribution of the genotoxicity of ochratoxin A to neoplasia in rats is unknown.

Ochratoxin A can cross the placenta and it is embryotoxic and teratogenic in rats and mice. It has been shown to have immunosuppressive effects in a number of species. Prenatal administration of ochratoxin A to rats caused immunosuppression, but perinatal administration stimulated certain aspects of the immune response in rats. Ochratoxin A inhibited the proliferation of B and T lymphocytes and affected the late stages of T-lymphocyte activation in vitro. However, both the immunological and teratogenic effects have been observed only at doses much higher than those that cause nephrotoxicity.

#### *Observations in humans*

Ochratoxin A has been found in human blood samples, most notably in a number of countries in the cool temperate climatic areas of the Northern Hemisphere; however, no cases of acute intoxication in humans have been reported. The Committee noted that ochratoxin A was found more frequently and at higher average concentrations in blood samples obtained from people living in regions where a fatal human kidney disease (known as Balkan endemic nephropathy) occurs and is associated with an increased incidence of tumours of the upper urinary tract. Nevertheless, similar average concentrations have been reported in several other European countries where this disease is not observed. The Committee concluded that the epidemiological and clinical data available do not provide a basis for calculating the likely carcinogenic potency in humans and that the etiology of Balkan endemic nephropathy may involve other nephrotoxic agents.

#### *Analytical methods*

Reliable, validated methods have been developed for the analysis of ochratoxin A in maize, barley, rye, wheat, wheat bran, wheat whole meal, roasted coffee, wine, and beer, which are based on liquid chromatography with fluorescence detection. The limit of quantification was 0.03  $\mu\text{g}/\text{kg}$  for wine and beer and 0.3–0.6  $\mu\text{g}/\text{kg}$  for other commodities. These methods have also been used successfully to analyse a

number of other cereals, cereal products, and dried fruit. Two certified reference materials (blank and naturally contaminated wheat) are available, which improve quality assurance in laboratories. Screening methods based on TLC are available but have been used in only a few laboratories. Data obtained by these analytical methods, with a limit of quantification greater than 5 µg/kg, were not considered in this evaluation, as this was the lower concentration for which the Codex Committee on Food Additives and Contaminants requested a risk assessment. Furthermore, enzyme-linked immunosorbent assay (ELISA) techniques had not been used to produce the survey data considered by the Committee.

There are no formally validated methods for the analysis of ochratoxin A in human blood. The available methods are based on liquid chromatography with fluorescence detection and have different limits of quantification, ranging from about 0.1 to 2 ng/ml.

#### *Sampling protocols*

Adequate sampling procedures should be used in future surveys of cereals and cereal products for ochratoxin A. For example, an acceptable sampling procedure was used in 10 of 22 studies on cereals submitted to this Committee, whereas no description was reported in the remaining 12. No sampling plans for the determination of ochratoxin A in foods have been published, and details of sampling variability have not been reported.

#### *Effects of processing*

Milling has been reported to reduce substantially the concentration of ochratoxin A in white flour, but it had little effect on levels in wholemeal flour. Milling is a physical process: the ochratoxin A removed from the grain in the production of white flour remains in bran and other fractions, some of which may be used in foods. Ochratoxin A is relatively stable to heat: at 100 °C, a 50% reduction in the concentration was achieved after 2.3 h in wet wheat and 12 h in dry wheat. The process involved in the manufacture of breakfast cereals and biscuits resulted in substantial reductions in ochratoxin content, but little or no reduction was found in the manufacture of egg noodles and pasta. Decaffeination of coffee reduced the ochratoxin concentration by about 90%. The reduction obtained by roasting coffee varies but may also be as much as 90%.

#### *Levels and patterns of contamination of food commodities*

The data on ochratoxin A over the past 5 years that were reviewed by the Committee originated mainly from Europe (85%); 7% came from South America, 6% from North America, 1% from Africa, and 1% from Asia. The concentrations of ochratoxin A in the different commodities were highly variable; 1.4% and 0.6% of samples contained more than 5 µg/kg and 20 µg/kg, respectively. Within the cereals, 1.2% and 0.3% of samples contained more than 5 µg/kg and 20 µg/kg of ochratoxin A, respectively. Within cereal products, 0.3% and 0.05% of samples contained more than 5 µg/kg and 20 µg/kg of ochratoxin A, respectively. The weighted mean concentrations of ochratoxin A that were used for estimating intake were: 0.94 µg/kg for cereals, 0.19 µg/kg for cereal products, 0.32 µg/kg for wine, 0.86 µg/kg for coffee, 2.3 µg/kg for dried vine fruit, and 0.44 µg/kg for grape juice. The incidence of samples

found to contain ochratoxin A depended on the commodity and was higher in the same commodity when analytical methods with lower limits of quantification were used.

#### *Food consumption/intake assessment*

Intake of ochratoxin A at the international level has been assessed on the basis of data on mean consumption combined with weighted mean levels of contamination. As ochratoxin A occurs mainly in the diet in European countries, data on food consumption in Europe obtained from the GEMS/Food database were considered the most relevant for risk assessment. The submitted data on levels of contamination were aggregated according to the recommendations of a FAO/WHO workshop to obtain a weighted mean. When this approach was used, the mean total intake of ochratoxin A was estimated to be 45 ng/kg bw per week, assuming a body weight of 60 kg.

Cereals and wine contributed about 25 and 10 ng/kg bw per week, respectively, to the mean intake, whereas grape juice and coffee each contributed 2–3 ng/kg bw per week. Other food products (dried fruits, beer, tea, milk, cocoa, poultry, and pulses) contributed less than 1 ng/kg bw per week. Most of the results submitted for pig meats and pig meat products were for samples of pig liver and kidney, whereas the figure for food consumption in the GEMS/Food database was based on pig meats. The resulting estimate of 1.5 ng/kg bw per week can therefore be considered a gross overestimate of intake.

A probabilistic approach was used to assess intake from cereals and cereal products, in which a simulated distribution of contamination and the distribution of cereal consumption in France were used. This example, which was considered to be realistic for European diets, showed that consumers of cereals at the 95th percentile would have an intake of ochratoxin A of 92 ng/kg bw per week. Use of a proposed maximum limit of 5 µg/kg as opposed to 20 µg/kg would have a statistically significant effect on intake of ochratoxin A only for consumers of quantities of cereals greater than the 95th percentile. However, the difference would be very small (84 vs 92 ng/kg bw per week at the 95th percentile) in view of the distribution of the level of contamination indicated by the available data.

#### *Prevention and control*

As formation of ochratoxin A depends on the fungal source, the type of crop, and its geographical location, control of ochratoxin A production by each fungal species was considered separately. Control of *A. ochraceus*, which occurs primarily in stored foods, consists of the standard methods for preventing growth of any fungus in dried foods. The major commodities in which *A. ochraceus* may produce ochratoxin A are stored grains. The traditional means of avoiding fungal growth in grains is to dry them rapidly and thoroughly and to keep them dry. Reduction of the moisture content of grains to provide a water activity below 0.8 is necessary to prevent formation of ochratoxin A by *A. ochraceus*. Further effective approaches to grain storage include fumigation, aeration and cooling, sealed storage, and controlled atmospheres, especially in tropical and subtropical regions where insect damage is a major problem. Controlled atmosphere storage is achieved by continuous application of atmospheres with a low oxygen or a high carbon dioxide concentration.

Modified atmospheres to control insects may contribute to controlling fungi. Some fumigants used for insect control may also control fungi.

As ochratoxin A is apparently formed in green coffee beans after harvest, agricultural practice has little or no influence on the concentration of the toxin in dried beans. Control measures for ochratoxin A in coffee are therefore based on good manufacturing practice, i.e. rapid and effective drying, good storage practices, and, in some countries, colour sorting to reject defective beans.

The available evidence indicates that *A. carbonarius* and *A. niger* are not pathogens on fruit such as grapes and hence cannot gain entry to sound fruit. However, mechanical or chemical damage to fruit or damage caused by insects or microorganisms may permit fungal invasion of fruit tissue. Controlling the growth of these species in grapes before harvest therefore relies on controlling pathogenic fungi, mechanical damage, and splitting due to rain just before harvest. The occurrence of ochratoxin A from *P. verrucosum* in Canadian and European grains was attributed to insufficient drying or inadequate storage. Analysis and segregation of defective lots could be used to reduce the concentration of ochratoxin A in dried grain used for human food.

## 9. EVALUATION

The Committee concluded that the new data raised further questions about the mechanisms by which ochratoxin A causes nephrotoxicity and renal carcinogenicity and the interdependence of these effects. The mechanism by which ochratoxin A causes carcinogenicity is unknown, although both genotoxic and non-genotoxic modes of action have been proposed. The Committee noted that studies to resolve these issues are in progress and would wish to review the results when they become available. The Committee retained the previously established PTWI of 100 ng/kg bw per week, pending the results of on-going studies on the mechanisms of nephrotoxicity and carcinogenicity, and recommended a further review of ochratoxin A in 2004. In reaching this conclusion, the Committee noted the large safety factor applied to the NOEL for nephrotoxicity in deriving the PTWI, which corresponds to a factor of 1500 applied to the NOEL for carcinogenicity in male rats, the most sensitive species and sex for this end-point.

The adverse effect at the lowest effective dose in several mammalian species is nephrotoxicity, and this is likely also to be true in humans. Although an association between the intake of ochratoxin A and nephropathy in humans has been postulated, causality has not been established. The Committee noted that the intake of ochratoxin A by 95th percentile consumers of cereals may approach the PTWI from this source alone. Given the distribution of ochratoxin A contamination of cereals, application of a limit of 5 or of 20 µg/kg would make no significant difference to the average intake. The estimated intake at the 95th percentile of cereal consumers on a European diet would be about 84 and 92 ng/kg bw per week, respectively. Intakes below the PTWI would not present an appreciable risk. The Committee was unable, on the basis of the available data, to arrive at a quantitative estimate of the risk for nephrotoxicity if the PTWI were to be exceeded. Efforts are needed to ensure that intakes of ochratoxin A do not exceed the PTWI, and this could best be achieved by lowering overall contamination by appropriate agricultural, storage, and processing practices.

*Recommendations*

- Studies should be conducted to clarify the mechanism by which ochratoxin A induces nephrotoxicity and carcinogenicity.
- Appropriate sampling procedures should be developed for food commodities likely to be contaminated with ochratoxin A.
- Better surveys are needed, particularly in regions of the world other than Europe, in order that intakes in those regions may be assessed.
- Epidemiological investigations should be encouraged to explore the role of ochratoxin A in chronic renal disease.
- Studies should be conducted to improve understanding of the occurrence and ecology of the fungi that produce ochratoxin A, especially in fresh produce.

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Appendix A

Results of surveys for ochratoxin A showing concentrations and distribution of contamination in food commodities

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 5 ≤ 20 (µg/kg)	n > 20 µg/kg)	References	Sampling procedure
<b>Wheat</b>											
Germany	Wheat grain	1995–98	35	0.01 <sup>a</sup>	21	0.11/0.65	0.26	0	0	P,S,A, Wolff et al. (2000)	
	Wheat flour < T550	1995–98	98	0.01 <sup>a</sup>	16	0.10/1	0.26	0	0		
		1995–98	83	0.01 <sup>a</sup>	6	0.20/1.73	0.59	0	0		
	Wheat wholemeal flour	1995–98	18	0.01 <sup>a</sup>	0	0.20/1.2	0.66	0	0		
	Wheat semolina	1995–98	25	0.01 <sup>a</sup>	15	0.41/2.58	1.49	0	0		
	Wheat bran	1995–98	25	0.01 <sup>a</sup>	22	0.22/1.59	0.72	0	0		
	Wheat germ	1995–98	19	0.01 <sup>a</sup>	13	0.11/0.45	0.27	0	0		
Netherlands	Wheat, domestic	1995	7	1.000	7	0/0	NR	0	0	P,S,A, Sizoo & van Egmond (1997)	
	Wheat, imported	1995	24	1.000	23	0.36/8.7	NR	1	0		
	White wheat flour	1999	31	0.250	30	0.048/1.5	NR	0	0		
	Whole-wheat meal	1999	19	0.250	19	0/0	NR	0	0		
Norway	Wheat	1990	138	0.3 <sup>a</sup>	122	0.17/1.5	NR	0	0	P,S,A, Langseth (1999)*	
		1993	7	0.2 <sup>a</sup>	6	0.77/4.7	NR	0	0		
		1994	24	0.2 <sup>a</sup>	20	0.3/3.4	NR	0	0		
		1995	32	0.25 <sup>a</sup>	20	0.15/0.57	NR	0	0		
		1996	28	0.3 <sup>a</sup>	28	0/0	NR	0	0		
		1997	25	0.01 <sup>a</sup>	22	0.22/3.5	NR	0	0		
		1998	35	0.05 <sup>a</sup>	30	0.7/20	NR	1	0		
		Total wheat samples	1990–98	289		248	0.26/20	NR	5		0
	Wheat, imported	1990	28	0.3 <sup>a</sup>	25	0.34/3.8	NR	0	0		
	1993	11	0.2 <sup>a</sup>	10	0.52/4.6	NR	0	0			

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples (µg/kg)	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 µg/kg)	References	Sampling procedure
Norway	Wheat, imported	1994	9	0.2 <sup>a</sup>	8	0.46/3.3	NR	0	0		
		1995	13	0.25 <sup>a</sup>	10	0.89/8.2	NR	1	0		
		1996	14	0.3 <sup>a</sup>	13	0.67/7.5	NR	1	0		
		1997	10	0.01 <sup>a</sup>	7	0.14/0.56	NR	0	0		
		1998	24	0.05 <sup>a</sup>	20	0.07/0.54	NR	0	0		
	Total imported wheat	1990–98	108		93	0.4/8.2	NR	2	0		
Sweden	Wheat grain	1996–98	57	0.1	41	0.24/2.3	0.84	0	0	P,S, Thuvander et al. (2000); A, Larsson & Møller (1996) <sup>*</sup>	Mills; 1 kg sampled in national pesticide control programme/
		1999	75	0.05	36	0.37/5.2	0.69	1	0	P, National Food Administration; S, Thuvander et al. (2000); A, Larsson (1996) <sup>b</sup>	
Brazil	Wheat grain	1988–90	16	5 <sup>a</sup>	15	2.5/40	0.00	0	1	P,S, Furlong et al. (1995a); A, Soares et al. (1985) <sup>c</sup>	From experimental plots; all grain within 3 m x 6 rows/ 3.0–10
		1990	20	5 <sup>a</sup>	20	0/0	0.00	0	0	P,S, Furlong et al. (1995); A, Soares et al. (1985) <sup>c</sup>	
	Wheat products	1991	38	5 <sup>a</sup>	38	0/0	0.00	0	0	P,S, Soares & Furlani (1996); A, Soares et al. (1985) <sup>c</sup>	
USA	Wheat grain	1997	383	0.03 <sup>a</sup>	327	NR/31.4	NA	3	1	P,S,A, Trucksess et al. (1999)	Sampled by GIPSA by unspecified USDA sampling plan

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 µg/kg)	References	Sampling procedure
Finland	Wheat grain	1996	34	0.800	32	18.2/430	0.00	0	2	P,A, Solfrizzo et al. (1998) <sup>d</sup>	Random 1-kg samples from farms
Denmark	Wheat grain	1986–92	402	0.05a	283	0.7/51	NR	6	3	P,S,A, Jorgensen et al. (1996) <sup>d</sup>	Random 1-kg samples from mills
	Wheat grain, organic	1986–92	73	0.05a	44	1.2/36	NR	3	1		
	Wheat grain	1986–92	45	0.05a	28	0.9/13	NR	1	0		
	Wheat bran	1986–92	120	0.05a	46	0.8/12	NR	2	0		
	Wheat bran, organic	1986–92	22	0.05a	7	0.6/2.6	NR	0	0		
Dubai	Wheat flour	NR	11	0.500	10	0.023/0.25	0.00	0	0	P,S,A, Rao (2000)	
United Kingdom	Wheat noodles	1995	4	0.1 <sup>a</sup>	3	0.1/0.4	0.28	0	0	P,S,A, Patel et al. (1996)	Purchased in specialist food shops
	Wheat grain	1993	384	0.1 <sup>a</sup>	NR	< 1/< 1	NR	0	0	P, Scudamore (1999)	
	Wheat grain stored	1992–93	25	0.1 <sup>a</sup>	NR	< 1/ 1	NR	0	0	A, Sharman et al. (1992)	
	Wheat from millers	1993	129	0.1 <sup>a</sup>	NR	NA/15	NR	2	0	S, NR	
		1994	250	0.1 <sup>a</sup>	NR	NA/32	NR	NR	3		
	Wheat grain	1997–98	148	0.200	126	0.3/9.2	NR	3	0	P,S,A, MAFF (1999a)	
		1996	76	0.200	74	0.042/2.4	0.00	0	0	P,S, MAFF (1997)	
Cereals and flours	1996	67	0.200	30	NA/6.4	NR	2	0	A, Sharman et al. (1992) P,S, MAFF (1996b) A, Sharman et al. (1992)		
Uruguay	Wheat grain	1993–95	123	50 <sup>a</sup>	123	0/0	0.00	0	0	P,S, Pineiro et al. (1996) A, Pineiro & Giribone (1994) <sup>c</sup>	

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples(µg/kg)	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 µg/kg)	References	Sampling procedure
<b>Buckwheat</b>											
Germany	Buckwheat	1995–98	23	0.01 <sup>a</sup>	13	0.07/0.59	0.02	0	0	P,S,A, Wolff et al. (2000)	
	Buckwheat flour	1995–98	14	0.01 <sup>a</sup>	3 <sup>a</sup>	0.96/12.1	0.04	1	0		
<b>Barley</b>											
Germany	Barley	1995–98	22	0.01 <sup>a</sup>	3	0.07/0.49	0.10	0	0	P,S,A, Wolff et al. (2000)	
	Pearl barley	1995–98	31	0.01 <sup>a</sup>	10	0.094/0.95	0.10	0	0		
Norway	Barley	1990	10	0.3 <sup>a</sup>	10	0/0	0.00	0	0	P,S,A, Langseth (1999) <sup>b</sup>	
	Barley groats	1990	10	0.3 <sup>a</sup>	10	0/0	0.00	0	0		
USA	Barley	1997	103	0.03 <sup>a</sup>	92	NA/17	NR	1	0	P,S,A, Trucksess et al. (1999)	Sampled by GIPSA with unspecified USDA sampling plan
United Kingdom	Barley	1997-98	131	0.1 <sup>a</sup>	96	0.7/17.8	NR	5	0	P,S,A, MAFF (1999a) P,S, MAFF (1997); A, Sharman et al. (1992) P, Scudamore (1999); A, Sharman et al. (1992); S, NR	
	Barley	1996	37	0.2 <sup>a</sup>	34	0.20/6.4	0.00	1	0		
	Barley stored in 1992	1993	73	0.1 <sup>a</sup>	NR	NA/<1	NR	0	0		
	Barley	1994	150	0.1 <sup>a</sup>	NR	NA/33	NR	0	1		
	Barley stored in 1993	1994	50	0.1 <sup>a</sup>	NR	NA/14	NR	1	1		
Uruguay	Barley and malt	1993-95	137	50 <sup>a</sup>	137	0/0	0.00	0	0	P,S, Pineiro et al. (1996), A, Pineiro & Giribone (1994) <sup>c</sup>	

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 µg/kg)	References	Sampling procedure	
Denmark	Barley kernel	1986–92	41	0.05 <sup>a</sup>	30	0.9/14	NR	3	0	P,S,A, Jorgensen et al. (1996) <sup>d</sup>	Random 1-kg samples from mills	
	Barley kernel, organic	1986–92	20	0.05 <sup>a</sup>	14	1.0/13	NR	2	0			
Finland	Barley kernel	1996	45	0.800	42	0.55/12.3	0.00	2	0	P,A, Solfrizzo et al. (1998) <sup>d</sup>	Random 1-kg samples from farms	
Canada	Barley cereals	1998–99	20	0.500	NR	NA/0.57	NR	0	0	P, Canada; S,A, NR <sup>b</sup>		
	Barley based cereals	1998–99	20	0.200	NR	NA/6.92	NR	NR	0			
	<b>Maize</b>											
Germany	Maize and popcorn	1995–98	31	0.01 <sup>a</sup>	12	0.17/3.35	0.26	0	0	P,S,A, Wolff et al. (2000)		
United Kingdom	Raw maize, imported	1998–99	139	0.200	125	NA/1.5	NR	0	0	P,S, Scudamore (2000), A, Howell & Taylor (1981)	At ports; from conveyor between silos and mill or from ships' holds	
Croatia	Raw maize	1996	105	0.2 <sup>a</sup>	95	3.61/224	0.00	0	2	P,S, Jurjevic et al. (1999); A, Solfrizzo et al. (1998) <sup>d</sup>	Random 1-kg samples from farms	
	Raw maize	1997	104	0.2 <sup>a</sup>	68	19.8/614	1.29	2	7			
Brazil	Raw maize	1991	130	5 <sup>a</sup>	130	0/0	0.00	0	0	P,S, Pozzi et al. (1995) A, Soares et al. (1985) <sup>c</sup>	Samples from stored material collected from 60-kg sacks at monthly intervals	
Brazil	Raw maize	1993–94	292	5 <sup>a</sup>	292	0/0	0.00	0	0	P,S, Gloria et al. (1997) A, Soares et al. (1985) <sup>c</sup>		
Uruguay	Maize and by-products	1993-95	147	50 <sup>a</sup>	147	0/0	0.00	0	0	P,S, Pineiro et al. (1996) A, Pineiro & Giribone (1994) <sup>c</sup>		

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 µg/kg)	References	Sampling procedure	
United Kingdom	Corn flour	1995	4	0.1 <sup>a</sup>	3	0.15/0.6	0.42	0	0	P,S,A, Patel et al. (1996)		
Sweden	<b>Oats</b> Oat grain	1996–98	23	0.1	16	0.32/3.6	0.81	0	0	P,S, Thuvander et al. (2000); A, Larsson & Møller (1996) <sup>b</sup>		
	Oat grain	1999	10	0.05	8	0.05/0.15	0.11	0	0	P, National Food Administration (2000); S, Thuvander et al (2000), A, Larsson & Møller (1996) <sup>b</sup>		
Norway	Oat groats	1990	20	0.3 <sup>a</sup>	14	0.26/0.9	NR	0	0	P,S,A, Langseth (1999) <sup>b</sup>		
	Oats	1990	20	0.3 <sup>a</sup>	17	0.44/5.8	NR	1	0			
	Oats	1993	3	0.2	2	0.17/0.26	NR	0	0			
	Oats	1994	3	0.2 <sup>a</sup>	2	3.47/10.2	NR	1	0			
	Oats	1995	21	0.25 <sup>a</sup>	20	0.32/4.2	NR	0	0			
	Oats	1996	14	0.3 <sup>a</sup>	14	0/0	0	0	0			
	Oats	1997	14	0.01 <sup>a</sup>	?	0.053/0.23	NR	0	0			
	Oats	1998	22	0.01 <sup>a</sup>	19	0.065/0.47	NR	0	0			
	Total oats samples	1990–98	97			0.46/10.2	NR	2	0			
Denmark	Oat kernels	1986–92	50	0.05 <sup>a</sup>	29	0.5/5.6	NR	1	0	P,S,A, Jorgensen et al. (1996) <sup>d</sup>	Random 1-kg samples from mills	
	Oat kernels, organic	1986–92	17	0.05 <sup>a</sup>	11	0.3/4.2	NR	0	0			
	Oat kernels, imported	1986–92	25	0.05 <sup>a</sup>	12	0.5/4.6	NR	0	0			

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 (µg/kg)	References	Sampling procedure
Finland	Oat kernels	1996	34	0.80	32	1.7/56.6	0.00	0	1	P,A, Solfrizzo et al. (1998) <sup>d</sup>	Random 1-kg samples from farms
United Kingdom	Oat kernels	1997–98	21	0.1 <sup>a</sup>	15	0.1/2.2	NR	0	0	P,S,A, MAFF (1999a)	
		1996	18	0.2 <sup>a</sup>	17	0.33/5.9	0.00	1	0	P,S, MAFF (1997) A, Sharman et al. (1992)	
Germany	Oats	1995–98	30	0.01 <sup>a</sup>	6	0.06/0.14	0.00	0	0	P,S,A, Wolff et al. (2000)	
Uruguay	<b>Rice</b> Rice	1993-95	62	50 <sup>a</sup>	62	0/0	0.00	0	0	P,S, Pineiro et al. (1996); A, Pineiro & Giribone (1994) <sup>c</sup>	Stratified random sampling to obtain 5-kg samples .
United Kingdom	Basmati rice	1995	4	0.1 <sup>a</sup>	4	0/0	0.00	0	0	P,S,A, Patel et al. (1996) <sup>b</sup>	
	Chinese rice	1995	4	0.1 <sup>a</sup>	4	0/0	0.00	0	0		
Dubai	Rice	NR	15	0.50	14	0.017/0.25	0.00	0	0	P,S,A, Rao (2000)	
Germany	Rice	1995–98	22	0.1 <sup>a</sup>	18	0.11/0.28	0.01	0	0	P,S,A, Wolff et al. (2000)	
	Parboiled rice	1995–98	21	0.1 <sup>a</sup>	21			0	0		
	Long-grain rice	1995–98	24	0.1 <sup>a</sup>	24			0	0		
	Round-grain rice	1995–98	15	0.1 <sup>a</sup>	15			0	0		

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 µg/kg)	References	Sampling procedure
Sweden	<b>Rye</b> Rye grain	1996–98	28	0.1	8	0.56/2.3	1.2	0	0	P,S, Thuvander et al. (2000); A, Larsson & Møller (1996) <sup>b</sup> P, National Food Administration, S, Thuvander et al. (2000), A, Larsson & Møller (1996) <sup>b</sup>	
		1999	19	0.05	6	1.8/27	1.5	0	1		
Norway	Rye, imported	1990	18	0.3 <sup>a</sup>	13	0.22/0.8	NR	0	0	P,S,A, Langseth (1999) <sup>b</sup>	
		1993	9	0.2 <sup>a</sup>	5	0.31/1.0	NR	0	0		
		1994	6	0.2 <sup>a</sup>	4	0.28/1.0	NR	0	0		
		1995	4	0.25 <sup>a</sup>	3	0.75/2.5	NR	0	0		
		1996	4	0.3 <sup>a</sup>	4	0/0	0.00	0	0		
	Total	1990–98	41		29	0.31/2.5	NR	0	0		
Denmark	Rye kernels	1986–92	503	0.05 <sup>a</sup>	326	1.2/121	NR	16	4	P,S,A, Jorgensen et al. (1996) <sup>d</sup>	Random 1-kg samples from mills
	Rye kernels, organic	1986–92	91	0.05 <sup>a</sup>	20	5.4/120	NR	12	4		
	Rye kernels, imported	1986–92	22	0.05 <sup>a</sup>	14	0.1/0.7	NR	0	0		
United Kingdom	Rye kernels	1996	22	0.2 <sup>a</sup>	21	0.05/1.1	0.00	0	0	P,S, MAFF (1997); A, Sharman et al. (1992)	
Germany	Rye	1995–98	37	0.01 <sup>a</sup>	23	0.11/0.8	0.01	0	0	P,S,A, Wolff et al. (2000)	
	Rye flour < T997	1995–98	26	0.01 <sup>a</sup>	6	0.42/6.4	0.87	1	0		
		1995–98	71	0.01 <sup>a</sup>	3	0.32/2.14	0.06	0	0		
	Rye wholemeal flour	1995–98	43	0.01 <sup>a</sup>	11	0.11/1.46	0.03	0	0		

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 (µg/kg)	References	Sampling procedure
Germany	<b>Sorghum</b> Sorghum	1995–98	26	0.01 <sup>a</sup>	3	0.11/0.83	0.01	0	0	P,S,A, Wolff et al. (2000)	
Germany	<b>Spelt</b> Spelt/spelt flour	1995–98	21	0.01 <sup>a</sup>	3	0.66/9.43	0.06	NR	0	P,S,A, Wolff et al. (2000)	
	<b>Cereal products</b>										
United Kingdom	Pitta bread	1995	4	0.1 <sup>a</sup>	NR	NA/0.8	NR	0	0	P,S,A, Patel et al. (1996) <sup>b</sup>	
	Chapatti	1995	4	0.1 <sup>a</sup>	NR	NA/0.9	NR	0	0		
	Nan bread	1995	4	0.1 <sup>a</sup>	4	0/0	000'	0	0		
	Poppadoms	1995	4	0.1 <sup>a</sup>	4	0/0	000'	0	0		
Tunisia	Cereal-derived food	1994	66	0.1 <sup>a</sup>	0	1715/12 770	4314.4	6	56	P,S,A, Maaroufi et al. (1995b)	From homes of nephro-pathy patients
Dubai	Mixed cereals	NR	28	0.500	25	NA/4.3	NR	0	0	P,S,A, Rao (2000)	
Canada	Mixed grain cereals	1998–99	31	0.200	NR	0.25/0.54	NR	0	0	P, Canada (2000); S	
	Mixed cereals	1998–99	19	0.500	19	0/0	000'	0	0	and A, NR <sup>b</sup>	
Europe, Tunisia	Bread	NR	141	0.008	0	NA/6.66	NR	NR	0	P, Burdaspal & Legarda (2000); A, Burdaspal & Legarda (2001); S, NR <sup>b</sup>	
Germany	Bread, wheat mix	1995–98	125	0.01a	15	0.19/2.09	000	0	0	P,S,A, Wolff et al. (2000)	

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 µg/kg)	References	Sampling procedure
Germany	Bread, rye mix	1995–98	128	0.01 <sup>a</sup>	7	0.24/2.24	0.04	0	0	P,S,A, Wolff et al. (2000)	
	White bread	1995–98	57	0.01 <sup>a</sup>	9	0.11/1.9	0.01	0	0		
	Toast bread	1995–98	59	0.01 <sup>a</sup>	7	0.081/0.58	0.20	0	0		
	Rye meal bread	1995–98	96	0.01 <sup>a</sup>	7	0.22/5.49	0.32	1	0		
	Milk–water bread roll	1995–98	89	0.01 <sup>a</sup>	10	0.09/0.52	0.21	0	0		
	Various cereals, bread	1995–98	49	0.01 <sup>a</sup>	1	0.24/1.76	0.73	0	0		
	Various cereals, bread +oilseed	1995–98	101	0.01 <sup>a</sup>	3	0.17/2.44	0.33	0	0		
	Crispbread	1995–98	87	0.01 <sup>a</sup>	25	0.076/0.44	0.01	0	0		
	Wheat whole bread	1995–98	13	0.01 <sup>a</sup>	0	0.134/0.40	0.02	0	0		
	Special bread	1995–98	64	0.01 <sup>a</sup>	9	0.145/2.23	0.02	0	0		
	Wholemeal bread roll	1995–98	31	0.01 <sup>a</sup>	0	0.17/0.77	0.02	0	0		
	Various cereals and muesli bread	1995–98	49	0.01 <sup>a</sup>	1	0.36/5.54	0.02	2	0		
	Rye bread roll	1995–98	38	0.01 <sup>a</sup>	1	0.16/0.44	0.03	0	0		
	Pasta without egg	1995–98	50	0.1 <sup>a</sup>	21	0.28/1.75	0.61	0	0		
	Pasta with egg	1995–98	84	0.1 <sup>a</sup>	57	0.199/0.95	0.04	0	0		
	Wholemeal pasta	1995–98	27	0.1 <sup>a</sup>	17	2.0/29.77	0.11	0	1		
	Oat flakes	1995–98	66	0.01 <sup>a</sup>	40	0.07/0.25	0.01	0	0		
	Oats bran	1995–98	26	0.01 <sup>a</sup>	12	0.089/0.33	0.01	0	0		
	Polenta	1995–98	29	0.01 <sup>a</sup>	23	0.20/1.53	0.03	0	0		
	Green corn	1995–98	17	0.01 <sup>a</sup>	15	0.07/0.10	0.01	0	0		
	Infant food	1995–98	97	0.01 <sup>a</sup>	31	0.12/2.13	0.01	0	0		
	Peas, lentil, beans	1995–98	103	0.01 <sup>a</sup>	102	ND/0.84		0	0		
	Soya beans	1995–98	31	0.01 <sup>a</sup>	5	0.06/0.10	0.01	0	0		
FSIS185 pulses	1998	50	0.2 <sup>a</sup>	50			0	0			
FSIS185 pulses	1997	29	0.2 <sup>a</sup>	27	1.1/15.4	< 0.2	2	0			

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 (µg/kg)	References	Sampling procedure
<b>Seeds</b>											
United Kingdom	Fennel	1995	3	0.1 <sup>a</sup>	3	0/0	0.00	0	0	P,S,A, Patel et al. (1996)	
	Sesame seeds	1995	3	0.1 <sup>a</sup>	3	0/0	0.00	0	0		
	Coriander	1995	3	0.1 <sup>a</sup>	2	1.33/4.0	3.20	0	0		
<b>Herbs and spices</b>											
Netherlands	Paprika powder	1996–98	12	0.250	3	1.7/9.8	NR	1	0	P,S,A, Inspectorate for Health Protection (personal communication, 1999)	
	Pepper	1996–97	14	0.250	7	3.73/14.5	NR	4	0		
United Kingdom	Chilli powder	1995	4	0.1 <sup>a</sup>	NR	NA/50.4	NR	NR	NR	P,S,A, Patel et al. (1996)	
	Curry powder	1995	10	0.1 <sup>a</sup>	NR	NA/21.3	NR	NR	NR		
	Tandoori	1995	3	0.1 <sup>a</sup>	NR	NA/23.9	NR	NR	NR		
	Ginger	1995	4	0.1 <sup>a</sup>	NR	NA/7.5	NR	NR	0		
	Garlic	1995	4	0.1 <sup>a</sup>	4	0/0	0.00	0	0		
	Five spices powder	1995	4	0.1 <sup>a</sup>	3	0.65/2.6	1.82	0	0		
Tunisia	Dried vegetables	1994	6	0.1 <sup>a</sup>	0	2934/7444	3426.6	0	6	P,S,A, Maaroufi et al. (1995b)	From homes of nephro- pathy patients
Dubai	Spices	NR	7	0.500	3	NA/3.56	NR	0	0	P,S,A, Rao (2000)	
<b>Pickles and pastes</b>											
United Kingdom	Chilli pickle	1995	4	0.1 <sup>a</sup>	NR	NA/1.2	NR	0	0	P,S,A, Patel et al. (1996)	
	Garlic pickle	1995	4	0.1 <sup>a</sup>	NR	NA/2.5	NR	0	0		

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 (µg/kg)	References	Sampling procedure
United Kingdom	Curry paste	1995	4	0.1 <sup>a</sup>	NR	NA/15.5	NR	NR	0	P,S,A, Patel et al. (1996)	
	Chilli sauce	1995	4	0.1 <sup>a</sup>	3	0.82/3.3	2.31	0	0		
United Kingdom	<b>Canned foods</b>										
	Canned foods	1995	8	0.1 <sup>a</sup>	NR	NA/0.3	NR	0	0	P,S,A, Patel et al. (1996)	
United Kingdom	<b>Oils</b>										
	Sesame oil	1995	3	0.1 <sup>a</sup>	2	0.13/0.4	0.32	0	0	P,S,A, Patel et al. (1996)	
Chili, almond oils	1995	4	0.1 <sup>a</sup>	4	0/0	0.00	0	0			
Uruguay	Oilseed	1993-95	80	50 <sup>a</sup>	80	0/0	0.00	0	0	P,S, Pineiro et al. (1996), A, Pineiro & Giribone (1994)	
Dubai	Oilseed <b>Olive</b>	NR	5	0.500	5	0/0	0.00	0	0	P,S,A, Rao (2000)	
Tunisia	Olives	1994	6	0.1 <sup>a</sup>	0	7809/46 830	32 782	2	1	P,S,A, Maaroufi et al. (1995b)	From homes of nephro- pathy patients
Uruguay	<b>Beans</b> Soya beans	1993-95	19	50 <sup>a</sup>	19	0/0	0.00	0	0	P,S, Pineiro et al. (1996), A, Pineiro & Giribone (1994)	

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 µg/kg)	References	Sampling procedure
Sweden	Brown beans	1996–98	20	0.1	18	0.18/1.9	0.07	0	0	P,S, Thuvander et al. (2000), A, Larsson & Møller (1996)	
United Kingdom	Baked beans	1996	50	0.2 <sup>a</sup>	49	0.006/0.3	0.00	0	0	P,S, MAFF (1997)	
	Butter beans	1996	12	0.2 <sup>a</sup>	11	1.14/13.7	0.00	1	0	A, Sharman et al. (1992)	
	<b>Pulses</b>										
Denmark	Pulses	1993–94	22	0.1 <sup>a</sup>	22	0/0	NA	0	0	P,S,A, Jorgensen (1998)	'Random samples' from retail shops
United Kingdom	Pulses	1998	50	0.200	50	0/0	NA	0	0	P,S,A, MAFF (1999b)	
	<b>Chickpeas</b>										
Sweden	Peas, dry	1996–98	30	0.1	28	0.12/1.2	0.08	0	0	P,S, Thuvander et al. (2000); A, Larsson & Møller (1996)	
United Kingdom	Dried chickpeas	1996	14	0.2 <sup>a</sup>	14	0/0	NA	0	0	P,S, MAFF (1997); A, Sharman et al. (1992)	
	<b>Lentils</b>										
	Dried lentils	1996	21	0.2 <sup>a</sup>	21	0/0	NA	0	0		
	<b>Soya</b>										
Canada	Soya-based cereals	1998–99	16	0.200	NR	NA/0.92	NR	0	0	P, Canada (2000); S,A, NR	
Japan	Soya sauce	1996	5	0.003 <sup>a</sup>	0	0.0068/ 0.026	NR	0	0	P,A, Ueno (1998); S, NR	

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples(µg/kg)	LOQ	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 µg/kg)	References	Sampling procedure
<b>Coffee</b>											
Netherlands	Roasted coffee	1999	22	0.1 <sup>a</sup>	13	0.45/4.5	NR	0	0	P,S,A, Inspectorate for Health Protection (per- sonal communication, 1999)	
USA	Green coffee	1997	19	0.03 <sup>a</sup>	10	NA/4.6	NR	0	0	P,S,A, Trucksess et al. (1999)	Sampled by GIPSA with unspecified USDA sampling plan
USA	Green coffee, imported	1995-99	180	4-10 <sup>a</sup>	174	0.353/19.2	0.00	3	0	P, Ochratoxin A Monitor- ing Program, S,A, NR <sup>c</sup>	
	Soluble coffee, imported	1995-99	23	4-10 <sup>a</sup>	23	0/0	0.00	0	0		
	Roasted coffee	1997	13	0.03 <sup>a</sup>	4	NA/1.2	NR	0	0		P,S,A, Trucksess et al. (1999)
Denmark	Roasted coffee	1993-94	11	0.1 <sup>a</sup>	0	0.51/3.2	NR	0	0	P,S,A, Jorgensen (1998)	'Random samples' from retail shops
United Kingdom	Green coffee, imported Coffee products	NR	291	0.260	181	NA/27.3	NR	11	2	P,S,A, MAFF (1996a)	
		1995	100	0.1 <sup>a</sup>	19	NA/8	NR	NR	0	P,S,A, Patel et al. (1997)	
Europe	Coffee products	1999	633	0.2-1 <sup>a</sup>	334	0.90/27.2	NR	3	1	P,S,A, Stegen et al. (1997)	
Eastern Europe	Adulterated soluble coffee	NR	15	0.2 <sup>a</sup>	0	5.9/15.9	1.40	6	0	P,S,A, Pittet et al. (1996)	

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples(µg/kg)	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 5 ≤ 20 (µg/kg)	n > 20 µg/kg)	References	Sampling procedure
World	Soluble coffee	NR	101	0.2 <sup>a</sup>	26	1.1/6.5	NA	NR	0	P,S,A, Pittet et al. (1996)	
Canada	Instant coffee	1997-98	30	0.1 <sup>a</sup>	NR	NA/3.1	NR	0	0	P, Canada (2000);S,A,	
	Coffee, ground and beans	1997-98	71	0.1 <sup>a</sup>	NR	NA/2.3	NR	0	0	NR	
Dubai	Coffee beans	NR	8	0.500	5	NA/7.46	NR	1	0	P,S,A, Rao (2000)	
Sweden	Green coffee	1999	45	0.05	23	0.53/12.1	0.74	1	0	P,S,A, National Food	
	Roasted coffee	1999	37	0.05	29	0.40/3.86	1.7	0	0	Administration	
	Coffee granulate	1999	6	0.05	0	0.50/0.79	0.68	0	0		
Spain	Coffee, roasted and soluble	1997	38	0.110	0	1.01/5.64	NR	NR	0	P, Burdaspal & Legarda (1998b), A, Pittet et al.	
	Decaffeinated coffee	1997	8	0.110	0	0.55/1.29	NR	0	0	(1996); S, NR	
Japan	Canned coffee	1996	10	0.003 <sup>a</sup>	1	0.028/0.133	NR	0	0	P,A, Ueno (1998);	
	Instant coffee	1996	12	0.06 <sup>a</sup>	0	0.018/0.063	NR	0	0	S, NR	
	Regular coffee	1996	10	0.06 <sup>a</sup>	10	0/0	000'	0	0		
European Union,	Roasted coffee	1995-96	86	NR	NR	0.8/NR	NR	NR	NR	Olsen (2000)	
	Roasted coffee	1995-97	504	NR	NR	0.8/NR	NR	NR	NR		
Switzerland	Roasted coffee	1996-98	232	NR	NR	0.6/NR	NR	NR	NR		
	Roasted coffee	1999	107	NR	NR	0.4/NR	NR	NR	NR		
Brazil	Roasted coffee	2000	34	0.2 <sup>a</sup>	11	0.93/6.5	000	1	0	P,A, Leoni et al. (2000);	
	Instant coffee	2000	16	0.2 <sup>a</sup>	0	2.17/5.10	0'05	1	0	S, NR	

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples(µg/kg)	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 µg/kg)	References	Sampling procedure
Germany	Roasted coffee	1997	34	0.3 <sup>a</sup>	10	1.43/7.54	NR	3	0	P,A, Koch et al. (1996); S, NR	
Germany	Roasted coffee	1995–98	113	0.3 <sup>a</sup>	61	0.61/6.32	2	1	0	P,S,A, Wolff et al. (2000)	
	Roasted coffee	1995–98	60	0.3 <sup>a</sup>	39	0.45/4.75	1	0	0		
	Roasted coffee	1995–98	67	0.3 <sup>a</sup>	35	0.56/3.34	2	0	0		
	Soluble coffee	1995–98	52	0.3 <sup>a</sup>	6	1.83/9.47	4	5	0		
	Soluble coffee	1995–98	32	0.3 <sup>a</sup>	13	0.59/1.8	2	0	0		
	Malt coffee	1995–98	33	0.3 <sup>a</sup>	28	< 0.3/0.96	1	0	0		
	Green coffee	1995–99	82	0.250	60	1.29/24.5	NR	NR	NR	P,S, Ottender & Majerus (2001); A, Entwisle et al. (2000b)	
	Roasted coffee	1995–99	419	0.250	228	0.99/12.1	NR	NR	0		
	Decaffeinated coffee	1995–99	71	0.250	45	0.49/2.7	NR	0	0		
	Instant coffee	1995–99	41	0.250	12	1.0/4.8	NR	0	0		
<b>Cocoa</b>											
Netherlands	Cocoa products	1996	19	0.250	19	0/0	0.00	0	0	P,S,A, Inspectorate for Health Protection (per- sonal communication, 1999)	
Uruguay	Cocoa beans and by-products	1993-95	91	50 <sup>a</sup>	91	0/0	0.00	0	0	P,S, Pineiro et al. (1996), A, Pineiro & Giribone (1994) <sup>c</sup>	
United Kingdom	Cocoa powder	1998	20	0.200	0	1.67/2.4	2.11	0	0	P,S,A, MAFF (1999b)	
	Cocoa powder	1996	20	0.2 <sup>a</sup>	3	0.67/1.1	1.00	0	0	P,S, MAFF (1997); A, Sharman et al. (1992)	

Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples (µg/kg)	LOQ	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 (µg/kg)	References	Sampling procedure
Germany	Cocoa	1995–98	40	0.01 <sup>a</sup>	0	NR/1.8	0.93	0	0	P,S,A, Wolff et al. (2000)	
	Cocoa powder	1995–98	56	0.01 <sup>a</sup>	5	NR/0.63	0.03	0	0		
	Cocoa drinks	1995–98	34	0.01 <sup>a</sup>	0	NR/0.05	0.00	0	0		
	FSIS185 powder	1998	20	0.01 <sup>a</sup>	0	1.7/2.4	NR	0	0		
	FSIS185 powder	1997	20	0.01 <sup>a</sup>	5	0.68/1.1	NR	0	0		
	<b>Chocolate</b>										
United Kingdom	Chocolate	1998	40	0.020	10	0.16/0.6	0.31	0	0	P,S,A, MAFF (1999b)	
Germany	Milk chocolate < 30%	1995–98	39	0.01 <sup>a</sup>	3	NR/0.41	0.01	0	0	P,S,A, Wolff et al. (2000)	
Germany	Plain chocolate > 60%	1995–98	78	0.01 <sup>a</sup>	0	NR/0.66	0.02	0	0		
Germany	Chocolate with nuts	1995–98	35	0.01 <sup>a</sup>	4	NR/0.16	0.01	0	0		
Germany	Filled chocolate	1995–98	58	0.01 <sup>a</sup>	3	NR/0.324	0.01	0	0		
Germany	FSIS185 milk chocolate	1998	28	0.01 <sup>a</sup>	9	0.15/0.6	NR	0	0		
Germany	FSIS185 plain chocolate	1998	12	0.01 <sup>a</sup>	1	0.27/0.6	NR	0	0		
	<b>Dried fruits</b>										
Uruguay	Dried fruits	1993–95	157	50 <sup>a</sup>	157	0/0	0.00	0	0	P,S, Pineiro et al. (1996), A, Pineiro & Giribone (1994) <sup>c</sup>	
USA	Raisins	NR	63	NR	14	1.56/11.5	3.48	5	0	P, Ochratoxin A Monitor- ing Program, S,A, NR	

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 (µg/kg)	<i>n</i> > 20 (µg/kg)	References	Sampling procedure
USA	Raisins	1998–99	133	NR	43	1.27/29	3.96	3	1	P, Ochratoxin A Monitoring Program; S,A, NR	
	Raisins	1998	114	NR	38	0.82/8.1	1.87	6	0		
	Raisins	1997	69	NR	19	0.42/3.1	0.92	0	0		
United Kingdom	Sultanas	1998	100	0.200	8	3.42/25.1	8.56	15	2	P,S,A, MAFF (1999b)	
	Raisins	1998	101	0.200	3	2.87/29.8	6.40	13	1		
	Currants	1998	100	0.200	4	4.97/40.8	11.47	20	5		
	Currants	1996	20	0.2 <sup>a</sup>	1	9.19/53.6	14.33	9	2		P,A, MacDonald et al. (1999); A, NR
United Kingdom	Apricots	1996	20	0.2 <sup>a</sup>	20	0/0	NA	0	0	P,S, MAFF (1997); A, Sharman et al. (1992)	
	Dried, fresh coconut	1996	20	0.2 <sup>a</sup>	20	0/0	NA	0	0		
	Dried dates	1996	20	0.2 <sup>a</sup>	19	0.01/0.2	0.00	0	0	P,A, MacDonald et al. (1999); A, NR	
	Raisins	1996	20	0.2 <sup>a</sup>	3	2.79/20	8.60	4	0		
	Sultanas	1996	20	0.2 <sup>a</sup>	3	4.86/18.1	11.35	7	0		
	Figs	1998	20	0.200	18	0.05/0.8	0.15	0	0		P,S,A, MAFF (1999b)
Germany	FSIS 185 currants	1998	100	0.01 <sup>a</sup>	4	NA/40.8	NA	29 (> 4)	5	P,S,A, Wolff et al. (2000)	
	FSIS 185 sultanas	1998	100	0.01 <sup>a</sup>	8	NA/53.6	NA	18 (> 4)	2		
	Dried raisins	1995–98	117	0.01 <sup>a</sup>	5	0.90/7.74	NA	2	6		
	FSIS 185 raisins	1998	101	0.01 <sup>a</sup>	3	NA/29.8	NA	19 (> 4)	1		
	Dried plums	1995–98	31	0.01 <sup>a</sup>	5	NR/0.07	0.00	0	0		
	Other dried fruit	1995–98	49	0.01 <sup>a</sup>	23	NR/0.09	0.00	0	0		
	Dried figs	1995–98	34	0.01 <sup>a</sup>	7	NR/3.95	0.02	0	0		

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 (µg/kg)	References	Sampling procedure
<b>Sweets</b>											
Germany	Marmelade	1995–98	42	0.01 <sup>a</sup>	42	0/0	NA	0	0	P,S,A, Wolff et al. (2000)	
	Nut-nugget cream	1995–98	33	0.01 <sup>a</sup>	2	0.06/0.27	0.00	0	0		
	Other puddings and creams	1995–98	32	0.01 <sup>a</sup>	25	< 0.01/0.09	0.00	0	0		
	Cocoa cream	1995–98	32	0.01 <sup>a</sup>	25	0.03/0.08	0.00	0	0		
<b>Milk and milk products</b>											
Germany	Milk and milk products	1995–98	264	0.01 <sup>a</sup>	242	<0.01/0.86	NA	0	0	P,S,A, Wolff et al. (2000)	
Norway	Milk	1995–98	87	0.01 <sup>a</sup>	76	NA/0.058	NA	0	0	P,S, Skaug (1999) A, Breitholtz-Emanuelsson (1993b)	
	Infant formula	1995–98	20	0.01 <sup>a</sup>	20	0/0	NA	0	0		
	Human milk	1995–96	80	0.01 <sup>a</sup>	63	0.006/0.18	NA	0	0		
Sierra Leone	Human milk	NR	113	0.2 <sup>a</sup>	73	7.9/337	NA	NR	4	P,S,A, Jonsyn et al. (1995)	
<b>Dried vegetables</b>											
Uruguay	Dried vegetables	1993–95	100	50 <sup>a</sup>	100	0/0	0.00	0	0	P,S, Pineiro et al. (1996), A, Pineiro & Giribone (1994) <sup>c</sup>	
<b>Oil and oilseeds</b>											
Germany	Sunflower seed	1995–98	34	0.01 <sup>a</sup>	14	NA/0.1	0.00	0	0	P,S,A, Wolff et al. (2000)	
	Sesame seed	1995–98	24	0.01 <sup>a</sup>	15	NA/0.86	1	0	0		

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 µg/kg)	References	Sampling procedure
Germany	Linseed	1995–98	24	0.01 <sup>a</sup>	12	NA/1.79	1	0	0	P,S,A, Wolff et al. (2000)	
	Poppy seed	1995–98	16	0.01 <sup>a</sup>	16	0/0	NA	0	0		
	Edible oils	1995–98	30	0.01 <sup>a</sup>	30	0/0	NA	0	0		
Uruguay	<b>Meat</b>										
	Meat products	1993–95	59	10 <sup>a</sup>	59	0/0	0.00	0	0	P,S, Pineiro et al. (1996); A, Pineiro & Giribone (1994) <sup>c</sup>	
Denmark	Pig kidney	1999	300	0.060	63	NA/14.72	1.5 <sup>b</sup>	5	0	P,S,A, Petersen (2000)	'Random samples' from slaughterhouses
	Pig meat	1999	300	0.090	227	NA/2.88	0.3 <sup>b</sup>	0	0		
	Pork	1993–94	76	0.02 <sup>a</sup>	12	0.11/1.3	NR	0	0	P,S, Jorgensen (1998); A, Jorgensen et al. (1996)	
	Pork, organic	1993–94	7	0.02 <sup>a</sup>	3	0.05/0.12	NR	0	0		
	Duck	1993–94	19	0.03 <sup>a</sup>	8	0.02/0.09	NR	0	0		
	Duck liver	1993–94	7	0.03 <sup>a</sup>	3	0.06/0.16	NR	0	0		
	Goose	1993–94	12	0.03 <sup>a</sup>	7	0.03/0.10	NR	0	0		
	Goose liver	1993–94	12	0.03 <sup>a</sup>	8	0.02/0.06	NR	0	0		
	Turkey	1993–94	17	0.03 <sup>a</sup>	7	0.02/0.11	NR	0	0		
	Turkey liver	1993–94	17	0.03 <sup>a</sup>	14	0.04/0.28	NR	0	0		
Chicken	1993–94	65	0.03 <sup>a</sup>	29	0.03/0.18	NR	0	0			
United Kingdom	Pork liver	1996	10	0.2 <sup>a</sup>	9	0.02/0.2	0.02	0	0	P,S, MAFF (1997); A, Sharman et al. (1992)	
	Pork salami	1996	9	0.2 <sup>a</sup>	9	0/0	NA	0	0		
Germany	Raw sausage	1995–98	56	0.01 <sup>a</sup>	28	0.04/0.27	0.00	0	0	P,S,A, Wolff et al. (2000)	
	Sausage	1995–98	40	0.01 <sup>a</sup>	26	0.02/0.18	0.00	0	0		

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 (µg/kg)	References	Sampling procedure
Germany	Sausage	1995–98	45	0.01 <sup>a</sup>	24	0.04/0.38	0.00	0	0	P,S,A, Wolff et al. (2000)	
	Liver sausage	1995–98	53	0.01 <sup>a</sup>	17	0.15/4.56	0.00	0	0		
	Blood sausage	1995–98	57	0.01 <sup>a</sup>	13	0.16/3.16	0.00	0	0		
	Other meat products	1995–98	21	0.01 <sup>a</sup>	18	0.01/0.04	0.00	0	0		
	Beef sausage	1995–98	31	0.01 <sup>a</sup>	26	0.02/0.19	0.00	0	0		
	Poultry sausage	1995–98	40	0.01 <sup>a</sup>	33	0.01/0.03	0.00	0	0		
	Beaf meat	1995–98	58	0.01 <sup>a</sup>	57	0.01/0.03	0.00	0	0		
	Pig meat	1995–98	58	0.01 <sup>a</sup>	48	0.02/0.14	0.00	0	0		
	Poultry meat	1995–98	41	0.01 <sup>a</sup>	41	0/0	NA	0	0		
	Pig kidney	1995–98	61	0.01 <sup>a</sup>	34	0.43/9.33	0.00	3	0		
Pig liver	1995–98	59	0.01 <sup>a</sup>	49	0.07/2.72	0.00	0	0			
France	Pig kidney	1997	300	1	297	0.01/1.4	< 0.5	0	0	P,S,A, Dragacci et al. (1999)	
	Pig kidney	1998	710	1	656	NA/5.0	NA	0	0		
	Nephropathic pig kidneys	1997	100	0	94	NA/0.48	NA	0	0		
Germany	<b>Snacks</b>									P,S,A, Wolff et al. (2000)	
	Bar	1995–98	32	0.01 <sup>a</sup>	4	NR/0.11	0.097	0	0		
	Bar with nuts	1995–98	47	0.01 <sup>a</sup>	7	NR/3.6	0.02	0	0		
	Muesli bar	1995–98	67	0.01 <sup>a</sup>	28	NR/1.72	0.01	0	0		
	Muesli	1995–98	115	0.01 <sup>a</sup>	44	NR/31.8	0.03	1	1		
	Breakfast cereals	1995–98	85	0.01 <sup>a</sup>	20	NR/0.94	0.02	0	0		
	Corn flakes	1995–98	38	0.01 <sup>a</sup>	26	NR/0.1	0.01	0	0		
	Biscuit	1995–98	102	0.01 <sup>a</sup>	20	NR/3.81	0.02	0	0		
Biscuit with chocolate	1995–98	67	0.01 <sup>a</sup>	2	NR/0.39	0.02	0	0			

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 (µg/kg)	References	Sampling procedure
Germany	Rusk	1995–98	37	0.01 <sup>a</sup>	5	NR/2.26	0.03	0	0	P,S,A, Wolff et al. (2000)	
	Rye biscuit	1995–98	31	0.01 <sup>a</sup>	8	NR/0.92	0.01	0	0		
	Chips, popcorn	1995–98	33	0.01 <sup>a</sup>	23	NR/2.1	0.01	0	0		
<b>Nuts</b>											
Netherlands	Roasted peanuts	1996	12	0.250	12	0/0	0.00'	0	0	P,S,A, Inspector- ate for Health Protection (personal communication, 1999)	
	Peanuts products	1996	4	0.250	4	0/0	0.00	0	0		
	Pistachio nuts	1996	3	0.250	3	0/0	0.00	0	0		
Germany	Hazelnuts	1995–98	32	0.01 <sup>a</sup>	13	NR/0.08	0.00	0	0	P,S,A, Wolff et al. (2000)	
	Groundnuts	1995–98	31	0.01 <sup>a</sup>	28	NR/0.08	0.00	0	0		
	Other nuts	1995–98	125	0.01 <sup>a</sup>	99	NR/0.27	0.024	0	0		
<b>Beer</b>											
Canada	Beer	NR	41	0.1 <sup>a</sup>	15	0.04/0.65	NA	0	0	P,S,A, Scott & Kanhere (1995)	
Denmark	Beer	1993–94	21	0.001 <sup>a</sup>	0	0.049/0.16	NR	0	0	P,S,A, Jorgensen (1998)	'Random samples' from retail shops
United Kingdom	Beer	1996	20	0.2 <sup>a</sup>	20	0/0	NA	0	0	P,S, MAFF (1997); A, Sharman et al. (1992)	
Spain	Beer	1997	40	0.004 <sup>a</sup>	1	0.024/0.075	NR	0	0	P,A, Burdaspal & Legarda (1998a); S, NR	
Europe	Beer	1997	40	0.004 <sup>a</sup>	0	0.025/0.121	NR	0	0		

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 (µg/kg)	References	Sampling procedure
Italy	Beer, imported	1999	61	0.01 <sup>a</sup>	31	0.017/0.135	0.05	0	0	P,S,A, Visconti et al. (2000b)	
Japan	Beer	1998	22	0.001 <sup>a</sup>	1	0.012/0.045	NR	0	0	P and A, Nakajima et al. (1999); S, NR	
	Beer, imported	1998	94	0.001 <sup>a</sup>	8	0.01/0.066	NR	0	0		
Germany	Pils beer	1995–98	135	0.01 <sup>a</sup>	34	0.026/0.137	0.058	0	0	P,S,A, Wolff et al. (2000)	
	Export beer	1995–98	31	0.01 <sup>a</sup>	6	0.027/0.123	0.059	0	0		
	Wheat beer	1995–98	30	0.01 <sup>a</sup>	7	0.031/0.293	0.041	0	0		
	Strong beer	1995–98	54	0.01 <sup>a</sup>	9	0.031/0.126	0.082	0	0		
	Beer, alcohol-free	1995–98	24	0.01 <sup>a</sup>	11	0.013/0.035	0.030	0	0		
	Light beer	1995–98	14	0.01 <sup>a</sup>	8	0.012/0.047	0.044	0	0		
	Malt beer	1995–98	30	0.01 <sup>a</sup>	16	0.016/0.081	0.033	0	0		
Germany	<b>Teas</b>										
	Black tea	1995–98	32	0.3 <sup>a</sup>	32	0/0	NA	0	0	P,S,A, Wolff et al. (2000)	
	Green tea	1995–98	32	0.3 <sup>a</sup>	31	< 0.3/1.33	NA	0	0		
Fruit tea	1995–98	32	0.3 <sup>a</sup>	32	0/0	NA	0	0			
Netherlands	<b>Wine</b>										
	Red wine	1999	150	0.100	90	0.22/3.1	1	0	0	P,S,A, Inspectorate for Health Protection and van Egmond (personal communication, 1999)	
	White wine	1999	20	0.100	18	0.12/2.1	0.00	0	0	P,S,A, van Egmond (personal communication, 1999)	

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 µg/kg)	References	Sampling procedure
Sweden	Wine	1998–99	32	0.005	3	0.21/2.5	0.45	0	0	P,S, A, National Food Administration	
United Kingdom	Red wine	1998	50	0.020	22	0.074/0.46	0.22	0	0	P,S,A, MAFF (1999b)	
	White wine	1996	10	0.2 <sup>a</sup>	6	0.38/1.1	1.10	0	0	P,S, MAFF (1997); A, Sharman et al. (1992)	
Switzerland	Wine	NR	18	0.005	5	NA/0.11	NR	0	0	P,A, Zimmerli & Dick (1995); S, NR	
Japan	Wine	1996	46	0.003 <sup>a</sup>	27	NA/0.245	NR	0	0	P,A, Ueno (1998), S, NR	
Italy	Red wine	1992–94	8	0.001	1	0.54/1.29	0.91	0	0	P, Pietri (2000); A, Zimmerli & Dick (1995), S, NR	
	Passito	1990–94	5	0.001	3	0.009/0.04	0.028	0	0		
	Red wine	1995	9	0.001	0	1.05/2.47	2.34	0	0		
	Passito	1995	2	0.001	1	1.92/3.86	3.47	0	0		
	Red wine	1996	23	0.001	7	0.54/1.78	1.55	0	0		
	Passito	1996	2	0.001	1	0.007/0.01	0.01	0	0		
	Passito	1997	5	0.001	1	1.42/3.48	3.07	0	0		
	Red wine	1997	13	0.001	0	0.76/2.15	1.41	0	0		
	Red wine	1998	18	0.001	4	0.66/3.17	0.09	0	0		
	Red wine	1999	115	0.01 <sup>a</sup>	12	2.10/15.61	7.12	18	0	P, Pietri (2001); A, Visconti et al. (1999); S, NR	
	White wine	1999	21	0.01 <sup>a</sup>	14	0.57/8.86	2.03	1	0		
	Rosé wine	1999	4	0.01 <sup>a</sup>	2	0.13/0.28	0.27	0	0		

Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples(µg/kg)	LOQ	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 µg/kg)	References	Sampling procedure
Italy	Red wine	1997–98	38	0.01 <sup>a</sup>	1	1.21/7.63	2.28	1	0	P,S,A, Visconti et al. (1999)	
	Rosé wine	1997–98	8	0.01 <sup>a</sup>	1	0.63/1.15	1.09	0	0		
	White wine	1997–98	9	0.01 <sup>a</sup>	0	0.16/0.97	0.43	0	0		
North Italy	Red wine	1997–99	8	0.01 <sup>a</sup>	4	0.102/0.54	NR	0	0	P,A, Ottender & Majerus (2000); S, NR	
South Italy	Red wine	1997–99	43	0.01 <sup>a</sup>	15	0.193/2.55	NR	0	0		
	Red wine	1997–99	20	0.01 <sup>a</sup>	1	1.153/3.31	NR	0	0		
North France	Red wine	1997–99	68	0.01 <sup>a</sup>	60	0.061/0.78	NR	0	0		
South France	Red wine	1997–99	40	0.01 <sup>a</sup>	19	0.07/0.47	NR	0	0		
Germany	White wine	1995–98	58	0.01 <sup>a</sup>	44	NA/1.4	0.00	0	0	P,S,A, Wolff et al. (2000)	
	Rosè wine	1995–98	51	0.01 <sup>a</sup>	33	NA/2.4	0.00	0	0		
	Red wine	1995–98	172	0.01 <sup>a</sup>	110	NA/7	1	1	0		
	FSIS 185 red wine	1998	50	0.01 <sup>a</sup>	22	0.08/0.8	NA	0	0		
	FSIS 185 red wine	1997	10	0.01 <sup>a</sup>	6	0.44/1.1	NA	0	0		
North Germany	Red wine	1997–99	30	0.01 <sup>a</sup>	23	0.022/0.23	NR	0	0	P,A, Ottender & Majerus (2000); S, NR	
South Germany	White wine	1997–99	26	0.01 <sup>a</sup>	22	0.012/0.04	NR	0	0		
Germany	White wine	1997–99	18	0.01 <sup>a</sup>	10	0.054/1.36	NR	0	0		
Germany	Red wine		40		20	0.17/1.90	NR	0	0	P,A, Lehtonen (1999); S, NR	
	Red wine		48		28	0.14/1.10	NR	0	0		
	White wine		7		5	0.08/0.35	NR	0	0		
France	Wine	1998	29	0.01 <sup>a</sup>	15	0.038/0.19	0.01	0	0	P,A, Ospital et al. (1998); S, NR	

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples(µg/kg)	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 (µg/kg)	References	Sampling procedure
World	Red wine	1997–99	305	0.01 <sup>a</sup>	140	0.20/3.31	NR	0	0	P,A, Ottender & Majerus (2000); S, NR	
	Rosé wine	1997–99	55	0.01 <sup>a</sup>	33	0.12/2.38	NR	0	0		
	White wine	1997–99	60	0.01 <sup>a</sup>	45	0.108/1.36	NR	0	0		
World	White wine	NR	41	0.00	27	NA/1.2	NA	0	0	P,S,A, Majerus & Ottender (1996)	
	Rosé wine	NR	14	0.00	8	NA/2.4	NA	0	0		
	Red wine	NR	89	0.00	49	NA/7.0	NA		0		
Europe	Wine	1998	40	NR	20	0.17/1.90	0.02	0	0	P, www.elintarvikevirasto. fi/; A, S, NR	
Europe	Red wine	1997	91	0.003 <sup>a</sup>	7	0.054/0.603	NR	0	0	P, Burdaspal & Legarda (1999);A, Zimmerli & Dick (1996);S, NR	
	Rosé wine	1997	32	0.003 <sup>a</sup>	3	0.031/0.161	NR	0	0		
	White wine	1997	69	0.003 <sup>a</sup>	24	0.020/0.267	NR	0	0		
	Aperitif wine	1997	47	0.003 <sup>a</sup>	12	0.04/0.254	NR	0	0		
	Sparkling wine	1997	12	0.003 <sup>a</sup>	2	0.012//0.037	NR	0	0		
	Dessert wine	1997	16	0.003 <sup>a</sup>	1	1.05/2.54	NR	0	0		
Europe	Red wine	1994–95	79	0.005	NR	0.039/0.39	NR	0	0	P,A, Zimmerli & Dick (1996);S, NR	
	Rosé wine	1994–95	15	0.005	NR	0.025/0.12	NR	0	0		
	White wine	1994–95	24	0.005	NR	0.011/0.18	NR	0	0		
	Special wines	1994–95	15	0.005	NR	NA/0.45	NR	0	0		
	<b><i>Vinegar and mustard</i></b>										
Germany	Apple and fruit vinegar	1995–98	18	0.01 <sup>a</sup>	17	NA/< 0.01	< 0.01	0	0	P,S,A, Wolff et al. (2000)	
	Wine vinegar	1995–98	38	0.01 <sup>a</sup>	19	NA/1.9	0.00	0	0		
	Balsam vinegar	1995–98	29	0.01 <sup>a</sup>	5	NA/4.35	3	0	0		
	Mustard	1995–98	4	0.01 <sup>a</sup>	1	NA/0.34	0.00	0	0		

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 5 ≤ 20 (µg/kg)	<i>n</i> > 20 (µg/kg)	References	Sampling procedure
United Kingdom	<b>Grape juice</b> Grape juices	1998	20	0.020	1	0.48/2.05	0.02	0	0	P,S,A, MAFF (1999b)	
Germany	White grape juice	1995–98	27	0.01 <sup>a</sup>	6	NA/1.3	1	0	0	P,S,A, Wolff et al. (2000)	
	Red grape juice	1995–98	64	0.01 <sup>a</sup>	8	NA/5.3	3	2	0		
	FSIS 185 white grape juice	1998	11	0.01 <sup>a</sup>	1	0.27/0.6	NA	0	0		
	FSIS 185 red grape juice	1998	9	0.01 <sup>a</sup>	0	0.76/2.05	NA	0	0		
	FSIS 185 grape juice	1998	20	0.01 <sup>a</sup>	1	0.48/2.05	1.73	0	0		
Europe	Grape juice	1994–95	8	0.005	3	0.137/0.31	0.30	0	0	P,A, Zimmerli & Dick (1996); S, NR	
Japan	Grape juice	1996	12	0.003 <sup>a</sup>	10	NA/0.006	NR	0	0	P,A, Ueno (1998); S, NR	
World	Grape juice Other juices	NR	20	000	6	NA/4.7	NA	0	0	P,S,A, Majerus & Ottender (1996)	
Germany	Apple juice	1995–98	33	0.01 <sup>a</sup>	33	0/0	NA	0	0	P,S,A, Wolff et al. (2000)	
	Orange juice	1995–98	30	0.01 <sup>a</sup>	30	0/0	NA	0	0		
	Blackcurrant juice	1995–98	19	0.01 <sup>a</sup>	16	NA/0.06	0.048	0	0		
	Tomato juice	1995–98	30	0.01 <sup>a</sup>	27	NA/0.032	000	0	0		
	Carrot juice	1995–98	18	0.01 <sup>a</sup>	17	NA/0.01	< 0.01	0	0		
	Other vegetable juice	1995–98	30	0.01 <sup>a</sup>	30	0/0	NA	0	0		

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 5 ≤ 20 (µg/kg)	n > 20 (µg/kg)	References	Sampling procedure
	<b>Seasonings</b>										
Germany	Ketchup	1995–98	57	0.01 <sup>a</sup>	41	NA/3.8	1	0	0	P,S,A, Wolff et al. (2000)	
	Herb sauce	1995–98	15	0.01 <sup>a</sup>	13	NA/0.25	0	0	0		
	Pepper sauce	1995–98	50	0.01 <sup>a</sup>	43	NA/0.72	0	0	0		
	<b>Fermented beverages</b>										
Japan	Fermented beverages	1996	15	0.003 <sup>a</sup>	15	0/0	0.00	0	0	P,A, Ueno (1998); S, NR	
	<b>Baby food</b>										
Canada	Baby food	1998–99	11	0.500	11	0/0	0.00	0	0	P, Canada (2000); S,A, NR	
	<b>Diet</b>										
United Kingdom	Normal diet	NR	32	0.002	0	0.025/0.073	0.03	0	0	P,S,A, MAFF (1999c)	
	Vegetarian diet	NR	11	0.002	0	0.045/0.114	0.09	0	0		
	Traditional diet	NR	7	0.002	0	0.029/0.066	0.05	0	0		
	<b>Dust</b>										
USA	Dust	2000	7	NR	0	278.8/ 1581.8	816	0	3	P,A, Richard et al. (1999); S, NR	

NA, not analysed; NR, not reported; MAFF, Ministry of Agriculture, Fisheries and Food (United Kingdom); USDA, Department of Agriculture of the USA; GIPSA, Grain Inspection, Packers and Stockyards Administration (United Kingdom)

<sup>a</sup> Limit of detection

<sup>b</sup> Sampling not described

<sup>c</sup> LOQ > 5 µg/kg; not used in calculation of weighted mean

<sup>d</sup> The number of samples was divided by a factor of 3 for calculation of the weighted mean.



## **TRICHOTHECENES**



## DEOXYNIVALENOL

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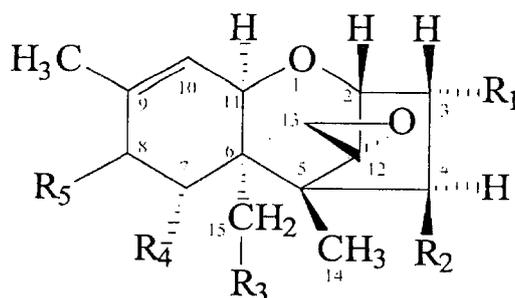
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## 1. EXPLANATION

Deoxynivalenol (DON, vomitoxin) is a type B trichothecene, an epoxy-sesquiterpenoid. This mycotoxin occurs predominantly in grains such as wheat, barley, oats, rye, and maize, and less often in rice, sorghum, and triticale. The occurrence of deoxynivalenol is associated primarily with *Fusarium graminearum* (*Gibberella zeae*) and *F. culmorum*, both of which are important plant pathogens which cause *Fusarium* head blight in wheat and *Gibberella* ear rot in maize. A direct relationship between the incidence of *Fusarium* head blight and contamination of wheat with deoxynivalenol has been established. The incidence of *Fusarium* head blight is strongly associated with moisture at the time of flowering (anthesis), and the timing of rainfall, rather than the amount, is the most critical factor. *F. graminearum* grows optimally at a temperature of 25 °C and at a water activity above 0.88. *F. culmorum* grows optimally at 21 °C and at a water activity above 0.87. The geographical distribution of the two species appears to be related to temperature, *F. graminearum* being the commoner species and occurring in warmer climates. Deoxynivalenol has been implicated in incidents of mycotoxicoses in both humans and farm animals. The Committee has not previously evaluated this toxin.

Most trichothecenes have a double bond at position C-9,10, a 12,13-epoxide ring, and a number of hydroxyl and acetoxy groups. The basic structure is shown in Figure 1. Trichothecenes can be divided into four types (A–D) according to characteristic functional groups. T-2 and HT-2 toxins are type A trichothecenes with an oxygen function different from a carbonyl function at the C-8 position. Type B trichothecenes have a carbonyl function at this position. The most frequently detected mycotoxin of this category is deoxynivalenol. Type C trichothecenes are characterized by a second epoxide function at C-7,8 or C-9,10, whereas type D include trichothecenes containing a macro cyclic ring between C-4 and C-15 with two ester linkages.

**Figure 1. Basic structure of trichothecenes**

Trichothecenes are stable at 120 °C, moderately stable at 180 °C, and decompose within 30–40 min at 210 °C.

## 2. BIOLOGICAL DATA

### 2.1 Biochemical aspects

#### 2.1.1 Absorption, distribution, and excretion

##### (a) Gastrointestinal metabolism

Cultures of 20% w/w suspensions of rat caecal contents were incubated anaerobically with [<sup>14</sup>C]deoxynivalenol at a concentration of 35 µg/ml for up to 24 h. A standard-co-elution method involving high-performance liquid chromatography (HPLC) was used to quantify the proportions of radiolabel associated with deoxynivalenol and with the de-epoxidated form. The latter represented 1.3% of the administered radiolabel immediately after addition of deoxynivalenol, 29% at 7 h, and 90% at 24 h; 60% co-eluted with deoxynivalenol at 7 h and 2% at 24 h (Worrell et al., 1989).

The metabolism of deoxynivalenol by intestinal flora was examined in extracts of porcine duodenum, jejunum, caecum, colon, and rectum after addition of 0.3 mg to 10 ml of pre-incubated suspensions containing 1 g of intestinal contents and anaerobic incubation for 24 h. The rate of recovery of deoxynivalenol was compared with that from inactivated suspensions that had been heated to 121 °C for 35 min before incubation with deoxynivalenol for 24 h. The only metabolite was identified by gas-liquid chromatography-mass spectrometry (MS) as a de-epoxy derivative. Differences in the rates of metabolism were seen between active and inactivated suspensions from caecum, colon, and rectum but not those from duodenum and jejunum. The greatest de-epoxidation activity was seen in colon, and only 1% of the applied dose was recovered as deoxynivalenol from the active suspension (Kollarzik et al., 1994).

Deoxynivalenol was not converted to the de-epoxy metabolite in cultures of the contents of pig large intestine (including caecum, but not otherwise specified) in another study in which 1 ml of a 1-µg/ml solution of deoxynivalenol was added to 2 g of large intestinal contents and incubated anaerobically for 96 h. Nearly complete

recovery of intact deoxynivalenol was reported. The intestinal contents of chickens treated identically showed nearly complete conversion of deoxynivalenol to the de-epoxy metabolite after 96 h. After 24 h of incubation, the rate of conversion was 56% of an applied concentration of 0.014 µg/ml, 69% of 0.14 µg/ml, and 70% of 1.4 µg/ml. Similarly, 35% of the applied deoxynivalenol was metabolized to the de-epoxy metabolite in bovine rumenal fluid after 96 h of incubation (He et al., 1992).

No metabolism of deoxynivalenol was observed in pigs dosed intragastrically with [<sup>14</sup>C]deoxynivalenol. The total recovery of radiolabel from faeces, urine, and bile of an intragastric dose of 0.6 mg/kg bw 24 h after dosing ranged from 82 to 100% for three pigs. No metabolites of deoxynivalenol were observed by gas chromatography (GC)–MS (Prelusky et al., 1988).

Deoxynivalenol did not disappear from 3-h cultures of sheep or cattle rumen after addition at a concentration of 2.5 mg/L to a 50% v/v solution of rumenal fluid and buffer (total volume, 10 ml), when metabolism was determined after incubation for 0.5, 1, 2, or 3 h. GC (flame ionization detector) was used to measure the disappearance of deoxynivalenol (Kiessling et al., 1984). Few details of the results were reported, and limitations in study design and analytical methods may have hampered observation of the metabolism of deoxynivalenol. The standard rate of recovery was reported to be only 43%, and the detection limit was 2 µg. The results of only three determinations after a 3-h incubation with sheep rumenal fluid were reported, and the results with bovine rumenal fluid were not reported.

In contrast, a decrease in deoxynivalenol concentration was reported after 6 h of incubation with bovine rumenal fluid, with nearly complete disappearance by 24 h of concentrations of 5 and 10 mg/L of culture medium. The concentrations also decreased after initial concentrations of deoxynivalenol of 50 and 100 mg/L in culture, but complete disappearance was not seen at 48 h. Analysis was conducted by HPLC and gas–liquid chromatography (King et al., 1984a).

#### *(b) Bioavailability*

Although deoxynivalenol appeared in the blood within 30 min after intake by sheep, the systemic bioavailability was only 7.5%. A single dose of 5 mg/kg bw of deoxynivalenol was administered by oral intubation to four 1-year-old male sheep, and repeated blood samples were taken over 30 h. Deoxynivalenol and the de-epoxy metabolite were determined by GC with electron capture detection (ECD). No deoxynivalenol or the de-epoxy metabolite could be detected in plasma within the 30-h observation period. Three male sheep were given a single intravenous dose of deoxynivalenol at 0.5 mg/kg bw, with blood sampling and analysis as after oral dosing. Systemic bioavailability was calculated from the ratio of the integrated area under the concentration–time curve times the dose for both oral and intravenous administration. Less than 0.3% of the oral dose and less than 2% of the intravenous dose was detected in plasma as the de-epoxy metabolite. Free deoxynivalenol accounted for an average of 24.8% of the absorbed dose measured in blood; the remainder was made up of the de-epoxy metabolite or the glucuronide conjugate of deoxynivalenol (Prelusky et al., 1985).

The oral absorption rate of deoxynivalenol in sheep was approximately 7% on the basis of recovery rates from urine and bile collected over 36 h from two sheep given 5 mg/kg bw of deoxynivalenol orally. Deoxynivalenol and the de-epoxy metabolite were analysed by GC-ECD. An average of 6.9% of the administered dose was recovered from urine and 0.11% from bile. Glucuronide-conjugated de-epoxy metabolite was the only form detected in bile (detection limit, 0.1 mg, corresponding to 0.04% of the administered dose). An average of 1.3% of the administered dose was recovered from urine as the de-epoxy metabolite or its conjugate, and 5.7% was recovered as deoxynivalenol or its conjugate (Prelusky et al., 1986a).

Rapid appearance of deoxynivalenol in blood was also observed in two dairy cows given a single oral dose of 920 mg of deoxynivalenol (equivalent to 1.84 mg/kg bw). Although the absolute bioavailability was not determined in this study, the serum concentrations (peak of 20 ng/ml at 4.7 h in one cow) and clearance from plasma to below the detection limit within 24 h suggested low systemic bioavailability. The concentrations of deoxynivalenol and the de-epoxy metabolite were measured by GC-MS (Prelusky et al., 1984).

In contrast to the low bioavailability seen in sheep and cows, relatively high bioavailability was observed in pigs. Blood, urine, bile, and faeces were collected over 24 h after an intragastric dose of 0.6 mg/kg bw of [<sup>14</sup>C]deoxynivalenol or an intravenous dose of 0.3 mg/kg bw. The proportions of radiolabel were assumed to represent those of administered deoxynivalenol, and the validity of this assumption was confirmed by GC-MS, which showed very little metabolism or conjugation. On the basis of measurements of the integrated area under the concentration-time curve for three animals treated intravenously and three treated intragastrically, the average systemic bioavailability of deoxynivalenol in pigs was estimated to be 55%. Approximately 95% of the administered dose was recovered as deoxynivalenol; the amount recovered as conjugated deoxynivalenol or as other metabolites was not reported quantitatively (Prelusky et al., 1988).

Although the absolute bioavailability of deoxynivalenol has not been measured in rats, 25% of an oral dose of 10 mg/kg bw was recovered in urine at 96 h, suggesting that the absorption rate in rats may be higher than in sheep or cows. HPLC and GC-MS analysis indicated that 25% of the radiolabel in 0-24-h urine was associated with unchanged deoxynivalenol and 10% with the de-epoxy metabolite (Lake et al., 1987). Similarly, 4.5 and 4.4% of an orally administered dose of 6 mg/kg bw was recovered in the urine of Wistar rats as free deoxynivalenol and the de-epoxy metabolite, respectively, within 96 h (Yoshizawa et al., 1983).

### (c) Distribution

The distribution of [<sup>14</sup>C]deoxynivalenol was measured after a single oral dose of 2.2 mg in chickens (equivalent to 1.3-1.7 mg/kg bw on the basis of the reported body weights). The average distribution, measured as disintegrations per minute per gram of wet tissue (dpm/g), was 416 in blood, 570 in plasma, and 4345 in bile, and 19 in cutaneous fat, 10 in abdominal fat, 5 in breast muscle, 5.3 in thigh muscle, 91 in spleen, 205 in liver, 27 in heart, 733 in kidney, 21 in brain, and 5 in oviduct at

3 h. At 72 h, the average distribution was 0 dpm/g in blood, 0 in plasma, and 661 in bile, and 10 in cutaneous fat, 9.8 in abdominal fat, 0.5 in breast muscle, 2 in thigh muscle, 8 in spleen, 10 in liver, 0 in heart, 18 in kidney, 0 in brain, and 2 in oviduct. Radiolabel was observed only in cutaneous fat, kidney, gizzard, and bile 96 h after administration of [<sup>14</sup>C]deoxynivalenol. The tissue distribution after continuous intake of 2.2 mg/bird per day from the feed for 2, 4, or 6 days was similar to that after a single oral dose (Prelusky et al., 1986b).

A single intravenous injection of deoxynivalenol at 1 mg/kg bw to pigs resulted in substantially higher initial concentrations in plasma, kidney, and liver than in other tissues. Analysis by HPLC–MS 3 h after injection showed concentrations of 550 ng/g of plasma, 930 ng/g of kidney, 440 ng/g of liver, 330 ng/g of abdominal fat, 130 ng/g of back fat, 140 ng/g of lymph, 78 ng/g of lung, 69 ng/g of adrenals, 74 ng/g of spleen, 54 ng/g of testis, 29 ng/g of brain, 11 ng/g of heart, 19 ng/g of muscle, 16 ng/g of skin, 5 ng/g of intestine, and 4 ng/g of pancreas. At 24 h after injection, the concentrations were 18 ng/g in plasma, 10 ng/g in kidney, 8.2 ng/g in liver, 3.4 ng/g in abdominal fat, 12 ng/g in back fat, 0.8 ng/g in lymph, and 1 ng/g in lung, with none in the other tissues examined (Prelusky & Trenholm, 1991).

#### (d) Excretion

Excretion of deoxynivalenol and the de-epoxy metabolite after a 10-mg/kg oral dose of [<sup>14</sup>C]deoxynivalenol was examined in male PVG rats. At 96 h, 25% of the administered radiolabel was recovered in urine, 64% in faeces, and 0.11% in expired air (Lake et al., 1987).

Rapid elimination was reported of an oral dose of 2.2 mg of [<sup>14</sup>C]deoxynivalenol in chickens (equivalent to 1.3–1.7 mg/kg bw on the basis of the reported body weights). Recovery of radiolabel in excreta accounted for 79, 92, and 98% of the administered dose by 24, 48, and 72 h, respectively (Prelusky et al., 1986b).

A plasma elimination half-time of 3.9 h was reported after intravenous administration of deoxynivalenol to pigs at 1 mg/kg bw. The compound was recovered in bile and urine, as analysed by HPLC–MS (Prelusky & Trenholm, 1991).

A single dose of 5 mg/kg bw of deoxynivalenol was administered by oral intubation to four 1-year old male sheep, and repeated blood samples were taken over 30 h. Analysis for deoxynivalenol and the de-epoxy metabolite by GC–ECD showed complete elimination from plasma within 30 h. In three male sheep given a single intravenous dose of 0.5 mg/kg bw, with blood sampling and analysis as above, the half-life of elimination from plasma was 100–125 min (Prelusky et al., 1985).

Urine and bile were collected over 36 h from two sheep dosed orally with deoxynivalenol at 5 mg/kg bw. Deoxynivalenol and the de-epoxy metabolite were determined by GC–ECD. An average of 6.9% of the administered dose was recovered from urine, 0.11% from bile, and 64% from faeces (Prelusky et al., 1986a).

Two ewes were given an intravenous dose of [<sup>14</sup>C]deoxynivalenol at 4 mg/kg bw, and excretion in urine and bile was monitored over 24 h. An average of 91% of

the administered radiolabel was recovered in urine and 6% in bile (Prelusky et al., 1987a).

Blood, urine, bile, and faeces were collected from pigs over 24 h after an intragastric dose of [<sup>14</sup>C]deoxynivalenol at 0.6 mg/kg bw or an intravenous dose of 0.3 mg/kg bw. After intravenous dosing, an average of 3.8% of the administered radiolabel was recovered in bile, < 0.3% in faeces, and 93% in urine. After oral dosing, bile accounted for an average of 2.5% of the administered dose, urine for 68%, and faeces for 20%. After oral intake, the peak concentration in plasma was reached within 15–30 min, remained elevated for about 9 h and then declined with a half-time of 7.1 h (Prelusky et al., 1988).

(e) *Transmission into eggs and milk*

Transmission of deoxynivalenol into eggs was studied in laying hens. Each hen was given a single oral dose of [<sup>14</sup>C]deoxynivalenol at 2.2 mg (equal to 1.3–1.7 mg/kg bw). The maximum amount of radiolabel in the first eggs laid within 24 h of dosing represented 0.087% of the administered dose (equal to 1.9 µg of deoxynivalenol or metabolites per egg). After repeated dosing for 6 days, the maximum amount of radiolabel per egg represented 0.19% of the administered daily dose (equal to 4.2 µg per egg of deoxynivalenol or metabolites) (Prelusky et al., 1987b).

Administration of feed containing [<sup>14</sup>C]deoxynivalenol at a concentration of 5.5 mg/kg over 65 days did not result in increased accumulation of deoxynivalenol or metabolites in chicken eggs. The maximum amount of radiolabel in eggs (equivalent to 1.7 µg of deoxynivalenol or metabolites per 60-g egg) was reached after 8 days of administration; the amount decreased slowly during subsequent weeks (Prelusky et al., 1989).

Two ewes were given [<sup>14</sup>C]deoxynivalenol intravenously at a dose of 4 mg/kg bw, and excretion of radiolabel into the milk was monitored every 4 h over 48 h. Oxytocin was used to stimulate lactation. Less than 0.25% of the administered dose was recovered. Gas–liquid chromatography–MS analysis showed that conjugated de-epoxy metabolite made up most of the recovered radiolabel. The highest concentration of deoxynivalenol was 61 ng/ml (comprising conjugated and unconjugated compound in an approximately 2:1 ratio). The highest concentration of the de-epoxy metabolite was 1200 ng/ml (with conjugated and unconjugated material in a 3:1 to 5:1 ratio) (Prelusky et al., 1987a).

Low concentrations of free and conjugated deoxynivalenol were also found in cows' milk collected twice daily after administration of a single oral dose of 920 mg of deoxynivalenol in *Fusarium*-contaminated maize. The amount of deoxynivalenol was quantified by HPLC. The highest concentration was 4 ng/ml, comprising both conjugated and free deoxynivalenol. The concentrations of the de-epoxy metabolite were not assessed (Prelusky et al., 1984).

Eighteen primiparous Holstein cows at 13–22 weeks of lactation were divided into six groups according to their stage of lactation and milk yield and were observed for 10 weeks to determine the effect of deoxynivalenol in the diet on milk yield and

transfer of deoxynivalenol and its de-epoxy metabolite to milk. Contaminated maize was added to the diets to provide concentrations of deoxynivalenol of 0, 5, and 12 mg/kg of dry matter concentrate, and daily intakes of 0.001, 0.085, and 0.21 mg/kg bw. The animals were weighed during weeks -2, 0, 2, 4, 6, 8, and 10. Feed intake was recorded daily, and pooled milk samples (from morning and evening milkings) were analysed every 14 days. The condition of the animals was scored at the beginning of week 8. Increasing concentrations of deoxynivalenol in the diet did not affect the feed intake or total milk output, but the output of milk fat and fat were reduced in both groups given deoxynivalenol, with the greatest effect at the intermediate dietary concentration. Overall energy efficiency was not affected because the reduced energy output in milk was compensated by increased body weight gain. No transfer of deoxynivalenol or the de-epoxy metabolite to milk was observed at the detection limit of 5 ng/ml by HPLC-MS (Charmley et al., 1993).

### 2.1.2 *Biotransformation*

De-epoxidation was shown in rats (Yoshizawa et al., 1983; Lake et al., 1987; Worrell et al., 1989) and in pigs (Kollarczik et al., 1994), whereas He et al. (1992) showed an absence of de-epoxidation in pigs. De-epoxidation and glucuronide conjugation were demonstrated in cows (Côté et al., 1986; Yoshizawa et al., 1986), and glucuronide conjugation was found in sheep (Prelusky et al., 1985).

The de-epoxy metabolite of deoxynivalenol was identified in the urine and faeces of male Wistar rats given oral doses of deoxynivalenol at 8–11 mg/kg bw (dose not otherwise specified; source of deoxynivalenol not specified). The compounds were quantified by gas-liquid chromatography and identified by GC-MS (Yoshizawa et al., 1983).

No microsomal metabolism of deoxynivalenol was observed in fractions of male rabbit or male Wistar rat liver (Ohta et al., 1978; Côté et al., 1987).

A single 5-mg/kg bw dose of deoxynivalenol was administered by oral intubation to four 1-year-old male sheep, and repeated blood samples were taken over 30 h. The presence of glucuronide-conjugated metabolites was deduced from an increase in the recovery of the de-epoxy metabolite or deoxynivalenol after treatment with  $\beta$ -glucuronidase. Three further male sheep were each given a single intravenous dose of deoxynivalenol at 0.5 mg/kg bw, with blood sampling and analysis as for oral dosing. Less than 0.3% of the administered oral dose and less than 2% of the intravenous dose was detected in plasma as the de-epoxy metabolite. Free deoxynivalenol accounted for an average of 25% and conjugated deoxynivalenol for 73% of the dose in blood during the observation period. In animals dosed intravenously, conjugated deoxynivalenol accounted for an average of 20% of the dose in blood. The clearance times for conjugated deoxynivalenol were considerably longer than those for free deoxynivalenol (elimination half-times, < 125 min for deoxynivalenol and > 6 h for conjugated deoxynivalenol after oral administration) (Prelusky et al., 1985).

Urine and bile were collected over 36 h from two sheep given deoxynivalenol orally at a dose of 5 mg/kg bw. Glucuronide-conjugated de-epoxy metabolite was the only form detected in bile (detection limit, 0.1 mg, corresponding to 0.04% of the

administered dose). In urine, an average of 1.3% of the administered dose was recovered as the de-epoxy metabolite, alone or conjugated, and 5.7% as parent or conjugated deoxynivalenol (Prelusky et al., 1986a).

Blood, urine, bile, and faeces were collected from pigs over 24 h after they were given [<sup>14</sup>C]deoxynivalenol intragastrically at a dose of 0.6 mg/kg bw or intravenously at a dose of 0.3 mg/kg bw. GC-MS analysis for deoxynivalenol and metabolites showed little metabolism or conjugation. About 95% of the administered dose was recovered as deoxynivalenol; the amounts recovered as conjugated deoxynivalenol or as other metabolites were not reported quantitatively (Prelusky et al., 1988).

### 2.1.3 Effects on enzymes and other biochemical parameters

#### (a) Effect on nutrients

During a 6-week feeding trial in groups of 10 male NMRI mice, the effects of dietary administration of deoxynivalenol at 0, 0.1, 1, or 10 mg/kg, equivalent to 0.014, 0.14, and 1.4 mg/kg bw, on food consumption and weight gain were investigated. Food intake was similar in the four groups, but the weight gain in the group receiving 10 mg/kg was significantly ( $p < 0.01$ ) reduced. At the end of the feeding period, the animals were killed, and absorption of water, D-glucose, L-leucine, L-tryptophan, 5-methyltetrahydrofolic acid, and iron was measured in perfused jejunal segments *in vitro*. No effects were observed on absorption of water, leucine, tryptophan, or iron, but at the dietary concentration of 10 mg/kg, a slight but significant ( $p < 0.05$ ) reduction in glucose transfer was measured. Furthermore, the transfer and the tissue accumulation of 5-methyltetrahydrofolic acid in the jejunal segment were significantly decreased, by up to 50%. When the heavy metal and trace element content of the liver, kidney, and small intestine was determined, the manganese and molybdenum content in liver was reduced at the deoxynivalenol concentration of 10 mg/kg of diet. The authors concluded that ingestion of feed containing deoxynivalenol at concentrations that occur in contaminated food and feed results in impairment of intestinal transfer and uptake of nutrients such as glucose and 5-methyltetrahydrofolic acid (Hunder et al., 1991).

#### (b) Effects on macromolecular synthesis

Most trichothecenes inhibit protein synthesis, their potency depending on structural substituents and requiring an unsaturated bond at the C9-C10 position and integrity of the 12,13-epoxy ring. Trichothecenes bind to the 60S subunit of eukaryotic ribosomes and interfere with the activity of peptidyltransferase. Deoxynivalenol, which lacks a substituent at C-4, inhibits chain elongation (Ehrlich & Daigle, 1987; Betina, 1989). Inhibition of protein synthesis is considered to be the primary toxic effect of trichothecenes, including deoxynivalenol. The  $ID_{50}$  for inhibition of protein synthesis in rabbit reticulocytes was 2  $\mu\text{g/ml}$ , while that for T-2 toxin was 0.03  $\mu\text{g/ml}$  (reviewed by Sato & Ueno, 1977). *In vitro*, deoxynivalenol is about 100 times less toxic than T-2 toxin, which has been more widely studied for its macromolecular effects. Owing to differences in lipophilicity and other possible effects, the toxicity of deoxynivalenol *in vivo* is greater than would be expected from its effects on protein synthesis *in vitro* (Sato & Ueno, 1977; Thompson & Wannemacher, 1986).

The effects of deoxynivalenol on synthesis of protein, DNA, and RNA (studied with radiolabelled amino acids, [<sup>14</sup>C]uridine, and [<sup>3</sup>H]thymidine) in spleen slices taken from 8–10-week-old rats and cultured for 90 min in Krebs Ringer phosphate buffer at pH 7.4 was studied at concentrations of 100, 1000, and 10 000 ng/ml. The minimum effective concentration for inhibition of protein and DNA synthesis was 1000 ng/ml (72% and 53% inhibition, respectively), whereas RNA synthesis was stimulated at this concentration (Friedman et al., 1996).

## 2.2 Toxicological studies

The toxicity of deoxynivalenol has been reviewed by WHO (1990), IARC (1993), and Rotter et al. (1996). Risk assessments that included toxicological reviews of deoxynivalenol have been published for Canada (Kuiper-Goodman, 1985), the Nordic Council (Eriksen & Alexander, 1998), The Netherlands (Pieters et al., 1999), and the European Union (Commission of the European Union, 1999).

### 2.2.1 Acute toxicity

The acute symptoms of poisoning with trichothecenes are characterized by skin irritation, feed refusal, vomiting, diarrhoea, haemorrhage, neural disturbance, abortion, and death. The LD<sub>50</sub> values for deoxynivalenol and its 3-acetyl and 15-acetyl metabolites administered orally are shown in Table 1. Studies of the emetic effects of deoxynivalenol are summarized in Table 2.

**Table 1. Acute oral toxicity of deoxynivalenol and metabolites**

Species and strain	Route	Compound	LD <sub>50</sub> (mg/kg bw)	Reference
Mouse, ddy, 6-week-old male	Oral	Deoxynivalenol	46	Yoshizawa & Morooka (1974)
	Intraperitoneal		70	
	Intraperitoneal		77	
Mouse, B6C3F <sub>1</sub> , weanling	Oral	Deoxynivalenol	78	Forsell et al. (1987)
	Intraperitoneal		49	
Mouse	Intraperitoneal	Deoxynivalenol	43	Thompson & Wannemacher (1986)
	Subcutaneous		45	
Mouse, ddy, 6-week-old male	Oral	3-Acetyldeoxynivalenol	34	Yoshizawa & Morooka (1974)
	Intraperitoneal		49	
	Intraperitoneal		47	
Mouse, B6C3F <sub>1</sub> , weanling	Oral	15-Acetyldeoxynivalenol	34	Forsell et al. (1987)
Chicken, Cobb, broiler, female	Oral	Deoxynivalenol	140	Huff et al. (1981)
Duck, Peking 10-day-old	Subcutaneous	Deoxynivalenol	27	Yoshizawa & Morooka (1974)

**Table 2. Results of studies of emesis in animals treated with deoxynivalenol**

Species	Route	Purity	ED <sub>50</sub> (mg/kg bw)	LOEL (mg/kg bw)	NOEL (mg/kg bw)	Reference
Pig, 9–10 kg	Feed Intraperi- toneal	Purified		0.100 0.050	0.075 0.025	Forsyth et al. (1977)
Pig, 28–51 kg	Feed	Purified	0.085 (estimated)	0.07 (2/6)	0.035	Young et al. (1983) and personal communication
Pig, 10–15 kg	Feed Intraperi- toneal	Purified		0.05 (1/3) 0.05 (1/3)	0.025 0.025	Pestka et al. (1987a)
Pig, 12–25 kg, fasted	Cannula Intravenous		0.075 (2/6) 0.020 (2/4)	0.05 (1/5) 0.02 (2/4)	0.025 0.015	Prelusky & Trenholm (1993) and personal communication
Pig, 7.5 kg	Feed	Contaminated corn		0.8	0.6	Young et al., (1983)
Pig, 34 kg	Feed	Inoculated corn			0.42	Friend et al. 1984)
Dog, 2–3 kg	Subcuta- neous	Purified		0.10		Yoshizawa & Morooka (1974)
Dog	Feed	Contaminated wheat		0.45	0.3	Hughes et al. (1999)
Cat	Feed	Contaminated wheat		0.4	0.3	Hughes et al. (1999)

<sup>a</sup> Additional data on emesis obtained by personal communication from D. Prelusky, Agriculture Canada, 1994

The minimum single dose (LOEL) of deoxynivalenol that induced vomiting (emesis) in groups of three to six pigs weighing 9–10 kg was 0.1 mg/kg bw when given by oral gavage (NOEL, 0.075 mg/kg bw) and 0.05 mg/kg bw when given intraperitoneally (NOEL, 0.025 mg/kg bw) (Forsyth et al., 1977).

The minimum single emetic doses of deoxynivalenol and 15-acetyldeoxynivalenol in groups of three Yorkshire pigs weighing 10–15 kg were 0.050 and 0.075 mg/kg bw, respectively, when given either by gavage or intraperitoneally. After gavage, three of 15 pigs given the 15-acetyl metabolite and four of 15 given deoxynivalenol showed emesis at all doses from 20 to 200 µg/kg bw. After intraperitoneal administration, nine of 15 pigs showed emesis at all doses. The NOELs were 0.025 mg/kg bw for deoxynivalenol and 0.050 mg/kg bw for the 15-acetyl metabolite after either oral intubation or intraperitoneal injection (Pestka et al., 1987a).

In a pilot study, the median emetic dose (ED<sub>50</sub>) of purified deoxynivalenol, administered by gavage to groups of two to six Yorkshire pigs weighing 28–51 kg was 0.088 mg/kg bw (Young et al., 1983). Additional data provided by D. Prelusky, Agriculture and Agrifoods, Canada, indicated that the LOEL in this study was 0.07 mg/kg bw (for the two pigs that responded, and the NOEL was 0.035 mg/kg bw).

The ED<sub>50</sub> values after single doses of deoxynivalenol (> 96% pure) administered by cannula into the stomach or intravenously to groups of four to six Yorkshire pigs, weighing 12–25 kg, which had fasted for 4 h, were 75 and 20 mg/kg bw by the two routes, respectively (Prelusky & Trenholm, 1993). Additional data provided by D. Prelusky, Agriculture and Agrifoods, Canada, indicated that after oral administration the LOEL was 0.05 mg/kg bw for the one pig that responded, and the NOEL was 0.025 mg/kg bw. After intravenous administration, the NOEL was 0.015 mg/kg bw.

In studies in which young pigs (7.5 kg) received feed containing heavily contaminated mouldy corn, a dietary concentration of approximately 20 mg/kg (equal to 0.8 mg/kg bw) caused emesis, whereas no emesis was observed at 12 mg/kg feed (equal to 0.6 mg/kg bw) (Young et al., 1983).

In a study of feed inoculated with corn, no emesis was observed in 34-kg pigs at a dietary concentration of 14 mg/kg (equal to 0.42 mg/kg bw) (Friend et al., 1984).

Similarly, in studies in dogs and cats given a diet containing contaminated wheat, emesis occurred at doses of 0.45 and 0.4 mg/kg bw, respectively. The NOEL was 0.3 mg/kg bw for both species (for further experimental details, see section 2.2.2) (Hughes et al., 1999).

The minimum single emetic doses (LOELs) of deoxynivalenol and 15-acetyl-deoxynivalenol given subcutaneously to 6-month-old dogs weighing 2–3 kg were 0.1 and 0.2 mg/kg bw, respectively (Yoshizawa & Morooka, 1974).

The Committee noted that, in the studies described above, emesis occurred in pigs at much lower doses when deoxynivalenol was given by gavage than when it was given in the feed. This difference was attributed to a bolus effect of gavage. The usual exposure of humans would be comparable to administration in the feed.

At a dose of 46 mg/kg bw given by gavage, deoxynivalenol damaged the cells lining the gastrointestinal tract of 4-week-old ICR mice. Ulcers and cell infiltration were observed in the forestomach, and necrosis of immature crypt cells, cell infiltration in the mucosa, and cystic changes in the crypts were observed in the small intestine (Ito et al., 1993).

### **2.2.2 Short-term studies of toxicity**

The results of these studies are summarized in Table 3.

*Mice:* In a study lasting about 18 weeks, groups of 80 Swiss-Webster weanling male mice were fed either an 'uncontaminated' wheat diet containing deoxynivalenol

**Table 3. Summary of short- and long-term studies of the toxicity of deoxynivalenol**

Species, strain, sex, age	Length of study (days)	No. per group	Dose		Route	Effect	LOEL (mg/kg bw per day)	NOEL (mg/kg bw per day)	Reference
			mg/kg of diet	mg/kg bw per day					
Mouse, Swiss Webster, m weanling	7-137	80	6.3	0.9	Diet, contam. wheat	Reduced body-weight gain	0.9		Arnold et al. (1986a)
Mouse, BALB/c	7	3 x 4	2.5, 5, 10, 20, 50	0.35, 0.67, 1.3, 2.7, 6.5	Diet	Reduced feed intake, body-weight gain, thymus weight; decreased cardiac protein synthesis	0.35	0.67	Robbana-Barnat et al. (1987)
	30	4	10	1.3		Cardiac lesions	1.3	0.67	
Mouse, ICR, f, m, 21 days	14	10-12	8, 12, 16	1.2, 1.8, 2.4	Diet	Reduced feed intake	< 1.2		Rotter et al. (1992)
	14		4, 8	0.6, 1.2		Reduced growth	< 0.6		
Mouse, Swiss Webster, m weanling	35	24		0.75, 2.5, 7.5	Diet	Reduced feed intake, decreased thymus weight; changes in spleen, thymus, lymph nodes, gut	0.75		Arnold et al. (1986a)
							2.5	0.75	
Mouse, B6C3F <sub>1</sub> , f, weanling	56	8	0.5, 2, 5, 10, 25	0.07, 0.28, 0.7, 1.4, 3.5	Diet	Reduced body-weight gain and liver, kidney weights	0.28	0.07	Forsell et al. (1986)
						0.7	0.28		
Mouse, B6C3F <sub>1</sub> , f, weanling	56	10	0.5, 2, 5, 10, 25	0.07, 0.28, 0.7, 1.4, 3.5	Diet, 15-acetyldeoxynivalenol	Reduced feed intake, body-weight gain; decreased kidney, spleen weights	0.7	0.28	Pestka et al. (1986)

Table 3 (contd)

Species, strain, sex, age	Length of study (days)	No. per group	Dose		Route	Effect	LOEL (mg/kg bw per day)	NOEL (mg/kg bw per day)	Reference
			mg/kg of diet	mg/kg bw per day					
Mouse, 22–25 g	56	24	0.04 2 x/ week	0.006	Diet	Reduced body-weight gain; intestinal necrosis, renal glomerular lesions; study inadequate	0.006		Bilgrami et al. (1993)
Mouse, NMRI, m, 18 g	42	10	0.1, 1, 10	0.014, 0.14, 1.4	Diet	Reduced body-weight gain; impaired uptake of nutrients	1.4	0.14	Hunder et al. (1991)
Mouse, ICR, 18 g	14	8	2, 4, 8 3, 6, 9	0.37–1.5 m 0.4–1.6 f	Diet	Reduced feed intake and growth	0.37 m	0.81 f	Rotter et al. (1994a)
Mouse, 3 strains	90	3–6	10	1.4	Diet	Adverse effects on epididymides	> 1.4		Sprando et al. (1999)
Mouse, B6C3F <sub>1</sub> , males females	730	50	1, 5, 10	0.1, 0.5, 1.1 0.1, 0.6, 1.4	Diet	Reduced body-weight gain; reduced tumour incidence	0.5	0.1	Iverson et al. (1995)
Rat, ICR, m	91	50	6.3	0.5	Diet	Reduced feed intake and body-weight gain	0.5		Arnold et al. (1986a)
Rat Sprague-Dawley, m, f, weanling	60 68	25 m 25 f		0.25, 0.5, 1	Diet	Reduced body-weight gain, reduced feed intake; decreased jejunum and spleen thymidine uptake	0.25 f 1 m	0.5 m 1 (f)/0.5 (m)	Arnold et al. (1986b)

Table 3 (contd)

Species, strain, sex, age	Length of study (days)	No. per group	Dose		Route	Effect	LOEL (mg/kg bw per day)	NOEL (mg/kg bw per day)	Reference
			mg/kg of diet	mg/kg bw per day					
Rat, Sprague-Dawley, m	90	10	20	1	Diet	Reduced body-weight gain	1		Morrissey et al. (1985)
Rat, Sprague-Dawley, m, f, 280 g	2	4 x 2	40 x 2		Diet, pure	Reduced feed intake (46% of control)	2		Vesonder et al. (1979)
Rat, Wistar, f, 139 g	8	5	40	2	Diet, contam. maize	Reduced feed intake, body-weight gain; decreased absolute liver and thymus weights, increased haemoglobin, haematocrit, serum parameters	2		Basilico et al. (1997)
			40	2	Detoxified	Only effect, reduced serum alkaline phosphatase activity			
Broiler chicks, m, 1 day of age at beginning	21		16	1.3	Diet, contam. wheat	Reduced feed efficiency	1.3		Kubena et al. (1989)
Broiler chicks, m, f, 1 day of age at beginning	35	240	0.1, 1.0, 2.1, 3.4 + 10% 3-acetyldeoxynivalenol	0.01, 0.1, 0.34	Diet, contam. oats	No effect on feed intake, weight gain, carcass weight, heart, or histological parameters		0.21, 0.34	Bergsjø & Kaldhusdal (1994)

Table 3 (contd)

Species, strain, sex, age	Length of study (days)	No. per group	Dose		Route	Effect	LOEL (mg/kg bw per day)	NOEL (mg/kg bw per day)	Reference
			mg/kg of diet	mg/kg bw per day					
Broiler chicks, m 1 day of age at beginning	21	36	16	1.5	Diet, contam. wheat	No effect on feed intake, body-weight gain, haematological, serum and histological parameters		1.5	Harvey et al. (1997)
Broiler chicks, m 1 day of age at beginning	21	36	15	1.3	Diet, contam. wheat	No effect on feed intake, body-weight gain, haematological or serum parameters; increased relative weight of heart, bursa, and gizzard	1.3		Kubena et al. (1997)
Broiler chicks, 1 day of age at beginning	37	45	1.8, 3.6, 5.3 + 50% other mycotoxins	0.14, 0.3, 0.46	Diet, contam. maize	No effect on body-weight gain, feed conversion, or serum parameters; increased heart weight: dose-related, significant at highest dose	0.46	0.3	Leitgeb et al. (1999)
Turkey poults, f 1 day of age at beginning	21	24	20	1.6	Semi-purified deoxynivalenol	No effect on feed intake, body-weight gain, haematological, most serum parameters, histology, heart or kidney weights; reduced serum calcium	1.6		Morris et al. (1999)

**Table 3** (contd)

Species, strain, sex, age	Length of study (days)	No. per group	Dose		Route	Effect	LOEL (mg/kg bw per day)	NOEL (mg/kg bw per day)	Reference
			mg/kg of diet	mg/kg bw per day					
Mallard duck, m, f, 1 year old	14	10	5.8	1.5	Diet, contam. wheat	No effect on serum, haematological, or histological parameters		1.5	Boston et al. (1996)
Shrimp	112	72	0.2, 0.5, 1	0.007, 0.018, 0.036	Diet, contam. wheat	Reduced growth rate, dose-related	0.007		Trigo-Stockli et al. (2000)
Cat, American shorthair, 1–9 years of age	14	2–7	1, 2, 4, 6, 8, 10	0.05, 0.1, 0.2, 0.3, 0.4, 0.5	Diet, contam. wheat	Emesis; reduced food intake	0.4	0.3	Hughes et al. (1999)
Dog, beagle or Brittany, 1–7 years of age	14	2–14	1, 2, 4, 6, 8, 10	0.075, 0.15, 0.3, 0.45, 0.6, 0.75	Diet, contam. wheat	Emesis, reduced food intake	0.45	0.3	Hughes et al. (1999)
Reduced body-weight		0.08 (1984)				Trenholm et al.	2	0.08	Diet, contam. gain
Pig									
8 kg	21	≥ 1	1–4.2	0.04, 0.09, 0.18	Diet, contam. wheat	Reduced feed intake and body-weight gain	0.18	0.09	Pollman et al. (1985)
60 kg	42						0.09	0.04	
Pig, 49 days, 14 kg, castrated m	28	6	4.5	0.2	Diet, contam. wheat	Reduced feed intake and body-weight gain; renal lesions; interaction with fumonisin B <sub>1</sub>	0.2		Harvey et al. (1996)

Table 3 (contd)

Species, strain, sex, age	Length of study (days)	No. per group	Dose		Route	Effect	LOEL (mg/kg bw per day)	NOEL (mg/kg bw per day)	Reference
			mg/kg of diet	mg/kg bw per day					
Pig, young	21		1.3, 12, 20, 43	0.06, 0.6, 0.8, 1.6	Diet, contam. maize	Emesis Feed refusal Reduced body-weight gain	0.8 0.6 0.06	0.6 0.06	Young et al. (1983)
Pig, 84 days, 38 kg	35	6	2.5	0.1	Diet, contam. maize	Reduced feed intake and body-weight gain	0.1		Friend et al. (1992)
Pig, Yorkshire, 6–7 weeks, 13 kg, castrated m	28	6–8	0.95, 1.8, 2.8	0.08, 0.13, 0.18	Diet, contam. maize, pair fed	Reduced body-weight gain; decreased thyroid weight, increased thyroxine serum albumin and albumin:globulin ratio, decreased $\alpha$ -globulin	0.08 (day 7) 0.13	0.08	Rotter et al. (1994b)
Pig, 18 kg, castrated m	42	8	4	0.26 initially, 0.16 at end	Diet	Reduced body-weight gain and feed intake; stomach corrugation; decreased serum protein	0.26, transient		Rotter et al. (1995)
Pig, 25 kg, f, castrated m	100	7–9	0.5, 1, 2, 4; control: 0.1–0.4	0.02, 0.04, 0.08, 0.16	Diet, contam. oats	Reduced body-weight gain and feed intake	0.16	0.08	Bergsjø et al. (1992)
Pig, f, castrated m, 21 kg, 59 days	95	7–11	0.7, 1.7, 3.5	0.04, 0.1, 0.2	Diet, contam. + 0.75 mg/kg zearalenone	Reduced feed uptake and body-weight gain, increased liver weight, decreased serum albumin	0.1	0.04	Bergsjø et al. (1993a)

Table 3 (contd)

Species, strain, sex, age	Length of study (days)	No. per group	Dose		Route	Effect	LOEL (mg/kg bw per day)	NOEL (mg/kg bw per day)	Reference
			mg/kg of diet	mg/kg bw per day					
Pig, castrated m, 27 kg	56	3	4.7	0.19	Diet, pure	Reduced feed intake (29%), reduced body-weight gain (27%)	0.19 (pure)		Foster et al. (1986)
			2.1–5.2	0.08–0.2	Diet, pure, compared to 16 samples of contam. maize	Greater reduced feed intake and body-weight gain	0.2		
Pig, Yorkshire, 10–13 kg, castrated m	32	6	1, 3	0.08, 0.24 0.09, 0.22	Diet, pure (P) Diet, contam. (N)	Reduced body-weight gain Reduced plasma $\alpha$ -globulin; cortisol	0.09 N 0.24 P	0.08 P	Prelusky et al. (1994)
			4, 9	0.17, 0.27 (75% bio-availability) 0.26, 0.53 Pig, 60 kg Lusky et al.	Diet, pure Intraperitoneal, pure 90	Reduced feed intake and body-weight gain Reduced feed intake and body-weight gain 3–6	0.17  1	  ~ 0.04	
reduced body-weight no clinical effects;			0.04 (1998)						
Pig, 10 kg, f	56	9	0.3, 0.6, 1.2	0.012, 0.024, 0.048	Diet, purified	No reduced body-weight gain		0.048	Götz-Schröm et al. (1998)

**Table 3** (contd)

Species, strain, sex, age	Length of study (days)	No. per group	Dose		Route	Effect	LOEL (mg/kg bw per day)	NOEL (mg/kg bw per day)	Reference
			mg/kg of diet	mg/kg bw per day					
Lambs, m, f, 3–6 months, 18 kg	28	3–4	16	0.94	Naturally contam. wheat	No effect on feed intake, body-weight gain, haematological, serum or histological end-points		0.94	Harvey et al (1986)
Horse, 12.5 years, m, f, 444 kg	40	5	40 mg/kg ration + hay 1.3 kg/day	0.11	Diet, contam. barley	No effect on feed intake, body-weight gain, or serum end-points		0.11	Johnson et al. (1997)
Steer calves, 293 kg	84	18	0.9, 3.7, 6.4, 9.2	0.01, 0.05, 0.07, 0.1	Diet, contam. barley	No effect on feed intake, weight gain, or serum end-points		0.1	Anderson et al. (1996)
Dairy cows, Holstein, early lactation	21	2	0, 2.1, 6.3, 8.5	0.075, 0.22, 0.3	Diet, contam. barley	No effect on feed intake, weight gain, rumenal pH, or milk production		0.3	Ingalls (1996)

m, male; f, female; contam., contaminated

at 0.05 mg/kg or a contaminated wheat diet containing 6.3 mg/kg, equivalent to approximately 0.9 mg/kg bw per day. The mice were killed serially between 7 and 137 days. A 10% decrease in body weight was seen which was related in part to decreased food intake. There were no significant pathological findings and only slight changes in haematological parameters, probably related to body weight. The NOEL was 0.9 mg/kg bw per day (Arnold et al., 1986a).

Groups of four BALB/c mice were fed diets containing deoxynivalenol at a concentration of 2.5, 5, 10, 20, or 50 mg/kg, equal to 0.35, 0.67, 1.3, 2.7, and 6.5 mg/kg bw per day, for 7 days (repeated in three replicate trials). Food intake was decreased at all doses. At 1.3 mg/kg bw, decreased body-weight gain, decreased thymus weight, and decreased cardiac protein synthesis were seen. When feeding was continued until day 30, cardiac lesions (calcified pericarditis foci) were also observed at this dose (Robbana-Barnat et al., 1987).

Groups of 10–12 young female ICR mice were fed diets containing deoxynivalenol at a concentration of 0, 4, 8, 12, or 16 mg/kg, equivalent to 0.6, 1.2, 1.8, and 2.4 mg/kg bw. Reduced feed intake was observed at the three higher doses and reduced growth at all doses (Rotter et al., 1992).

Groups of eight male and eight female outbred ICR mice aged 3 weeks and weighing 16–18 g, housed singly, were fed diets containing deoxynivalenol at a concentration of 0, 2, 4, or 8 mg/kg for 14 days. The authors calculated that the actual intakes were 0, 0.37, 0.76, and 1.5 mg/kgbw per day for the males and 0, 0.41, 0.81, and 1.6 mg/kg bw per day for the females. Food consumption was measured on days 7 and 14 of the experiment, and individual body weights were recorded on days 0, 7, and 14. Feed efficiency was calculated for both weeks of the experiment. Blood samples were collected at the end of the experiment and analysed for erythrocyte count, haemoglobin concentration, erythrocyte volume fraction, mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin concentration. The feed consumption of the deoxynivalenol-fed animals was significantly reduced ( $p < 0.05$ ), especially for the males, during each of the two 7-day test periods. For animals of each sex and during both weeks, the amounts consumed by animals at 2 and 4 mg/kg were similar. The weight gains of all treated males were significantly reduced after 7 days of exposure ( $p < 0.001$ ), but only the males fed 8 mg/kg of diet gained less weight than the controls during week 2. Females at this concentration weighed less than the controls after the first week of exposure but gained more weight than the controls during the second week. The erythrocyte counts of treated animals were significantly lower than in the controls ( $p < 0.05$ ), but the effect was seen primarily at 2 mg/kg of diet. The LOEL was 0.37 mg/kg bw per day and the NOEL was 0.81 mg/kg bw per day (Rotter et al., 1994a).

Groups of 24 Swiss-Webster-derived male weanling mice were given deoxynivalenol at a dose of 0, 0.75, 2.5, or 7.5 mg/kg bw per day by gavage for 35 days. Two control groups were given the solvent or were untreated. Most of the animals given the two higher doses died during the study. At 2.5 mg/kg bw per day, lesions were found in the spleen, thymus, lymph nodes, and gastrointestinal tract. Bone-marrow and haematological parameters were also affected at this dose. Decreased food consumption, decreased body weight, decreased relative weights of the thymus

and heart, and increased relative weight of the stomach were seen at all doses (Arnold et al., 1986a).

Groups of eight weanling female B6C3F<sub>1</sub> mice were fed diets containing deoxynivalenol at a concentration of 0.5, 2, 5, 10, or 25 mg/kg, equivalent to 0.07, 0.28, 0.7, 1.4, and 3.5 mg/kg bw per day for 56 days. Decreased body-weight gain was seen at doses  $\geq$  0.28 mg/kg bw per day. The liver and kidney were affected at doses  $\geq$  0.7 mg/kg bw per day (Forsell et al., 1986).

Groups of 10 weanling female B6C3F<sub>1</sub> mice were fed diets containing 15-acetyldeoxynivalenol at a concentration of 0.5, 2, 5, 10, or 25 mg/kg, equivalent to 0.07, 0.28, 0.7, 1.4, and 3.5 mg/kg bw per day for 56 days. Decreased body-weight gain was seen at doses  $\geq$  0.7 mg/kg bw per day, and the weights of the spleen and kidney were decreased (Pestka et al., 1986).

*Rats:* Groups of 50 male Sprague-Dawley rats were fed either an 'uncontaminated' wheat diet containing deoxynivalenol at 0.05 mg/kg or a contaminated wheat diet containing 6.23 mg/kg, estimated to be equivalent to 0.5 mg/kg bw. Rats were killed serially up to 91 days. A 10% decrease in body weight was observed which was related in part to decreased food intake. There were no significant pathological findings; the slight changes in haematological parameters were probably related to the changes in body weight (Arnold et al., 1986a).

Groups of 25 male and female weanling Sprague-Dawley rats were fed diets containing purified deoxynivalenol to provide a dose of 0.25, 0.5, or 1 mg/kg bw per day for 60 days. Body weight decreases, attributed in part to reduced food intake, were seen at all doses in females and at the highest dose in males (both 5% less than controls, but statistically significant). Thymidine uptake was decreased in the jejunum and spleen of males at the two highest doses, although the effect was significant only at the highest dose. The average decreases in spleen were 2.6% for male controls, 2.8% at 0.25 mg/kg bw per day, 1.8% at 0.5 mg/kg bw per day, and 0.9% at 1.0 mg/kg bw per day; and 19%, 17%, 12%, and 9.2%, respectively, in the jejunum. No notable differences in thymidine labelling were observed in the oesophagus of males or in the spleen, jejunum, or oesophagus of females. No changes were observed in organ weights, haematological or bone-marrow variables, sequential multichannel autoanalyser variables, or histological appearance (Arnold et al., 1986b).

In a 90 day-study, groups of 10 male Sprague Dawley rats weighing 200 g were fed diets containing deoxynivalenol at 20 mg/kg, equivalent to about 1 mg/kg bw per day. At this dose, there was no feed refusal, but a 10% decrease in body-weight gain was observed. Serum enzyme activity, haematological end-points, histopathological appearance, and liver detoxication systems were unaffected (Morrissey et al., 1985).

*Poultry:* Chickens tolerate deoxynivalenol at a concentration of at least 5 mg/kg of diet, equivalent to about 0.45 mg/kg bw per day. At concentrations up to 5 mg/kg feed, in fact, some beneficial effects on food consumption and weight gain were observed in Leghorn chickens (up to 28 days) and broilers (up to 45 days). When

laying Leghorn hens were fed diets containing deoxynivalenol at 0.7 mg/kg for 70 days, no effect was found on feed intake, body weight, egg production, egg yield, or the number of cracked eggs. With increasing doses of deoxynivalenol up to 5.2 mg/kg of feed for 168 days, egg and shell weight and shell thickness decreased. Turkey poults given feed containing deoxynivalenol at 0.5 mg/kg for 14 days showed slightly reduced feed intake and weight gain, which were not statistically significant (Trenholm et al., 1984). The Committee noted that insufficient experimental detail was provided.

Many studies have shown that the performance of chicken broilers and turkey poults is little affected by concentrations of deoxynivalenol up to about 16 mg/kg; some of the more recent studies are summarized in Table 3.

*Mink:* Mink given a choice between uncontaminated feed and deoxynivalenol-contaminated feed displayed a preference for the uncontaminated feed at a concentration of deoxynivalenol as low as 0.28 mg/kg. However, when no choice was available, the mink readily consumed feed containing deoxynivalenol at concentrations up to 1.2 mg/kg (equal to 0.20 mg/kg bw per day) with no apparent ill effects over a 28-day period. The study suggests that the sensitivity of mink is close to that of pigs and greater than that of rats and chickens (Gibson et al., 1993).

*Cats:* Wheat naturally contaminated with deoxynivalenol to a concentration of 37 mg/kg was used to manufacture feed containing the toxin at 0, 1, 2, 4, 6, 8, or 10 mg/kg, equivalent to 0, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/kg bw per day and fed to groups of two to seven mature American shorthair cats, 1–9 years old, for 14 days. The wheat also contained 15-acetyldeoxynivalenol at 1 mg/kg, but none of the other *Fusarium* toxins (detection limits, 0.1–0.5 mg/kg). The concentration of deoxynivalenol in the feed was unchanged after manufacture, indicating that the toxin was stable during conventional extrusion processing. The feed intake of the cats was significantly reduced when it contained deoxynivalenol at a concentration  $> 7.7 \pm 1.1$  mg/kg (equivalent to 0.38 mg/kg bw per day). Vomiting was common at the highest concentration (Hughes et al., 1999).

*Dogs:* The same diets were fed to groups of 2–14 mature male and female beagle or Brittany dogs, 1–7 years old for 14 days, equivalent to doses of deoxynivalenol of 0, 0.075, 0.15, 0.3, 0.45, 0.6, and 0.75 mg/kg bw per day. Dogs previously fed deoxynivalenol-contaminated food preferentially selected uncontaminated food, whereas dogs not previously exposed to deoxynivalenol-contaminated food consumed equal quantities of contaminated and uncontaminated food. The digestibility of the food was not affected when it contained 6 mg/kg. Feed intake was significantly reduced at concentrations of deoxynivalenol  $> 4.5 \pm 1.7$  mg/kg, equivalent to 0.34 mg/kg bw per day. Vomiting was common at the two higher doses (Hughes et al., 1999).

### *Pigs*

#### *Naturally contaminated wheat, maize, and oats*

Maize infected with *Gibberella zeae*, in which about 25% of the kernels were seen to be damaged and containing deoxynivalenol at a concentration of 12 mg/kg, was added to the feed of pigs weighing 20–45 kg. Feed consumption was reduced,

by 20% at a concentration of 3.6 mg/kg to 90% at 40 mg/kg. Weight loss was associated with the feed refusal. Feed was refused more frequently when it contained naturally contaminated maize than when equal concentrations of the pure compound. This indicates that additional factors are involved in the feed refusal response of pigs (Forsyth et al., 1977).

Pigs of two age groups, weighing 8 and 60 kg, were fed rations containing wheat naturally contaminated with deoxynivalenol at a concentration of 1–4.2 mg/kg (equal to 0.036, 0.09, and 0.18 mg/kg bw per day) for 21 days and 42 days, respectively. The younger animals had decreased feed intake at the highest dose, but their weight gain was not affected. In the older animals, feed intake and body-weight gain were affected at 0.09 mg/kg bw. Histological examination revealed no significant lesions or abnormalities in the tissues examined (Pollman et al., 1985).

Groups of six 49-day-old, 14-kg, castrated male pigs were fed a ration to which naturally contaminated wheat had been added, resulting in a deoxynivalenol concentration of 5 (actual, 4.5) mg/kg of feed, equal to 0.2 mg/kg bw per day (using a factor of 0.05 rather than 0.04), for 28 days. Feed intake and growth were not decreased, but mild renal nephrosis was observed in two pigs. An additive or greater interaction was observed when the diets also contained fumonisin B<sub>1</sub> at 100 (actual, 47–56) mg/kg of feed (Harvey et al., 1996).

In young pigs weighing 30–80 kg and pregnant gilts, ingestion of a diet containing deoxynivalenol at concentrations  $\geq 2$  mg/kg of feed, equivalent to  $\geq 0.08$  mg/kg bw per day, resulted in decreased feed consumption and reduced weight gain. Pigs could ingest feed containing deoxynivalenol at up to 2 mg/kg of feed without serious adverse effects (Trenholm et al., 1984). The Committee noted that insufficient experimental details were provided for an assessment of the study.

Four trials were conducted in young pigs to evaluate the effect of deoxynivalenol-contaminated maize on performance. Mouldy maize containing deoxynivalenol at 875 mg/kg and zearalenone at 3.9 mg/kg was mixed with clean maize and other ingredients to provide feeds containing deoxynivalenol at concentrations ranging from 0.14 mg/kg (control) to 230 mg/kg. A dietary concentration of approximately 20 mg/kg caused emesis, 12 mg/kg caused almost complete feed refusal, and 1.3 mg/kg of feed (equivalent to 0.06 mg/kg bw per day) caused a significant reduction in feed intake and rate of weight gain. No lesions attributable to deoxynivalenol were observed in pigs fed up to 43 mg/kg feed for 21 days. Alterations in various serum characteristics were observed in pigs fed deoxynivalenol, but the effects could not be separated from those that result from low intake of food (Young et al., 1983).

Groups of six 84-day-old, 38-kg pigs were fed a ration to which naturally contaminated maize had been added, resulting in a deoxynivalenol concentration of 2.5 mg/kg, equivalent to 0.1 mg/kg bw per day, for 35 days. Decreased feed intake and growth were observed (Friend et al., 1992).

Groups of six to eight castrated male Yorkshire pigs, 6–7 weeks old and weighing 13 kg, were fed diets containing deoxynivalenol at a concentration of 0 (control), 0.95, 1.8, or 2.8 mg/kg for 28 days, equal to 0, 0.08, 0.13, and 0.18 mg/kg bw per

day. The deoxynivalenol in the diet was from naturally contaminated maize, which also contained 15-acetyldeoxynivalenol (at about 25% the concentration of deoxynivalenol) and zearalenone (at about 4%). Feed consumption and body weight were recorded on days 2, 4, 7, 14, 21, and 28 of the study. Blood samples, collected on days 0, 7, and 28, were analysed for thyroxine, tri-iodothyronine uptake, cortisol, and haematological parameters. Serum electrophoresis was conducted on samples collected on day 28. At sacrifice, the weights of the thyroid, thymus, spleen, and kidneys were recorded, and the stomachs were scored for colour, thickness, and inflammation. During the first 2 weeks of the experiment and also overall, food intake was decreased as the dietary deoxynivalenol concentration increased. Intake during the last 7 days was similar in all groups except that receiving 2.8 mg/kg of diet. A dose-related reduction was seen in weight gain in treated pigs during the first 7 days, but the overall daily gain over 28 days was similar in all groups. The absolute and relative weights of the thyroid were significantly lower ( $p < 0.02$  and  $p < 0.05$ , respectively) in pigs given the diets containing 1.8 and 2.8 mg/kg. The thyroxine concentration increased in response to increasing dietary concentrations of deoxynivalenol after 7 and 28 days ( $p < 0.017$ ), and the albumin concentration increased in pigs fed increasing concentrations of deoxynivalenol at 28 days ( $p = 0.013$ ). The  $\alpha$ -globulin concentration showed a dose-related linear decrease ( $p = 0.016$ ), and the albumin:globulin ratio in treated pigs was higher than in controls ( $p = 0.009$ ). The NOEL was 0.08 mg/kg bw per day (Rotter et al., 1994b).

The effects of feeding a diet contaminated with deoxynivalenol at 4 mg/kg on performance and blood parameters were studied for 42 days in groups of eight male castrated Yorkshire pigs weighing 18 kg. On the basis of feed intake, the intake of deoxynivalenol was 0.26 and 0.16 mg/kg bw per day at the beginning and end of the experiment, respectively. Blood samples were collected weekly from all animals. Controls fed *ad libitum* and in pairs with treated animals were used to distinguish between differences in feed intake and effects of the deoxynivalenol-containing diet. Pigs fed the contaminated diet had on average a 20% lower feed intake and 13% lower weight gain than the controls fed *ad libitum*, but these parameters were similar in the pair-fed groups. At necropsy, no differences were found in absolute and relative organ weights, but the fundic region of the stomach of pigs fed the deoxynivalenol diet was more corrugated than that of either of the controls. When compared with both set of controls, the serum protein concentration and  $\beta$ -globulin levels were reduced, although these differences had disappeared by the end of the 6-week experiment (Rotter et al., 1995).

A 100-day feeding trial was conducted to evaluate the effect of including deoxynivalenol-contaminated oats in the feed of groups of seven to nine growing pigs with initial weight of 25 kg to provide concentrations of 0.5, 1, 2, and 4 mg/kg in the complete diets, equivalent to 0.02, 0.04, 0.08, and 0.16 mg/kg bw per day. Performance was recorded as weight gain, feed intake, efficiency of feed use, and carcass quality. Restricted feeding was compared to feeding *ad libitum*. At the highest concentration of deoxynivalenol, feed intake, weight gain, and efficiency of feed use were decreased throughout the experiment. The groups fed diets containing the two highest concentrations of deoxynivalenol showed a dose-related decrease in weight gain during the first 8 weeks on experimental diets. No effects were observed in groups fed diets containing deoxynivalenol at 0.5 or 1 mg/kg. The carcass quality

was not affected at any concentration (Bergsjø et al., 1992). Since the control diet contained deoxynivalenol at 0.1–0.4 mg/kg, changes due to low added concentrations of deoxynivalenol could not be detected.

Oats naturally contaminated with deoxynivalenol were included in feed mixtures at graded levels and given to groups of 7–11 female or castrated male growing pigs (59 days old, 21 kg) for 95 days. The concentrations of deoxynivalenol were 0, 0.7, 1.7, and 3.5 mg/kg of complete feed mixture given *ad libitum*, equal to 0.04, 0.1, and 0.2 mg/kg bw per day. Feed consumption, body-weight gain, weight at slaughter, biochemical and haematological data including serum immunoglobulin (Ig) A, clinical condition, and pathological and histopathological effects *post mortem* were recorded. The group that received the highest dose had significantly decreased body-weight gain throughout the experiment, decreased weight at slaughter, and reduced feed use efficiency. At the same concentration, the weight of the liver was increased, the concentrations of serum protein and albumin were decreased, and packed blood cell volume and serum calcium and phosphorus concentrations fell transiently. At the two higher doses, a statistically significant, dose-related decrease in daily feed consumption was observed. No other effects on haematological, biochemical, or immunological parameters were observed. Carcass quality was not affected in any group. The authors concluded that significant effects in growing pigs can be observed at a dietary deoxynivalenol concentration of 1.7 mg/kg, originating from naturally contaminated oats included in a diet that was otherwise adequate and contained only minor traces of other mycotoxins (zearalenone, 0.75 mg/kg of feed) (Bergsjø et al., 1993a).

#### *Deoxynivalenol from naturally contaminated maize or purified*

Groups of three castrated male Yorkshire pigs weighing 28 kg were given diets containing pure deoxynivalenol at a concentration of 4.7 mg/kg, maize inoculated with various strains of *F. graminearum* providing deoxynivalenol at 2.1–5.2 mg/kg of diet, uncontaminated maize, or naturally contaminated wheat *ad libitum* for 56 days. The feed intake of pigs receiving the diet containing pure deoxynivalenol at 0.19 mg/kg was reduced by 29% after 1 week and 18% after 7 weeks, and their weight gain was reduced by 27% and 20%, respectively, although the differences were not significant. The reductions in feed consumption and weight gain were generally greater in pigs given the diets inoculated with maize, reaching 40% and 37% after 7 weeks on a diet containing deoxynivalenol at 5.2 mg/kg (0.2 mg/kg bw per day). The difference was attributed to factors such as other fungal metabolites and differences in storage of the maize. No emesis occurred (Foster et al., 1986).

The toxic effects of deoxynivalenol were examined in castrated male Yorkshire pigs weighing 10–13 kg given feed into which deoxynivalenol was incorporated at a concentration of 0, 1, or 3 mg/kg, either as the purified toxin or as naturally contaminated maize, for 32 days. The estimated intakes of deoxynivalenol were 0.08 and 0.24 mg/kg bw per day of purified toxin and 0.09 and 0.22 mg/kg bw from the naturally contaminated feed. The diet also contained 7% 15-acetyldeoxynivalenol and 3% nivalenol. Growth performance and blood biochemical and haematological parameters were monitored throughout the study. At the higher concentrations, significantly reduced feed consumption and body-weight gain were evident soon after the start of feeding. While the weight gain of pigs fed the diet containing purified

deoxynivalenol recovered after several days, the values for pigs fed the naturally contaminated diet remained depressed throughout the study. These observations might reflect the presence of other, unidentified toxic compounds in the naturally contaminated grain. Generally, the blood chemical parameters of pigs fed the contaminated diets were not different from those of controls, with the exception of reduced serum concentrations of  $\alpha$ -globulin (significant at the highest concentration of either pure deoxynivalenol or naturally contaminated maize at day 32) and possibly increased cortisol concentrations in animals receiving the highest concentration in either diet. The effect of deoxynivalenol on the  $\alpha$ -globulin fraction might have been independent of the feed refusal syndrome associated with this toxin. Alterations in several haematological end-points, including a higher erythrocyte count, erythrocyte volume fraction, and platelet count, occurred sporadically at 3 mg/kg of either diet; however, these effects could not be separated from the influence of decreased feed intake and were of limited value in diagnosing the effects of dietary deoxynivalenol in pigs (Prelusky et al., 1994).

#### *Purified deoxynivalenol*

Groups of three to six pigs weighing 60 kg were used to study the health effects of purified deoxynivalenol and ochratoxin A in their feed, singly or in combination, and the presence of residues 90 days after intake. The pigs received diets containing ochratoxin A at 0.1 mg/kg with deoxynivalenol at 1 mg/kg, equivalent to 0.004 mg of ochratoxin A and 0.04 mg of deoxynivalenol per kg bw, respectively; ochratoxin A alone at 0.1 mg/kg; or deoxynivalenol alone at 1 mg/kg. Two controls received feed containing neither ochratoxin A nor deoxynivalenol. The pigs that received mycotoxins in their feed did not show clinical or haematological changes. The pigs that received both mycotoxins had hyperaemia in the gastric mucosa, and changes in the tubular epithelium were observed in one animal in each treated group. Few pathological lesions were found, but the Committee noted that there were few animals in the study. The observed antibody titres against pseudorabies (Aujeszky disease or 'mad itch'), as a measure of effects on the immune system, suggest that non-specific defence mechanisms were not affected. The mean concentration of ochratoxin A in the kidneys of animals treated with both toxins was about 50% higher than that in the group given ochratoxin A alone, indicating a possible interaction. The concentration of ochratoxin A also appeared to be slightly increased in muscle of animals receiving both mycotoxins (Lusky et al., 1998).

Semi-synthetic potato-based, grain-free diets containing deoxynivalenol purified from inoculated rice cultures at a concentration of 0, 0.3, 0.6, or 1.2 mg/kg were fed with restriction to groups of nine sows weighing 10 kg over a period of 8 weeks, equivalent to 0, 0.012, 0.024, and 0.048 mg/kg bw per day. Body weight, biochemical and haematological end-points including serum IgA and insulin-like-growth factor-I, crude protein content in faeces, general condition, and pathological and histological findings *post mortem* were recorded. No significant effect was seen on weight gain, and the maximum body weight in all groups was about 30 kg. These results correspond to the results of clinical chemical and histological investigations. Serum IgA levels were increased by about 30% at the two higher doses, but these changes were not statistically significant (Götz-Schröm et al., 1998). Additional details were provided by M. Lauber (University of Hohenheim), who noted that the water content of the diet was high and may have affected feed consumption and weight gain.

The same group conducted three further unpublished trials. In the first, groups of five castrated male pigs weighing 10 kg were fed, with restriction, semi-synthetic potato-based, grain-free diets containing deoxynivalenol purified from inoculated rice cultures at a concentration of 0 or 4 mg/kg or wheat-based diets consisting of clean wheat or wheat inoculated with *Fusarium culmorum*, for 4 weeks, equivalent to an intake of deoxynivalenol of 0 or 0.16 mg/kg bw per day. The inoculated wheat also contained 5.8–12% 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and small quantities of nivalenol. The second and third trials were similar to the first, except that the diets were fed *ad libitum* and the dietary concentrations of deoxynivalenol were 0, 4, or 6 mg/kg diet, equivalent to 0, 0.16, and 0.24 mg/kg bw per day. The results were presented in graphical form. In the first trial, the maximum weight at the end of 4 weeks was about 28 kg in all groups; in the second trial, a dose-related decrease in body-weight gain was seen at each week, with weights of 35 kg for controls, 30 kg at 4 mg/kg, and 26 kg at 6 mg/kg at the end of 4 weeks of dosing. In the third trial, the maximum weight at the end of 4 weeks was about 30 kg in all groups. De-epoxidation of deoxynivalenol to the de-epoxy metabolite in faeces was more extensive with the wheat-based than the potato-based diets (information provided by M. Lauber, University of Hohenheim). The Committee noted that data on food intake were not provided, and only limited statistical analysis was done. Animals gained less weight on the potato-based diet than on the wheat-based diet, even when fed *ad libitum*.

**Ruminants:** The feed consumption of 10 non-lactating dairy cows decreased slightly when a wheat–oats diet containing deoxynivalenol at 6 mg/kg was fed at a rate of 1 mg/kg bw per day, with hay offered *ad libitum*. In surveys of Canadian grains carried out during the early 1980s, the deoxynivalenol content (maximum, 8.5 mg/kg) in eastern Canadian wheat was probably not high enough to account for reports of feed refusal, vomiting, and reproductive problems in livestock operations. This conclusion is based partly on the fact that formulated diets contain a maximum of 70–80% wheat. Consequently, the actual deoxynivalenol content of diets fed to farm animals would be much lower (Trenholm et al., 1984). The Committee noted that insufficient experimental details were provided.

Ruminants tend to be less susceptible to the effects of deoxynivalenol in feed concentrate, which is usually provided in addition to hay. Recent studies are summarized in Table 3.

**Equidae:** The results of one study are shown in Table 3.

**Primates:** In a limited study, changes in haemostasis were seen after single oral administration of deoxynivalenol at 1, 5, 10, 25, or 50 mg/kg bw or oral administration of deoxynivalenol at 1 or 5 mg/kg bw per day for 2 weeks to groups of one or two *Macaca rhesus* monkeys, with normalization of blood coagulation parameters within 45–60 days (Fomenko et al., 1991).

Groups of two male and two female infant cynomolgus monkeys were given pure deoxynivalenol at a dose of 0, 1, 2, or 5 mg/kg bw per day in milk by gavage for 200 days. Two males at the highest dose died during the first week of the study. In these animals, the histological effects, including atrophy of the thymus and spleen,

were similar to those seen in rodents given deoxynivalenol, and the relative weight of the thymus was reduced. No significant pathological findings were observed in any other monkeys (F. Iverson, Health Canada, personal communication, 1986).

### 2.2.3 Long-term studies of toxicity and carcinogenicity

*Mice:* In a 2-year study, groups of 50 male and 50 female B6C3F<sub>1</sub> mice were given diets containing deoxynivalenol (purity, > 95%; no 3-acetyl- or 15-acetyldeoxynivalenol) at a concentration of 0, 1, 5, or 10 mg/kg, equal to 0, 0.1, 0.5, and 1.1 mg/kg bw per day in males and 0, 0.1, 0.7, and 1.6 mg/kg bw per day in females. Survival was not significantly affected. Average daily food consumption was unchanged in females, but that of males was significantly reduced by about 8% at the two higher doses. The graphical presentation of body-weight changes indicated that the decreases in body weight (and in body-weight gain) at 500 days were 8.7% (13%) at 1 mg/kg, 21% (32%) at 5 mg/kg, and 38% (56%) at 10 mg/kg of diet in females and 1% (1.6%), 6.8% (11%), and 21% (33%) for males, respectively. Females, showed a 56% increase in serum IgA and a < 10 % increase in IgG at 5 and 10 mg/kg of feed, and there were sporadic changes in haematological and clinical chemical end-points; however, these changes were considered not to be biologically relevant. The relative weight of the liver was decreased in males at 5 and 10 mg/kg; at 10 mg/kg, the relative weight of the spleen was decreased and the relative weight of the testis significantly increased. No increase in the incidence of preneoplastic or neoplastic changes was observed. In fact, there was a statistically significant, dose-related decrease in the incidences of preneoplastic and neoplastic lesions in the liver and in that of non-neoplastic lesions affecting large islets of the pancreas. In the liver, this negative trend probably resulted from the known positive correlation between body weight and the appearance of spontaneous hepatic neoplasms in this strain of mouse. The NOEL was 1 mg/kg of diet, equal to 0.1 mg/kg bw per day (Iverson et al., 1995).

Deoxynivalenol was tested for its potential to initiate or promote skin tumours in a two-stage regimen in female Sencar mice. Initiation was tested by applying a single topical dose of 200 µg followed by multiple treatments with the promoter 12-O-tetradecanoylphorbol 13-acetate. The test for promotion involved initiation with the carcinogen 7,12-dimethylbenz[*a*]anthracene followed by multiple treatments with 50 µg of deoxynivalenol. Appropriate control groups were included. The mice were observed for 26 weeks, and skin tumours were counted. Deoxynivalenol was neither an initiator nor a promoter. When it was tested as an initiator, no statistically significant difference was found in the cumulative number of tumours or the number of tumour-bearing mice. When it was administered as a promoter, no tumours were observed. Histopathological examination of the skin showed that deoxynivalenol induced mild diffuse squamous hyperplasia, but there was no progression of the lesion to neoplasia (Lambert et al., 1995).

### 2.2.4 Genotoxicity

The results of studies for genotoxicity summarized in Table 4 indicate that deoxynivalenol did not cause gene mutation *in vitro* but that it caused chromosomal aberration *in vitro* and *in vivo*.

**Table 4. Results of assays for the genotoxicity of deoxynivalenol**

End-point	Test object	Concentration	Results	Reference
<i>in vitro</i>				
Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537 <sup>a</sup>	0.4–400 mg/plate	Negative	Wehner et al. (1978); Kuczuk et al. (1978)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100 <sup>a</sup>	0.7–500 mg/plate	Negative	Knasmuller et al. (1997)
Chromosomal aberration	<i>E. coli</i> PQ37 <sup>a</sup>	5–500 mg/assay	Negative	Knasmuller et al. (1997)
Gene mutation	Chinese hamster lung V79 cells at <i>Hprt</i> locus <sup>b</sup>	1–3 mg/mL <sup>c</sup>	Negative	Rogers & Heroux-Metcalf (1983)
Unscheduled DNA synthesis	Rat primary hepatocytes	0.1–1000 mg/mL	Negative	Bradlaw et al. (1985)
DNA repair	<i>E. coli</i> K12 (2 strains)	0.7–500 mg/mL	Negative	Knasmuller et al. (1997)
Chromosomal aberration <sup>d</sup>	Chinese hamster V79 cells	≤ 1 mg/mL	Positive (7-fold)	Hsia et al. (1988)
Chromosomal aberration Micronucleus formation	Rat primary hepatocytes	0.001–100 mg/ml Max at 1 mg/ml	Positive (6-fold) Negative	Knasmuller et al. (1997)
Gap-junctional communication	Chinese hamster V79 cells	0.1–0.5 mg/mL	Inhibited	Jone et al. (1987)
Cell transformation	BALB/c3T3 cells	0.1–1.6 mg/mL Min at 0.2 mg/mL at cell passage 9	Positive	Sheu et al. (1988)
<i>In vivo</i>				
Chromosomal aberration	Mouse bone-marrow cells	3 mg/kg bw 2 x/ week by gavage for 8 weeks 0.06 mg/kg bw per day in diet	Positive (3-fold <sup>e</sup> ) Negative	Bilgrami et al. (1993)

<sup>a</sup> With and without activation with S9

<sup>b</sup> With and without activation with hepatocytes

<sup>c</sup> Reduced colony size at 1 mg/mL; 90% cell lethality at 10 mg/mL

<sup>d</sup> Mainly chromatid breaks

<sup>e</sup> Dubious, since gaps were included in the analysis

### 2.2.5 Reproductive toxicity

#### (a) Effects on reproductive organs

*Mice:* The potential of deoxynivalenol to affect testicular morphology and testicular and epididymal sperm counts was assessed in three strains of mice: IL-6KO [B6129-IL6 (tmlKopf) (IL-6 gene-deficient)], WT [B6129F2 (wild-type to B6129-IL6 with an intact IL-6 gene)], and B6C3F<sub>1</sub> mice. The treated mice received deoxynivalenol at a concentration of 10 mg/kg in their diet (equivalent to 1.5 mg/kg bw per day) for 90 days. The body weight of treated animals was significantly lower than that of controls. Slight, not statistically significant changes were observed in relative testis weight and testicular spermatid counts, but no histological changes were seen. The diameter of the seminiferous tubules, the height of the seminiferous epithelium, and the number of Sertoli cell nucleoli per cross-sectioned seminiferous tubule in the treated groups were not significantly different from those of their respective untreated controls. IL-6KO and B6C3 F<sub>1</sub> mice had significantly lower caudal epididymal weights than controls. These changes were not due to decreased sperm counts, and the finding suggests that deoxynivalenol may have an adverse affect on the epididymides (Sprando et al., 1999).

*Pigs:* Diets containing uncontaminated wheat, wheat contaminated with deoxynivalenol at 3.7 mg/kg, or maize inoculated with *Fusarium* at 4.2 mg/kg was fed to groups of 12–18 23-kg male and female Yorkshire pigs (equivalent to 0, 0.14, and 0.17 mg/kg bw per day) for 7 weeks. The diets caused a 23–29% reduction in feed consumption. The weight gain of animals on the diets containing contaminated wheat or maize was 30% and 72% less than that of controls, respectively, suggesting that there were additional metabolites in the maize diet. Histological examination of the testis (seminiferous epithelium) and ovary (follicle) revealed no significant differences in sexual development attributable to the diet (Friend et al., 1986a).

#### (b) Multigeneration studies

The results of these studies are summarized in Table 5.

*Mice:* Groups of 15 weanling mice of each sex (F<sub>0</sub>) were fed diets containing deoxynivalenol at concentrations that resulted in a dose of 0 or 2 mg/kg bw per day, and groups of seven male and 10–20 female mice received diets providing a dose of 0, 0.38, 0.75, or 1.5 mg/kg bw per day. The diets were fed continuously to the F<sub>0</sub> parents and their progeny for the duration of the two experiments. After 30 days, the mice were allowed to mate within their experimental groups for a maximum of three 5-day trials. Females found to have mated successfully were allowed to litter normally. The F<sub>1a</sub> progeny of 10 dams in the control group and that receiving deoxynivalenol at 1.5 mg/kg bw per day were cross-fostered at birth, whereas the remaining F<sub>1a</sub> progeny were reared by their natural dams. The offspring were examined up to 21 days of age and were then discarded. The F<sub>0</sub> mice were re-bred to produce F<sub>1b</sub> litters, which were killed on day 19 of gestation, and the fetuses were examined for gross, visceral, and skeletal malformations. The feed and water intakes and body weights of male and female F<sub>0</sub> mice were reduced, as were the numbers of live pups and postnatal survivors, the postnatal body weight of F<sub>1a</sub> progeny, the number

**Table 5. Reproductive and developmental effects of deoxynivalenol (purified, unless otherwise specified)**

Species, strain, sex, age	Study	No. per group	Dose		Route	Effect	LOEL (mg/kg bw per day)	NOEL (mg/kg bw per day)	Reference
			mg/kg of diet	mg/kg bw per day					
Mouse, Swiss Webster, weanling	Reproductive toxicity, 1 generation, <i>in utero</i> ; 2 litters	7-20		0.38, 0.75, 1.5, 2	Diet	Maternal toxicity and/or embryotoxicity	0.38		Khera et al. (1984)
Rat, Sprague Dawley, 30 days	Reproductive toxicity, 1 generation, <i>in utero</i> ; 65 days	15 m, 15 f		0.25, 0.5, 1	Diet	Maternal toxicity and/or embryotoxicity		1	Khera et al. (1984)
Rat, Sprague Dawley, 165 g	Reproductive toxicity	10 m, 25 f	20	2	Diet	Reduced fertility	2		Morrissey & Vesonder (1985)
Mouse, Swiss Webster, 30 g	Developmental toxicity, days 8-11	15-19		0.5, 1, 2.5, 5, 10, 15	Gavage	Teratogenic effects, increased resorptions; skeletal abnormalities	5 1	2.5 0.5	Khera et al. (1982)
Rat, Fischer 344	Developmental toxicity, days 1-21	23 f	0.5, 2, 5	0.025, 0.1, 0.25	Diet	No teratogenic or reproductive effects; decreased dam weight	0.1	0.025	Morrissey (1984)
Rat	Developmental toxicity, days 7-15			0.2, 1, 5, 10	Gavage	Fetotoxic effects; delayed ossification	1	0.2	Tutel'ian et al. (1991)

Table 5 (contd)

Species, strain, sex, age	Study	No. per group	Dose		Route	Effect	LOEL (mg/kg bw per day)	NOEL (mg/kg bw per day)	Reference
			mg/kg of diet	mg/kg bw per day					
Rabbit	Developmental toxicity, days 0-30	13-15	0, 7.5, 15, 30, 60, 120, 240	0.3, 0.6, 1, 1.6, 1.8, 2	Diet	Increased fetal resorption; reduced maternal and fetal weights	1	0.6	Khera et al. (1986)
White Leghorn laying hens, 20-23 weeks old	Developmental toxicity, 70 days	12	0.12, 2.5, 3.1, 4.9 + 12% 3-acetyl-deoxynivalenol	0.006, 0.12, 0.15, 0.25	Diet, naturally contaminated oats	No effect on food intake, weight gain, egg production, fertility, hatchability, perinatal mortality, chick viability, body weight; developmental anomalies: delayed ossification, cloacal atresia, cardiac anomalies	0.12		Bergsjø et al. (1993b)
Pig, Yorkshire, 178 days old	Developmental toxicity, days 1-54 of gestation	8-10	0.13, 1.7, 3.5	0.003, 0.04, 0.07	Diet, naturally contaminated	Maternal toxicity; decreased fetal weight; no gross malformations	0.07	0.04	Friend et al. (1983)
Pig, Yorkshire, 90 kg	Developmental toxicity, throughout gestation and lactation	6-10	0.2, 3.8, 6.2	0.003, 0.06, 0.09	Diet, naturally contaminated, restricted feed intake	No maternal toxicity, no fetal effects or effects on piglets except transiently decreased kidney weights		0.09	Friend et al. (1986b)

of live fetuses, and the mean weight of F<sub>1b</sub> fetuses. No adverse effects were found on the fertility of male and female F<sub>0</sub> mice, and there were no major malformations in the F<sub>1b</sub> generation. Cross-fostering of the offspring of control dams and those at 1.5 mg/kg bw per day adversely affected postnatal survival and body weight after prenatal exposure or after combined pre- and postnatal exposure (Khera et al., 1984).

*Rats:* Groups of 15 male and 15 female Sprague-Dawley rats, 30 days of age, were fed diets containing deoxynivalenol (purity, 99%) to deliver a dose of 0, 0.25, 0.5, or 1 mg/kg bw per day. After 6 weeks of feeding, the rats were bred within groups, and the males were then discarded. The mated females were maintained on their respective diets throughout gestation and were killed on the last day of gestation; the fetuses were evaluated for effects on prenatal development. No reproductive effects were noted. Except for dilatation of the renal pelvis and urinary bladder, the significance of which was unclear, no other adverse effects were observed in the pups. Decreased body weight, related to decreased food consumption, was observed in dams at the two higher doses (Khera et al., 1984).

Groups of 10 male and 25 female Sprague-Dawley rats weighing 165 g were fed a diet containing purified deoxynivalenol at a concentration of 20 mg/kg (equal to about 2 mg/kg bw) for 60 and 15 days, respectively, before mating. Rats that ate the deoxynivalenol-supplemented diet throughout gestation and lactation showed no clinical signs of toxicity but had lower body weights than pair-fed rats. Only 50% of the matings between treated rats resulted in pregnancy, compared with 80% in controls fed *ad libitum* or pair-fed. No differences were seen in the sex ratio, survival rate, or average number and weight of litters. The weight gains of pups were comparable in all groups up to postnatal day 14, but between days 14 and 21, control male and female pups had significantly better weight gain than pups of treated dams. No treatment-related histological abnormalities were found in the testes or ovaries of treated pups (Morrissey & Vesonder, 1985).

*Pigs:* From the time of breeding at an average age of 178 days and a body weight of 121 kg, three groups of 12 Yorkshire gilts were offered *ad libitum* one of three diets containing 70% wheat and deoxynivalenol at a concentration of 0.1, 1.7, or 3.5 mg/kg from naturally contaminated wheat, equal to 0.002, 0.04, and 0.07 mg/kg bw per day. The gilts were housed and fed individually and were slaughtered on day 50–54 of gestation. The reproductive tract, oesophagus, stomach, large intestine, liver, heart, kidney, bladder, and adrenals were examined. The growth rate of gilts given the highest concentration of deoxynivalenol was significantly less ( $p < 0.01$ ) than that of other gilts, probably as a result of reduced feed intake. The differences in organ weights were not significant. The fetal mortality rate, although lowest for gilts fed the highest concentration of deoxynivalenol, was not significantly different among the groups. There were significant linear trends towards lower fetal weight, decreased fetal length, and reduced osmolality of allantoic fluid with increasing concentration of deoxynivalenol which could not be attributed to a direct physiological or toxicological effect of deoxynivalenol. There was no increase in the frequency of fetal resorptions, and no gross malformations were observed, but the fetuses were not examined microscopically (Friend et al., 1983).

Groups of 6–10 Yorkshire gilts weighing 91 kg were fed restricted quantities (2 kg/day) of wheat diets containing deoxynivalenol at a concentration of 0.2, 3.8, or 6.2 mg/kg from naturally contaminated wheat, equal to 0.003, 0.06, and 0.09 mg/kg bw per day, respectively until farrowing, and unrestricted quantities of the same diets thereafter until weaning (total, 114 days). No effects on maternal weight gain or on the number and size of piglets at weaning or at time of market were observed (Friend et al., 1986b).

(c) *Developmental toxicity*

*Mice:* Deoxynivalenol dissolved in distilled water was given by oesophageal intubation to groups of 15–19 pregnant Swiss Webster mice on days 8–11 of gestation. The incidence of resorptions was 100% at 10 or 15 mg/kg bw per day and 80% at 5 mg/kg bw per day. At the lowest dose, the number of live fetuses and the average fetal weight were below those in controls. Visceral anomalies considered to be teratogenic effects were observed mainly in this group and included exencephaly (26%), syndactyly (19%), and hypoplastic cerebellum (93%). Low incidences of skeletal and visceral anomalies were found in the fetuses of dams at 1, 2.5, and 5 mg/kg bw per day. The skeletal malformations occurred in a dose-related manner and included lumbar vertebrae with fused arches or partly absent centra and absent or fused ribs attributed to retarded ossification (biologically not significant). There was no apparent maternal toxicity. No teratogenic or embryotoxic effects were seen in mice at 0.5 mg/kg bw per day (Khera et al., 1982).

*Rats:* The teratogenic potential of purified deoxynivalenol was studied by feeding a certified rat feed to which deoxynivalenol was added at a concentration of 0.0, 0.5, 2.0, or 5.0 mg/kg, equivalent to 0.025, 0.1, and 0.25 mg/kg bw per day *ad libitum* to groups of 23 female Fischer 344 rats throughout gestation. There were no overt signs of toxicity in the dams and no statistically significant differences in feed consumption in comparison with the control group. The dams receiving the two higher concentrations of deoxynivalenol tended to weigh less at term than other females, and their carcass weights were significantly lower (by 5%) than those of the control group after removal of the pups and uterus. The weights of the pups were unaffected by maternal treatment. Deoxynivalenol had no statistically significant adverse effects on the incidence of gross, skeletal, or visceral abnormalities, and neither dams nor pups showed any significant histopathological changes (Morrissey, 1984).

Rats were given an aqueous solution of deoxynivalenol by stomach tube, providing a dose of 0.2, 1, 5, or 10 mg/kg bw per day, on days 7–15 of gestation. On the basis of fetotoxic effects (skeletal abnormalities such as delayed ossification), the NOEL was 0.2 mg/kg bw per day (Tutel'ian et al., 1991).

*Rabbits:* Groups of 13–15 adult female New Zealand white rabbits were fed diets containing deoxynivalenol (purity, > 98%) at a concentration of 0, 7.5, 15, 30, 60, 120, or 240 mg/kg throughout gestation, equal to 0, 0.3, 0.6, 1, 1.6, 1.8, and 2 mg/kg bw per day. An additional pair-fed control group was added for the group given 1.6 mg/kg bw per day. The incidence of fetal resorption was 100% in the females fed 1.8 or 2 mg/kg bw per day; although an increased incidence of resorption

was also observed at 1 and 1.6 mg/kg bw per day, it was similar to that in the pair-fed controls. Maternal weight was decreased in the pair-fed controls and in rabbits receiving 1 or 1.6 mg/kg bw per day, with associated reductions in mean fetal weight of 7%, 7%, and 28%, respectively. No teratogenic effects were observed (Khera et al., 1986).

### 2.2.6 Special studies

#### (a) Immunotoxicity

##### (i) Altered host resistance and humoral and cell-mediated responses

*Mice:* The studies in mice summarized in this section are shown in Table 6.

Groups of 12 male weanling Swiss Webster mice were given deoxynivalenol by gavage at a dose of 0.75 or 2.5 mg/kg bw per day for 5 weeks. The antibody response to sheep red blood cells was suppressed, and the lower dose decreased the weights of the spleen and thymus (Tryphonas et al., 1984).

In a follow-up study by the same group, immune function was studied in groups of 6–10 male weanling Swiss Webster mice fed purified deoxynivalenol at 0, 0.25, 0.5, or 1 mg/kg bw per day for 5 weeks. Spleen plaque-forming cells and serum antibody responses to sheep red blood cells were unaffected at any dose. At the two higher doses, deoxynivalenol induced a dose-related reduction in the time to death after a challenge with *Listeria monocytogenes* and increased proliferative capacity in splenic lymphocytes stimulated with phytohaemagglutinin. No effects were observed at 0.25 mg/kg bw. The authors estimated that the NOEL for immunotoxicity in mice was 0.25–0.5 mg/kg bw per day (Tryphonas et al., 1986).

After 2–3 weeks on a diet containing deoxynivalenol at 25 mg/kg, equal to 5 mg/kg bw per day, groups of five female B6C3F<sub>1</sub> mice showed depressed plaque-forming cell response to sheep red blood cells, delayed hypersensitivity response to keyhole limpet haemocyanin, and reduced ability to clear *L. monocytogenes* in comparison with pair-fed controls, whereas a diet containing 5 mg/kg, equal to 1 mg/kg bw per day, had no effect on these parameters. The effects on resistance to *Listeria* and delayed hypersensitivity seen after 2–3 weeks disappeared when feeding was extended to 8 weeks; however, the effects on the plaque-forming cell response were detected after both 2 and 8 weeks of ingestion of the mycotoxin. The NOEL for deoxynivalenol was 1.0 mg/kg bw per day (Pestka et al., 1987b).

Groups of eight female B6C3F<sub>1</sub> mice were fed AIN 76A semi-purified diets containing purified deoxynivalenol at 0, 0.5, 2, 10, or 25 mg/kg (equal to 0, 0.1, 0.4, 1, 2, and 5 mg/kg bw per day) for 6 weeks. Leukocyte counts were depressed at doses  $\geq 10$  mg/kg of diet. The NOEL for this parameter was 1 mg/kg per day (Forsell et al., 1986).

Groups of 4–17 male BALB/c mice, 4–6 weeks old, were fed diets containing deoxynivalenol at a concentration of 0, 2.5, 5, 10, 20, or 50 mg/kg, equivalent to 0, 0.37, 0.75, 1.5, 3, and 7.5 mg/kg bw per day, for 1 or 2 weeks. Control animals were pair-fed at the highest dose. At concentrations  $\geq 10$  mg/kg of diet, reductions were

**Table 6. Studies on the immunotoxicity of purified deoxynivalenol in mice**

Length of study	Route	Effect	LOEL		NOEL		Reference
			mg/kg bw per day	mg/kg of diet	mg/kg bw per day	mg/kg of diet	
5 weeks	Gavage	Antibody response	0.75		ND		Tryphonas et al. (1984)
5 weeks	Diet	Host resistance, lymphocyte proliferation	0.5		0.25		Tryphonas et al. (1986)
2-4 weeks	Diet	Host resistance, delayed-type hypersensitivity, antibody response	5 <sup>a</sup>	25	1 <sup>a</sup>	5	Pestka et al. (1987b)
1-2 weeks	Diet	Antibody response, lymphocyte proliferation	1.5 <sup>a</sup>	10	0.75 <sup>a</sup>	5	Robbana-Barnat et al. (1988)
4 weeks	Drinking-water	Host resistance	0.12		0.024		Sugita-Konishi et al. (1998)
1 week	Gavage	Host resistance	6.25		ND		Atroschi et al. (1994)
6 weeks	Diet	Serum immunoglobulin A	0.4 <sup>a</sup>	2	0.1 <sup>a</sup>	0.5	Forsell et al. (1986)
4-12 weeks	Diet	Serum immunoglobulin A	2 <sup>a</sup>	10	0.4 <sup>a</sup>	2	Greene et al. (1994)
12 weeks	Diet	Kidney immunoglobulin A deposition, immunoglobulin A-associated nephropathy	0.4 <sup>a</sup>	2	ND		Greene et al. (1994)
2-7 days	Gavage	Cytokine expression	2		0.5		Zhou et al. (1998)

<sup>a</sup> Calculated from feed intake and body weight

seen in the response to sheep red blood cells, splenic leukocyte responses to phytohaemagglutinin and lipopolysaccharide, and thymic responses to phytohaemagglutinin; the weight of the thymus was reduced, with extensive atrophy. The NOEL was 5 mg/kg of diet, equivalent to 0.75 mg/kg bw per day (Robbana-Barnat et al., 1988).

Groups of 10 male BALB/c mice, 7 weeks of age, were given deoxynivalenol in their drinking-water at a concentration of 0, 0.2, 1, or 3 mg/L for 4 weeks, equivalent to intakes of 0, 0.024, 0.12, and 0.36 mg/kg bw per day, and resistance to *Salmonella enteritidis* was evaluated on day 14. These concentrations of deoxynivalenol did not cause refusal of water or feed. Deaths due to *S. enteritidis* infection were observed at 1 and 3 mg/L but not at 0.2 mg/L. In mice given drinking-water containing deoxynivalenol at 2 mg/L, both IgM antibody ( $p < 0.005$ ) (humoral) and delayed-type hypersensitivity ( $p < 0.05$ ) (cell-mediated) responses to *S. enteritidis* were significantly suppressed. The authors reported a LOEL, based on water intake, of 0.12 mg/kg bw per day (Sugita-Konishi et al., 1998). The Committee noted that the data on *S. enteritidis* infectivity were presented in a descriptive fashion without statistical analysis.

Groups of five lactating, inbred Han:NMR1 mice were given deoxynivalenol at 12.5 mg/kg bw for 1 day or at 6.25 mg/kg bw for 7 consecutive days by gavage, and their resistance to the mastitic pathogens *Staphylococcus hyicus* and *Mycobacterium avium* was examined. No suppression of the immune response was observed. Rather, both treatments enhanced resistance to *S. hyicus* but not to *M. avium*. In mice infected with *S. hyicus*, administration of deoxynivalenol for 1 day increased total serum IgA, whereas administration for 7 days increased IgA, IgM, and IgG (Atroshi et al., 1994). Enhanced host resistance is seen frequently when trichothecenes are administered just before challenge with a model pathogen (Bondy & Pestka, 2000).

*Chickens:* Groups of 10 female white Leghorn chicks, 1 day old, were fed diets containing uncontaminated wheat or naturally contaminated wheat containing deoxynivalenol at a concentration of 18 mg/kg, equivalent to 2.25 mg/kg bw per day, for 18 weeks. The contaminated diet resulted in a suppressed antibody response to Newcastle disease vaccine given at week 14. When groups of three 1-day-old broilers were fed a diet containing 50 mg/kg, equivalent to 6.25 mg/kg bw per day, a suppressed lymphocyte blastogenesis response was seen (Harvey et al., 1991).

*Pigs:* Groups of six to eight castrated male Yorkshire pigs (6–7 weeks old, weighing 13 kg) were given diets amended with maize naturally contaminated with deoxynivalenol at a concentration of 0, 0.95, 1.8, or 2.8 mg/kg of feed, equal to 0.08, 0.13, and 0.18 mg/kg bw per day for 28 days. The diet also contained 15-acetyldeoxynivalenol at about 25% the concentration of deoxynivalenol and zearalenone at about 4%. Antibody responses to sheep red blood cells were delayed in animals exposed to the two highest concentrations; the results for pair-fed controls indicated that this was not solely a nutritional effect. The treatments had no effect on peripheral blood mononuclear cell proliferative responses to the mitogens concanavalin A, phytohaemagglutinin, and pokeweed mitogen. At the end of the experiment, the total leukocyte count was found to be increased with increasing deoxynivalenol concentration, apparently due to increases in segmented and band

neutrophil counts. No alterations were seen in monocyte and eosinophil counts. The NOEL for the most sensitive immune parameter was 0.08 mg/kg bw per day (Rotter et al., 1994b). It should be noted that castration might alter the sensitivity of pigs, as was seen in mice (Greene et al., 1994).

The effects on the immune response of diets containing naturally contaminated oats containing deoxynivalenol at a concentration of 0.6 (control), 1.8, or 4.7 mg/kg of feed (equivalent to 0.024, 0.072, and 0.2 mg/kg bw) for 9 weeks were investigated in groups of eight male and female growing Norwegian Landrace pigs. The immune response was evaluated on the basis of primary and secondary antibody titres after injection of five antigens: human serum albumin, sheep red blood cells, paratuberculosis vaccine, tetanus toxoid, and diphtheria toxoid. Tests for delayed hypersensitivity and lymphocyte stimulation were also performed. A significant, dose-dependent reduction in secondary antibody response to tetanus toxoid was observed. A slightly higher mitogen response after phytohaemagglutinin stimulation was seen in lymphocytes from animals given the two higher doses of deoxynivalenol when compared with the group given the lowest dose after 9 weeks, but this result was considered inconclusive. No other indication of dose-dependent inhibition or stimulation of immune response was found, and there was no evidence for the presence of IgA-associated nephropathy (Øvernes et al., 1997). The Committee noted that this study was limited by the absence of a toxin-free control group, and no LOEL or NOEL could be identified.

(ii) *Altered serum IgA levels*

*Mice:* Groups of eight weanling female B6C3F<sub>1</sub> mice were fed AIN 76A semi-purified diet containing purified deoxynivalenol at a concentration of 0, 0.5, 2, 10, or 25 mg/kg, equal to 0, 0.1, 0.4, 2, and 5 mg/kg bw per day, for 6 weeks. The serum IgA levels were increased at doses  $\geq 0.4$  mg/kg bw per day, with no effect at 0.1 mg/kg bw per day. The serum IgM level was decreased in animals at 5 mg/kg bw per day. The NOEL was 0.1 mg/kg bw per day (Forsell et al., 1986).

The same group subsequently reported that the serum IgA level could be induced maximally in female B6C3F<sub>1</sub> mice by feeding them a diet containing deoxynivalenol at 25 mg/kg, equal to 5 mg/kg bw per day. The effect was detectable after 4 weeks of treatment, and the level increased to 17 times the control level after 24 weeks. Concurrent decreases were seen in serum IgM and IgG. Comparison with diet-restricted controls showed that these effects on the Ig isotype were not due solely to reduced food intake (Pestka et al., 1989).

In a later study, the same group compared the sensitivity of groups of seven to nine male and female B6C3F<sub>1</sub> mice, 8–10 weeks old, and found that a dietary concentration of deoxynivalenol of at least 10 mg/kg was necessary to induce consistent, significant increases in serum IgA level in males and females at 4, 8, and 12 weeks; 2 mg/kg had no effect. This would indicate a NOEL for deoxynivalenol of 0.4 mg/kg bw per day (Greene et al., 1994).

In a 2-year study, B6C3F<sub>1</sub> mice were given diets containing purified deoxynivalenol at a concentration of 0, 1, 5, or 10 mg/kg of feed, equal to 0, 0.1, 0.5, and

1.1 mg/kg bw per day in males and 0, 0.1, 0.7, and 1.6 mg/kg bw per day in females. A linear, dose-related increase in serum IgA and IgG levels was observed in female but not male mice. The increase seen, about 1.5-fold, was much smaller than those found in studies of shorter duration from the laboratory of Pestka. The authors suggested that feeding deoxynivalenol for 2 years might have allowed for adaptation, thus masking earlier effects (Iverson et al., 1995). The Committee suggested that differences in the diets used (Purina certified feed in the last study and AIN-76A semi-purified diet in the previous studies) might also have played a role.

*Pigs:* In two studies (see section 2.2.2), groups of 7–11 female or castrated male growing Norwegian Landrace pigs were fed diets amended with oats naturally contaminated with deoxynivalenol at a concentration of 0, 0.7, 1.7, or 3.5 mg/kg, equal to 0.04, 0.1, and 0.2 mg/kg bw per day. No differences in serum IgA levels was detected, and no evidence for IgA-associated nephropathy was found (Bergsjø et al., 1993a).

The mechanistic basis for the increase in serum IgA induced by deoxynivalenol has been examined in detail, typically by giving a single concentration of 10–25 mg/kg of feed, equal to 2–5 mg/kg bw per day, to 8–10-week-old B6C3F<sub>1</sub> mice to achieve the maximal response. Peyer's patch lymphocytes and, to a lesser extent, splenic lymphocytes isolated from female B6C3F<sub>1</sub> mice fed purified deoxynivalenol at 25 mg/kg of feed produced significantly more IgA than cultures derived from mice receiving diets *ad libitum* or restricted control diets. These results suggest that deoxynivalenol enhances differentiation to IgA secreting cells at the level of Peyer's patches, which affects the systemic immune compartment (Pestka et al., 1989, 1990a,b; Bondy & Pestka, 1991).

With an *ex-vivo* approach and neutralizing antibodies, it was found that the potential for enhanced IgA production exists in mouse lymphocytes as early as 2 h and as late as 24 h after a single oral exposure to purified deoxynivalenol at 5 or 25 mg/kg bw (Yan et al., 1997). This effect may be related to an increased capacity to secrete the helper cytokines interleukin (IL)-2, IL-5, and IL-6. Both CD4<sup>+</sup> and macrophage cells appear to be involved in this process. Thus, increased cytokine expression may be partly responsible for upregulation of IgA secretion in mice exposed orally to deoxynivalenol (Yan et al., 1998).

### (iii) *IgA-associated nephropathy*

An increase in serum IgA level in female B6C3F<sub>1</sub> mice after ingestion of a diet containing deoxynivalenol at 25 mg/kg, equal to 5 mg/kg bw day, for 24 weeks resulted in marked deposition of IgA in the kidney mesangium, mimicking common human glomerulonephritis (Pestka et al., 1989; Dong & Pestka, 1993). This effect has since been observed in several strains of mice. These IgA deposits can persist in the kidney for at least 16 weeks after 8 weeks' feeding of deoxynivalenol (Dong & Pestka, 1993).

Administration to groups of seven to nine male and female B6C3F<sub>1</sub> mice of a diet containing purified deoxynivalenol at a concentration of 2 or 10 mg/kg of feed (equal to 0.4–2 mg/kg bw per day) resulted at week 12 in a significant increase in

renal mesangial IgA, in a dose-related manner, and to a greater extent in male mice. Other effects included haematuria and increased IgA immune complexes (Greene et al., 1994).

Another common feature of human IgA-associated nephropathy and the deoxynivalenol–mouse model is the involvement of polyvalent ‘natural’ IgA, which may be associated with immune complex formation and subsequent glomerulonephritis (Rasooly & Pestka, 1992, 1994; Rasooly et al., 1994; Yan et al., 1997).

Intermittent dietary intake by 8–10-week-old female mice of purified deoxynivalenol was less effective in inducing IgA-associated nephropathy than continuous exposure, perhaps due to the ability of mice to stop eating contaminated feed until it is replaced with control feed (Banotai et al., 1999a).

The presence of purified deoxynivalenol at 5 or 10 mg/kg of feed, equal to 1 or 2 mg/kg bw per day, induced IgA-associated nephropathy in murine models of systemic lupus erythematosus but did not exacerbate the manifestations of lupus (Banotai et al., 1999b).

#### *(iv) Cytokine expression*

The ability of deoxynivalenol to alter the expression of cytokines transiently is important because such effects can disrupt normal regulation of a wide variety of immune functions. Deoxynivalenol can up-regulate cytokine production in murine models *in vitro* and *in vivo* (Heller et al., 1990; Dong et al., 1994; Warner et al., 1994; Azcona-Olivera et al., 1995a,b; Ouyang et al., 1995, 1996a; Ji et al., 1998; Wong et al., 1998). The concentrations required for effects *in vitro* (50–1000 ng/ml) are readily attained within minutes in plasma, lymph, and other tissues of mice given 5 or 25 mg/kg bw by gavage and can last for several hours (Azcona-Olivera et al., 1995a). Thus, *in-vitro* approaches are suitable for exploring the mechanisms of action of deoxynivalenol *in vivo*.

Superinduction of cytokine gene expression by deoxynivalenol is mediated by both transcriptional and post-transcriptional mechanisms. For example, transcriptional mechanisms involving NF- $\kappa$ B and AP-1 have been described for IL-2 in T-cell lines (Ouyang et al., 1996b; Li et al., 2000) and IL-6/TNF- $\alpha$  in cloned macrophage cell lines at deoxynivalenol concentrations of 100–250 ng/ml (Wong, 2000). With transcriptional inhibitors, superinduction of IL-2 mRNA expression by deoxynivalenol was found to be due partly to markedly increased IL-2 mRNA stability in T cells (Li et al., 1997) and IL-6/tumour necrosis factor (TNF)- $\alpha$  mRNA stability in macrophages (Wong, 2000).

The effects of deoxynivalenol on cytokine mRNA expression in groups of three male B6C3F<sub>1</sub> mice were investigated after a single oral dose of deoxynivalenol at 0, 0.1, 0.5, 1, 5, or 25 mg/kg bw. The abundance of cytokine mRNA in spleen and Peyer’s patches (indicators of the systemic and mucosal immune compartments, respectively) were assessed 2 h after exposure by reverse transcriptase-polymerase chain reaction in combination with hybridization analysis. At 5 and 25 mg/kg bw, deoxynivalenol significantly induced the mRNAs for the proinflammatory cytokines

IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ; the T helper 1 cytokines interferon (IFN)- $\gamma$  and IL-2; and the T helper 2 cytokines IL-4 and IL-10, whereas lower doses had no effect. IL-12p40 mRNA was also induced. but IL-12p35 mRNA was not. The effects were more pronounced in spleen than in Peyer's patches. IL-5 and TGF- $\alpha$  mRNAs were expressed constitutively in spleen and Peyer's patches but were not affected by deoxynivalenol. The NOEL was 1 mg/kg bw per day (Zhou et al., 1997).

The same authors subsequently examined the effects of repeated doses of deoxynivalenol on cytokine expression. Groups of three male B6C3F<sub>1</sub> mice, 8–10 weeks old, were given oral doses of purified deoxynivalenol at 0, 0.5, 2, or 5 mg/kg bw per day for 2, 4, or 7 days, and cytokine mRNAs were assessed 2 h after the last treatment in spleen and Peyer's patches. After administration of 2 or 5 mg/kg bw per day, the relative abundance of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-12 p35, IL-12p40, IL-2, and IL-10 mRNAs increased in a dose-related manner, whereas IFN- $\gamma$  and IL-4 mRNAs were unaffected. The NOEL was 0.5 mg/kg per day (Zhou et al., 1998).

#### (v) *Apoptosis in lymphoid tissue*

High doses of trichothecenes promote rapid onset of leukocyte apoptosis (programmed cell death), which is manifested as immunosuppression. Flow cytometry was used to demonstrate that deoxynivalenol inhibits or enhances apoptosis in a concentration-dependent manner in T cells, B cells, and IgA<sup>+</sup> cells isolated from spleen, Peyer patches, and thymus. Induction of apoptosis was dependent on the lymphocyte subset, tissue source, and glucocorticoid induction (Pestka et al., 1994).

Deoxynivalenol-induced apoptosis was observed in murine macrophage cells *in vitro*. These results are relevant to whole animals, since administration of trichothecenes to rodents *in vivo* results in apoptosis in thymus, spleen, bone marrow, and liver (Ihara et al., 1997; Shinozuka et al., 1997; Ihara et al., 1998; Miura et al., 1998; Shinozuka et al., 1998).

#### (b) *Neurotoxicity*

##### (i) *Biogenic amines*

Groups of six male Sprague-Dawley rats weighing 180 g and 4-week-old white Leghorn cockerels were given purified deoxynivalenol at 2.5 mg/kg bw once by gavage. Whole brains collected 2, 6, 12, 24, and 48 h after treatment did not show altered concentrations of monoamine neurotransmitters or their metabolites. In a second experiment, in which brains collected 24 h after dosing were dissected into five regions, biogenic monamines were assayed by HPLC with ECD. Deoxynivalenol significantly increased the concentrations of serotonin (102–180% greater than control) and 5-hydroxyindole-3-acetic acid (27–79% greater than control) in all regions in rats, but did not significantly change the regional concentrations of noradrenaline and dopamine. In poultry, however, the treatment decreased the concentrations of noradrenaline in the hypothalamus and hippocampus and decreased those of dopamine in the pons and medulla oblongata. These results suggest that deoxynivalenol influences the metabolism of biogenic amines in brain and that there may be intraspecies differences in the central effects of this mycotoxin (Fitzpatrick et al., 1988a,b).

The effect of deoxynivalenol on brain amine concentrations was investigated in pigs, in which the toxin causes suppression of feed intake (anorexia) in susceptible animals. After administration of a single dose of deoxynivalenol at 0.25 mg/kg bw intravenously to groups of eight male castrated Yorkshire pigs, aged 10–13 weeks and weighing 15–23 kg, the concentrations of the endogenous catecholamines noradrenaline, dopamine, 3,4-dihydroxyphenyl acetic acid, and homovanillic acid and the indoleamines serotonin and 5-hydroxyindoleacetic acid were determined by HPLC with electrochemical detection in five brain regions periodically over 24 h after dosing. The effects of deoxynivalenol were specific to each transmitter, time, and brain region. The concentrations of the main transmitters (noradrenaline, dopamine, and serotonin) were statistically significantly different from those of controls in the hypothalamus, frontal cortex, and cerebellum up to 8 h after dosing. Overall, the concentration of noradrenaline in the hypothalamus was increased twofold within 1 h, decreasing thereafter, and that of dopamine in these regions was depressed; the concentration of serotonin, which increased initially (by 1 h) in the hypothalamus, dropped significantly below that of controls in both the hypothalamus and frontal cortex at 8 h. The authors considered that these were not neurochemical changes associated with chemical-induced anorexia but that the neurochemical effects of a single exposure to deoxynivalenol are due to peripheral toxicological events, such as vomiting, which can overwhelm the more subtle central feed refusal activity (Prelusky et al., 1992).

Groups of four castrated male specific pathogen-free Yorkshire pigs, aged 8–12 weeks and weighing 15–20 kg, were fitted with an indwelling catheter in the foramen magnum of the brain and given six doses of deoxynivalenol at 0 (saline), 10 (intravenous), or 30 (intra-gastric)  $\mu\text{g}/\text{kg}$  bw at 30-min intervals; the concentrations of neurotransmitters were measured in cerebrospinal fluid, as a reflection of brain activity. The cerebrospinal fluid was collected twice a day for 3 days before dosing and at 2-h intervals for 28 h and twice a day for an additional 3 days after dosing. The pigs showed no overt clinical signs of toxicosis, and the doses given did not induce emesis. The spinal fluid samples were analysed for the presence of 3-methoxy-4-hydroxyphenylethylene, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindole acetic acid, homovanillyl alcohol, and homovanillic acid. Analyses for dopamine, normetanephrine, metanephrine, methoxytyramine, serotonin, and vanillic acid showed that they were present in quantities below the detection limit. A rapid, sustained increase (by about 50% in all four pigs) was found in the concentration of 5-hydroxyindole-3-acetic acid after intra-gastric administration, which remained elevated for up to 20 hours after oral dosing, and to a lesser extent after intravenous dosing, for up to 6 h (LOEL, 0.18 mg/kg bw after gavage and 0.06 mg/kg bw after intravenous administration). The author considered that these results indicated increased serotonergic activity in the central nervous system and supported the theory of a link between elevated brain serotonin turnover and decreased feed intake. Alterations in dopamine metabolism were also observed. After intra-gastric dosing, a delayed increase in homovanillyl alcohol concentration (about 20% in three of four pigs) was seen, which suggested to the authors that the dopaminergic response was enhanced as serotonergic activity diminished. After intravenous dosing, a rapid drop in homovanillic acid concentration (about 30% in three of four pigs) was observed, returning to normal within 36 h after dosing. The author suggested that this result indicated a direct toxic effect, possibly by inhibition of dopamine-metabolizing enzymes (Prelusky, 1993).

(ii) *Effects of anti-emetic compounds and receptor-specific compounds*

The effects of deoxynivalenol were investigated on gastric emptying and intestinal propulsion in groups of eight male ICR mice weighing 25–30 g and groups of eight male Wistar rats weighing 150–200 g and on gastrointestinal myoelectric activity in rats. Gastric emptying and intestinal transit were evaluated after gavage with a milk meal containing a marker ( $^{51}\text{CrO}_4\text{Na}_2$ ), and radiolabel was measured in the stomach and in 10 segments of the small intestine. The myoelectric activity of the antrum, duodenum, and jejunum was assessed by implanting electrodes for long-term electromyographic recording. Deoxynivalenol given orally at 10–1000  $\mu\text{g}/\text{kg}$  bw 10 min before the test meal inhibited gastric emptying in a dose-related manner, but administration of 5  $\mu\text{g}/\text{kg}$  bw intracerebrovascularily had no effect. Intestinal propulsion was reduced at the highest dose only. The inhibition of gastric emptying induced by deoxynivalenol was antagonized by ondansetron and granisetron given subcutaneously (50  $\mu\text{g}/\text{kg}$  bw) but not by ondansetron given intracerebrovascularily at 10  $\mu\text{g}/\text{kg}$  bw. The anti-emetic compounds metaclopramide and domperidone at 1 mg/kg bw subcutaneously and methylsergide, ritanserine, and cisapride at 2 mg/kg bw subcutaneously did not modify the deoxynivalenol-induced inhibition of gastric emptying. In rats, gavage with a 2.5-ml milk meal increased the frequency of antral spike bursts from  $1.9 \pm 0.9/\text{min}$  in the fasted state to  $4.7 \pm 0.4/\text{min}$ , and disrupted intestinal migrating motor complexes for  $85 \pm 11$  min. Oral administration of deoxynivalenol at 50–100  $\mu\text{g}/\text{kg}$  bw 10 min before the meal did not modify the frequency of antral spike bursts but induced migrating motor complexes on the small intestine after the meal. This effect was reversed by ondansetron at 10  $\mu\text{g}/\text{kg}$  bw subcutaneously. The authors concluded that, in rodents, deoxynivalenol inhibits gastric emptying by inducing intestinal migrating motor complexes through a peripheral action at the serotonin 3 receptors (Fioramonti et al., 1993).

In an investigation of the efficacy of several classes of receptor-specific antagonists in blocking the action of neurotransmitters possibly involved in the emetic effect of deoxynivalenol, groups of two castrated male specific pathogen-free Yorkshire pigs aged 6–8 weeks and weighing 15–20 kg were fitted with cannulae in the jugular vein and stomach. Each pig was used in up to four randomly assigned trials over a 4-week period, the time between trials ranging from 5 to 7 days. The  $\text{ED}_{50}$  in pigs given deoxynivalenol (purity, > 96%) was 75  $\mu\text{g}/\text{kg}$  bw when it was administered intragastrically and 20  $\mu\text{g}/\text{kg}$  bw intravenously. After anti-emetic pretreatment, the pigs were given deoxynivalenol at 80  $\mu\text{g}/\text{kg}$  bw intravenously or 300  $\mu\text{g}/\text{kg}$  bw intragastrically, and the onset of emesis was monitored. Certain specific antagonists of the serotonin<sub>3</sub> receptor (ICS 205-930, BRL 43694 A) were found to prevent deoxy-nivalenol-induced vomiting, indicating that serotonin plays an important role in chemically induced emesis. The serotonin<sub>2</sub>-receptor antagonists cyproheptadine and sulpiride were also moderately effective, but only at high doses. Compounds with strong anticholinergic activity were effective but apparently act directly at the emetic centre and can thus prevent emesis regardless of the cause. No effect was seen with antihistaminic and antidopaminergic anti-emetics, except those that also have considerable anticholinergic activity, or with intravenously administered chlorpromazine, which has been speculated to block specific receptors in the brain chemoreceptor trigger zone reportedly involved in initiating emesis (Prelusky & Trenholm, 1993).

Groups of four specific pathogen-free castrated male Yorkshire pigs, 8–12 weeks old and weighing 15–20 kg, were fitted with cannulae in the jugular vein and the fundic region of the stomach. The effect of deoxynivalenol (purity, > 96%) on the plasma concentrations of serotonin, its metabolite 5-hydroxyindole-3-acetic acid, and their precursor tryptophan was investigated, as a reflection of an induced peripheral serotonergic system. Typical values for the plasma concentrations of the three compounds were established, and changes were measured for 8 h after administration of deoxynivalenol intragastrically at 30 or 300  $\mu\text{g}/\text{kg}$  bw or intravenously at 10 or 100  $\mu\text{g}/\text{kg}$  bw. No effect was found, even at doses sufficient to induce emesis. The author concluded that deoxynivalenol has no peripheral effect that could account for the increased serotonergic activity reflected by altered feeding behaviour or emesis (Prelusky, 1994).

The competitive potency of deoxynivalenol against several radioactive ligands that have a high affinity for serotonin receptor subgroups, such as [ $^{125}\text{I}$ ]lysergic acid, was investigated in a membrane receptor-binding assay *in vitro*. The brains were removed from 16 castrated male Yorkshire pigs, 12–14 weeks old and weighing 18–22 kg, and dissected on ice. The densities of receptor sites and the displacement profiles in 12 regions of the brain were investigated. Overall, deoxynivalenol had only minimal ability to block the serotonin ligands tested. The median concentration for inhibition of binding was at least 5 mmol/L, and a concentration of 100 mmol/L was ineffective in certain regions. In contrast, several standard serotonin antagonists had a  $10^3$ – $10^5$  times greater ability than deoxynivalenol to displace binding of these ligands. As these results indicated that deoxynivalenol has only weak affinity for the serotonin receptor subtypes investigated, the author suggested that, unless relatively high concentrations of the toxin are present, its pharmacological effects *in vivo* may be mediated by mechanisms other than a functional interaction with serotonergic receptors at the central level (Prelusky, 1996).

The ability of cyproheptadine, a serotonin antagonist at the serotonin<sub>2</sub> receptor and a known appetite stimulant, to attenuate the adverse effect of deoxynivalenol was investigated in three trials with 21-day-old male ICR mice weighing 15–18 g. Groups of 10 mice received diets containing combinations of cyproheptadine and deoxynivalenol (purity, 99%), providing doses of deoxynivalenol of 4–16 mg/kg (equivalent to 0.6–2.4 mg/kg bw per day) and of cyproheptadine of 1.2–20 mg/kg (equivalent to 0.19–3 mg/kg bw). Cyproheptadine was administered in the feed for 2 days before addition of deoxynivalenol, and the two agents were then administered concurrently for 12 days. Cyproheptadine effectively offset the reduction in feed intake caused by deoxynivalenol, but only at certain doses. At a dose of deoxynivalenol of 4 mg/kg of feed, the optimal dose of cyproheptadine was 1.2–2.5 mg/kg of feed; at 8 mg/kg of feed, cyproheptadine was required at 2.5 mg/kg of feed; at 12 mg/kg of feed, the required dose of cyproheptadine was 2.5–5.0 mg/kg feed; and at 16 mg/kg of feed, cyproheptadine at 5–10 mg/kg feed was required. At lower doses of cyproheptadine ( $\leq 5$  mg/kg of feed), alone or in combination with the lowest dose of deoxynivalenol tested, a modest increase in weight gain was noted, but this was not seen at higher concentrations of deoxynivalenol. The authors concluded that serotonergic mechanisms probably mediate the deoxynivalenol-induced reduction in feed intake. The finding that cyproheptadine significantly attenuated the effect of deoxynivalenol indicates the involvement of the serotonin<sub>2</sub> receptor in this process (Prelusky et al., 1997).

*(iii) Role of the chemosensitive area postrema*

Conditioned aversion to the taste of saccharin was used to assess the aversive effects of deoxynivalenol in rats and to examine the putative role of the chemosensitive area postrema in the brain. In the first experiment, groups of seven adult male Long Evans rats weighing 330–370 g drank a 0.15% saccharin solution and then received an intraperitoneal injection of deoxynivalenol (purity, 98%) at 0.125 mg/kg bw or the vehicle, propylene glycol, at 0.5 ml/kg bw. In subsequent tests, the rats conditioned with deoxynivalenol had significant ( $p < 0.01$ ), two- to fourfold lower absolute and relative intakes of saccharin than control rats, which showed a strong preference for the saccharin solution. In the second experiment, the area postrema of the brainstem was ablated by cauterization in groups of six adult male rats; another six rats received sham lesions. After a 10-day recovery, all rats drank the 0.15% saccharin solution for 2 days and were then given an intraperitoneal injection of deoxynivalenol at 0.125 mg/kg bw. In subsequent preference tests, the sham-operated rats showed a significant ( $p < 0.01$ ) aversion to saccharin, while the area postrema-ablated rats showed a preference for the saccharin solution. The authors concluded that systemic administration of deoxynivalenol to rats after a novel taste induces conditioned taste aversions, which are mediated by the area postrema (Ossenkopp et al., 1994).

*(c) In-vitro studies*

The haemolytic effects of deoxynivalenol on rat erythrocytes were studied at concentrations of 130, 200, and 250  $\mu\text{g/ml}$  over 11 h. Complete haemolysis was achieved at the two higher concentrations by 10 and 7 h, but the lower concentration induced insignificant haemolysis. This finding suggests that there is a threshold below which the lytic reaction does not occur. An additional test conducted in the presence of mannitol, glutathione, ascorbic acid,  $\alpha$ -tocopherol, and histidine showed that these compounds inhibited the haemolytic reaction to the toxins. The authors suggested that deoxynivalenol acts on prokaryotic cells in three ways: by penetrating the phospholipid bilayer and acting at the subcellular level; by interacting with cellular membranes; and by free radical-mediated phospholipid peroxidation. More than one mechanism probably operates at the same time (Rizzo et al., 1992).

Deoxynivalenol, fusarenon-X, and nivalenol suppressed the growth of a human hepatoblastoma cell line (HuH-6KK) at 0.15 mg/L in a serum-free medium without an extracellular matrix ( $1 \times 10^5$  cells/ml), while aflatoxins did not inhibit growth even at 5 mg/L. The medium contained insulin, transferrin, ethanolamine, and selenite. The viability and growth of cells was evaluated in a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] reduction assay. In a chemiluminescence assay, the  $\text{IC}_{50}$  for deoxynivalenol was 1.1 mg/L (Isshiki et al., 1992, 1995).

Cellular injury caused by deoxynivalenol was assessed by means of a series of enzymic and functional indexes in 4-h-old cultures of primary rat hepatocytes exposed for 24 h to deoxynivalenol at 10–2500 ng/ml. Clear evidence of cytotoxicity was obtained, as leakage of lactate dehydrogenase and alanine and aspartate aminotransferases was significantly increased ( $p = 0.05$ ), while intracellular protein concentration and cell viability were significantly decreased ( $p < 0.01$ ). The severity of morphological effects was dose-related, and the lesions were seen at concentra-

tions  $\geq 10$  ng/ml. Cytotoxic effects occurred in a dose-dependent manner, with a threshold at 50 ng/ml. Cell viability, measured as reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), and the intracellular protein content were affected at 10 ng/ml, with an  $IC_{50}$  of 1200 ng/ml (Tseng, 1998).

The effects of a 2-week exposure to low concentrations of deoxynivalenol on the structural and functional characteristics of the human colonic adenocarcinoma cell lines Caco-2 and T84 were examined. Scanning electron microscopic analysis of the apical surfaces of Caco-2 cells showed reduction or abnormal formation of brush borders in the presence of deoxynivalenol at 50, 100, or 200 ng/ml. Monolayer integrity, measured as the transepithelial electrical resistance of tight junctions, was studied in Caco-2 and T84 cells cultured on permeable membranes. The integrity of Caco-2 cells was significantly reduced at all three doses of deoxynivalenol. A dose-related increase in permeability to Lucifer yellow, significant at a dose of 100 ng/ml of deoxynivalenol, was also observed in these cells. The integrity of T84 cells was significantly reduced at 100 and 200 ng/ml, and permeability to Lucifer yellow was significantly increased at 200 ng/ml. Alkaline phosphatase activity in Caco-2 cells was reduced from day 6 to day 15 of culture in the presence of 100 or 200 ng/ml of deoxynivalenol, whereas adding 50 or 100 ng/ml of deoxynivalenol for 15 or 20 days significantly decreased sucrase-isomaltase activity. The protein content was attenuated only by treatment with 200 ng/ml throughout the experiment. The results indicate that deoxynivalenol interferes with structural and functional characteristics of differentiation in enterocytes at low doses (Kasuga et al., 1998).

The human haematopoietic progenitors, granulocyte-monocyte colony-forming units (CFU-GM), were grown in culture in the presence of deoxynivalenol at 3, 90, or 300 ng/ml for up to 14 days. Complete inhibition of growth was observed at the highest dose at all times; partial inhibition was seen at 90 ng/ml, and at 3 ng/ml colonies were inhibited at day 7, with some recovery during the next 7 days. The authors proposed that the haematological lesions observed during human intoxication are due to destruction of haematopoietic progenitors such as CFU-GM (Parent-Massin et al., 1994).

Rat CFU-GM were cultured in the presence of deoxynivalenol at 3, 30, or 300 ng/ml for up to 14 days. Complete inhibition of growth was observed at the highest dose at all times. With 30 ng/ml, increased growth of colonies was observed; no toxicity was observed at 3 ng/ml (Parent-Massin & Thouvenot, 1995).

CFU-GM from human umbilical cord blood or from rat bone marrow were cultured in the presence of deoxynivalenol at 4–400 ng/ml for 14 days. Deoxynivalenol rapidly inhibited human and rat CFU-GM in a concentration-dependent manner at doses of 100–400 ng/ml. The  $IC_{50}$  values for human CFU-GM were 12 ng/ml on days 7 and 10 and 16 ng/ml on day 14; those for rat CFU-GM were 100 ng/ml on day 7, 60 ng/ml on day 10, and 64 ng/ml on day 14, the human cells being significantly more sensitive than rat cells. The authors reported that previous studies in their laboratory had shown that deoxynivalenol was about 10 times less toxic to human CFU-GM and about 100 times less toxic to rat CFU-GM than T-2 or HT-2 toxin (Lautraite et al., 1997).

As trichothecenes are known to induce haematological disorders such as neutropenia, thrombopenia, and aplastic anaemia in human and animals, the same group also studied the effect of deoxynivalenol at concentrations of 3–75 ng/ml and other trichothecenes in a model of human erythroblastic progenitors. Human cells were as sensitive to trichothecenes as human CFU-GM, except that deoxynivalenol caused only a slight decrease in proliferation at the highest dose (Rio et al., 1997).

The effects of deoxynivalenol at a concentration of 0, 30, 60, or 400 ng/ml for up to 72 h on the expression of the sequentially expressed activation markers CD69, CD25, and CD71 and on proliferation of human CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes provided by three healthy women were studied in culture. After 6 h at the highest concentration, less CD69 was expressed in exposed cultures than in controls, but after 24 and 48 h of exposure, an increased frequency of cells expressing CD69 was found, indicating a delay in down-regulation of CD69 expression. At 24 h, stimulation of CD25 expression was observed at doses below the IC<sub>50</sub> value, while suppression was found at higher doses. The pattern differed from that of CD69 expression, in that increased expression of CD25 did not occur after exposure to the highest concentration of the toxin and no stimulation was found after 48 h of exposure, indicating that the response was inhibited and not delayed. The effects of the toxin on CD71 expression were similar to those on CD25 expression. The authors concluded that deoxynivalenol exerts its main antiproliferative action early in the cell cycle, before or in conjunction with CD25 expression. Cell proliferation, measured by bromodeoxyuridine (BrdU) flow cytometry, was inhibited by 8%, 19% and 99% at the three concentrations of deoxynivalenol, respectively (Johannisson et al., 1999). The Committee noted that inclusion of results obtained with a concentration of about 200 ng/ml would have been informative.

Individual differences in sensitivity and the individual and combined effects of deoxynivalenol, T-2 toxin, diacetoxyscirpenol, and nivalenol on production of IgM, IgA, and IgG were studied in human lymphocytes *in vitro*. All four trichothecenes effectively inhibited mitogen-induced lymphocyte proliferation and immunoglobulin production in a dose-dependent manner, with limited variation in sensitivity between individuals. The IC<sub>50</sub> values for immunoglobulin production by deoxynivalenol (approximately 120 ng/ml) were similar to those in the tests for proliferation. Greater IgA synthesis than in controls was observed in cell cultures exposed to the lower doses of deoxynivalenol. Combined exposure to two of the toxins resulted mainly in additive or antagonistic effects, although synergistic effects could not be excluded. The authors concluded that the total intake of type A and B trichothecenes should be taken into account in risk assessments (Thuvander et al., 1999).

### **2.3 Observations in domestic animals/veterinary toxicology**

#### **2.3.1 Pigs**

The history of mycotoxicosis due to scabbed grains, which cause feed refusal and emesis, in monogastric animals was reviewed by Vesonder & Hesseltine (1980). Feed refusal by pigs and equines fed scabbed barley was reported in 1928. In 1972, maize naturally contaminated with *F. graminearum* resulted in feed refusal

and emesis in pigs, and led to the identification of deoxynivalenol (Vesonder et al., 1973).

In 1984, complete feed refusal was observed in weanling pigs on a farm in Queensland, Australia, which was associated with the presence of *F. graminearum* in wheat, triticale, and barley. These grains contained deoxynivalenol at 34, 10, and < 0.1 mg/kg, respectively, and zearalenone was found at concentrations of 6.2, 2.8, and 0.1 mg/kg of feed. The feed intake of growing pigs was reduced, and young gilts were found to have red, swollen vulvas, indicating that both mycotoxins played a role in this outbreak (Moore et al., 1985).

Feed refusal by pigs was observed in 1986 in Argentina. The concentrations of deoxynivalenol were found to be 1–40 mg/kg in wheat and 1.7–8 mg/kg in formulated feed (Marpegan et al., 1988).

### **2.3.2 Chickens and other birds**

An episode of suboptimal growth, poor feathering, and behavioural abnormalities in broilers in Scotland during the winter of 1980–81 was considered to be associated with mould-contaminated maize and wheat in the feed, from which fusaria were isolated in persistently high numbers. Four species, *F. culmorum*, *F. tricinctum*, *F. nivale*, and *F. moniliforme*, were identified. Chloroform extracts of the raw materials and of an artificial medium in which three of the *Fusarium* species were cultured proved toxic to cultures of a human epithelial cell line (HEp II). The mycotoxins deoxynivalenol, zearalenone, and diacetoxyscirpenol were identified by thin-layer chromatography (TLC) in some extracts, and several other areas of the chromatograms were found to be toxic to the HEp II cell system. These may have contained toxins for which standards were not available or, alternatively, previously uncharacterized fungal metabolites. The authors concluded that the toxins produced by the fusaria were major contributing factors to the symptoms in the birds (Robb et al., 1982).

In 1987, an epizootic at a wildlife research centre in Maryland, USA, caused symptoms in 80% of 300 captive whooping cranes (*Grus americana*) and sandhill cranes (*G. canadensis*) and the deaths of 15 of these birds. Gross examination revealed dehydration, atrophy of fat, renal insufficiency, and small spleens, which were considered inconclusive findings. Extensive testing resulted in isolation of *Fusarium* spp. from constituents of the grain-based diet, and low concentrations of T-2 toxin (1–2 mg/kg) and deoxynivalenol (0.4 mg/kg) were isolated from pelleted feed (Olsen et al., 1995).

## **2.4 Observations in humans**

### **2.4.1 Clinical observations**

The acute effects of deoxynivalenol—nausea, vomiting, diarrhoea, abdominal pain, headache, dizziness, and fever—can develop within 30 min of exposure and are difficult to distinguish from gastrointestinal conditions attributed to microbes, such as the preformed emetic toxins from *Bacillus cereus*. No deaths attributed to deoxynivalenol have been reported in humans (Luo, 1988).

### 2.4.2 Epidemiological studies

Acute outbreaks of red mould toxicosis affecting humans and involving *F. graminearum* have been reported in China, India, and Japan. Deoxynivalenol has also been investigated for its possible role in chronic diseases such as oesophageal cancer, stomach cancer, liver cancer, and a form of osteoarthritis. Some of these studies were reviewed previously (IARC, 1993; Kuiper-Goodman, 1994; Wild & Hall, 1996).

About 35 outbreaks of acute human illness were reported in China between 1961 and 1985 that were attributed to consumption of scabby wheat and mouldy maize, with at least 7818 victims. Typically, the persons became ill 5–30 min after consumption, with symptoms of nausea, vomiting, diarrhoea, abdominal pain, headache, dizziness, and fever. No deaths were reported. In an outbreak in 1984 in Xingtai County, 362 of 383 (94%) persons who ate mouldy maize became ill. Analysis by TLC of five samples associated with symptoms in this outbreak, with approximate limits of detection (LODs) of 0.1, 0.04, and 0.05 mg/kg for deoxynivalenol, T-2 toxin, and zearalenone, respectively, indicated the presence of deoxynivalenol at 3.8–93 mg/kg and zearalenone at 0.13–0.59 mg/kg in four samples; one sample contained deoxynivalenol at 0.34 mg/kg and zearalenone at 0.004 mg/kg; neither T-2 toxin nor nivalenol was found. The authors reported the presence of deoxynivalenol at a concentration of 1–40 mg/kg in scabby wheat collected from three villages and significantly higher concentrations of deoxynivalenol in wheat samples collected during the food poisoning incident than in samples not associated with the incident (Luo, 1988). The Committee noted that deoxynivalenol was probably responsible for the mycotoxicoses involving scabby wheat and mouldy maize, as zearalenone is relatively non-toxic after a single exposure.

No cases of acute illness were observed in Henan, China, in 1985 among 191 peasant families who ate scabby wheat containing deoxynivalenol at a concentration of 0.016–3.3 mg/kg (mean, 0.92 mg/kg) and nivalenol at a mean concentration of 0.13 mg/kg (both measured by GC–ECD). On the basis of a loss of deoxynivalenol during processing of about 30% and an intake of 560 g/person, the authors estimated an intake of deoxynivalenol of 380–520 µg/adult, which, for a body weight of 50 kg, would give an intake of 7.5–10 µg/kg bw. The authors indicated that the concentrations of deoxynivalenol in the following year were 0.007–0.18 mg/kg (Guo et al., 1989).

In Linxian, China, a high-risk area for oesophageal cancer (mortality rate, 132 per 100 000), no cases of acute disease were observed among oesophageal cancer patients who consumed a staple diet containing maize and maize meal containing deoxynivalenol at concentrations of 0.36–13 mg/kg (mean, 5.4 mg/kg) and nivalenol at 0.054–2.8 mg/kg (mean, 0.76 mg/kg), both analysed by TLC and HPLC, in 1985–86 (Hsia et al., 1988). The Committee noted that, as the intake of maize was not given, the intake of deoxynivalenol could not be estimated.

Studies conducted in 1989 to compare maize samples collected randomly from families with oesophageal cancer patients in Linxian with samples from families with no such cancer in Shangqiu (mortality rate from this cancer, 16 per 100 000) indicated that the mean concentration of deoxynivalenol in maize was 0.57 mg/kg

(range, 0.017–3.5 mg/kg) in Linxian and 0.099 mg/kg (range, 0.011–0.61 mg/kg) in Shangqiu. The samples were analysed by GC–ECD (Luo et al., 1990).

The natural occurrence of mycotoxins was compared in staple foods from high- and low-risk areas for oesophageal cancer in China. A total of 54 samples of maize and 40 samples of wheat intended for human consumption were collected during January and February 1995 from Linxian and Shangqiu counties in Henan Province, high- and low-risk areas for oesophageal cancer, and were analysed for fumonisins and trichothecenes by GC–MS and for zearalenone by HPLC. The prevalence of trichothecenes and zearalenone in maize samples from the high-risk area was 3.7- and 11-fold higher ( $p < 0.01$ ), respectively, than that in maize from the low-risk area. Significantly higher mean concentrations were found in the high-risk area than in the low-risk area, for deoxynivalenol (0.4 versus 0.05 mg/kg), 15-acetyldeoxynivalenol (0.24 versus undetected), nivalenol (0.086 versus 0.059), and zearalenone in maize and of deoxynivalenol (0.08 versus 0.04 mg/kg) and nivalenol in wheat. Fumonisins were found in 79% of maize samples from the high-risk area and 50% of samples from the low-risk area, but the concentration was similar: about 3.4 mg/kg. The authors concluded that the concentrations of trichothecenes and zearalenone correlated with the incidence of oesophageal cancer (Gao & Yoshizawa, 1997).

Fungal and mycotoxin contamination of 220 maize or wheat samples and 34 maize samples from four locations in Cixian County, an area with a high incidence of oesophageal carcinoma, was analysed in 1990–94; 26 maize samples collected from an area with a relatively low incidence of oesophageal carcinoma in Zanhuang County, with similar dietary customs, were analysed for mycotoxins in 1990. The maize and wheat were severely contaminated, *F. moniliforme* and *Penicillium* spp. being the predominant fungi in maize and these as well as *Alternaria alternata* and *Aspergillus flavus* in wheat. HPLC showed low concentrations of deoxynivalenol (0.05–0.17 mg/kg) in the four areas with a high incidence of oesophageal carcinoma and none in the control area. The authors concluded that fungal and mycotoxin contamination of foodstuffs in Cixian County is common (Zhang et al., 1998). The Committee noted that the units may be in error since the values appear to be lower than expected.

Consumption of fermented maize pancakes was associated with increased rates of death from stomach cancer in rural Linqu County in Shandong Province, China. To determine whether mycotoxins accounted for the increased risk, specimens of maize, maize meal, unfermented and fermented pancake batter, and cooked fermented pancakes from 16 households in five villages in Linqu County were obtained in 1996. The samples were analysed for aflatoxins, fumonisins, zearalenone, deoxynivalenol, and 15-acetyldeoxynivalenol, the limit of detection for the last two compounds being 0.5 mg/kg. No aflatoxin was detected, but fumonisins were detected in 6–19% of the maize products at concentrations of 0.6–7.2 mg/kg. Deoxynivalenol and 15-acetyldeoxynivalenol were detected in 58 and 17% of the raw maize specimens, respectively, and zearalenone was detected in 15% of the maize meal specimens. Analysis for deoxynivalenol showed that 33% of the raw maize samples and 21% of the cooked pancakes contained > 1 mg/kg (maximum, 2.7 mg/kg and 1.5 mg/kg, respectively). The concentrations of mycotoxins did not increase with fermentation. The authors concluded that the concentrations were similar to or lower

than those found in the USA, and did not increase the risk for gastric cancer among people who consumed fermented pancakes (Groves et al., 1999). The Committee noted that differences in the rate of stomach cancer among the five villages were not reported and a control area with a low rate of stomach cancer was not included. The detection limits for the mycotoxins were high. The amount and frequency of pancakes consumed was not given, and this could be considerably greater than that in the USA.

The natural occurrence of aflatoxin B<sub>1</sub>, fumonisins, and trichothecenes was investigated in maize samples harvested in areas of low and high risk of primary liver cancer, Penlai (Sandong) and Haimen (Jiangsu), respectively, in China, during 1993. In Haimen, 40 samples contained a mean concentration of deoxynivalenol of 0.89 mg/kg, and in Penlai, 13 kernel samples contained a mean of 0.49 mg/kg. The authors concluded that co-contamination of Chinese maize with aflatoxin B<sub>1</sub>, fumonisins, and deoxynivalenol is common (Wang et al., 1995).

A total of 35 maize and wheat samples from several areas of high and low incidence of Kashin-Beck disease (endemic osteoarthritis deformans) in China were surveyed in 1989 for contamination with mycotoxins, including fusarochromanone, produced by *F. equiseti*. Trichothecenes were analysed by GC-ECD (LOD, 0.005 mg/kg; recovery, > 96%). Deoxynivalenol was found at significantly higher concentrations in all high-incidence areas (range, 0.005–3.9 mg/kg) than in low-incidence areas (range, 0.002–0.7 mg/kg), with mean concentrations of 0.25 versus 0.05 mg/kg, 1.0 versus 0.27 mg/kg, and 0.51 versus 0.18 mg/kg in three areas of comparison. In addition, the concentrations of 15-acetyldeoxynivalenol and 3-acetyldeoxynivalenol, while somewhat lower than those of deoxynivalenol and nivalenol (which were about 10% those of deoxynivalenol) were also significantly higher in all high-incidence areas than in low-incidence areas. Fusarochromanone was not identified, regardless of the incidence of the disease (Luo et al., 1992).

In 1987, an acute outbreak of disease, affecting about 50 000 persons in the Kashmir valley in India, was attributed to consumption of bread made from wheat damaged by rain. The wheat was reported to contain several trichothecenes, including deoxynivalenol (0.34–8.4 mg/kg in 11 of 24 samples), acetyldeoxynivalenol (0.6–2.4 mg/kg in 4 of 24 samples), nivalenol (0.03–0.1 mg/kg in 2 of 24 samples), and T-2 toxin (0.55–4 mg/kg in 3 of 24 samples) (Bhat et al., 1989a,b). Interviews with about 150 affected families revealed that only persons who had eaten wheat products had become ill, showing mainly mild gastrointestinal tract symptoms for about 2 days. The authors estimated a NOEL of 0.44 µg/kg bw per day, on the basis of the lowest concentration of deoxynivalenol found in wheat (0.34 mg/kg), an average intake of 67 g of wheat products, and a mean body weight of 52 kg (Bhat et al., 1987). The Committee noted that, as samples were not collected until 4 months after the outbreak, a clear association of specific samples with specific cases of illness or lack of illness was not established. Furthermore, many of the collected samples contained other, more toxic trichothecenes, which may have contributed to the overall disease profile. Therefore, the actual NOEL for trichothecene-associated human illness, expressed as equivalents of deoxynivalenol, could not be identified from these studies.

### 3. ANALYTICAL METHODS

#### 3.1 Chemistry

Deoxynivalenol has a 12,13-epoxy group, three OH functions, and an  $\alpha,\beta$ -unsaturated keto group. Its chemical name is therefore 12,13-epoxy-3 $\alpha$ ,7 $\alpha$ ,15-trihydroxy trichothec-9-ene-8-one. Its molecular formula, C<sub>15</sub>H<sub>20</sub>O<sub>6</sub>, shows that its relative molecular mass is 296.3. Deoxynivalenol crystallizes as colourless needles, with a melting-point of 151–153 °C. Its specific rotation has been determined to be  $[\alpha]^{20}_D = +6.35$ . The  $\alpha,\beta$  unsaturated keto function results in absorption of ultra-violet (UV) radiation of short wavelength, but the UV spectrum of deoxynivalenol is not characteristic. As it is a type B trichothecene, deoxynivalenol is soluble even in water and in polar solvents such as aqueous methanol, aqueous acetonitrile, and ethyl acetate. Deoxynivalenol is stable in organic solvents (Shepherd & Gilbert, 1988), but ethyl acetate and acetonitrile are the most suitable solvents, particularly for long-term storage (Pettersson, 2000).

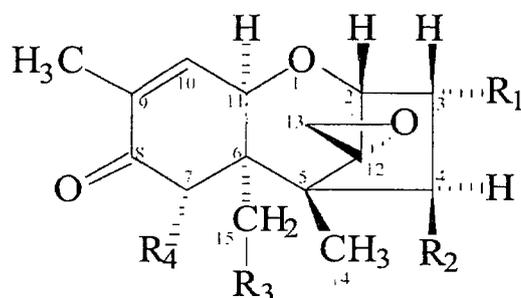
The 12,13-epoxy group is extremely stable to nucleophilic attack, and deoxynivalenol is stable at 120 °C and is not decomposed under mildly acidic conditions. The three hydroxyl groups can be derivatized (e.g. esterified), for instance before GC analysis.

Figure 2 gives the structures of the five common type B trichothecenes, deoxynivalenol, nivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and fusarenol X, with the typical basic tetracyclic, sesquiterpenoid ring system.

#### 3.2 Analysis of cereals and derived products

As the 12,13-epoxy trichothecenes are closely related, physicochemical analytical methods are usually designed to determine more than one trichothecene. However, the naturally occurring trichothecenes in cereals can be divided into polar substances carrying a keto group at C<sub>8</sub> (type B trichothecenes) and less polar type A toxins which contain no keto function at C<sub>8</sub> and generally fewer free hydroxyl groups. Hence, the analytical procedures usually differ in the extraction, clean-up, and end determination steps, depending on which group of trichothecenes is to be analysed.

**Figure 2. Structure of type B trichothecenes**



Deoxynivalenol ( $R_1 = \text{OH}$ ,  $R_2 = \text{H}$ ,  $R_3 = \text{OH}$ ,  $R_4 = \text{OH}$ ); nivalenol ( $R_1 = \text{OH}$ ,  $R_2 = \text{OH}$ ,  $R_3 = \text{OH}$ ,  $R_4 = \text{OH}$ ); 3-acetyldeoxynivalenol ( $R_1 = \text{OAc}$ ,  $R_2 = \text{H}$ ,  $R_3 = \text{OH}$ ,  $R_4 = \text{OH}$ ); 15-acetyldeoxynivalenol ( $R_1 = \text{OH}$ ,  $R_2 = \text{H}$ ,  $R_3 = \text{OAc}$ ,  $R_4 = \text{OH}$ ); and fusarenol X ( $R_1 = \text{OH}$ ,  $R_2 = \text{OAc}$ ,  $R_3 = \text{OH}$ ,  $R_4 = \text{OH}$ )

Several methods for trichothecenes based on TLC are included in the Official Methods of Analysis of AOAC International. HPLC and GC methods were reviewed in detail by Scott et al. (1993) and Langseth & Rundberget (1998). The analysis of mycotoxins, including trichothecenes, has also been reviewed by Cole (1986), WHO (1990), Chu (1991), Steyn et al. (1991), Gilbert (1992a), Scott (1995), Trucksess (1995), and Lawrence & Scott (2000). Various immunochemical methods, especially enzyme-linked immunosorbent assays (ELISAs), have been established for the determination of selected trichothecenes, as reviewed by Morgan (1989), Candlish (1991), and Park & Chu (1996). The method chosen depends on the instrumentation available, the required detection limit, the matrix composition, and the analyte properties.

The choice of analytical method also depends on the availability of appropriate calibrants of defined concentration. Comparative studies (Pettersson, 1998; Josephs et al., 2001) clearly show that determination of the concentration of calibrant solutions is still a serious problem in the analysis of deoxynivalenol, resulting in a coefficient of variation (CV) > 20% (Schuhmacher et al., 1996). Studies in which a common calibrant was provided to participants resulted in much better agreement for naturally contaminated samples (CV = 19%). A major task of the Standard, Measurement and Testing project of the European Union (Pettersson, 1998) was to check the purity of calibrants for trichothecenes and to develop a common procedure.

The purity of trichothecenes is often claimed to be high (> 95%), but the bound solvent or water is not always taken into account. The concentration of dissolved trichothecene calibrants should therefore be checked, e.g. by spectrophotometry. Owing to the extensive absorption below  $\lambda = 220$  nm, methanol is not suitable for UV determination, especially of type A trichothecenes, which have an absorption maximum around 205 nm in methanol. Therefore, for spectrophotometric determination of both A and B trichothecenes, acetonitrile is the recommended solvent (Pettersson, 1998). Values for the molar absorptivity of B trichothecenes ranging from 4500 to 7040 are found in the literature, but the Standard, Measurement and Testing project suggested use of 6400 as the molar absorptivity for all type B trichothecenes in acetonitrile, and a spectrophotometric method for assessing the purity of solid A and B trichothecenes has been developed within the framework of the project. In addition to the UV method, HPLC separation is recommended for determination of impurities, with a final comparison of the concentrations of new and old calibrants by GC. Owing to the lack of an UV-absorbing keto-group and with the double bond between C-9 and C-10, which allow B trichothecenes to absorb at 219 nm relatively well, spectrophotometric determination of concentrations cannot be recommended for A trichothecenes.

### 3.2.1 Screening tests

The commonest methods for final separation and detection of mycotoxins such as trichothecenes in agricultural commodities are still based on TLC. This method is simple and economical, and, with the introduction of high-performance TLC and scanning instruments, the efficiency and precision of separation are comparable to those achieved with other types of chromatography. TLC is still commonly used for the final separation step in the determination of deoxynivalenol. Reagents such as sulfuric acid and *para*-anisaldehyde are required, however, to visualize the non-fluorescent, short wavelength ( $\lambda_{\max} = 220$  nm) absorbing deoxynivalenol (Scott et

al., 1970; Ueno et al., 1973). Besides the spray reagents which are specific for the 12,13-epoxy group of the trichothecenes (e.g. 4-*para*-nitrobenzylpyridine or nicotinamide in combination with 2-acetylpyridine),  $\text{AlCl}_3$  is probably the most useful reagent for visualizing deoxynivalenol and other type B trichothecenes.  $\text{AlCl}_3$  has been tested in a collaborative study for the determination of deoxynivalenol by AOAC International (Eppley et al., 1986).

ELISAs have been developed for rapid screening or quantification of trichothecenes in cereals, and both ELISA and TLC are used for rapid detection of deoxynivalenol. In less complex matrices, such as pure cereals, these methods can also be used for quantification of deoxynivalenol. ELISAs involve use of specific antibodies, which are derived by a series of complex procedures including immunogen synthesis, immunization of animals, isolation, and characterization of antibodies. ELISAs are selective, sensitive, rapid, and easy to use, and clean-up, if any, is minimal. Owing to the multiple properties of trichothecenes, however, production of useful antibodies against these compounds has proved difficult. A wide range of ELISAs has been developed that involve polyclonal IgG and IgY antibodies, and monoclonal antibodies have been elicited for use in direct and indirect competitive assays. Usually, acetonitrile and water, methanol and water, pure methanol, or water is used as the extraction solvent.

A rapid method has been introduced in which Mycosep is used for clean-up, with fluorimetric detection of deoxynivalenol derivatized with zirconylnitrate and ethylenediamine in methanol (Malone et al., 1998).

Trichothecenes Appendix 1 lists the performance of tests that have been developed to screen for deoxynivalenol. The most sensitive ELISA methods have been developed for 3-acetyldeoxynivalenol, with an LOD of 0.3–1 ng/g, which requires acetylation of the toxin in cereal extract before assay for deoxynivalenol and therefore results in determination of the sum of deoxynivalenol and its acetylated derivatives. ELISAs have also been developed to enable direct determination of deoxynivalenol, but with less sensitivity (LOD = 20–300 ng/g). Accurate quantification of deoxynivalenol by immunological assays is often limited because of the remarkable cross-reactivity of deoxynivalenol-related compounds (Xu et al., 1988; Park & Chu, 1996). Nevertheless, when chromatographic instruments are not available, ELISAs are an interesting alternative for determining deoxynivalenol. Typical detection limits for deoxynivalenol by TLC are 20–300 ng/g.

The one-step solid-phase extraction clean-up and subsequent fluorimetric analysis of deoxynivalenol in grains allows detection within less than 30 min with an LOD of 100 ng/g. No values have yet been published, however, for the recovery or the precision of the method.

A comparative study organized by Schuhmacher et al. (1996) showed that participating laboratories in which ELISAs were used had difficulty in determining deoxynivalenol in pure organic solvents, with a CV of 21%, for example. In addition, overestimates and matrix dependence were observed. In a study carried out 1998 (Josephs et al., 2001), laboratories in which ELISA methods were used to determine deoxynivalenol found significantly higher concentrations than those in which HPLC and GC were used. The reason for the higher values found in maize samples in one study was additional contamination of the sample with 15-acetyldeoxynivalenol at a

concentration of 477 ng/g, determined by GC–ECD. This compound cannot be distinguished from deoxynivalenol with most ELISA systems.

### 3.2.2 Quantitative methods

#### (a) Extraction

The relatively polar deoxynivalenol and other type B trichothecenes are usually extracted by mechanical shaking or blending with aqueous acetonitrile or aqueous methanol. Other solvents, such as water and polyethylene glycol, chloroform and ethanol, and chloroform and methanol have also been used. Use of aqueous acetonitrile provides cleaner extracts than use of aqueous methanol. In an assessment of extraction procedures for deoxynivalenol, longer extraction times were required for naturally contaminated samples than for those that had been spiked (Trenholm et al., 1985). It is therefore recommended that extraction efficiency be evaluated with naturally contaminated samples when possible.

#### (b) Clean-up

While clean-up is usually not required for immunoassays, physicochemical methods commonly involve extensive clean-up procedures. The main methods used in the analysis of trichothecenes are liquid–liquid partitioning, solid-phase extraction, column chromatography, immunoaffinity columns, and multifunctional clean-up columns. If necessary, interfering lipids can be removed by extracting the sample with *n*-hexane or another non-polar solvent before further clean-up.

Liquid–liquid partitioning is conventionally performed by shaking the sample extract with a non-miscible solvent in a separation funnel. For this type of clean-up, the aqueous phase can be supported on a column packed with solid hydrophilic matrix (ClinElut® or Extrelut®) and the organic solvent percolated down the column to elute the purified toxin.

Column chromatography can involve use of various stationary phases (silica gel, aluminium oxide, Florisil, charcoal, and C<sub>8</sub> or C<sub>18</sub> reversed phases) corresponding to the required polarity range of the adsorbent. The column packing material used most frequently for deoxynivalenol is a mixture of charcoal, alumina, and Celite. Modern solid-phase extraction columns are a potential alternative to the conventional column chromatographic methods. Solid-phase extraction columns are generally delivered prepacked in disposable plastic cartridges and are available with the adsorbents listed above. Gel permeation chromatography has also been used for clean-up in the analysis of deoxynivalenol.

Application of immunoaffinity columns for purification before instrumental methods has been used successfully for several mycotoxins. When the aqueous sample extract is applied to the immunoaffinity column, the analyte molecules are bound to antibodies which are linked to an organic carrier material. Before washing, the toxins can be eluted by denaturing the antibodies with pure organic solvents. However, immunoaffinity columns for most trichothecenes are not available commercially, and the columns are applicable to only one toxin. Furthermore, low recovery of deoxynivalenol was found with a commercial immunoaffinity column (Scott & Trucksess, 1997). An HPLC method involving both a charcoal–alumina–Celite column and a commercial immunoaffinity column gave reasonable recoveries, with an LOQ of 50 ng/g for cereals (Reutter, 1999).

Supercritical-fluid extraction combined with GC–ECD appears to be suitable for the determination of deoxynivalenol and related trichothecenes (Josephs et al., 1998; Krska, 1998). The heavy investment and maintenance costs for a supercritical-fluid extraction apparatus and the poor recovery of deoxynivalenol (50%) remain problems, however, which limit the applicability of these methods in routine analysis.

Another development in clean-up methods is the simple, rapid, multifunctional clean-up column MycoSep™ (Romer, 1986; Weingärtner et al., 1997). These columns consist of packing material containing various adsorbents, such as charcoal, Celite, and ion-exchange resins, which are housed in a plastic tube between filter discs, with a rubber flange at the lower end containing a porous frit and a one-way valve. When the column is inserted into a culture tube, the flange seals tight, thus forcing the extract through the packing material. The pure extract appears at the top of the plastic tube. The Mycosep® column allows sample purification within 10–30 s. A major advantage of this column is that no time-consuming rinsing steps are required, as in solid-phase extraction. In addition, nearly all the interfering substances are retained on the column, and the trichothecenes are not adsorbed onto the packing material. Mycosep® columns are among the most frequently used commercial columns for clean-up of deoxynivalenol in combination with HPLC and GC.

### (c) Separation and detection

ELISA and TLC are used for the quantitative determination of deoxynivalenol. GC is widely used, particularly for simultaneous determination of several trichothecenes as their trimethylsilyl, pentafluoropropionyl, heptafluorobutyryl, and trifluoroacetyl derivatives, with ECD, flame ionization detection, MS, or tandem MS detection. The choice of derivatization reagent depends on the type of trichothecene to be analysed and the method of detection. The conjugated carbonyl group makes type B trichothecenes sensitive to ECD, while enhanced sensitivity of type A trichothecenes, which lack this group, is obtained when fluoroacylation is used. Trimethylsilylation allows more selective derivatization of type B trichothecenes, with less background interference than with fluoroacylation or derivatization with heptafluorobutyryl- or pentafluoropropionylimidazole (Poole, 1978). Trimethylsilyl ethers are made by derivatizing all hydroxyl groups with reagents such as *N,O*-bis(trimethylsilyl)acetamide, trimethylchlorosilane, and trimethylsilylimidazole. When a single compound is used, however, two peaks may arise for type B trichothecenes, owing to incomplete derivatization. Such problems can be avoided by using derivatization mixtures such as TRI-SIL TBT® and Sylon BTZ, which contain trimethylsilylimidazole (40 ± 5%), *N,O*-bis(trimethylsilyl)acetamide (35 ± 5%), and trimethylchlorosilane (25 ± 5%).

Several suitable HPLC methods have been published for the determination of type B trichothecenes in food and cereals. Separation is usually achieved on a C<sub>18</sub> reversed-phase column with methanol–water mixtures as the mobile phase. Use of acetonitrile (UV cut-off, 190 nm) instead of methanol (UV cut-off, 210 nm) in aqueous mixtures is preferable, however, in view of the convenient cut-off value and better transmission at the important 220-nm wavelength. Deoxynivalenol and nivalenol have been determined by HPLC with UV detection at 222 nm. Nevertheless, time-consuming clean-up is required for the determination of deoxynivalenol in complex matrices, such as food and feed. A German standard method for determination of deoxynivalenol, which is being validated in a second round trial, involves an HPLC

method that includes a time-consuming three-step clean-up with liquid–liquid partitioning with petroleum ether, solid-phase extraction with a charcoal–alumina–Celite mixture, and use of an immunoaffinity column.

Analytical performance characteristics comparable to those of GC methods can be achieved by HPLC combined with pre- or post-column derivatization (Maycock & Utley, 1985). The post-column derivatization procedure that has been developed by Sano et al. (1987) involves alkaline decomposition of deoxynivalenol and nivalenol to generate formaldehyde, and subsequent reaction with acetoacetate and ammonium acetate to form a fluorescent derivative. In view of the problems associated with use of GC, this method is an interesting alternative for reliable, accurate determination of deoxynivalenol, even at the lower range of nanograms per gram. LC–MS instruments, particularly those with atmospheric pressure chemical ionization interfaces, have been used for the determination and identification of trichothecenes, including deoxynivalenol, at trace concentrations (Razzazi-Fazeli et al., 1999). Owing to the decreasing cost of suitable LC–MS instruments, this technique is also being used for the determination of mycotoxins. ECD has also been described for the determination of deoxynivalenol (Sylvia et al., 1986).

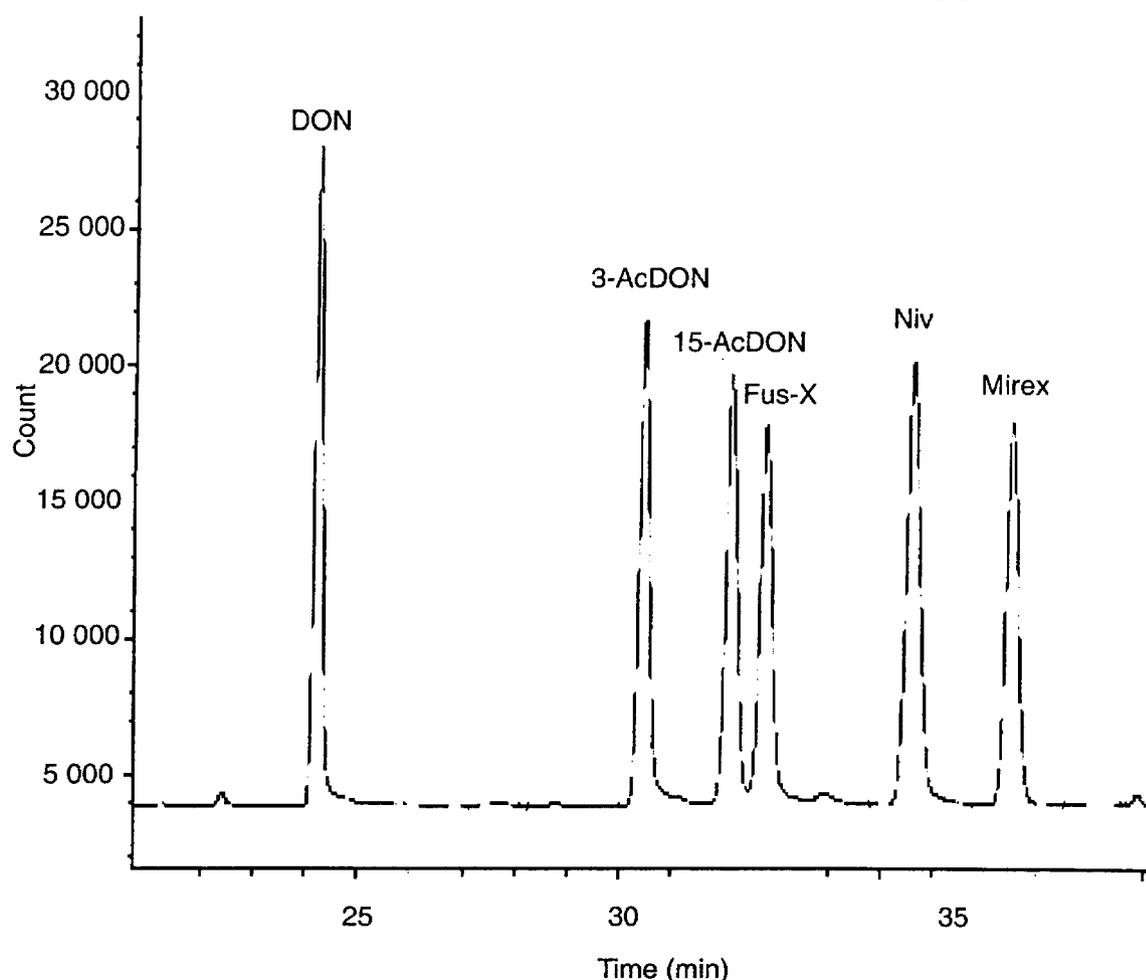
The method used in a recent comparative study (Josephs et al., 2001) reflects the techniques being used by most European laboratories involved in analysing deoxynivalenol. The trial for the determination of deoxynivalenol in cereals comprised 28 laboratories in Europe, Singapore, and the USA. Deoxynivalenol was purified from the raw extracts by solid-phase extraction on MycoSep™ or Florisil-active charcoal columns (nine laboratories). Two participants used immunoaffinity columns, and one used the Extrelut® technique in combination with solid-phase extraction on Fluorisil for clean-up. Deoxynivalenol was determined by GC–ECD (five laboratories), GC–MS (two laboratories), HPLC–UV–diode array detection (six laboratories), and HPLC–fluorescence detection (one laboratory). Four laboratories used ELISA for determination of deoxynivalenol. It should be emphasized that the ELISA methods used for determination of deoxynivalenol cannot distinguish between deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and 3,15-diacetyldeoxynivalenol, since the antibodies are elicited against 3,7,15-triacetyldeoxynivalenol. The LODs of the methods used by the participants ranged from 0.30 to 110 ng/g, the recoveries varied from 60 to 100%, and the reported CVs of the methods ranged from 3 to 15%.

In a comparative study organized by Gilbert (1992a), the participants who used TLC for determination of deoxynivalenol found interference in extracts of maize. Accordingly, most of the participants changed from TLC to HPLC.

#### *(d) Performance characteristics*

Trichothecenes Appendix 2 lists the performance characteristics of quantitative methods for trichothecenes. Figure 3 shows a typical chromatogram obtained after separation and detection with GC–ECD after trimethylsilylation of five B trichothecenes. The method thus allows simultaneous quantification of several B trichothecenes. The typical detection limits of quantitative methods for the determination of deoxynivalenol in cereals are 100–1600 ng/g (HPLC–UV), 6–40 ng/g (HPLC–MS), 20 ng/g with HPLC–fluorescence detection) 20–50 ng/g and even lower with GC–ECD, and down to approximately 5 ng/g with GC–MS with heptafluorobutyryl and pentafluoropropionyl derivatives. Typical recoveries were 70–110%. The results

Figure 3. Gas chromatogram for wheat spiked with deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), fusarenon X (Fus-X), and nivalenol (Niv) at a concentration of about 1000 ng/g



The halogenated pesticide Mirex was used as the internal standard to control the electrochemical detector response.

of the comparative studies (Schuhmacher et al., 1996; Pettersson, 1998; Josephs et al., 2001) indicate that the analysis of the most relevant *Fusarium* mycotoxin, deoxynivalenol, is still not satisfactory. This is clear in the comparison of determinations of deoxynivalenol within the framework of the Standard, Measurement and Testing project organized by Pettersson (1998) involving 20 participants from 12 countries, in which no common calibrant was provided and for which the CV was 60%. The values for a naturally contaminated wheat sample ranged from 185 to 1100 ng/g, after rejection of outliers. In general, the results of the study showed relatively wide variation in both repeatability and reproducibility for all trichothecenes analysed. The Community Bureau of Reference comparative studies (Pettersson, 1998) and studies for the certification of deoxynivalenol in wheat and maize were different (Gilbert, 1992a,b), since mainly HPLC methods were used.

The main objective of the project funded by the Commission of the European Union is to improve the analysis of trichothecenes with GC methods and to obtain better agreement among laboratories. The main methodological problems in the project coordinated by Pettersson (1998) were:

- differences in the response to trichothecenes of pure calibrants and matrix-assisted calibrants;
- non-linearity of calibration curves;
- too high a recovery;
- drifting response for trichothecenes;
- high variation in repeatability of runs with MS detection;
- carry-over or 'memory' effects from previous samples; and
- matrix interference.

Although a standardized GC method for deoxynivalenol and other trichothecenes is needed, these problems and sources of variation must be addressed. Otherwise, the method will not be robust enough and will result in wide variations in reproducibility and repeatability.

#### 4. SAMPLING PROTOCOLS

Generation of meaningful survey data requires collection of representative samples from carefully selected batches of food which, in turn, are representative of clearly defined locations (e.g. country, region within a country). For general aspects of the sampling protocols see Trichothecenes Appendix 6.

The variability of measurements of deoxynivalenol in barley and wheat has been studied by Freese et al. (2000) and Whitaker et al. (2000), respectively. Freese et al. collected 225-kg bulk samples from six batches of barley and riffle-divided each sample into 16 samples of 0.1 kg, 16 samples of 0.8 kg, and 16 samples of 7 kg. The samples were comminuted in a Romer mill, and 50-g portions were taken from each sample for determination of deoxynivalenol. An evaluation of the analytical results indicated that the variation associated with sample preparation and analysis was of greater significance than sampling variance for all sample sizes and that the variation was not significantly reduced by increasing the sample size. In a further experiment, 10 samples of about 2.5 kg were taken from each of 10 truck loads of barley, by sampling methods prescribed by the Grain Inspection, Packers and Stockyards Administration (1995). Each 2.5-kg sample was comminuted in a Romer mill, and two 50-g portions were taken from each sample. A single determination of deoxynivalenol was made on the extract from the first portion, whereas duplicate determinations were performed on the second portion, by an ELISA procedure. In this instance, an evaluation of the variance associated with the sampling, sample preparation, and analytical steps indicated an approximately equal contribution from each step. It was concluded that sample sizes of 100–200 g were adequate, assuming that they were obtained by riffle division of a large bulk sample. However, it was also concluded that batches might be stratified to different degrees, depending on the amount of mixing during handling, and that stratification could have a significant effect on sampling variance.

Whitaker et al. (2000) adopted a similar approach in studying the variances in sampling, sample preparation, and analysis during determination of deoxynivalenol in wheat. A 20-kg bulk sample was taken from each of 24 commercial batches, and each sample was riffle-divided into 32 samples weighing 0.45 kg (1lb). Each 0.45-kg sample was finely comminuted in a Romer mill, which was set to produce a representative, comminuted 25-g portion automatically. Deoxynivalenol was determined in each of 768 (24 x 32) 25-g portions by the Romer FluoroQuant™

fluorimetric procedure, and the analytical data were used to determine total variance (sampling, sample preparation, and analysis). Twenty comminuted samples, with a wide range of concentrations of deoxynivalenol, were selected from the residual 768 comminuted samples, and eight 25-g portions were taken from each sample by riffle division. The Romer method was then used to determine the deoxynivalenol concentration in four replicate extracts prepared from each 25-g portion. The combined variance for sample preparation and analysis and the analytical variance alone were estimated by the SAS procedures (Statistical Analysis System Institute, Inc., 1997). The total CV varied from 260 (batch concentration, 0.02 mg/kg deoxynivalenol) to 7.9 (14 mg/kg). For a batch concentration of deoxynivalenol of 5.0 mg/kg, the CVs associated with sampling, sample preparation, and analysis were 6.3, 10, and 6.3%, respectively. The sampling variance was specific to a 0.45-kg sample, the sample preparation variance to a Romer mill and a 25-g analytical sample, and the analytical variance to the Romer FluoroQuant method. The total variance was 13%. The low variance associated with the sampling step (relative to those of other mycotoxins and other commodities) is due partly to the small kernel count of wheat (about 30 kernels per gram), which is about 10 times higher than that of shelled maize and 30 times higher than that of shelled groundnuts.

## 5. EFFECTS OF PROCESSING

During preharvest of crops with *Fusarium* head blight, the fungi kill some developing seeds, resulting in shrunken, shrivelled kernels of low weight. In general, at concentrations up to 1 mg/kg mycotoxins such as deoxynivalenol are typically found near the surface of the kernel, whereas at high concentrations they may be more evenly distributed (Charmley & Prelusky, 1994). Many of the shrivelled kernels can be removed by the use of gravity separators, which separate particles on the basis of differences in specific gravity, size, shape, and surface texture. The concentrations of deoxynivalenol after storage were always lower in samples from which the infected kernels were removed before storage than in samples that contained infected kernels (Wilcke et al., 1999). The disappearance of deoxynivalenol during cleaning of grain, such as removal of infected kernels and washing, has been reported to be up to 74% (Charmley & Prelusky, 1994); however, the degree of decontamination varies among studies. Washing barley and maize three times in distilled water reduced the deoxynivalenol concentration by 65–69%. Using 1 mol/L sodium carbonate solution for the first wash reduced the concentration by 72–74%. Soaking barley, maize, or wheat in a 0.1 mol/L sodium carbonate solution for 24–72 h caused a 42–100% reduction in toxin concentration.

Natural degradation of deoxynivalenol has been observed in cereal grain both in the field and during storage. Several explanations have been put forward for how the concentrations of mycotoxins are decreased in a natural system. The concentration of free mycotoxin in natural ecosystems may be the result of concurrent synthesis, transport, conjugation, release from bound forms, and degradation by the plant or by other microbes (Karlovski, 1999).

The effectiveness of milling practices in reducing trichothecene concentrations in flour fractions from that in whole grain has been reviewed (Patey & Gilbert, 1989; Scott, 1991; Charmley & Prelusky, 1994). Milling usually resulted in a higher concentration of deoxynivalenol in bran, shorts, and feed flour and lower concentra-

tions in straight-grade flour. The distribution of deoxynivalenol in the various milling fractions of wheat depends to a large extent on the degree of fungal penetration of the endosperm (Nowicki et al., 1988). In other words, milling grain in which the deoxynivalenol contamination is located predominantly at the surface of the kernel would result in flour with a low deoxynivalenol concentration. The ability of fungi to penetrate the kernel appears to vary among different varieties of wheat. Dry milling of maize containing deoxynivalenol gave a higher concentration in germ meal than wet milling, after which the highest concentrations were in steep liquor and gluten.

The trichothecenes are stable at 120 °C, moderately stable at 180 °C, and decompose within 30–40 min at 210 °C (Kamimura, 1989). Neira et al. (1997) showed an average reduction of 44% in the deoxynivalenol concentration in dough and in the final baked products. Approximately half of the reduction occurred after the dough fermentation step.

In a study of the effect of high-temperature and high-pressure processing of foods spiked with deoxynivalenol, no significant reduction was found after processing in extruded maize grits, extruded dry dog food, or autoclaved moist dog food. Autoclaved cream-style maize showed a reduction of only 12% (Wolf-Hall et al., 1999).

During cooking of noodles and spaghetti, trichothecenes may leach into the boiling water to a considerable extent (Scott, 1991).

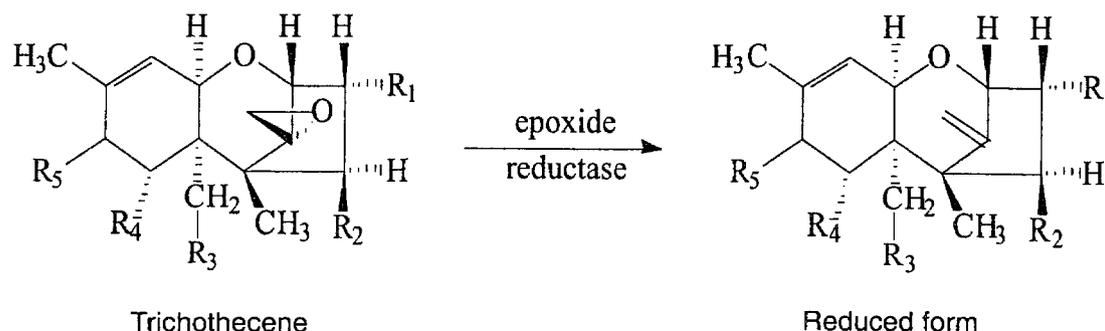
Trichothecenes are not stable in the presence of alkali. Only 18–28% of deoxynivalenol in maize was retained during tortilla fabrication, in which the maize was first boiled in calcium hydroxide solution (Abbas et al., 1988).

Deoxynivalenol was not metabolized by yeast strains of technological relevance (Böswald et al., 1995).

Microbiological transformation could be used to transform deoxynivalenol to less toxic metabolites such as the de-epoxy metabolite (Yoshizawa et al., 1986; Figure 4). However, only mixed cultures have been used in the microbiological methods, whereas a defined isolate is a requirement for feed additives.

Several studies were conducted to investigate the ability of microorganisms isolated from rumen fluid and soil to degrade or biotransform deoxynivalenol under anaerobic or aerobic conditions in liquid culture (King et al., 1984a). He et al. (1992) detected transformation of 50% of deoxynivalenol to the de-epoxy metabolite in the first culture of soil microorganisms but no further transformation in later subcultures. Anaerobic degradation of deoxynivalenol has been studied extensively (Binder et

**Figure 4. Microbial transformation of trichothecenes into their de-epoxylated forms**



al., 1998), and rumenal bacteria capable of transforming deoxynivalenol and 3-acetyldeoxynivalenol to a the de-epoxy metabolite have been isolated. The rumenal contents of a fistulated cow were used as a natural habitat for the enrichment and isolation of anaerobic bacteria (Binder et al., 2000). The active bacterium is a gram-positive, non-spore-forming, strictly anaerobic, irregular rod belonging to the genus *Eubacterium*. The efficiency of this bacterium as a feed additive, commercially available as such in e.g. South America, has been tested in feeding trials with pigs and chickens. Microbes in rumenal fluid had no effect on the concentration of deoxynivalenol (Kiessling et al., 1984).

Mouldy maize was detoxified microbially by incubation with the contents of the large intestine of chickens, and the response of young pigs fed this detoxified deoxynivalenol-contaminated feed was evaluated. A significant amount of the deoxynivalenol in the maize had been transformed to the de-epoxy metabolite. Microbial inocula from rumenal fluid, soil and the contents of the large intestines of chickens and of pigs were tested for their ability to transform deoxynivalenol in vitro. Microorganisms in the chicken intestinal contents completely transformed pure deoxynivalenol, and this activity was retained through six serial subcultures. No alteration of the toxin was detected after incubation with pig intestinal contents, whereas 35% of the deoxynivalenol was metabolized in the original culture of rumenal fluid and 50% was metabolized by the soil sample. About 50% of the deoxynivalenol in mouldy maize in culture medium was transformed by microorganisms from chicken intestine (He et al., 1993).

A mixed microbial culture capable of metabolizing deoxynivalenol was obtained from soil samples by an enrichment culture procedure. A bacterium isolated from the enrichment culture completely removed exogenously applied deoxynivalenol from the culture medium after incubation for 1 day (Shima et al., 1997). The main metabolite was identified as 3-keto-4-deoxynivalenol. This compound had remarkably less (one-tenth) immunosuppressive toxicity than deoxynivalenol, indicating that the 3-OH group on deoxynivalenol is likely to be involved in its immunosuppressive effects.

The concentration of deoxynivalenol remained stable when deoxynivalenol-containing wort was fermented with strains of *Saccharomyces cerevisiae* for 7–9 days (Scott et al., 1992).

In a study of the transmission of deoxynivalenol from naturally contaminated barley and wheat malts into beer, the deoxynivalenol content increased markedly during mash production. The increase was suggested to be due to enzymatic activity in the malt. Deoxynivalenol was stable after the wort was boiled for 90 min. Fermentation did not affect the initial toxin content of the wort. The concentration in finished beer was similar to that in the respective malt (Niessen & Donhauser, 1993).

## **6. LEVELS AND PATTERNS OF CONTAMINATION OF FOOD COMMODITIES**

### **6.1 Results of surveys**

Argentina, Brazil, Canada, China, Finland, Germany, Italy, Norway, Sweden, the United Kingdom, Uruguay, and the USA submitted data on contamination of grains and food products with deoxynivalenol to FAO/WHO. Other data on

contamination of food by these toxins were taken from the published literature for 1990 through early 2000. Previous data were reviewed in *Environmental Health Criteria* 105 (WHO, 1990). As the information available was not complete in many cases, most of the authors were contacted; many of them sent details on sampling and analytical methods and some also sent unpublished data.

The data on the natural occurrence of deoxynivalenol are summarized in Appendix A. The criteria for accepting data on food contamination included the date of the report between 1990 and 2000, use of adequate analytical methods for this toxin, and random collection of samples. Details of the sampling procedures are given in Appendix A and in Trichothecenes Appendix 6. The data were analysed on a case-by-case basis. When no information was provided about sampling or analytical methods, the data were not included. The Appendix includes the primary reference (P), the reference for sampling (S), and the references for the analytical method (A) given by the authors. Mean values were calculated when the mean of positive samples was given or values for 'not detected' were given as half the detection limit. The mean values in the Appendix therefore indicate the mean of all samples, with those below the limit of detection taken as 0, with some exceptions, as shown.

In the decade 1980–90, deoxynivalenol occurred commonly, particularly in wheat and maize, at concentrations usually < 1000 µg/kg (WHO, 1990). This value was therefore chosen for comparison with concentrations reported in the decade 1990–2000. A concentration of 100 µg/kg was used to compare the concentration of deoxynivalenol with those of T-2 and HT-2 toxins.

The analytical methods used to derive the data included in Appendix A were GC–ECD, GC–MS, TLC, HPLC, and ELISA, and the details are described in Trichothecenes Appendix 5. Methods for ELISAs were included in Trichothecenes Appendix 5 only when the paper provided information on recovery or when the extraction solvent was not methanol–water. In approximately half of the reports, deoxynivalenol was quantified by GC with ECD or MS, and in one-fourth of the reports by TLC. The recovery of the toxin in various substrates sometimes affected the data on occurrence more than the sensitivity of the method. When the extraction and clean-up steps are effective, low limits of detection or quantification can be obtained even with low-cost methods such as TLC.

Some authors defined LODs and LOQs in different ways. The LOD was sometimes expressed as two or three times the noise over background, and sometimes as the smallest quantity of the standard that could be detected. The LOQ was sometimes defined as five or six times the noise (measured in standard solutions), without taking into account recovery in the matrix, while other investigators included the data on recovery in their estimation of the LOQ. Investigators should report the method by which they calculated the LOD and LOQ, in order to standardize the procedure. Intake can best be estimated by considering studies in which the recovery was > 60% the LOQ. Analytical methods, particularly with regard to recovery, in different matrixes require constant improvement.

No specific sampling method has been reported for these toxins. Those used are described in Trichothecenes Appendix 6.

Some interesting studies were not included in Appendix A, as they could not be used to estimate the total dietary intake of the toxin. These include reports of the occurrence of the toxin in areas where disease was found, estimates of variations in the concentration of deoxynivalenol in different lots, studies on genetic variation, samples of grains with scab symptoms, and comparisons of the presence of deoxy-

nivalenol in mouldy and healthy grains or in different parts of the plant. In other cases, the results reported were insufficient to be entered in Appendix A, as for example when deoxynivalenol was expressed as total type B trichothecenes obtained after hydrolysis (Perkowski et al., 1990; Luo et al., 1992; Okoye, 1993; Chu & Gy, 1994; Trigo-Stockli et al., 1995; Menna et al., 1997; Perkowski et al., 1997; Li et al., 1999; Perkowski, 1999; Sohn et al., 1999; Wetter et al., 1999; Chelkowski et al., 2000; Freese et al., 2000).

In one study, beans were found to be contaminated by trichothecenes (deoxynivalenol, diacetoxyscirpenol, and T-2 toxin). As the samples were not collected at random, the data were not included in Appendix A. However, the quantities of toxins detected were substantial and warrant investigation in other legumes (Tseng et al., 1995).

Deoxynivalenol was also detected in soya beans, with discolouration damage, and in some soya bean products from a pilot plant. The concentrations ranged from not detectable to 490  $\mu\text{g}/\text{kg}$  in whole soya beans, from  $< 10 \mu\text{g}/\text{kg}$  to 420  $\mu\text{g}/\text{kg}$  in hulls, from  $< 5 \mu\text{g}/\text{kg}$  to 600  $\mu\text{g}/\text{kg}$  in meal, and from not detectable to 30  $\mu\text{g}/\text{kg}$  in crude unrefined oil (Jacobsen et al., 1995). In Appendix A, only five samples of soya beans were found to have no detectable deoxynivalenol.

In the United Kingdom, samples of traditional foods were reported to be contaminated with deoxynivalenol at concentrations similar to those found in wheat (Patel et al., 1996).

As shown in Appendix A, deoxynivalenol was a frequent contaminant of cereal grains such as wheat (11 444 samples, 57% contaminated), maize (5349 samples, 40% contaminated), oats (834 samples, 68% contaminated), barley (1662 samples, 59% contaminated), rye (295 samples, 49% contaminated), and rice (154 samples, 27% contaminated). It has also been detected in some wheat and maize products, such as wheat flour, bread, breakfast cereals, noodles, baby and infant foods, and cooked pancakes. It has been reported in barley products and beer: more than 50% of 321 beer samples analysed were contaminated with deoxynivalenol (Niessen et al., 1993; Scott et al., 1993; Moltó et al., 2000).

In some countries, such as Germany and Italy, the presence of deoxynivalenol was reported in grains grown organically and by conventional techniques.

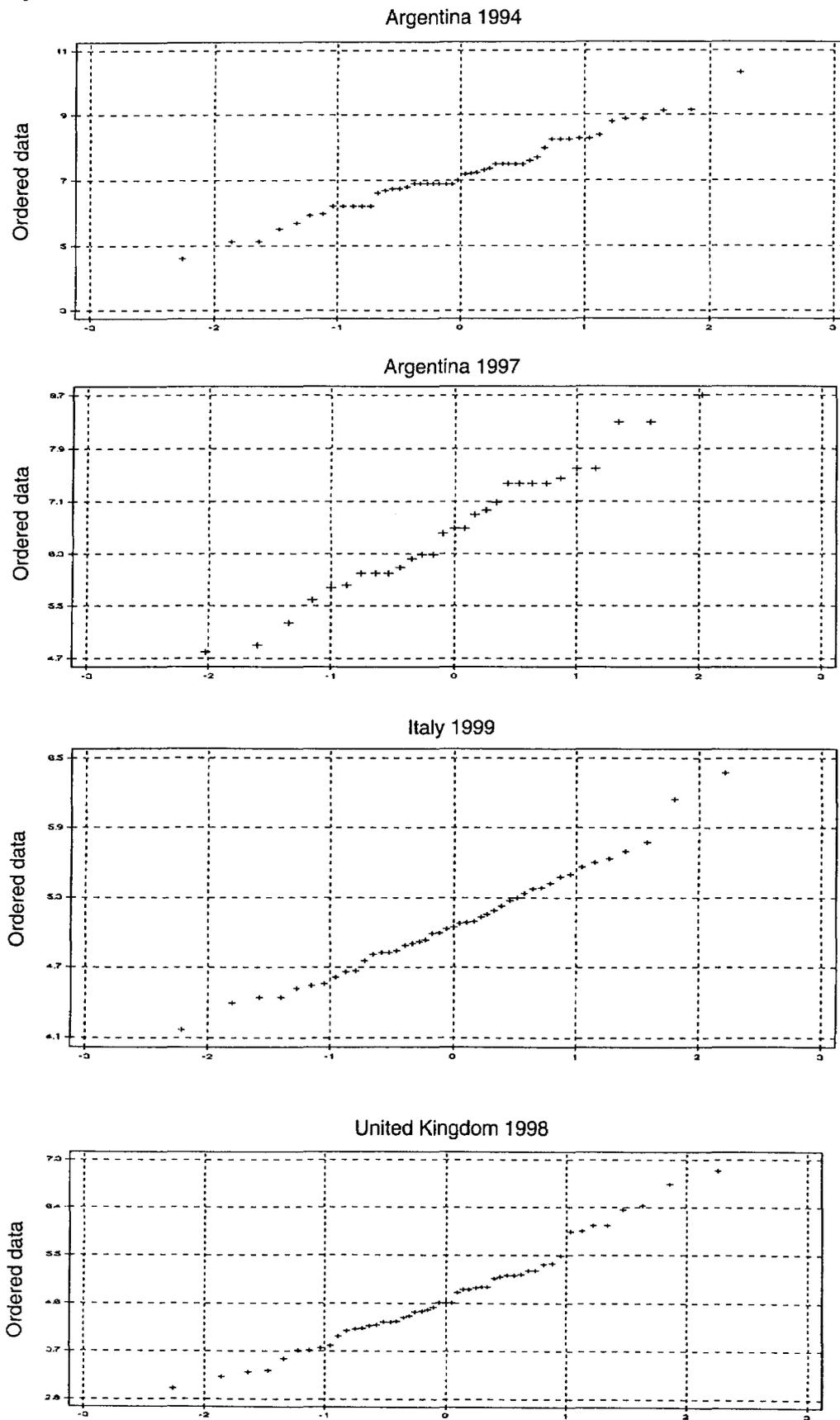
Although a large amount of data was summarized (23 380 samples), information on some products in various parts of the world is still lacking.

## 6.2 Distribution curves

Some authors presented data on distribution in the form of frequency tables or histograms (for example Müller et al., 1997a,b). Fontán et al. (1994) showed that the Fisher–Tippett distribution could be adjusted adequately for the data on the 1990–92 wheat harvests in Argentina. Some individual data were almost discrete, owing to rounding, and could not be used for the distribution analysis. For this purpose, only data on deoxynivalenol contamination of wheat were available.

Figure 5 presents Q–Q plots of the log-transformed data for the 1994 and 1997 harvests in Argentina, the 1999 harvest in Italy, and the 1998 harvest in the United Kingdom. Similar patterns were found for the harvest in Finland in 1998 (data not shown). For the analysis, only values greater than 20  $\mu\text{g}/\text{kg}$  (LOD) for the United Kingdom, 50  $\mu\text{g}/\text{kg}$  (LOD) for Italy, and 48  $\mu\text{g}/\text{kg}$  (LOQ) for Argentina were considered. In all cases, goodness-of-fit tests were applied. The *p* values obtained ( $\geq 0.5$ ) indicated that the log-normal distribution provided an adequate model for these data.

Figure 5. Q-Q plots of log-transformed data of distribution of concentrations of deoxynivalenol in cereals



The distribution function for processed products such as bread could be different from that adjusted for raw cereal, as contamination tends to be more homogeneous during processing. The adjusted distribution function could therefore belong to other distribution families (Schollenberger et al., 2000a).

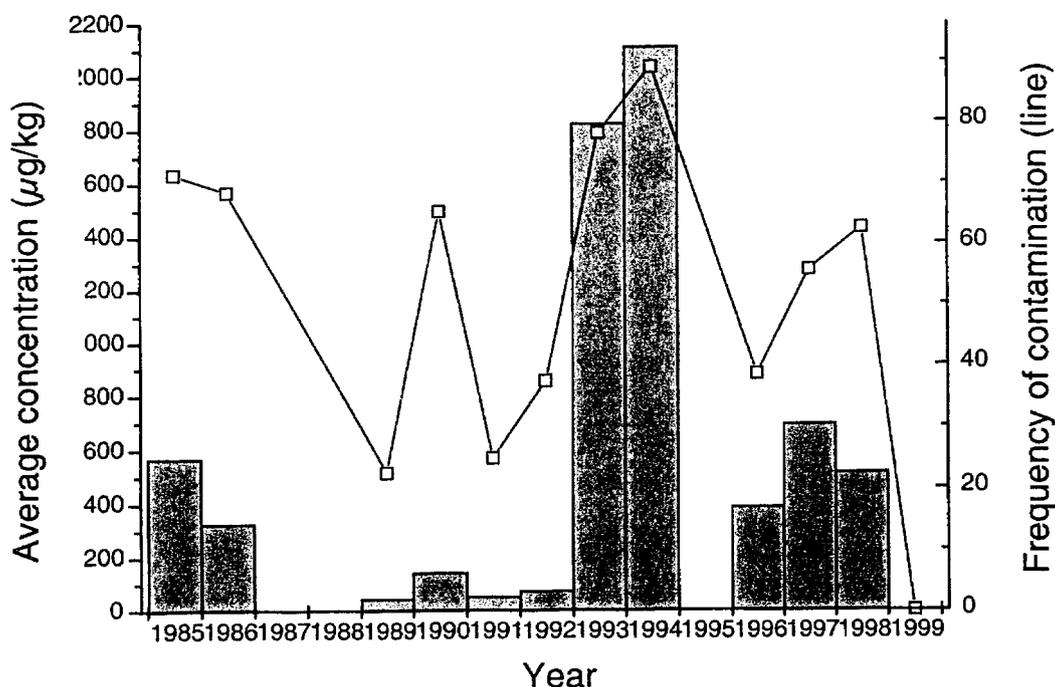
### 6.3 Annual variation

Most of the information on contamination with deoxynivalenol has been reported for wheat (11 000 samples) and maize (6000 samples). These large data sets allow determinations of annual variation in concentrations. For instance, wheat harvested in Germany, Norway and the Russian Federation in various years showed similar annual variations in contamination (Appendix A). Data from Canada obtained in consecutive years showed that the average contamination in hard wheat was lower than that in soft wheat but that the annual variation in the two products was similar. Figure 6 shows the annual variation in the average contamination of wheat and the frequency of contamination in Argentina from a survey conducted in the main production area between 1985 and 1999.

The annual variation in the average concentration of deoxynivalenol in maize in Canada and New Zealand in 1987–94 is shown in Figure 7.

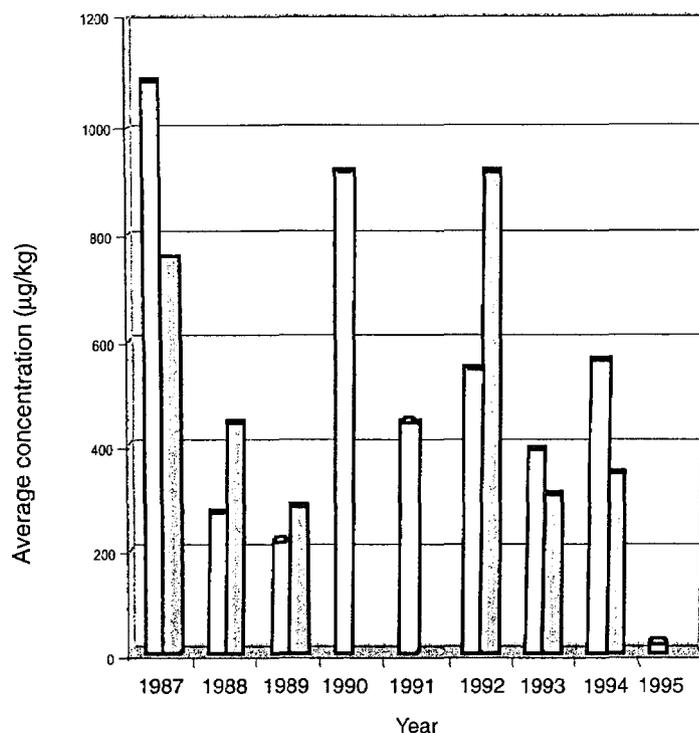
Although there were annual variations, the mean concentrations of deoxynivalenol in maize in Argentina, Canada, and Uruguay over the past 5 years were < 120 µg/kg (Appendix A). Oats and barley also showed annual variations in contamination with

**Figure 6. Annual variation in concentration of deoxynivalenol in wheat in Argentine wheat, 1985, 1986, 1989, 1990–94, 1996–1999**



From Quiroga et al. (1995), González et al. (1996), Dalcero et al. (1997), and Resnik & Pacin (2000)

**Figure 7. Annual variation in the average concentration of deoxynivalenol in maize in Canada (light bars) and New Zealand (dark bars), 1987–94**



Data from Lauren et al. (1991,1996) and Scott (1997). No data were available from New Zealand for 1990,1991, and 1995.

deoxynivalenol (Appendix A). These are clearly observed when the frequency of contamination is high, which has been the case for barley for some years. These results emphasize the need for regular screening for deoxynivalenol in cereal crops. The annual variation observed could be due mainly to heavy rainfall between flowering and harvest, which facilitates *F. graminearum* infection and accumulation of deoxynivalenol (Trigo-Stockli et al., 1998).

## 7. FOOD CONSUMPTION AND DIETARY INTAKE ESTIMATES

The dietary intake of deoxynivalenol was assessed according to the recommendations of a FAO/WHO workshop on methods for assessing exposure to contaminants and toxins, which was held in Geneva in June 2000 (WHO, 2000). The workshop recommended that the median concentration be given when data on individual samples were available, whereas a mean should be given when only pooled or aggregated data were available. In the case of commodities that contribute significantly to intake, distribution curves should be generated to allow risk managers to determine the effects on dietary intake of different maximum levels.

The workshop further recommended that international estimates of dietary intake should be calculated by multiplying the mean or median concentration by the values for consumption of the commodity in the five GEMS/Food regional diets (WHO, 1998). The diets (African, European (which includes Australia, Canada, New Zealand, and the USA), Far Eastern, Latin American, and Middle Eastern) were established on the basis of information on food balance sheets compiled by FAO. As such

information is available for most countries, the data are comparable across countries and regions of the world. The regional diets represent the average availability of food commodities per capita rather than actual food consumption.

The report of the workshop noted that national intake estimates should also be reported when available, as they may provide information about intake by specific population subgroups or extreme consumers, which cannot be derived from GEMS/Food regional diets.

## 7.1 Methods

For this assessment, concentrations of deoxynivalenol in food commodities and in some processed foods were reported to FAO/WHO or were obtained from the literature. The quality and reporting of the data are discussed in the previous section. Since the dietary intakes were based on the GEMS/Food regional diets, which include information on consumption of raw or minimally processed foods, concentrations of deoxynivalenol in processed foods were not used to estimate dietary intake.

Information was available on the concentrations of 10 commodities: barley, maize, popcorn, oats, rice, rye, sorghum, triticale, wheat, and other cereals. Data were received from 28 countries, representing four of the five GEMS/Food regional diets (Table 7); no data were reported for the Middle Eastern diet. Of the 10 commodities for which data were available for the intake assessment, data on barley, maize, and wheat predominated, with limited reports on popcorn, oats, rice, rye, sorghum, triticale, and other cereals (Table 8).

Most of the data available for this evaluation were pooled; that is, each data point represented the mean concentration in a number of individual samples. In calculating the mean values, samples in which the concentration was below the LOQ or LOD were assumed to have a value of zero. The maximum analytical value and the number of samples with concentrations below the LOD or LOQ were also reported for each data point. A total of 375 data points (mean values) representing about 23 000 individual samples were included in the intake assessment (Table 8). Of those 375 data points, 243 were reported from countries represented by the

**Table 7. Countries for which information on deoxynivalenol concentrations were available (by GEMS/Food regional diet)**

African	European	Far Eastern	Latin American
South Africa	Austria	China	Argentina
	Bulgaria	India	Brazil
	Canada	Indonesia	Chile
	Finland	Japan	Uruguay
	Germany	Korea, Republic of	
	Italy	Papua New Guinea	
	Netherlands	Philippines	
	New Zealand	Thailand	
	Norway	Viet Nam	
	Poland		
	Russian Federation		
	Sweden		
	United Kingdom		
	USA		

**Table 8. Numbers of countries for which data on concentrations of deoxynivalenol were available, by commodity**

Commodity	No. of countries	Data used in intake estimates	
		No. of data points	No. of individual samples represented
Barley	11	33	1 778
Maize	17	78	5 719
Oats	7	28	834
Popcorn	2	2	50
Rice	4	13	203
Rye	4	12	295
Sorghum	1	1	15
Triticale	1	1	10
Wheat	18	209	14 200
Other cereals	2	4	254

GEMS/Food European diet. The remaining 132 data points represented intake of the nine commodities for the other regional diets.

For each commodity, the data were sorted according to the country groupings of the GEMS/Food regional diets. The number of data points reported, the number of individual samples represented, the highest maximum analytical value reported, the proportion of samples with concentrations below the LOD or LOQ, and the average of all mean values are summarized in Table 9. For each commodity, the mean of all data, weighted by sample size, is also reported.

## 7.2 Concentrations in foods

The concentrations of deoxynivalenol used in estimating dietary intakes are summarized by commodity and region in Table 9.

*Barley:* Data on the concentrations of deoxynivalenol in barley were received from 11 countries. Of the 1778 samples analysed, 41% had concentrations below the LOD or LOQ. The unweighted means by region ranged from 130 µg/kg in the Far Eastern diet to 860 µg/kg in the European diet. The weighted mean for all samples combined was 720 µg/kg, and the maximum analytical value reported was 34 000 µg/kg.

*Maize:* Seventeen countries reported data on a total of 5719 samples of maize. Of these, 52% contained concentrations below the LOD or LOQ. The unweighted means across regional diets ranged from 66 µg/kg in the Latin American to 640 µg/kg in the European diet. The weighted mean of all samples combined was 180 µg/kg, and the maximum analytical value reported was 19 000 µg/kg.

*Popcorn:* Only two countries (Argentina and USA) reported data on popcorn. Of the 50 samples, 92% had concentrations below the LOQ. The weighted mean for all samples was 310 µg/kg, and the maximum analytical value reported was 4500 µg/kg.

*Oats:* Seven countries representing only two regional diets submitted data on a total of 834 samples of oats. Most of the data came from countries with the European

**Table 9. Summary of data on concentrations of deoxynivalenol in grains in GEMS/  
Food regional diets**

Commodity	Far Eastern	African	Latin American	European	Total
<i>Barley</i>					
No. of data points	9		9	15	33
No. of individual samples	309		842	627	1 778
Unweighted mean ( $\mu\text{g}/\text{kg}$ )	130		370	860	530
Maximum value ( $\mu\text{g}/\text{kg}$ )	3 800		34 000	26 000	34 000
% < LOD or LOQ	34		53	28	41
Weighted mean, all samples: 718 $\mu\text{g}/\text{kg}$					
<i>Maize</i>					
No. of data points	23	4	21	30	78
No. of individual samples	1 110	683	2 421	1 300	5 719
Unweighted mean ( $\mu\text{g}/\text{kg}$ )	200	130	66	640	330
Maximum value ( $\mu\text{g}/\text{kg}$ )	6 500	2 800	4 300	19 000	19 000
% < LOD or LOQ	35	13	93	11	52
Weighted mean, all samples: 175 $\mu\text{g}/\text{kg}$					
<i>Popcorn</i>					
No. of data points			1	1	2
No. of individual samples			42	8	50
Unweighted mean ( $\mu\text{g}/\text{kg}$ )			0	2 000	980
Maximum value ( $\mu\text{g}/\text{kg}$ )			0	4 500	4 500
% < LOD or LOQ			100	50	92
Weighted mean, all samples: 310 $\mu\text{g}/\text{kg}$					
<i>Oats</i>					
No. of data points			1	27	28
No. of individual samples			6	828	834
Unweighted mean ( $\mu\text{g}/\text{kg}$ )			0	140	130
Maximum value ( $\mu\text{g}/\text{kg}$ )			0	2 600	2 600
% < LOD or LOQ			100	3	32
Weighted mean, all samples: 89 $\mu\text{g}/\text{kg}$					
<i>Rice</i>					
No. of data points			11	2	13
No. of individual samples			173	30	203
Unweighted mean ( $\mu\text{g}/\text{kg}$ )			33	2 600	430
Maximum value ( $\mu\text{g}/\text{kg}$ )			960	9 500	9 500
% < LOD or LOQ			86	43	80
Weighted mean, all samples: 150 $\mu\text{g}/\text{kg}$					
<i>Rye</i>					
No. of data points				12	12
No. of individual samples				295	295
Unweighted mean ( $\mu\text{g}/\text{kg}$ )				39	39
Maximum value ( $\mu\text{g}/\text{kg}$ )				1 300	1 300
% < LOD or LOQ				51	51
Weighted mean, all samples: 65 $\mu\text{g}/\text{kg}$					

Table 9 (contd)

Commodity	Far Eastern	African	Latin American	European	Total
<i>Sorghum</i>					
No. of data points			1		1
No. of individual samples			15		15
Unweighted mean ( $\mu\text{g}/\text{kg}$ )			0		0
Maximum value ( $\mu\text{g}/\text{kg}$ )			0		0
% < LOD or LOQ			100		100
Weighted mean, all samples: 0					
<i>Triticale</i>					
No. of data points				1	1
No. of individual samples				10	10
Unweighted mean ( $\mu\text{g}/\text{kg}$ )				92	92
Maximum value ( $\mu\text{g}/\text{kg}$ )				200	200
% < LOD or LOQ				20	20
Weighted mean, all samples: 92 $\mu\text{g}/\text{kg}$					
<i>Wheat</i>					
No. of data points	19		32	158	209
No. of individual samples	1 354		2 081	10 765	14 200
Unweighted mean ( $\mu\text{g}/\text{kg}$ )	560		490	310	360
Maximum value ( $\mu\text{g}/\text{kg}$ )	20 000		30 000	21 000	30 000
% < LOD or LOQ	19		40	44	38
Weighted mean, all samples: 390 $\mu\text{g}/\text{kg}$					
<i>Other cereals</i>					
No. of data points	1			3	4
No. of individual samples	29			225	254
Unweighted mean ( $\mu\text{g}/\text{kg}$ )	330			56	120
Maximum value ( $\mu\text{g}/\text{kg}$ )	2 300			570	2 300
% < LOD or LOQ	59			55	55
Weighted mean, all samples: 46 $\mu\text{g}/\text{kg}$					

diet; only one record representing six samples (all with concentrations below the LOQ) was reported for the Latin American diet. Thirty-two percent of all samples had concentrations below the LOQ. The maximum analytical value reported was 2600  $\mu\text{g}/\text{kg}$ .

*Rice:* Only four countries representing two regional diets submitted data on rice, representing a total of 203 samples. Overall, 80% of the samples had concentrations below the LOD or LOQ. The maximum analytical value reported was 9500  $\mu\text{g}/\text{kg}$ . The weighted mean concentration in all samples was 150  $\mu\text{g}/\text{kg}$ .

*Rye:* Four countries, all with the European regional diet, submitted data on a total of 295 samples of rye. Just over half (51%) of the samples had concentrations below the LOQ. The maximum analytical value reported was 1300  $\mu\text{g}/\text{kg}$ , and the unweighted mean was 39  $\mu\text{g}/\text{kg}$ ; the weighted mean for all samples was 65  $\mu\text{g}/\text{kg}$ .

*Sorghum*: Only one country reported data on 15 samples of sorghum. Deoxynivalenol was not detected in any of the samples.

*Triticale*: Only one data point representing 10 samples was reported for triticale. The mean value was 92 µg/kg; the maximum analytical value was 200 µg/kg.

*Wheat*: Eighteen countries with three regional diets reported data on 14 200 samples of wheat. Overall, 38% of the samples had concentrations below the LOD or LOQ. A maximum analytical value of 30 000 µg/kg was reported for two sample sets. The unweighted means ranged from 310 µg/kg for the European diet to 560 µg/kg for the Far Eastern. The weighted mean for all samples was 390 µg/kg.

*Other cereals*: Only two countries submitted data on a total of 254 samples of cereal grains other than those specified above. Overall, 55% of the samples had concentrations below the LOD or LOQ. The maximum analytical value reported was 2300 µg/kg. Both the unweighted and the weighted means for all samples were 46 µg/kg.

### **7.3 Estimates of dietary intake at the international level**

The average intakes of deoxynivalenol were calculated by multiplying the weighted mean concentration of each commodity by the corresponding amount of each commodity consumed in each of the five GEMS/Food regional diets (Table 10). As limited data were available on the concentrations of all commodities in diets other than the European one, a mean concentration for each commodity could not be derived for each region.

Intakes were estimated per person per day and converted to intake per kilogram of body weight per day, assuming a body weight of 60 kg, as recommended by FAO/WHO for international intake assessments (WHO, 1985). The results are reported separately for each GEMS/Food regional diet in Tables 11–15. The intakes from the different diets are compared in Table 16.

The total intake of deoxynivalenol in micrograms per kilogram of body weight per day was estimated to be 0.78 from the African diet, 1.2 from the Latin American diet, 1.4 from the European diet, 1.6 from the Far Eastern diet, and 2.4 from the Middle Eastern diet. The main source of intake in Europe, Latin America, and the Middle East was wheat (64–88% of total intake), whereas the sources in the other two regions were more varied: wheat, rice, and maize in the African region and wheat and rice in the Far East. The estimates of average intake were based on the assumption that consumers choose foods randomly with respect to the distribution of concentrations of contaminants and will, therefore, be exposed to an approximation of the mean of that distribution over time. It should be noted that any reduction in the concentration of deoxynivalenol as a result of processing has not been taken into consideration in this assessment.

The distribution of dietary intake could not be assessed from the available data. Nonetheless, high intakes can be approximated by multiplying the average intake by a factor of two for a single food and by a factor of three for the total diet (WHO, 1985).

**Table 10. Grain consumption (g per person per day) in GEMS/Food regional diets**

Commodity	African	European	Far Eastern	Latin American	Middle Eastern
Barley	1.8	20	3.5	6.5	1
Maize	110	8.8	31	42	48
Oats	0.2	2	0	0.8	0
Popcorn	0.2	0.2	0.2	0.2	0.2
Rice	100	12	280	86	49
Rye	0	1.5	1	0	0
Sorghum	27	0	9.7	0	2
Triticale	0	0	1	0	0
Wheat	28	180	110	120	330
Other cereals	0	4.3	1.3	0	0.8

**Table 11. Estimated intakes of deoxynivalenol from the GEMS/Food African diet**

Commodity	Weighted mean concentration ( $\mu\text{g}/\text{kg}$ )	Consumption (g/person per day)	Intake			% of total intake
			ng/person per day	$\mu\text{g}/\text{person}$ per day	$\mu\text{g}/\text{kg}$ bw per day	
Barley	720	1.8	1 300	1.3	0.022	3
Maize	180	110	19 000	19	0.31	40
Oats	89	0.2	18	0	< 0.001	< 1
Popcorn	310	0.2	62	0.1	0.001	< 1
Rice	150	100	16 000	16	0.26	33
Rye	65	0	0	0	0	0
Sorghum	0	27	0	0	0	0
Triticale	92	0	0	0	0	0
Wheat	390	28	11 000	11	0.18	24
Other cereals	46	0	0	0	0	0
Total intake			46 000	46	0.78	100

**Table 13. Estimated intakes of deoxynivalenol from the GEMS/Food European diet**

Commodity	Weighted mean concentration ( $\mu\text{g}/\text{kg}$ )	Consumption (g/person per day)	Intake			% of total intake
			ng/person per day	$\mu\text{g}/\text{person}$ per day	$\mu\text{g}/\text{kg}$ bw per day	
Barley	720	20	14 000	14	0.24	16
Maize	180	8.8	1 500	1.5	0.026	2
Oats	89	2.0	180	0.2	0.003	< 1
Popcorn	310	0.2	62	0.1	0.001	< 1
Rice	12	12	1 800	1.8	0.030	2
Rye	65	1.5	97	0.1	0.002	< 1
Sorghum	0	0	0	0	0	0
Triticale	92	0	0	0	0	0
Wheat	390	180	69 000	69	1.2	79
Other cereals	46	4.3	200	0.2	0.003	< 1
Total intake			87 000	87	1.4	100

**Table 15. Estimated intakes of deoxynivalenol from the GEMS/Food Far Eastern diet**

Commodity	Weighted mean concentration (µg/kg)	Consumption (g/person per day)	Intake			% of total intake
			ng/person per day	µg/person per day	µg/kg bw per day	
Barley	720	3.5	2 500	2.5	0.042	3
Maize	180	31	5 500	5.5	0.091	6
Oats	89	0	0	0	0	0
Popcorn	310	0.2	62	0.1	0.001	< 1
Rice	150	280	42 000	42	0.70	44
Rye	65	1.0	65	0.1	0.001	< 1
Sorghum	0	9.7	0	0	0	0
Triticale	92	1.0	92	0.1	0.002	< 1
Wheat	390	110	45 000	45	0.74	47
Cereal, other	46	1.3	60	0.1	0.001	< 1
Total intake			95 000	95	1.6	100

**Table 12. Estimated intakes of deoxynivalenol from the GEMS/Food Latin American diet**

Commodity	Weighted mean concentration (µg/kg)	Consumption (g/person per day)	Intake			% of total intake
			ng/person per day	µg/person per day	µg/kg bw per day	
Barley	720	6.5	4 700	4.7	0.078	7
Maize	180	42	7 300	7.3	0.12	10
Oats	89	0.8	71	0.1	0.001	< 1
Popcorn	310	0.2	62	0.1	0.001	< 1
Rice	150	86	13 000	13	0.22	18
Rye	65	0	0	0	0	0
Sorghum	0	0	0	0	0	0
Triticale	92	0	0	0	0	0
Wheat	390	120	45 000	45	0.76	64
Other cereals	46	0	0	0	0	0
Total intake			70 000	70	1.2	100

**Table 14. Estimated intakes of deoxynivalenol from the GEMS/Food Middle Eastern diet**

Commodity	Weighted mean concentration (µg/kg)	Consumption (g/person per day)	Intake			% of total intake
			ng/person per day	µg/person per day	µg/kg bw per day	
Barley	718	1.0	720	0.7	0.012	1
Maize	175	48	8 500	8.5	0.14	6
Oats	89	0	0	0	0	0
Popcorn	312	0.2	62	0.1	0.001	0
Rice	150	49	7 300	7.3	0.12	5
Rye	65	0	0	0	0	0
Sorghum	0	2.0	0	0	0	0
Triticale	92	0	0	0	0	0
Wheat	388	330	127 000	130	2.1	88
Other cereals	46	0.8	37	< 0.1	0.001	< 1
Total intake			144 000	140	2.4	100

**Table 16. Comparison of intakes of deoxynivalenol in the GEMS/Food regional diets**

Commodity	African		European		Far Eastern		Latin American		Middle Eastern	
	µg/kg bw	% total intake	µg/kg bw	% total intake	µg/kg bw	% total intake	µg/kg bw	% total intake	µg/kg bw	% total intake
Barley	0.022	3	0.24	16	0.042	3	0.078	7	0.012	1
Maize	0.31	40	0.026	2	0.091	6	0.12	10	0.14	5.9
Oats	< 0.001	< 1	0.003	< 1	0	0	0.001	< 1	0	0
Popcorn	0.001	< 1	0.001	< 1	0.001	< 1	0.001	< 1	0.001	0
Rice	0.26	33	0.03	2	0.70	44	0.22	18	0.12	5
Rye	0	0	0.002	< 1	0.001	< 1	0	0	0	0
Sorghum	0	0	0	0	0	0	0	0	0	0
Triticale	0	0	0	0	0.002	< 1	0	0	0	0
Wheat	0.18	24	1.2	79	0.74	47	0.76	64	2.1	88
Other cereals	0	0	0.003	< 1	0.001	< 1	0	0	0.001	< 1
Total intake	0.78	100	1.4	100	1.6	100	1.2	100	2.4	100

#### 7.4 National estimates of dietary intake

Information on food consumption patterns or deoxynivalenol intakes was submitted by Argentina, Norway, Sweden, the United Kingdom, and the USA. When only data on food consumption were reported, the weighted mean concentrations of deoxynivalenol were used to estimate dietary intake.

Information on the consumption of maize meal was reported from Argentina. The intake of deoxynivalenol was calculated by multiplying these amounts by the weighted concentration in maize (Table 17) (Solovey et al., 1999).

**Table 17. Estimated intake of deoxynivalenol from maize meal in Argentina, assuming a deoxynivalenol concentration of 180 µg/kg (weighted mean of all data)**

Population	Body weight (kg)	Eaters only			Per capita		
		Maize meal consumption (g/person per day) <sup>a</sup>	Deoxynivalenol intake µg/person per day	µg/kg bw	Maize meal consumption (g/person per day) <sup>a</sup>	Deoxynivalenol intake µg/person per day	µg/kg bw
Males, 1–5 years	14	200	35	2.5	15	2.7	0.19
Males, 15–25 years	61	230	40	0.66	7.5	1.3	0.02
Males, 26–55 years	78	250	44	0.56	20	3.5	0.04
Females, 1–5 years	14	200	35	2.5	13	2.3	0.17
Females, 15–25 years	56	220	38	0.68	11	1.9	0.03
Females, 26–55 years	58	220	38	0.65	12	2.0	0.04

<sup>a</sup> Data from Solovey et al. (1999)

Norway submitted information on the consumption of oats, rye, and wheat by eight population subgroups, and deoxynivalenol intake was calculated from the weighted mean concentrations in these commodities (Table 18). Estimates of deoxynivalenol intake from grain were provided for two population subgroups in Sweden (Table 19).

The United Kingdom provided the mean, median, and 97.5th percentile consumption of grains by two population subgroups. The intakes of deoxynivalenol were calculated from the weighted mean concentrations in each of the grains (Table 20).

Table 21 shows the intake of deoxynivalenol from grain in the USA. The intakes were based on data on consumption collected in a nationwide survey (US Department of Agriculture, 1996). The amount of each grain (barley, maize, oats, popcorn, rice, rye, and wheat) consumed was multiplied by the weighted mean concentration of deoxynivalenol. The total intake is the sum of the intakes from all grains.

## **8. PREVENTION AND CONTROL**

### **8.1 Pre-harvest control**

The occurrence of deoxynivalenol is associated primarily with *F. graminearum* (*Gibberella zeae*) and *F. culmorum*, which cause *Fusarium* head blight in wheat and *Gibberella* ear rot in maize. *F. graminearum* grows optimally at a temperature of 25 °C and will grow down to about 0.88 water activity ( $w_a$ ). *F. culmorum* grows optimally at 21 °C and will grow down to 0.87  $w_a$ . The geographical distribution of the two species appears to be related to temperature (Pitt & Hocking, 1997).

The incidence of *Fusarium* head blights in grain is most closely related to moisture at the time of flowering (anthesis), and the timing of rainfall, rather than the amount of rain, is the most critical factor. All *Fusarium* spp. can survive saprophytically on crop debris, and this is considered to be the principal reservoir of inoculum; other important sources are grass and broad-leaved weeds (for reviews, see Miller, 1994; Parry et al., 1995; D'Mello et al., 1997). A direct relationship between the incidence of ear blight and the deoxynivalenol contamination of wheat has been established (Snijders & Perkowski, 1990; Dill-Macky & Jones, 2000). Consequently, measures taken to control or minimize *Fusarium* infection will also reduce the formation of deoxynivalenol. Such measures include culture techniques, growing resistant cultivars, and use of fungicides or biological antagonists. The measures were summarized by Parry et al. (1995) and are briefly described below.

Culture control techniques include suitable crop rotation, appropriate use of fertilizers, irrigation, and weed control. Maize–wheat rotation increases the incidence of *Fusarium* head blight and should be avoided, whereas removal or ploughing in of crop debris reduces the incidence in wheat. Direct drilling or minimal cultivation increases the risk of infection when *Fusarium*-contaminated debris is present. High concentrations of nitrogen fertilizer may increase plant water stress, but the effect on *Fusarium* head blight is unclear. Effective weed control may be useful in reducing *Fusarium* inoculum, but the efficacy of weed control in reducing *Fusarium* head blight is debated. Irrigation may avoid water stress and reduce the severity of

**Table 18. Intake of deoxynivalenol from grains in Norway**

Population	Food	Deoxynivalenol concentration <sup>a</sup> (µg/kg)	Body weight (kg)	Median consumption			95th percentile consumption		
				Grain (g/person per day)	Deoxynivalenol		Grain (g/person per day)	Deoxynivalenol	
					µg/person per day	µg/kg bw per day		µg/person per day	µg/kg bw per day
Males and females, 6 years	Oats	89	23	6.2	0.55	0.02	26	2.3	0.10
	Rye	65	23	13	0.82	0.04	25	1.6	0.07
	Wheat	390	23	180	69	3.0	380	150	6.4
Males and females, 10 years	Oats	89	35	8.2	0.73	0.02	34	3.0	0.09
	Rye	65	35	16	1.0	0.03	32	2.1	0.06
	Wheat	390	35	230	89	2.5	490	190	5.4
Males, 16–29 years	Oats	89	75	7.5	0.67	0.01	76	6.8	0.09
	Rye	65	75	15	1.0	0.01	31	2.0	0.03
	Wheat	390	75	280	110	1.4	700	270	3.6
Males, 30–59 years	Oats	89	83	7.7	0.69	0.01	63	5.6	0.067
	Rye	65	83	14	0.93	0.01	28	1.8	0.02
	Wheat	390	83	240	92	1.1	570	220	2.6
Males, 60–79 years	Oats	89	79	6.5	0.58	0.01	67	6.0	0.08
	Rye	65	79	13	0.84	0.01	25	1.6	0.02
	Wheat	390	79	190	75	0.95	720	280	3.5

**Table 18** (contd)

Population	Food	Deoxynivalenol concentration <sup>a</sup> (µg/kg)	Body weight (kg)	Median consumption			95th percentile consumption		
				Grain (g/person per day)	Deoxynivalenol		Grain (g/person per day)	Deoxynivalenol	
					µg/person per day	µg/kg bw per day		µg/person per day	µg/kg bw per day
Females, 16–29 years	Oats	89	63	6.3	0.56	0.01	45	4.0	0.06
	Rye	65	63	11	0.72	0.01	19	1.2	0.02
	Wheat	390	63	190	76	1.2	440	170	2.7
Females, 30–59 years	Oats	89	65	5.8	0.52	0.01	46	4.1	0.06
	Rye	65	65	10	0.66	0.01	18	1.2	0.02
	Wheat	390	65	170	68	1.0	390	150	2.3
Females, 60–79 years	Oats	89	69	5.1	0.46	0.01	56	5.0	0.07
	Rye	65	69	10	0.65	0.01	17	1.1	0.02
	Wheat	390	69	160	61	0.88	360	140	2.0

Sources of information on food consumption and body weight: children 6 and 10 years, Norkost (1997); males and females ≥ 16 years, Langseth et al. (2000)

<sup>a</sup> Weighted mean of all data

**Table 19. Intake of deoxynivalenol from cereals by children and adults in Sweden (eaters only)**

Population	Food	Consumption (g/person per day)		Deoxynivalenol concentration (µg/kg)	Deoxynivalenol intake (ng/kg bw per day) <sup>a</sup>	
		Mean	95th percentile		Mean	95th percentile
Children, 7–14 years	Wheat	76	120	70	140	230
	Rye	27	58	14	10	22
	Oats	28	64	8.5	6.3	14
Adults	Wheat	78	140	70	78	140
	Rye	36	77	14	7.5	16
	Oats	34	75	8.5	4.1	9.1

From Olsen et al. (1998)

<sup>a</sup> Based on individual body weights

*Fusarium* foot rot in wheat, which may serve as an inoculum for the development of head blight. Overhead irrigation has been shown to increase the severity of the disease.

Differences between cultivars in susceptibility to *Fusarium* head blight has been recognized for more than 100 years. Most cultivars of wheat are susceptible, and only a few are moderately resistant. Few reports of immune species exist. Limited work has been done on breeding resistance into species other than wheat.

The effect of previous crop residues and tillage on *Fusarium* head blight in wheat were examined by Dill-Macky & Jones (2000), who confirmed that the incidence and severity of the disease were greatest when wheat followed maize and least when wheat followed soya beans. In addition, the incidence and severity were lower in moldboard plow plots than in either chisel (reduced-till) or no-till plots. They suggested that changes in tillage practices, principally the move to conservation tillage and reduced-till systems, contributed to the recent epidemics of *Fusarium* head blight in midwestern USA.

Experimental work in plots revealed significant differences in the activity of fungicides against the *Fusarium* head blight pathogens (D'Mello et al., 1998). Those that effectively controlled the mycotoxin-producing pathogens also decreased the concentrations of deoxynivalenol in grain. Other reports indicated, however, that use of certain fungicides under certain circumstances increased the toxin concentration in the grain (Milus & Parsons, 1994). Homdork et al. (2000) found that application of tebuconazole, which was also used by Milus & Parsons (1994), before infection allowed good control of *Fusarium* head blight and reduced the formation of deoxynivalenol. Jennings et al. (2000) found that tebuconazole, metconazole, and carbendazim effectively controlled the mycotoxin-producing *F. graminearum* and *F. culmorum*. Use of these products also decreased the concentrations of deoxynivalenol in the grain. Azoxystrobin appeared to be less effective and even stimulated deoxynivalenol production.

**Table 20. Estimated intake of deoxynivalenol in the United Kingdom (eaters only)**

Population	Food	Deoxynivalenol concentration <sup>a</sup> (µg/kg)	% eaters	Consumption (g/person per day) <sup>b</sup>			Deoxynivalenol intake (µg/person per day)		
				Mean	Median	97.5th percentile	Mean	Median	97.5th percentile
Children, 1.5–4.5 years	Barley	720	< 1	1.1	0.6	2.1	0.79	0.43	1.5
	Maize	180	66	10	6.6	37	1.8	1.2	6.4
	Oats	89	25	4.1	2.2	18	0.37	0.20	1.6
	Rye	65	1	2.0	1.7	4.9	0.13	0.11	0.32
	Wheat	390	99	47	45	100	18	17	39
Adults, 16–64 years	Barley	720	< 1	4.9	4.7	8.7	3.5	3.4	6.2
	Maize	180	50	12	7.6	50	2.2	1.3	8.8
	Oats	89	25	12	7.6	41	1.0	6.8	3.6
	Rye	65	9	7.4	3.7	39	0.48	2.4	2.5
	Wheat	390	99	130	120	250	49	47	97

<sup>a</sup> Weighted mean of all data<sup>b</sup> From Gregory et al. (1990, 1992)

**Table 21. Estimated intake of deoxynivalenol from grains in the USA (3-day average)**

Population	Intake									
	µg/person per day					µg/kg bw per day <sup>a</sup>				
	Mean	50th percentile	90th percentile	95th percentile	97.5th percentile	Mean	50th percentile	90th percentile	95th percentile	97.5th percentile
Males and females, 1–6 years	25	25	40	45	51	1.5	1.4	2.3	2.7	3.0
Males and females, 7–12 years	35	33	53	60	66	1.0	0.97	1.6	1.8	2.0
Males, 13–19 years	46	42	71	80	90	0.72	0.65	1.2	1.4	1.5
Females, 13–19 years	35	31	53	61	80	0.63	0.55	1.0	1.2	1.5
Males, ≥ 20 years	53	44	95	120	150	0.66	0.54	1.2	1.5	1.9
Females, ≥ 20 years	31	29	53	62	71	0.49	0.43	0.85	1.0	1.2

From US Department of Agriculture (1996); grains comprised barley, maize, oats, popcorn, rice, rye, and wheat

<sup>a</sup> Based on reported body weights

Parry et al. (1995) suggested that biological control measures could be a useful alternative to fungicide treatment, since the period during which the cereals are sensitive to the disease is short. There are few reports of such control of *Fusarium* head blight, and none seems to have been used in practice. Experimental use of biological control against diseases attributed to *Fusarium* spp. has been reported (Kempf & Wolf, 1989; Mao et al., 1998; Hoefnagels & Linderman, 1999).

## 8.2 Decontamination

Numerous chemicals have been tested for their ability to decontaminate trichothecene-contaminated grain or feed. Sodium bisulfite treatment of grain results in the greatest reduction in deoxynivalenol (Young et al., 1986). The concentrations of the reagents used and the treatment time appeared to affect the reduction; however, the concentrations would not be suitable for baked products for human consumption as they would change the rheological properties of the flour. Flour derived from sodium bisulfite-tempered wheat contained only low concentrations of deoxynivalenol, but the baking of that flour increased the amount of deoxynivalenol. This result was due partially to the fact that sodium bisulfite reacts with deoxynivalenol to form deoxynivalenol-sulfur adducts, which are unstable to high temperatures and basic pH. Accerbi et al. (1999) investigated the effect of combining sodium bisulfite and extrusion processing on deoxynivalenol concentrations in wheat grain and milled fractions. The extrusion process did not change the concentrations from those in unextruded milled flour or wholemeal flour.

Gaseous chemicals have also been tested for their ability to decontaminate deoxynivalenol-contaminated maize and wheat. In the laboratory, chemical treatment with moist ozone, ammonia, and microwave and convection heat treatment reduced the deoxynivalenol concentrations in mouldy grain (Young et al., 1986).

Treatment of deoxynivalenol in methanol with hypochlorite bleach containing added sodium hydroxide gave rise to a single major product, the 9 $\alpha$ ,10 $\alpha$ ,12 $\beta$ ,13 $\beta$ -diepoxy-8,15-hemiketal (Burrows & Szafraniec, 1987).

Natural and modified clay minerals (e.g. bentonite, zeolithe, and diatomite) showed little or no binding to deoxynivalenol, in contrast to their extensive binding to aflatoxin B<sub>1</sub> (Thimm et al., 2000).

The chemical binding agent polyvinylpyrrolidone had no effect on the reduced feed intake and body-weight gain of pigs fed diets containing deoxynivalenol (Friend et al., 1984).

## 9. COMMENTS

### *Toxicological studies*

Deoxynivalenol is metabolized in particular by de-epoxidation and glucuronidation, generally to less toxic metabolites.

It may have adverse health effects after single, short-term, or long-term administration. After single administration, deoxynivalenol has two characteristic

toxicological effects: decreased feed consumption (anorexia) and emesis (vomiting). Both effects have been linked to increased central serotonergic activity. Single doses of deoxynivalenol also damage rapidly dividing cells, such as those of the gastrointestinal tract. These characteristic effects have been observed with other trichothecenes, although differences in potency were seen.

Many early studies were conducted in which deoxynivalenol-contaminated cereal was incorporated into the feed of livestock. In later studies, purified deoxynivalenol was generally administered to experimental animals. In studies in livestock, feed naturally contaminated with deoxynivalenol tended to be more toxic than feed to which purified deoxynivalenol had been added. This result was attributed to the presence of additional fungal metabolites. Low concentrations of zearalenone or the 3- or 15-acetyldeoxynivalenol precursors were found in some cases.

After short- or long-term administration, one of the most consistent effects observed in most species was reduced growth. This was often the most sensitive parameter in routine studies of toxicity. At higher doses, the thymus, spleen, heart, and liver were affected. In a 2-year study in mice, a slight reduction in body weight observed at the lowest dose (0.1 mg/kg bw per day) was considered not to be biologically significant. Since no other changes were seen at this dose, the NOEL was 0.1 mg/kg bw per day.

A working group convened by IARC in 1993 placed deoxynivalenol in Group 3, 'not classifiable as to its carcinogenicity to humans'. A study of carcinogenicity in mice conducted since that time showed fewer tumours of the liver in treated male mice than in controls. The Committee concluded that the lower incidence was due to the reduced body-weight of the treated animals. No significant difference in tumour incidence was seen in female mice.

Deoxynivalenol was not mutagenic in bacteria, but chromosomal aberrations were observed both in vitro and in vivo, suggesting that deoxynivalenol is genotoxic. However, in the one study conducted in vivo, most of the aberrations consisted of gaps, and the overall significance of the results was considered to be equivocal.

Deoxynivalenol was teratogenic but not maternally toxic when given to pregnant mice at 5 mg/kg bw per day by gavage over a short critical period (days 8–11) of gestation, but not when given at 2.5 mg/kg bw per day. When deoxynivalenol was administered in the feed, the NOEL for maternal toxicity and fetotoxicity was 0.38 mg/kg bw per day.

The results of two studies in mice suggested that deoxynivalenol can suppress host resistance to *Listeria monocytogenes* and *Salmonella enteritidis*, with a NOEL of 0.25 mg/kg bw per day in the first study and a LOEL of 0.12 mg/kg bw per day in the second. Antibody responses were also affected by deoxynivalenol, the NOEL being 1 mg/kg bw per day in mice. In pigs given naturally contaminated feed, the NOEL was 0.08 mg/kg bw per day.

#### *Observations in humans*

Many outbreaks of acute human disease involving nausea, vomiting, gastrointestinal upset, dizziness, diarrhoea, and headache have been reported in Asia. These outbreaks have been attributed to consumption of *Fusarium*-contaminated grains and, more recently, to the presence of deoxynivalenol at reported concentrations of 3–93 mg/kg in grain for human consumption. Occasionally, other trichothecenes were present as well, but at much lower incidence and much lower

concentrations. When the contaminated food was replaced with uncontaminated food, the signs and symptoms disappeared. In one study, these effects were not observed after consumption of grain containing deoxynivalenol at reported concentrations of 0.4–13 mg/kg, but there may have been underreporting or false-negative results at the higher concentrations. In two studies, none of the health effects described above were observed after consumption of grain containing deoxynivalenol at 0.02–3.5 mg/kg. Most of the studies on acute effects in humans were population-based or ecological studies.

#### *Sampling protocols and analytical methods*

Studies of variations during sampling for deoxynivalenol have been reported. In one study, 225-kg bulk samples were collected from six batches of barley, and each was riffle-divided into 16 test samples of 0.1 kg, 16 test samples of 0.8 kg, and 16 test samples of 7 kg and analysed. The results indicated that the variation associated with sample preparation and the analytical steps were of greater significance than the sampling variance for all sizes of test sample and that the variation was not substantially reduced by increasing the test sample size. In another study with a similar approach to studying variance in sampling, sample preparation, and analysis associated with the determination of deoxynivalenol in wheat, a 20-kg bulk sample was taken from each of 24 commercial batches, and each bulk sample was riffle-divided into 32 test samples of 0.45 kg each. For a batch concentration of deoxynivalenol of 5.0 mg/kg, the coefficient of variation was 6.3% for sampling, 10% for sample preparation, and 6.3% for analytical steps. The total variation was 13%. The low variation associated with the sampling step (relative to that for other mycotoxins and other commodities) is due partly to the high kernel count of wheat (about 30 kernels per gram), which is about 10 times higher than that of shelled maize and 30 times higher than that of shelled peanuts.

Official methods and other validated methods have been developed for the analysis of deoxynivalenol in cereals and foodstuffs. The introduction of improved clean-up columns based on charcoal, alumina, and modified diatomaceous earth before determination by TLC, gas, or liquid chromatography has simplified and accelerated the analysis of deoxynivalenol. Use of these columns in combination with GC and ECD or MS detection after derivatization of deoxynivalenol is the commonest technique for quantification. This technique allows simultaneous determination of deoxynivalenol and other trichothecenes at concentrations of a few nanograms per gram, even in complex food matrices. However, matrix problems may occur in GC analysis. LC with fluorescence detection after post-column derivatization or UV detection in combination with rigorous clean-up is a suitable alternative. LC with MS detection can be used for direct, simultaneous determination of several trichothecenes, but its high cost prohibits its routine use. TLC, particularly high performance, is still a convenient method for quantifying deoxynivalenol. TLC and ELISA methods are also good means for screening for deoxynivalenol.

Interlaboratory comparisons clearly showed that further improvements are needed in analytical methods for deoxynivalenol with respect to recovery, accuracy, and precision of measurements. Wider availability of reference materials for deoxynivalenol and regular international comparative studies are needed to improve internal and external quality assurance.

*Levels and patterns of contamination of food commodities*

Data on the concentrations of deoxynivalenol in food commodities were received from Argentina, Brazil, Canada, China, Finland, Germany, Italy, the Netherlands, Norway, Sweden, the United Kingdom, Uruguay, and the USA and were also obtained from the literature. Gas chromatography with electron capture or mass spectrometric detection was the commonest technique used for the quantification of deoxynivalenol, followed in order by thin-layer chromatography, liquid chromatography, and ELISA. Data were excluded from the evaluation when no information was provided on the analytical method or sampling protocol. The remaining data were used only if the samples had been collected at random and if the analytical methods used were considered to be adequate.

Deoxynivalenol was found to be a frequent contaminant of cereal grains, such as wheat (11 444 samples, 57% positive), maize (5349 samples, 41% positive), oats (834 samples, 68% positive), barley (1662 samples, 59% positive), rye (295 samples, 49% positive), and rice (154 samples, 27% positive). It was also detected in buckwheat, popcorn, sorghum, triticale and in some processed food products such as wheat flour, bread, breakfast cereals, noodles, baby and infant foods, and cooked pancakes. In addition, it has been reported in barley products, malt, and beer. The mean concentrations in data sets in which samples containing deoxynivalenol were found were 4–9000 µg/kg for barley, 3–3700 µg/kg for maize, 4–760 µg/kg for oats, 6–5100 µg/kg for rice, 13–240 µg/kg for rye, and 1–5700 µg/kg for wheat.

The submitted data showed wide annual variation in the deoxynivalenol concentrations in most of the cereals tested. These results emphasize the need for regular screening for deoxynivalenol in cereal crops.

Carry-over of deoxynivalenol to food products of animal origin does not appear to be of concern because animals refuse feed when the mycotoxin is present at high concentrations, and deoxynivalenol undergoes rapid metabolism and elimination in livestock species.

*Food consumption/dietary intake assessment*

The average intake of deoxynivalenol at the international level can be estimated by multiplying the average concentration by the estimated average food consumption. The GEMS/Food regional diets (Africas, European, Far Eastern, Latin American, and Middle Eastern), which are based on the average consumption of commodities, were used in the dietary intake assessment. Most of the data on mean concentrations of deoxynivalenol that were available for this evaluation were pooled; that is, each data point represented the mean concentration in a number of individual samples.

Data on processed food products were excluded from estimates of dietary intake. A total of 375 data points representing about 23 000 individual samples were included in the assessment. Of these data points, 243 were reported from countries represented by the GEMS/Food European-type diet. The remaining 132 data points represented the nine commodities in the other four geographical regions. As few data were available on the concentrations of deoxynivalenol in all commodities in regions other than Europe, a single mean concentration weighted by sample size was calculated for each commodity from the available data. The weighted mean concentration for each commodity (barley, maize, oats, rice, rye, wheat, popcorn,

sorghum, and triticale) was multiplied by the respective value for consumption in each of the five GEMS/Food regional diets.

The total intake of deoxynivalenol was estimated to range from 0.77 µg/kg bw per day in the African diet to 2.4 µg/kg bw per day in the Middle Eastern diet. The major source of intake in three of the five regional diets (European, Latin American, and Middle Eastern) was wheat (64–88% of total intake), whereas the sources in the other two regional diets were more varied (wheat and rice in the Far Eastern and wheat, rice, and maize in the African diet). These estimates of average intake were based on the assumption that consumers choose foods randomly with respect to the distribution of the concentration of the contaminant and will, therefore have an intake that approximates the mean of that distribution over time. Although it was not possible to estimate high intakes from the available data, they may be approximated by multiplying the average intake by a factor of two for a single food and three for the total diet. Possible reductions in the concentrations of deoxynivalenol resulting from processing were not taken into consideration in this assessment.

In general, more data on the occurrence of deoxynivalenol in food products are required for better estimates of intake. The Committee noted that the distribution of concentrations of contaminants in processed products might differ from that in raw cereals; contamination tends to be more homogeneous after processing. Despite the uncertainty associated with the data on both concentration and food consumption, they provide useful preliminary estimates of contamination and intake at the international level.

#### *Prevention and control*

Preharvest measures to control *Fusarium* infection can also reduce the formation of deoxynivalenol. Reducing the inoculum of *Fusarium* in host debris and other reservoirs in the field seems to be one important control measure. Consequently, reduced tillage seems to increase the concentrations of deoxynivalenol in subsequent crops. Crop rotation is also important in reducing the inoculum, and rotation of wheat and maize with non-host crops has been recommended. Use of appropriate fungicides and the timing of their application is another important measure for controlling *Fusarium* head blight. Good agricultural practice, such as immediate drying after harvest and proper storage, prevents further contamination with deoxynivalenol.

Physical, chemical, and biological methods have been used to decontaminate grains containing trichothecenes. Some of the treatments reduce the concentration of toxin, while others are ineffective. Cleaning methods, such as gravity separation and washing procedures, can reduce the concentrations of deoxynivalenol in wheat and maize. The effectiveness of milling practices for reducing the concentration of deoxynivalenol in flour depends to a large extent on the degree of fungal penetration of the endosperm. Thermal processing is usually ineffective. Chemical and biological decontamination processes cannot yet be applied on a commercial scale.

## **10. EVALUATION**

The results of a 2-year feeding study in mice did not suggest that deoxynivalenol presents a carcinogenic hazard. The Committee considered that this study was

appropriate for evaluation of other long-term effects. Although the mean body weight of animals at the lowest dose was lower than that of controls, the difference was considered not to be biologically significant, and no toxicological changes were observed at this dose. The Committee established a provisional maximum tolerable daily intake (PMTDI) of 1 µg/kg bw on the basis of the NOEL of 100 µg/kg bw per day in this study and a safety factor of 100. The Committee concluded that intake at this level would not result in effects of deoxynivalenol on the immune system, growth, or reproduction.

The Committee recognized that deoxynivalenol can cause outbreaks of acute illness in humans; however, the available data did not permit the establishment of a level below which no acute effects would be expected to occur.

Estimation of the dietary intake of deoxynivalenol on the basis of the single weighted mean concentrations and the GEMS/Food regional diets resulted in values that exceeded the PMTDI for four of the five regional diets. The Committee noted that there was considerable uncertainty in the intake estimates because of uncertainties in the values for concentration and consumption used in the assessment. Furthermore, food processing would be expected to reduce the levels of deoxynivalenol to varying extents, which would result in lower estimates of dietary intake.

#### *Recommendations*

- The results of comparative studies of toxicity and toxicokinetics would help to clarify species differences in sensitivity to deoxynivalenol.
- Studies are needed on the combined effects of deoxynivalenol and other trichothecenes that may be present in human food. As the trichothecenes have similar toxic properties, albeit with different potencies, the Committee recommended that toxic equivalency factors be developed for the trichothecenes, if sufficient data become available. Since deoxynivalenol is the most extensively studied trichothecene, the Committee further recommended that toxic equivalency factors be established relative to deoxynivalenol.
- In view of the widespread human exposure to deoxynivalenol, further studies on the genotoxicity of deoxynivalenol should be conducted, as well as a study of carcinogenicity in a second species (rat).
- More detailed, analytical epidemiological studies of human disease should be conducted in those areas of the world where the presence of scabby wheat or mouldy maize is a cyclic, endemic event. Such data would help to establish a dose–response relationship between the intake of deoxynivalenol (and other trichothecenes) and acute illness and allow the identification of a NOEL based on human data.
- For surveys of concentrations of deoxynivalenol, the accuracy and comparability of analytical measurements of the toxin in processed foods should be improved.
- Additional data on the distribution of contamination and national food consumption patterns, particularly in countries where deoxynivalenol is prevalent, are needed.
- Information on the effects of processing and its impact on levels of contamination by deoxynivalenol are needed for better estimates of dietary intake.
- Better tools should be developed for the prevention of *Fusarium* plant diseases that result in production of deoxynivalenol in cereal crops.

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## Appendix A.

**Results of surveys for deoxynivalenol showing concentrations and distribution of contamination in food commodities**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
<b>Africa</b>											
South Africa	Yellow maize	1993	236	150 <sup>a</sup>		82/1092				P,S, Rava et al. (1996); A, Scott et al. (1986), Truck- sess et al. (1987), Marasas et al. (1979)	Samples collected at harvest from silos in main production zones
	White maize	1994–95	143	150 <sup>a</sup>		157 <sup>b</sup> /2750				P,S, Rava (1996); A, Scott et al. (1986)	Samples collected from mills throughout country
	Yellow maize	1994–95	148	150 <sup>a</sup>		169 <sup>b</sup> /1800					
	Maize products	1994–95	156	150 <sup>a</sup>	92	116/850					
<b>Americas</b>											
Argentina	Maize	1987–89	100	100 <sup>a</sup>	67	129/1200			2	P,Saubois et al. (1992); S, Junta Nacional de Granos (1984), COPANT (1998), Jewers (1987); A, Trucksess et al. (1984)	Samples, 10 kg; analytical sample, 50 g
	Wheat	1985	123	30 <sup>a</sup>	35	571/1730					
		1986	261	30 <sup>a</sup>	82	329/2400				P, Quiroga et al. (1995); S, Apro et al. (1987); A, Truck- sess et al. (1984)	See Trichothecenes Appendix 6
		1989	102	30 <sup>a</sup>	79	47/400	197	23	0		
		1990	159	30 <sup>a</sup>	55	146/672	400	89	0		
		1991	189	30 <sup>a</sup>	142	55/515	200	37	0		
		1992	222	30 <sup>a</sup>	139	75/505	258	72	0		
	Wheat	1993	40	100 <sup>a</sup>	8	846/4500	2640	32	13	P,S, Dalcero et al. (1997); A, Trucksess et al. (1984)	

Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 100 (µg/kg)	n > 1000 (µg/kg)	References	Sampling procedure
Argentina (contd)	Wheat	1993	44	48	11	2594/30000	8000	33	24	P, González et al. (1996); S, Apro et al. (1987); A, Trucksess et al. (1984)	See Trichothecenes Appendix 6
	Wheat	1993	17	48	3	2147/8000	5200	14	13	S, Resnik & Pacin (2000); P, Pacin & Resnik (2000); A, Trucksess et al. (1984)	See Trichothecenes. Appendix 6. Laboratory sample, 100 g
	Wheat flour	1994	61	48	15	1042/9000	2000	46	23	P,S,A, Pacin et al. (1997)	Purchased at mills (1 kg)
	Wheat	1994	73	48	8	2114/30000	5720	65	38	P,S,A, Pacin et al. (1997); S, Apro et al. (1987)	See Trichothecenes Appendix 6
	Bread	1994	20	17	0	637/2800	1432	20	3	P,S,A, Neira et al. (1997)	Purchased at bakeries; 20 samples of ~25 g
	Maize	1994-95	30	48	30					P, González et al. (1999a); S, Apro et al. (1987); A, Solovey et al. (1999)	See Trichothecenes
	Wheat flour	1995	14	17	0	293/436	410			P,S,A, Neira et al. (1997)	Purchased at bakeries (1 kg)
	Maize	1995	197	48	190	26/2250		6	2	S, Resnik & Pacin (2000); P, Pacin & Resnik (2000); A, Solovey et al. (1999)	See Trichothecenes Appendix 6
	Maize flour	1995	7	48	7					P, Pacin & Resnik(2000); A, Solovey et al. (1999)	
	Wheat flour	1996	5	48	5					Resnik & Pacin (2000); P, Resnik et al. (2000); A, Solovey et al. (1999)	See Trichothecenes Appendix 6
	Maize	1996	59	48	59					Pacin & Resnik (2000); Solovey et al. (1999)	
	Wheat	1996	10	48	10					S, Resnik & Pacin (2000); P, González et al. (1999b); A, Solovey et al. (1999)	See Trichothecenes Appendix 6 ; sample, 1 kg
Wheat	1996	60	48	33	455/6400						

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Argentina (contd)	Barley	1997	3	48	3					P, Pacin & Resnik (2000); A, Solovey et al. (1999)	
	Corn flakes	1997	17	48	17					P,S,A, Solovey et al. (1999)	
	Maize flour	1997	22	48	22						
	Wheat flour	1997	33	17	17	56/193	171	10	0	P,S, Samar et al. (2000); A, Neira et al. (1997)	
	Maize	1997	268	48	268					S, Resnik & Pacin (2000);	See Trichothecenes Appendix 6
	Wheat	1997	52	48	23	698/6000	1920	29	13	P, Pacin & Resnik (2000);	
	Wheat	1998	8	48	3	516/3600		5	1	A, Solovey et al. (1999)	
	Maize	1998	116	48	113	4.6/250		2	0		
	Coffee	1998	3	48	3					P,S, Pacin & Resnik (2000);	
	Oats	1998	6	48	6					A, Solovey et al. (1999)	
	Rice	1998	6	48	6					P,S, Broggi et al. (1999a,b); A,Solovey et al. (1999)	
	Rice	1998	5	48	5					P,S, Pacin & Resnik (2000); A, Solovey et al. (1999)	
	Soya bean	1998	5	17	5					P,S, Boca et al. (2000); A, Neira et al. (1997)	
	Maize	1998	34	48	31	78.4/1000	333	3	2	S, Resnik & Pacin (2000); P, Broggi et al. (2000); A, Solovey et al. (1999)	See Trichothecenes Appendix 6
	Maize	1999	363	48	355	9.4/1000		8	1	S, Resnik & Pacin (2000); P, Resnik et al. (2000); A, Solovey et al. (1999)	See Trichothecenes Appendix 6
	Wheat flour	1999	3	48	0	905/1963		3	1	P,S, Pacin & Resnik (2000); A, Solovey et al. (1999)	
Rice, husked	1999	43	48	43					P,S, Broggi et al. (1999b); A, Solovey et al. (1999)		

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Argentina (contd)	Rice, polished	1999	6	48	6					P,S, Broggi et al. (1999b); A, Solovey et al. (1999)	See Trichothecenes Appendix 6
	Wheat	1999	6	48	6					P, Resnik & Pacin (2000); S, Pacin & Resnik (2000); A, Solovey et al. (1999)	
	Maize flour	1999	4	48	4					P,S, Broggi et al. (1999b); A, Solovey et al. (1999)	
	Maize	1999	73	48	73					P, Broggi et al. (2000); S, Resnik & Pacin (2000); A, Solovey et al. (1999)	See Trichothecenes Appendix 6
	Popcorn	1999	42	48	42					P,S, de Souza et al. (2000); A, Solovey et al. (1999)	Collected at harvest in zigzag from interior of block
	Sorghum	1999	15	48	15					P, Pacin & Resnik(2000); S, Resnik & Pacin (2000); A, Solovey et al. (1999)	See Trichothecenes Appendix 6
	Maize	2000	1025	48	967	35.5/2700		49	10		
	Barley	2000	29	48	29					P,S, Martínez et al. (2000); A, Solovey et al. (1999)	Collected from beer indus- try; sample size, ≥ 1 kg
Beer	1997	50	2	28	14/221	34.7			P,S, Moltó et al (2000); A, Scott et al. (1993)	Collected at markets; sample size, ≥ 2 bottles or cans	
Brazil	Wheat	1990	20	200 <sup>a</sup>	16	110/590	578	4	0	P,S, Furlong et al. (1995); A, Furlong & Valente Soares (1995)	Samples from experimen- tal plots in wheat-growing areas of São Paulo, 3–10 kg; laboratory samples, 1kg
Brazil Wheat	Wheat 2000	1999 108	67 100	100 14	1 198/8500	238/15 950		1 6	1 1	P,S,A, Mallman (2000)	Samples, 200–1000 g

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Brazil (contd)	Wheat and wheat products	1991	38	200 <sup>a</sup>	38					P,S, Soares & Furlani (1996); A, Furlong & Valente Soares (1995)	Samples (1 kg) purchased in organic food shops
Brazil, Paraná	Maize	1994–95	80	111	75	?/542				P, Prado et al. (1997); S, Fonseca (1991); A, Trucksess et al. (1984)	
Brazil, Goiás	Maize	1994–96	8	111	8	8				P, Prado et al. (1997); S, Fonseca (1991); A, Trucksess et al. (1984)	
Canada, Ontario	Soft winter wheat	1979	4	4 <sup>a</sup>	0	60/130				P,S, Scott (1997); A, Scott et al. (1981)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Soft winter wheat	1980	49	10 <sup>a</sup>	1	421/3580					
	Soft winter wheat	1981	101	10 <sup>a</sup>	0	250/3240					
	Soft winter wheat	1982	129	25 <sup>a</sup>	1	744/5670					
	Soft winter wheat	1983	13	30 <sup>a</sup>	7	32/110					
	Soft winter wheat	1984	22	10 <sup>a</sup>	3	129/830					
	Soft winter wheat	1985	45	50–100 <sup>a</sup>	35	15/160					
Canada, Ontario	Soft winter wheat	1986	25	100 <sup>a</sup>	13	274/1730				P,S, Scott (1997); A, Scott et al. (1986)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Soft winter wheat	1987	24	100 <sup>a</sup>	14	313/1650					
	Soft winter wheat	1988	20	100 <sup>a</sup>	20						

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Canada, Ontario	Soft winter wheat	1989	28	100 <sup>a</sup>	13	263/1360				P,S, Scott (1997); A, Scott et al. (1989)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Soft winter wheat	1990	28	100 <sup>a</sup>	19	42/170					
	Soft winter wheat	1991	22	100 <sup>a</sup>	8	204/1160					
	Soft winter wheat	1992	17	100 <sup>a</sup>	8	127/390					
	Soft winter wheat	1993	15	100 <sup>a</sup>	1	317/910					
	Soft winter wheat	1994	18	100 <sup>a</sup>	5	339/1540					
Canada, western	Soft spring wheat	1981	49	30 <sup>a</sup>	24	90/1040				P,S, Scott (1997); A, Scott et al. (1981)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Soft spring wheat	1982	35	5 <sup>a</sup>	27	30/?					
	Soft spring wheat	1983	15	30 <sup>a</sup>	13	7.8/60					
	Soft spring wheat	1984	20	10 <sup>a</sup>	9	49.5/280					
	Soft spring wheat	1985	14	50–100 <sup>a</sup>	9	40/260					
Canada, western	Soft spring wheat	1986	16	100 <sup>a</sup>	14	80/1050				P,S, Scott (1997); A, Scott et al. (1986)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Soft spring wheat	1987	15	100 <sup>a</sup>	12	14/100					
	Soft spring wheat	1988	12	100 <sup>a</sup>	12						

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Canada, western	Soft spring wheat	1989	29	100 <sup>a</sup>	19	52/240				P,S, Scott (1997); A, Scott et al. (1989)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Soft spring wheat	1990	16	50 <sup>a</sup>	10	156/1510					
	Soft spring wheat	1991	15	50 <sup>a</sup>	14	13/190					
	Soft spring wheat	1993	18	50 <sup>a</sup>	5	267/1300					
	Soft spring wheat	1994	4	20 <sup>a</sup>	4						
Canada, western	Hard wheat	1979	19	10–40 <sup>a</sup>	17	4/60				P,S, Scott (1997); A, Scott et al. (1981)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Hard wheat	1980	67	10 <sup>a</sup>	63	1.2/35					
	Hard wheat	1981	66	30 <sup>a</sup>	64	1.8/70					
	Hard wheat	1982	135	30 <sup>a</sup>	135						
	Hard wheat	1983	57	30 <sup>a</sup>	57						
	Hard wheat	1984	201	10 <sup>a</sup>	114	134/10500					
Canada, western	Hard wheat	1985	142	50–100 <sup>a</sup>	109	58/3800				P,S, Scott (1997); A, Scott et al. (1986)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Hard wheat	1986	147	100 <sup>a</sup>	94	267/7120					
	Hard wheat	1987	121	100 <sup>a</sup>	100	80/2100					
Canada, western	Hard wheat	1988	82	100 <sup>a</sup>	82					P,S, Scott (1997); A, Scott et al. (1989)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Hard wheat	1989	80	100 <sup>a</sup>	79	2.4/190					
	Hard wheat	1990	69	50 <sup>a</sup>	69						
	Hard wheat	1991	97	50 <sup>a</sup>	78	92/3380					
	Hard wheat	1992	70	50 <sup>a</sup>	53	75/1170					
	Hard wheat	1993	91	50 <sup>a</sup>	70	136/2800					
	Hard wheat	1994	43	20 <sup>a</sup>	35	108/1800					
Hard wheat	1995	6	20 <sup>a</sup>	4	127/480						

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Canada, Ontario	Maize	1980	43	25 <sup>a</sup>	2	524/2240				P,S, Scott (1997); A, Scott et al. (1981)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Maize	1981	26	25 <sup>a</sup>	0	340/620					
	Maize	1982	36	30 <sup>a</sup>	0	200/880					
	Maize	1983	18	30 <sup>a</sup>	1	170/1190					
	Maize	1984	13	10 <sup>a</sup>	0	510/1020					
	Maize	1985	16	50–100 <sup>a</sup>	2	1172/2280					
Canada, Ontario	Maize	1986	16	100 <sup>a</sup>	4	1072/3050				P,S, Scott (1997); A, Scott et al. (1986)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Maize	1987	18	100 <sup>a</sup>	1	1086/4090					
	Maize	1988	10	100 <sup>a</sup>	7	273/1590					
Canada, Ontario	Maize	1989	11	100 <sup>a</sup>	3	218/650				P,S, Scott (1997); A, Scott et al. (1989)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Maize	1990	15	50–100 <sup>a</sup>	0	920/2200					
	Maize	1991	10	50–100 <sup>a</sup>	2	448/1500					
	Maize	1992	12	100 <sup>a</sup>	0	550/1530					
	Maize	1993	20	100 <sup>a</sup>	8	396/1800					
	Maize	1994	11	100 <sup>a</sup>	3	567/1970					
	Maize	1995	8	100 <sup>a</sup>	7	20/160					
Canada	Wheat foods	1980–81	10	4 <sup>a</sup>	1	63/140				P,S, Scott (1997); A, Scott et al. (1981)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Wheat foods	1982–83	270	25 <sup>a</sup>	119	140/4060					
	Wheat foods	1983–84	155	10–100 <sup>a</sup>	54	143/1150					
	Wheat foods	1984–85	167	10–100 <sup>a</sup>	92	81/1150					
Canada	Wheat foods	1985–86	87	10–50 <sup>a</sup>	56	61/750				P,S, Scott (1997); A, Scott et al. (1986)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Wheat foods	1986–87	91	30–280 <sup>a</sup>	60	126/1600					
	Wheat foods	1987–88	42	100 <sup>a</sup>	33	122/1030					
	Wheat foods	1988–89	55	100 <sup>a</sup>	50	53/1080					
	Wheat foods	1989–90	2	100 <sup>a</sup>	1	110/220					
Canada	Wheat foods	1990–91	14	100 <sup>a</sup>	11	66/700				P,S, Scott (1997); A, Scott et al. (1989)	Collected immediately after harvest or at mills; sample size, 2–5 kg

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Canada	Wheat foods	1991–92	7	50–100 <sup>a</sup>	6	19/130				P,S, Scott (1997); A, Scott et al. (1989)	Collected immediately after harvest or at mills; sample size, 2–5 kg
	Wheat foods	1992–93	57	50–100 <sup>a</sup>	36	192/1700					
	Wheat foods	1993–94	54	20–100 <sup>a</sup>	36	57/400					
	Wheat foods	1994–95	59	20–100 <sup>a</sup>	33	93/1000					
	Wheat foods	1995–96	187	20–100 <sup>a</sup>	124	108/2750					
Canada	Beer	1993	33	0.1 <sup>a,c</sup>	13	3.7/503 <sup>c</sup>	11.4 <sup>c</sup>			P,S,A, Scott et al. (1993)	
Chile	Maize	1995–96	68	10 <sup>a</sup>	68					P,A, Vega et al. (1998); S, Olavarria (1992)	See Trichothecenes Appendix 6
Uruguay	Wheat and by-products	1993–94	40	40 <sup>a</sup>	15	345/4000	667	20	1	P, Piñeiro et al. (1994, 1996), Piñeiro & Silva (1997); Piñeiro (2000); S, FAO (1994), UNEP/FAO/WHO (1988); A, Trucksess et al. (1984), Eppley et al. (1986)	
	Barley and by-products	1993–94	99	40 <sup>a</sup>	58	1220/4000	1200	24	10		
Uruguay	Maize and by-products	1993–94	10	40 <sup>a</sup>	8	158/1500	80		1	P, Piñeiro et al. (1996), Piñeiro & Silva (1997), Piñeiro (2000); S, UNEP/FAO/WHO (1988); FAO (1994); A, Trucksess et al. (1984), Eppley et al. (1986)	
Uruguay	Rice and by-products	1993–94	3	40 <sup>a</sup>	2	100/300			1	P, Piñeiro et al. (1994, 1996), Piñeiro & Silva (1997), Piñeiro (2000); S, UNEP/FAO/WHO (1988), FAO (1994); A, Trucksess et al. (1984), Eppley et al. (1986)	

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure		
Uruguay	Wheat and by-products	1994–95	168	40 <sup>a</sup>	81	297/4000	844	58	13	P, Piñeiro et al (1996), Piñeiro & Silva (1997), Piñeiro (2000); S, UNEP/FAO/WHO (1988), FAO (1994); A, Trucksess et al. (1984), Eppley et al. (1986)			
	Barley and by-products	1994–95	51	40 <sup>a</sup>	45	23/400	80	4					
	Maize and by-products	1994–95	7	40 <sup>a</sup>	4	43/120	100	1					
	Rice and by-products	1994–95	10	40 <sup>a</sup>	8	32/167	152	2					
	Wheat and by-products	1995–96	15	40 <sup>a</sup>	6	164/945	440	6					
	Barley and by-products	1995–96	77	40 <sup>a</sup>	39	54/287	160	18					
	Maize and by-products	1995–96	6	40 <sup>a</sup>	3	33/120	40	1					
	Rice and by-products	1995–96	10	40 <sup>a</sup>	8	15/96	52						
	Wheat and by-products	1996–97	27	40 <sup>a</sup>	3	325/1668	834	17	2				
	Barley and by-products	1996–97	53	40 <sup>a</sup>	16	152/1704	319	18	2				
	Rice and by-products	1996–97	31	40 <sup>a</sup>	18	184/956	574	13					
	Uruguay	Wheat and by-products	1997–98	91	40 <sup>a</sup>	32	316/5536	769	34		6	P, Piñeiro (2000); S, UNEP/FAO/WHO (1988), FAO (1994); A, Trucksess et al. (1984), Eppley et al. (1986)	
		Barley and by-products	1997–98	241	40 <sup>a</sup>	58	1823/344624781		77		60		
Maize and by-products		1997–98	8	40 <sup>a</sup>	1	697/4308	862	3	1				

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples(µg/kg)	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Uruguay	Rice and by-products	1997–98	26	40 <sup>a</sup>	21	29/431	48	2		P, Piñeiro (2000); S, UNEP/FAO/ WHO (1988), FAO (1994); A, Trucksess et al. (1984), Eppley et al. (1986)	
	Wheat and by-products	1998–99	11	20 <sup>a</sup>	1	126/528	225	4			
	Barley and by-products	1998–99	136	20 <sup>a</sup>	62	63/862	161	18			
	Maize and by-products	1998–99	7	20 <sup>a</sup>	3	111/651	81	1			
	Rice and by-products	1998–99	19	20 <sup>a</sup>	19						
	Wheat and by-products	1999–2000	9	20 <sup>a</sup>	3	62/246	173	2			
	Barley and by-products	1999–2000	153	20 <sup>a</sup>	133	4/98	21				
	Maize and by-products	1999–2000	9	20 <sup>a</sup>	4	43/176	84	1			
	Rice and by-products	1999–2000	14	20 <sup>a</sup>	13	6/80					
USA	Maize	1989	8	1000 <sup>a</sup>	1	1575/3000				P,S,A, Abouzied et al. (1991)	Grain-based food prod ucts bought from retail outlets and natural-food shops (unit packages of various sizes)
	Wheat	1989	12	1000 <sup>a</sup>	11	158/1900					
	Rice	1989	4	1000 <sup>a</sup>	1	5100/9500					
	Oats	1989	5	1000 <sup>a</sup>	3	760/2600					
	Wheat and oat biscuits	1989	18	1000 <sup>a</sup>	12	933/5400					
	Maize chips	1989	6	1000 <sup>a</sup>	4	833/3000					
	Popcorn	1989	8	1000 <sup>a</sup>	4	1950/4500					

Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 100 (µg/kg)	n > 1000 (µg/kg)	References	Sampling procedure
USA	Wheat flour muffin mix	1989	17	1000 <sup>a</sup>	4	3059/5800				P,S,A, Abouzied et al. (1991)	Grain-based food products bought from retail outlets and natural-food shops (unit packages of various sizes)
		Maize meal		1989	11	1000 <sup>a</sup>	4	3691/19000			
USA	Mixed-grain Wheat	1989	3	1000 <sup>a</sup>	2	5333/16000				P,S,A, Fernandez et al. (1994)	Samples collected by Federal Grain Inspection, Department of Agriculture
		1991	81	40 <sup>a</sup>	unk	1570 <sup>b</sup> /9330					
USA	Barley Malting barley	1993	118	500	39	3000/14 000				P,S, Trucksess et al. (1995); A, Veratox	Samples collected from 25 states by Federal Grain Inspection, Department of Agriculture. Each consisted of subsamples from various sites, which were pooled and mixed; about 100 g of each composite was sent to the Food and Drug Administration for analysis. Analytical sample, 50 g
		1993	29	500	0	9000/25800					
	Hard spring wheat	1993	201	500	21	3700/18400					
	Hard winter wheat	1993	194	500	100	800/7600					
	Mixed wheat	1993	1	500	0	2300/2300					
	Soft winter wheat	1993	59	500	9	1400/14600					
	Soft white wheat	1993	28	500	20	100/700					
	White flour	1994	89	20 <sup>a</sup>		500/1700		36	3		
USA	White flour	1994	23	20 <sup>a</sup>	23	0		0	0	P,S,A, Trucksess et al. (1996)	
		1994	160	20 <sup>a</sup>		420/2630		105	25		
		1994	54	20 <sup>a</sup>		530/3800		24	12		
		Whole-wheat flour	1994	29	20 <sup>a</sup>		600/1400		7		2
		Whole-wheat flour	1994	7	20 <sup>a</sup>		410/680		5		0

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
USA	Wheat brans	1994	63	20 <sup>a</sup>		940/2580		32	11	P,S,A, Trucksess et al. (1996)	
	Wheat brans	1994	32	20 <sup>a</sup>		600/600		1	0		
	Wheat brans	1994	68	20 <sup>a</sup>		460/2920		49	9		
	Wheat products	1994	5	20 <sup>a</sup>		420/1200		3	0		
	Wheat products	1994	18	20 <sup>a</sup>		0/0		0	0		
USA	Wheat products	1994	14	20 <sup>a</sup>		320/1160		7	2		
USA	Wheat	1996	14	500	1	5719/11 900	11 800	12	10	P,S, Hart (1998); A, Veratox	Samples collected at three elevators and from 14 trucks containing newly harvested wheat by inserting a metal probe (2 m, 3.5 cm outside diameter) at random to remove 500–800 g; 10 probes from each were bagged separately. From five trucks chosen at random, five 50-g subsamples or whole kernels were collected from each of the 10 probes, ground in a coffee grinder and analysed. The remaining grain was milled with a Romer mill at the finest setting to produce a mixture of flour and bran, and a 5–12% continuous substream was extracted from the milled stream.

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
<b>Europe</b>											
Austria	Maize autumn	1996,	85	81 <sup>a</sup>	4	694/2435				P,S, Ellend et al. (1997); A, Weingaertner et al. (1997), Scott et al. (1986)	Samples > 2 kg
Austria	Maize	1996	51	100	2	662/2570				Lew et al. (2000a); A, ELISA	Samples of about 60 kg collected as composites, then homogenized and quartered to 6-kg samples. Ground in a home mill (1 kg)
Austria	Maize	1996 1997 1998	46 58 48	50 50 50	5 20 12	575/2810 91/580 285/1360	1380 190 710	32 25 31	10 0 2	Lew et al. (2000a); A, Lew et al. (2000b)	Samples of about 60 kg collected as composites, then homogenized and quartered to 6-kg samples. Ground in a home mill (1 kg)
Austria	Wheat	1998	15	50	7	52.7/145	123	4	0	Lew et al. (2000a); A, Wein- gärtner et al. (1997)	Samples of about 30 kg collected as composites, then homogenized and quartered to 8-kg samples. Ground in a home mill (1 kg)
Bulgaria	Wheat	1993	44	5 <sup>a</sup>	7	37.9/137	91.5	3	0	P,S, Atanassov et al. (1995); A, Luo et al. (1990)	
Bulgaria	Wheat	1995	140	50 <sup>a</sup>	46	120.6/1800				P,S,A, Vrabcheva et al. (1996), Ridascreen™	Median

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Finland	Oats	1987–88	21	5 <sup>a</sup>	0	168/861				P,S,A, Hietaniemi & Kumpulainen (1991)	2–3-kg samples collected from Finnish State Granaries and private farmers
	Barley	1987–88	30	5 <sup>a</sup>	3	70/202					
	Wheat	1987–88	40	5 <sup>a</sup>	3	75/356					
	Rye	1987–88	31	5 <sup>a</sup>	7	40/93					
Finland	Wheat	1998	31	10	5	29.6/190	57	2	0	P,S,A, Eskola et al. (2000a,b)	See Trichothecenes Appendix 6
	Rye	1998	49	10	9	24.5/144	52	2	0		
	Barley	1998	15	10	6	20.7/55.9	40	0	0		
	Oats	1998	10	10	2	138.6/955	955	1	0		
Germany	Bread and related products Noodles Breakfast cereals Baby and infant foods Rice Cereal foods	Jan–Jun 1998	96	23	16	76.4/788				P,S, Schollenberger et al. (1999); A, Schollenberger et al. (1998)	Samples dried at 40 °C and ground in a home mill (1.5 mm); 25 g taken for analysis
			29	23	2	146.9/1670					
			32	23	14	42/238					
			25	23	10	36.6/314					
			26	23	12	106.5/305					
			29	23	14	71.8/505					
			56	23	4	647/7730	805	43	4		
Germany	White wheat flour (ash content, 40–55 mg/kg)	1999	28	23	0	239/965				P,S, Schollenberger et al. (2000b); A, Schollenberger et al. (1998)	5-kg samples collected at random from stores, mixed, and 10 g taken for analysis

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	n < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	n > 100 (µg/kg)	n > 1000 (µg/kg)	References	Sampling procedure
Germany	White wheat flour (ash con- tent, 105 mg/kg)	1999	13	23	1	216/756				P,S, Schollenberger et al. (2000b); A, Schollenberger et al. (1998)	5-kg samples collected at random from stores, mixed, and 10 g taken for analysis
	White wheat flour (ash con- tent, 160–170 mg/kg)	1999	19	23	0	404/1379					
Germany	Bread, white	1999	55	23	2	176/584				P,S, Schollenberger et al. (2000c); A, Schollenberger et al. (1998)	Samples > 100 g dried at 40 °C and ground in a home mill (1.5 mm); 10 g taken for analysis
	Bread, whole grain	1999	52	23	7	103/690					
	Noodles, white flour	1999	27	23	1	220/1000					
	Noodles, whole-grain flour	1999	12	23	3	791/4840					
Germany	Wheat	1987	84	1 <sup>a</sup>	3	1574/20 538		49	21	P,S, Müller & Schwadorf (1993); A, Schwadorf & Müller (1991)	Samples collected ran- domly 1–4 weeks after harvest from farms by Governmental Advisory Board
Germany	Wheat	1989	78	1–5 <sup>a</sup>	24	105/1187		24	8	P,S, Müller et al. (1997a); A, Schwadorf & Müller (1991)	Samples of 0.7–1 kg col- lected randomly 1–4 weeks after harvest from farms by Governmental Advisory Board
	Wheat	1990	80	1–5 <sup>a</sup>	3	573/8969		59	13		
	Wheat	1991	80	1–5 <sup>a</sup>	3	346/4627		37	7		
	Wheat	1992	78	1–5 <sup>a</sup>	4	318/5412		43	2		
	Wheat	1993	45	1–5 <sup>a</sup>	2	374/6165		22	3		
Germany	Oats	1987	56	3 <sup>a</sup>	18	91.9/1480				P,S, Müller et al. (1998); A, Schwadorf & Müller (1991)	Samples of 0.7–1 kg col- lected randomly 1–4 weeks after harvest from farms by Governmental Advisory Board

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Germany	Oats	1989	56	3 <sup>a</sup>	15	99.7/536				P,S, Müller et al. (1998); A, Schwadorf & Müller (1991)	Samples of 0.7–1 kg col- lected randomly 1–4 weeks after harvest from farms by Governmental Advisory Board
	Oats	1990	54	3 <sup>a</sup>	26	26.8/203					
	Oats	1991	51	3 <sup>a</sup>	26	107.3/857					
	Oats	1992	55	3 <sup>a</sup>	8	256.9/1224					
Germany	Barley	1987	44	3 <sup>a</sup>	1	391/4764		18	4	P,S, Muller et al. (1997b); A, Schwadorf & Müller (1991)	Samples of 0.7–1 kg col- lected randomly 1–4 weeks after harvest from farms by Governmental Advisory Board
	Barley	1989	40	3 <sup>a</sup>	11	75/483		16	0		
	Barley	1990	47	3 <sup>a</sup>	13	54/300		9	0		
	Barley	1991	51	3 <sup>a</sup>	15	38/530		4	0		
	Barley	1992	58	3 <sup>a</sup>	6	38/486		3	0		
Germany	Rye, conven- tional	1991	50	50 <sup>a</sup>	30	64/1250				P,S,A, Marx et al. (1995)	
	Rye, organic	1991	50	50 <sup>a</sup>	22	239/500					
	Wheat, con- ventional	1991	51	50 <sup>a</sup>	6	370/1200					
	Wheat, organic	1991	50	50 <sup>a</sup>	12	369/1000					
Germany	Beer	1993	67	50 <sup>a,c</sup>	17	183/569 <sup>c</sup>				P,S,A, Niessen et al. (1993)	
	Beer	1993	123	50 <sup>a,c</sup>	88	42/478 <sup>c</sup>					
	Beer	1993	6	50 <sup>a,c</sup>	6						
Italy, north	Durum wheat	1994–95	64	500 <sup>a</sup>	50	204/3085	800		3	P,S, Lops et al. (1998); A, Romer Labs Inc. (1995), Vicom (1996)	Various hybrids and varieties, 200-g laboratory samples
	Soft wheat	1994–95	77	500 <sup>a</sup>	71	59/920	0		0		
Italy, north	Soft wheat	1998	42	50 <sup>a</sup>	37	17/330	72.5	2	0	P,S, Pascale et al. (2000a); A, Vicam (1996)	Various hybrids and varieties, 1-kg batch; com- bined sample, 3 kg; ana- lytical sample, 25 g

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Italy, north	Durum wheat	1998	26	50 <sup>a</sup>	2	186/1000	463	19	1	P,S, Pascale et al. (2000a); A, Vicam (1996)	Various hybrids and varieties, 1-kg batch; com- bined sample, 3 kg; ana- lytical sample, 25 g
	Barley	1998	20	50 <sup>a</sup>	18	80/1540		1	1		
	Triticale	1998	10	50 <sup>a</sup>	2	92/200	197	5	0		
	Soft wheat, organic	1998	35	50 <sup>a</sup>	17	36/105	89	2	0		
	Spelt wheat, organic	1998	20	50 <sup>a</sup>	2	172/350	317	14	0		
Italy, north	Soft wheat, organic	1999	48	50 <sup>a</sup>	1	173/529	282	36	0	P,S, Pascale et al. (2000b); A, Vicam (1996)	Various hybrids and varieties, 200-g laboratory
	Spelt wheat, organic	1999	30	50 <sup>a</sup>	0	148/452	230	25	0		
	Soft wheat	1999	112	50 <sup>a</sup>	44	133/956	390	37	0		
	Durum wheat	1999	111	50 <sup>a</sup>	22	202/1206	446	78	1		
Nether- lands	Wheat products	1999	20	32	2	134/250	220	15	0	P,S,A, Spanjer (2000)	See Trichothecenes Appendix 6
	Wheat	1999	54	32	4	358/1900	640	49	3		
	Wheat flour	1999	24	32	4	193/460	330	20	0		
	Maize and by-products	1999	3	32	0	110/130		2	0		
	Malt	1999	2	32	2	0/0		0	0		
	Various grains and by-products	2000	171	32	91	55/570	128	32	0		
Norway (imported)	Wheat	1990	27	30 <sup>a</sup>	19	98/660	570	4	0	P,S, Langseth & Elen (1997); A, Langseth & Elen (1996)	See Trichothecenes Appendix 6

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Norway (imported)	Wheat	1992	16	30 <sup>a</sup>	4	403/1300	950	10	2	P,S, Langseth & Elen (1997); A, Langseth & Clasen (1992)	See Trichothecenes Appendix 6
	Wheat	1993	29	30 <sup>a</sup>	15	383/2500	936	14	3		
	Wheat	1994	30	30 <sup>a</sup>	18	69/610	177	5	0		
	Wheat	1995	40	30 <sup>a</sup>	18	148/650	527	3	0		
	Wheat	1996	34	20 <sup>a</sup>	20	328/2700	625	6	1		
Norway (imported)	Wheat	1997	10	20 <sup>a</sup>	3	353/1900	784	4	1	S, Langseth (2000); P and A, Langseth (2000), Langseth et al. (2000)	See Trichothecenes Appendix 6
	Wheat	1998	24	20 <sup>a</sup>	6	74/233	204	5	0		
Norway	Wheat	1990	138	30 <sup>a</sup>	28	101/890	215	50	0	P,S, Langseth & Elen (1997); A, Langseth & Elen (1996)	See Trichothecenes Appendix 6
	Wheat	1991	107	30 <sup>a</sup>	20	81/310	182	31	0		
Norway	Wheat	1992	112	30 <sup>a</sup>	16	189/900	449	71	0	P,S, Langseth & Elen (1997); A, Langseth & Clasen (1992)	See Trichothecenes Appendix 6
	Wheat	1993	102	30 <sup>a</sup>	37	89/560	198	36	0		
	Wheat	1994	112	30 <sup>a</sup>	86	29/370	93	11	0		
	Wheat	1995	26	30 <sup>a</sup>	15	19.5/170	41	1	0		
	Wheat	1996	28	20 <sup>a</sup>	28						
	Wheat	1997	25	20 <sup>a</sup>	19	8/73	28	0	0		
	Wheat	1998	35	20 <sup>a</sup>	32	4/85	0	0	0		
Norway (imported)	Rye	1990	18	30 <sup>a</sup>	12	15/50	46.5	0	0	P,S, Langseth & Elen (1997); A, Langseth & Elen (1996)	See Trichothecenes Appendix 6
Norway (imported)	Rye	1993	12	30 <sup>a</sup>	9	14/62	51	0	0	P,S, Langseth & Elen (1997); A, Langseth & Clasen (1992)	See Trichothecenes Appendix 6
	Rye	1994	12	30 <sup>a</sup>	8	16/60	53	0	0		
	Rye	1995	11	30 <sup>a</sup>	8	12.5/57	50	0	0		
	Rye	1996	10	20 <sup>a</sup>	10						
Norway	Barley	1990	10	20 <sup>a</sup>	2	30/60	51			P,S, Langseth & Elen (1997); A, Langseth & Elen (1996)	See Trichothecenes Appendix 6
	Barley	1990	10	20 <sup>a</sup>	9	5.5/55					

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Norway	Oats	1990	20	30 <sup>a</sup>	0	262/690	590	18	0	P,S,Langseth & Elen (1997); A,Langseth & Elen (1996)	See Trichothecenes Appendix 6
Norway	Oats	1993	3	30 <sup>a</sup>	0	470/670				P,S, Langseth & Elen (1997); A, Langseth & Clasen (1992)	See Trichothecenes Appendix 6
	Oats	1994	3	30 <sup>a</sup>	0	538/1300					
	Oats	1995	26	30 <sup>a</sup>	10	54/230	135	6	0		
	Oats	1996	14	20 <sup>a</sup>	5	101/266	244	7	0		
Norway	Oats	1997	14	20 <sup>a</sup>	0	58/110	101	2	0	S, Langseth (2000); P,A, Langseth (2000), Langseth et al. (2000)	See Trichothecenes Appendix 6
	Oats	1998	22	20 <sup>a</sup>	10	129/850	309	8	0		
	Oats	1999	20	20 <sup>a</sup>	17	4/28	23	0	0		
Poland	Wheat	1993	78	10 <sup>a</sup>	39	10.5/102		1	0	P,S,A, Golinski et al. (1996)	
Russian Federation	Wheat	1986	14	50 <sup>a</sup>	0	590/2510	2310		6	P,S,A,Tutelyan et al. (1990)	Samples obtained from 40 grain stores in north Caucasus; subsample size, 1 kg; analytical sample, 25 g
	Wheat	1987	90	50 <sup>a</sup>	34	260/9090	1460		14		
	Wheat	1988	120	50 <sup>a</sup>	8	1130/13900	3800		62		
Russian Federation	Wheat, freshly harvested	1989	251	50 <sup>a</sup>	0	680/5800	1200			P,S, Tutelyan (1998); A, Tutelyan et al. (1990)	Representative 2-kg samples obtained from grain stores in 10 regions
		1990	214	50 <sup>a</sup>	90	570/3520	660				
		1991	159	50 <sup>a</sup>	39	520/3800	650				
		1992	311	50 <sup>a</sup>	3	880/8600	1600				
		1993	543	50 <sup>a</sup>	4	710/4000	1200				
		1994	154	50 <sup>a</sup>	118	80/950	180				
	Wheat, food grain	1989	57	50 <sup>a</sup>	42	230/6650	440				
		1990-91	67	50 <sup>a</sup>	63	20/740	0				
		1992	190	50 <sup>a</sup>	118	330/5630	1060				
		1993	169	50 <sup>a</sup>	139	200/3950	630				
		1994	267	50 <sup>a</sup>	249	30/1130	0				

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Sweden	Wheat	1990	88	10	63	9.5/90	35.5	0	0	P,S, Pettersson (2000); A, Pettersson (1993)	Samples of about 1 kg collected from trials and plots were dried and milled; subsamples of 20 g analysed
	Oats	1990	71	10	28	25/375	65	3	0		
	Barley	1990	39	10	12	16/45	40	0	0		
	Rye	1990	5	10	0	22/30		0	0		
	Wheat	1991	92	10	40	17.7/240	43.5	2	0		
	Oats	1991	38	10	28	6/95	16.5	0	0		
	Wheat	1992	13	10	7	8/30		0	0		
	Oats	1992	2	10	0	40/40		0	0		
	Wheat	1993	2	10	1	10/20		0	0		
	Oats	1993	10	10	6	14.4/89		0	0		
	Oats	1994	34	10	2	136/538	269	18	0		
	Oats	1996	80	10	9	47/365	146	10	0		
	Oats	1997	84	10	5	86/406	195	30	0		
	Oats	1998	33	10	18	14/120	45.8	1	0		
Sweden	Barley	1998	10	10	8	31/243		1	0	P,S, Thuvander et al. (2000a,b); A, Möller & Gustavsson (1992)	Samples of about 1 kg collected as composites at inflow of cereals to mill, during storage in mill, or in production flow. Analytical sample, 50 g
	Wheat	1996–98	59	10	43	69/2153	173	7	1		
	Wheat	1999	75	10	16	53/346	120	15	0		
	Oats	1996–98	23	10	17	9.3/70	42	0	0		
	Oats	1999	10	10	6	6/19	18.9	0	0		
	Rye	1996–98	28	10	26	13/351		0	0		
United Kingdom	Rye	1999	19	10	9	13/47	41	0	0	P,S,A, Home Grown Cereals Authority (1999)	
	Wheat	1998	53	20 <sup>a</sup>	2	175/1202	432	27	1		
	Wheat	1999	201	20 <sup>a</sup>	3	86/600		52	0		
	Barley	1999	106	20 <sup>a</sup>	31	38/370		3	0		
	Oats	1999	13	20 <sup>a</sup>	3	39/108		1	0		

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
<b>Asia and Australasia</b>											
China, Linxian	Wheat	1995	25	5 <sup>a</sup>	4	70/193		5	0	P,S, Gao & Yoshizawa (1997); A, Luo et al. (1990)	
China, Shangqiu	Wheat	1995	15	5 <sup>a</sup>	7	21/125		1	0	P,S, Gao & Yoshizawa (1997); A, Luo et al. (1990)	
China, Linxian	Maize	1995	34	5 <sup>a</sup>	9	294/1160		17	2	P,S, Yoshizawa & Gao (1999); A, Luo et al. (1990)	
China, Shangqiu	Maize	1995	20	5 <sup>a</sup>	16	10/87		0	0	P,S, Yoshizawa & Gao (1999); A, Luo et al. (1990)	
China, Linxian	Maize	1997	15	5 <sup>a</sup>	1	120/393		7	0	P,S, Yoshizawa & Gao (1999); A, Luo et al. (1990)	
China, Shangqiu	Maize	1997	20	5 <sup>a</sup>	11	40/171		4	0	P,S, Yoshizawa & Gao (1999); A, Luo et al. (1990)	
China, Linxian	Wheat	1997	25	5 <sup>a</sup>	8	19/138		1	0	P,S, Yoshizawa & Gao (1999); A, Luo et al. (1990)	
China, Shangqiu	Wheat	1997	15	5 <sup>a</sup>	15			0	0	P,S, Yoshizawa & Gao (1999); A, Luo et al. (1990)	
China, Linqu	Maize, raw	1996	12	500 <sup>a</sup>	5	817/2700	2370		5	P,S,A, Groves et al. (1999)	Three households selected randomly from among those known to prepare sour pancakes in seven randomly selected villages; 5 specimens representing successive stages of pro- cessing collected in each household
	Maize meal	1996	13	500 <sup>a</sup>	5	615/1600	1560		6		
	Cooked pancake	1996	14	500 <sup>a</sup>	10	314/1500	1300		3		

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
China, Anhui Province	Wheat	1983	40	50 <sup>a</sup>	0	1161/			9	P,S, Lu et al. (1994); A,FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
	Wheat	1986	182	50 <sup>a</sup>	100	312/			13		
	Wheat	1989	81	50 <sup>a</sup>	0	2640/			65		
	Wheat	1991	26	50 <sup>a</sup>	0	2106/			15		
China	Wheat grain summer	1986,	214	100	48	211.3/1200	428.6			P, Cao et al. (1994); S, China (undated), FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
China	Wheat grain	No data	99	100	92	697.3/20000	1600			P,S, Guo et al. (1995); A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
China	Wheat flour	1988, summer	30	100	13	83.3/400	200			Lu et al. (1992); S,China (undated); A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
China	Wheat flour	1989, autumn	50	100	2	561/1428	1250			P, Cao et al. (1994); S, China (undated); A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
China	Wheat flour	1989, summer	70	100	0	1403/3130	2500			P, Lu et al. (1992); S,China (undated); A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
China	Wheat flour	1990, summer	50	100	32	56.2/173	173			P, Luo & Li (1991); S,China (undated); A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
	Wheat flour	1991, summer	50	100	31	302/1000	800				
China	Wheat grain	1996, summer	100	100	60	272.7/2322.1	917.9			P, Li (1997); S, China (undated); A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
China	Maize kernel	1988/?	101	100	21	287/2500	625			P, Cao et al. (1994), Guo et al. (1995); S, China (undated); A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g

**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
China	Maize kernel	1988, autumn	55	100	23	177.5/1100	432			P, Liu et al. (1992); S, GB5009; A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
China	Maize kernel	1989, summer	100	100	68	241.3/4000	800			P, Guo et al. (1995); S, GB5009; A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
China	Maize kernel	1989, autumn	140	100	111	35.2/800	130			P, Cao et al. (1994), Liu et al. (1992), Lu et al. (1992); S, GB5009; A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
China	Maize kernel	1989/?	50	100	12	136/1000	200			P, Guo et al. (1995); S, GB5009; A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
China	Maize kernel	1990, summer	85	100	62	69.2/48	240			P, Lu et al. (1992); S, GB5009; A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
China	Maize kernel	1990, winter	20	100	15	163/1500	765			P, Luo et al. (1991); S, GB5009; A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
China	Maize kernel	1991, summer	50	100	33	505/3200	1600			P, Luo et al. (1991); S, GB5009; A, FAO/WHO (2000)	National standard methods for food chemistry; 15 subsamples of 100 g
India	Maize	1994–97	197	100 <sup>a</sup>	195	0.19/21				P, S, Janardhana et al. (1999); A, Trucksess et al. (1984)	Samples representing different cultivars collected from farmers, production plots, and regulating mar- kets in 14 districts

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Indonesia	Maize	1995	16	10 <sup>a</sup>	14	3.3/32				P,S,A, Ali et al. (1998)	Samples collected from three locations 60 km apart; ground samples, 200 g; analytical samples, 20 g
Indonesia	Maize	1992–94	12	10 <sup>a</sup>	12					P,S, Yamashita et al. (1995), A, Luo et al. (1990)	Samples for animal and human consumption collected at random from stores of wholesalers and retailers, university farms, and local farmers
Japan	Wheat	1989–94	151	5 <sup>a</sup>	69	87/1620	198			P,S, Yoshizawa (1997), Yoshizawa & Jin (1995); A, Luo et al. (1990)	
	Barley	1989–94	94	5 <sup>a</sup>	23	205/3780	280				
Japan, Hokkaido	Wheat	1991–92	79	5	28	393/13002	554	31	5	P, Yoshizawa (2000); S and A, Luo et al. (1990)	
Japan	Wheat flour	1996–99	52	5	25	178.6/1884	639	15	2	P, Yoshizawa (2000); S and A, Luo et al. (1990)	
	Barley, pressed	1996–99	20	5	12	37.2/223	159	3	0		
	Barley flour, parched	1996–99	30	5	28	6.77/190	0	1	0		
Korea, Republic of	Barley, husked	July 1990	10	2 <sup>a</sup>	1	237/677	667	5	0	P,S,A, Park et al. (1992)	Samples collected from farms in four provinces
	Barley, naked	July 1990	27	2 <sup>a</sup>	3	189/645	501	18	0	P,S,A, Park et al. (1992)	

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	<i>n</i> > 1000 (µg/kg)	References	Sampling procedure
Korea, Republic of	Barley	July 1990	39	5 <sup>a</sup>	4	153/1051	152.56			P,S,A, Kim et al. (1993)	Samples collected from farmers' stocks in four provinces
Korea, Republic of	Maize	Nov 1990–91	46	5 <sup>a</sup>	16	202/2752	202.17			P,S,A, Kim et al. (1993)	Samples collected from six counties in one province
Korea, Republic of	Barley	July 1992	30	5 <sup>a</sup>	10	70.6/361				P,S,A, Ryu et al. (1996)	Samples collected from six provinces
Korea, Republic of	Maize	March 1992	15	5 <sup>a</sup>	5 <sup>a</sup>	1	135/442				
Korea, Republic of	Barley	1993	48	5 <sup>a</sup>	20	120/1522				P,S, Lee et al. (1995); A, Kim et al. (1993)	100-g samples collected from five provinces
Korea, Republic of	Barley	1993	11	5 <sup>a</sup>	3	190/955					
Korea, Republic of	Beer	1995	42	0.5 <sup>a,c</sup>	34	0.59/5.3 <sup>c</sup>	2.7			P,S, Shim et al. (1997); A, Scott et al. (1993)	
New Zealand	Maize	1987	29	30	8	760/3500		13		P,S, Lauren et al. (1991); A, Lauren & Agnew (1991)	
	Maize	1988	34	30	5	446/1120		16			
	Maize	1989	28	30	5	286/710		16			
New Zealand	Maize	1992	178	10 <sup>a</sup>	0	920/8500	2265	145	45	P,S, Lauren et al. (1996); A, Lauren & Agnew (1991)	
	Maize	1993	162	10 <sup>a</sup>	12	310/3370	800	90	11		
	Maize	1994	276	10 <sup>a</sup>	31	350/4790	730	162	16		
Papua New Guinea	Cereals, food	1991	29	10	17	329/2270	1720	7	4	P,S, Yuwai et al. (1994); A, Tanaka et al. (1985)	
Philippines	Maize	1992–94	50	10 <sup>a</sup>	50					P,S, Yamashita et al. (1995), A, Luo et al. (1990)	Samples for animal and human consumption collected at random from stores of wholesalers and retailers, university farms, and local farmers

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ ( $\mu\text{g}/\text{kg}$ )	$n < \text{LOQ}$	Mean/Max ( $\mu\text{g}/\text{kg}$ )	90th %ile ( $\mu\text{g}/\text{kg}$ )	$n > 100$ ( $\mu\text{g}/\text{kg}$ )	$n > 1000$ ( $\mu\text{g}/\text{kg}$ )	References	Sampling procedure
Thailand	Maize	1992–94	27	10 <sup>a</sup>	27					P,S, Yamashita et al. (1995), A, Luo et al. (1990)	Samples for animal and human consumption collected at random from stores of wholesalers and retailers, university farms, and local farmers
Viet Nam	Maize	1993	15	100 <sup>a</sup>	15					P,S, Wang et al. (1995);	
	Maize powder	1993	17	100 <sup>a</sup>	13	746/6510		4	4	A, Tanaka et al. (1985)	

LOQ, limit of quantification

Mean, true mean for  $n$  analytical values; the true mean is the sum  $X_i/n$ , where  $X_i$  is the value of each analytical result

References: P, parent reference; S, sampling method; A, analytical method

<sup>a</sup> Detection limit

<sup>b</sup> Mean of positives samples

<sup>c</sup> ng/ml



## T-2 AND HT-2 TOXINS

First draft prepared by

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## 1. EXPLANATION

T-2 and HT-2 toxins are type-A trichothecene mycotoxins, which are closely-related epoxy sesquiterpenoids. Surveys have revealed the presence of T-2 and HT-2 toxins in grains such as wheat, maize, oats, barley, rice, beans, and soya beans as well as in some cereal-based products. T-2 and HT-2 toxins have been reported to be produced by *Fusarium sporotrichioides*, *F. poae*, *F. equiseti*, and *F. acuminatum*. The most important producer is *F. sporotrichioides*, a saprophyte (i.e. not pathogenic to plants) which grows at  $-2$  to  $35$  °C and only at high water activities (above 0.88). In consequence, T-2 and HT-2 toxins are not normally found in grain at harvest but result from water damage to the grain such as may occur when it remains for extended periods in the field at or after harvest, especially in cold weather, or in grain that becomes wet during storage.

T-2 toxin is the trivial name for 4 $\beta$ ,15-diacetoxy-3 $\alpha$ ,dihydroxy-8 $\alpha$ -[3-methylbutyryloxy]-12,13-epoxytrichothec-9-ene, which is shown in Figure 1. Corresponding to the molecular formula  $C_{24}H_{34}O_9$ , its relative molecular mass is 466.5 g/mol. 15-Acetoxy-3 $\alpha$ ,4 $\beta$ -dihydroxy-8 $\alpha$ -[3-methylbutyryloxy]-12,13-epoxytrichothec-9-ene is the systematic name of HT-2 toxin. The molecular formula is  $C_{22}H_{32}O_8$ , and the relative molecular mass is 424.5 g/mol. The structures of T-2 and HT-2 toxins differ only in the functional group at the C-4 position. As T-2 toxin is readily metabolized to HT-2 toxin, these two mycotoxins were evaluated together.

Neither T-2 nor HT-2 toxin has been evaluated previously by the Committee.

## 2. BIOLOGICAL DATA

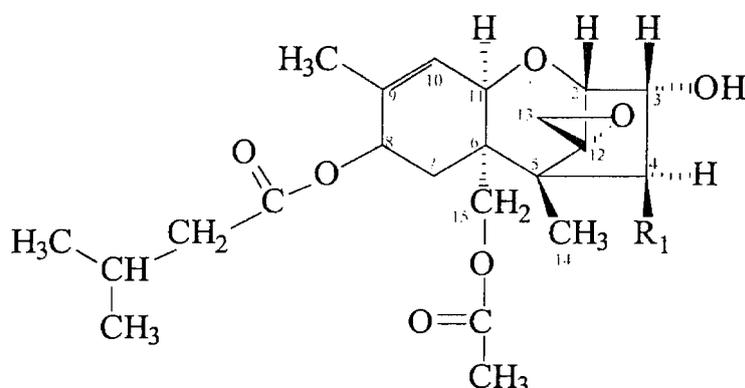
### 2.1 Biochemical aspects

#### 2.1.1 Absorption, distribution, and excretion

##### (a) Gastrointestinal metabolism

T-2 toxin is metabolized extensively in the small intestine. [ $^3$ H]T-2 toxin at a dose of 5 nmol/L (2.3  $\mu$ g) or 500 nmol/L (230  $\mu$ g T-2 toxin plus 2.3  $\mu$ g [ $^3$ H]T-2 toxin), both in 0.5 ml of an ethanol:saline solution, was injected into semi-intact isolated jejunal

**Figure 1. Structure of type A trichothecenes: T-2 ( $R_1 = \text{OAc}$ ) and HT-2 ( $R_1 = \text{OH}$ ) toxins**



loops from female Sprague-Dawley rats. Blood draining from the loops was collected and analysed by gas-liquid chromatography-mass spectroscopy at various times. Both T-2 and HT-2 toxin were found in the blood within 1 min after injection of T-2 toxin into the lumen of the intestinal loop. Only 2% of the dose of 5 nmol/L was recovered in plasma as unchanged T-2 toxin after 50 min, while 25% was recovered as HT-2 toxin, 4–7% as 3'-OH-HT-2 toxin, and smaller amounts as 3'-OH-T-2 toxin, T-2 tetraol, and 4-deacetylneosolaniol. No glucuronide or sulfate conjugates were detected. Similarly, less than 1% of the dose was recovered as T-2 toxin in the intestinal lumen, while 15% was recovered in the intestinal lumen as HT-2 toxin and 5% as 3'-OH-HT-2 toxin. The dose of 500 nmol/L caused extensive tissue damage, but the ability to metabolize T-2 toxin was retained. Intestinal fluid extracted from the jejunal loops was incubated with T-2 toxin at either 5 or 500 nmol/L for 50 min. Less metabolism was seen than in intact preparations: after 50 min of incubation with intestinal fluid alone, 14% of the administered dose was recovered as HT-2 toxin at the 5 nmol/L concentration and 12% at 500 nmol/L. The rest of the dose was recovered as T-2 toxin, and no other metabolites were observed. Filtration (0.45- $\mu\text{m}$  pore) to remove bacteria from the intestinal fluid did not appreciably reduce the amount of T-2 toxin that was deacetylated to HT-2 toxin in 50 min (Conrady-Lorck et al., 1988).

T-2 toxin was also deacetylated to HT-2 toxin in sheep rumen. When T-2 toxin at a dose of 0.02 mg/mL was incubated with sheep rumenal fluid for 0.5, 1, 2, or 3 h, it was deacetylated at a rate of 1.7 mg/L-h. The proportion of a 100- $\mu\text{g}$  dose of T-2 toxin recovered as HT-2 toxin increased over 0.5, 1, 2, and 3 h of incubation with sheep rumenal fluid, to approximately 50% HT-2 toxin by 3 h. Gas chromatography (with a flame ionization detector) was used to detect T-2 toxin and HT-2 toxin. HT-2 toxin was the only T-2 toxin metabolite analysed in the culture medium (Kießling et al., 1984).

Intestinal de-epoxidation of T-2 toxin also occurs, with deacetylation to HT-2 toxin in rats. De-epoxy HT-2 toxin was the predominant metabolite observed when T-2 toxin (at 0.1 mg/mL) was incubated with rat caecal microorganisms for 4 days. T-2 tetraol, 4-deacetylneosolaniol, T-2 toxin, HT-2 toxin, and T-2 triol were not found in the culture medium after 4 days (Swanson et al., 1987).

(b) *Bioavailability*

No studies of the systemic bioavailability of T-2 or HT-2 toxin were available. As T-2 toxin is extensively metabolized in the small intestine, however, the bioavailability of unchanged T-2 toxin may be quite low and the predominant absorbed chemical species may be HT-2 toxin (Conrady-Lorck et al., 1988; Kiessling et al., 1984; Swanson et al., 1987).

(c) *Distribution*

Placental transfer of T-2 toxin to fetal tissues was observed after intravenous injection of [<sup>14</sup>C]T-2 toxin to dams. Preferential distribution of T-2 toxin to thymus and spleen rather than liver was observed in fetuses after intraperitoneal administration of T-2 toxin to dams (Lafarge-Frayssinet et al., 1990).

In 6-week-old broiler chicks fed a diet containing T-2 toxin at 2 mg/kg for 5 weeks and then intubated with a single dose of [<sup>3</sup>H]T-2 toxin at 0.5 mg/kg bw, the radiolabel reached a maximum concentration in most tissues 4 h after dosing; the exceptions were muscle, skin, and bile, in which the maximum level was reached after 12 h. After 48 h, the chicks contained the equivalent of 39 µg/kg of T-2 toxin and/or its metabolites in the muscle and 40 µg/kg in liver, as calculated on the basis of the specific activity of the radiolabelled T-2 toxin administered (Chi et al., 1978a).

In a weanling cross-bred pig (weighing 7.5 kg bw) intubated with [<sup>3</sup>H]T-2 toxin at a dose of 0.1 mg/kg bw, the percentage of administered radiolabel 18 h after dosing was 0.7% in muscle, 0.43% in liver, 0.08% in kidney, 0.06% in bile, 22% in urine, and 25% in faeces. The percentage of administered radiolabel 18 h after dosing in another pig intubated with T-2 toxin at 0.4 mg/kg bw was 0.7% in muscle, 0.29% in liver, 0.08% in kidney, 0.14% in bile, 18% in urine, and 0.86% in faeces (Robison et al., 1979a).

Four hours after intravenous administration of [<sup>3</sup>H]T-2 toxin to pigs, the largest amount of radiolabel was located in the gastrointestinal tract (15–24% of the dose), and 4.7–5.2% of the dose was found in the remaining tissues; muscle accounted for 2.9–3.2% of the dose and liver for 0.7–1.7% (Corley et al., 1986).

The fate and distribution of an intramuscular dose of 1.04 mg/kg bw of [<sup>3</sup>H]T-2 toxin was studied in guinea-pigs. Except in the large intestine and bile, the amount of radiolabel had peaked by 30 min and rapidly declined, with no measurable long-term accumulation. The tissue distribution was expressed in intervals over a 30-min to 672-h period, with six animals per time, as picomoles per milligram of tissue. Three hours after dosing, the distribution of T-2 toxin-derived radiolabel corresponded to 850 pmol/mg of tissue (wet weight) in kidney, 970 pmol/mg in liver, 490 pmol/mg in lung, 570 pmol/mg in spleen, 280 pmol/mg in adrenals, 630 pmol/mg in fat, 230 pmol/mg in heart, 430 pmol/mg in muscle, 440 pmol/mg in testis, and 160 pmol/mg in brain (equal to 400, 450, 230, 260, 130, 290, 110, 200, 200, and 77 µg/g of tissue). At 3 h, the distribution of T-2 toxin-derived radiolabel corresponded to 290 pmol/ml of plasma and 2700 pmol/ml of bile. At 72 h after dosing, the distribution of T-2 toxin-derived radiolabel corresponded to 90 pmol/mg in kidney, 100 pmol/mg

in liver, 61 pmol/mg in lung, 59 pmol/mg in spleen, 49 pmol/mg in adrenals, 39 pmol/mg in fat, 36 pmol/mg in heart, 51 pmol/mg in muscle, 41 pmol/mg in testis, and 24 pmol/mg in brain (equal to 43, 47, 28, 28, 23, 18, 17, 24, 19, and 11  $\mu\text{g/g}$  of tissue). At this time, the distribution of radiolabel corresponded to 88 pmol/ml of plasma and 39 000 pmol/ml of bile (equal to 41 and 18 000  $\mu\text{g/L}$ ) (Pace et al., 1985).

In pigs given T-2 toxin at 1.2 mg/kg bw by intra-aortal injection, the plasma and tissue concentrations of T-2 toxin decreased rapidly. By 4 h after dosing, no T-2 toxin was detected in any of the tissues examined (analysis by gas-liquid chromatography; limit of quantification, 40 ng/g). However, at 1, 2, and 3 h after injection, larger amounts of T-2 toxin were seen in spleen than in either kidney or muscle. T-2 toxin was not detected in liver at any time (Beasley et al., 1986).

A lactating Jersey cow weighing 375 kg was given 180 mg of T-2 toxin orally, equal to 0.48 mg/kg bw per day, by capsule for 3 days and was then given 160 mg of [ $^3\text{H}$ ]T-2 toxin, equal to 0.42 mg/kg bw. Although almost all the administered dose was eliminated within 72 h, measurable tritium remained in the bile, liver, and kidney (equivalent, respectively, to 27, 18, and 14  $\mu\text{g/kg}$  of tissue) 3 days after dosing. These concentrations are higher than those in whole blood (13  $\mu\text{g/kg}$ ), plasma (10  $\mu\text{g/kg}$ ), and other tissues, including the spleen (9.4  $\mu\text{g/kg}$ ), heart (10  $\mu\text{g/kg}$ ), mammary gland (11  $\mu\text{g/kg}$ ), ovaries (11  $\mu\text{g/kg}$ ), muscle (8.8  $\mu\text{g/kg}$ ), and fat (4.7  $\mu\text{g/kg}$ ) (Yoshizawa et al., 1981).

#### (d) Excretion

Excretion was measured in faeces and urine collected for 6 days after oral, intravenous, and dermal administration of [ $^3\text{H}$ ]T-2 toxin to rats at a dose of 0.15 or 0.6 mg/kg bw. After oral administration, > 95% of the administered radiolabel was excreted within 72 h. After intravenous administration, similarly rapid elimination of the dose of 0.15 mg/kg bw was seen, but < 80% of the dose of 0.6 mg/kg bw was eliminated within 72 h. Extensive vascular damage was seen at the higher dose in these animals. After dermal application, < 60% of either dose was excreted within 72 h. More radiolabel was excreted in faeces than in urine with all routes and both doses. After oral administration, faecal excretion accounted for approximately 80% of the administered dose. A similar ratio (80:20, faeces:urine) was seen with the dose of 0.15 mg/kg given intravenously (Pfeiffer et al., 1988).

In 6-week-old broiler chicks given [ $^3\text{H}$ ]T-2 toxin by crop intubation at a dose of 0.5 mg/kg bw, the radiolabel peaked at 4 h in most tissues but at 12 h in muscle, skin, and bile. Bile contained the highest concentration of radiolabel of the tissues examined over the 48-h observation period. The pattern of distribution and excretion indicated that biliary excretion dominated (Chi et al., 1978a).

Biliary excretion was also found after intramuscular injection of [ $^3\text{H}$ ]T-2 toxin at a dose of 1 mg/kg bw to guinea-pigs. The concentration in bile at 12 h was 540 000 pmol/ml (equal to 250 000  $\mu\text{g/L}$ ), whereas that in plasma was 290 pmol/ml (equal to 140  $\mu\text{g/L}$ ). Over the first 5 days 75% of the administered radiolabel was excreted in urine and faeces at a ratio of 4:1. The amount peaked in urine at 24 h; 99% of the administered dose was eliminated by 28 days (Pace et al., 1985).

(e) *Transmission (excretion into eggs and milk)*

Radiolabel was transmitted into the eggs of laying hens that had been intubated gastrically with a single or several doses of [<sup>3</sup>H]T-2 toxin. In birds given a single dose of 0.25 mg/kg bw, the maximum residues were found in eggs 24 h later; the yolk contained 0.04% of the total dose and the white contained 0.13%. In birds given 0.1 mg/kg bw per day for 8 days, radiolabel accumulated in eggs until the fifth day of dosing, remained unchanged until the last day of dosing, and rapidly decreased thereafter. The maximum percentage of administered radiolabel recovered in egg white and yolk after the repeated doses was 0.41% and 0.28%, respectively. Assuming that birds weighing 1.6 kg consumed 100 g of the diet containing T-2 toxin at 1.6 mg/kg each day, the concentration of residues (T-2 toxin and/or its metabolites) in the contaminated eggs would be about 0.9 µg/egg (Chi et al., 1978b).

A pregnant Holstein cow was given 182 mg of T-2 toxin, equal to 0.5 mg/kg bw per day, for 15 days, and milk was sampled on days 2, 4, 5, 8, 10, and 12 after initiation of treatment. T-2 toxin was detected at concentrations of 10–160 ng/kg of milk. A sow was given T-2 toxin in feed at a concentration of 12 mg/kg, equivalent to 0.48 mg/kg bw per day, for 220 days, and its milk was analysed on day 190 of treatment. T-2 toxin was found at a concentration of 76 ng/g. No analysis for metabolites was reported (Robison et al., 1979b). The Committee noted that the purity and source of the T-2 toxin were not reported.

T-2 toxin was found at a concentration of 2 ng/ml in cows' milk after administration of 160–180 mg/day for 4 days (equivalent to 0.42–0.48 mg/kg bw per day) (Yoshizawa et al., 1981).

### 2.1.2 *Biotransformation*

Metabolic transformations of T-2 toxin and HT-2 toxin that have been demonstrated in animals are shown in Table 1.

Male Sprague-Dawley rats received [<sup>3</sup>H]T-2 toxin at a dose of 0.15 or 0.6 mg/kg bw by oral, intravenous, or dermal administration, and 16 peaks in urine and faecal extracts were compared with known metabolite standards by high-performance liquid chromatography (HPLC) to identify radiolabel associated with T-2 toxin, HT-2 toxin, 3'-OH-HT-2, 3'-OH-T-2, neosolaniol, T-2 tetraol, and 4-deacetylneosolaniol. Averaged over all doses and routes, 68% of the recovered radiolabel in urine was associated with T-2 toxin (5.6%), HT-2 toxin (8.9%), 3'-OH-HT-2 (29%), 3'-OH-T-2 (3.2%), and T-2 tetraol (21%). On the basis of retention times, de-epoxy 3'-OH-T-2 triol, de-epoxy 3'-OH-HT-2, and 3'-OH-T-2 triol were also tentatively identified in excreta. The relative conversion rates to the identified metabolites were not affected by dose, but higher relative rates of conversion to T-2 tetraol, HT-2 toxin, and de-epoxy tetraol and lower rates of conversion to 3'-OH-HT-2 were seen after intravenous than after oral administration. More de-epoxy 3'-OH-HT-2 was found in orally dosed animals than those treated intravenously. In general, de-epoxidation appeared to be an important route of detoxication. T-2 toxin represented a greater percentage of the recovered radiolabel in urine over time, suggesting slow release from cutaneous fat

**Table 1. Transformation of T-2 toxin**

Animal	Transformation reaction	References
Rat	De-epoxidation, hydrolysis, 3'-hydroxylation, glucuronide conjugation	Pace (1986); Pfeiffer et al. (1988); Yoshizawa et al. (1985a,b); Gareis et al. (1986)
Guinea-pig	Hydrolysis, 3'-hydroxylation	Pace et al. (1985)
Chicken	Hydrolysis, 3'-hydroxylation	Yoshizawa et al. (1980); Visconti & Mirocha (1985)
Dog	Hydrolysis, glucuronide conjugation	Sintov et al. (1986, 1987, 1988)
Pig	Hydrolysis, 3'-hydroxylation, de-epoxidation, glucuronide conjugation	Corley et al. (1985, 1986)
Cow	Hydrolysis, 3'-hydroxylation, 7'-hydroxylation, de-epoxidation	Yoshizawa et al. (1981, 1982a,b); Pawlosky & Mirocha (1984); Chatterjee et al. (1986)
Monkey	Hydrolysis, 3'-hydroxylation	Yoshizawa et al. (1984)

stores. An increase over time in the percentage of recovered radioactivity as the de-epoxy metabolites was also observed (Pfeiffer et al., 1988).

C-4 deacetylation of T-2 toxin to HT-2 toxin was demonstrated in microsomal preparations of liver, kidney, and spleen from various species. The reaction rates of hepatic microsomes from various species, expressed in nmol/mg protein per 10 min, were: rabbit, 3000; human, 330; mouse, 75; chicken, 55; rat, 36; and guinea-pig, 14 (equal to 1400, 150, 35, 26, 17, and 6  $\mu\text{g}/\text{mg}$  of protein) (Ohta et al., 1977; Johnsen et al., 1986).

Metabolism of T-2 toxin to hydrolysates at the C-4, C-8, and C-15 positions and hydroxylation at the C-3' position by liver homogenates from rats, mice, and monkeys were demonstrated *in vitro*. Hydrolytic transformation was also observed in hepatic homogenates from rabbits, pigs, and cows (Yoshizawa et al., 1984, 1985b).

Enzymic conversion of T-2 toxin to HT-2 toxin was examined in liver homogenates from male Wistar rats, in studies in which 0.5  $\mu\text{mol}$  (230  $\mu\text{g}$ ) of T-2 toxin were added to 2 ml of a cytosol/microsomal fraction. Complete conversion to HT-2 toxin was demonstrated within 60 min, and no other metabolites were reported (analysis by gas chromatography (GC)–mass spectrometry (MS)). There was much less conversion to HT-2 toxin in liver cytosol, and no metabolism was reported in plasma. Co-incubation of T-2 toxin in a liver homogenate with paraoxon showed that inhibition of serine esterases completely blocked conversion of T-2 toxin to HT-2 toxin. Further analysis with serine esterases showed that carboxylesterase but not cholinesterase was effective in converting T-2 toxin to HT-2 toxin (Johnsen et al., 1986)

Inhibition of carboxylesterase with tri-*ortho*-cresyl phosphate (TOCP) in groups of 10 mice (strain and sex unspecified) given T-2 toxin at 0.5, 1, 2, 3, or 4 mg/kg bw intravenously led to the death of all animals at 2, 3, and 4 mg/kg bw, but not at 0.5 or 1 mg/kg bw (Johnsen et al., 1986).

Glucuronide-conjugated HT-2 toxin was the main metabolite recovered from bile collected from isolated, perfused male Wistar rat livers. Only trace amounts of T-2 toxin were recovered as the glucuronide conjugate. Analysis was performed with gas-liquid chromatography-mass spectroscopy.  $\beta$ -Glucuronidase was used to determine glucuronide conjugates (Gareis et al., 1986).

Glucuronide conjugates of T-2 toxin and metabolites were identified in the urine and bile of two sows after intravenous administration of [ $^3\text{H}$ ]T-2 toxin at 0.15 mg/kg bw. Urine was collected hourly for 4 h through a catheter, and bile was collected after the animals were killed at 4 h. High-performance thin-layer chromatography with comparison to standards was used to identify and quantify the metabolites. Glucuronide conjugates were determined with  $\beta$ -glucuronidase for T-2 toxin, 3'-OH-T-2, neosolaniol HT-2, 3'-OH-HT-2, T-2 triol, 4-deacetylneosolaniol, and T-2 tetraol. De-epoxy derivatives were not determined. Less than 1% of the recovered T-2 toxin and < 10% of the recovered HT-2 toxin was unconjugated in either bile or urine. The main metabolite recovered in bile was conjugated T-2 toxin, followed by conjugated HT-2 toxin. Conjugated metabolites represented an average of 77% of the recovered radiolabel in bile, and all metabolites accounted for an average of 92% of the radiolabel. In urine, conjugated metabolites represented an average of 63% of the recovered radiolabel, with an average of 90% of the recovered radiolabel accounted for by metabolites (Corley et al., 1985).

### **2.1.3 Effects on enzymes and other biochemical parameters**

#### *(a) Effect on nutrients*

The effect of T-2 toxin on intestinal absorption of monosaccharides was studied in rats. The absorption of 3-*O*-methylglucose was reduced one- to threefold after injection of T-2 toxin into the jejunal lumen or after intravenous injection. Jejunal function was impaired by specific damage to the active transport and diffusional movement of monosaccharides (Kumagai & Shimizu, 1988).

Three groups of 1-day-old chicks (10 chicks per group) were given diets containing T-2 toxin at a concentration of 0.5–15 mg/kg, equivalent to 0.06–1.9 mg/kg bw, for 3 weeks. Plasma vitamin E activity and hepatic vitamin A content were measured. A dose-dependent decrease in plasma vitamin E activity was observed, with a 65% decrease from that of controls in chicks fed the diet containing 15 mg/kg. This decrease was considered to be due to a reduction in the plasma concentration of lipoproteins, which are required for the transport of vitamin E (Coffin & Combs, 1981).

Several authors reported changes in the serum concentrations of glucose and essential elements in animals given T-2 toxin in the feed (for example, Rafai et al., 1995a; Weaver et al., 1978). The Committee noted that, as feed refusal was observed

at all doses tested and decreased feed intake would result in changes in metabolism and energy use, observations such as these are difficult to interpret. Experimental designs that control for feed intake are needed before changes in metabolism and energy use can be assessed.

(b) *Inhibition of protein synthesis*

The effects of T-2 toxin on protein synthesis were studied in Swiss mice and hepatoma cell cultures. T-2 toxin was given as an intraperitoneal dose of 0.75 mg/kg bw per day for 3 or 7 days. Protein synthesis was inhibited in the animals and in cells obtained from bone marrow, spleen, and thymus. Protein synthesis was also inhibited in vitro in hepatoma cell cultures and phytohaemagglutinin-stimulated lymphocytes (Rosenstein & Lafarge-Frayssinet, 1983).

Chinese hamster ovary and African green monkey kidney (Vero) cell cultures were exposed to T-2 toxin at 0.01 or 1 ng/ml for 1 or 12 h. The cells showed morphological changes indicative of inhibition of protein synthesis, including disassociation of polysomes and matrix density, ballooning of the intracristal space, and malalignment of cristae in mitochondria. The Chinese hamster ovary cells had bleb formations of the plasma membrane, which indicates inhibition of protein synthesis (Trusal, 1985).

Protein inhibition was also observed in muscle, heart, liver, and spleen of rats that received intraperitoneal injections of T-2 toxin at 0.3, 0.75, or 2 mg/kg bw (Thompson & Wannemacher, 1990).

The effects of T-2 toxin on rat hepatocytes in culture were studied by adding it at 0.01 or 1 ng/ml for 1 or 12 h. The lower concentration caused 75% inhibition of protein synthesis within 1 h. At the higher concentration, the hepatocytes recovered from a 1-h but not a 12-h exposure. Cell damage (release of lactate dehydrogenase) lagged behind inhibition of protein synthesis, which was 90% at 1 ng/ml. Ultrastructural alterations were present in the endoplasmic reticulum and mitochondria. The rough endoplasmic reticulum showed degranulation, and mitochondria had translucent foci and electron-dense cores (Trusal & O'Brien, 1986).

T-2 toxin induced disaggregation of polysomes in HeLa cells (Liao et al., 1976). It interacted with the peptidyl transferase centre on the 60S ribosomal subunit and inhibited transpeptidation of peptide-bond formation. These results were consistent with the effects of an inhibitor of prolongation or termination of protein synthesis (Stafford & McLaughlin, 1973).

The ribosomal subunits of *Myrothecium verrucaria*, a producer of macrocyclic trichothecenes, were resistant to T-2 toxin. The authors concluded that the 60S subunits of eukaryotes are responsible for the sensitivity to T-2 toxin (Hobden & Cundliffe, 1980).

(c) *Inhibition of nucleic acid synthesis*

The incorporation of [<sup>3</sup>H]thymidine into DNA in cell lines from thymus was strongly inhibited by T-2 toxin at 10 ng/ml and slightly inhibited at 0.1–10 ng/ml. Concentrations

of 0.1–1 ng/ml caused a transient increase in DNA polymerase and  $\alpha$ - and  $\beta$ -terminal deoxynucleotidyl transferase activity, whereas concentrations > 1 ng/ml caused strong inhibition (Munsch & Mueller, 1980)

A single dose, three daily doses, or seven daily doses of T-2 toxin at 0.75 mg/kg inhibited DNA synthesis *ex vivo* in cell cultures from the spleen, thymus, and bone marrow of treated mice. T-2 toxin also inhibited DNA synthesis *in vitro* in cultures of hepatoma cells and in phytohaemagglutinin-stimulated lymphocytes (Rosenstein & Lafarge-Frayssinet, 1983).

The effects of T-2 toxin on DNA synthesis in phytohemagglutinin-stimulated human peripheral blood lymphocytes was assayed by incorporation of [ $^3$ H]thymidine. Total inhibition was obtained with 8 ng/ml and 80% inhibition with 1.5 ng/ml (Cooray, 1984).

In synchronously dividing *Tetrahymena* cells, incorporation of radiolabelled thymidine and uracil into DNA and RNA, respectively, was inhibited. Neither DNA nor RNA synthesis nor RNA hybridase activity was altered by T-2 toxin *in vitro* in isolated nuclei from normal cells or from cells pretreated with T-2 toxin (Iwahashi et al., 1982).

Thymidine uptake and incorporation into DNA was biphasic, with increased uptake at 0.4 pg/ml reduced uptake at 4 pg/ml, and reduced incorporation at 40 pg/ml (Bunner & Morris, 1988).

#### (d) Alterations of cellular membranes

At a concentration of T-2 toxin of 20  $\mu$ g/ml, no entry of [ $^{14}$ C]sucrose or [ $^3$ H]inulin was observed in bovine erythrocytes *in vitro*. Very little radiolabel was bound to bovine erythrocytes, and the binding was independent of the T-2 toxin concentration. The toxin had no effect on the entrapment of sucrose or inulin. Carrier erythrocytes retained 85% of [ $^{14}$ C]sucrose and only 18% of [ $^3$ H]T-2 toxin. Thus, T-2 toxin diffused from carrier cells more rapidly than sucrose. The authors concluded that the interaction of T-2 toxin with bovine erythrocytes was minimal and intercalation with the inner bilayer was unlikely, because the increase in cell volume that would have resulted did not occur (DeLoach et al., 1987).

The effects of T-2 toxin on membrane function were studied in L-6 myoblasts. The minimal effective concentration of T-2 toxin for reducing uptake of calcium and glucose and for reducing uptake of leucine and tyrosine and their incorporation into protein was 4 pg/ml. The uptake of rubidium was increased at 0.4 pg/ml and reduced at 4 pg/ml or more. Calcium efflux was reduced after 1, 5, and 15 min of exposure to T-2 toxin at a concentration of 40 pg/ml. The authors concluded that that T-2 toxin has multiple effects on membrane function at low concentrations and that these effects are independent of inhibition of protein synthesis (Bunner & Morris, 1988).

The effect of T-2 toxin on lipid peroxidation and the antioxidant status of liver and blood was examined in three species of fowl. As measured by malondialdehyde concentrations in liver homogenate and blood plasma, lipid peroxidation was increased in ducks, chickens, and geese given feed containing T-2 toxin at concentrations of 0.2–0.6 mg/kg (Mezes et al., 1999).

## 2.2 Toxicological studies

### 2.2.1 Acute toxicity

The LD<sub>50</sub> values for T-2 toxin and HT-2 toxin in several animal species are summarized in Tables 2 and 3. Strain and sex differences in death rates were seen.

Groups of five to seven male and female mice of strains ICR:CD1, BALB/c, C57Bl/6, and DBA/2 were given single doses of T-2 toxin at 2.5, 5, or 10 mg/kg bw by oral gavage. T-2 toxin was more lethal in C57Bl/6 and BALB/c strains and more lethal in the females of those strains. Five of six C57Bl/6 females and three of seven BALB/c females at 10 mg/kg bw but none of the males at this dose had died by 24 h, whereas one of six male and female DBA/2 mice at 10 mg/kg bw, one of six female BALB/c mice at 5 mg/kg bw, and none of the ICR:CD1 mice had died by 24 h. At 48 h, none of the male DBA/2 mice given the same doses of T-2 toxin had died. The mitotic index of intestinal crypt epithelial cells was depressed at all doses in all strains, with no difference by sex, strain, or dose. The apoptotic index in these cells varied by dose, was higher in C57Bl/6 mice than in the other three strains, and was reportedly generally greater in females (Li et al., 1997; Shinozuka et al., 1997a,b).

Sex differences were also observed after exposure by inhalation. When 9-week-old Swiss ICR mice weighing 15–20 g were exposed to T-2 toxin at a concentration of 120 µg/L of air for 10 min (dose per kg bw not estimated), adrenal lesions in the zona fasciculata were observed in 11 of 11 females and in none of 10 males. Extensive necrosis of cortical lymphocytes and necrosis of lymphoid cells in the follicles of the spleen were observed in both males and females (Thurman et al., 1986).

Adaptation to the local and systemic acute effects of T-2 toxin was observed in groups of eight CD-1 mice after dermal application to shaved areas on the back of 10 or 25 µg per animal weekly for 22 weeks. Initially, T-2 toxin caused necrosis, scarring, and sloughing of skin. Application of 10 µg caused reactions that healed within 2–3 weeks, and application 4 or 7 days later and weekly thereafter caused minor or no irritation. Furthermore, a lower acute mortality rate than expected was observed in the group given the higher dermal dose: at 25 µg, only 2/20 mice died after an intraperitoneal LD<sub>50</sub> dose (0.4 mg/kg bw), while 10/16 mice given the same intraperitoneal LD<sub>50</sub> dose died after having received 10 µg of T-2 toxin (Lindenfelser et al., 1974).

Newborn mice appeared to be more sensitive than adults to a single subcutaneous dose of T-2 toxin (Ueno et al., 1973a); however, the LD<sub>50</sub> after oral administration did not differ with age in poultry. The birds died within 48 h of receiving T-2 toxin. Within 4 h, they showed asthenia, inappetence, diarrhoea, and panting. The abdominal cavities of birds given lethal doses contained a white chalk-like material that covered much of the viscera (Chi et al., 1977b).

Inhaled T-2 toxin was at least 10 times more toxic than orally administered material (Creasia et al., 1987). Surprisingly, no epithelial necrosis was reported after exposure to T-2 toxin by inhalation, although inflammation and necrosis were seen after dermal application or ingestion of high doses. The LC<sub>50</sub> of an aerosolized saline solution of T-2 toxin was 0.035 mg/L of air in mice (Creasia & Thurman, 1993).

**Table 2. Results of studies of the acute toxicity of T-2 toxin**

Species	Sex	Route	LD <sub>50</sub> (mg/kg bw)	Reference
Mice	Male	Oral	10	Ueno (1984)
Mice	Male	intraperitoneal	5.2	Ueno (1984)
Mice	Female	Intraperitoneal	14	Fairhurst et al. (1987)
Mice	Male	Intraperitoneal	5.3	Yoshizawa et al. (1982b)
Mice	Male	Intraperitoneal	9.1	Thompson & Wannemacher (1986)
Mice	Female	Intraperitoneal	0.4	Lindenfelser et al. (1974)
Mice	Male	Subcutaneous	2.1	Ueno (1984)
Mice	Female	Subcutaneous	6.4–8.0	Fairhurst et al. (1987)
Mice	Male	Subcutaneous	3.3	Thompson & Wannemacher (1986)
Mice	Male	Intravenous	4.2	Ueno (1984)
Mice	Female	Intravenous	11	Fairhurst et al. (1987)
Rats	Male	Intraperitoneal	1.5	Creasia et al. (1990)
Rats	Male	Intraperitoneal	0.9	Martin et al. (1986)
Rats	Female	Intraperitoneal	1.3	Fairhurst et al. (1987)
Rats	NS	Intraperitoneal	2.2	Brennecke & Neufeld (1982)
Rats	Male	Subcutaneous	1	Bergmann et al. (1985)
Rats	Female	Subcutaneous	2	Fairhurst et al. (1987)
Rats	NS	Subcutaneous	0.56	Brennecke & Neufeld (1982)
Rats	Male	Intramuscular	0.85	Chan & Gentry (1984)
Rats	NS	Intramuscular	0.47	Brennecke & Neufeld (1982)
Rats	Female	Intravenous	0.9	Fairhurst et al. (1987)
Rats	Male	Intravenous	0.74	Feuerstein et al. (1985)
Rats	Male	Inhalation	0.05	Creasia et al. (1990)
Guinea-pigs	Male	Intraperitoneal	1.2	Creasia et al. (1990)
Guinea-pigs	Female	Subcutaneous	1-2	Marrs et al. (1985)
Guinea-pigs	Male	Intravenous	1-2	Fairhurst et al. (1987)
Guinea-pigs	Male	Intravenous	1.3	Feuerstein et al. (1985)
Guinea-pigs	Male	Inhalation	0.4	Creasia et al. (1990)
Rabbits	Male/ Female	Intramuscular	1.1	Chan & Gentry (1984)
7-day-old broiler chicks	Male	Oral	4	Hoerr et al. (1981)
Laying hens	Female	Oral	6.3	Chi et al. (1977a)
Day-old cockerels	Male	Oral	1.84	Lansden et al. (1978)
Day-old broiler chicks	NS	Oral	5	Chi et al. (1978a)
Pigs		Intravenous	1.21	Weaver et al. (1978)

NS, not specified

Intraperitoneal injection of T-2 toxin caused myocardial damage in rats. A single dose of 2 mg/kg bw or four doses of 0.3 mg/kg bw per day caused focal necrosis of endothelial cells and other histological signs of cell damage (Yarom et al., 1987). Two months after the last of 10 daily intraperitoneal injections of T-2 toxin at 0.3 mg/kg bw, hypertrophy, focal fibrosis, and abundant cellularity were seen (Yarom et al., 1983).

**Table 3. Results of studies of the acute toxicity of HT-2 toxin**

Species	Sex	Route	LD <sub>50</sub> (mg/kg bw)	Reference
Mice	Male	Intraperitoneal	10	Thompson & Wannemacher (1986)
Mice	Male	Intraperitoneal	6.5	Yoshizawa et al. (1982b)
Mice	Male	Subcutaneous	6.7	Thompson & Wannemacher (1986)
Rats	Male	Subcutaneous	1	Bergmann et al. (1988)
1-day-old broiler chicks	Not specified	Oral	7.2	Chi et al. (1978)

The LD<sub>50</sub> of T-2 toxin dissolved in ethanol and administered intravenously was 1.2 mg/kg bw in normal, healthy, cross-bred pigs weighing 3–50 kg. Soon after administration, emesis was followed by eager consumption of feed, moderate posterior paresis, staggering gait, extreme listlessness, and frequent defaecation of normal stools. Between 1 and 6 h, severe posterior paresis, knuckling-over of the rear feet, and extreme lethargy were observed. These signs were followed by severe posterior paresis, frequent falling because of hind-quarter weakness, and dragging of both rear legs. Twenty-four hours after administration, the surviving pigs appeared normal (Weaver et al., 1978; Coppock et al., 1985).

Similar clinical signs were observed in pigs exposed to T-2 toxin by inhalation. Necrosis was present in the epithelial cells of the mucosa and in the crypt cells of the jejunum and ileum, the Peyer's patches of the ileum, the lymphoid elements of the caecum, the lymphoid follicles in the spleen, and the germinal centre of the mesenteric lymph node (Weaver et al., 1978; Pang et al., 1988).

Eighteen white, cross-bred female pigs weighing 40–60 kg, immunized against *Erysipelothrix rhusiopathiae*, were given purified T-2 toxin dissolved in 70% ethanol intravenously at a dose of 0 (five pigs), 0.6 (five pigs), 1.2 (one pig), 4.8 (five pigs), or 5.4 (two pigs) mg/kg bw. The animals at 4.8 or 5.4 mg/kg bw died 5–10.5 h later, and the other animals were killed 12–24 h after treatment. Gross lesions were seen in pigs given doses  $\geq$  1.2 mg/kg bw, consisting of oedema, congestion, and haemorrhage of the lymph nodes and pancreas and congestion and haemorrhage of the gastrointestinal mucosa, subendocardium, adrenal glands, and meninges. The histological alterations paralleled the gross lesions. Other lesions were widespread degeneration and necrosis of lymphoid tissue and the surface and crypt epithelium of the intestines. Scattered foci of necrosis were present in the pancreas, myocardium, bone marrow, adrenal cortex, and the tubular epithelium of the renal medulla. Most of the lesions were dose-dependent. The T-2 toxin-induced lesions in the lymphoid and gastrointestinal tract of pigs were similar to those described in other species. The heart and pancreas were additional target organs in pigs (Pang et al., 1987a).

Groups of 17 castrated male, cross-bred, specific pathogen-free pigs, 9–11 weeks of age, were used to characterize pulmonary and systemic responses to nebulized T-2 toxin (mixed with 100–200  $\mu$ Ci of technetium) at a dose of 9 mg/kg bw given by endotracheal intubation. The animals were exposed to aerosols in pairs, one animal

receiving T-2 toxin and the other acting as a control. The pigs retained 20–30% of the T-2 toxin. Five pairs of animals were killed 1, 3, and 7 days after dosing; two pairs in which one treated pig died and the other was killed in a moribund state 0–10 h after dosing were designated 0.33-day groups. The treated pigs vomited after exposure and showed cyanosis, anorexia, and lethargy; they then became laterally recumbent. Alveolar macrophages showed reduced phagocytosis, and the blastogenic responses of pulmonary lymphocytes, but not peripheral blood lymphocytes, to mitogen were reduced. The lesions in the pigs that died included multifocal interstitial pneumonia, necrosis of lymphoid tissue, necrohaemorrhagic gastroenteritis, oedema of gall-bladder mucosa, and multifocal areas of necrosis in the heart and pancreas. Inhalation of T-2 toxin produced a clinical and morphological syndrome resembling that caused by intravenously administered T-2 toxin at doses  $\geq 1.2$  mg/kg bw (approximate LD<sub>50</sub>) or death. The lesions produced after inhalation were more severe than those seen after intravenous administration (Pang et al., 1987a).

### **2.2.2 Short-term studies of toxicity**

#### *Rats*

Wistar rats fed T-2 toxin (purity not specified) at a concentration of 5, 10, or 15 mg/kg of feed, equivalent to 0.25, 0.5, and 0.75 mg/kg bw per day, for 4 weeks showed gastric lesions, which were diffuse and severe in the rats at the highest dose, focal but definite in those at 0.5 mg/kg bw per day, and negligible in rats at the lowest dose (Ohtsubo & Saito, 1977). The Committee noted that few experimental details were provided, e.g. on the purity of the T-2 toxin used, the way in which it was incorporated into the diet, and the number of rats per dose.

Twenty-four male Wistar rats received subcutaneous injections of T-2 toxin at 0.05 mg/kg bw per day for 28 days, and groups of six were examined at 7, 14, 21, and 28 days. Changes ranging from dystrophia or necrosis to hyperplasia were observed in the liver, kidney, and heart, with progression of severity with duration of exposure. Necrotic changes were observed from the third week. The changes in the kidney appeared to be more severe and occurred earlier (Sinovec & Jovanovic, 1993).

Groups of five to six female Holtzman albino rats were given diets containing T-2 toxin (purity not specified) for 3 weeks to 8 months at a concentration of 5 or 15 mg/kg (equivalent to 0.25 and 0.75 mg/kg bw per day) for up to 3 weeks and 10 mg/kg (equivalent to 0.5 mg/kg bw per day) during alternate 4-week periods for 8 months (4 weeks on the T-2 toxin-containing diet alternating with 4 weeks on control diet). The body weight of rats fed a diet containing 15 mg/kg feed for 19 days was markedly reduced. Slight growth depression was reported in rats fed a diet containing T-2 toxin at 5 mg/kg for 3 weeks. No gastric lesions were observed in any of the treated animals. Focal changes and cytoplasmic degradation (but no macroscopic abnormalities) were seen in the livers of four rats fed diets containing 5 mg/kg feed for 3 weeks followed by 15 mg/kg feed for 3 weeks. Severe inflammation around the nose and mouth were also seen at this time. After 8 months of alternating 4-week exposures to 10 mg/kg of diet, histopathological examination of the livers

showed no evidence of toxic hepatitis, cirrhosis, neoplasia, or hyperplasia of either hepatocytes or cholangioles (Marasas et al., 1969).

### Poultry

Chickens were fed a diet containing T-2 toxin at a concentration of 1–16 mg/kg for 3 weeks. The T-2 toxin was extracted from a *F. tricinctum* culture and purified by the method of Burmeister (1971) to yield a crystalline product melting at 148–150 °C. Birds at 4, 8, and 16 mg/kg of diet showed reduced growth and developed yellow–white lesions in the mouth consisting of a fibrinous surface layer and a heavy infiltration of the underlying tissues by granular leukocytes. *Escherichia coli* and *Staphylococcus epidermis* were isolated from the lesions (Wyatt et al., 1972).

Groups of 36 broiler chicks aged 1 day to 9 weeks received a diet containing T-2 toxin (purity not specified) at a concentration of 0.2, 0.4, 2, or 4 mg/kg, equivalent to 0.025, 0.05, 0.25, and 0.5 mg/kg bw per day. Those at the highest concentration had reduced body-weight gain and feed consumption and developed oral lesions characterized by circumscribed, proliferating, yellow caseous plaques at the margin of the beak, the mucosa of the hard palate, and the tongue and the angle of the mouth. No lesions were observed in the bone marrow or in peripheral blood (Chi et al., 1977a).

In 1-day-old broiler chicks fed a diet containing T-2 toxin at 1, 2, 4, 8, or 16 mg/kg (equivalent to 0.125, 0.25, 0.5, 1, and 2 mg/kg bw per day) for 3 weeks, the growth rate, the weight of the pancreas, and the weight of the spleen were decreased at concentrations  $\geq 4$  mg/kg. The T-2 toxin was extracted from *F. tricinctum* culture and purified by the method of Burmeister (1971) to yield a crystalline product melting at 148–150 °C. Oral lesions were seen at concentrations  $\geq 1$  mg/kg (Wyatt et al., 1973).

T-2 toxin was administered to 10 laying hens at a concentration of 20 mg/kg of feed for 3 weeks, equivalent to 0.86–0.91 mg/kg bw on the basis of the reported weekly feed consumption during the study. The T-2 toxin was extracted from *F. tricinctum* culture and purified by the method of Burmeister (1971) to yield a crystalline product melting at 148–150 °C. In comparison with untreated controls, the T-2 toxin-treated hens had reduced feed consumption, body weight, and egg production and thinner egg shells (Wyatt et al., 1975).

Single-comb white Leghorn hens were fed a diet containing T-2 toxin (purity not specified) at 0.5, 1, 2, 4, or 8 mg/kg for 8 weeks. Feed consumption, egg production, and shell thickness were significantly decreased in hens fed the highest concentration. Furthermore, the hatchability of fertile eggs of hens fed 2 or 8 mg/kg of diet was lower than that of hens fed the control diet (Chi et al., 1977b).

Feed consumption and egg production were also reduced in groups of 10 laying hens, 33 weeks old, fed a diet containing T-2 toxin (purity not specified) at 2 mg/kg for 24 days. The total amount of T-2 toxin consumed per chicken per day was 0.2 mg on the basis of feed intake rates and the concentration of T-2 toxin in the feed. Body weights were not reported; however, the authors stated that there was no reduction in body weight (Diaz et al., 1994).

### Cats

T-2 toxin was administered to four groups of four to six cats orally in gelatin capsules on alternate days at a dose of 0.06, 0.08, or 0.1 mg/kg bw per day, until death. The animals survived for 6–40 days. Emesis, anorexia, bloody diarrhoea, and ataxia were observed. The cats lost weight and became emaciated. The gross lesions observed included multiple petechiae to ecchymotic haemorrhages of the intestinal tract, lymph nodes, and heart. The lumen of the gut contained copious amounts of dark-red material. The microscopic lesions included haemorrhages in the gut, lymph nodes, heart, and meninges, necrosis of the gastrointestinal epithelium, and decreased cellularity of the bone marrow, lymph nodes, and spleen. The mean survival time was inversely related to the dose of T-2 toxin (Lutsky et al., 1978). The Committee noted that, given the substantial detoxication of T-2 toxin by glucuronide conjugation in rats, dogs, and pigs (Corley et al., 1985; Gareis et al., 1986; Sintov et al., 1986, 1987), the severe effects in cats at this low dose were likely to be a result of deficient glucuronide conjugation in this species. For example, as stated in Annex 1, reference 123, p. 40: “The urinary metabolites of benzoic acid were determined after oral administration of <sup>14</sup>C-benzoic acid, mostly at a dose of 50 mg/kg bw, to a large variety of species, including primates, rodents, carnivores, reptiles, and birds.... Substantial amounts of the glucuronide of benzoic acid, in addition to hippuric acid, were detected in the urine of carnivores, except in cats (Bridges et al., 1970).” and “An outbreak of poisoning affected 28 cats that had eaten meat containing 2.39% benzoic acid. The effects were nervousness, excitability, and loss of balance and vision. Convulsions occurred, and 17 cats died or were killed. Autopsies showed damage to the intestinal mucosa and liver. The sensitivity of cats may be due to their failure to form benzoyl glucuronide, ... (Bedford & Clarke, 1971).”

### Pigs

Groups of 10 pigs were fed a diet containing T-2 toxin at 0.0, 0.5, 1, 2, 3, 4, 5, 10, or 15 mg/kg for 3 weeks. On the basis of feed intake and weekly measurement of the T-2 toxin concentration in the feed, the 3-week average daily intakes were equal to 0.029, 0.062, 0.10, 0.13, 0.1, 0.08, 0.09, and 0.23 mg/kg bw per day, respectively. The T-2 toxin was extracted from a culture of *F. tricinctum* and determined to be > 90% pure by gas and liquid chromatography. Feed was refused at all concentrations tested. Decreased weight gain was observed at concentrations  $\geq 1.0$  mg/kg of feed. Hyper- and parakeratosis, acanthosis, erosion, and inflammatory-cell infiltration were observed on histological examination of the skin near the mouth and snout of pigs at all doses. Clinical haematological parameters, such as serum enzyme activities and urea and glucose concentrations, were affected at 0.029 and 0.062 mg/kg bw per day (Rafai et al., 1995a). The Committee noted that the changes in clinical haematological parameters were not clearly dose-related and may have been due in part to reduced feed intake.

Nine young male pigs were fed a diet containing T-2 toxin (purity, 99%) at a concentration of 8 mg/kg for 30 days, equivalent to 0.64 mg/kg bw per day. Feed intake, weight gain, and haemoglobin concentrations were reduced in treated pigs. Serum alkaline phosphatase activity was also significantly decreased (Harvey et al., 1994).

### *Ruminants*

Groups of five male Suffolk-Finn-Columbian lambs were fed diets containing T-2 toxin (purity, 99%) to give a dose of 0.0, 0.3, or 0.6 mg/kg bw per day for 21 days. All treated lambs developed focal hyperaemia and dermatitis at the mucocutaneous junction of the commissure of the lips, diarrhoea, leukopenia, lymphopenia, and lymphoid depletion of the mesenteric lymph nodes and spleen (Friend et al., 1983a).

Four calves were given T-2 toxin at a dose of 0.08, 0.16, 0.32, or 0.6 mg/kg bw per day orally in capsules for 30 days. The calf at the highest dose developed a hunched stance and died on day 20. Some evidence of mild enteritis was seen at all doses. Bloody faeces were observed at doses  $\geq$  0.32 mg/kg bw per day. At necropsy, abomasal ulcers were present in the calf at 0.16 mg/kg bw per day and rumenal ulcers in the two calves given the two higher doses. Prothrombin times and the activity of serum aspartate aminotransferase were increased in calves at the two higher doses (Pier et al., 1976).

### **2.2.3 Long-term studies of toxicity and carcinogenicity**

No standardized long-term studies of HT-2 toxin were available.

Groups of 50 male and 50 female weanling CD-1 mice were fed a semi-synthetic diet containing T-2 toxin (purity, 99%) at 1.5 or 3 mg/kg, equivalent to 0.22 and 0.45 mg/kg bw per day, for 71 weeks. The survival rate (reported in a graph) was consistently lower in the control groups of both males and females, beginning from about week 35; the survival rate of treated males at 71 weeks was 75% or more, whereas that of the controls was about 62%. No statistically significant differences among the groups were found in feed consumption or body-weight gain. A dose-related increase in heart weight was seen in males receiving T-2 toxin, being statistically different from controls at the high dose. No changes were seen in the heart weights of females, and no treatment-related weight changes were reported for other organs in either males or females. No treatment-related changes were reported in haematological parameters or in the response to sheep red blood cell challenge. A dose-related increase in the frequency of squamous mucosa hyperplasia was found in the forestomach of both male and female mice, with an increased frequency of hyperkeratosis. The incidence of pulmonary adenoma increased in a dose-related manner in males (10, 15, and 23% for controls and at the low and high dose, respectively) but not in females. The incidences of pulmonary adenomas and hepatic adenomas in males at the high dose were statistically significantly higher than in controls. The incidence of pulmonary adenocarcinoma was 5% in control males and 6% in males at the high dose (Schiefer et al., 1987). The Committee noted the lower survival rate of control rats.

Dermal application of 100  $\mu$ g of 7,12-dimethylbenz[*a*]anthracene (DMBA) to 45 BALB/c mice, followed 1 week later by T-2 toxin at 0.5  $\mu$ g, three times a week for 26 weeks, resulted in papillomas in eight mice and a carcinoma in one, with one papilloma in 15 mice that received DMBA alone. In the same study, 35 BALB/c mice were given dermal applications of 5  $\mu$ g of T-2 toxin for 6 days, followed 1 week later by application of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or acetone three times a week for 26 weeks. No tumours were observed in either group. In 21 BALB/c

mice, no papillomas were seen after DMBA or T-2 toxin alone [dosing regimen not translated from Chinese], but papillomas occurred in two of 22 mice treated with DMBA followed by T-2 toxin and four of 21 mice treated with T-2 toxin followed by TPA. One skin carcinoma was observed in mice treated with DMBA followed by T-2 toxin (Yang & Xia, 1988a,b).

Dermal application of 5, 10, or 20 µg of T-2 toxin to the backs of 20 mice (strain not specified) followed after 2 weeks by croton oil twice weekly for 10 weeks did not produce papillomas. Two of 20 mice developed papillomas after dermal treatment with DMBA followed 2 weeks later by 10 µg of T-2 toxin twice weekly for 10 weeks; however, the papilloma rate was not statistically different from that in DMBA-treated controls (Marasas et al., 1969). The Committee noted that the statistical test and the results for DMBA-treated mice were not reported.

In a promotion–initiation study in groups of eight CD-1 mice, an initial dose of DMBA, aflatoxin B<sub>1</sub>, or T-2 toxin (25 µg) was applied to shaved areas on the back and was followed 4 days later by weekly applications of 10 or 25 µg of T-2 toxin to the same area for 22 weeks. One of eight mice treated with DMBA once and 25 µg of T-2 toxin for 22 weeks developed papillomas. No papillomas were observed in any other T-2 toxin-treated groups. Adaptation to the dermal effects of T-2 toxin was observed: the initial T-2 toxin treatment caused severe skin reactions (necrosis and sloughing), while applications 4 or 7 days later and weekly thereafter caused only minor or no irritation. The authors concluded that T-2 toxin was not initiating but was a weak promoter (Lindenfelser et al., 1974)

Fifty male Kunming mice received T-2 toxin in ethanol:saline at a dose of 0.1 mg/kg bw per day three times per week by oral gavage for up to 25 weeks. Forestomach papillomas occurred in five of 35 treated animals, with one each at weeks 6 and 20 and three at week 25 of exposure. No papillomas of the forestomach were observed in 30 control mice (Yang & Xia, 1988a). The Committee noted that few experimental details were provided.

Groups of 16–22 female DDD mice were given feed containing T-2 toxin at a concentration of 0, 10, or 15 mg/kg, equivalent to 0, 1.5, and 2.2 mg/kg bw per day, for 12 months. Two mice from each group were killed at 3, 6, and 9 months. At 12 months, all controls were killed, and five mice in each treated group were fed a T-2 toxin-free diet for 3 months. Lesions were observed in the oesophageal region of the stomach of mice fed T-2 toxin, which included hyperkeratosis, acanthosis, and papillomatosis with inflammatory-cell infiltration of the squamous epithelium. These changes were found 13 weeks after the start of treatment and persisted throughout the 12-month feeding; however, most had subsided by 3 months after cessation of treatment. One adenocarcinoma of the glandular stomach and two hepatocellular carcinomas were observed in mice at the high dose. No papillomas of the forestomach were found (Ohtsubo & Saito, 1977). The Committee noted that few experimental details were provided, including the purity of the T-2 toxin used and the way in which it was incorporated into the diet.

A working group convened by IARC (1993) evaluated the experimental data on the carcinogenicity of T-2 toxin and concluded that it was not classifiable as to its carcinogenicity to humans (Group 3).

### 2.2.4 Genotoxicity

The results of studies on the genotoxicity of T-2 toxin are summarized in Table 4.

Hydroxyurea did not alter unscheduled DNA synthesis in cells treated with T-2 toxin or HT-2 toxin at 6 ng/ml; however, the combination of rat liver microsomes and hydroxyurea increased the rate of unscheduled DNA synthesis in cells exposed to HT-2 toxin at 100 µg/L. The authors concluded that the microsomal drug-metabolizing enzyme system participates in the induction of DNA damage by T-2 toxin (Agrelo & Schoental, 1980).

### 2.2.5 Reproductive toxicity

No data were available on the reproductive toxicity of HT-2 toxin.

#### (a) Multigeneration study

A two-generation study of reproductive and developmental toxicity was conducted in which 90 female CD-1 mice were fed a semi-synthetic diet containing T-2 toxin (purity, 99%) at a concentration of 1.5 or 3 mg/kg, equivalent to 0.22 and 0.45 mg/kg bw per day. The body-weight gain of dams was similar in all groups. T-2 toxin had minimal, if any, effects on female reproduction and fetal development and was not teratogenic or fetotoxic. Offspring of dams at the higher concentration (through milk) had an initial depression of weight gain, but the weights by 6 weeks of age were reported to be within the normal range for Swiss outbred mice. The weights of the spleen of male offspring of treated dams were greater than that of male offspring of control dams (Rousseaux et al., 1986).

#### (b) Developmental toxicity

Groups of five female B6C3F<sub>1</sub> mice received T-2 toxin by gavage at a dose of 1.2 or 1.5 mg/kg bw per day on days 14–17 of gestation. On day 18, fetal liver cells were collected and pooled per dam for staining and flow cytometry. Depletion of cells expressing CD44 and CD45 antigens was observed. Subsequent analysis of a prolymphocyte-enriched culture of fetal liver cells exposed to T-2 toxin also showed selective elimination of a subpopulation of lymphocytes suggested to be of the CD45<sup>+</sup> B-lineage. Similar reductions were found in CD44<sup>lo</sup> and CD45R<sup>+</sup> bone-marrow cells of adult mice exposed to T-2 toxin by gavage at 1.8 mg/kg bw per day, suggesting that B-cell precursors are a sensitive target for T-2 toxin (Holladay et al., 1995).

T-2 toxin was injected intraperitoneally at a dose of 0.5, 1, or 1.5 mg/kg bw into pregnant mice on day 7, 8, 9, 10, or 11 of gestation (number of mice per dose and per gestation day not reported). The two higher doses caused significant maternal mortality, fetal deaths, and fetal body-weight loss. Gross malformations were seen in 37% of the fetuses of dams given 1 mg/kg bw (eight litters) or 1.5 mg/kg bw (four litters) on day 10. The most frequent anomalies were bent, shortened, or missing tails and limb malformations including oligodactyly and syndactyly. Exencephaly, open eyes, retarded jaw, and skeletal malformations of the rib or vertebrae were also found (Stanford et al., 1975). The Committee noted that intraperitoneal injection of T-2 toxin might have resulted in high concentrations in the conceptus and that the malformations occurred at doses that were maternally toxic.

**Table 4. Results of assays for genotoxicity with T-2 toxin**

Test system	Test object	Concentration	Results	Reference
<i>In vitro</i>				
Reverse mutation <sup>a</sup>	<i>S. typhimurium</i> , TA100, TA1535, TA1537, TA98	50 µg/ml	Negative <sup>a</sup>	Wehner et al. (1978)
	<i>S. typhimurium</i> , TA1535, TA1537, TA1538	50 000 ng/ml	Negative <sup>a</sup>	Kuczuk et al. (1978)
	<i>S. typhimurium</i> , TA100	100 µg/plate	Negative <sup>a</sup>	Takahashi et al. (1992)
SOS DNA repair (spot test)	<i>E. coli</i> PQ37	Not specified	Negative <sup>a</sup>	Auffray & Boutibonnes (1986)
SOS DNA repair (Chromotest)	<i>E. coli</i> PQ37	1000 ng/ml	Negative <sup>a</sup>	Krivobok et al. (1987)
Differential toxicity	<i>B. subtilis</i> rec strains	100 µg/plate	Negative	Ueno & Kubota (1976)
Mitotic cross-ing-over	<i>Saccharomyces cerevisiae</i> ade2 locus	100 µg/plate	Negative <sup>a</sup>	Kuczuk et al. (1978)
Petite forward mutation	<i>Saccharomyces cerevisiae</i>	50 000 ng/ml	Negative	Schappert & Khachatourians (1986)
DNA single-strand breaks	BALB/c mouse primary hepatocytes	5 ng/ml	Weakly positive	Lafarge-Frayssinet et al. (1981)
	BALB/c mouse spleen lymphocytes	5 ng/ml	Positive	
	BALB/c mouse thymic lymphocytes	5 ng/ml	Positive	
Gene mutation	Chinese hamster V79 fibroblasts, thioguanine	100 ng/ml	Positive <sup>b</sup>	Zhu et al. (1987)
Sister chromatid exchange	Chinese hamster V79 fibroblasts	2300 ng/ml	Weakly positive <sup>a</sup>	Thust et al. (1983)
	Chinese hamster V79 fibroblasts	100 ng/ml	Weakly positive <sup>b</sup>	Zhu et al. (1987)
	Human lymphocytes	3 ng/ml	Negative <sup>a</sup>	Cooray (1984)
Chromosomal aberrations	Chinese hamster V79 fibroblasts	500 ng/ml	Positive <sup>a</sup>	Thust et al. (1983)
	Chinese hamster V79 fibroblasts	5 ng/ml	Positive	Hsia et al. (1986)
	Chinese hamster V79 fibroblasts	50 ng/ml	Weakly positive <sup>a</sup>	Zhu et al. (1987)
	Chinese hamster V79 fibroblasts	1 ng/ml	Positive	Hsia et al. (1988)
	Human lymphocytes	0.1 ng/ml	Positive	Hsia et al. (1986)

Table 4 (contd)

Test system	Test object	Concentration	Results	Reference
Micronucleus formation	Chinese hamster V79 fibroblasts	50 ng/ml	Positive <sup>a</sup>	Zhu et al. (1987)
Unscheduled DNA synthesis	Human fibroblasts	5 ng/ml	Positive	Oldham et al. (1980)
Inhibition of intercellular communication	Chinese hamster V79 cells	3 ng/ml	Positive	Jone et al. (1987)
<i>In vivo</i>				
Polyploidy induction	<i>Allium cepa</i>	20 000 ng/ml	Positive	Linnainmaa et al. (1979)
Sex-linked recessive lethal mutations	<i>Drosophila melanogaster</i>	63 000 ng/ml	Weakly positive	Sorsa et al. (1980)
	<i>Drosophila melanogaster</i>	100–1000 mg/kg in feed, 2–3 days	Negative	
Sex-linked chromosomal loss	Adult <i>Drosophila melanogaster</i>	20 mg/kg in feed, 48 h	Positive	Sorsa et al. (1980)
DNA single-strand breaks	BALB/c mouse liver	3 mg/kg bw, intraperitoneally	Negative	Lafarge-Frayssinet et al. (1981)
	BALB/c mouse spleen	3 mg/kg bw, intraperitoneally	Positive	
	BALB/c mouse thymus	3 mg/kg bw, intraperitoneally	Weakly positive	
Micronucleus induction	Chinese hamster bone marrow	3 mg/kg bw, intraperitoneally	Negative	Norppa et al. (1980)
Chromosomal aberrations	Chinese hamster bone marrow	1.7 mg/kg bw, intraperitoneally	Weakly positive	Norppa et al. (1980)
	Mice	0.1 mg/kg of feed	Positive	Bilgrami et al. (1995)

Adapted from IARC (1993)

<sup>a</sup> With and without metabolic activation

<sup>b</sup> With metabolic activation

Pregnant CD-1 mice (18 litters per treatment group) were given T-2 toxin dissolved in propylene glycol intraperitoneally at a dose of 0.5 mg/kg bw on day 8 or 10 of gestation. Treatment induced grossly malformed fetuses, principally with tail and limb anomalies. A higher incidence of malformations was observed when T-2 toxin was combined with ochratoxin A at 4 mg/kg bw (Hood et al., 1978). The Committee noted that intraperitoneal injection of T-2 toxin might have resulted in high concentrations in the conceptus.

T-2 toxin dissolved in a 1:1 mixture of propylene glycol and 0.1 N sodium bicarbonate was administered intraperitoneally at a dose of 0.5 mg/kg bw alone or in combination with rubratoxin B at 0.4 mg/kg bw to pregnant CD-1 mice on day 1 of gestation. Only T-2 toxin caused gross malformations. The combination of toxins increased the adverse effects on fetal body weight and mortality rate but not the incidence or severity of the gross malformations (Hood, 1986). The Committee noted that intraperitoneal injection of T-2 toxin might have resulted in high concentrations to the conceptus.

Groups of 10–29 CD-1 mice received crystalline T-2 toxin dissolved in propylene glycol orally by gavage at a dose of 0, 0.5, 1, 2, 3, 3.5, or 4 mg/kg bw on day 9 of gestation. One control group was fed normally and another was starved for 36 h after injection of the vehicle. Fetal morphology and implantation loss were examined on day 18 of gestation. Three of 20 starved controls and 0/19, 1/10, 0/20, 3/20, 9/25, 10/16, and 21/29 of mice given T-2 toxin at 0.0, 0.5, 1, 2, 3, 3.5, and 4 mg/kg bw, respectively, died. T-2 toxin at doses of 3.5 and 4 mg/kg caused more maternal deaths than in the starved controls. Over the 9-day period, reduced feed consumption and weight gain were seen at 4 mg/kg bw, and reduced weight gain alone was seen at 3.5 mg/kg bw. The rate of fetal resorption was 6% in the starved control group and 100%, 73%, and 4% at 4, 3.5, and 0 mg/kg bw, respectively. More skeletal abnormalities were seen in the starved controls and T-2 toxin-treated mice at 3 mg/kg bw.

In a second experiment, a single dose of T-2 toxin at 3 mg/kg bw was given by oral gavage on day 6, 7, 8, 9, 10, 11, or 12 of gestation to CD-1 mice. Fetal morphology and implantation loss were examined on day 18 of gestation. Control groups for each treated group were starved for 36 h after administration of the vehicle. The fetuses of 279 dams were examined, but the number of dams per treated and control group and the number of animals that died before examination were not reported. The treated females lost more fetuses than controls, and there were more dead fetuses among litters treated on day 9 of gestation than on other days. Major skeletal defects (not defined) were reported to be more numerous in mice treated on day 7 of gestation than in those treated on other days or in starved controls. The authors concluded that a single oral dose of T-2 toxin in propylene glycol was primarily maternally toxic and embryo-lethal; the defective development was possibly secondary to the maternal toxicity (Rousseaux & Schiefer, 1987).

Wag rat dams were treated on days 14–20 of gestation with T-2 toxin at 0.1, 0.2, or 0.4 mg/kg bw per day by intraperitoneal injection or at 0.1 or 0.4 mg/kg bw per day in the diet. Feed intake, weight gain, and the numbers of treated and control dams were not reported. Four to 29 rat pups per dose and time of sacrifice were examined; no mention was made of efforts to control for litter effects. Differences in thymus weight were seen in all treated groups. In rat pups examined 1 day after birth, the thymus weights, expressed as a percentage of total body weight, were 30–39% lower in treated rats, with the exception of those treated at 0.1 mg/kg bw per day intraperitoneally, which had thymus weights 11% lower than those of controls. Liver weight as a percentage bw was reported to be higher in 1-day-old pups of dams dosed with 0.4 mg/kg bw per day intraperitoneally. No differences were seen in liver or spleen weights at other doses and times. One week after parturition, thymic atrophy was less marked, suggesting that the effect was transient. The

lymphoblastic response of spleen T and B cells (but not thymus T cells) to phytohaemagglutinin, concanavalin A, and lipopolysaccharide was reduced in 4- and 6-day-old offspring of dams that received T-2 toxin at 0.2 mg/kg bw per day intraperitoneally on days 18–20 of gestation (Lafarge-Frayssinet et al., 1990). The Committee noted the lack of detail in reporting and the lack of statistical analysis of the any of the reported differences. In general, the differences were difficult to interpret.

(c) *Reproductive endocrine effects*

Groups of 10 New Zealand white rabbits were fed a diet consisting of 'naturally infected' wheat containing T-2 toxin at a concentration of 0.19 mg/kg, equivalent to 0.008 mg/kg bw per day, for 32 days. A control group was fed uncontaminated wheat. The treated animals were then given gonadotropin-releasing hormone to induce false gestation plus T-2 toxin for a total duration of T-2 toxin treatment of 50 days; progesterone levels were monitored during this time. The concentrations of T-2 toxin and zearalenone were determined by HPLC; no other toxins were assayed. Two animals died during the 32-day treatment period, and one animal died during the subsequent treatment, each of *Staphylococcus aureus* infection. No control animals died. No gross morphological differences were found in three treated and three control animals killed after 32 days. Three of the five animals given T-2 toxin and gonadotropin-releasing hormone showed abnormal progression of progesterone concentrations, and one of these animals died about 1 week after initiation of hormone treatment. Serum creatinine and alanine aminotransferase activity were higher in treated than in control animals, and the serum cholinesterase concentration was decreased. No differences in feed intake were seen. Body weights were not reported (Fekete & Huszenicza, 1993). The Committee noted the poor description of the experimental design, that naturally infected feed was used, and that the process of infection and the organism used were not described.

Perturbation of progesterone progression after stimulation of ovarian activity was reported in groups of four to five ewes given T-2 toxin (purity, 95%) at 0.005 or 0.015 mg/kg bw per day for 21 days orally by intubation. The authors concluded that progesterone cycling was affected at the higher dose (Huszenicza et al., 2000). The Committee noted that the report did not permit evaluation of the results. Examples of progesterone profiles were given, but no statistical analysis was reported.

Four heifers fed a diet that stimulated ruminal acidosis were given T-2 toxin (purity, 95%) at a dose of 0.025 mg/kg bw per day by oral intubation for 20 days after initiation of ovulation. Ruminal acidosis was induced in order to investigate an increased rate of abortions that had been observed in a dairy herd on a similar diet contaminated with T-2 toxin. Three heifers were fed the acidosis-stimulating diet without T-2 toxin; no heifers were fed diets that did not stimulate ruminal acidosis. The ovarian activity of the heifers was synchronized by administering 25 mg of prostaglandin F<sub>2α</sub> intramuscularly 23 and 12 days before T-2 toxin and on the day of initiation of T-2 toxin treatment. Plasma progesterone was sampled on alternate days starting 12 days before T-2 toxin treatment and continuing 10 days afterwards. The authors reported that ultrasonography showed no difference in the number of antral follicles or the size of the dominant follicle. All the treated heifers ovulated, but

the mean time to ovulation was significantly greater in T-2 toxin-treated animals when compared with their pre-treatment ovulation time and with the ovulation time of controls that did not receive T-2 toxin. The progesterone concentrations remained low for about 2 days longer after the last prostaglandin  $F_{2\alpha}$  injection in the T-2 toxin-treated heifers, suggesting that, under acidotic conditions, a low dose of T-2 toxin can retard follicle maturation and ovulation (Huszenicza et al., 2000).

T-2 toxin inhibited testosterone secretion in gerbil testicular interstitial cells *in vitro*. The median inhibitory dose was 0.042 nmol/L, equivalent to 0.02 ng/mL (Fenske & Fink-Gremmels, 1990).

### 2.2.6 *Special studies*

#### (a) *Cell proliferation and apoptosis in dermal tissues*

Hairless WBN/ILA-Ht rats were given single applications of 10  $\mu$ L of a 0.5 mg/mL solution of T-2 toxin on each of four dorsal skin areas. Control animals were treated with the vehicle, a 20% ethanol solution. Biopsies of treated skin were taken 3, 6, 12, and 24 h after application, and the number of cells staining for proliferating cell nuclear antigen (PCNA) was used as a measure of proliferation. Less epidermal basal-cell proliferation was seen in treated than control animals 3 h after treatment, and the number continued to decrease during the observation period. No degranulation of mast cells was seen, but the number of mast cells increased during the observation period, especially around the small vessels of the dermis. Basal cells showing acidophilic degeneration were noted 12 h after treatment. DNA fragmentation was analysed by tritiated thymidine-mediated dUTP-biotin nick end-labelling (TUNEL). Basal cells showing acidophilic degeneration were generally positive, indicating apoptosis. The number of these cells increased at the 12- and 24-h observation times (Albarenque et al., 1999).

#### (b) *Haematological effects*

Groups of five male CD-1 mice were given T-2 toxin orally by gavage at a dose of 0.1, 0.5, or 2.5 mg/kg bw per day for 2 weeks. Increased formation of macrophage colonies from cultured bone marrow was observed, but no change was seen in the number of granulocyte or granulocyte–macrophage (GM) colonies. At 0.5 mg/kg bw per day, no change in macrophage colonies was observed in bone-marrow culture, but an increased number of granulocyte colonies and a decreased number of GM colonies were observed. At 2.5 mg/kg bw per day, there was no change in the number of granulocyte colonies, but an increased number of macrophages and a decreased number of GM colonies were seen. Increased spleen weight and decreased thymus weight (both relative to body weight) and a decreased red blood cell count were observed at the highest dose. No change was seen in the leukocyte count at any dose. Exposure of gGM progenitor cells from unexposed mice to T-2 toxin inhibited their proliferation into granulocyte or GM colonies at concentrations  $\geq 1$  nmol/L (0.47 ng/ml) and inhibited their proliferation to macrophage colonies at concentrations  $\geq 3$  nmol/L (1.4 ng/ml) (Dugyala et al., 1994).

Groups of 10 male BALB/c mice received T-2 toxin by subcutaneous injection at a dose of 0.17, 0.3, 0.44, 0.66, 1, or 1.5 mg/kg bw 1 h before an intraperitoneal

injection of  $^{59}\text{Fe}$ . Blood was drawn 24 and 72 h after  $^{59}\text{Fe}$  injection. Dose-dependent inhibition of  $^{59}\text{Fe}$  uptake into erythrocytes was seen at doses of T-2 toxin  $\geq 0.3$  mg/kg bw at both 24 and 72 h. The mean response to the lowest dose was also lower than that of controls but was not statistically significant. The leukocyte count was affected in a dose- and time-dependent manner. In a separate experiment, groups of four to six male BALB/c mice received T-2 toxin at the same doses, followed 3, 24, or 72 h later by withdrawal of blood for leukocyte counting. Dose-dependent leukocytosis was observed 3 h after injection of T-2 toxin, with increases of several hundred per cent in the count at doses  $\geq 0.66$  mg/kg bw. Conversely, 24 h after injection, the leukocyte count was less than 50% that of controls at the same doses. By 72 h after T-2 toxin injection at doses  $\geq 0.3$  mg/kg bw, the leukocyte count was numerically lower but was not significantly lower than that of controls. The erythrocyte count was not affected at the doses tested, nor was the serum Fe concentration changed by treatment, suggesting that the effect on Fe incorporation occurred at the level of erythrocyte formation. The lowest statistically significant level of effect on  $^{59}\text{Fe}$  uptake in circulating erythrocytes was 0.3 mg/kg bw (Faifer & Godoy, 1991).

The proliferation of colony-forming units (CFU) GM colonies was assessed in the bone marrow of groups of eight to ten male BALB/c mice that received a single subcutaneous injection of T-2 toxin at 0.17, 0.3, 0.6, or 1 mg/kg bw. All doses caused a decrease in the number of colonies 1 h after dosing. The effect was transient, with a slight increase 72 h after exposure. Significant inhibition of  $^{59}\text{Fe}$  uptake in circulating erythrocytes was seen at doses  $\geq 0.3$  mg/kg bw 1 h after dosing, with non-significant inhibition at 0.17 mg/kg bw. A significant rebound to levels of  $^{59}\text{Fe}$  uptake above that of controls was seen even at the lowest dose 72 h after treatment (Faifer et al., 1992).

Recovery after T-2 toxin treatment occurred more readily in spleen than in bone marrow in male BALB/c mice. A single subcutaneous dose of 2 mg/kg bw reduced  $^{59}\text{Fe}$  uptake into erythrocyte precursors in bone marrow and spleen. The uptake into spleen returned to control levels by 3 days, but that into bone marrow was depressed for 15–21 days. The cellularity of spleen and femur marrow had decreased 1 day after T-2 toxin injection, but that of the femur returned to normal and that of the spleen was increased to 200% of the control value by day 6. By day 35, the cellularity of the spleen had returned to normal (Velazco et al., 1996).

Repeated doses of T-2 toxin at 2 mg/kg bw per day for 3 days to groups of five or more male BALB/c mice did not cause additional damage to their blood-forming capacity over that seen with single doses. The repeated doses caused similar cellular depletion in the spleen and femur marrow, with a similar recovery period (Velazco et al., 1996). Furthermore, repeated doses did not inhibit the haematopoietic response to bleeding, consisting in removal of approximately one-third of the total blood volume on each of two successive days, the volume being replaced by saline. Rather, there was an increased response, measured as total nucleated cellularity and total erythroid cellularity in spleen up to 35 days after T-2 toxin treatment (Godoy et al., 1997).

Twenty male weanling outbred Swiss mice were fed a dry diet containing purified crystalline T-2 toxin at a concentration of 20 mg/kg for 41 days, and another group of four animals received the same diet for 21 days, followed by control diet for 7 days. One control group of 20 animals was maintained on restricted diet; a second

control group consisting of eight animals was given the diet *ad libitum*; and 12 animals were killed at day 0. Haematological examinations were made weekly of treated and control animals. During the first 3 weeks of treatment, lymphoid tissues, bone marrow, and splenic red pulp became hypoplastic, resulting in anaemia, lymphopenia, and eosinopenia. Subsequently, during continued treatment, regeneration occurred, leading to hyperplasia of the haematopoietic cells by 6 weeks. All treated animals also developed perioral dermatitis and ulceration of the gastric mucosa. The authors concluded that T-2 toxin is irritating and suppresses haematopoiesis; however, the haematopoietic effects were transient at the dose tested and did not lead to haematopoietic failure (Hayes et al., 1980).

Incubation of CFU-GM from the bone marrow of rats with T-2 toxin and HT-2 toxin at 1 nmol/L (equivalent to 0.47 ng/ml) for 7, 10, or 14 days inhibited the growth of the cells (Parent-Massin & Thouvenot, 1995).

Hartley guinea-pigs (number not stated) given T-2 toxin dissolved in ethanol by intramuscular injection at the 24-h LD<sub>50</sub> dose of 1 mg/kg bw showed decreased activities of all coagulation factors except fibrinogen. Platelet aggregation in the whole blood response to ADP and collagen was depressed. The animals also showed an initial rise, followed by a fall in erythrocyte volume fraction, leukocytosis, and a fall in the platelet count. These changes, which were found within a few hours of administration, reached a maximum at 24 h and returned to normal over the next 2 days. Pretreatment with vitamin K<sub>1</sub> did not prevent the effects of T-2 toxin on coagulation. Addition of the toxin to plasma and blood of untreated guinea-pigs at a concentration of 1 mg/mL did not affect clotting times or platelet aggregation, indicating that T-2 toxin itself did not have a direct effect on the activity of coagulation factors (Cosgriff et al., 1984).

Eight New Zealand white rabbits were given T-2 toxin dissolved in dimethyl sulfoxide by intravenous injection at 0.5 mg/kg bw; and five rabbits were given a single oral dose of 2 mg/kg bw by gavage. The rabbits treated intravenously showed reductions in both packed cell volume and total leukocyte count, but no significant alterations in haematological parameters were seen in rabbits given T-2 toxin orally. In another study, nine New Zealand white rabbits were given a single intravenous injection of T-2 toxin dissolved in dimethyl sulfoxide at a dose of 0.5 mg/kg bw; five animals received daily subcutaneous injections of vitamin K at a dose of 0.5 mg/kg bw per day for 5 days before administration of the same dose of T-2 toxin for the subsequent 4 days. Two groups of eight rabbits served as controls. Blood samples were taken from each animal before treatment with dimethyl sulfoxide and 6–96 h later. The concentrations of coagulation factors VII, VIII, IX, X, and XI were decreased by about 40% within 6 h of administration of T-2 toxin, and the fibrinogen content was elevated at 24 h. The reduction in coagulation factors did not induce clinical haemorrhage, however, and administration of vitamin K did not alter the effects of T-2 toxin, indicating that the effect of the toxin on coagulation was not due to antagonism of vitamin K (Gentry & Cooper, 1981).

Cats given T-2 toxin subcutaneously or in the feed showed leukocytosis followed by leukopenia. The frequency of dosing and the dose were different for individual cats, and there were few cats per dose, so that the dose–response relationship could not be analysed. When three cats were given T-2 toxin subcutaneously at

0.05 mg/kg bw per day for 12 days, a transient increase in leukocyte count was seen in two cats within the first few days. This transient increase was followed by a decrease in all cats, to < 20% of the initial values by the last day of dosing. Of the three cats, two died within 35 days after the last injection. Deaths occurred at all doses tested (Sato et al., 1975).

Extracted, purified T-2 toxin was administered to cats in gelatin capsules every 2 days at a dose of 0.08 mg/kg bw per day (six cats) or 0.1 mg/kg bw per day (four cats). Pancytopenia and death occurred within 6–24 days for all cats (Lutsky et al., 1978).

Ten male cats were anaesthetized with ketamine and given T-2 toxin orally by capsule at a dose of 0.08 mg/kg bw every 48 h until death; two cats received the vehicle only. Blood was drawn at each administration. All 10 treated cats but neither of the controls died within 32 days of initiation of treatment, with an average survival time of 21 days. The clinical signs included vomiting, bloody faeces, weakness, lassitude, ataxia, dyspnoea, dehydration, loss of weight, and pre-terminal anorexia. Progressive decreases were observed in total leukocyte count, erythrocyte volume fraction, haemoglobin, and thrombocyte count during treatment. Initial leukocytosis was observed in most cats. On gross examination, subcutaneous petechiae, haemorrhagic lymph nodes, and multiple haemorrhagic erosions were observed in the gastric and intestinal mucosa. Petechial haemorrhages and ecchymoses were observed on the myocardium (Lutsky & Mor, 1981).

The effects of T-2 toxin on blood coagulation were studied in groups of 40-day-old chickens fed diets containing T-2 toxin at a concentration of 1, 2, 4, 8, or 16 mg/kg, equivalent to 0.12, 0.25, 0.5, 1, and 2 mg/kg bw. Forty birds served as controls. The activities of factor X, prothrombin, and fibrinogen were reduced only at the highest concentration. The activity of factor VII was reduced at the three higher concentrations (Doerr et al., 1981).

In a study of the effects of ochratoxin and T-2 toxin, nine young pigs were given feed containing T-2 toxin (purity, 99%) at 8 mg/kg, equivalent to 0.64 mg/kg bw per day, for 30 days. Treatment reduced the haemoglobin concentration and serum alkaline phosphatase activity. No effect was reported on the erythrocyte volume fraction or mean cell volume (Harvey et al., 1994).

The erythrocyte volume fraction was reduced in young pigs fed a diet containing T-2 toxin extracted from a culture of *F. tricinctum* (> 90% pure by gas and liquid chromatography) at a concentration of 3 mg/kg of diet, equivalent to 0.13 mg/kg bw per day. Groups of two 7-week-old pigs (sex unspecified) received diets containing T-2 toxin at concentrations equivalent to doses of 0, 0.029, 0.062, 0.10, or 0.13 mg/kg bw per day for 21 days. A dose-related reduction in leukocyte count was observed at all doses. The haemoglobin concentration was also decreased in a dose-related manner, at doses  $\geq$  0.062 mg/kg bw per day. A reduction in the erythrocyte count was observed at 0.10 and 0.13 mg/kg bw per day (Rafai et al., 1995b). The Committee noted that the changes observed might have been due to reduced feed intake.

Two calves were given T-2 toxin by stomach tube at a dose of 0.2 mg/kg bw per day for 11 days. There were no controls. The treated animals developed clinical

signs of weakness and inappetence, and one died. Prothrombin time was prolonged in both animals, and one had marked neutrophilia. No haemorrhagic syndrome was found (Patterson et al., 1979).

Nine male cynomolgus monkeys received a single intramuscular injection of T-2 toxin at a dose of 0.65 mg/kg bw (LD<sub>20</sub> dose). Three monkeys served as controls. Haematological parameters were measured before injection and 0, 6, 12, and 24 h and 2, 3, and 7 days after treatment. Samples of 8 ml of blood were drawn through a heparinized catheter implanted surgically 7 days before treatment. The monkeys wore leather restraints for the duration of surgery, recovery, and the observation period; they were observed for signs of toxicity and particularly for evidence of haemorrhage. Three of the treated monkeys died < 24 h after treatment, and two more died during the observation period; none of the controls died. The animals that died were necropsied, and necrosis of lymphoid tissues and petechial haemorrhage of the colon and heart were observed. A parallel decline in erythrocyte volume fraction (10–25% over 3 days) was observed in both the surviving treated and control monkeys. Transient leukocytosis was seen, the neutrophil and lymphocyte counts in treated animals being four to five times those before treatment and in controls. The lymphocyte counts returned to normal within 1 day and those of neutrophils within 3 days. Slight lymphopenia was seen at days 2 and 3. Prolongation of prothrombin and activated thromboplastin times and decreased activities of multiple coagulation factors were also observed within hours of administration, which reached a maximum at 24 h and returned to normal over the next 3 days. Fibrin–fibrinogen degradation products were not detected at any time. The platelet counts of treated animals varied nonsignificantly over the observation period but were significantly increased in controls. Despite the changes in haematological parameters, none of the surviving animals had clinical signs of haemorrhage (Cosgriff et al., 1986).

Three male and two female adult rhesus monkeys were given T-2 toxin in 20 ml of milk, initially at 1 mg/kg bw per day for 4 days and at 0.5 mg/kg bw per day on days 5–15. Three males and three females served as controls. All three treated males died of respiratory failure between days 0 and 15. After 30 days' recovery, the two treated females and two additional males received T-2 toxin at 0.1 mg/kg bw per day for 15 days. All monkeys given 1 mg/kg per day showed signs of toxicity similar to those of alimentary toxic aleukia in humans, i.e. vomiting, apathy, and weakness of the lower limbs. The signs were more severe in males, which also developed petechial haemorrhages on the face. All males developed severe leukocytopenia, follicular atrophy of the spleen and lymph nodes, and pneumonia, suggesting involvement of the immune system. Bone-marrow changes were not found at necropsy. All animals at 0.1 mg/kg per day developed leukocytopenia and mild anaemia after 15 days of treatment (Rukmini et al., 1980).

Development of burst-forming unit–erythroid (BFU-E) colonies was scored in cells harvested from human umbilical cord after addition of T-2 toxin or HT-2 toxin to the culture medium for 14 days. The size of the colonies was measured microscopically, and the degree of cell differentiation was measured from the total porphyrin and haemoglobin content. The concentrations tested were 0.1, 0.5, 2.2, and 10 nmol/L (equivalent to 0.047, 0.23, 1.1, and 4.7 ng/ml) for T-2 toxin and 0.1, 0.5, 2.5, and 100 nmol/L (equivalent to 0.042, 0.21, 1.1, and 42 ng/ml) for HT-2

toxin. Significant differences in colony growth were observed at 0.1–2.5 nmol/L of HT-2 toxin, with no difference in porphyrin or haemoglobin concentrations, even though growth was inhibited. No colonies survived at the highest dose of HT-2 toxin (100 nmol/L). No differences in growth were observed with T-2 toxin up to a concentration of 2.2 nmol/L, but the highest concentration caused complete loss of BFU-E colonies. Intermediate doses did change the porphyrin or haemoglobin concentrations, although no dose–response relationship was evident. At the lowest dose of T-2 toxin (0.1 nmol/L), the haemoglobin concentration was greater than that of controls, and at 0.5 nmol/L a decrease in porphyrin concentration was observed (Rio et al., 1997). The Committee noted that the number of individuals on which the mean values were based was not reported.

In cells harvested from human umbilical cord, inhibition of CFU-GM colony growth was observed after 7 days of culture with T-2 toxin at a concentration of 0.1 nmol/L (equivalent to 0.047 ng/ml); however, no inhibition was observed after 10 and 14 days. At 2 nmol/L, T-2 toxin inhibited colony growth at 7, 10, and 14 days. With HT-2 toxin, a concentration of 1 nmol/L (equivalent to 0.42 ng/ml) inhibited growth at 7 days of culture, with no inhibition at day 10 or 14. At 10 nmol/L (equivalent to 4.2 ng/ml), HT-2 toxin inhibited growth at 7, 10, and 14 days (Parent-Massin et al., 1994).

Similar results were obtained for growth of rat and human CFU-GM colonies. GM progenitors were collected from the marrow of rats and from the umbilical cord of humans and were cultured and incubated with T-2 toxin and HT-2 toxin for 14 days. The median inhibitory concentrations ( $IC_{50}$ ) for rat CFU-GM at 14 days were 2.6 nmol/L for T-2 toxin (equivalent to 1.2 ng/ml) and 2.2 nmol/L for HT-2 toxin (equivalent to 0.93 ng/ml), and the values for human CFU-GM were 1.4 nmol/L (equivalent to 0.65 ng/ml) for T-2 toxin and 1.8 nmol/L (equivalent to 0.76 ng/ml) for HT-2 toxin (Lautraite et al., 1995, 1996).

When platelets isolated from 12 healthy volunteers were incubated with T-2 toxin at doses of 5–500  $\mu\text{g}/10^9$  platelets for 20 min, a concentration-related inhibition of platelet aggregation was seen with various activators, including adrenaline, arachidonic acid, and collagen, with release of dense bodies consisting mainly of serotonin-containing granules. There was also a change in membrane permeability but no change in shape. No parallel inhibition of thromboxane synthesis or significant alterations in platelet calcium content were observed. The microtubular system was unaffected (Yarom et al., 1984a).

### (c) *Effect on vascular parameters*

Intravenous administration of T-2 toxin to pigs at a dose of 4 or 8 mg/kg bw resulted in a shock syndrome characterized by reductions in cardiac output and blood pressure and increased plasma concentrations of adrenaline, noradrenaline, thromboxane B<sub>2</sub>, 6-keto-prostaglandin F<sub>1a</sub>, and lactate. The pigs given the higher dose showed signs including persistent vomiting, watery diarrhoea, abdominal straining, cold extremities, coma, and death (Lorenzana et al., 1985).

In the bovine ear perfusion system *in vitro*, T-2 toxin caused dose-dependent vasoconstriction of the peripheral vasculature but was a less potent vasoconstrictor

than histamine or noradrenaline. The presence of known histamine or noradrenergic antagonists did not affect the response to T-2 toxin (Wilson & Gentry, 1985). T-2 toxin administered systemically markedly increased peripheral vascular resistance in conscious rats. The cardiac output gradually decreased, eventually resulting in cardiovascular collapse and death (Feuerstein et al., 1985).

Lethal intravenous doses of T-2 toxin to male Sprague-Dawley rats reduced blood flow and increased vascular resistance in hindquarter, mesenteric, and renal vascular beds. The mean arterial pressure and heart rate were not significantly altered after an intravenous dose of 1 mg/kg bw. Two of five rats died within 5 h, and only one animal survived to 24 h after treatment. The maximum fall in blood flow in mesenteric and renal vascular beds occurred 4 h after injection of T-2 toxin (Siren & Feuerstein, 1986).

(d) *Immunotoxicity*

*Mice*

(i) *Effects of T-2 toxin on spleen and thymus cellularity and lymphoproliferative response*

A single dose of T-2 toxin at 10 mg/kg bw given to female ICR:CD1 mice by oral gavage induced apoptosis in thymus and spleen, as measured by TUNEL. The spleen and thymus weights were decreased, as were the lymphocyte and platelet counts in blood. Hypocellularity was observed in bone marrow and spleen, with specific depletion of myelocytes in bone marrow due to loss of immature granulocytes, erythroblasts, and lymphocytes (Shinozuka et al., 1997a,b, 1998, 1999). TUNEL, indicating apoptosis, was also seen after treatment of female ICR:CD1 mice with T-2 toxin at 2.5 mg/kg bw by oral gavage (Shinozuka et al., 1997a,b) or intraperitoneal injection of female BALB/c mice at 2.5 or 5 mg/kg bw (Ihara et al., 1997; Sugamata et al., 1998).

Thymic atrophy and apoptosis were observed in groups of female BALB/c mice after a single intraperitoneal dose of 1.8 or 3.5 mg/kg bw. A dose of 0.35 mg/kg bw per day did not affect thymus weight or cellularity. The percentage of DNA fragmentation, as a measure of apoptosis, was increased with T-2 toxin treatment at 1.8 mg/kg bw per day but was reduced in animals that received a protein synthesis inhibitor (cycloheximide) 5 min after T-2 toxin, suggesting that protein synthesis may be necessary for the toxicity of T-2 toxin to thymus cells (Islam et al., 1998a).

Depletion of fetal liver B lymphocytes was observed in the offspring of female B6C3F<sub>1</sub> mice exposed on days 14–17 of gestation to T-2 toxin by oral gavage at a dose of 1.2 or 1.5 mg/kg bw per day for 4 days. Fetal livers were collected on day 18 of gestation for cell flow cytometric analysis. A specific subpopulation of CD44<sup>lo</sup> lymphocytes (CD44<sup>lo</sup>) was reduced in fetal livers of dams at 1.5 mg/kg bw per day. The number of CD45R<sup>+</sup> lymphocytes was decreased at both 1.2 and 1.5 mg/kg bw per day. Subsequent analysis of a prolymphocyte-enriched culture of fetal liver cells exposed to T-2 toxin *in vitro* suggested selective elimination of a subpopulation of B-lineage lymphocytes, CD45R<sup>+</sup>. Similar reductions were seen in CD44<sup>lo</sup> and CD45R<sup>+</sup> bone-marrow cells in adult mice exposed to T-2 toxin by gavage at 1.8 mg/kg bw

per day, suggesting that B-cell precursors represent a sensitive target for T-2 toxin (Holladay et al., 1993).

The proliferative response of lymphocytes to phytohaemagglutinin and lipopolysaccharide was examined in male French IC mice treated with T-2 toxin (purity unspecified) at doses representing one-half or one-quarter of the LD<sub>50</sub> for 2 days or one-twelfth of the LD<sub>50</sub> for 15 days. No other information on doses was reported. Stimulation of both T and B cells was inhibited reversibly, and the ability to synthesize antibodies to sheep red blood cells was suppressed. The effects on lymphocytes and fibrosarcoma cells in culture included a direct cytostatic action at high concentrations and stimulation at low concentrations. The histological observations included severe lymphoid damage in the thymus and spleen. The immune system therefore appears to be sensitive to the trichothecenes and is impaired at doses that do not inhibit other organs (Lafarge-Frayssinet et al., 1979).

The proliferative response of spleen lymphocytes of female BALB/c mice to concanavalin A, phytohaemagglutinin, pokeweed mitogen, and lipopolysaccharide was examined in vitro and in vivo. Spleen lymphocytes from five mice were incubated with T-2 toxin at a concentration of 0, 0.25, 0.5, 0.75, 1, or 2.5 nmol/L (equivalent to 0.12, 0.23, 0.35, 0.47, and 1.2 ng/ml) and the mitogens. The lymphoproliferative response in vitro depended on the concentration of T-2 toxin and the mitogen; the response was increased with T-2 toxin at 0.5 nmol/L and concanavalin A and decreased with T-2 toxin at 2.5 nmol/L alone or with concanavalin A; decreased with T-2 toxin at concentrations  $\geq 0.75$  nmol/L T-2 toxin and phytohaemagglutinin, pokeweed mitogen, or lipopolysaccharide. For exposure in vivo, groups of three mice were given intraperitoneal injections of T-2 toxin at 0, 0.8, or 1.6 mg/kg bw per day on days 0, 7, and 14; the spleens were harvested on day 15, and lymphocytes cultured with mitogen. In T-2 toxin treated groups, more lymphoproliferation was seen at 0.08 mg/kg bw per day (a 1000% increase) and less at 1.6 mg/kg bw per day, when compared with controls. When lymphocytes were cultured with mitogen, the lymphoproliferative response was decreased with T-2 toxin at 1.6 mg/kg bw per day plus phytohaemagglutinin, pokeweed mitogen, or lipopolysaccharide. In a separate experiment in which groups of three mice were immunized with keyhole limpet haemocyanin and *Bordetella pertusis* antigen by subcutaneous injection on days 1 and 8 and treated as in the initial experiment, the lymphoproliferative response was increased with T-2 toxin at 1.6 mg/kg bw per day and concanavalin A or phytohaemagglutinin (Paucod et al., 1990).

Male Swiss mice were fed a diet containing T-2 toxin (purity, 99%) at 5, 10, or 20 mg/kg, equivalent to 0.75, 1.5, and 3.0 mg/kg bw per day, for up to 48 days. A control group fed the same amount of food as that consumed by the mice at 20 mg/kg of diet was used to determine the effects of dietary restriction caused by feed refusal in the treated groups; an additional control group was fed *ad libitum*. Eight mice from each control and treated group were killed and examined after 7, 14, 21, 28, and 48 days of treatment. After 7 days, the spleen cell count was significantly lower in the pair-fed control group than in the group fed T-2 toxin at 20 mg/kg of diet, and was lower in all T-2 toxin-treated groups than in controls fed *ad libitum*. At subsequent times, the mean count in pair-fed controls was no different from that of the group given T-2 toxin at the highest concentration, and both were lower than in controls

fed *ad libitum*. The lymphoproliferative response of the spleen to concanavalin A and lipopolysaccharide at the same times was significantly lower at 20 mg/kg of diet and in pair-fed controls than in the group fed *ad libitum*. The mean proliferative response to both mitogens was lower in the group given T-2 toxin at 20 mg/kg of diet than in the pair-fed control group, but the difference was not significant (Friend et al., 1983b).

The T-2 toxin metabolites 3'-OH-T-2, HT-2 toxin, 3'-OH-HT-2, neosolaniol, and T-2 tetraol were tested for their ability to induce apoptosis in the thymus, assessed by DNA fragmentation analysis after an intravenous dose of 1.6 mg/kg bw to female BALB/c mice. 3'-OH-T-2, T-2 toxin, HT-2 toxin, and 3'-OH-HT-2 caused a significant increase in DNA fragmentation. On this basis, 3'-OH-T-2 was as effective as T-2 toxin in causing thymic apoptosis, while HT-2 toxin and 3'-OH-HT-2 were less effective. Neosolaniol and T-2 tetraol did not cause thymic cell apoptosis (Islam et al., 1998b).

#### (ii) Antibody formation

Four male Swiss IC mice received T-2 toxin by intraperitoneal injection at a dose of 0.75 mg/kg bw per day for 7 days. On day 3 of treatment, the mice were immunized with sheep erythrocytes, and all animals were killed 5 days later. T-2 toxin decreased the antibody titres, as measured by haemagglutination stimulation, and reduced the thymic weight. In a second study, seven groups of five mice received T-2 toxin intraperitoneally at a dose of 0.5, 0.6, 0.75, 1, or 2 mg/kg bw per day for 7 days. The mice were immunized on day 3 and killed 5 days later. Antibody-producing cells from the spleen were counted as the number of plaque-forming cells on sheep erythrocytes. A dose-dependent inhibition of plaque-forming cells was observed in T-2 toxin-treated mice, with total suppression of the immune response at 2 mg/kg bw per day. A dose-dependent reduction in thymus weight and antibody titre to sheep red blood cells was seen at 0.5–1 mg/kg bw per day. The immune suppressive effect disappeared within 6 days after cessation of treatment.

In another experiment, 36 Swiss IC mice were treated intraperitoneally with T-2 toxin at 0.75 mg/kg bw per day for 7 days, and groups of four mice were killed at nine times over the subsequent 83 days. The mice were immunized with sheep red blood cells 5 days before being killed. The thymus weight and antibody titre to sheep red blood cells titre returned to the range seen in controls as early as 6 days after cessation of T-2 toxin treatment (Rosenstein et al., 1979).

#### (iii) Graft rejection

Inhibition of cellular immunity by trichothecenes included effects on grafting. The mean length of survival of skin grafted from C57Bl/6 mice onto Swiss mice was 8.7 days in control recipients and 12 days in recipients treated with T-2 toxin at 0.75 mg/kg bw per day for 7 days before skin grafting and then three times a week for 20 days, indicating that T-2 toxin suppressed immunity, resulting in allograft rejection. The areas of the graft in T-2 toxin-treated mice lacked the typical cellular infiltrates of a cell-mediated immune response of macrophages and lymphocytes (Rosenstein et al., 1979).

(iv) *Delayed hypersensitivity*

Delayed hypersensitivity is an immune response mediated by sensitized T lymphocytes. The effect of T-2 toxin on T lymphocyte responses was studied in female BDF<sub>1</sub> mice sensitized by subcutaneous injection of sheep red blood cells followed by measurement of foot-pad swelling. No appreciable effect on delayed hypersensitivity was observed when mice received T-2 toxin at 3 mg/kg bw before or on the day of sensitization. However, when T-2 toxin was administered 2 or 3 days after sensitization, marked enhancement of the response was seen. Since the lifetime of the effective T-2 toxin dose in vivo was short and the optimal timing of injection corresponded to the time of appearance of suppresser cells, it was presumed that trichothecenes interfere with the proliferation of suppresser T cells that appear in mice tolerant to delayed hypersensitivity (Masuko et al., 1977; Otokawa et al., 1979).

(v) *Resistance to infection*

*Mice*

C3H/HeN mice resistant to *Salmonella* were used to examine interactions between T-2 toxin and *Salmonella* infection. Groups of 30 female mice were given T-2 toxin at 1 mg/kg bw by oral gavage every 2 days for 3 weeks, for 11 doses over 21 days. *S. typhimurium* was inoculated at a dose of  $1 \times 10^7$ ,  $2.5 \times 10^6$ , or  $5 \times 10^6$  CFU on day 2 of the 3-week exposure. Two control groups were used: one given the vehicle only and one given the vehicle by gavage plus *S. typhimurium* inoculation. The body-weight gains of the groups receiving T-2 toxin or the vehicle only were equivalent, but that of the group receiving T-2 toxin plus *S. typhimurium* was significantly lower than that of the other groups by day 5. Survival was monitored for up to 30 days (9 days after the end of T-2 toxin treatment). No deaths occurred with T-2 toxin alone, with the vehicle, or at the lowest dose of *S. typhimurium* alone; the mortality rate was significantly greater in all groups receiving T-2 toxin plus *S. typhimurium* than in that given *S. typhimurium* plus vehicle.

In the same study, the mortality rate of groups of 10 animals given T-2 toxin at a dose of 0.1, 0.25, 0.5, or 1 mg/kg bw per day plus *S. typhimurium* at  $3 \times 10^5$  CFU was also examined over a 30-day period. Three deaths occurred in the group given vehicle plus *S. typhimurium*, and a statistically significant increase in the mortality rate (7/10) was observed with T-2 toxin at 0.5 mg/kg bw per day. At 0.1 and 0.25 mg/kg bw per day, the mortality rate was 3/10 and 6/10, respectively. In mice inoculated with  $3 \times 10^5$  CFU *S. typhimurium* and given the vehicle or T-2 toxin at 1 mg/kg bw per day (11 treatments over 21 days), the spleen weight and accumulation of *S. typhimurium* in the spleen were affected by T-2 toxin treatment. Five or more mice given T-2 toxin or the vehicle were killed and their spleens examined on days 2, 5, 7, 12, 15, 18, and 23. The spleen weight was lower by day 12, and remained lower, in T-2 toxin-treated mice than in controls given *S. typhimurium* plus vehicle. The *S. typhimurium* count in the spleen was higher in T-2 toxin-treated mice on day 2, no different from that in vehicle controls on days 5–12, and again higher in T-2 toxin-treated mice on days 15–23. In mice treated with *S. typhimurium* at doses of  $3 \times 10^2$  to  $3 \times 10^6$  CFU per mouse and examined on day 10 after inoculation, those treated with T-2 toxin at 1 mg/kg bw per day on alternate days had significantly higher *S. typhimurium* counts in the spleen. However, the spleen weight in mice treated with T-2 toxin plus *S. typhimurium* was higher than that of mice treated with *S. typhimurium*

at  $3 \times 10^2$  CFU per mouse plus vehicle, and no different from that of mice treated with *S. typhimurium* at  $3 \times 10^3$  to  $3 \times 10^6$  CFU plus vehicle (Tai & Pestka, 1988).

Groups of 10 male BALB/c mice, 7 weeks of age, were given T-2 toxin in their drinking-water at a concentration of 0, 0.2, 1, or 6 mg/L for 4 weeks (equivalent to 0, 0.024, 0.12, and 0.72 mg/kg bw per day), and were given *S. enteritidis* ( $1 \times 10^7$  cells in 0.2 ml) by gastric intubation on day 14. The concentrations of T-2 toxin were insufficient to cause water or food refusal. The survival ratio of T-2 toxin treated to control animals after exposure to *S. enteritidis* was expressed as  $< 0.5$ ,  $< 1.2$ , or  $> 2$ . The ratio was  $< 1.2$  with T-2 toxin at concentrations of 1 and 6 mg/L (i.e. no effect) but was  $> 2$  at 0.2 mg/L (improved survival). (Sugita-Konishi et al., 1998). The Committee noted that the data were presented as ranges of ratios and a statistical analysis was not reported.

Groups of 10–14 ddY male mice weighing 18–20 g were inoculated intravenously with mycobacteria into the tail vein. In a first study, the mice received 0.01 mg of tubercle bacteria (species not indicated) and 0.1 mg of T-2 toxin orally 12 times, starting the day before inoculation, seven times at 1-day intervals, and then five times daily. For comparison, a group of mice was given 5 mg of cortisone acetate intraperitoneally on a similar schedule. A third group was inoculated with tubercle bacteria only. At the end of the 20-day observation period, the mice in the first group had a lower spleen weight and a higher tubercle bacteria count in the spleen than those in the other two groups, indicating greater depression of resistance with T-2 toxin than with cortisone.

In a second study, two groups of animals were inoculated with 0.25 mg of a culture of *Mycobacterium bovis*. One group was given 0.1 mg of T-2 toxin daily for 6 days, starting 8 days after injection, and there were two groups of controls. The average length of survival of the T-2 toxin-treated group was 19 days, whereas that of the untreated group was 35 days, indicating decreased resistance (Kanai & Kondo, 1984).

Single or 7 days of administration of T-2 toxin by gavage to female Han:NMRI mice reduced the virulence of *Staphylococcus hyicus* and *Mycobacterium avium* in mastitis infections. In the single-dose experiment, mice received T-2 toxin at 2.6 mg/kg bw, followed 6 h later by surgical removal of the mammary gland tips and inoculation of the glands with either  $5 \times 10^9$  CFU of *S. hyicus* or  $6 \times 10^7$  CFU of *M. avium*. In the 7-day experiment, mice received T-2 toxin at 0.75 mg/kg bw per day by gavage, followed by infection with  $5 \times 10^9$  CFU of *S. hyicus*. The severity of the mastitis resulting from the infection was scored 48 h after inoculation with *S. hyicus* and 17 days after inoculation with *M. avium*. The infection was generally less severe in animals treated with T-2 toxin than in controls; this difference was more readily apparent in the *S. hyicus*-treated mice. The mice were killed after assessment of the severity of mastitis and serum immunoglobulin (Ig)M, IgG, and IgA were determined. With both protocols of exposure to T-2 toxin, the serum IgA level was significantly increased in infected T-2 toxin-treated animals over that in controls infected with either *S. hyicus* or *M. avium*. The IgM and IgG levels were significantly increased only in *S. hyicus*-infected mice that had received 7 days' treatment with T-2 toxin at 0.75 mg/kg bw per day (Atroschi et al., 1994).

The effects of T-2 toxin on cell-mediated resistance were studied in female ICR mice infected with *Listeria monocytogenes*. Groups of 17 animals (10 in the control group) were inoculated intraperitoneally with  $4 \times 10^5$  (LD<sub>50</sub>) or  $4 \times 10^4$  CFU of *L. monocytogenes*, given a single oral dose of T-2 toxin at 4 mg/kg bw, and observed for 15 days. The bacteria multiplied rapidly in the spleen after T-2 toxin treatment, and the mortality rate was increased in both treated groups. Necrosis and depletion of lymphoid tissue were observed in the thymus, the periarteriolar lymphoid sheaths, and the lymphoid follicles of the spleen. The cellular response to *L. monocytogenes* in the spleen and liver was decreased by treatment with T-2 toxin, and the lesions were sparsely populated with mononuclear cells. The foci of necrosis were larger, with numerous colonies of bacteria. The influx and number of lymphocytes and macrophages were greater in *Listeria*-elicited peritoneal exudates. The immunotoxic effects of T-2 toxin were comparable with those produced by cyclophosphamide and were attributed to depletion of T lymphocytes and subsequent failure of T cell-dependent macrophages to clear the host of bacteria (Corrier & Ziprin, 1986a).

In a continuation of this study, groups of 17 female ICR mice were inoculated intraperitoneally on day 1 with  $4 \times 10^5$  (LD<sub>50</sub>) or  $4 \times 10^4$  CFU of bacteria, treated orally on days 0, 1, 2, and 3 with T-2 toxin at 0, 1, or 2 mg/kg bw per day, and observed for 15 days. Suppression of resistance was indicated by rapid multiplication of *L. monocytogenes* in the spleen and an increased mortality rate of mice in both groups treated with 2 mg/kg bw per day. The thymuses and spleens of T-2 toxin-treated mice showed necrosis and depletion of lymphoid cells. Foci of necrosis induced by *Listeria* infection in the spleen and liver were larger in treated mice, and the inflammatory reaction was sparse (Corrier & Ziprin, 1986a,b).

Increased resistance to *L. monocytogenes* infection was, however, observed by the same group in mice given T-2 toxin several days before inoculation of bacteria. Groups of 16 female ICR mice were given T-2 toxin by stomach tube at a dose of 0, 0.5, 1, or 2 mg/kg bw on days -5, -4, -3, -2, -1, +1, and +3. On day 0, half of each treated group was inoculated intraperitoneally with  $10^6$  (LD<sub>100</sub>) and half with  $10^5$  (LD<sub>50</sub>) *L. monocytogenes*, respectively. An additional 20 mice were given T-2 toxin alone at 2 mg/kg bw per day on the same days. Although the cytotoxic effect of T-2 toxin on lymphoid tissue was marked, enhanced resistance to *Listeria* infection was seen by a decrease in the number of deaths due to listeriosis (in both bacteria-exposed groups) in a T-2 toxin dose-dependent manner. No specific cause for the increased resistance seen with T-2 toxin treatment prior to bacterial infection was identified (Corrier & Ziprin, 1986b).

Young male white Swiss mice received diets containing T-2 toxin (purity, 99%) at a concentration of 10 or 20 mg/kg, equivalent to 1.5 and 3.0 mg/kg bw per day, for 2–3 weeks. The mice were then inoculated intraperitoneally with *Herpes simplex* virus (HSV-1). Mice fed the high concentration of T-2 toxin were highly susceptible to HSV-1 infection, and about 75% died with extensive hepatic and adrenal necrosis and little or no inflammatory cellular reaction in the affected tissues or the central nervous system. No necrotizing encephalitis was found in treated mice. Mice fed the lower concentration of T-2 toxin had lesions of intermediate severity between those seen at the high dose and in the virus-infected controls (Friend et al., 1983c). Feeding of diets containing T-2 toxin at 5, 10, or 20 mg/kg, equivalent to 0.75, 1.5, and 3 mg/kg bw per day, for 3–6 weeks did not reactivate the virus in mice latently infected with HSV-1 (Friend et al., 1983b).

A study of the effect of T-2 toxin and aflatoxin B<sub>1</sub> on activation of toxoplasmosis in mice suggested that T-2 toxin precipitates *Toxoplasma gondii* cyst rupture. A 3 x 3 study design was used to test aflatoxin B<sub>1</sub>, T-2 toxin, and *T. gondii* infection in 30 female CF-1 mice. Groups of five mice were infected with *T. gondii* and 1 month later were given T-2 toxin as an intragastric dose of 0.5 mg/kg bw per day for 50 days. When compared with a control group of five mice that received *T. gondii* alone, T-2 toxin-treated mice had more severe brain lesions and focal proliferation of glial cells and an increased degree of perivascular cuffing and necrotic and degenerate changes in neurons. The number and percentage of both ruptured and unruptured *T. gondii* cysts was greater in T-2 toxin-treated animals. Furthermore, the body-weight gain of the T-2 toxin-treated, *T. gondii*-infected mice was lower than that of untreated and *T. gondii*-infected control mice; no difference in body-weight gain was observed between T-2 toxin-treated and control mice (Venturini et al., 1996).

### Rats

Groups of four male Sabra rats received T-2 toxin as a single intraperitoneal injection of 1 mg/kg bw or as 0.5 mg/kg bw per day for 5 days. Twenty-four hours after the last T-2 toxin injection, the rats were inoculated intramuscularly with 0.1 ml of a medium containing *Staphylococcus aureus* at 10<sup>9</sup>/ml. Rats given multiple intraperitoneal injections of T-2 toxin showed more oedema and myofibril necrosis at the site of injection of the bacteria than vehicle-injected controls, and the cellular infiltrate in T-2 toxin-treated rats was sparse and bacteria were abundant.

In separate experiments, reduced leukocyte counts were observed 24 h after intraperitoneal injection of T-2 toxin at 0.5 mg/kg bw as a single dose or after up to five daily doses. The number of myeloid cells in the femur marrow was also markedly decreased by single or up to three daily intraperitoneal injections of T-2 toxin at 0.5 mg/kg bw (Yarom et al., 1984b).

### Chickens

Groups of 200 broiler chicks were fed a diet containing T-2 toxin at 16 mg/kg, equivalent to 2 mg/kg bw per day, for 3 weeks or control diet. The T-2 toxin was extracted from *F. tricinctum* culture and purified by the method of Burmeister (1971) to yield a crystalline product melting at 148–150 °C. After 1 week, groups of 40 chicks in each group were inoculated orally with either *Salmonella worthington*, *S. thompson*, *S. derby*, or *S. typhimurium*. Two to seven chicks exposed to T-2 toxin and *Salmonella* died, whereas no deaths were observed after exposure to *Salmonella* or T-2 toxin alone. The weight gain of T-2 toxin-treated chicks was markedly reduced: for example, 420 g for controls and 260 g for treated chicks at 3 weeks. The weight gain was not affected by *Salmonella*. The T-2 toxin-treated chicks also had lower relative weights of the spleen and bursa of Fabricius. *Salmonella* increased the relative spleen weight of both T-2 toxin-treated and control birds but did not affect the relative weight of the bursa (Boonchuvit et al., 1975).

### Pigs

Six weaned pigs received diets containing T-2 toxin at a concentration of 5 mg/kg, equivalent to 0.2 mg/kg bw per day, for 25 days, and their immune response was evaluated in vitro by testing for blast transformation, immune-rosette formation, and

IgG-positive cell counts. T-2 toxin caused a 40–50% reduction in immune responsiveness and a decreased total leukocyte count but an increase in adrenocortical activity. The neutralizing antibody titres to enteric B vaccine were lower in the treated pigs. It was concluded that T-2 toxin had a distinct immunosuppressive effect during the early phase of immune induction by altering the function of both T and B lymphocytes (Rafai & Tuboly, 1982).

Groups of 10 pigs (sex not specified), 7 weeks of age, were fed a diet containing T-2 toxin at a concentration of 0, 0.5, 1, 2, or 3 mg/kg for 3 weeks, equal on the basis of feed intake and weekly measurements of T-2 toxin in feed to 0.029, 0.062, 0.10, and 0.13 mg/kg bw per day. The T-2 toxin was extracted from a culture of *F. tricinctum* and determined to be > 90% pure by gas and liquid chromatography. On the first and fourth days of exposure to T-2 toxin, the pigs were immunized by an intramuscular injection of 5 mL of horse globulin. Blood was drawn before the first immunization and on days 7, 14, and 21 and used to determine the antibody titre, the lymphoproliferative response to mitogens in vitro, and the immune complex, cytotoxic reaction, and phagocytic activity of circulating granulocytes. Blood drawn on day 21 was also used to assess the erythrocyte count, erythrocyte volume fraction, mean cell volume, haemoglobin concentration, leukocyte count, and proportion of T lymphocytes. A T-2 toxin dose-related decrease in feed intake was observed, with average daily intakes over the 3-week period of 820, 710, 770, 660, and 480 g/day at 0, 0.5, 1, 2, and 3 mg/kg of diet, respectively. The titre of antibodies to horse globulin was significantly lower in T-2 toxin-treated than control pigs at all doses tested at 14 and 21 days. The leukocyte count and the proportion of T lymphocytes were lower in all treated groups, and a dose-related decrease in the size of the thymus lobules and spleen follicles was noted, although no statistical analysis of trend or differences from control was presented. A decreased proliferative response of lymphocytes to phytohaemagglutinin and concanavalin A was observed at all concentrations of T-2 toxin at 21 days but not at earlier times (Rafai et al., 1995b). The Committee noted that, as pair-fed animals were not used, the potential confounding effects of differences in feed intake and weight gain on the end-points cannot be evaluated.

### Cows

Alterations in the levels of several serum proteins and immunoglobulins were reported in calves given T-2 toxin orally at 0.6 mg/kg per day for 43 days. The total protein, albumin, and immunoglobulin fractions, including the  $\alpha$ - $\beta$ 1- and  $\beta$ 2-globulin fractions and IgA and IgM and complement protein values, were decreased in T-2 toxin-treated calves (Mann et al., 1983).

Lymphocytes from calves given a diet containing T-2 toxin at 0.6 mg/kg for up to 43 days had a decreased proliferative response to phytohaemagglutinin on days 1, 8, and 29 after administration and decreased proliferative responses to concanavalin A and pokeweed mitogen on day 29 after dosing (Buening et al., 1982).

In five calves treated orally with T-2 toxin at 0.3 mg/kg bw per day for 56 days, neutrophil function and the cutaneous reaction to injected phytohaemagglutinin were reduced. In a second study, six calves were given T-2 toxin at a dose of 0.5 mg/kg

bw per day for 28 days. The number of B lymphocytes and the response of the B-cell-enriched fraction to phytohaemagglutinin were increased by treatment. Exposure of mononuclear cells, B-cell-enriched or T-cell enriched fraction, to T-2 toxin in vitro at 1.4 ng/ml reduced the lymphoblastic response to mitogens by 50% (Mann et al., 1984).

### *Monkeys*

Seven male rhesus monkeys (*Macaca mulatta*) were given T-2 toxin by stomach tube at a dose of 0.1 mg/kg bw per day for 4–5 weeks; no vehicle controls were used. Three animals died. Although the cause of death was not reported, acute fibrous pericarditis and pneumonitis were observed in the one animal that was autopsied. The surviving monkeys showed a reduction in leukocyte count, a reduction in the bactericidal activity of neutrophils (phagocytosis of *E. coli*), a reduction in the transformation of lymphocytes by mitogens, and a reduction in the numbers of C-cell and T-cell lymphocytes (Jagadeesan et al., 1982). The Committee noted that no statistical analysis of the results was reported and the results were reported only as mean plus or minus standard error.

### *Human lymphocytes in vitro*

Apoptosis of human peripheral lymphocytes was observed after exposure in vitro to T-2 toxin at a concentration of 0.1, 1, 10, or 100 ng/mL of culture medium. Apoptosis was measured by flow cytometry with propidium iodide staining of peripheral lymphocytes after culture for various times with T-2 toxin. A concentration- and time-dependent apoptotic response was observed that was inhibited by chelating intracellular calcium with BAPTA-AM, a chelator activated by cytosolic esterases. No response was seen with T-2 toxin at 0.01 or 0.1 ng/mL after up to 5 days of incubation. Increased apoptotic cell counts were observed after 3 or 5 days of incubation with concentrations  $\geq 1$  ng/mL. Apoptosis was observed in all lymphocyte types (Yoshino et al., 1997).

Addition of T-2 toxin at 1.6 ng/ml to culture medium inhibited the proliferative response of human peripheral lymphocytes to concanavalin A, and 2.4 ng/ml inhibited the responses to phytohaemagglutinin and pokeweed mitogen. Addition of T-2 toxin at 2 ng/ml to human peripheral lymphocytes at the same time as any of the three mitogens caused maximal inhibition of the proliferative response, but inhibition was still observed when T-2 toxin was added as long as 66 h after addition of mitogen. Conversely, T-2 toxin stimulated lymphocyte proliferation when added > 16 h after initiation of control cultures without addition of mitogen (Tomar et al., 1988).

Inter-individual differences and the effects of combined exposure to four trichothecenes on the mitogen-stimulated lymphocyte proliferation response were studied. The mean  $IC_{50}$  for inhibition of proliferation of lymphocytes from 10 women was 1.3 nmol/L for phytohaemagglutinin and 1.2 nmol/L for pokeweed mitogen (equivalent to 0.61 and 0.56 ng/ml). The mean  $IC_{50}$  for inhibition of proliferation of lymphocytes from five men was 1.4 nmol/L for phytohaemagglutinin and 0.9 nmol/L for pokeweed mitogen (equivalent to 0.65 and 0.42 ng/ml). The difference in the  $IC_{50}$  for phytohaemagglutinin and pokeweed mitogen between men was significant. No significant differences were reported between men and women for any measure.

A three- to fourfold difference in the  $IC_{50}$  for inhibition of proliferation was observed in lymphocytes from six male and 16 female subjects. Combinations of T-2 toxin, diacetoxyscirpenol, deoxynivalenol, and nivalenol had either additive or marginally antagonistic interactions. The  $IC_{50}$  values for inhibition by T-2 toxin of IgA, IgG, and IgM production were slightly higher than those for inhibition of mitogen-stimulated proliferation. Increased immunoglobulin production was observed at the lower doses tested (a U-shaped dose-response curve). At 0.2 nmol/L (equivalent to 0.093 ng/ml), T-2 toxin caused an increase in IgA and IgM (but not IgG) production in vitro, while 2 nmol/L (equivalent to 0.93 ng/ml) decreased the IgM and IgA levels (Thuvander et al., 1999).

Human lymphocyte proliferation after mitogen stimulation was examined in cell cultures by flow cytometry and measurement of bromodeoxyuridine (BrdU) incorporation. T-2 toxin at 1–2 nmol/L (equivalent to 0.47–0.93 ng/ml) in the culture medium inhibited cell proliferation and altered the proportions of cells (increases and decreases at different times) expressing CD69, CD25, and CD71 markers in CD4<sup>+</sup> and CD8<sup>+</sup> cells. The authors concluded that T-2 toxin and deoxynivalenol inhibited the cell cycle in a similar way and that proliferation was affected early in the cell cycle, before CD25 expression (Johannisson et al., 1999)

The initial hydrolysis of T-2 toxin to HT-2 toxin and hydroxylation to 3'-OH T-2 slightly decreased inhibition of the lymphoproliferative response, as measured by tritiated thymidine uptake in human lymphocyte cultures; however, metabolism to 3'-OH HT-2, T-2 triol, and T-2 tetraol affected the response more dramatically. The  $IC_{50}$  values for inhibition of mitogen-induced proliferation of cultured human lymphocytes by T-2 toxin, HT-2 toxin, 3'-OH T-2, 3'-OH HT-2, T-2 triol, and T-2 tetraol were 1.5, 3.5, 4.0, 50, 150, and 150 ng/ml, respectively (Forsell et al., 1985). The  $IC_{50}$  values for inhibition of tritiated thymidine uptake in mitogen-stimulated human lymphocytes were higher for the trichothecenes fusarenon-X, nivalenol, deoxynivalenol, and 15-acetyldeoxynivalenol, at 18, 72, 140, and 240 ng/ml, respectively. These results suggest that the lymphotoxicity of trichothecenes is related to the C-4 substituent (Forsell & Pestka, 1985).

#### (e) Neurotoxicity

Four-week-old male broiler chickens were intubated with a single dose of T-2 toxin at 2.5 mg/kg bw, and the brain concentrations of dopamine, noradrenaline, serotonin, and selected blood components were determined 4–48 h after administration. A significant increase in the dopamine concentration and a reduction in the noradrenaline concentration in the brain were found. The brain serotonin content did not change (Chi et al., 1981).

Weanling male Wistar rats were given T-2 toxin orally at 2 mg/kg bw, and the concentrations of neurotransmitters were determined. T-2 toxin increased the concentrations of tryptophan, serotonin, and dopamine in the brain but decreased that of 3,4-dihydroxyphenylacetic acid (MacDonald et al., 1988).

In male Sprague-Dawley rats given T-2 toxin orally at 22 mg/kg bw, the concentrations of serotonin and 5-hydroxy-3-indoleacetic acid were significantly

increased in all regions of the brain examined, whereas those of noradrenaline and dopamine were unaltered (Fitzpatrick et al., 1988). In male Sprague-Dawley rats that received T-2 toxin at 1 mg/kg bw by intravenous injection, the concentrations of vasopressin, oxytocin, and leucine enkephalin decreased in the posterior pituitary, and the concentrations of methionine enkephalin increased (Zamir et al., 1985).

Changes in monoamine neurotransmitter levels were also observed in groups of 10 male Sprague-Dawley rats that received feed containing T-2 toxin (purity unspecified) at a concentration of 2.5 or 10 mg/kg, equal to 0.26 and 0.63 mg/kg bw per day for 7 days and 0.24 and 0.71 mg/kg bw per day for 14 days on the basis of reported feed consumption and body weights. Feed consumption, feed use efficiency, and weight gain were decreased at both doses. When specific brain areas were assessed in a slice–micropunch procedure, decreased noradrenaline in the substantia nigra and decreased 3,4-dihydroxyphenylacetic acid in the paraventricular nucleus and medial forebrain bundle were observed. Increased concentrations of serotonin, 5-hydroxy-3-indoleacetic acid, dopamine, and noradrenaline were found in the nucleus raphe magnus and increased adrenaline in the substantia nigra (Wang et al., 1993a).

Neurotransmitters were also assessed by a slice–micropunch procedure in discrete brain regions of groups of 10 male Sprague-Dawley rats 2–10 h after administration of a single dose of T-2 toxin at 0.1, 1, or 2.5 mg/kg bw by gavage. The serotonin concentration was increased in the nucleus raphe magnus, the medial forebrain bundle, and the paraventricular nucleus, that of 3,4-dihydroxyphenylacetic acid in the medial forebrain bundle and paraventricular nucleus, and that of noradrenaline in the locus coeruleus at all doses. The concentration of noradrenaline was decreased in the substantia nigra at 0.1 mg/kg bw (Wang et al., 1998a).

The permeability of the blood–brain barrier was assessed in 10 male Sprague-Dawley rats by measuring the intracerebral recovery of labelled, systemically administered mannitol or dextran 2 h after intraperitoneal injection of T-2 toxin (purity unspecified) at 0.2 or 1 mg/kg bw or after 7 days on a diet containing T-2 toxin at a concentration of 10 mg/kg, equal to 0.93 mg/kg bw per day on the basis of reported feed consumption and weight gain. Monoamine oxidase activity and protein synthesis (labelled leucine incorporation) were also measured, except in the group given the low intraperitoneal dose. A dose-related increase in mannitol uptake was seen after intraperitoneal injections of T-2 toxin, but a statistically significant difference from control was seen only at the highest dose; mannitol uptake was also increased in some areas of the brain by administration of 10 mg/kg of diet. Dextran uptake was not affected. Protein synthesis in brain tissue was increased by the intraperitoneal dose of 1 mg/kg bw but not by intake of T-2 toxin in the diet. Monoamine oxidase activity was not affected by T-2 toxin given intraperitoneally but was decreased by administration of T-2 toxin in the feed at a concentration of 2.5 or 10 mg/kg, equal to 0.32 and 0.88 mg/kg bw per day on the basis of the reported feed consumption and weight gain. The Committee noted that the reduction in monoamine oxidase activity was the same at both concentrations. Effects on feed consumption and weight gain were seen with the 7-day dietary exposure. The authors noted that addition of a pair-fed control group would have allowed control for the possible effects of reduced

feed intake on brain protein synthesis and enzyme activity in the rats treated in the diet (Wang et al., 1998b).

The effects of T-2 toxin were assessed in male Wistar rats given a single dose of 0.4 or 2 mg/kg bw by oral gavage on motor performance, nociceptor measures, open field behaviour, passive avoidance 4 and 8 h after dosing, sequential performance on an elevated plus-maze, rotarod, horizontal bridges, and passive avoidance or reaction to a hot plate 4–8.5 h after dosing. No effects on behaviour were observed at 0.4 mg/kg bw. At 2 mg/kg bw, body-weight gain was decreased 7 days after dosing, recumbancy was more prevalent, and sniffing was decreased. Passive avoidance was impaired, suggesting an effect on learning; and step-through latency was shortened. The latency of the response to a hot plate was not affected (Sirkka et al., 1992).

### **2.3 Observations in humans**

#### **2.3.1 Clinical observations**

Effects of crude extracts of fungal cultures or solutions containing T-2 toxin on the skin have been reported (Bamburg & Strong, 1971; Saito & Ohtsubo, 1974). All of the incidents were accidental and involved few persons, who developed severe irritation, loss of sensitivity, and desquamation. Despite the presence of T-2 toxin in the contact material, the involvement of other compounds could not be ruled out.

#### **2.3.2 Epidemiological studies**

Alimentary toxic aleukia was reported in the former USSR during the period 1931–47, which was attributed to the presence of toxic *Fusarium* species in mouldy over-wintered grain. An association was established with the ingestion of grain invaded by some moulds, in particular *F. poae* and *F. sporotrichioides*. The main pathological changes were necrotic lesions of the oral cavity, oesophagus, and stomach and, in particular, pronounced leukopenia. The primary lesions were bone-marrow hypoplasia and aplasia. The disease was lethal in a high proportion of cases (Sarkisov et al., 1944; Bilai, 1977; Leonov, 1977; Joffe, 1986; Beardall & Miller, 1994).

The clinical symptoms reported in alimentary toxic aleukia and the identification of *Fusarium* in foods suggested that it was associated with mycotoxins, identified years later in fungal cultures of *Fusarium* species under laboratory conditions, including T-2 toxin (Mirocha & Pathre, 1973) and wortmannin (Mirocha & Abbas, 1989).

Scabby grain toxicosis, a disease of both humans and farm animals, was reported from Japan and Korea during 1946–63. The commonest clinical symptoms were nausea, vomiting, diarrhoea, and abdominal pain. All the cases were acute, with recovery within a few days; none was lethal. *Fusarium* fungi, *F. graminearum* in particular, were isolated from suspected cereals (Tochinai, 1933; Hirayama & Yamamoto, 1948, 1950; Nakamura et al., 1951; Tsunoda et al., 1957; Cho, 1964; Ogasawara, 1965; Chung, 1975).

Three investigations of outbreaks of trichothecene-related disease involving either T-2 toxin or deoxynivalenol have been reported, two in China involving maize, wheat, or rice (Luo, 1988; Wang et al., 1993b) and one in India involving wheat (Bhat et al., 1989). Each involved 100 or more cases.

During the first incident, outbreaks of poisoning with mouldy maize and scabby wheat were reported. Among approximately 600 persons who ate mouldy cereals, there were 463 cases of poisoning (77%). The latency for the onset of symptoms was 5–30 min. These included nausea, vomiting, abdominal pain, diarrhoea, dizziness, and headache. No deaths occurred. Pigs and chicks fed the same mouldy cereals were also affected. Analysis of five samples of mouldy maize by GC–MS and radioimmunoassay revealed the presence of deoxynivalenol at 0.34–93 mg/kg and zearalenone at 0.004–0.59 mg/kg; neither T-2 toxin nor nivalenol was found. Analysis by TLC of 19 samples of scabby wheat collected from affected and unaffected families showed a deoxynivalenol content of 1–40 mg/kg, which was significantly higher than that in the non-scabby wheat samples. Zearalenone was detected in two samples at 0.25 and 0.5 mg/kg. No T-2 toxin was found (Luo, 1988).

A similar outbreak was reported in Kashmir, India, in 1987, which was ascribed to the consumption of bread made from flour that had become mouldy during storage following unseasonal rains in the wheat-harvesting season. *Fusarium* spp. were grown from the wheat and found to contain mycotoxins. Of the 224 persons investigated randomly, 97 had symptoms including abdominal pain (100%), throat irritation (63%), diarrhoea (39%), blood in stools (5%), and vomiting (7%). Symptoms developed 15 min to 1 h after consumption of locally baked bread. The following concentrations of mycotoxins were found in 12 of 24 samples of refined wheat flour used in the preparation of bread: deoxynivalenol at 0.35–8.4 mg/kg, acetyl-deoxynivalenol at 0.64–2.5 mg/kg (no details of the analysis of this derivative were provided), nivalenol at 0.03–0.1 mg/kg, and T-2 toxin at 0.55–0.8 mg/kg. Deoxynivalenol, its acetyl derivative, and nivalenol were measured quantitatively by HPLC and T-2 toxin by TLC, but no rigorous confirmation of identity was undertaken (Bhat et al., 1987).

The most recent report of a trichothecene-related outbreak involved 97 persons with symptoms out of an estimated 165 persons who had eaten polished rice from which *F. heterosporum* and *F. graminearum* were isolated. Of 29 persons examined, 28 reported nausea with lesser incidences of dizziness, vomiting, chills, abdominal pain and distention, thoracic constriction, and diarrhoea. The latent period for symptoms was 10–30 min after ingestion of the rice. Fungal culture of suspected rice demonstrated the presence of *F. heterosporum* and *F. graminearum*, and ELISA was used to show the presence of T-2 toxin. No analysis was conducted to determine whether other mycotoxins were present. T-2 toxin was present in three samples of rice at 0.18–0.42 mg/kg (Wang et al., 1993b).

### 3. ANALYTICAL METHODS

#### 3.1 Chemistry

T-2 toxin forms white needles, with a melting-point of 151–152 °C, and its specific rotation has been determined as  $[\alpha]_{D}^{26} = +15$  ( $c = 2.58$ , ethanol). All trichothecenes are stable under the usual conditions of storage for long times, and the epoxide

group at the C-12,13 position is extremely stable to nucleophilic attack (Shepherd & Gilbert, 1988). Biochemically, all tricothecenes are derived from isoprenoid pathways, as are many secondary metabolites. Trichodiene is an intermediate compound derived from farnesol.

### 3.2 Screening tests

Immunoassay and TLC are the only screening tests for T-2 toxin- and HT-2 toxin that are applicable for routine analysis of cereals. Extraction is usually performed with acetonitrile and water, methanol, or chloroform and ethanol. Immunoassays for the determination of T-2 toxin and HT-2 toxin are based mainly on monoclonal antibodies.

Tricothecenes Appendix 3 gives the performance characteristics of the screening tests that have been developed for the detection of T-2 toxin and HT-2 toxin. The detection limits of the assays for T-2 toxin range from 0.2 to 50 ng/g. TLC methods allow detection of T-2 toxin down to 100 ng/g.

### 3.3 Quantitative methods

Various combinations of solvents, usually acetonitrile and water and methanol and water, have been used to extract type-A tricothecenes from grain, food, and feeds. Extraction is performed mainly by high-speed blending or mechanical shaking. Subsequent clean-up is done on prepacked silica gel, Florisil, or cyano and C<sub>18</sub> solid-phase extraction cartridges. Multifunctional MycoSep™ columns, which contain activated charcoal, alumina, and Celite are also being used for the determination of type-A tricothecenes.

Generally, type-A tricothecenes are less polar than type-B toxins, as they have fewer free hydroxyl groups and lack a keto group at C-8 position. Therefore, different methods are used to extract these two types. For example, HPLC with detection of ultra-violet absorbance is not applicable to type-A tricothecenes owing to the lack of a conjugated keto group at the C-8 position. HPLC methods for T-2 toxin and HT-2 toxin toxins are being developed in which a variety of derivatization reagents are used to allow detection by fluorescence (Lawrence & Scott, 1993).

Although TLC is also used for quantitative determination of T-2 toxin and HT-2 toxin, GC analysis is the method of choice for the determination of type-A tricothecenes. Most GC methods are based on derivatization of hydroxyl groups in order to increase volatility and sensitivity. Both trimethylsilylation and fluoroacylation are currently used for derivatization. MS detection and use of ECD or flame ionization detection, after derivatization with a fluoroacylation reagent are recommended for reliable detection of type-A tricothecenes. The formation of fluoroacyl derivatives by trifluoroacetic anhydride, pentafluoropropionyl, or heptafluorobutyryl derivatives is also commonly used in order to increase the sensitivity of type-A tricothecenes to ECD (Langseth & Rundberget, 1998). LC with atmospheric pressure chemical ionization and MS has also been used for the determination of T-2 and HT-2 toxins (Berger et al., 1999).

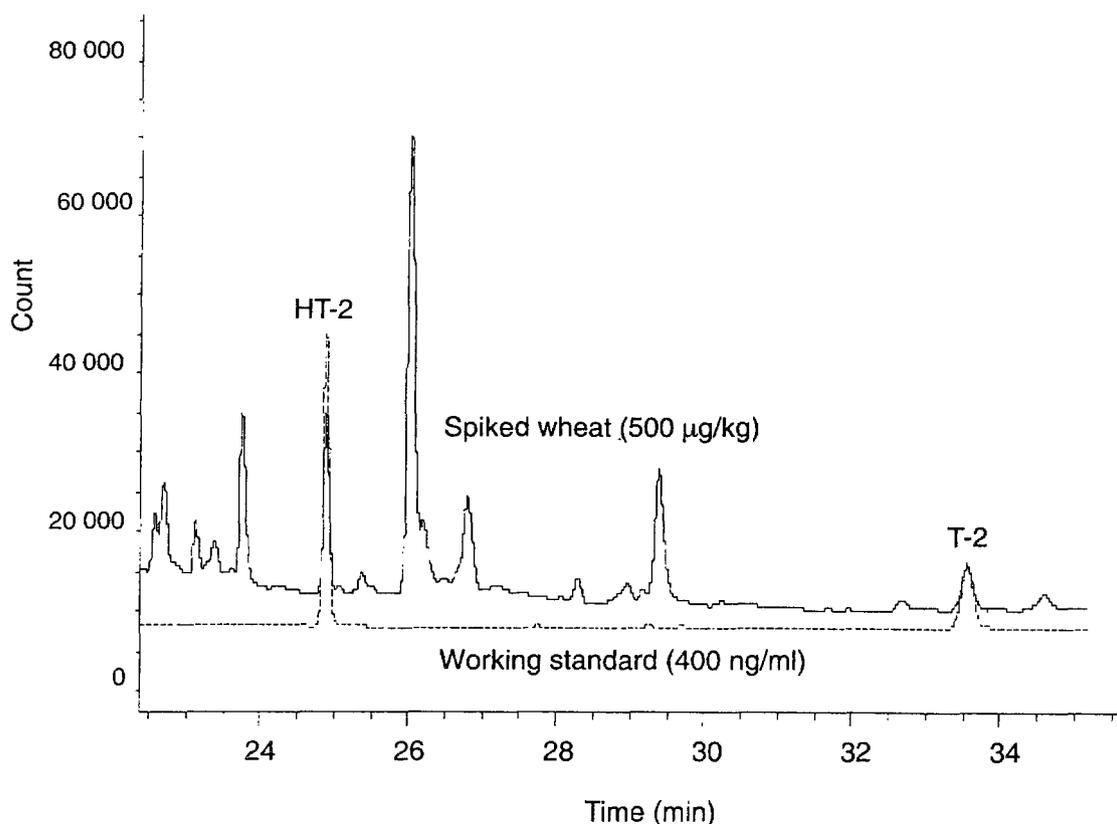
Tricothecenes Appendix 4 lists the performance characteristics of the quantitative methods that have been developed for the determination of T-2 toxin and HT-2

toxin. Figure 2 shows a typical chromatogram obtained after separation and detection by GC with ECD of a mixture of HT-2 and T-2 in a standard solution of 400 ng/ml overlaid with a wheat sample spiked with HT-2 and T-2 at 500 ng/ml. The trace shows the advantage of this method for sensitive, simultaneous quantification of both trichothecenes. The typical LODs of quantitative methods for the determination of T-2 toxin and HT-2 toxin in cereals are 3 ng/g for T-2 toxin and 1 ng/g for HT-2 toxin by LC with MS; 10 ng/g for T-2 toxin and HT-2 toxin by GC with MS; and 10 ng/g for T-2 toxin and HT-2 toxin with GC and ECD. The typical recovery is 70–120%.

#### 4. SAMPLING PROTOCOLS

There are no published sampling plans for the determination of T-2 toxin and HT-2 toxin in foods, and no details of the sampling variability of these toxins have been reported. The generation of meaningful data requires the collection of representative samples from carefully selected batches of food which, in turn, are representative of clearly defined locations (e.g. country, region within a country).

**Figure 2. Gas chromatogram of a HT-2 and T-2 mixture in a standard solution of 400 ng/ml (dotted line) overlaid with a wheat sample (solid line) spiked with HT-2 and T-2 at 500 ng/ml**



## 5. EFFECTS OF PROCESSING

In wheat contaminated at low concentrations (up to 1 mg/kg), the mycotoxins are typically found near the surface of the kernel, whereas at high levels of contamination the mycotoxins may be more evenly distributed (Charmley & Prelusky, 1994). Many infected kernels can be removed in gravity separators, which separate particles on the basis of differences in specific gravity, size, shape, and surface texture.

Natural degradation of T-2 toxin and HT-2 toxin has been observed in cereal grain both in the field and during storage. It is difficult to understand how the concentrations of mycotoxin are decreased in a natural system, although several explanations have been put forward. The concentration of free mycotoxin in a natural ecosystem may be the result of concurrent synthesis, transport, conjugation, release from bound forms, and degradation by the plant or by other microbes (Karlovski, 1999).

The effectiveness of milling practices in reducing trichothecene concentrations in the flour fractions from that of the whole grain were reviewed by Patey & Gilbert (1989), Scott (1991), and Charmley & Prelusky (1994). Most of the studies concern deoxynivalenol, and few are available on T-2 toxin and HT-2 toxin. In a laboratory-scale experiment, wet milling of maize containing T-2 toxin resulted in the loss of two-thirds in the steep or process water and a high concentration in the germ (Patey & Gilbert, 1989).

The trichothecenes are stable at 120 °C, moderately stable at 180 °C, and decompose within 30–40 min at 210 °C (Kamimura, 1989). T-2 toxin and HT-2 toxin were reported to be relatively stable during baking processes (Patey & Gilbert, 1989).

During cooking of noodles and spaghetti, considerable amounts of trichothecenes may leach into the boiling water (Scott, 1991).

T-2 toxin was deacetylated to HT-2 toxin by rumen microbes (Kiessling et al., 1984). In another study, rumen microorganisms transformed T-2 toxin to HT-2 toxin, T-2 triol, de-epoxy HT-2, and de-epoxy T-2 triol (Swanson et al., 1987).

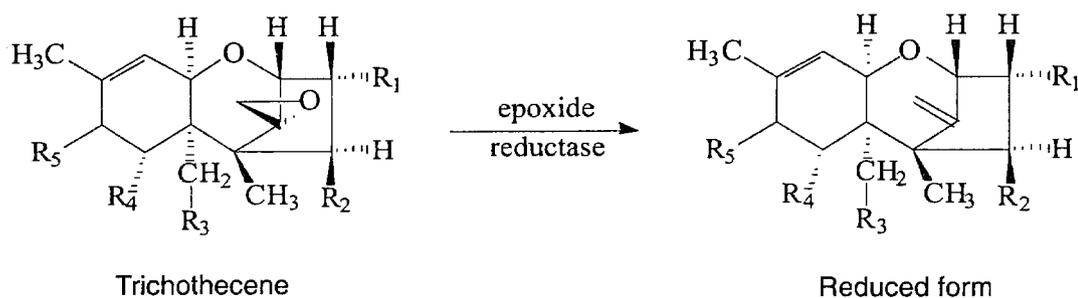
Bacterial communities isolated from soil and water samples readily detoxified T-2 toxin (Beeton & Bull, 1989). The main degradation pathway in most isolates involved side-chain cleavage of acetyl moieties to produce HT-2 toxin and T-2 triol. In all cases, the complete communities were more active against T-2 toxin in terms of rates of degradation than any single bacterial component. Figure 3 shows the microbial transformation of trichothecenes to their de-epoxylated forms.

## 6. LEVELS AND PATTERNS OF CONTAMINATION OF FOOD COMMODITIES

### 6.1 Results of surveys

Data on contamination of grains and food products with T-2 toxin and HT-2 toxin were submitted to the Committee by Brazil, China, Finland, Germany, Norway, Sweden, and the United Kingdom. Other data on contamination of food with these

**Figure 3. Microbial transformation of trichothecenes to their de-epoxylated forms**



toxins were taken from literature published between 1990 and early 2000. Previous data were reviewed in *Environmental Health Criteria* 105 (WHO, 1990). As the information available was not complete in many cases, many authors were contacted and asked to send details of their sampling and analytical methods and additional data; for example, the 90th percentile was generally not included in the papers.

Surveys for T-2 toxin and HT-2 toxin revealed their presence in grains such as wheat, maize, oats, barley, rice, beans, and soya beans as well as in some cereal-based products. There have been occasional reports of the presence of T-2 toxin and HT-2 toxin in human foods. Bread, breakfast cereals, and other cereal foods were found to be contaminated with T-2 toxin, and infant foods, bread, noodles, and cereal foods were found to contain HT-2 toxin (Patel et al., 1996; Schollenberger et al., 1999, 2000a,b).

The results of surveys for T-2 toxin and HT-2 toxin are presented in Appendices A and B, respectively. The references in these tables include the primary reference (P), the reference for the sampling method (S), and the reference for the analytical method (A) given by the authors. The analytical methods are described in Trichothecenes Appendix 5, and the sampling methods are described in Trichothecenes Appendix 6. The data were included on a case-by-case basis: when there was no information about sampling or analytical method, the data were not included. Mean values were recalculated when they were given as the mean for positive samples or when 'undetected' was considered to be half the LOD. The mean values shown are therefore the means of all samples, with those below the LOD taken as zero.

Although soya bean samples were contaminated at concentrations ranging from not detectable to 1100 µg/g (Jacobsen et al., 1995), these data were not included in the Appendices because they represented soya beans that had been refused or heavily discounted by local grain merchants, and the concentration of toxins was analysed after hydrolysis and reported as T-2 toxin equivalents.

WHO (1990) reported that T-2 toxin was present at concentrations > 100 µg/kg in only a few of 999 samples. This value was therefore chosen to represent contamination with T-2 toxin and HT-2 toxin for the decade 1990–2000. In a total of 8918 samples, T-2 toxin was found in 64% and HT-2 toxin in 36%. Concentrations > 100 µg/kg were found for T-2 toxin in only 37 and HT-2 toxin in 77 samples. Occasionally, high levels of both toxins were found; for T-2 toxin, for example, 820 µg/kg in wheat in Asia (Chen et al., 1995), 1700 µg/kg in oats in Europe (Müller

et al., 1998), and 2400 µg/kg in maize in America (Saubois et al., 1992); and for HT-2 toxin, 2000 µg/kg in oats in Europe (Müller et al., 1998). When the LOD or LOQ was > 100 µg/kg, therefore, the chance of detecting the toxins was quite low. These data were included in the Appendix in order to illustrate this point. As the LOQ diminishes, there is an incremental increase in the frequency of contamination and an incremental increase in the average values. For example, when the LOQ is between 1 and 50 µg/kg, the mean concentration in oats is up to 200 µg/kg for HT-2 toxin (Langseth, 2000) and 76 µg/kg for T-2 toxin (Müller et al., 1998). In other studies, the LOQ was > 100 µg/kg, and the toxins were not detected or the average concentration was low. For example, a mean concentration of 13 µg/kg was reported in heavily contaminated wheat in 1986 (LOQ = 500 µg/kg; Quiroga et al., 1995).

The analytical method used most commonly for these trichothecenes was GC-MS or GC-ECD followed by ELISA, with an LOD of 1–1000 µg/kg. Different authors defined the LOD and LOQ in different ways. The LOD was sometimes expressed as two or three times the background and sometimes as the smallest quantity of the standard that could be detected; the LOQ was sometimes defined as five to six times the 'noise' in standard solutions, without taking into account recovery in the matrix under study. Some investigators included recovery values in their estimate of the LOQ. It would be advisable if investigators reported the method by which the LOD and LOQ were calculated, in order to standardize this procedure.

Intake is best estimated by considering results in which the recovery at the LOQ is > 60%. Analytical procedures must be improved to allow better quantification at lower cost and to improve the recovery of both mycotoxins in most matrices.

During 1990–2000 (WHO, 1990), 7% of 999 samples were reported to be contaminated with T-2 toxin, but variable methods were used to derive the data. The vast majority of studies did not provide evidence that the methods used had been tested rigorously and that the confirmation of identity was adequate. Few of the reported data have been corroborated. In the analyses in Europe, 52% of samples containing T-2 toxin and 88% of those containing HT-2 toxin were detected at LOQ or LOD values ≤ 100 µg/kg, and the frequency of contamination was 11% and 14%, respectively. HT-2 toxin appears to be a more frequent contaminant, but the relationship varies among studies. Although there are inadequate data for tropical and subtropical zones, where, for example, maize, beans, and rice are grown, the few samples from those regions that have been analysed show contamination.

## **6.2 Distribution curves**

Confirmed information on the distribution of these toxins has been reported in the form of a frequency distribution table for all type A trichothecenes (Park et al., 1996). As dried extracts were hydrolysed before analysis, the individual distribution cannot be ascertained.

Few of the data submitted to the Committee could be used to make a distribution analysis, and the high percentage of 'undetected' entries obviated estimation of a distribution curve. Although the number of positive samples was high in some sets of data, the figures were rounded in such a way that any of the distribution functions recommended in the literature for other mycotoxins provided a suitable adjustment.

### 6.3 Annual variation

The literature contains few data for an analysis of annual variation in contamination with T-2 toxin and HT-2 toxin, because studies with a LOQ < 10 µg/kg are required for this purpose. Nevertheless, annual variations can be seen in Appendices A and B. For example, a survey in Sweden showed annual variations in the average contamination with T-2 toxin and HT-2 toxin in oats (Pettersson, 2000).

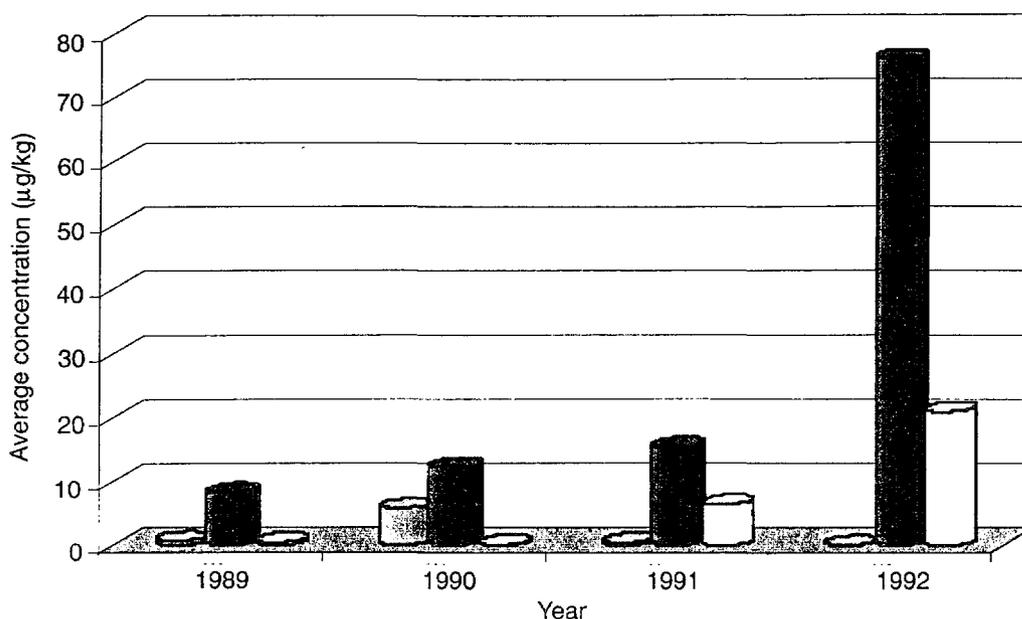
Figure 5 shows the annual variation in T-2 toxin contamination in Germany in three substrates: wheat, oats, and barley. All samples were analysed by the same persons by a method with a LOQ of < 5 µg/kg. Although the samples were of feed, the results clearly illustrate not only the annual variations that can occur in contamination but also differences with respect to substrates. Oats appeared to be more sensitive to contamination with T-2 toxin than wheat or barley, which is consistent with results (Appendix A) from Finland (Hietaniemi & Kumpulainen, 1991), Norway (Langseth, 2000), and Sweden (Thuvander et al., 2000).

The influence of climatic conditions on the amount and frequency of T-2 toxin and HT-2 toxin contamination is difficult to understand (Müller et al., 1998). A larger number of samples would be necessary to determine the effect of such conditions on the behaviour of *Fusarium* species and accumulation of the toxins.

## 7. FOOD CONSUMPTION AND INTAKE ASSESSMENTS

The dietary intake of T-2 and HT-2 toxins was assessed according to the recommendations of a FAO/WHO workshop on methods for assessing exposure to contaminants and toxins, which was held in Geneva in June 2000 (WHO, 2000). The workshop recommended that the median concentration be given when data on

**Figure 4. Annual variations in contamination with T-2 toxin in Germany**



From Müller et al. (1997a,b, 1998). Wheat (grey), oats (black), barley (white)

individual samples were available, whereas a mean should be given when only pooled or aggregated data were available. In the case of commodities that contribute significantly to intake, distribution curves should be generated to allow risk managers to determine the effects on dietary intake of different maximum levels.

The workshop further recommended that international estimates of dietary intake should be calculated by multiplying the mean or median concentration by the values for consumption of the commodity in the five GEMS/Food regional diets (WHO, 1998). The diets (African, European (which includes Australia, Canada, New Zealand, and the USA), Far Eastern, Latin American, and Middle Eastern) were established on the basis of information on food balance sheets compiled by FAO. Since such information is available for most countries, the data are comparable across countries and regions of the world. The regional diets represent the average availability of food commodities per capita rather than actual food consumption.

The report of the workshop noted that national intake estimates should also be reported when available, as they may provide information about intake by specific population subgroups or heavy consumers, which cannot be derived from GEMS/Food regional diets.

### 7.1 Methods

For this assessment, concentrations of T-2 and HT-2 toxins in food commodities and in some processed foods were submitted to the Committee or were obtained from the literature. The quality and reporting of the data are discussed in the previous section. As the dietary intakes are based on the GEMS/Food regional diets, which include information on consumption of raw or minimally processed foods, concentrations of T-2 and HT-2 toxins in processed foods were not used to estimate dietary intake.

Information was available on the concentrations of six commodities: barley, maize, oats, rice, rye, and wheat, and other cereals. Data on T-2 toxin were received from

**Table 5. Countries for which data on concentrations of T-2 and HT-2 toxins were available, by geographical regional diet**

Toxin	Far Eastern	African	Latin American	European
T-2	China India Republic of Korea	South Africa	Argentina Brazil Chile Ecuador	Austria Bulgaria Canada Finland Germany Norway Sweden United Kingdom
HT-2	China Republic of Korea		Brazil Chile	Austria Canada Finland Germany Norway Sweden United Kingdom

16 countries, representing four of the five GEMS/Food regional diets, while data on HT-2 toxin were received from 11 countries (Table 5); no data were reported for the Middle Eastern diet.

Most of the data available for this evaluation were pooled; that is, each data point represented the mean concentration in a number of individual samples. In calculating the mean values, samples in which the concentration was below the LOQ or LOD were assumed to have a value of zero. The maximum analytical value and the number of samples with concentrations below the LOD or LOQ were also reported for each data point. For T-2 and HT-2 toxins, a total of 175 data points (mean values) representing 8410 individual samples were included in the intake assessment (Table 6). Of those 175 data points, 147 were reported from countries represented by the GEMS/Food European diet. The remaining 28 data points represented intake of the six commodities in the other regional diets.

For each commodity, the data were sorted according to the country groupings of the GEMS/Food regional diets. The number of data points reported, the number of individual samples represented, the highest maximum analytical value reported, the proportion of samples with concentrations below the LOD or LOQ, and the average of all mean values are summarized in Table 7.

## 7.2 Concentrations of T-2 and HT-2 toxins in foods

### 7.2.1 Mean and maximum values

The concentrations of T-2 toxin and HT-2 toxin available for this assessment are summarized by commodity and region in Table 7. In view of the limited amount of data on these toxins in diets other than the European diet, only the latter were used in the dietary intake estimates. The mean of all the data for each commodity in the European diet, weighted by sample size, was used as the basis for the intake estimates.

**Table 6. Numbers of countries, data points, and samples from which information on concentrations of T-2 and HT-2 was available**

Toxin	Commodity	No. of countries	Data used in intake estimates	
			No. of data points (means)	No. of individual samples
T-2	Barley	6	10	372
	Maize	9	16	1239
	Oats	4	21	758
	Rice	2	2	125
	Rye	3	4	83
	Wheat	10	38	2564
HT-2	Barley	4	10	364
	Maize	5	9	292
	Oats	4	21	758
	Rice	1	1	26
	Rye	3	5	87
	Wheat	3	35	1740

**Table 7. Summary of data on concentrations of T-2 and HT-2 toxins in grains in GEMS/Food regional diets**

Commodity	Far Eastern	African	Latin American	European
<b>T-2</b>				
<i>Barley</i>				
No. of data points	1			9
No. of individual samples	30			342
Unweighted mean ( $\mu\text{g}/\text{kg}$ )	0			3.1
Maximum value ( $\mu\text{g}/\text{kg}$ )	0			310
% < LOD or LOQ	100			90
Weighted mean, all samples: 4.6 $\mu\text{g}/\text{kg}$				
<i>Maize</i>				
No. of data points	3	4	5	4
No. of individual samples	40	685	346	168
Unweighted mean ( $\mu\text{g}/\text{kg}$ )	0	0	39	3
Maximum value ( $\mu\text{g}/\text{kg}$ )	0	0	2400	260
% < LOD or LOQ	100	100	92	98
Weighted mean, all samples: 3.2 $\mu\text{g}/\text{kg}$				
<i>Oats</i>				
No. of data points				21
No. of individual samples				758
Unweighted mean ( $\mu\text{g}/\text{kg}$ )				18
Maximum value ( $\mu\text{g}/\text{kg}$ )				530
% < LOD or LOQ				71
Weighted mean, all samples: 21 $\mu\text{g}/\text{kg}$				
<i>Rice</i>				
No. of data points			1	1
No. of individual samples			99	26
Unweighted mean ( $\mu\text{g}/\text{kg}$ )			27	0.7
Maximum value ( $\mu\text{g}/\text{kg}$ )			NA	19
% < LOD or LOQ			94	96
Weighted mean, all samples: 0.7 $\mu\text{g}/\text{kg}$				
<i>Rye</i>				
No. of data points				4
No. of individual samples				83
Unweighted mean ( $\mu\text{g}/\text{kg}$ )				0.1
Maximum value ( $\mu\text{g}/\text{kg}$ )				17
% < LOD or LOQ				99
Weighted mean, all samples: 0.2 $\mu\text{g}/\text{kg}$				
<i>Wheat</i>				
No. of data points	2		3	33
No. of individual samples	512		319	1 733
Unweighted mean ( $\mu\text{g}/\text{kg}$ )	22		24	1.1
Maximum value ( $\mu\text{g}/\text{kg}$ )	122		800	249
% < LOD or LOQ	4		93	96
Weighted mean, all samples: 1.6 $\mu\text{g}/\text{kg}$				

Table 7 (contd)

Commodity	Far Eastern	African	Latin American	European
<b>HT-2</b>				
<i>Barley</i>				
No. of data points	1			9
No. of individual samples	30			334
Unweighted mean ( $\mu\text{g}/\text{kg}$ )	0			3.8
Maximum value ( $\mu\text{g}/\text{kg}$ )	0			290
% < LOD or LOQ	100			91
Weighted mean, all samples: 4.4 $\mu\text{g}/\text{kg}$				
<i>Maize</i>				
No. of data points	3		1	5
No. of individual samples	40		68	184
Unweighted mean ( $\mu\text{g}/\text{kg}$ )	0		0	7
Maximum value ( $\mu\text{g}/\text{kg}$ )	0		0	230
% < LOD or LOQ	100		100	97
Weighted mean, all samples: 2.9 $\mu\text{g}/\text{kg}$				
<i>Oats</i>				
No. of data points				21
No. of individual samples				758
Unweighted mean ( $\mu\text{g}/\text{kg}$ )				44
Maximum value ( $\mu\text{g}/\text{kg}$ )				2 000
% < LOD or LOQ				67
Weighted mean, all samples: 35 $\mu\text{g}/\text{kg}$				
<i>Rice</i>				
No. of data points				1
No. of individual samples				26
Unweighted mean ( $\mu\text{g}/\text{kg}$ )				0
Maximum value ( $\mu\text{g}/\text{kg}$ )				0
% < LOD or LOQ				100
Weighted mean, all samples: 0 $\mu\text{g}/\text{kg}$				
<i>Rye</i>				
No. of data points				5
No. of individual samples				87
Unweighted mean ( $\mu\text{g}/\text{kg}$ )				0.01
Maximum value ( $\mu\text{g}/\text{kg}$ )				23
% < LOD or LOQ				99
Weighted mean, all samples: 0.01 $\mu\text{g}/\text{kg}$				
<i>Wheat</i>				
No. of data points	2		2	31
No. of individual samples	39		58	1 643
Unweighted mean ( $\mu\text{g}/\text{kg}$ )	0		0	1.6
Maximum value ( $\mu\text{g}/\text{kg}$ )	0		0	310
% < LOD or LOQ	100		100	90
Weighted mean, all samples: 1.9 $\mu\text{g}/\text{kg}$				

*Barley:* Information on the concentrations of T-2 toxin in barley was received from six countries with two regional diets (the Far Eastern and European). A total of 372 samples were analysed, of which 342 were for the European diet. Of the samples for the European diet, the results for 90% were below the LOD or LOQ. The weighted mean value was 4.6 µg/kg.

Information on the concentrations of HT-2 toxin in barley were received from four countries with the same two diets. Of the 364 samples analysed, 334 were for the European diet. The maximum value reported was 290 µg/kg, and the weighted mean for the European diet was 4.4 µg/kg.

*Maize:* Nine countries representing four regional diets reported information on the concentration of T-2 toxin in maize, in a total of 1239 samples. Of these, 97% had results below the LOD or LOQ. Of the 168 samples for the European diet, the results for 98% were below the LOD or LOQ. The weighted mean in samples for the European diet was 3.2 µg/kg.

Five countries representing three regional diets reported information on the concentrations of HT-2 toxin in maize. Of the 292 samples, 184 were for the European diet. The results for all samples from the Far Eastern and Latin American diets were below the LOD or LOQ. The weighted mean of values for the European diet was 2.9 µg/kg. The maximum individual value reported was 230 µg/kg.

*Oats:* Four countries representing the European diet provided data on the concentration of T-2 toxin in a total of 758 samples of oats. The values for 71% of all samples were below the LOD or LOQ. The maximum individual value reported was 530 µg/kg, and the weighted mean was 21 µg/kg.

Four countries representing the European diet submitted data on the concentration of HT-2 toxin in 758 samples of oats. The results for 67% were below the LOD or LOQ. The maximum individual value reported was 2000 µg/kg, and the weighted mean was 35 µg/kg.

*Rice:* Only two countries (Ecuador and Germany) reported data on T-2 toxin in rice. Of the 26 samples representing the European diet, the results for 96% were below the LOD or LOQ. The maximum analytical value reported was 19 µg/kg, and the weighted mean for the European diet was 0.7 µg/kg.

Data on HT-2 toxin in rice were submitted by one country with the European diet, for only 26 samples. None of the samples contained quantifiable concentrations.

*Rye:* Three countries, all with the European diet, submitted data on the concentration of T-2 toxin in rye. The results for 99% of the 83 samples were < LOD/LOQ. The maximum individual value reported was 17 µg/kg, and the weighted mean was 0.2 µg/kg.

Three countries with the European diet submitted data on the concentrations of HT-2 toxin in 87 samples of rye. The results for 99% of the samples were < LOD/LOQ. The maximum individual value reported was 23 µg/kg, and the weighted mean of all samples was 0.01 µg/kg.

*Wheat:* Ten countries with three regional diets reported data on the T-2 toxin concentrations in 2564 samples of wheat. Of those representing the European diet, 96% had values below the LOD or LOQ; the weighted mean was 1.6 µg/kg.

Three countries with three regional diets provided data on HT-2 toxin concentrations in wheat. Of 1740 samples, 1643 represented the European diet. For 90% of these, the results were below the LOD or LOQ. A maximum value of 310  $\mu\text{g}/\text{kg}$  was reported for this diet, and the weighted mean of all samples was 1.9  $\mu\text{g}/\text{kg}$ .

### 7.3 Estimates of intake at the international level

As noted above, dietary intakes were estimated only for the European diet, as limited information was available on T-2 toxin and HT-2 toxin in other regional diets. The average intakes of the two toxins were calculated by multiplying the weighted mean concentration of each commodity times the corresponding amount consumed in the GEMS/Food European diet. Intakes were calculated per person per day and converted to intake per kilogram of body weight per day, assuming a body weight of 60 kg, as recommended for international intake assessments (WHO, 1985).

The average intake of T-2 toxin in the European diet was estimated to be 7.6 ng/kg bw per day, while that of HT-2 toxin was estimated to be 8.7 ng/kg bw per day (Table 8). These estimates are based on the assumption that consumers choose foods randomly with respect to the distribution of concentrations of contaminants and are, therefore, exposed to an approximation of the mean of that distribution over time.

The distributions of dietary intakes could not be constructed from the available data. Nonetheless, intakes at high levels can be approximated by multiplying the average intake by a factor of two for a single food commodity and three for the total diet (WHO, 1985).

### 7.4 National estimates of intake

Information on food consumption patterns at the national level was submitted by Norway and the United Kingdom. The intakes of T-2 toxin and HT-2 toxin were

**Table 8. Estimated intakes of T-2 and HT-2 toxins in the European diet**

Toxin	Commodity	Weighted mean ( $\mu\text{g}/\text{kg}$ )	Consumption (g/person per day)	Intake			% total intake
				ng/person per day	$\mu\text{g}/\text{person}$ per day	ng/kg bw per day	
T-2	Barley	4.6	20	91	0.09	1.5	20
	Maize	3.2	8.8	28	0.03	0.5	6
	Oats	21	2.0	42	0.04	0.7	2
	Rice	0.7	12	8	0.01	0.1	2
	Rye	0.2	1.5	0	0	0	0
	Wheat	1.6	180	280	0.28	4.7	63
	Total intake			450	0.45	7.6	100
HT-2	Barley	4.4	20	87	0.09	1.5	17
	Maize	2.9	8.8	26	0.03	0.4	5
	Oats	35	2.0	70	0.07	1.2	13
	Rice	0	12	0	0	0	0
	Rye	0.01	1.5	0	0	0	0
	Wheat	1.9	180	340	0.34	5.6	65
	Total intake			520	0.52	8.7	100

calculated by multiplying the national food consumption values by the weighted mean concentrations of T-2 toxin in samples from the European diet. Norway submitted information on the median and 95th percentile consumption of oats, rye, and wheat by eight population subgroups. Males aged 16–29 had the highest consumption of these foods and the highest intakes of the toxins on the basis of kilograms of body weight (Table 9). Children 6 years of age had the lowest intakes per kilogram of body weight.

The United Kingdom provided information on the mean, median, and 97.5th percentile consumption of grains by two population subgroups, children aged 1.5–4.5 years and persons aged 16–64 years. The estimated intakes are reported in Table 10.

## **8. PREVENTION AND CONTROL**

### **8.1 Pre-harvest control**

*Fusarium* species infect grain and produce mycotoxins when in the field. Measures taken to control or minimize *Fusarium* infection may also reduce the possibility of formation of T-2 toxin and HT-2 toxin. Such measures include culture control techniques, growing resistant cultivars, and the use of fungicides or biological antagonists. The measures, summarized by Parry et al. (1995), are described briefly below.

Culture control techniques include suitable crop rotation, appropriate use of fertilizers, irrigation, and weed control. Maize–wheat rotation increases the incidence of *Fusarium* head blight and should be avoided, whereas removal or ploughing in of crop debris reduces the incidence in wheat. Direct drilling or minimal cultivation increases the risk of infection when *Fusarium*-contaminated debris is present. High concentrations of nitrogen fertilizer may increase plant water stress, but the effect on *Fusarium* head blight is unclear. Effective weed control may be useful in reducing *Fusarium* inoculum, but the efficacy of weed control in reducing *Fusarium* head blight is debated. Irrigation may avoid water stress and reduce the severity of *Fusarium* foot rot in wheat, which may serve as an inoculum for the development of head blight. Overhead irrigation has been shown to increase the severity of the disease.

Differences between cultivars in susceptibility to *Fusarium* head blight has been recognized for more than 100 years. Most cultivars of wheat are susceptible, and only a few are moderately resistant. Few reports of immune species exist. Limited work has been done on breeding resistance into species other than wheat.

The effect of previous crop residues and tillage on *Fusarium* head blight in wheat were examined by Dill-Macky & Jones (2000), who confirmed that the incidence and severity of the disease were greatest when wheat followed maize and least when wheat followed soya beans. In addition, the incidence and severity were lower in moldboard plow plots than in either chisel (reduced-till) or no-till plots. They suggested that changes in tillage practices, principally the move to conservation tillage and reduced-till systems, contributed to the recent epidemics of *Fusarium* head blight in midwestern USA.

Sub-lethal doses of certain fungicides have been shown to stimulate T-2 toxin production, while other fungicides may be highly effective inhibitors of T-2 toxin synthesis (D'Mello et al., 1998).

**Table 9. Intake of T-2 and HT-2 toxins from grains in Norway**

Population group	Grain	Concentration of toxin ( $\mu\text{g}/\text{kg}$ )	Body weight (kg)	Median consumption			95th percentile consumption		
				Grain (g/person per day)	Toxin intake		Grain (g/person per day)	Toxin intake	
					$\mu\text{g}/\text{person}$ per day	$\mu\text{g}/\text{kg}$ bw per day		$\mu\text{g}/\text{person}$ per day	$\mu\text{g}/\text{kg}$ bw per day
<b>T-2</b>									
Males, females	Oats	21	23	6.2	0.13	0.006	26	0.54	0.02
6 years	Rye	0.2	23	13	< 0.01	< 0.001	25	0.01	< 0.001
	Wheat	1.6	23	180	0.28	0.012	380	0.60	0.026
Males, females	Oats	21	35	8.2	0.17	0.0005	34	0.71	0.020
10 years	Rye	0.2	35	16	< 0.01	< 0.001	32	0.01	< 0.001
	Wheat	1.6	35	230	0.37	0.010	490	0.79	0.022
Males	Oats	21	75	7.5	0.16	0.002	76	1.6	0.021
16–29 years	Rye	0.2	75	15	< 0.01	< 0.001	31	0.01	< 0.001
	Wheat	1.6	75	280	0.44	0.006	700	1.1	0.015
Males	Oats	21	83	7.7	0.16	0.002	63	1.3	0.016
30–59 years	Rye	0.2	83	14	< 0.01	< 0.001	28	0.01	< 0.001
	Wheat	1.6	83	240	0.38	0.005	570	0.91	0.011
Males	Oats	21	79	6.5	0.14	0.002	67	1.4	0.018
60–79 years	Rye	0.2	79	13	< 0.01	< 0.001	25	0.01	< 0.001
	Wheat	1.6	79	190	0.31	0.004	720	1.2	0.015
Females	Oats	21	63	6.3	0.13	0.002	45	0.94	0.015
16–29 years	Rye	0.2	63	11	< 0.01	< 0.001	19	< 0.01	< 0.001
	Wheat	1.6	63	190	0.31	0.005	440	0.71	0.011
Females	Oats	21	65	5.8	0.12	0.002	46	0.96	0.015
30–59 years	Rye	0.2	65	10	< 0.01	< 0.001	18	< 0.01	< 0.001
	Wheat	1.6	65	170	0.28	0.004	390	0.62	0.010
Females	Oats	21	69	5.1	0.11	0.002	56	1.2	0.017
60–79 years	Rye	0.2	69	10.0	< 0.01	< 0.001	17	< 0.01	< 0.001
	Wheat	1.6	69	160	0.25	0.004	360	0.58	0.008

Table 9 (contd)

Population group	Grain	Concentration of toxin ( $\mu\text{g}/\text{kg}$ )		Body weight (kg)	Median consumption			95th percentile consumption		
					Grain (g/person per day)	Toxin intake		Grain (g/person per day)	Toxin intake	
						$\mu\text{g}/\text{person}$ per day	$\mu\text{g}/\text{kg}$ bw per day		$\mu\text{g}/\text{person}$ per day	$\mu\text{g}/\text{kg}$ bw per day
<b>HT-2</b>										
Males, females 6 years	Oats	35	23	6.2	6.2	0.22	0.015	26	0.91	0.039
	Rye	0.01	23		12.6	< 0.01	< 0.001	25	< 0.01	< 0.001
	Wheat	1.9	23		180	0.34	0.015	380	0.72	0.031
Males, females 10 years	Oats	35	35	8.2	8.2	0.29	0.008	34	1.2	0.034
	Rye	0.01	35		16	< 0.01	< 0.001	32	< 0.01	< 0.001
	Wheat	1.9	35		230	0.44	0.012	490	0.93	0.027
Males 16–29 years	Oats	35	75	7.5	7.5	0.26	0.003	76	2.6	0.035
	Rye	0.01	75		15.4	< 0.01	< 0.001	31	< 0.01	< 0.001
	Wheat	1.9	75		280	0.52	0.007	700	1.32	0.018
Males 30–59 years	Oats	35	83	7.7	7.7	0.27	0.003	63	2.2	0.026
	Rye	0.01	83		14	< 0.01	< 0.001	28	< 0.01	< 0.001
	Wheat	1.9	83		240	0.45	0.005	570	1.1	0.013
Males 60–79 years	Oats	35	79	6.5	6.5	0.23	0.003	67	2.3	0.030
	Rye	0.01	79		13	< 0.01	< 0.001	25	< 0.01	< 0.001
	Wheat	1.9	79		190	0.37	0.005	720	1.4	0.017
Females 16–29 years	Oats	35	63	6.3	6.3	0.22	0.003	45	1.6	0.025
	Rye	0.01	63		11	< 0.01	< 0.001	19	< 0.01	< 0.001
	Wheat	1.9	63		190	0.37	0.006	440	0.84	0.013
Females 30–59 years	Oats	35	65	5.8	5.8	0.20	0.003	46	1.6	0.025
	Rye	0.01	65		10	< 0.01	< 0.001	18	< 0.01	< 0.001
	Wheat	1.9	65		170	0.33	0.005	390	0.74	0.011
Females 60–79 years	Oats	35	69	5.1	5.1	0.18	0.003	56	2.0	0.028
	Rye	0.01	69		10	< 0.01	< 0.001	17	< 0.01	< 0.001
	Wheat	1.9	69		160	0.30	0.004	360	0.69	0.010

Sources of information on food consumption and body weight: Children 6–10 years, Norkost (1997); males and females  $\geq 16$  years, Langseth (2000). Toxin concentrations, weighted mean of European data

**Table 10. Estimated intake of T-2 and HT-2 toxins by British children aged 1.5–4.5 years and British adults aged 16–64 years (eaters only)**

Age group (years)	Grain	Toxin (µg/kg) <sup>a</sup>	% eaters	Consumption (g/person per day)			Toxin intake (µg/person per day)		
				Mean	Median	97.5th %ile	Mean	Median	97.5th %ile
<b>T-2</b>									
1.5–4.5	Barley	4.6	< 1	1.1	0.6	2.1	0.005	0.003	0.010
	Maize	3.2	66	10	6.6	37	0.032	0.021	0.12
	Oats	21	25	4.1	2.2	18	0.085	0.046	0.37
	Rye	0.2	1	2.0	1.7	4.9	< 0.001	< 0.001	0.001
	Wheat	1.6	99	47	45	100	0.076	0.072	0.16
16–64	Barley	4.6	< 1	4.9	4.7	8.7	0.023	0.022	0.040
	Maize	3.2	50	12	7.6	50	0.040	0.024	0.16
	Oats	21	25	12	7.6	41	0.24	0.16	0.85
	Rye	0.2	9	7.4	3.7	39	0.001	0.001	0.008
	Wheat	1.6	99	130	120	250	0.20	0.19	0.40
<b>HT-2</b>									
1.5–4.5	Barley	4.4	< 1	1.1	0.6	2.1	0.005	0.003	0.009
	Maize	2.9	66	10	6.6	37	0.029	0.019	0.11
	Oats	35	25	4.1	2.2	18	0.14	0.077	0.62
	Rye	0.01	1	2.0	1.7	4.9	< 0.001	< 0.001	< 0.001
	Wheat	1.9	99	47	45	100	0.090	0.085	0.19
16–64	Barley	4.4	< 1	4.9	4.7	8.7	0.022	0.021	0.038
	Maize	2.9	50	12	7.6	50	0.036	0.022	0.15
	Oats	35	25	12	7.6	41	0.40	0.26	1.4
	Rye	0.01	9	7.4	3.7	39	< 0.001	< 0.001	< 0.001
	Wheat	1.9	99	130	120	250	0.24	0.23	0.48

Sources of information on consumption: Gregory et al. (1990, 1992)

<sup>a</sup> Weighted mean of data for European diet

Parry et al. (1995) suggested that biological control measures could be a useful alternative to fungicide treatment, since the period during which the cereals are sensitive to the disease is short. There are few reports of such control of *Fusarium* head blight, and none seems to have been used in practice. Experimental use of biological control against diseases attributed to *Fusarium* spp. has been reported (Kempf & Wolf, 1989; Mao et al., 1998; Hoefnagels & Linderman, 1999).

## 8.2 Decontamination

Numerous chemicals have been tested for their ability to decontaminate trichothecene-contaminated grain or feed. Calcium hydroxide monomethylamine effectively decontaminated feeds containing T-2 toxin (Bauer et al. 1987), but the efficiency was dependent on the moisture content of the feed and the processing temperature. A moisture content of 25% and a temperature of 100 °C also had to be maintained for 1 h in order to destroy the less toxic HT-2 toxin, which was formed during the detoxication process as a result of alkaline hydrolysis of T-2 toxin.

Bentonite and spent canola oil bleaching clays appear to exert beneficial effects by adsorbing T-2 toxin present in the diet and inhibiting its adsorption from the

gastrointestinal tract (Carson & Smith, 1983). Bentonite fed at 10% was the most effective treatment in overcoming feed refusal and growth depression in rats.

A hydrated sodium calcium aluminosilicate protected some animals against the adverse effects of some *Fusarium* mycotoxins (Charmley & Prelusky, 1994); however, incorporation of this compound into contaminated diets had no effect on the toxicity of T-2 toxin in poultry (Kubena et al., 1990).

Superactivated charcoal had little effect in alleviating mycotoxicosis when T-2 toxin was fed to broiler chicks (Edrington et al., 1997).

Administration of monoclonal antibodies specific for T-2 toxin neutralized the inhibitory effects of the toxin on protein synthesis in vitro (Feuerstein et al., 1988).

## 9. COMMENTS

### *Absorption, distribution, metabolism, and excretion*

T-2 toxin is readily metabolized by mammalian gut microflora to several metabolites. HT-2 toxin is a primary metabolite in the gut and is absorbed into the blood after ingestion of T-2 toxin. Metabolism continues in the liver (with biliary excretion), resulting in a substantial, combined first-pass effect in the gut and liver. Metabolites of T-2 toxin include HT-2, 3'-hydroxy-HT-2, 3'-hydroxy-T-2, T-2 tetraol, de-epoxy 3'-hydroxy-T-2 triol, de-epoxy 3'-hydroxy-HT-2, and 3'-hydroxy-T-2 triol. Glucuronide conjugates are also formed extensively in most species (with the exception of cats). T-2 toxin and its metabolites are eliminated rapidly. In rats, more than 95% of a radioactively labelled oral dose of 0.15 mg/kg bw per day was excreted within 72 h. In the same study, a dose of 0.6 mg/kg bw per day was eliminated more slowly, suggesting potentially saturable metabolism or elimination pathways at doses that are relevant to those used in the studies of toxicity considered in this evaluation.

### *Toxicological studies*

T-2 toxin is a potent inhibitor of protein synthesis both in vivo and in vitro. The effective concentration for protein inhibition in vitro is lower than the effective concentrations for all other effects that have been demonstrated.

The metabolites of T-2 toxin have not been studied in detail, but several primary metabolites were less toxic than the parent compound in vitro. Furthermore, T-2 toxin was 10 times more toxic when inhaled than after oral intake, suggesting that the first-pass effect reduces the toxicity, at least after acute exposure. The Committee noted that, although it is generally assumed that T-2 toxin is considerably (e.g. 10-fold) more toxic than deoxynivalenol, a comparison of the LOELs for similar species and end-points (see monograph on deoxynivalenol) suggests that these trichothecenes have roughly similar toxicity when they are ingested with food.

Strain and sex differences in susceptibility to the toxicity of T-2 toxin have been observed in mice given single oral doses by gavage in studies designed to evaluate this variation. A sex difference was also observed after administration by inhalation. The cause of the differences has not been identified. Differences in susceptibility to T-2 toxin among species were also suggested from a comparison of the results of short-term studies. Severe toxic effects, including haemorrhage in the intestinal tract, lymph nodes, and heart, that led to death within weeks were observed in cats that received a dose of T-2 toxin as low as 0.06 mg/kg bw per day in a gelatin capsule. In

contrast, relatively mild effects were observed in 7-week-old pigs given T-2 toxin in the diet at doses up to 0.13 mg/kg bw per day for 3 weeks and in mice given 0.22 mg/kg bw per day in the diet in a 71-week study. The greater susceptibility of cats was to be expected in view of the demonstrated deficiencies in conjugation reactions in this species. Humans would not be expected to be similarly susceptible.

Little direct information was available on the toxicity of HT-2 toxin alone. The few comparative data available on T-2 and HT-2 toxins indicate that they induce adverse effects with similar potency. Furthermore, because T-2 toxin is rapidly converted to HT-2 toxin (and other metabolites common to T-2 and HT-2 toxins) in the gut, the toxicity of T-2 toxin in vivo can be considered to include that of HT-2 toxin. Hence, the results of studies of T-2 toxin can be used to approximate the effects of HT-2 toxin.

Information on the toxicity of T-2 toxin when ingested daily was limited largely to studies of less than 1 month. The immune system is a primary target of T-2 toxin, and the effects include changes in leukocyte count, delayed hypersensitivity, depletion of selective blood cell progenitors, depressed antibody formation, allograft rejection, and a blastogenic response to lectins. Either increased or decreased leukocyte counts were observed, depending at least in part on the dose and the time after administration of T-2 toxin when the leukocytes were counted. Similarly, both decreased and increased resistance to microbial infection has been observed in a number of studies. For example, in separate experiments in several laboratories, decreased resistance (leading in most cases to greater mortality rates) was observed in mice exposed to T-2 toxin at the time of infection with *Salmonella typhimurium*, *Salmonella enteritidis*, *Mycobacterium bovis*, *Herpes simplex*, *Toxoplasma gondii*, or *Listeria monocytogenes*. However, increased resistance leading to a reduced mortality rate was observed when mice were treated with T-2 toxin before infection with *Listeria monocytogenes*.

Feed refusal, reduced weight gain, and changes in organ weights, which are sensitive end-points, have been observed in most studies of feeding of T-2 toxin in which these parameters were recorded.

A 3-week study was conducted in which 7-week-old pigs were fed a diet providing an average T-2 toxin intake equal to 0.029, 0.062, 0.10, or 0.13 mg/kg bw per day. On the first and fourth days of administration, the pigs were immunized by an intramuscular injection of horse globulin. The titre of antibodies to this antigen was significantly lower in T-2 toxin-treated than in control pigs at 14 and 21 days at all doses tested. The leukocyte count and the proportion of leukocytes made up by T-lymphocytes was lower in all treated groups. A decreased proliferative response to phytohaemagglutinin and concanavalin A was observed at all doses of T-2 toxin at 21 days. A dose-related decrease in feed intake was observed at all doses, and decreased weight gain was observed at doses of 0.062 mg/kg bw per day and above. The haemoglobin concentration was decreased in a dose-related manner at these doses. A reduction in erythrocyte count was observed at 0.10 and 0.13 mg/kg bw per day, and the erythrocyte volume fraction was reduced at 0.13 mg/kg bw per day. The Committee noted that, as pair-fed animals were not used as controls, the potential confounding effects of feed intake and differences in weight gain on the observed end-points could not be evaluated. A NOEL was not identified.

In a 71-week bioassay of the carcinogenicity of T-2 toxin administered in the feed of mice, the incidence of pulmonary adenomas and hepatic adenomas was statistically significantly increased at the end of the study in males at the highest

dose, with no increase in tumour incidence in female mice. However, an increase in the incidence of benign tumours of the liver or lung in mice of one sex in a single study constitutes, at most, weak evidence of carcinogenicity. A dose-related increase in heart weight was seen in males (but not females) receiving T-2 toxin. No other treatment-related changes were reported. Studies of cancer initiation and promotion in mice suggest that T-2 toxin is not likely to be a potent carcinogen. A working group convened by IARC in 1993 evaluated the same experimental data and concluded that T-2 toxin is not classifiable with regard to its carcinogenicity to humans (Group 3).

Tests for genotoxicity with T-2 toxin in microorganisms gave uniformly negative results. In cultured mammalian cells, however, low concentrations of T-2 toxin induced DNA strand breaks, unscheduled DNA synthesis, gene mutation, chromosomal aberrations, and inhibition of gap-junctional intercellular communication. There was also evidence that DNA strand breaks and chromosomal aberrations are induced *in vivo*. It was unclear whether these effects are a consequence of interaction of T-2 toxin with genetic material or are secondary to inhibition of protein synthesis by T-2 toxin.

No embryotoxicity or gross fetal malformations were seen at intraperitoneal doses lower than 0.5 mg/kg bw per day. Continuous administration in the feed at concentrations of T-2 toxin equivalent to 0.22 and 0.45 mg/kg bw per day did not result in reproductive or gross developmental effects in CD-1 mice, although increased spleen weights were observed in male offspring of exposed dams at both doses.

The Committee noted that reduced feed intake is a potential confounder in studies of the toxicity of T-2 and HT-2 toxins. For example, in one study in mice in which pair-fed controls were used, changes in spleen weight, cell counts, and lymphoproliferative response were observed that paralleled those in mice given 3 mg/kg bw per day. The spleen weight, cell counts, and lymphoproliferative response were significantly lower in both the T-2 toxin-treated and pair-fed groups than in control mice fed *ad libitum*.

#### *Observations in humans*

The studies of adverse health effects in human populations were limited to a few investigations of outbreaks of acute poisoning, in which the effects reported included nausea, vomiting, pharyngeal irritation, abdominal pain and distension, diarrhoea, bloody stools, dizziness, and chills. In subsequent investigations, analyses of limited numbers of suspected food or grain samples indirectly linked the outbreaks to T-2 toxin. The concomitant occurrence of T-2 toxin with deoxynivalenol, acetyldeoxynivalenol, and nivalenol was reported in one of these outbreaks and the presence of these or other trichothecenes could not be ruled out in other studies. A series of food-related poisoning incidents referred to as alimentary toxic aleukia that occurred in 1931–47 in the former Soviet Union was associated with ingestion of grain infected with moulds, in particular *F. poae* and *F. sporotrichioides*. The dominant pathological changes were necrotic lesions of the oral cavity, oesophagus, and stomach and, in particular, pronounced leukopenia consisting primarily of bone-marrow hypoplasia and aplasia. The disease was lethal in a high proportion of cases. In investigations conducted three decades later, cultures implicated in the outbreak were shown to produce T-2 toxin.

### *Sampling protocols and analytical methods*

No sampling plans for the determination of T-2 and HT-2 toxins in foods have been published, and details on variation in the sampling of these toxins have not been reported. Furthermore, no official methods for the determination of T-2 and HT-2 toxins have been published, although some methods that have been validated in collaborative studies have been reported.

The introduction of improved clean-up columns based on charcoal, alumina, and modified diatomaceous earth before chromatographic determination has simplified and accelerated the analysis of T-2 and HT-2 toxins. Use of these columns in combination with gas chromatography and electron capture detection or mass spectrometry after derivatization of T-2 and HT-2 toxins is the commonest technique for their quantification. These techniques allow determination of concentrations of a few nanograms per gram, even in complex food matrices. Effective clean-up and derivatization are required for quantification by liquid chromatography with ultra-violet or fluorescence detection. Liquid chromatography with mass spectrometric detection is a potentially useful technique for determining T-2 and HT-2 toxins directly and simultaneously; however, its high cost prohibits its use as a routine method. Thin-layer chromatography, particularly high-performance, can also be used for the determination of T-2 and HT-2 toxins. A few enzyme-linked immunosorbent assay methods have been developed for screening T-2 toxin.

Although a variety of analytical methods is available for quantifying T-2 and HT-2 toxins, inter-laboratory comparisons have clearly shown that appropriate screening methods and better analytical methods are needed for these mycotoxins, particularly with respect to the recovery, accuracy and precision of measurements. More widely available reference materials and regular international comparative studies are required for these mycotoxins in order to improve internal and external quality assurance.

### *Levels and patterns of contamination of food commodities*

Data on the concentrations of T-2 and HT-2 toxins in food commodities were submitted by Brazil, China, Finland, Germany, Norway, Sweden, and the United Kingdom, and others were obtained from the literature. Gas chromatography with electron capture detection was the technique used most commonly for quantification of these toxins, followed in order by thin-layer chromatography and enzyme-linked immunosorbent assay.

Data from studies in which information on the sampling protocol or the analytical method was not provided were excluded from the evaluation. The remaining data were used only if the samples had been collected at random and if the analytical methods used were considered to be adequate.

Data were available on barley, oats, rice, rye, and wheat. Most of the 8918 samples were collected in Europe. The frequency of occurrence of contaminated samples was 11% for T-2 toxin and 14% for HT-2 toxin; high concentrations of the two toxins were occasionally found together. Annual variation was observed in the degree of contamination of oats, wheat, and barley. The mean concentrations in samples in which T-2 was found were 0.1–21 µg/kg in barley, 1.3–6.0 µg/kg in maize, 2.3–26 µg/kg in oats, 2.7–27 µg/kg in rice, 0.6 µg/kg in rye, and 0.1–60 µg/kg in wheat. The mean concentrations in samples in which HT-2 was found were

0.4–15 µg/kg in barley, 2.4–14 µg/kg in maize, 3.7–20 µg/kg in oats, 26–100 µg/kg in rice, 0.03 µg/kg in rye, and 0.2–20 µg/kg in wheat.

As T-2 and HT-2 toxins were not detected in a large proportion of samples from the United Kingdom, the distribution could not be derived. The use of distribution functions was also not practicable for the other data sets, mainly because of the way in which the data were reported.

#### *Food consumption/dietary intake assessment*

The average intakes of T-2 and HT-2 toxins can be estimated by multiplying the average concentrations in food commodities by estimates of the average food consumption. For the latter, the GEMS/Food regional diets were used. Most of the data on average concentrations of T-2 and HT-2 toxins that were available for the evaluation were pooled; that is, each data point represented the mean concentration of a number of individual samples. Data on processed food products were excluded from estimates of dietary intake. A total of 175 data points representing 8410 individual samples were used for the intake assessment. Of these, 147 were reported from countries in Europe; the remaining 28 data points represented only three commodities in the other four geographical regions. In view of the limited amount of information on these toxins in regions other than Europe, the dietary intakes of T-2 and HT-2 toxins were estimated from the GEMS/Food European-type diet only. The mean concentrations of T-2 and HT-2 toxins, weighted by sample size, were calculated for each commodity (barley, maize, oats, rice, rye, and wheat) from the data submitted, and the intakes of T-2 and HT-2 toxins were estimated by multiplying the weighted mean concentration in each commodity by the respective value for consumption in the GEMS/Food European diet.

The total intake of T-2 toxin was estimated to be 7.6 ng/kg bw per day, wheat and barley being the major dietary sources. The total intake of HT-2 toxin was estimated to be 8.7 ng/kg bw per day, wheat, barley, and oats being the most important dietary sources. These estimates were based on the assumption that consumers choose food randomly with respect to the distribution of concentrations of contaminants, which will approximate the mean over time.

In general, more data on the occurrence of T-2 and HT-2 toxins in food commodities, particularly from geographical regions other than Europe, are required to allow better estimates of intake. The Committee noted that the distribution of contamination in processed products could differ from that in raw cereals, as contamination tends to be more homogeneous after processing. Despite the limited amount of data on concentrations of T-2 and HT-2 toxins, the preliminary estimates of average contamination and dietary intake based on the GEMS/Food European diet proved to be useful. However, significant data gaps were identified in the assessment, with respect to both the quality and the geographical representativeness of the available data. Although it was not possible to estimate intakes at high consumption levels from the available data, such intakes may be approximated by multiplying the average intake by a factor of two for a single food commodity and three for the total diet.

#### *Prevention and control*

Preharvest measures taken to control or minimize *Fusarium* infection may also reduce the possibility of formation of T-2 and HT-2 toxins. Reducing the inoculum of

*Fusarium* in host debris and other reservoirs in the field appears to be an effective control measure. Practices such as reduced tillage have been shown to increase the incidence of other trichothecenes and may also affect those of T-2 and HT-2 toxins. Good agricultural practice, such as immediate drying after harvesting and proper storage, prevents further contamination with T-2 and HT-2 toxins.

Physical, chemical, and biological methods have been used to decontaminate grain containing trichothecenes, but few studies were available on any reduction in the concentration of T-2 or HT-2 toxins. Thermal processing is usually ineffective.

## 10. EVALUATION

The Committee concluded that there was substantial evidence for the immunotoxicity and haematotoxicity of T-2 toxin in several species, and that these are critical effects after short-term intake. Only one long-term study was available, and that study alone was not suitable for establishing a tolerable intake. Nonetheless, on the basis of the critical effects seen in several short-term studies, the Committee concluded that the safety of food contaminated with T-2 toxin could be evaluated from the LOEL of 0.029 mg/kg bw per day for changes in white and red blood cell counts identified in the 3-week dietary study in pigs. This LOEL was the lowest LOEL for adverse effects in the studies on T-2 toxin. It was considered to be close to a NOEL, as the effects on blood cell counts were subtle and reversible. Furthermore, other studies in pigs showed no effects at this dose.

The Committee used this LOEL and a safety factor of 500 to derive a provisional maximum tolerable daily intake (PMTDI) for T-2 of 60 ng/kg bw per day. The safety factor of 500 was used because there was no clear NOEL in the 3-week study in pigs and there were deficiencies in the database, including insufficient study of long-term administration of T-2 toxin and sex, species, and individual variations in sensitivity.

The Committee further concluded that the toxic effects of T-2 toxin and its metabolite HT-2 toxin could not be differentiated, and that the toxicity of T-2 toxin *in vivo* might be due at least partly to effects of HT-2 toxin. Hence, HT-2 toxin was included in the PMTDI, resulting in a group PMTDI of 60 ng/kg bw per day for T-2 and HT-2 toxins, alone or in combination.

### *Recommendations*

- Further studies are needed to reduce the uncertainty in the evaluation of the carcinogenic potential of T-2 toxin. Standard bioassays in rats and mice, with pair-fed controls, would be preferred. Also, a longer-term study in pigs is needed in which a NOEL is identified, control groups are used to account for the potential effects of reduced feed consumption, and relevant, sensitive end-points of haematotoxicity and immunotoxicity are measured. In order to clarify differences among species, comparative studies of toxicity and toxicokinetics should be carried out in rodents, cats, and pigs. Studies are needed on the combined effects of other trichothecenes that contaminate foods consumed by humans.
- As little information on the concentrations of T-2 and HT-2 toxins in food commodities was available from geographical regions other than Europe, dietary intake was estimated only on the basis of the GEMS/Food European regional diet. Dietary intake in other geographical regions should be evaluated when

more data on the concentrations of T-2 and HT-2 toxin become available. The average intake of T-2 toxin was estimated to be 8 ng/kg bw per day, and that of HT-2 toxin was estimated to be 9 ng/kg bw per day. The total was therefore not expected to exceed the group PMTDI of 60 ng/kg bw per day. Nonetheless, more accurate information on human intake of T-2 toxin in various regions of the world and improved analytical methods and reference materials for the determination of both toxins are needed.

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## Appendix A.

**Results of surveys for T-2 toxin showing concentrations and distribution of contamination in food commodities**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	References	Sampling procedure
<b>Africa</b>										
South Africa	Yellow maize	1993	236	250 <sup>a</sup>	236				P,S, Rava et al. (1996); A, Sydenham & Thiel (1987)	Samples collected at harvest from silos in main production zones
South Africa	White maize	1994–95	143	250 <sup>a</sup>	143				P,S, Rava (1996); A, Sydenham & Thiel (1987)	Samples collected from mills throughout country
	Yellow maize		148	250 <sup>a</sup>	148					
	Maize products		158	250 <sup>a</sup>	158					
<b>Americas</b>										
Argentina	Maize	1987–89	100	100 <sup>a</sup>	85	163/2400			P, Saubois et al. (1992); S, Junta Nacional de Granos (1984), COPANT (1998), Jewers (1987); A, Kamimura et al. (1981)	Samples, 10 kg; analytical sample, 50 g
Argentina	Wheat	1986	261	500 <sup>a</sup>	241	13/NA			P, Quiroga et al. (1995); S, Apro et al. (1987); A, Trucksess et al. (1984)	See Trichothecenes Appendix 6
Brazil	Wheat	1990	20	100 <sup>a</sup>	18	60/800	360	2	P,S, Furlong et al. (1995); A, Furlong & Valente Soares (1995)	Samples from experimental plots in wheat-growing areas of São Paulo; 3–10 kg; laboratory sample, 1 kg

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	References	Sampling procedure
Brazil	Wheat and wheat products	1991	38	100 <sup>a</sup>	38				P,S, Soares & Furlani (1996); A, Furlong & Valente Soares (1995)	1-kg samples purchased from health-food shops
Brazil, Paraná	Maize	1994–95	80	50 <sup>a</sup>	79	1.3/104		1	P, Prado et al. (1997); S, Fonseca (1991); A, ELISA	See Trichothecenes Appendix 6
Brazil, Goiás	Maize		8	50 <sup>a</sup>	8					
Canada	Durum wheat	1987	29	400	29				P,S, Scott (1997); A, Scott et al. (1989)	2–5-kg samples collected immediately after harvest or at mills
	Soft winter wheat	1988	17	400	17					
	Maize	1989	16	400	16					
	Western hard wheat	1991	108	400	108					
Canada	Wheat	1990	29	150 <sup>a</sup>	29				P,S,A, Stratton et al. (1993)	3-kg samples collected at harvest and ground in Romer mill; subsamples, 250 g
	Barley	1990	23	150 <sup>a</sup>	23					
Chile	Maize	1995–96	68	10 <sup>a</sup>	68				P,A, Vega et al. (1998); S, Olavarría (1992)	See Trichothecenes Appendix 6
Ecuador	Rice	1992–94	99	50	93	27/NA			P, Mühlemann et al. (1997a,b); S, Mühlemann et al. (1997a); A, Veratox™	Mixed samples of 1.2–3 kg collected at random from various climatic regions taking into account variety and type of storage
	Beans		76	50	65	30/NA				
	Maize		90	50	78	30.7/NA				

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	References	Sampling procedure
<b>Europe</b>										
Austria	Maize	1996	46	100	45	5.6/260		1	Lew et al. (2000a); A, Lew et al. (2000b)	Composite samples of about 60 kg collected, homogenized, and reduced to 6-kg; 1 kg ground in home mill
	Maize	1997	58	100	58					
	Maize	1998	48	100	46	6/150		2		
Bulgaria	Wheat	1995	140	40 <sup>a</sup>	139	0.39/55			P,S,A, Vrabcheva et al. (1996)	
Finland	Oats	1987–88	21	15 <sup>a</sup>	19	5.6/73			P,S,A, Hietaniemi & Kumpulainen (1991)	2–3-kg samples collected from Finnish State granaries and private farmers
	Barley		30	15 <sup>a</sup>	30					
	Wheat		40	15 <sup>a</sup>	40					
	Rye		31	15 <sup>a</sup>	30					
Finland	Oats	1998	10	20	9	2.3/23	23	0	P,S,A, Eskola et al. (2000a,b)	See Trichothecenes Appendix 6
Germany	Wheat	1987	84	1–3 <sup>a</sup>	62	21/249			P,S, Müller & Schwadorf (1993); A, Schwadorf & Müller (1991)	Samples collected randomly from farms 1–4 weeks after harvest by Governmental advisory board
Germany	Wheat	1989	78	1–5 <sup>a</sup>	73	0.66/12			P,S, Müller & Schwadorf (1993); A, Schwadorf & Müller (1991)	Samples of 700 g to 1 kg collected randomly from farms 1–4 weeks after harvest by Governmental advisory board
	Wheat	1990	80	1–5 <sup>a</sup>	71	5.7/136				
	Wheat	1991	80	1–5 <sup>a</sup>	77	0.4/16				
	Wheat	1992	78	1–5 <sup>a</sup>	78					
	Wheat	1993	45	1–5 <sup>a</sup>	27	8/94				

Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	References	Sampling procedure
Germany	Barley	1987	44	1-5 <sup>a</sup>	40	0.5/9			P,S, Muller et al. (1997b); A, Schwadorf & Müller (1991)	Samples of 700 g to 1 kg collected randomly from farms 1-4 weeks after harvest by Governmental advisory board
	Barley	1989	40	1-5 <sup>a</sup>	39	0.4/16				
	Barley	1990	47	1-5 <sup>a</sup>	46	0.1/6				
	Barley	1991	51	1-5 <sup>a</sup>	40	6.3/110				
	Barley	1992	58	1-5 <sup>a</sup>	41	21/300				
Germany	Oats	1987	56	1-5 <sup>a</sup>	30	10/57			P,S, Müller et al. (1998); A, Schwadorf & Müller (1991)	Samples of 700 g to 1 kg collected randomly from farms 1-4 weeks after harvest by Governmental advisory board
	Oats	1989	56	1-5 <sup>a</sup>	39	8.2/86				
	Oats	1990	54	1-5 <sup>a</sup>	21	12/26				
	Oats	1991	51	1-5 <sup>a</sup>	37	15/220				
	Oats	1992	55	1-5 <sup>a</sup>	38	76/1700				
Germany	Wheat	1998	56	6	56				P,S, Schollenberger et al. (2000c); A, Schollenberger et al. (1998)	Samples collected at random from storage; subsamples of 100 g milled (1 mm) and 10 g taken for analysis
Germany	White wheat flour (ash con- tent, 400 and 550 mg/100 g)	1999	28	6	28				P,S, Schollenberger et al. (2000a); A, Schollenberger et al. (1998)	Samples of 5 kg collected at random from shops, mixed, and 10 g taken for analysis
	White wheat flour (ash con- tent, 1100 mg/ 100 g)	1999	13	6	13					
	White wheat flour (ash con- tent, 1600-1700 mg/100 g)	1999	19	6	18	0.21/4				

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	References	Sampling procedure																																																																																																																																
Germany	Bread	1999	107	6	105	0.07/4			P,S, Schollenberger et al. (2000b); A, Schollenberger et al. (1998)	Samples > 100 g dried at 40 °C, ground in home mill (1.5 mm), and 10 g taken for analysis																																																																																																																																
	Noodles	1999	39	6	36	0.8/12					Germany	Bread and related products	Jan–July 1998	96	6	95	0.04/4			P,S, Schollenberger et al. (1999); A, Schollenberger et al. (1998)	Samples dried at 40 °C, ground in home mill (1.5 mm), and 25 g taken for analysis	29	6	29					Germany	Breakfast cereals	Jan–July 1998	32	6	30	0.19/7			P,S, Schollenberger et al. (1999); A, Schollenberger et al. (1998)	Samples ground in home mill (1.5 mm), and 25 g taken for analysis	25	6	25					26	6	25	0.73/19				29	6	23	3.8/39				Norway	Oats, home grown	1996–1999	14	30 <sup>a</sup>	9	41/320	95	2	S, Langseth (2000); P and A, Langseth (2000); Langseth & Rundberget (2000)	See Trichothecenes Appendix 6	14	30 <sup>a</sup>	6	47/195	113	3	22	30 <sup>a</sup>	18	7/42	38	0	20	30 <sup>a</sup>	12	26/91	79	0	Wheat, home grown	1996–1998	34	30 <sup>a</sup>	34						10	30 <sup>a</sup>	10						24	30 <sup>a</sup>	24						Wheat, home grown	1996–1998	28	20 <sup>a</sup>	28						25	20 <sup>a</sup>	25						35	20 <sup>a</sup>	35		
Germany	Bread and related products	Jan–July 1998	96	6	95	0.04/4			P,S, Schollenberger et al. (1999); A, Schollenberger et al. (1998)	Samples dried at 40 °C, ground in home mill (1.5 mm), and 25 g taken for analysis																																																																																																																																
			29	6	29							Germany	Breakfast cereals	Jan–July 1998	32	6	30	0.19/7			P,S, Schollenberger et al. (1999); A, Schollenberger et al. (1998)	Samples ground in home mill (1.5 mm), and 25 g taken for analysis	25	6	25								26	6	25	0.73/19						29	6	23	3.8/39				Norway	Oats, home grown	1996–1999	14	30 <sup>a</sup>	9	41/320	95	2	S, Langseth (2000); P and A, Langseth (2000); Langseth & Rundberget (2000)	See Trichothecenes Appendix 6	14				30 <sup>a</sup>	6	47/195	113	3	22			30 <sup>a</sup>	18	7/42	38	0	20	30 <sup>a</sup>	12	26/91	79	0	Wheat, home grown	1996–1998	34	30 <sup>a</sup>	34								10	30 <sup>a</sup>	10						24	30 <sup>a</sup>	24						Wheat, home grown	1996–1998	28	20 <sup>a</sup>	28								25	20 <sup>a</sup>	25						35	20 <sup>a</sup>	35					
Germany	Breakfast cereals	Jan–July 1998	32	6	30	0.19/7			P,S, Schollenberger et al. (1999); A, Schollenberger et al. (1998)	Samples ground in home mill (1.5 mm), and 25 g taken for analysis																																																																																																																																
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Norway	Oats, home grown	1996–1999	14	30 <sup>a</sup>	9	41/320	95	2	S, Langseth (2000); P and A, Langseth (2000); Langseth & Rundberget (2000)	See Trichothecenes Appendix 6																																																																																																																																
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**Appendix A (contd)**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	References	Sampling procedure
Sweden	Wheat	1990	88	50	88				P,S, Pettersson (2000); A, Pettersson (1992)	Samples of about 1 kg collected from trials and plots and dried and milled; subsamples of 20 g analysed
	Oats	1990	71	50	44	43/430	118	17		
	Barley	1990	39	50	39					
	Rye	1990	5	50	5					
	Wheat	1991	92	50	92					
	Oats	1991	38	50	33	10/90		0		
	Wheat	1992	13	50	13					
	Oats	1992	2	50	2					
	Wheat	1993	2	50	2					
	Oats	1993	10	50	10					
	Oats	1994	34	30	25	34/532	82	3		
	Oats	1996	80	30	71	18/271	39	6		
	Oats	1997	84	20	65	8/94	26.5	0		
	Oats	1998	33	20	23	10/58	43.2	0		
Sweden	Barley	1998	10	20	10				P,S, Thuvander et al. (2000); A, Möller & Gustavsson (1992)	Composite samples of about 1 kg collected at the inflow of cereals to mill, during storage in mill, or in production; analytical sample, 50 g
	Wheat	10/1996–59	59	10	59					
	Oats	06/1998	23	10	23					
	Rye		28	10	28					
	Wheat	1999	75	25	75					
	Oats	1999	10	25	10					
United Kingdom	Rye	1999	19	25	19				P,S,A, Home Grown Cereals Authority (2000)	
	Wheat	1999	53	20 <sup>a</sup>	53					

## Appendix A (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	References	Sampling procedure
<b>Asia</b>										
Korea, Republic of	Maize, barley, rice, millet	1989	28	15 <sup>a</sup>	28				P,S,A, Park et al. (1991)	Samples collected from farms in four provinces
Korea, Republic of	Barley	July 1992	30	5 <sup>a</sup>	30				P,S, Ryu et al. (1996); A, Tanaka et al. (1985)	Samples collected from six provinces
	Maize	March 1992	15	5 <sup>a</sup>	15					
India	Maize	1994–97	197	100 <sup>a</sup>	188	NA/40			P,S, Janardhana et al. (1999); A, ELISA	Samples from 14 districts of Karnataka representing different cultivars collected directly from farmers, production plots, and regulating markets
China, Linxian	Wheat	1995	25	100 <sup>a</sup>	25				P,S, Gao & Yoshizawa (1997); A, Luo et al. (1990)	
China, Linqu County	Raw maize	1996	12	500 <sup>a</sup>	12				P,S,A, Groves et al. (1999)	3 households in seven villages selected at random from among those known to prepare sour pancakes; in each household, 5 specimens collected to represent successive stages of processing
	Maize meal	1996	13	500 <sup>a</sup>	13					
	Cooked pancake		1996	14	500 <sup>a</sup>	14				

**Appendix A** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	$n < \text{LOQ}$	Mean/Max (µg/kg)	90th %ile (µg/kg)	$n > 100$ (µg/kg)	References	Sampling procedure
China	Wheat grain	Summer 1990	330	1	66	53/120	130		P,A, Yang et al. (1992); S, GB5009	National standard methods for food chemistry; 15 subsamples of 100 g
China	Wheat grain	Summer 1992	147	1	34	NR/820	NR		P, Chen et al. (1995); A, Yang et al. (1992); S, GB5009	National standard methods for food chemistry; 15 subsamples of 100 g
China	Wheat grain	Summer 1994	157	1	62	14/100	47		P, He et al. (1998); A, Yang et al. (1992); S, GB5009	National standard methods for food chemistry; 15 subsamples of 100 g

NR, not reported

<sup>a</sup>Limit of detection

## Appendix B.

**Results of surveys for HT-2 toxin showing concentrations and distribution of contamination in food commodities**

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	References	Sampling procedure
<b>America</b>										
Brazil	Wheat	1990	20	500 <sup>a</sup>	20				P,S, Furlong et al. (1995); A, Furlong & Valente Soares (1995)	
Brazil	Wheat and wheat products	1991	38	500 <sup>a</sup>	38				P,S, Soares & Furlani (1996); A, Furlong & Valente Soares (1995)	
Canada	Soft winter wheat	1988	17	100	16	5.9/100			P,S, Scott (1997); A, Scott et al. (1989)	2–5-kg samples collected immediately after harvest or at mills
	Maize	1989	16	100	15	14/230				
	Western hard wheat	1991	108	100	106	5/310				
Chile	Maize	1995–96	68	10 <sup>a</sup>	68				P,A, Vega et al. (1998); S, Olavarría (1992)	See Trichothecenes
<b>Europe</b>										
Austria	Maize	1996	46	50	45	2.4/110		1	P,S,A, Lew et al. (2000a); A, Lew et al. (2000b)	Composite samples of about 60 kg collected, homogenized, and reduced to 6-kg samples; ground in home mill (1 kg)
	Maize	1997	58	50	58					
	Maize	1998	48	50	46	4/120		1		

## Appendix B (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	References	Sampling procedure
Finland	Oats	1987–88	21	15 <sup>a</sup>	19	3.7/44			P,S,A, Hietaniemi & Kumpulainen (1991)	2–3-kg samples collected from Finnish State granaries and private farmers
	Barley		30	15 <sup>a</sup>	30					
	Wheat		40	15 <sup>a</sup>	40					
	Rye		31	15 <sup>a</sup>	30		0.03/23			
Finland	Barley	1998	15	20	13	4.1/41	20	0	P,S,A, Eskola et al. (2000a,b)	See Trichothecenes
	Oats	1998	10	20	7	21/95	95	0		
Germany	Wheat	1987	84	1–3 <sup>a</sup>	78	0.7/20			P,S, Müller & Schwadorf (1993); A, Schwadorf & Müller (1991)	Samples collected randomly from farms 1–4 weeks after harvest by Governmental advisory board
Germany	Wheat	1989	78	1–5 <sup>a</sup>	72	1.4/22			Müller et al. (1997a); A, Schwadorf & Müller (1991)	Samples of 700 g to 1 kg collected randomly from farms 1–4 weeks after harvest by Governmental advisory board
	Wheat	1990	80	1–5 <sup>a</sup>	79	0.2/17				
	Wheat	1991	80	1–5 <sup>a</sup>	80					
	Wheat	1992	78	1–5 <sup>a</sup>	73	3/150				
	Wheat	1993	45	1–5 <sup>a</sup>	45					
Germany	Oats	1987	56	1–5 <sup>a</sup>	40	59/2000			P,S, Müller et al. (1998); A, Schwadorf & Müller (1991)	As above
	Oats	1989	56	1–5 <sup>a</sup>	52	21/520				
	Oats	1990	54	1–5 <sup>a</sup>	54					
	Oats	1991	51	1–5 <sup>a</sup>	51					
	Oats	1992	55	1–5 <sup>a</sup>	55					
Germany	Barley	1987	44	1–5 <sup>a</sup>	42	1.0/32			P,S, Muller et al. (1997b); A, Schwadorf & Müller (1991)	As above
	Barley	1989	40	1–5 <sup>a</sup>	37	1.1/18				
	Barley	1990	47	1–5 <sup>a</sup>	45	0.4/10				
	Barley	1991	51	1–5 <sup>a</sup>	51					
	Barley	1992	58	1–5 <sup>a</sup>	53	13/290				

## Appendix B (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	References	Sampling procedure
Germany	Wheat	1998	56	18	44	3.7/51	12	0	P,S, Schollenberger et al. (2000c); A, Schollenberger et al. (1998)	Samples collected at random from storage; subsample, 100 g milled (1 mm) and 10 g taken for analysis
Germany	White wheat flour (ash content, 400 and 550 mg/100 g)	1999	28	18	28				P,S, Schollenberger et al. (2000a); A, Schollenberger et al. (1998)	Samples of 5 kg collected at random from shops, mixed, and 10 g taken for analysis
Germany	White wheat flour (ash content, 1000 mg/100 g)	1999	13	18	12	0.9/12			P,S, Schollenberger et al. (2000a); A, Schollenberger et al. (1998)	Samples of 5 kg collected at random from shops, mixed, and 10 g taken for analysis
	White wheat flour (ash content, 1600–1700 mg/100 g)	1999	19	18	16	1.9/12				
Germany	Bread	1999	107	18	105	0.22/12			P,S, Schollenberger et al. (2000b); A, Schollenberger et al. (1998)	Samples > 100 g dried at 40 °C, ground in home mill (1.5 mm), and 10 g taken for analysis
	Noodles	1999	39	18	37	0.6/12				
Germany	Bread and related products	Jan–July 1998	96	18	83	2.2/32			P,S, Schollenberger et al. (1999); A, Schollenberger et al. (1998)	Samples dried at 40 °C, ground in home mill (1.5 mm), and 25 g taken for analysis
	Noodles		29	18	21	3.6/25				
	Breakfast cereals			32	18	22	4/22			
	Infant foods		25	18	24	0.5/12				
	Rice		26	18	26					
	Cereal foods		29	18	18	8/51				

**Appendix B** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	References	Sampling procedure
Norway	Rye, imported	1996	4	20 <sup>a</sup>	4				S, Langseth (2000); P,A, Langseth (2000), Langseth & Rundberget (2000)	See Trichothecenes <i>Appendix 6</i>
	Wheat, imported	1996	34	20 <sup>a</sup>	33	0.6/20				
	Wheat, imported	1997	10	20 <sup>a</sup>	10					
	Wheat, imported	1998	24	20 <sup>a</sup>	24					
	Oats, home grown	1996	14	20 <sup>a</sup>	0	140/400	295	8		
	Oats, home grown	1997	14	20 <sup>a</sup>	0	200/710	546	7		
	Oats, home grown	1998	22	20 <sup>a</sup>	6	64/520	93	2		
	Oats, home grown	1999	20	20 <sup>a</sup>	3	91/240	190	8		
	Wheat, home grown	1996	28	20 <sup>a</sup>	28					
	Wheat, home grown	1997	25	20 <sup>a</sup>	25					
	Wheat, home grown	1998	35	20 <sup>a</sup>	35					

## Appendix B (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	References	Sampling procedure
Sweden	Wheat	1990	88	30	88				P,S, Pettersson (2000); A, Pettersson (1992)	Samples of about 1 kg collected from trials and plots, dried, and milled; subsample, 20 g
	Oats	1990	71	30	35	70/330	230	18		
	Barley	1990	39	30	33	15/140		3		
	Rye	1990	5	30	5					
	Wheat	1991	92	10	51	12/70	33.5	0		
	Oats	1991	38	10	13	95/365	291	13		
	Wheat	1992	13	10	13					
	Oats	1992	2	10	0	40/40		0		
	Wheat	1993	2	10	2					
	Oats	1993	10	10	10					
	Oats	1994	34	20	22	30/339	91.5	3		
	Oats	1996	80	10	48	23/303	75.7	6		
	Oats	1997	84	10	45	21/175	78	4		
	Oats	1998	33	10	22	12/92	37.2	0		
	Sweden	Barley	1998	10	10	10				
Wheat		10/1996–	59	10	59					
Oats		06/1998	23	10	23					
Rye			28	10	28					
Wheat		1999	75	25	75					
Oats		1999	10	25	10					
United Kingdom	Rye	1999	19	25	19				P,S,A, Home Grown Cereals Authority (2000)	
	Wheat	1999	53	20 <sup>a</sup>	36	20/170	70	3		

**Appendix B** (contd)

Country/ Region	Commodity	Year/ Season	No. of samples	LOQ (µg/kg)	<i>n</i> < LOQ	Mean/Max (µg/kg)	90th %ile (µg/kg)	<i>n</i> > 100 (µg/kg)	References	Sampling procedure
<b>Asia</b>										
Korea, Republic of	Barley	July 1992	30	5 <sup>a</sup>	30				P,S, Ryu et al. (1996); A, Tanaka et al. (1985)	
	Maize	March 1992	15	5 <sup>a</sup>	15					
China, Linxian	Wheat	1995	25	100 <sup>a</sup>	25				P,S, Gao & Yoshizawa (1997); A, Luo et al. (1990)	
China, Linqu County	Raw maize	1996	12	500 <sup>a</sup>	12				P,S,A, Groves et al. (1999)	3 households in 7 villages selected at random from among those known to prepare sour pancakes; at each household, 5 specimens collected to represent successive stages of processing
	Maize meal	1996	13	500 <sup>a</sup>	13					
	Cooked pancake		1996	14	500 <sup>a</sup>	14				

<sup>a</sup> Limit of detection

## Trichothecenes Appendix 1.

Methods for screening for B-trichothecenes and particularly for deoxynivalenol<sup>a</sup>

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Technique	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	CV (%)
Malone et al. (1998)	Wheat, barley, maize, bran, oats	25	CH <sub>3</sub> CN/H <sub>2</sub> O	Mycosep	Fluorometric determination after derivatization	DON	100	500–	NA 50 000	NA
Trucksess et al. (1987) 78–89	Corn, wheat, barley	50	CH <sub>3</sub> CN:H <sub>2</sub> O: MeOH (70:25:5)	Carbon column	TLC fluoro- C18 column	DON	NA	200 NIV	81–88	2–20 100
			200ml, shake 60 min			FX		50	61–108	
Trucksess et al. (1984)	Wheat, maize	50	CH <sub>3</sub> CN/H <sub>2</sub> O	Charcoal/ alumina/celite	TLC	DON	20/40 (wheat) 20/100 (maize)	100–1000 (wheat) 100–1000 (maize)	85–93 77–80	3–17 6–12
Fernandez et al. (1994)	Wheat	50	CH <sub>3</sub> CN/H <sub>2</sub> O	Charcoal/ alumina/ celite	TLC	DON	40/NA	200 400 800	83 82 72	NA
Eppley et al. (1986)	Wheat	50	CH <sub>3</sub> CN/H <sub>2</sub> O	Charcoal/ alumina/celite	TLC	DON	50/NA	50 100 300 1000	90 96 95 78	30–87
Eppley et al. (1984)	Wheat	50	CH <sub>3</sub> CN/H <sub>2</sub> O	Charcoal/ alumina	TLC	DON	NA/200	200–5000	80	NA

**Tricothecenes Appendix 1 (contd)**

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Technique	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	CV (%)
Romer (1986)	Foods, feeds		CH <sub>3</sub> CN/H <sub>2</sub> O	Charcoal/alumina	TLC	DON NIV FX	300/NA 500/NA 300/NA	900–1100 750–1100 900–1100	99 92 99	8 15 9
Romer (1999) Accutox™			Distilled water		ELISA	DON		500		
Xu et al. (1988)	Wheat, maize		CH <sub>3</sub> CN/H <sub>2</sub> O	None	ELISA (acetylated)	DON	NA	10–1000	102 100	12 15
Ridascreen™ DON (2000)	Cereals, feeds	2	CH <sub>3</sub> CN/H <sub>2</sub> O		ELISA (acetylated)	DON 3-AcDON 15-AcDON	1.25/NA	NA	80–90	
Sinha et al. (1995)	Wheat, maize	1	MeOH/ H <sub>2</sub> O		ELISA	DON 15-AcDON	250/NA	NA	NA	NA
Abouzied (2000) Veratox™ (1998)	Cereals Wheat Corn Barley Oat	NA 10	H <sub>2</sub> O H <sub>2</sub> O	None None	ELISA CD-ELISA	DON DON	20/NA 300/500	NA NA	NA NA	NA NA
Schneider et al. (2000)	Wheat	10	CH <sub>3</sub> CN/H <sub>2</sub> O	None	ELISA (IgY)	DON	160/NA	525	104-120	6
Mills et al. (1990)	Wheat	NA	60% MeOH	None	ELISA	DON	100/NA	NA	NA	NA
Usleber et al. (1992)	Wheat	2	MeOH/ H <sub>2</sub> O	Ethyl acetate	ELISA	DON	4.5 ng/ml/ 200	200–1000	71–79	3.6–4.9

<sup>a</sup> For definitions of abbreviations, see end of Tricothecenes Appendix 6.

<sup>b</sup> The references are listed in the monograph on deoxynivalenol.

*Trichothecenes Appendix 2.**Quantitative methods for B-trichothecenes and particularly for deoxynivalenol<sup>a</sup>*

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Technique assayed	Toxins ng/g	LOD/LOQ levels	Spiking (%) (ng/g)	Recovery (%)	CV (%)
Veldman et al. (1992)	Feed	20	CH <sub>3</sub> CN/H <sub>2</sub> O	Celite/cahrcoal/ aluminium oxide	HPLC-UV	DON	100-500/NA	500-600	60-86	NA
Rajakylä et al. (1987)	Wheat, rye	20	CH <sub>3</sub> CN	Bond Elut	HPLC-MS	DON	250-1000/NA	NA	75	10
Zabe et al. (2000)	Food	NA	NA	Immunoaffinity columns	HPLC-NA	DON	NA	0-5	80	NA
Josephs et al. (1998)	Wheat	1	SFE	SFE	GC-ECD	DON	1600/5350	2350-16 750	53	9.6
Pineiro et al. (1996)	Liquid culture	50	MeOH	SPE-C18	HPLC-UV	DON	NA	NA	96	NA
Sano et al. (1987)	Maize, wheat, barley	20	CH <sub>3</sub> CN/H <sub>2</sub> O	Florisil and CN-Sep Pak	HPLC-FLD (methyl acetate)	DON NIV FX	20/NA	1000	> 70	NA
Schuhmacher et al. (1996)	Wheat	15	CH <sub>3</sub> CN/H <sub>2</sub> O	Mycosep	HPLC-DAD	DON	500/2000	1000-50 000	79	9.6
Berger et al. (1999)	Wheat	10	CH <sub>3</sub> CN/H <sub>2</sub> O	Mycosep 227, 216	LC-MS	DON FX 3-AcDON	6/50 3/40 3/25	NA	86 91 98	3.6 3.4 2.6

*Trichothecenes Appendix 2* (contd)

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Technique assayed	Toxins ng/g	LOD/LOQ levels	Spiking (%) (ng/g)	Recovery (%)	CV (%)
Cahill et al. (1999)	Wheat	50	H <sub>2</sub> O/PEG	DONtest	HPLC-UV	DON	100/NA	100-10 000	100-81	NA
Razzazi-Fazeli et al. (1999)	Wheat	25	CH <sub>3</sub> CN/H <sub>2</sub> O	Mycosep	HPLC-APCI-MS	DON NIV	NA/40 NA/50	50-500	86 70	3.5-7.7
Reutter (1999)	Cereals, feed	25	CH <sub>3</sub> CN/H <sub>2</sub> O	Charcoal/ alumina/ celite/immuno- affinity column	HPLC-UV	DON	NA/50 NA/100	NA	NA	NA
Mulders et al. (1986)	Cereals	25	CHCl <sub>3</sub> /EtOH	Silica gel	GC-ECD (HFB) GC-MS (HFB)	DON	20/NA	100	99	11
Scott & Kanhere (1986)	Cereals	50	CH <sub>3</sub> OH/H <sub>2</sub> O	Silica gel	GC-ECD (TMS)	DON	20/NA	200 1000	121 93 (barley)	NA
Tacke & Casper (1996)	Wheat, barley, malt	5	CH <sub>3</sub> CN/H <sub>2</sub> O	C <sub>18</sub> /alumina	GC-ECD (TMS)	DON	50/NA	500-20 000	94-100	
Weingärtner et al. (1997)	Wheat	25	CH <sub>3</sub> CN/H <sub>2</sub> O	MycoSep'	GC-ECD (TMS/ TBT)	DON 3-AcDON 15-AcDON FX NIV	35/123 41/143 35/123 35/124 33/117	100-2000	102 96 95 97 66	9.5-18

*Trichothecenes Appendix 2* (contd)

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Technique	Toxins assayed	LOD/LOQ ng/g	Spiking levels (ng/g)	Recovery (%)	CV (%)
Schollenberger et al. (1998)	Cereals, food, feed	10	CH <sub>3</sub> CN/H <sub>2</sub> O	Florisil/Bond Elut CBA	GC-MS (TFA)	DON FX 15-AcDON 3-AcDON NIV	7/23 6/21 7/22 5/16 12/38	50-500	83-98 82-120 73-108 88-110 71-107	NA
Croteau et al. (1994)	Maize	50	CH <sub>3</sub> CN/H <sub>2</sub> O, shake 2.5 h	Charcoal/ alumina	GC-ECD (DMAP, HFBA)	DON	NA/50	100 250 1000	73 108 81	NA
Adler et al. (1992)	Wheat	20	MeOH/ H <sub>2</sub> O	Silica gel/C <sub>18</sub>	GC-ECD (TMS)	DON	NA	NA	NA	NA
Blaas et al. (1984)	Cereals	20	MeOH/ H <sub>2</sub> O, hexane	Florisil	GC-MS (TMS)	DON NIV	10/NA	NA	NA	NA
Seidel et al. (1993)	Wheat, maize	10	MeOH/ H <sub>2</sub> O	Florisil/C <sub>18</sub>	GC-ECD (HFB)	DON	3.5/NA	NA	75-80	5
van Egmond (2000)	Wheat	25	CH <sub>3</sub> CN/H <sub>2</sub> O	Mycosep	GC-FID	DON NIV FX 3-AcDON	25/75	300	96 96 98 102	4.8 3.8 2.9 0.7
Young & Games (1993)	Liquid culture	NA	NA	NA	SFC-UV SFC-MS	DON 3-AcDON ZEN	150/NA 100/NA 250/NA	NA	NA	NA

**Trichothecenes Appendix 2 (contd)**

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Technique	Toxins assayed	LOD/LOQ ng/g	Spiking levels (ng/g)	Recovery (%)	CV (%)
Groves et al. (1999)	Maize	5	CH <sub>3</sub> CN/H <sub>2</sub> O	Aluminium oxide/C18	GC-MS (TMS)	DON NIV FX 3-AcDON 15-AcDON	500/NA	1000	80-100	NA
Petterson (1992)	Barley, oats, wheat	20	CH <sub>3</sub> CN/H <sub>2</sub> O	Charcoal/alumina	GC-ECD (TMS)	DON NIV	10/NA	NA	NA	NA
Stenwig et al. (1992)	Barley, oats	50	CH <sub>3</sub> CN/H <sub>2</sub> O	Charcoal/alumina/ celite	GC-ECD (TMSI)	DON	10/NA	NA	90	NA
Lauren & Agnew (1991)	Maize	20	CH <sub>3</sub> CN/ MeOH/H <sub>2</sub> O	Alumina/cation resins/celite GC-ECD (TFAA)	HPLC-UV	DON NIV	30/NA 20/NA (GC)	NA	55-70 73-103	NA
Scudamore et al. (1998)	Maize	30	CH <sub>3</sub> CN/4% KCl	Charcoal/alumina	GC-MS (TFAA)	DON NIV FX	10/NA 10/NA 10/NA	100	72-80	10-16
Mirocha et al. (1998)	Wheat, barley	25	CH <sub>3</sub> CN/H <sub>2</sub> O	C18/alumina	GC-MS (TMSI, TCMS)	DON	NA	1 000 5 000 10 000	97 88 87	NA
Liu et al. (1997)	Wheat, barley, oats		CH <sub>3</sub> CN/H <sub>2</sub> O	Charcoal/alumina/ celite	GC-ECD (TMSI)	DON	30/NA 3-AcDON NIV	NA 30/NA 50/NA	NA	11

*Trichothecenes Appendix 2* (contd)

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Technique	Toxins assayed	LOD/LOQ ng/g	Spiking levels (ng/g)	Recovery (%)	CV (%)
Park et al. (1991)	Cereals	50	CH <sub>3</sub> CN/H <sub>2</sub> O	Florisil	GC-MS (TMI/TMA/TMCS)	DON NIV	15/NA	1000	83 89	NA
Tanaka et al. (1986)	Wheat, barley	20	CH <sub>3</sub> CN/H <sub>2</sub> O	Florisil/C18	GC-ECD (TMS)	DON NIV	2/NA	NA	NA	NA
Swanson et al. (1986)	Milk	40 mL	Chem tube/ ethyl acetate	C18	GC-ECD (TMS, HFB)	DON	1 ng/mL/NA	2.5-10 ng/mL	82	10
Dahlem et al. (1986)	Urine	9 mL	Clin-Elut/ethyl acetate		GC-ECD (TMS)	DON	50/NA	200-5000 ng/mL	86	8.5
	Faeces	12 g	MeOH/H <sub>2</sub> O/ Clin-Elut	C18					87	10
Kiessling et al. (1984)	Rumen fluid	5 mL	MeOH/0.1% NaCl	C18	GC-FID	DON	2/NA	NA	43	NA
Scott et al. (1993)	Beer	50 mL	Chem-Elut/ ethyl acetate/ MeOH	C18	GC-MS (HFB)	DON NIV ZEN	1.5 ng/mL 0.3 ng/mL 2 ng/mL/NA	5-20 ng/mL	103 90 97	NA
Tanaka et al. (1990)	Cereals	50	CH <sub>3</sub> CN/H <sub>2</sub> O	Florisil	GC-ECD (TMS)	DON NIV ZEN	2/NA 2/NA 1/NA	300 300 500	88 88 91	1.9-11
Scott & Trucksess (1997)	Wheat, maize	NA	NA	Charcoal/C18	GC-MS (TMS, HFB)	DON NIV	7-100/NA	500-1000 500-1000	81-96 48-73	NA

*Trichothecenes Appendix 2* (contd)

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Technique	Toxins assayed	LOD/LOQ ng/g	Spiking levels (ng/g)	Recovery (%)	CV (%)
Scott et al. (1989)	Wheat	50	MeOH/ H <sub>2</sub> O	C18/alumina/ carbon	GC-ECD (HFB, TMS)	DON NIV	20-50/NA 200/NA	200-1000 200-1000	81-108 71-102	NA
Luo et al. (1990)	Wheat, maize	40	CH <sub>3</sub> CN/H <sub>2</sub> O	Florisil	GC-ECD (TMS)	DON NIV	5/NA 5/NA	200 200	97-103	NA
Möller & Gustavsson (1992)	Cereals	50	CH <sub>3</sub> CN/ EtOAc/H <sub>2</sub> O	Florisil-Sep Pak	GC-ECD (Tri-syl/ TBT)	DON NIV FX 3-AcDON	NA	25-250	86-111 72-110 84-95 91-106	NA
Onji et al. (1998)	Cereals	15	CH <sub>3</sub> CN/H <sub>2</sub> O	Bond Elut florisil	GC-MS (on-column)	DON FX 3-AcDON	100-500/NA	60	75 49 82	22 29 28
Krska et al. (1997)	Wheat	25	CH <sub>3</sub> CN/H <sub>2</sub> O	Mycosep	GC-ECD (Tri-syl/TBT)	DON	NA	100-2000	96	9.5
Krska (1998)	Wheat	25	CH <sub>3</sub> CN/H <sub>2</sub> O	Mycosep	GC-ECD (Tri-syl/TBT)	DON	40/NA	100-2000	96	9.5
Josephs (1999)	Wheat	25	CH <sub>3</sub> CN/H <sub>2</sub> O	Mycosep	GC-ECD (Tri-syl/ TBT)	DON NIV FX 3-AcDON 15-AcDON	28/57 41/82 28/56 23/47 23/46	257-2060 204-1438 280-2240 255-2040 280-2240	103 76 106 107 107	4.3-7.8

**Trichothecenes Appendix 2** (contd)

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Technique	Toxins assayed	LOD/LOQ ng/g	Spiking levels (ng/g)	Recovery (%)	CV (%)
Kotal et al. (1999)	Cereals	5	CH <sub>3</sub> CN/H <sub>2</sub> O	GPC (BioBeads S-X3)	GC-ECD (TFAA)	DON	NA/50	2000	88	NA
			CH <sub>3</sub> CN/			NIV	NA/40		87	
			MeOH			FX	NA/100		78	

<sup>a</sup> For definitions of abbreviations, see end of Trichothecenes Appendix 6.

<sup>b</sup> For references, see the monograph on deoxynivalenol.

*Trichothecenes Appendix 3.**Methods for screening for A-trichothecenes and particularly for T-2 and HT-2 toxins<sup>a</sup>*

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Technique	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	CV (%)
Romer (1986)	Foods, feeds		CH <sub>3</sub> CN/H <sub>2</sub> O	Charcoal/alumina	TLC	T-2 HT-2	500(TLC)/NA	850-1200	98	12 13
Kawamura et al. (1990)	Cereals	20	CH <sub>3</sub> CN/H <sub>2</sub> O	Florisil	icELISA	T-2	0.2/NA	NA	NA	NA
Märtlbauer et al. (1991)	Milk	NA	MeOH	None	ELISA	T-2	0.5/NA	NA	68-98	5-20
Barna-Vetro et al. (1997)	Cereals		89% CH <sub>3</sub> CN	None	ELISA	T-2	50/NA	NA	85	3-15
Yang & Luo (2000)	Wheat	20	CHCl <sub>3</sub> /EtOH	Charcoal/alumina	ELISA	T-2	NA	NA	NA	NA

From Hack (1994), Gendloff (1987), and Hunter (1985), with sensitivities of 5 pg/ml (0.25 pg/assay), 10 ng/ml (0.5 ng/assay), and 50 ng/assay, respectively. Park & Chu (1996) reported the efficiency of ELISA and RIA for the analysis of T-2 toxin and of 3-AcDON in naturally contaminated maize samples. A homogeneous competition inhibition immunoassay for T-2 toxin was reported by Ligler et al. (1987).

<sup>a</sup> For definitions of abbreviations, see end of Trichothecenes Appendix 6.

<sup>b</sup> For references, see monograph on T-2 and HT-2 toxins

*Trichothecenes Appendix 4.**Quantitative methods for A-trichothecenes and particularly for T-2 and HT-2 toxins<sup>a</sup>*

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Technique	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	CV (%)
Berger et al. (1999)	Wheat	10	CH <sub>3</sub> CN/H <sub>2</sub> O	Mycosep 227, 216	LC-MS	T-2 HT-2	3/60 1/10		87 90	4.6 1.7
Rajakylä et al. (1987)	Wheat, rye	20	CH <sub>3</sub> CN	Bond Elut	HPLC-MS	T-2 HT-2	250-1000/NA	NA	89 90	6.3-7.7
Möller & Gustavsson (1992)	Cereals	50	CH <sub>3</sub> CN/ EtOAc/H <sub>2</sub> O	Florisil-Sep Pak	GC-ECD (Tri-syl/ TBT)	T-2	NA HT-2	250-2500	76-93	NA 71-94
Rosen & Rosen (1983)	Maize	10	MeOH	Silica gel/C18	GC-MS	T-2 HT-2	20/50 5/20	111 46	80 91	14 8.7
Schollenberger et al. (1998)	Cereals, food, feed	10	CH <sub>3</sub> CN/H <sub>2</sub> O	Florisil/Bond Elut CBA	GC-MS (TFA)	T-2 HT-2	2/6 5/18	50-500	87-110 74-109	NA
Onji et al. (1998)	Cereals		CH <sub>3</sub> CN/H <sub>2</sub> O	Bond Elut florisil	GC-MS (on-column)	T-2	100-500/NA	60	98	22
Croteau et al. (1994)	Maize	50	CH <sub>3</sub> CN/H <sub>2</sub> O, shake 2.5 h	Charcoal/ alumina	GC-ECD (DMAP, HFBA)	T-2 HT-2	NA/100 NA/50	100 250 1000	70/121 NA/95 83/99	NA
Plattner et al. (1989)	Cultures		Ethyl acetate	charcoal	GC-MS (Tri-syl/TBT)	T-2	NA	NA	NA	NA
Scott & Kanhere (1986)	Standards	NA	NA	NA	GC-ECD (TMS, HFB)	T-2 HT-2	NA	NA	NA	NA

**Trichothecenes Appendix 4** (contd)

Reference	Matrix	Amount of sample (g)	Extraction	Clean-up	Technique	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	CV (%)
Groves et al. (1999)	Maize	5	CH <sub>3</sub> CN/H <sub>2</sub> O	Aluminium oxide/C18	GC-MS (TMS)	T-2 HT-2	500/NA	1000	80-100	NA
Petterson (1992)	Barley, oats, wheat	20	CH <sub>3</sub> CN/H <sub>2</sub> O	Charcoal/alumina	GC-ECD (TMS)	T-2 HT-2	50/NA 10/NA	NA	NA	NA
Scudamore et al. (1998)	Maize	30	CH <sub>3</sub> CN/4 KCL	Charcoal/alumina	GC-MS (TFAA)	T-2 HT-2 DAS	10/NA 10/NA 10/NA	100 100 100	0-103 21-53 64-73	20-38
Hietaniemi & Kumpulainen (1991)	Cereals, feed	20	MeOH/ H <sub>2</sub> O	Silica gel/ Florisil/C18	GC-MSD (TMSI)	T-2	15/NA HT-2	NA	72-130	NA
Park et al. (1991)	Cereals	50	CH <sub>3</sub> CN/H <sub>2</sub> O	Florisil	GC-MS (TMI/TMA/TMCS)	T-2	15/NA	1000	83	NA
Kiessling et al. (1984)	Rumen fluid	5 mL	MeOH/0.1% NaCl	C18	GC-FID (?)	T-2 HT-2	10/NA 5/NA	NA NA	67 NA	NA
Scott (1997)	Wheat, maize	NA	NA	Charcoal/C18	GC-MS (TMS, HFB)	T-2 HT-2	NA	4000 1200-2400	94 88-93	NA
Scott et al. (1989)	Wheat	50	MeOH/ H <sub>2</sub> O	C18/alumina/ carbon	GC-ECD (HFB, TMS)	T-2 HT-2	400/NA 100-200/NA	200-4000 200-4000	66-91 72-110	NA

**Trichothecenes Appendix 4 (contd)**

Reference	Matrix	Amount of sample (g)	Extraction	Clean-up	Technique	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	CV (%)
Luo et al. (1990)	Wheat, maize	40	CH <sub>3</sub> CN/H <sub>2</sub> O	Florisil	GC-ECD (TMS)	T-2 HT-2	100/NA 100/NA	200 200	97-103	NA
Josephs (1999)	Wheat	25	CH <sub>3</sub> CN/H <sub>2</sub> O	Mycosep	GC-ECD (HFBI)	T-2 HT-2	10/37 10/38	51-1027 52-1047	100 117	2.2
Kotal et al. (1999)	Cereals	5	CH <sub>3</sub> CN/H <sub>2</sub> O CH <sub>3</sub> CN/MeOH	GPC (Bio Beads S-X3)	GC-ECD (TFAA)	T-2 HT-2	NA/200 NA/100	2000	91 105	NA

<sup>a</sup> For definitions of abbreviations, see end of Trichothecenes Appendix 6.

<sup>b</sup> For references, see monograph on T-2 and HT-2 toxins

**Trichothecenes Appendix 5.**

**Analytical methods used in surveys summarized in Appendix A of the monograph on deoxynivalenol and Appendices A and B of the monograph on T-2 and HT-2 toxins<sup>a</sup>**

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Method of quantification	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	RSD <sub>r</sub>	RSD <sub>R</sub>
Abouzied et al. (1991)	Grain foods	25	CH <sub>3</sub> CN:H <sub>2</sub> O 9:1		ELISA	DON	1000				
Ali et al. (1998)	Maize	20	CH <sub>3</sub> CN:H <sub>2</sub> O 3:1 100 ml	Florisil column	GC-MS TMS derivatives	DON NIV	10/NA 10/NA				
Eskola et al. (2000a,b)	Cereals			Mycosep 227 column	GC-MS TMS derivatives	DON 3-AcDON NIV FX DAS HT-2 T-2	NA/10    NA/20 NA/20				
FAO/WHO (2000)	Cereals  Cereal products	20	H <sub>2</sub> O 8 ml  Chloroform+ ethanol (8+2) 100 ml	Petroleum ether:ethanol: H <sub>2</sub> O	TLC						
Fernández et al. (1994)( modified from Truck- sess et al., 1984)	Wheat	50	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16 200 ml	Charcoal/celite/ alumina column	HPTLC Chloroform: acetone:2- propanol 8:1:1	DON	40/NA	200 400 800	83 82 72		

*Trichothecenes Appendix 5 (contd)*

Reference <sup>b</sup> Matrix	Amount of sample (g)	Extraction	Clean-up	Method of quantification	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	RSD <sub>r</sub>	RSD <sub>R</sub>
Furlong & Valente Soares (1995)	Wheat, maize, wheat products	Methanol:4% KCl 9:1	Chloroform Charcoal/celite/ alumina column	GC-FID HFB derivatives	DON	200/NA		85		
					NIV	100/NA		87		
					DAS	100/NA		83		
					T-2	100/NA		87		
					HT-2	500/NA		91		
					T-2 triol	300/NA				
				T-2 tetrol	400/NA					
Golinski et al. (1996)	Wheat	20	Tanaka et al. (1985)	Tanaka et al. (1985)	HPLC C-R4A C-18 column UV (229 nm) methanol water 20%	DON 10/NA NIV				
Groves et al. (1999)	Maize, maize products	5	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, 20 ml shake 60 min	Mixture of neutral aluminum oxide and C-18 packing material	GC-MS TMS derivatives	17 toxins 500/NA	1000	80-100		
Hietaniemi & Kumpulainen (1991)	Grains, feeds	20	CH <sub>3</sub> CN:H <sub>2</sub> O 95:5, 100 ml, homogenize 5 min	Hexane isobutanol Silica gel column Florisil or Sep Pak C18 column	GC-MS TMS derivatives	DON 5 DAS 5 3-AcDON 5 FX 5 NIV 5 T-2 15 HT-2 15		72-128 (mean, 100)		

*Trichothecenes Appendix 5 (contd)*

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Method of quantification	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	RSD <sub>r</sub>	RSD <sub>R</sub>	
Home Grown Cereals Authority (1999, 2000)	Wheat	20	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, 100 mL shake 120 min	Mycosep No. 225 column	GC-MS TMS derivatives	DON	20/NA					
						T-2	20/NA					
						HT-2	20/NA					
Kamimura et al. (1981)	Maize	100	CH <sub>3</sub> OH:H <sub>2</sub> O 95:5, 200 mL shake 30 min	1-Amberlite XAD-4 column 2-Florisil	TLC Developing solvents: 1-CHCl <sub>3</sub> : methanol 93:7 2-Toluene: acetone: methanol 5:3:2	DON	20-50/NA	1000	93			
						T-2	100-500/ NA	2000	83			
						HT-2	100-500/ NA	2000	92			
						NIV	20-50/NA	1000	82			
						FX	20-50/NA	1000	90			
						ZEN	10-50/NA	2000	88			
						DAS	100-500/ NA	2000	102			
						NEO	100-500/ NA	2000	108			
						Buteno- lide	100-500/ NA	2000	92			
						Monili- form	200-500/ NA	2000	76			
Kim et al. (1993) modified from Tanaka, 1985)	Barley, maize	40	CH <sub>3</sub> CN:H <sub>2</sub> O 3:1, 160 ml 30 min	Hexane Florisil column	GC-MS TMS derivatives	DON	5	200	92-105 (barley) 87-95 (maize)			
						3-AcDON	5					
						15-AcDON	5					
						NIV	5					
						4-ANIV	5					

**Trichothecenes Appendix 5 (contd)**

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Method of quantification	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	RSD <sub>r</sub>	RSD <sub>R</sub>
Langseth (2000a,b)	Cereals	25	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, homogenize	Mycosep 225	GC-MS PFFPA derivatives	DON	20-30	400	90		
						3-AcDON		400	72		
						NIV		400	90		
						FX		400	91		
						T-2	30	400			
HT-2	20	400									
						Scirpentriol					
						MAS					
						DAS					
Langseth & Claasen (1992)	Cereals	25	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, homogenize	Charcoal/celite/alumina column	GC-ECD TMS derivatives	DON	10/30	400	80-110		
						3-AcDON		400			
						NIV		400			
						FX		400			
Langseth & Elen (1996)	Cereals	25	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, homogenize	Charcoal/celite/alumina column, hexane, silica cartridge	GC-ECD TMS derivatives						
Lauren & Agnew (1991)	Grains	20	CH <sub>3</sub> CN:CH <sub>3</sub> OH:H <sub>2</sub> O, 80:5:15 100 ml, 2 h	Alumina-carbon cation exchange resin, hydrolysis	HPLC C-8 GC-ECD TFAA derivatives	DON	10*				
Lew et al. (2000)	Maize	25	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, 100 mL shake 80 min	Mycosep No. 227 column	GC-ECD Type B, TMS derivatives Type A, HFBI derivatives	DON NIV 3-AcDON 15-AcDON	50 50 50 50				

*Trichothecenes Appendix 5 (contd)*

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Method of quantification	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	RSD <sub>r</sub>	RSD <sub>R</sub>
Luo et al. (1990)	Maize, wheat	40	CH <sub>3</sub> CN:H <sub>2</sub> O 3:1, 160 ml shake 30 min	Hexane Florisil column	GC-MS TMS derivatives	DON	5		97-103		
						NIV	5		97-104		
						T-2	100		97-105		
						15-AcDON	100		97-106		
						HT-2	100		97-107		
Mallman (2000)	Cereals	50	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, 100 mL	Mycosep No. 224, automatic	HPLC C-18	DON	100				
Marasas et al. (1979)	Maize	50	Hexane, 100 mL methanol:1% KCl, 55:45 200 mL	Hexane chloroform	GC-MS TMS derivatives	DON	100/NA		60		
						DAS	100/NA		90		
						T-2	100/NA		87		
Marx et al. (1995)		20	CH <sub>3</sub> CN:H <sub>2</sub> O 3:1, 200 mL	Hexane Florisil Sep-pak	HPTLC	DON	50				
Möller & Gustavsson (1992)	Cereals	50	Ethyl acetate: acetonitrile 4:1 250 ml + 10 ml water, shake 30 min	Hexane Sep Pak Florisil cartridge	GC-ECD TMS derivatives	DON		250	89		
						FX		250	87		
						NIV		250	72		
						3-AcDON		250	93		
						DAS		250	89		
						Tricho- thecins		250	115		
						NEO		250	97		
						HT-2		250	94		
						T-2		250	84		

**Tricothecenes Appendix 5 (contd)**

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Method of quantification	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	RSD <sub>r</sub>	RSD <sub>R</sub>
Möller & Gustavsson (1992)	Cereals	50	Ethyl acetate: acetonitrile 4:1 250 ml + 10 ml water, shake 30 min	Hexane Sep Pak Florisil cartridge	GC-ECD TMS derivatives	DON	250	250	86		
						FX			84		
						NIV			68		
						3-AcDON			89		
						DAS			105		
						Tricho- thecenes			81		
						NEO			93		
						HT-2			77		
T-2	79										
Neira et al. (1997)	Wheat products	Pacin et al. (1997)	Pacin et al. (1997)	Pacin et al. (1997)	GC-ECD HFB derivatives	DON	6/17				
Niessen et al. (1993)	Beer	20 ml			ELISA	DON	50 ng/ml	50	45		
								500	77		
								1000	83		
Pacin et al. (1997) (modified Romer® MY 8402s)	Wheat products (with fat)	25	1- <i>n</i> -hexane, 2-acetonitrile:H <sub>2</sub> O 84:16, 100 mL blend 3 min	Mycosep No. 225	TLC	DON	25/48	100	102		
								250	87		
								500	92.4		
Park et al. (1992)	Barley	Luo et al. (1990)	Luo et al. (1990)	Florisil silica cartridge	GC-ECD TMS derivatives	DON NIV	2		88–90		

**Trichothecenes Appendix 5 (contd)**

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Method of quantification	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	RSD <sub>r</sub>	RSD <sub>R</sub>
Pettersson (1993)	Barley, oats, wheat	20	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16	Charcoal/alumina column	GC-ECD TMS derivatives	DON NIV T-2 HT-2 DAS 3-AcDON	10  20-50 10/30				
Romer Labs Inc. (1994); Lops et al. (1998)	Wheat	25	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, 100 ml blend 3 min	Mycosep No. 224	TLC, toluene: ethyl acetate: formic acetate 5:4:1	DON	500/NA		> 85		
Ryu et al. (1996)	Barley, maize	20	CH <sub>3</sub> CN:H <sub>2</sub> O 3:1, 200 ml shake 30 min	Hexane Florisil column	GC-MS TMS derivatives	DON T-2 HT-2 NIV FX 3-AcDON	5 5 5 5 5 5		83.8 88.2 91.3 91.8 90.9 87.9		
Schollenberger et al. (1998)	Cereals, cereal foods	10	Tanaka et al. (1985)	Hexane Florisil cation exchange cartridge	GC-MS TFAA derivatives	DON T-2 HT-2 NIV FUS 15-AcDON 3-AcDON DAS	7/23 2/6 5/18 12/38 6/21 7/22 5/16 7/22	50-500 50-500 50-500 50-500 50-500 50-500 50-500 50-500	83-98 87-110 74-109 71-107 82-120 73-108 88-110 82-116		

**Trichothecenes Appendix 5 (contd)**

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Method of quantification	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	RSD <sub>r</sub> , RSD <sub>R</sub>
Schwadorf & Müller (1991)	Barley, oats, wheat	20	Tanaka et al. (1985)	Tanaka et al. (1985)	GC-MS TFA derivatives	NIV	15		84.7	
						DON	15		89.1	
						3Ac-DON	15		81.3	
						FX	15			
						HT-2	15		79.6	
						T-2	15		80.2	
						DAS	15		77.8	
15-AcDON	15		83.2							
Scott et al. (1981)	Cereals	50	Methanol:H <sub>2</sub> O 1+1, 250 mL	Partition: aqueous ammonium sulfate 30%/ ethyl acetate, silica gel column	GC-ECD HFBI derivatives	DON	10	100 (wheat flour) 1000 (wheat flour) 100 (wheat) 500 (wheat) 1000 (wheat)	72, 73, 86, 70 85, 59, 78, 68 101, 102 77, 88 78, 86	
Scott et al. (1986)	Cereals	50	Methanol:H <sub>2</sub> O 7:3, 150 ml	Partition: aqueous ammonium sulfate 30%/ ethyl acetate, silica gel column	GC-ECD TMS derivatives	DON NIV	150/NA			
Scott et al. (1989)	Cereals	50	Methanol:H <sub>2</sub> O 7:3, 150 mL	Partition: aqueous ammonium sulfate 30%/ ethyl acetate, silica gel column	GC-ECD TMS derivatives: DON and NIV HFBI derivatives: other trichothecenes	DON NIV T-2 HT-2 DAS	20/50 200 200/400 50/200 200	800 (wheat) 4000 (wheat) 2000 (wheat)	81 86 82	

**Trichothecenes Appendix 5 (contd)**

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Method of quantification	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	RSD <sub>r</sub> , RSD <sub>R</sub>
Scott et al. (1993)	Beer	10	Chem Elut CE 1020, Varian	C-18 SPE (Supelclean, ) Supelco	GC-MS HFB derivatives	DON NIV	0.1-1.5 0.01-0.3	5-20 ng/ml	103	
Solovey et al. (1999) (modified Romer® MY 8402s)	Maize, wheat, maize and wheat products	25	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, 100 mL blend 3 min	Mycosep No. 225	TLC	DON	25/48	100 300	82 86	
Spanjer (2000)	Cereals, cereal products	25	PEG:H <sub>2</sub> O 5 g/400 ml	immunoaffinity column DON test™ HPLC (VICAM)	HPLC-UV RP18	DON	16/32	146-1070	92-103	13
Stratton et al. (1993)	Wheat, barley	50	Acetonitrile:H <sub>2</sub> O 21:4, 250 ml shake 2 h	<i>n</i> -Hexane LC-NH <sub>2</sub> solid phase extraction column Charcoal/celite/ alumina column	HPLC C-18 column UV	T-2 DON HT-2 DAS	150/NA 50/NA 120/NA 70/NA	400 (wheat) 100 (wheat) 400 (wheat)	84.7 89.8 84.9	
Sydenham & Thiel (1987)	Maize, oats	50	Methanol:water 1:1, shake 30 min	Extrelut 20 column Silica gel cartridge	GC-ECD HFBI derivatives	T-2 DAS	100/NA 100/NA		88.0 88.2	5 4.4

**Trichothecenes Appendix 5 (contd)**

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Method of quantification	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	RSD <sub>r</sub>	RSD <sub>R</sub>
Tanaka et al. (1985)	cereal	20	CH <sub>3</sub> CN:H <sub>2</sub> O 3:1, 200 ml shake 30 min	Hexane Florisil Sep-pak	GC-ECD TMS derivatives	DON NIV	2/NA 2/NA	300 300	87		
									86		
Trucksess et al. (1984); Eppley et al. (1986)	Wheat, maize	50	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, 200 mL	Charcoal/celite/ alumina column	TLC chloroform: acetone:2- propanol 8:1:1	DON	50/NA		77-93	50 ppb, 45	87
										300 ppb, 39	40
										1000 ppb, 64	64
Trucksess et al. (1987)	Wheat	50	Acetonitrile: H <sub>2</sub> O:methanol 70:25:5, 200 mL shake 60 min	Lead acetate Charcoal/celite/ alumina column Octadecylsilane column	HPTLC 1-chloroform: acetone:2- propanol 8:1:1 2-chloroform: acetone:2- propanol 7:1.5:1.5	DON NIV FX	50	50 100 200	61.3 85 81.5		
Trucksess et al. (1996)	White flour	25	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, 100 mL blend 3 min or shake 30 min	Mycosep No. 225 column	HPLC Novapack C <sub>18</sub> column	DON	20/500	500 1000 2000	95 84 85	7.52 5.72 3.14	11.46 18.98 12.72

**Trichothecenes Appendix 5 (contd)**

Reference <sup>b</sup> Matrix	Amount of sample (g)	Extraction	Clean-up	Method of quantification	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	RSD <sub>r</sub>	RSD <sub>R</sub>
Tutelyan et al. (1990)	Wheat	25	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, 125 mL	Charcoal/alumina column	HPLC, silica gel column, UV (224 nm) mobile phase, hexane:isopropanol: H <sub>2</sub> O 75:25:1.5	DON	50/NA	500	90	
Vega et al. (1994) (Romer method®)	Wheat	25	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, 100 mL blend 3 min	Mycosep No. 227	HPTLC tolueno:ethyl acetate: formyl acetate 6:3:1	DON DAS HT-2 T-2	10			
Veratox® (1998); Trucksess et al. (1995)	Wheat, barley	50	H <sub>2</sub> O, 250 ml blend 2 min		ELISA (Veratox method)	DON	300/500	1000 2000 4000 1000 2000 4000	65 70 78 40 70 68	
Vicam® (1996); Lops et al. (1998)	Wheat	25	Polyethylene-glycol 5 g/400 mL H <sub>2</sub> O	Immunoaffinity column, DON test™ HPLC (VICAM)	HPLC Supelcosil LC-18 DAD 220 nm acetonitrile: H <sub>2</sub> O 10:90	DON	50	500 1000 2000	75 84 85	

**Trichothecenes Appendix 5 (contd)**

Reference <sup>b</sup>	Matrix	Amount of sample (g)	Extraction	Clean-up	Method of quantification	Toxins assayed	LOD/LOQ (ng/g)	Spiking levels (ng/g)	Recovery (%)	RSD <sub>r</sub>	RSD <sub>R</sub>
Vrabcheva et al. (1996)	Wheat	2	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, 10 mL stir 120 min		ELISA (Ridascreen DON method) acetylate toxin before assay	DON	50		70–110		
		2	Methanol:H <sub>2</sub> O 70:30, 8 mL, stir 60 min CH <sub>3</sub> OH:PBS 70:30, stir 30 min			3-AcDON 15-AcDON	≥ 40				
Weingärtner et al. (1997)	Wheat	25	CH <sub>3</sub> CN:H <sub>2</sub> O 84:16, 100 mL shake 90 min	Mycosep No. 225 column derivatives	GC–ECD TMS	DON NIV 3-AcDON 15-AcDON FX	36/124 33/144 41/123 35/124 35/117	DON 100 DON 1000 DON 2000	98 80 81		3.95
Yang et al. (1992)	Cereals, cereal products	20	H <sub>2</sub> O, 8 ml Chloroform + ethanol (8 + 2) 100 ml	Charcoal/ alumina column	ELISA	T-2					

\* Limit of detection

<sup>a</sup> For definitions of abbreviations, see end of Trichothecenes Appendix 6.

<sup>b</sup> For references, see monographs on deoxynivalenol and T-2 and HT-2 toxins

*Trichothecenes Appendix 6.*

*Sampling methods used in surveys summarized in Appendix A of the monograph on deoxynivalenol and Appendices A and B of the monograph on T-2 and HT-2 toxins*

Reference <sup>a</sup>	Grain	No. of samples	Site of collection	Lot size	Method of collection	Sample size (kg)				
						Sample	Combined first sub-sample	Combined second sub-sample	Laboratory sample	Analysed sample
Apro et al. (1987)	All	Proportional to total production of each area			Portions ≤ 50 g Automatic sampling	Small grains (wheat, sorghum, oat, maize) = 10; peanut, soya bean, sunflower = 50; cotton = 60	3 (wheat) 5 (maize)	1 (wheat) 2 (maize)	0.2	0.025
Eskola (2000a,b)	Cereals		Mills and malt houses	4–1000 t	Industry: Automatic sampling equipment: 100–200 g every 50–1000 kg Farmers: Grain handling equipment. Several subsamples	3–100  0.5–1			0.5–1	
Fonseca (1991)	Peanuts, maize, rice, beans	Proportional to size of lot	Bags or in bulk	20 t	Minimum no. of bags or points (bulk) to be sampled is a function (square root) of the lot size, portions of 200 g	Homogenized and quartered into 4-kg samples				

*Trichothecenes Appendix 6* (contd)

Reference <sup>a</sup>	Grain	No. of samples	Site of collection	Lot size	Method of collection	Sample size (kg)				
						Sample	Combined first sub-sample	Combined second sub-sample	Laboratory sample	Analysed sample
Langseth & Elen (1997)	Barley, oats, wheat	Proportional to production and level of <i>Fusarium</i> infection	Grain loads, silos		Grain loads and silos: Automatic sampling equipment Farmers: Grain handling equipment				0.3–0.8	0.025
Olavarría (1992)	Maize, wheat, rice	Proportional to total production of each area	Harvest	8–12 t truck	Grain handling equipment (metal probe, 130 cm); 12–15 portions ≤ 300 g	5–6				0.025
Quiroga et al. (1995)	Wheat	Proportional to total production of each area	Harvest	max 2000 ha	Portions ≤ 50 g Automatic sampling or grain-handling equipment	10 Homogenized and quartered	3 14-mesh sieve	1 20-mesh sieve	0.2	0.025
Resnik & Pacin (2000)	Wheat, maize	Proportional to total production of each area	Harvest	max 2000 ha	Portions ≤ 50 g Automatic sampling or grain-handling equipment	10 Homogenized and quartered	3 (wheat) 5 (maize) 14-mesh sieve Romer mill			0.05

**Trichothecenes Appendix 6 (contd)**

Reference <sup>a</sup>	Grain	No. of samples	Site of collection	Lot size	Method of collection	Sample size (kg)				
						Sample	Combined first sub-sample	Combined second sub-sample	Laboratory sample	Analysed sample
Spanjer (2000)	Wheat, rye, buck-wheat, maize, rice, oats, millet, barley, grain products	Imported and domestic	Mills, silos, retail shops		Automatic and hand sampling	3–5 kg for grains	Slurry prepared with water in ratio 2:3		0.0625 of slurry	0.025

Abbreviations for Trichothecenes Appendixes 1–6: AcDON, acetylated deoxynivalenol; 4-ANIV, 4-acetylnivalenol; APCI, atmospheric pressure chemical ionization; CBA, carboxylic acid; CD, competitive direct; DAD, diode array detection; DAS, diacetoxyscirpenol; DMAP, 4-dimethylaminopyridine; DON, deoxynivalenol; ECD, electron capture detection; ELISA, enzyme-linked immunosorbent assay; FID, flame ionization detection; FLD, fluorimetric detection; FX, fusarenon X; GC, gas chromatography; GPC, gel permeation chromatography; HFB, heptafluorobutanoyl; HFBA, heptafluorobutyric anhydride; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; ic, intracerebrally; Ig, immunoglobulin; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; MAS, monoacetoxyscirpenol; MS, mass spectroscopy; NEO, neosolaniol; NIV, nivalenol; NA, not available; PEG, polyethylene glycol; RSD<sub>r</sub>, relative standard deviation for repeatability; RSD<sub>R</sub>, relative standard deviation for reproducibility; SFC, supercritical fluid chromatography; SFE, supercritical fluid extraction; SPE, solid-phase extraction; TBT, tributyl tin; TCMS, trichloromethylsilane; TFA, trifluoroacetic acid; TFAA, trifluoroacetic anhydride; TLC, thin-layer chromatography; TMA, trimethylaluminium; TMCS, trimethylchlorosilane; TMI, trimethylindium; TMS, trimethylsilyl; TMSI, trimethylsilylimidazole; UV, ultra-violet radiation; ZEN, zearalenone

<sup>a</sup> For references, see monographs on deoxynivalenol and T-2 and HT-2 toxins

## **ANNEXES**



## ANNEX 1

### **REPORTS AND OTHER DOCUMENTS RESULTING FROM PREVIOUS MEETINGS OF THE JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES**

1. **General principles governing the use of food additives** (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
2. **Procedures for the testing of intentional food additives to establish their safety for use** (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).
3. **Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants)** (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as **Specifications for identity and purity of food additives**, Vol. I. **Antimicrobial preservatives and antioxidants**, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).
4. **Specifications for identity and purity of food additives (food colours)** (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as **Specifications for identity and purity of food additives**, Vol. II. **Food colours**, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).
5. **Evaluation of the carcinogenic hazards of food additives** (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).
6. **Evaluation of the toxicity of a number of antimicrobials and antioxidants** (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).
7. **Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents** (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).
8. **Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants** (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).
9. **Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants**. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).
10. **Specifications for identity and purity and toxicological evaluation of food colours**. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.

11. **Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases** (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).
12. **Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases.** FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29.
13. **Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances** (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.
14. **Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non nutritive sweetening agents** (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.
15. **Toxicological evaluation of some flavouring substances and non nutritive sweetening agents.** FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.
16. **Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents.** FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.
17. **Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics** (Twelfth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.
18. **Specifications for the identity and purity of some antibiotics.** FAO Nutrition Meetings Series, No. 45A, 1969; WHO/Food Add/69.34.
19. **Specifications for the identity and purity of food additives and their toxicological evaluation: some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances** (Thirteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 46, 1970; WHO Technical Report Series, No. 445, 1970.
20. **Toxicological evaluation of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances.** FAO Nutrition Meetings Report Series, No. 46A, 1970; WHO/Food Add/70.36.
21. **Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives.** FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.
22. **Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and certain other substances; and a review of the technological efficacy of some antimicrobial agents.** (Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.
23. **Toxicological evaluation of some extraction solvents and certain other substances.** FAO Nutrition Meetings Report Series, No. 48A, 1971; WHO/Food Add/70.39.
24. **Specifications for the identity and purity of some extraction solvents and certain other substances.** FAO Nutrition Meetings Report Series, No. 48B, 1971; WHO/Food Add/70.40.

25. **A review of the technological efficacy of some antimicrobial agents.** FAO Nutrition Meetings Report Series, No. 48C, 1971; WHO/Food Add/70.41.
26. **Evaluation of food additives: some enzymes, modified starches, and certain other substances: Toxicological evaluations and specifications and a review of the technological efficacy of some antioxidants** (Fifteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 50, 1972; WHO Technical Report Series, No. 488, 1972.
27. **Toxicological evaluation of some enzymes, modified starches, and certain other substances.** FAO Nutrition Meetings Report Series, No. 50A, 1972; WHO Food Additives Series, No. 1, 1972.
28. **Specifications for the identity and purity of some enzymes and certain other substances.** FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.
29. **A review of the technological efficacy of some antioxidants and synergists.** FAO Nutrition Meetings Report Series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.
30. **Evaluation of certain food additives and the contaminants mercury, lead, and cadmium** (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.
31. **Evaluation of mercury, lead, cadmium and the food additives amaranth, diethylpyrocarbamate, and octyl gallate.** FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.
32. **Toxicological evaluation of certain food additives with a review of general principles and of specifications** (Seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 53, 1974; WHO Technical Report Series, No. 539, 1974, and corrigendum (out of print).
33. **Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers, and thickening agents.** FAO Nutrition Meetings Report Series, No. 53A, 1974; WHO Food Additives Series, No. 5, 1974.
34. **Specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers.** FAO Food and Nutrition Paper, No. 4, 1978.
35. **Evaluation of certain food additives** (Eighteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 54, 1974; WHO Technical Report Series, No. 557, 1974, and corrigendum.
36. **Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain other food additives.** FAO Nutrition Meetings Report Series, No. 54A, 1975; WHO Food Additives Series, No. 6, 1975.
37. **Specifications for the identity and purity of some food colours, enhancers, thickening agents, and certain food additives.** FAO Nutrition Meetings Report Series, No. 54B, 1975; WHO Food Additives Series, No. 7, 1975.
38. **Evaluation of certain food additives: some food colours, thickening agents, smoke condensates, and certain other substances.** (Nineteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 55, 1975; WHO Technical Report Series, No. 576, 1975.
39. **Toxicological evaluation of some food colours, thickening agents, and certain other substances.** FAO Nutrition Meetings Report Series, No. 55A, 1975; WHO Food Additives Series, No. 8, 1975.

40. **Specifications for the identity and purity of certain food additives.** FAO Nutrition Meetings Report Series, No. 55B, 1976; WHO Food Additives Series, No. 9, 1976.
41. **Evaluation of certain food additives** (Twentieth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Food and Nutrition Meetings Series, No. 1, 1976; WHO Technical Report Series, No. 599, 1976.
42. **Toxicological evaluation of certain food additives.** WHO Food Additives Series, No. 10, 1976.
43. **Specifications for the identity and purity of some food additives.** FAO Food and Nutrition Series, No. 1B, 1977; WHO Food Additives Series, No. 11, 1977.
44. **Evaluation of certain food additives** (Twenty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 617, 1978.
45. **Summary of toxicological data of certain food additives.** WHO Food Additives Series, No. 12, 1977.
46. **Specifications for identity and purity of some food additives, including antioxidant, food colours, thickeners, and others.** FAO Nutrition Meetings Report Series, No. 57, 1977.
47. **Evaluation of certain food additives and contaminants** (Twenty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 631, 1978.
48. **Summary of toxicological data of certain food additives and contaminants.** WHO Food Additives Series, No. 13, 1978.
49. **Specifications for the identity and purity of certain food additives.** FAO Food and Nutrition Paper, No. 7, 1978.
50. **Evaluation of certain food additives** (Twenty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 648, 1980, and corrigenda.
51. **Toxicological evaluation of certain food additives.** WHO Food Additives Series, No. 14, 1980.
52. **Specifications for identity and purity of food colours, flavouring agents, and other food additives.** FAO Food and Nutrition Paper, No. 12, 1979.
53. **Evaluation of certain food additives** (Twenty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 653, 1980.
54. **Toxicological evaluation of certain food additives.** WHO Food Additives Series, No. 15, 1980.
55. **Specifications for identity and purity of food additives (sweetening agents, emulsifying agents, and other food additives).** FAO Food and Nutrition Paper, No. 17, 1980.
56. **Evaluation of certain food additives** (Twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 669, 1981.
57. **Toxicological evaluation of certain food additives.** WHO Food Additives Series, No. 16, 1981.
58. **Specifications for identity and purity of food additives (carrier solvents, emulsifiers and stabilizers, enzyme preparations, flavouring agents, food colours, sweetening agents, and other food additives).** FAO Food and Nutrition Paper, No. 19, 1981.
59. **Evaluation of certain food additives and contaminants** (Twenty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 683, 1982.
60. **Toxicological evaluation of certain food additives.** WHO Food Additives Series, No. 17, 1982.

61. **Specifications for the identity and purity of certain food additives.** FAO Food and Nutrition Paper, No. 25, 1982.
62. **Evaluation of certain food additives and contaminants** (Twenty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 696, 1983, and corrigenda.
63. **Toxicological evaluation of certain food additives and contaminants.** WHO Food Additives Series, No. 18, 1983.
64. **Specifications for the identity and purity of certain food additives.** FAO Food and Nutrition Paper, No. 28, 1983.
65. **Guide to specifications General notices, general methods, identification tests, test solutions, and other reference materials.** FAO Food and Nutrition Paper, No. 5, Rev. 1, 1983.
66. **Evaluation of certain food additives and contaminants** (Twenty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 710, 1984, and corrigendum.
67. **Toxicological evaluation of certain food additives and contaminants.** WHO Food Additives Series, No. 19, 1984.
68. **Specifications for the identity and purity of food colours.** FAO Food and Nutrition Paper, No. 31/1, 1984.
69. **Specifications for the identity and purity of food additives.** FAO Food and Nutrition Paper, No. 31/2, 1984.
70. **Evaluation of certain food additives and contaminants** (Twenty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 733, 1986, and corrigendum.
71. **Specifications for the identity and purity of certain food additives.** FAO Food and Nutrition Paper, No. 34, 1986.
72. **Toxicological evaluation of certain food additives and contaminants.** WHO Food Additives Series, No. 20. Cambridge University Press, 1987.
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74. **Toxicological evaluation of certain food additives and contaminants.** WHO Food Additives Series, No. 21. Cambridge University Press, 1987.
75. **Specifications for the identity and purity of certain food additives.** FAO Food and Nutrition Paper, No. 37, 1986.
76. **Principles for the safety assessment of food additives and contaminants in food.** WHO Environmental Health Criteria, No. 70. Geneva, World Health Organization, 1987 (out of print). The full text is available electronically at [www.who.int/pes](http://www.who.int/pes).
77. **Evaluation of certain food additives and contaminants** (Thirty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 759, 1987 and corrigendum.
78. **Toxicological evaluation of certain food additives.** WHO Food Additives Series, No. 22. Cambridge University Press, 1988.
79. **Specifications for the identity and purity of certain food additives.** FAO Food and Nutrition Paper, No. 38, 1988.
80. **Evaluation of certain veterinary drug residues in food** (Thirty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 763, 1988.
81. **Toxicological evaluation of certain veterinary drug residues in food.** WHO Food Additives Series, No. 23. Cambridge University Press, 1988.
82. **Residues of some veterinary drugs in animals and foods.** FAO Food and Nutrition Paper, No. 41, 1988.

83. **Evaluation of certain food additives and contaminants** (Thirty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 776, 1989.
84. **Toxicological evaluation of certain food additives and contaminants**. WHO Food Additives Series, No. 24. Cambridge University Press, 1989.
85. **Evaluation of certain veterinary drug residues in food** (Thirty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 788, 1989.
86. **Toxicological evaluation of certain veterinary drug residues in food**. WHO Food Additives Series, No. 25, 1990.
87. **Residues of some veterinary drugs in animals and foods**. FAO Food and Nutrition Paper, No. 41/2, 1990.
88. **Evaluation of certain food additives and contaminants** (Thirty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 789, 1990, and corrigenda.
89. **Toxicological evaluation of certain food additives and contaminants**. WHO Food Additives Series, No. 26, 1990.
90. **Specifications for identity and purity of certain food additives**. FAO Food and Nutrition Paper, No. 49, 1990.
91. **Evaluation of certain veterinary drug residues in food** (Thirty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 799, 1990.
92. **Toxicological evaluation of certain veterinary drug residues in food**. WHO Food Additives Series, No. 27, 1991.
93. **Residues of some veterinary drugs in animals and foods**. FAO Food and Nutrition Paper, No. 41/3, 1991.
94. **Evaluation of certain food additives and contaminants** (Thirty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 806, 1991, and corrigenda.
95. **Toxicological evaluation of certain food additives and contaminants**. WHO Food Additives Series, No. 28, 1991.
96. **Compendium of Food Additive Specifications**. Joint FAO/WHO Expert Committee on Food Additives (JECFA). Combined specifications from 1st through the 37th Meetings, 1956-1990. FAO, 1992 (2 volumes).
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98. **Toxicological evaluation of certain veterinary residues in food**. WHO Food Additives Series, No. 29, 1991.
99. **Residues of some veterinary drugs in animals and foods**. FAO Food and Nutrition Paper, No. 41/4, 1991.
100. **Guide to specifications—General notices, general analytical techniques, identification tests, test solutions, and other reference materials**. FAO Food and Nutrition Paper, No. 5, Ref. 2, 1991.
101. **Evaluation of certain food additives and naturally occurring toxicants** (Thirty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series No. 828, 1992.
102. **Toxicological evaluation of certain food additives and naturally occurring toxicants**. WHO Food Additive Series, No. 30, 1993.
103. **Compendium of food additive specifications: Addendum 1**. FAO Food and Nutrition Paper, No. 52, 1992.
104. **Evaluation of certain veterinary drug residues in food** (Fortieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 832, 1993.

105. **Toxicological evaluation of certain veterinary drug residues in food.** WHO Food Additives Series, No. 31, 1993.
106. **Residues of some veterinary drugs in animals and foods.** FAO Food and Nutrition Paper, No. 41/5, 1993.
107. **Evaluation of certain food additives and contaminants** (Forty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 837, 1993.
108. **Toxicological evaluation of certain food additives and contaminants.** WHO Food Additives Series, No. 32, 1993.
109. **Compendium of food additive specifications: Addendum 2.** FAO Food and Nutrition Paper, No. 52, Add. 2, 1993.
110. **Evaluation of certain veterinary drug residues in food** (Forty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 851, 1995.
111. **Toxicological evaluation of certain veterinary drug residues in food.** WHO Food Additives Series, No. 33, 1994.
112. **Residues of some veterinary drugs in animals and foods.** FAO Food and Nutrition Paper, No. 41/6, 1994.
113. **Evaluation of certain veterinary drug residues in food** (Forty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 855, 1995, and corrigendum.
114. **Toxicological evaluation of certain veterinary drug residues in food.** WHO Food Additives Series, No. 34, 1995.
115. **Residues of some veterinary drugs in animals and foods.** FAO Food and Nutrition Paper, No. 41/7, 1995.
116. **Evaluation of certain food additives and contaminants** (Forty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 859, 1995.
117. **Toxicological evaluation of certain food additives and contaminants.** WHO Food Additives Series, No. 35, 1996.
118. **Compendium of food additive specifications: Addendum 3.** FAO Food and Nutrition Paper, No. 52, Add. 3, 1995.
119. **Evaluation of certain veterinary drug residues in food** (Forty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 864, 1996.
120. **Toxicological evaluation of certain veterinary drug residues in food.** WHO Food Additives Series, No. 36, 1996.
121. **Residues of some veterinary drugs in animals and foods.** FAO Food and Nutrition Paper, No. 41/8, 1996.
122. **Evaluation of certain food additives and contaminants** (Forty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 868, 1997.
123. **Toxicological evaluation of certain food additives.** WHO Food Additives Series, No. 37, 1996.
124. **Compendium of food additive specifications, addendum 4.** FAO Food and Nutrition Paper, No. 52, Add. 4, 1996.
125. **Evaluation of certain veterinary drug residues in food** (Forty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 876, 1998.
126. **Toxicological evaluation of certain veterinary drug residues in food.** WHO Food Additives Series, No. 38, 1996.
127. **Residues of some veterinary drugs in animals and foods.** FAO Food and Nutrition Paper, No. 41/9, 1997.

128. **Evaluation of certain veterinary drug residues in food** (Forty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 879, 1998.
129. **Toxicological evaluation of certain veterinary drug residues in food**. WHO Food Additives Series, No. 39, 1997.
130. **Residues of some veterinary drugs in animals and foods**. FAO Food and Nutrition Paper, No. 41/10, 1998.
131. **Evaluation of certain food additives and contaminants** (Forty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 884, 1999.
132. **Safety evaluation of certain food additives and contaminants**. WHO Food Additives Series, No. 40, 1998.
133. **Compendium of food additive specifications: Addendum 5**. FAO Food and Nutrition Paper, No. 52, Add. 5, 1997.
134. **Evaluation of certain veterinary drug residues in food** (Fiftieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 888, 1999.
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145. **Compendium of food additive specifications, addendum 7**. FAO Food and Nutrition Paper, No. 52, Add. 7, 1999.
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148. **Residues of some veterinary drugs in animals and foods**. FAO Food and Nutrition Paper, No. 41/13, 2000.
149. **Evaluation of certain food additives and contaminants** (Fifty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 901, 2001.

150. **Safety evaluation of certain food additives and contaminants.** WHO Food Additives Series, No. 46, 2001.
151. **Compendium of food additives specifications, addendum 8.** FAO Food and Nutrition Paper, No. 52, Add. 8, 2000.
152. **Evaluation of certain mycotoxins in food** (Fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, in press.



## ANNEX 2

### ABBREVIATIONS USED IN THE MONOGRAPHS

AcDON	acetylated deoxynivalenol
ADI	acceptable daily intake
4-ANIV	4-acetylivalenol
AOAC	formerly, Association of Official Analytical Chemists; now, AOAC International
APCI	atmospheric pressure chemical ionization
AT1	anion transporter 1
$a_w$	water activity
BFE-E	burst-forming unit–erythroid
BrDU	bromodeoxyuridine
bw	body weight
CBA	carboxylic acid
CD	competitive direct
CEN	European Committee for Standardization
CFU	colony-forming unit
CV	coefficient of variation
CYP	cytochrome P450
DAD	diode array detection
DAS	diacetoxyscirpenol
dpm	decays per minute
DMAP	4-dimethylaminopyridine
DMBA	7,12-dimethylbenz[a]anthracene
DON	deoxynivalenol
ECD	electron capture detection
ED <sub>50</sub>	median emetic dose
ELISA	enzyme-linked immunosorbent assay
FAO	Food and Agricultural Organization of the United Nations
FDA	Food and Drug Administration (USA)
FID	flame ionization detection
FLD	fluorimetric detection
FX	fusarenon X
GC	gas chromatography
GM	granulocyte–monocyte
GPC	gel permeation chromatography
GST	glutathione S-transferase
GST-P	glutathione S-transferase, placental form
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCV	hepatitis C virus
HFB	heptafluorobutanoyl
HFBA	heptafluorobutyric anhydride
HORRAT	ratio of RSD <sub>r</sub> in trial to predicted RSD <sub>r</sub>

HPLC	high-performance liquid chromatography
HPTLC	high-performance thin-layer chromatography
HSV	<i>Herpes simplex</i> virus
HT-2	15-Acetoxy-3 $\alpha$ ,4 $\beta$ -dihydroxy-8 $\alpha$ -[3-methylbutyryloxy]-12,13-epoxytrichothec-9-ene
IARC	International Agency for Research on Cancer
ic	intracerebrally
IC <sub>50</sub>	median inhibitory concentration
ID <sub>50</sub>	median inhibitory dose
Ig	immunoglobulin
IPCS	International Programme on Chemical Safety
IUPAC	International Union of Pure and Applied Chemistry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
K <sub>m</sub>	Michaelis constant
LC	liquid chromatography
LD <sub>50</sub>	median lethal dose
LOD	limit of detection
LOQ	limit of quantification
MAFF	Ministry of Agriculture, Fisheries and Food
MAS	monoacetoxyscirpenol
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MVK	Moolgavkar–Venzon–Knudson
NA	not available
NEO	neosolaniol
NIV	nivalenol
NOEL	no-observed-effect level
NR	not reported
OR	odds ratio
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG	polyethylene glycol
PKC	phosphokinase C
PMTDI	provisional maximal tolerable daily intake
RSD <sub>r</sub>	relative standard deviation for repeatability
RSD <sub>R</sub>	relative standard deviation for reproducibility
SD	standard deviation
SD <sub>r</sub>	standard deviation for repeatability
SD <sub>R</sub>	standard deviation for reproducibility
SFC	supercritical fluid chromatography
SFE	supercritical fluid extraction
SPE	solid-phase extraction
T-2	4 $\beta$ ,15-diacetoxy-3 $\alpha$ ,dihydroxy-8 $\alpha$ -[3-methylbutyryloxy]-12,13-epoxytrichothec-9-ene
TBT	tributyl tin

TCMC	trichloromethylsilane
TDI	tolerable daily intake
TFA	trifluoroacetic acid
TFAA	trifluoroacetic anhydride
TGF	transforming growth factor
TLC	thin-layer chromatography
TMA	trimethylaluminium
TMCS	trimethylchlorosilane
TMI	trimethylindium
TMS	trimethylsilyl
TNF	tumour necrosis factor
TOCP	tri- <i>ortho</i> .cresyl phosphate
TPA	12- <i>O</i> -tetradecanoylphorbol 13-acetate
TUNEL	tritiated thymidine-mediated dUTP-biotin nick end-labelling
UV	ultra-violet radiation
$V_{\max}$	maximum velocity
WHO	World Health Organization
ZEN	zearalenone



### ANNEX 3

#### JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

Geneva, 6–15 February 2001

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## CORRIGENDA

### WHO Food Additives Series 35: Toxicological evaluation of certain food additives and contaminants, 1996

*Page 186, paragraph 3, line 2:*

Replace 'both male and female rats' with 'both male and female mice'.

### WHO Food Additives Series 44: Safety evaluation of certain food additives and contaminants, 2000

*Page 371, Table 8:*

Replace lines 1 and 2 under 'Estimated intake of mercury' by:

	Middle Eastern	Far Eastern	African	Latin American	European
Total ( $\mu\text{g}/\text{person per day}$ )	2.6	7.0	7.2	9.0	9.2
Total ( $\mu\text{g}/\text{person per week}$ )	18	49	50	63	64





This volume contains monographs prepared at the fifty-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Geneva, Switzerland, from 6 to 15 February 2001. Five mycotoxins or groups of mycotoxins that contaminate food commodities were evaluated at the meeting: aflatoxin M<sub>1</sub>, fumonisins B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub>, ochratoxin A, deoxynivalenol and T-2 and HT-2 toxins. The monographs in this volume summarize the data that were reviewed on these contaminants, including information on metabolism and toxicity, epidemiology, analytical methods for their measurement in food commodities, sampling protocols, effects of processing, levels and patterns of contamination of food commodities, food consumption, and prevention and control. Based upon this information the Committee assessed the risks associated with intake of these mycotoxins.

This volume and other documents produced by JECFA contain information that is useful to the Codex Alimentarius, government and food regulatory officers who are involved with controlling contaminants in food, industrial testing laboratories, toxicological and analytical laboratories, and universities