CODEX ALIMENTARIUS COMMISSION E

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DISCUSSION PAPER ON PYRROLIZIDINE ALKALOIDS

(Prepared by Electronic Working Group led by The Netherlands)

BACKGROUND

1. The 4th CCCF agreed to establish an electronic Working Group led by the Netherlands to develop a Discussion Paper on Pyrrolizidine Alkaloids (PAs) in Food and Feed and Consequences for Human Health, with an aim to gather information on the chemistry of PAs, their toxicity, what methods of analysis are available, occurrence in plants, food and feed and the carry-over from feed to food.

2. The electronic working group was established and members included: Apimondia, Australia, Brazil, European Union, FAO, France, Germany, Hungary, Indonesia, Japan, Jordan, New Zealand, Sweden, Switzerland and Togo (see Annex VII). Comments were received from Apimondia, Australia, Brazil, FAO, France, Germany, Japan, New Zealand, and Switzerland. A discussion paper was prepared following the CCCF directions for its content. In addition, information on management practices was added to the paper.

INTRODUCTION

3. Pyrrolizidine alkaloids (PAs) are toxins found naturally in a wide variety of plant species. PAs are probably the most widely distributed natural toxins and affect wildlife, livestock and humans. Outbreaks of toxicity in farm animals cause severe economic losses to farmers and rural communities and there is the possibility of risk to humans from the intake of PA contaminated food of botanical or animal origin. Direct human cases of poisoning are well-documented,, such as in the direct and deliberate use of toxic plant species as herbal teas or traditional medicines which in some cases have resulted in deaths.. Also consumption of grain or grain products (flour or bread) contaminated with PA-containing seeds has been involved in outbreaks of poisonings affecting rural populations in Afghanistan, India, South Africa and the former USSR. No reported cases of poisonings as a result from PA-containing animal products are known (WHO, 1988; FAO, 2010).

4. FAO (2010) indicated that major plant families that contain PAs are the *Asteraceae* (*Compositae*), the *Boraginaceae* and *Fabaceae* (*Leguminosae*). Of the *Asteraceae* the genera of *Senecio* and *Eupatorium* are by far the mostly known to contain PAs, while most genera of the *Boraginaceae*, e.g. *Heliotropium* or *Echium* species, are known to contain PAs. Of the *Fabaceae,* the *Crotalaria* genus hosts the majority of PA-containing species. Over 6 000 plant species are expected to contain PAs, although direct poisonings in man and animals seem to be associated with only a few species. Poisoning caused by these toxins is associated with acute and chronic liver damage, for some PAs with pulmonary arterial hypertension (in animals), and can result in death. In this discussion paper, a list of PA containing plants, including their common names, based on references found, is presented in ANNEX I.

CHEMISTRY OF PAS

5. PAs are heterocyclic compounds and most of them are derived from four necine bases:

retronecine, heliotridine, otonecine and platynecine; the platynecine type PAs are considered non-toxic (Hartmann and Witte, 1995, see also Figure 1). Retronecine and heliotridine are diastereomers at the C7 position. Over 350 different tertiary amine PA structures are known (Hartmann and Witte, 1995). Most of the naturally occurring PAs in plants are esterified necines or alkaloid N-oxides (except for the otonecine-type alkaloids), whereas non-esterified PAs occur less frequently in plants (such as in Australian *Crotalaria*

medicaginea and *Crotalaria aridicola*, common names unknown). The esters can be divided in monoesters, non-macrocyclic diesters and macrocyclic diesters of a necine base. Macrocyclic diesters are most commonly found in *Senecio* (which include ragwort) and *Crotalaria* (which include rattleweed) species. The PAs found in *Eupatorium* and *Boraginaceae* species are in general of the mono and diester type (Hartmann and Witte, 1995).

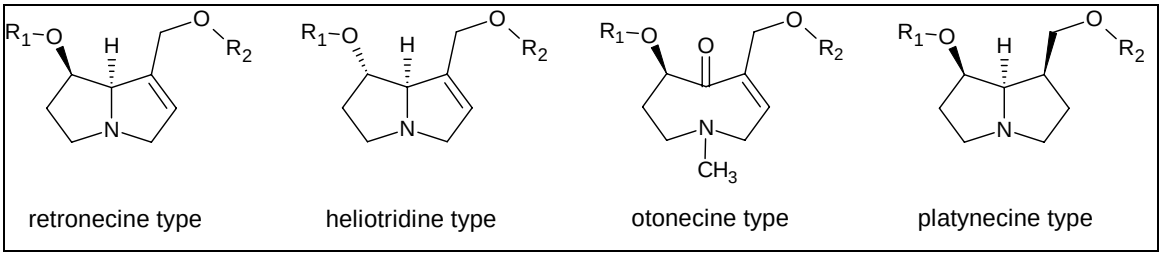

Figure 1: Basic structure of the four necine base types that are found in the large majority of pyrrolizidine alkaloids. The retronecine, heliotridine and otonecine type PAs are toxic, the platynecine type PAs are considered non-toxic (Hartmann and Witte, 1995)

6. The minimum structural requirements for toxicity are:

1. a Δ^3-pyrroline (3,4-didehydropyrrolidine) ring;

2. one or preferably two hydroxyl groups, each attached to the pyrroline ring via one carbon atom;

3. at least one of the hydroxyls is esterified;

4. the acid moiety has a branched chain (Australia New Zealand Food Authority, 2001)[1].

7. It can be generalized that macrocyclic PAs are more toxic than diester PAs, which are more toxic than monoester PAs. Liver enzymes can convert the otonecine ring of otonecine type PAs into a retronecine ring through oxidative N-demethylation.

REGULATION OF PAS IN FOOD AND FEED

8. Standards for the control of PAs concentrate on the control of the toxic plants and plant parts. The regulatory situation regarding PAs in food and feed has been summarized by FAO (2010) and Kempf et al. (2010). Details of existing regulations and recommendations are presented below.

CODEX ALIMENTARIUS

9. For grains and pulses, the Codex Alimentarius states that toxic seeds in wheat should not be present in amounts that represent a hazard to health and mentions specifically the presence of Crotolaria. These standards are (FAO, 2010):

- Maize (corn) CODEX STAN 153-1985

- Certain pulses CODEX STAN 171-1989

- Sorghum grains CODEX STAN 172-1989

- Wheat and durum wheat CODEX STAN 199-1995

- Oats CODEX STAN 201-1995

Australia

10. In Australia, comfrey (*Symphytum* spp.) is included in Appendix C (Substances, other than those included in Schedule 9, of such danger to health as to warrant prohibition of sale, supply and use) of the

[1] The Australia New Zealand Food Authority (ANZFA) became Food Standards Australia New Zealand (FSANZ) on 1 July 2002.

Poisons Standard 2010, for therapeutic or cosmetic use except for dermal use, for which it is included in Schedule 5 of the Poisons Standard (DOHA, 2010).

11. The *Joint Food Standards Code (ANZJFSC)*, which applies to both Australia and New Zealand, lists PA-containing plant species under *Schedule 1: 'Prohibited Plants and Fungi* of Standard 1.4.4. The most important are the following plants: *Borago officinalis*; *Crotolaria* spp; *Echium plantagineum*; *Echium vulgare*; *Heliotropium* spp; *Senecio* spp; *Symphytum asperum*; *Symphytum officinale*; and *Symphytum x uplandicum'*. A plant or fungus, or a part or a derivative of a plant or fungus listed in Schedule 1, or any substance derived there from, must not be intentionally added to food or offered for sale as food.

12. There are also various Australian state regulations detailing limit for seeds in stock feed from: *Heliotropium* spp; *Echium* spp; *Amsinckia* spp; and *Crotalaria* spp.

13. In 2001, Food Standards Australia New Zealand (FSANZ) established a Provisional Tolerable Daily Intake (PTDI) for PAs at 1 µg/kg bw/day. In 2004, FSANZ advised honey processors in Australia/New Zealand to continue their practice of blending honeys that are mainly derived from Paterson's curse or Salvation Jane (*Echium plantagineum*) with other honeys to reduce the PAs to a safe level (FSANZ, 2004).

Austria

14. In Austria only a few PA-containing plants are authorized for herbal remedies. These plants or preparations thereof can only be marketed if they are analyzed by a ''.. state of the art detection method'' which proves that ''.. the final product does not contain pyrrolizidine alkaloids.'' (Bundesgesetzblatt, 1989, as cited by Kempf et al., 2010).

Belgium

15. In Belgium, borage (*Borago oSfficinalis*) is prohibited for use in food. Oil of borage may be used in food supplements, if it can be shown that it is free of PAs using an appropriate detection method with sufficient LOD (Koninklijk besluit, 1997). Recently, the maximum level of 1 µg/kg as regulated by The Netherlands was adopted, but again, the detection method and LOD should be reported.

Canada

16. Health Canada has advised Canadian consumers not to use or to ingest the herb comfrey (*Symphytum officinale*) or any health products that contain comfrey. As a precaution, consumers were advised not to topically apply comfrey-containing products to broken skin. This advisory applied to both approved and unapproved products (Health Canada, 2003).

EU

17. The EU has regulated alkaloids in feed by setting a maximum content of 3000 mg of weed seeds and unground and uncrushed fruits containing alkaloids, glucosides or other toxic substances and fruits/kg total feed (relative to a feeding stuff with a moisture content of 12 %). The maximum content related to *Crotolaria* spp. is 100 mg seeds/kg total feed (EU, 2010).

Germany

18. Since 1992, PA-containing phytopharmaceuticals are regulated in a Federal Pharmaceutical Ordinance. According to these regulations, only a few proven active PA plants and preparations thereof, which are listed by name, can be marketed. With regard to the PA content the following limits were established: at customary oral intake the total amount of 1,2 unsaturated PAs (including the PA-N-oxides (PANOs)) must not exceed 1 µg per day. If the application is more than 6 wks, the limit is further reduced to 0.1 µg per day. In addition, the package insert for orally used products needs to contain the warning notice ''do not use during pregnancy or lactation'' (Bundesgesundheitsamt, 1992, cited by Kempf et al., 2010).

Japan

19. In Japan, comfrey (*Symphytum* spp.) and products thereof cannot be marketed, in accordance with Article 6.2 of the Food Sanitation Law. For precaution, the decision was made by the Ministry of Health, Labour and Welfare in June 2004, due to incident of adverse effects reported overseas, to ban the sale of any comfrey and comfrey containing foods for consumption.

New Zealand

20. For contaminants in food, the *Australia New Zealand Foods Standards Code (ANZFSC)*, described under the heading Australia, covers both Australia and New Zealand.

The Netherlands

21. PAs are regulated in The Netherlands for herbal preparations or herbal extracts. The total PA content (including PANOs) of these products must not exceed 1 µg/kg or 1 µg/L, respectively (Warenwetbesluit Kruidenpreparaten, 2001). In the Annex of the Warenwetbesluit Kruidenpreparaten, an overview is given of all plants and fungi of which it is presumed that they contain PAs.

South Africa

22. The South African Ministry of Agriculture, Forestry and Fisheries reiterated in 2008 that the sale of comfrey (*Symphytum spp.*) as a foodstuff had been prohibited by the Regulations Relating to the Prohibition of the Sale of Comfrey, Foodstuffs Containing Comfrey and Jelly Confectionery Containing Konjac, which were published in the Government Gazettee on 10 October 2003, to prohibit the use of comfrey as a foodstuff (DAFF, 2008). Regulations as regards to comfrey containing products with medicinal claims were under development at the time.

Switzerland

23. In Switzerland the same regulations for phytopharmaceuticals as in Germany are in force. (Ordinance on complementary and herbal medicinal products (SR 812.212.24), Annex 6; http://www.swissmedic.ch/produktbereiche/00445/00454/index.html?lang=en&download=NHzLpZeg7t,lnp6I0NTU042l2Z6ln1ad1IZn4Z2qZpnO2Yuq2Z6gpJCDdIR3gGym162epYbg2c_JjKbNoKSn6A--).

UK

24. In the past, similar to the German regulation for herbal remedies, comfrey (*Symphytum* spp.) and preparations thereof were banned in the UK (Kempf et al., 2010, no original references found).

USA

25. The Food and Drug Administration issued a letter to industry in 2001 communicating concern about the safety of supplement products containing comfrey (*Symphytum* spp.) because of its PAs. FDA further recommended that firms immediately stop marketing comfrey-containing supplements and alert consumers to stop using the products. Finally, FDA urged manufacturers to identify and report any adverse events, including liver disorders, which had been associated with comfrey and other ingredients containing PAs (FDA, 2001).

26. In the United States, animals presented for slaughter that show signs of PA-related disease, are condemned and not allowed to enter the food supply (USDA, 2007).

ISO

27. In addition to these regulations/recommendations, ISO has a condition implemented for PAs: The ISO Specification for Wheat allows a maximum of 0.05% PA containing seeds and provides a test method for toxic seeds in samples of wheat for human consumption (ISO, 2000, cited by FAO, 2010). This tolerance appears to be close to the limit of determination of the method.

TOXICITY

28. In this chapter, evaluations of IPCS and other risk assessment bodies have been summarized per topic. In addition, recent available studies are summarized to give an overview of the additional beyond the scope of this discussion paper information to the risk assessments above. The validity of these studies should however be separately assessed, which is a risk assessment task and therefore beyond the scope of this discussion paper.

Acute toxicity

29. IPCS (WHO, 1988) concluded that ‘in man, PA poisoning is usually manifested as acute veno-occlusive disease (VOD) characterized by a dull dragging ache in the right upper abdomen, rapidly filling ascites resulting in marked distension of the abdomen, and sometimes associated with oliguria, and massive

pleural effusion. It can also manifest as sub-acute disease with vague symptoms and persistent hepatomegaly. Children are particularly vulnerable. Many cases progress to cirrhosis and, in some cases, a single episode of acute disease has been demonstrated to progress to cirrhosis, in spite of the fact that the patient has been removed from the source of toxic exposure and has been given symptomatic treatment. Mortality can be high with death due to hepatic failure in the acute phase or due to hematemesis resulting from ruptured oesophageal varices caused by cirrhosis.'

30. IPCS (WHO, 1988) and COT (2008) listed the available LD50-values of several PAs in the rat after intraperitoneal (ip) injection. Oral LD50-values are not available. While the relevance of ip injection to oral exposure is low, these ip LD50-values are presented in Table 1 below. The ip LD50s indicate that there is considerable difference in acute toxicity between PAs when administered by ip injection, however it should also be noted that substantial differences in susceptibility to PAs have been noted between different strains of rat.

Table 1 LD50-values of several PAs in the rat after intraperitoneal injection (WHO, 1988; COT, 2008)

PA	**LD50 (mg/kg)**
Echimidine	200
Echinatine	350
Europine	>1000
Heleurine	140
Heliosupine	60
Heliotrine	300
Heliotridine	1200
Intermedine	1500
Jacobine	77 (mouse)
Jaconine	168 (female rat)
Lasiocarpine	77
Lasiocarpine *N*-oxide	547
Lycopsamine	1500
Monocrotaline	175
Retrorsine	34
Retrorsine *N*-oxide	250
Riddelliine	105 (mouse)
Senecionine	50 (also quoted as 85)
Seneciphylline	77
Senkirkine	220
Spectabiline	220
Supinine	450
Symphytine	130 (also quoted as 300)

31. To determine whether a single high dose or repeated small doses would result in poisoning and to evaluate if resistance could be induced by smaller repeated doses over time, Anjos et al. (2010) investigated the toxic effects of the seeds of wedge-leaved crotalaria (*Crotalaria retusa*, containing 6.84% monocrotaline) on sheep. They found that sheep are highly susceptible to acute (and chronic) intoxication after a single dose of approximately 205 mg monocrotaline/kg. In addition, sheep that ingested non-toxic doses of 136.8 mg/kg daily became resistant to toxic doses of 273 mg/kg and 342 mg/kg. As possible explanations for this resistance, the authors suggested based on other studies that the production of the metabolite dehydromonocrotaline by microsomal enzymes could be decreased, or that the detoxification of PAs by rumen microbes could be increased. The authors also indicated that sheep can acquire resistance by a rapid adaptation of ruminal microbes to metabolise monocrataline.

Chronic toxicity

Statements on chronic toxicity

32. As to chronic toxicity, IPCS (WHO, 1988) concluded that 'megalocytosis, the presence of enlarged hepatocytes containing large, hyper-chromatic nuclei, is a characteristic feature of pyrrolizidine alkaloid-induced chronic hepatotoxicity in experimental animals'. The enlarged hepatocytes arise through the powerful antimitotic action of the pyrrole metabolites of pyrrolizidine alkaloids. This change has not been observed in the human liver, though human fetal liver cells *in vitro* culture become enlarged when exposed to PAs, indicating susceptibility to the antimitotic effect of the alkaloids.

33. IPCS concluded that a daily intake of PAs as low as the equivalent of 0.01 mg/kg heliotrine may cause disease in humans. They based this on data from Ridker et al. (1985), who reported that 0.015 mg/kg of echimidine and related alkaloids (equivalent to 0.009 mg/kg bw/day heliotrine using the LD50-data) led to comfrey poisoning. Furthermore, if expressed in terms of equivalent doses of heliotrine, the estimated total doses in the known outbreaks or case reports ranged from 1 to 167 mg/kg bw. When comparing these figures with the total lethal dose of several PAs in rats, IPCS stated that this would seem to indicate that man is markedly more sensitive to PA poisoning than rat. However, they stated, that "it should be noted that these estimates are based on limited raw data and a number of assumptions, and so are of uncertain reliability". Still, it was recommended to minimize exposure if possible (WHO, 1988).

34. The ANZFA (2001) concluded that the target organ for PA toxicity in both experimental animals and humans is the liver. In animals, this toxicity is manifested as anti-mitotic activity leading to extensive fibrosis, nodular regeneration, parenchyma and cancer, whereas in humans the major effects are hepatocellular injury, cirrhosis and veno-occlusive disease. They proposed a provisional tolerable daily intake of 1 µg/kg/day based on veno-occlusive disease of the liver as the major toxicological effect of chronic exposure. According to the Authority, "the available data on cases of veno-occlusive disease in humans indicate that a tentative no-observed-effect level (NOEL) of 10 µg/kg bw/day can be established. If an uncertainty factor of 10 to account for human variability is applied to this NOEL, the provisional tolerable daily intake (PTDI) for PAs in humans is 1 µg/kg bw/day". Further characterization of the potential human health risk from exposure to PAs in food was considered not possible because of the inadequate dietary exposure information.

35. The EFSA concluded in 2007 that the major signs of toxicity observed in animals, include various degrees of progressive liver damage, and veno-occlusive diseases associated with a direct effect of the toxic metabolites on endothelial cells. Moreover, proliferation of the bile ducts, hepatic megalocytosis and liver fibrosis, renal injury, pulmonary hypertension and secondary cardiac right ventricular hypertrophy (only for a small number of PAs), as well as weight loss, anorexia and depression were regularly reported. In humans, the most sensitive endpoint of PA toxicity was identified to be veno-occlusive disease. They further point out to significant differences that were noted regarding species-specific sensitivity to PAs. That is, it is generally recognized that rodents, as well as pigs, poultry, cattle and horses are very sensitive to PA intoxication, whereas sheep, goats and rabbits are not (EFSA, 2007).

36. The Federal Institute of Risk Assessment (Bundesinstitut für Risikobewertung, BfR, Germany) performed a risk assessment of common groundsel (*Senecio vulgaris*) as a contaminant of salad mixes in 2007. They indicated that cases of poisoning had shown that PAs can cause life-threatening liver damage in humans and animals. Based on an exposure assessment it was concluded that acute to medium-term liver damage due to the intake of salad mix contaminated with common groundsel could not be excluded. They stated that a portion size below which the 1,2-unsaturated genotoxic PAs do not constitute a health risk could not be determined on the basis of the data available and therefore a tolerable intake can also not be derived. As a preventive measure, the intake of PAs should be avoided as much as possible. For an adult weighing 60 kg, the long-term intake would entail an estimated 220 to 349 µg of unsaturated PAs per day and therefore would far exceed the tolerated daily intake of 0.1 µg 1,2-unsaturated PAs for medicinal products without therapeutic indications or without restriction of intake to 6 weeks. The possible intake of unsaturated PAs through other foodstuffs had not been taken into account due to a lack of data, but they advocated an examination of the possible increased consumer exposure to unsaturated PAs that could enter foods such as honey; and milk, meat and eggs through animal feed (BfR, 2007a).

37. The UK Committee on Toxicity (COT, 2008) concluded that PAs were known to cause veno-occlusive disease in humans. They concluded that 0.1 µg riddelliine/kg bw/day would not be expected to result in non-cancer effects. As there were no reliable human studies, they derived this value from the NOAEL of 0.01 mg/kg bw/day for hepatocyte cytomegaly in rats resulting from oral exposure to riddelliine (NTP, 2003), and applying uncertainty factors of 10 for interspecies and 10 for within species variability. They indicated that the ratio of LD50 values could be used to convert other PAs to riddelliine equivalents for comparison with this dose. They further concluded that PAs in milk were unlikely to be a human health concern, while more information was needed on levels of PAs in grain and honey. Data were not available on concentrations of PAs in grain, eggs or meat on the UK market and, therefore, an assessment of UK consumer exposure from these foodstuffs could not be carried out (COT statement, 2008).

38. In 2005, the Dutch National Institute for Public Health and the Environment (RIVM) concluded that the toxic effects of PAs manifest mostly in liver, but also in lungs and kidney, and are a result of the reactive properties of the pyrrole metabolites. They calculated for non-cancer effects a tolerable daily intake (TDI) for ridelliine of 0.1 µg/kg bw per day, based on a NOAEL for non-neoplastic lesions in rats of 0.01 mg/kg per day (NTP, 2003), using an uncertainty factor of 100. The Dutch Food and Consumer Product Safety Authority (VWA) concluded in 2007 that high consumers with a prolonged intake of types of honey with high levels of PAs had an elevated risk to develop cancer and possibly acute effects. However, they indicated that it was not known to what extent this situation actually occurred, and also concluded that when honey consumers vary in their consumption between different types of honey, there was no extra cancer risk.

39. An overview of identified reference values for non-cancer endpoints is presented in Table 2 below. In general, health based guidance values for individual alkaloids could not be derived, due to lack of good oral toxicity data. Only for riddelliine a separate health based guidance value was derived. Also, availability of pharmacokinetic data for PAs and robust comparative animal toxicity data is limited.

Table 2: Identified reference values for non-cancer endpoints from risk assessment bodies.

	Health based guidance value (HBGV)	**Value HBGV (µg/kg bw/day)**	**Reference**
Total PAs	PTDI	1	FSANZ/ANZFA, 2001
Riddelliine	TDI	0.1	RIVM, 2005
Riddelliine**	TDI	0.1	COT, 2008
1,2-unsaturated PAs	TDI	0.1* (>6 wks) 1* (<6 wks)	BfR, 1992

* in µg/person/day

** COT concluded that the ratio of LD50 values could be used to convert other PAs to riddelliine equivalents for comparison with this dose.

Recent studies on hepatotoxicity

40. Copple et al. (2006) demonstrated that hypoxia occurred in the livers of monocrotaline-treated rats (300 mg/kg). Since this hypoxia occurred before significant hepatic parenchymal cell (HPC) injury has arisen, this might indicate that hypoxia contributes to damage to these HPCs. Hypoxia and HPC injury were reduced by approximately 70% after warfarin administration, suggesting that fibrin deposition could be a causal factor in the development of hypoxia. However, since warfarin could not prevent the hypoxia completely other factors will play a role. A non-specific inhibitor of nitric oxide synthases on the other hand enhanced monocrotaline-induced hypoxia and also HPC injury. The authors stated that these results suggest a relationship between hypoxia and HPC injury.

41. Mingatto et al. (2008) found that in fed rats, monocrotaline increased glycogenolysis and glycolysis, whereas in livers of fasted rats, it decreased gluconeogenesis and urea synthesis. These metabolic alterations were only observed after pre-treatment with phenobarbital, an inducer of metabolism, which indicates that the effects are due to the metabolites of monocrotaline. Since monocrotaline can influence energy-linked hepatic metabolism and thereby several other processes, the authors concluded that this may contribute to monocrotaline hepatotoxicity.

42. The effects of isoline, clivorine and monocrotaline on the livers of mice were compared with or without an esterase-inhibitor or a P450 inhibitor by Tang et al. (2007). Even at the highest dose tested (164 mg/kg bw for 3 consecutive days), monocrotaline had no effect on the mouse liver. Clivorine on the other hand was lethal at that dose, just like isoline which was already lethal after 2 days. When the esterase-inhibitor was given concurrently, isoline appeared to be already lethal at the mid dose (100 mg/kg bw) and displayed effects on liver enzymes even at the lowest dose tested (50 mg/kg bw). Also the toxicity of clivorine was increased, whereas that of monocrotaline was not affected. In combination with the P450 inhibitor, the increases in liver enzymes induced by isoline were either completely abolished or partially suppressed dependent on the applied dose. The authors concluded that these results show that, in mice, isoline is more hepatotoxic than clivorine or monocrotaline, and that the toxic effects of isoline are dependent on metabolic activation by P450 enzymes.

Pulmonary toxicity

43. PAs have been shown to produce pulmonary hypertension (PH) with associated vascular changes in the pulmonary circulation in several animal species as well as in non-human primates both directly and indirectly. Several studies and one human case report are summarized in the WHO report of 1988 (WHO, 1988). PH may also occur without significant effects on the liver under certain circumstances. Monocrotaline is one of the PAs known to cause primary PH in rats.

44. Recently, Shimzu et al. (2008) examined the effect of hemin treatment on monocrotaline (60 mg/kg bw)-induced PH and concluded that hemin significantly ameliorated the PH as well as pulmonary inflammation. The authors stated that this was possibly mediated by the induction of pulmonary heme oxygenase-1 1 which may lead to the attenuation of pulmonary inflammation.

Cardiotoxicity

45. Pulmonary toxicity may subsequently result in cardiac right ventricular hypertrophy as is described, amongst others, by Burns (1972, cited by WHO, 1988) and Allen and Chesney (1972, 1973, cited by WHO, 1988) who reported signs of PH and *cor pulmonale* in rats and non-human primates.

46. Recently, Akhavein et al. (2007) reported that the administration of monocrotaline (50 mg/kg) to rats resulted in a direct cardiotoxic effect, independent of the degree of PH, as shown by decreased left ventricular function, myocarditis and coronary arteriolar medial thickening.

Genotoxicity and carcinogenicity

47. Many PAs belonging to the retronecine-, heliotridine-, or otonecine-type of PAs exhibit genotoxicity. Examples include riddelliine, retrorsine, and monocrotaline (retronecine-type), clivorine and senkirkine (otonecine-type), and lasiocarpine and heliotrine (heliotridine-type) (Fu et al., 2007).

Statements on carcinogenicity of PAs

48. In 1988, the IPCS published their evaluation of PAs (WHO, 1988). Concerning carcinogenicity they concluded that, since various PAs have been shown to be mutagenic in several cell culture systems and carcinogenic in experimental animals, a potential cancer risk for human beings should be seriously considered. However, they found that no information was available on the long-term follow-up of humans exposed to and suffering from PA toxicity. Because of this lack of knowledge, it was not possible to make an evaluation of the cancer risk of PAs to humans.

49. Several PAs have been evaluated by the International Agency for Research on Cancer (IARC). Lasiocarpine, monocrotaline and riddelliine have been classified as Group 2B (possibly carcinogenic to humans) while hydroxysenkirkine, isatidine, jacobine, retrorsine, seneciphylline, senkirkine and symphytine have been classified as Group 3 (not classifiable as to its carcinogenicity to humans, IARC, 1976, 1983, 2002).

50. The ANZFA (2001) concluded that there was convincing evidence that chronic PA exposure is carcinogenic for rats and some other laboratory animal species, but noted that there were also no known reports of cancer in domesticated animals caused by exposure to PAs in their diet. Based on an analysis of Culvenor (1983) of the estimated dietary intake of PAs by humans during outbreaks compared to the effect dose range in rats for carcinogenicity in rats, they concluded that there was no evidence from the known significant poisoning outbreaks that PAs cause liver cancer in humans.

51. In 2007, EFSA concluded that epidemiological data did not provide any evidence for an increased risk of cancer in human populations exposed to PAs. Experimental animal data, in which very high concentrations were used, indicated the ability of some PAs to induce DNA adducts, DNA cross-links and DNA-protein cross links, as well as mutagenic and genotoxic effects. They concluded that various PAs had proven to be carcinogenic in rodent assays at high, experimental dosages.

52. The BfR reevaluated in 2007 the zero tolerance principle of the EU for certain compounds and compound classes in food and feeding stuff. Concerning the occurrence of genotoxic compounds as natural ingredients of traditional food in general, BfR recommended a case by case risk assessment which may lead to a recommendation of reduced intake of special food items. With regard to the possible addition of isolated genotoxic botanical ingredients to food or in respect to genotoxic contaminant of plant origin, referring specifically to the contamination of salad by PA containing parts of *Senecio vulgaris* explicitly, they generally recommend applying a zero tolerance to these (BfR, 2007).

53. Based on an NTP study of orally administrated riddelliine in rats, the Dutch National Institute for Public Health and the Environment (RIVM) calculated a virtually safe dose (VSD) of 0.00043 µg/kg bw/day, leading to an increased risk of at most one person in a million developing cancer (RIVM, 2005). This VSD is based on the lowest dose leading to tumor development in the NTP study, which was 1 mg/kg/day (Chan et al., 2003).

54. The UK Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC) reviewed in 2008 the mutagenicity and carcinogenicity of 7 PAs and 2 proposed metabolites. The COC concluded that riddelliine was genotoxic and carcinogenic and that it would be prudent to assume at least part of the carcinogenic effect was through a genotoxic mechanism. Similarly, they concluded that lasiocarpine was possibly a genotoxic carcinogen and that there were sufficient data to conclude that monocrotaline was carcinogenic. There was limited evidence of carcinogenicity for clivorine, petasitenine and symphytine while for senkirkine there was insufficient evidence of carcinogenicity. A limited number of studies using non-oral routes of administration had been carried out on the two metabolites, dehydroretronecine and dehydroheliotridine. On balance, these did not indicate carcinogenic activity of the metabolites (COC, 2008, cited by COT, 2008). The Committee further agreed that a "Cumulative Assessment Group" approach, as described in the opinion of an EFSA Scientific Panel on methodologies for the assessment of cumulative and synergistic risks from pesticides, would be appropriate for pyrrolizidine alkaloids in view of the evidence for a common tumor pattern for several of these compounds (COC, 2008).

55. The UK Committee on Toxicity (COT) endorsed in their Statement on Pyrrolizidine Alkaloids in Food in 2008 the COC recommendation to assess all PAs as a cumulative assessment group using the BMDL10 with an adequate MOE, while acknowledging the precautionary nature of this approach. A BMDL10 of 0.073 mg/kg bw/day was derived from a 2-year carcinogenicity study of lasiocarpine and should be used to assess exposure for any PA. Allowing an MOE of at least 10,000 indicated that PA doses of up to 0.007 µg/kg bw/day were unlikely to be of concern for cancer risk. Such doses would also not be expected to result in non-cancer effects (COT, 2008).

56. An overview of identified reference values for cancer endpoints is presented in Table 3 below.

Table 3: Identified reference values for cancer endpoints from risk assessment bodies.

	Health based guidance limit (HBGL)	**Value HBGL (µg/kg bw/day)**	**Reference**
Riddelliine	VSD	0.00043	RIVM, 2005
Total PAs	'unlikely to be of concern for cancer risk'	0.007	COT, 2008

Mechanism of carcinogenicity

57. PAs have long been known to be carcinogenic to animals (WHO, 1988). The parent alkaloids are chemically non-reactive, but following ingestion PAs undergo hepatic metabolism leading to (geno)toxic metabolites. The critical step for toxicity is the formation of the reactive bifunctional pyrrolic derivates, the 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizidine (DHP). DHP reacts with cellular proteins and

DNA to form DNA-adducts, DNA cross links and DNA-protein cross links. This has been well established in the past decades (WHO, 1988, ANZFA, 2001). In addition, Yang et al. (2001) confirmed that riddelliine induces liver tumors mediated by DHP-derived DNA adduct formation. That is, riddelliine was metabolized by male and female rat liver microsomes (more in males than in females, metabolism specifically by CYP3A4 which is found in both rats and humans) to form DHP, which, because of its strong binding capacity to DNA, lead to a set of eight DHP-derived DNA adducts, which resulted in DNA damage. They further obtained a clear dose-response relationship between the dose and level of total DNA adducts. In 2005, Wang et al. (2005b) underlined that PANOs could be genotoxic due to the formation of DHP-DNA adducts via the reductive conversion of the parent compound.

Recent studies on genotoxicity or carcinogenicity

Relative genotoxic potency

58. The same DHP-derived DNA adducts as induced by riddelliine were formed by metabolic activation of lasiocarpine, heliotrine, retronecine, retronecine N-oxide, retrorsine, retrorsine N-oxide, riddelliine N-oxide, monocrotaline, and monocrotaline N-oxide as found both *in vitro* and *in vivo* by Xia et al. (2006), Xia et al. (2008), Yan et al. (2008) and Wang et al. (2005a, 2005b), but to a lesser extent than riddelliine. From these four studies, the following order of potency for DNA-adduct formation can be deducted: riddelliine ≈ lasiocarpine > retrorsine > monocrotaline ≈ retrorsine N-oxide ≥ riddelliine N-oxide > heliotrine ≈ retronecine > retronecine N-oxide. Details of these studies are given in ANNEX II.

Relative genotoxic potency of plant materials

59. Extract of (common) comfrey root (*Symphytum officinale*) is found to show a mutagenic potential comparable to riddelliine in the rat liver *in vivo* as reported by Mei et al. (2005), Mei et al. (2006), and Guo et al. (2007). Both display a similar mutational spectrum with G:C→ T:A transversion as the predominant mutation. Chou and Fu (2006) investigated both *in vivo* the formation of DHP-derived DNA adducts from comfrey (*Symphytum officinale*), coltsfoot (*Tussilago farfara* L.) and coltsfoot flower (*Flos farfara*) in the liver by adding them to the scompounds other than PAs might be involved.

Carcinogenicity in humans

60. As the statements of the risk assessment bodies indicate, it has not yet been unequivocally demonstrated that PAs are carcinogenic in humans. However, IPCS indicated in 1988 that this should be very seriously considered. Recent studies which provide indications on the possible carcinogenicity in humans are summarized here.

61. In 2003, it was reported by Xia et al. that riddelliine can be metabolized by human liver microsomes to form DHP and riddelliine N-oxide. This study also showed that, when the human CYP3A4 enzyme is inhibited by the CYP inhibitor troleandomycin (TAO), the formation of DHP and riddelliine N-oxide was strongly reduced. In addition, the kinetic parameters V_{max} and K_m from human liver microsomal metabolism were comparable to those from rat liver microsomal metabolism. Authors concluded that this indicates that in humans the same metabolic liver enzyme is involved in DHP formation as in rats, which might be an indication that similar carcinogenic processes may occur.

62. Similar enzymatic action was further seen in the studies of Wang et al. (2005b) and Xia et al. (2006) with retrorsine, retrorsine N-oxide, riddelliine N-oxide, monocrotaline N-oxide, and lasiocarpine. In these studies, the human CYP3A inhibitors ketoconazole and TAO significantly inhibited DHP formation, whereas Xia et al. also showed that the CYP3A inducer dexamethasone led to an increase in DHP formation.

Immunotoxicity

63. Dehydroheliotridine (DHH), a pyrrolic metabolite, has a significant immunosuppressant activity on the primary response in young mice; when injected ip shortly before the antigenic stimulus (Percy & Pierce, 1971, cited by WHO, 1988). The secondary response to antigenic stimuli; as measured by the reduction in the number of 7S and 19S specific antibody-synthesizing cells of the spleen, was suppressed when DHH was administered at the time of secondary stimulus, but not when it was given 24 or 36 h after the antigenic stimulus. It was suggested that dehydroheliotridine selectively destroys or inactivates cells involved in the initial stages of antigen recognition and processing.

64. Recently, monocrotaline was investigated with respect to immunotoxicity by Hueza et al. (2009). Rats that received 3.0 mg/kg bw monocrotaline showed diminished bone marrow cellularity, but no other parameters such as splenic and thymic indices were affected. Furthermore, a reduction in NO production by macrophages was measured (after 1.0, 3.0 and 5.0 mg/kg bw monocrotaline) which according to the authors, might be a critical factor underlying the increased risk of pulmonary hypertension and anomalous inflammatory responses. However, they indicate that further studies are needed to elucidate this.

Reproductive toxicity

65. IPCS concluded in 1988 that the ability of PAs to cross the placental barrier in the rat and to induce premature delivery or death of litters had been demonstrated. They concluded that the embryo in utero appeared to be more resistant to the toxic effects of pyrrolizidine alkaloids than the neonate and that PAs were known to have passed through the mother's milk to the sucklings. IPCS was not aware of data on the teratogenic/fetotoxic effects of PAs on human beings and was therefore unable to evaluate the potential for these effects in PA exposure (WHO, 1988).

66. COT (2008) indicated, based on information provided by two paediatric liver centres to the Committee that paediatric veno-occlusive disease was rare in the UK, and that cases were almost always attributable to other causes and therefore unlikely to be related to PA exposure.

67. A human case report (Rasenack et al., 2003) indicating fetal effects of PAs is described below in the section ‘recent case reports – humans’.

Recent case reports

68. Case reports that have not been included in earlier risk assessments are summarized here. Case reports of animal intoxications indicate possible sources of PA contamination and possible livestock species which could be relevant for human consumption of contaminated animal products.

Animals - Livestock

69. Poisoning of ostriches was reported on 12 ostrich farms in the Pomona farming district, 35 km north of Harare, Zimbabwe (Cooper, 2007). Amongst other toxic plants, part of the poisoning was caused by the ingestion of Rhodesia ragwort *(Senecio sceleratus*) which resulted in skin haemorrhages and bleeding in the tracheal mucous membranes, the pericardium, diaphragm and interperitoneal membrane. Furthermore, the liver was enlarged, soft and jaundiced, and the lungs and thoracic and abdominal cavities were filled with clear fluid.

70. In England, four out of thirteen horses that had access to the same batch of hay showed signs of diarrhoea after eating the hay for six months during 2007. Blood tests showed that these horses had raised liver enzyme activities. Since liver problems are associated with PA poisoning, samples of the batch of hay were examined for their PA content and compared to hay samples from a control farm. Whereas in the control samples no PAs were detected, the approximate concentrations of PAs measured in the hay samples were up to 10 mg/kg (Crews & Andersen, 2009).

71. In Sierra Norte (Seville, Spain), ten 1-year-old fighting bulls from a herd of 700 animals died (Moyano et al., 2006). All animals had grazed in the same pasture containing large quantities of vipers' bugloss (*Echium vulgare* 80%) and common groundsel (*Senecio vulgaris* 15%) and were also fed with a light supplement of alfalfa hay. Drinking water was obtained from creeks running through the farm. Symptoms, biochemical data and the observed lesions found were similar to those found by other authors in cases of bovine PA poisoning, although not much information is available about spontaneous bovine poisoning by *Echium* spp. or *Senecio* spp. in Spain.

72. In June 2006, a 5-year-old red cross-breed bull was presented to the Ontario Veterinary College because of lethargy for 4 weeks (Walsh and Dingwell, 2007). After physical examination, in which amongst other things ventral abdominal swelling was reported, the diagnosis included peritonitis, pericardial effusion, gastrointestinal parasitism, and infection with *Bovine viral diarrhea virus*. Two days later, a yellow brockle-face cross-bred cow was found dead on the same farm. The cow had no previous history of disease, but the owner reported that this cow appeared to have a similar lethargy and depression as the bull. Based on post mortem examination, the diagnosis was blue-green algae poisoning and samples from several organs were sent for histopathologic examination. A second cow died 4 days later and was submitted for examination, but

could not be diagnosed. Meanwhile, the results of the histopathological examination revealed the first cow died from toxic hepatotoxicity consistent with pyrrolizidine alkaloidosis. The farm pasture on which the herd was grazing appeared to contain preflowering tansy or common ragwort (*Jacobaea vulgaris*, formerly known as *Senecio jacobaea*). This plant was spread throughout the low areas of the pasture at a density estimated to be 3 to 5 plants/m^2. The bull was euthanized and the post mortem examination showed in both this animals and in another animal that died, severe hepatopathy and lesions consistent with PA toxicity.

73. In early 2010, 35 horse deaths occurred in central-western Queensland, Australia (Williamson, 2010). In these cases, hepatic necrosis was accompanied by a predominantly neutrophilic inflammatory response, hepatic megalocytosis and nodular regeneration in some areas of the liver. This together with elevated bilirubin and liver enzymes was consistent with the ingestion of pyrrolizidine alkaloids. An investigation of the responsible plants is on-going.

Humans

74. Since 1974, several outbreaks of veno-occlusive disease (VOD) have been reported in Afghanistan, with the latest in February 2008 in the Gulran district of Herat Province, Western Afghanistan (Kakar et al., 2010). The consumption of bread made from wheat contaminated with seeds of a local weed called charmac (*Heliotropium popovii*) appears to be strongly associated with the outbreaks. In the 2008 outbreak, heliotrine, lasiocarpine and heliotrine-N-oxide were detected in all flour samples with higher levels of heliotrine found in samples from the houses of cases compared with non-cases. A secondary, though minor, likely source was the qurut (a whey product from the milk of goats which have ingested plants containing PAs while grazing). In qurut, also heliotrine, lasiocarpine and heliotrine N-oxide were found but in a reversed ratio. Also, although not present in the charmac samples, trichodesmine was found in the qurut. Water samples were also investigated, but their PA content was zero.

75. Two persons were hospitalised in China after prolonged use of oral decoctions of *Gynura segetum* (common name unknown, 3 g/day) as traditional medicine (Dai et al., 2006). Physical examinations revealed in both cases ascites and hepatomegaly. Based on hepatic biopsy revealing thickening of hepatic venular walls, dilation of sinusoids, and necrosis of hepatocytes, the diagnosis veno-occlusive disease (VOD) could be made.

76. In January 2006, a 62-year-old woman was admitted to the hospital in Hangzhou, China (Dai et al. (2007). She presented with abdominal distension after eating, hepatomegaly and ascites. Based on clinical and histological findings, HVOD was diagnosed. It appeared that the woman had ingested gynura root for 3 months before admission in dosages of approximately 2 g/day. She had put 3 slices of fresh gynura root a day soaked in rice wine and steamed. Then she ate the root and drank the wine. The root was identified as *Gynura segetum* (common name unknown), and seneciphylline and related PAs were identified in this species (Qi et al., 2009a and 2009b).

77. Thirteen cases with HVOD caused by traditional Chinese medicine were analysed retrospectively and the analysis revealed that 11 of the 13 cases were caused by gynura root, in dosages ranging from 150 g to 1800 g for over 10 days to 4 months (Wang et al., 2008, from abstract). One of the 13 cases was caused by Qianliguang (*Senecio scandens*), a Chinese herb, and one was caused by tea-thirsty (*unclear what species is meant*). The main symptoms included abdominal distension and pain, ascites, hepatomegaly and jaundice. Liver function appeared abnormal and GGT and ALP were increased. Of all cases, 2 patients improved following medical treatment, 2 received a liver transplant and 5 cases died.

78. A 66-year-old woman with known arterial hypertension, non-insulin-dependent diabetes, moderate adiposity and mild renal insufficiency was admitted to a hospital in Switzerland in November 2006 because of progressive dyspnoea (Györik and Stricker, 2009). On examination, she was tachydyspnoeic and blood gas analysis showed severe partial respiratory insufficiency. Echocardiography revealed she was suffering from a moderate pulmonary hypertension. Despite extensive investigations, no explanation could be found for the PH. Later on, when the woman was specifically asked for the use of alternative medicines, she reported that she used a mixture of several herbs to make tea and drank between one and one and a half litres a day in the months prior to her hospitalisation. These herbs were checked for their relationship to PH and comfrey (*Symphytum officinale*) and although not proven, the authors believed the PH to be possibly caused by the prolonged use of large quantities of boiled herbal remedies containing comfrey. The woman however refused to stop using the herbs as ingredients for her tea and was hospitalized again in March 2008 with severe right heart failure. After treatment, she could be discharged.

79. One hundred kilometres south-west of Johannesburg, a one-month-old set of twins was admitted to the hospital with a history of three weeks of pale stools, dark urine and a gradually distending abdomen (Conradie et al., 2005). Both twins were orally administered with traditional medicine. Ultrasonography showed severe ascites and fatty changes in the liver. Liver biopsy confirmed VOD. Both twins were treated and discharged. Another set of twins of one-month old were admitted to the hospital in Johannesburg with jaundice following the administration of a traditional remedy 48 hours previously. One twin died within 24 hours due to respiratory problems. After one week, the surviving twin showed a distended abdomen with splenomegaly but no hepatomegaly. This child subsequently died, after which an autopsy confirmed that this was a case of VOD with secondary necrosis.

80. In both cases, the parents provided samples of the traditional medicines used which were analysed by GC/MS. This revealed that retrorsine was present in both herbal remedy suspensions, with much lower levels in the sample from the fatal case than from the survivors. These results, while seemingly contradictory, indicated that the twin who died may have been dosed with higher or more frequent doses. No biological fluids or organs were examined due to the absence of consent.

81. There is one case report on an incidence of VOD in a human fetus caused by PA intake by the mother. A preterm neonate who was symptomatic with hepatomegaly and ascites was delivered by caesarean section for threatening fetal asphyxia and died shortly afterwards. Post mortem examination revealed veno-occlusive disease typical for pyrrolizidine alkaloid poisoning. The content of pyrrolizidine alkaloids in the liver was confirmed. Analysis of a herbal mixture which was used for cooking in the family revealed high amounts of the respective alkaloids clarifying the source of the poison and the causal relationship. Authors estimated mean daily intake of 20-30 µg PAs (Rasenack et al., 2003).

82. A 55-year-old woman suffered from breathlessness and unproductive cough (De et al., 2005). Biopsy of the lung revealed a granulomatous lung reaction compatible with the inhalation of foreign material. About one year before presenting the symptoms, she removed wet, black ragwort weeds (*unclear what species is meant*) in bundle to protect her horses and was exposed to rotten smelly pollen from it. Within a few days she had developed a dry cough, which later on developed into breathlessness on exertion. According to the authors, this is the first reported case suspecting ragwort weed (*unclear what species is meant*) to affect human lung. It is unknown if other possible causes have been investigated.

METHODS OF SAMPLING AND ANALYSIS

Sampling

83. The distribution of the concentration of PAs in foods and feeds is an important factor to be considered, when samples are taken for analysis. Whereas in the EU official limits exist for botanical impurities in feed (ranging from 0.01 -0.3 % for weed seed and unground and uncrushed fruits containing alkaloids, Directive 2002/32/EC), there is no specific mentioning or guidance on effective sampling procedures (EU, 2010). In general, the larger the sample size, the better its representativeness. Sampling of fluid samples (see below) seems less of a problem with respect to possible heterogeneity.

Analysis

84. Methods of analysis for PAs in foods and feeds and biological fluids (milk, blood plasma, bile, urine) have been reviewed by Roeder (1999), the European Food Safety Authority (EFSA, 2007) and recently by Crews et al. (2010). EFSA (2007) notes that various analytical techniques, particularly chromatographic methods in conjunction with mass spectrometry can be used to detect PAs in plants or plant derived products. None of these methods, however, has been validated for the analysis of (mixed) food and feed samples. EFSA (2007) also notes ongoing research into the use of ELISA techniques. There are no official methods for the detection or determination of PAs in foods and feeds and there is apparently no systematic analysis for PAs in grains entering the food supply (Australia New Zealand Food Authority, 2001, cited by FAO, 2010). A method for the determination of toxic seeds in samples of wheat for human consumption is given in the ISO Specification for Wheat (ISO, 2000, cited by FAO, 2010). A useful review of screening methods and confirmation assay development for plant-associated toxins in animal feed is provided by Than et al. (K.A. Than, 2005, cited by FAO, 2010).

85. A crucial point in quantitative assessment of PA-contamination is the limited availability of analytical standards of PAs and PANOs (Raezke et al., 2010, Kempf et al., 2011). Results of most of the methods mentioned in the following correspond to the sum of results for single compounds. Due to the lack

of standards the total PA-content maybe underestimated. Recently, the determination of retronecine type PAs as a sum parameter (reduced to their common core structure) was presented by Kempf et al. (2008). A similar approach has been described by Zhang et al. (2007). However, these methods do not include otonecine bases.

Methods for extraction of PAs

86. For PA analysis in (parts) of plants and herbal products, the plant material is often air or freeze dried, ground and homogenized. PAs are relatively polar compounds with a basic nature. Soxhlet extraction has been used often in the past, but requires relatively long extraction times and may lead to partial decomposition of the PANOs (Hartmann and Toppel, 1987; Hösch et al, 1996). Good extraction recoveries of PA tertiary amines and PANOs are obtained when the material is extracted with polar organic solvents such as methanol or with acidic aqueous solutions or mixtures thereof (Crews et al., 2010). An overview of used extraction methods for PAs from different plants and foodstuffs is presented in ANNEX III.

Methods for extract clean-up

87. Before the sample extract can be analysed for PA content a clean-up and/or concentration step is often required. Liquid/liquid extraction (LLE) and solid phase extraction (SPE) are the most commonly applied techniques (Crews et al, 2010). SPE is often used in the preparation of honey samples. Strong cation exchange (SCX) columns are most commonly used to trap PA tertiary amines and their N-oxides. An overview of used clean-up methods is presented in ANNEX IV.

Methods for separation and detection

Thin-layer chromatography

88. Thin-layer chromatography (TLC) on silica and aluminium sorbents has been used for qualitative analysis (Molyneux and Roitman, 1980; Roeder, 1999, Crews et al, 2010). Erlich's reagent is often used to visualize the PAs (Mattocks, 1967). Specifics on limits of detection (LOD) are hard to find, but are probably above 1 µg/g.

Gas chromatography

89. Gas chromatography (GC) in combination with flame-ionization detection (FID), nitrogen-phosphorus detection (NPD) and mass spectrometry (MS) are often used analytical methods (Roeder, 1999; Crews et al, 2010). Advantages of GC separation are the high resolution of GC columns and the availability of Retention Indices and electron impact mass spectra for a large set of PAs (Witte et al., 1993). A major limitation is that the PANOs are too polar and thermally unstable compounds to be analyzed by GC techniques. PANOs can therefore only be analyzed in an indirect way by means of reduction to the corresponding tertiary amines. Limits of detection are in the order of 10-100 ng/g. An overview of used GC methods is given in ANNEX V.

Liquid chromatography

90. Liquid chromatography (LC) in combination with UV detection is somewhat hampered by the absence of chromophores in most PAs. This requires detection at low, non-specific wavelengths (Crews et al, 2010). Zhang et al (2007) and Xiong et al (2009) have performed pre-column derivatisation of the PAs to improve the UV characteristics and lower detection limits. The advantage of the method of Zhang et al. (2007) is that it provides quantitative analysis of total retronecine PAs as a common derivative produced from all different RET-PAs. This method can be used in combination with both HPLC-UV and LC-MS.

91. An important advantage of LC compared to GC is that tertiary bases and N-oxides can be analysed simultaneously (Hösch et al, 1996; Brown et al, 1994). Of the many mono and diester PAs, several enantiomers and diastereoisomers have been identified. A separation of the enantiomers lycopsamine and intermedine by chiral HPLC has been reported by Pawar et al. (2010). Limits of detection (LOD) were reported above 100 ng/ml in extracts.

Liquid chromatography-mass spectrometry

92. LC in combination with mass spectrometry offers many possibilities for separation and detection and is nowadays the most used technique. The most commonly used applied mass spectrometers are of the triple quadrupole (LC-MS/MS) and ion-trap (IT) type. (Crews et al. 2010). Applications of time-of-flight (ToF)

MS are still relatively rare (Crews et al. 2009, 2010). Most investigators use positive electrospray ionisation (ESI) to generate ions for mass spectrometric detection. Separation of the PAs and PANOs is achieved under reversed phase (RP) chromatographic conditions. LODs vary with the matrix and the application. LODs typically range from (sub) ng/mL levels for detection in milk and honey up to several 100 ng/g in plant material. An overview of LC-MS methods and references is given in ANNEX V.

Fast screening methods for PAs

93. Several studies have been published that describe the development of enzyme-linked immunosorbent assays (ELISA) for the detection of one or more PAs. Roseman et al. (1992) developed a competitive ELISA against retronecine (as a class specific PA assay). The assay was used for the analysis of alkaline hydrolysates of PA-containing plants. Roseman et al (1996) also developed an ELISA against retrorsine, which displayed a LOD in the sub-ng/well level for retrorsine and retrorsine N-oxide. A sensitive competitive ELISA against senecionine was developed by Langer et al. (1996). The assay displayed cross reactivity against integerrimine and seneciphylline. The LOD for senecionine was 23 pg/well (equivalent to 0.45 ng/ml in diluted sample extract). Two different ELISA assays against riddelliine were developed by Lee et al. (2001). The first one showed cross reactivity against other senecionine type PAs such as senecionine, retrorsine, seneciphylline with LODs in the 18-110 pg/well (0.36-2.2 ng/ml) range. The second assay showed a good cross reactivity against riddelliine N-oxide, seneciphylline and seneciphylline N-oxide, with LODs in the 30-530 pg/well (0.6-10.6 ng/ml) range. The assay was used to analyze riddelliine in bovine blood and plant material. In plasma the LOD obtained for riddelliine was 25 ng/ml. Monoclonal antibodies raised against retrorsine (Zündorf et al., 1998) also showed good sensitivity against a set of structurally related senecionine type PAs. Cavallaro et al. (2004) developed a competitive ELISA against heliotrine. The ELISA showed good cross reactivity against lasiocarpine, europine, heleurine and to a lesser extent to heliotrine N-oxide and lasiocarpine N-oxide. LODs were in the range 8-300 pg/well. The assay was used to analyse wheat samples for the presence of heliotropium PAs.

94. One of the current activities in method development for PAs, is the EU-funded project CON*ff*IDENCE. This Large Collaborative Project in the Food, Agriculture, Fisheries and Biotechnology Area of the EC's 7th Framework Programme, runs from 2008-2012 under coordination of RIKILT Institute of Food Safety, Wageningen, the Netherlands. The project's focus is on the development of simple, fast, multi-analyte, multi-class detection for a variety of analytes, including some PAs. In Workpackage 4a (alkaloids) of the project, antibody-based dipstick methods are being developed. For the PAs, the focus is currently on the detection of jacobine and lycopsamine in honey and feed. The antibodies also cross react with several other PAs which can therefore, in principle, be detected as well. It is anticipated that prototype dipsticks will be extensively tested in 2011, followed by inter-laboratory validations later in 2011 and in 2012. The project will end in the course of 2012. Dipstick tests are particularly useful for field testing. The targeted limit of detection is 50 µg/kg.

OCCURRENCE IN FEED AND FOOD

95. Humans are thought to be exposed to PAs through plant products (mainly via herbal products and contaminated crops), or animal-derived products such as honey, milk, eggs and offal (ANZFA, 2001). Levels of PAs and other information on occurrence as collected for risk assessments are summarized here. Details of recent studies are shown in ANNEX VI.

Plants in general

96. The PA content of plants has been reported as generally varying from 100 mg/kg dry weight to 40,000 mg/kg dry weight, although the highest reported is 180,000 mg/kg dry weight in Riddell's ragwort (*Senecio riddelli*). The amount of PAs present in a plant depends on the season and locality. In addition, various parts of plants have different levels of PAs, often the majority of which is present as PANOs (WHO, 1988, ANZFA, 2001). A list of PA containing plants including their common names and PAs, based on references found for this discussion paper, is presented in ANNEX I.

Food

Cereals

97. Products of PA-containing plants, generally seeds, may contaminate staple foods and may be eaten over long periods of time (WHO, 1988). Quantitative data was not presented by IPCS.

98. The main source of intoxication by PAs in underdeveloped countries is grain, due to contamination with seeds of PA producing plants, including for example: narrow-leaved ragwort (*Senecio inaequidens*), *Senecio ilicifolius (*common name unknown*)*, charmac (*Heliotropium popovii*), European heliotrope (*Heliotropium europaeum*) and rattle weed (*Crotalaria*) species (EFSA, 2007).

99. Substantial contamination of grain commodities has been recorded in various countries due to both contamination by seeds of PA-containing weeds growing in the crop as well as plant dust fragments from the same plants. The levels of PAs found in various grain commodities in Australia has ranged from <50 to >6000 µg/kg, but there has been no systematic analysis of the levels in grains entering the food supply. There was no data available to indicate whether PAs occur in oilseed crops (ANZFA, 2001).

Recent studies

100. During an outbreak of VOD in Western Afghanistan (2008), wheat flour samples from case households had PA levels of heliotrine 0.16 mg/kg, heliotrine-N-oxide 5.4 mg/kg and lasiocarpine 0.045 mg/kg. PA levels in samples from control households were two-fold lower than unaffected households (Kakar et al., 2010).

101. In an Iranian study, PAs were qualitatively and quantitatively determined in wheat and flour samples from farms in the Mazandaran province. Common groundsel (*Senecio vulgaris*) was abundant at those farms and was determined to be the source of detected PAs in wheat and flour samples (Azadbakht & Talavaki, 2003).

Herbal preparations

102. An overview of some plants containing (or suspected of containing) PAs, which have been used by people either as herbal medicines or foods was compiled by IPCS, but no information on PA content was given (WHO, 1988).

103. Exposure to PAs may also result from the intentional consumption of herbal medicinal products, food supplements, herbal teas, or leaves of PA-containing plants that are used in salads or otherwise. Examples are species of the genus *Cynoglossum* (hounds-tongue (*Cynoglossum officinale*)), *Heliotropium* (European heliotrope (*Heliotropium europaeum*)), *Symphytum* (common comfrey (*Symphytum officinale*)), *Senecio* spp. (doronic ragwort (*Senecio doronicum*), tansy ragwort (*Jacobaea vulgaris*), common groundsel (*Senecio vulgaris*)), *Adenostyles* spp. (*Adenostyles alliariae* (*Grey adinostyle*)), *Petasites* spp. (common butterbur (*Petasites hybridus*), white butterbur (*Petasites albus*)) and *Crotalaria* spp. (wedge-leaved crotalaria (*Crotalaria retusa*)). The plant that has been most widely used as therapeutic agent is comfrey. However, in consideration of its hepatotoxicity, use of comfrey in medical applications has been restricted in most countries and its use is permitted only for local, cutaneous preparations (EFSA, 2007).

Salad

104. In Germany, common groundsel (*Senecio vulgaris*) was found as a contaminant in a salad mix. BfR made their risk assessment using the detected amount of 1.7 % common groundsel in salad mix and a study in which PA levels in flower heads of common groundsel were determined. Based on measurement of PA levels in flower heads 630 to 1000 µg PA/g wet weight, it was calculated that the salad contained 10.7-17 µg PA/g salad. The uncertainty was related to plant material, other than the flower heads, present in the salad (BfR, 2007a).

Milk

105. Milk concentrations of PAs are only available from carry over experiments. Cow's milk had PA levels ranging between 0.47 and 0.84 mg/L when cows were exposed to dried tansy ragwort (*Jacobaea vulgaris*) with a PA content of 0.16% (dry weight) at a dosage rate of 10 g/kg body weight per day over 125 days (Dickinson et al., 1976, cited by EFSA, 2007). Milk from goats receiving the same dosing regime had average PA concentrations (total PAs not specified) of 381 µg/L (225 - 530 µg/L, Dickinson, 1980, cited by WHO, 1988). In another experiment, goat's milk contained an average concentration of 7.5 ng PAs/g dry weight during exposure of the goats to a diet containing 25 % (about 123 g/day per goat) of dried tansy ragwort, The dry matter content of the milk was 12 %, leading to an average PA content of 62.5 µg/kg milk (Goeger et al., 1982, cited by WHO, 1988). However, this study does not mention any corrections for the usually low recovery rate of about 20 % of PAs in milk (EFSA, 2007).

Eggs

106. In Australian eggs, PA levels due to contaminated feed were found ranging from 5 to 168 µg/kg (Edgar & Smith, 2000; ANZFA, 2001).

Meat

107. No occurrence data were found on PAs in meat, only data from carry-over experiments were found (see paragraph 8.2).

108. It is unlikely that significant levels of free, unbound PAs are detectable in meat (muscle tissue), although it is possible that PA-protein-adducts may be present. As metabolism of PAs occurs largely in the liver, this organ could contain significant levels of PA-adducts to proteins and DNA. It is not known whether these adducts may pose a toxicological risk for the consumer and if they can be released under physiological conditions or in the intestinal tract. An analytical method for the detection of PA-adducts in blood and liver tissue has been described (Mattocks and Jukes, 1990, 1992; Stegelmeier et al, 1996), and applied to limited animal surveys (see Section 8.2).

Honey

109. In the USA, Deinzer et al. (1977) reported the presence of all PAs contained in tansy ragwort (*Jacobaea vulgaris*) in the honey secreted by bees feeding on the plant. The total alkaloid content ranged from 0.3 to 3.9 mg/kg. It has been estimated that an average annual human intake of honey (600 g) at the highest alkaloid level quoted would contain less than 3 mg of PAs (Mattocks, 1986). Culvenor et al. (1981) and Culvenor (1983, 1985) drew attention to the same potential hazard in honey from Paterson's curse/Salvation Jane (*Echium plantagenium*), a weed that grows widely in Southern Australia. Echimidine is the major component of the alkaloids of *Echium* spp., which are present in concentrations of up to 1 mg/kg. Culvenor (1983) estimated that individuals could consume up to 80 g honey/day with a corresponding alkaloid intake of 80 µg/day, if only *Echium* honey were used. No reports of acute human toxicity through this source are available (WHO, 1988). It is possible that there is a relevant number of still unknown PA containing plants which are of importance for contamination of honey with PAs.

110. In Australian honey, levels of alkaloid up to 1 mg/kg have been recorded from hives where bees foraged exclusively on *Echium* spp. (Culvenor, 1983, cited by ANZFA, 2001). In 2004, FSANZ reported that Australian honey samples had levels up to 2 mg/kg PAs though it was noted that blending could substantially reduce this level. The highest levels were found in honey from Paterson's Curse/Salvation Jane (*Echium plantagenium*) (ANZFA, 2001).

111. In a 1994 UK survey, honey samples were collected from hives placed close to ragwort (*unclear which species was meant*), or obtained from farmgate producers and a small independent retailer. Eight of 23 honey samples contained ragwort pollen and six of these had detectable levels of PAs. The two honey samples with the highest levels were dark, waxy samples, which were considered unpalatable and would not be used for blending with other honeys. Excluding these two samples, the highest detected level of PAs, was 0.06 mg/kg though the method used for this analysis was not reported. Using data on maximum honey consumption at any one time for adults (93g), children (60g) and infants (32g), the authors concluded that PA consumption from locally produced honey was not a cause for concern (MAFF, 1995 cited by COT, 2008).

112. A 2002 review of PAs in honey noted that the highest identified level of 3.9 mg PAs/kg was in honey reported to be from tansy ragwort (*Jacobaea vulgaris*). (Edgar et al., 2002 cited by COT, 2008).

113. The study of Edgar et al. (2002) was also used by EFSA (2007) to review the PA concentrations found in honey. In honey derived mainly from tansy ragwort (*Jacobaea vulgaris*) up to 3.9 µg/g PAs (uncorrected for extraction efficiency, which is estimated to be 50 – 70 %) were found. The (uncorrected) highest recorded level of PAs in honey, derived from Paterson's curse/ Salvation Jane (*Echium plantagenium*) was reported to be 0.95 µg/g. In addition, it was found that genera that contain PA producing plants represent a significant part of all plants used in honey production. In Europe, honey originating from the following plant genera is of importance: *Borago, Cynoglossum, Echium, Myosotis, Petasites, Senecio* and *Tussilago*. In countries outside of the European Community, also plants from the genera *Ageratum, Chromolaena, Crotalaria, Eupatorium* and *Heliotropium* are potential sources of honey contamination.

114. The Dutch Food and Consumer Product Safety Authority (VWA) analyzed honey samples for PA content of which 171 were retail samples of Dutch or imported origin and 8 were from hives deliberately placed in areas with high occurrence of tansy ragwort (*Jacobaea vulgaris*). Of the retail samples, 28% contained PAs at levels between 0.001 and 0.365 mg/kg. Four of the eight non-retail samples had detectable levels of PAs with the highest at 0.010 mg/kg. Pollen counts indicated that the bees had foraged on many other plants not just the groundsel (VWA, 2007).

Recent studies

115. Very recently, Kempf et al. (2010a,b) reviewed available occurrence data on PAs in honey and honey products. In 17 out of 55 commercial pollen products, that were analysed by the HRGC-ESI_MS sum parameter method (LOQ 10 µg/kg retronecine equivalents, equal to approximately 22 µg/kg PAs), PAs were detected. The detected concentrations ranged from 1.08 mg/kg to 16.35 mg/kg retronecine equivalents (equal to approximately 2.2 to 35 mg/kg PAs) (Kempf et al. 2010a). Kempf et al (2008) analysed 216 honeys collected in Europe and from internet stores by means of the sum parameter method. In 19 samples PAs were detected in the range of 19 to 120 µg/kg retronecine equivalents (equal to approximately 40 to 250 µg/kg PAs). A set of selected Echium and Eupatorium honeys was analysed by the sum parameter method and with an HPLC-ESI-MS/MS method in which individual PAs were quantified (Kempf et al. 2011). A good correlation was found between both methods.

116. In addition, commercial analyses in Germany of 8000 imported, raw honey samples (Raezke, 2010, see ANNEX VI), revealed that echimidine, lycopsamine and lycopsamine-N-oxide are the PAs most often found in honey. The average PA amount in raw honey samples was 36 µg/kg, while the maximum found was 3.3 mg/kg. In 4 different surveys of German retail honeys the average PA amount detected was between 9.1 and 22.9 µg/kg, while the maximum found varied between 31 and 150 µg/kg.

Feed

117. IPCS did not present any information on PA levels in feed in their evaluation (WHO, 1988).

118. In the EFSA evaluation, the data available for farm animal species did not allow tolerance levels to be set for individual PAs in feed materials. The exact level of exposure of livestock could not be estimated due to the high variability in the content of alkaloids in plants, as well as the variability in animal diets (EFSA, 2007).

Recent studies

119. Mulder et al., (2009) conducted a survey on the occurrence of pyrrolizidine alkaloids in animal forage produced in The Netherlands, in the period 2006-2008. Categories of animal forage sampled were grass silage, hay, (artificially) dried grass and alfalfa. In total 147 samples were analysed for PA content by LC-MS/MS. The focus was on PAs typical for tansy ragwort (*Jacobaea vulgaris*), narrow-leafed ragwort (*Senecio inaequidens*) and common groundsel (*Senecio vulgaris*). Detected PA amounts ranged from less than detection limit (10 µg/kg) up to 5401 µg/kg for alfalfa. Mean concentrations ranged between less than detection limit and 476 µg/kg. High PA concentrations were found in alfalfa, with an average of 455 µg/kg, which is 30 times or more the average concentration obtained for silage, dried grass and hay.

120. A comparison with samples of reference plants revealed that in most instances the forage samples were contaminated with common groundsel (*Senecio vulgaris*). Only in two occasions was there evidence for other ragwort species (*Senecio* spp.) present in the forage. The contaminated hay sample contained PAs that could not be directly linked to one of the three ragwort species included in this study, but a related ragwort species appeared likely. The contaminated dried grass sample contained a mix of mostly common groundsel together with a smaller amount of tansy ragwort (*Jacobaea vulgaris*).

Analysis of the occurrence data

121. A first analysis has been made of the major plants and PAs found in studies describing PA contamination of different feed and food categories in mostly recent studies. The results are summarized in Table 4 below. It should be noted that this analysis is influenced by the earlier mentioned limited availability of analytical reference standards. It might be possible that other PAs for which no reference standards were available (or used) are of relevance too.

Table 4: Plants and their PAs found in different commodities of food and feed

Food /feed	Commodity	Plant Genera/ species	Major PAs	References (selection)
Food (or traditional medicine)	Herbal remedies and (bush) teas	*Symphytum* *Tussilago* *Senecio* *Crotalaria* *Heliotropium*	(Acetyl)lycopsamine (Acetyl)intermedine Symphytine Echimidine Adonifoline Monocrotaline Fulvine Senecionine Senkirkine Heliotrine	Roeder, 1995 Roeder, 2000 Coulombe, 2003 Fu et al, 2007 Wiedenfeld and Edgar, 2010
Food	Wheat, cereals	*Heliotropium* *Crotalaria* *Senecio*	Heliotrine Lasiocarpine Europine Monocrotaline Trichodesmine Senecionine	Stewart and Steenkamp, 2001 Coulombe, 2003 Wiedenfeld and Edgar, 2010
Food	Salads	*Senecio vulgaris*	Senecionine Seneciphylline Retrorsine	BfR, 2007
Food	Milk	*Senecio jacobaea*	Jacoline	Dickinson et al, 1976 Hoogenboom et al, 2011
Food	Eggs	*Senecio* *Heliotropium*	Senecionine Heliotrine	Edgar and Smith, 2000
Food	Honey Honey pollen	*Echium* *Cynoglossum* *Borago* *Eupatorium* *Symphytum* *Senecio*	(Acetyl)lycopsamine (Acetyl)echimidine Senecionine Seneciphylline Retrorsine	Edgar et al, 2002 VWA, 2007 Kempf et al, 2008 Raezke et al, 2010a
Animal feed	Hay, grass, compound feed	*Senecio* *Echium* *Heliotropium* *Crotalaria* *Amsinckia*	Senecionine Seneciphylline Retrorsine Jacobine Erucifoline Lycopsamine Echimidine Heliotrine Monocrotaline Retusamine	EFSA, 2007 Mulder et al, 2009 Wiedenfeld and Edgar, 2010 Fletcher et al, 2011
Animal feed	Alfalfa	*Senecio vulgaris*	Senecionine Seneciphylline Retrorsine	Mulder et al, 2009

122. Combining the above available data results in a list of mostly detected PAs which is presented below[2]. It should be noted that PAs of importance will vary according to the country/region and the prevalence of PA containing plants. Much of the data included in the table above comes from Europe which may have limited relevance to other regions. Moreover, this list may be incomplete as there have been no contributions from other major grain and meat producing nations. Also it should be noted that this list is based on occurrence only, toxicity has not been taken into account.

[2] Although herbal remedies are included in this discussion paper for information purposes, these are considered medicinal applications and therefore fall outside the scope of CODEX and the related PAs are not included in the list of PAs to be evaluated.

In food:	In feed:
(Acetyl)echimidine	Echimidine
(Acetyl)lycopsamine	Erucifoline
Europine	Heliotrine
Heliotrine	Jacobine
Jacoline	Lycopsamine
Lasiocarpine	Monocrotaline
Monocrotaline	Retrorsine
Retrorsine	Retusamine
Senecionine	Senecionine
Seneciphylline	Seneciphylline
Trichodesmine	

ANIMAL MEDIATED CONTAMINATION OF FOOD

Milk

123. Most studies on carry-over of PAs from feed to milk have been performed over 20 years ago. Evaluations of risk assessment bodies mostly refer to the studies of Schoental, 1959, Dickinson et al., 1976, Eastman et al., 1982, Goeger et al., 1982, Panter and James, 1990, and Molyneux and James, 1990.

124. IPCS concluded in 1988 that PAs are transmitted from the feed into milk based on the studies in rats, goats and cows, and can cause toxic damage via this route in the suckling young. However, they stated that 'no reports of cases of acute toxicity caused by consumption of contaminated dairy products were available to the Task Group' (WHO, 1988).

125. Based on studies devoted to the carry-over of PAs from feed into edible tissues of farm animals (dairy cows and lactating sheep), it is likely that no more than about 0.1% of the ingested alkaloid base will be excreted in milk. PAs and PANOs are known to be excreted in cow's milk, therefore milk can be a relevant source of PAs when obtained from a single animal which has ingested considerable amounts of PAs. Due to milk bulking, it is unlikely that significant exposures would come from this source. In relation to human milk, PAs had been found in human milk during PA poisoning epidemics and cases of veno-occlusive disease have occurred in both neonates and other infants by this means (ANZFA, 2001, EFSA, 2007).

126. In a UK survey, 21 retail bulked samples of milk were analysed. Milk samples were taken from an area with highest reported incidence of ragwort poisoning in cattle in the 2 years previous to the survey. No senecionine, seneciphylline or jacobine were detected in any sample and it was concluded that detectable levels were unlikely to be present elsewhere in the UK. COT stated that it was common commercial practice in the UK to bulk milk samples from all the cows at one farm and then also at the dairy, which results in dilution of the PAs if present (MAFF, 1988, cited by COT, 2008).

127. In a study not included in the above risk assessments, the transfer of radiolabeled PAs into cow's milk was investigated. One dairy cow received a single oral dose of 1 mg/kg bw radiolabeled seneciphylline. Blood levels of seneciphylline reached a maximum value of 120 ng / mL within 1 hour (calculated from radioactivity levels), remained in this range for about 20 hours, and then decreased rapidly to 11 ng/mL after 54 hours. Milk levels were found to be 11 ng/mL after 2 hours and reached a maximum of 102 ng/mL 14 hours later. Sixty-four hours after administration, still 5 ng/mL could be detected. The total calculated amount of seneciphylline found in milk was 900 µg or 0.16% of the dose given. At 16 hours, 11.3% of the radioactivity was extractable as free alkaloids and 2.9% as N-oxides. At 27 hours, these values were 15.1% and 11.2%, respectively (Candrian et al., 1991).

Recent study

128. Hoogenboom et al. (2011) studied the possible transfer of PAs from contaminated feed to milk in cows. To investigate the possible transfer of PAs from contaminated feed to milk, cows were put for 3 weeks on a ration with increasing amounts (50-200 g/day) of dried ragwort. Milk was collected and sampled twice a day; faeces and urine twice a week. For milk, a dose-related occurrence of PAs was found. Jacoline was the major component in milk despite being a minor component in the ragwort material. Practically no N-oxides were observed in milk, notwithstanding the fact that they constituted over 80% of the PAs in ragwort. The

overall carry-over of the PAs was estimated to be only around 0.1%, but for jacoline 4%. The authors stated that analysis of the faeces and urine samples indicated that substantial metabolism of PAs was taking place.

Meat and organs

129. The possible presence of PAs or their metabolites in the meat of animals fed PA-containing material before slaughter was evaluated previously (WHO, 1988, ANZFA, 2001, EFSA, 2007 and COT, 2008), and the possibility of toxicity being caused through this medium was considered to be low.

130. In livers and kidneys for human consumption from domesticated animals, PA levels (unknown if PA-adduct or unbound) of <0.010 to 0.073 mg/kg were found (ANZFA, 2001).

131. In the study of Candrian et al. (1991, described under 'milk'), the dairy cow was slaughtered 21 days after the seneciphylline administration and the liver was examined. The radioactivity measured corresponded to a seneciphylline concentration of 40 ng/g in the fresh liver. This was 0.06% of the dose (1 mg/kg bw) or 340 µg of PA. No radioactivity was measured in the water removed (LOD = 0.5 ng/mL).

Recent study

132. Fletcher et al. (2011) investigated the risks from PA-containing plants for livestock and meat quality in Northern Australia. They looked at different rattlepod species (*Crotalaria* spp.), blue heliotrope (*Heliotropium amplexicaule*) and fireweed (*Senecio brigalowensis*). *C. novae-hollandiae* subsp. *novae-hollandiae* (common name unknown) chemotype 2 was fed to weaned calves (110-120 kg) for six weeks at 5.5 mg/kg bw/day. Total PAs in blood generally plateaued around days 7-28 with levels up to 150 µg/kg. Muscle and liver total PAs paralleled this trend with maximum levels up to 250 µg/kg and 2500 µg/kg, respectively. Furthermore, all PAs present in the plant were detected in tissues, but at varying levels not reflecting the relative alkaloid levels in the plant material. PA-adducts were also detected in all tissues in the order: liver > kidney ≈ heart > muscle.

133. Blue heliotrope (*Heliotropium amplexicaule*) was fed to weaned calves for six weeks at 15 mg/kg bw/day. PAs in tissues were at the LOQ (1 µg/kg) or less. PA-adducts were however detectable in the following order liver > kidney ≈ heart ≈ muscle. Also blood samples of cattle grazing among blue heliotrope (*Heliotropium amplexicaule*) on properties involved in a biological control program were taken. Indicine and heliospathine were detected at trace levels (1-3 µg/kg) in whole blood from 4 of 10 animals on one of these 6 properties. Indicine N-oxide (2 µg/kg) was detected in whole blood from only one animal on a different property, and PA-adducts were detected in almost all of these blood samples. The last study regarding blue heliotrope, was a survey of 50 cattle from 10 properties where blue heliotrope was considered a serious pest. PA-adducts were detected at trace levels in liver samples of 9 out of 10 animals from one property and one out of one from a second property, but not in livers of animals from other properties. The authors consider it very likely that, in areas such as these where animals are continually exposed to blue heliotrope, there will be some adaptation by the animal, e.g. an increased destruction of toxin in the rumen and in the liver.

134. Fireweed (*Senecio brigalowensis*) was fed to weaned calves for six weeks at 2.5 mg/kg bw/day. Alkaloids present in the plant were identified as sceleratine (predominantly present as N-oxide), senkirkine, otosenine, desacetyldorinine, florosenine and dorinine. Free PAs were detected in blood and liver, tending to plateau after 2 to 3 weeks with levels up to 90 µg/kg in blood and 400 µg/kg in liver, but then to decrease at the end of the trial to 30 and 40 µg/kg, respectively. Muscle levels followed a similar trend. The PAs identified were all of the otonecine type with neither sceleratine nor its N-oxide detected in tissue, although this was the main PA in the plant. PA-adducts were found in all tissues in the order: liver > kidney > heart ≈ muscle. In addition, a survey for PA residues in meat from cattle originating from areas where fireweed was prevalent was done. Low concentrations of PA-adducts were detected in livers of 80% of the animals assayed, at levels of approximately 1-10% of that measured in livers of calves in the feeding trial.

Eggs

135. IPCS did not report any information on possible carry-over of PAs from feed to eggs (WHO, 1988).

136. Both EFSA (2007) and COT (2008) reviewed a Turkish study on possible carry-over of PAs from feed to eggs. Free PAs were not detected in the eggs of laying hens fed up to 4% Eastern groundsel (*Senecio vernalis*). The original authors considered this may have been due to residues being below the level of detection, stated as 0.4 mg dissolved residue/ml, or the PAs being bound to egg protein, but they noted that

reduced feed intake and egg production occurred at 2 and 4% of feed levels (Eröksüz et al. 2003, cited by EFSA, 2007 and COT, 2008).

137. A contrasting study was found by COT (2008), where in eggs from chickens fed contaminated wheat containing 26 mg/kg of PAs (heliotrine, europine and lasiocarpine), up to 0.168 mg/kg PAs were detected (Edgar and Smith, 2000, cited by COT 2008). As the original study could not be obtained, it is unknown if the PAs were present as PANOs in the feed and/or animal tissues.

Recent study

138. Eröksüz et al. (2008) performed a carry-over study of PAs in quail. In all, 160 Japanese quail (80 male and 80 female) were divided into 4 groups (3 test groups and 1 control group). The test groups were fed a diet containing aerial parts (leaves, stems, and flowers) of Eastern groundsel (*Senecio vernalis*, SV group), *Heliotropium dolosum* (HD group, common name unknown), or *Heliotropium circinatum* (HC group, common name unknown) at the level of 30% for 6 weeks, and the control group was fed 0% in order to evaluate parental and progenial toxicity, along with the transference of alkaloid residues to their eggs. The PA content in the feed was 390 mg/kg in the HD group, 450 mg/kg in the HC group, and 420 mg/kg in the SV group. No clinical signs or death occurred in the test groups; however, egg production and hatchability significantly decreased in all test groups, as compared to the control group. In spite of the occurrence of specific biochemical and histopathological changes in parental quail, no remarkable changes were observed in their progeny on post-hatching days 0, 10, 20, 30, or 40. Gas chromatography and mass spectrometry (GC-MS) analysis of the eggs indicated the presence of 8.66 µg/g of the PA europine in the HD group, 19.05 µg/g of europine and 1.46 µg/g of heliotrine in the HC group, and 3.21 µg/g of senecionine in the SV group at the end of study. The authors concluded that the results of the study provided experimental evidence that alkaloids transferred to the eggs of quail fed high doses of PA-containing plant material.

Honey

139. There are many data demonstrating the presence of PAs in honey (see Section 7.3.7). IPCS (WHO, 1988) concluded from one instance of large-scale contamination that the source of PAs in the honey was nectar and pollen from a common weed rich in PAs on which the bees foraged (WHO, 1988).

140. The number of plant sources is a considerable factor in the PA content of honeys. PA-levels in honey derived from a single plant species can contain up to several µg of PAs per gram and particularly unifloral honeys are at risk, where in multifloral honeys a certain dilution reduces the PA concentrations in the end product (Edgar et al, 2002, cited by EFSA, 2007). This is supported by the findings in Australian honey, where levels of alkaloids up to 1 mg/kg have been recorded from hives where bees foraged exclusively on *Echium* spp. (ANZFA, 2001).

141. COT (2008) reviewed a Food Standards Agency funded project T01037 "Collection and Analysis of Honey Samples Potentially Contaminated with Pyrrolizidine Alkaloids from Ragwort and Borage". This project investigated the potential for PA contamination of honey if bees forage on flowers of borage (*Borago officinalis*) and tansy ragwort (*Jacobaea vulgaris*)[3]. While the PA concentrations in honey could not be quantified due to a lack of analytical standards, they could be compared from one honey sample to another and relative to the amount of PAs in a fixed weight of plant material. Six locations with either tansy ragwort or borage occurrence were sampled several times throughout the 2005 season. Concerning tansy ragwort, it was concluded that even under unfavorable conditions this plant would be of no concern, since neither pollen analysis nor analytical results showed significant amounts of PAs. Honeys originating from Borago sites showed significant pollen counts and could be correlated with the detection of a PA (lycopsamine or intermedine). A reliable quantification of the corresponding PAs was not achieved, however COT indicted that 'this was a preliminary project to determine whether further quantitative analysis would be required for risk assessment. A standard for lycopsamine is now commercially available and the Food Standards Agency plans to fund further work to assess the levels of PAs in borage honey' (LGC, 2007, cited by COT, 2008).

[3] It should be noted that honey produced from borage (*Borago officialis*) is often called borage honey. This should not be confused with honey produced in New Zealand from Vipers bugloss (also called Alpine borage, *Echium vulgare*), which is called borage, blue borage or alpine borage honey. Australia doesn't refer to its Echium plantagenium honey as blue borage but as Paterson's curse or Salvation Jane.

Recent study

142. In feeding experiments with honey bees (*Apis mellifera*), a mix of tertiary PAs and the corresponding N-oxides from Eastern groundsel (*Senecio vernalis*), pure monocrotaline, and 1,2-dehydromonocrotaline was tested in concentrations of 0.02, 0.2 and 2.0%. Metabolism of PAs by the bees, deterrent effects, and horizontal transfer of PAs between bees were determined (Reinhard et al., 2009). The bees were not able to detoxify PAs through N-oxidation. PANOs were detected at concentrations of >0.2%, whereas 1,2-unsaturated tertiary PAs were toxic at high concentrations. 1,2-dehydromonocrotaline revealed no toxic effects. Levels of less than 50 µg 1,2-unsaturated tertiary PAs were well tolerated, while higher levels caused mortality. Authors stated that since in this study no noticeable effects in repellency or mortality were observed in the experiments with the 0.2% PA diet, bees can deal safely with PA concentrations found in their environment (0.2% expected in flower heads). A horizontal transfer experiment showed that bees without direct PA contact contained approximately 4% (2% PA diet) and 15% (0.2% diet) of the PA load that was found in bees that had direct PA contact. The authors concluded that horizontal transfer of PA contaminated food is thus possible.

MANAGEMENT PRACTICES

143. For this discussion paper, a first survey was done of possible management practices for the prevention and reduction of contamination of food and feed with PAs. These have been summarized below; details are presented in ANNEX VII. It should be noted that the management practices described in this section and in ANNEX VII are very much focussed on ragwort species. This information was readily available and could be gathered in the limited timeframe of this electronic working group. It is provided to give an indication of possible management measures. However, for a full overview of possible risk management strategies, management practices should be gathered for other plant species and countries/regions.

144. For tansy ragwort, control is obligatory in some countries and is included in the legislation. In the UK: ‘‘Weeds Act 1959’’ and ‘‘Ragwort Control Act 2003’’, in Ireland: ‘‘Noxious Weed Act 1936’’, in New Zealand: ‘‘Biosecurity Act 1993’’, and in Friesland, province of the Netherlands ‘‘Verordening Jakobskruiskruid 2007’’ (Leiss, 2010). In Australia, various federal, state and territory legislation have been enacted for the control of plants containing pyrrolizidine alkaloids, including: ragwort (*Senecio jacobaea*); Paterson’s curse/Salvation Jane (*Echium plantagineum*); viper’s bugloss (*Echium vulgare*); blue and common heliotrope (*Heliotropium amplexicaule, H. europaeum*), fireweed (*Senecio madagascariensis*), African daisy (*Senecio pterophorus*), rattlepod (*Crotalaria* spp.) and/or yellow burrweed (*Amsinckia* spp., DSEWPC, 2010).

145. Management practices can be aimed at

- Measures for prevention of spreading of PA-containing plants, on a regional level (mainly found for ragwort). Knowledge of ecology is important here, as it is known that different PA containing plant species have different ecology, even within one genus. For instance, in a risk assessment of ragwort species, it was shown that tansy ragwort (*Jacobaea vulgaris*) is more likely to grow in damaged pastures. Common groundsel (*Senecio vulgaris*) is a typical crop weed and was detected in alfalfa crops (VWA, 2010), and as earlier indicated in this discussion paper, also found in salad (BfR, 2007a);
- Measures for prevention of contact of food producing animals with these plants, as PAs are being carried over from feed to food. This includes the transport of PA containing pollen by bees;
- Prevention of contamination of food products (such as salads) with PAs;
- Practices for reduction of PAs in contaminated feed and food.

146. It should be noted that the effectiveness of these measures has not been assessed in this discussion paper as this was beyond the scope of this electronic working group. A comprehensive analysis of all available management practices (including for other plants, food products and regions) is needed including an evaluation of their effectiveness. Their value as a component of a possible risk management strategy could then be assessed.

RESEARCH CURRENTLY UNDER WAY

Methods of sampling and analysis

147. As described in the chapter on Methods of sampling and analysis, new fast screening methods for the detection of PAs are currently being developed in the EU funded program Con*ff*idence.

148. In Germany, the BfR is developing methods for the quantitative determination of PA in food and feed aiming at the generation of occurrence data and at the evaluation of different approaches for quantification. It is intended that the validated methods will be used for monitoring.

Occurrence data

149. In The Netherlands the monitoring of PAs in animal feeds will be continued. Monitoring will include lycopsamine, echimidine and heliotrine-like PAs.

150. In The Netherlands, a survey is being conducted of PA levels in total diet studies. Results are expected in the course of 2011.

Animal mediated contamination of food

151. As indicated in the chapter on Carry over, the UK Food Standards Agency plans to fund further work to assess the levels of PAs in borage honey.

152. In The Netherlands a second carry-over study of PAs from feed to milk is being carried out in cows (end 2010). The transfer and metabolism of PAs present in tansy ragwort (*Jacobaea vulgaris*), common groundsel (*Senecio vulgaris*) and viper's bugloss (*Echium vulgare*) are being investigated. Cheese will be produced from milk containing PA metabolites to study their transfer into milk derived products.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Toxicity

153. PAs have a common toxicity profile; liver is the main target organ of toxicity. Major signs of toxicity in all animal species include various degrees of progressive liver damage (centrolobular hepatocellular necrosis), and veno-occlusive disease. Furthermore bile duct proliferation, hepatic megalocytosis, and liver fibrosis are reported. Also effects on other organs such as lungs (pulmonary hypertension), the cardiovascular system (cardiac right ventricular hypertrophy) and degenerative injury in the kidneys are seen. Acute intoxications have been described in livestock and humans, for livestock fatal cases have been seen. PAs may differ in potency, the relative potencies are currently not known due to lack of oral toxicity data on individual PAs, which hampers risk assessment for PAs.

154. There is still some discussion on the possible carcinogenicity of PAs in humans, IARC has classified three PAs, lasiocarpine, monocrotaline and riddelliine, as 'possibly carcinogenic to humans' (Group 2B).

155. IPCS concluded in 1988 that dietary exposure to PAs should be as low as possible, and other risk assessment bodies reached the same conclusion in later years. Only a few health based guidance values have been identified which are presented in the table below. Several recent case reports in humans and animals indicate that PAs nowadays still pose an actual health risk.

	Health based guidance value (HBGV)	**Value HBGV (µg/kg bw/day)**	**Reference**
1,2-unsaturated PAs	TDI	0.1* (>6 wks) 1* (<6 wks)	BfR, 1992
Total PAs	PTDI	1	FSANZ/ANZFA, 2001
Riddelliine	TDI	0.1	RIVM, 2005
Riddelliine	TDI	0.1**	COT, 2008
Riddelliine	VSD	0.00043	RIVM, 2005

Total PAs	'unlikely to be of concern for cancer risk'	0.007	COT, 2008

** in µg/person/day*

*** COT concluded that the ratio of LD50 values could be used to convert other PAs to riddelliine equivalents for comparison with this dose*

156. Data on toxicity of individual PAs and their relative potencies is still very limited. This is also the case for pharmacokinetic data and (species) comparative animal toxicity.

Analytical methods

157. Several analytical methods are available, but LC-MS is the predominant method in current use. Limits of detection for LC-MS typically range from (sub) ng/ml levels for detection in milk and honey up to several 100 ng/g in plant material.

158. The unavailability of reference standards is limiting method development and validation. Due to the lack of these standards, many papers report in equivalents of another reference standard, but the reliability of this is often unclear.

159. New and fast screening methods are being developed. New occurrence data in feed, borage honey and in total diet studies are currently being generated.

Occurrence

160. PA-containing plants, mostly comfrey, have been banned from use by humans either in food or in medicinal products. Some regional or national maximum limits have been installed for PAs in food products, 1 µg/kg in herbs (The Netherlands and Belgium).

161. Recent occurrence data are available for different foods and feeds. Foods that can be directly contaminated with PAs are contaminated cereals, salads (and possibly other crops) and herbal remedies in which PA-containing plants have been used.

162. Food products for humans that can be indirectly contaminated are honey, milk, eggs and possibly meat and offal. Animals that are in contact with PAs either through consumption of PA-containing plants or contaminated feed (livestock) or pollen (bees), pass the PAs on to their product. In feed, there are regional maximum limits for PA-containing seeds in feed, e.g. 3000 mg (100 mg for *Crotalaria* spp.) per kg (EU, 2010).

163. A first analysis of the occurrence and experimental data indicate that the PAs which are the mostly detected in food and feed are[4]

In food:	In feed:
(Acetyl)echimidine	Echimidine
(Acetyl)lycopsamine	Erucifoline
Europine	Heliotrine
Heliotrine	Jacobine
Jacoline	Lycopsamine
Lasiocarpine	Monocrotaline
Monocrotaline	Retrorsine
Retrorsine	Retusamine
Senecionine	Senecionine
Seneciphylline	Seneciphylline
Trichodesmine	

[4] Although herbal remedies are included in this discussion paper for information purposes, these are considered medicinal applications and therefore fall outside the scope of CODEX and the related PAs are not included in the list of PAs to be evaluated.

164. It should be noted that this list is based on occurrence only, toxicity has not been taken into account. Also, this analysis is influenced by the limited availability of analytical reference standards. It might be possible that other PAs for which no reference standards were available (or used) are of relevance too. In addition, it should be noted that importance of PAs will vary according to the country/region and the prevalence of PA containing plants. The above list is based on data from Europe which may have limited relevance to other regions, more data from other regions is necessary for a more accurate overview.

Management practices

165. Several management practices have been applied to reduce possible exposure; such as controlling of spreading of ragwort and preventing cattle from ingesting PA-containing plants. The management practices found were very much focussed on ragwort species and additional practices should be gathered for other plant species, food products and countries/regions for a complete overview. It should be noted that the effectiveness of the practices included in this discussion paper has not been assessed as this was beyond the scope of this electronic working group. A comprehensive analysis of all available management practices (including for other plants, food products and regions) is needed including an evaluation of their effectiveness. Their value as a component of a possible risk management strategy could then be assessed.

166. Management practices can be aimed at

- Measures for prevention of spreading of PA-containing plants, on a regional level (mainly found for ragwort spp. (*Senecio* spp.). Knowledge of ecology is important here, as it is known that different PA containing plant species have different ecology, even within one genus;
- Measures for prevention of contact of food producing animals with these plants, either directly or via dried feed, as PAs are being carried over from food to feed. This includes bees;
- Prevention of contamination of food products (such as salads) with PAs;
- Practices for reduction of PAs in contaminated feed and food.

Recommendations

Analytical reference standards

167. To gather more insight in occurrence of Pas in food and feed, the working group recommends that CCCF encourages Codex members and observers to develop more analytical reference standards for PAs and generate more occurrence data in food and feed.

Risk assessment

168. As there have been many studies performed on PAs since the evaluation of IPCS in 1988, several of which are very recent, it is recommended that the evaluation of PAs is updated by JECFA.

169. Therefore the working group recommends that CCCF request JECFA to evaluate which Pas in food and feed (via carry over to animal products) are of key interest for human health, taking into account the list of Pas as summarized in the recommendations under Para. 163, and to perform a full risk assessment for the resulting Pas. Should a full risk assessment not be possible, JECFA is requested to identify which data gaps need to be filled.

Code of practice

170. Although there were gaps in the information available on the toxicity and relative potency of individual Pas, IPCS concluded in 1988 that dietary exposure to Pas should be as low as possible. Other risk assessment bodies reached the same conclusion in later years. Although it is recognized that the JECFA results would give further guidance on effectivity of management practices, the working group recommends CCCF to already start work on the development of a ‘Codex Recommended Code of Practice for the prevention and reduction of contamination of food products with pyrrolizidine alkaloids’. For this work, a further review of existing management practices in all regions and on other PA containing plants than ragwort species would be necessary. The exact subject of the work remains to be discussed at the CCCF plenary, this could vary from weed management to management practices in honey production.

Maximum levels (MLs)

171. It was suggested by IPCS in 1988 that 'The setting of regulatory tolerance levels for certain food products may be required in some situations'. However, as there is no recent JECFA evaluation available, and there is still limited information on levels of PAs in food products, the working group recommends not to start work on an ML for PAs in food or feed.

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ANNEX I: Overview of PA-containing plants

Family:	**Genus:**	**Species:**	**English common name:**	**Pyrrolizidine alkaloids:**	**Products:**	**Reference:**
Asteraceae (Compositae)	Adenostyles	*Adenostyles alliariae*	Grey adinostyl	senecionine integerrimine (acetyl)seneciphylline spartioidine	herbal (medicinal) products or tea food supplements	Langel et al, 2011 Röder, 1995 *cited by EFSA, 2007*
	Ageratum				honey	Edgar et al., 2002 EFSA, 2009
	Brachyglottis	*Brachyglottis repanda*	Rangiora Bushman's friend	senecionine senkirkine	herbal medicinal products	WKB, 2001 Langel et al, 2011
	Chromolaena			(acetyl)rinderine	honey	Edgar et al., 2002
	Erechtites	*Erechtites hieracifolia*	Fireweed	senecionine seneciphylline		WKB, 2001 Langel et al, 2011
	Eupatorium	*Eupatorium aromaticum*	Pool-root			WKB, 2001
		Eupatorium cannabinum	Hemp-agrimony Thoroughwort	intermedine lycopsamine amabiline supinine rinderine echinatine	honey pollen	Boppré et al., 2008 Kempf et al., 2010a, 2010b Röder, 1995 *cited by EFSA, 2007* WKB, 2001 EFSA, 2009
		Eupatorium purpureum (Eutrochium purpureum)	Queen of the meadow Trumpet weed Sweet Joe-Pye weed			WKB, 2001 EFSA, 2009
	Gynura	*Gynura segetum* *Syn. Gynura japonica*		senecionine (acetyl)seneciphylline senecicannabine	food herbal product	Dai et al., 2006, 2007 Qi et al., 2009 WHO, 1988 Langel et al, 2011
	Petasites	*Petasites albus*	White butterbur	senecionine senkirkine	herbal products or tea food supplements	Langel et al, 2011
		Petasites hybridus	Common butterbur	senecionine	herbal products or tea	Chizzola et al., 2000 *cited by*

		syn. Petasites vulgaris *syn. Petasites officinalis* *syn. Tussilago petasites*	Pestilence-wort	integerrimine senkirkine	food supplements	*EFSA, 2007* Röder, 1995 *cited by EFSA, 2007* WKB, 2001 EFSA, 2009 Langel et al, 2011
		Petasites spurious	Spurious pestilence-wort	senkirkine (and non-toxic PAs)		Langel et al, 2011 Röder, 1995 *cited by EFSA, 2007*
	Senecio	*Senecio adonidifolius* *syn. Jacobaea adonidifolia*	Adonis-leaved ragwort	senecionine seneciphylline adonifoline		WKB, 2001 Langel et al, 2011
		Senecio alpinus *syn. Jacobaea alpina*	Alpine ragwort	senecionine integerrimine (acetyl)seneciphylline jacobine jacozine jaconine jacoline	feed	Langel et al, 2011
		Senecio aquaticus *syn. Jacobaea aquatica*	Marsh ragwort	senecionine senecionine integerrimine seneciphylline (acetyl)erucifoline otosenine florosenine	feed	Christov et al., 2002 *cited by EFSA, 2007* Kirk et al., 2004 *cited by EFSA, 2007* WKB, 2001 Langel et al, 2011
		Senecio aureus *syn. Packera aurea*	Golden ragwort	senecionine otosenine floridanine		WKB, 2001 Langel et al, 2011
		Senecio bicolor *syn. Senecio cineraria* *syn. Jacobaea maritima* *syn. Cineraria maritima*	Dusty miller Cineraria ragwort Silver groundsel	senecionine integerrimine seneciphylline retrorsine jacobine jacozine		Röder, 1995 *cited by EFSA, 2007* WKB, 2001 EFSA, 2009 Langel et al, 2011

				jaconine jacoline otosenine		
		Senecio brigalowensis	Fireweed	sceleratine senkirkine otosenine (deasacetyl)doronine florosenine	feed food (meat and organs)	Fletcher et al., 2011
		Senecio doronicum	Doronic ragwort	doronenine bulgarsenine	herbal product or tea food	Röder, 1995 *cited by EFSA, 2007* WKB, 2001 Langel et al, 2011
		Senecio erucifolius *syn. Jacobaea erucifolia*	Hoary ragwort	senecionine integerrimine (acetyl)seneciphylline spartioidine retrorsine (acetyl)erucifoline senecivernine jacobine jaconine		Langel et al, 2011 Witte et al., 1992 *cited by EFSA, 2007*
		Senecio ilicifolius		senecionine integerrimine seneciphylline retrorsine	food (grain)	EFSA, 2007 Langel et al, 2011 WHO, 1988
		Senecio inaequidens *syn. Senecio burchellii*	Narrow-leaved ragwort	senecionine integerrimine seneciphylline spartioidine retrorsine senkirkine otosenine florosenine floridanine doronine	feed	EFSA, 2007 Mulder et al., 2009 WHO, 1988 Langel et al, 2011

		Senecio incanus *syn. Jacobaea incana*	Grey alpine groundsel	senecionine integerrimine seneciphylline jacobine jacozine jaconine jacoline		Langel et al, 2011 WKB, 2001
		Senecio jacobaea *syn. Jacobaea vulgaris*	Tansy ragwort Common ragwort	senecionine integerrimine senecivernine (acetyl)seneciphylline spartioidine retrorsine usaramine riddelliine jacobine jacozine jacoline jaconine (acetyl)erucifoline	herbal product or tea feed (bovine urine, faeces) food (bovine, goat milk) honey pollen	COT, 2008 Crews et al., 2009 Deinzer et al., 1977 Dickinson et al., 1976, 1980 (milk) Edgar et al., 2002 Kempf et al., 2010a, 2010b Kirk et al., 2004 *cited by EFSA, 2007* Macel et al., 2004 *cited by EFSA, 2007* Mulder et al., 2009 Pelser et al., 2005 *cited by EFSA, 2007* Röder, 1995 *cited by EFSA, 2007* Vrieling and Derridj, 2003 *cited by EFSA, 2007* Walsh and Dingwell, 2007 WKB, 2001 WHO, 1988 Witte et al., 1992 *cited by EFSA, 2007* EFSA, 2009 Langel et al, 2011
		Senecio longilobus *syn. Senecio flaccidus*	Thread-leaf groundsel	senecionine integerrimine seneciphylline spartioidine retrorsine usaramine	food	Anonymous, 1988 *cited by ANZFA, 2001* Langel et al, 2011

				riddelliine		
		Senecio madagascariensis	Madagascar ragwort Madagascar groundsel Fireweed	senecionine integerrimine senecivernine retrorsine usaramine (acetyl)senkirkine otosenine florosenine doronine	feed	Gardner et al., 2006 Langel et al, 2011
		Senecio nemorensis *syn. Senecio ovatus*	Wood ragwort	senecionine fuchsisenecionine triangularine retroisosenine dororenine nemorensine	honey pollen	Boppré et al., 2008 Kempf et al. 2010b EFSA, 2009 Langel et al, 2011
		Senecio riddellii	Riddell groundsel	retrorsine riddelliine	feed	ANZFA, 2001 COT, 2008 Langel et al, 2011
		Senecio scandens	Qian li guang	senecionine seneciphylline usaramine senkirkine jacobine jacozine erucifoline adonifoline	herbal product or tea honey	Li et al., 2008 Wang et al., 2008 Zhang et al., 2008 Langel et al, 2011
		Senecio sceleratus *syn. Senecio latifolius*	Rhodesia ragwort	seneciphylline retrorsine sceleratine	feed	Cooper, 2007 Langel et al, 2011
		Senecio vernalis	Eastern groundsel	senecionine integerrimine senecivernine seneciphylline riddelliine	food (eggs) honey pollen	Eröksuz et al., 2003 *cited by EFSA, 2007* Eröksüz et al., 2008 Kempf et al., 2010a Reinhard et al., 2009

				retrorsine (hydroxy)senkirkine		Skaanild et al., 2001 *cited by EFSA, 2007* Langel et al, 2011
		Senecio vulgaris	Common groundsel	senecionine integerrimine seneciphylline spartioidine retrorsine usamarine riddelliine	herbal product or tea feed food (salad)	BfR, 2007 Frischknecht et al., 2001 *cited by EFSA, 2007* Moyano et al., 2006 Mulder et al., 2009 Röder, 1995 *cited by EFSA, 2007* WKB, 2001 EFSA, 2009 Langel et al, 2011
	Solanecio	*Solanecio gigas*		senecionine integerrimine seneciphylline spartioidine usaramine neosenkirkine bulgarsenine	herbal (medical) product honey	Asres et al., 2007
		Solanecio angulatus *Senecio subscandens*		senecionine integerrimine retrorsine	honey (pollen)	Asres et al., 2008 Langel et al, 2011
		Solanecio mannii *syn. Senecio mannii*		no toxic PAs identified	herbal product	Asres et al., 2008
		Solanecio tuberosus		senecionine integerrimine eruciflorine seneciphylline retrosine jacobine jaconine bulgarsenine retroisosenine	herbal product	Asres et al., 2008 Langel et al, 2011
	Tussilago	*Tussilago farfara*	Coltsfoot	senkirkine		Jiang et al., 2009

				senecionine		Röder, 1995 *cited by EFSA, 2007* WKB, 2001 EFSA, 2009 Langel et al, 2011
Boraginaceae	Alkanna	*Alkanna tinctoria* *syn. Anchusa tinctoria*	Alkanet, Dyers' bugloss	triangularine		WKB, 2001 EFSA, 2009
	Anchusa	*Anchusa arvensis*	Small bugloss	echinatine?		WKB, 2001
		Anchusa italica	Italian bugloss			WKB, 2001
		Anchusa officinalis	Common bugloss	(7-acetyl)lycopsamine intermedine		Röder, 1995 *cited by EFSA, 2007* WKB, 2001 EFSA, 2009
	Borago	*Borago officinalis*	(Common) Borage	(7-acetyl)lycopsamine (7-acetyl)intermedine supinine	food honey (pollen)	ANZFSC Edgar et al., 2002 COT, 2008 Koninklijk besluit, 1997 Röder, 1995 *cited by EFSA, 2007* WKB, 2001 Wretensjö and Karlberg, 2003 EFSA, 2009
	Cynoglossum	*Cynoglossum officinale*	(Common) Hounds-tongue	(12-acetyl)heliosupine 7-angeloylheliotridine echinatine viridiflorine	herbal product or tea honey	Edgar et al., 2002 Knight et al., 1984 *cited by EFSA, 2007* Röder, 1995, 2000 *cited by EFSA, 2007* WHO, 1988 WKB, 2001 EFSA, 2009
	Echium	*Echium amoenum*		echimidine (isomer) 7-angeloyl retronecine 7-tigloylretronecine	herbal product	Mehrabani et al., 2006

		Echium plantagineum	Salvation Jane Paterson's curse	echimidine echiumine echiuplatine	honey pollen food (grain)	ANZFA, 2001 ANZFSC, FSANZ 2004 Beales et al., 2004 Betteridge et al., 2005 Boppré et al., 2008 Culvenor et al., 1981 Culvenor 1983, 1985 Edgar et al., 2002 Kempf et al., 2010b EFSA, 2009
		Echium vulgare	Vipers' bugloss	echivulgarine (acetyl)vulgarine (acetyl)echimidine leptanthine echimiplatine uplandicine echiuplatine	honey pollen food	ANZFSC Beales et al., 2004, 2007 Betteridge et al., 2005 Boppré et al., 2008 Edgar et al., 2002 Edgar and Smith, 2005 Kempf et al., 2010a, 2010b Moyano et al., 2006 EFSA, 2009
	Heliotropium	*Heliotropium amplexicaule*	Blue heliotrope	indicine heliospathine	honey feed food (meat and organs)	Beales et al., 2004 Fletcher et al., 2011 Kempf et al., 2010b
		Heliotropium arborescens *Heliotropium peruvianum*	Common heliotrope	(12-acetyl)indicine		Röder, 1995 *cited by EFSA, 2007* WKB, 2001
		Heliotropium circinatum		europine heliotrine heulerine lasiocarpine	food (eggs)	Eröksüz et al., 2008
		Heliotropium dolosum		europine	food (eggs)	Eröksüz et al., 2008
		Heliotropium europaeum	European heliotrope	lasiocarpine europine heliotrine	food (grain) herbal product or tea honey	Beales et al., 2004 Edgar and Smith, 2000 EFSA, 2007

				supinine heleurine		Kempf et al., 2010b WHO, 1988 WKB, 2001 EFSA, 2009
		Heliotropium indicum	Indian heliotrope	indicine echinatine supinine heleurine lasiocarpine		WKB, 2001 EFSA, 2009 Hartmann and Witte, 1995
		Heliotropium popovii	Charmac	heliotrine lasiocarpine	food (grain, wheat) feed (goat milk)	Kakar et al., 2010 WHO, 1988
	Lithospermum	*Lithospermum officinale*	Gromwell European stoneseed	(12-acetyl)lithosenine		Röder, 1995 *cited by EFSA, 2007* WKB, 2001 EFSA, 2009
	Myosotis	*Myosotis palustris* *syn. Myosotis scorpioides*	Water or true forget-me-not	myoscorpine (7-acetyl)scorpioidine symphytine	honey	Edgar et al., 2002 Röder, 1995 *cited by EFSA, 2007*
		Myosotis sylvatica	Wood forget-me-not	(3-acetyl)heliosupine		Hartmann and Witte, 1995
	Pulmonaria	*Pulmonaria officinalis*	Lungwort			EFSA, 2009
	Symphytum	*Symphytum asperum*	Rough comfrey Prickley comprey	asperumine echiumine symlandine symphytine myoscorpine echinatine echimidine	herbal product or tea food	Hartmann and Witte, 1995
		Symphytum officinale	Common comfrey	(7-acetyl)intermedine (7-acetyl)lycopsamine echimidine symlandine symviridine myoscorpine symphytine	herbal product or tea food	ANZFSC Couet et al., 1996 *cited by EFSA, 2007* Mei et al., 2005 Röder, 1995 *cited by EFSA, 2007* Oberlies et al., 2004 *cited by*

						EFSA, 2007 WKB, 2001 EFSA, 2009
		Symphytum tuberosum	Tuberous comfrey	amadoline (7-acetyl)lycopsamine symphytine echimidine		EFSA, 2009 Hartmann and Witte, 1995
		Symphytum x uplandicum syn. Symphytum peregrinum	Russian comfrey	echimidine (7-acetyl)intermedine (7-acetyl)lycopsamine uplandicine symlandine symviridine myoscorpine symphytine	herbal product or tea food	Röder, 1995 *cited by EFSA, 2007*
Fabaceae (Leguminosae)	Crotalaria	*Crotalaria alata*		monocrotaline fulvine	rangeland spp.	Fletcher et al., 2009
		Crotalaria aridicola		no toxic PAs identified	rangeland spp.	Fletcher et al., 2009
		Crotalaria brevis		monocrotaline fulvine	rangeland spp.	Fletcher et al., 2009
		Crotalaria crispata		monocrotaline fulvine crispatine	rangeland spp.	Fletcher et al., 2009
		Crotalaria cunninghamii		retusamine	rangeland spp.	Fletcher et al., 2009
		Crotalaria dissitiflora			rangeland spp.	Fletcher et al., 2009
		Crotalaria goreensis		no toxic PAs identified	rangeland spp.	Fletcher et al., 2009
		Crotalaria grahamiana		monocrotaline grahamine	rangeland spp.	Fletcher et al., 2009
		Crotalaria incana		integerrimine usaramine	rangeland spp.	Fletcher et al., 2009

		Crotalaria juncea.		senecionine integerrimine junceine trichodesmine	rangeland spp.	Ji et al., 2005
		Crotalaria lanceolata			rangeland spp.	Fletcher et al., 2009
		Crotalaria medicaginea		no toxic PAs identified	rangeland spp.	Fletcher et al., 2009
		Crotalaria mitchellii		retusamine crosemperine de-ethylretusamine	rangeland spp.	Fletcher et al., 2009
		Crotalaria montana		fulvine	rangeland spp.	Fletcher et al., 2009
		Crotalaria nana		crotananine cronaburmine	food	Anonymous, 1988 *cited by ANZFA, 2001*
		Crotalaria novae-hollandiae		retusamine crosemperine croaegyptine monocrotaline crispatine trichodesmine	rangeland spp. food (meat and organs)	Fletcher et al., 2009
		Crotalaria pallida		usaramine integerrimine	rangeland spp.	Fletcher et al., 2009
		Crotalaria ramosissima		fulvine monocrotaline crispatine	rangeland spp.	Fletcher et al., 2009
		Crotalaria retusa	Wedge-leaved crotalaria	monocrotaline spectabiline retusine	herbal product or tea food (grain) rangeland spp.	Anjos et al., 2010 Fletcher et al., 2009 Hooper and Scanlann, 1977 *cited by EFSA, 2007*
		Crotalaria spectabilis		monocrotaline	rangeland spp.	Fletcher et al., 2009

				spectabiline retusine		Hooper and Scanlann, 1977 *cited by EFSA, 2007* Ji et al., 2005 EFSA, 2009
		Crotalaria verrucosa		(acetyl)crotaverrine fulvine pumiline A	rangeland spp.	Fletcher et al., 2009
		Crotalaria zanzibarica		usaramine integerrimine	rangeland spp.	Fletcher et al., 2009
	Trichodesma	*Trichodesma incanum*		trichodesmine		EFSA, 2009

ANNEX II: Details on genotoxicity studies

Substance (purity)	Test	Concentration	Outcome	Reference
Riddelliine (purity confirmed) Comfrey (root extract, compound oil, leaves, consoude) Coltsfoot (root extract, tussilage) Flos farfara	^{32}P-postlabeling/HPLC analysis of DHP-derived DNA adducts in female F344 rat livers (For confirmation, in vitro rat liver microsomal metabolism was conducted, but not described)	Riddelliine: 1 nmol Comfrey root extract: equivalent to 2.28 nmol riddelliine Comfrey compound oil: eq. to 13.1 nmol riddelliine Comfrey leave, tablet: no PAs comfrey leave, pepsin: no PAs Coltsfoot root extract: eq. to 1.86 nmol riddelliine Coltsfoot tussilage: eq. to 2.66 nmol riddelliine Flos farfara: eq. to 6.66 nmol riddelliine (comfrey consoude not mentioned)	Riddelliine (pos contr):1350±127 add/10^8 nucl Comfrey root extract: 22.0±3.8 add/10^8 nucl Comfrey compound oil: 31.9±5.1 add/10^8 nucl Coltsfoot root extract: 12.9±7.3 add/10^8 nucl Flos farfara: 7.4±3.2 add/10^8 nucl No DHP-derived DNA adducts detected in comfrey leaves, comfrey consoude, or coltsfoot	Chou and Fu, 2006

			tussilage (*In vitro* metabolism displayed similar results)	
Riddelliine Comfrey (purity and precise composition unknown)	Expression of genes and processes of biological functions in rat liver were compared with microarray analysis and ingenuity analysis pathway software, respectively	1 mg/kg riddelliine (5x/week) for 12 weeks 8% comfrey root diet for 12 weeks	Comparable mutation spectra between comfrey and riddelliine Strong correlations between gene expression alterations caused by riddelliine and comfrey, especially regarding drug metabolizing genes and cancer-related genes	Guo et al. 2007
Comfrey (symphytine, 7-acetyllycopsamine, 7-acetylintermedine) (precise composition unknown)	Determination of mutant frequencies (MF) in male rat liver Type of mutations induced by comfrey compared with riddelliine (Mei et al. 2004)	2% comfrey root diet for 12 weeks	$MF_{comfrey} = 146 \pm 15 \times 10^{-6}$ $MF_{control} = 30 \pm 16 \times 10^{-6}$ No significant difference between mutation spectra (mainly G:C→T:A transversion) induced by comfrey and riddelliine Strong suggested that mutations induced by comfrey are due to PAs in comfrey	Mei et al. 2005
Comfrey (precise composition unknown)	Determination of mutant frequencies (MF) and sequence analysis in male rat liver (and compared with Mei et al. 2005)	8% comfrey root diet for 12 weeks	$MF = 139 \pm 35 \times 10^{-6}$ (comparable with 2% comfrey diet) Mutation spectrum of 8% diet differed significantly from control, but not from mutation spectrum of 2% diet	Mei et al. 2006

	Microarray analysis for studying gene expression alterations		Comfrey exposure altered the expression of genes involved in metabolism, injury of endothelial cells, and liver injury and abnormalities, including liver fibrosis and cancer development	
Riddelliine (>97%)	Microarray analysis for studying gene expression profiles of female rat livers	1 mg/kg bw (5 days/week) for 12 weeks	Genes that were altered by riddelliine mainly involved in cancer, cell death, tissue development, cellular movement, tissue morphology, cell-to-cell signaling and interaction, and cellular growth and proliferation	Mei et al. 2007a
Comfrey (symphytine, 7-acetyllycopsamine, 7-acetylintermedine) (precise composition unknown)	Determination of mutant frequencies (MF) and sequence analysis in male rat lung	8% comfrey root diet for 12 weeks	$MF_{comfrey} = 47.7 \pm 8.9 \times 10^{-6}$ $MF_{control} = 33.8 \pm 9.6 \times 10^{-6}$ Mutation spectrum of comfrey (mainly G:C→T:A transversion) differed significantly from that of control, but comparable to mutation spectrum of riddelliine and mutation spectra of comfrey found in rat liver However, other mutations were observed too suggesting also other compounds in comfrey might be involved in lung mutagenicity	Mei and Chen 2007b
Retrorsine (purity unknown)	In vitro metabolism of retrorsine in female rat liver, lung, kidney, and spleen microsomes in presence or absence of triacetylolandomycin (TAO, liver enzyme CYP3A	*In vitro* Retrorsine: 2 μmol	*Liver microsomes* Rate of DHP formation from metabolism of retrorsine in presence 1.7-fold higher than in absence of dexamethasone	Wang et al. 2005a

	inhibitor) and dexamethasone (liver enzyme inducer) ^{32}P-postlabeling/HPLC analysis of DHP-derived DNA adducts in female rat liver (in vivo) and in rat liver microsomes (in vitro)	*In vivo* 1.0 mg/kg/day for three consecutive days	DHP formation 67% (with dexamethasone) and 77% (no dexamethasone) reduced in presence of TAO Retrorsine N-oxide formation 29% (with dexamethasone) and 30% (no dexamethasone) reduced in presence of TAO The same DHP-derived DNA adducts were found with retrorsine microsomal metabolism as with riddelliine (positive control) *Extrahepatic microsomes* Retrorsine metabolizing enzyme activities from both dexamethasone-induced as control rat microsomes much lower than compared with liver microsomes *In vivo* The same DHP-derived DNA adducts were found with retrorsine in vivo metabolism as with riddelliine (positive control)	

Riddelliine (N-oxide) Retrorsine (N-oxide) Monocrotaline (N-oxide) (purities confirmed)	In vitro metabolism in female rat and human liver microsomes under oxidative and hypoxic conditions and with or without troleandomycin (TAO, liver enzyme CYP3A inhibitor) ^{32}P-postlabeling/HPLC analysis of DHP-derived DNA adducts in female rat liver (in vivo)	*In vitro* Monocrotaline N-oxide: 1.5 µmol Riddelliine N-oxide and retrorsine N-oxide: 1.4 µmol *In vivo* Riddelliine, retrorsine and monocrotaline N-oxide: 2.9 µmol/kg/day for three consecutive days	*In vitro* Both DHP and the corresponding parent PA are major metabolites of rat and human liver microsomal metabolism of PA N-oxides Under oxidative conditions, reduction of PA-N-oxide to parent compound is inhibited, while under hypoxic conditions DHP formation is decreased TAO inhibited DHP formation by >70%, while N-oxide reduction was not affected Level of microsomal enzyme activity comparable between human liver and rat liver Rate of in vitro metabolism follows the order: riddelliine ≥ retrorsine > monocrotaline *In vivo* Levels of DHP-derived DNA adducts (in vivo): Riddelliine:118±17add/10^7nucl Retrorsine:110±18add/10^7nucl	Wang et al., 2005b

		Monocrotaline: 3.0 µmol/kg/day Riddelliine N-oxide and retrorsine N-oxide: 2.7 µmol/kg/day	Monocrotaline:55.2±1add/10^7nucl Riddelliine N-oxide: 39.9±0.6add/10^7nucl Retrorsine N-oxide: 11.5±0.1add/10^7nucl Monocrotaline N-oxide: 9.2±0.1add/10^7nucl	
Riddelliine (purity unknown)	Metabolism of riddelliine by rat and human liver microsomes in presence or absence of triacetyloleandomycin (TAO, liver enzyme CYP3A inhibitor) ^{32}P-postlabeling/HPLC analysis of DHP-derived DNA adducts formed from rat and human liver microsomal metabolism	0.1 mM	Major metabolites of riddelliine were riddelliine N-oxide and DHP as a result of metabolism by both rat and human liver microsomes The kinetic parameters, V_{max} en K_m, from human liver microsomal metabolism were comparable to those from rat liver microsomal metabolism The same set of eight DHP-derived DNA adducts were detected after rat and human liver microsomal metabolism In presence of TAO, the formation of DHP and riddelliine N-oxide was reduced by 48 and 92%, respectively	Xia et al. 2003

Lasiocarpine (>99%) Riddelliine (purity unknown)	In vitro metabolism of lasiocarpine in male and female rat liver microsomes in presence or absence of ketoconazole or troleandomycin (TAO) (both liver enzyme CYP3A inhibitor) and dexamethasone (liver enzyme inducer) ^{32}P-postlabeling/HPLC analysis of DHP-derived DNA adducts formed by rat liver microsomal metabolism	0.2 mM	The same DHP-derived DNA adducts were formed by metabolism of lasiocarpine as with riddelliine (pos. control) Level of DHP-derived DNA adducts: Riddelliine:10.2±3.3add/10^6nucl Lasiocarpine:10.1±3.3dd/10^6nucl The formation of DHP by liver microsomes after dexamethasone treatment 1.6- and 3.47-fold higher for male and female rat, respectively Both ketoconazole and TAO reduced DHP formation in the range of 85-92%	Xia et al. 2006
Heliotrine Riddelliine (N-oxide) Retrorsine (N-oxide) Monocrotaline (purity unknown)	^{32}P-postlabeling/HPLC analysis of DHP-derived DNA adducts formed from male rat liver microsomal metabolism	0.2 mM	Same set of DHP-derived DNA adducts was formed from metabolism of heliotrine compared with riddelliine Relative levels of DHP-DNA adduct formation: Riddelliine:38.5±23.2add/10^8nucl ≈ Lasiocarpine > Retrorsine:196±22.8add/10^8nucl > Monocrotaline:88.7±2.7add/10^8nucl ≈ Retrorsine N-oxide: 76±8.5add/10^8nucl ≥ Riddelliine N-oxide:66±10.8add/10^8nucl >	Xia et al. 2008

			Heliotrine 23.1±8.3add/10^8nucl	
Retronecine (N-oxide) Riddeliine (N-oxide) Dehydroretronecine (DHR/ (DHP)) (purity unknown)	^{32}P-postlabeling/HPLC analysis of DHP-derived DNA adducts formed from female rat liver microsomal metabolism (in vitro) and from metabolism in vivo	*In vitro* Riddelliine, riddelliine N-oxide, retronecine, retronecine N-oxide: 0.31 mg in 50 µL DMSO *In vivo* Riddelliine and riddelliine N-oxide: 3 µmol/kg/day for three consecutive days Retronecine and retronecine N-oxide: 6 µmol/kg/day for three consecutive days	The metabolism of retronecine, retronecine N-oxide, riddelliine, and riddelliine N-oxide in vitro and in vivo resulted in comparable DHP-DNA adduct profiles Relative levels of DHP-DNA adduct formation: DHR > riddelliine > riddelliine N-oxide >> retronecine > retronecine N-oxide	Yan et al. 2008
Riddelliine (purity unknown)	In vitro metabolism in female rat liver microsomes in presence or absence of Phenobarbital (metabolic activation) ^{32}P-postlabeling/HPLC analysis of DHP-derived DNA adducts formed from female rat liver microsomal metabolism (in vitro) and in vivo metabolism	*In vitro* 2 µmol in 50 µL DMSO *In vivo* 0.01, 0.033, 0.1, 0.33, 1.0 mg/kg/day (5 days/week) for 3 or 6 months	*In vitro* Metabolism of riddelliine was enhanced after phenobarbital treatment The same eight DNA adducts were formed after riddelliine metabolism as after DHR reaction with DNA *In vivo* A similar DNA adduct profile was found in vivo as in vitro A dose-response relationship was obtained between the dose and level of total DNA adducts	Yang et al. 2001

ANNEX III: Extraction methods for PAs in plants, food and feed

Product	Extraction method	Remarks	Reference
Plant			
Tansy ragwort	At room temperature by 2% formic acid solution in a 1.2:100 weight/volume ratio for 1 h and by 0.25 M sulphuric acid in a 1.5:100 weight/volume ratio for 1 h. Comparable extraction yields were obtained for both solvents.	Used in combination with two detection methods (GC-NPD and LC-MS/MS). Formic acid has as advantage that it is compatible with LC-MS/MS analysis	Joosten et al., 2010
Common groundsel	0.05M sulphuric acid in 30 min at room temperature		Hartmann and Toppel, 1987
Comfrey leaves	Steeping in hot (90^{o}C) water for 5 min in a 1:100 weight/solvent ratio.	PA N-oxides were efficiently extracted by this method	Oberlies et al, 2004
Comfrey	Methanol/water 1:1 in a 1:60 weight/volume ratio at 65^{o}C for 1 hour.	The extraction was also carried out with a pressurized hot water extraction system, but this resulted in lower yields.	Liu et al., (2009)
Comfrey root powder	Methanol/water 30/70 v/v containing 5% acetic acid using an ultrasonic bath at 45^{o}C for 45 min. A 1:50 weight/solvent ratio was used.		Altamirano et al. 2005
Symphytum, Tussilago, Petasites, Emiila and Doronicum species	Methanol containing 1% tartaric acid solution for 2 h at reflux temperature Or: decoction of leaves in boiling water for 15 min in a 1:40 weight/solvent ratio.		Mroczek et al, 2002
Coltsfoot	Highest extraction yields were obtained with a mixture of methanol/water 50/50 acidified with citric acid to pH 2-3. Plant material was extracted for 15 min at reflux temperature in a 1:60 weight/volume ratio.	Different extraction solvents (water, methanol, and in combination with acidic and alkaline modifiers) were tested to optimise the extraction of PAs	Lebada et al., 2000
Coltsfoot	Best results were obtained with a mixture of methanol/water 1:1, acidified with hydrochloric acid to pH 2-3.	Comparison of microwave-assisted extraction (15 min), pressurized hot water extraction (50 min) and extraction under reflux (60 min). For all methods similar recoveries were obtained.	Jiang et al ., 2009
Qianliguang (Chinese herbal medicine)*	Ultrasonic extraction using 0.2% HCl for 40 min in a 1.2:100 weight/solvent ratio.		Zhang et al., 2008
Chinese herbal medicines*	Methanol/water 1:1, acidified with citric acid to pH 2-3 for 30 min at reflux temperature in a 1:60 weight/volume ratio.		Yu et al., 2005
Gynura segetum (traditional Chinese	Methanol/water 1:1, acidified with citric acid (pH 2-3) at room temperature for 30 min with a 1:50		Qi et al., 2009a

medicine)*	weight/solvent ratio.		
Honey and pollen			
Honey	0.05 M sulphuric acid/methanol mixtures in a 1:1.5 weight/volume ratio.	Addition of a high percentage of methanol resulted in a precipitate.	Betteridge et al., 2005
Honey	Dilution with water in a 1:2 weight/volume ratio.		Beales et al., 2004
Honey	0.05 M sulphuric acid in a 1:1.5 weight/volume ratio.		Kempf et al., 2008
Honey	Dilution with 0.05 M sulphuric acid in a 2:1 weight/volume ratio for 1 h at room temperature.		Crews et al, 1997
Echium honey pollen	0.05 M sulphuric acid in a 1:100 weight/volume ratio for 4 h at ambient temperature.		Boppré et al., 2005
Honey pollen	0.05 M sulphuric acid in an approx. 1:30 weight/volume ratio for 16h. Pollen containing low levels of PAs were extracted in a 1:10 weight/volume ratio for 30 h.		Boppré et al., 2008
Honey	Dilution with water in a 1:4 weight/volume ratio and liquid-liquid extraction with acetonitrile after addition of QuEChERS salt.		Kempf et al., 2011
Ground pollen	0.05 M sulphuric acid to extract in a 1:15 weight/volume ratio for 24 h.		Kempf et al., 2010
Feed			
Animal forage	Extraction with 2% formic acid for 30 min at room temperature in a 1:20 weight/solvent ratio.		Mulder et al., 2009
Other			
Bovine faeces	Extraction with 2% formic acid for 30 min at room temperature in a 1:20 weight/solvent ratio.		Hoogenboom et al. 2011

*Compilations of Chinese herbal medicines containing PAs are available (Roeder, 2000; Fu et al, 2007; Roeder and Wiedenfeld, 2009).

ANNEX IV: Methods used for clean-up of extracts containing PAs

Clean-up method	Type of extract	Remarks	Reference
Liquid liquid extraction (LLE)			
Reducing agents used were zinc dust and sodium metabisulphite. LLE with dichloromethane on Extrelut columns	Tansy ragwort	LLE with chlorinated solvents such as dichloromethane requires reduction of the polar PA N-oxides to the tertiary amines prior to LLE. Somewhat higher yields were obtained with zinc dust compared to sodium metabisulphite.	Joosten et al., 2010
Zinc dust reduction of the plant extract in 0.05M sulphuric acid, followed by LLE on Extrelut column with dichloromethane	Various ragwort species		Hartmann and Toppel., 1987, Hartmann and Witte, 1992, Hartmann and Dierich, 1998
Reduction with zinc dust, followed by LLE on Extrelut column with dichloromethane/methanol (95:5)	Honey		Crews et al, 1997.
Solid phase extraction			
Strong cation exchange (SCE) SPE for PAs and PA N-oxides	Symphytum, Tussilago, Petasites, Emiila and Doronicum species		Mroczek et al, 2002
SCE cartridges used to extract the PAs and PA N-oxides from acidic aqueous extracts	Salvation Jane/ Paterson's Curse		Colegate et al., 2005
SCE cartridges used to extract the PAs and PA N-oxides from acidic aqueous extracts	Honey		Beales et al., 2004, Betteridge, 2005
SPE on SCE cartridges	Vipers' bugloss pollen and other species		Boppré et al., 2005, Boppré et al., 2008
SCE cartridges	Honey and pollen		Kempf et al., 2008, 2010
Online-SPE C18 guard columns	Honey		Kempf et al., 2011
Neutral polymeric SPE cartridges	Animal forage		Mulder et al. 2009
Neutral polymeric SPE cartridges	Bovine urine and faeces		Hoogenboom et al. 2011

ANNEX V: Separation and detection methods for Pas

	Method	Original product	PA	LOD	Remarks	Reference
GC						
Herbal product	GC-EI-MS of derivatised PAs	Borage oil and borage plant parts	PAs from *Borago officinalis*	20 ng/g		Wretensjö and Karlberg, 2003
Honey and pollen	GC-MS method. The various PAs are first reduced to their necine core structure and subsequently derivatized and analysed.	Honey and pollen	Retronecine, the necine core base of many PAs: Sum determination of PAs	3 ng/g (LOD) 10 ng/g (LOQ)	The method is not applicable to otonecine type PAs.	Kempf et al (2008, 2010)
Animal tissue	GC method. Derivatisation prior to analysis is required to volatilize the necine base.	Sheep rumen fluid	Retronecine, the necine core base of many PAs	90 ng/ml		Hovermale and Graig, 1998
LC-MS						
Herbal product	LC-MS/MS with bridged C18 RP HPLC column run at pH 11	Tansy ragwort	PAs and PA N-oxides of *Senecio jacobaea*	200-500 ng/g	Practically no sample pre-treatment required	Joosten et al (2010)
	LC-ToF-MS with C18 RP HPLC column	Tansy ragwort	PAs and PA N-oxides of *Senecio jacobaea*	Approx 1000 ng/g	Semiquantitative method to monitor degradation of PAs upon composting.	Crews and Anderson, 2009, Crews et al. 2009, Hough et al, 2010

	LC-IT-MS with C18 RP HPLC column	Coltsfoot	PAs of *Tussilago farfara*	Approx. 500 ng/g		Jiang et al, 2009
	LC-IT-MS with C18 RP HPLC column	Comfrey	PAs and PA N-oxides of Symphytum	LOQ 100 ng/g		Liu et al (2009
	LC-IT-MS with C18 RP HPLC column	Russian comfrey root	PAs and PA N-oxides of *Symphytum*	??	Method for identification of new PAs	Wuilloud et al (2004)
	LC-IT-MS with C18 RP HPLC column	Commercial comfrey products	PAs and PA N-oxides of *Symphytum*	??	Qualitative analysis	Altamirano et al (2005)
	LC-IT-MS with C18 RP HPLC column at high pH	two Onosma and Emilia species	PAs and PA N-oxides of *Emilia* and *Onosma*	??	Method for identification of new PAs	Mroczek et al, 2004
	LC-IT-MS with Aqua C8 RP HPLC column	Salvation Jane/ Paterson's curse	PAs and PA N-oxides of *Echium*	??	Method for identification of new PAs	Colegate et al (2005)
Herbal product	LC-IT-MS with C18 RP HPLC column	*Gynura segetum* (Chinese medicinal herb)	PAs and PA N-oxides of *Gynura segetum*	??	Method for identification of new PAs	Qi et al (2009)
	LC-MS/MS with C18 RP HPLC column at high pH	Qianliguang (Chinese medicinal herb)	PAs and PA N-oxides of *Senecio scandens*	690 ng/g		Li et al, 2008;

	LC-IT-MS with C18 RP HLPC column	Qianliguang	PAs and PA N-oxides of *Senecio scandens*	50-100 ng/g		Zhang et al, 2008
	LC-IT-MS with Aqua C18 RP HPLC column	Herbal products	PAs and PA N-oxides of *Symphytum* and *Senecio scandens*	1 ng/ml in extract		Cao et al (2008)
Honey and pollen	LC-MS with polymeric HPLC column	Honey	PAs of *Senecio jacobaea*	2 ng/g	PA N-oxide reduction prior to analysis	Crews et al, 1997
	LC-IT-MS with C8 RP HPLC column	Honey	PAs from *Echium* and *Heliotropium*	Approx. 1.5 ng/g	PA N-oxide reduction prior to analysis	Beales et al, 2004;
	LC-IT-MS with Aqua C18 RP HPLC column	Honey	PAs and PA N-oxides from Echium	Approx. 1.5 ng/g		Betteridge et al, 2005
	LC-IT-MS with Aqua C18 RP HPLC column	Pollen	PAs and PA N-oxides from *Echium, Eupatorium, Senecio jacobaea* and *S. ovatus* pollen	??	Method for identification of new PAs	Boppré et al, 2005; Boppré et al 2008
	LC-MS/MS with bridged C18 RP UPLC column at pH 11	Animal forage	PAs and PA N-oxides of *Senecio jacobaea, S. vulgaris* and *S. inaequidens*	5-10 ng/g		Mulder et al., 2009

Animal product	LC-MS/MS with bridged C18 RP UPLC column	Bovine milk	PAs and PA N-oxides of *Senecio jacobaea*	LOQ 0.05-0.2 ng/ml		Hoogenboom et al., 2011
Other	LC-ToF-MS and LC-IT-MS with C18 RP HPLC column	Rat serum, urine and bile	Metabolites of PAs from *Senecio scandens*	LOQ 0.6-1.2 ng/ml	Method for identification of new PAs and PA metabolites	Xiong et al, 2009a, 2009b
	LC-MS/MS with bridged C18 RP UPLC column run at pH 11	Bovine urine, faeces	PAs and PA N-oxides of *Senecio jacobaea*	LOQ 0.2-0.5 ng/ml		Hoogenboom et al., 2011

ANNEX VI: Occurrence data of PAs in food groups

	PA plants (tested PA)	Product	Origin	Mean concentration	Minimal concentration	Maximal concentration	Analytical method used (LOD/LOQ)	Reference
Feed and rangeland spp.	Total PA	Grass silage	The Netherlands	<10 µg/kg	-	28 µg/kg	LC-MS/MS based (LOD= 10 µg/kg)	Mulder et al., 2009
		Prewilted silage		<10 µg/kg	-	<10 µg/kg		
		Hay		<10 µg/kg	-	<10 µg/kg		
		Hay nature reserve		22 µg/kg	-	549 µg/kg		
		Dried grass		<10 µg/kg	-	<10 µg/kg		
		Pellets, crumb grass		21 µg/kg	-	288 µg/kg		
		Dried/hay alfalfa		411 µg/kg	-	3524 µg/kg		
		Pellets, crumb alfalfa		476 µg/kg	-	5401 µg/kg		
	Madagascar ragwort (*Senecio madagascariensis*) (Total PA)	Stems, leafs, flowers	Hawaii, Maui, Hawaiian Islands	874 µg/g	217 µg/g	1990 µg/g	GC-MS (LOD and LOQ unknown)	Gardner et al., 2006
	Sunn hemp (*Crotalaria juncea*) (Total PAs of which only junceine and trichodesmine were detected)	Seeds	Southeast Asia, South America, Africa, Europe	1.87 µmol/g	0.8 µmol/g	3.8 µmol/g	HPLC-UV-ELSD (LOD= 40 µg), HPLC-UV-MS (LOD= 1 µg), NRM spectroscopy	Ji et al., 2005
	Showy rattlebox (*Crotalaria spectabilis* Roth.)		Nigeria	96 µmol/g	-	-		
			USA	60 µmol/g				

	Crotalaria taxa (Total PA) *C. alata* Buch.-Ham. ex D.Don	Plant material	Queensland, Northern Territory, Western Australia	(PA content in retrorsine equivalents) 0.03 mg/g	0 mg/g	0.06 mg/g	GC-MS (LOD and LOQ unknown)	Fletcher et al., 2009
	C. aridicola subsp. *densifolia* A.E.Holland			13.6 mg/g	7 mg/g	22 mg/g		
	C. brevis Domin			0.03 mg/g	0 mg/g	0.15 mg/g		
	C. crispata F.Muell. ex Benth.			7.7 mg/g	3 mg/g	21 mg/g		
	Crotalaria cunninghamii R.Br. subsp. *cunninghamii*			0.06 mg/g	0.04 mg/g	0.08 mg/g		
	C. dissitiflora Benth. subsp. *dissitiflora*			<LOD	<LOD	<LOD		
	C. goreensis Guill. & Perr.			4.5 mg/g	0.3 mg/g	14 mg/g		
	C. grahamiana Wight & Arn.			1.3 mg/g	1.3 mg/g	1.3 mg/g		
	C. incana L. subsp. *incana*			0.04 mg/g	0.04 mg/g	0.04 mg/g		
	C. incana subsp. *purpurascens* (Lam.) Milne-Redh.			0.03 mg/g	0.03 mg/g	0.03 mg/g		
	C. lanceolata E.Mey. subsp. *Lanceolata*			<LOD	<LOD	<LOD		
	C. medicaginea var. *neglecta* (Wight & Arn.) Baker (chemotype 1)			2.2 mg/g	0.7 mg/g	4 mg/g		
	C. medicaginea var. *neglecta* (Wight & Arn.) Baker (chemotype 2)			4.6 mg/g	0 mg/g	11 mg/g		

	C. medicaginea var. *neglecta* (Wight & Arn.) Baker (chemotype 3)			6.8 mg/g	6.8 mg/g	6.8 mg/g		
	C. mitchellii Benth. subsp. *mitchellii*			0.5 mg/g	0.3 mg/g	0.7 mg/g		
	C. montana var. *angustifolia* (Gagnep.) Niyomdham			0.1 mg/g	0 mg/g	0.3 mg/g		
	C. montana var. *exserta* (Domin) A.E.Holland			<LOD	<LOD	<LOD		
	C. novae-hollandiae subsp. *crassipes* (Hook.) A.E.Holland			2.3 mg/g	2.3 mg/g	2.3 mg/g		
	C. novae-hollandiae subsp. *lasiophylla* (Benth.) A.T.Lee			0.2 mg/g	0 mg/g	0.6 mg/g		
	C. novae-hollandiae DC. subsp. *novae-hollandiae* (chemotype 1)			0.6 mg/g	0.1 mg/g	1.4 mg/g		
	C. novae-hollandiae DC. subsp. *novae-hollandiae* (chemotype 2)			6.0 mg/g	0.3 mg/g	23 mg/g		
	C. novae-hollandiae DC. subsp. *novae-hollandiae* (chemotype 3)			0.4 mg/g	0.2 mg/g	0.7 mg/g		
	Crotalaria pallida var. obovata (G.Don) Polhill			0.1 mg/g	0 mg/g	0.2 mg/g		
	C. ramosissima Roxb.			22.3 mg/g	11 mg/g	71 mg/g		
	C. retusa L. var. *retusa*			12.2 mg/g	0.5 mg/g	31 mg/g		
	Crotalaria spectabilis Roth.			0.6 mg/g	0.6 mg/g	0.6 mg/g		

	Crotalaria verrucosa L.			0.01 mg/g	0 mg/g	0.05 mg/g		
	Crotalaria zanzibarica Benth.			0.3 mg/g	0.3 mg/g	0.3 mg/g		
Herbal remedy	*Senecio scandens* (Adonifoline)	Herb, flower, slim stem, thick stem, root, leaf	Different localities in China	32.56 µg/g (herb)		109.9 µg/g (herb)	HPLC-MS (LOD= 0.5 ng/ML; LOQ= 1.0 ng/mL)	Zhang et al., 2008
				261.6 µg/g (flower, n=1)	4.000 µg/g (herb)			
				85.90 µg/g (slim stem, n=1)				
				43.81 µg/g (thick stem, n=1)				
				31.48 µg/g (root, n=1)				
				15.61 µg/g (leaf, n=1)				
Cereal	Charmac (*Heliotropium popovii*) (Heliotrine (N-oxide), lasiocarpine)	Flour (contaminated)	Western Afghanistan	Heliotrine 0.16 mg/kg Heliotrine N-oxide 5.4 mg/kg Lasiocarpine 0.045 mg/kg			LC/MS/MS	Kakar et al., 2010
		Flour (control)	Western Afghanistan	Heliotrine 0.07 mg/kg Heliotrine N-oxide 2.6 mg/kg Lasiocarpine 0.025 mg/kg				Kakar et al., 2010
Honey	Eastern groundsel (*Senecio vernalis*) (Total PAs)	Pollen, flowers or flower heads	Germany, From market places and drug stores in Europe, via internet from USA, Mexico, New Zealand, Asia	4.1 mg/g (pollen) 2.3 mg/g (flowers or flower heads)		5.2 mg/g (pollen, flowers)	GS-MS (floral pollen) High resolution GC-MS (pollen products; LOD= 0.003 µg/g; LOQ= 0.01 µg/g)	Kempf et al., 2010a
	Tansy ragwort (*Jacobaea vulgaris*) (Total PAs)			3.3 mg/g (pollen) 3.4 mg/g (flowers)		-		
	Hemp-agrimony (*Eupatorium cannabinum*) (Total PAs)			0.6 mg/g (pollen) 4.2 mg/g (flowers)	2.9 mg/g (pollen) 1.0 mg/g (flowers)	-		

	Vipers' bugloss (*Echium vulgare*) (Total PAs)			0.9 mg/g (pollen) 2.0 mg/g (flowers)	-	-		
	Orchids (*Phalaenopsis hybrids*) (Total PAs)			0.6 mg/g (pollen) 4.4 mg/g (flowers)	-	-		
	Pollen products (Total PAs)			5.17 µg/g	-	16.35 µg/g		
	Tansy ragwort (*Jacobaea vulgaris*) (Total PAs)	Honey	-	3.4 µg/g	-	3.9 µg/g	GC-MS (Deinzer et al., 1977)	Kempf et al., 2010b
				0.02 µg/g	1.08 µg/g	0.06 µg/g	LC-MS (MAFF, 1995; Crews et al., 1997)	
				<0.002 µg/g	0.3 µg/g	-		
				0.95 µg/g	-	1.5 µg/g		
	Salvation Jane/ Paterson's Curse (*Echium plantagineum*) (Total PAs)			0.58 µg/g	-	0.95 µg/g	GC-MS (Culvenor et al., 1981)	
				0.91 µg/g	-	2.63 µg/g	LC-MS (Beales et al., 2004; Beales et al., 2007)	
				0.27 µg/g	-	1.03 µg/g		
	Blue heliotrope (*Heliotropium amplexicule*) (Total PAs)			0.81 µg/g	-	1.65 µg/g	LC-MS (Beales et al., 2004; Beales et al., 2007)	
	European heliotrope (*Heliotropium europaeum*) (Total PAs)			0.19 µg/g	-	0.25 µg/g	LC-MS (Beales et al., 2004; Beales et al., 2007)	
	Vipers' bugloss (*Echium vulgare*) (Total PAs)			0.39 µg/g	-	1.3 µg/g	LC-MS (Beales et al., 2004; Beales et al., 2007)	
	Vipers' bugloss (*Echium vulgare*)			0.79 µg/g	-	2.85 µg/g	LC-MS (Betteridge et al.,	

	(Total PAs)						2005)	
	Tansy ragwort (*Jacobaea vulgaris*) (Total PAs)	Floral pollen, pollen loads	-	0.175 mg/g (floral pollen, senecionine eq.)	-	-	GC-MS (Budde et al., 2004)	
				0.8 mg/g (floral pollen, lasiocarpine eq.)	-	-	LC-MS (Boppré et al., 2008)	
				0.1 mg/g (pollen loads, lasiocarpine eq.)	-	-		
	Salvation Jane/ Paterson's Curse (*Echium plantagineum*) (Total PAs)			0.028 mg/g (pollen loads, lasiocarpine eq.)	-	-	LC-MS (Boppré et al., 2008)	
				0.006 mg/g (pollen loads, lasiocarpine eq.)	-	-		
	Vipers' bugloss (*Echium vulgare*) (Total PAs)			8.2 mg/g (floral pollen, lasiocarpine eq.)	-	-	LC-MS (Boppré et al., 2005)	
				14 mg/g (floral pollen, lasiocarpine eq.)	-	-		
				0.35 mg/g (pollen loads, lasiocarpine eq.)	-	-	LC-MS (Boppré et al., 2008)	
	Hemp-agrimony (*Eupatorium cannabinum*) (Total PAs)			0.120 mg/g (pollen loads, lasiocarpine eq.)	-	-	LC-MS (Boppré et al. 2008)	
	Alpine ragwort (*Senecio alpinus*) (Total PAs)			0.155 mg/g (floral pollen, lasiocarpine eq.)	-	-	LC-MS (Boppré et al. 2008)	
				0.07 mg/g (pollen loads, lasiocarpine eq.)	-	-		
	Total PA (≥2 µg/kg)	Honey	The Netherlands	6.8 µg/kg	-	365 µg/kg	LC-MS/MS based (LOD= 2 µg/kg) (van Rhijn, 2007)	RIVM, 2007 (VWA)

	Total 16 PAs and N-oxides (≥1 µg/kg)	Honey (raw)	Imported	36 µg/kg	-	3.3 mg/kg	LC-MS/MS (LOD = 0.5 µg/kg; LOQ = 1 µg/kg)	Raezke, unpublished data 2010; Kempf et al., 2011
	Total 16 PAs and N-oxides (≥1 µg/kg)	Honey (retail)	Germany	9.1 - 22.9 µg/kg (4 surveys)	-	31 – 150 µg/kg	LC-MS/MS (LOD = 0.5 µg/kg; LOQ = 1 µg/kg)	Raezke 2010a, unpublished data Intertek Food Services GmbH, 2010
Milk	Tansy ragwort (*Jacobaea vulgaris*) (0.16% PA, 10 mg/kg/day)	Milk (cows)	-	0.840 mg/L (highest mean)	-	-	-	Dickinson et al., 1976, cited by WHO 1988
	Tansy ragwort (*Jacobaea vulgaris*) (0.16% PA, 10 mg/kg/day)	Milk (goats)	-	0.381 mg/L	<2 µg/kg	0.530 mg/L	-	Dickinson et al., 1980, cited by WHO 1988
Meat	*Crotalaria novae-hollandiae* subsp. *novae-hollandiae* Chemotype 2 (Total PA and adducts)	Liver, kidney, heart, muscle (calves)	Northern Australia	-	-	250 µg/kg (muscle) 2500 µg/kg (liver) (kidney and heart in between)	LCMSMS	Fletcher et al., 2010
	Blue heliotrope (*Heliotropium amplexicaule*) (Total PA and adducts)	Liver, kidney, heart, muscle (calves)	Northern Australia	-	-	<LOQ (1 µg/kg)	LCMSMS	

	Fireweed (*Senecio brigalowensis*) (Total PA and adducts)	Liver, kidney, heart, muscle (calves)	Northern Australia	-	-	400 µg/kg after 3 weeks, 40 µg/kg after 6 weeks (liver) (kidney, heart, muscle below that)	LCMSMS	
	Total PA	Liver and kidney (domesticated animals)	-	-		73 µg/kg	-	Edgar, unpublished data, cited by ANZFA 2001
Eggs	Total PA in wheat	Eggs (chicken)	-	-	-	0.168 mg/kg	-	Edgar and Smith, 2000, cited by COT 2008

ANNEX VII Management practices

Management practices can be aimed at

- Measures for prevention of spreading of PA-containing plants, on a regional level (mainly found for ragwort species);
- Measures for prevention of consumption of these plants by livestock, as PAs are being carried over from food to feed;
- Practices for reduction of PAs in contaminated feed and food.

Details on management practices found have been summarized below. The measures found for weed management are mainly aimed at the control of tansy ragwort.

Prevention of spreading of PA-containing plants

The principal control measure is weed control in accordance with Good Agricultural Practices. According to FAO (FAO, 2007, cited by FAO, 2010), methods most used for weed control include:

- Preventative methods (legal and quarantine procedures, and others at the farm level);
- Cultural methods (crop rotation, land preparation, use of cover crops, polycropping, mulching, water management);
- Mechanical weeding (manual or using machines);
- Chemical methods (use of herbicides);
- Biological methods (classical methods through the introduction of exotic natural enemies and increasing the population of already existing natural enemies);
- Other non-conventional methods (soil solarization, use of hot water, and others in development).

Preventative weed control

Prevention of tansy ragwort (*Jacobaea vulgaris*) requires fewer resources than the control of infestations and is therefore a good investment (McLaren & Faithfull, 2004[5]). According to the authors, prevention should include the following steps:

- Destroying isolated plants with herbicides or manually, before they set seed;
- Ensuring that hay and other fodder and planting seed is not contaminated. If contaminated fodder must be used, it should be fed out only in already infested areas or in a defined area that can be readily treated should an outbreak occur;
- Applying the same standards to soil, sand and gravel as are applied to fodder;
- Quarantine stocks that have grazed in infested areas as seed could be carried on the hooves and coats, and in the digestive tracts of livestock. Inspect quarantine area regularly for ragwort plants;
- Cleaning vehicles, machinery and equipment after being used in infested areas;
- Taking internal quarantine measures for infested properties. Produce from infested areas should be separated from that grown in clean areas. Maintain weed-free buffer zones between infested and uninfested land. Permanently or temporarily fence wet areas to exclude grazing until drier months;
- Designing areas such as gullies and shelter belts from which stock are excluded to enable effective weed treatment;

[5] This article was written based on the situation for the state of Victoria in Australia. As the practises may apply to other regions, the information was taken up in this Annex.

- Training contractors, roadside maintenance staff etc. to identify and report infestations, to treat them at the appropriate time, and to manage them in a way that will prevent spread;
- Bagging all material when removing plants in flower or seed, as seed are easily detached from the head. Seed heads and seed-contaminated material should be destroyed using fire or deep burial.

Prioritization of control measures could be done using the system as proposed by Neumann et al. (2009). Based on distance of tansy ragwort (*Jacobaea vulgaris*) to a field or pasture, three risk zones are identified. When the risk is classified as high (0-50 meters), immediate action on controlling ragwort (*Senecio*) species is recommended. At medium risk (50-100 meters), a control policy should ensure that changes from medium to high risk are anticipated and effectively dealt with. No immediate actions would be required at low risk (> 100 meters).

In 2004 the Department for Environment, Food and Rural Affairs (Defra) of the United Kingdom published a Code of Practice on How to Prevent the Spread of Ragwort. The aim of the CoP is to control the spread of tansy ragwort where there is an identifiable risk to vulnerable animals including through the production of forage (Defra, 2004, cited by COT, 2008).

Cultural weed control

Pastures

The infestation or presence of ragwort species in pastures may be controlled by:

- Promoting a dense, continuous and competitive pasture sward (Leiss, 2010). Swards with a high percentage of uncovered soil (>25%) carried a 40-fold higher risk on the occurrence of ragwort species than swards with less than 25% uncovered soil (Suter et al., 2007).
- Appropriate stocking densities and grazing regimes and/or irrigation and fertilization of pastures. Rotational grazing is better than continuous grazing because the latter leads to a significantly higher risk of infestations with ragwort species (Suter et al., 2007). Ruminant stock such as sheep can become 'adapted' to PA containing plants, and it has been suggested that 'adapted' sheep can be used to control such pest species as *Crotalaria retusa* (Anjos, 2010).
- Fertilization of pastures with superphosphate or urea (Thompson & Saunders, 1986 cited by Leiss, 2010). High nitrogen application, doubling nitrogen from 50 to 100 kg per hectare per year, reduces the occurrence of tansy ragwort (*Jacobaea vulgaris*) fivefold and that of marsh ragwort (*Senecio aquaticus*) threefold (Suter et al., 2007; Suter & Lüscher, 2008 cited by Leiss, 2010).
- Combining high mowing frequencies with the use of additional nitrogen. This leads to the promotion of fast growing grass species, which resist frequent defoliation and are strong competitors. This way germination and establishment of tansy ragwort (*Jacobaea vulgaris*) is strongly impaired (Crawley & Nachapong, 1985 cited by Leiss, 2010).
- Cutting tansy ragwort (*Jacobaea vulgaris*) at the start or end of anthesis, this reduces the number of flower heads with 87% (Siegrist-Maag et al., 2008 cited by Leiss, 2010). It is recommended to do the first mowing when 50% of the plants start anthesis and the second mowing when half of the re-established plants start anthesis again.

Crops

Wheat fields, millet fields, etc., should be weeded prior to planting and periodically during the first six weeks of the growth cycle. A final weeding about two weeks before harvest significantly reduces to possibility of contamination of the harvest with toxic seeds. In legume crops, mechanical or manual weeding may be the only option (FAO, 210).

In weeding, attention should be paid to areas bordering the crop or pasture, as these may constitute a reservoir for the weeds and create year-after-year problems. Long-term measures may include biological pest control but this requires extensive research and evaluation of the environmental impact of the introduced species (North West Weeds, 2007, cited by FAO, 2010).

Another effective method in controlling tansy ragwort (*Jacobaea vulgaris*) is deep ploughing of non-agricultural land followed by cultivations in summer and autumn (McLaren & Faithfull, 2004). But this is only advised when it is done systematically and when suppressing tansy ragwort which is germinating from the soil seed bank.

Mechanical weed control

Effective manual control requires removal of the crown and all larger roots. Therefore, this may be only effective for seedlings and rosettes in contrast to bigger plants, which normally develop deep roots. On the other hand, disturbance of the soil may lead to more germination since buried seeds get exposed to (sun) light.

Another form of mechanical removal is slashing or mowing. Nevertheless, tansy ragwort (*Jacobaea vulgaris*) has got the ability to grow back in a few weeks and might switch to vegetative reproduction forming multiple crowns extending their lifespan (van der Meijden & van der Waals-Kooi, 1979; Wardle, 1987 cited by Leiss, 2010).Therefore, slashing or mowing should be followed up with chemical application and/or cultivation.

Flaming killed 93% of tansy ragwort in the seeding stage whereby the burned plants did not retain their viability (Wardle, 1987 cited by Leiss, 2010).

Chemical weed control

The effectiveness of several management interventions to control ragwort (*Senecio*) species was analysed (Roberts & Pullin, 2007). Application of the herbicides 2,4-D, Asulam, Clopyralid, and MCPA is effective at reducing ragwort densities. However, when considering individual species, 2,4-D and MCPA are only effective against tansy ragwort (*Jacobaea vulgaris*), while Asulam was only effective against marsh ragwort (*Senecio aquaticus*).

Because tansy ragwort (*Jacobaea vulgaris*) has a large proportion of biomass in the crown and root system below ground level, it is a challenge to get an effective amount into the crowns and roots. For the best achievements, herbicidal control should be applied when the plants are not under stress (e.g. from drought or extreme temperatures) (McLaren & Faithfull, 2004). Furthermore, the effective concentration will be reduced when rain falls within 5 hours of application, as is observed with 2,4-D and MCPA (Coles, 1967; Forbes et al., 1980 cited by Roberts & Pullin, 2007).

Biological weed control

Natural enemies *Longitarsus jacobaeae* (ragwort flea beetle) and a combination of *Longitarsus jacobaeae* and *Tyria jacobaeae* (cinnabar moth) appear to have the potential to reduce tansy ragwort (*Jacobaea vulgaris*) densities. That is, the combination of flea beetles and the cinnabar moth reduces tansy ragwort with an average of 99.5%. The application of *Tyria jacobaeae* alone does not appear to significantly reduce tansy ragwort densities, but does reduce the number of capitula per plant, seeds per capitula, viability of seeds, and dry weight of the plants (Roberts & Pullin, 2007).

Cochylis atricapitana, a ragwort stem and crown boring moth from Europe, was released as a biocontrol agent in Australia and New Zealand. As a result, a 40% reduction in plant height of flowering plants and a significant reduction in both size and survival of rosettes have been observed (McLaren et al., 2000; Gourlay, 2007a).

The most recent used bio control agent used is the ragwort plume moth (*Platyptillia isodactyla*), which has as common host marsh ragwort (*Senecio aquaticus*). This plume moth has been released in Australia in 1999 and in New Zealand in 2006. Marsh ragwort densities were reduced by 60-80% (Gourlay, 2007b).

In Australia, biological control of blue heliotrope (*Heliotropium amplexicaule*) has been trialed. Blue heliotrope is an introduced weed in Australia of South American origin and target biological control agents were sourced from similar origin with selection based on host specificity (Briese & Walker, 2002).

Other weed control

Tansy ragwort (*Jacobaea vulgaris*) control in very poor accessible areas may be best achieved by fencing off the area or let it return to bush. Shading, plant competition and reduction in seed dispersal due to windbreak effects are the main control parameters. For plant competition, radiata pine is the most effective plantation species but eucalyptus plantations can also successfully suppress tansy ragwort (McLaren & Faithfull, 2004).

Another measure proposed for areas that are difficult to access is sheep grazing. Sheep grazing leads to a lesser number of flowering and seeding plants and may lead to a reduction in the seed bank over time. However, caution should be taken when sheep are used because of the risk on liver damage (McLaren & Faithfull, 2004).

Prevention of ingestion of PAs by livestock

Prevention of grazing

PA-containing plants are usually unpalatable to livestock. Most cases of poisoning with fresh plant material occur when pastures are overgrazed or if there is a limited supply of forage (EFSA, 2007). In the case of depletion of pastures during drought, alternative animal feeding measures should be implemented by national and local authorities to the extent of their capabilities (FAO, 2010).

Where feed is preserved, contamination with PA-containing plant material is not readily recognised by animals. Experiments carried out on hay indicate that the concentration of PAs does not decrease with storage when crops are dried (WHO, 1988).

Crotolaria and other PA-bearing plants are sometimes used as ground cover, soil improvers (rattleweed species are legumes) and as animal feed. The safe use of these plants as animal feed depends on the fact that the leaves and stalks of the plant contain less PAs than the flowering parts and the seeds. This should be taken into account when allowing animals to graze on these plants. Also, the relative susceptibility of animals to PA intoxication (cattle being less sensitive that sheep, for example) should be taken into account (FAO, 2010).

Investigations into rumen microbes with the potential to detoxify PAs has led to the suggestion of the use of a concoction of such microbes as a probiotic therapy for livestock (Lodge-Ivey et al., 2005), and that resistence in animals can be induced by prior exposure, for example by the administration of small daily doses of wedge-leaved crotalaria (*Crotalaria Retusa*) seed before the introduction of sheep into fields invaded by the plant wedge-leaved crotalaria (Anjos, 2010).

Removing PA material from feed

Sieving

The provisions in the Codex Standards for cereals and pulses (CODEX STAN 153-1985

CODEX STAN 171-1989, CODEX STAN 172-1989, CODEX STAN 199-1995, CODEX STAN 201-1995) for the presence of toxic seeds should be applied before the crop is milled or distributed for human consumption. The test method contained in the ISO standard is easily applied and does not require elaborate laboratory equipment or extensive training of operators. It can also be applied to the Codex Standard, bearing in mind the nature of the reporting. In some cases, sieving can be used to separate the PA-bearing weed seeds on the basis of size (FAO, 2010).

Composting/silage

For contaminated feed, silage may be an option for reduction of PAs. It was demonstrated that composting tansy ragwort (*Jacobaea vulgaris*) in black bin bags in the direct sunlight in the field leads to a breakdown of PAs within four weeks and a complete loss within 10 weeks (Crews et al., 2009). It is also possible to use the growth of tansy ragwort in a digester.

Awareness

One of the most significant control measures is awareness and the spread of knowledge about PAs and the implications of PA-bearing weeds and seeds in products destined for human consumption or as animal feeds. Rural radio programmes, extension services and farmers' associations should be considered as means of educating food producers about the presence of PAs in foods and feeds (FAO, 2010).

ANNEX VII

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