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28

RESIDUE EVALUATION OF CERTAIN VETERINARY DRUGS

Joint FAO/WHO Expert Committee on Food Additives

94th Meeting (Virtual) 16–27 May 2022

Food and Agriculture Organization of the United Nations

World Health Organization

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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
WORLD HEALTH ORGANIZATION
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Virtual Meeting, 16–27 May 2022

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Abbreviations

ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
ARfD	acute reference dose
AUC	area under the curve
bw	bodyweight
CAC	Codex Alimentarius Commission
CAS	Chemical Abstracts Service
CCβ	detection capability
CCRVDF	Codex Committee on Residues of Veterinary Drugs in Foods
Cl_T	total body clearance
C_{max}	maximum concentration (plasma)
CV	coefficient of variation
CVMP	Committee for Medicinal Products for Veterinary Use, European Medicines Agency
d	day
DAD	diode array detector (in HPLC)
EC	electrochemical detection (liquid chromatography)
EDI	estimated daily intake
EMA	European Medicines Agency (formerly EMEA)
EMEA	European Agency for the Evaluation of Medicinal Products
eq	equivalent
ESI	electrospray interface (in LC/MS and LC-MS/MS)
FAO	Food and Agriculture Organization of the United Nations
GC	gas chromatography
GC-MS	gas chromatography/mass spectrometry
GCP	good clinical practice
GEADE	global estimate of acute dietary exposure
GECDE	global estimate of chronic dietary exposure
GLP	good laboratory practice
GVP	good veterinary practice
h	hour

HPLC/FL	high performance liquid chromatography with fluorescence detection
i.m.	intramuscular [injection]
INN	International Non-proprietary Name
IT	ion trap (mass spectrometer)
IUPAC	International Union of Pure and Applied Chemistry
i.v.	intravenous [injection]
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
kg	kilogram (10^3 g)
L	litre
LC	liquid chromatography
LC-MS	high performance liquid chromatography/mass spectrometry
LC-MS/MS	high performance liquid chromatography/tandem mass spectrometry
LLOQ	lower limit of quantification
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LOQ	limit of quantification
LSC	liquid scintillation counter
µg	microgram (10^{-6} g)
mg	milligram (10^{-3} g)
min	minimum or minute
mL	millilitre
mM	milliMolar
MR	marker residue
MRL	maximum residue limit
MRT	mean residence time
MS	mass spectrometry
MSPD	matrix solid phase dispersion
MW	molecular weight
m/z	mass to charge ratio
ND	not detectable
NOAEL	no observed adverse effect level
NQ	non-quantifiable
NS	Not sampled/ no sample analysed

PLE	pressurized liquid extraction
QC	quality control
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
rsd	relative standard deviation
rpm	revolutions per minute
SD	standard deviation
SPE	solid phase extraction
$t_{½}$	half life
$T_{½}\beta$	plasma elimination half-life
T½Ka	half-life of distribution rate constants
t_{max}	time to peak plasma concentration
TLC	thin layer chromatography
TMDI	theoretical maximum daily intake
TR	total residue
TRR	total radioactive residues
ULOQ	upper limit of quantification
USDA	United States Department of Agriculture
UV	ultraviolet
$Vd_{(ss)}$	volume of distribution at steady state
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products
WHO	World Health Organization

Introduction

This volume of FAO JECFA Monographs contains residue evaluation of certain veterinary drugs prepared at the 94th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), held virtually, from 16 to 27 May 2022. This JECFA meeting was convened specifically to consider residues of veterinary drugs in food-producing animal species. The tasks for the Committee were to further elaborate principles for evaluating the safety of residues of veterinary drugs in food and for establishing acceptable daily intakes (ADIs) and/or acute reference doses (ARfDs), and to recommend maximum residue limits (MRLs) for substances on the agenda when they are administered to food-producing animals in accordance with good veterinary practice in the use of veterinary drugs. The enclosed monographs provided the scientific basis for the recommendations of MRLs.

Background

In response to the growing use of veterinary medicines in food animal production systems internationally and the potential implications for human health and fair-trading practices, a Joint FAO/WHO Expert Consultation on Residues of Veterinary Drugs was convened in Rome in November 1984 (FAO and WHO, 1985). One of the major recommendations of this consultation was the establishment of the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) and the periodic convening of an appropriate expert body to provide independent scientific advice to this Committee and to member countries of FAO and WHO. At its first session, in Washington, DC, in November 1986, the CCRVDF reaffirmed the need for such a scientific body and made a number of recommendations and suggestions to be considered by JECFA (FAO and WHO, 1986). In response to these recommendations, the 32nd JECFA meeting was devoted entirely to the evaluation of residues of veterinary drugs in food - a new responsibility for the Joint FAO/WHO Expert Committee on Food Additives.

94th meeting of JECFA

The present volume contains monographs on the evaluations of residue data of *four* substances scheduled for evaluation at the 94th Meeting of the Committee: imidacloprid, ivermectin, nicarbazin and selamectin. The monographs are prepared in a uniform format consistent with the data provided and the specific request for risk assessment by CCRVDF. The format includes identity of substance, residues in food and their evaluation, metabolism studies, tissue residue depletion studies, methods of residue analysis, a final appraisal of the study results, and if appropriate, recommendations on MRLs. A summary of the recommendations on compounds on the agenda and further information required is included in Annex 1. In addition, a summary of JECFA evaluations of residues of veterinary drugs in foods from the 32nd meeting to the present 94th meeting can be found in Annex 2.

The monographs of this volume must be considered in the context of the full report of the meeting, which will be published in the WHO Technical Report Series.

On-line editions of residues of some veterinary drugs in animals and foods are available online at https://www.fao.org/food-safety/resources/publications/en/, where JECFA documents can be found listed under categories. The database containing the most recent information on veterinary drugs and their residues in foods as evaluated by JECFA can be found at https://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-vetdrugs/en/.

Contact and feedback

More information on the work of the Committee is available from FAO at https://www.fao.org/food-safety/scientific-advice/en/.

Please send questions and feedback to jecfa@fao.org.

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Imidacloprid (fin fish)

First draft prepared by

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Identity

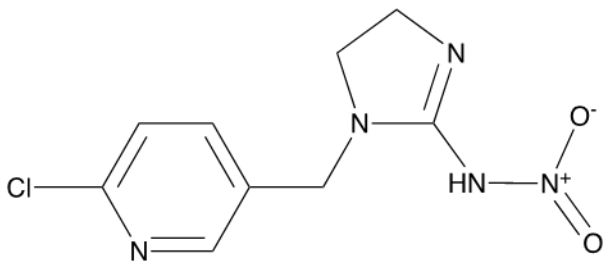
International Non-proprietary Names (INN): Imidacloprid

Synonyms: N-{1-[(6-Chloro-3-pyridyl) methyl]-4,5-dihydroimidazol-2-yl}nitramide; IV-38.

IUPAC name: (E)-1-(6-chloro-3-pyridinylmethyl)-N-nitroimidazolidin-2-ylideneamine

Chemical abstract service N°: 138261-41-3

Structural formula:



Molecular formula: $C_9H_{10}ClN_5O_2$

Molecular weight: 255.661 g mol^{-1}

Other information on identity and properties

Appearance: Colourless crystals or cream powder

Impurities: Impurity A (N-nitroguanidine): maximum 0.7 percent
Impurity B (N-[4,5-dihydro-1H-imidazol-2-yl] nitramide: maximum 0.25 percent

Melting point: 136.4–143.8°C

Solubility: 0.61 g/L (20°C) water

Organic solvents (all at 20°C):

<0.1 g/L n-hexane
0.69 g/L toluene
2.3 g/L 2-propanol
6.7 g/L ethyl acetate
50 g/L acetonitrile
50 g/L acetone
67 g/L dichloromethane
>200 g/L dimethylformamide
>200 g/L dimethylsulfoxide.

Log K_{ow}: 0.57 (21°C)

Log P_{ow}: 0.6 (24°C)

Relative density: 1.41 g/cm^3 (20°C)

Vapour pressure: 3 x 10^{12} mmHg (20°C)

Henry's constant: 1.7 x 10^{-10} Pa.m^3/mol

Refractive index: 1.713

Residues in food and their evaluation

Conditions of use

Imidacloprid is registered in a veterinary medicinal product, Ectosan Vet 1000 mg/g powder for treatment solution for fish (hereafter referred to as Ectosan) which contains 100 percent imidacloprid. This product is currently authorised for use in Norway (Ectosan Vet SPC, 2021; MTnr. 20-13358, 2021) and is indicated for the treatment of pre-adult and adult salmon lice (*Lepeophtheirus salmonis*) infestation in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). It is a bath treatment for use in closed containment vessels (well-boats) only, due to environmental concerns. The product has an approved withdrawal period of 98 degree-days for both Atlantic salmon and trout.

Dosage

The authorised dosing regimen is 20 mg imidacloprid per litre of sea water for a period of 60 minutes in a well boat.

It has been noted that the duration of immersion in the treatment baths might be inadvertently extended to up to 6 hours because of the method used to administer the product.

Pharmacokinetics and metabolism

Pharmacokinetics in laboratory animals

Rats

Studies on pharmacokinetics in rats were reviewed by FAO and WHO (2002) and by the EPA (US EPA, 1993) and the results published, but this work was not repeated by the sponsor. The following is a summary of those data.

Absorption

The absorption of imidacloprid was studied in male and female rats in a GLP-compliant study. Groups of 5 male and 5 female rats were given a single intravenous dose of 1 mg/kg bw or a single oral dose of 1 or 20 mg/kg bw of imidacloprid labelled with ^{14}C in the methylene or imidazolidine ring. Other groups were given 14 oral doses of unlabelled compound at 1 mg/kg bw/day once per day; 24 hours after the final dose, the animals were given a single oral dose of the radiolabeled compound at 1 mg/kg bw. Radioactivity was determined in plasma and excreta, and following sacrifice 48 hours after the last administration, the concentration of total radioactivity was determined in organs and tissues. A further group of 5 male rats was given a single oral dose of radiolabeled imidacloprid at 20 mg/kg bw orally and $^{14}CO_2$ was measured over the ensuing 48 hours. Additionally, four groups of 5 male rats were given a single oral dose of 20 mg/kg bw imidacloprid. These were sacrificed after 40 minutes and 1.5, 3 and 6 hours; radioactivity was determined in individual organs. A further group consisted of 5 male rats with bile duct fistulas. These were given a single intraduodenal dose of 1 mg/kg bw of radiolabeled imidacloprid in order to determine the rate and extent of biliary excretion (FAO and WHO, 2002).

After oral administration of 1 or 20 mg/kg bw of ^{14}C-methylene imidacloprid, the radioactivity was extensively absorbed and distributed from plasma into tissues. It was also readily eliminated. After intravenous administration of 1 mg/kg bw, around 92 percent of the radioactivity was excreted in urine and faeces within 48 hours. The urine:faeces ratio was 4:1. Following oral administration, around 96 percent of the dose was excreted in urine within 48 hours. There were no differences between male and female rats. More than 90 percent of the urinary radioactivity was excreted within 24 hours after administration. The average residual radioactivity in the body, excluding gastrointestinal tract, was around 0.5 percent while that in the gastrointestinal tract was about 0.06 percent. In the rats with bile duct fistulas, only 4.7 percent of the administered dose was found in faeces, with 56 percent in urine and 36 percent in bile, suggesting significant enterohepatic circulation. There was no significant excretion in expired air. The elimination of total radioactivity from plasma approximately followed a two-compartment model with half-lives of 2.6–3.6 and 26–118 hours (FAO and WHO, 2002).

Distribution

Male rats were treated with ^{14}C-methylene imidacloprid in a GLP-compliant study using a single oral dose of 20 mg/kg bw. The distribution of radioactivity was determined by whole-body autoradiography on X-ray film at 1, 2, 4, 24 and 48 hours after administration. One

animal was treated with an intravenous dose of the radiolabeled drug at 20 mg/kg bw and sacrificed after 5 minutes to permit determination of distribution immediately after treatment.

The radiolabel was rapidly absorbed after oral administration and distributed to tissues and organs. Radioactivity was noted in all parts of the body except for the fatty tissues, the central nervous system, and the mineral parts of bone, following the intravenous injection and 1 hour after oral administration. Higher concentrations were found in the thyroid and the adrenals, but after 24 hours, the concentrations in all other organs and tissues were low. Concentrations of radioactivity in the kidney over the first 24 hours after oral administration were very high, reflecting urinary excretion. The concentrations of radioactivity in the fatty tissues and in the central nervous system were very low throughout the duration of the study (FAO and WHO, 2002).

In a GLP-compliant study, male and female rats were given oral doses of 1 mg/kg bw of imidacloprid labelled with ^{14}C in the imidazolidine ring. A further group of male rats was given a dose of 150 mg/kg bw. Radioactivity was determined in plasma and excreta and in organs and tissues at sacrifice 48 hours after administration. Absorption was rapid and distribution followed a similar pattern to that noted with the methylene labelled compound described in the previous study. Excretion was rapid and mainly renal (FAO and WHO, 2002).

Metabolism

In the major study of absorption described above, urine was collected separately from each rat at intervals of 0–4, 4–8, 8–24 and 24–48 hours, while faeces were collected at intervals of 0–24 and 24–48 hours after administration. Metabolites were extracted and identified by comparison to known reference compounds in two independent chromatographic techniques or identified by ^{1}H-NMR or mass spectroscopy.

The main metabolites, only found in urine, were 6-chloronicotinic acid and its glycine conjugate. All identified metabolites were found in all dosage groups and in animals of both sexes. Some unchanged parent compound and monohydroxylated (5-hydroxyimidacloprid) and olefinic metabolites were noted. All other metabolites were found at very low concentrations.

Two main metabolic routes were identified. The first was oxidative cleavage leading to the formation of 6-chloronicotinic acid, which is subsequently conjugated with glycine to produce a hippuric acid conjugate. These two metabolites represented about 30 percent of the recovered radiolabel. Some dechlorination of the pyridinyl group occurred producing 6-hydroxy nicotinic acid and its mercapturic acid conjugate, but this was a minor metabolite, and it was converted to 6-methylmercapturic acid, and finally, to the glycine conjugate, which accounted for 5.6 percent of the recovered radiolabel. The second major pathway involved hydroxylation of the imidazolidine ring at the 4 or 5 position to yield 4- or 5-hydroximidacloprid and this accounted for about 16 percent of the recovered radiolabel. The loss of water from the hydroxyl derivative yields the olefinic derivative of imidacloprid. These products of metabolism and unchanged parent drug were found in urine and faeces. A very minor compound, the guanidine type derivative, was found only in faeces. Parent drug was eliminated to the extent of about 14 percent (FAO and WHO, 2002).

The distribution of metabolites was investigated in liver and kidney following a single oral dose of imidacloprid to rats. The metabolites found in the kidney, as might be predicted, were identical to those found in urine. Triazinone, was not found in excreta and may have been subject to further metabolism prior to urinary or biliary excretion. The relative amounts of metabolites formed from the oxidative pathway (6-chloronicotinic acid) increased in the liver over the study duration. In the kidney, the relative amounts of the more polar substances (6-chloronicotinic acid and its glycine conjugate) decreased over time. The amounts of the olefinic derivative and 4-hydroxyimidacloprid showed relative increases while the proportion of parent drug decreased slowly (FAO and WHO, 2002).

Treatment of female rats with 20 mg/kg bw imidacloprid as a single oral dose in corn oil revealed the major blood metabolite to be 6-chloronicotinic acid, with a lower concentration of 6-hydroxynicotinic acid. Imidacloprid was the major blood component. The major urinary component was 6-chloronicotinic acid with a lower concentration of 6-hydroxynicotinic acid. Imidacloprid was a minor urinary component. Smaller concentrations of all three metabolites were found in faeces. Blood clearance of imidacloprid was 204.9 mL/hour. AUC values for imidacloprid, 6-chloronicotinic acid and 6-hydroxynicotinic acid were 97.90, 115.96 and 176.51 µg/mL/h (0–48 h) respectively. Low concentrations of all 3 substances were found in brain, liver, and ovary, but higher concentrations were found in kidney, reflecting urinary excretion. Inhibition of brain and plasma acetylcholinesterase occurred. In brain, this ranged from 22 to 30 percent over the 48 hours following administration (Kapoor *et al*., 2014).

A minor constituent arising in plants treated with imidacloprid is the nitroso metabolite, 1-(6-chloro-3-pyridylmethyl)-N-nitroso(imidazolidine-2-ylidene)amine (also referred to as WAK 3839). However, this metabolite was not identified in rat excreta in the studies already described. When rats were given a single high dose of 150 mg/kg bw imidacloprid, no nitroso compound was formed. However, when imidacloprid was given to rats at 1800 ppm in the diet for a year, and then given a single oral dose of ^{14}C-methylene imidacloprid, 9.3 percent of urinary metabolite was attributable to the nitroso compound, equivalent to 6.8 percent of the administered dose. It was also detected in the urine of mice given 2000 ppm dietary imidacloprid for around one year (FAO and WHO, 2002). The proposed metabolic pathway for imidacloprid in rats is shown in Figure 1.

Excretion

As already described, the major route of elimination of imidacloprid and its metabolites is urinary in the rat. The ratio of urine: faeces excretion is approximately 4:1. Excretion is rapid following oral administration of imidacloprid (FAO and WHO, 2002).

Human (*in vitro*)

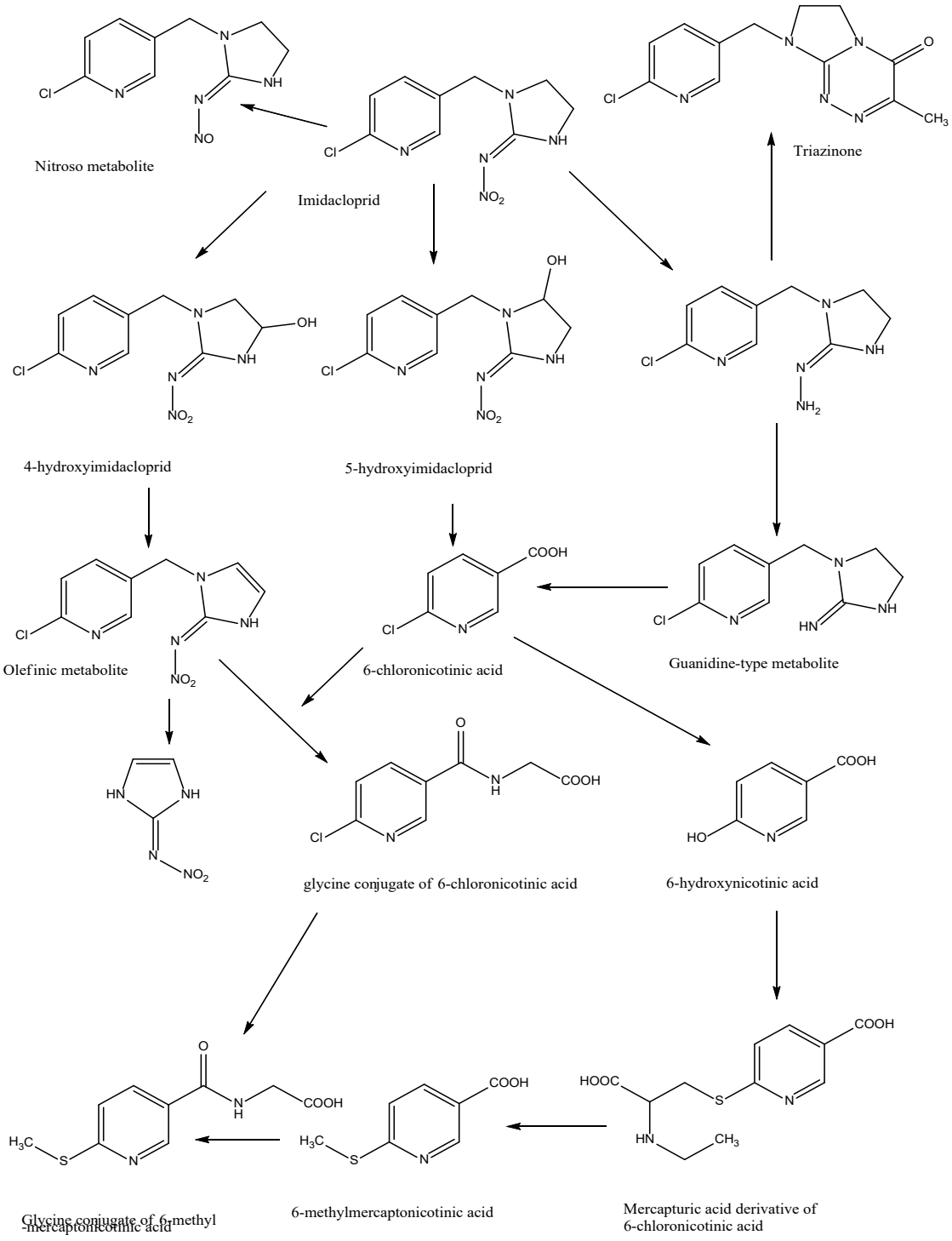
Schulz-Jander and Casida, 2002: *Imidacloprid insecticide metabolism: human cytochrome P450 isozymes differ in selectivity for imidazolidine oxidation versus nitroimine reduction.*

A study using individual recombinant cytochrome P450 isozymes from human liver demonstrated that the major metabolite of imidacloprid was the 5-hydroxy compound, with the olefinic derivative as a minor metabolite. Metabolites derived from reduction and cleavage of

the imidazolidine and the nitrosoimine led to formation of the guanidine compound and the urea metabolite (1-(6-chloro-3-pyridylmethyl)-2-imidazolidinone; imidacloprid-urea).

A single isozyme, CYP3A4 was responsible for oxidation and reduction of imidacloprid at the imidazolidine and nitroimine moieties, respectively. This *in vitro* study demonstrates some similarities with the metabolism of imidacloprid in the rat.

Figure 1. Proposed metabolic pathway of imidacloprid in rats



Pharmacokinetics in food-producing animals

Salmon

Hobbs, 2015: *The metabolism of [^{14}C] IV-38 in Atlantic salmon (Salmo salar L.).*

IV-38 is a code used for imidacloprid. This was a GLP-compliant study that investigated the tissue distribution and metabolism of ^{14}C radiolabeled imidacloprid in Atlantic Salmon. It was carried out in accordance with VICH GL46 (EMA, 2011). The label was placed in the methylene moiety, in between the two rings.

The test item was dissolved in seawater at the test site to create the treatment bath. The concentration of imidacloprid in the bath solution was measured to be 20.3–20.6 mg/L throughout the treatment and was homogenously dissolved in the treatment solution. The purity was measured to be 97.5–97.7 percent throughout the treatment (the supplied radiochemical (specific activity 141 µCi/mg or 5.22 MBq/mg) was diluted using non-labelled imidacloprid. The resultant specific activity was calculated to be 39.9 µCi/mg (or 1.48 MBq/mg).

Twenty-six fish (~1 year old) were held in the exposure bath (temperature 7–8°C) for a 60–minute period and then removed and returned to the holding tank containing fresh seawater. Samples of salmon fillet (containing muscle and skin in natural proportions), liver (excluding the gall bladder), spleen, gut, gills, kidney, and the residual carcass were collected from 6 fish each at 5 h, 25 h, 5 days, and 26 days post-exposure. Fish weights and weights of individual samples were recorded. Tissue samples from six fish at each time point were pooled. Samples were stored frozen at approximately -20°C and subsequently shipped to the laboratory on dry ice for analysis. There are no data provided regarding the residual carcasses, other than their weights.

Sub-samples of the fish samples were taken for initial Total Radioactive Residue (TRR) determination employing sample oxidation with Liquid Scintillation Counting (LSC) analysis. Representative combined samples of fillet and liver were extracted with acetonitrile and the extractable residues analysed by HPLC-UV to determine the nature of the residues. The LOD of the method was 10 µg/kg for all tissue matrices.

TRR declined progressively from 5 hours to 26 days after treatment in all sampled tissues. Residues in fillet declined from 359 µg eq/kg at 5 hours after treatment to 13 µg eq/kg at 26 days following treatment.

Radioactive residues were readily extracted from the liver and fillet, with <6.6 percent TRR remaining following the extraction procedure at 5 hours, 25 hours, and 5 days after treatment. At 26 days after treatment the extractability had reduced (77.6 and 83.7 percent TRR for the fillet and liver respectively) although total unextractable residues were low (≤4 µg eq/kg).

The parent substance was the major residue detected in all samples analysed, accounting for 69.4–95.2 percent TRR in fillet (341–9 µg eq/kg) and 77.7–95.2 percent TRR, in liver (767–20 µg eq/kg). An unknown metabolite, which was not present in the treatment solution, was detected in all extractable residues at low concentrations (≤8.2 percent TRR, ≤22 µg eq/kg). This metabolite was isolated and identified by LC-MS analysis (of a fillet sample from day 5)

as hydroxylated imidacloprid (although it was not clear whether this was the 4-hydroxy or 5-hydroxy imidacloprid).

Table 1. Distribution of metabolites in fillet and liver (percent TRR)

Time post dose	Fillet			Liver		
	Imidacloprid (%)	Hydroxy-imidacloprid (%)	Other (%)	Imidacloprid (%)	Hydroxy-imidacloprid (%)	Other (%)
5 h (1.5 dd)	95.2	1.1	0.7	93.6	2.7	0.3
25 h (8 dd)	94.6	1.4	ND	95.2	2.0	0.6
5 d (37.5 dd)	89.4	4.0	ND	90.4	4.7	0.6
26 d (195 dd)	69.4	8.2	ND	77.7	2.2	3.8

Notes(s): ND = not detected
Source: Adapted from Hobbs, G. 2015. The metabolism of [^{14}C]-IV-38 in Atlantic salmon (*Salmo salar* L.) Report No. IV38-GBR-012-2014-GLP, Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, UK. Sponsor submitted.

Results:
The tables below show the time course of the total radioactive residues in muscle + skin, liver spleen, kidney, gills, and gut.

Table 2. Total radioactive residues in Atlantic salmon tissues following treatment with ^{14}C–imidacloprid

Tissue	TRR (µg eq/kg)			
	5 hours	25 hours	5 days	26 days
Liver	820	699	403	26
Muscle + skin	359	329	182	13
Spleen	450	485	294	28
Kidney	1405	1326	980	125
Gills	304	245	112	12
Gut	337	295	183	10

Source: Adapted from Hobbs, G. 2015. The metabolism of [^{14}C]-IV-38 in Atlantic salmon (*Salmo salar* L.) Report No. IV38-GBR-012-2014-GLP, Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, UK. Sponsor submitted.

Table 3. Total radioactive residues in Atlantic salmon fillet following treatment with ^{14}C–imidacloprid

Time point		5 h	25 h	5 days	26 days
Extracts[a]	Total µg	3.519	3.422	1.813	0.991
	mg eq/kg	0.348	0.316	0.170	0.010
	% of total	97.0	96.0	93.4	77.6
Unextracted residue[b]	Total µg	0.108	0.141	0.129	0.286
	mg eq/kg	0.011	0.013	0.012	0.003
	% of total	3.0	4.0	6.6	22.4

Total[c]	Total µg	3.628	3.563	1.942	1.278
	mg eq/kg	0.359	0.329	0.182	0.013

Notes(s): a = samples were extracted twice with acetonitrile (3:1; v/w); b = The unextracted residues were obtained by combusting the pellet remaining after extraction; c = total extractable + unextracted ^{14}C residues in the sample.

Source: Adapted from Hobbs, G. 2015. The metabolism of [^{14}C]-IV-38 in Atlantic salmon (*Salmo salar* L.) Report No. IV38-GBR-012-2014-GLP, Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, UK. Sponsor submitted.

Frew *et al.*, 2018: *Toxicokinetics of the neonicotinoid insecticide imidacloprid in rainbow trout (Oncorhynchus mykiss).*

In vivo time-course studies were conducted to study the distribution and elimination of imidacloprid in rainbow trout. Animals confined to respirometer-metabolism chambers were injected with a low (47.6 µg/kg), medium (117.5 µg/kg) or high (232.7 µg/kg) dose directly into the arterial bloodstream and allowed to depurate. Temperature was held at 11 ± 1°C. The fish were then sampled to characterize the loss of imidacloprid from plasma and its appearance in expired water (all dose groups) and urine (medium dose group only).

Blood samples (50 or 100 µL) were collected before dosing to assess background imidacloprid concentrations. Additional samples were then collected at 1, 2, 4, 8, 16, 24, 36 and 48 h post injection (low and high dose groups), or at 0.5, 1, 2, 4, 6, 8, 12, 16, 20, 24 and 36 h post injection (medium dose group).

The plasma time-course data indicated an early (< 12 h) distributional phase followed by a log-linear terminal elimination phase. Mean total clearance (CLT) values determined by non-compartmental analysis were 21.8, 27.0 and 19.5 mL/h/kg for the low, medium, and high dose groups, respectively. Estimated half-lives for the same groups were 67.0, 68.4, and 68.1 h, while mean fitted values for the steady-state volume of distribution (VSS) were 1.72, 2.23, and 1.81 L/kg.

Measured branchial elimination rates were much lower than expected, suggesting that imidacloprid is highly bound in blood. Renal clearance rates were greater than measured rates of branchial clearance (60 percent of CLT in the medium dose group), possibly indicating a role for renal membrane transporters. There was no evidence for hepatic biotransformation of imidacloprid in trout (from an *in vitro* study using trout liver S9 fractions). Collectively, these findings suggest that imidacloprid would accumulate in trout in continuous waterborne exposures.

Table 4. Distribution of imidacloprid to selected tissues and organs of chambered rainbow trout at the end of depuration following bolus intra-arterial injection. Data presented as mean ± SD

Sample	**Low dose 47.6 µg/kg (48 h depuration, n = 5)**		**Medium dose 117.5 µg/kg (36 h depuration, n = 8)**		**High dose 232.7 µg/kg (48 h depuration, n = 5)**	
	IMI concentration	**Tissue:plasma ratio**	**IMI concentration**	**Tissue:plasma ratio**	**IMI concentration**	**Tissue:plasma ratio**
Plasma	13.3 ± 2.9	-	33.4 ± 13.0	-	76.4 ± 8.1	-
Brain	19.6 ± 2.6	1.4 ± 0.1	54.0 ± 14.0	1.8 ± 0.4	98.5 ± 16.9	1.3 ± 0.1

Kidney	44.7 ± 6.0	3.2 ± 0.3	292.9 ± 102.7	9.8 ± 4.6	257.9 ± 32.3	3.4 ± 0.4
Liver	41.9 ± 7.9	3.0 ± 0.4	1143 ± 21.4	3.9 ±1.5	228.9 ± 32.3	3.0 ± 0.3
Muscle	22.4 ± 1.8	1.7 ± 0.3	67.0 ± 18.1	2.2 ± 0.7	118.8 ± 12.4	1.6 ± 0.1
Bile	69.3 ± 5.2	5.1 ± 1.0	230.8 ± 57.4	7.9 ± 3.1	442.2 ± 75.5	5.6 ± 0.5
Urine	NA	NA	1185 ± 40.4	5.0 ± 1.9	NA	NA

Source: Adapted from Frew, J.A., Brown, J. T., Fitzsimmons, P. N., Hoffman, A. D., Sadilek, M., Grue C. E. & Nichols, J. W. 2018. Toxicokinetics of neonicotinoid insecticide imidacloprid in rainbow trout (*Onchrhynchus mykiss*). *Comp. Biochem. Physiol. C Toxicol. Pharmacol.*, 205:34-42. doi.org/10.1016/j.cbpc.2018.01.002

Iturburu *et al.*, 2017: *Uptake, distribution in different tissues, and genotoxicity of imidacloprid in the freshwater fish Australoheros facetus.*

Adult *Australoheros facetus* were sourced from fresh water and acclimatised for two months in freshwater tanks held at 15°C and a pH of 8.5. A bioaccumulation bioassay was designed using 5 fish per treatment. Fish were exposed individually in aquaria with 3 l of medium, and the concentration of imidacloprid in the medium (100mg/L, 300mg/L, and 2 500 mg/L) was tested at 0 h, 24 h, and 48 h to check the stability.

Samples of brain, muscle, gills, gut, liver, and blood were analysed. The extraction of imidacloprid from brain (10 mg), muscle (135 mg), gills (45 mg), gut (20 mg), liver (35 mg), and blood (7 mg) was carried out and the determination and quantification of imidacloprid were performed by LC-MS/MS. The limit of detection was 1 ng/mL. The limit of quantification was 5 ng/mL.

The concentrations of imidacloprid in brain, muscle, gills, gut, liver, and blood are shown in Figure 2. Most of the tissues showed the same pattern of concentration: the longer the time of exposure, the higher the concentration in tissues. In liver, gills, gut, and muscle, the imidacloprid concentration was higher after 48 h than after 24 h, whereas in brain and blood the levels of imidacloprid were similar at both times.

The fact that imidacloprid was found in brain tissue, independent of the concentration in the exposure medium, indicates that imidacloprid crossed the blood-brain barrier.

Although there was no accumulation, only uptake, of imidacloprid, genotoxicity was observed. In fish exposed to an imidacloprid formulation, increased micronucleus frequency at 100 µg/L and 1000 µg/L was detected.

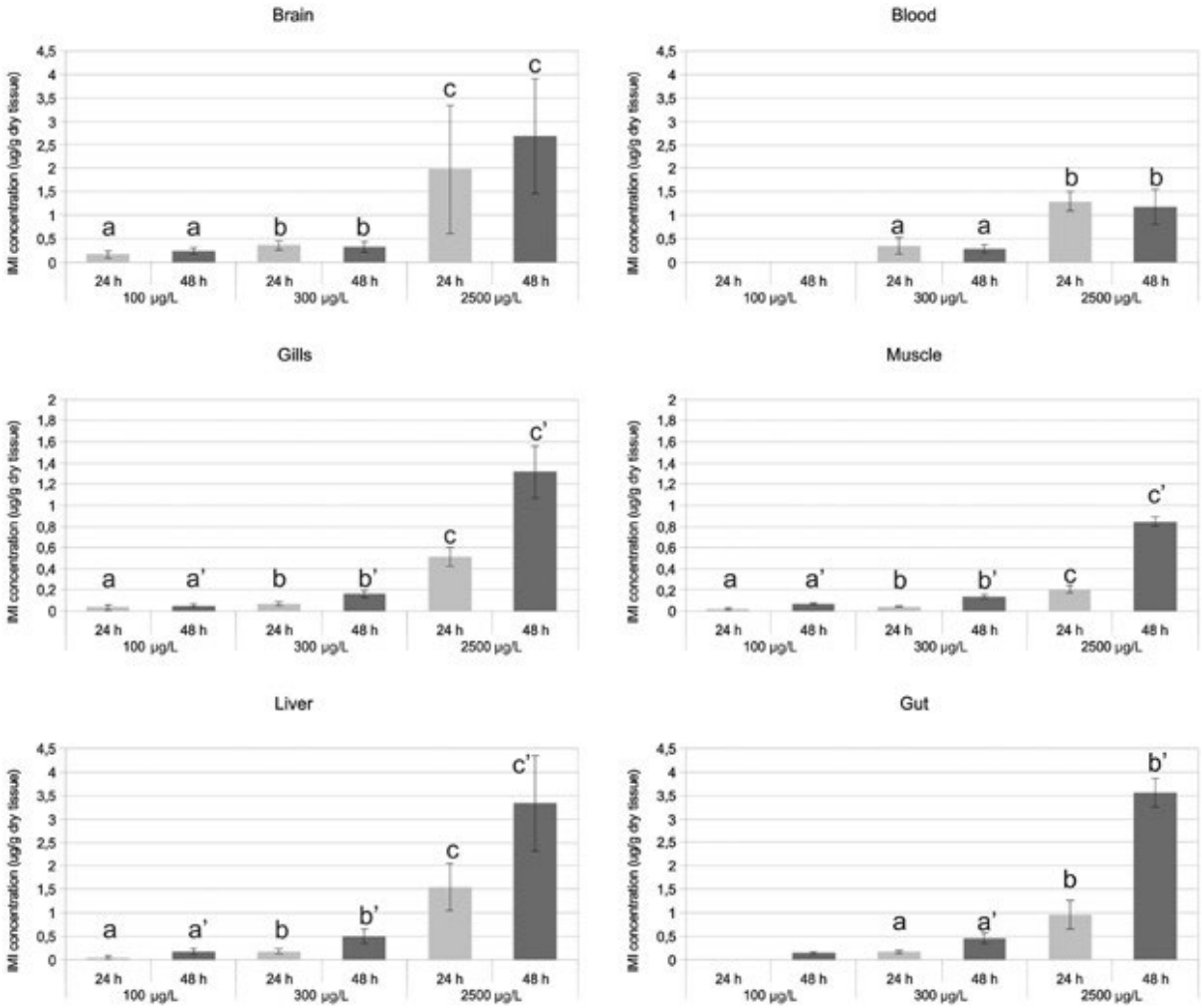
Comparative metabolism

The sponsor did not conduct a comparative metabolism study. From the total residue study above (Hobbs, 2015), there is only one substance (imidacloprid) that represents more than 10 percent of TRR, or greater than 0.1 mg/kg TRR (the thresholds for metabolite identification as per VICH GL 46) in either fillet or in liver (EMA, 2011). The hydroxyl metabolite is below these thresholds for identification.

The sponsor also refers to the metabolism studies conducted in rats and reviewed by the US EPA (US EPA, 31 Mar 1993) and FAO and WHO (2002). The review indicated that that there was extensive metabolism, and one of the metabolites was 5-hydroxy imidacloprid (identified

as WAK 4103). Therefore, there is autoexposure during *in vivo* rodent toxicity studies to the metabolite observed in salmon fillet.

Figure 2. Imidacloprid concentrations in brain, blood, gills, muscle, liver, and gut of *Australoheros facetus* fish exposed to 100 mg/L, 300 mg/l, and 2500 mg/L at 24 h and 48 h



Notes(s): Different letters indicate significant differences among exposure concentrations at the same time. a versus a', b versus b', and c versus c' indicate significant differences between times at the same exposure concentration

Source: Iturburu, F.G., Zömisch, M., Panzeri, A.M., Crupkin, A.C., Contardo-Jara, V., Pflugmacher, S. & Menone, M.L. 2017. Uptake, distribution in different tissues, and genotoxicity of imidacloprid in the freshwater fish *Australoheros facetus*. *Environ. Toxicol. Chem.*, 36(3):699–708. doi.org/ 10.1002/etc.3574

The following was found as part of the published literature search:

Kolanczyk *et al*., 2020: *In vitro metabolism of imidacloprid and acetamiprid in rainbow trout and rat.*

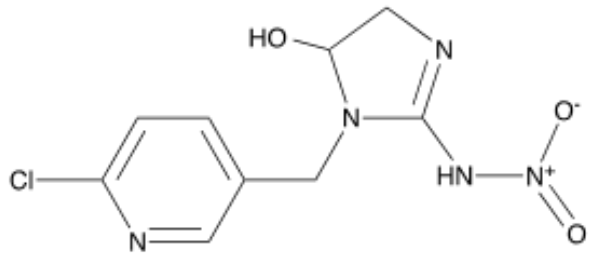
While the mammalian metabolism of neonicotinoids has been studied extensively, there is a lack of understanding of their metabolism in fish species. While considered slightly or not acutely toxic to fish, chronic effects such as genotoxicity, oxidative stress and early life stage developmental toxicity caused by imidacloprid have been observed for fish, in the range of 0.1

to 15 mg/L. A greater understanding of metabolism in fish is needed to determine whether bioactivation to more toxic metabolites acting through the nAChR occurs.

The current study was undertaken to identify and compare neonicotinoid metabolic pathways of *in vitro* microsomal systems in the rainbow trout and rat. Immature rainbow trout (*Oncorhynchus mykiss*, RBT), Erwin/Arlee strain, were used for production of liver slices and subcellular microsome fractions. Trout were kept at 11°C, as were the liver samples. Analysis resulted in the detection of a single metabolite, 5-hydroxy imidacloprid, in addition to the parent chemical in trout.

Ultimately, the formation of the metabolite 5-hydroxy-imidacloprid was conserved across rainbow trout and rat species in both microsomal and liver slice assays.

Figure 3. The likely only metabolite formed in salmon and trout (5-hydroxy imidacloprid)



Source: Adapted from Kolanczyk, R.C., Tapper, M.A., Sheedy, B.R. & Serrano, J.A. 2020. *In vitro* metabolism of imidacloprid and acetamiprid in rainbow trout and rat. *Xenobiotica*, 50(7):805–814. doi.org/10.1080/00498254.2019.1694197

Tissue residue depletion studies

Radiolabeled residue depletion studies

There were no additional radiolabeled studies available, other than the paper by Hobbs, 2015, reported above. It was demonstrated that the parent imidacloprid would be suitable as the sole marker residue, and that a MR:TRR of 0.7 would be appropriate when calculating the dietary exposure.

Residue depletion studies with non-radiolabeled drug

Salmon

Controlled studies:

Three studies conducted under controlled conditions have been performed to examine the depletion of residues in Atlantic salmon (Table 5).

Table 5. Controlled residues depletion studies with imidacloprid

Reference	GLP status	Title	Objective
Bancroft 2016a	GLP	IV-38: Atlantic Salmon Low and High Temperature Residue Depletion Study	To determine the residue depletion profile in Atlantic salmon after a 60-min bath treatment of imidacloprid at 20mg/l in sea water at 7°C and 15°C.
Auchinachie 2019	Non-GLP	Extended Ectosan Bath Exposure and Sampling of Atlantic Salmon (*Salmo salar*) for Residue Analysis.	To determine the residue levels in Atlantic salmon after a 60-, 180- or 360-min bath treatment of imidacloprid at 20 mg/l in sea water at 12°C at 1 day and 350 degree -days.
Longshaw 2020	GLP	Extended Ectosan Bath Exposure and Sampling of Atlantic Salmon (*Salmo salar*) for Residue Analysis.	To determine the residue depletion profile in Atlantic salmon after a 60-, 180- or 360-min bath treatment of imidacloprid at 20mg/l in sea water at 15°C.

Bancroft 2016a: *IV-38: Atlantic salmon low and high temperature residue depletion study. Test facility study no. 225411.*

This study was claimed to be conducted in accordance with VICH GL49 and GLP (EMA, 2015). The objective of the study was to perform an exposure study on Atlantic Salmon in sea water for a 28-day period at 15°C and a 60-day period at 7°C and to determine the residue depletion in muscle, liver, skin, and fillet. The parent compound was selected as the marker residue as it was the major residue detected in a previous study (Hobbs, 2015).

The salmon were exposed to 20 mg/L imidacloprid in sea water, for approximately 1 hour (the authorised dosing regimen). At each of the five sampling points during the 28 days post exposure for 15°C and 60 days post exposure for 7°C, 10 treated fish were removed and samples of muscle, liver, skin, and fillet (muscle and skin) from each fish were collected.

The concentrations of imidacloprid from filtered and unfiltered water samples from replicate exposure tanks at 7°C ranged from 19.2 to 20.5 mg/L, and from 18.5 to 21.0 mg/L from replicate exposure tanks at 15°C. The muscle, liver, skin, and fillet samples from one fish taken from the control tank and five fish taken from each replicate exposure tanks per timepoint and temperature were analysed.

The study used three tanks of 37 fish at a temperature of 7 ± 1 °C and two tanks of 44 fish and one of 45 fish at a temperature of 15 ± 1°C. Two of the tanks at each temperature were designated as replicate exposure tanks and one tank was designated as a control tank. Tank volumes were approximately 900 l and used sea water (salinity 34 ± 2 g/l) to maintain flow rates of 5–7 l/minute (once the 60-minute treatment period was complete).

The fish at 7 ± 1°C had an average weight of 576.93 g (average across all fish sampled) at the first sample point (day 1). At the end of the study phase (day 60) the fish averaged 761.06 g (average across all remaining fish).

The fish at 15 ± 1°C had an average weight of 383.47 g (average across all fish sampled) at the first sample point (day 1). At the end of the study phase (day 28) the fish averaged 505.07 g (average across all remaining fish).

Table 6. The study design

Water temperature	Tissue	Slaughter time points	Samples taken at each point	Total control samples to be analysed	Total treated samples to be analysed
7°C	Muscle	Day 1, 7, 21, 35 & 60	5 fish from each tank (control & 2 exposure)	5	50
	Liver			5	50
	Skin			5	50
	Muscle + skin			5	50
15°C	Muscle	Day 1, 7, 14, 21 & 28	5 fish from each tank (control & 2 exposure)	5	50
	Liver			5	50
	Skin			5	50
	Muscle + skin			5	50

Source: Bancroft. K. 2016a. IV-38: Atlantic salmon low and high temperature residue depletion study. Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, UK, Report No. 37362. Sponsor submitted.

The effects of storing sea water and salmon tissue samples frozen in a freezer set to maintain -20°C for a storage period of 98 days, which covers the longest interval between sampling and extraction of a sample observed in this study, was determined.

Each analytical batch included solvent calibration standards injected at the start and end of each analytical batch, at least three replicate procedural recovery samples at the validated fortification levels (25 µg/kg, 50 µg/kg, and 100 µg/kg), a control tissue sample, a control tissue sample fortified with internal standard (imidacloprid-d4), and tissue samples from two or three timepoints detailed in Table 6.

Table 7. Mean results

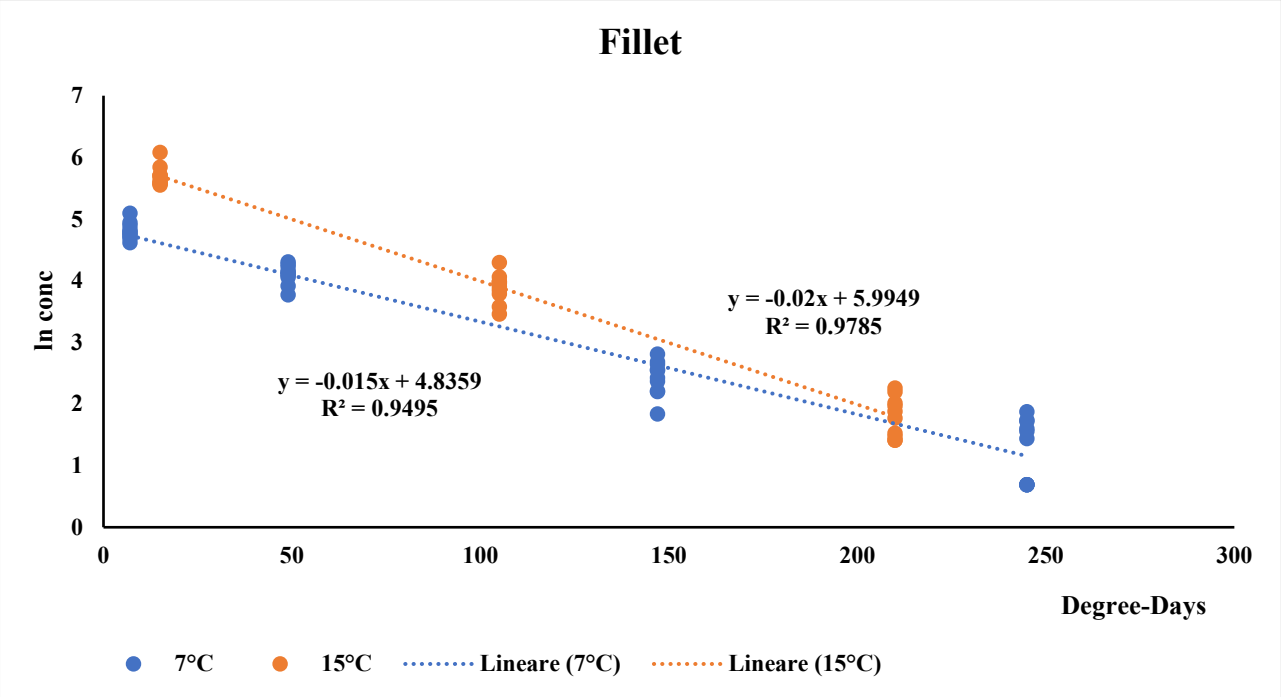
Water temperature	Timepoint	Tank ID	Mean concentration (µg/kg)			
			Muscle	Liver	Skin	Fillet
7°C	Day 1	07-05[a]	NQ	ND	NQ	ND
		07-06	141	305	96.6	135
		07-07	119	272	92.4	112
	Day 7	07-05[a]	NQ	NQ	NQ	ND
		07-06	63.3	126	47.6	59.9
		07-07	64.8	139	49.6	62.5
	Day 21	07-05[a]	ND	ND	ND	ND
		07-06	13.2	28.0	10.1	12.7
		07-07	10.7	20.5	8.05	10.8
	Day 35	07-05[a]	ND	ND	NQ	ND
		07-06	5.35	12.3	4.21	5.68
		07-07	5.05	8.60	4.80	4.89
	Day 60	07-05[a]	ND	ND	ND	ND
		07-06	NQ	NQ	NQ	NQ
		07-07	NQ	NQ	NQ	NQ

Water temperature	Timepoint	Tank ID	Mean concentration (µg/kg)			
			Muscle	Liver	Skin	Fillet
15°C	Day 1	06-05[a]	NQ	ND	NQ	ND
		06-06	325	720	251	318
		06-07	290	647	247	286
	Day 7	06-05[a]	ND	ND	NQ	ND
		06-06	49.5	90.5	37.0	52.0
		06-07	46.5	87.9	35.4	48.0
	Day 14	06-05[a]	NQ	NQ	NQ	NQ
		06-06	5.94	9.16	4.79	5.92
		06-07	6.68	12.0	5.19	6.66
	Day 21	06-05[a]	ND	ND	NQ	ND
		06-06	NQ	NQ	NQ	NQ
		06-07	NQ	NQ	NQ	NQ
	Day 28	06-05[a]	ND	ND	NQ	ND
		06-06	NQ	NQ	NQ	NQ
		06-07	NQ	NQ	NQ	NQ

Notes(s): a: Tissues from one fish were analysed from the control tanks, therefore the result is from one fish and is not a mean; ND: No residue detected, LOD is 0.131 µg/kg for muscle, 0.300 µg/kg for skin and 0.145 µg/kg for liver; NQ: Not quantifiable, as residue found is less than the LOQ of 4 µg/kg.

Source: Bancroft. K. 2016a. IV-38: Atlantic salmon low and high temperature residue depletion study. Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, UK, Report No. 37362. Sponsor submitted.

Figure 4. Depletion of imidacloprid concentrations in fillet of Atlantic salmon treated at 7°C and 15°C (timepoints in degree-days)



Notes(s): At 98 dd, residue concentration at 7°C was estimated to be 29 µg/kg; at 15°C it was estimated to be 56.5 µg/kg.

Source: Bancroft. K. 2016a. IV-38: Atlantic salmon low and high temperature residue depletion study. Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, UK, Report No. 37362. Sponsor submitted.

The study design is suitable to derive reliable estimates for residue concentrations in 'muscle plus skin in natural proportions' (fillet). Concentrations for muscle, skin, and liver were also measured separately. Residue concentrations deplete continuously with time in all analysed tissues and at both temperatures.

The bodyweights of animals treated at 7°C differed from those treated at 15°C (577 g compared to 383 g at day 0), which might have an impact on absorption of imidacloprid and hence on residue concentrations, as smaller animals have larger body surfaces in relation to their body weights. However, the sponsor has explained that this will have little effect on the residue measured, as the mode of uptake of imidacloprid is mainly via the gills, as opposed to via dermal absorption.

At the higher water temperature (15°C), initial residue concentrations were much higher and residue depletion over time was faster compared to residue concentrations at 7°C. At the lower temperature absorption of imidacloprid from the immersion treatment is lower, but also depletion of residues is much slower compared to the fish treated at 15°C.

The analytical method used was based on LC-MS/MS and is described later in this report. Concurrent validation data for accuracy and precision were provided, which indicate that the method was working suitably.

Table 8. A summary of the procedural recoveries at each level per matrix

Matrix	Fortification level (µg/kg)	Recovery range (%)	Mean recovery (%)	CV (%)
Muscle	25.0	88.0–108	98.6	4.79
	50.0	81.4–99.3	90.0	4.90
	100	87.6–112	99.3	7.48
Liver	25.0	89.3–123	104	8.15
	50.0	80.9–105	93.8	8.88
	100	88.9–114	102	7.24
Skin	25.0	83.9–185	110	28.1
	50.0	77.9–92.8	88.1	4.35
	100	87.2–104	96.0	4.79
Fillet	25.0	88.1–108	98.7	5.39
	50.0	80.6 –109	90.0	8.08
	100	88.1–110	99.2	6.17

Source: Bancroft. K. 2016a. IV-38: Atlantic salmon low and high temperature residue depletion study. Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, UK, Report No. 37362. Sponsor submitted.

The LOQ was 4 µg/kg for muscle, skin, and liver; the LOD was 0.131 µg/kg for muscle, 0.300 µg/kg for skin, and 0.145 µg/kg for liver.

Auchinachie 2019: *Extended ECTOSAN bath exposure and sampling of Atlantic salmon (Salmo salar) for residue analysis.*

The objective of this (non-GLP) pilot study was to provide samples for residue analysis to determine residue depletion times for the final formulation of imidacloprid when administered as an extended bath treatment to Atlantic salmon for up to 6 hours.

Field trials of Ectosan on commercial Atlantic salmon farms using a well boat revealed that the residence time of some of the fish in the treatment well can be up to 6 hours due to the length

of time required to discharge fish over the dewatering system. The usual residence time is between 4 and 5 hours.

This study was conducted in June/July 2018 and was designed as a target animal safety and efficacy study, but also as a residue depletion study. Treatment was given on 14 June, and 5 fish per tank were analysed: fillets (muscle and skin) and livers were sampled 1 day (15 June) and 26 days later (10 July), at 354.7 degree-days.

There were four treatment groups of 10 fish each, with an average weight of 162.3 ± 4.8 g. The temperature of the water used was 12 ± 1°C (although this was amended to 14 ± 2°C, it appears that the target 12°C was achieved). The fish were exposed for 61, 179, and 360 minutes (approximately 1 hour, 3 hours and 6 hours) to the proposed treatment dose of 20 µg/mL However, HPLC analysis (methodology not described) of the treatment water samples showed that target concentrations were not achieved during the exposure period, but they were within 80.43–91.37 percent of the target dose.

Tanks were provided with a continuous supply of seawater at a flow rate greater than 5 l/min at a target temperature of 14 ± 2 °C (overall range was 10.6–17.3°C; acclimation and post treatment ranges were 10.9–16.5 °C and 10.6–17.3 °C, respectively, and treatment range was 12.2–12.9°C. For the purposes of monitoring accumulated degree-days, the temperature was recorded twice daily. Salinity range was 35.0–37.0 g/L during the trial.

Tissue samples were stored in a nominal -20°C freezer until shipping. There were temperature fluctuations recorded and a deviation report raised. No concerns were raised in the report. Additional validation of stability of frozen samples (809 days in a freezer set to maintain -20°C) for Atlantic salmon fillet was demonstrated.

Results:

The samples were analysed using an LC-MS/MS method (described later) and reporting was in accordance with GLP. No imidacloprid was found in the control fillet group; however, residues were found in one sample of liver from the same group. This was attributed to cross contamination either at sampling or analysis, as all other samples from the control liver group analysed were below the limit of quantification (LOQ = 4 µg/kg).

Table 9. Results of the analyses for imidacloprid in salmon fillet after treatment for 60 min

Timepoint	Fish number	Results (µg/kg)
Day 1	1	270
	2	267
	3	244
	4	339
	5	301
Day 26	1	4.25*
	2	<LOQ
	3	<LOQ
	4	<LOQ
	5	<LOQ

Notes(s): * = original value 4.08 µg/kg. Repeat results <LOQ and 4.42 µg/kg. Mean of quantifiable data reported.

Source: Auchinachie, N. 2019. Extended ECTOSAN bath exposure and sampling of Atlantic salmon (*Salmo salar*) for residue analysis. Ardtoe Marine Laboratory, Ardtoe, Argyll, UK, Report ARD-0013. Sponsor submitted.

Table 10. Results of the analyses for imidacloprid in salmon fillet after treatment for 180 min

Timepoint	Fish number	Results (µg/kg)
Day 1	1	544
	2	636
	3	830
	4	675
	5	672
Day 26	1	<LOQ
	2	<LOQ
	3	<LOQ
	4	<LOQ
	5	6.04

Source: Auchinachie, N. 2019. Extended ECTOSAN bath exposure and sampling of Atlantic salmon (*Salmo salar*) for residue analysis. Ardtoe Marine Laboratory, Ardtoe, Argyll, UK, Report ARD-0013. Sponsor submitted.

Table 11. Results of the analyses for imidacloprid in salmon fillet after treatment for 360 min

Timepoint	Fish number	Results (µg/kg)
Day 1	1	1520
	2	1420
	3	1200
	4	1230
	5	1480
Day 26	1	5.64
	2	7.16
	3	<LOQ
	4	8.36
	5	<LOQ

Source: Auchinachie, N. 2019. Extended ECTOSAN bath exposure and sampling of Atlantic salmon (*Salmo salar*) for residue analysis. Ardtoe Marine Laboratory, Ardtoe, Argyll, UK, Report ARD-0013. Sponsor submitted.

Table 12. Results of the analyses for imidacloprid in salmon liver after treatment for 60 min

Timepoint	Fish number	Results (µg/kg)
Day 1	1	750
	2	640
	3	515
	4	754
	5	810
Day 26	1	<LOQ
	2	<LOQ
	3	<LOQ
	4	<LOQ
	5	<LOQ

Source: Auchinachie, N. 2019. Extended ECTOSAN bath exposure and sampling of Atlantic salmon (*Salmo salar*) for residue analysis. Ardtoe Marine Laboratory, Ardtoe, Argyll, UK, Report ARD-0013. Sponsor submitted.

Table 13. Results of the analyses for imidacloprid in salmon liver after treatment for 180 min

Timepoint	Fish number	Results (µg/kg)
Day 1	1	1280
	2	1330
	3	1680
	4	1550
	5	1400
Day 26	1	<LOQ
	2	4.61
	3	4.85
	4	<LOQ
	5	9.79

Source: Auchinachie, N. 2019. Extended ECTOSAN bath exposure and sampling of Atlantic salmon (*Salmo salar*) for residue analysis. Ardtoe Marine Laboratory, Ardtoe, Argyll, UK, Report ARD-0013. Sponsor submitted.

Table 14. Results of the analyses for imidacloprid in salmon liver after treatment for 360 min

Timepoint	Fish number	Results (µg/kg)
Day 1	1	2490
	2	2690
	3	2320
	4	2022
	5	3030
Day 26	1	11.0
	2	11.5
	3	5.08
	4	15.1
	5	6.76

Source: Auchinachie, N. 2019. Extended ECTOSAN bath exposure and sampling of Atlantic salmon (*Salmo salar*) for residue analysis. Ardtoe Marine Laboratory, Ardtoe, Argyll, UK, Report ARD-0013. Sponsor submitted.

No statistical analyses could be conducted on these results, as there were only two sampling points.

Longshaw, 2020: *Extended ECTOSAN bath exposure and sampling of Atlantic salmon (Salmo salar) for residue analysis.*

The objective of this GLP study was to provide samples for residue analysis to determine residue depletion times for the final formulation of imidacloprid when administered as an extended bath treatment to Atlantic salmon for up to 6 hours. It was conducted in accordance with VICH GL57 and VICH GL49 (EMA, 2015; 2019).

The intended method of treating Atlantic salmon with imidacloprid in a commercial setting involves the use of a well boat. Whilst achieving a 1-hour exposure with a conventional tarpaulin treatment, where the tarpaulin encloses the fish in their pen and can be quickly removed at treatment end, is readily achievable, this is not necessarily the case with a well boat treatment. A variety of factors can influence the loading and discharge speeds of fish from the

treatment well and consequently some fish may be exposed for longer than 1 hour. Field trials on commercial Atlantic salmon farms using a well boat have revealed that the residence time of some of the fish in the treatment well can be up to 6 hours.

Salmon (408.8 g (± 2.70 g), aged ~1 year) were exposed to between 20.9–21.8 mg/L imidacloprid for 60, 196 and 360 minutes at 15.2–16.0°C. The time to deplete to below the LLOQ in fillet was 21 days (313.2 degree-days) after a 60 min exposure, 28 days (422.1 degree-days) after a 180 min exposure and 33 days (508.6 degree-days) after a 360 min exposure.

A similar pattern was seen in the liver samples, LLOQ was reached at 28 days following the 60-min exposure, 33 days for 180-min exposure and 33 days for the 360-min exposure.

Long-term stability of imidacloprid in sea water and salmon livers was assessed. Imidacloprid was found to remain stable when frozen in seawater for up to 202 days and stable in frozen salmon livers for up to 198 days. Stability of imidacloprid in frozen Atlantic salmon fillets for 809 days was previously demonstrated in Bancroft, 2016a.

There were 4 treatment groups of 72 fish per group and a control group of 36 fish. The temperature was maintained at 15.2–16.0°C during the treatment, and then went to 14.8–17.0°C post treatment. The fish were allocated to 750 L tanks with a stocking density of <25 kg/m^3 and a flow rate of >5 L/min of seawater. Oxygen saturation was kept around 5–10 mg/L.

Samples were frozen at -20°C until transport to the analytical facility. The maximum time elapsed from sampling to extraction for the determination of imidacloprid residues in fillet was 204 days storage in a freezer set to maintain -20°C. The maximum time elapsed from sampling to extraction for the determination of Imidacloprid residues in liver was 181 days storage in a freezer set to maintain -20°C.

Results:

Table 15. Concentration in fillet (µg/kg) after 360 min treatment

Tank no.	Fish no.	Concentration in fillet (µg/kg) 360 min treatment					
		Day 1	Day 7	Day 14	Day 21	Day 28	Day 33
4.1	1	1350	244	68.7	14.1	<LOQ	<LOQ
	2	1420	309	63.5	8.07	<LOQ	<LOQ
	3	1440	403	58.2	11.5	<LOQ	<LOQ
	4	1430	288	54.3	5.28	<LOQ	<LOQ
	5	1540	271	86.5	6.61	<LOQ	<LOQ
	6	1310	317	35.0	12.1	<LOQ	<LOQ
4.2	1	1260	319	33.4	5.55	7.02	<LOQ
	2	1430	299	68.9	10.2	5.17	<LOQ
	3	1250	273	55.2	16.8	<LOQ	<LOQ
	4	1230	272	42.9	6.18	<LOQ	<LOQ
	5	1560	367	52.6	7.02	<LOQ	<LOQ
	6	1250	187	37.5	11.4	<LOQ	<LOQ
Mean ± SD		**1372 ± 114**	**296 ± 56**	**54.73 ± 15.90**	**9.57 ± 3.70**	**6.1 ± 1.3**	**N/A**

Source: Longshaw, M. 2020. Extended ECTOSAN® bath exposure and sampling of Atlantic salmon (*Salmo salar*) for residue analysis. Ardtoe Marine Laboratory, Ardtoe, Argyll, UK, Report ARD-0014. Sponsor submitted.

Table 16. Concentration in fillet (µg/kg) after 196 min treatment

Tank No.	Fish no.	Concentration in fillet (µg/kg) 196 min treatment					
		Day 1	Day 7	Day 14	Day 21	Day 28	Day 33
4.4	1	697	176	42.3	<LOQ	<LOQ	<LOQ
	2	727	187	28.5	5.05	<LOQ	<LOQ
	3	616	142	16.1	11.1	<LOQ	<LOQ
	4	780	158	16.9	5.91	<LOQ	<LOQ
	5	757	160	19.5	6.13	<LOQ	<LOQ
	6	632	158	17.0	5.55	<LOQ	<LOQ
4.7	1	792	171	34.4	7.27	<LOQ	<LOQ
	2	771	145	27.1	5.93	<LOQ	<LOQ
	3	673	186	21.8	9.10	<LOQ	<LOQ
	4	907	187	24.8	6.93	<LOQ	<LOQ
	5	702	151	38.4	4.25	<LOQ	<LOQ
	6	821	104	42.6	6.22	<LOQ	<LOQ
Mean ± SD		**740 ± 83**	**160 ± 24**	**27.45 ± 9.87**	**6.29 ± 2.28a**	**N/A**	**N/A**

Source: Longshaw, M. 2020. Extended ECTOSAN® bath exposure and sampling of Atlantic salmon (*Salmo salar*) for residue analysis. Ardtoe Marine Laboratory, Ardtoe, Argyll, UK, Report ARD-0014. Sponsor submitted.

Table 17. Concentration in fillet (µg/kg) after 60 min treatment

Tank No.	Fish no.	Concentration in fillet (µg/kg) 60 min treatment					
		Day 1	Day 7	Day 14	Day 21	Day 28	Day 33
4.3	1	414	68.1	10.8	<LOQ	<LOQ	<LOQ
	2	295	79.5	13.8	<LOQ	<LOQ	<LOQ
	3	487	71.5	10.1	<LOQ	<LOQ	<LOQ
	4	414	45.8	10.8	<LOQ	<LOQ	<LOQ
	5	282	65.3	5.15	<LOQ	<LOQ	<LOQ
	6	384	73.1	12.1	<LOQ	<LOQ	<LOQ
4.6	1	385	65.5	9.95	<LOQ	<LOQ	<LOQ
	2	340	106	14.5	<LOQ	<LOQ	<LOQ
	3	291	65.3	19.7	<LOQ	<LOQ	<LOQ
	4	388	81.6	14.3	<LOQ	<LOQ	<LOQ
	5	312	50.9	14.1	<LOQ	<LOQ	<LOQ
	6	321	80.3	17.5	<LOQ	<LOQ	<LOQ
Mean ± SD		**359 ± 63**	**71 ± 15**	**12.73 ± 3.81**	**N/A**	**N/A**	**N/A**

Source: Longshaw, M. 2020. Extended ECTOSAN® bath exposure and sampling of Atlantic salmon (*Salmo salar*) for residue analysis. Ardtoe Marine Laboratory, Ardtoe, Argyll, UK, Report ARD-0014. Sponsor submitted.

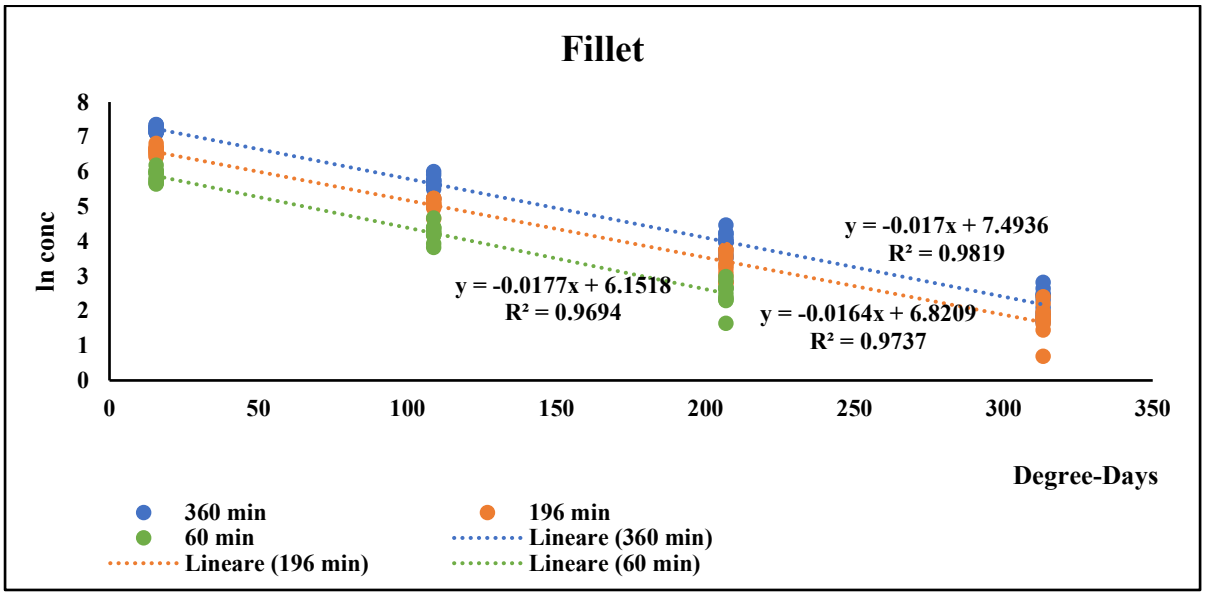
Table 18. Mean concentration (± SD) of Imidacloprid (µg/kg) in Atlantic Salmon fillet 1, 7, 14, 21, 28 and 33 days post exposure

Days post treatment (degree-days)	T01 60 mins Mean	T01 196 mins Mean	T01 360 mins Mean
1 (15.6)	359 ± 63	740 ± 83	1372 ± 114
7 (108.8)	71 ± 15	160 ± 24	296 ± 56
14 (206.8)	12.73 ± 3.81	27.45 ± 9.87	54.73 ± 15.90
21 (313.2)	<LLOQ	6.29 ± 2.28[a]	9.57 ± 3.70
28 (422.1)	<LLOQ	<LLOQ	2.68 ±1.64[a]
33 (508.6)	<LLOQ	<LLOQ	<LLOQ

Notes(s): LLOQ = lower limit of quantification (4 µg/kg); a = Where one or more samples were <LLOQ, the value was taken as 2 µg/kg (1/2 LLOQ) in order to calculate mean; b = original result 5.60 µg/kg, repeat results 6.37 µg/kg and 6.83 µg/kg (median reported)

Source: Longshaw, M. 2020. Extended ECTOSAN® bath exposure and sampling of Atlantic salmon (*Salmo salar*) for residue analysis. Ardtoe Marine Laboratory, Ardtoe, Argyll, UK, Report ARD-0014. Sponsor submitted.

Figure 5. Residue depletion pattern of imidacloprid when exposed to the treatment bath for either 60, 196, or 360 minutes (timepoints in degree-days)



Source: Longshaw, M. 2020. Extended ECTOSAN® bath exposure and sampling of Atlantic salmon (*Salmo salar*) for residue analysis. Ardtoe Marine Laboratory, Ardtoe, Argyll, UK, Report ARD-0014. Sponsor submitted.

Field Studies

Two field studies were performed that included residue sampling, one in Atlantic salmon and one in Rainbow trout. The residue results from these two field trials are provided in a single summary report (Gaffney, 2019). Both field trials were conducted in the same way.

Gaffney, 2019: *The depletion of Ectosan from the edible tissues of Atlantic salmon and rainbow trout after bath treatment under commercial conditions - summary report.*

This study aimed to investigate the rate of depletion of imidacloprid from the edible tissues of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) treated under commercial conditions in Norway. The study was designed to conform with VICH GL49 (EMA, 2015)

Atlantic salmon and rainbow trout were treated using Ectosan (the product authorised in Norway). This was done using wellboats to perform a bath treatment at a concentration of 20 mg/L (± 10 percent) for 60 minutes (± 5 minutes). A total of 8 Atlantic salmon sites and 4 trout sites were treated. Dose concentration was confirmed by sea water analysis conducted on the neighbouring CleanTreat vessel.

A whole side fillet was removed from each fish and these fillets were taken for residue testing from 5 fish from 2 pens per site. These were taken at the following time points:

- Immediately prior to treatment (to provide a baseline level of imidacloprid from other sources, e.g., feed).
- Immediately after treatment
- 24 hours after treatment
- 3–5 days post-treatment
- 8–10 days post-treatment
- 19–21 days post-treatment
- 350 degree-days post treatment

Degree-days were calculated with temperatures from specific pens, where possible, or with site specific data.

The samples were frozen in a temperature logged freezer (-20°C) and shipped on dry ice for analysis by LC-MS/MS. The analysis of the samples was performed to GLP.

Results (Salmon):

Table 19. Summary of details of the 8 commercial Atlantic salmon aquaculture sites used in this study

Site and pen no.	Pen no.	Mean fish weight (kg)	Mean treatment time (minutes)	Mean treatment temperature (°C)	Mean dose (mg/l)
Site 1-31397	OT02	2.98	60	9.85	13.60
	OT12	3.16	60	9.40	14.10
Site 2-29756	IB06	4.04	61	7.47	19.62
	IB15	3.86	61	7.43	18.25
Site 3-34137	SÅ06	nd[1]	64	6.00	17.70
	SÅ05	nd[i]	60	5.50	19.67
Site 4-17077	TV01	1.30	60	6.00	19.26
	TV07	nd[1]	60	6.00	18.57
Site 5-25055	BK02	1.19	66	11.00	23.54
	BK10	1.26	63	11.50	24.36
	HJ02	2.22	67	15.30	28.09

Site and pen no.	Pen no.	Mean fish weight (kg)	Mean treatment time (minutes)	Mean treatment temperature (°C)	Mean dose (mg/l)
Site 6-13643	HJ05	1.54	71	13.00	21.11
Site 7-13236	IN01	2.89	62	15.25	22.59
	IN06	3.01	62	15.75	22.46
Site 8-14081	TV01	1.41	62	16.45	22.96
	TV12	1.42	61	17.05	22.25

Notes(s): Treatment time and dose is the average across all the wells used. Treatment temperature is the mean water temperature in the wells at the start of treatment; nd = no data; i = weight not recorded.

Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

Before treatment

Table 20. Mean residues in salmon fillet samples taken prior to treatment. Seawater temperature was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (°C)	Degree-days	Mean residue (µg/kg)
Site 1-31397:OT02	9.9	0	<LOQ
Site 1-31397:OT12	9.9	0	<LOQ
Site 2-29756:IB06	6.5	0	<LOQ
Site 2-29756:IB15	6.4	0	<LOQ
Site 3-34137:SÅ06	4.9	0	<LOQ
Site 3-34137:SÅ05	5.2	0	<LOQ
Site 4-17077:TV01	5.8	0	<LOQ
Site 4-17077:TV07	5.8	0	<LOQ
Site 5-25055:BK02	11.4	0	<LOQ
Site 5-25055:BK10	11.2	0	<LOQ
Site 6-13643:HJ02	11.5	0	<LOQ
Site 6-13643:HJ05	10.5	0	<LOQ
Site 7-13236:IN04	14.5	0	<LOQ
Site 7-13236:IN06	14.3	0	<LOQ
Site 8-14081:TV01	13.3	0	<LOQ (2.68)[i]
Site 8-14081:TV12	12.7	0	8.18[i]

Notes(s): i = includes results <LOQ and >LOQ. <LOQ = <4 µg/kg.
Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

Immediately post treatment

Table 21. Mean residues in salmon fillet samples taken immediately post-treatment. Seawater temperature was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (°C)	Degree-days	Mean residue (µg/kg)
Site 1-31397:OT02	9.9	0	90.46
Site 1-31397:OT12	9.9	0	nd[i]
Site 2-29756:IB06	6.5	0	72.76
Site 2-29756:IB15	6.4	0	62.02
Site 3-34137:SÅ06	4.9	0	122.26
Site 3-34137:SÅ05	5.2	0	143.40
Site 4-17077:TV01	5.8	0	100.72
Site 4-17077:TV07	5.8	0	87.40
Site 5-25055:BK02	11.4	0	100.46
Site 5-25055:BK10	11.2	0	87.18
Site 6-13643:HJ02	11.5	0	149.56
Site 6-13643:HJ05	10.5	0	106.98
Site 7-13236:IN04	14.5	0	103.36
Site 7-13236:IN06	14.3	0	71.90
Site 8-14081:TV01	13.3	0	131.34
Site 8-14081:TV12	12.7	0	154.02

Notes(s): nd = no data; i = Sample not taken due to weather; <LOQ = <4 µg/kg.
Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

24 hours post treatment

Table 22. Mean residues in salmon fillet samples taken 24 hours post-treatment. Seawater temperature was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (°C)	Degree-days	Mean residue (µg/kg)
Site 1-31397:OT02	9.9	9.9	nd[i]
Site 1-31397:OT12	9.5	18.0	nd[i]
Site 2-29756:IB06	6.5	6.5	111.42
Site 2-29756:IB15	6.3	6.4	105.40
Site 3-34137:SÅ06	5.0	10.1	239.76
Site 3-34137:SÅ05	5.0	5.2	282.40

Site and pen no.	Seawater temperature (°C)	Degree-days	Mean residue (µg/kg)
Site 4-17077:TV01	6.7	5.8	251.20
Site 4-17077:TV07	6.7	5.8	142.28
Site 5-25055:BK02	11.2	11.4	222.00
Site 5-25055:BK10	12.0	11.2	126.54
Site 6-13643:HJ02	11.8	11.5	164.08
Site 6-13643:HJ05	10.8	10.5	171.80
Site 7-13236:IN04	14.3	14.5	133.02
Site 7-13236:IN06	13.4	14.3	272.20
Site 8-14081:TV01	14.3	13.3	291.20
Site 8-14081:TV12	13.3	12.7	300.20

Notes(s): nd = no data; i = samples lost due to mislabelling; <LOQ = <4 µg/kg.

Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

5 days post treatment

Table 23. Mean residues in salmon fillet samples taken 5 days post-treatment. Seawater temperature was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (°C)	Degree-days	Mean residue (µg/kg)
Site 1-31397:OT02	9.5	57.6	59.42
Site 1-31397:OT12	9.5	57.6	nd[i]
Site 2-29756:IB06	6.3	32.2	122.14
Site 2-29756:IB15	6.4	38.4	50.92
Site 3-34137:SÅ06	5.6	26.7	176.60
Site 3-34137:SÅ05	5.4	27.4	182.80
Site 4-17077:TV01	5.5	29.1	184.40
Site 4-17077:TV07	5.5	29.1	180.06
Site 5-25055:BK02	12.0	58.2	133.92
Site 5-25055:BK10	11.5	58.8	106.38
Site 6-13643:HJ02	10.3	64.2	118.80
Site 6-13643:HJ05	11.5	55.2	76.50
Site 7-13236:IN04	16.0	72.6	91.88
Site 7-13236:IN06	16.2	74.1	119.50
Site 8-14081:TV01	8.1	71.9	183.20

Site and pen no.	Seawater temperature (°C)	Degree-days	Mean residue (µg/kg)
Site 8-14081:TV12	13.7	70.9	133.80

Notes(s): nd = no data; i = Sample not taken due to weather; <LOQ = <4 µg/kg.
Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

10 days post treatment

Table 24. Mean residues in salmon fillet samples taken 10 days post-treatment. Seawater temperature was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (°C)	Degree-days	Mean residue (µg/kg)
Site 1-31397:OT02	8.5	127.6	48.18
Site 1-31397:OT12	8.5	88.0	50.82
Site 2-29756:IB06	6.1	64.3	63.22
Site 2-29756:IB15	6.18	63.2	47.18
Site 3- 34137:SÅ06	5.8	54.0	134.32
Site 3-34137:SÅ05	5.7	54.9	144.00
Site 4-17077:TV01	5.9	57.6	57.34
Site 4-17077:TV07	5.9	57.6	135.00
Site 5-25055:BK02	12.7	121.0	51.86
Site 5-25055:BK10	10.3	122.3	34.72
Site 6-13643:HJ02	14.4	117.7	24.22
Site 6-13643:HJ05	10.3	119.4	33.10
Site 7-13236:IN04	17.6	155.5	28.08
Site 7-13236:IN06	17.6	141.0	47.84
Site 8-14081:TV01	16.1	120.8	25.08
Site 8-14081:TV12	16.2	117.3	24.22

Notes(s): <LOQ = <4 µg/kg
Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

21 days post treatment

Table 25. Mean residues in salmon fillet samples taken 21 days post-treatment. Seawater temperature was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (°C)	Degree-days	Mean residue (µg/kg)
Site 1-31397:OT02	7.9	183.5	13.38
Site 1-31397:OT12	7.9	143.9	6.88
Site 2-29756:IB06	6.0	131.4	35.58
Site 2-29756:IB15	5.8	129.7	26.52
Site 3-34137:SÅ06	7.1	120.9	33.36
Site 3-34137:SÅ05	7.2	123.2	46.68
Site 4-17077:TV01	6.2	128.3	20.36
Site 4-17077:TV07	6.2	128.3	21.52
Site 5-25055:BK02	9.3	245.6	6.01
Site 5-25055:BK10	9.1	243.5	10.05
Site 6-13643:HJ02	16.8	289.8	10.80
Site 6-13643:HJ05	15.4	263.1	5.74[i]
Site 7-13236:IN04	16.6	328.3	<LOQ (3.32)[i]
Site 7-13236:IN06	16.6	313.8	7.51[i]
Site 8-14081:TV01	16.2	302.3	3.61[i]
Site 8-14081:TV12	16.4	298.6	4.08[i]

Notes(s): i = includes results <LOQ and >LOQ; <LOQ = <4 µg/kg
Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

350 degree-days post treatment

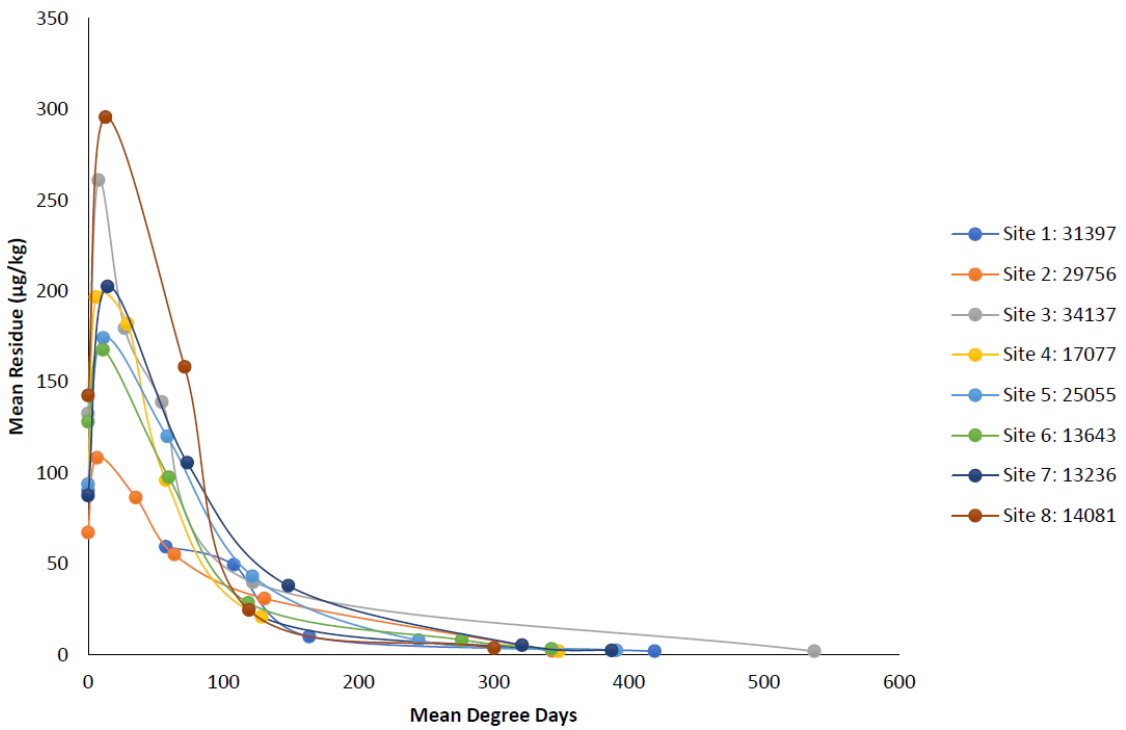
Table 26. Mean residues in salmon fillet samples taken at the 350 degree-days post-treatment time point. Seawater temperature was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (°C)	Degree-days	Mean residue(µg/kg)
Site 1-31397:OT02	5.8	438.9	<LOQ
Site 1-31397:OT12	5.9	399.3	<LOQ
Site 2-29756:IB06	4.3	352.8	2.51[i]
Site 2-29756:IB15	4.3	333.4	<LOQ
Site 3-34137:SÅ06	10.5	541.1	<LOQ
Site 3-34137:SÅ05	10.5	533.6	<LOQ

Site and pen no.	Seawater temperature (°C)	Degree-days	Mean residue(µg/kg)
Site 4-17077:TV01	9.1	348.3	<LOQ
Site 4-1707:TV07	9.1	348.3	<LOQ
Site 5-25055:BK02	14.7	389.2	<LOQ
Site 5-25055:BK10	14.5	392.5	<LOQ
Site 6-13643:HJ02	16.7	340.7	<LOQ (3.28)[i]
Site 6-13643:HJ05	16.8	345.0	<LOQ (3.22)[i]
Site 7-13236:IN04	16.6	394.6	<LOQ
Site 7-13236:IN06	16.6	380.1	<LOQ (2.98)[i]
Site 8-14081:TV01	nd[ii]	nd[ii]	nd[ii]
Site 8-14081:TV12	nd[ii]	nd[ii]	nd[ii]

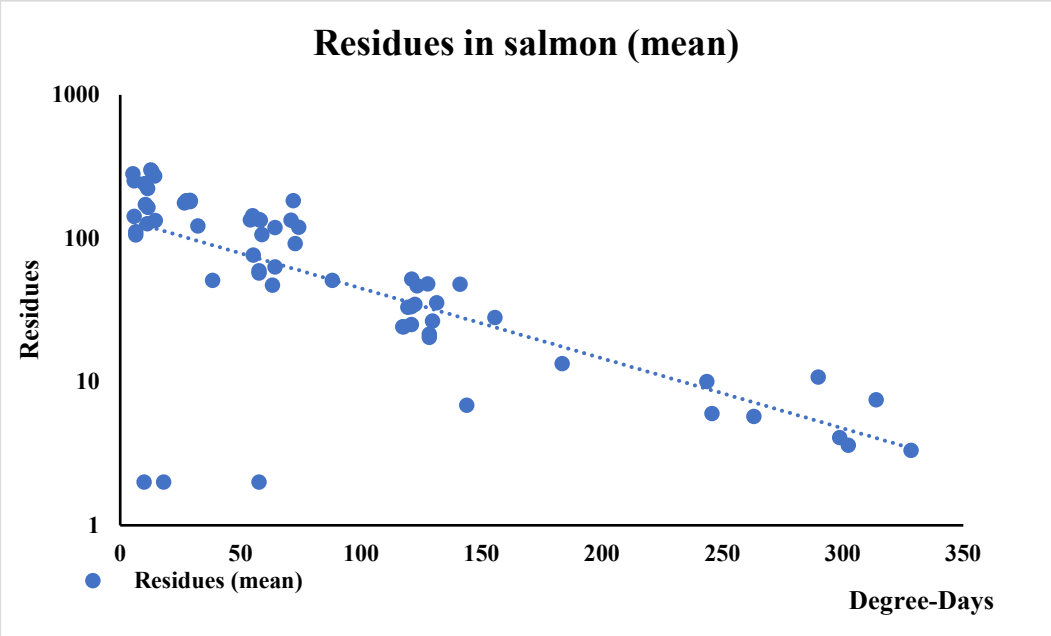
Notes(s): i = includes results <LOQ and >LOQ; ii = Sample not taken as coincided with 21 day post-treatment sample. <LOQ = 4 µg/kg. Site staff calculated a predicted 350 degree -day timepoint, table values represent actual degree days elapsed since treatment.
Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

Figure 6. Mean residues in fillet of Atlantic Salmon from the 8 different study locations at mean degree-days after bath treatment. Means below LOQ have been plotted as half LOQ (2 µg/kg)



Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

Figure 7. Log mean residues in fillet of Atlantic Salmon from the 8 different study locations at mean degree-days after bath treatment. Means below LOQ have been plotted as half LOQ (2 µg/kg)



Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

The first two timepoints (pre-treatment and immediately post treatment) are excluded as the depletion phase had not yet started.

Results (trout):

Table 27. Summary of details of the 4 commercial rainbow trout aquaculture sites used in this study. Bodyweight was recorded during the pre-study veterinary inspection. Treatment time and dose is the average across all the wells used. Treatment temperature is the average temperature in the wells at the start of treatment

Site and pen no.	Pen no.	Mean fish weight (kg)	Mean treatment time	Mean treatment temperature (ºC)	Mean dose (mg/L)
Site 1-12077	01	2.319	57	16.4	24.08
	02	2.084	154	16.4	20.65
	08	2.226	65	16.2	30.31
Site 2-13541 Tx1	01	1.993	69	16.0	25.19
	04	1.568	82	15.8	24.01
Site 3-14799	02	1.664	67	15.2	24.04
	04	1.710	68	15.3	20.14
Site 4-13541	04	2.012	66	13.4	22.07
	05	1.833	68	13.5	23.95

Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

Before treatment

Table 28. Mean residues in trout fillet samples taken prior to treatment. Seawater temperature on day of treatment was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (ºC)	Degree-days	Mean residue(µg/kg)
Site 1-12077 - 01	16.0	0	<LOQ
Site 1-12077 - 02	16.0	0	<LOQ
Site 1-12077 - 08	16.0	0	<LOQ
Site 2-13541 Tx 1 - 01	16.3	0	<LOQ
Site 2-13541 Tx 1 - 04	15.8	0	<LOQ
Site 3-14799 - 02	15.8	0	<LOQ
Site 3-14799 - 04	15.4	0	<LOQ
Site 4-13541 Tx2 - 04	12.9	0	<LOQ
Site 4-13541 Tx2 - 05	12.9	0	<LOQ

Notes(s): <LOQ = <4 µg/kg

Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

Immediately post treatment

Table 29. Mean residues in trout fillet samples taken immediately post-treatment. Seawater temperature was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (ºC)	Degree-days	Mean residue(µg/kg)
Site 1-12077 - 01	16.0	0	82.00
Site 1-12077 - 02	16.0	0	373.00
Site 1-12077 - 08	16.0	0	142.80
Site 2-13541 Tx 1 - 01	16.3	0	255.00
Site 2-13541 Tx 1 - 04	15.8	0	284.80
Site 3-14799 - 02	15.4	0	172.60
Site 3-14799 - 04	15.4	0	268.20
Site 4-13541 Tx2 - 04	12.9	0	175.48
Site 4-13541 Tx2 - 05	12.9	0	207.40

Notes(s): <LOQ = <4 µg/kg.

Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

24 hours post treatment

Table 30. Mean residues in trout fillet samples taken 24 hours after treatment. Seawater temperature was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (ºC)	Degree-days	Mean residue(µg/kg)
Site 1-12077 - 01	16.5	16.0	139.28
Site 1-12077 - 02	16.5	16.0	292.00
Site 1-12077 - 08	16.0	16.0	195.80
Site 2-13541 Tx 1 - 01	16.0	16.3	257.40
Site 2-13541 Tx 1 - 04	16.3	15.8	196.60
Site 3-14799 - 02	15.4	15.8	269.80
Site 3-14799 - 04	15.4	15.4	268.60
Site 4-13541 Tx2 - 04	12.9	12.9	231.20
Site 4-13541 Tx2 - 05	13.0	12.9	210.20

Notes(s): <LOQ = <4 µg/kg

Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

5 days post treatment

Table 31. Mean residues in trout fillet samples taken 5 days post-treatment. Seawater temperature was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (ºC)	Degree-days	Mean residue(µg/kg)
Site 1-12077 - 01	15.0	80.5	22.30
Site 1-12077 - 02	15.0	80.5	67.96
Site 1-12077 - 08	16.0	80.5	48.36
Site 2-13541 Tx 1 - 01	15.5	78.5	68.60
Site 2-13541 Tx 1 - 04	15.4	78.9	54.10
Site 3-14799 - 02	15.3	77.4	35.84
Site 3-14799 - 04	15.2	76.9	28.58
Site 4-13541 Tx2 - 04	13.0	77.8	50.78
Site 4-13541 Tx2 - 05	12.9	77.9	67.32

Notes(s): <LOQ = <4 µg/kg

Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

10 days post treatment

Table 32. Mean residues in trout fillet samples taken 10 days post-treatment. Seawater temperature was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (°C)	Degree-days	Mean residue(µg/kg)
Site 1-12077 - 01	15.0	154.5	3.84[i]
Site 1-12077 - 02	15.0	154.5	10.06
Site 1-12077 - 08	13.0	157.5	8.22
Site 2-13541 Tx 1 - 01	15.2	155.5	7.66[i]
Site 2-13541 Tx 1 - 04	15.2	156.1	4.75[i]
Site 3-14799 - 02	14.3	152.6	<LOQ
Site 3-14799 - 04	14.3	151.1	Nd[ii]
Site 4-13541 Tx2 - 04	12.2	143.2	14.94
Site 4-13541 Tx2 - 05	12.2	142.4	16.41[i]

Notes(s): nd = no data; i= includes results <LOQ and >LOQ; <LOQ = <4 µg/kg; ii = Sample not taken

Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

21 days post treatment

Table 33. Mean residues in trout fillet samples taken 21 days post-treatment. Seawater temperature was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (°C)	Degree-days	Mean residue (µg/kg)
Site 1-12077 - 01	14.0	309.5	6.04[1]
Site 1-12077 - 02	14.0	309.5	<LOQ
Site 1-12077 - 08	13.0	312.5	<LOQ
Site 2-13541 Tx 1 - 01	13.0	298.8	<LOQ
Site 2-13541 Tx 1 - 04	13.0	301.6	<LOQ
Site 3-14799 - 02	13.3	291.7	<LOQ
Site 3-14799 - 04	12.9	289.2	<LOQ
Site 4-13541 Tx2 - 04	11.4	268.4	<LOQ (2.94)[i]
Site 4 v Tx2 - 05	10.6	266.8	<LOQ (2.48)[i]

Notes(s): i = includes results <LOQ and >LOQ. <LOQ = <4 µg/kg

Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

350 degree-days post treatmentTreatment

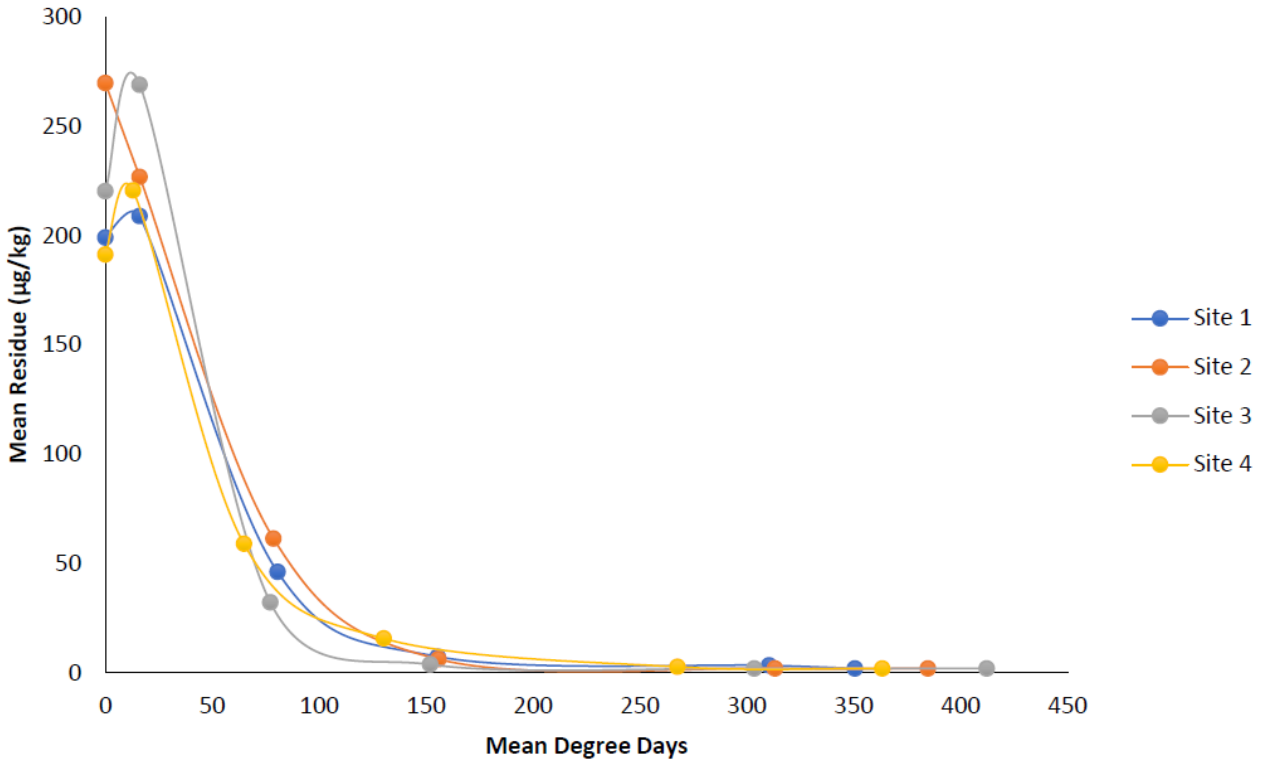
Table 34. Mean residues in trout fillet samples taken at the 350 degree-day post-treatment time point. Seawater temperature was recorded by site staff or taken from Barents Watch

Site and pen no.	Seawater temperature (°C)	Degree-days	Mean residue (µg/kg)
Site 1-12077 - 01	13.0	350.5	<LOQ
Site 1-12077 - 02	13.0	350.5	<LOQ
Site 1-12077 - 08	13.0	366.5	<LOQ
Site 2-13541 Tx 1 - 01	12.9	376.8	<LOQ
Site 2-13541 Tx 1 - 04	12.9	392.6	<LOQ
Site 3-14799 - 02	13.0	420.1	<LOQ
Site 3-14799 - 04	13.0	404.3	<LOQ
Site 4-13541 Tx2 - 04	10.9	428.6	<LOQ
Site 4-13541 Tx2 - 05	10.9	415.6	<LOQ

Notes(s): <LOQ = <4 µg/kg

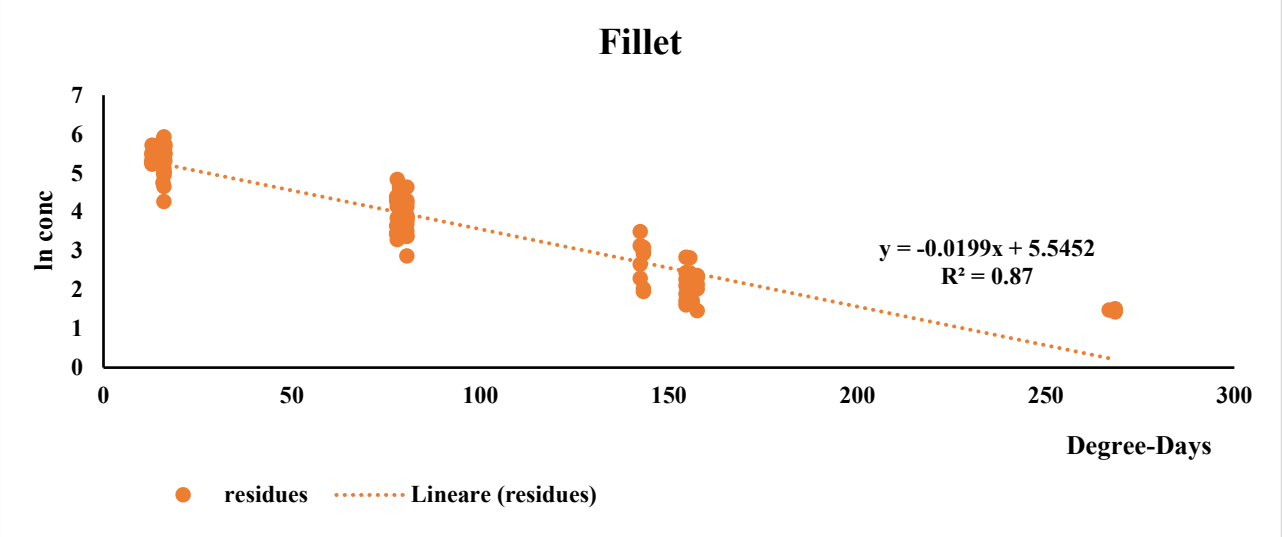
Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

Figure 8. Mean residues in fillet of rainbow trout from the 4 study sites at mean degree-days after bath treatment. Means below LOQ have been plotted as half LOQ (LOQ = 4 µg/kg)



Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

Figure 9. Log mean residues in fillet of rainbow trout from the 4 study sites at mean degree-days after bath treatment. Means below LOQ have been plotted as half LOQ (LOQ = 4 µg/kg)



Source: Gaffney, K. 2019. The Depletion of Ectosan from the Edible Tissues of Atlantic Salmon and Rainbow Trout after Bath Treatment under commercial conditions - Summary Report. Benchmark Animal Health Ltd. Study No. ECT-NOR-004-2018-GCP and ECT-NOR-005-2016-GCP. Sponsor submitted.

The first two timepoints (pre-treatment and immediately post treatment) are excluded as the depletion phase had not yet started.

Methods of analysis for residues in tissues

Methods suitable for screening samples for potential non-compliant residues are available. In addition, quantitative methods based on liquid chromatography with tandem mass spectrometry (LC-MS/MS) were developed and validated in compliance with GLP.

Screening methods

The following papers were found as part of the published literature search, and are included for information only:

ELISA

Frew and Grue, 2012: *Development of a new method for the determination of residues of the neonicotinoid insecticide imidacloprid in juvenile chinook (Oncorhynchus tshawytscha) using ELISA detection.*

Quantification of imidacloprid residues in tissue can be used for determining salmonid exposure. Refinement of an existing protocol using LC-MS/MS detection would provide the low limits of quantification, given the relatively small tissue sample sizes necessary for determining exposure in individual fish.

A new sample preparation protocol was developed for use with a commercially available enzyme-linked immunosorbent assay (ELISA) for the quantification of imidacloprid, thereby providing a low-cost alternative to LC-MS/MS. Extraction of the analyte from the

salmonid brain tissue was achieved by homogenization in Triton X-100, followed by incubation at 50–55°C. Centrifugal ultrafiltration and reversed phase solid phase extraction were used for sample clean-up. The limit of quantification for an average 77.0 mg whole brain sample was calculated at 18.2 µg/kg (ppb) with an average recovery of 79 percent. This relatively low limit of quantification allows for the analysis of individual fish.

GC-MS/MS

Buchweitz *et al.*, 2019: *Qualitative identification of imidacloprid in postmortem animal tissue by gas chromatography-tandem mass spectrometry.*

This method was developed to analyse samples from wild birds that had been poisoned. The procedure relies on a combined Food Emergency Response Network (FERN) and QuEChERS (*Qu*ick, *E*asy, *Ch*eap, *E*ffective, *R*ugged, and *S*afe) approach to sample extraction followed by qualitative analysis by gas chromatography-tandem mass spectrometry. Since imidacloprid is not amenable to the conditions of gas chromatography, a trimethylsilyl derivative was produced and characterized. Proposed mechanisms for the formation of this derivative and its mass spectrum are described. Since this method is not specifically for fish samples, no additional details will be provided here.

Quantitative methods

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The analytical method for the detection of residues used in the residue depletion studies is based on liquid chromatography with mass spectrometry (LC-MS/MS) detection for imidacloprid. The marker residue is the parent, imidacloprid. The methods have been validated for salmon and trout tissues, in accordance with GLP.

The methods are identical for both species. In summary, samples mechanically homogenised, spiked with internal standard (imidacloprid-d4) and extracted with methanol, followed by SPE. Imidacloprid is quantified by LC-MS/MS (*m/z* 256/209), using a solvent calibration curve and peak area ratios. Since the methods use LC-MS/MS quantitation, a confirmatory transition (*m/z* 256/175) is included in the detection parameters. This transition was not evaluated during method validation but could be employed to confirm the presence of imidacloprid.

The sponsor has provided several references describing the processes and validation of an analytical method to determine imidacloprid in various tissues of salmon and trout.

Table 35. Studies performed for the validation of the salmon and trout tissue residue LC-MS/MS method validation

Study	Purpose	Reference
Salmon tissue studies		
Development and Validation of an Analytical Method for the Determination of Imidacloprid in Sea Water and Atlantic Salmon Liver, Muscle and Skin	Development and validation of the analytical method at 25, 50, and 100 µg/kg for salmon muscle, skin, and liver and 10, 20 and 40 mg/L for sea water. Validation for: • Specificity (matrix) • Accuracy	Bancroft 2016b Amendment: Bancroft 2016c

	• Precision
	• Matrix dilution
	• Linearity
	• Limit of detection,
	• Limit of quantification
	• Stability
	  o frozen tissue samples
	  o Freeze/thaw cycles
	  o Stability of extracted samples	

Supplementary Validation: Specificity of an Analytical Method for the Determination of the Marker Residue of Imidacloprid from Other Anti Sea Lice Compounds and LC-MS/MS Robustness	Supplementary validation for tissue and sea water methods:
	• Robustness
	• Interference from other compounds
	  o Azamethiphos
	  o Cypermethrin
	  o Deltamethrin
	  o Emamectin Benzoate
		Bancroft 2016d

Supplementary Validation: Additional Validation of an Analytical Method for the Determination of Imidacloprid in Atlantic Salmon Liver, Muscle and Skin to Extend the Validated Range	• Validation of the limit of quantitation (LOQ) as 4 µg/kg and validation at 600, 1200, and 2400 µg/kg • Robustness testing of sample preparation steps	Bancroft 2016e
Analysis of Atlantic Salmon Fillet (Muscle with Skin) Samples for Imidacloprid by LC-MS/MS and an Assessment of the Frozen Storage Stability of Imidacloprid in Atlantic Salmon Fillet	• Validation of the extended frozen salmon fillet storage time. Samples initially stored as part of Bancroft 2016a study were re-analysed This study was performed as part of the field trial residue sample analysis.	Harris 2019a
Final analytical method	SOP of the final method	Bancroft 2016f
Trout tissue studies		

Validation of an Analytical Method for the Determination of Imidacloprid in Rainbow Trout Fillet (Muscle and Skin)	Validation of the analytical method at 4, 25, 50, 100, 600, 1200, and 2400 µg/kg for trout fillet.
	Validation for:
	• Specificity
	• Accuracy
	• Precision
	• Linearity
	• Limit of detection,
	• Limit of quantification
	• Stability
	  o frozen tissue samples
	  o Freeze/thaw cycles
	  o Stability of extracted samples
		Harris 2019b

Final analytical method	SOP of the final method	Harris 2019c

Bancroft, 2016f: *Analytical procedure for the determination of imidacloprid in salmon tissue*

This analytical method was developed for the determination of imidacloprid residue in salmon muscle, skin, and liver. It has been validated over the range 4–2400 µg/kg for all tissues. The validation was in accordance with GLP.

Samples were stored at -20°C until analysis, at which point, they were processed into a fine homogenous powder with dry ice.

Samples are extracted in methanol with mechanical agitation prior to SPE clean up. Final extracts are produced by reducing the eluent from the clean up to dryness under nitrogen gas, reconstitution in acetonitrile/water (50/50, v/v) and diluted 20-fold with acetonitrile/water (50/50, v/v) when required. An aliquot of the extract is then analysed by liquid chromatography with mass spectrometry (LC-MS/MS).

Extraction Procedure

1. Control matrix should be removed from the freezer and allowed to thaw at ambient temperature.
2. For control and QC samples, weigh control matrix into the bottom of a centrifuge tube. For test samples, weigh sample into the bottom of a centrifuge tube.
3. At least one double blank sample (no internal standard or test item) and one single blank sample (containing internal standard only, no test item) should be extracted with each batch.
4. Fortify quality control samples with QC solutions and the internal standard solution. Fortify test and single blank samples with internal standard solution.
5. Immediately after spiking, vortex mix and leave to stand.
6. Add three steel balls and methanol to all samples.
7. Mix samples then centrifuge at ambient temperature.
8. Transfer supernatant into a new centrifuge and repeat steps 6 and 7, with only one steel ball added to each sample.
9. Combine supernatant for each sample and make up to 8mL with methanol. Vortex mix and transfer 4 mL of the extract into a centrifuge tube.
10. Evaporate the extract to dryness under a steady flow of nitrogen and reconstitute in water.
11. Condition SPE cartridges with methanol followed by water.
12. Load all of the sample from step 10 onto SPE cartridges and elute by gravity, if possible, but vacuum can be used if necessary.
13. Wash SPE cartridges water followed by water/ methanol and elute by gravity, if possible, but vacuum can be used if necessary. Allow eluate to go to waste. Dry under full vacuum.
14. Elute SPE cartridge with methanol by gravity, if possible, but vacuum can be used if necessary, collecting eluate in a centrifuge tube. Dry under full vacuum.
15. Evaporate samples to dryness under a flow of nitrogen.
16. Reconstitute in acetonitrile/water (50/50, v/v) and vortex mix.
17. Dilute the samples with acetonitrile/water (50/50, v/v).
18. Transfer an aliquot into an HPLC vial for analysis.
19. Analyse by LC-MS/MS.

Test samples with determined or suspected concentrations greater than the upper limit of linearity of 50 ng/mL (equivalent to 400 µg/kg) should be diluted up to 10-fold with control matrix prior to extraction in order to bring the determined concentration of Imidacloprid within the linear range of the assay.

The test item and internal standard peak responses are calculated for each of the calibration standards, quality control samples, controls and unknown test samples. A calibration curve is then obtained by plotting the internal standard response ratio vs the concentration of the compound in each calibration standard. The calculated concentration in each sample is corrected for the dilutions performed during the analytical procedure and any unit conversions to yield a residue in µg/kg.

Table 36. Summarized validated method performance

Parameter		Muscle	Liver	Skin
System linearity			0.5 - 50 ng/mL	
Theoretical LOD		0.131 µg/kg	0.145 µg/kg	0.300 µg/kg
Theoretical LOQ		0.276 µg/kg	0.283 µg/kg	0.589 µg/kg
Validated LOQ		4 µg/kg	4 µg/kg	4 µg/kg
Validated assay range			4 - 2400 µg/kg	
Inter-day accuracy (%) and Precision (CV%)	25 µg/kg	102 (4.43)	106 (4.86)	96.3 (9.07)
	50 µg/kg	94.9 (6.21)	94.9 (8.77)	92.0 (5.90)
	100 µg/kg	103 (3.98)	104 (4.46)	96.8 (6.88)
	600 µg/kg	105 (3.42)	103 (5.33)	102 (3.58)
	1200 µg/kg	104 (3.53)	104 (3.91)	102 (4.35)
	2400 µg/kg	103 (2.85)	102 (4.53)	99.7 (6.13)
Matrix dilution Accuracy (%) and Precision (CV%)	10-Fold	102 (6.19)	109 (6.09)	109 (4.32)
	5-Fold	107 (2.47)	106 (3.04)	104 (7.41)
Room temperature stability (25 - 2400 µg/kg)			4 hours	
Freeze/thaw stability			3 freeze/thaw cycles	
Autosampler stability	25 - 100 µg/kg	108 hours	87 hours	105 hours
	600 - 2400 µg/kg	153 hours	115 hours	129 hours
Extended frozen storage stability	25 - 100 µg/kg	98 days	98 days	98 days
	2400 µg/kg	30 days	30 days	30 days
Assay specificity		Assay is specific no significant interfering peaks eluting at the same retention time as Imidacloprid and IS		

Source: Bancroft. K. 2016f. Analytical procedure for the determination of imidacloprid in salmon tissue. Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, UK, Charles River analytical procedure AP.225406B.03. Sponsor submitted.

Harris, 2019c: *Analytical procedure for the determination of imidacloprid in rainbow trout fillet.*

This analytical procedure was developed for the determination of imidacloprid in rainbow trout fillet (muscle with skin) and is suitable for the determination of imidacloprid in rainbow trout fillet over the range of 4–2 400 µg/kg. The validation was in accordance with GLP.

Samples are extracted in methanol with mechanical agitation prior to SPE clean up. Final extracts are produced by reducing the eluent from the SPE clean up to dryness under nitrogen gas and reconstituted in acetonitrile/water (50:50, v/v). Samples are diluted 20-fold with

acetonitrile:water (50:50, v/v) when required. An aliquot of the extract is then analysed by liquid chromatography with mass spectrometry (LC-MS/MS).

This is the same method as described in Bancroft, 2016f.

Table 37. Summary of the analytical results of validation

Information	Data
Analyte	Imidacloprid
Internal Standard (IS)	Imidacloprid-d4
Matrix	Rainbow trout fillet (muscle with skin)
Extraction technique	Solvent extraction followed by clean-up using reversed-phase SPE
Calibration curve concentration range	0.25 to 50.0 ng/mL (accuracy and precision batches, then 0.5 to 50.0 ng/mL)
Validated Quality Control (QC) samples concentrations	4.00 µg/kg, 25.0 µg/kg (low level), 50 µg/kg, 100 µg/kg, 600 µg/kg, 1200 µg/kg, and 2400 µg/kg (high level).
Regression type	Weighted linear (1/concentration)
Quantitation method	Peak area ratio
Stock solution stability	67 days at a concentration of 1000 µg/mL in acetonitrile and 49 days at concentrations of 100 and 0.025 µg/mL in acetonitrile:water (50:50, v/v) in a refrigerator set to maintain 4°C. 24 hours at a concentration of 1000 µg/mL in acetonitrile and 24 hours at concentrations of 100 and 0.025 µg/mL in acetonitrile:water (50:50, v/v) ambient room temperature unprotected from light
Internal standard stock solution stability	67 days at a concentration of 150 µg/mL in acetonitrile and 49 days at concentrations of 96 and 0.6 µg/mLin acetonitrile:water (50:50, v/v) in a refrigerator set to maintain 4°C. 24 hours at a concentration of 150 µg/mL in acetonitrile and 24 hours at concentrations of 96 and 0.6 µg/mL in acetonitrile:water (50:50, v/v) ambient room temperature unprotected from light
Specificity	No significant interference at the retention time of the analyte or IS
Matrix effects	No obvious differences observed when quantifying matrix samples with either a solvent or matrix-matched calibration standards
Inter-assay precision and accuracy range (solvent calibration standards)	% CV: 2.6 to 6.9% % Accuracy: 97.7 to 102.2%
Estimated Limit of Detection	0.413 µg/kg
Estimated Limit of Quantification	1.38 µg/kg
Dilution (single blank extract)	10 000 µg/kg extract diluted 10-fold and 20-fold
Freeze-thaw matrix stability	3 freeze-thaw cycles in a freezer set to maintain -20°C

Information	Data
Short-term matrix stability	4 hours at ambient room temperature
Long-term matrix stability	It was demonstrated that rainbow trout fillet fortified with Imidacloprid was stable for at least 242 days when stored in a freezer set to maintain -20°C.
Re-injection reproducibility	119 hours at injection tray temperature (*ca* 4°C)

Source: Harris, J. 2019b. Validation of an analytical method for the determination of imidacloprid in rainbow trout fillet (muscle and skin). Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, UK, Report No. 40587. Sponsor submitted.

Other techniques

The following reports were found as part of the published literature search:

Dufour *et al.*, 2021: *Miniaturization of an extraction protocol for the monitoring of pesticides and polar transformation products in biotic matrices.*

This study investigated the development of a miniaturized extraction protocol for the monitoring of small organisms, based on only 30 mg of matrix. The miniaturized sample preparation was developed using fish and macroinvertebrate matrices. It allowed the characterization of 41 pesticides and transformation products (log P from -1.9 to +4.8) in small samples with LC-MS/MS, based on European guidelines. Quantification limits ranged from 3 to 460 µg/kg dry weight (dw) for fish and from 0.1 to 356 µg/kg dw for invertebrates, with most below 60 µg/kg dw. Extraction rates ranged from 70 percent to 120 percent for 35 molecules in fish. Recoveries ranged from 70 percent to 120 percent for 37 molecules in macroinvertebrates. Inter-day precision was below 30 percent for 32 molecules at quantification limits. The method was successfully applied to 17 fish and 19 macroinvertebrates collected from two ponds of the French region of Dombes in November and May 2018, respectively. Both sample matrices were nearly always contaminated with benzamide, imidacloprid-desnitro, and prosulfocarb at respective concentrations of 42 –237, 3, and 30–165 µg/kg dw in fish, and 62–438, 2–6, and 15–29 µg/kg dw in macroinvertebrates. Results show that this method is an effective tool for characterizing polar pesticides in small biotic samples.

Xiao *et al.*, 2013: *Determination of neonicotinoid insecticides residues in eels using subcritical water extraction and ultra-performance liquid chromatography-tandem mass spectrometry.*

This study presented a new high-throughput methodology for the simultaneous determination of multiple neonicotinoid insecticide residues (including imidacloprid) in eel samples, based on subcritical water extraction followed by UPLC-MS/MS. The average recoveries of the various analytes varied from 84.6 percent to 102 percent, with relative standard deviations (RSD) lower than 10.8 percent. The method was shown to be sensitive, with limits of detection of 0.12–0.36 µg/kg and limits of quantification of 0.42–1.12 µg/kg for the various neonicotinoids. The procedure was found to be a sustainable and efficient method for the analysis of neonicotinoid residues in aquatic products.

Stability of residues

Bancroft, 2016b: *Development and validation of an analytical method for the determination of imidacloprid in sea water and Atlantic salmon liver, muscle and skin.*

Freeze/thaw stability

The effect of repeatedly freezing and thawing samples was investigated by preparing replicate matrix samples fortified with imidacloprid at suitable concentrations and repeatedly freezing and thawing prior to extraction. Samples of sea water were prepared at 10 mg/L and 40 mg/L and samples of salmon muscle, liver and skin were prepared at the lower and upper fortification levels.

To define the reference (initial) concentrations, replicate (n=3 at each level) aliquots of each matrix were prepared and assayed, together with a solvent calibration curve series of standard solutions, on the day of preparation. The back-calculated concentration for each replicate sample was determined and defined as the reference concentration.

To determine the freeze/thaw stability of imidacloprid in each matrix, three sets of replicate aliquots (n=3 at each level) of each matrix were prepared (fortified with solutions prepared independently from the calibration standard solutions). Following fortification of the aliquots, the samples were stored frozen in a freezer set to maintain -20ºC for a minimum of 24 hours.

The samples were then thawed at room temperature until they reached ambient temperature and then re-frozen for a minimum of 24 h. Samples were subjected to one, two and three freeze/thaw cycles. The samples were then assayed along with n=3 freshly fortified recovery samples of the same matrix fortified at the same nominal concentrations, together with a solvent calibration curve series of standard solutions.

The freeze/thaw stability of imidacloprid was defined as the percentage difference between the reference concentration and the stability timepoint concentration. Imidacloprid was deemed to be stable after the appropriate number of freeze/thaw cycles if the stability timepoint concentration was ± 20 percent of reference concentration.

Imidacloprid was found to be stable for up to three freeze/thaw cycles in sea water and salmon muscle, liver, and skin.

Solution stability

The effects of storing calibration standard solutions, quality control stock solutions, quality control solutions, and internal standard solutions of imidacloprid-d4 (IS) in a fridge set to maintain +4°C was investigated by the comparison of freshly prepared standard solutions with those from previously prepared standard solutions.

The test internal standard extracts were prepared in triplicate and contained and aliquot of the same freshly prepared standard solution to allow a comparison by peak area ratio.

The stability of calibration standard, quality control and internal standard solutions of imidacloprid and IS were considered to be stable if the mean peak area ratio for the stored solutions is within 100 ± 10 percent of the mean peak area ratio of the freshly prepared solutions, and the precision should be ≤10 percent.

Calibration and quality control solutions of imidacloprid were determined to be stable for 22 days when stored in a fridge set to maintain +4°C. Quality control stock solutions were determined to be stable for 28 days when stored in a fridge set to maintain +4°C. Internal standard stock solutions were determined to be stable for 22 days and working internal standard solution were determined to be stable for at least 20 days when stored in a fridge set to maintain +4°C.

Extended frozen storage stability

The effects of storing samples frozen in a freezer set to maintain -20°C was investigated by preparing sea water samples fortified at 10 mg/L and 40 mg/L, and salmon tissue samples fortified at lower and upper fortification levels. Sufficient samples were prepared in each matrix to permit (n=3) replicate samples at each level, which had not been thawed since preparation, to be analysed after at least 30 and 98 days frozen storage.

To define the reference (initial) concentrations, replicate (n=3 at each level) aliquots of each matrix were prepared and assayed, together with a solvent calibration curve series of standard solutions, on the day of preparation.

To determine the storage stability of imidacloprid in each matrix at ca -20°C after at least 30 and 98 days' frozen storage, replicate (n=3 at each level) samples were thawed and assayed along with n=3 freshly fortified recovery samples of the same matrix fortified at the same nominal concentrations, together with a calibration curve series of standard solutions.

The stability of imidacloprid was defined as the percentage difference between the reference concentration and the stability timepoint. Imidacloprid was deemed to be stable in each matrix in a freezer set to maintain -20°C if the stability concentration was ± 20 percent of reference concentration. Accuracy and precision for the stability samples should also meet the acceptance criteria.

Imidacloprid was determined to be stable in sea water and salmon tissues for at least 98 days when stored in a freezer set to maintain -20°C.

Harris, 2019a: *Analysis of Atlantic salmon fillet (muscle with skin) samples for imidacloprid by LC-MS/MS and an assessment of the frozen storage stability of imidacloprid in Atlantic salmon fillet.*

As the time samples had been kept frozen exceeded that previously validated (98 days in salmon muscle and 98 days in salmon skin as part of Bancroft, 2016b), it was decided to reanalyse 20 Atlantic salmon fillet samples previously analysed in Bancroft, 2016a for imidacloprid content and the results compared with the original values.

As the frozen storage stability analysis met the acceptance criteria, it was concluded that imidacloprid is stable in samples of Atlantic salmon fillet stored in a freezer set to maintain -20°C for at least 809 days.

Harris, 2019b: *Validation of an analytical method for the determination of imidacloprid in rainbow trout fillet (muscle and skin)*

Freeze/thaw stability

The effects of repeatedly freezing and thawing samples stored in a freezer set to maintain -20°C was investigated by preparing replicate samples fortified with imidacloprid at the lower and upper fortification levels and repeatedly freezing and thawing prior to extraction. Following fortification, the samples were stored frozen for a minimum of 24 h. The samples were thawed at room temperature until they reached room temperature and then refrozen for a minimum of 24 h. Samples were then subjected to 3 freeze/thaw cycles.

The samples were assayed together with n≥3 freshly fortified recovery samples of the same matrix fortified at the same nominal concentrations together with a solvent calibration curve series of standard solutions.

The freeze/thaw stability of Imidacloprid in matrix was assessed on the percentage difference between the reference concentration and the stability timepoint concentration. Imidacloprid was determined to be stable after 3 freeze/thaw cycles if the stability timepoint concentration is ± 20 percent of reference concentration.

It was demonstrated that rainbow trout fillet fortified with Imidacloprid was stable after at least 3 cycles of freeze/thaw where the frozen cycles were carried out in a freezer set to maintain -20°C.

Extended frozen storage stability

The effects of storing trout fillet samples frozen in a freezer set to maintain -20°C (range -18 to -22°C) was investigated by preparing samples fortified with imidacloprid at the lower and upper fortification levels).

To determine the storage stability of imidacloprid in the matrix at ca -20°C after approximately 8 months, replicate (n=3 at each level) samples were thawed and assayed together with n≥3 freshly fortified recovery samples of the same matrix fortified at the same nominal concentrations, together with a solvent calibration curve series of standard solutions.

The stability of imidacloprid in matrix was defined as the percentage difference between the reference concentration and the stability timepoint.

It was demonstrated that rainbow trout fillet fortified with imidacloprid was stable for at least 242 days when stored in a freezer set to maintain -20°C.

Appraisal

An acceptable daily intake (ADI) was set by the Joint FAO/WHO Meeting on Pesticide Residues (FAO and WHO, 2002) at 0.06 mg/kg bw in 2001, and acute reference dose (ARfD) 0.4 mg/kg bw in 2002. However, JECFA could not establish either an ADI or an ARfD due to a data gap on disruption of the human microbiome and because of this, a microbiological ADI/ARfD could not be established.

Pharmacokinetics

The sponsor provided proprietary data supplemented with published literature in order to demonstrate the pharmacokinetic profiles of imidacloprid when administered to Atlantic salmon and rainbow trout in a treatment bath consisting of 20 mg imidacloprid per litre of seawater for a duration of up to 360 minutes. The Committee also conducted a published literature search for useful data in the public domain, and any relevant data from papers found have been summarized in this report.

The JMPR assessed the toxicology and pharmacology of imidacloprid for use in plant protection products and published a summary in 2002 (FAO and WHO, 2002). The sponsor has referred to that report to support the proposed metabolic pathways in laboratory species.

The metabolism in Atlantic salmon has been investigated using radiolabeled ^{14}C-imidacloprid (Hobbs, 2015). The parent substance was the major radiolabeled substance found (between 77 percent and >90 percent TRR), with one minor metabolite, which could either be the 4-hydroxy or the 5-hydroxy metabolite, at a much lower concentration (≤8.2 percent TRR).

The temperature range used in this study (7 to 8°C) was not fully representative of real-world conditions, and temperature usually has a significant effect on absorption, metabolism, and clearance times in fish.

No information on metabolism of imidacloprid at higher temperatures is available and it is unknown whether the ratio of imidacloprid to its metabolite differs at various temperatures. Nonetheless, the study temperature does conform to the requirements of VICH GL57 (5 to 10°C), which is intended to provide guidance for studies used to determine the withdrawal period of a product (EMA, 2019). This study was well conducted and sufficient to determine the marker residue, i.e., parent imidacloprid. Because the main metabolite is not considered to be a major part of the residues to which humans would be exposed (i.e., less than 10 percent), it does not need to be a part of the marker residue definition.

The sponsor provided a published paper by Frew *et al*. (2018). The main finding of this study was that there was no *in vitro* hepatic metabolism of imidacloprid detected in rainbow trout liver S9 fractions. This demonstrates that the same marker residue (parent imidacloprid only) could be used for both salmon and trout.

In an *in vivo* experiment in rainbow trout, imidacloprid was quickly distributed into tissues and was eliminated almost entirely by excretion of parent compound across the gills or into urine. Plasma clearance half-life was around 68 hours. It is highly bound in trout blood, which suggests that it binds specifically to molecular components of blood. This finding suggests that imidacloprid may accumulate in trout in cases of continuous or repeated exposure (Frew *et al*., 2018).

As part of the published literature search, a study using *Australoheros facetus* fish was found which showed the distribution of the compound into different tissues after a freshwater bath exposure (Iturburu *et al*., 2017). The distribution was very similar to that seen in the study of trout by Frew *et al*. (2018), with distribution seen to all tissues including brain, liver, kidney, and guts.

The sponsor provided no data looking at the comparative metabolism across species, but did provide a good justification for the absence of such data by providing separate data on rats

(FAO and WHO, 2002), humans (*in vitro*; Schulz-Jander and Casida, 2002), and Atlantic salmon (Hobbs, 2015). The mammals have quite extensive metabolic pathways, whereas the salmon only metabolize the substance to a small degree, with only one identified metabolite. The same was also seen in rainbow trout in a study found in the published literature (Kolanczyk *et al*., 2020). Rats also produce this metabolite, via hydroxylation of the imidazolidine ring at the 4 or 5 position, to yield 4- or 5 hydroxy imidacloprid, which accounted for about 16 percent of the recovered radiolabel, whereas in salmon it was ≤8.2 percent. It can therefore be concluded that the toxicology studies in which rats were used would also take into account any mammalian toxicity related to the metabolite seen in salmonids.

The pharmacokinetics in fish appears to be similar in all species for which data are available. Imidacloprid is distributed to all tissues in various proportions, and hardly metabolized. The only metabolite seen is 5-hydroxy imidacloprid.

Based on the results of these studies the Committee identified imidacloprid as the sole marker residue in salmon and rainbow trout fillet and determined that a value of 0.7 was appropriate for the marker residue to total recovered radioactivity ratio (MR:TRR). This was based on two main factors. Firstly, that there were no sample points between day 5 and day 26 (approximately 37–195 degree-days) of the TRR study in salmon, although the withdrawal period is likely to fall between those two time points. Secondly that the TRR study was conducted at a relatively low temperature (7–8°C) whereas it has been seen in some of the residue depletion studies that water temperature can reach 15–17°C under field conditions. It is known that water temperature affects the metabolic rate in fish, so it is possible that this increased metabolic rate at higher temperatures may result in a lower MR:TRR ratio. Thus, with a choice between MR:TRRs of 0.9 determined at five days post treatment, or 0.7 determined at 26 days post treatment, the Committee considered that the lower value would lead to a more conservative approach and was therefore chosen in this case.

Residue depletion

The authorised dosing regimen is 20 mg/l administered in a treatment bath for 60 minutes; however, in field conditions it has been noted that it is not always possible to remove the fish from the treatment baths within a reasonable time, which is why data from studies using increased durations of immersion (up to 360 mins) have been provided.

The sponsor provided three studies conducted under laboratory conditions in Atlantic salmon. Two of them were conducted to GLP and provided robust data. The third, non-GLP study (Auchinachie, 2019a), provided no useful information.

The first GLP study, Bancroft, 2016a, was conducted in accordance with VICH GL49 and GLP (EMA, 2015). It was an exposure study conducted using Atlantic salmon in sea water for a 28-day period at 15°C and a 60-day period at 7°C and to determine the residue depletion in muscle, liver, skin, and fillet. The dosing regimen was 20 mg imidacloprid per litre of seawater, with the fish exposed for about 60 minutes. The parent compound was selected as the marker residue as it was the major residue detected in a previous study (Hobbs, 2015). The results demonstrate that absorption of imidacloprid is increased at the higher temperature when compared to the lower temperature and is excreted slower at the lower temperature when compared to the higher temperature.

The second GLP study, Longshaw, 2020a, was conducted in accordance with VICH GL49 and GLP (EMA, 2015). Because the product needs to be administered in a well-boat to prevent environmental exposure, it can sometimes mean that fish are exposed for more than 60 minutes, and up to 6 hours (360 minutes). As such, this study investigated the effect of extended treatment bath exposure on residue depletion from Atlantic salmon, using the same dosing regimen of 20 mg imidacloprid per litre of seawater. Three groups of fish were exposed to the treatment bath for 60, 196 and 360 minutes at 15.2 to 16.0°C. The results demonstrate that longer exposure times lead to increased residues levels and longer depletion times in the fish.

The worst-case scenario from all studies is where the fish are treated for extended durations at a relatively high temperature, and so the data from Longshaw (2020a) should be used as the pivotal data to establish the MRL.

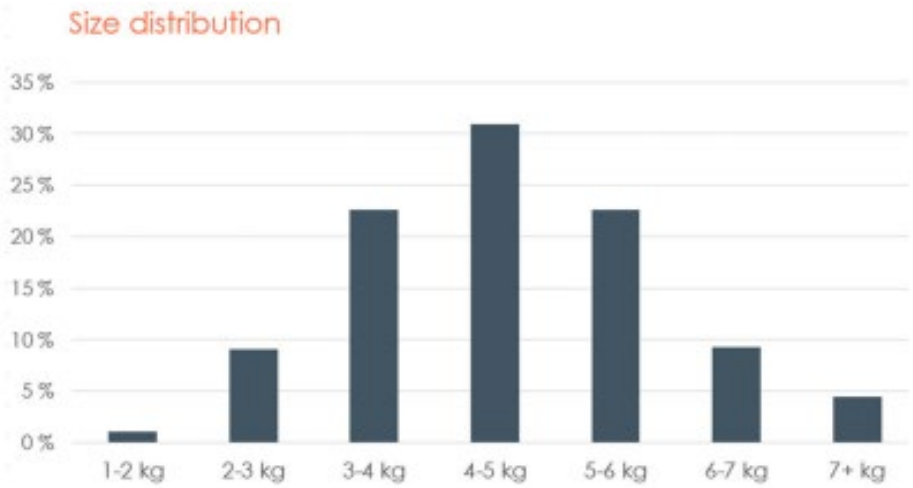
The sponsor also provided two residue depletion studies conducted under real-life conditions in the field. One study looked at residue depletion in Atlantic salmon and the other in rainbow trout. Both studies were reported in Gaffney, 2019. The fish were treated using well-boats to perform a bath treatment at a concentration of 20 mg/L (± 10 percent) for 60 minutes (± 5 minutes). A total of 8 Atlantic salmon sites and 4 trout sites were treated. The seawater temperature was carefully monitored in this study, in order to accurately determine the timepoints in degree-days. Seawater temperatures fluctuated between the different sites where the salmon study was conducted, with a range of 5.0 to 17.6°C. The temperature range for the trout sites was smaller, at 10.9 to 16.5°C.

For salmon, it was noted that the water temperature used in some sites was high in comparison to guidance found in VICH GL57 for salmonids (5-10°C), and when using the data from 5 to 10°C sites grouped together, and those sites where the water was >10°C, there was a large difference between the median values calculated at 98 degree-days (EMA, 2019). For the sites that were <10°C, it was 44 µg/kg, and for those >10°C, it was 67 µg/kg. This indicates that the water temperature significantly affects the absorption and excretion, and thereby the residue depletion profile, of imidacloprid in fish.

The committee noted that for all the available studies in salmon, the size/weight of the fish was quite small (Bancroft, 2016a: 383.5–577 g; Longshaw, 2020a: 408.8 g), whereas salmon are usually harvested around 4–5 kg. It is not clear whether larger fish would absorb as much imidacloprid per kg bw than smaller fish.

Figure 10 shows Norwegian harvest distribution for 2020, with the harvest size of 4–5 kg being the most frequent.

Figure 10. Norwegian salmon harvest distribution for 2020



Source: MOWI. 2022. *Salmon Farming Industry Handbook 2022*. Cited 27 May 2022. mowi.com/wp-content/uploads/2022/07/2022-Salmon-Industry-Handbook-1.pdf

VICH GL57 states: 'Study animals should be representative of the commercial species and representative of the target animal population that will be treated. The bodyweight ranges should be consistent with the intended product label for the proposed use. If the product is intended to be used at various stages of development, then the study should be conducted in animals representing the highest development stage (the stage that has a metabolic state that is representative of market size) (EMA, 2019).

The Committee recommends that VICH GL57 is followed when evaluating products containing imidacloprid for approval in a Member State, taking the harvest weights into account; however, water temperatures used should be representative of local conditions, and err on the higher side (EMA, 2019).

The Committee considered the residue depletion study by Longshaw (2020) to be the pivotal study, as this gave the worst-case results in terms of extent and persistence of imidacloprid residues in salmon fillet. It was noted that this was because the salmon had been exposed to the treatment solution for longer than the approved duration (approximately 6-fold longer); however, it was also noted that this was a practical consideration since the salmon had to be treated in well-boats in order to prevent exposure of the environment to imidacloprid. As a result, in some cases it can take longer than the approved treatment time to remove the salmon from the treatment bath. It was also noted that this was the study used to set the withdrawal period for the one approved product in a Member State.

It should be noted that there are no data available for repeated treatments, but imidacloprid would be expected to accumulate in fish tissues should repeated treatments occur too close together.

Analytical methodology

The sponsor used the same analytical method for all proprietary residue depletion studies provided, for both salmon and trout tissues. Samples are extracted in methanol with mechanical agitation prior to SPE clean up. Final extracts are produced by reducing the eluent from the clean up to dryness under nitrogen gas, reconstitution in acetonitrile/water (50/50, v/v) and diluted 20-fold with acetonitrile/water (50/50, v/v) when required. An aliquot of the extract is then analysed by liquid chromatography with mass spectrometry (LC-MS/MS).

The method was appropriately validated to determine sufficiently accurate and precise levels of imidacloprid residue in tissue samples from both species. The range over which it was validated as giving a linear response was 25–2 400 µg/kg for muscle, skin, liver, and fillet matrices. The LOQ of the method was determined to be 4 µg/kg.

Storage stability

The sponsor provided data that demonstrated the stability of imidacloprid in various media, including sea water (the treatment solution), stock solutions, and incurred salmon tissue matrices (muscle, skin, liver, and fillet), under various storage conditions (Bancroft, 2016b; Harris, 2019a).

The sponsor also determined the stability of incurred residues in rainbow trout fillet (Harris, 2019b).

Imidacloprid was found to be stable for up to three freeze/thaw cycles in sea water, salmon muscle, liver, and skin, and trout fillet.

It was also found to be stable in calibration standard solutions, quality control stock solutions, and quality control solutions, as were internal standard solutions of imidacloprid-d4 (IS), in a fridge set to maintain +4°C.

Additionally, it was found to be stable in sea water and salmon tissues (muscle, liver, and skin) for at least 98 days when stored in a freezer set to maintain -20°C, and in samples of Atlantic salmon fillet stored in a freezer set to maintain -20°C for at least 809 days.

It was demonstrated that rainbow trout fillet fortified with imidacloprid was stable for at least 242 days when stored in a freezer set to maintain -20°C.

These periods of time adequately cover the time the actual samples were stored between sampling and analysis in all the studies reviewed.

In summary, the analytical method was shown to have been appropriately validated, the marker residue is parent imidacloprid, the MR:TRR is 0.7, and the only withdrawal period authorized is 98 degree-days.

Considerations regarding the production of salmon oil for use in supplements for human consumption

The sponsor states that 'for both salmon and trout, the only edible tissue is fillet (muscle and skin in natural proportions), and this is the commodity which is exported/imported for human consumption.' However, there are supplements available for sale that are marketed as containing 'salmon oil', which is derived from the pressing of the residual carcass of the salmon after the fillets are removed (GOED, 2022). It is noted that the residual carcass in the residue studies provided was either not analysed, or the results of the analyses were not included in the report; however, the liver was routinely sampled and analysed, demonstrating high levels when compared to e.g., fillet. It has also been seen from the data provided in the pharmacology section that imidacloprid distributes freely around the other organs of the fish, including the brains and kidneys.

In response to a question from the assessor, the sponsor provided some information on the processing of salmon carcasses into salmon oil for use in supplements for human use. The information provided describes the production processes in the EU, for instance, there is a European pharmacopeia monograph for salmon oil produced from farmed fish (European Pharmacopeia Monograph, 2015), and so may differ in other regions.
The process includes the following steps:

(1) Cooking to coagulate the proteins which releases the bound water and oil.
(2) Separating the coagulate to split the material into a solid and liquid phase which includes the released oils, this can be done either by centrifugation or pressing.
(3) Separation of the oil from the suspended solids either by centrifugation or by cooling and filtering (winterisation).
(4) Polishing/refining to remove impurities.

At the end of the process, there is a highly refined and purified oil, which is unlikely to contain any contaminants, including residues of veterinary drugs. It can therefore be concluded that there will be no additional exposure to imidacloprid for consumers of salmon oil supplements.

Dietary exposure assessment

Estimates of dietary exposure to imidacloprid residues

Dietary exposure to imidacloprid may occur through its use as a veterinary drug or as discussed above, its multiple registered uses as a pesticide.

Dietary exposure to imidacloprid (in some cases in combination with other neonicotinoid insecticides) has been estimated in several studies. FAO and WHO (2012) estimated exposure to be 2–5 percent of an ADI of 0.06 mg/kg bw from residues potentially occurring in plant and animal commodities.

EFSA (2019) recently estimated chronic and acute dietary exposure for all uses of imidacloprid. Dietary exposures calculated were compared with the toxicological reference value derived by EFSA. The highest chronic exposure estimate represented 7 percent of the ADI. Exceedance of the EFSA ARfD was identified for some commodities. A second exposure

calculation was therefore performed, considering fallback Good Agricultural Practice for these commodities. According to the revised calculation, the highest chronic exposure estimates declined to 6 percent of the EFSA ADI and the highest acute exposure (cucumbers) was 76 percent of the EFSA ARfD.

Crépet *et al*. (2021) estimated acute dietary exposure to several pesticide residues, including imidacloprid, using probabilistic methods. They estimated that imidacloprid exposure for adults ranged from 0.07 to 0.78 µg/kg bw, depending on consumption patterns in six different countries. For children the range was 0.26–1.9 µg/kg bw. They concluded that estimated acute dietary exposures were much lower than the ARfD of 400 µg/kg bw established by JMPR.

Dietary exposure was estimated based on the on the following scenarios and assumptions for occurrence of imidacloprid residues in Atlantic salmon muscle only and in all fin fish: MR:TRR = 0.7. Adjusted median residue levels in Atlantic salmon (muscle, adult fish) were 486 µg/kg at a withdrawal period of 98 degree-days post dose UTL 95/95 residue in Atlantic salmon (muscle, adult fish) was 859 µg/kg at a withdrawal period of 98 degree-days post dose. No ADI, ARfD, or MRL was recommended.

It should be noted that, as theoretical scenarios have been used for estimating dietary exposure, there is a high level of uncertainty for any proposed MRL based on these estimates. A final exposure estimate will be made once GVP, an ADI and ARfD have been established.

Chronic dietary exposure estimates

When used as a veterinary drug, chronic dietary exposure was estimated based on the potential occurrence of imidacloprid residues in Atlantic salmon muscle. The adjusted (MR:TRR = 0.7) mean residue level in Atlantic salmon (fillet) was 486 µg/kg. This value relates to a withdrawal period of 98 degree-days. No ADI was available.

Based on incurred residues in Atlantic salmon (fillet) and a withdrawal period of 98 degree-days, the global estimate of chronic dietary exposure (GECDE) for adults and the elderly is 1.0 µg/kg bw per day. For children and adolescents, the GECDE is 2.7 µg/kg bw per day. For infants and toddlers, the GECDE is 0.9 µg/kg bw per day (Table 38).

Based on incurred residues in fish meat and a withdrawal period of 98 degree-days, the GECDE for adults and the elderly is 1.8 µg/kg bw per day. For children and adolescents, the GECDE is 3.8 µg/kg bw per day. For infants and toddlers, the GECDE is 1.2 µg/kg bw per day (Table 38).

The consumption figures used were highest reliable percentile consumption figures based on consumers only considered from the available dataset, Chronic individual food consumption database, Summary statistics (CIFOCOss)[1].

Table 38. Global estimates of chronic dietary exposure (GECDE*) to imidacloprid residues at 98 degree-days

Food	Adults and elderly	Children and adolescents	Infants and toddlers
	GECDE µg/kg bw per day	GECDE µg/kg bw per day	GECDE µg/kg bw per day
Atlantic salmon fillet	1.0	2.7	0.9
Fin fish	1.8	3.8	1.2

Notes(s): *the GECDE is the sum of the highest exposure at the highest reliable percentile of consumption for a food and the mean dietary exposures of the other foods.

Acute dietary exposure assessment

Acute dietary exposure (global estimate of acute dietary exposure, GEADE) was estimated for consumption of Atlantic salmon using food consumption values from the FAO/WHO large portion (97.5th percentile, one day) database and 95/95 upper tolerance limit (UTL) concentrations for imidacloprid. Acute dietary exposure was also assessed based on total fish consumption using the same residue data. Data was taken GEMS/Food large portion size database (97.5th percentile, one day; see JECFA, 2013).

Acute dietary exposures were assessed at 98 degree-days post dose. The adjusted (MR:TRR = 0.7) 95/95 UTL concentrations used were 859 µg/kg. No ARfD was available. Estimates were made for both children and the general population (Table 39).

The GEADE based on consumption of Atlantic salmon was 6.2 and 6.6 µg/kg bw for adults and children respectively. The GEADE based on consumption of all fin fish was 34.1 and 23.8 µg/kg bw for adults and children respectively (Table 39).

Table 39. Global Estimates of Acute Dietary Exposure (GEADE#) for imidacloprid residues at two upper tolerance limits in Atlantic salmon at 98 degree-days

Food	General population	Children
	GEADE µg/kg bw	GEADE µg/kg bw
Atlantic salmon fillet	6.2	6.6
Fin fish	34.1	23.8

Notes(s): #the GEADE uses food consumption of a large portion (97.5th percentile, one day) and residues concentration at the upper tolerance limit to estimate acute exposure; ^UTL: upper tolerance limit

Maximum Residue Limits

In recommending MRLs for imidacloprid in fin fish fillet, the Committee considered the following factors:

- An ADI for imidacloprid could not be established by the Committee.
- An ARfD for imidacloprid could not be established by the Committee.
- Imidacloprid is used as a pesticide and a veterinary drug.
- Imidacloprid is authorized for use in salmon and trout in one Member State. The maximum recommended treatment regimen is 20 mg/L, once, via immersion in a seawater treatment bath for 60 minutes. The approved withdrawal period is 98 degree-days for Atlantic salmon and rainbow trout.
- Under field conditions it may not be possible to remove all the fish from the treatment bath immediately after 60 minutes, so exposures of up to 360 minutes were considered.
- Imidacloprid is the marker residue in salmon and trout fillet.
- The ratio of marker residue to total residue concentrations was established at 0.7.
- Residue data for salmon and trout were provided using a validated analytical method to quantify imidacloprid in fillet.
- A validated analytical method for determining imidacloprid in salmon and trout fillet is available and may be used for monitoring purposes.

As the Committee could not determine an ADI or an ARfD, an MRL could not be recommended.

When considering the possibility of recommending the extrapolation of MRLs, the Committee referred to the discussion at the CCRVDF (see link here) on the 'Proposed approach for the extrapolation of maximum residue limits of veterinary drugs to one or more species'.

Were an MRL to be recommended in salmon and trout, the Committee could recommend extrapolation of the MRL to all salmonids, and potentially to all fin fish.

Although there are no pharmacokinetic or residue depletion data currently available for species other than salmon and trout, experience shows that fish do not metabolize pharmaceutical compounds to a great extent. As such, it is considered to be unlikely that the MR:TR in non-salmonid fin fish species would be much different from that seen in Atlantic salmon.

It should be noted, however, that water temperature has a significant effect on the extent of absorption, extent of metabolism and rate of elimination in fish. The data available do not address rate or extent of metabolism at higher water temperatures. Many other farmed fin fish species are kept at higher temperatures than salmonid species, and so might have different metabolic profiles, including an increased metabolism which would lead to a lower MR:TR being calculated, leading to an underestimate of human dietary exposure.

Nonetheless, the Committee considered that, as the worst-case scenario had been used for estimating the likely dietary exposure, there would be a margin of safety for any proposed MRL that could take into account slight differences in metabolism between salmonids and non-salmonids. A final recommendation will be made once an ADI and ARfD have been established.

Interested parties may wish to provide data on the MR:TR when fish are exposed to higher water temperatures (that is, greater than 10°C) to allow the Committee to make a more informed recommendation.

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Ivermectin

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Background

Ivermectin (CAS No. 70288-86-7)[1] is a macrocyclic lactone belonging to the avermectin family, widely used as a broad-spectrum antiparasitic drug against nematodes and arthropods in food-producing animals. It is also used in human medicine to treat a variety of internal nematode infections, including onchocerciasis, strongyloidiasis, ascariasis, cutaneous larva migrans, filariasis, gnathostomiasis, and trichuriasis, as well as for oral treatment of ectoparasitic infections, such as pediculosis and scabies (Ottesen and Campbell, 1994).

Ivermectin is a chemically modified fermentation product of *Streptomyces avermitilis*, consisting of a mixture of two homologous compounds 22,23-dihydroavermectin B1a (H_2B_{1a}, not less than 80 percent) and 22,23-dihydroavermectin B1b (H_2B_{1b}, not more than 20 percent).

The Committee previously evaluated ivermectin at is 36th (FAO and WHO, 1990), 40th (FAO and WHO, 1993), 54th (FAO and WHO, 2000), 58th (FAO and WHO, 2002), 75th (FAO and WHO, 2012), 78th (FAO and WHO, 2014), 81st (FAO and WHO, 2016) and 88th (FAO and WHO, 2020) meetings.

At its 36th meeting the Committee evaluated radiolabeled depletion studies in cattle, sheep and pigs and recommended an MRL for all species for the marker residue H_2B_{1a} of 15 µg/kg for liver and 20 µg/kg for fat.

[1](1'R,2R,4'S,10'E,14'E,16'E,21'R)-6-(butan-2-yl)-21',24'-dihydroxy-12'-{[(2R,4S,6S)-5-{[(2S,4S,6S)-5-hydroxy-4-methoxy-6-methyloxan-2-yl]oxy}-4-methoxy-6-methyloxan-2-yl]oxy}-5,11',13',22'-tetramethyl-3',7',19'-trioxaspiro[oxane-2,6'tetracyclo[15.6.1.1;{4,8}.0;{20,24}]pentacosane]-10',14',16',22'-tetraen-2'-one

At its 40th meeting, the Committee established an ADI of 0–1 µg/kg bw based on the developmental toxicity of ivermectin in CF-1 mice and recommended MRLs of 40 µg/kg for fat and 100 µg/kg for liver for residues of ivermectin in cattle, using the marker residue ivermectin B_{1a}.

At its 54th meeting, the Committee recommended a temporary MRL of 10 µg/kg for cattle milk, expressed as ivermectin B_{1a}, which was confirmed at the 58th Meeting of the Committee.

At its 78th meeting, the Committee recommended an MRL of 4 µg/kg for cattle muscle, determined as ivermectin B_{1a}, based on the depletion data contained in the residue monographs prepared by the 36th and 40th meetings of the Committee and on the value of two times the limit of quantification of the analytical method.

At its 81st meeting, the Committee was made aware that the MRL for ivermectin in bovine muscle recommended at the 78th meeting was in some cases ≥ 2.5 times lower than the MRL established in some countries where ivermectin was used. At the meeting, the Committee received new residue depletion data and recommended the following MRLs for cattle tissues: 400 µg/kg for fat, 100 µg/kg for kidney, 800 µg/kg for liver, and 30 µg/kg for muscle. Also, at this meeting, an ADI of 0–10 µg/kg bw was established based on a NOAEL of 0.5 mg/kg bw per day for neurological effects (mydriasis) and retardation of weight gain in a 14-week dog study, with the application of an uncertainty factor of 50. At this meeting, the Committee also established an ARfD of 0.2 mg/kg bw, based on a NOAEL of 1.5 mg/kg bw (WHO, 2016).

At its 88th meeting, the Committee received a data set from one Member State, including two residue depletion studies in sheep. No residue depletion data were received for pigs or goats. At this meeting, the Committee recommended MRLs in sheep tissues: 15 µg/kg for kidney and 10 µg/kg for muscle. The Committee also confirmed the existing MRLs for fat at 20 µg/kg and for liver at 15 µg/kg. In addition, the Committee recommended maintaining the existing MRLs in pig fat (20 µg/kg) and pig liver (15 µg/kg) tissues and extending the MRLs for sheep muscle to pig muscle (10 µg/kg) and sheep kidney to pig kidney (15 µg/kg). This extension was made considering the limited residue data for pigs and the similarity of the overall tissue distribution and residue depletion in both species. As no residue depletion data for ivermectin were received to calculate MRLs for goats and based on the similarity of the residue distribution and depletion in different animal species, the Committee recommended extrapolation of the MRLs for sheep and pig tissues to goat tissues (10 µg/kg for muscle, 15 µg/kg for liver, 15 µg/kg for kidney and 20 µg/kg for fat).

The 25th session of the CCRVDF requested that JECFA revaluate the MRLs for ivermectin for pigs and sheep/goat muscle, liver, kidney, and fat. Member States expressed concern about the MRLs established at the 88th JECFA meeting, considering that the values are considerably lower than those established in many Member States, and while not representing a safety concern, the MRLs for sheep, goat and pig tissues could pose trade difficulties.

The MRLs of ivermectin in these species currently adopted in some Member States are shown in Table 1. These MRLs are based on the marker residue H_2B_{1a}.

Table 1. MRLs for ivermectin residues in sheep, pigs and mammals adopted in some Member States

Jurisdiction	MRL in µg/kg (marker residue H_2B_{1a})						
	Animal species	Muscle	Liver	Fat	Kidney	Edible offal	Injection site
Codex[a]	Sheep	10	15	20	15		
	Goat	10	15	20	15		
	Pig	10	15	20	15		
European Union	Mammalian	30	100	100	30		1300
United States of America	Sheep		30				
	Pig	20	20				
Canada[b]	Sheep	10	30	120	180		
	Pig	10	15	100	150		
Australia	Sheep	20	15		10		
	Pig	20	10		10		
Brazil[c]	Sheep	10	15	20	15		
	Goat	10	15	20	15		
	Pig	10	15	20	15		
Japan	Sheep	10	15	20	10	15	
	Pig	20	15	20	10	10	

Notes(s): a: 88[a] JECFA evaluation (2020); b: May 2021 (https://www.canada.ca/en/health-canada/services/drugs-health-products/veterinary-drugs/maximum-residue-limits-mrls/list-maximum-residue-limits-mrls-veterinary-drugs-foods.html); c: December 2019 (https://www.in.gov.br/web/dou/-/instrucao-normativa-n-51-de-19-de-dezembro-de-2019-235414514).

The current Committee received data set from three sources, including residue depletion studies in pigs (one study with radiolabeled ivermectin and two with non-radiolabeled ivermectin), sheep (two studies with non-radiolabeled ivermectin), and goats (one study with non-radiolabeled ivermectin) and metabolism study in pigs (one study) and sheep (one study). One of these studies had been previously assessed by JECFA Committee, but at that time, only the summary was available. The Committee also received data on residues of ivermectin in animal tissues from one chemical residue monitoring programme reported by the competent national authority of one Member State.

Metabolism in food-producing animals

Pigs

A metabolism and depletion study, GLP compliance not stated, (Chiu and Lu, 1982) of tritium-labelled ivermectin in pigs not previously evaluated by the Committee was reviewed. Twelve pigs (body weight not provided) received tritium-labelled ivermectin (tritium in the position C_{22}-C_{23}) via a single subcutaneous injection at a dose of 0.4 mg/kg bw. Three animals per group were slaughtered at 1, 7, 14, and 28 days post dose. Muscle, liver, kidney, and fat tissues were collected and were assayed for H2B1a and H2B1b by reverse isotope dilution assay (RIDA). The composite liver of pigs slaughtered 7 and 14 days after dosing was examined for the presence of drug metabolites by use of solvent fractionation and a combination of reversed-phase and normal-phase HPLC (Table 2).

Radioactive residues in the liver were identified or classified based on chromatography polarity. Two metabolites were identified by comparison with the metabolites generated by *in vitro* swine liver microsome incubations. The fat tissues of one pig slaughtered 14 days after dosing was analysed similarly to the liver for the presence of drug metabolites.

Table 2. Metabolite characterization (polarity) in pig liver and fat

Polarity/ metabolites	Solvent fractions	Metabolite	TRR (%) Liver[a]	TRR (%) Fat[b]
Polar	Ethyl acetate/methanol eluate of silica gel	At least 6 compounds	2	19
Polar	Methanol eluate of Sep-Pak		3	
Drug like metabolites	HPLC	D5	2	-
		D4	8	2
		D3	12	6
		D2	12	12
		D1	4	1
Non-polar	Dichloromethane eluate and HPLC	Ivermectin	45	37
Non-polar	Dichloromethane eluate and HPLC	At least 6 compounds	12	26

Notes(s): a: Animals slaughtered 7- and 14-days post dose. b. animal slaughtered 14 days post dose.

Source: Chiu, S-H.L & Lu, A.Y. 1982. Metabolism of ivermectin (MK-0933) in swine. Unpublished report study RN-194, Merck Sharp & Dohme., Rahway, New Jersey, USA, submitted to FAO by Boehringer Ingelheim Vetmedica GmbH, EU.

The total radioactive residues were extractable in organic solvents, which indicates that no covalently bound residues need to be considered. The authors stated that the unaltered drug (H2B1a, H2B1b) accounts for about 42 percent and 28 percent of the TRR in the liver at 7- and 14-days post dose, respectively. In fat, these values were 51 percent and 37 percent, respectively, for the same days.

Due to the low levels of the radioactive residues in the tissues, the isolation and purification of the metabolites for structural elucidation were not possible. *In vitro* incubations of ivermectin with pig liver microsomes were performed for metabolite identification. The metabolites were purified by reversed-phase and normal phase HPLC, and the products were analysed by UV, NMR and Fast Atom Bombardment-Mass Spectrometry.

Among the metabolites isolated and purified from the *in vitro* study, two metabolites were characterized and suggested by the authors: 3"-O-desmethyl-H_2B_{1a} and 3"-O-desmethyl-H_2B_{1b}. These metabolites were considered by the Committee at the 88th JECFA Meeting. Based on the quality of the spectra provided, the Committee was unable to confirm the indicated metabolites.

Sheep

A metabolism study, GLP compliance not stated, (Chiu and Jacob, 1981) in sheep using tritium labelled ivermectin ([22,23-^{3}H]-ivermectin) was reviewed by the Committee. Four groups of

three sheep each were dosed into the rumen, once, with ivermectin at 0.3 mg/kg bw. Animals were sacrificed on days 1, 3, 5, or 7 post dose. Tissue samples were taken from the liver, kidney, fat, and muscle to measure the radioactive residues. Edible tissues were assayed for both components of the unaltered drug (H2B1a, H2B1b) either by RIDA or by direct fluorescence assay. By a combination of solvent fractionations and reverse-phase HPLC, the radioactive residue in the liver and fat was identified or classified based on chromatographic polarity. The unaltered H2B1a (marker) and H2B1b were the major radioactive residue components at all slaughter timepoints. The radioactive residues in the tissues were extractable in organic solvents (methylene chloride), indicating that are no significant bound residues. The liver of one animal slaughtered on day 5 post dose was assayed for the presence of metabolites. The analysis included solvent extraction and liquid chromatography. The radioactive residues were identified based on chromatographic polarity. Overall, 68 percent of the TRR in the liver was identified. The remaining 32 percent of the residue consisted of at least two products less polar than the parent compound and the other seven compounds more polar than ivermectin. At least four more polar metabolites than the parent compound were identified by co-chromatography with in vitro metabolites prepared from steer liver microsome incubation of ivermectin (Table 3). The fat tissues of two animals slaughtered 5 and 7 days post dose contained the unaltered drug at 29.9 percent and 30.5 percent, respectively.

Table 3. Metabolite identification in sheep liver

Polarity	Solvent fractions	Liver			Fat	
		Metabolites (liver)	Identity	TRR (%) Liver[a]	TRR (%) Fat[b5]	Fat[b7]
Very polar	SPE, methanol		unidentified	8.9	1.6	1.3
Polar	HPLC CH_3CN: CH_3OH:H_2O, 36:24:40 v/v/v	At least two compounds	unidentified	1.6	17	2.6
Polar	HPLC CH_3CN: CH_3OH:H_2O, 36:24:40 v/v/v	A	24-OH-H_2B_{1b}-monosaccharide[c]	1.5		
		B1	24-OH-H_2B_{1a}-monosaccharide	3.9		
		B2	24-OH-H_2B_{1b}	3.2		
		C	24-OH-H_2B_{1a}	11.4		
Polar	HPLC	At least four compounds	unidentified	13		
Non-polar	HPLC	Unaltered drug	Ivermectin	48	29.9	30.5
Non-polar	HPLC		unidentified	8.1	6.9	6.3
Non-polar	SPE, CH_2Cl_2		unidentified	1.4	4.7	4.3

Notes(s): a: Animal slaughtered 5 days post dose; b5: animal slaughtered 5 days post dose; b7: animal slaughtered 7 days post dose; c: suggested but not confirmed.

Source: Chiu, S.H.L. & Jacob, T.A. 1981. Metabolism of Ivermectin (MK-0933) in Sheep. Unpublished report study RN-197, Merck Sharp & Dohme., Rahway, New Jersey, USA, submitted to FAO by Boehringer Ingelheim Vetmedica GmbH, EU.

The distribution of polar metabolites in sheep liver is like those in cattle and rat livers.

The profile of the radioactive metabolites in sheep fat indicated that most of the metabolites were less polar than the parent drug. A decline in the proportion of the polar residues was also observed accompanied by an increase of the non-polar metabolites with increasing time post dose.

The identified metabolites corroborate the findings reported by Chiu and Lu (1989), in the *in vivo* liver metabolism study of tritium-labelled ivermectin in sheep, swine, cattle and rats. The Committee had reviewed this study at the 88th meeting.

Goats

No metabolism studies were available to the Committee for ivermectin in goats.

Tissue residue depletion studies

Radiolabeled residue depletion studies

One sponsor provided two residue depletion studies using radiolabeled ivermectin, one in sheep and another in pigs, for evaluation at the present meeting.

The radiolabeled studies were carried out using [22,23-^{3}H]-ivermectin. The tritium label is stable on volatility (Chiu *et al*., 1990a).

Pigs

One study (Chiu and Lu, 1982), GLP compliance not stated, using [22,23-^{3}H]-ivermectin in pigs was reviewed by the current Committee. Twelve pigs (body weight not stated) were dosed with 0.4 mg/kg bw tritium-labelled ivermectin via single subcutaneous injection. The purity, specific activity, and solvent in which the drug was prepared were not reported.

Three animals were slaughtered at 1, 7, 14 and 28 days post dose and tissues were collected. Concentrations of H_2B_{1a}, and H_2B_{1b} concentrations were determined using RIDA (Table 4). The LOD was 1.2 µg/kg for liver and fat, 0.8 µg/kg for kidney and 3 µg/kg for muscle.

Table 4. Radiolabeled residues in pig tissues after subcutaneous administration of [22,23-^{3}H]-ivermectin at a dose of 0.4 mg/kg bw

Time post-dose (days)	Radiolabeled residues, TRR, H_2B_{1a} and H_2B_{1b} in µg eq/kg											
	Muscle			Liver			Kidney			Fat		
	TRR	H_2B_{1a}	H_2B_{1b}	TRR	H_2B_{1a}	H_2B_{1b}	TRR	H_2B_{1a}	H_2B_{1b}	TRR	H_2B_{1a}	H_2B_{1b}
1	43	15	6.5	199	48	22	106	31	11.5	384	51.5	19
7	25	11	2.95	112	39	24	55	24.5	6.6	152	80.5	20.5
14	4	-	-	22	5.4	1.95	10	5	1.5	28	6.45	2.35
28	-	-	-	3	-	-	1	-	-	6.3	-	-

Source: Chiu, S-H.L & Lu, A.Y. 1982. Metabolism of ivermectin (MK-0933) in swine. Unpublished report study RN-194, Merck Sharp & Dohme., Rahway, New Jersey, USA, submitted to FAO by Boehringer Ingelheim Vetmedica GmbH, EU.

Concentrations of H_2B_{1a} were at least 2–3 times higher than the homologue H_2B_{1b} in all tissues analysed. The ratios of mean marker residue to total radioactive residues (MR:TRR) varied with time post dose, and are shown in Table 5. The Committee noted that the changes in MR:TRR with time were not monotonic, except in kidney.

Table 5. Mean marker residue (H_2B_{1a}) to TRR at different times post dose in pigs

Time post dose (days)	MR:TRR			
	Muscle	Liver	Kidney	Fat
1	0.35	0.24	0.29	0.13
7	0.44	0.35	0.45	0.53
14	-	0.25	0.50	0.20

Sheep

A radiolabeled study (GLP compliance not stated) of ivermectin in sheep was considered by the Committee at this meeting. In this study (Chiu and Jacob, 1981), four groups of three sheep each were dosed into the rumen, once, with ivermectin at 0.3 mg/kg bw. Sheep were sacrificed on days 1, 3, 5, or 7 post dose. Liver, kidney, fat, and muscle were sampled and assayed for H2B1a and H2B1b either by RIDA or by direct fluorescence assay (Table 6).

Table 6. Radiolabeled residues in sheep tissues after subcutaneous administration of [22,23-^{3}H]-ivermectin at a dose of 0.3 mg/kg bw

Time post-dose (days)	Radiolabeled residues, TRR (µg eq/kg), H_2B_{1a} and H_2B_{1b} in %											
	Muscle[a]			Liver[a]			Kidney[b]			Fat[b]		
	TRR	H_2B_{1a}	H_2B_{1b}	TRR	H_2B_{1a}	H_2B_{1b}	TRR	H_2B_{1a}	H_2B_{1b}	TRR	H_2B_{1a}	H_2B_{1b}
1	55	67	22	238	54	51	72	51	22	307	71	24
3	50	52	22	125	51	27	46	44	26	153	51	8.5
5	9	54	3	25	56	12	12	8	<0.5	63	25	5
7	10	-	-	44	-	-	13	-	-	73	19	3

Notes(s): Analyses by a: RIDA; b: fluorescence.

Source: Chiu, S.H.L. & Jacob, T.A. 1981. Metabolism of Ivermectin (MK-0933) in Sheep. Unpublished report study RN-197, Merck Sharp & Dohme., Rahway, New Jersey, USA, submitted to FAO by Boehringer Ingelheim Vetmedica GmbH, EU.

Fat presented the highest residues at all time points.

The mean ratios of the marker residues to TRR calculated with the values given in Table 6 are shown in Table 7.

Table 7. Mean marker residue (H_2B_{1a}) to TRR at different times post dose in sheep

Time post dose (days)	MR:TRR			
	Muscle	Liver	Kidney	Fat
1	0.67	0.54	0.51	0.71
3	0.52	0.51	0.44	0.51
5	0.54	0.56	0.08	0.25

Residue depletion studies with non-radiolabeled drug

Five residue depletion studies with non-radiolabeled ivermectin in pigs (two studies), sheep (two studies) and goats (one study) from three different sources were reviewed by the Committee at the present meeting.

At the 88th JECFA meeting, the Committee used data from the residue depletion study of ivermectin in pigs carried out by Wood *et al*., 1981, and reported in FAO (1991). However, only a summary of the study was available at that meeting. At this meeting, the Committee received the data from that study.

Pigs

In a residue depletion study (Wood *et al*., 1981), 35 pigs (22.8–32.3 kg, barrows, and gilts) received a single subcutaneous dose of ivermectin of 0.4 mg/kg bw. The drug formulation contained 40 percent v/v glycerol formal and propylene glycol to make up 100 percent. The indicated withdrawal period for this formulation is 14 days. The animals were slaughtered in groups of five at 1, 3, 5, 7, 10, 14 and 28 days post dose and the concentration of H_2B_{1a} was determined in the edible tissues and in an injection site sample using HPLC with a fluorescence detector (HPLC-FLD). The results presented in Table 8 have not been corrected for recovery. The recovery was assessed by fortification of H_2B_{1a} blank tissues at four concentration levels (10, 20, 50 and 100 µg/kg). The mean recoveries were: 88 percent for muscle, 82 percent for liver, 87 percent for kidney and 90 percent for fat. The limit of the detection of the method was in the range of 1-2 µg/kg, and the limit of quantitation 10 µg/kg.

Table 8. Mean concentrations ± SD of H_2B_{1a} in muscle, liver, kidney, fat, and injection site from pigs after a single subcutaneous injection of ivermectin at a dose of 0.4 mg/kg bw. Values corrected for recovery

Time post dose (d)	Mean concentration ± SD of H_2B_{1a} (µg/kg)				
	Muscle	Liver	Kidney	Fat	Injection site
1	25.6 ± 9.7	81.7 ± 42.1	54.3 ± 31.6	82.2 ± 35.3	139333.3 ± 5856.6
3	35.9 ±. 9.0	84.4 ± 11.2	55.4 ± 17.4	121.8 ± 8.7	5715.6 ± 5757.2
5	23.0 ± 3.2	64.6 ± 12.7	36.6 ± 8.1	101.3 ± 9.7	1233.3 ± 647.8
7	14.5 ± 3.4	49.8 ± 21.1	26.7 ± 9.1	81.1 ± 26.1	2519.1 ± 3626.8
10	9.8 ± 2.5	28.3 ± 8.9	16.1 ± 8.6	51.8 ± 12.4	2760.0 ± 4318.9
14	4.1 ± 2.4	15.4 ± 3.8	5.7 ± 2.2	26.9 ± 9.0	260.0 ± 254.5
28	nd	nd	nd	nd	nd

Notes(s):nd: not detected (the lowest limit of detection is 1-2 µg/kg).

Source: Wood, J.S., Baylis, F.P. & Stauffer, S.C. 1981. Ivermectin (MK-0933): Tissue residue in swine dosed subcutaneously. Unpublished report study SW304 (0.4 mg/kg Formulation B), Merck &Co. Inc., Rahway, New Jersey, USA, submitted to FAO by Boehringer Ingelheim Vetmedica GmbH, EU.

Besides the injection site, the highest ivermectin concentrations were determined in fat and liver. By 28-days post dose, all residues were lower than 2 µg/kg. Injection site residues stayed relatively high even at 14 days post dose but were negligible at 28 days post dose.

In another residue depletion study (GLP-compliant), (Brennan, 1999) 22 pigs (11 barrows, 11 gilts; weight range 92.4 to 122.2 kg on Day -1) received a single subcutaneous injection of ivermectin at a dose of 0.3 mg/kg bw. Groups of four animals were slaughtered at 7, 14, 21, 28 and 35 days post dose and tissues (muscle, liver, kidney, fat and the injection site) collected. Ivermectin quantitation was carried out using a validated HPLC-FLD method (Hughes *et al.,* 2000). The limit of detection (LOD) of ivermectin for the method was 0.11 µg/kg for muscle and liver, 0.56 µg/kg for fat; and 0.10 µg/kg for the kidney. The limit of quantitation (LOQ) of ivermectin for this method is 2 µg/kg for all tissues. Recoveries of ivermectin from fortified blank samples at different concentration levels were 91 percent (standard deviation: 8.1 percent) for muscle, 86 percent (standard deviation: 5.5 percent) for liver, 91 percent (standard deviation: 7.3 percent) for kidney and 96 percent (standard deviation: 8.1 percent) for fat. Individual dosing data were not provided and therefore the Committee could not confirm the actual dose administered to each animal. The results of the residues determined in the tissues are shown in Table 9. These values have been corrected for recoveries in each tissue. For the statistical treatment of the data, the concentration of one animal (tissue injection site, 1719 µg/kg at 14 days post dose) was eliminated from the data set because it was considered an outlier by the Committee.

Table 9. Mean concentrations ± SD of H_2B_{1a} in muscle, liver, kidney, fat, and injection site of pigs after single subcutaneous injections of ivermectin at a dose of 0.3 mg/kg bw. Values corrected for recovery

Time post dose (d)	Mean concentration ± SD of H_2B_{1a} (µg/kg)				
	Muscle	Liver	Kidney	Fat	Injection site
7	14.0 ± 8.4	44.3 ± 22.2	25.1 ± 14.1	112.8 ± 63.8	26.6 ± 17.5
14	7.7 ±. 6.0	18.8 ± 5.6	11.1 ± 3.0	72.9 ± 20.6	16.1 ± 7.9
21	2.3 ± 1.5	11.1 ± 4.1	12.6 ± 11.1	37.0 ± 12.3	8.0 ± 3.3
28	<LOQ	4.2 ± 0.8	3.7 ± 1.8	6.7 ± 3.8	2.8 ± 1.04
35	<LOQ	<LOQ	1.8 ± 0.9	3.8 ± 2.2	<LOQ

Source: Brennan, J.J., Lothrop, R., Cahil, L., Hancey, B. & Wilson J. 1999. Ivermectin injection. Tissue residue study in pigs following subcutaneous administration of ivermectin injection at the recommended dose. Unpublished report study 9813 Final report, Cross Vetpharm Group Ltd., Broomhill Rd. Tallaght, Dublin, Ireland, submitted to FAO by Bimeda Animal Health Limited, Stillorgan, Dublin, Ireland.

Sheep

In a residue depletion study, GLP-compliance not stated, (Wood *et al*., 1984), 24 wether and ewe lambs (39.6–58.1 kg) received a subcutaneous dose of ivermectin of 0.3 mg/kg bw once a week for three weeks. The drug formulation contained 40 percent v/v glycerol formal and propylene glycol to make up 100 percent. The indicated withdrawal period is 22 days. The animals were slaughtered in groups of four or five at 3, 7, 10, 14 and 28 days post dose, and the concentration of H_2B_{1a} was determined in the edible tissues and the injection site by HPLC-FLD.

The results presented in Table 10 had been corrected for recovery. The recovery was assessed by fortification of blank tissues at four concentration levels (10, 20, 50 and 100 µg/kg). The mean recoveries were: 93 percent for muscle, 89 percent for liver, 87 percent for kidney and

95 percent for fat. The limit of the quantitation of the method was about 10 µg/kg. The residue depletion curves of ivermectin in sheep are presented in Figure 3. In these depletion curves, the concentration of the marker residue was corrected for recoveries.

Table 10. Mean concentrations ± SD of H_2B_{1a} in muscle, liver, kidney, fat, and injection site of pigs after three weakly subcutaneous injections of ivermectin at a dose of 0.3 mg/kg bw. Values corrected for recovery

Time post dose (d)	Mean concentration ± SD of H_2B_{1a} (µg/kg)				
	Muscle	Liver	Kidney	Fat	Injection site
3	68.4 ± 13.3	179.8 ± 27.5	51.7 ± 2.6	246.3 ± 34.6	18357.9 ± 18612.6
7	80.6 ±. 26.6	211.2 ± 57.0	69.0 ± 11.6	326.3 ± 107.3	3098.9 ± 1787.2
10	51.6 ±2 5.9	108.8 ± 54.0	33.3 ± 11.0	186.3.3 ± 75.7	2471.6 ± 1464.5
14	32.0 ± 14.7	61.6 ± 28.3	22. 5± 6.2	104.0 ± 54.6	480.0 ± 294.9
28	3.5 ± 2.4	7.9 ± 6.4	2.6 ± 1.7	13.9 ± 8.2	235.3 ± 185.4

Source: Wood, J.S., Baylis, F.P. & Gawlik, G. 1984. Ivermectin (MK-933): Study in sheep (SH256) dosed three times with IVOMEC at 0.3 mg/kg to determine tissue residues. Merck &Co. Inc., Rahway, New Jersey, USA, submitted to FAO by Boehringer Ingelheim Vetmedica GmbH, EU.

Another residue depletion study (GLP compliant) of ivermectin in sheep was considered by the Committee at this Meeting (McHardy *et al*., 2005). Twenty Suffolk cross ewe (52–89 kg) received a single subcutaneous dose of ivermectin of 0.2 mg/kg bw. The animals were slaughtered in groups of five at 28, 35, 42, and 49 days post dose, and the concentration of H_2B_{1a} was determined in muscle, liver, kidney, fat and injection site by and validated HPLC-FLD method (Quinn, 2004). The analytical method involved the extraction of ivermectin and the internal standard abamectin from tissues with acetonitrile (acetonitrile and hexane in equal proportions were used for the extraction from fat), followed by C_{18} solid phase extraction. Eluants were dried under nitrogen before reconstitution and derivatized before injection unto the HPLC fitted with fluorescence detection (excitation wavelength: 365 nm and emission wavelength: 475 nm). The calibration was in the range of 5 to 200 µg/kg, and the limit of quantitation of the method was 5 µg/kg for all tissues.

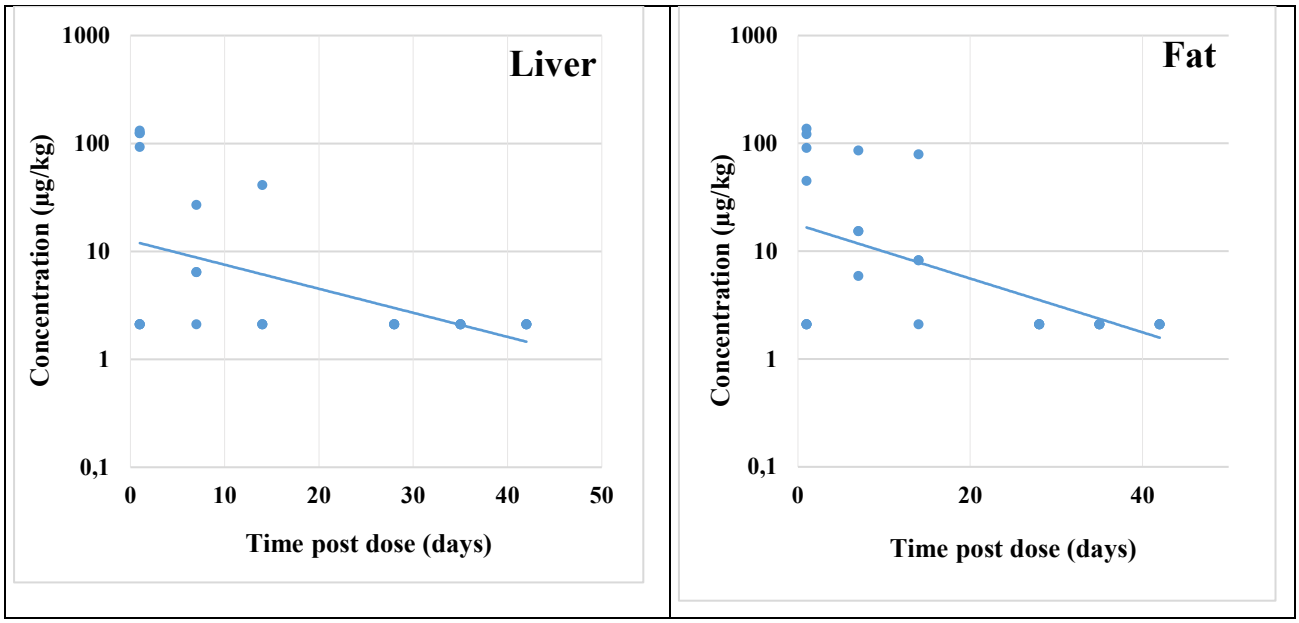
All 100 tissue samples analysed contained ivermectin concentrations lower than the LOQ with the exceptions of seven samples collected at 28 days post dose (kidney: 5.2 µg/kg, liver 14.3 µg/kg, fat: 30.8 and 12.0 µg/kg, and injection site: 5.7 µg/kg) and 49 days post dose (injection site: 44.9 µg/kg and 6.4 µg/kg). The Committee could not establish depletion curves for the four tissues due to a lack of data above the LOQ.

Goats

In a residue depletion study, GLP-compliance not stated, (Ferguson, 1986), 12 bucks and 12 does (18–36 kg) received a single subcutaneous dose of ivermectin of 0.2 mg/kg bw (Ferguson, 1986). The animals were slaughtered in groups of four at 1, 7, 21, 28, 35- and 42 days post dose and the concentration of H_2B_{1a} was determined in the liver and fat tissues by HPLC-FLD (Leavitt and Bunkelmann, 1987). Data on ivermectin residues in the kidney and muscle were not reported. Quantitation was performed using a solvent calibration curve, and not all the method validation parameters were reported. An LOD of 4.2 µg/kg of the marker residue was

reported. The mean recoveries were: 90 percent for muscle, 78 percent for liver, 76 percent for kidney and 85 percent for fat. The documentation presented is incomplete. The incurred depletion study samples were stored for a longer period than the amount of time for which stability was tested. The residue depletion curves of ivermectin in goat liver and fat are presented in Figure 1.

Figure 1. Depletion curves of ivermectin in goat liver and fat. Values were corrected for recoveries



Method of analysis for residues in tissues

A great number of analytical methods have been reported in the literature for the determination of residues of avermectins in biological fluids, animal tissues, and foods. A comprehensive review of the developments in the analysis of residues of avermectins in food is reported by Danaher *et al*. (2012).

The analytical methods for the determination of the marker residue H_2B_{1a} in animal tissues were reviewed at the 88th JECFA Meeting.

The Committee noted that the validation data provided in the studies for the determination of the marker residue in pig tissues (Wood *et al.*, 1981) and sheep tissues (Wood *et al.,* 1984) were incomplete. The Committee assessed the validation data against the analytical requirements as published in CAC/GL71-2009 (FAO and WHO, 2014). The technique used for the determination of the marker residue in the residue depletion studies was high-performance liquid chromatography with a fluorescence detector (HPLC-FLD). The analytical method provided by the sponsor for the determination of the marker residue in tissues comprises about 48 steps, which are not clearly described. The laborious work in so many steps would likely introduce a great uncertainty in the result. Briefly, the tissues were homogenized with acetone-water and the marker residue extracted with isooctane. Following removal of the isooctane, solvent-solvent distributions into acetonitrile out of hexane and into hexane out of

acetonitrile-water are performed. The solvent is removed, and a derivatization reaction at 95 ºC using acetic anhydride, methyl imidazole, and dimethylformamide is performed. After adding chloroform, the reaction mixture is cleaned through a silica gel solid-phase extraction cartridge and HPLC-FLD quantifies the fluorescent compound. There are some minor modifications of the method in terms of dependency of the tissue. All centrifuge tubes were silylated before use. Calibrations curves for H_2B_{1a} were prepared at five concentration levels in methanol (50, 100, 150, 200, 250 and 500 ng/mL). Each calibration standard solution was added to the derivatization mixture and cleaned up onto de solid-phase extraction cartridge as performed for the tissue samples. The HPLC-FLD method reported used a Zorbax ODS-C_{18} column (150 mm x 4.6; 5 µm), maintained at 30ºC, and a mobile phase of water:methanol (flow rate of 1.8 mL/min) for the separation of the marker residue (H_2B_{1a}). The excitation and emission wavelength of the fluorescence detector were 365 nm and 428 nm. The validation parameters of this method were poorly described and comprised recovery, linear range, the limit of detection (LOD) and limit of quantitation (LOQ). Recovery was evaluated by fortifying swine or sheep control tissues at four concentration levels (Table 11). The calibration curves were obtained by dissolution the standard in methanol and then following the sample preparation procedure (derivatization reaction and clean-up using solid-phase extraction). The LOD and LOQ were reported as 1–2 µg/kg and 10 µg/kg, respectively. The limits were established by visual inspection of the chromatograms registered on charts. It is described that at the level of the LOQ a chromatographic peak of about 20 percent of the chart or 5 cm in height is observed using the most sensitive scale. For the LOD is described that at this concentration level, discernible peaks are observed.

Table 11. Validation parameters of the HPLC-FLD method for the determination of ivermectin in swine and sheep tissues

Parameter	Muscle	Liver	Kidney	Fat
Intraday and interday precision (CV, %)	nr	nr	nr	nr
Accuracy	nr	nr	nr	nr
LOQ (µg/kg)	10	10	10	10
LOD	2	2	2	2
Analytical range (ng/mL)	50–500	50–500	50–500	50–500
Linearity (r)	nr	nr	nr	nr
Specificity/selectivity	nr	nr	nr	nr
Extraction recovery[a] mean ±SD (%) - *swine*	86.8 ± 12.8	81.5 ± 10.9	87.1 ± 10.4	88.3 ± 8.9
Extraction recovery[b] mean ±SD (%) - *sheep*	93.0 ± 1.7	88.5 ± 8.3	87.0 ± 2.0	95.2 ± 9.7

Note(s): nr: not reported a: recovery evaluated in the concentration range of 10 to 100 µg/kg; b: recovery evaluated in the concentration range of 9.2 to 91.9 µg/kg.

Sources: Wood, J.S., Baylis, F.P. & Stauffer, S.C. 1981. Ivermectin (MK-0933): Tissue residue in swine dosed subcutaneously. Unpublished report study SW304 (0.4 mg/kg Formulation B), Merck &Co. Inc., Rahway, New Jersey, USA, submitted to FAO by Boehringer Ingelheim Vetmedica GmbH, EU.

Wood, J.S., Baylis, F.P. & Gawlik, G. 1984. Ivermectin (MK-933): Study in sheep (SH256) dosed three times with IVOMEC at 0.3 mg/kg to determine tissue residues. Merck &Co. Inc., Rahway, New Jersey, USA, submitted to FAO by Boehringer Ingelheim Vetmedica GmbH, EU.

The other method, reported by Hughes *et al.,* 2000, was considered by the Committee for the analysis of ivermectin in pig tissues. The technique used for the determination of ivermectin was HPLC-FLD. Briefly, 5 g of each tissue sample (muscle, liver or kidney) were weighed into 50 mL polypropylene centrifuge tubes. The samples were homogenized and extracted with acetonitrile. The kidney tissue samples were homogenised while in an ice bath. The acetonitrile extract was added to a conical flask containing ABC reagent water and triethylamine. The aqueous acetonitrile with triethylamine solution was cleaned by passing it through a C_8 SPE column and eluting it with acetonitrile. The acetonitrile was evaporated to dryness and reconstituted in methanol. The methanol was then evaporated to dryness and derivatized with DMF/acetic anhydride/methylimidazole, at 95°C for 1 hour. The derivatized sample was cleaned by passing it through a Waters Sep-Pak Silica Cartridge and washed with chloroform. The chloroform was then evaporated to dryness and reconstituted in methanol for HPLC, analysis with fluorescence detection. The sample preparation for fat followed the method described for the other tissues, except for these necessary steps. The homogenized samples were extracted in acetonitrile and hexane in equal proportions. The bottom layer, hexane saturated acetonitrile, was removed and blown down to a suitable level to remove any trace of hexane. The acetonitrile was then added to a conical flask containing ABC reagent water and triethylamine. The extraction from this point on was as described for muscle, liver, and kidney. Calibrations curves for H_2B_{1a} were prepared in the concentration range of 5–500 ng/mL. Each calibration standard solution was added to the derivatization mixture and cleaned up onto de solid-phase extraction cartridge as performed for the tissue samples. The chromatographic separation was done using a Phenomenex Phenosphere column (250 mm x 4.6; 5 µm), maintained at 40 ºC, and a mobile phase of ABS reagent water: methanol (flow rate of 1.5 mL/min) for the separation of the marker residue (H_2B_{1a}). The excitation and emission wavelength of the fluorescence detector were 365 nm and 470 nm.

The Committee noted that the validation was also not completely provided (Table 12).

Table 12. Validation parameters of the HPLC-FLD method for the determination of ivermectin in pig tissues

Parameter	Muscle[a]	Liver[b]	Kidney[c]	Fat[d]
Precision (CV, %)	7.6	10.5	14.4	12.0
Accuracy	nr	nr	nr	nr
LOQ (µg/kg)	2	2	2	2
LOD	0.11	0.11	0.10	0.56
Analytical range (ng/mL)	5–500	5–500	5–500	5–500
Linearity (r)	nr	nr	nr	nr
Specificity/selectivity	nr	nr	nr	nr
Extraction recovery mean ±SD (%)	91 ± 8.1	86 ± 5.5	91 ± 7.3	96 ± 8.1

Note(s): nr: not reported; recoveries evaluated in the range: a) 2.02–40.4 µg/kg; b) 2.12–424 µg/kg; c) 2.12–424 µg/kg and d) 2.02–404 µg/kg.

Another analytical method (McHardy *et al.,* 2005) was reviewed by the Committee for the determination of ivermectin in sheep tissues. The analytical method involved the extraction of ivermectin and internal standard abamectin from tissues with acetonitrile (acetonitrile and hexane in equal proportions were used for the extraction from fat) followed by C_{18} solid-phase extraction. Eluants were dried under nitrogen before reconstitution, and derivatized before

injection onto the HPLC fitted with fluorescence detection (excitation wavelength λ: 365 nm, emission wavelength λ: 475 nm). This method was validated in sheep liver, kidney, fat, and muscle over the concentration range 5–200 µg/kg. The validation parameters are presented in Table 13.

Table 13. Validation parameters of the HPLC-FLD method for the determination of ivermectin in sheep tissues

Parameter	Muscle	Liver	Kidney	Fat
Precision (CV, %)[a]		6.81% (15 µg/kg) 3.40% (50 µg/kg) 6.75% (150 µg/kg)		
Accuracy[a]		102.75% (15 µg/kg) 92.29% (50 µg/kg) 103.08% (150 µg/kg)		
LOQ (µg/kg)	5	5	5	5
LOD	2	2	2	2
Analytical range (ng/mL)	5–200	5–200	5–200	5–200
Linearity (r)	>0.99	>0.99	>0.99	>0.99
Extraction recovery mean ±SD (%)	86.8 ± 12.8	81.5 ± 10.9	87.1 ± 10.4	88.3 ± 8.9

Note(s): a: two replicates for each tissue and calculated with a n=10.

The last method (Ferguson, 1986), considered by the Committee, was used for the determination of ivermectin in goat tissues. This analytical method is almost the same as described previously for swine and sheep tissues (Wood *et al.,* 1981 and Wood *et al.,* 1984). Quantitation of H_2B_{1a} was performed by a standard graph plotting peak height (mm) versus concentration of the standard (ng/mL). The HPLC-FLD method reported used a Zorbax ODS-C_{18} column (150 mm x 4.6; 5 µm), maintained at 30 ºC, and a mobile phase of water:methanol (flow rate of 1.8 mL/min) for the separation of the marker residue (H_2B_{1a}). The excitation and emission wavelength of the fluorescence detector were 365 nm and 428 nm. The reported validation parameters are described in Table 14.

Table 14. Validation parameters of the HPLC-FLD method for the determination of ivermectin in goat tissues

Parameter	Muscle	Liver	Kidney	Fat
Intraday and interday precision (CV, %)	nr	nr	nr	nr
Accuracy	nr	nr	nr	nr
LOQ (µg/kg)	nr	nr	nr	nr
LOD	4.2	4.2	4.2	4.2
Analytical range (ng/mL)	nr	nr	nr	21–105
Linearity (r)	nr	nr	nr	nr
Specificity/selectivity	nr	nr	nr	nr
Extraction recovery[b] mean ±SD (%)	90.0 ±.7.0	78.4 ±.8.3	76.0 ± 8.5	84.6 ±.14.0

Note(s): nr: not reported; a: recovery evaluated in the concentration range of 10 to 100 µg/kg; b: recovery evaluated in the concentration range of 10.5 to 20.9 µg/kg.

The validation parameters presented for the determination of the marker residue in all studies

in swine, sheep and goat tissues are incomplete and did not fulfil the analytical requirements recommended in the document CAC/GL71-2009 (FAO and WHO, 2014).

Monitoring data

One competent national authority for residues control has reported findings of residues of ivermectin in pigs, goat, and lambs. Samples of liver and muscle were collected between 2015 and 2021 and analysed using LC-MS/MS or HPLC-FLD (LOQ of 2 µg/kg). Of the samples tested, only 15 out of 4 634 (0.32 percent) samples of pig tissues and two out of 1471 samples of lamb tissues (0.14 percent) were positive for 22,23-dihydro-avermectin B1a residues. For goat tissues, no residues of the marker residue were detected in the 123 samples analysed. The highest concentration (7.4 µg/kg) was determined in a sample of pig liver.

Stability of residues

Analysis of pig tissues (muscle, kidney, and fat) stored at -20 ºC for 6.5 months demonstrated that the marker residue is stable in the three tissues. In this study, the liver was not included. However, data from radioactive studies showed that the marker residue in the liver stored for about two years remained unchanged (Wood *et al*., 1981).

Ivermectin residues showed to be stable in sheep tissues (muscle, liver, kidney, and fat) when stored at -20 ºC for at least 2 months (Wood *et al.,* 1984).

Appraisal

Ivermectin is an old drug with a long history of use and has been previously reviewed several times by the Committee. Ivermectin is a chemically modified-fermentation product belonging to the macrocyclic lactone class of endectocides, consisting of a mixture of two homologous compounds 22,23-dihydroavermectin B1a (H2B1a, not less than 80 percent) and 22,23-dihydroavermectin B1b (H_2B1b, not more than 20 percent).

Ivermectin is registered in many Member States for use in cattle, sheep, goats, pigs, horses, and reindeer. It is available as injectable, topical (pour-on), premix and drenches formulations.

The recommended dose of ivermectin for sheep varies between 0.2 and 0.63 mg/kg bw, and for pigs between 0.3 and 0.63 mg/kg bw.

Ivermectin is a lipophilic compound and tends to accumulate in fat tissues. It persists in the body for a prolonged time. The kinetics are characterized by a slow absorption process, limited metabolism and slow excretion in the different species studied.

Tissue distribution of residues of ivermectin was similar in sheep and pigs, with the highest residue levels in fat and liver tissues, comparable to those described in cattle.

The Committee confirmed that 22,23-dihydroavermectin B1a (H2B1a) is the marker residue in muscle, liver, kidney and fat for the use in pigs, sheep and goats.

Radiolabeled data are available for the depletion of ivermectin in pigs. The Committee identified H2B1a as the marker residue in edible tissues and determined the ratios of the marker residue to total residue in pigs of 0.20 in fat, 0.50 in kidney, 0.25 in liver and 0.44 in muscle.

Radiolabeled data are available for the depletion of ivermectin in sheep. The Committee used the ratios of the marker residue to total residue in sheep of 0.25 in fat, 0.08 in kidney, 0.56 in liver and 0.54 in muscle.

The MRLs recommended for pig tissues are based on the upper limit of the one-sided 95 percent confidence interval over the 95th percentile of residue concentrations (95/95 UTL) for the day 14 post-treatment data from the non-radiolabeled residue depletion study. The tolerance limits for ivermectin in pig tissues are shown in Figure 2.

The MRLs recommended for sheep tissues are based on the upper limit of the one-sided 95 percent confidence interval over the 95th percentile of residue concentrations (95/95 UTL) for the day 22 post-treatment data from the non-radiolabeled residue depletion study. The tolerance limits for ivermectin in sheep tissues are shown in Figure 3.

Due to data limitations, the single study provided by one sponsor using subcutaneous administration of ivermectin in goats was not sufficient to derive UTLs. However, the study confirmed the similarity of the tissue distribution and residue depletion in pigs and sheep, which was also consistent with information available from the previous JECFA assessment.

Dietary exposure

Chronic dietary exposure assessment

Dietary exposure to ivermectin may occur only through its use as a veterinary drug. There is no registered use for ivermectin as a pesticide. When used as a veterinary drug, dietary exposure was estimated based on the potential occurrence of ivermectin residues in cattle, sheep, pig and goat tissues.

Median residue levels in cattle tissues (muscle, liver, kidney and fat) were taken from the evaluation carried out at the eighty-first meeting of the Committee. These values relate to a withdrawal period of 14 days.

For pigs and sheep, several residue depletion studies were available. However, a range of different dosing regimens were used and for the current exercise a conservative approach was taken, with the study that reported the highest tissue residue levels being used to determine chronic dietary exposure.

For sheep, residue levels were derived from the study of Wood *et al.* (1984). Data were taken from 22 days post dose (0.3 mg/kg bw administered subcutaneously, once a week for three weeks). In this study, tissues were analysed at 14 and 28 days post final dose, but not at 22 days. Residue concentrations for dietary exposure assessment were determined by linear regression. The study of Chiu and Jacob (1982) determined MR:TRR ratios at 1, 3, and 5 days post dose (0.3 mg/kg bw subcutaneously). Ratios at day 5 were applied to the day 22 residue concentrations. The ratios were: 0.54 for muscle, 0.56 for liver, 0.08 for kidney and 0.25 for fat.

Figure 2. Tolerance limits considerations for ivermectin in pig tissues. Regression line (blue), UTL 95/95 regression line (orange), UTL 95/99 regression line (yellow) and UTL 99/99 regression line (gray)

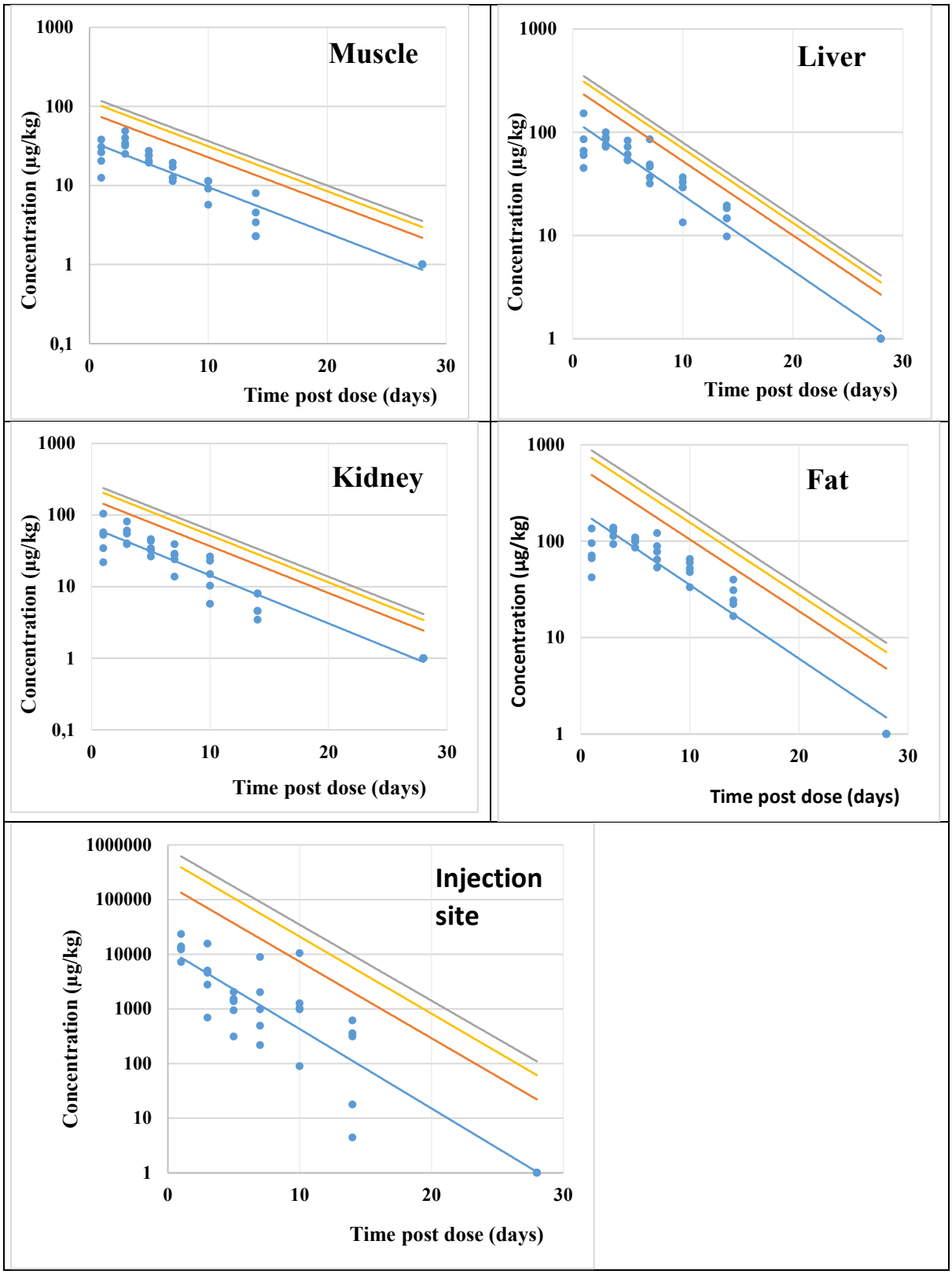
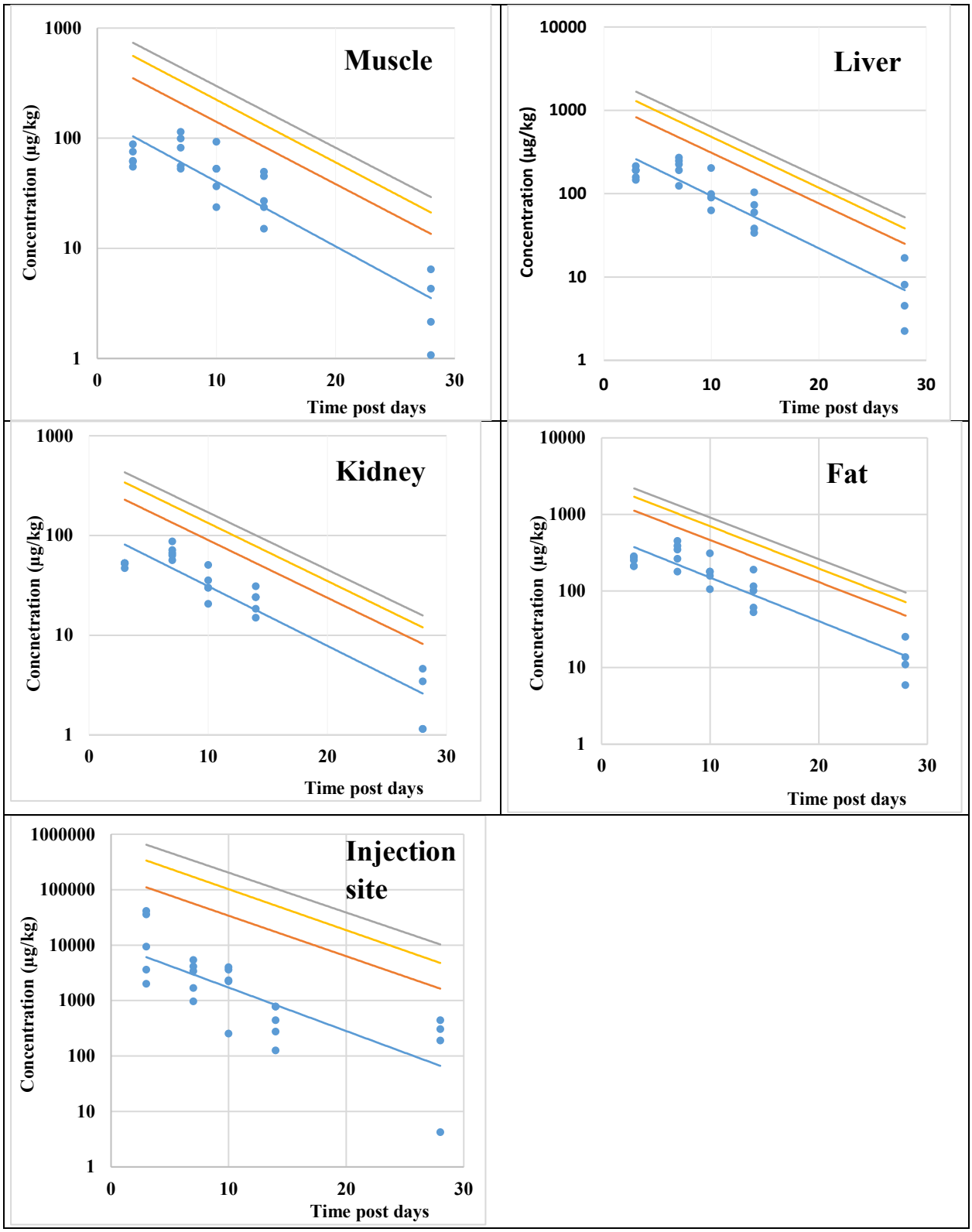


Figure 3. Tolerance limits considerations for ivermectin in sheep tissues. Regression line (blue), UTL 95/95 regression line (orange), UTL 95/99 regression line (yellow) and UTL 99/99 regression line (gray).



For pigs, residue data were available from the study by Wood *et al.* (1981). Data were taken from 14 days post dose (0.4 mg/kg bw, subcutaneously), the shortest withdrawal period reported for ivermectin use in pigs. The study by Chiu and Lu (1982) determined MR:TRR ratios at 1, 7, and 14 days post dose (0.4 mg/kg bw, subcutaneously). Ratios for day 14 were applied to the day 14 median residue concentrations, except for muscle for which only day 1 and day 7 ratios were available, so the day 7 ratio was used. The ratios were: 0.44 for muscle, 0.25 for liver, 0.50 for kidney and 0.20 for fat.

No suitable residue data were available for ivermectin in goat tissues and the values derived for sheep were used as surrogates.

The Committee had previously evaluated milk residue data and recommended an MRL of 10 µg/kg for milk in cattle, expressed as H_2B_{1a}. However, there are currently no approvals for the application of ivermectin formulations to lactating dairy cattle and dietary exposure to ivermectin residues in milk was not considered in the current evaluation. There are no MRLs for ivermectin residues in milk from other species.

Based on incurred residues in cattle, sheep, pig and goat tissues (muscle, liver, kidney and fat) and a withdrawal period of 14 days for cattle and pigs and 22 days for sheep and goats, the global estimate of chronic dietary exposure (GECDE) for adults and the elderly is 0.72 µg/kg bw per day, which represents 7.2 percent of the upper bound of the ADI of 10 µg/kg bw. For children and adolescents, the GECDE is 0.93 µg/kg bw per day, which represents 9.3 percent of the upper bound of the ADI. For infants and toddlers, the GECDE is 0.48 µg/kg bw per day, which represents 4.8 percent of the upper bound of the ADI (Table 15).

Table 15. Global estimate of chronic dietary exposure (GECDE) for ivermectin in cattle, sheep, swine and goat tissues

Category	Type	Median concentration[i] (µg/kg)	Mean consumption, whole population[ii] (g/kg bw per day)	HRP consumption, consumers only[iii] (g/kg bw per day)	MR:TR ratio	Exposure µg/kg bw per day		GECDE[iv]	
						mean	HRP	µg/kg bw per day	%ADI
Adults and elderly									
Mammalian muscle	Cattle and other bovines	6.3	1.5	6.8	0.67	0.014	0.064	0.014	
Mammalian offal	Bovine liver	78	0.06	2.8	0.37	0.013	0.60	0.60	
Mammalian offal	Bovine kidney	12.5	0.001	0.004	0.54	<0.001	<0.001	<0.001	
Mammalian trimmed fat	Bovine fat	46.7	0.15	0.58	0.18	0.039	0.15	0.039	
Mammalian muscle	Sheep and other ovines	7.95	0.26	3.8	0.54	0.004	0.056	0.004	
Mammalian offal	Ovine liver	16.5	0.03	0.57	0.56	0.001	0.017	0.001	
Mammalian offal	Ovine kidney	5.9	0.02	0.22	0.08	0.001	0.016	0.001	
Mammalian trimmed fat	Ovine fat	31.1	-	-	0.25	-	-	-	
Mammalian muscle	Pork and other porcines	3.4	1.3	6.1	0.44	0.010	0.047	0.010	
Mammalian offal	Pig liver	14.6	0.05	1.7	0.25	0.003	0.10	0.003	
Mammalian offal	Pig kidney	24.4	0.01	0.84	0.50	<0.001	0.007	<0.001	
Mammalian trimmed fat	Pig fat	26.9	0.30	4.7	0.20	0.036	0.57	0.036	
Mammalian muscle	Goats and other caprines	7.95	0.72	2.2	0.54	0.011	0.031	0.011	
Mammalian offal	Goat liver	16.5	-	-	0.56	-	-	-	
Mammalian offal	Goat kidney	5.9	-	-	0.08	-	-	-	
Mammalian trimmed fat	Goat fat	31.1	<0.01	0.026	0.25	<0.001	0.003	<0.001	
TOTAL								**0.72**	**7.2**

Children and adolescents									
Mammalian muscle	Cattle and other bovines	6.3	5.2	10.5	0.67	0.048	0.099	0.048	
Mammalian offal	Bovine liver	78	0.05	1.1	0.37	0.011	0.23	0.011	
Mammalian offal	Bovine kidney	12.5	0.01	-	0.54	<0.001	-	<0.001	
Mammalian trimmed fat	Bovine fat	46.7	0.28	1.3	0.18	0.072	0.34	0.072	
Mammalian muscle	Sheep and other ovines	7.95	0.25	9.4	0.54	0.004	0.14	0.004	
Mammalian offal	Ovine liver	16.5	0.01	0.86	0.56	<0.001	0.025	<0.001	
Mammalian offal	Ovine kidney	5.9	-	-	0.08	-	-	-	
Mammalian trimmed fat	Ovine fat	31.1	-	-	0.25	-	-	-	
Mammalian muscle	Pork and other porcines	3.4	2.1	10.7	0.44	0.016	0.083	0.016	
Mammalian offal	Pig liver	14.6	0.10	2.1	0.25	0.006	0.12	0.006	
Mammalian offal	Pig kidney	24.4	0.04	-	0.50	<0.001	-	<0.001	
Mammalian trimmed fat	Pig fat	26.9	1.1	6.3	0.20	0.13	0.77	0.77	
Mammalian muscle	Goats and other caprines	7.95	0.24	6.3	0.54	0.004	0.0.093	0.004	
Mammalian offal	Goat liver	16.5	-	-	0.56	-	-	-	
Mammalian offal	Goat kidney	5.9	-	-	0.08	-	-	-	
Mammalian trimmed fat	Goat fat	31.1	<0.001	-	0.25	<0.001	-	<0.001	
TOTAL								**0.93**	**9.3**
Infants and toddlers									
Mammalian muscle	Cattle and other bovines	6.3	4.5	26.5	0.67	0.043	0.25	0.043	
Mammalian offal	Bovine liver	78	0.3	1.5	0.37	0.063	0.32	0.063	
Mammalian offal	Bovine kidney	12.5	0.04	-	0.54	<0.001	-	<0.001	
Mammalian trimmed fat	Bovine fat	46.7	0.19	1.3	0.18	0.050	0.33	0.33	

Mammalian muscle	Sheep and other ovines	7.95	0.39	7.4	0.54	0.006	0.11	0.006	
Mammalian offal	Ovine liver	16.5	0.01	-	0.56	<0.001	-	<0.001	
Mammalian offal	Ovine kidney	5.9	-	-	0.08	-	-	-	
Mammalian trimmed fat	Ovine fat	31.1	-	-	0.25	-	-	-	
Mammalian muscle	Pork and other porcines	3.4	2.2	13.7	0.44	0.017	0.11	0.017	
Mammalian offal	Pig liver	14.6	0.07	2.0	0.25	0.004	0.12	0.004	
Mammalian offal	Pig kidney	24.4	0.001	-	0.50	<0.001	-	<0.001	
Mammalian trimmed fat	Pig fat	26.9	0.11	1.1	0.20	0.013	0.13	0.013	
Mammalian muscle	Goats and other caprines	7.95	0.04	-	0.54	<0.001	-	<0.001	
Mammalian offal	Goat liver	16.5	-	-	0.56	-	-	-	
Mammalian offal	Goat kidney	5.9	-	-	0.08	-	-	-	
Mammalian trimmed fat	Goat fat	31.1	-	-	0.25	-	-	-	
TOTAL								**0.48**	**4.8**

Note(s): MR: marker residue, TR: total residue, HRP: highest reliable percentile, GECDE: global estimates of chronic dietary exposure
i = Median or mean concentration of the marker residue at the specified times after the end of treatment; ii = Highest mean consumption figures based on whole population considered from the available dataset. Inclusion of "-" indicates that consumption of the tissue was not reported for any country; iii = Highest reliable percentile food consumption figures based on consumers only considered from the available dataset. Inclusion of "-" indicates that no HRP could be identified for the tissue in any country; iv = GECDE is the sum of the highest exposure at the highest reliable percentile of consumption for a food and the mean dietary exposures of the other foods.

Maximum Residue Limits

In recommending MRLs for ivermectin in pigs, sheep and goats the Committee considered the following factors:

- The ADI previously established by the Committee was 0–10 µg/kg bw.
- The ARfD previously established by the Committee was 200 µg/kg bw.
- Ivermectin B1a (synonym for 22,23-dihydroavermectin B1a or H2B1a) is the marker residue in pigs, sheep and goats.
- Ivermectin is authorized for use in sheep, goats and pigs in many Member States.
- Data on the metabolism of ivermectin in pigs and sheep were provided by one sponsor (two studies). No metabolism data were received for goats.
- Tissue distribution of ivermectin residues was similar in pigs and sheep, with the highest residue levels in fat and liver tissues comparable to those described in cattle.
- The ratios of marker residue to total residue in pigs of 0.20 in fat, 0.50 in kidney, 0.25 in liver and 0.44 in muscle were used (day 14 post dose for all tissues except muscle, day 7 post dose for muscle).
- The ratios of marker residue to total residue in sheep of 0.25 in fat, 0.08 in kidney, 0.56 in liver and 0.54 in muscle were used (all day 5 post dose).
- One complete study was available for deriving upper tolerance limits (UTLs) in pig tissues. The animals were dosed once (0.4 mg/kg bw) with a 1 percent ivermectin formulation; the indicated withdrawal period for this formulation is 14 days.
- One complete study was available for deriving UTLs in sheep tissues. The animals were dosed (0.3 mg/kg bw) three times at weekly intervals with a 1 percent ivermectin formulation; the indicated withdrawal period for this formulation is 22 days. The Committee noted that the dose administered in this study is not the indicated dosing regimen for this product, which is a single injection.
- The analytical methods used for the residue depletion studies in pigs, sheep and goats were adequate for the time that they were used, however, they are not fully validated based on current requirements. Validated analytical methods for the determination of ivermectin in all edible tissues of all the species considered are available and are suitable for monitoring purposes.

MRLs were calculated on the basis of the upper limit of the one-sided 95 percent confidence interval over the 95th percentile of total residue concentrations (95/95 UTL) in pig and sheep tissues derived from the data provided.

The Committee recommended the following MRLs in pig tissues: 15 µg/kg for muscle, 30 µg/kg for liver, 20 µg/kg for kidney and 50 µg/kg for fat, based on the UTLs at 14 days.

The Committee recommended the following MRLs in sheep tissues: 30 µg/kg for muscle, 60 µg/kg for liver, 20 µg/kg for kidney and 100 µg/kg for fat, based on the UTLs at 22 days. MRLs based on UTLs for shorter withdrawal periods were not recommended because estimates of acute exposure based on injection site residues resulted in an exceedance of the ARfD (130 percent).

The residue depletion study of ivermectin in goats was incomplete. Data were provided for liver and fat only, and it was not possible to derive UTLs. Based on the similarities between small ruminant species, the Committee recommended extension of the MRLs for sheep to goat tissues.

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Nicarbazin

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Identity

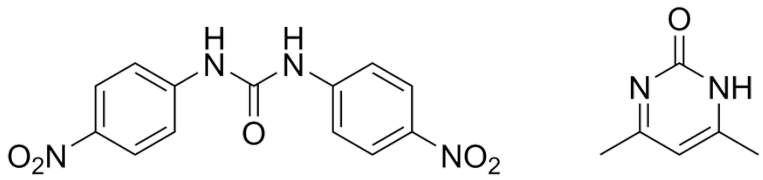
International Non-proprietary Names (INN): Nicarbazin,
Nicarbazin comprises a 1:1 molar complex of 4,4'-dinitrocarbanalide (DNC) and 2 hydroxy-4,6-dimethylpyrimidine (HDP). Weight ratio = 2.43:1 (DNC:HDP)

Synonyms: Nicarbazine, Nicoxin, Nicrazin
(of components) DNC: N,N bis (4 nitrophenyl) urea (BNPU)
HDP: 4,6- dimethylpyrimidin-2-ol

IUPAC name: N,N′-Bis(4-nitrophenyl)urea-4,6-dimethylpyrimidin-2(1H)-one (1/1)

Chemical abstract service N°: 330-95-0

Structural formula:



Molecular formula: $C_{19}H_{18}N_6O_6$

Molecular weight: 426.39 g/mol total
302.25 g/mol DNC
124.14 g/mol HDP

Other information on identity and properties

Appearance: Pale yellow powder

Melting point: 265–275 °C

Solubility: in water: almost insoluble (DNC: < 0.02 mg/L, HDP: appr. 70 g/L), complex dissociates slowly in alcohol, ether and chloroform very slightly soluble,

complex dissociates slowly in dilute acids: almost insoluble, complex dissociates rapidly in dimethylformamide: soluble (1:700) with dissociation of complex in dimethylsulfoxide: soluble, with dissociation of complex

pH: 5–7 (1 percent suspension in water)

Optical rotation: No

UV_{max}: (conc. H_2SO_4): 298 nm (A 1 percent /1 cm 670)

Impurities: ≤ 1 percent p-nitroaniline max. 0.5 percent

Log K_{ow}: 3.6 at pH 5–9 DNC

-0.94 at pH 5–9 HDP

Background

Nicarbazin is a carbanilide used for the prevention of faecal and intestinal coccidiosis in chickens, as well as in some other poultry species. Nicarbazin is registered as a feed additive or as a veterinary drug for use in feed in several countries in Europe, North and South Amerika, Africa and Asia. Nicarbazin may be used in combination with ionophore coccidiostatics like narasin.

The equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) is practically insoluble, but will dissociate completely in aqueous conditions, such as the digestive tract (Martínez-Larrañaga and Anadón, 2014). The two components then behave independently of each other. DNC is the active anticoccidial component while HDP has no anticoccidial activity. The absorption of DNC is greatly enhanced when the two components are complexed together. DNC is highly insoluble in water while HDP is soluble and effectively acts as a carrier to transport DNC through the digestive tract, where DNC is absorbed following dissociation (Rogers *et al*., 1983). The mode of action (MOA) of DNC is unclear but may involve the inhibition of mitochondrial electron transport.

Nicarbazin was evaluated for toxicology and residues by the Committee at its fiftieth meeting (FAO and WHO, 1998). An acceptable daily intake (ADI) of 0–400 µg/kg bw nicarbazin (24 mg/person for a 60 kg person) was set. The No Observed Effect Level (NOEL) was 200 mg/kg bw per day, selected from a developmental toxicity study in rats. A safety factor of 500 was applied, chosen to account for the limitations in the available data. MRLs for chicken muscle, liver, kidney and skin/fat (in natural proportions) were established at 200 µg/kg bw nicarbazin, using DNC as the marker residue. The marker residue accounts for 65 percent of the total residues in muscle and 45 percent, 15 percent and 90 percent of the total residues in liver, kidney and skin with adhering fat, respectively. The MRLs are twice the limit of quantification (LOQ 100 µg/kg) for the method evaluated in 1998. From these MRLs, the theoretical maximum daily intake of residues as nicarbazin equivalents is 214 µg, based on a daily food intake of 300 g of muscle, 100 g of liver, and 50 g each of kidney and fat. At the time of the last JECFA assessment, nicarbazin withdrawal times ranged from 5 to 7 days globally.

More recent ADI and MRL values for nicarbazin are available from the European Food Safety Authority (EFSA) and the US Center for Veterinary Medicine (CVM): EFSA established an ADI of 0.77 mg/kg bw for DNC. This ADI was based on a NOAEL of 154 mg DNC/kg bw, selected from a two year dog toxicology study and using an uncertainty factor of 200 (EFSA, 2010a). MRLs for chicken tissues of 15 000, 6 000, 4 000 and 4 000 µg DNC/kg in liver, kidney, muscle and skin/fat, respectively, were established (EFSA, 2010b). CVM also used DNC as the marker residue and an ADI of nicarbazin (DNC+HDP) of 0.2 mg/kg bw/day was established. This ADI was based on a NOEL of 30 mg/kg bw selected from the developmental toxicity in rabbits and using a 200x safety factor. A tolerance of 52 000 µg DNC/kg was established for chicken liver (CVM, 2018). Table 1 presents a comparison of results from JECFA, EFSA and CVM assessments.

Table 1. Comparison of results from nicarbazin evaluations from JECFA, EFSA and CVM

	Marker residue	ADI based on	ADI	MRL based on	MRL
JECFA 1998	DNC	Nicarbazin	0–400 µg/kg bw	Nicarbazin (M:T)	200 µg/kg liver 200 µg/kg kidney 200 µg/kg muscle 200 µg/kg skin + fat
EFSA 2010	DNC	DNC	0–770 µg/kg bw	DNC	15 000 µg/kg liver 6 000 µg/kg kidney 4 000 µg/kg muscle 4 000 µg/kg skin + fat
CVM 2018	DNC	DNC+HDP	0–200 µg/kg bw	DNC	52 000 µg/kg liver

The sponsor provided unpublished proprietary studies as well as data from studies in the published literature to support the assessment.

Residues in food and their evaluation

Conditions of use

Nicarbazin is used in the European Union, United States of America, Canada, Malaysia, Australia, New Zealand, China, Taiwan Province of China, India, Republic of Korea, Japan, Brazil, Thailand as a feed-additive or as a veterinary drug for use in poultry for prevention of infection by enteric protozoan parasites. Overall, nicarbazin has been used as a coccidiostat for more than 50 years.

In chicken (broiler) production as well as in other poultry species, nicarbazin is used for the prevention of infections caused by *Eimeria tenella*, *Eimeria necatrix*, *Eimeria brunetti*, *Eimeria maxima*, and *Emeria acervulina* (Rogers *et al.*, 1983). Nicarbazin also has wide-spectrum anti-parasitic properties approved for veterinary use, effective on *Toxocara canis*, *Toxascaris leonina*, *Ancylostoma caninum*, *Uncinaria stenocephala*, *Trichuris vulpis*,

Dipylidium caninum, and *Taenia sp.* and *Mesocestoides sp*. In addition, it is used as a contraceptive for population control in birds (e.g. feral pigeons).

Dosage

The inclusion rate for nicarbazin provided by the sponsor was 125 mg/kg nicarbazin per day in complete feeding stuffs (0.01–0.0125 percent in feed) to be used in chickens for fattening. For this dose, withdrawal periods range from 1 to 10 days for edible tissues. A higher inclusion rate of 200 mg/kg feed is approved in at least one Member State, with a withdrawal period of five days, but no residue data were provided for this dosing regimen. When used in combination with either narasin or monesin the nicarbazin inclusion rate is lower at 50 mg/kg feed and withdrawal periods range from 0 to 8 days (EFSA, 2017, 2018). Products are not intended for use in animals producing eggs for human consumption.

The sponsor provided a partial list of approved uses, dosages, and withdrawal periods in several Member States in Tables 2 and 3. No information on the treatment period was provided.

Table 2. Products containing nicarbazin and doses recommended

Product name	Company	Country	Active Ingredients	Dose
Nicarb	Phibro	United States of America	Nicarbazin	125 mg/kg
Koffogran	Phibro	European Union	Nicarbazin	125 mg/kg
Maxiban	Elanco	United States of America/ European Union	Nicarbazin + Narasin	50 mg/kg + 50 mg/ kg
Carbogran	Elanco	United States of America (not marketed)	Nicarbazin	50 mg/kg
Monimax	Huevapharma	European Union	Nicarbazin + Narasin	50 mg/kg + 50 mg/kg

Table 3. Withdrawal periods for 2 products as provided by the sponsor

Country	Withdrawal Time for Maxiban®	Withdrawal Time for Koffogran®	Regulations
European Union	0 days	1 days	Local
United States of America	0 days	4 days	Local
Canada	0 days	4 days	Local
Malaysia	0 days	4 days	Local
Australia	0 days	1 days	Local
New Zealand	0 days	Not marketed	Local
China/ Taiwan Province of China	5 days	4 days in China	Codex
India	5 days	Not marketed	Codex
Republic of Korea	5 days	Not marketed	Codex

Japan	7 days	Not marketed	7 day withdrawal applies to all coccidiostats
Brazil	8 days	10 days	In process to update to zero or 1 day withdrawal
Thailand	4 days	Not marketed	Codex

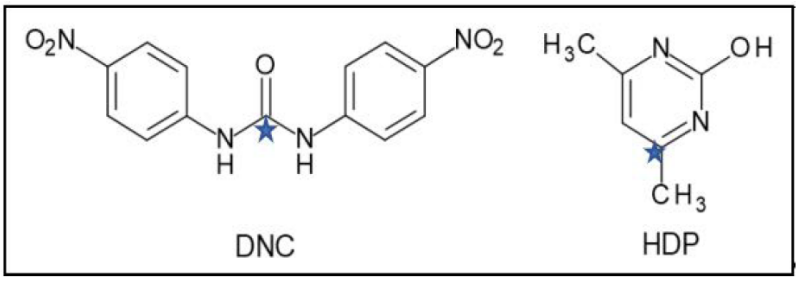
Nicarbazin is not currently used as a plant protection product or as a human medicine.

Pharmacokinetics and metabolism

Pharmacokinetics and metabolism studies were conducted using radiolabeled and unlabelled nicarbazin in rats and in chickens.

Where radiolabeled nicarbazin is used, separate radiolabels are required on each of the moieties as nicarbazin is a chemical complex, composed of 4,4'-dinitrocabanalide (DNC) and 2-hydroxy-4,6 dimethylpyrimidine (HDP). To label the DNC portion of the complex, the [^{14}C] label is placed on the carbonyl of the bis-4-nitrophenyl urea. To label the HDP portion of the complex, the [^{14}C] label is placed on the 2 position of the pyrimidinone ring. The molecular labelling sites for these preparations are shown in Figure 1.

Figure 1. Location of [^{14}C] label in radiolabeled nicarbazin studies



Pharmacokinetics in laboratory animals

Rats

In a study in 54 male rats the relative bioavailability and pharmacokinetics of DNC administered alone, mixed with HDP and as nicarbazin (DNC+HDP complexed) was evaluated (Lloyd, 2009). Animals were administered medication via oral gavage in three treatment groups. The first group received nicarbazin, the second group received a simple mixture of DNC and HDP and the final group received DNC only. Blood samples were collected at 0.5, 1, 2, 4, 8, 12, 24, 36, 48 and 72 hours. Equivalent doses between groups were selected based on the molecular weight of DNC. For example, 35 mg/kg of DNC would be equivalent to 50 mg/kg of nicarbazin, 106 mg/kg of DNC would be equivalent to 150 mg/kg of nicarbazin and 319 mg/kg of DNC would be equivalent to 450 mg/kg of nicarbazin. DNC concentrations were quantified and the pharmacokinetics were analysed for each group.

Table 4. Mean concentration of DNC in plasma of rats orally administered either DNC, DNC+HDP or nicarbazin, expressed as mg/kg

Molecule/ Dose	0.5 h	1 h	2 h	4 h	8 h	12 h	24 h	36 h	48 h	72 h
Nicarbazin										
35 mg/kg	164.2	303.2	595.4	456.8	193.56	49.92	7.96	2.18	13.42	1.17
106 mg/kg	268.6	451.8	847.4	789.6	402.6	141.06	52.44	84.87	1.52	0.4
319 mg/kg	244.64	446.8	721.6	872.6	463.8	428.8	420.6	135.26	12.53	0.97
DNC + HDP										
35 mg/kg	2.65	3.51	3.09	4.68	2.31	1.18	5.57	0.67	0.12	0.14
106 mg/kg	4.71	7.35	9.24	7.95	4.75	7.05	3.76	1.53	0.71	0.16
319 mg/kg	6.9	10.63	13.11	14.38	18.3	5.43	2.23	0.74	0.23	0.55
DNC only										
106 mg/kg	2.24	2.45	6.89	2.97	2.74	1.6	4.16	0.57	0.17	0.18
319 mg/kg	9.37	17.36	16.36	11.24	10.49	4.65	4.33	0.7	0.61	1.09
638 mg/kg	9.72	20.86	30.22	22.76	12.72	6.19	3.74	2.17	3.54	1.0

Source: Lloyd, Z. 2009. Pilot laboratory study: relative bioavailability of DNC in rats administered alone, mixed with HDP and as nicarbazin. Study No. 130-136. MPI Research Inc, Mattawan, MI 49071-9399, USA. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

The total amount of DNC is at least 60x higher in rats administered nicarbazin when compared to administration of DNC alone or to a simple mixture of DNC+HDP (Table 4).

Pharmacokinetic parameters were also estimated for each treatment group: T_{max} was similar for each group, between 2 and 24 hours. $T^1/_2$ was also similar for each group, roughly between 6 and 27 hours. Systemic exposure to DNC was much higher with nicarbazin, where C_{max} and AUC were significantly higher for the nicarbazin group when compared to the DNC+HDP or the DNC only group (Table 5).

Table 5. Pharmacokinetic parameters in rats orally administered nicarbazin, DNC+HDP and DNC alone

Molecule/ Dose	Mean T1/2 (h)	SD	Mean Tmax (h)	Mean Cmax (µg/mL)	SD	AUCinf (h*ng/mL)(SD)	SD
Nicarbazin 35 mg/kg	13.84	9.33	2.4	604.4	113	4 140	741
Nicarbazin (106 mg/kg	11.13	13.7	2.8	883	99.7	8 518	4 621
Nicarbazin (319 mg/kg)	5.98	0.939	14	989.8	314	16 340	12 316
DNC + HDP (35 mg/kg)	6.48	4.11	24.6	6.08	1.12	134	60.1
DNC + HDP (106 mg/kg)	9.53	3.29	3.8	11.22	4.98	202	90.8
DNC + HDP (319 mg/kg)	17.27	14.9	5.6	236.6	7.46	251	83

DNC only (106 mg/kg)	19.28	15.5	8.4	10.88	11.1	209	226
DNC only (319 mg/kg)	8.71	3.69	3.2	22.16	4.51	269	59
DNC only (638 mg/kg)	26.38	14.9	2.4	30.54	8.92	422	276

Source: Lloyd, Z. 2009. Pilot laboratory study: relative bioavailability of DNC in rats administered alone, mixed with HDP and as nicarbazin. Study No. 130-136. MPI Research Inc, Mattawan, MI 49071-9399, USA. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

Relative bioavailability (percent F) was calculated using nicarbazin as the reference dose using the following equation: percent F= 100*AUC_{inf} (indicated group)/AUC_{inf} (nicarbazin dose group). The bioavailability was less than 5 percent when dosed as DNC alone or as DNC mixed with HDP (Table 6).

Table 6. Bioavailability of DNC relative to nicarbazin

Test compound	Dose Level (expressed as mg DNC/ kg bw)	%F
DNC	106	2.45
	319	1.65
DNC + HDP	35	3.24
DNC + HDP	106	2.38
DNC + HDP	319	1.54

Source: Lloyd, Z. 2009. Pilot laboratory study: relative bioavailability of DNC in rats administered alone, mixed with HDP and as nicarbazin. Study No. 130-136. MPI Research Inc, Mattawan, MI 49071-9399, USA. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

The addition of HDP as a simple mixture does not appear to alter the bioavailability of DNC. Overall, when considering the bioavailability, the pharmacokinetic parameters and the overall DNC concentrations, the administration of nicarbazin results in significantly higher absorption of DNC compared to the administration of DNC alone or when mixed with HDP.

Pharmacokinetics in food-producing animals

Data on pharmacokinetics in chickens are available from studies using nicarbazin either non-radiolabeled or radiolabeled.

Chicken

Pharmacokinetics of nicarbazin in chickens was examined by administering the compound in various doses and formulations, both as nicarbazin and as the individual components of DNC and HDP. Analyses were conducted in samples from plasma, intestinal contents, as well as liver and muscle tissues. DNC absorbs faster when administered as a nicarbazin complex, compared to administration as DNC alone or as a simple mixture with HDP. HDP is rapidly absorbed and eliminated, while DNC is more slowly absorbed and eliminated. Another finding is that both HDP and DNC residues declined rapidly after withdrawal of medication in tissues,

with HDP undetectable after 24 hours after administration and DNC undetectable after 48 hours of withdrawal using a colorimetric analytical method (LOQ not reported) (Porter and Gilfillan, 1955).

In another study, chickens were administered 125 mg/kg nicarbazin with a radiolabel on either the HDP or DNC moiety of the molecule (Nessel, 1977). Concentrations in plasma were highest for the HDP moiety after two days (2.1 mg/kg), and highest for the DNC moiety after four days (3.8 mg/kg). The plasma clearance value for DNC was much lower than that of HDP, i.e. HDP declined more quickly than DNC in plasma after withdrawal.

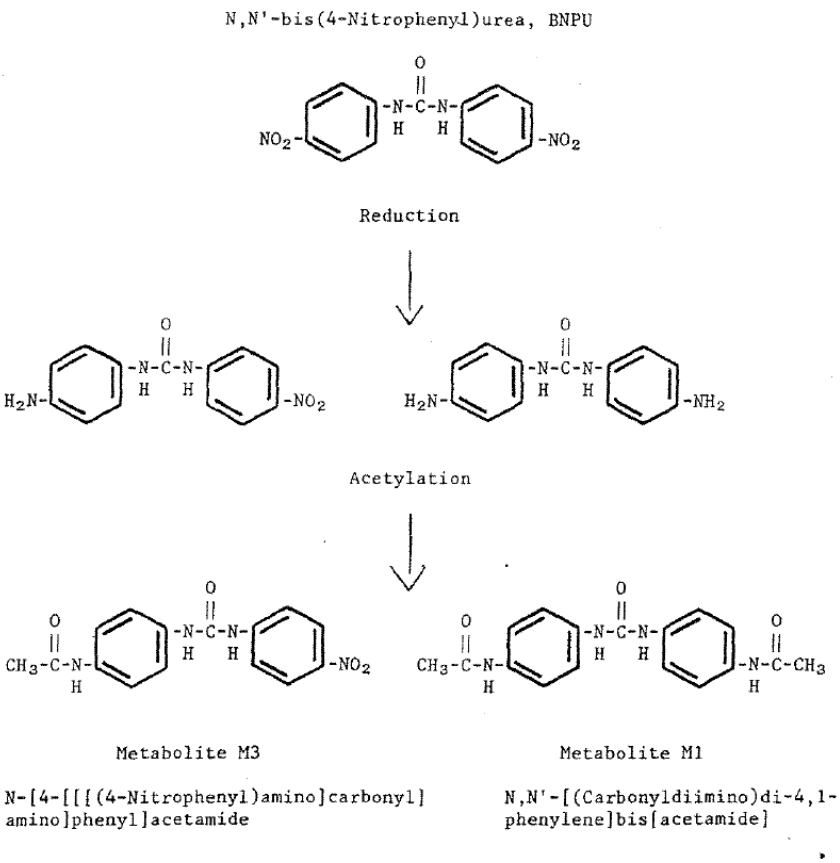
In another study conducted in 18 broiler chickens at three weeks of age, animals were fed 125 mg radiolabeled nicarbazin/kg for 7 consecutive days (King and Walker, 2007). Blood samples were collected at 24 hour intervals and examined for [^{14}C]-DNC via combustion analysis. Mean plasma total radioactivity ranged from 1.212 to 2.822 µg eq/g throughout the dosing period. At 9 days withdrawal, mean plasma concentrations of DNC had decreased to 0.002 µg eq/g. Steady state was reached after 6 days of administration.

Metabolism in laboratory animals

Rat

Six young mature rats (3 male/3 female) were orally administered with nicarbazin radiolabeled at the DNC moiety at a dose of 1 mg/kg/day for five consecutive days. Urine and faeces were collected and analysed for metabolites. This study found two metabolites identical to those identified in chickens (M1: DNC with one nitro group reduced and acetylated, M3: DNC with both nitro groups reduced and acetylated) (Figure 2). No differences in metabolism between male and female animals was found. The authors concluded that the same metabolites occur in both rats and chickens (Manthey, 1985).

Figure 2. Proposed structure for [^{14}C] DNC metabolites in rats



Source: Manthey, J.A. 1985. ^{14}C-Nicarbazin metabolism in orally dosed rats. Study No. ABC 0313. Lily Research Laboratories. Greenfield, IN. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

Metabolism in food-producing animals

Studies on metabolism of nicarbazin report concordantly that the substance is entirely split in the intestinal tract of birds into its two constituents, DNC and HDP. Therefore, nicarbazin itself does not appear as a residue in tissues and only its two individual components may generate residues.

Chicken

Chickens were administered 125 mg/kg nicarbazin with a radiolabel on either the HDP or DNC moiety of the molecule (Nessel, 1977, GLP-compliance not stated). The medication was administered in feed for 2–7 days and radioactivity was measured in plasma, faeces, urine and tissues. Radioactivity concentrations were measured on days 2, 3, 4, 5 and 7 during dosing or after 0, 5, 8, 11, 14 or 21 days withdrawal.

Urine is the main excretion pathway for HDP (90 percent). HDP is well absorbed and rapidly eliminated (83 percent eliminated by the third day). HDP residues were relatively comparable in all tissues, but quantitatively highest in the kidney. DNC is primarily eliminated through the

faeces (90 percent) at a slower rate than HDP. DNC residues were highest in the liver and kidney, with concentrations 10 times higher than HDP. DNC is not rapidly eliminated by the kidneys, with urinary concentrations only 5–10 percent of that of HDP.

After withdrawal, no HDP or DNC was found in tissues after five days, with the exception of liver, which continued to have DNC residues up to eight days after withdrawal.

Another study was conducted to demonstrate the rate and magnitude of excretion of [^{14}C] radioactivity in the excrement of broiler chickens. Three animals were administered a single dose of 125 mg/kg nicarbazin radiolabeled on the DNC moiety in capsules. Measurement of radioactivity in excreta for 10 days indicated that nicarbazin was rapidly eliminated. Within three days post dosage, 95.5–98.8 percent of the radioactivity had been excreted (Manthey *et al.*, 1984).

In a similar study, chickens were administered nicarbazin radiolabeled on the HDP moiety at an inclusion rate of 125 mg/kg feed for six days. Tissues were collected at the end of dosing and excreta collected between day 3 and 6 of dosing. Analysis for radioactivity found that 85 percent, 89 percent and 84 percent of the radiolabel was present as unchanged HDP in the excreta, liver and kidney, respectively. At zero withdrawal, radioactivity concentrations (as mg nicarbazin equivalents/kg) were: kidney 1.48, liver 0.85, muscle 0.81, skin 0.29 and fat 0.06. Results of the study indicate that the HDP portion of the nicarbazin molecule is excreted primarily as parent HDP (Manthey and Donoho, 1986).

Two more recent studies using radiolabeled nicarbazin, with one examining the HDP moiety and one examining the DNC moiety were provided by the sponsor and are briefly described below.

A study on absorption, distribution, metabolism and excretion of [^{14}C]-HDP following multiple administrations of nicarbazin containing [^{14}C]-HDP was conducted in 24 broiler chickens at three weeks of age (McLellan and Coyle, 2007). Animals were fed 125 mg nicarbazin/kg administered twice daily for 7 days in gelatine capsules. Excreta and plasma were collected during treatment in one group, and tissue samples were collected on 1 (24 hours post final administration), 2, 3 and 9 days withdrawal. Samples were examined for radioactive residues via combustion analysis and metabolic profiling was conducted on pooled tissue samples by HPLC.

Elimination of total radioactivity was rapid, with a mean of 96.71 percent (range: 92.85–99.05 percent) of the total dose recovered within 16 h of the last dose administration. The overall mean recovery at 384 h post first dose (240 h post last morning dose) was 100.85 percent (range: 98.44–104.31 percent).

Concentration of total radioactivity in plasma was low for each time point, ranging from 0.036 to 0.093 µg HDP-eq/g. The highest mean concentration was observed at 144 hours post initial dose.

Tissue residue concentrations were highest in the kidney (0.134 µg HDP-eq/g), followed by skin with fat (0.106 µg HDP-eq/g), liver (0.095 µg HDP-eq/g) and muscle (0.084 µg HDP-

eq/g) at one day withdrawal. Total radioactivity decreased with each timepoint, with values almost undetectable at 9 days withdrawal. Mean concentrations are shown in Table 7.

Table 7. Mean concentration of total radioactivity in tissues following oral administration of nicarbazin ([^{14}C]-HDP) to broiler chickens, expressed as µg HDP-eq/g

Sample	24 hour post last dose	72 hour post last dose	120 hour post last dose	240 hour post last dose
Kidney	0.134	°0.005	°0.002	°0.002
Liver	0.095	0.008	0.006	°0.002
Muscle	0.084	°0.003	°0.002	°0.000
Skin plus Fat	0.106	0.027	°0.017	°0.006

Note(s): °=Mean includes results calculated from data less than 30 d.p.m. above background

Source: McLellan, G. & Coyle, D. 2007. The adsorption, distribution, metabolism and excretion of [^{14}C]-HDP following multiple administrations of nicarbazin containing [^{14}C]-HDP in broiler chickens. Study No. 805286, Report No. 24715, by Charles River Laboratories, Tranent EH33 2NE, Scotland, UK. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

Additional HPLC analysis indicated that the principal component of the total radioactivity was HDP at 24 hours last dose. Overall, HDP residues were extremely low in plasma and all tissues, and parent HDP was the principal component of all residues examined.

A study on residue depletion and metabolic identification of [^{14}C]-DNC following multiple administrations of nicarbazin containing [^{14}C]-DNC was conducted in 18 broiler chickens at three weeks of age (King and Walker, 2007). Animals were fed 125 mg nicarbazin/kg feed administered as 62.5 mg/kg twice daily for 7 days in gelatine capsules. Excreta, blood samples and cage wash were collected at 24 hr intervals from prior to first dose until sacrifice in one group, and tissue samples were collected on 1 (24 hours post final administration),4 and 9 days withdrawal. Samples were examined for radioactive residues via combustion analysis and metabolic profiling was conducted on pooled tissue samples by HPLC/LC-MS/MS.

Tissue DNC residue concentrations were highest in the liver (27.797 µg DNC-eq/g), followed by the kidney (16.776 µg DNC-eq/g), skin with fat (5.122 µg DNC-eq/g) and muscle (4.431 µg DNC-eq/g) at one day withdrawal. Total radioactivity decreased over time with very low levels present at 9 days withdrawal. Values are shown in Table 8.

Table 8. Mean concentration of total radioactivity in tissues following oral administration of nicarbazin ([^{14}C]-DNC) to broiler chickens, expressed as µg eq/g

Sample	24 hour post last dose	120 hour post last dose	240 hour post last dose
Kidney	16.776	0.369	0.033
Liver	27.797	0.608	0.050
Muscle	4.431	0.069	°0.002
Skin plus Fat	5.122	0.151	°0.024

Note(s): °=Mean includes results calculated from data less than 30 d.p.m. above background

Source: King, N. & Walker, A. 2007. The residue depletion and metabolic identification of [^{14}C]-DNC in chickens following repeated administrations of nicarbazin containing [^{14}C]-DNC. Study No. 805129, Report No. 24697, by Charles River Laboratories, Tranent EH33 2NE, Scotland, UK. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

Mean plasma total radioactivity ranged from 1.212 to 2.822 µg DNC-eq/g throughout the dosing period. At 9 days withdrawal, mean plasma concentrations had decreased to 0.002 µg DNC-eq/g. Steady state was reached in 6 days of administration. Elimination was through excreta with a mean of 98.74 percent of the total dose recovered by 4 days post last dose.

To characterize the nature of nicarbazin residues, edible tissues (liver, kidney, muscle and skin with fat) were pooled (3 birds) and subject to methanol solvent extraction with subsequent sample clean-up to enable HPLC analysis. Post extracted solids (tissue pellet) were further extracted with pepsin and protease enzymes. Extraction solutions were split, with an aliquot measured by Liquid Scintillation Counting (LSC) to determine the amount of radioactivity extracted (LOQ was 30 d.p.m.) and an aliquot measured by HPLC with on-line radiodetection. A standard mix of non-radiolabeled DNC and non-radiolabeled HDP were prepared and used to confirm retention times of DNC and HDP. Recovery of radioactivity from the HPLC column was checked by quantifying the radioactivity from the system to the level injected. Excreta recovery was 108.2 percent, liver recovery was 105.8 percent, kidney recovery was 101.2 percent, muscle recover was 106.6 percent and skin with fat recovery was 108.7 percent.

Extraction in all tissues was >70 percent of total radioactive residues in all tissues at 24 hour withdrawal, shown in Table 9. Further enzymatic processing released an additional 5–20 percent of the total radioactive residues in the tissues. The amount of radioactivity extracted did decrease in the 120 hours withdrawal group and even further in the 240 hours withdrawal group.

Table 9. Mean percentage of total radioactive residues measured after methanol extraction in each tissue at 24 hour withdrawal in chickens

Tissue matrix	Percent TRR
Liver	94.17
Kidney	78.38
Muscle	82.68
Skin plus fat	90.53

As nicarbazin is also used in combination with other active ingredients, studies to investigate whether co-administration with e.g. narasin might have an influence on metabolism and/or effects on nicarbazin concentrations in edible tissues:

In one study nicarbazin, radiolabeled at the DNC moiety, was administered to chickens at 50 mg/kg, with and without narasin at 50 mg/kg, in feed for 5 days (Manthey and Zornes, 1985). Tissues were collected at zero days and analysed for radioactive DNC residues and metabolite identification. Recovery was favourable and was between 98–103 percent for excreta and all tissues.

Residue concentrations were highest in the liver, followed by kidney, muscle and skin with fat. The concentrations were not significantly different between the group treated with nicarbazin and the group treated with the nicarbazin/narasin combination. Parent DNC was the principal component in excreta at 40 percent, in liver at 79 percent, but only 6 percent of residues in the kidney.

Three DNC metabolites were qualified in this study, but not fully quantified. These metabolites were isolated by reverse phase HPLC then subjected to mass spectrometric analysis to determine structure. Metabolite M1 was identified as monoacetylamino-DNC corresponding to the reduction and acetylation of one nitro group and was found in both the liver and kidney. Metabolite M2 was identified as N,N'-1,4-phenylenebis(acetamide) resulting from the split, reduction and acetylation of the molecule. This metabolite was only identified in the excreta. Metabolite M3 was identified as diacetylamino-DNC resulting from the reduction and acetylation of both nitro groups. It was identified in the liver at the highest concentration, but still less than 10 percent of TRR.

Similar results were derived from another tissue residue depletion study conducted in broiler chickens, dosed with medicated rations which contained [^{14}C]-narasin or [^{14}C]-nicarbazin (radiolabeled at the DNC moiety) alone, or in combination with unlabeled nicarbazin or narasin, respectively (Manthey, 1982). The chickens were given the test ratios containing the narasin and/or nicarbazin at concentrations of 50 mg/kg for five days. The chickens were sacrificed at zero withdrawal. Tissue specimens of liver, muscle, kidney, fat, and skin from three chickens of each treatment group were pooled per tissue and assayed for [^{14}C] radioactivity. There were no significant effects upon either narasin or nicarbazin tissue residue levels when the two were fed in combination or when each drug was fed alone.

An additional study, with the radiolabel on the HDP moiety of nicarbazin given at the same dose, further confirmed that the coadministration of narasin had no effect on nicarbazin concentrations in tissues (Kennington and Darby, 1994).

Comparative metabolism

From an EFSA report (EFSA, 2017), a summary of an *in vitro* study on the metabolic fate of DNC and HDP in chicken, turkey and rat cryopreserved hepatocytes based on a liquid chromatography (HPLC) - high resolution mass spectrometry (HRMS) method is available. Seven to eight metabolites were isolated from hepatocyte incubations of the three species, separated and identified. The main metabolites produced *in vivo* and already described were also identified *in vitro*. Hydroxylation (position not established) followed by glucuronidation or sulfation and glucuronidation of secondary amine function were also identified. No significant difference was observed in the amount of DNC metabolized over time between chicken and turkey. The *in vitro* metabolism of HDP by chicken, turkey and rat cryopreserved hepatocytes was investigated in the same study using the same experimental design and the analytical approaches used to characterize DNC and metabolites. Only unreacted HDP was detected in any incubation analysed, indicating the absence of significant biotransformation of HDP in the three species.

Tissue residue depletion studies

Radiolabeled residue depletion studies

Chicken

Three radiolabeled residue depletion studies were available for evaluation. In one study nicarbazin radiolabeled either on the HDP or the DNC moiety was used. The second and the third studies used either [^{14}C]-DNC or [^{14}C]-HDP.

The study conducted by Nessel (1977; GLP-compliance not stated) concerns ADME data, which are summarized in the section above, as well as tissue residue depletion data, which are covered here. Chickens were administered nicarbazin with a radiolabel on either the HDP or DNC moiety of the molecule for 2–7 consecutive days at 125 mg/kg feed.

The overall averages of the [^{14}C] concentrations in chicken tissues were lower in birds fed nicarbazin [^{14}C] labelled at the HDP moiety, than in those given the drug with the [^{14}C] label in the DNC moiety. Specifically, [^{14}C] residues averaged 1.7, 1.7, 2.8 and 2.0 µg/ml or mg of plasma, muscle, kidney and liver, respectively, in the [^{14}C]-HDP group, as compared to values of 3.0, 4.8, 22.4 and 29.6 mg/ml or mg in these same respective tissues from the [^{14}C]-DNC group. The highest average [^{14}C] residue values were obtained at day 4 during treatment with the [^{14}C] on the DNC moiety, but at day 2 during treatment with the [^{14}C] on the HDP moiety.

No [^{14}C] residues were found by the 5th day after withdrawal of nicarbazin labelled with [^{14}C] in the HDP moiety. In the chicks previously fed the [^{14}C] in the DNC label of nicarbazin, only liver retained detectable [^{14}C] up to day 8 after withdrawal. No [^{14}C] were found in the other tissues by the 5th day after withdrawal.

The second study (GLP-compliant) was conducted to provide total residue depletion data in tissue samples following multiple oral administrations of nicarbazin containing [^{14}C]-DNC to 18 broiler chickens (King and Walker, 2007). Animals were dosed for seven days with nicarbazin at a target inclusion rate of 125 mg/kg feed. Doses were prepared in gelatine capsules and administered twice daily.

Excreta was collected daily, blood was collected daily and tissues were collected at 1 (24 hours), 4 and 9 days withdrawal. Radioactivity was measured at each timepoint, and DNC concentrations were measured by HPLC. Each six birds were sacrificed at days 0, 4 and 9 after withdrawal and samples from liver, kidneys, skin with fat and muscle (250 g composite of breast and thigh) were retained for analysis. Tissues for animals were pooled into groups of three, with two groups at each timepoint. Radiation was not detected in animals past the 240 hours withdrawal group.

Concentrations of total radioactive residues in plasma were consistently low. The highest mean concentration of total radioactivity in plasma was 2.822 µg eq/g observed at 168 h post first dose administration. By 192 h post first dose administration (48 h post last dose) the concentrations in plasma decreased to a mean level of 1.303 µg eq/kg and continued to decrease to a mean level of 0.002 µg eq/kg at 384 h post first dose administration.

The major route of radioactivity elimination was via excreta with a mean total of 99.58 percent (range; 95.96 –102.19 percent) of the total dose administered recovered by 384 h post first dose administration (240 h post last dose). Throughout the 7 day dosing period the mean levels of total radioactivity excreted ranged from 8.80 percent to 14.47 percent daily. By 168 h post first dose (24 h post last dose) a mean of 85.04 percent of the administered dose was recovered (range; 80.80–89.56 percent). Radio-HPLC and LC/MS analysis confirmed that the major component in all pooled excreta samples was 4,4-dinitrocarbanilide (parent DNC) and represented ca 90 percent of the extracted radioactivity.

A similar pattern of radioactive residue distribution was observed in all birds (Table 10). Highest mean total radioactive residues in tissue samples were observed in the liver followed by kidney, skin with fat and muscle at all time points. The mean total radioactive residues in the liver at 24 h post last dose (0 day withdrawal) was 27.797 µg DNC-eq/g (range; 25.869–30.259 µg DNC-eq/g). Levels of total radioactive residues decreased to 0.050 µg DNC-eq/g (range; 0.038–0.076 µg DNC-eq/g) at 240 h post last dose (9 days withdrawal).

Table 10. Concentration of total radioactivity in tissues following multiple oral administrations of [^{14}C]-DNC to chickens, results expressed as µg DNC-eq/g

Group 10 day withdrawal (24 h post last am dose)								
Sample	**001**	**002**	**003**	**004**	**005**	**006**	**Mean**	**SD**
Kidneys	15.526	17.116	18.619	17.378	15.217	16.802	16.776	1.254
Liver	27.765	27.257	28.244	30.259	27.391	25.889	27.797	1.445
Muscle	4.022	4.774	5.130	4.914	3.849	3.895	4.431	0.571
Skin with Fat	5.461	5.233	4.672	5.425	4.823	5.120	5.122	0.320
Group 24 day withdrawal (120 h post last am dose)								
Sample	**007**	**008**	**009**	**010**	**011**	**012**	**Mean**	**SD**
Kidneys	0.538	0.336	0.453	0.185	0.322	0.383	0.36	0.121
Liver	0.865	0.572	0.721	0.287	0.624	0.6	0.608	0.198
Muscle	0.062	0.070	0.081	0.041	0.074	0.084	0.069	0.016
Skin with Fat	0.198	0.155	0.169	0.080	0.146	0.162	0.151	0.039
Group 39 day withdrawal (240 post last am dose)								
Sample	**013**	**014**	**015**	**016**	**017**	**018**	**Mean**	**SD**
Kidneys	0.025	0.026	0.028	0.037	0.054	0.031	0.033	0.011
Liver	0.043	0.044	0.038	0.055	0.078	0.042	0.050	0.014
Muscle	*0.003	*0.001	*0.002	*0.001	0.005	*0.001	°0.002	°0.001
Skin with Fat	0.015	0.023	0.02	0.031	0.040	0.016	0.024	0.010

Note(s): * = Results calculated from data less than 30 d.p.m. above background; ° = Mean includes results calculated from data less than 30 d.p.m. above background

Source: King, N. & Walker, A. 2007. The residue depletion and metabolic identification of [^{14}C]-DNC in chickens following repeated administrations of nicarbazin containing [^{14}C]-DNC. Study No. 805129, Report No. 24697, by Charles River Laboratories, Tranent EH33 2NE, Scotland, UK. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

HPLC analysis of residues in tissues revealed the following results (all expressed as µg DNC-equivalents):

Liver samples in two study groups from 24 h post last am dose (0 day withdrawal) and 120 h post last am dose (4 day withdrawal) showed that 48.72 percent (13.523 µg eq/g), 37.84 percent (10.534 µg eq/g), 23.29 percent (0.167 µg eq/g) and 47.78 percent (0.237 µg eq/g) TRR was present as parent DNC, respectively.

Kidney samples in two study groups from 24 h post last am dose (0 day withdrawal) showed that 42.18 percent (7.207 µg eq/g) and 31.12 percent (5.124 µg eq/g) of the extracted radioactivity was present as parent DNC, respectively. Only kidney samples from one group taken at 120 h post last am dose (4 day withdrawal) showed the presence of parent DNC and accounted for 4.73 percent (0.021 µg eq/g) TRR. No parent DNC was observed in the 120 h pool.

Skin with fat samples from 2 study groups at 24 h post last am dose (0 day withdrawal) showed that 39.60 percent (2.028 µg eq/g) and 54.62 percent (2.798 µg eq/g) TRR was present as parent DNC, respectively. Samples from two groups at 120 h post last am dose (4 days withdrawal) showed 19.33 percent (0.034 µg eq/g) and 9.23 percent (0.012 µg eq/g) TRR was present as parent DNC, respectively.

Muscle samples from two groups taken at 24 h post last am dose (0 days withdrawal) showed that 27.15 percent (1.260 µg eq/g) and 21.18 percent (0.894 µg eq/g) TRR was present as parent DNC. Muscle samples at 120 h post last am dose (4 day withdrawal) showed that 18.24 percent (0.012 µg eq/g) of the extracted radioactivity was present as parent DNC although no parent DNC was observed in at 120 h post last am dose (4 day withdrawal).

Radio-HPLC and LC/MS analysis confirmed that the major component in all pooled tissue samples at 24 h post last am dose (0 day withdrawal) was 4,4-dinitrocarbanilide (parent DNC). Results of HPLC analysis are summarized in Table 11.

Table 11. Summary of marker residue (DNC) concentrations after oral administration of 125 mg/kg nicarbazin ([^{14}C]-DNC) in feed for 7 days

Time post dose (h)	Animal group	Liver	Kidney	Muscle	Skin plus Fat
		µg eq/kg			
24	Animal 1-3	13 520	7 207	1 260	2 028
24	Animal 4-6	10 534	5 124	895	278
Mean		12 027	6 165.5	1 077.5	2 413
120	Animal 7-9	719	21	174	ND
120	Animal 7-9	497	ND	129	12
Mean		608	21	151.5	12

Source: King, N. & Walker, A. 2007. The residue depletion and metabolic identification of [^{14}C]-DNC in chickens following repeated administrations of nicarbazin containing [^{14}C]-DNC. Study No. 805129, Report No. 24697, by Charles River Laboratories, Tranent EH33 2NE, Scotland, UK. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

A radiolabeled study with nicarbazin labelled at the HDP moiety is described in detail above in the section on metabolism (McLellan and Coyle, 2007). Hence, each component of nicarbazin was examined in a separate radiolabeled study at the intended nicarbazin dose of 125 mg/kg feed nicarbazin for seven days, resulting in the mean total residue concentrations as listed in table 12.

Table 12. Mean total residue concentrations from radiolabeled studies

Tissue	DNC at 24 hours (mg eq DNC/kg)[i]	HDP at 24 hours (mg eq HDP/kg)[ii]
Liver	27.79 ± 1.445.	0.095 ± 0.041
Kidney	16.776 ± 1.257	0.134 ± 0.061
Muscle	4.431 ± 0.571	0.084 ± 0.037
Skin/Fat	5.122 ± 0.32	0.106 ± 0.036

i (King and Walker, 2007); ii (McLellan and Coyle, 2007)

*Source*s King, N. & Walker, A. 2007. The residue depletion and metabolic identification of [^{14}C]-DNC in chickens following repeated administrations of nicarbazin containing [^{14}C]-DNC. Study No. 805129, Report No. 24697, by Charles River Laboratories, Tranent EH33 2NE, Scotland, UK. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

McLellan, G. & Coyle, D. 2007. The adsorption, distribution, metabolism and excretion of [^{14}C]-HDP following multiple administrations of nicarbazin containing [^{14}C]-HDP in broiler chickens. Study No. 805286, Report No. 24715, by Charles River Laboratories, Tranent EH33 2NE, Scotland, UK. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

As HDP represents less than 1 percent of the total radioactive nicarbazin residues at 24 hours after treatment, its contribution does not need to be taken into account to calculate the marker to total ratio. Therefore, marker to total ratios as listed in Table 13 are based on data from the DNC total residue study conducted by King and Walker (2007) at 24 hours withdrawal. Marker to total ratio is calculated as DNC concentration divided by the mean concentration of total radioactivity expressed as µg eq/kg.

Table 13. DNC marker to total ratios in edible tissues at 24 hours after treatment

Tissue	Mean concentration of total radioactivity (µg eq/kg)	Pooled DNC concentrations measured by HPLC as µg/kg	DNC concentration/ total DNC radioactivity (marker:total)
Liver	2 777	1 2028.5	0.43
Kidney	16 776	6 165.5	0.37
Muscle	4 431	1 077	0.24
Skin with fat	5 122	2 413	0.47

Source: King, N. & Walker, A. 2007. The residue depletion and metabolic identification of [^{14}C]-DNC in chickens following repeated administrations of nicarbazin containing [^{14}C]-DNC. Study No. 805129, Report No. 24697, by Charles River Laboratories, Tranent EH33 2NE, Scotland, UK. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

Residue depletion studies with non-radiolabeled drug

Chicken

Two residue depletion studies using unlabelled nicarbazin were provided. In one study, nicarbazin was administered at a target inclusion rate of 125 mg/kg feed and in the other study, nicarbazin was administered in combination with narasin, each at a target inclusion rate of 50 mg/kg feed.

In the GLP-compliant study using nicarbazin only, 36 one day old chickens (18 male/18 female) received feed containing nicarbazin at 125 mg/kg feed for 28 days, then six animals were sacrificed on each of days 1, 5, 6, 9, 11 and 14 (Cairns and Davidson, 2006b).

The concentrations of DNC in edible tissues (skin with fat, fat, kidneys, liver and muscle) were determined by a validated HPLC and LC-MS/MS method (Cairns and Davidson, 2006a). Samples were extracted with acetonitrile. The mean recovery for the method was between 70–110 percent with coefficients of variation that were ≤15 percent for concentrations ≥100 µg/kg. The LOD for the method was 4.06, 4.89, 4.22, 0.54 and 1.18 µg/kg for liver, kidney, muscle, skin with fat and fat respectively. The LOQ for the method was 50 µg/kg for liver and fat, 100 µg/kg for kidney and 25 µg/kg for muscle and skin with fat.

DNC was detected in all tissues at day 1 following withdrawal of the test diet. Residues detected ranged from: 7 564–12 595 µg/kg in liver, 1 194–4 110 µg/kg in kidney, 1 342–2 688 µg/kg in muscle, 1 678–2 798 µg/kg in skin with fat, and 1 811–2 866 µg/kg in fat.

At day 5 post withdrawal, residues for kidney had declined below the LOQ in all birds tested. The ranges at day 5 were 411–544 µg/kg in liver, 33.4–56.6 µg/kg in muscle, 90.3–176 µg/kg in skin with fat, and 62.9–93.5 µg/kg in fat residues. At days 7, 9 and 11 post withdrawal, residues in liver, kidney, muscle and fat had declined to below the LOQ in all birds. At these samplings times residue levels in skin with fat ranged from below the LOQ to 41.7 µg/kg. At day 14 post withdrawal, all residues were below the LOQ in all tissues and for all birds. DNC concentrations in edible tissues are summarized in Table 14.

Table 14. DNC concentrations in edible tissues from broilers treated with nicarbazin at an inclusion rate of 125 mg/kg feed for 28 days

Days withdrawal	Liver		Muscle		Kidney		Skin with fat		Fat	
	Mean (µg/kg)	SD	Mean (µg/kg)	SD	Mean (µg/kg)	SD	Mean (µg/kg)	SD	Mean (µg/kg)	SD
1 (24 h)	9249	1647	2110	462	3007	999	2327	432	2368	355
5	453	43	45.1	8	<LOQ	-	131	30	77.6	11
7	<LOQ	-	<LOQ	-	<LOQ	-	<27*	4	<LOQ	-
9	<LOQ	-	<LOQ	-	<LOQ	-	<29.6*	-	<LOQ	-
11	<LOQ	-	<LOQ	-	<LOQ	-	<26.9*	-	<LOQ	-
14	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-

Note(s): LOQ = 100 µg/kg for kidney, 50 µg/kg for liver and fat, 25 µg/kg for muscle and skin with fat; *For concentrations <LOQ, the LOQ was used in the calculation of the mean

Source: Cairns, S.D. & Davidson, J. 2006b. Residue depletion of Koffogran (nicarbazin) in broiler chickens. Study No. 207188, Report No. 25651, by Inveresk, Tranent EH33 2NE, Scotland, UK. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

In another GLP-compliant residue depletion study, nicarbazin was administered in combination with narasin (50 mg/kg feed each) to 24 broiler chickens for 35 days (Johnston, 2008). Three females and three males were necropsied at day 0 (immediately after feed was withdrawn) and at days 3, 5, and 7 post withdrawal, and tissues including the kidneys, liver, muscle, skin with fat, and the abdominal fat pad were removed from each bird.

DNC concentrations in samples were analysed using a validated LC-MS/MS method (LOQ: 50 µg/kg for liver, 100 µg/kg for kidney and 25 µg/kg for muscle and fat). Tissues were extracted with acetonitrile and quantitated over the nominal range of 15–1600 µg/kg in muscle and skin with fat, 30–3 200 µg/kg in liver and 60–6 400 µg/kg in kidney. Recoveries were between 70–110 percent of the theoretical value for fortified samples.

Individual residues of DNC in tissues were below 750 µg/kg at five days following the last dose. The relative concentrations of DNC in the tissues at five days withdrawal were: liver > skin with fat > muscle > kidney. The concentrations at seven days after the last dose were less than 87.8 µg/kg for liver, less than 25.9 µg/kg for skin with fat, less than the LOQ (25 µg/kg) for muscle and less than the LOQ (100 µg/kg) for kidney (Table 15).

Table 15. DNC concentrations in edible tissues from broilers treated with nicarbazin in combination with narasin (50 mg/kg feed each) for 35 days

Days withdrawal	Liver		Muscle		Kidney		Skin with fat	
	Mean (µg/kg)	SD	Mean (µg/kg)	SD	Mean (µg/kg)	SD	Mean (µg/kg)	SD
0	9 190	917	1 610	136	4 290	945	2 040	438
3	2 450	261	187	25	25	101	313	38
5	355	65	<27.8*	3.6	<LOQ	-	5.6	15
7	<87.8*	56	<LOQ	-	<LOQ	-	<25.9*	2

Note(s): LOQ = 50 µg/kg for liver, 100 µg/kg for kidney, 25 µg/kg for muscle and skin/fat; *For concentrations <LOQ, the LOQ was used in the calculation of the mean

Source: Johnston, D.L. & Roberts, S. 2008. Residue depletion of nicarbazin and narasin in edible tissues from chickens following administration of Maxiban® G160 via Feed. Study No. 285266, Report No. 28890, by Charles River Laboratories, Cumbria, CA8 1LE, UK. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

Methods of analysis for residues in tissues

Several methods for the determination of nicarbazin in samples of animal origin have been reported. As this monograph on nicarbazin is focused on residues in chicken tissues, only analytical methods developed and validated for edible tissues of this species are described here.

The Committee assessed the validation data against the requirements for analytical methods published in the Codex Guideline CAC/GL 71-2009 (FAO/WHO, 2014).

Methods suitable for screening samples for potential non-compliant residues are available. In addition, quantitative methods based on high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) were developed and validated in compliance with GLP. LC-MS/MS has been considered the method of choice.

As available data indicate that nicarbazin that from the chemical complex composed of 4,4'-dinitrocabanalide (DNC) and 2-hydroxy-4,6 dimethylpyrimidine (HDP), DNC residues reach a greater concentration and deplete more slowly (Cairns and Davidson, 2006a; King and Walker, 2007; McLellan and Coyle, 2007; Nessel, 1977). DNC does undergo some metabolism and metabolites other than DNC have been identified. But as they are present at <10 percent of the total residues, analytical methods focus on DNC only. Only methods validated for analysis of DNC in edible tissues are included here.

Dropping mercury electrode (DME) polarography

The initial regulatory method utilized for nicarbazin was dropping mercury electrode (DME) polarography which measures the current change in a sample as the voltage is swept across a given range in a closed electrochemical cell. A drop of mercury is used as the working electrode and is discarded (into the bottom of the cell) after every measurement, to avoid fouling of the electrode with the products of the electrochemical reaction. In this method the reduction of the two aromatic nitro groups of nicarbazin into aromatic amines is monitored (Elanco and Phibro Animal Health Collaboration, 2017).

The polarography method uses a triple ethyl acetate extraction followed by an acetonitrile:hexane liquid-liquid partitioning. This technique was utilized in a number of studies to support nicarbazin residue studies throughout the 1970s and early 1980s. By applying pulse polarography at the dropping mercury electrode to acetonitrile extracts of animal tissues and eggs, a detection limit of 50 µg/kg and mean recoveries of 79 percent were obtained (Knupp et al., 1987). This method lacks selectivity and does not meet the requirements of CAC-GL71-2009.

High-performance liquid chromatography-UV detection (HPLC-UV)

A HPLC-UV method has been developed for the determination of DNC residues and described by several working groups (Capurro *et al*., 2005; Hurlbut *et al*., 1985; Macy and Loh, 1984). It uses the same extraction steps as the polarography method with an added alumina B cleanup step. Reported limits of quantitation were between 12.5 µg/kg and 25 mg/kg. Sensitivity was reported to be higher compared to the polarography method.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The recent method used for determination and confirmation of the nicarbazin marker residue DNC is the AOAC Official Method 2013.07 based on a liquid chromatography followed by tandem mass spectrometry (AOAC International, 2021).

Poultry tissue is cryogenically homogenized with solid sodium sulphate, and then extracted twice with acetonitrile (ACN). Extracts are combined, filtered, and diluted accordingly based on the regulatory limits being targeted and the working concentrations of the standards used for LC/MS/MS analysis. Confirmation of identity is accomplished by comparing the product ions measured in the samples to those present in the standard injections in mass and relative intensity, and comparison of chromatographic retention times between samples and standards.

Nicarbazin determination and confirmation is based on the DNC portion of the molecule as are the regulatory limits and tolerances. Concentrations are determined by LC/MS/MS using a matrix-matched standard curve and DNC-d8 internal standard (Coleman *et al*., 2014).

The method of analysis was validated for the marker residue DNC with respect to the following aspects.

Specificity: Specificity for the LC-MS/MS method was evaluated in chicken tissues with lasalocid, salinomycin, narasin, tilmicosin, tylosin and monensin. Control tissues were fortified with DNC at 100 µg/kg and every other compound tested was fortified at 50 µg/kg. Samples were assayed to verify non-interference, which were defined at either no peak(s) attributable to the compound or the detected peak was baseline resolved from the DNC peak. If an interfering peak was observed, it needed to be demonstrated that the peak did not contribute more than 10 percent of the measured DNC concentration. The results indicated that none of these molecules were observable when quantifying DNC. Presence of each compound was verified by LC-MS/MS (Rodewald, 2014).

Linearity: For evaluation of linearity standard curve solutions in chicken liver extract with internal standard (DNC-d8 ISTD) were analysed in triplicate and a 1/x weighted regression was performed. The highest residual percentages occurred in the lowest two concentrations All residuals were within the acceptable range, ranging from -0.07 to 6.0 percent (Coleman et al., 2014).

A supplemental dilution linearity validation study was conducted in poultry liver and kidney for the AOAC First Action Method 2013.07 (Edwards, 2019). Both incurred and fortified tissues were evaluated. Liver samples were fortified with DNC at 10 000 µg/kg and 20,000 µg/kg. Kidney samples were fortified with DNC at 5 000 µg/kg and 10 000 µg/kg. Following sufficient extraction, liver samples were diluted 1:50 and 1:100 with control matrix extract, and kidney samples were diluted 1:20. The same dilutions were conducted with incurred liver and kidney samples. This study indicated that dilution pre-extraction (with control tissue) or dilution post extraction (with control matrix extract) were equivalent.

All fortified samples for liver and kidney met the acceptance criteria. The mean accuracy was between 80–110 percent at each concentration, with a percent CV of less than 10 percent.

Accuracy and precision: Precision was determined by testing six replicates at each fortification in three independent trials, which included at least two operators and on at least two different days. For the method with standard solutions and without ISTD, intra-trial RSDr percent values ranged from 2.5 to 11.3 percent across all tissue types. For eggs at 50 ng/g DNC, RSDr percent values ranged from 2.1 to 12.1 percent. In incurred tissues RSDr percent values ranged from 0.53 to 2.5 percent. Average recoveries ranged between 82–98 percent for all tissues (Coleman *et al.*, 2014).

For the final version of the method (matrix-matched and DNC-d8 as ISTD) chicken liver was fortified at six concentrations and six replicates at each concentration were processed and analysed on 2 different days. Results for RSDr percent values ranged from 1.8 to 6.2 percent.

In order to achieve final action status through AOAC, the performance of the AOAC first action method was reviewed by retroactively analysing results from 10 studies performed at four separate laboratories is listed in Table 16 (AOAC International, 2021). In all cases, repeatability and reproducibility were within acceptable limits.

Table 16. Relative recovery and relative standard deviation (RDS)

Tissue	Relative recovery % (95% CI)	RSDr, % (95% CI)	RSDr, %
Muscle	90.4 (83.8, 97.5)	5.4 (3.8, 9.2)	7.9
Liver	94.5 (91.1, 98.0)	5.8 (4.1, 9.9)	6.8
Kidney	91.5 (85.3, 98.1)	5.2 (3.7, 8.8)	9.0
Skin with fat	94.5 (89.2, 100.1)	8.9 (6.3, 15.1)	8.9

Source: AOAC International. 2021. AOAC Official Method 2013.07 Determination and Identification of Nicarbazin in Chicken Tissues: Liquid Chromatography with Tandem Mass Spectrometry (final action pre publication).

Limit of Detection and Limit of Quantification: Limit of detection (LOD) was determined in the AOAC Official method after analyzing 20 replicate test portions of control tissue in each tissue type. The determination was defined as the mean result plus three standard deviations. An estimated LOD could not be determined for liver and muscle as all results were 0.00 ng/g. LODs are listed in Table 17. LOQ was determined in the AOAC Official method by fortifying control matrices at 1/10 the CODEX MRL. Ten samples were extracted and analysed with appropriate precision (Coleman et al., 2014).

Table 17. Calculated LOD and LOQ

Tissue	Calculated LOD	LOQ	RSDr, % for LOQ
Liver	ND*	16.5	2.1
Kidney	2.9	12.5	15.1
Muscle	ND*	17.7	2.5
Skin with fat	1.0	17.9	2.0

Note(s): ND* = not determined

Source: Coleman, M.R., Rodewald, J.M., Brunelle, S.L., Nelson, M., Bailey, L., & Burnett, T.J. 2014. Determination and confirmation of nicarbazin, measured as 4,4-dinitrocarbanilide (DNC), in chicken tissues by liquid chromatography with tandem mass spectrometry: First Action 2013.07. *J. AOAC INTERNATIONAL*, 97 (2):630–640. doi.org/10.5740/jaoacint.13-197

Practicability and applicability: Robustness was evaluated in chicken muscle. Method parameters including tissue weight, sodium sulphate weight, vortex time, shaking time, tissue temperature, fortification residence time, and injection volume were analysed according to the Plackett-Burman design. None of the parameters evaluated were found to have a significant impact on the recoveries of the method.

Transferability: This parameter has been demonstrated by the successfully use of this nicarbazin method in multiple residues in four different laboratories, over a five-year period, in ten separate trials. The analysis concluded that the method is repeatable and reproducible in several different laboratories, confirming that this method is appropriate for use as a surveillance method (AOAC International, 2021).

Overall comment on validation of the LC-MS/MS method

In validation, the determination of specificity is done in an odd way. The definition that we have used is the following: the ability of a method to distinguish the analyte from other substances. We have so far understood that "other substances" are all substances that might interfere with the measurement of the analyte in question. To do this, what we usually do is to analyse an appropriate number of representative blank samples ($n \geq 20$) and check for possible interferences (signals, peaks, ionic traces) in the area where the analyte is expected to elute. Thus, the determination made for specificity, while meeting the spirit of the definition, is too restrictive.

There is a mixture of data obtained with the first version of the method (standard solutions and without ISTD) and another with the final version described (matrix-matched and DNC-d8 as ISTD). For example, repeatability and reproducibility were determined for the final version of the method in liver only.

The MRLs that are now proposed (at the mg/kg level) and those for which the method was designed (µg/kg), leads to a "mismatch" of the method. This is why a dilution of the extracts before analysis by LC-MS/MS is recommended, for several reasons, the main one being the possibility of "carry-over" phenomena. This may compromise its applicability in routine controls.

Stability of residues

As part of the validation of analytical methods, there is a requirement to demonstrate that there is no loss of analyte(s) in the samples over the period in which they are stored, in between the slaughter of the sampled animals, and the time of analysis. The conditions of storage should mimic those of the samples analysed, in particular they should be treated in exactly the same manner prior to storage and stored at the same temperature. There are three periods where stability needs to be demonstrated (in accordance with VICH GL49):

• The time of sample storage, between taking of the samples and the analysis of the samples

• The time between extracting the analyte(s) from the samples and analysing the extracts (e.g. could be stored for several days in the fridge at +4°C)

• The time where the samples for analysis are waiting to be analysed (e.g. could be stored in an autosampler for several hours at room temperature)

There is also the requirement to demonstrate that the analytes are stable in samples when being repeatedly frozen and thawed out (three freeze-thaw cycles are usually needed).

For the LS-MS/MS method, stability of DNC was evaluated in tissues, extracts, and standard curve solutions. Stability is defined as less than a 20 percent change in mean concentration or calibration slope.

Fortified tissue stability: Fortified tissue was frozen at -20°C and tested at 0, 14 and 28 days of storage. Mean DNC was compared to freshly fortified tissue, and stability was defined as less than a 20 percent change in mean concentrations. Stability was demonstrated at 28 days where all tissues had a change under 5 percent, except for kidney, which changed by 9.8 percent. This was repeated with fortified tissue extracts with acceptable stability. Fortified tissues were also subjected to three freeze thaw cycles at -20°C for 18 hours, then placed on the benchtop. The stability of DNC was appropriate through three freeze thaw cycles (Coleman *et al.*, 2014).

A supplemental stability study tested fortified tissue liver at 2 000, 4 000, and 8 000 ng/g (Mizinga, 2017a). Samples were pooled and stored at -80°C. An acceptance range for these fortified levels were between -20 percent to +10 percent. Each mean recovery value for fortified stability samples met the acceptance criterion and each percent of initial met the acceptance criterion except for the 8 week sample, which exceeded the +10 percent criteria. Recovery was appropriate for chicken livers out to 18 weeks at -80C° (Table 18).

Table 18. Nicarbazin stability results in control and fortified chicken liver stored at -80C°

Sample fortification (ng/g)	Timepoint	Average results (ng/g)	Average recovery (%)	%Initial
Control	All timepoints	<LOQ	NA	NA
2000	Initial	1 910	95.9	NA
	2 weeks	1 860	93	97.4
	4 weeks	1 870	93.3	97.9
	8 weeks	2 150	108	113
	13 weeks	2 000	99.7	105
	18 weeks	1 830	91.8	95.8
4000	Initial	3 880	96.7	NA
	2 weeks	3 530	88.1	91
	4 weeks	3 900	97.4	101
	8 weeks	4 420	110	114
	13 weeks	3 990	99.6	103
	18 weeks	3 900	97.5	101
8000	Initial	7 740	96.8	NA
	2 weeks	7710	96.2	9.6
	4 weeks	7820	97.6	101
	8 weeks	8630	108	111
	13 weeks	8510	106	110
	18 weeks	7830	97.7	101

Source: Mizinga, K.M. 2017a. Determination of Narasin and Nicarbazin Stability in Chicken Tissues. Study No. ELA1600366. Covance Laboratories Inc. Greenfield, Indiana. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

An additional stability study analysed stability in fortified chicken liver at 100, 2 000, 4 000, or 8 000 ng/g (Mizinga, 2017b). Liver extracts were pulled at day 2, 4 and 7 days and analysed by the AOAC first action method. Samples were stored at either ambient or refrigerated conditions (2-8C°). All samples through 7 days met the acceptance criterion of -20 percent to +10 percent.

Incurred tissue stability: Stability testing for incurred nicarbazin residues in chicken liver was conducted out to 20 weeks (Mizinga, 2017a). Tissues were stored at -80C°, and samples were pulled at 0, 2, 4, 8, 13, 20 weeks. An acceptance range for these fortified levels were between -20 percent to +10 percent of the initial results (Table 19). The percent of initial ranged from 87.9 to 125 percent. Additionally, benchtop stability was 93.3 percent of the initial when held at room temperature for 5 hours.

Table 19. Nicarbazin stability results in incurred chicken liver stored at -80°C

Timepoint	Average results (ng/g)	%RSD	%Initial
Initial	5 200	5.4	NA
2 weeks	4 570	6.6	87.
4 weeks	5 240	5.9	100.8
8 weeks	6 510	3.2	125
13 weeks	5 570	3.8	107
20 weeks	5 840	3.4	112

Source: Mizinga, K.M. 2017a. Determination of Narasin and Nicarbazin Stability in Chicken Tissues. Study No. ELA1600366. Covance Laboratories Inc. Greenfield, Indiana. Submitted to FAO/WHO by Elanco Animal Health, Greenfield, IN 46140, USA. (Unpublished)

In an additional study, incurred chicken liver was obtained, pooled and cryo-prepared (Mizinga, 2017b). Triplicate samples were analysed on Day 0, 2, 4 and 7 days. Tissues were stored at ambient conditions and refrigerated conditions (2–8°C). The stability was considered acceptable if the sample agreed with day 0 from 20 percent to +10 percent. For samples stored at ambient conditions, the percent of initial ranged from 98.8 to 98.9 percent, and for samples stored at refrigerated conditions, the percent of the initial ranged from 95.6 to 98.8 percent. All samples met the acceptance criteria at 7 days of stability.

Solution stability: Standard curve solutions were subjected to stability testing at 2-8°C at 0, 1, 3, 7 and 14 days. The standard curve solutions met acceptance criteria for stability (less than 20 percent difference), with the exception of the 2.5 ng/mL standard solution. The standard curve slopes were less than 20 percent different and met the acceptance criteria (Coleman *et al.*, 2014).

Overall comment on storage stability

The sponsor has demonstrated that DNC is stable in frozen tissue for up to 28 days and up to 3 freeze-thaw cycles; in chilled tissue extract for up to 3 days; and in chilled standard solutions for up to 14 days.

In line with VICH GL 49, the stability of DNC in raw matrices as well as in processed samples was demonstrated (EMA, 2015).

Appraisal

Introduction:

Nicarbazin (IUPAC name 1,3-bis(4-nitrophenyl)urea;4,6-dimethyl-1H-pyrimidin-2-one; Chemical Abstract Service No. 330-95-0) is a carbanilide used for the prevention of faecal and intestinal coccidiosis in chickens, as well as in some other poultry species. Nicarbazin is used as a feed additive or as a veterinary drug for oral use in feed.

The equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) is practically insoluble, but will dissociate completely in aqueous conditions, such as the digestive tract. DNC is the active anticoccidial component while HDP has no anticoccidial activity. The absorption of DNC is greatly enhanced when the two components are complexed together. The mode of action (MOA) of DNC is unclear but may involve the inhibition of mitochondrial electron transport.

Nicarbazin was evaluated for toxicology and residues by the Committee at its fiftieth meeting (FAO and WHO, 1998). An acceptable daily intake (ADI) of 0–400 µg/kg bw nicarbazin (24 mg/person for a 60 kg person) was set. MRLs for chicken muscle, liver, kidney and skin/fat (in natural proportions) were established at 200 µg/kg bw nicarbazin, using DNC as the marker residue.

The Committee evaluated nicarbazin at the present meeting at the request of the twenty-fifth session of the Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF) with a view to recommending maximum residue limits (MRLs) for edible chicken tissues. A toxicological re-evaluation was also undertaken to establish health-based guidance values due to the time that had elapsed since its last review. The sponsor provided unpublished proprietary studies as well as data from studies in the published literature to support the assessment.

The inclusion rate for nicarbazin provided by the sponsor was 125 mg/kg nicarbazin per day in complete feeding stuffs to be used in chickens for fattening. For this dose, withdrawal periods range from 1 to10 days for edible tissues. The Committee noted that a higher inclusion rate of 200 mg/kg feed is approved in at least one Member State, with a withdrawal period of five days, but no residue data were provided for this dosing regimen. When used in combination with either narasin or monesin the nicarbazin inclusion rate is lower at 50 mg/kg feed and withdrawal periods range from 0 to 8 days. Products are not intended for use in animals producing eggs for human consumption.

Nicarbazin is not currently used as a plant protection product or as a human medicine.

Metabolism:

The sponsor had proposed a possible metabolic pathway in rats and chickens based on radiolabeled studies. In the intestinal tract nicarbazin is entirely dissociated into its two components, DNC and HDP. Nicarbazin parent does not appear as a residue in tissues.

HDP is excreted much faster than DNC and primarily as parent HDP. The DNC component is metabolized, and three resulting metabolites have been identified. Metabolite M1 was identified as monoacetylamino-DNC, corresponding to the reduction and acetylation of one nitro group. Metabolite M2 was identified as N,N′-1,4-phenylene-bis(acetamide) resulting from the cleavage, reduction and acetylation of the molecule. Metabolite M3 was identified as diacetylamino-DNC, resulting from the reduction and acetylation of two nitro groups.

Residue depletion:

Three radiolabeled residue depletion studies were available for evaluation. In one study nicarbazin radiolabeled either on the HDP or the DNC moiety was used. The second and the third studies used either [^{14}C]-DNC or [^{14}C]-HDP.

In one study (Nessel, 1977; GLP-compliance not stated) chickens were administered nicarbazin with a radiolabel on either the HDP or DNC moiety of the molecule for 2–7 consecutive days at 125 mg/kg feed. No radiolabeled residues were found five days post withdrawal of nicarbazin labelled on the HDP moiety. In the chickens fed nicarbazin labelled on the DNC moiety only, liver retained detectable radioactivity up to day 8 post withdrawal. Other tissues were clear of radioactivity by day 5 post withdrawal.

The second study (GLP-compliant) was conducted to provide total residue depletion data in tissue samples following multiple oral administrations of nicarbazin containing [^{14}C]-DNC to 18 broiler chickens (King and Walker, 2007). Animals were dosed for seven days with nicarbazin at a target inclusion rate of 125 mg/kg feed. Doses were prepared in gelatine capsules and administered twice daily. The major route of elimination for radioactivity was via excreta. The major component in all pooled excreta samples was parent DNC which represented approximately 90 percent of the extracted radioactivity. The highest mean total radioactive residues at all time points were observed in the liver, followed by kidney, skin with fat, and muscle. The mean total radioactive residues of DNC in the liver at 24 hours post dose (one day withdrawal) was 27.8 mg DNC-eq/kg (range 25.9–30.3 mg DNC-eq/kg). Levels of total radioactive residues decreased to 0.05 mg DNC-eq/kg (range 0.04–0.08 mg DNC-eq/kg) at 240 hours post dose (nine days withdrawal). Analysis by radio-HPLC (high performance liquid chromatography) and LC-MS/MS confirmed that the major component in all pooled tissue samples at 24 hours after the last morning dose (zero day withdrawal) was 4,4 dinitrocarbanilide, that is parent DNC). The highest concentration of DNC residues for each tissue was observed in tissues collected at 12 hours withdrawal. The highest mean concentration was measured in liver, followed by kidney, skin with fat, and muscle.

The third study (GLP-compliant) evaluated residue depletion after oral administration of nicarbazin containing [^{14}C]-HDP, at a target inclusion rate of 125 mg/kg feed (McLellan and Coyle, 2007). Doses were prepared in gelatine capsules and administered twice daily for seven days. Elimination of total radioactivity was rapid, with a mean of 96.7 percent (range 92.9–99.1 percent) of the total dose recovered within 16 hours of the final dose. Concentrations of total radioactivity from HDP in plasma were low at each time point, ranging from 0.036 to 0.093 mg HDP-eq/kg. Tissue residue concentrations at one day withdrawal were highest in the kidney (0.13 mg HDP-eq/kg), followed by skin with fat (0.11 mg HDP-eq/kg), liver (0.095 mg

HDP-eq/kg), and muscle (0.084 mg HDP-eq/kg). Total radioactivity decreased with each timepoint, with radioactivity almost undetectable at nine days withdrawal. Additional HPLC analysis indicated that the principal component of the total radioactivity was HDP at 24 hours post final dose. Overall, HDP residues were extremely low in plasma and all tissues, and parent HDP was the principal component of all residues examined.

From the radiolabeled residue depletion studies, it can be concluded that HDP residues deplete quickly, while DNC residues reach greater tissue concentrations and deplete more slowly. Radioactive HDP represents less than 1 percent of the total radioactive nicarbazin residues at 24 hours withdrawal. Other metabolites have been identified but are present at less than 10 percent of the total residues. DNC is the most appropriate marker residue. Liver is the target tissue based on the distribution and decline of the [^{14}C]-DNC administered to chickens. For DNC at 24 hours withdrawal, marker residue to total recovered radioactivity (MR:TRR) ratios of 0.43, 0.36, 0.24, and 0.47 were calculated for liver, kidney, muscle and skin with fat respectively.

Two residue depletion studies using unlabelled nicarbazin were provided. In one study, nicarbazin was administered at a target inclusion rate of 125 mg/kg feed and in the other study, nicarbazin was administered in combination with narasin, each at a target inclusion rate of 50 mg/kg feed.

In the GLP-compliant study using nicarbazin only, chickens received feed containing nicarbazin at 125 mg/kg feed for 28 days, then six animals were sacrificed on each of days 1, 5, 6, 9, 11 and 14 (Cairns and Davidson, 2006b). DNC was detected in all tissues at day 1 following withdrawal of the test diet. Limits of quantitation (LOQs) for the LC-MS/MS method used were 50, 100, 25, 25 and 50 µg/kg in liver, kidney, muscle, skin with fat and fat, respectively. Residues of DNC ranged from: 7 564–12 595 µg/kg in liver, 1 194–4 110 µg/kg in kidney, 1 342–2 688 µg/kg in muscle, 1 678–2 798 µg/kg in skin with fat, and 1 811–2 866 µg/kg in fat. At day 5 post withdrawal, DNC residues for kidney had declined below the LOQ in all birds tested. The DNC ranges at day 5 were 411–544 µg/kg in liver, 33.4–56.6 µg/kg in muscle, 90.3–176 µg/kg in skin with fat, and 62.9–93.5 µg/kg in fat residues. At days 7, 9 and 11 post withdrawal, DNC residues in liver, kidney, muscle and fat had declined to below the LOQ in all birds. At these samplings times residue levels in skin with fat ranged from below the LOQ to 41.7 µg/kg. At day 14 post withdrawal, all residue concentrations were below the LOQ in all tissues and for all birds.

In another GLP-compliant residue depletion study, nicarbazin was administered in combination with narasin (50 mg/kg feed each) to broiler chickens (Johnston, 2008). Three females and three males were necropsied at day 0 (immediately after feed was withdrawn) and at days 3, 5, and 7 post withdrawal, and tissues including the kidneys, liver, muscle, skin with fat, and the abdominal fat pad were removed from each bird. DNC concentrations in samples were analysed using a validated LC-MS/MS method (LOQ: 50 µg/kg for liver, 100 µg/kg for kidney and 25 µg/kg for muscle and fat). Individual residues of DNC in tissues were below 750 µg/kg at five days following the last dose. The relative concentrations of DNC in the tissues at five days withdrawal were: liver > skin with fat > muscle > kidney. The DNC concentrations at seven days after the last dose were less than 87.8 µg/kg for liver, less than 25.9 µg/kg for

skin with fat, less than the LOQ (25 µg/kg) for muscle and less than the LOQ (100 µg/kg) for kidney.

Using either treatment regimen, DNC residue concentrations were consistently highest in liver tissues. The Committee considered both residue depletion studies as suitable to derive 95/95 UTLs (upper tolerance limits) for the two dosage regimens used in veterinary practice.

Analytical method:

The Committee assessed the validation data against the requirements for analytical methods as published in the Codex Guideline CAC/GL 71-2009.

An LC-MS/MS method has been developed and validated for the nicarbazin marker residue (DNC) depletion studies in chickens. The LOQ of the method is 17 µg/kg for liver tissues and 13, 18, and 18 µg/kg for kidney, muscle, and skin with fat, respectively. The stability of samples was adequately demonstrated for normal conditions of laboratory handling.

Estimated dietary exposure

Chronic dietary exposure assessment

When used as a veterinary drug, dietary exposure was estimated based on the potential occurrence of DNC residues in chicken tissues. Residue concentrations were taken from measurements made at 24 hours withdrawal (day 1) for an inclusion rate of 125 mg/kg feed (Cairns and Davidson, 2006), or at day 0 for nicarbazin at an inclusion rate of at 50 mg/kg feed (Johnston, 2008). These studies reported residue concentrations in terms of DNC (the marker residue).

The above studies provide residue data for both chicken liver and kidney. However, the available food consumption data are for chicken offal, without further distinction. Residue data from the tissue with the higher residue concentrations (chicken liver) were used for the dietary exposure assessment.

Based on incurred DNC residues at 24 hours withdrawal time in chicken muscle, offal, and skin with fat (125 mg/kg feed) the global estimates of chronic dietary exposure (GECDE) for the adults and the elderly, children and adolescents, and for infants and toddlers were 120, 160 and 210 µg/kg bw per day, respectively, which represent 13 percent, 18 percent and 23 percent respectively of the upper bound of the acceptable daily intake (ADI) of 900 µg/kg bw.

Based on incurred DNC residues in chicken muscle, offal, and skin with fat at zero days withdrawal time (50 mg/kg feed) the GECDE for the adults and the elderly, children and adolescents, and infants and toddlers were 95, 120 and 160 µg/kg bw per day, respectively, which represent 11 percent, 14 percent and 18 percent respectively of the upper bound of the ADI of 900 µg/kg bw.

As part of the GECDE methodology, further estimates of chronic dietary exposure were carried out. Instead of using the highest mean and the highest 97.5th percentile consumption across

surveys, the calculations were carried out using the mean and the highest reliable percentile for each individual national survey from available datasets (CIFOCOss). The highest GECDE for each age class for each country was determined.

For the inclusion rate of nicarbazin at 125 mg/kg feed, the mean (range) of 35 country-specific estimates for DNC dietary exposure for adults and the elderly at 24 hours withdrawal was 32 (4–100) µg/kg bw per day, or 3.5 percent (0.4–11.1 percent) of the upper bound of the ADI. The mean (range) of 26 country-specific estimates of DNC dietary exposure for children and adolescents at 24 hours withdrawal was 53 (2–160) µg/kg bw per day, or 5.9 percent (0.2–17.9 percent) of the upper bound of the ADI. The mean (range) of 19 country-specific estimates of DNC dietary exposure for infants and toddlers at 24 hours withdrawal was 67 (10–210) µg/kg bw per day or 7.4 percent (1.1–23.4 percent) of the upper bound of the ADI.

For the inclusion rate of nicarbazin at 50 mg/kg feed, the mean (range) of 35 country-specific estimates of DNC dietary exposure for adults and the elderly at 0 days withdrawal was 25 (3–76) µg/kg bw per day or 2.7 percent (0.3–8.4 percent) of the upper bound of the ADI. The mean (range) of 26 country-specific estimates of DNC dietary exposure for children and adolescents at 0 days withdrawal was 41 (2–120) µg/kg bw per day or 4.5 percent (0.2–13.5 percent) of the upper bound of the ADI. The mean (range) of 19 country-specific estimates of DNC dietary exposure for infants and toddlers at 0 days withdrawal was 51 (7–160) µg/kg bw per day or 5.7 percent (0.8–17.8 percent) of the upper bound of the ADI.

As no ARfD was necessary, acute dietary exposure (global estimate of acute dietary exposure; GEADE) was not assessed for nicarbazin.

Table 20. Global estimate of chronic dietary exposure (GECDE) for nicarbazin (as DNC) in chicken tissues

Category	Type	Median concentration[i] (µg/kg)	Mean consumption, whole population[ii] (g/kg bw per day)	HRP consumption, consumers only[iii] (g/kg bw per day)	MR:TR ratio	Exposure µg/kg bw/day		GECDE[iv]	
						mean	HRP	µg/kg bw/day	%ADI
125 mg/kg nicarbazin - 24 hours post withdrawal									
Adults and the elderly									
Poultry muscle	Chicken muscle	2140	1.25	11.2	0.24	11	100	100	
Poultry offal	Chicken offal	8830	0.88	1.59	0.43	18	33	18	
Poultry trimmed fat	Chicken fat	2350	0.06	0.35	0.47	0.3	1.7	0.3	
TOTAL								**118**	**13**
Children and adolescents									
Poultry muscle	Chicken muscle	2140	2.75	18.0	0.24	25	161	161	
Poultry offal	Chicken offal	8830	0.06	2.04	0.43	1.3	42	1.3	
Poultry trimmed fat	Chicken fat	2350	0.12	0.65	0.47	0.6	3.2	0.6	
TOTAL								**162**	**18**
Infants and toddlers									
Poultry muscle	Chicken muscle	2140	3.96	23.6	0.24	35	211	211	
Poultry offal	Chicken offal	8830	0.06	3.89	0.43	1.2	80	1.2	
Poultry trimmed fat	Chicken fat	2350	0.10	0.63	0.47	0.5	3.1	0.5	
TOTAL								**212**	**24**
50 mg/kg nicarbazin - 0 days post withdrawal									
Adults and the elderly									
Poultry muscle	Chicken muscle	1630	1.25	11.2	0.24	8.4	76	76	
Poultry offal	Chicken offal	9290	0.88	1.58	0.43	19	34	19	
Poultry trimmed fat	Chicken fat	2060	0.06	0.35	0.47	0.3	1.5	0.3	
TOTAL								**95**	**11**

Children and adolescents									
Poultry muscle	Chicken muscle	1630	2.75	18.0	0.24	19	122	122	
Poultry offal	Chicken offal	9290	0.06	2.04	0.43	1.3	44	1.3	
Poultry trimmed fat	Chicken fat	2060	0.12	0.65	0.47	0.5	2.8	0.5	
TOTAL								**124**	**14**
Infants and toddlers									
Poultry muscle	Chicken muscle	1630	3.96	23.6	0.24	27	160	160	
Poultry offal	Chicken offal	9290	0.06	3.89	0.43	1.3	84	1.3	
Poultry trimmed fat	Chicken fat	2060	0.10	0.63	0.47	0.4	2.7	0.4	
TOTAL								**161**	**18**

Note(s): MR: marker residue, TR: total residue, HRP: highest reliable percentile, GECDE: global estimates of chronic dietary exposure

i = Median concentration of the marker residue at the specified times after the end of treatment expressed as DNC; ii = highest mean consumption figures based on whole population considered from the available dataset; iii = highest reliable percentile food consumption figures based on consumers only considered from the available dataset; iv = GECDE is the sum of the highest exposure at the highest reliable percentile of consumption for a food and the mean dietary exposures of the other foods

Table 21. National estimates of chronic dietary exposure to nicarbazin (as DNC) median total residues in chicken tissues, based on GECDE methodology

Country	Estimate of chronic dietary exposure (µg/kg bw per day)					
	125 mg/kg, 24 hours post-withdrawal			50 mg/kg, 0 days post-withdrawal		
	Adults and elderly	Children and adolescents	Infants and toddlers	Adults and elderly	Children and adolescents	Infants and toddlers
Argentina	68.7	-	-	52.1	-	-
Austria	30.3	36.2	-	23.0	27.5	-
Bangladesh	18.9	-	-	14.3	-	-
Belgium	30.0	63.9	9.7	22.8	48.5	7.4
Bolivia (Plurinational State of)	22.9	24.6	-	22.6	18.6	-
Bulgaria	-	38.2	42.1	-	42.3	32.1
Burkina Faso	20.6	61.4	-	15.6	46.6	
China	36.3	85.0	69.3	27.5	64.5	52.6
Croatia	36.0	-	-	27.3	-	-
Cyprus	30.5	72.4	97.3	23.2	54.9	73.8
Czechia	26.4	67.9	-	28.3	51.5	-
Democratic Republic of the Congo	3.5	-	-	2.6	-	-
Denmark	9.1	18.2	19.6	6.9	13.8	14.9
Finland	32.2	41.9	51.5	24.4	31.8	39.1
France	23.5	51.5	19.2	17.8	39.1	14.6
Greece	26.3	30.5	-	20.0	23.2	-
Guatemala	43.6	-	-	33.0	-	-
Hungary	21.4	-	-	16.4	-	-
Ireland	24.1	-	-	18.3	-	-
Italy	32.9	52.3	48.4	29.1	39.7	36.7
Lao People's Democratic Republic	25.0	42.5	23.3	19.0	32.4	24.2
Latvia	34.8	64.3	68.7	26.5	48.8	52.3
Malaysia	31.7	-	-	24.1	-	-
Mexico	64.3	160.7	210.9	48.8	121.9	160.1
Mozambique	40.8	2.1	-	31.0	1.6	-
Netherlands (Kingdom of the)	27.8	39.7	42.1	21.1	30.1	32.0
Pakistan	5.3	-	-	4.0	-	-
Philippines	57.4	-	-	43.6	-	-
Portugal	40.1	59.4	100.2	30.5	45.1	76.0
Republic of Korea	100.1	118.5	96.3	76.0	90.0	73.0

Country	Estimate of chronic dietary exposure (µg/kg bw per day)					
	125 mg/kg, 24 hours post-withdrawal			50 mg/kg, 0 days post-withdrawal		
	Adults and elderly	Children and adolescents	Infants and toddlers	Adults and elderly	Children and adolescents	Infants and toddlers
Romania	23.3	-	-	24.6	-	-
Sao Tome and Principe	-	32.9	120.7		25.0	91.6
Slovenia	29.6	39.8	66.3	22.5	30.2	50.3
Spain	27.4	66.5	93.1	20.8	50.5	70.6
Sweden	21.0	24.8	-	15.9	18.8	-
United Kingdom of Great Britain and Northern Ireland	16.9	28.2	39.2	12.8	21.4	29.7
United States of America	21.9	52.9	55.6	16.7	40.2	42.3
Mean (%ADI)	**32 (3.5)**	**53 (5.9)**	**67 (7.4)**	**25 (2.7)**	**41 (4.5)**	**51 (5.7)**
Minimum (%ADI)	**3.5 (0.4)**	**2.1 (0.2)**	**9.7 (1.1)**	**2.6 (0.3)**	**1.6 (0.2)**	**7.4 (0.8)**
Maximum (%ADI)	**100 (11)**	**160 (18)**	**210 (23)**	**76 (8.4)**	**120 (14)**	**160 (18)**

Maximum Residue Limits

In recommending MRLs for nicarbazin in chickens, the Committee considered the following factors:

- An ADI, expressed as DNC, of 0.9 mg/kg bw per day was established by the Committee.
- Withdrawal periods range from 1 to 10 days for use of nicarbazin at an inclusion rate of 125 mg/kg feed in chickens for fattening. Withdrawal periods range from 0 to 8 days for use of nicarbazin at an inclusion rate of 50 mg/kg feed when applied in combination with narasin or monensin.
- Nicarbazin is not intended for use in laying hens.
- Nicarbazin is an equimolar complex of DNC and HDP that fully dissociates in aqueous conditions. While HDP residues quickly deplete, DNC residues reach a greater concentration in tissues and deplete more slowly. Neither component of nicarbazin is extensively metabolized in chickens; metabolites are present at less than 10 percent of the total residues.
- DNC is the marker residue and is considered to be suitable for residue monitoring purposes.
- The non-radiolabeled nicarbazin marker residue depletion data were sufficient to determine mean and 95/95 UTL concentrations in chicken muscle, liver, kidney, and skin with fat, at 24 hours withdrawal for use of nicarbazin only (125 mg/kg feed), and at 0 hours withdrawal for use in combination with narasin at the lower inclusion rate of 50 mg/kg feed.
- The residue of concern (DNC) can be estimated from the non-radiolabeled residue depletion data, along with MR:TRR data.
- A validated analytical method (LC-MS/MS) for the determination of nicarbazin marker residue (DNC) in chicken liver, kidney, muscle, and skin with fat is available and may be used for monitoring purposes.

Available residue depletion data are not suitable for linear regression analysis. Quantifiable residue values (below the LOQ) were measured in all edible tissues only at 24 hours and five days after withdrawal of treatment after use of nicarbazin at an inclusion rate of 125 mg/kg feed (Cairns and Davidson, 2006b) and at zero days and three days after use of nicarbazin at an inclusion rate of 50 mg/kg feed (Johnston, 2008).

Alternatively, tolerance limits were calculated based on the one-sided tolerance interval calculation (Odeh *et al*., 1977). Upper tolerance limits of DNC residues at one day were calculated for the use of nicarbazin at an inclusion rate of 125 mg/kg feed, as well as at zero days for the use of nicarbazin in combination with narasin at an inclusion rate of 50 mg/kg feed for each. Upper tolerance limits were highest in liver tissues and in the same order of magnitude for both patterns of use.

Maximum residue limits were calculated based on the upper limit of the one-sided 95 percent confidence interval over the 95th percentile of marker residue concentrations (95/95 UTL) in chicken liver, kidney, muscle and skin with fat.

The Committee recommended MRLs based on the marker residue DNC, as the toxicological evaluation is based on DNC, and DNC is the residue of concern. The Committee recommended increasing the MRLs set in 1998 to 15 000, 8 000, 4 000, and 4 000 µg/kg for DNC residues in chicken liver, kidney, muscle, and skin with fat, respectively. These MRLs are based on nicarbazin inclusion rates in feed of 125 and 50 mg/kg and withdrawal periods of 1 and 0 days respectively. As no residue data were available for other inclusion rates, the Committee could not assess whether these recommended MRLs are compatible with such inclusion rates and corresponding GVPs.

Table 22. Upper tolerance limit calculations for DNC residues in chicken tissues after administration of nicarbazin at 125 mg/kg feed at one day withdrawal (DNC concentrations from Cairns and Davidson, 2006b) and after administration of 50 mg/kg feed with 50 mg/kg feed at 0 days withdrawal (DNC concentrations from Johnston, 2008) and proposed MRLs for edible tissues

Dose	Withdrawal period		Liver	Kidney	Muscle	Skin with fat
Nicarbazin at 125 mg/kg feed	1 day	Mean DNC concentration (µg/kg) $n = 6$	9 249	3 007	2 110	2 327
		Standard deviation	1 804	1 094	506	473
		95/95 UTL (µg/kg)	**15 937**	**7 065**	**3 988**	**4 081**
Nicarbazin at 50 mg/kg feed	0 days	Mean DNC concentration (µg/kg) $n = 6$	9 193	4 293	1 610	2 043
		Standard deviation	953	1 036	149	480
		95/95 UTL (µg/kg)	**12 727**	**8 133**	**2 163**	**3 822**
Proposed MRLs (based on DNC)			**15 000**	**8 000**	**4 000**	**4 000**

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Selamectin

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Addendum to the monograph prepared by the 88th meeting of the Committee and published in the FAO JECFA Monograph 24

Identity

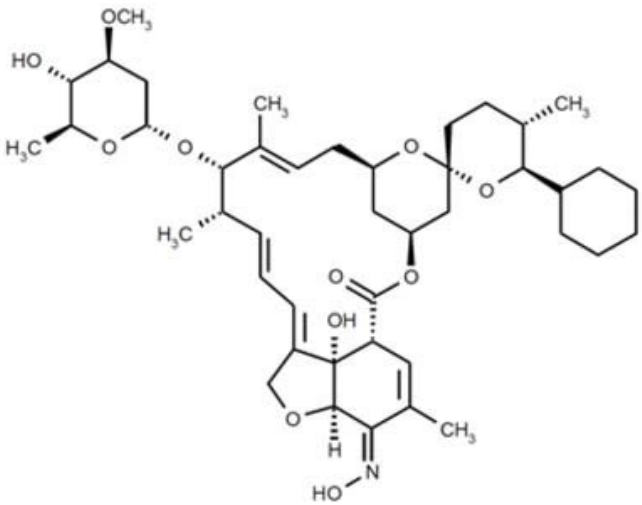
International Non-proprietary Names (INN): selamectin

Synonyms: UK-124114, PF-02636204, ZTS-00010302

IUPAC name: (1*R*,4*S*,5'*S*,6*R*,6'*S*,8*R*,10*E*,12*S*,13*S*,14*E*,16*E*,20*R*,21*E*,24*S*)-6'-cyclohexyl-24-hydroxy-21-hydroxyimino-12-[(2*R*,4*S*,5*S*,6*S*)-5-hydroxy-4-methoxy-6-methyloxan-2-yl]oxy-5',11,13,22-tetramethylspiro[3,7,19-trioxatetracyclo[15.6.1.1^{4,8}.0^{20,24}]pentacosa-10,14,16,22-tetraene-6,2'-oxane]-2-one

Chemical abstract service N°: 165108-07-6

Structural formula:



Molecular formula: $C_{43}H_{63}NO_{11}$

Molecular weight: 770 g/mol

Other information on identity and properties

Appearance: white to off-white solid

Solubility in water: 0.435 mg/L

Melting point: 221.9°C

Solubility in organic solvents:

n-Pentane	0.0252 mg/mL
Toluene	1.3 mg/mL*
Acetonitrile	12 mg/mL*
Methanol	30.7 mg/mL
Acetone	41 mg/mL*
Dichloromethane	60 mg/mL*
Methyl tertiary-butyl ether	>592 mg/mL
t-Amyl alcohol	>424 mg/mL
Methanol: water 90:10 v/v	12.2 mg/mL
Propan-2-ol (IPA)	>483 mg/mL
Dipropylene glycol methyl ether (DPM)	>568 mg/mL
IPA:DPM 94:6 v/v	>500 mg/mL
IPA:DPM 88:12 v/v	>489 mg/mL
Acetonitrile: water 80:20 v/v	47 mg/mL*
Acetonitrile: water 58:42 v/v	15 mg/mL*
Methanol: water 75:25 v/v	2.75 mg/mL
Dichloromethane: ethyl acetate 50:50 v/v	45 mg/mL*

Note(s): * selamectin slowly forms a gel over 24 hours with these solvents at higher concentrations

Log K_{ow}: 3.15 at 25°C

LogP: 4.423 ± 0.790 (25°C)

LogD(7.4): 7.22

Optical rotation: -206° (25°C/365 nm), 5 percent w/v in methanol

Background

Selamectin was reviewed previously by the Committee at its 88th meeting (FAO and WHO, 2020a,b). The Committee established an ADI of 0–0.01 mg/kg bw, and an ARfD of 0.4 mg/kg bw. MRLs could not be recommended for selamectin due to the lack of several key items: characterization and metabolite profile of residues in tissues, data necessary to establish MR:TRR ratios, data from a marker residue depletion study, and an analytical method for monitoring. The suitability of the proposed marker residue for selamectin could not be confirmed without further characterization of the residues. Before re-evaluation of selamectin with the aim of recommending permanent MRLs in tissues of Atlantic salmon, the Committee would need the following information in order to complete the residue assessment:

1. Characterization of the residues in tissues in order to establish a MR:TRR
2. A marker residue depletion study under conditions of use
3. Information on an analytical method suitable for monitoring purposes.
4. Information on the proposed withdrawal period
5. Confirmation of the stability of the radiolabel in tissues.

The current Committee received additional information from the sponsor for evaluation. In the dossier, they also submitted information previously submitted and evaluated by the 88th Committee. For completeness of the evaluation, those studies are re-summarized below. The newly submitted studies were as follows: a pharmacokinetic study in Atlantic salmon comparing two selamectin formulations, residue profiling of samples from the radiolabeled residue depletion study, a non-radiolabeled residue depletion study in Atlantic salmon, an in vitro study assessing stability of tritium labelled selamectin ([^{3}H]-selamectin) that was used in the radiolabeled study, and an analytical method. The studies previously submitted and evaluated by the 88th Committee were as follows: a radiolabeled depletion study in Atlantic salmon (TRR counts only), a study attempting to identify a major metabolite in semi-solid effluent (faeces and uneaten feed), and an in vitro comparative metabolism study with liver microsomes.

Residues in food and their evaluation

Conditions of use

Selamectin is not currently approved for use in food-producing animals. JECFA evaluated selamectin as a pilot programme in which it conducts a parallel review at the same time as the sponsor pursues approval in the proposed species with national authorities, as discussed at the Twenty-fourth Session of CCRVDF (FAO and WHO, 2018). Selamectin is intended as an in-feed ectoparasiticide additive for the treatment and prevention of all parasitic stages of sea lice (*Lepeophtheirus* sp. and *Caligus* sp.) on Atlantic salmon (*Salmo salar*), ranging from smolts to market weight fish, in seawater. The product is to be administered in-feed to fish at an appropriate feeding rate for 7 days to yield a dose rate of 100 µg selamectin/kg biomass/day.

Dosage

The proposed dosing regimen is 100 micrograms selamectin/kg biomass/day via feed for 7 consecutive days.

Pharmacokinetics and metabolism

Pharmacokinetics in food-producing animals

Salmon

In a GLP compliant study, the pharmacokinetics of selamectin in salmon from two different formulations were compared (Bassett, 2021). Atlantic salmon (*Salmo salar*, mean mass 406 g at dosing) held in seawater with a salinity range of 31–33 g/L and temperature range of 9–16°C, were fed one of two medicated feed formulations: feed top coated with either Revolution® 12 percent (treatment 1, T01) or Selarma® 12 percent (treatment 2, T02) in cod liver oil, at a rate of 100 µg/kg body weight/day for 7 days. A formulation comparison is provided in Table 1. Four fish per tank (14 tanks, 7 tanks/treatment group) were harvested on days 0, 3, 6, 7, 14, 28, 42, 56, 70, 98, and 112, and blood collected for plasma analysis and analysed using LC-MS/MS. Tank level data was used to estimate pharmacokinetic parameters. The mean C_{max} was 871 ng/mL (90 percent CI: 799, 950) for T01 (Revolution®) and was 843 ng/mL (774, 919) for T02 (Selarma®). Mean $AUC_{0\text{-}t(last)}$ of 436 000 and 402 000 ng•h/mL for T01 and T02, respectively. The mean $t_{1/2}$ values were also similar across groups at 501 hours (20.9 days) and 417 hours (17.4 days) for T01 and T02, respectively.

Table 1. Formulation comparison

Component	Revolution® formulation (%)	Selarma® formulation (%)
Selamectin	12	12
Buylated hydroxytoluene (BHT)	0.8	0.8
Dipropylene glycol monomethyl ether (DPGMME)	12 (v/v)	85.36
Isopropanol	Approx. 88	0

Metabolism in food-producing animals

Salmon

In a GLP-compliant study, previously submitted and evaluated by the Committee at its 88th meeting (FAO and WHO, 2020a), 120 Atlantic salmon (*Salmo salar*), were used in a total residue and metabolism study (Roberts and Fox, 2015). The fish weighed an average of 585 g at Day -11. Fish were maintained at 8°C in three identical 850 L tanks (one control; two treatment tanks) containing re-circulating seawater. After an acclimatization period of 11 days,

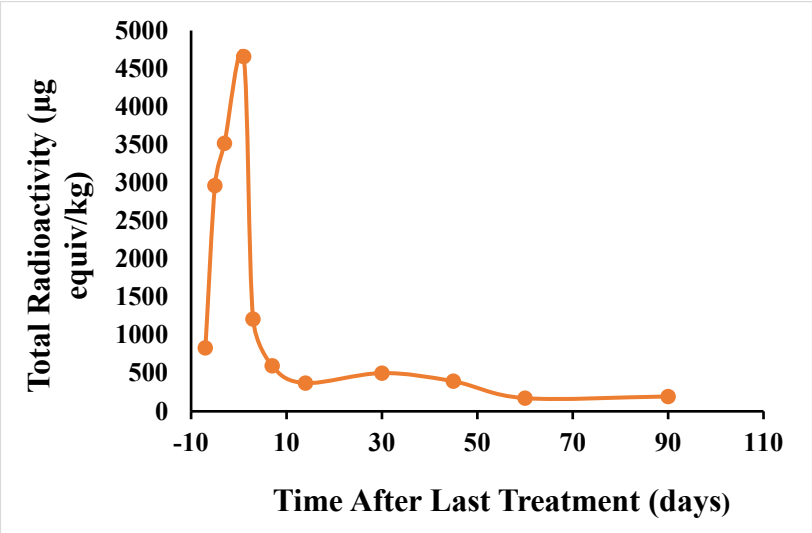
80 fish (40/tank) were given [^{3}H]-selamectin formulated in feed to provide a nominal dose of 100 µg/kg of live weight/day for 7 consecutive days, based on a feeding rate of 0.19 percent biomass/day. The daily dose was split in half and administered in two feedings, "am" and "pm". After the treatment period, all fish were put back onto standard smolt 200 diet (a commercial fish feed) for the remainder of the study, where they were fed 3 times daily at a rate of 0.25–0.30 percent biomass/day until Day 57 and then at a rate of 0.30–0.40 percent biomass/day thereafter.

The treated feed was prepared by mixing a feed pre-mix containing the [^{3}H]-selamectin (99 percent radiopurity), a commercial fish feed (smolt 200 feed), and fish oil. Analysis of the treated feed before and after dosing confirmed that the concentration of selamectin was 52.6 µg/g.

Three fish/tank were euthanized at 3 and 12 h, and at 1, 3, 7, 14, 30, 40, 60, and 90 days post-final dose. Semi-solid effluent (faecal material and any uneaten food) samples were collected from each tank on the first day of treatment (day -7 post final dose), treatment days 3 and 5 (days -5 and -3 post final dose, respectively), and at 1, 3, 7, 14, 30, 45, 60, and 90 days post-final dose. Fish were weighed and measured, and samples of liver, kidney, fillet, were collected individually. Carcass (defined as bones, head, any meat which did not come off in the filleting, all the viscera, scales and the fins which were removed during filleting) and gut contents were pooled from each tank at each time point listed above. Control fish (3/timepoint) were euthanized at the same timepoints as treated fish, but samples were pooled by matrix and post-dose interval.

For the semi-solid effluent samples, average total radioactive residues (TRR) increased during the treatment period of the medicated diet, peaked at 24 hours after the last treatment, then rapidly declined over the following 2 weeks and a continual slow decline through 90 days post last dose (Figure 1). There was considerable variability between the two tanks. The maximum TRR in semi-solid effluent samples were measured 0.5 days post-last dose for Tank 5-02 and 3 days post-last dose for Tank 5-04.

Figure 1. Total radioactivity (µg equivalent/kg) in semi-solid effluent from salmon treated with [^{3}H]-selamectin in feed at 100 µg/kg body weight/day for 7 consecutive days



A prominent metabolite (≥ 10 percent the total) of selamectin was found in gut contents, semi-solid effluent, and liver samples. Presence of the metabolite in the excreta suggests that it may be released into the environment after treatment of animals. A separate non-GLP compliant study was conducted to identity the metabolite in support of environmental assessment (Killmer, 2018). This was previously submitted and reviewed at the 88th meeting (FAO and WHO, 2020a), and resubmitted by the sponsor for the current meeting.

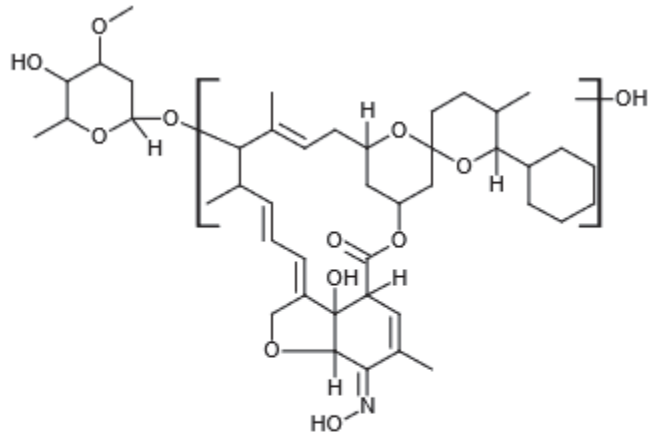
In this study, three strategies for identification of the metabolite were attempted:
1. Extracts of semi-solid effluent samples were analysed by UPLC-MS/MS (tandem quadrupole) for identification of the metabolite.
2. Generation of the metabolite by liver microsomes was attempted using dog, mouse, rat, and salmon liver microsomes in the absence and presence of an NADPH regeneration system.
3. Semi-solid effluent samples were extracted and analysed by UPLC-MS/MS (quadrupole orbitrap) at two separate test sites for identification of the metabolite.

Direct injection of sample extracts using a tandem quadrupole MS/MS instrument or a linear ion-trap instrument proved unsuccessful for identification purposes. Rat, dog, or salmon liver microsomal incubations also failed to produce a signal corresponding to the metabolite. Utilizing different accurate mass UPLC-MS/MS instruments for the analyses, which offered greater sensitivity and rapid scanning, making them better suited for identification of metabolites present at low concentrations provided successful identification.

The chromatograms from assay of each sample from the first facility showed peaks for selamectin at 47.25 min (*m/z* 770, $[M+H]^+$) and the metabolite of interest at 38.5 min (*m/z* 786, $[M+H]^+$). Based on the accurate mass measurements of the molecular ions, the molecular formulas for selamectin and the metabolite were $C_{43}H_{63}NO_{11}$ and $C_{43}H_{63}NO_{12}$, respectively.

Fragmentation of selamectin yielded 4 major fragments at *m/z* 626, 608, 333, and 276, and 2 minor fragments at *m/z* 113 and 69. Fragmentation of the metabolite, similar to that of selamectin, yielded 7 major fragments at *m/z* 642, 624, 606, 349, 331, and 276, and 2 minor fragments at *m/z* 113 and 69. The fragmentation pattern of the metabolite did not allow for precise determination of the site of oxidation. However, the presence of fragments at *m/z* 333 (selamectin) and 349 (metabolite) indicates that hydroxylation occurred in the region of the molecule shown in Figure 2 below in brackets.

Figure 2. Proposed metabolic structure from test site 1

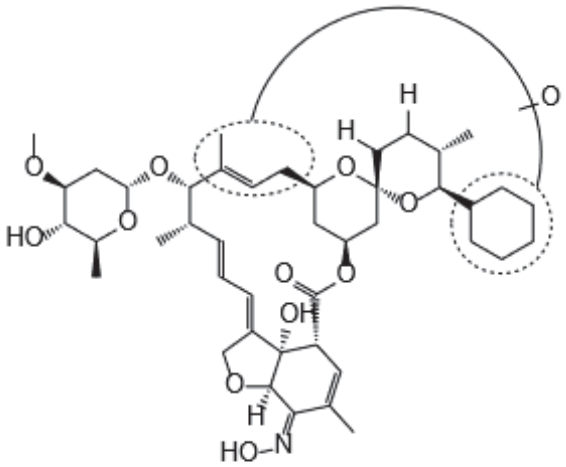


$C_{43}H_{63}NO_{12}$

m/z 786.4423 ($[M+H]^+$)

Chromatogram from assay of each sample at the second facility showed peaks for selamectin at 44.2 min (*m/z* 770.4472, $[M+H]^+$) and the metabolite of interest at 35.6 min (*m/z* 786.4423, $[M+H]^+$). Based on the accurate mass measurements of the molecular ions, the formulas for the $[M+H]^+$ molecular ions of selamectin and the metabolite were $C_{43}H_{64}O_{11}N$ and $C_{43}H_{64}O_{12}N$, respectively. Fragmentation of selamectin and the metabolite yielded multiple fragments. The precise site of oxidation was not determined; however, the presence of fragments at *m/z* 333 and 626 (selamectin), 349 and 642 (metabolite), and 276 and 406 (selamectin and metabolite) indicates that hydroxylation occurred in one of the 2 regions of the molecule shown in Figure 3 (encircled by dashed lines).

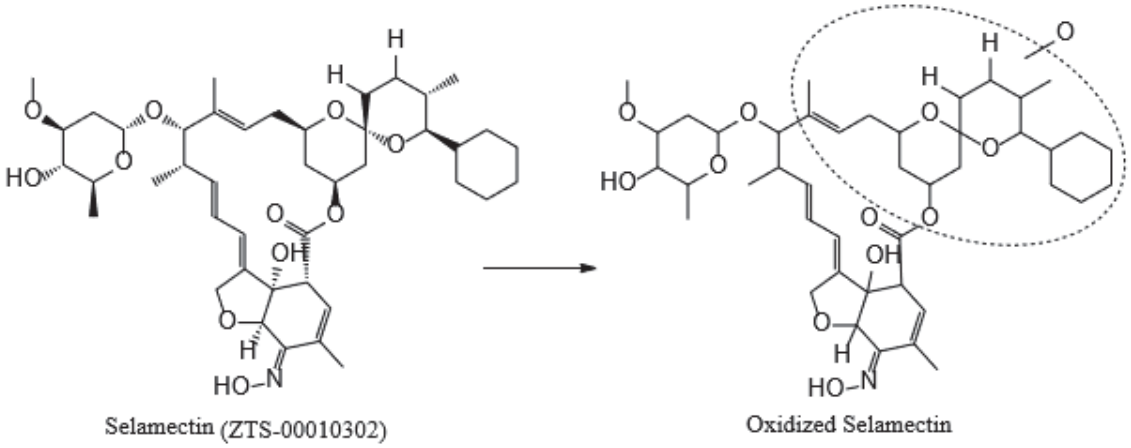
Figure 3. Proposed metabolite structure from test site 2



Chemical Formula: $C_{43}H_{63}NO_{12}$
m/z: 786.4423 ($[M+H]^+$)

The difference of 16 amu suggests that the metabolite is a mono oxidation product of selamectin. The site of oxidation was localized to the region within the metabolite shown in Figure 4. This structure is supported by the presence of fragments *m/z* 349 and 642 in the metabolite spectrum, fragments *m/z* 333 and 626 in the selamectin spectrum, and fragments *m/z* 276 and 406 (406 fragment seen only at one location) in both spectra. The fragments in common between the 2 spectra indicate that oxidation did not occur in these 2 regions of the molecule. Fragments *m/z* 349 and 642 in the metabolite spectrum, 16 amu greater than fragments *m/z* 333 and 626 in the selamectin spectrum, indicate oxidation within the region of the molecule that corresponds to these 2 fragments. There are 2 sites within the oxidized region of the metabolite that do not overlap with the unoxidized fragments *m/z* 276 and 406. Because the *m/z* 406 fragment was only seen from one location and stereochemistry cannot be determined by mass spectral data, the structure in Figure 4, showing a larger region of oxidation and no stereochemistry, is proposed.

Figure 4. Selamectin and proposed metabolite structures



Comparative metabolism

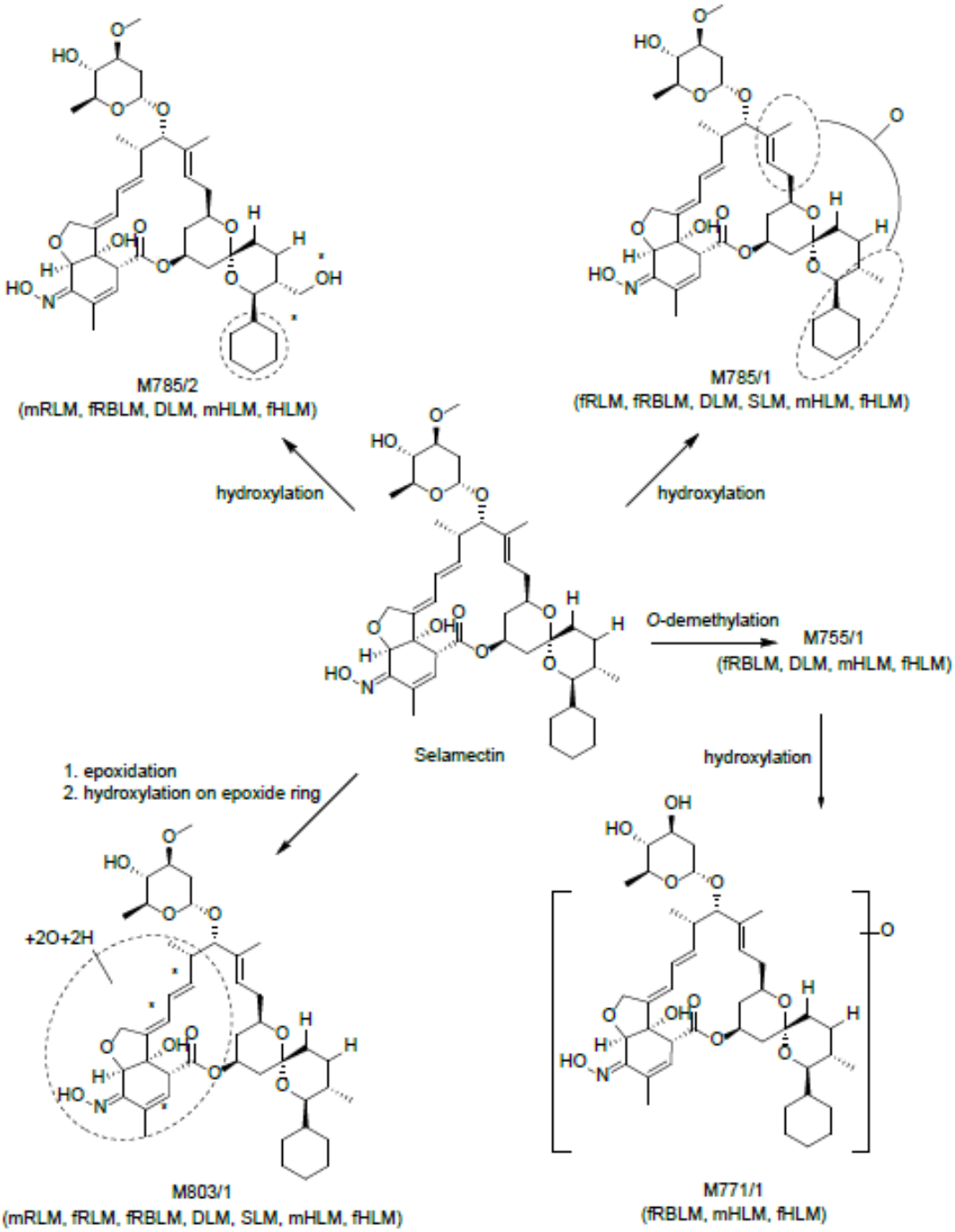
The comparative metabolism study was at the 88th meeting (FAO and WHO, 2020a) and resubmitted by the sponsor for the current meeting. In a GLP compliant study (Chao, 2018), [^{3}H]-selamectin at final nominal concentrations of 1 and 10 µM was incubated in duplicate with liver microsomes from rats, rabbits, dogs, salmon and humans at *ca* 37°C (25°C for salmon). Metabolite characterization and identification were accomplished by LC/MS with online radiodetection. Structures of metabolites were proposed by interpretation of their mass spectral fragmentation patterns and comparisons with available reference standards.

Unchanged selamectin accounted for >70 percent of the total radioactivity (TRA) at the end of the incubation in liver microsomes from male rats, female rats, female rabbits, dogs, salmon, male humans, and female humans. In addition to unchanged selamectin, 5 common metabolites were tentatively identified by LC/MS in selected liver microsomal extracted samples. Two of the metabolites were detected in fish and there were no unique metabolites in fish. Among the 5 metabolites, one hydroxylated metabolite M785/2 was the most abundant metabolite in male rats (3.31–3.88 percent of TRA), female rabbits (5.61–11.86 percent of TRA), male humans (4.42–6.80 percent TRA) and female humans (4.76–8.68 percent of TRA). M785/2 was not observed in liver microsomes from female rats and salmon. In dog liver microsomes, M755/1 was the most abundant metabolite and accounted for 2.01–4.66 percent of TRA. M785/1 and M803/1 were the only two observed metabolites in liver microsomes from female rats and salmon (Table 2). Proposed metabolic pathways are summarized in Figure 5.

Table 2. Metabolite summary for selamectin in liver microsomes from rats, female rabbits, dogs, salmon, and humans

Compound	Biotransformation	Rat	Rabbit	Dog	Salmon	Human
Selamectin	Parent	+	+	+	+	+
M755/1	$-CH_3$	-	+	+	-	+
M771/1	$+O-CH_3$	-	+	+	-	+
M785/1	+O	+ (females)	+	-	+	+
M785/2	+O	+ (males)	+	+	-	+
M803/1	+2H+2O	+	+	+	+	+

Figure 5. Proposed metabolic pathways of selamectin in liver microsomes from male rats, female rats, female rabbits, dogs, salmon, male humans and female humans



Note(s): * Possible site of metabolism. fRLM:female rat liver microsomes; mRLM: male rat liver microsomes; fRBLM: female rabbit liver microsomes; DLM: dog liver microsomes; SLM: salmon liver microsomes; mHLM: male human liver microsomes; fHLM: female human liver microsomes

Source: Chao, P. 2018. In Vitro Comparative Metabolism of [^{3}H]-Selamectin in Liver Microsomes from Rats, Rabbits, Dogs, Salmon, and Humans. XenoBiotic Laboratories, Inc., Plainsboro, NJ, USA. Zoetis Reference Number A4X4N-US-16-333, Xenobiotics Study Number 16009, Report Number RPT04196. Sponsor submitted.

Tissue residue depletion studies

Radiolabeled residue depletion studies

Salmon

One radiolabeled study in fish was provided by the sponsor. The study summarizing the total radioactive residues (Roberts and Fox, 2015) was previously submitted and reviewed by the Committee at its 88th meeting (FAO and WHO, 2020a), and resubmitted for the current meeting. Atlantic Salmon (*Salmo salar*) maintained in tanks of seawater at 8°C were dosed with [^{3}H]-selamectin in feed at a nominal rate of 100 µg/kg of live weight/day for 7 consecutive days (Roberts and Fox, 2015). Samples were collected from 6 fish each at 3 and 12 h, and at 1, 3, 7, 14, 30, 40, 60, and 90 days post-final dose. Liver, kidney, and fillet were collected individually. Carcass (defined as bones, head, any meat which did not come off in the filleting, all the viscera, scales and the fins which were removed during filleting), and gut contents were pooled from each tank at each time point listed above and analysed for total radioactivity.

Highest concentrations of total radioactivity in tissues were measured in liver, with peak TRR occurring at 12 hours withdrawal. Highest concentrations in kidney were also at 12 hours withdrawal, with the highest mean concentrations in fillet occurring at 7 days withdrawal (Table 3). For all tissues, there was considerable variability in the reported TRR concentrations, both within each tank and between the two tanks. Mean TRR in fillet for each tank is presented in Table 4. The Committee noted that the actual dose received by the fish was not confirmed.

Table 3. Mean total radioactivity (µg eq/kg, ± SD) in fillet, liver, and kidney from salmon treated with [^{3}H]-selamectin in feed at 100 µg/kg body weight/day for 7 consecutive days

Withdrawal time (days)	Mean TRR concentration (µg eq/kg, ± SD)		
	Liver	Kidney	Fillet
0.125	1455 ± 770	587.9 ± 276.7	284.9 ± 157.4
0.5	2948 ± 1367	1275 ± 453.7	515.2 ±204.5
1	1840 ± 906	892.7 ± 446	395.8 ± 219.4
3	1636 ± 1118	710.7 ± 459.4	404.1± 281.5
7	2095 ± 602	978.7 ± 208.8	568.9 ± 138.3
14	1693 ± 450	667.9 ± 105.1	383.4 ± 59.7
30	1109 ± 569	498.1 ± 240.9	276.4 ± 144.6
45	767 ± 228	335.3 ± 93.8	200.9 ± 56.3
60	762 ± 436	356.3 ± 189.8	190.2 ± 128.3
90	591 ± 281	248.2 ± 118.8	140.8 ± 72.9

Table 4. Mean TRR concentrations (µg eq/kg, ± SD) in fillet for each tank from salmon treated with [^{3}H]-selamectin in feed at 100 µg/kg body weight/day for 7 consecutive days

Withdrawal time (days)	Treatment tank 1	Treatment tank 2
0.125	345.1 ± 129	224.7 ± 186
0.5	576.2 ± 220	454.1 ± 212
1	374.3 ± 148	417.3 ± 312
3	400.5 ± 346	407.7 ± 280
7	610.7 ± 202	531.4 ± 55.0
14	387.9 ± 49.3	379.0 ± 80.1
30	176.2 ± 129	376.6 ± 74.5
45	169.5 ± 39.5	232.3 ± 58.3
60	84.8 ± 73	295.5 ± 50.3
90	191.4 ± 63	90.1 ± 40

Mean total residue concentrations in carcass were highest at 12 hours withdrawal, with a subsequent gradual decline. In gut content samples, selamectin residue concentrations peaked at 24 hours after the last treatment, then rapidly declined over the following two weeks (Table 5).

Table 5. Mean total radioactivity (µg eq/kg) in carcass and gut contents in carcass and gut contents of salmon treated with [^{3}H]-selamectin in feed at 100 µg/kg body weight/day for 7 consecutive days (N=2, pooled samples, one from each treatment tank)

Withdrawal time (days)	Mean TRR concentration (µg eq/kg)	
	Carcass	Gut contents
0.125	596.8	2971
0.5	793.2	3237
1	612.9	4538
3	491.6	1271
7	649.2	621.7
14	457.6	548.8
30	300.2	296.2
45	206.5	306.9
60	116.7	192.5
90	169.6	202.7

For metabolite profiling, the Committee evaluated a newly submitted study (Killmer, 2020), in which samples from the TRR study (Roberts and Fox, 2015) were analysed. Samples were extracted with acetonitrile/water (90:10) and then the extractant was put through solid phase extraction prior to being injected onto the HPLC system. Fractions of the eluate were collected and analysed using a Perkin Elmer TopCount NXT HTS system and recompiled into a radio-chromatogram to show the radioactivity profile of each extract. Extraction efficiency was

monitored throughout the procedure using LSC of aliquots. Major metabolites were considered those with peaks present at a mean of ≥10 percent TRR at ≥ 1 timepoint. Due to the extended time between initial sampling and final profiling (~360 to 1700 days from TRR analysis to final profiling), values were corrected for radioactive decay based on the half-life of tritium. Extraction efficiency averaged 96 percent over all steps for fillet, and ≥ 90 percent for all other tissues.

Radioprofiling results showed that parent selamectin was the predominant component in all samples across all time points with a mean of 90.5 percent of the TRR in fillet, 72 percent in liver, 76 percent in kidney, 87 percent in remaining carcass, 62 percent in gut contents, and 67 percent in semi-solid effluent samples.

A total of 6 metabolites were identified based on the criteria above and given the identifiers "SM1" through "SM6" for salmon metabolite 1 through 6. The number represents the order of chromatographic elution. Metabolite SM1 is the mono-oxidative product of selamectin previously identified (Killmer, 2018). No major metabolites were seen in the fillet samples. No major metabolites were seen in kidney or carcass tissues. In liver, 2 major metabolites, SM1 and SM3, were seen. Metabolite SM1 was also a major metabolite in excreta (i.e., gut contents and semi-solid effluent). Metabolite SM1 was detected in liver (5.3–10.1 percent of TRR), gut contents (0.33–33.6 percent of TRR), and semi-solid effluent (0.6–23.8 percent of TRR). It was identified as the mono-oxidative product of selamectin. Metabolite SM3 was found as a major metabolite in liver, and it met the criteria for a major metabolite, ≥10 percent, at a single timepoint, 3 days post-treatment, at 10.14 percent of TRR. This metabolite was not identified because it was not present in an edible tissue (liver is not considered an edible tissue for fish) or excreta. Two minor metabolites, SM3 (<1–3 percent TRR) and SM4 (1.1–5.2 percent TRR) were found in fillet. Metabolites SM1 and SM3 were identified in liver. Metabolite SM1 also was found in gut contents and semi-solid effluent.

Selamectin concentration in fillet was determined using two methods. First, it was calculated from the peak area percent in the radio-chromatogram multiplied by the total radioactivity of the sample. Second, it was measured using a validated LC-MS/MS analytical procedure. Selamectin to TRR ratios ranged from 80.5 to 107 percent using marker data from LC-MS/MS analysis and 83.8 percent to 94.5 percent using radio-chromatograms (Table 6).

Table 6. Selamectin concentration in fillet and M:T

Withdrawal time (days)	Mean TRR (µg eq/kg)	Mean selamectin (µg/kg) from radio-chromatograms	M:T using radio-chromatograms	Mean selamectin (µg/kg) from LC- MS/MS	Marker/Total ratio using marker data from LC-MS/MS
0.125	285	266	0.914	299	1.05
0.5	515	489	0.943	545	1.07
1	396	370	0.919	378	0.916

3	404	370	0.896	364	0.931
7	569	538	0.945	539	0.934
14	383	358	0.932	383	0.992
30	276	254	0.875	264	0.994
45	201	183	0.909	190	0.946
60	220	198	0.877	192	0.805
90	141	119	0.838	132	0.970

One study newly submitted for evaluation by the current Committee (Janes, 2021) examined the stability of tritium labelled selamectin ([^{3}H]-selamectin) that was used in the radiolabeled Roberts and Fox, 2015, study to confirm the TRR counts measured in tissues are valid. In this non-GLP study, stability of [^{3}H]-selamectin in acetonitrile:water (50:50, v/v, ambient temperature), and methanol:water:TFA (50:50:0.1, v/v/v, 60°C) was evaluated at 1.5, 5, and 24 hours by comparison of the radiochromatogram at each timepoint to the respective 0-hour radiochromatogram. Tritium exchange with water was evaluated by comparing solvent front fractions to positive (tritiated water) and negative control samples. No tritium exchange with water occurred under either condition tested, and no significant degradation of [^{3}H]-selamectin was observed over 24 hours when exposed to acetonitrile:water (50:50, v/v). When exposed to methanol:water:TFA (50:50:0.1, v/v/v) at 60°C, 1.45 percent radiochemical degradation was observed after 1.5 hours. The maximum amount of time [^{3}H]-selamectin was exposed to such conditions in the previous studies was <1 hour.

Residue depletion studies with non-radiolabeled drug

Salmon

In a partially GLP-compliant study, post-smolt (mean weight 331 g at start of dosing) and adult (mean weight 3.07 kg at start of dosing) Atlantic salmon (*salmo salar*) were used to examine the residue depletion of selamectin in tissues following treatment of a target dose of 100 µg/kg biomass/day for 7 consecutive days. Fish were maintained in tanks with seawater supplied in a flow-through system with no recirculation between tanks. Salinity ranged from 24 to 35 parts per thousand (ppt) throughout the study, with the exception of a four-day period (Study Days 50–53 for adults and Days 49–52 for post-smolts) in which only freshwater was supplied to the tanks for approximately 44 hours, resulting in daily salinity of 0 and 15 ppt before returning to 34 ppt. Water temperature was maintained at 10±1°C during the acclimation and treatment periods, then adjusted to reflect seasonal norms for the rest of the study. The average water temperature decreased during the post treatment period from 10.9°C to 3.7°C on Study Day 77. On the last study day (Day 94), the water temperature was 4.7°C. The Committee noted that because fish were not fed during this time and housed in different salinity, the growth rate and therefore tissue residue concentrations may be affected at the later time points.

Fish were sampled from each tank at 2-, 4-, 10-, 22-, 58- and 88-days post treatment. Fifteen post-smolts per tank (4 tanks, 60 post smolts total) and 12 adult fish per tank (2 tanks, 24 adults total) were collected on each sampling day. Blood, liver, and fillet samples were collected, and plasma and fillet analysed for selamectin using a validated LC-MS/MS analytical procedure

(analysis of liver was not done). Generally, plasma concentrations were higher in fish that had higher tissue residue concentrations.

The actual dose of selamectin ingested was substantially lower than the target dose of 100 µg/kg biomass/day in two of the post-smolt treatment tanks (67.4 percent, 87.6 percent of target) and both adult treatment tanks (80.2 percent, 78.8 percent of target). Mean concentrations of selamectin in fillet for each tank at each time point are listed in Table 7. Values listed in the analytical report as below the calibration range were excluded from the calculation.

Table 7. Mean concentrations of selamectin (ug/kg) in fillet of fish treated with a target dose of 100 µg/kg biomass/day for 7 days

Withdrawal period (days)	Post-smolts				Adults	
	Tank 7	Tank 8*	Tank 9	Tank 10*	Tank X*	Tank Y*
2	428	330	436	404	365	298
4	400	308	368	361	306	315.5
10	370	267	396	289	292	287
22	281	230	317	330	150	234
58	255	178	209	240	173	177
88	169.5	107	161	193	141**	115.5***

Note(s): *Received substantially less than target dose; **n=5 fish total; ***n=3 fish

Methods of analysis for residues in tissues

Methods of analysis from public literature

Selamectin is a semisynthetic macrocyclic lactone compound of the avermectin class, a large family of broad-spectrum topical parasiticides, and is widely used as an endectocide against nematode and arthropod parasites in dogs and cats. A few published reports describing analysis of selamectin were available in the public literature; however, none were for analysis of samples in fish.

One analytical method for the detection of selamectin in dog and cat plasma was developed (Walker and Fenner, 2000). The method involves solid phase extraction followed by chemical derivatisation using triethylamine and trifluoroacetic anhydride. This reaction yields a highly fluorescent product who can be measured by fluorescence detection after HPLC separation. The assay has been validated over a concentration range of 0.2–40 ng/ml. Another described a procedure by LC/MSD after liquid-liquid extraction with acetonitrile for determination of some avermectins compounds including selamectin in animal sera and liver (Rudik *et al*., 2002). A detection limit of 50 ppb for selamectin was obtained.

Several multi-residue methods have been reported in the literature. A multi-residue method was developed for determination of five macrocyclic lactone residues in milk, using selamectin

as an internal standard by LC-MS/MS (Daeseleire *et al.*, 2004). Another uses an LC-MS/MS method in positive and negative ionization mode to measure some avermectins and moxidectin in milk using selamectin as internal standard (Durden, 2007). For selamectin the positive ionization mode shown to be the best solution. One method used HPLC-MS/MS for the simultaneous determination of seven avermectin, including selamectin, in aquatic products (Liu *et al.,* 2017). For extraction QuEChERS was used. The limit of quantification for selamectin was 5 µg/kg. A multiresidue method was developed and validated for the determination of 40 anthelmintic compounds in surface and groundwater samples (Mooney *et al*., 2019). Analytes were extracted from unfiltered water samples using polymeric divinylbenzene solid phase extraction (SPE) cartridges and eluted with methanol: acetone (50:50, v/v). Purified extracts were concentrated, filtered and injected for UHPLC-MS/MS determination. Selamectin was used as internal standard for the determination of the macrocyclic lactone's compounds.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

An analytical method report was provided by the sponsor (Zakowicz, 2019). Samples of salmon fillet (muscle with skin in natural proportions) and liver were fortified with solutions of selamectin and internal standard (selamectin D_2 ^{15}N). They were then extracted by shaking in acetonitrile:water (70:30, v/v). The supernatants were partitioned on 2 occasions with hexane, and then centrifuged. A portion of the extract was cleaned-up by SPE (Oasis HLB), and the eluate evaporated to dryness, before being reconstituted in acetonitrile:water (80:20, v/v). Samples were injected into LC-MS/MS equipped with an C18 column maintained at 50ºC. Elution was carried out using water:formic acid (100:0.3 v/v) as mobile phase A and acetonitrile:formic acid (100:0.3) as mobile phase B with a flow rate of 0.65 mL/min.

Analysis was performed by liquid chromatography with mass spectrometry (LC-MS/MS) using a positive ion multiple-ion reaction monitoring (MRM). The electrospray ionization was used in positive ion mode. Quantification of selamectin (*m/z* 770 → *m/z* 608) was performed using selamectin D_2 ^{15}N as internal standard (*m/z* 773 → *m/z* 612). The measured peak area ratios for selamectin/internal standard for standard solutions were used to generate a calibration curve. A linear regression with $1/x^2$ weighting factor was applied to the calibration line. These data were used to calculate the LOD and LOQ for each matrix (Table 8).

The method of analysis was validated in house for research purposes for the marker residue, selamectin, with respect to system suitability, linearity, limit of detection and quantification, intra- and inter-day assay accuracy and precision, specificity, selectivity, matrix effects, recovery, robustness, storage at room temperature and extended frozen stability, freeze/thaw stability, extract and solution stability, determination of matrix dilution.

Suitability was demonstrated with replicate injections of selamectin standard solution at 0.4 ng/mL (n=10). Precision of peak area, retention time and peak area ratio were determined. The low coefficient of variation (CV percent) for each of the parameters evaluated suggests, that the system is suitable for this assay.

Linearity was demonstrated in solvent calibration standards over the range of 0.4 to 32 ng/mL, equivalent to 16 to 1280 µg/kg in tissue. Matrix matched calibration standards were also prepared for each occasion of accuracy and precision.

Precision was demonstrated in terms of the peak area, peak area ratio and retention time for the test item and internal standard. The intra-day assay accuracy and precision was determined at low fortification level (25 µg/kg), 3x low fortification level (75 µg/kg), mid fortification level (250 µg/kg) and high fortification level (1000 µg/kg). The recoveries and precision (CV percent) for intra-day assay were calculated using solvent calibration standards and matrix-matched calibration standards.

Limit of Detection and Limit of Quantification were determined as described in Annex 2 of VICH GL49 (EMA, 2015). Calibration solvent standards ranged from 0.02 ng/mL to 0.8 ng/mL were prepared and analysed. Data from the regression were used to calculate the estimated LOD and LOQ values. In a next step salmon fillet and liver aliquots (n=7) were fortified with selamectin. Data were quantified using both calibration ranges. These data were used to calculate the estimated values of LOD and LOQ for each matrix.

Specificity of the assay was examined by the extraction and analysis of aliquots of control matrix from 6 individual sources. One double blank and one single blank sample were prepared for each source. The assay was demonstrated to be specific for the test item and the internal standard.

Selectivity of the assay was evaluated for potential interference from the following exogenous compounds; abamectin, doramectin, eprinomectin, ivermectin and milbemycin. No significant interfering signal (>20 percent peak area of the selamectin individual solvent standard) eluting at the same retention time as selamectin was seen from the exogenous compounds.

Matrix effects was assessed at 75 µg/kg and 1000 µg/kg for Atlantic salmon fillet and liver using 6 lots of blank matrix from individual donors. No matrix effects were found for selamectin in salmon fillet and liver.

Recovery was demonstrated with the mean CV (percent) of the internal standard normalised recovery samples prepared at 75 µg/kg and 1000 µg/kg. No formal acceptance criteria were put in place for recovery; however, the IS mean normalized recoveries at each QC level assessed were 104 percent (range 88.3–119 percent) for fillet and 103 percent (range 97.4–116 percent) for liver. In each assessment, the CV was <15 percent. Thus, the assay recovery was deemed acceptable.

Robustness was demonstrated by varying 6 parameters, inclusion of metal beads, varying the composition of the extraction solvent, utilizing different homogenization techniques, varying the centrifugation speed, utilizing different SPE drying techniques, and varying the SPE elution volume. The assay robustness was successfully demonstrated for salmon liver only. The

salmon fillet replicates were within acceptance criteria for the assay robustness, except for 1 sample.

Matrix dilution with 2 dilution factors (10 and 20) was successfully demonstrated for fillet and liver samples fortified at 10 000 µg/kg.

Stability of residues

Stability of residues during storage

Storage stability of selamectin in salmon fillet and liver was investigated at two fortified concentrations of 75 µg/kg and 1000 µg/kg. Stability was demonstrated to be acceptable at room temperature (ca 4 h) and for 3 freeze/thaw cycles for the samples stored at ca -10ºC and ca -80ºC. Extract stability for at least 118 hours for salmon fillet samples and at least 72 hours for salmon liver samples was demonstrated for the final (vialled) extracts stored in a refrigerator set to maintain +4ºC. Stability of selamectin and Internal Standard solutions was tested. Stock solutions and dilutions were stable during 65- and 42-days storage at ca +4ºC, when not in use. Data obtained in the validation procedure are summarized in Table 8.

Table 8. Validation summary table

Analyte: selamectin (ZTS-00010302)		
	Matrix	
Parameter	**Salmon fillet**	**Salmon liver**
Linearity R^2	0.9914–0.9996	0.9926–0.9980
Range (µg/kg equivalent tissue concentration)	16.0–1280 (0.4–32.0 ng/mL)	16.0–1280 (0.4–32.0 ng/mL)
Calculated LOD (µg/kg)	0.943	1.28
Calculated LOQ (µg/kg)	2.83	3.85
Between-run accuracy(% bias) validated LOQ* 25 µg/kg 75 µg/kg 250 µg/kg 1 000 µg/kg	**NA** 95 .2 99 .6 103 99.8	**NA** 9 5.1 97.2 103 101
Between-run precision (% CV) validated LOQ* 25 µg/kg 75 µg/kg 250 µg/kg 1 000 µg/kg	**NA** 9.61 7.46 3.39 4.77	**NA** 9.22 7.14 5.31 5.98
Dilution integrity (parallelism)	10-fold, 20-fold	10-fold, 20-fold
Matrix effect	Not significant	Not significant
Stability-short term in matrix (ambient)	At least 4 hours	At least 4 hours

Stability-long term in matrix (freezer)	At least 6 months at *ca* -10°C and *ca* -80°C	At least **1** month at *ca* -10°C At least 6 months at *ca* -80°C
Stability- extracts (+4°C)	At least 118 hrs	At least 72 hrs
Stability- freeze thaw (-10°C)	At least 3 cycles at *ca* -10°C and *ca* -80°C	At least 3 cycles at *ca* -10°C and *ca* -80°C
Specificity (at least 6 pools of control samples)	≤20% of lowest standard	≤20% of lowest standard
Selectivity against:		
• Abamectin	Demonstrated (No significant peak found)	Demonstrated (No significant peak found)
• Doramectin	Demonstrated (No peak found)	Demonstrated (No peak found)
• Eprinomectin	Demonstrated (No peak found)	Demonstrated (No peak found)
• lvermectin	Demonstrated (No peak found)	Demonstrated (No peak found)
• Milbemycin	Demonstrated (No peak found)	Demonstrated (No peak found)
Ruggedness with respect to:		
• inclusion of metal beads	Demonstrated	Demonstrated
• composition of the extraction solvent	Demonstrated	Demonstrated
• homogenization technique	Demonstrated for flat bed and GenoGrinder (not sonication)	Demonstrated for flat bed, sonication and GenoGrinder
• centrifugation speed	Demonstrated	Demonstrated Demonstrated
SPE drying technique	Demonstrated for ±vacuum (not aspirate-wet)	Demonstrated
• SPE elution volume	Demonstrated	Demonstrated
• HPLC column	Demonstrated	
Stock solution stability calibration and QC fortification solutions (40–40,000 ng/mL): 42 days; Selamectin stock solution (1,000 µg/mL): 65 days; Selamectin D2 ^{15}N working solutions (1.0–16 µg/mL): 42 days; Selamectin D2 ^{15}N stock solution (1 000 µg/mL): 65 days		

Note(s): *Fillet estimated LOQ level = 2.20 µg/kg, Liver estimated LOQ level = 2.20 µg/kg; **NA**= Not applicable

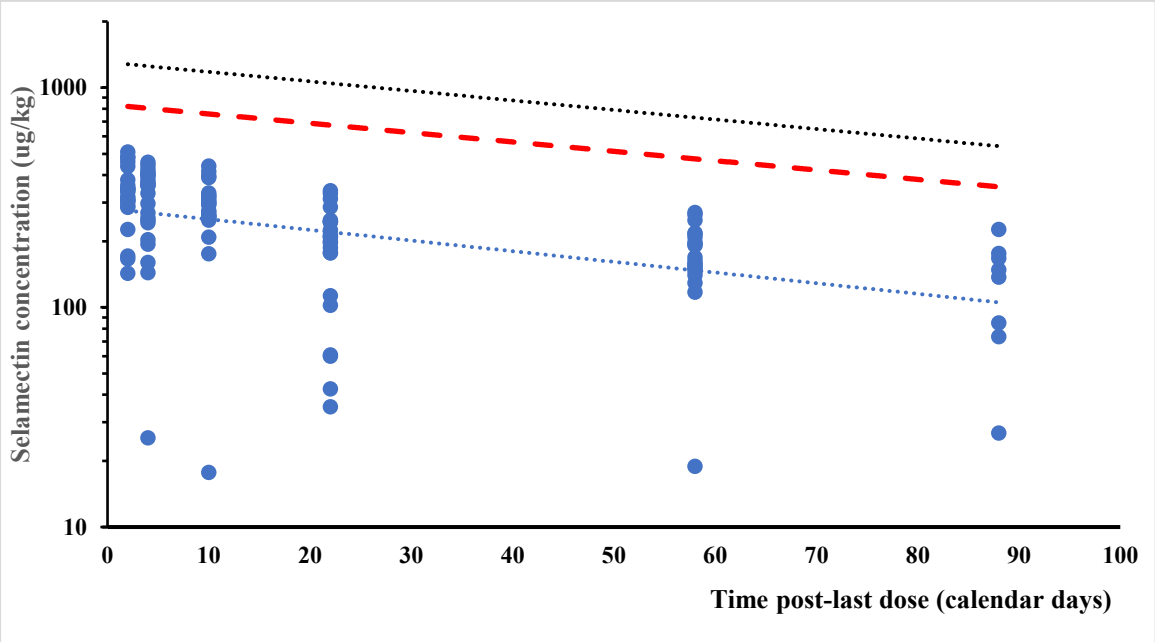
Appraisal

Selamectin is not currently approved for use in animals for human consumption. JECFA evaluated selamectin at the 88th (FAO and WHO, 2020b) and present meetings as a pilot programme in which it conducts a parallel review while the sponsor concurrently pursues approval in the proposed species with national authorities, as discussed at the Twenty-fourth Session of CCRVDF (FAO and WHO, 2018). Selamectin is intended as a 7-day, in-feed ectoparasiticide additive for treatment and prevention of all parasitic stages of sea lice (*Lepeophtheirus* sp. and *Caligus* sp.) on Atlantic salmon (*Salmo salar*), ranging from smolts to market weight fish, in seawater. The product is to be administered in-feed to fish for 7 days at an appropriate feeding rate to yield a dose rate of 100 µg selamectin/kg biomass/day. A withdrawal period has not currently been established by any Member State.

A radiolabeled study in salmon demonstrated that selamectin is the marker residue in fillet and is the primary component in all tissues across all time points (Roberts and Fox, 2015). The Committee considered that the lowest fillet MR:TRR value of 0.8 would lead to a more conservative approach, but was considered appropriate due to uncertainties in the provided residue data. Residue data were obtained from a depletion study treating Atlantic salmon with non-radiolabeled selamectin in medicated feed. Selamectin concentrations in fillet were determined using a validated LC-MS/MS method.

Based on the lack of a registration in a Member State and lack of GVP, and the study deficiencies noted above, specific MRLs could not be recommended. Based on the information the Committee received regarding preliminary proposed GVP, the following ranges could be considered. These were based on the upper limit of the one-sided 95 percent confidence interval over the 95th percentile (UTL 95/95) and UTL 99/95 from the non-radiolabeled residue depletion study using the treated adult fish, as they represent those which will enter the market for human consumption. They correspond to withdrawal periods of 22 degree-days (two calendar days; the earliest withdrawal time point in the residue depletion study) and 544 degree-days (88 calendar days; the longest withdrawal time point). Preliminary proposed MRLs at 22 degree-days would be in the range of 900 to 1300 µg/kg, and those at 544 degree-days would be in the range of 400 to 600 µg/kg (Figure 6).

Figure 6. Tolerance limits for selamectin (marker residue) in salmon fillet. Regression line (median residues, blue dots), 95/95 UTL regression line (red dash), 99/95 UTL regression line (black dots)



Dietary exposure assessment

Dietary exposure to selamectin may occur only through its use as a veterinary drug. There is no registered use for selamectin as a pesticide, consequently no GVP has been established. Dietary exposure was assessed for some possible scenarios, however, MRLs were not recommended. Consequently, estimates are for guidance only and need to be reassessed when real-world scenarios based on GVP and proposed MRLs can be established.

Dietary exposure was estimated based on the following scenarios and assumptions for occurrence of selamectin residues in Atlantic salmon muscle only:

- MR:TRR = 0.8
- median total residue levels in Atlantic salmon (muscle, adult fish) were 410 and 179 µg/kg at withdrawal periods of 22 and 544 degree-days respectively
- UTL 95/95 total residue levels in Atlantic salmon (muscle, adult fish) were 1 068 and 454 µg/kg at withdrawal periods of 22 and 544 degree-days respectively
- UTL 99/95 total residue levels in Atlantic salmon (muscle, adult fish) were 1 653 and 695 µg/kg at withdrawal periods of 22 and 544 degree-days respectively
- ADI of 50 µg/kg bw
- ARfD of 400 µg/kg bw

Chronic dietary exposure estimates

Based on incurred residues in Atlantic salmon muscle (muscle) and a withdrawal period of 22 and 544 degree-days (shown in square brackets) for Atlantic salmon, the global estimate of chronic dietary exposure (GECDE, Table 9) for adults and elderly is 0.8 [0.4] µg/kg bw per day, which represents 2 percent [1 percent] of the upper bound of the ADI of 50 µg/kg bw. For children and adolescents, the GECDE is 2.2 [1.0] µg/kg bw per day, which represents 4 percent [2 percent] of the upper bound of the ADI of 50 µg/kg bw. For infants and toddlers, the GECDE is 0.8 [0.3] µg/kg bw per day, which represents 2 percent [1 percent] of the upper bound of the ADI of 50 µg/kg bw. The consumption figures used were highest reliable percentile Atlantic salmon consumption figures based on consumers only considered from the available dataset, Chronic individual food consumption database, Summary statistics (CIFOCOss) (WHO, 2018).

Table 9. Global estimates of chronic dietary exposure (GECDE*) to selamectin residues in Atlantic salmon at 22 and 544 degree-days

Degree-days	Adults and elderly		Children and adolescents		Infants and toddlers	
	GECDE µg/kg bw per day	% ADI	GECDE µg/kg bw per day	% ADI	GECDE µg/kg bw per day	% ADI
22	0.8	2	2.2	4	0.8	2
544	0.4	1	1.0	2	0.3	1

Note(s): *the GECDE is the sum of the highest exposure at the highest reliable percentile of consumption for a food and the mean dietary exposures of the other foods

Acute dietary exposure assessment

Acute dietary exposure (global estimate of acute dietary exposure; GEADE) was assessed for consumption of Atlantic salmon muscle (adult fish) using food consumption values from the FAO/WHO large portion (97.5th percentile, one day) database and 95/95 and 99/95 upper tolerance limit (UTL) concentrations for selamectin residues. The ARfD of 400 µg/kg bw was used to calculate percentage exposure.

Adjusted (MR:TRR = 0.8) UTL 95/95 residue levels in Atlantic salmon (muscle, adult fish) were 1068 and 454 µg/kg, relating to withdrawal periods of 22 and 544 degree-days respectively. Adjusted (MR:TRR = 0.8) UTL 99/95 residue levels in the same tissue were 1 653 and 695 µg/kg, also relating to withdrawal periods of 22 and 544 degree-days respectively.

The GEADE at 22 degree-days post-dose (Table 10) based on UTL 95/95 and 99/95 (shown in square brackets) was 7.7 [12.0] and 8.2 [12.7] µg/kg bw (2 percent [3 percent] of the ARfD) from consumption of Atlantic salmon muscle for adults and children, respectively.

The GEADE at 544 degree-days post-dose (Table 10) based on UTL 95/95 and 99/95 (shown in square brackets) was 3.3 [5.0] and 3.5 [5.3] µg/kg bw (1 percent [1 percent] of the ARfD) from consumption of Atlantic salmon muscle for adults and children, respectively.

Table 10. Global Estimates of Acute Dietary Exposure (GEADE[#]) for selamectin residues at two upper tolerance limits in Atlantic salmon at 22 and 544 degree-days

Degree-days	General population				Children			
	GEADE µg/kg bw	ARfD %	GEADE µg/kg bw	ARfD %	GEADE µg/kg bw	ARfD %	GEADE µg/kg bw	ARfD %
UTL[^]	95/95		99/95		95/95		99/95	
22	7.7	2	12.0	3	8.2	2	12.7	3
544	3.3	1	5.0	1	3.5	1	5.3	1

Note(s): [#]the GEADE uses food consumption of a large portion (97.5th percentile, one day) and residues concentration at the upper tolerance limit to estimate acute exposure; [^]UTL: upper tolerance limit

Maximum Residue Limits

Selamectin is not yet approved for use in salmon in any Member State. Using the process followed by the Committee, specific MRLs cannot be recommended at this time due to a lack of established GVP in at least one Member State. In evaluating a range of potential MRLs for selamectin in salmon, the Committee considered the following factors:

- Selamectin is not approved for use in any Member State and was evaluated by the Committee under the pilot parallel review programme as requested by CCRVDF.
- An ADI 0–0.05 mg/kg bw was established by the Committee.
- An ARfD of 0.4 mg/kg bw was previously established by the Committee.
- Selamectin is predominantly unmetabolized.
- Selamectin is the marker residue.
- The ratio of the concentration of marker residue to the concentration of total residue is 0.8 in fillet in salmon.
- A non-radiolabeled depletion study in post-smolt and adult Atlantic salmon was available. The Committee noted several deficiencies in the study, including most fish not receiving the target dose.
- A validated analytical method for the determination of selamectin in fillet of salmon is available and may be used for monitoring purposes.

Based on the lack of a registration in a Member State and lack of GVP, and the study deficiencies noted above, specific MRLs could not be recommended. Based on the information the Committee received regarding preliminary proposed GVP, the following ranges could be

considered. These were based on the upper limit of the one-sided 95 percent confidence interval over the 95th percentile (UTL 95/95) and UTL 99/95 from the non-radiolabeled residue depletion study using the treated adult fish, as they represent those which will enter the market for human consumption. They correspond to withdrawal periods of 22 degree-days (two calendar days; the earliest withdrawal time point in the residue depletion study) and 544 degree-days (88 calendar days; the longest withdrawal time point). Preliminary proposed MRLs at 22 degree-days would be in the range of 900 to 1300 µg/kg, and those at 544 degree-days would be in the range of 400 to 600 µg/kg.

No further recommendations can be made without full registration in a Member State, including GVP.

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Annex 1 - Summary of recommendations from the 94th JECFA on compounds on the agenda and further information required

Imidacloprid (parasiticide)

Acceptable daily intake

In view of the absence of a study to assess the impact of imidacloprid on representative human intestinal microbiota, it was not possible to determine an mADI, thus the Committee was unable to establish an ADI for imidacloprid.

The Committee established a toxicological acceptable daily intake (tADI) of 0–0.05 mg/kg bw on the basis of a NOAEL of 5.25 mg/kg body weight (bw) per day for decreased body weight (bw) gain in the extended one-generation reproduction study, with the application of a safety factor of 100 to allow for interspecies and intraspecies differences.

Acute reference dose

In view of the absence of a study to assess the impact of imidacloprid on representative human intestinal microbiota, it was not possible to determine an mARfD, thus the Committee was unable to establish an ARfD for imidacloprid.

The Committee established a toxicological acute reference dose (tARfD) of 0.09 mg/kg bw based on a BMD05 of 9 mg/kg bw reported by Cal EPA for an acute neurotoxicity study in rats and a safety factor of 100 to allow for interspecies and intraspecies differences. This value was supported by a NOAEL of 7.5 mg/kg bw per day for tremors in a 90-day toxicity study in dogs occurring during the first week of treatment, although it is not known whether tremors occurred after the first dose.

Estimated chronic dietary exposure

While estimates of dietary exposure were derived, there are no HBGVs with which to compare them.

Based on incurred residues in Atlantic salmon (fillet) and a withdrawal period of 98 degree-days:

The global estimate of chronic dietary exposure (GECDE) for adults and the elderly is 1.0 µg/kg bw per day.

The GECDE for children and adolescents is 2.7 µg/kg bw per day.

The GECDE for infants and toddlers is 0.9 µg/kg bw per day.

Based on incurred residues in fish meat and a withdrawal period of 98 degree-days:

The GECDE for adults and the elderly is 1.8 µg/kg bw per day.

The GECDE for children and adolescents is 3.8 µg/kg bw per day.

The GECDE for infants and toddlers is 1.2 µg/kg bw per day.

Estimated acute dietary exposure

Acute dietary exposures were assessed at 98 degree-days post dose. The adjusted (MR:TRR = 0.7) 95/95 UTL concentrations used were 859 µg/kg. No ARfD was available.

Based on consumption of Atlantic salmon:

The GEADE for adults is 6.2 µg/kg bw per day.

The GEADE for children is 6.6 µg/kg bw per day.

Based on consumption of all fin fish:

The GEADE for adults is 34.1 µg/kg bw per day.

The GEADE for children is 23.8 µg/kg bw per day.

Residue definition

The marker residue for imidacloprid in fillets of salmonids is the parent molecule, imidacloprid.

Maximum residue limits

As the Committee could not establish an ADI or an ARfD, an MRL could not be recommended for imidacloprid.

Future work and recommendations

Further information required to complete the residue assessment:

- Disruption of the colonization barrier and on the selection for and emergence of, resistance in the microbiota in the gastrointestinal tract.

Ivermectin (broad-spectrum antiparasitic agent)

Acceptable daily intake

The Committee established an ADI of 0–10 µg/kg body weight at the eighty-first meeting.

Acute reference dose

The Committee established an ARfD of 200 µg/kg body weight at the eighty-first meeting.

Estimated chronic dietary exposure

The GECDE for adults and the elderly is 0.72 µg/kg bw per day, which represents 7.2 percent of the upper bound of the ADI of 10 µg/kg bw.

The GECDE for children and adolescents is 0.93 µg/kg bw per day, which represents 9.3 percent of the upper bound of the ADI of 10 µg/kg bw.

The GECDE for infants and toddlers is 0.48 µg/kg bw per day, which represents 4.8 percent of the upper bound of the ADI of 10 µg/kg bw.

Estimated acute dietary exposure

The GEADE for cattle muscle, applicable to children and the general population, is 69 µg/kg bw, which represents 35 percent of the ARfD of 200 µg/kg bw.

The GEADE for sheep muscle, applicable to children and the general population, is 73 µg/kg bw, which represents 37 percent of the ARfD of 200 µg/kg bw.

The GEADE for pig muscle, applicable to children and the general population, is 30 µg/kg bw, which represents 15 percent of the ARfD of 200 µg/kg bw.

Residue definition

The marker residue in sheep, pigs and goats is ivermectin B1a (H2B1a, or 22,23-dihydroavermectin B1a).

Recommended maximum residue limits (MRLs)

Species	Muscle (µg/kg)	Liver (µg/kg)	Kidney (µg/kg)	Fat (µg/kg)
Pig	15	30	20	50
Sheep and goat	30	60	20	100

Nicarbazin (coccidiostat)

Toxicological effects

The NOAEL was 60 mg/kg bw per day (equivalent to 42.5 mg/kg bw per day of DNC) due to prominent liver lobulation, observed in a study of developmental toxicity in the rabbit.

Uncertainty factor

When considering nicarbazin it is DNC that is the toxic component, and its absorption alone or in a mixture with HDP is substantially less (<5 percent) than when formed from ingested nicarbazin. As DNC is the residue of concern and there is no nicarbazin in products from treated animals, the Committee concluded that despite limitations in the database, a reduction in the default safety factor of 100 used to account for interspecies and intraspecies variability, would be justified. The Committee was unable to quantify just how much of a reduction would be appropriate, but concluded that 50 could certainly be supported, and would still result in a conservative evaluation.

Toxicological ADI

The tADI for nicarbazin was established at 0–0.9 mg/kg bw (DNC).

Microbiological effects

Nicarbazin and/or its metabolites show no antimicrobial activity towards representative bacteria of the human intestinal microbiota

Microbiological ADI

The Committee concluded that it was not necessary to establish an mADI for nicarbazin.

Acceptable daily intake

The ADI for nicarbazin was established at 0–0.9mg/kg bw based on toxicological effects.

Acute reference dose

The Committee concluded that it was not necessary to establish an ARfD for nicarbazin.

Residue definition

The marker residue in chickens is DNC.

Estimated dietary exposure

Based on incurred DNC residues in chicken muscle, offal, and skin with fat, at 24 hours withdrawal time and 125 mg/kg feed:

The global estimate of chronic dietary exposure (GECDE) for adults and the elderly is 120 µg/kg body weight (bw) per day, which represents 13 percent of the upper bound of the ADI of 900 µg/kg bw.

The GECDE for children and adolescents is 160 µg/kg bw per day, which represents 18 percent of the upper bound of the ADI of 900 µg/kg bw.

The GECDE for infants and toddlers is 210 µg/kg bw per day, which represents 23 percent of the upper bound of the ADI of 900 µg/kg bw.

Based on incurred DNC residues in chicken muscle, offal, and skin with fat, at zero days withdrawal time and 50 mg/kg feed:

The GECDE for adults and the elderly is 95 µg/kg bw per day, which represents 11percent of the upper bound of the ADI of 900 µg/kg bw.

The GECDE for children and adolescents is 120 µg/kg bw per day, which represents 14 percent of the upper bound of the ADI of 900 µg/kg bw.

The GECDE for infants and toddlers is 160 µg/kg bw per day, which represents 18 percent of the upper bound of the ADI of 900 µg/kg bw.

Recommended maximum residue limits (MRLS)

Species	Muscle (µg/kg)	Liver (µg/kg)	Kidney (µg/kg)	Skin with fat (µg/kg)
Chicken	4 000	15 000	8 000	4 000

Selamectin (broad-spectrum parasiticide)

Acceptable daily intake

The Committee withdrew the previous ADI and established an ADI of 0–0.05 mg/kg bw, based on a NOAEL of 5 mg/kg bw per day for increased liver and uterus/cervix weights at 15 mg/kg bw per day in a one-year rat study, with application of a safety factor of 100 to account for interspecies and intraspecies variability. Although the NOAEL for effects seen in a 13-week dietary neurotoxicity/toxicity study in rats, assessed by the Committee at its last meeting was 1 mg/kg bw per day, the LOAEL at 15 mg/kg bw per day, and the effects observed were the same as those on which the ADI is based. The Committee concluded that the ADI established at the present meeting would be sufficiently protective of these findings.

Acute reference dose

The Committee concluded that the ARfD of 0.4 mg/kg bw established at the eighty-eighth meeting was still appropriate.

Residue definition	The marker residue in Atlantic salmon fillet is selamectin.
Estimated dietary exposure	Dietary exposure was assessed for some possible scenarios, but no GVP has been established.
Maximum residue limits	Specific MRLs could not be recommended at this time due to a lack of established GVP.

Future work and recommendations

Further information required to complete the residue assessment:

- Full registration in a Member State, including GVP.

Annex 2 - Summary of JECFA evaluations of veterinary drug residues from the 32nd meeting to the present

The following table summarizes the veterinary drug evaluations conducted by JECFA at the 32nd (1987), 34th (1989), 36th (1990), 38th (1991), 40th (1992), 42nd (1994), 43rd (1994), 45th (1995), 48th (1997), 50th (1998), 52nd (1999), 54th (2000), 58th (2002), 60th (2003), 62nd (2004), 66th (2006), 70th (2008), 75th (2011), 78th (2013), 81st (2015), 85th (2017), 88th (2019) and 94th (2022) meetings. These meetings were devoted exclusively to the evaluation of veterinary drug residues in food. This table must be considered in context with the full reports of these meetings, published as WHO Technical Report Series.

Some notes regarding the table:

- The “ADI/ArfD” column provides the ADI and, when applicable, the ArfD established by the Committee. When no ARfD is stated, an ArfD has not been established.
- The “ADI Status” column refers to the ADI and indicates whether an ADI was established; if a full ADI was given, or if the ADI is temporary (T).
- Where an MRL is temporary, it is indicated by “T”.
- Where a compound has been evaluated more than once, the data given are for the most recent evaluation, including the 78th meeting of the Committee.

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Abamectin	ADI: 0–1 (JMPR 1995)	Full	47 (1996)	100 50	Liver, Fat Kidney	Cattle	Avermectin B_{1a}
Albendazole	ADI: 0–50	Full	34 (1989)	100 5000	Muscle, Fat, Milk Liver, Kidney	Cattle, Sheep	MRLs analysed as 2-amino-benzimidazole, expressed as albendazole equivalents
Amoxicillin	ADI: 0–0.7	Full	75 (2011)	50	Muscle, Liver, Kidney, Fat Milk	Cattle, Pig, Sheep Cattle, Sheep	Amoxicillin
	ADI: 0–2	Full	85 (2017)	4 50	Fillet, Muscle[b]	Finfish[a]	
Ampicillin	ADI: 0–3	Full	85 (2017)	50	Muscle[b]	Finfish[a]	Ampicillin
Apramycin	ADI: 0–30	Full	75 (2011)	5000	Kidney	Cattle, Chicken	Apramycin
Avilamycin	ADI: 0–2 000 (as avilamycin activity)	Full	70 (2008)	200 300	Muscle, Kidney, Skin/Fat Liver	Pig, Chicken, Turkey, Rabbit Pig, Chicken, Turkey, Rabbit	Dichloroisoeverninic acid (DIA), expressed as avilamycin equivalents
Azaperone	ADI: 0–6	Full	52 (1999)	60 100	Muscle, Fat Liver, Kidney	Pig	Sum of azaperone and azaperol
Benzylpenicillin	ADI: <30µg/person/day of the penicillin moiety	Full	36 (1990)	50 4	Muscle, Liver, Kidney Milk	All species	Benzylpenicillin
Bovine Somatotropins	ADI: Not specified	Full	78 (2013)	Not specified	Muscle, Liver, Kidney, Fat, Milk	Cattle	
Carazolol	ADI: 0–0.1 ARfD: 0.1	Full	52 (1999)	5	Muscle, Fat/Skin		The Committee noted that the concentration of carazolol at the injection site may exceed the ADI that is based on the acute

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
				25	Liver, Kidney	Pig	pharmacological effect of carazolol.
Carbadox	No ADI or ARfD		60 (2003)	No MRL			The Committee decided that quinoxaline-2-carboxylic acid is not an appropriate marker residue
Ceftiofur	ADI: 0–50	Full	48 (1997)	1 000	Muscle	Cattle, Pig	Desfuroylceftiofur
				2 000	Liver, Fat		
				6 000	Kidney		
				100	Milk	Cattle	
Cefuroxime	No ADI or ARfD		62 (2004)	No MRL			
Chloramphenicol	No ADI or ARfD		62 (2004)	No MRL			
Chlorpromazine	No ADI or ARfD		38 (1991)	No MRL			
Chlortetracycline, Oxytetracycline, Tetracycline	ADI: 0–30 (group ADI)	Full	58 (2002)	200	Muscle	Cattle, Pig, Sheep, Poultry	Parent drugs, either singly or in combination
				600	Liver		
				1 200	Kidney		
				400	Eggs	Poultry	
				100	Milk	Cattle, Sheep	
				200	Muscle	Fish, giant prawn	Oxytetracycline only
Clenbuterol	ADI: 0–0.004	Full	47 (1996)	0.2	Muscle, Fat	Cattle, Horse	Clenbuterol
				0.6	Liver, Kidney	Cattle, Horse	
				0.05	Milk	Cattle	
Closantel	ADI: 0–30	Full	40 (1992)	1 000	Muscle, Liver	Cattle	Closantel
				3 000	Kidney, Fat		
				1 500	Muscle, Liver	Sheep	
				5 000	Kidney		
				2 000	Fat		
Colistin	ADI: 0–7	Full	66 (2006)	150	Muscle, Liver, Fat	Cattle, Sheep, Goat, Chicken, Turkey, Pig,	Residue definition is the sum of Colistin A and colistin B. The MRL includes skin + fat where appropriate (chicken, turkey, pigs).
				200	Kidney	Rabbit	
				50	Milk	Cattle, Sheep	

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
				300	Eggs	Chicken	
Cyfluthrin	ADI: 0–20	Full	48 (1997)	20 200 40	Muscle, Liver, Kidney Fat Milk	Cattle	Cyfluthrin
Cyhalothrin	ADI: 0–5	Full	62 (2004)	20 400 20 50 30	Muscle, Kidney Fat Liver Liver Milk	Cattle, Sheep, Pig Cattle, Sheep, Pig Cattle, Pig Sheep Cattle, Sheep	Cyhalothrin
Cypermethrin α-Cypermethrin	ADI: 0–20 (group ADI)	Full	62 (2004)	50 1 000 100 Milk	Muscle, Liver, Kidney Fat	Cattle, Sheep Cattle, Sheep Cattle	Total of cypermethrin residues (resulting from the use of cypermethrin or α-cypermethrin as veterinary drugs)
Danofloxacin	ADI: 0–20	Full	48 (1997)	200 400 100 100 50 200 100	Muscle Liver, Kidney Fat Muscle Liver Kidney Fat	Cattle, Chicken Pig	Danofloxacin For chicken fat/skin in normal proportions
Deltamethrin	ADI: 0–10 (1982 JMPR)	Full	60 (2003)	30 50 500 30 30	Muscle Liver, Kidney Fat Milk Eggs	Cattle, Chicken, Sheep, Salmon Cattle, Sheep, Chicken Cattle Chicken	Deltamethrin
Derquantel	ADI: 0–0.3	Full	78 (2013)	0.3 0.4 7 0.8	Muscle Kidney Fat Liver	Sheep	Derquantel
Dexamethasone	ADI: 0–0.015	Full	70 (2008)	1 2 0.3	Muscle, Kidney Liver Milk	Cattle, Pig, Horse Cattle, Pig, Horse Cattle	Dexamethasone

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Diclazuril	ADI: 0–30	Full	50 (1998)	500	Muscle	Sheep, Rabbit, Poultry	Diclazuril Poultry skin + fat
				3 000	Liver		
				2 000	Kidney		
				1 000	Fat		
Diflubenzuron	No ADI or ARfD		81 (2015)	No MRL			Diflubenzuron
	ADI: 0–20 No ARfD		88 (2019)	10	Muscle plus skin	Salmon	
Dihydro-streptomycin Streptomycin	ADI: 0–50 (group ADI)	Full	58 (2002)	600	Muscle, Liver, Fat	Cattle, Pig, Chicken, Sheep	Sum of dihydrostreptomycin and streptomycin
				1 000	Kidney		
				200	Milk	Cattle, Sheep	
Dimetridazole	No ADI or ARfD		34 (1989)	No MRL			
Diminazene	ADI: 0–100	Full	42 (1994)	500 12 000 6 000	Muscle Liver Kidney	Cattle	Diminazene
				150	Milk		
Doramectin	ADI: 0–1	Full	62 (2004)	10 5	Muscle Muscle	Cattle Pigs	Doramectin
				100	Liver	Cattle, Pigs	
				30	Kidney	Cattle, Pigs	
				150	Fat	Cattle, Pigs	
				15	Milk	Cattle	
Emamectin benzoate	ADI: 0–0.5	Full	78 (2013)	100	Muscle	Salmon	Emamectin B1a
					Fillet (muscle with skin)	Trout	
Enrofloxacin	ADI: 0–2	Full	48 (1997)	No MRL			
Eprinomectin	ADI: 0–10	Full	50 (1998)	100 2 000	Muscle Liver	Cattle	Eprinomectin B1a
				300	Kidney		
				250	Fat		
				20	Milk		
Erythromycin	ADI: 0–0.7	Full	66 (2006)	100	Muscle, Liver, Kidney, Fat/Skin	Chicken, Turkey	Erythromycin A

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
				50	Eggs	Chicken	
Estradiol-17β	ADI: 0–0.05	Full	52 (1999)	Not specified	Muscle, Liver, Kidney, Fat	Cattle	
Ethion	ADI: 0–2 ARfD: 20		88 (2019)	No MRL			
Febantel, Fenbendazole, Oxfendazole	ADI: 0–7 (group ADI)	Full	50 (1998)	100	Muscle, Kidney, Fat	Cattle, Goat, Horses,	Sum of febantel, fenbendazole and oxfenbendazole, expressed as oxfendazole sulfone equivalents
				500	Liver	Pig, Sheep	
				100	Milk	Cattle, Sheep	
Fenbendazole (see Febantel)							
Fluazuron	ADI: 0–40	Full	48 (1997)	200 500 7000	Muscle Liver, Kidney Fat	Cattle	Fluazuron
Flubendazole	ADI: 0–12	Full	40 (1992)	10	Muscle, Liver	Pig	Flubendazole
				200	Muscle	Poultry	
				500	Liver	Poultry	
				400	Eggs	Poultry	
Flumequine	ADI: 0–30	Full	66 (2006)	500	Muscle	Cattle, Sheep, Pig, Chicken	Flumequine
				1 000	Fat		
				500	Liver		
				3 000	Kidney	Trout	The MRLs are temporary for Black Tiger Shrimp and Shrimp. The MRLs for shrimp applies to all fresh water and marine shrimp.
				500	Muscle	Black Tiger Shrimp	
				500T	Muscle	Shrimp	
Flumethrin	ADI: 0–4	Full	85 (2017)	6		Honey	Flumethrin (trans-Z1 and trans Z2 diastereomers at a ratio of approximately 60:40).
	ADI: 0–4 ARfD: 5	Full	88 (2019)	No MRL			
Fosfomycin	No ADI ARfD: 80		88 (2019)	No MRL			

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Furazolidone	No ADI or ARfD		40 (1992)	No MRL			
Gentamicin	ADI: 0–20	Full	50 (1998)	100 2 000 5 000 200	Muscle, Fat Liver Kidney Milk	Cattle, Pig Cattle	Gentamicin
Gentian violet	No ADI or ARfD		78 (2013)	No MRL			
Halquinol	ADI: 0–200 ARfD: 300	Full	88 (2019)	40 350 500 9 000	Muscle Skin plus fat Liver Kidney	Swine	Sum of 5-chloroquinolin-8-ol (5-CL), 5,7-dichloroquinolin-8-ol 5,7-DCL (5,7-DCL) and their glucuronide metabolites: 5-CLG (expressed as 5-CL equivalents) and 5,7-DCLG (expressed as 5,7-DCL equivalents)
Imidacloprid	No ADI or ARfD		94 (2022)	No MRL			Imidacloprid
Imidocarb	ADI: 0–10	Full	60 (2003)	300 1 500 2 000 50	Muscle Liver Kidney Fat, Milk	Cattle	Imidocarb, free base
Ipronidazole	No ADI or ARfD		34 (1989)	No MRL			
Isometamidium	ADI: 0–100	Full	40 (1992)	100 500 1 000	Muscle, Fat, Milk Liver Kidney	Cattle	Isometamidium
Ivermectin	ADI: 0–10 ARfD: 200	Full	81 (2015)	30 800 100 400 15 20 10	Muscle Liver Kidney Fat Liver Fat Milk	Cattle Pig, Sheep Cattle	Ivermectin B_{1a}. The Committee considers that the presence of high concentrations of ivermectin residues at the injection site is product dependent and must be assessed on a case-by-case basis during marketing authorization by comparison of suitable acute dietary exposure estimates with the ARfD.
	ADI: 0–10 ARfD: 200	Full	88 (2019)	20 15 15 10	Fat Kidney Liver Muscle	Sheep, pigs and goats	The MR in sheep, pigs and goats in ivermectin B_{1a} (H_2B_{1a}, or 22,23-dihydroavermectin B_{1a})

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
	ADI: 0–10 ARfD: 200	Full	94 (2022)	15 30 20 50 30 60 20 100	Muscle Liver Kidney Fat Muscle Liver Kidney Fat	Pigs Sheep, goats	
Lasalocid sodium	ADI: 0–5	Full	81 (2015)	400 1 200 600 600	Muscle Liver Kidney Fat/Skin	Chicken, Turkey, Quail, Pheasant	Lasalocid A
Levamisole	ADI: 0–6	Full	42 (1994)	10 100	Muscle, Kidney, Fat Liver	Cattle, Sheep, Pig, Poultry	Levamisole
Lincomycin	ADI: 0–30	Full	62 (2004)	200 500 1 500 500 100 150	Muscle Liver Kidney Kidney Fat Milk	Chicken, Pig Chicken, Pig Pig Chicken Chicken, Pig Cattle	Lincomycin A separate MRL of 300 µg/kg for skin with fat adhering fat for pigs was recommended to reflect the concentrations found in skin of pigs. MRL was also extended skin/fat for chicken.
Lufenuron	ADI:0–20	Full	85 (2017)	1350	Muscle[b]	Finfish[a]	Lufenuron
Melengestrol Acetate	ADI: 0–0.03	Full	66 (2006)	1 10 2 18	Muscle Liver Kidney Fat	Cattle	Melengestrol acetate
Metronidazole	No ADI or ARfD		34 (1989)	No MRL			
Monensin	ADI: 0–10	Full	70 (2008) 75 (2011)	10 10 20 100 100	Muscle, Liver, Kidney Muscle, Kidney Liver Liver Fat	Chicken, Turkey, Quail Cattle, Sheep, Goat Sheep, Goat Cattle Cattle, Sheep, Goat,	Monensin Cattle liver MRL revised at 75 JECFA

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
						Chicken, Turkey, Quail	
				2	Milk	Cattle	
Monepantel sulfone	ADI: 0–20	Full	78 (2013)	500	Muscle	Sheep	Monepantel sulfone
				7 000	Liver		
				1 700	Kidney		
				13 000	Fat		
	ADI: 0–20	Full	85 (2017)	300	Muscle	Cattle	Monepantel sulfone
				2 000	Liver		
				1 000	Kidney		
				7 000	Fat		
Moxidectin	ADI: 0–2	Full	50 (1998)	20	Muscle	Cattle, Deer	Moxidectin
				50	Muscle	Sheep	The Committee noted very high concentrations and great variation in the residue levels at the injection site in cattle over a 49-day period after dosing.
				100	Liver	Cattle, Deer, Sheep	
				50	Kidney	Cattle, Deer, Sheep	
				500	Fat	Cattle, Deer, Sheep	
Narasin	ADI: 0–5	Full	70 (2008)	15	Muscle, Kidney	Chicken, Pig	Narasin A
			75 (2011)	50	Liver, Fat	Chicken, Pig	Temporary MRLs for cattle, replaced with full MRLs in cattle tissue
				15	Muscle, Kidney	Cattle	
				50	Liver, Fat	Cattle	
Neomycin	ADI: 0–60	Full	60 (2003)	500	Muscle, Fat, Liver	Cattle, Chicken, Sheep, Turkey Goat, Pig, Duck	Neomycin
				10 000	Kidney		
				1 500	Milk	Cattle	
				500	Eggs	Chicken	
Nicarbazin	ADI: 0–400	Full	50 (1998)	200	Muscle, Liver, Kidney, Fat/Skin	Chicken (broilers)	N,N'-bis(4-nitrophenyl)urea
	ADI: 0–900 No ARfD		94 (2022)	4 000	Muscle	Chicken	4,4'-dinitrocarbanilide (DNC)
				15 000	Liver		
				8 000	Kidney		

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
				4 000	Skin plus fat		
Nitrofurazone/ Nitrofural	No ADI		40 (1992)	No MRL			
Olaquindox	No ADI or ARfD		42 (1994)	No MRL			The Committee recommended no MRLs but noted that 4µg/kg in muscle of pigs of the metabolite MQCA (3-Methylquinoxaline-2-carboxylic acid) is consistent with Good Veterinary Practice.
Oxfendazole (See Febantel)							
Oxolinic acid	No ADI or ARfD		43 (1994)	No MRL			
Oxytetracycline See chlortetracycline							
Permethrin	No ADI or ARfD		54 (2000)	No MRL			
Phoxim	ADI: 0–4	Full	62 (2004)	50 400	Muscle, Liver, Kidney Fat	Goat, Pig, Sheep	Phoxim
Pirlimycin	ADI: 0–8	Full	62 (2004)	100 1 000 400 100	Muscle, Fat Liver Kidney Milk	Cattle	Pirlimycin
Porcine Somatotropin	ADI: Not Specified		52 (1999)	Not Specified	Muscle, Liver, Kidney, Fat	Pig	
Procaine benzylpenicillin	ADI: <30µg/ person/day of the penicillin moiety	Full	50 (1998)	50 4	Muscle, Liver, Kidney Milk	All species	Benzylpenicillin
Progesterone	ADI: 0–30	Full	52 (1999)	Not Specified	Muscle, Liver, Kidney, Fat	Cattle	
Propionyl-promazine	No ADI or ARfD		38 (1991)	No MRL			

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Ractopamine hydrochloride	ADI: 0–1	Full	66 (2006)	10	Muscle, Fat	Cattle, Pig	Ractopamine
				40	Liver		
				90	Kidney		
Ronidazole	No ADI or ARfD		42 (1994)	No MRL			
Sarafloxacin	ADI: 0-0.3	Full	50 (1998)	10	Muscle	Chicken, Turkey	Sarafloxacin
				80	Liver, Kidney		
				20	Fat/skin		
Selamectin	ADI: 0–10 ARfD: 400	Full	88 (2019)	No MRL			
	ADI: 0–50 ARfD: 400	Full	94 (2022)	No MRL			
Spectinomycin	ADI: 0–40	Full	50 (1998)	500	Muscle	Cattle, Chicken, Pig, Sheep	Spectinomycin
				2 000	Liver, Fat		
				5 000	Kidney	Chicken	
				2 000	Eggs	Cattle	
				200(µg/L)	Milk		
Spiramycin	ADI: 0–50	Full	48 (1997)	200	Muscle	Cattle, Chicken, Pig	For cattle and chicken, MRLs are expressed as the sum of spiramycin and neospiramycin.
				600	Liver	Cattle, Chicken, Pig	
				300	Kidney		
				800	Kidney	Cattle, Pig Chicken	
				300	Fat	Cattle, Chicken, Pig	For pigs, the MRLs are expressed as spiramycin equivalents (antimicrobial active residues).
				200(µg/L)	Milk	Cattle	
Streptomycin (See dihydro-treptomycin)							
Sulfadimidine (Sulfamethazine)	ADI: 0–50	Full	42 (1994)	100	Muscle, Liver, Kidney, Fat	Cattle, Sheep, Pig, Poultry	Sulfadimidine
				25	Milk	Cattle	
Sulfathiazole	No ADI or ARfD		34 (1989)	No MRL			
Teflubenzuron	ADI: 0–5	Full	81 (2015)	400	Muscle	Salmon	Teflubenzuron
				400	Muscle plus skin	Salmon	

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
					in natural proportion		
Testosterone	ADI: 0–2	Full	52 (1999)	Not specified	Muscle, Liver, Kidney, Fat	Cattle	
Tetracycline (See chlortetracycline)							
Thiamphenicol	ADI: 0–5	Full	58 (2002)	No MRL			
Tiabendazole (Thiabendazole)	ADI: 0–100	Full	58 (2002)	100	Muscle, Liver, Kidney, Fat	Cattle, Pig, Goat, Sheep	Sum of tiabendazole + 5-hydroxy tiabendazole
				100	Milk	Cattle, Goat	
Tilmicosin	ADI: 0–40	Full	70 (2008)	100	Muscle, Fat	Cattle, Pig, Sheep	Tilmicosin
				1 000	Liver	Cattle, Sheep	
				1 500	Liver	Pig	
				300	Kidney	Cattle, Sheep	
				1 000	Kidney	Pig	
				150	Muscle	Chicken	
				100	Muscle	Turkey	
				2 400	Liver	Chicken	
				1 400	Liver	Turkey	
				600	Kidney	Chicken	
				1 200	Kidney	Turkey	
				250	Skin/Fat	Chicken, Turkey	
Trenbolone acetate	ADI: 0–0.02	Full	34 (1989)	2	Muscle	Cattle	β Trenbolone for muscle
				10	Liver		α-Trenbolone for liver
Trichlorfon (Metrifonate)	ADI: 0–2	Full	66 (2006)	50	Milk	Cattle	Trichlorfon
				50	Muscle, Liver, Kidney, Fat		Guidance MRLs at the limit of quantitation of the analytical method for monitoring purposes. No residues should be present in tissues when used with Good Veterinary Practice.

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Triclabendazole	ADI: 0–3	Full	70 (2008)	250	Muscle	Cattle	Keto-triclabendazole
				850	Liver	Cattle	
				400	Kidney	Cattle	
				200	Muscle	Sheep	
				300	Liver	Sheep	
				200	Kidney	Sheep	
				100	Fat	Sheep, Cattle	
Tylosin	ADI: 0–30	Full	70 (2008)	100	Muscle, Liver, Kidney	Cattle, Pig, Chicken	Tylosin A
				100	Fat	Cattle, Pig	
				100	Skin/Fat	Chicken	
				100	Milk	Cattle	
				300	Eggs	Chicken	
Xylazine	No ADI or ARfD		47 (1996)	No MRL			

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1. Combined compendium of food additive specifications, JECFA specifications monographs from the 1st to the 65th meeting. Vol. 1: Food additives A–D; Vol. 2: Food additives E–O; Vol. 3: Food additives P–Z; Vol. 4: Analytical methods, test procedures and laboratory solutions.
2. Residue evaluation of certain veterinary drugs, Joint FAO/WHO Expert Committee on Food Additives, 66th meeting 2006
3. Compendium of food additive specifications, Joint FAO/WHO Expert Committee on Food Additives, 67th meeting 2006
4. Compendium of food additive specifications, Joint FAO/WHO Expert Committee on Food Additives, 68th meeting 2007
5. Compendium of food additive specifications, Joint FAO/WHO Expert Committee on Food Additives, 69th meeting 2008
6. Residue evaluation of certain veterinary drugs, Joint FAO/WHO Expert Committee on Food Additives, 70th meeting 2008
7. Compendium of food additive specifications, Joint FAO/WHO Expert Committee on Food Additives, 71st meeting 2009
8. Safety evaluation of certain contaminants in food, Joint FAO/WHO Expert Committee on Food Additives, 72nd meeting 2010. Joint FAO/WHO publication: WHO Food Additives Series No. 63/FAO JECFA Monographs 8
9. Residue evaluation of certain veterinary drugs, Joint FAO/WHO Expert Committee on Food Additives, Meeting 2010, Evaluation of data on ractopamine residues in pig tissues
10. Compendium of food additive specifications, Joint FAO/WHO Expert Committee on Food Additives, 73rd meeting 2010
11. Compendium of food additive specifications, Joint FAO/WHO Expert Committee on Food Additives, 74th meeting 2011
12. Residue evaluation of certain veterinary drugs, Joint FAO/WHO Expert Committee on Food Additives, 75th meeting 2011
13. Compendium of food additive specifications, Joint FAO/WHO Expert Committee on Food Additives, 76th meeting 2012
14. Compendium of food additive specifications, Joint FAO/WHO Expert Committee on Food Additives, 77th meeting 2013
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19. Compendium of Food Additive Specifications, Joint FAO/WHO Expert Committee on Food Additives, 82nd Meeting 2015.
20. Compendium of Food Additive Specifications, Joint FAO/WHO Expert Committee on Food Additives, 84th Meeting 2017.
21. Residue Evaluation of Certain Veterinary Drugs, Joint FAO/WHO Expert Committee on Food Additives, 85th meeting 2017.
22. Compendium of Food Additive Specifications, Joint FAO/WHO Expert Committee on Food Additives, 86th Meeting 2018.
23. Compendium of Food Additive Specifications, Joint FAO/WHO Expert Committee on Food Additives, 87th Meeting 2019.
24. Residue Evaluation of Certain Veterinary Drugs, Joint FAO/WHO Expert Committee on Food Additives, 88th meeting 2019.
25. Compendium of Food Additive Specifications, Joint FAO/WHO Expert Committee on Food Additives, 89th Meeting 2020.
26. Compendium of Food Additive Specifications, Joint FAO/WHO Expert Committee on Food Additives, 91st meeting 2021.
27. Compendium of Food Additive Specifications, Joint FAO/WHO Expert Committee on Food Additives, 92nd meeting 2021.

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RESIDUE EVALUATION OF CERTAIN VETERINARY DRUGS

Joint FAO/WHO Expert Committee on Food Additives

94th Meeting (Virtual) 16–27 May 2022

This volume of FAO JECFA Monographs contains residue evaluation of certain veterinary drugs prepared at the 94th Meeting of the Joint FAO/ WHO Expert Committee on Food Additives (JECFA), held virtually, 16–27 May 2022. This JECFA meeting specifically convened to consider residues of veterinary drugs in food. The Committee elaborated principles for evaluating the safety of residues of veterinary drugs in food, for establishing acceptable daily intakes (ADIs) and acute reference doses (ARfDs) and for recommending maximum residue limits (MRLs) for such residues when the drugs under consideration are administered to food-producing animals in accordance with good practice in the use of veterinary drugs (GVP). The enclosed monographs provide the scientific basis for the recommendations of MRLs, including information on chemical identity and properties of the compounds, pharmacokinetics and metabolism, residue depletion studies and analytical methods validated and used for the detection and quantification of the compounds.
This publication and other documents produced by JECFA contain information that is useful to all those who work with or are involved with recommending or controlling maximum residue limits for veterinary drugs in food.

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