EUROPEAN COMMISSION
DIRECTORATE-GENERAL FOR HEALTH AND FOOD SAFETY

Safety of the Food Chain
Pesticides and Biocides

COMMISSION WORKING DOCUMENT[1]

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Nature of pesticides residues in fish

The contents of this working document have been finalised in the meeting of the Standing Committee on Plants, Animals, Food and Feed on 22/23 February 2021. It becomes only applicable upon its inclusion in the Commission Communications 2013/C 95/01[2] and 2013/C 95/02[3] and will then be completed with an implementation schedule.

1 This document has been conceived as Working Document of the Commission Services which was elaborated in co-operation with the Member States. It does not represent the official position of the Commission. It does not intend to produce legally binding effects. Only the European Court of Justice has jurisdiction to give preliminary rulings concerning the validity and interpretation of acts of the institutions of the EU pursuant to Article 267 of the Treaty.

2 Commission Communication in the framework of the implementation of Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market.

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Contents

Abbreviations

ASE	Accelerated solvent extraction
BMF	Biomagnification factor
DM	Dry matter
GC	Gas chromatography
HPLC	High performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
log P_{OW}	Logarithm of the *n*-octanol/water partition coefficient
MRL	Maximum residue level
MS	Mass spectrometry
NMR	Nuclear magnetic resonance spectrometry
OECD	Organisation for Economic Co-operation and Development
SFE	Supercritical fluid extraction
STMR	Supervised trials median residue
STMR-P	Supervised trials median residue in processed commodity
TLC	Thin layer chromatography
TRR	Total radioactive residue

1. Introduction

Uptake of pesticides by fish, leading to the occurrence of residues in fish products, can occur as a consequence of environmental exposure from water. This mainly concerns persistent organic pollutants arising from historic pesticide uses. For example, a couple of outdated pesticides such as the persistent organic substances chlordane, DDT, dieldrin, lindane, toxaphene, hexachlorobenzene, mirex, and bromocyclen are occasionally found in fish. Moreover, some currently used pesticides have been found in fish such as chlorpyrifos, pendimethalin, trifluralin or the feed additive ethoxyquin. Besides environmental exposure from water, a second and increasingly important pathway of exposure has to be taken into account which is the ingestion of feed containing a pesticide residue. Consequently, residues in products of fish origin need to be evaluated.

This working document focusses on residues originating from exposure via feed. The objective of the studies described herein is to determine the nature and distribution of the residue in fish. Bioconcentration studies carried out in accordance to OECD TG 305 (OECD, 2012) exposing fish to the radiolabelled active substance in water can be considered on a case-by-case basis, but in general they are not suitable as substitutes for nature of residue studies as they investigate a different route of ingestion leading to different organ exposure and a potentially different metabolic pattern.

Fish metabolism data quantify total residues and characterise the chemical nature of residues which may occur in edible tissues of fish exposed to pesticides. Such studies are required when pesticide use may lead to significant residues of an active substance or a major metabolite in the total diet (considered to be ≥ 0.1 mg/kg[4] feed on a dry matter basis) which also have the potential to accumulate (considered where the logarithm of the *n*-octanol/water partition coefficient (log Pow) is > 3).

A fish metabolism study using ^{14}C radiolabelled test substance should primarily identify the definition and expression of the residue, and the distribution of residues. Hence, analytical methods are needed to determine residues in fish matrices. A fish metabolism study should also include the development of suitable extraction/residue release procedures which allow the efficient extraction of the various components of the residue.

This working document should only be used in connection with residues in fish feed. Exposure from environmental contaminations of water bodies, the direct treatment of water bodies or spray drift/run-off/drainage after treatment along water bodies and the possibly resulting fish

[4] The trigger value of 0.1 mg/kg feed (dry matter basis) in the total diet differs from the corresponding trigger value of 0.004 mg/kg bw/day for poultry, ruminant and pig metabolism studies as defined in Regulation (EU) No 283/2013. This is because fishes grow faster than poultry, ruminants or pigs. While the amount of feed is adjusted with time to satisfy their needs it would not be practical to weigh the fishes during the study.

exposure are not within the scope of this document. Furthermore, the document was not developed to cover active substances administered for other purposes such as veterinary uses.

2. Objectives

The objectives of these studies are:

- to provide an estimate of total radioactive residues in fish under steady state conditions;
- to determine the distribution of residues in fish under steady state conditions;
- to identify the major components of the total radioactive residue in edible fish commodities;
- to quantify the major components of the residue in edible fish commodities and to show the efficiency of extraction procedures for these components;
- to elucidate the metabolic pathway(s);
- to generate data from which a decision on the need for fish feeding studies can be made;
- to provide information to decide on the definition and expression of a residue for both risk assessment and enforcement;
- to establish MRLs (if possible without a fish feeding study).

3. Formation of metabolites in fish

Ingestion of pesticides and their metabolites present in fish feed may result in uptake and metabolism of the ingested compounds. Residues in fish will be available as parent compound, free metabolites, conjugates and/or unextractable / bound residues. Free metabolites (Phase 1 metabolites) may be formed by hydrolysis, oxidation or reduction.

Conjugates (Phase 2 metabolites) may be formed by reaction of primary metabolites (exocons) with substances occurring naturally in fish (endocons). Conjugated compounds are usually more polar than “Phase 1 metabolites” and dissolve in water or other polar solvents. Conjugates can be formed with glucuronic acid, glutathione, amino acids and other natural components.

The exocon (the metabolite of the active substance) as well as the parent compound, can also be bound covalently to insoluble components. According to the International Union of Pure and Applied Chemistry (IUPAC), “a xenobiotic bound residue is a residue which is associated with one or more classes of endogenous macromolecules. It cannot be disassociated from the natural macromolecule using exhaustive extraction or digestion without significantly changing the nature of either the exocon or the associated endogenous macromolecule”.

Residues that are not readily solubilised in solvents at room temperature should not be directly characterised as “bound residues”. They should be further investigated to define the nature of the binding by using different procedures such as enzyme treatments. The residues

should then be termed as 'unextractable' or 'non-extractable' and the procedure used should be specified (Skidmore, 2000; Skidmore et al., 1998).

More extensive degradation may lead to the release of carbon dioxide or other low-molecular weight fragments (e.g. formate and acetate). In some cases, these fragments are incorporated into naturally occurring substances which are of no toxicological concern.

Fish metabolism studies should be conducted using radiolabelled test compound. The desired goal of the study is the identification and characterisation of at least 90 % of the total radioactive residue (TRR) in fillet incl. skin and liver. In many cases it may not be possible to identify significant portions of the TRR especially when low total amounts of residue are present, when incorporated into biomolecules, or when the pesticide is extensively metabolised to numerous low level components. In the latter case it is important for the applicants to demonstrate clearly the presence and levels of the components, and if possible, attempt to characterise them.

4. Extent of data required

The potential of pesticide residues to accumulate in fish tissue is determined to a significant extent by the lipophilicity of the active substance (expressed as the *n*-octanol-water partition coefficient, P_{OW}). The accumulation of compounds of relatively low lipophilicity (log $P_{OW} \leq 3$) via the diet is known to be negligible as far as reported residues in fish are taken into account. Fish metabolism studies are therefore required only for active substances where the log P_{OW} is > 3. If a plant metabolite accounts for a major portion of the fish exposure or if fish is not exposed to the parent compound but to a plant metabolite, which is not formed in the fish itself, a fish metabolism study involving dosing with the plant metabolite may be needed (see chapter 5.1). In these cases the requirement of a fish metabolism study depends on log P_{OW} of the metabolite. Registrants are encouraged to consult with the regulatory authority if guidance is needed on the appropriate test item(s) to be used.

Studies should be carried out with one species of edible fish of a weight sufficient for determination of parent and/or metabolites. Important aquaculture species reared for human consumption such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) or common carp (*Cyprinus carpio*) are the recommended species (Schlechtriem et al., 2016). These fresh water and marine species were chosen here as they represent inland aquaculture and different diets (omnivorous vs. carnivorous), are easily reared under laboratory conditions and have been used widely as model species in research. Other species may however be used as well if justification is provided. It is not necessary but possible to choose the fish species for testing for which the highest dietary burden was calculated. Results gained on the metabolism in fresh-water species are extrapolated to marine species and vice versa.

5. Experimental design and reporting requirements

5.1. Test substance

Experiments should be carried out with radiolabelled substances - preferably ^{14}C, although ^{35}S, ^{32}P and other radioisotopes may be more appropriate in some cases. As animals are living in water and tritium is known for its potential for hydrogen exchange, this isotope should not be used. If a potentially labile side chain is chosen, a metabolism study will be considered adequate only if all significant radioactivity in the animal is identified and found to be associated with the pesticide, and not related to loss of the label from the basic structure of the pesticide molecule.

The substance should be labelled in a sufficiently stable position in the molecule to allow all significant metabolites to be tracked and identified or characterised. Aromatic rings, other cyclic systems and atoms with several substituent groups usually provide suitable positions for labelling. The specific activity of the labelled material should be high enough to assure acceptable limits of detection for radioactive residues. Reports must state: the position of radiolabelling, the radiochemical purity (desirable > 95 %) and the specific activity.

If an active substance contains multiple ring structures or significant side chains it may be necessary to conduct separate experiments using more than one radiolabel; e.g., labelling synthetic pyrethroids on either side of the ester bond, or both rings of active substances with one phenyl ring and one heterocyclic ring etc. A scientifically sound justification must be presented for using only one radiolabel (e.g. if no cleavage is anticipated).

Occasionally, a fish metabolism study may need to be conducted with a metabolite rather than the parent active substance. If a plant metabolism study and/or supervised field residue trial indicates that residues in feed comprise negligible amounts of the active substance (i.e. below the trigger value), but contain a major metabolite, which has a $\log P_{ow} > 3$ and a fish dietary intake ≥ 0.1 mg/kg, then this metabolite should be used as the test substance in the fish metabolism studies. Although information on the lipophilicity (log Pow) of the specific metabolite may not always be available, it can easily be generated. Furthermore, a prediction of its fat solubility could be supported by information derived from the livestock metabolism studies (see chapter "Fat-soluble chemicals and additional considerations" in OECD Guideline 505 (OECD, 2007)).

5.2. Test animals

Healthy fish demonstrating normal feed consumption and behaviour should be used in the study. Fish may be either all male or all female, or a mix of gender may be used. Individual fish should be of similar size/weight (± 20%) at the beginning of the dosing phase to ensure consistent feeding and to minimise aggression. Weighing of fish should be conducted a few days before dosing to ensure fish recover prior to treatment/dosing. During the study, the fish

should be observed regularly and any changes in health should be noted and reported. Mortalities must be justified within the report. Reports should specify species and bodyweight at the commencement of dosing and at the time of sacrifice.

5.3. Housing conditions

An acclimatisation period of at least one week prior to the beginning of dosing is recommended. During this period, fish will be maintained on the unfortified diet and monitored for health, feeding rate and growth rate. The unfortified diet must be nutritionally balanced to ensure optimal growth. Reports should state: date of study, location and conditions (i.e., water source and chemical properties, water temperature, water exchange rate, dissolved oxygen, pH, light regime) of tank.

A flow-through test system that provides a sufficient volume of dilution water to the test tanks should be used to avoid the accumulation of pesticide residues excreted by the test animals into the test water (Schlechtriem et al., 2016). The flow rates should be recorded. Suitable filtration systems are recommended to avoid the accumulation of dissolved test item and metabolites in recirculated water. Water contaminated with radioactive substances must be disposed of in accordance with the local regulations. The capacity of the tank should be in compliance with a maximum loading rate of 1 g of fish (wet weight) per litre of water per day. The filter in used filter systems should be controlled and changed as necessary. The frequency of changes depends on the type of filtration system used.

Water temperatures should be in the range of 20-25 °C for common carp and of 12-16 °C for rainbow trout and Atlantic salmon, respectively. A 12 to 16 hour photoperiod is recommended. Details of lighting should be reported. Partial tank coverings should be provided to cover sheltering areas which reduce stress for the test animals during the light photoperiod.

5.4. Calculation of dietary burden

The calculation of the dietary burden is based on the supervised trials median residue values (STMR, STMR-P) as appropriate for the feed commodities. These values are derived from supervised crop residue trials and/or processing studies. For detailed information on the calculation it is referred to the Working Document on the Fish dietary burden calculator (European Commission, 2021).

5.5. Fish feeding and calculation of dose rate

The daily dosage of fish must at least match the maximum daily exposure based upon the calculation of dietary burden. In practice, there may be analytical problems if dose rates of less than 10 mg/kg feed DM are used. If necessary for identification of metabolites, administration of higher doses may be useful. However, the test substance's toxicity should always be taken into account. The toxicological safety of the calculated dose rate should be

always confirmed in a pre-test. Fish are fed at a fixed ration following the feeding recommendations provided by the food manufacturer (approximately 3 % of wet body weight per day). The nominal feeding rate based on the amount of feed applied during the experiment must be recorded. The feed ration should be adjusted after 50 % of the intended experimental period is completed to account for the expected growth increment of the animals. Expected growth can be calculated based on feed conversion ratios usually stated for most commercial fish diets.

The feed conversion ratio (FCR) is the quantity of feed fed divided by fish weight gain over a specific time period. More thoroughly, the expected growth of the experimental animals should be determined prior to the fish metabolism study during the adaptation phase. Weighing of the fish during the study should be avoided because any disturbance of the animals may affect the ingestion of the experimental diet. Feeding must be constantly observed to ensure that the fish are visibly consuming all of the food presented. If food is consistently being left uneaten, it may be advisable to spread the dose over an extra feeding period in the feeding schedule (e.g. feeding half the amount twice daily). Faeces should as far as possible be removed from the tank. The acceptability of the experimental diet should be tested in advance to make sure it is palatable.

Reports should state: nominal dose rate (mg/kg dry feed and feeding rate in % of wet body weight), number and timing of treatments, method of dosing and amount of feed consumed as well as the weights of individual fish at start and end of dosing phase.

Due to the specific experimental conditions applied during a fish metabolism study, the determination of a mass balance is of limited value and thus not required.

5.6. Stability of fortified feed

When spiking the feed with the test substance, all possible efforts should be made to ensure homogeneity throughout the test feed. Fortified feed pellets should be stabilised by surface coating (e.g. corn oil or calcium alginate) to avoid leaching losses prior to the ingestion of the experimental diet (Goeritz et al., 2014). The methodology for preparing the dose and the extent of leaching from the treated pellets should be described in the report. Registrants should demonstrate prior to the initiation of dosing that leaching losses occurring prior to uptake of the daily ration do not exceed a maximum of 10 % of the applied dose.

5.7. Treatment

Owing to the practical difficulties associated with oral dosing of individual fish with radiochemicals contained in capsules, it is recommended that the dose should be administered via commercial fish feed (floating and/or slow sinking pelletised diet) that has previously been fortified with the radiochemical. The feed should have a uniform pellet size to increase the efficiency of the feed exposure and should be appropriate for the size of the

fish used. The time taken by the fish to consume the feed should be reported. If any food remains in the experimental tank, it should be removed as far as possible shortly after feeding (approx. after 10 minutes). The tank should be cleaned approx. 60 minutes after feeding to remove faeces as far as possible. Both cleaning steps are necessary to avoid recontamination of the water with the radiolabelled substance.

5.8. Pre-Test

Pre-testing is needed to confirm the palatability of the experimental diet (see chapter 5.5). Furthermore, a pre-test is recommended to verify that at the concentration level planned for the main test no adverse or toxic effects to the fish are to be expected. The same fish species envisaged for the main test should be used for this pre-test. Fish selected for the pre-test should have a minimum weight of 50 g at the onset of dosing. It is recommended that a minimum of nine fish should be dosed to prove steady-state conditions. Fish should be fed a commercial fish feed of appropriate pellet size that has previously been fortified with the radiochemical. Fish are fed at a fixed ration following the feeding recommendations provided by the food manufacturer (approximately 3 % of wet body weight per day). The daily dosage of fish must at least match the maximum daily exposure concentration level envisaged for the main test (e.g. 10 mg/kg feed dry matter (DM)). The length of the pre-test should be comparable to the main test and should last for no longer than 10-14 consecutive days (see also chapter 5.9). Housing conditions are the same as for the main test (see chapter 5.3). If toxic effects are observed, the pre-test should be repeated with a lower test concentration. Fish collected at the end of the pre-test can be analysed for concentrations of parent and TRR in fillet (incl. skin), liver and carcass (after removal of gastrointestinal tract) to elucidate whether steady-state conditions were reached after 10-14 days of exposure. A steady-state is reached in fish when in three successive analyses of fish fillet (incl. skin), liver and carcass made on three samples from individual fish (n=3) taken at intervals of at least two days (e.g. day 10, 12, 14) concentrations are within ± 20% of each other, and there is no significant increase of concentration between the first and last successive analysis. Steady-state concentrations in whole fish (calculated based on concentrations measured in the different tissue compartments) should be further confirmed by applying a calculated BMF derived from the uptake rate of parent concentrations and TRR during the pre-test and an estimated depuration rate based on empirical relationships (Annex 1). Furthermore, the anticipated duration of the uptake phase to reach steady-state conditions may be calculated according to equations given in OECD TG 305. Testing of highly lipophilic substances (e.g. log $P_{OW} > 5$) may require uptake phases beyond the maximum study period of 14 days to reach steady state concentrations. If a steady state is not reached within the metabolism study, it cannot serve as a surrogate for a feeding study (e.g. for MRL setting). In this case, steady-state concentrations are derived from fish feeding studies if required.

5.9. Main test

Fish selected for the study should have a minimum weight of 250 g at the onset of dosing. The number of fish to be dosed should provide sufficient material to ensure the objectives of the study are met. Five fish are often appropriate. If a higher number of fish is needed in order to generate enough material for analysis, the amount of test animals should be adjusted accordingly. Mortalities must be justified within the report.

Fish should be fed a commercial fish feed of appropriate pellet size that has previously been fortified with the radiochemical. The daily dosage of fish must at least match the maximum daily exposure based upon the calculation of dietary burden, but will often be higher (see chapter 5.4 for further details).

5.10. Sampling

The experimental animals should be exposed to the fortified diet until the end of the uptake period. Experimental animals should be sacrificed 6-12 hours after the last feeding event. An overview of sampling is depicted in Table 1. Carcass must be collected for TRR determination. The total weight of each sacrificed fish should be recorded. The tissues to be sampled are muscle (fillet) including skin, liver tissue and carcass. Tissue samples should be dissected and the weight of each tissue recorded in the raw data. If the samples cannot be analysed immediately, they should be stored frozen at or below –18 °C.

Table 1 Fish sampling

Sample Material	**Sampling Method**	**Analytical Sample Preparation**
Fillet + Skin	Collect the whole fillet from both body sides including the entire skin of the fillet. Take weight of individual samples.	Homogenise each sample.
Liver	Collect the entire organ (trout, Atlantic salmon) or the hepatopancreatic tissue (carp). Take weight of individual samples.	Homogenise each sample.
Carcass	Collect all remains, including feed and faeces free intestines and except fillet/skin and liver. Take weight of individual samples.	Homogenise each sample.

Reports should state: nature, number and size/weight of samples taken. Furthermore, storage duration and temperature prior to analysis should be stated.

5.11. Analysis of samples

In the analytical phase of a fish metabolism study, the animal parts to be analysed are sampled, chopped or homogenised and the total radioactive residues (TRR) determined in all individual samples including fillet (combined sample containing muscle and skin in natural proportions), liver tissue, and carcass. Full accountability of all radioactivity in collected samples must be ensured in the analysis.

The lipid content of the individual homogenised muscle (fillet incl. skin) samples should be analysed to allow normalisation of the measured concentrations to average lipid content, if required (e.g. to decide if the trigger for conducting a feeding study is met). If the metabolism study serves as a surrogate for a feeding study, requirements of the Working Document on the Magnitude of pesticide residues in fish (European Commission, 2021a) have to be met. Suitable methods should be used for determination of lipid content. The chloroform/methanol extraction technique according to Bligh and Dyer (1959) or the Smedes (1999) method using non-chlorinated solvents should be used, as explained by Schlechtriem et al. (2012).

Metabolite patterns need to be analysed in muscle (fillet incl. skin) and liver. Metabolite determination in carcass is not required. For metabolite identification, tissue samples of the same type may be pooled.

Samples are extracted with a series of solvents and/or solvent systems (including aqueous) with various polarities and other characteristics depending on the nature of the expected residues. These initially obtained residues are defined as extractable residues. The requirements for characterisation and/or identification of extractable residues are summarised in Table 2.

A strategy for identification/characterisation of residue components is detailed in chapter 5.12.

Identification refers to the exact structural determination of components of the total radioactive residue. Typically, identification is accomplished either by co-chromatography of the metabolite with known standards using two dissimilar systems or by techniques capable of positive structural identification such as mass spectrometry (MS), nuclear magnetic resonance spectrometry (NMR), etc. In the case of co-chromatography, chromatographic techniques utilising the same stationary phase with two different solvent systems are not considered to be an adequate two-method verification of metabolite identity, since the methods are not independent. Identification by co-chromatography should be obtained using two dissimilar, analytically independent systems such as reverse and normal phase thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Provided the chromatographic separation is of suitable quality, additional confirmation by spectroscopic means is not necessary. Unambiguous identification can also be obtained using methods

providing structural information such as: liquid chromatography/mass spectrometry (LC-MS), or liquid chromatography/tandem mass spectrometry (LC-MS/MS), gas chromatography/mass spectrometry (GC-MS), and NMR. If the metabolite is determined to be of minimal importance due to its low absolute level (less than 0.05 mg/kg tissue) or percentage of the TRRs (less than 10 % of the TRRs), identification by co-elution with putative synthetic metabolites as reference standards using one chromatographic technique e.g., reverse phase HPLC, will be acceptable.

Characterisation refers to the elucidation of the general nature/characteristics of the radioactive residue short of metabolite identification. Terms used to characterise residues include organosoluble, water soluble, neutral, acidic or basic, polar, non-polar, non-extractable, etc. Characterisation may also involve description of chemical moieties known to be present in the molecule based on conversion to a common structure or due to reactivity with particular reagents. The degree of characterisation refers to how close the assignment comes to structural identification.

New extraction and analysis techniques may be appropriate as a substitute for the techniques mentioned above. Alternate extraction procedures such as supercritical fluid extraction (SFE), microwave extraction and accelerated solvent extraction (ASE) can be used. In any case, the best available technique should be used to fully elucidate the metabolic pathway.

Reports should include: full details of the methods of analysis (including extraction scheme), recoveries of radioactivity in relation to the measured radioactivity in the specific tissue, and sufficient representative chromatograms and spectra to illustrate the quality of the data and to indicate the confidence which can be attached to characterisations and identifications.

5.12. Strategy for identification/characterisation of residue components

The approach described below should be viewed as a broad outline of the type of information needed. Different procedures and methodologies may be appropriate in certain circumstances. The basic concepts regarding trigger values for identification of residue components, methodologies for characterisation and/or identification of residue components, and steps to be taken for adequate release of unextractable residues have to be observed. The trigger values (see Table 2) are meant as rough guidance and may not apply to situations where a metabolite is suspected to be of particular toxicological concern, or where less than 10 % of the TRR represents a high absolute residue level.

When identification of radioactive residues is not accomplished, the degree of characterisation required for a portion of the total radioactivity will depend on several factors including the amount of residue present, the amount of the TRR already identified, the toxicological concern over a class of compounds, the suspected significance of the residue as determined by characterisation already performed, the results obtained in other livestock

metabolism studies and the capability of analytical methods to detect characterised but unidentified residues.

When radioactive residues greater than 0.01 mg/kg tissue are observed in fish commodities from ingestion of the pesticide at levels expected in feed items, thorough identification of the residues is generally necessary. The procedure for identification of residues outlined in Table 2 should be followed.

The threshold values shown in Table 2 reflect the minimum level of characterisation and/or identification required for each tissue or organ following application of the radiolabelled test compound. If the TRR in an animal commodity is 0.01 mg/kg or less, no differentiation of compounds would be necessary unless toxicological concerns over residues occurring at lower levels have been identified. For TRR greater than 0.01 mg/kg, the sample should be extracted with solvents or solvent mixtures of various polarities. The components of the extractable radioactive residue (parent compound and metabolites) should be quantified by chromatographic analysis (TLC, HPLC) to determine the degree of characterisation that is needed.

For extractable TRR of 0.01-0.05 mg/kg tissue, the chromatographic behaviour of this residue can be compared to that of the parent pesticide and likely metabolites (characterisation and/or identification).

When the extractable radioactive residue exceeds 0.05 mg/kg or 10 % of the TRR, whichever is greater, characterisation and identification should be attempted in each fraction. In case of toxicity concerns characterisation and identification may be needed at lower levels.

Low levels (in terms of both mg/kg and % of TRR) of individual residues do not typically need to be identified if the major components of the residue have been identified. For example, if the total radioactive residue in a tissue (or organ) is 3 mg/kg and 75 % of that has been firmly identified, it is unlikely that identification of a series of individual residues in the 0.05-0.1 mg/kg range would be required. On the other hand, extensive efforts towards identification of 0.05-0.1 mg/kg residues would be expected when the total radioactive residue is only 0.3 mg/kg. It should be noted that the trigger values (on a concentration basis) are not absolute standards, but intended to provide guidance as to how much characterisation is adequate. However, in many cases, a potentially important metabolite may partition into multiple fractions because of solubility characteristics, and/or because it is present in both free and conjugated forms. In order to apply the trigger values, particularly in cases where the TRRs are distributed among numerous fractions, it should be demonstrated (e.g., by chromatographic analysis of each fraction) that no single metabolite is distributed among the various fractions in such amounts that the combined level of this component significantly exceeds the trigger value.

The relative amounts of radioactive residues as shown in Table 2 apply, regardless of the dose rate used in fish metabolism studies. If doses lower than 10 mg/kg feed DM are used, this is often insufficient to provide adequate absolute amounts for characterisation and/or identification of residues. Additional studies will be needed to provide sufficient amounts of radioactive residues by appropriate means, e.g., by increased specific radioactivity, suitable time of sacrifice or exaggerated doses. Furthermore, in-vitro investigations using microsomes/hepatocytes might be provided (Bischof et al., 2016).

Table 2 Strategy for identification and characterisation of residues in fish

Relative amount (% TRR)	Concentration (mg/kg tissue)	Required Action
< 10	< 0.01	No action if no toxicological concern.
< 10	0.01 – 0.05	Characterise, only attempt to confirm identity if straightforward (e.g., a reference compound is available or the identification is known from a previous study).
< 10	> 0.05	Characterisation/identification needs to be decided on a case- by- case basis taking into account how much has been identified.
> 10	< 0.01	Characterise. Only attempt to confirm identity if straightforward (e.g., a reference compound is available or the identification is known from a previous study).
> 10	0.01 – 0.05	Significant attempts to achieve identification should be made especially if needed to establish a pathway, ultimately characterisation might be accepted.
> 10	> 0.05	Identify using all possible means.
> 10	> 0.05 unextracted radioactive residue	Unextractable radioactive residue – See Figure 1.

The trigger values shown in Table 2 are meant to waive the need for characterisation and/or identification of metabolites present at very low and insignificant levels.

There might be cases where even adhering to these trigger values leads to a higher characterisation/identification effort than necessary. For example, in metabolism studies in which highly exaggerated feeding levels are employed and low radioactivity levels result in tissues, characterisation and/or identification requirements should be less stringent than when the expected dietary burden already leads to significant radioactivity in fish products. To illustrate this example: if the anticipated dietary burden to fish is about 0.1 mg/kg dry feed, 10 mg/kg radiolabelled compound (dry matter basis) is fed (100X dose), and total radioactivity in tissues is less than 0.1 mg/kg tissue, minimal characterisation and/or identification of residues should be adequate (unless for substances of toxicological concern).

On the other hand, there are many cases, where a metabolite partitions into multiple fractions because of solubility characteristics, and/or because it is present in both free and conjugated forms. In these cases the combined levels have to be taken into account. To justify waiving of identification/characterisation requirements it should be demonstrated (e.g. by HPLC analysis of each fraction) that no single metabolite is distributed among the various fractions in such amounts that the combined level of this component significantly exceeds the trigger value.

5.13. Non-extractable residues

There are three situations in which radioactive residues are observed to be non-extractable in fish:

- Incorporation into biomolecules (i.e., amino acids, sugars, etc.). This occurs when the test compound is degraded into small (usually one or two) carbon units which enter the carbon pool of endogenous compounds used in the biosynthesis of new cell constituents by the animal.

- Chemical reaction or physico-chemical tight-binding with appropriate moieties in biomolecules to form bound residues, which can be released via other chemical reactions (e.g., enzymatic or acid/base hydrolysis).

- Physical encapsulation (trapping) or integration of radioactive residues into fish matrices.

Release of residues in this situation may require solubilisation of the tissue, usually by drastic treatment with base, although use of surfactants may allow the radioactive residue to be released under less severe conditions.

The following general road map for dealing with non-extractable residues is intended to provide guidance and clarification regarding characterisation and/or identification of these residues. Figure 1 provides a visual description of the steps discussed below.

The extracted solid animal material should be assayed and, if the radioactive residue exceeds the trigger values of 0.05 mg/kg or 10 % of the TRR, release of the radioactivity should be

attempted (see Table 2). It is emphasised that, in case of toxicity concerns, attempts to release, characterise and identify residues may have to be conducted already for lower levels.

At each step of the evaluation of non-extractable residues (Figure 1), the total radioactivity in the released residues should be quantified. With respect to characterisation, it should be emphasised that the chromatographic behaviour of the released radioactive residue (including water soluble materials) should be compared to that of the parent and likely metabolites, which are close in structure to the parent. This will indicate whether the released radioactive residue is chemically different from the parent molecule. If the remaining unextracted radioactive residue after a given procedure is $\leq$ 0.05 mg/kg and $\leq$ 10 % of the TRR, further attempted release of radioactive residues is not necessary.

Treatments may be performed sequentially or on sub-samples. The types of treatments include addition of dilute acid and base at ambient temperature (note that these procedures should be employed initially for both metabolism and method development considerations), or the use of surfactants, enzymes, and 6N acid and/or 10N base with reflux. It should be kept in mind that the milder procedures provide more accurate assignments of metabolite structures released, i.e., acid/base reflux would probably release moieties as their final hydrolysis products, which could have only a minor or remote relationship to the conjugated non-extractable form of the radioactive residue.

An ambient temperature acid treatment followed by ambient temperature base treatment will provide a mild hydrolysis of conjugated moieties, and again possibly release any biomolecules containing incorporated radioactive residues. The use of surfactants may release physically encapsulated or membrane bound residues. Because membrane and/or cell wall disruption may improve substrate accessibility to the enzyme, a sonication step should be employed followed by a carefully chosen enzymatic battery. (Note: The activity of each enzyme utilised should be confirmed.) These steps could release chemically-bound residues including any biomolecules containing incorporated radioactive residues.

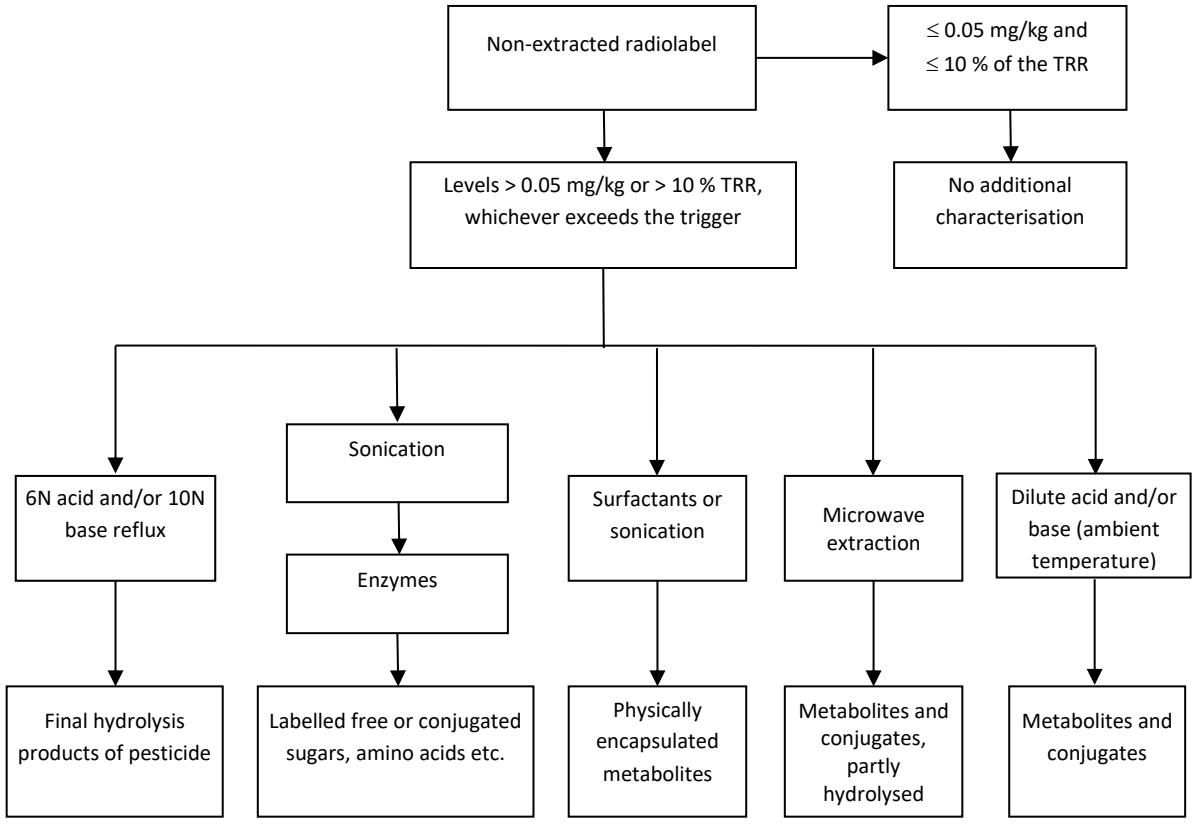
The final release steps would involve reflux acid and base hydrolysis, which will likely solubilise the animal tissue. Radioactive residues released at this time would probably reflect amino acids, sugars and encapsulated or conjugated compounds, which may or may not have any relationship to the original non-extractable/encapsulated structures. However, this step does provide evidence that residues of the pesticide can be released, and may provide data on incorporated radioactive residues and limited information about the nature of the metabolites. In all cases, samples, homogenates and extracts should be maintained at low temperatures except during hydrolytic steps in order to reduce degradation/artefact formation. A variant is the microwave extraction. It is more rapid than Soxhlet extraction, but analyte degradation can occur as well.

Identification of specific radiolabelled endogenous compounds like amino acids, sugars, phenolic compounds, nucleotides, etc. may alleviate the need for further characterisation

and/or identification of non-extractable residues in many instances, since this usually means that the pesticide has been degraded into small carbon units which have entered the carbon pool. This conclusion does not, however, apply to pesticides in which the ^{14}C label is incorporated at a labile site in the molecule. This conclusion also does not apply in cases where a single released metabolite making up a significant portion of the TRR (greater than 10 % of the TRR or greater than 0.05 mg/kg) has not been identified.

The evaluation scheme for non-extractable residues is depicted in Figure 1.

Figure 1 Evaluation scheme for non-extractable residues



At each step in Figure 1, the radioactivity of the released residues should be quantified. If the trigger values shown in Table 2 for extractable residues are met, the radioactive residue should again be partitioned against various solvents/solvent mixtures and characterised and/or identified as required. With respect to characterisation, it should be emphasised that the chromatographic behaviour of the released radioactive residue (including water soluble materials) should be compared to that of the parent and likely metabolites, which are close in structure to the parent. This will indicate whether the released radioactive residue is chemically different from the parent molecule. If the remaining unextracted radioactive

residue after a given procedure is less than 0.05 mg/kg or less than 10 % of the TRR, further attempted release is not necessary.

5.14. Storage stability

Determinations as to whether sample integrity was maintained during collection, sample preparation, and storage should be made. Such analyses should show that the basic profile of radiolabelled residues has not changed throughout the duration of the study. Storage stability data is not normally necessary for samples analysed within six months of collection, provided evidence is given that attempts were made to limit degradation of residues by appropriate storage of matrices and extracts during the analytical part of the study.

If instability of the active ingredient is observed or suspected based on other information, steps should be taken to safeguard the integrity of the study. In those cases where a metabolism study cannot be completed within six months of sample collection, evidence should be provided that the identity of residues did not change during the period between collection and final analysis. This can be done by analyses of representative matrix samples early in the study and at its completion. The matrix sample should be the item stored, i.e., if the matrix extract is used throughout the study and the matrix is not extracted later in the study, the stability of the extract should be shown.

If changes are observed (e.g., disappearance of a particular HPLC peak or TLC spot), additional analyses or another metabolism study with shorter collection to analysis intervals may be necessary. For reasons of animal welfare, repetition of a vertebrate study should however be well justified.

Ideally samples should be stored at/or below –18 °C. Storage under any other conditions needs to be recorded and justified.

6. Interpretation

Full scientific justification should be given for the duration chosen based on the pre-test results. In some cases, a fish metabolism study conducted at the equivalent of a 10X dose, where the 1X dose is the anticipated dietary burden, may be used to indicate that transfer of residues is minimal (e.g. low lipophilic residues) and that the expected dietary exposure will generate residues less than the limit of determination in a feeding study at the 1X dose. In such cases a feeding study that estimates MRLs is unnecessary. When a plateau cannot be achieved during the metabolism study, the feeding study cannot be waived.

In case of non-extractable residues, the question remains as to whether such residues will be available to biological systems, including humans, when persisting in certain constituents of feed or food. If biological unavailability of residues can be demonstrated by showing that the

residues are not affected by any of the procedures envisaged for the evaluation of non-extractable residues (Figure 1), such residues can be considered non-significant.

It may be necessary to evaluate the bio-availability of conjugated metabolites in the same way as non-extractable residues. The extractability of residues should be discussed in relation to the proposed analytical methodology.

Where significant residues in fish are anticipated and MRLs are likely to be established, the extraction efficiency of residue analytical methods has to be assessed. In the respective Technical Guideline (EU, 2017) the inclusion of a general extraction efficiency module in a metabolism study is recommended for such cases.

A schematic diagram of the metabolic pathway with a brief explanation of the distribution and chemical changes involved should be provided in the report.

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Annex 1 Confirmation of steady-state tissue concentrations (pre-test)

Steady-state concentrations (definition)

The uptake phase should be run for no longer than 14 days. A steady-state is reached when three successive analyses of tissue concentrations (C_f) made on samples (fillet incl. skin, liver, carcass) taken at intervals of at least two days (e.g. day 10, 12, 14) are within ± 20% of each other, and there is no significant increase of C_f in time between the first and last successive analysis. A steady-state concentration of the whole fish can be calculated as described in the following chapter.

Approximation of concentration in the fish at steady-state (whole fish)

Biomagnification factors can be determined according to the technical guidance document OECD 305 (OECD, 2012) as a dynamic (kinetic) value derived from the assimilation efficiency (α) of the test substance in the fish feed and the rate constant k_2:

$$BMF = \frac{I \cdot \alpha}{k_2}$$ [equation A1.1]

The assimilation efficiency (α) can be calculated as:

$$\alpha = \frac{C_{fish} * k_2}{1 * C_{food}} * \left(\frac{1}{1 - e^{(-k_2 * t)}}\right)$$ [equation A1.2]

where:

α = assimilation efficiency (absorption of test substance across the gut);

C_{fish} = concentration in fish tissue at the end of the uptake phase (mg/kg);

k_2 = overall (not growth-corrected) depuration rate constant (day^{-1});

I = food ingestion rate (g food/g fish/day);

C_{food} = concentration in fish feed (mg/kg feed);

t = duration of the feeding period (d)

An estimate of k_2 (depuration rate) may be obtained from empirical relationships between k_2 and the *n*-octanol/water partition coefficient (P_{OW}). For example, the following empirical relationships could be used:

$log_{10}(k_2)$ = -0.414 * $log_{10}(P_{ow})$ + 1.47 (r^2 = 0.95) [equation A1.3]

BMF × C_{food} can be used as an approximation to the concentration in the fish tissue (whole fish) at steady-state ($C_{f,s}$). Steady-state tissue concentrations of fish samples taken at the end of the uptake phase (equation A1.1) and the approximated concentration can be compared. Values should not deviate from each other by more than 20%. It should be realised, however, that the equations in this section only apply when uptake and depuration follow first-order kinetics.

Prediction of the time required to reach steady-state tissue concentrations

Testing of highly lipophilic substances (e.g. log P_{OW} > 5) may require uptake phases beyond the maximum study period of 14 days to reach steady state concentrations.

The duration of the uptake phase required to reach a steady-state during biomagnification studies can be predicted based on the bioaccumulation kinetics of the test item (first-order kinetics) according to OECD TG 305:

$C_f = \frac{k_1}{k_2} * C_{food}$ (1-e^{-k2t}) [equation A1.4]

When steady-state is approached (t→∞), equation A1.4 may be reduced to:

$C_f = \frac{\alpha * I}{k_2} * C_{food}$ [equation A1.5]

or

$\frac{C_f}{C_{food}} = \frac{k_1}{k_2}$ = BMF [equation A1.6]

Equation A1.4 may be transcribed to:

$C_f = C_{f,s}$ * (1-e^{-k2t}) [equation A1.7]

or

$\frac{C_f}{C_{f,s}}$ = 1-e^{-k2t} [equation A1.8]

Applying equation A1.8, the time to reach some percentage of steady-state may be predicted when k_2 is pre-estimated using equation A1.3.

As a guideline, the statistically optimal duration of the uptake phase for the production of statistically acceptable data (BMF_K) is that period which is required for the curve of the logarithm of the concentration of the test substance in whole fish plotted against linear time to reach its mid-point, or $1.6/k_2$, or 80 % of steady-state but not more than $3.0/k_2$ or 95 % of steady-state.

Using equation A1.8, the time to reach 80 % of steady-state (t_{80}) is:

$$0.80 = 1 - e^{-k_2 t_{80}}$$ [equation A1.9]

or

$$t_{80} = \frac{-\ln(0.20)}{k_2} = \frac{1.6}{k_2}$$ [equation A1.10]

Similarly, the time to reach 95 % of steady-state (t_{95}) is:

$$t_{95} = \frac{-\ln(0.05)}{k_2} = \frac{3.0}{k_2}$$ [equation A1.11]

For example, the duration of the uptake phase (t_{uptake}) for a test substance with log P_{OW} = 4 would be (using equations A1.3, A1.10 and A1.11):

$$\log_{10}(k_2) = -0.414\ (4) + 1.47$$

k_2 = 0.652 day^{-1}

$$t_{uptake}(80\,\%) = \frac{1.6}{0.652} = 2.45 \text{ days (59 hours)}$$ or

$$t_{uptake}(95\,\%) = \frac{3.0}{0.652} = 4.60 \text{ days (110 hours)}$$

Alternatively, the expression:

$$t_{eq} = 6.54 \cdot 10^{-3} \cdot P_{OW} + 55.31 \text{ (hours)}$$ [equation A1.12]

may be used to calculate the time for steady-state (t_{eq}) to be reached.