OECD/OCDE **503**

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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Metabolism in Livestock

INTRODUCTION

1. Metabolism in Livestock studies are used to determine the qualitative and quantitative metabolism and/or degradation of the active ingredient resulting from pesticide use in feedstuffs, direct application to livestock, or premise treatment. Metabolism in Livestock studies are generally carried out on lactating ruminants and poultry.

2. Livestock metabolism studies are complex. The scientific techniques used to study xenobiotic metabolism and conjugate formation, isolation of animal macromolecules and procedures for generating monomers/oligomers are constantly advancing. It is, therefore, the responsibility of the applicant to utilise state-of-the-art techniques and provide citations of such techniques when used.

PURPOSE

3. Livestock metabolism studies fulfil several purposes:

- Provide an estimate of total residues in the edible livestock commodities, as well as the excreta.
- Identify the major components of the terminal residue in the edible tissues, thus indicating the components to analyzed in residue quantification studies (i.e., the residue definitions for both risk assessment and enforcement).
- Elucidate a metabolic pathway for the pesticide in ruminants and poultry.
- Provide evidence whether or not a residue should be classified as fat soluble.

CONDUCT OF STUDIES

General considerations

4. Metabolism in Livestock studies are needed to elucidate the absorption, and disposition of active ingredients whenever a pesticide use may lead to residues entering the human food chain. In addition, *in vitro* data are useful to show if the pesticide is likely to undergo hydrolysis (acid, base, or enzymatic), oxidation or reduction, photolysis, or other changes.

5. In the case of direct application of pesticides to livestock, registrants are advised to consult with their national authority to determine if such uses are regulated as pesticides or veterinary drugs. Harmonized guidance for veterinary use of direct application is also under development internationally[1].

6. Applicants should provide the proposed metabolic pathway, including a table with associated chemical structures and names (Chemical Abstract Service (CAS) and International Union of Pure and Applied Chemistry (IUPAC) as available). Any postulated intermediates/metabolites should also be indicated in the pathway. The capability of the analytical methods utilized in the metabolism study to determine the components of the residue, whether free, conjugated, or unextractable, should be clearly specified.

7. Applicants should always be alerted to the possibility of new and unexpected metabolites of the pesticide, which may affect future maximum residue limit (MRL) proposals. Where the structure of a metabolite or alteration product is identical to that of another registered pesticide chemical and the information is in the public domain, the applicant should state this fact.

8. For each set of experimental conditions for pesticides (dermal *vs.* oral application or for each radiolabelled position), the following number of animals should be used. A ruminant metabolism study can be carried out on a single animal. For poultry, the use of ten birds per experiments (or dose) is recommended. It should be noted that the objective of these studies is not to do statistical analyses of the radioactive residue in edible matrices but rather to provide a qualitative appreciation of the absorption, translocation and disposition of the residue. Applicants are free to include additional animals if they feel that it is scientifically required.

9. If the applicant wishes to request a waiver of the data elements associated with the livestock feeding by using the Metabolism in Livestock study, inclusion of a second animal (or group of animals in the case of poultry) treated with a realistic dose is strongly recommended. In addition, the applicant may wish to extend the dosing period for the second animal if it is suspected that a plateau is not likely to be reached. The proposed waiver for the feeding studies would require fully adequate scientific reasoning, especially if a plateau has not been reached in milk or eggs in the metabolism study.

10. Livestock metabolism studies should be conducted using radiolabelled test compound. The desired goal of a livestock metabolism study is the identification and characterization of at least 90% of the total radioactive residue (TRR) in each edible tissue, milk or eggs. In many cases it may not be possible to identify significant portions of the TRRs especially when low total amounts of residue are present, when incorporated into biomolecules, or when the pesticide is extensively metabolized 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 characterize them.

NATURE OF THE RESIDUE IN LIVESTOCK

11. Data on the metabolism of a pesticide in laboratory animals that are needed in the toxicology section of these guidelines (i.e., OECD Test Guideline 417 Toxicokinetics) will generally not substitute for metabolism data on livestock. The kinetic information derived from these laboratory studies can, however, play a significant role in the design of the livestock metabolism studies. In some exceptional cases

[1] At the time this guideline was written, the expert group was aware of the VICH process (International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products) and CCRVDF (Codex Committee on Residues of Veterinary Drugs in Food) and its scientific advisory panel JECFA.

laboratory animal metabolism data may be used to supplement livestock metabolism studies in which complete characterization and/or identification of the residue is not attained.

12. In general, separate metabolism studies should be conducted for ruminants and poultry. The species of choice are lactating goats and chickens (laying hens). Non-ruminant (swine) metabolism studies may be necessary if the rat metabolism is significantly different than the ruminant or poultry metabolism. Such differences may include (but are not limited to) the following:

- Differences in the extent of the metabolism;
- Differences in the nature of the observed residue; and
- The appearance of metabolites with sub-structures, which are of known potential toxicological concern.

13. It is not necessary to include control animals in livestock metabolism studies. The acclimatization period should be such to ensure that the livestock maintain good levels of milk and egg production prior to dosing in the study.

14. All estimates of relative dose used in animal metabolism studies should be based on a dry weight basis. It should be noted that the use of percent crop treated information and median residue values are not acceptable to determine the dose level in these experiments.

15. The minimum dosage used in livestock oral metabolism studies should approximate the level of exposure expected from the feeding of treated crops with the highest observed residues. In the case of dermal application the minimum dose should be the maximum concentration from the label. Exaggerated dosages are usually needed to obtain sufficient residue in the tissues for characterization and/or identification. Regardless, for oral studies, livestock should be dosed at least at a level of 10 mg/kg in the diet. Animals should be dosed orally. Ruminants and swine should be dosed daily for at least five days, and poultry for at least seven days.

16. Livestock should not be pre-dosed with the test compound for the following reasons:

- Predosing may result in the induction of enzymatic pathways, and
- Predosing may lead to changes in the specific radioactivity of the parent and metabolites, which may result in low levels of radioactivity in tissues, milk, and eggs masking the degree of residue transfer and thus precluding the identification of the components of the terminal residue.

Also, the resulting differences in specific radioactivities of components of the TRR may make the comparison of relative amounts of parent and metabolites problematic.

17. During the conduct of the metabolism study in livestock, applicants need to keep in mind future issues that may arise with regard to the ability of analytical methods (enforcement and data collection) to efficiently extract the residues defined for purposes of MRL/tolerances or dietary risk assessment. Preferably, samples of liver and milk should be used; however, if specific metabolites accumulate in specific organs, samples of these organs should also be retained. Therefore, radiolabelled samples may need to be retained for future analyses by the subsequently developed methods (sometimes referred to as “radiovalidation” of methods). However, if the extraction procedures in the analytical methods mirror those used in the radiolabelled studies, such data would generally not be necessary.

DISCUSSION OF THE TEST METHOD

Dosing of Radiolabelled Pesticide

18. Radiolabelled active ingredients are required to allow for quantification of the total, extractable and unextractable residues. The active ingredient should be labelled so that the degradation pathway can be traced as far as possible. The radiolabel should be positioned in the molecule so that all significant moieties or degradation products can be tracked. If multiple ring structures or significant side chains are present, separate studies reflecting labelling of each ring or side chain will normally be required if it is anticipated that cleavage between these moieties may occur. A scientifically based rationale may be submitted in lieu of conducting studies with multiple radiolabels if no cleavage is anticipated. However, if cleavage of the molecule is evident, the applicant may be required to conduct an additional study with a radiolabel that tracks the portion of the molecule that is cleaved.

19. In choosing the position to be labelled, assurance is required that a stable position is chosen. The preferred radioisotope is ^{14}C, although ^{32}P, ^{35}S, or other radioisotopes may be more appropriate if no carbons or only labile carbon side chains exist in the molecule. The use of tritium (^{3}H) as a label is strongly discouraged due to the possibility of hydrogen exchange with endogenous materials. If a potentially labile side chain or tritium labelling 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.

20. The specific activity of the radiolabeled active ingredient should be adequate to meet the data requirements of the livestock metabolism study (quantitation of 0.01 mg/kg total TRR in edible tissues, milk or eggs).

21. The national or international authority(ies) might not accept situations where the exaggeration rate is used to calculate "trigger values" (see paragraph 30 and Table 1). For example, if an animal is dosed with radiolabelled material at an exaggerated rate (e.g., 5X), the resulting radioactivity levels should not be divided by the degree of exaggeration (i.e., 5) to arrive at trigger values. Therefore, the studies with 1X application rate are generally necessary for the decision of exceeding or not exceeding the threshold levels. However, dosing with an exaggerated rate is recommended when low residue levels are anticipated by the applicant, which in turn may result in a lack of data to define the metabolic pathways (from the 1X treatment). It should be noted that the calculation of 1X level residues from trials conducted at exaggerated rates are subject to individual regulatory authorities and may only be acceptable when these authorities have decided which identified residues are to be of regulatory concern.

22. The use of stable isotopes such as ^{13}C, ^{15}N, or ^{2}D (nonexchangeable) together with the radiolabeled isotope to aid in identification of metabolites by various spectroscopic methods (mass spectrometry (MS) or nuclear magnetic resonance (NMR)) is encouraged.

23. Livestock metabolism studies should reflect feeding of one compound, usually the parent. The dosing material for oral studies should not be a mixture of active ingredient and plant metabolites. If the plant metabolites are also found to be animal metabolites, then additional livestock metabolism experiments that involve dosing with plant metabolites will not generally be needed. If a plant metabolite comprises a major portion of the TRR on a feed item, a livestock metabolism study involving dosing with the plant metabolite may be needed. If a unique plant metabolite is observed, livestock metabolism studies may be required. Applicants are advised to consult the appropriate regulatory authority if guidance is needed on the appropriate active ingredient.

24. When the dose is administered orally, livestock should be dosed via a balling gun, capsule or gavage to ensure complete administration of the active ingredient.

25. Preferentially, when dermal metabolism studies are considered, the same species of animal should be used as in the oral study. Additionally, the vehicle, the number of administrations, the dose level, and type of treatment(s) should be described. A comparison of the treatments to those proposed for use on the animals, with particular attention to and explanation of any differences in the formulation, dosing level, or other experimental parameters, should be provided. In cases where low levels of radioactivity are observed even at exaggerated rates, utilisation of adjuvants or typical inerts may enhance the dermal absorption of the active ingredient into the animal. Applicants should describe the precautions taken to assure that dermally applied pesticide is not orally taken up due to grooming; this is particularly important for ruminants.

Time of Sacrifice

26. One of the main objectives of livestock metabolism studies is to determine the identity of the residue in the edible tissues, milk and eggs at sacrifice. The applicant will be asked to provide the scientific basis, which justifies the time of sacrifice chosen for the species used in these studies. This time should be preferentially 6 to 12 hours after the last dose. However, under no circumstances should the time of sacrifice be later than 24 hours after the last dose. [See section: Considerations for Data Reporting – Data – Materials/methods – e) Time of sacrifice].

Sampling of Animal Parts

27. Excreta, milk and eggs should be collected twice daily (if applicable). Tissues to be collected should include at least muscle (loin and flank muscles in ruminant and leg and breast muscle in poultry), liver (whole organ for the goat and poultry and representative parts of the different lobes of the liver if cattle or swine are used), kidney (ruminants only), and fat (renal, omental, subcutaneous). The TRR should be quantified for all tissues, excreta, milk, and eggs. For milk the fat fraction should be separated from the aqueous portion by physical means and the TRR in each fraction quantified. Characterization and identification of the residue in urine and feces frequently facilitates characterization of the lower levels of residue found in tissue, but is not required. The radioactivity in the different muscle and fat types should be quantified separately. If the concentration of radioactivity is similar within tissue type, the samples may be pooled before metabolite analysis. However, applicants should ensure that the relative proportion of the different muscle types and fat types in these pooled samples are representative of the relative importance of these tissues within the animal. If they are significantly different, metabolite analysis should be carried out on the different muscle and fat types. Gross pathology of the collected organs should be performed. Abnormalities should be recorded and reported.

Analytical Phase

28. In the analytical phase of a livestock metabolism study, the animal parts to be analyzed are sampled, chopped or homogenized and the TRR determined. Full accountability of all radioactivity must be ensured.

29. 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 characterization and/or identification of extractable residues are summarized in Table 1.

30. The pathway described below should be viewed as a broad outline of the type of information needed to determine that a livestock metabolism study is acceptable. Different procedures and

methodologies may be appropriate in a given circumstance. The basic concepts regarding "trigger" values for identification of radioactivity, methodologies required for characterization and/or identification of radioactivity, and steps that should be taken to assure adequate release of unextractable residues should be observed to assure that the submitted study is adequate. These trigger values (see Table 1) 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 percent of the TRR represents a high absolute residue level.

31. **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 MS, NMR, etc. In the case of co-chromatography, chromatographic techniques utilizing 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 layer chromatography (HPLC). Provided that the chromatographic separation is of suitable quality, then 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) or percentage of the TRRs (less than 10 percent 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.

32. **Characterization** refers to the elucidation of the general nature/characteristics of the radioactive residue short of metabolite identification. Terms used to characterize residues include organosoluble, water or aqueous soluble, neutral, acidic or basic, polar, nonpolar, nonextractable, etc. Characterization may also involve descriptions 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 characterization refers to how close the assignment comes to structural identification.

33. When identification of radioactive residues is not accomplished, the degree of characterization 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 importance of the livestock commodity as a food, toxicological concern over a class of compounds, the suspected significance of the residue as determined by characterization already performed and the capability of analytical methods to detect characterized but unidentified residues.

34. In the metabolism studies in which highly exaggerated feeding levels are employed and low radioactivity results in tissues, characterization and/or identification requirements should be less stringent than when expected dietary burdens lead to significant radioactivity in animal products. For example, if the anticipated dietary burden to livestock is about 0.01 mg/kg, 10 mg/kg radiolabelled compound is fed (1,000X), and total radioactivity in tissues, milk, or eggs is less than 0.1 mg/kg, minimal characterization and/or identification of residues should be adequate (unless toxicologists express a special concern with residues at this level). Such situations often arise with early season herbicides having low application rates.

35. When radioactivity concentrations greater than 0.01 mg/kg are observed in livestock commodities from ingestion of the pesticide at levels expected on feed items, thorough identification of the residues is generally necessary. This is likely when pesticides are applied to crops at high rates late in the growing season. The procedure outlined in Table 1 should be followed.

36. The stereochemistry of metabolites generally does not need to be determined. If identified metabolites with stereochemical centers are to be included in the residue definition and have toxicological concerns, the ratio of the stereosisomers may need to be addressed in the supervised field studies.

37. New extraction and analysis techniques may be appropriate to utilize 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 technology should be used to fully elucidate the metabolic pathway.

Table 1. Strategy for Identification and Characterization of Extractable Residues from Metabolism in Livestock Studies

Relative amount (%)	Concentration (mg/kg)	Required Action
< 10	< 0.01	No action if no toxicological concern.
< 10	0.01 – 0.05	Characterize, 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	Characterization/identification needs to be decided on a case- by- case basis taking into account how much has been identified.
> 10	< 0.01	Characterize. 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 identify should be made especially if needed to establish a pathway, ultimately characterization might be accepted.
> 10	> 0.05	Identify using all possible means.
> 10	> 0.05 Unextracted radiolabel	Unextractable radiolabel – See paragraphs 43 ff and Figure 1.

Strategy for determining when identification of metabolites is needed

38. The radioactivity threshold values shown in Table 1 reflect the minimum level of characterization and/or identification required for each tissue or organ following application of the radiolabelled test compound. If the total radioactivity in an animal commodity is 0.01 mg/kg or less, no differentiation of the radioactivity would be necessary unless toxicological concerns over residues occurring at lower levels are identified. For radioactivity greater than 0.01 mg/kg, the sample should be extracted with solvents or solvent mixtures of various polarities. The components of the extractable radioactivity should then be quantitated by chromatographic analysis (TLC, HPLC) to determine the degree of characterization that is needed.

39. If the extractable radioactivity represents 0.01mg/kg or less, it need not be examined further. For extractable radioactivity of 0.01-0.05 mg/kg, the chromatographic behavior of this radioactivity can be compared to that of the parent pesticide and likely metabolites (characterisation and/or identification).

40. When the extractable radioactivity exceeds 0.05 mg/kg or 10% of the TRR, whichever is greater, characterization and identification should be attempted in each fraction. The exception for this would be toxicology concerns over potential residues which might occur at lower levels.

41. Low levels (in terms of both mg/kg and percent of total residue) 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 radioactivity in a tissue (or organ) is 3 mg/kg and 75 percent 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 toward identification of 0.05-0.1 mg/kg residues would be expected when the total radioactivity is only 0.3 mg/kg. It should be noted that the trigger values (on a concentration basis) are not absolute standards, but approximate guides as to how much characterization 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 for the trigger values to apply, 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 (sum) of this component significantly exceeds the trigger value.

42. The radioactivity levels shown in Table 1 apply regardless of the dose rate used in livestock metabolism studies. If dosages are used which are insufficient to provide adequate radioactivity for characterization and/or identification of residues, additional studies will be needed to provide sufficient amounts of radioactivity by appropriate means, e.g., increased specific radioactivity, suitable time of sacrifice or exaggerated dose.

Release of Unextractable Residues

43. There are three situations in which radioactive residues are observed to be non-extractable in livestock:

- 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 livestock 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.

44. The following general road map for dealing with non-extractable residues is intended to provide guidance and clarification regarding characterization and/or identification of these residues.

45. The extracted solid animal material should be assayed and, if radioactivity exceeds the trigger values of 0.05 mg/kg or 10 percent of the TRR, whichever is greater, release of the radioactivity should be attempted (see Table 1). It is emphasized that, if toxicologists express concerns over potential residues at lower levels; the trigger values will not necessarily apply.

46. At each step in Table 1, the total radioactivity in the released residues should be quantitated. With respect to characterization, it should be emphasized that the chromatographic behavior of the released radioactivity (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 radioactivity is chemically different from the parent molecule. If the remaining unextracted radioactivity after a given procedure is less than 0.05 mg/kg or less than 10 percent of the TRRs, further attempted release of radioactivity is not necessary.

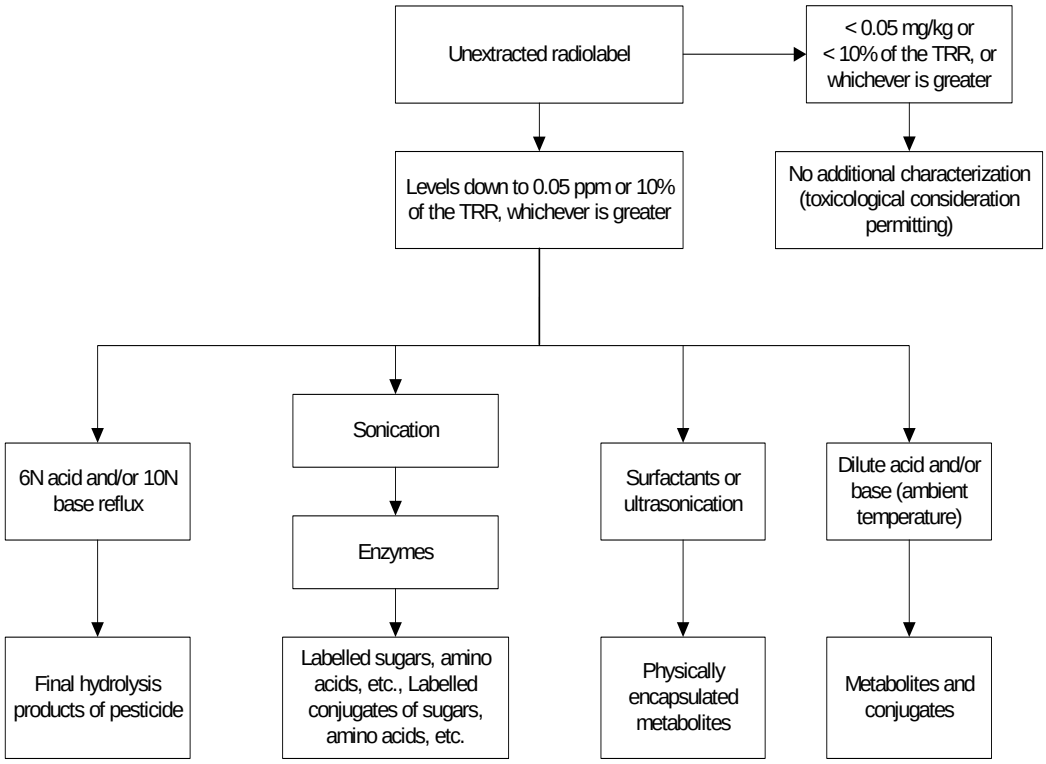
47. Treatments may be performed sequentially or on sub-samples. The types of treatments include addition of dilute acid and base at 37 °C (note that these procedures should be employed initially for both metabolism and method development considerations), or the use of surfactants, enzymes, and 6N acid 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 radioactivity.

48. 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 radioactivity. 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: In each case the activity of each enzyme utilized should be confirmed.) These steps could release chemically-bound residues including any biomolecules containing incorporated radioactivity.

49. The final release steps would involve reflux acid and base hydrolysis, which will likely solubilise the animal tissue. Radioactivity 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 radioactivity 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/artifact formation (see the discussion in paragraphs 52-55 regarding storage stability). Figure 1 provides a visual description of the steps discussed above.

50. Identification of specific radiolabelled endogenous compounds like amino acids, sugars, phenolic compounds, nucleotides, etc. may alleviate the need for further characterization 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 tritium labelled compounds, or to pesticides in which the ^{14}C label is incorporated at a labile site in the molecule. This conclusion would also not apply in cases where a single released metabolite, which comprises a significant portion of the TRR (greater than 10 percent of the TRR or greater than 0.05 mg/kg), has not been identified.

Figure 1 Characterization and Identification of Non-Extractable and Bound Residues



51. Comments on Table 1 and Figure 1:

- At each step in Figure 1, the radioactivity of the released residues should be quantitated. If the trigger values shown in Table 1 for extractable residues are met, the radioactivity should again be partitioned against various solvents/solvent mixtures and characterized and/or identified as required. With respect to characterization, it should be emphasized that the chromatographic behavior of the released radioactivity (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 radioactivity is chemically different from the parent molecule. If the remaining unextracted radioactivity after a given procedure is less than 0.05 mg/kg or less than 10 percent of the TRR, further attempted release of radioactivity is not necessary.

- The trigger values shown in Table 1 are meant to negate the need for characterization and/or identification of metabolites present at very low and insignificant levels. 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 for the trigger values to apply, particularly in cases where the TRR is distributed among numerous fractions, 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 so that the combined level (sum) of this component significantly exceeds the trigger value.

Storage Stability

52. 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. It is impossible to spike samples before the identity of the residue and the length of time needed for metabolism studies are known. Storage stability data are not normally necessary for samples analyzed 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 portion of the study.

53. If instability of the active ingredient is suspected or observed, 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 substrates early in the study and at its completion. The substrate 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.

54. If changes are observed (e.g., disappearance of a particular HPLC peak or TLC spot), additional analyses or another metabolism study with a shorter collection to analysis interval may be necessary.

55. Ideally metabolism samples should be stored at/or below –18 ^{o}C. Storage under any other conditions needs to be recorded and justified.

Clarifications

56. In chromatography (e.g., HPLC, TLC) of radioactive residues, the polarity of the solvent system should be governed by the polarity of the compounds being analyzed, i.e., the solvent polarity should be adjusted to the compounds of interest.

57. With regard as to whether the specific radioactivity should be reported as megabequerels per milligram (MBq/mg) instead of disintegrations per minute per gram or Curies/mole, any units that would permit calculation of radioactivity concentration using reported counts are acceptable. Sufficient information on counts should be provided so that the appropriate regulatory authority can verify the concentration (in mg/kg) reported for animal tissues and the various chromatographic fractions thereof. Regardless of the unit used, a sample calculation should be provided showing how the analyst arrived at concentrations (in mg/kg) from the experimental data.

58. Clear images or radio-analytical imaging detection of TLC plates or autoradiograms that were critical to the identification should be provided. If HPLC coupled to a detector capable of measuring radioactivity was employed, then appropriate radiochromatograms should be submitted. Regardless of the chromatographic technique used, chromatograms showing the behavior of the analytical standards should also be included in the report.

59. At a minimum, applicants should report the total mg/kg radioactivity concentration (usually in equivalents of parent pesticide) for each livestock tissue that could be used for food. For those studies where the radioactivity is measured in all animal parts/tissues, it would be useful to report the percent of total radioactivity in each tissue.

60. The radiovalidation of the extraction process of analytical methods should be submitted as part of the report on the analytical method, the metabolism report or it may stand by itself as a report. The cover letter or summary of the full data package should indicate where it has been placed.

CONSIDERATIONS FOR DATA REPORTING

Data

61. The following elements should be considered during the design, conduct and reporting of the study.

General Considerations

(i) Radiolabelling techniques to include dose level, method of administration or application.

(ii) Extraction, fractionation, and characterization techniques employed for the identification of residue components whether free or non-extractable for each sample.

(iii) Definition of total terminal residues, to include data for all major components of the total terminal residue reflecting their distribution within the tissue or organ expressed as both percentage of the total recovered radioactivity and concentration (in mg/kg) found at the time of sacrifice.

(iv) A detailed discussion, preferably accompanied by a flowsheet of the possible routes of degradation or pathways of metabolism observed. Postulated (but not identified) metabolites/ degradates should be clearly indicated.

(v) During the conduct of the metabolism study in animals, applicants need to keep in mind future issues that may arise with regard to the ability of analytical methods (enforcement and data collection) to efficiently extract the residues defined for purposes of MRL/tolerances or dietary risk assessment. Preferably, samples of liver and milk should be used, however if specific metabolites accumulate in specific organs, samples of these organs should also be retained. Therefore, radiolabelled samples may need to be retained for future analyses by the subsequently developed methods (sometimes referred to as “radiovalidation” of methods). However, if the extraction procedures in the analytical methods mirror those used in the radiolabelled studies, such radiovalidation data would generally not be necessary. The cover letter or summary of the full data package should indicate where it has been placed.

Summary/Introduction.

(i) The purpose of the study, to include testing strategies employed and the rationale for the selection of these strategies.

(ii) The overall experimental procedure employed, to include a discussion, if applicable, of unusual experimental problems encountered, attempts made to alleviate these problems which resulted in deviations from the intended test protocol and the effects, if any, of those deviations on the results of the study.

(iii) The modes and routes of metabolism observed including a complete description of the identity and quantity (both free and non-extractable) of all major components of the terminal residue and their distribution within edible animal tissues and organs.

(iv) A conclusion concerning the qualitative nature of the terminal residue in tissues analyzed as well as milk and eggs should be included.

(v) When enforcement analytical methodology has been developed, these methods should be validated with radiolabelled samples derived from the animal metabolism study, accompanied by a statement made as to their capability to determine the identified major components of the terminal residue, whether free, non-extractable/conjugated, and all components of the residue of concern (ROC). The statement should also indicate the detection limits, precision, and accuracy of the methodology employed. Note that if the specified statement/information is provided elsewhere, it need not be reiterated in this section, but should be referenced.

Materials/methods

a) Active ingredient

(i) Identification of the test pesticide active ingredient (a.i.), including chemical name; common name, American National Standards Institute (ANSI), British Standards Institution (BSI), or International Standards Organization (ISO) names; company developmental/experimental name; and Chemical Abstracts Service (CAS) number and IUPAC. A certificate of chemical and radiochemical purity should be included.

(ii) Chemical structure(s) for the parent compound and metabolites constituting the residue should be provided and a cross reference of all different developmental or experimental names should be provided in either an overview document or as an appendix to the study. Certificates of analysis describing the purity and the identity of standards used in the identification process should be provided if available.

(iii) Information on relevant formulation parameters as pertinent (e.g., nature of the solvent, carrier, or other matrix in which the radiolabelled pesticide was applied).

(iv) For radiolabelled test material, report the radiochemical purity, nature of the radiolabel and its source. The identity of radiolabeled impurities, if any, derived from the test material should also is reported. The site(s) of labelling in the molecule for radiolabeled test material should be provided. A rationale provided for selection of radiolabels other than ^{14}C and for site(s) of labelling in the molecule (where possible, emphasis is placed on labelling the ring position).

(v) With regard to the specific radioactivity, it should be reported as MBq/mg with a sample calculation to show how the analyst arrived at radioactivity concentrations (mg/kg) from the experimental data. Sufficient information on radioactivity counts should be provided so that the relevant regulatory authority can verify the concentration reported for tissues and organs, and the various chromatographic fractions.

(vi) All additional information applicants consider appropriate and relevant to provide a complete and thorough description of the test chemical, such as physical/chemical properties (e.g., solubility, etc.).

b) Test conditions and animal health

(i) A detailed description of the overall testing environment utilized for the study, e.g., animal housing conditions.

(ii) The developmental stage and or age of the animals used.

(iii) Description of animal health throughout the study; observations of changes in animal health status during the study; changes/observations in animal liver and kidney especially when such

observations were reported in studies with laboratory animals. For dermal application, the degree of skin hydration should be noted and reported.

(iv) Body weights and egg/milk production.

(v) An explanation or rationale provided by applicants if the reported testing conditions are not representative or differs significantly from expected practices under which livestock are likely to be treated.

c) Representative animal sample collection.

(i) A rationale or statement provided by the applicant for selection of a test animal other than those specified. In this section a rationale or statement as to why a metabolism study in either ruminant or poultry was not carried out should be provided.

(ii) Identification of specific animal parts collected and subjected to radioactive residue determination and for further analysis and identification of the TRR.

(iii) All additional information the applicant considers appropriate and relevant to provide a complete and thorough description of the experiments.

(iv) Collection procedures for each animal fraction (meat, milk, eggs, offal, fat, excreta, etc.).

d) Administration of the pesticide

(i) A detailed description of the method of pesticide administration used (i.e., dose administered by balling gun, applied on feed, dermal application, etc.), including the vehicle (i.e., sprayed on feed item, commercial formulation used in dermally applied pesticides, etc.) in which the radiolabeled pesticide was administered to the test animals.

(ii) The actual dosage rates used in the study, in mg/kg bodyweight (bw). This information should be related to the expected 1X level in the animal diet and should be expressed on a dry weight basis, or expressed as the maximum concentration of the active applied to the animal on a mg/kg bw basis.

(iii) Number and timing of dosing, interval between doses or applications, number of applications and time of sacrifice.

e) Time of sacrifice

(i) The kinetic information available as well as typical slaughter intervals are two factors that are available when deciding on a time of sacrifice. This time should be preferentially 6 to 12 hours after the final dose. However under no circumstances should the time be later than 24 hours. A scientific basis that justifies the time of sacrifice should be provided.

(ii) In any case at the time of sacrifice sufficient bioincurred radioactive material for a comprehensive metabolite identification/characterization should be available. If at the time of sacrifice chosen, an insufficient amount of radioactive material is anticipated, the determination of a maximum tissue load as a prerequisite of a suitable time of sacrifice can be determined from kinetic experiments. The maximum tissue load will in most cases occur close to the time point of peak plasma or blood concentration (C_{max}) assuming a rapid distribution of radioactivity between the central compartment (blood) and peripheral compartments (organs, tissues). This

information can be derived directly from the test animal during the course of the experiment. Blood samples (e.g., from the ear vein) at certain times after the first dose can be obtained in order to determine the blood kinetics. From the resulting curve a conclusion can be drawn as to the possible level of tissue concentrations at various times after dosing. Also, experience has shown that in many cases the biokinetic behavior of the orally administered pesticide is similar in rats, ruminants, and, in large part, poultry. In such estimations data should be adjusted by allometric coefficients. Therefore, another option to determine the time of maximum blood concentration (C_{max}) is the information contained in the rat biokinetic study developed in accordance with accepted guidelines and submitted as part of the toxicology data set. This study will, in many cases, provide the needed amount of detailed information as well as a description of the biokinetic behavior of the total radioactivity in the plasma following oral administration. In addition, these studies may also contain information on the distribution of radioactivity among tissues and organs from a quantitative or qualitative whole-body autoradiography study. Animals should not be slaughtered before C_{max} is reached to avoid over-emphasising the contribution of the unchanged parent compound in the residue definition. Slaughter at a later time point (but no later than 24 hours after the last dose) is acceptable, if typical slaughter intervals are much longer than the time it takes to reach C_{max}.

(iii) Finally it should be mentioned that kinetic information can also provide assurances that the metabolites included in the residue of concern are, in fact, terminal metabolites.

(iv) A rationale for the time of sacrifice is needed. Apart from the consideration of typical slaughter intervals this should be a summary of kinetic information obtained either from the test animal itself or from the rat metabolism studies submitted to fulfil data requirements for toxicology. A summary of the available information should be provided and the supporting information can be submitted as an appendix. An explanation or rationale by applicants for any significant deviation in the dose administered or any other pertinent deviation from accepted protocols should be provided.

f) Sample handling and storage stability (additional information is provided in the OECD Guidance Document on Overview of Residue Chemistry Studies). The report should contain the following information:

(i) A detailed description of the handling, preshipping storage, and shipping procedures, as applicable, for collected samples.

(ii) A detailed description of the conditions and length of storage of collected samples following their receipt in the laboratory.

(iii) A detailed description of the conditions and length of storage of extracts prior to identification of residues.

g) Analytical methods used for the analyses of radioactive residues

(i) Applicants should specify the capability of the analytical methods utilized in the metabolism study to determine the components of the residue, whether free, conjugated, or non-extractable.

(ii) A method for quantitation and distribution of total recovered radioactivity in the collected animal parts should be provided in narrative, tabular format, or as a figure.

(iii) A detailed description of sample preparation prior to oxidative combustion/liquid scintillation analyses.

(iv) A quantitative accountability of the majority of total radioactivity recovered from the treated animals.

(v) Details of analytical method parameters including descriptions of equipment used for determining total radioactivity in each sample.

(vi) Details of radioactive counting data for selected representative samples to include counting times, total dpm recorded, concentration equivalents found, sensitivity, and limit of detection including representative calculations.

(vii) Radioassay methods using quench correction (automated or not) should describe quench correction methodology and report methods applied to decrease quench.

h) Extraction and fractionation of radioactivity

(i) A discussion of and rationale for the selection and extraction sequence for the extracting solvent (polar *vs.* nonpolar) used and extraction procedures (i.e. blending, maceration, partitioning, Soxhlet) employed, including use of additional techniques (i.e., decomplexing reagents, ultrasonics, etc.).

(ii) A description of conditions employed for the acidic, alkaline and/or enzymatic hydrolysis of the filter cake or residue remaining from previously extracted tissue and/or water soluble extracts to release conjugated residues from these samples. Specific information on the source, purity, specificity, and activity of all enzymatic preparations utilized for hydrolysis.

(iii) Calculations showing the ratio and/or amounts of total free *vs.* conjugated parent compound and/or metabolites in each extracted sample matrix.

(iv) Applicants should provide a quantitative estimate of residual radioactivity (i.e., non-extractable) remaining in the extracted sample matrix following both exhaustive solvent extractions and hydrolytic treatments. The residual radioactivity reported should be expressed as both percentage and concentration (as parent equivalents) of total recovered radioactivity. Any attempts at releasing unextracted radiolabel should also be reported by applicants and a rationale for their use given.

(v) Radiochemical extraction efficiencies calculated and reported for all harvested animal tissues.

(vi) Data to account for or track the loss of radioactivity in each subsequent step of the fractionation and isolation procedure and attempts made by applicants to minimize these losses should be discussed.

(vii) Applicants should report detailed procedures for the fractionation of unextractable or bound radioactivity in animal tissues into proteins, fat, etc.

(viii) Applicants should then report if significant quantities of the original radioactive residue characterized as unextractable have been incorporated into natural products.

(ix) The amount of radioactivity in each sample fraction (e.g., water soluble, organosoluble, released by hydrolysis, etc.) should be quantified and reported in terms of total radioactive counts, and as both percentage and mg/kg (as parent equivalents) of total radioactivity recovered in the original sample matrix analyzed.

i) Characterization and/or identification of radioactivity

(i) A complete tabular listing and description of all known metabolites and proposed intermediates of the parent compound (model compounds, including their structure and purity) used to facilitate the characterization and/or identification of unknown sample metabolites.

(ii) Calculations and data for both sample and reference Rf values on TLC radioautograms and for relative retention times on GC and HPLC columns. Unexpected deviations or variances observed from expected values including loss of sample resolution between analytes (samples) in subsequent chromatographic analyses should be reported and steps taken to correct these problems should be discussed.

(iii) Images (or radioanalytical imaging detection) of TLC plates, autoradiograms, or output from other appropriate imaging systems that were critical to the identification. Samples or reproductions of HPLC/GLC chromatograms including mass spectral scans, etc., should also be submitted. Regardless of the chromatographic technique used, chromatograms showing the behavior of the analytical standards should also be included in the report.

(iv) Complete details of additional confirmatory analytical procedures used to separate and characterize/identify metabolites (i.e., high voltage electrophoresis, ion exchange, or exclusion chromatography, derivatisation, etc.) and determinative methods (i.e., mass spectroscopy) used for ultimate identification of metabolites.

(v) A complete description of all instrumentation, equipment, and reagents used, including operating conditions of the instrumentation utilized for the separation, characterization, and identification of radioactive residues.

(vi) Explanation for all lost or unaccounted radioactivity in each extract or fraction from all samples collected should be given. The amount reported should be expressed as both percentage and concentration (as parent equivalents in mg/kg) of total radioactivity recovered from the particular animal part or fraction analyzed.

(vii) A report of data/information delineating attempts made to characterize/identify chemically any conjugated or complex non-extractable chemical species originating from the parent pesticide in edible animal organs and tissues.

(viii) All radioactivity should be reported either as:

1. Free metabolites - normally extractable by organic solvents that do not require chemical treatment to be released.

2. Conjugated metabolites - Conjugates are made up of two parts, one derived from the pesticide, called the exocon, and one from the animal, called the endocon. The endocon is often glucuronic acid, but there are other possibilities, e.g., sulfates, amino acids, glutathione. Identification of the exocon is not normally possible without cleavage of the conjugate bond. This is normally done by acid, base, or enzymatic hydrolysis. After hydrolysis, the pesticide or pesticide metabolite, free of the conjugating moiety, is usually soluble in organic solvents.

3. Unextracted radiolabel, i.e., metabolites binding covalently with cellular components to yield products that cannot be removed from the matrix by exhaustive extraction with polar and nonpolar solvents. If these residues are removed chemically, e.g., by acid, base, or enzymatic hydrolysis, a subclass of non-extractable residues should be established.

4. Natural constituent - applies to a pesticide that has been degraded into small fragments that have been channeled into anabolic cycles and is incorporated into cell constituents. If the natural constituents are unextractable, they are difficult to distinguish from bound metabolites. This may lead to the misclassification of these residues as bound pesticide residues, when they are not pesticide residues at all. It may be desirable to establish that radioactive residues are natural constituents, particularly if these residues are thought to comprise a large portion of the terminal radioactivity.

(ix) All additional information applicants consider appropriate and relevant in order to provide a complete and thorough description of the conduct of the livestock metabolism study and the determination of the TRRs.

Results and Discussion

(i) Test strategies. This portion of the report should include a discussion of deviations made from the intended testing protocols or strategies as a result of unusual experimental problems or conditions encountered. These include difficulties in extraction, fractionation, and characterization of residues and, if applicable, specific extraction and characterization strategies employed for unextractable or bound residues. It should include a discussion of the impact or effects, if any, of those deviations on the results of the study.

(ii) Metabolic pathways. If possible, a detailed discussion, accompanied by a flowsheet format, of the routes of degradation or pathways of metabolism observed in animals. For discussion purposes, the observed metabolic routes in the tissue may be compared and contrasted to the results obtained n the rat metabolism study as well as to the results observed in plant metabolism studies conducted with the subject chemical if these are available at the time of conduct of the livestock metabolism study. Based on the results of the characterization and/or identification studies, the chemical definition of the metabolic pathway should be proposed, including a table with associated chemical structures and names (CAS and IUPAC as available, including CAS numbers). Any postulated (but not identified) intermediates/ metabolites should also be clearly indicated in the pathway.

(iii) Characterization and/or identification and distribution of TRR.

1. Use a tabular or graphic format. Identify all major components of TRR, both free and conjugated and non-extractable, including name, structure, and quantity (expressed both as percentage of TRR and concentration as parent equivalents), and report their distribution within each animal fraction. All radioactivity should be reported as free, conjugated or non-extractable metabolites or natural constituents.

2. Applicants should provide as much information as possible on all significant unidentifiable and/or uncharacterisable components of the terminal residue, their quantities, and their distribution within all animal fractions.

3. Statistical treatment(s). Include representative examples of any statistical tests applied to the raw data obtained during sampling/analyses in the course of the livestock metabolism study. Provide the LOQ for radioactivity determination and chromatographic separation.

(iv) All additional information applicants consider appropriate and relevant to provide a complete and thorough description of the Metabolism in Livestock study including quality control measures/precautions taken to ensure validity of all aspects of the study.

Conclusion

(i) The routes or pathways, mechanisms involved and extent or degree of metabolism observed in the animals.

(ii) The nature, amount, and distribution of the TRR in the sampled tissues, eggs and milk at the time sample collection.

(iii) Based on the results of studies conducted on radiolabelled animal samples to prove the extraction efficiency, the capability of developed and available enforcement analytical methodology to determine the identified components of the terminal residue, whether free or non-extractable/conjugated, and the capability of the same or modified analytical methodology to determine all components of the ROC, whether free or non-extractable/conjugated in the animal tissue, milk or eggs.

(iv) All conclusions that the applicants consider appropriate and relevant to provide a complete and thorough understanding of the kinetic and metabolic processes occurring in livestock.

Tables/Figures

(i) Tables (for example):

1. Name, structure, purity, for all reference standards and metabolites utilized in study.

2. HPLC/GLC retention times and TLC Rf values for parent compound, metabolites, related compounds and model compounds under the applied chromatographic conditions.

3. Properties, characteristics, quantities and distribution within milk, eggs, edible tissues and organs of all significant unidentified components of the terminal residue.

(ii) Figures (for example):

1. Overall extraction and fractionation strategies or scheme employed for each sample matrix analyzed.

2. Distribution of radioactivity in various ion exchange (exclusion) or preparative HPLC/GLC fractions.

3. Metabolism flow diagrams or charts.

References

Appendices

(i) Representative chromatograms, spectra, etc. (as applicable).

(ii) Reports that contain any supporting kinetic information that was used.

(iii) Other. Any relevant material not fitting in any of the other sections of this report.

Study Report

62. The following section can serve as a checklist to ensure that all pertinent information is included in the study report:

- Identification of the test pesticide active ingredient (a.i.), including chemical name; common name (American National Standards Institute (ANSI), British Standards Institution (BSI), or International Standards Organization (ISO)); company developmental/experimental name; and CAS number and IUPAC chemical name.

- A description of the radiolabelled active ingredient(s) and a justification for the site(s) of radiolabelling, the radiochemical purity, nature of the radiolabel, specific radioactivity (reported as MBq/mg), source, identity of significant radiolabelled impurities, if any.

- Name, structure, and purity of reference standards and metabolites utilized in the study.

- A description of the overall testing environment utilized for the study (i.e., animal housing conditions).

- Description of the animals selected (breed, age, stage of development, health) and rationale for selection of a test animal other than those specified.

- Description of the method of pesticide administration used (dermal, balling gun, etc.), the formulation, actual dosage rate (in milligrams per kilogram of body weight) and actual amounts of radiolabelled and unlabeled pesticide in each dose, number and timing of doses and interval between doses, time from final dose to sacrifice and rationale for that interval.

- Description of types of samples and the handling and storage of those samples (urine, feces, eggs, milk) collected pre-sacrifice.

- A description of samples collected at sacrifice, including a description of the handling and storage of the samples prior to laboratory preparation.

- A description of the preparation and analysis of animal tissues, eggs, milk, and other samples (feces, cage wash) for TRR determinations.

- A careful and full description of the extraction and fractionation of radioactivity in the various animal matrices, including reports on the amount of radioactivity in each sample fraction, including residual radioactivity in the post-extraction solids, quantified in terms of total radioactive counts and as both percentage and concentration (mg/kg, as parent equivalents) in the original sample matrix analyzed.

- A complete description of all instrumentation, equipment, and reagents used, including operating conditions of the instrumentation utilized for the separation, characterization, and identification of radioactive residues.

- Characterization and/or identification of radioactive residues, to include data for all major components, whether free, conjugated, non-extractable, or natural constituent, and to reflect their presence within the tissue, eggs, or milk expressed as both percentage of the TRR (% TRR) and concentration (in mg/kg).

- Description of the chromatographic behavior (HPLC and/or GLC retention times, TLC Rf values) and spectroscopic behavior (MS ions and abundances) of radioactive residues in the extracts of animal matrices, parent, metabolites, and reference standards.

- Information of the storage stability for all major components of the TRRs in the various animal matrices.

- Quantitative information on the recovery of the radioactive residue from the animal tissues, milk, and eggs via the extraction methods used, especially as related to (probable) enforcement analytical methods.

- A detailed discussion, accompanied by a metabolic pathway, of the routes of degradation or metabolism observed in the animal, including a comparison to the metabolism/degradation observed in other livestock studies, in rats, and in plant metabolism studies if these are available at the time of conduct of the livestock metabolism study.

- Conclusions on: (a) pathways or routes of metabolism and extent of metabolism observed for the animal; (b) nature, amount, and distribution of the TRR in the animal tissues, milk, and eggs; and (c) results of validation studies conducted on animal tissues, milk, and/or eggs to demonstrate the degree of capability of available enforcement analytical methodology to extract/release the identified components of the residue definition.

LITERATURE

The source material for this guideline is the following set of documents:

(1) OECD Guidance Document on Overview of Residue Chemistry Studies (2006)

(2) OECD Guidance Document on the Definition of Residue (2006)

(3) Commission of the European Communities (1997). Document 7030/VI/95 - Rev.3 (22/7/1997); Appendix F: Metabolism and Distribution in Domestic Animals.

(4) Food and Agriculture Organization of the United Nations (FAO) (2002). Submission and Evaluation of Pesticide Residues Data for the Estimation of Maximum Residue Levels in Food and Feed. Rome.

(5) Food and Agricultural Organization of the United Nations (FAO) (1996). Guidelines on Pesticide Residue Trials to Provide Data for the Registration of Pesticides and the Establishment of Maximum Residue Limits, Section 2.1 Radiolabelled Studies (Metabolism Studies). Rome.