OECD/OCDE **502**

Adopted:
8 January 2007

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Metabolism in Rotational Crops

INTRODUCTION

1. The field use of most pesticides results in some soil contact of the pesticide, whether it is from intentional application to the soil, e.g., for pre-emergent weed or soil insect control, or inadvertent such by the overspray of foliage, foliage runoff, etc. The rotational crop studies are used to assess the potential for the pesticide and its soil metabolites or soil degradates to accumulate in a rotational crop. Rotational food or feed crops, also referred to as succeeding or following crops, are defined as any field or aquatic crops, which may be planted after the harvest of a pesticide treated primary crop or replanted crop after failure of the pesticide treated primary crop. This guideline describes the test methods to assess the degree of pesticide accumulation and the steps necessary to identify or characterize any significant residues accumulated in rotational crops.

2. Metabolism in rotational crops studies are complex. The scientific techniques used to study xenobiotic metabolism and conjugate formation, isolation of plant 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 they are used.

PURPOSE

3. Metabolism in rotational crops studies are conducted to determine the nature and amount of pesticide residue uptake in rotational crops that are used as human food or as livestock feed. Specifically the studies fulfill these purposes:

- Provide an estimate of total radioactive residues (TRRs) in the various raw agricultural commodities (RACs) via soil uptake.

- Identify the major components of the terminal residue in the various RACs, thus indicating the components to be analyzed for in residue quantification studies (i.e., the residue definition(s) for both risk assessment and enforcement).

- Elucidate the degradation pathway of the active ingredient in rotated crops.

- Provide data to determine appropriate rotational intervals (time from pesticide application to a time when crops can be rotated) and/or rotational crop restrictions based on residue uptake levels.

- Provide information for determining if limited field trials for rotational crops should be performed.

CONDUCT OF STUDIES

General Considerations

4. Metabolism in rotational crops studies are generally not required for uses of pesticides on permanent or semi-permanent crops including, but not limited to, the following commodities or crop groups: asparagus, avocado, banana, berries crop group, citrus fruit crop group, coconut, cranberry, dates, fig, ginseng, globe artichoke, grapes, guava, kiwi fruit, mango, mushrooms, olives, papaya, passion fruit, pineapple, plantain, the pome fruits crop group, rhubarb, the stone fruits crop group, and the tree nuts crop group.

5. If the pesticide is to be applied primarily to paddy rice the applicant should consult the appropriate regulatory agencies to determine if an alternative study design, such as aging the pesticide under paddy conditions prior to rotation, may be required.

6. The desired goal of a metabolism study is the identification and characterization of at least 90% of the TRR in each RAC of the rotated crop. 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 natural products, 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.

7. The determination of whether the residue has been sufficiently characterized and/or identified will depend on the level of radioactivity remaining unidentified, the importance of the plant commodity containing the unidentified residue as a food or livestock feed, the chemical structure of the active ingredient and identified metabolites, and the toxicity of chemicals that are structurally similar to potential metabolites. Where the structure of a metabolite or alteration product is identical to another registered pesticide chemical and the information is in the public domain, the applicant should state this fact.

8. During the conduct of the metabolism study in rotational crops, 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 maximum residue limits (MRL) or dietary risk assessment. 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 residue definition is the same as in the primary crops or if the extraction procedures in the analytical methods mirror those used in the radiolabelled studies, such data would generally not be necessary. The radiovalidation of the extraction process of analytical methods should be submitted as part of the report on the analytical method, or it may stand by itself as a report, or in the metabolism report itself. The cover letter or summary of the full data package should indicate where it has been placed.

DISCUSSION OF THE TEST METHOD

Isotopic Labelling of the Active Ingredient

9. Radiolabelled active ingredients are required to allow for quantification of the total, extractable and unextracted radiolabel. 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.

10. 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 plant 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.

11. The specific activity of the radiolabelled active ingredient should be adequate to meet the data requirements of the crop rotation study (quantitation of 0.01 mg/kg TRR in crop matrices. In cases where the radiochemical purity at the time of application is below 95% justification should be given.

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

Application Parameters

13. The study should be performed using a sandy loam soil that has been treated with the radiolabelled test substance applied at a rate equivalent to the maximum seasonal rate (1X). However, if the pesticide label instructions of the product limit its use to one soil type other than sandy loam, then the study should be conducted with the soil type specified on the pesticide label. In either case, the soil should not be sterilized. Also, if the maximum seasonal application rate can only be attained by multiple treatments under actual use conditions, e.g., many foliar insecticides and fungicides, the radiolabelled material may be applied to the soil in a similar manner or in a reduced number of applications. For example, if the label allows nine applications at weekly intervals of 1 kg active ingredient *per* hectare, the metabolism in rotational crops study could be conducted with one application of 9 kg active ingredient *per* hectare or three applications of 3 kg active ingredient *per* hectare or other application scheme as long as the maximum seasonal rate was met. In all such cases, the aging period for the soil will be considered to start at the last application.

14. The soil should be treated with radiolabelled pesticide active ingredient, preferably containing formulation ingredients typical of an end use product as applied in the field. If parent is applied in a solution (solvent carrier only), then the applicant should ensure that the solvent or an additive in the solvent is not used if it is a photosensitizer, e.g., acetone. Direct application of the radiolabelled pesticide in solution is acceptable provided justification is given, for example, the test substance is soluble in the spray tank under actual commercial use conditions and means of application, or it is difficult to formulate on a small scale.

15. Following application to the soil, the pesticide may be incorporated into the soil if this represents typical agricultural practice.

16. Sampling of the soil is not required, but may be performed at the discretion of applicant.

Rotational Intervals

17. Representative rotational crops should be planted at three appropriate rotational intervals, e.g., 7-30 days for assessing circumstances of crop failure or closely rotated crops, 60-270 days to reflect a typical rotation after harvest of the primary crop and 270-365 days for crops rotated the following year. The

rotational intervals selected should be based on the expected agricultural use for the pesticide and typical rotational practices. The applicant should provide justifications if fewer than three rotational intervals are studied.

18. In cases where the pesticide applied (e.g., certain herbicides) results in excessive phytotoxicity to rotational crops at 7-30 days, an alternative timing for the first rotational interval should be studied. Information regarding planting restrictions due to phytotoxicity should be provided.

Representative Rotational Crop Groups

19. Rotational crops should be representative of each of the following crop groupings: root and tuber vegetable, e.g., radish, beets or carrots; small grain, e.g., wheat, barley, oats or rye; and leafy vegetable, e.g., spinach or lettuce. Where possible, crops should include those expected in the rotational schedule on the label, if known. The representative root crop, however, should not be a bulb vegetable, such as onions or garlic. Soybeans may be substituted for a leafy vegetable due to the importance of this crop in certain rotational practices. Control crops are not required to be grown, but may be planted in untreated soils, to determine possible background or other interferences in the analysis.

Crop In-life Phase

20. The study may be performed either in a greenhouse or in an outdoor plot or container or a combination of the two, e.g., rotated crops can be grown under greenhouse conditions in soils that were treated and aged under outdoor or field conditions. Growing a primary crop in the soil during the aging period is not precluded, provided that the soil is treated prior to planting. If a primary crop is grown, it should be maintained and harvested according to typical agronomic practices. Soil treated and planted with rotational crops at early rotational intervals can also be replanted with rotational crops at the longer rotational intervals if so desired.

Sampling of Crop Parts

21. The three rotated crops should be harvested and the appropriate RACs for human and livestock feed plant parts should be sampled. Samples should also be collected on selected crops at multiple intervals if both immature and mature crops are normally harvested in the course of usual agricultural practices. Samples harvested should include forage, hay, straw and grain for cereal crops; an immature and mature leafy vegetable sample and both the root or tuber and the aerial (leafy) portion of the root crop, even if the leafy portion is not a RAC of the actual root crop planted. Data from the leafy portion of the root crop and the immature leafy vegetable are needed due to the three crops in the study being used as models to extrapolate to wide range of food crops. In addition, due to the increase in the culinary use of immature greens, an immature leafy vegetable sample is needed. Immature leafy vegetable is defined as the crop stage representing approximately 50% of the normal time period for the plant to reach full maturity.

ANALYSIS

22. In the initial stages of the analytical phase of a Metabolism in Rotational Crops study, the crop parts to be analysed are sampled, chopped or homogenized, and the TRR determined. Full accountability of all radioactivity must be ensured.

23. At this point, if each of the three crops demonstrate a TRR of < 0.01 mg/kg in edible portions (food or livestock feed) at all of the rotational intervals, then further characterisation work is usually not required, except in rare cases where regulatory authorities may have concerns regarding the presence of a

pesticide or metabolite at levels < 0.01 mg/kg. Determination of the presence (or absence) of specific pesticide or metabolites of concern at levels < 0.01 mg/kg may be required in these cases. In cases where TRRs exceeds the trigger value (0.01 mg/kg) in a RAC from various crops, then the nature of the residues in those test crops having a TRR > 0.01 mg/kg will normally need to be determined. Criteria for characterization and identification of residues are discussed below. Special attention should be paid to residues of toxicological concern that could be present in soil and taken up by the rotated crops.

24. Samples are extracted with a series of solvents or solvent mixtures with various polarities and other characteristics depending on the nature of the expected residues. The resultant extracts are defined as the extractable residues. The required characterization and/or identification of extractable residues and of unextracted radiolabel are summarized in Table 1 and Figure 1, respectively.

25. Identification refers to the exact structural determination of components of the TRRs. Characterization refers to the elucidation of the general nature/characteristics of the radioactive residues. Terms used to characterize residues include organosoluble, water or aqueous soluble, neutral, acidic or alkaline, 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.

26. 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 crop part as a food or feed item, 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 (i.e., by conversion to a common moiety). Conversion to a common moiety is acceptable for the characterization of multiple low concentration components. However, conversion to the common moiety to alleviate identification of a significant portion of the residues is not an acceptable approach.

27. 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 (NMR), etc. In the case of co-chromatography, chromatographic techniques utilizing the same stationary phase with two different solvent systems are not adequate for the verification of the 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) or TLC and high performance liquid chromatography (HPLC). Provided that the chromatographic separation is of suitable quality, then additional confirmation by spectroscopy is not required. Unambiguous identification can also be obtained using methods providing structural information such as gas chromatography/mass spectrometry (GC-MS), liquid chromatography/mass spectrometry (LC-MS), or liquid chromatography/ tandem mass spectrometry (LC-MS/MS), and NMR. If the metabolite is determined to be of minimal importance due to its low absolute level (<0.05 mg/kg) or percentage of the TRRs (<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. These trigger values are meant as rough guidance and may not apply to situations where a metabolite is suspected to be of particular toxicological concern, or where <10 percent of the TRRs represents a high absolute residue level.

28. 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.

29. 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. However, state of the art technology should be used, as appropriate, to fully elucidate the metabolic pathway.

Table 1. Strategy for Identification and Characterization of Extractable Residues from Metabolism in Rotational Crop Studies Metabolism in Rotational Crop 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	Characterisation/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 characterisation might be accepted
> 10	> 0.05	Identify using all possible means
> 10	> 0.05 Unextracted radiolabel	Unextractable residues – See paragraphs 39-46 and Figure 1

Characterization / Identification of Extractable Residues

30. The radioactivity threshold values shown in Table 1 reflect the characterisation or identification required for each RAC following application of the radiolabelled test compound at the 1X application rate. If TRR in a crop part, is 0.01 mg/kg or less, no differentiation of the radioactivity would be needed, unless there are toxicological concerns over residues occurring at lower levels.

31. If the TRR is greater than 0.01 mg/kg, the crop part should be extracted with solvents or solvent mixtures of various polarities. The components of extractable radioactivity should then be quantitated by chromatographic analysis to determine the degree of characterization that is needed.

32. If the extractable radioactivity represents 0.01 mg/kg or less, it will not require further analysis. If the extractable radioactivity is higher than 0.01 mg/kg, refer to Table 1 for trigger values relating to the identification/characterisation of extractable residues. The exception for this would be toxicological concerns over potential residues, which might occur at lower levels, this includes polar fractions. However, low-level individual residues in terms of both mg/kg and percent of total 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 crop part 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 range of 0.05-0.1 mg/kg would be needed. 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.

33. It should be noted that trigger values expressed 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 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.

Release and Characterization / Identification of Unextractable Radiolabel

34. There are three situations in which unextracted radioalabel may be observed in crops:

- Incorporation into biomolecules (i.e., amino acids, sugars, etc.) which occurs when the active ingredient is degraded into small carbon units (usually 1 or 2), that enter the pool of endogenous compounds used in the synthesis of new cell constituents by the plant.

- Chemical reaction with or physicochemical tight-binding to appropriate moieties in biomolecules (such as cellulose, hemicellulose, lignin) to form "unextractable" radiolabel, which can be released only via other chemical reactions (e.g., enzymatic or acid/alkaline hydrolysis).

- Physical encapsulation (trapping) or integration of radioactive residues into crop matrices (such as cellulose and lignin). Release of unextracted radiolabel in this situation may require solubilization of the tissue, usually by drastic treatment with alkaline, although use of surfactants may allow the radioactive residue to be released under less severe conditions.

35. The extracted solid crop material should be assayed and, if radioactivity is present in the unextracted radiolabel down to the trigger values of 0.05 mg/kg or 10 percent of the TRRs, whichever is greater, release of the radioactivity should be attempted for further identification (see Figure 1). All unsuccessful attempts at releasing unextracted radiolabel and characterizing and/or identifying the TRR should be documented and submitted in order to demonstrate diligence.

36. At each step in Figure 1, the total radioactivity released should be quantitated. With respect to characterization, it should be emphasized that the chromatographic behaviour of the released radioactivity, including water soluble materials, should be compared to that of the active ingredient and available reference compounds. If the remaining unextracted radiolabel after a given procedure is <0.05 mg/kg or <10 percent of the TRR, further attempts to release radioactivity are not necessary.

37. Treatments may be performed sequentially or in parallel. Types of treatments include addition of dilute acid and alkaline at 37 ^{o}C, use of surfactants, enzymes, and 6N acid and/or 10N alkali with reflux. It should be kept in mind that the milder procedures provide more accurate assignments of metabolite structures released. Exhaustive extraction such as acid/alkaline reflux would probably release moieties as their final hydrolysis products, which may have little structural relationship to the original unextracted radiolabel.

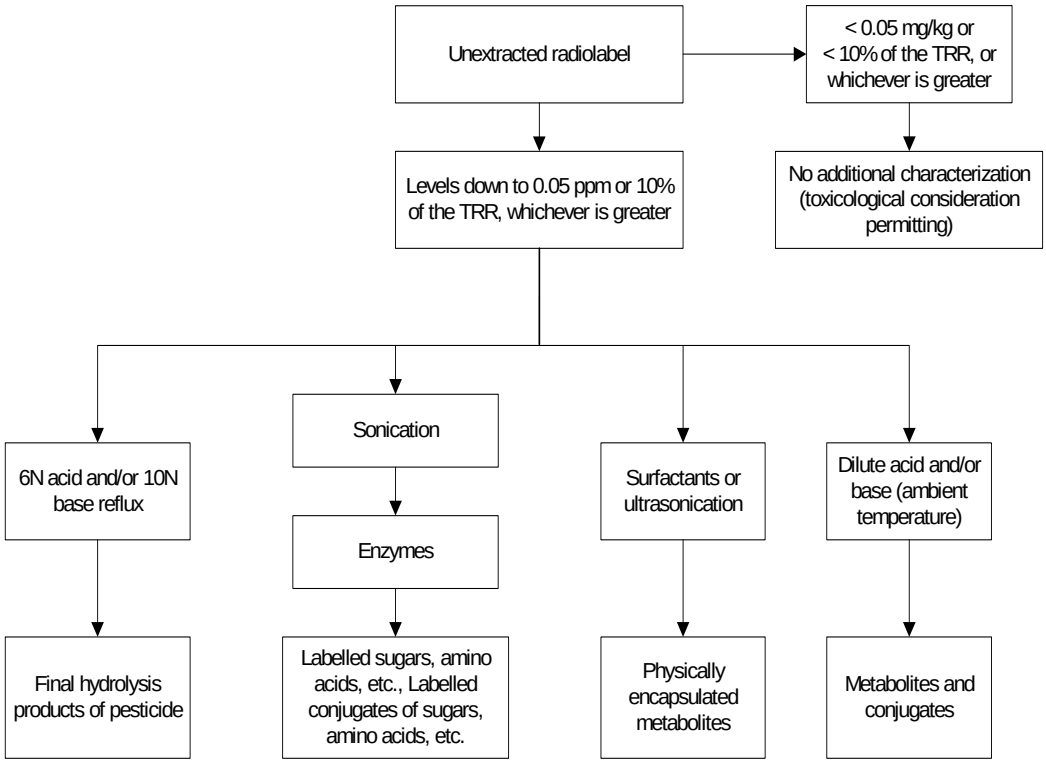
38. Mild acid or alkaline treatment may hydrolyse conjugated moieties, and possibly release any biomolecules containing incorporated radioactivity. The use of surfactants may release physically encapsulated or membrane bound residues. Since membrane and/or cell wall disruption may improve substrate accessibility to the enzyme, a sonication step could be employed followed by a carefully chosen enzymatic battery. 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.

39. The final release steps could involve reflux acid and alkaline hydrolysis, which will likely solubilize the plant matrix. 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 unextracted radiolabel or encapsulated structures. However, this step can 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 buffered and maintained at low temperatures except during hydrolytic steps in order to reduce degradation/artefact formation.

40. Identification of specific radiolabelled 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 would not apply in cases where a single released metabolite, which comprises a significant portion of the total radioactive residue, >10 percent of the TRR or >0.05 mg/kg, has not been identified.

41. The points described above should be viewed as a broad outline of the type of information needed to determine that a crop 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 appropriate steps to release non-extractable/bound residues should be observed to assure that the submitted study is adequate.

FIGURE. 1. Characterization / Identification of Unextracted Radiolabel



STORAGE STABILITY

42. 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 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 portion of the study.

43. 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.

44. 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.

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

CONSIDERATIONS FOR DATA REPORTING

Data

46. The following elements should be considered during the design conduct and reporting of the study:

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-extracted radiolabel) of all major components of the terminal residues and their distribution within the RAC. It is preferable that the foregoing information be summarized in a narrative form with tables and/or figures.

(i) Information on the metabolic pathway of the pesticide in soil, especially in relation to the residues, if any, taken up into the rotational crops.

(ii) A conclusion concerning the qualitative nature of the terminal residues in the RAC at time of harvest or when utilized for livestock feed.

(iii) If residue in crop rotation (limited field) studies are conducted, extraction efficiency may need to be validated with radiolabelled samples derived from the confined crop rotation study or a plant metabolism study (if the metabolites/analytes are similar). The extraction efficiency may be described here in this report, the residues in rotational crops report, as part of the analytical method report or as a stand-alone report.

Materials/Methods

a) Test substance

(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); company developmental/experimental name or number; and Chemical Abstracts Service (CAS) number and IUPAC chemical name.

(ii) Chemical structure(s) for the active ingredient 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, bait, adjuvant, or other matrix in which the radiolabelled pesticide was applied).

(iv) For radiolabelled test material, report the radiopurity, nature of the radiolabel and its source. The identity of significant radiolabelled impurities (>5%), if any, derived from the test material should also be reported. The site(s) of labelling in the molecule for radiolabelled 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 activity, it should be reported as megabequerels per milligram (MBq/mg), with a sample calculation to show how the analyst arrived at radioactivity concentrations (mg active substance per kg) from the experimental data. Sufficient information on counts should be provided so that the relevant regulatory authority can verify the mg active substance per kg reported for crop parts, and in the various chromatographic fractions.

(vi) Any additional information the applicant considers 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 site

A detailed description of the overall testing environment utilized for the study (i.e. outdoor test plots, greenhouse, or plant growth chambers) including, as appropriate, a record of environmental conditions experienced during the course of the study (i.e. temperature, rainfall, sunlight) and documentation of soil characteristics i.e., % sand, % silt, % clay, % organic matter, pH, cation exchange capacity, and moisture capacity). A description of the means by which the test material is confined in the areas surrounding the test plantings should also be provided and any meteorological abnormalities that may have impacted the study should also be explained.

c) Test crop and sample harvesting (collection)

(i) Identification of the rotated test crops, and primary crop if applicable, including type/variety and crop group classification.

(ii) A rationale or statement provided by applicants for selection of test crops other than root crop, leafy vegetable and small grain.

(iii) A description of the procedure used in planting the rotational crops, the number of days between treatment of the soil with the pesticide and planting of the rotational crops, and a description of all procedures used in the maintenance of the rotational crops. If a primary crop was treated, a description of the maintenance and disposition of the primary crop prior to planting of rotational crops should be reported.

(iv) Identification of specific crop part(s) harvested and subjected to 14C-residue analysis for the determination of the TRRs.

(v) The developmental stage(s), general condition (immature/mature, green/ripe, fresh/dry, etc.) and size of the test crop at time of pesticide applications and at harvesting.

(vi) Harvest procedures (method of harvesting or collection (mechanical/hand, from the plant/ground/flotation, etc.); type of equipment used; number/weight of samples collected per replication and number of replications per treatment level; sample coding/labelling). The sampling procedure used to obtain representative samples should be clearly stated.

(vii) A detailed description of additional relevant information on the growing of the test crop, applications of the maintenance pesticides, and harvesting of samples. Any phytotoxicity to the rotational crop observed should also be reported.

d) Application of the pesticide

(i) A description of the radiolabelled pesticide application to the soil including the formulation (i.e. solvent, carrier, bait, adjuvant, or other matrix) and any post application activities (soil incorporation, tilling, etc.).

(ii) The actual application rates to the soil used in the study expressed as kilograms of active ingredient per hectare or pounds of active ingredient per acre.

(iii) Number and timing of applications, reported as days between application intervals, and days from last application to the planting of the rotational crops.

(iv) Dates of planting/sowing/transplanting, as applicable, and other significant dates in the growing of the crop (e.g., harvesting of immature crop to obtain specific crop parts which may be utilized for animal feed); maintenance pesticide applications; and harvest of mature crop). All dates should be provided in terms of days from the last radioactive pesticide application.

(v) An explanation or rationale by applicants for any significant deviation in either the rate or mode of application to the test crop from the intended use pattern.

e) Sample handling and storage stability

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

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

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

f) 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 residues, whether free, conjugated, or unextracted radiolabel.

(ii) Method for quantification and distribution of TRR in the rotational crop for all plant parts sampled, including fractions which may be processed into food or feed, at time of normal harvest or at a stage of development when normally utilized for animal feed provided in narrative, tabular format, or figure.

(iii) A description of sample preparation (i.e. grinding, lyophilisation, etc.) prior to oxidative combustion/liquid scintillation analyses.

(iv) Details of analytical method parameters including descriptions of equipment used for determining total radioactivity in each sample. Radioassay methods using quench correction (automated or not) should describe quench correction methodology and report methods applied to decrease quench.

(v) Details of radioactive counting data for selected representative samples to include mg/kg equivalents found, limit of detection including representative calculations should be reported.

(vi) Extraction efficiency, using the proposed data collection/ enforcement analytical methodology with radiolabelled samples derived from the metabolism study, accompanied by a statement made as to their capability to determine (extract) all components of the TRRs whether free or bound/conjugated in the RAC (if reported here).

g) Extraction and fractionation of radioactivity

(i) A complete description, preferably accompanied by a flowsheet or diagram depicting the overall extraction and fractionation strategies (schema) employed for each sample matrix analyzed.

(ii) A discussion of and rationale for the 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, ultrasonic or microwave extraction, etc.) should be provided.

(iii) A description of conditions employed for the acidic, basic and/or enzymatic hydrolysis of (the filter cake or residue remaining from) previously extracted plant tissue and/or water soluble plant extracts to release conjugated residues from these samples. Specific information on the source,

purity, specificity, and activity of all enzymatic preparations utilized for hydrolysis should also be provided.

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

(v) The applicants should provide a quantitative estimate of residual radioactivity (i.e. unextracted radiolabel) 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 mg/kg (as parent equivalents) of total recovered radioactivity. Attempts at releasing unextractable radiolabel by exotic or other procedures, or extractions following repeated treatments with concentrated acids and/or bases at elevated temperatures should also be reported by the applicant and a rationale for their use provided.

(vi) Radiochemical extraction efficiencies calculated and reported for all harvested crop tissues that are analyzed.

(vii) The efficiency of separation and purification for all fractionation and isolation techniques employed in the study (i.e., solvent partitioning, high voltage electrophoresis, ion exchange, or exclusion column chromatography, HPLC using gradient elution, 2-dimensional thin layer radioautography employing multiple solvent systems) should be reported for a representative sample.

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

(ix) The applicants should report detailed procedures for the fractionation of unextracted radiolabel in plant tissues into natural constituents such as proteins, starch, lignin, cellulose, etc.

(x) The applicants should then report if significant quantities of the original radioactive residues characterized as unextracted radiolabel have been incorporated into natural products.

(xi) The amount of radioactivity in each sample fraction should be quantified and reported in terms of total radioactivity (Bq), and as both percentage and mg/kg (as parent equivalents) of total radioactivity recovered in the original sample matrix analyzed.

h) Characterization and/or identification of radioactivity

(i) A complete tabular listing and description of all known and suspected metabolites of the parent compound (model compounds, including their structure, chemical name (CAS), 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) Photographs (or radioanalytical imaging detection) of thin-layer chromatographic (TLC) plates, auto-radiograms, or output from other appropriate imaging systems that were critical to the identification should be provided. 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 behaviour of the analytical standards should also be included in the report.

(iv) Details of additional confirmatory analytical procedures used to separate and characterize/ identify metabolites (i.e., various chromatographic techniques, derivatisation, etc.) and determinative methods (i.e. mass spectroscopy, NMR) used for ultimate identification of metabolites should be provided.

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

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

(vii) A report of each of the major metabolite components and, if possible, provide information on the chemical nature of discrete (minor) metabolite components.

(viii) A report of data/information delineating attempts made to characterize/identify chemically any conjugated or unextracted radiolabel originating from the parent pesticide in edible plant parts used for food or animal feed should be included.

Results and Discussion

a) 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 in growing, treating, or sampling the test crop to include difficulties in extraction, fractionation, and characterization of residues and, if applicable, specific extraction and characterization strategies employed for unextracted radiolabel. It should include a discussion of the impact or effects, if any, of those deviations on the results of the study.

b) Metabolic pathways

If possible, a detailed discussion, accompanied by a flowsheet format, of the metabolism pathways observed in the subject RACs should be provided. For discussion purposes, the observed metabolic routes in the subject RAC may be compared and contrasted to known and previously reported metabolic pathways in other RACs or observed in animal metabolism studies conducted with the subject chemical. Data on soil residues is not required to collected for this study, however, information on the metabolic behavior of the pesticide in soil may be included here, if it impacts the nature and amount of residues found in the rotational crops. Based on the results of the characterization and/or identification studies, the chemical definition of the metabolic pathway should be proposed in each plant type, including a table with associated chemical structures and names (CAS and IUPAC as available). Any postulated (but not identified) intermediates/metabolites should also be clearly indicated in the pathway.

c) Characterization and/or identification and distribution of TRRs

(i) Use a tabular or graphic format. Identify all major components of TRR in the RAC, both free and conjugated metabolites, natural constituents and unextracted radiolabel, including name, structure, and quantity (expressed both as percentage of TRR and mg/kg (as parent equivalents), and report their distribution within the RAC crop parts.

(ii) If the immature RAC (including plant parts and processed fractions thereof) is normally utilized for as a human food item or as an animal feed, then identification and quantification of all major components of the residues present at that stage of plant development must also be reported.

(iii) Applicants should provide as much information as possible on all significant unidentifiable and/or uncharacterizable components of the terminal residues, their quantities, and their distribution within the RAC.

(iv) Statistical treatment(s). Include representative examples of any statistical tests applied to the raw data obtained during sampling/analyses in the course of the rotational crop study. Provide the limit of quantification for radioactivity determination and chromatographic separation.

(v) Any additional information applicants consider appropriate and relevant to provide a complete and thorough description of the accumulation in confined rotational crop study including quality control measures/precautions taken to ensure validity of all aspects of the study.

Conclusion

The following items should be discussed:

(i) The potential for the accumulation of TRRs from the soil into rotational crops after the proposed use of the pesticide in relation to the various rotational intervals.

(ii) The nature, amount and distribution of the TRR in the food or feed commodity at the time of harvest.

(iii) The routes or pathways, mechanisms involved and extent or degree of metabolism observed in the subject RACs.

Tables/Figures

a) Tables (for example):

(i) Weather and/or environmental data.

(ii) Distribution and quantity of radioactivity in various harvested plant parts.

(iii) Name, structure, purity, for all reference standards and metabolites utilized in study.

(iv) HPLC/GLC retention times and TLC Rf values for parent compound, metabolites, related compounds and model compounds under different column, solvent (elution) conditions.

(v) Name, structure, quantity and location in the RAC of all major identified components of terminal residue.

(vi) Properties, characteristics, quantities and distribution within RAC of all significant unidentified components of the terminal residue.

b) Figures (for example):

(i) Discussion or diagram of location, topography, and size of outdoor test plot(s).

(ii) Overall extraction and fractionation strategies or schema employed for each sample matrix analyzed.

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

(iv) Metabolism flow diagrams or charts.

References

Appendices

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

(ii) Sample Calculations and representative raw data

(iii) Cited or reference reprints of published and unpublished literature, company reports, letters, analytical methodology, etc., used by the applicants (unless physically located elsewhere in the overall data report, in which case cross referencing will suffice).

(iv) Other. Any relevant material not fitting in any of the other sections of this report should be appended.

Study Report

47. The study report should contain the following information:

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

- A description of the radiolabelled test substance(s) and a justification for the site(s) of radiolabelling, the radiopurity, nature of the radiolabel, specific activity (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. outdoor test plots, greenhouse, or plant growth chambers) including, as appropriate, a record of environmental conditions experienced during the course of the study (i.e. temperature, rainfall, sunlight) and documentation of soil characteristics (i.e., % sand, % silt, % clay, % organic matter, pH, cation exchange capacity, and moisture).

- A description of the application parameters: type(s) of pesticide application to the soil; formulation in which the radiolabelled pesticide was applied; method of application; rate(s) of application; number and timing of applications.

- A description of the means by which the test substance is confined in the areas surrounding the test plantings.

- A description of the rotational intervals (in days from the last pesticide application) and rationale for selection.

- A description of the growing of the rotational crops, including harvesting techniques, plant growth stage and size at harvest, crop parts harvested, and handling and shipping and storage of the harvested crop parts. Any phytotoxicity to the rotated crop observed should also be reported.

- A description of the preparation and analysis of crop parts for TRR determinations.

- A careful and full description of the extraction and fractionation of radioactivity in the various plant matrices, including reports on the amount of radioactivity in each sample fraction, 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, unextracted radiolabel, or natural constituent, and to reflect their distribution within the RAC expressed as both percentage of the TRR (% TRR) and concentration (in mg/kg).

- A description of the chromatographic behaviour (e.g., HPLC and/or GC retention times, TLC reference (Rf) values) of parent, metabolites, and related reference standards and a comparison to the chromatographic behaviour of extracted radioactive residues from the RACs. Representative radiochromatograms of sample extracts and chromatograms of the analytical standards, as well as any spectral data supporting the identity of metabolites should also be included.

- Information on the storage stability for all major components of the residue.

- A detailed discussion, accompanied by a metabolic pathway of the pesticide observed in the subject RACs. Information on the metabolic pathway of the pesticide in soil, especially in relation to the residues, if any, taken up into the rotational crops should be included.

- A conclusion discussing: (1) the potential for the accumulation of TRRs from the soil into rotational crops after the proposed use of the pesticide in relation to the various rotational intervals, (2) the nature, amount and distribution of the TRR in the food or feed commodity at the time of harvest; and (3) the routes or pathways, and extent or degree of metabolism observed in the subject RACs.

LITERATURE

(1) U.S. Environmental Protection Agency. (1996). OPPTS Harmonized Test Guideline 860.1850. Confined Accumulation in Rotational Crops. EPA Report No. 712-C-96-188, August 1996.

(2) U.S. Environmental Protection Agency. (1996). OPPTS Harmonized Test Guideline 860.1900. Field Accumulation in Rotational Crops. EPA Report No. 712-C-96-189, August 1996.

(3) U.S. Environmental Protection Agency. (1996), OPPTS Harmonized Test Guideline 860.1300. Nature of the Residue – Plants, Livestock. EPA Report No. 712-C-96-172, August 1996.

(4) Canada Pest Management Regulatory Agency (PMRA) (1998). Residue Chemistry Guidelines, Directive 98-02.

(5) European Commission (1997), Appendix C – Testing of plant protection products in rotational crops. Document 7524/VI/95 rev. 2, 22/7/97, Directorate General for Agriculture VI B II-1. http://europa.eu.int/comm/food/plant/protection/resources/publications_en.htm

(6) Food and Agricultural Organization of the United Nations (FAO) (1986). 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.

(7) Food and Agricultural 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, 2002.