OECD/OCDE **501**

Adopted:
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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Metabolism in Crops

INTRODUCTION

1. The desired goal of a Metabolism in Crops study is the identification and characterisation of at least 90% of the total radioactive residue (TRR) in each raw agricultural commodity (RAC) of the treated 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 biomolecules, or when the active ingredient is extensively metabolised to numerous low level components. In the latter case it is important for the applicants to demonstrate clearly the presence and levels of the components, and if possible, attempt to characterise them.

2. Metabolism in 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 utilize state of the art techniques and provide citations of such techniques when used.

PURPOSE

3. Studies of Metabolism in Crops are used to elucidate the degradation pathway of the active ingredient and require the identification of the metabolism and/or degradation products when a pesticide is applied to a crop directly or indirectly.

4. Studies of Metabolism in Crops fulfill several major purposes:

- Provide an estimate of the total residues in the various RACs after crop treatment, which allows determination of the distribution of residues within the crop, e.g., whether the pesticide is absorbed through roots or foliage or whether translocation occurs;

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

- Elucidate the metabolic pathway of the active ingredient in treated crops.

CONDUCT OF STUDIES

General Considerations

5. Metabolism in Crops studies are conducted to show the fate of the active ingredient in the crops. In addition, *in vitro* data are useful to show if the active ingredient is likely to undergo hydrolysis (acid, alkaline, or enzymatic), oxidation or reduction, photolysis, or other changes. Supplementary techniques,

such as metabolism of the active ingredient in tissue cultures, excised crops, or analysis of immature or non-edible crop parts such as apple leaves, can also be used to aid in the identification and characterization of the residues.

6. Consultation with a regulatory authority on the particulars of a metabolism in crops study and/or when issues arise from the use of this guideline is always encouraged. Some issues may be decided by consultation before starting the study, e.g., which representative crops to use or how to address special crops that may not easily fit into a crop group. Other issues may need to be addressed as the particular study progresses, such as the adequacy of the degree of characterization and/or identification.

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 crop commodity containing the unidentified residue as a food or feed item, 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 that of another registered active ingredient chemical and the information is in the public domain, the applicant should provide this information.

Crop Groups

8. A Metabolism in Crops study should be submitted for each type of crop group for which use is proposed. Crops can be considered to belong to one of five categories for crop metabolism studies: root vegetables, leafy crops, fruits, pulses and oilseeds, and cereals (see Annex 1). One crop from a group will cover the entire group for purposes of metabolism in those crops within the group. For those crops which do not fit into the five categories, the applicant should consult the “Miscellaneous section” in Annex 1 for instructions. In order to extrapolate metabolism of a pesticide to all crop groupings, metabolism studies on a minimum of three representative crops (from the five different crop categories) should be conducted. If the results of these three studies indicate a comparable metabolic route, then additional studies will not be needed.

9. The studies should reflect the intended use pattern of the active ingredient such as foliar, soil/seed, or post-harvest treatments. If, for instance, three studies have been conducted using foliar application and at a later date soil application (e.g., seed treatment, granular, or soil drench) is proposed, then an additional study reflecting soil application should be conducted. Provided that the results from this study are similar to the foliar results, then no further work is needed. However, if both foliar and soil treatments are proposed at the same time, and two studies are for foliar application and the third is for soil treatment, and the results from these three studies on three crops from different crop groups are similar, then no further studies are needed.

10. Differences in the quantities of metabolites belonging to the same pathway will not trigger the need for additional studies. However, if different metabolic routes are observed among the representative crops from studies conducted in a similar manner (e.g., foliar spray with similar pre-harvest interval (PHI) and growth stages), further studies should be conducted for uses on crops in the remaining categories. This is best illustrated by a few examples.

Example 1

Foliar application of an active ingredient to a leafy vegetable, a fruit, and a cereal grain shows that the only metabolic pathway entails hydroxylation of a phenyl ring followed by conjugation to glucose. In lettuce, a leafy vegetable, the hydroxyl metabolite and its conjugate each represent about 10% of the TRR. In the wheat RACs, the metabolite and its conjugate are found at 20%

and 30% of the TRR, respectively. On apples the hydroxyl metabolite is observed at 10% of the TRR, while the conjugate represents 60% of the total residues. In this scenario, additional metabolism studies would not be needed for uses on the two remaining crop categories, root vegetables and pulses/oilseeds, since the same metabolic route is being observed in three diverse crops. The differences in the levels of the metabolite and its conjugate will be taken into consideration by regulatory authorities when determining the definition of the residue for purposes of maximum residue limit (MRL) enforcement and dietary risk assessment. The criteria considered in that process are described in the OECD Guidance Document on Definition of the Residue (1).

Example 2

Pre-plant application of an herbicide to the soil reveals that the metabolic pathway in sugar beets (a root vegetable) and tomatoes (fruit category) is hydroxylation of various alkyl groups. No separation of an aromatic ring from the remainder of the molecule is observed in these two crops. However, in wheat (cereal crop), extensive loss of the aromatic ring from the herbicide is observed, i.e., 30-70% of the TRR represents loss of that ring. In this situation additional metabolism studies would be needed for uses in the remaining crop categories leafy vegetables and pulses/oilseeds.

11. In example 2, it is clear that the unique route in wheat represents a major metabolic pathway. In those cases where a metabolic process seen in only one representative crop is found to be minor, generating less than 10% of the TRR or 0.05 ppm residues, whichever is greater, additional studies would not normally be triggered unless the resulting residues are considered to be significantly more toxic than the active ingredient. It is not feasible to address in this guideline all the possible situations which may arise with respect to this issue.

12. There are situations where an intended use is unique, in terms of the crop and/or its growing conditions, for which a metabolism study would be necessary, in addition to the three representative crops. For example, if a use on paddy rice is intended, a metabolism study should be submitted for paddy rice, regardless of other available metabolism studies.

Post-harvest Uses

13. Post-harvest uses require at least one study if no other appropriate foliar metabolism study is available. A foliar study can substitute for a post-harvest study if the mature commodity was present and exposed at application. If post-harvest uses are proposed on a number of commodities from different crop groupings (see Annex 1), then additional studies should be conducted up to a maximum of three.

Genetically Modified Crops (GM)

14. For genetically modified crops that do not involve the insertion of a gene conveying resistance by means of metabolism, no additional metabolism studies are needed. However, the rationale for concluding that the gene does not alter metabolism should be detailed. When a gene is inserted that conveys active ingredient resistance due to pesticide metabolism, then a Metabolism in Crops study should be conducted for each crop grouping (Annex 1) to which the genetically modified crops belong. If one such study shows a similar metabolism to conventional crops, however, no additional studies would be needed. If a different metabolic route is observed, then two additional studies should be conducted.

DISCUSSION OF THE TEST METHOD

Isotopic Labelling of the Active Ingredient

15. Radiolabelled active ingredients are required to allow quantification of the total, extractable and unextracted radiolabel 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, it may be necessary to conduct an additional study with a radiolabel that tracks the portion of the molecule that is cleaved.

16. In choosing the position to be labelled, assurance is needed that a stable position is selected. The preferred isotope 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 if all significant radioactivity in the crop is identified and found to be associated with the active ingredient, and not related to loss of the label from the basic structure of the active ingredient molecule.

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

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

Application Parameters

19. The method of application and the application rate of radiolabelled active ingredient to be used should be considered in designing a metabolism in crop study. Since the primary purpose of a metabolism study is to identify the chemical components of the residue, the maximum application rate (the proposed Good Agriculture Practice (GAP) application rate) should be utilized to allow for characterization and/or identification of the residue. Where the number of applications in the proposed GAP exceeds three, the number of applications of the active ingredient may be reduced to three or one-third of the total number of single applications, whichever is greater, as long as 1) the total seasonal application to the crop, timing of the initial application, if less than or equal to three months before final application, and PHI are met and 2) the single application rate does not exceed three times the proposed GAP single rate and/or phytotoxicity is not observed. Where the time interval from initial to final application is greater than three months, the intervals among the last few applications should be considered more critical than the initial to final interval, and such situations should be considered on a case-by-case basis.

20. When low residue levels in crops are expected from the maximum application rate, experiments at exaggerated rates may be needed to aid metabolite identification. The decision as to what exaggerated rate to utilize is contingent upon several factors. For example, in the case of herbicides, phytotoxicity may stress or even kill the crops, thus limiting the exaggerated rate that can be used.

21. In the case where only an exaggerated rate is used, then this exaggeration factor cannot be used to calculate "trigger values" (see paragraph 30 and Table 1). For example, if a crop is treated 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 the trigger values for identification and characterization. However, if trials are conducted at both 1X and an exaggerated rate, then the trial at the 1X application rate should be used to decide whether or not the "trigger values" for identification and characterization have been exceeded.

22. The crop should be treated with radiolabelled active ingredient, preferably containing formulation ingredients typical of an end-use product as applied in the field. If the use of an adjuvant is specified in the proposed GAP, then attempts should be made to use the adjuvant in the test formulation. If the active ingredient 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 radiolabeled pesticide in solution is acceptable provided a reasonable justification is given; for example, the active ingredient 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.

23. Selection of specific crops and use patterns consistent with intended GAP should reflect the situation where the highest amount of radioactivity resulting from metabolism would be expected in the edible portions of the crop at harvest. The highest application rate and shortest treatment intervals should be used (but see paragraph 19). The PHI should be selected to reflect the proposed use pattern. The growth stage of the crops at application and sampling should be similar to that of the anticipated field use of the pesticide.

Test Facility

24. Crops may be grown for metabolism studies in outdoor test plots, greenhouse, or plant growth chambers. A description of the physical facility (totally enclosed glass structure with or without environmental controls, polytunnel, closed system with recirculating atmosphere) and a record of environmental conditions during the course of the study, (e.g., temperature, rainfall, lighting), at the testing site should be documented. When a study is conducted in a growth chamber or greenhouse, the potential for photodegradation of the pesticide should be considered.

Sampling of Crop Parts

25. Samples of all RACs should be obtained for characterization and/or identification of residues. For crops that are sometimes consumed at an immature stage, such as baby corn or leafy salads, samples should be taken of such commodities for analysis. Analysis of immature crop parts may also facilitate characterization and/or identification of residues in cases where the "trigger" values are exceeded, but the residues give rise to unusual difficulties in characterization and/or identification due to low residue levels or the nature of the metabolites. Such data may provide adequate information to allow conclusions to be drawn about the identity of residue in mature parts of the crop. [Refer to Annex 3 "RACs to be Analyzed for Metabolism in Crops and Rotational Crop Studies" in the Overview of Residue Chemistry Studies Document (2)].

26. If applicants wish to use mature but inedible crop parts (e.g., apple leaves, potato foliage) to help identify residues on the mature RAC, evidence of similar chromatographic profiles for mature edible and inedible crop portions is necessary.

27. If more than one use pattern is anticipated, extra samples need to be taken to reflect, for example, the different PHIs. Samples should be profiled to show comparability. Identification should be conducted on the most appropriate samples.

Analysis

28. In the initial stages of the analytical phase of a Metabolism in 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. Samples may also be surface washed before sample preparation since this can help in estimating the level of penetration.

29. In commodities with inedible peel such as oranges, melons, and bananas, the distribution of the residue between peel and pulp should be determined.

30. 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.

31. Identification refers to the exact structural determination of components of the TRR. Characterization refers to the elucidation of the general nature/characteristics of the radioactive residue. Terms used to characterize residues include organosoluble, water or aqueous soluble, neutral, acidic or alkaline, polar, non-polar, unextracted radiolabel, 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 complete structural identification.

32. 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.

33. 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 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), 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 (less than 0.05 mg/kg) or percentage of the TRR (less than 10 percent of the TRR), 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 less than 10 percent of the TRR represents a high absolute residue level.

34. 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 stereoisomers may need to be addressed in the supervised field studies.

35. New extraction and analysis techniques may be substituted 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.

36. During the conduct of the Metabolism in Crops study, 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 or dietary risk assessment. Therefore, radiolabelled samples may need to be retained for future analyses by the methods developed subsequently (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. The radiovalidation of the extraction process of analytical methods should be submitted as part of the report on the analytical method. It may stand by itself as a report, or it may be placed in the metabolism report itself. The cover letter or summary of the full data package should indicate where it has been placed.

Characterisation / Identification of Extractable Residues

37. The radioactivity threshold values (“trigger”) shown in Table 1 reflect the characterization or identification needed for each RAC following application of the radiolabelled test compound at the 1X application rate. If the 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 regarding residues occurring at lower levels.

38. 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.

39. If the extractable radioactivity represents 0.01 mg/kg or less, it will not require further analysis. If the extractable radioactivity is greater 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 toxicology concerns regarding potential residues which might occur at lower levels, including 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 conclusively 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.

40. 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.

Table 1. Strategy for Identification and Characterisation of Extractable Residues from Metabolism in Crops

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 radiolabel – See paragraphs 42-46 and Figure 1.

Release and Characterization / Identification of Unextracted Radiolabel

41. There are three situations in which unextracted radiolabels are 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 crop.

- Chemical reaction with or physicochemical tight-binding to appropriate moieties in biomolecules, such as cellulose, hemicellulose, lignin, to form “unextracted radiolabel” residues, 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 residues in this situation may require solubilisation of the tissue, usually by drastic treatment with alkali, although use of surfactants may allow the radioactive residue to be released under less severe conditions.

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

43. At each step in Figure 1, the total radioactivity released should be quantified. 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 less than 0.05 mg/kg or less than 10 percent of the TRR, further attempts to release radioactivity are not necessary.

44. Treatments may be performed sequentially or in parallel. Types of treatments suggested 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.

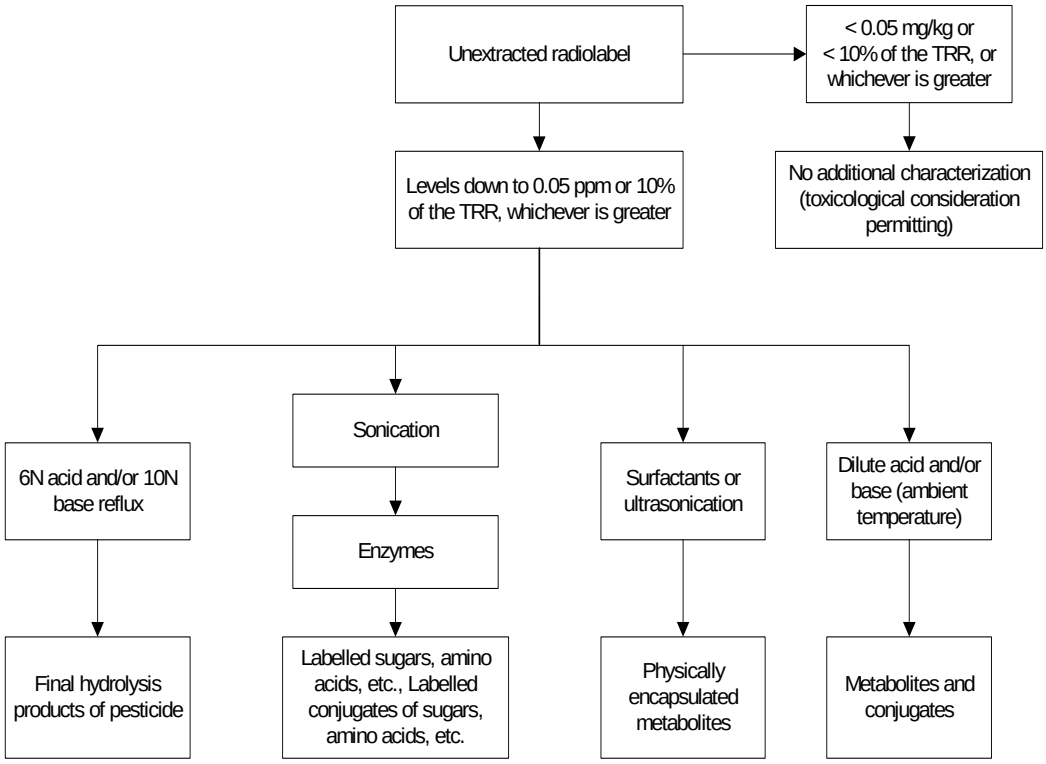
45. Mild acid or alkaline treatment may hydrolyse conjugated moieties, and possibly release any biomolecules containing incorporated radioactivity. The use of surfactants may release physically encapsulated or membrane unextracted radiolabel. Since membrane and/or cell wall disruption may improve substrate 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 unextracted radiolabel including any biomolecules containing incorporated radioactivity.

46. The final release steps could involve reflux acid and alkaline hydrolysis, which will likely solubilise the crop 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.

47. Identification of specific radiolabelled amino acids, sugars, phenolic compounds, nucleotides, etc. may alleviate the need for further characterization and/or identification of unextracted radiolabel 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, greater than 10 percent of the TRR or greater than 0.05 mg/kg, has not been identified.

48. 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 unextracted radiolabel should be observed to assure that the submitted study is adequate.

Figure 1. Characterisation and Identification of Unextracted Radiolabel



Storage Stability

49. 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 6 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.

50. 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.

51. 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.

52. 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

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

Summary / Introduction

(i) Testing strategies employed and the rationale for the selection of these strategies.

(ii) The overall experimental procedure employed should 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 should include a complete description of the identity and quantity (both free and unextracted radiolabel) of all major components of the total radioactive residues. It is preferable that the foregoing information be summarized in a narrative form with tables and/or figures.

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

(v) When enforcement and/or data collection analytical methodology has been developed, it should be validated with samples derived from the crop metabolism study, accompanied by a statement made as to their capability to extract and determine all components of the TRRs, whether free or unextracted radiolabel/conjugated in the RAC. The statement should also indicate the detection limits, precision, and accuracy of the methodology employed. Extraction efficiency may be reported here or submitted as part of the analytical method report, as a stand-alone report, or in the metabolism report

Materials/Methods

1. 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) names; company developmental/experimental name; 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 purity, nature of the radiolabel and its source. If radioactive impurities are at significant levels (i.e., > 5%) then the identity of radiolabelled

impurities, 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 should be 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 counts should be provided so that the relevant regulatory authority can verify the concentration, expressed as mg active substance/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

(i) 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 (not required for materials applied to foliage) at the testing site.

(ii) An explanation or rationale provided by the applicant if the reported testing environment, including testing media, employed in the metabolism study is not representative of or differs significantly from expected cultural practices or environmental conditions under which the test crop would normally be grown.

Explain any meteorological abnormalities that may have impacted the study.

c) Test crop and sample harvesting (collection)

(i) Identification of the test crop including type/variety and crop group classification.

(ii) A rationale or statement provided by the applicant for selection of a test crop other than that for which use is proposed

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

(iv) 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. Any and all additional information the applicants consider appropriate and relevant to provide a complete and thorough description of the test crop.

(v) Harvest procedures (method of harvesting or collection (mechanical/ hand, from the crop/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.

(vi) A description of additional relevant information on the growing of the test crop, applications of the pesticide formulated products, and harvesting of samples.

d) Application of the pesticide.

(i) A description of the type of pesticide application to the test crop (i.e. pre-plant soil incorporated, over the top post emergent foliar application, bait application, etc.), including the formulation, i.e., solvent, carrier, bait, adjuvant, or other matrix, in which the radiolabelled pesticide was applied and the method of application i.e. hand sprayer, topical, soil injection, etc.

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

(iii) Number and timing of applications, between application intervals, and treatment to sampling intervals (TSI) or pre-harvest interval (PHI).

(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; pesticide applications; and harvest of mature crop.

(v) An explanation or rationale 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, pre-shipping 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.

f) Analytical methods used for the analyses of radioactive residues

(i) The capability of the analytical methods utilized in the metabolism study to determine the components of the residue, whether free, conjugated, or unextracted radiolabel.

(ii) Method for quantitation and distribution of total recovered radioactivity in the treated crop for all crop 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) A quantitative accountability of the majority of the total radioactivity recovered from the treated crop at times of sampling or harvest as a result of aggregate sample analyses. Significant losses should be discussed.

(v) 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.

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

(vii) Extraction efficiency, using the proposed enforcement analytical extraction conditions with radiolabelled samples derived from the crop metabolism study, accompanied by a statement made as to their capability to extract the relevant components of the TRRs whether free, conjugated, or unextracted radiolabel in the RAC.

g) Extraction and fractionation of radioactivity

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

(ii) A discussion of and rationale for the selection and extraction sequence for the extracting solvent (polar vs. non-polar) used and extraction procedures i.e. blending, maceration, partitioning, Soxhlet, employed, including use of additional techniques i.e. decomplexing reagents, ultrasonic, etc., should be provided.

(iii) A description of conditions employed for the acidic, alkaline and/or enzymatic hydrolysis of (the filter cake or residue remaining from) previously extracted crop tissue and/or water-soluble crop 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 active ingredient and/or metabolites in each extracted sample matrix.

(v) 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 of total radioactive residue, and mg/kg of total recovered radioactivity. Attempts at unextracted radiolabel extraction by exotic or other procedures, or extractions following repeated treatments with concentrated acids and/or alkalines at elevated temperatures should also be reported by the applicant and a rationale should be provided for their use.

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

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

(viii) The applicants should report detailed procedures for the fractionation of unextracted radiolabel in crop tissues into proteins, starch, lignin, cellulose, etc.

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

(x) 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 active ingredient equivalents) of total radioactivity recovered in the original sample matrix analyzed. For those studies where the

radioactivity is measured in all crop parts, it would be useful to report the percent of total crop radioactivity in each part, but this is not required.

h) Characterization and/or identification of radioactivity

(i) A tabular listing and description of all known and suspected metabolites of the active ingredient (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) Photographs (or radioanalytical imaging detection) of thin-layer chromatographic (TLC) plates, radioautograms, 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. high voltage electrophoresis, ion exchange, or exclusion chromatography, derivatisation, etc., and determinative methods i.e. mass spectroscopy in electron impact (EI) and chemical ionization (CI) modes, used for ultimate identification of metabolites.

(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. The amount reported should be expressed as both percentage and mg/kg (as active ingredient equivalents) of total radioactivity recovered from the particular crop part or fraction analyzed.

(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 complex unextracted radiolabel originating from the active ingredient in edible crop parts used for food or animal feed.

Results and Discussion

1. Test strategies

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.

2. Metabolic pathways

Discussion, accompanied by a flow sheet format, of the routes of degradation or pathways of metabolism observed in the subject RAC 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. Based on the results of the characterization and/or identification studies, the chemical definition of the metabolic pathway should be proposed in each crop 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.

3. 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, unextracted radiolabel, natural constituents including name, structure, and quantity (expressed both as percentage of TRR and mg/kg as active ingredient equivalents), and report their distribution within the RAC crop parts.

(ii) If the immature RAC (including crop parts and processed fractions thereof) is normally utilized for animal feed, then identification and quantification of all major components of the residue present at that stage of crop development must also be reported.

(iii) The 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 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 crop metabolism study. Provide the limit of quantification for radioactivity determination and chromatographic separation.

(v) Any and all additional information the applicants consider appropriate and relevant to provide a complete and thorough description of the crop metabolism 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 when the subject RAC is grown to maturity or harvest.

(ii) The nature, amount, and distribution of the TRRs in the RAC at the time of harvest or when normally utilized for animal feed resulting from the proposed use of the pesticide.

(iii) The results of validation studies conducted on radiolabelled crop samples, if conducted, should also be discussed, including, the capability of developed and available enforcement analytical methodology to determine the identified components of the residue definition.

Tables/Figures

a) Tables (for example):

(i) Weather and/or environmental data.

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

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

(iv) HPLC/GLC retention times and TLC Rf values for active ingredient, 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) Cite 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).

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

Study Report

54. The study report should contain the following information:

– Identification of the test 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 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 for 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 (not required for materials applied to foliage) at the testing site.

- Identification of the test crop including type/variety and crop group classification.

- A detailed description of the application parameters: type(s) of pesticide application to the test crop; formulation in which the radiolabelled pesticide was applied; method of application; rate(s) of application; number and timing of applications (including growth stage at application(s)); preharvest interval(s).

- A description of the harvest, including technique, crop growth stage and size at harvest, crop parts harvested, and handling and shipping and storage of the harvested crop parts.

- A description of the preparation and analysis of crop parts for total radioactive residue determinations.

- A careful and full description of the extraction and fractionation of radioactivity in the various crop matrices, including reports on the amount of radioactivity in each sample fraction, quantified in terms of total radioactivity (Bq or dpm) and as both percentage and concentration (mg/kg, as active ingredient 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 total radioactive residue (% 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 of the storage stability for all major components of the total radioactive residues.

- Quantitative information on the recovery of the radioactive residue 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 subject RAC.

- Conclusions on: (a) pathways or routes of metabolism and extent of metabolism observed for the subject RAC at maturity or harvest; (b) nature, amount, and distribution of the TRR in the RAC at harvest or when utilized as an animal feed; and (c) results of validation studies conducted on crop metabolism crop samples 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 following set of documents:

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

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

(3) U.S. Environmental Protection Agency (1996). OPPTS Test Guidelines Series 860, Residue Chemistry, Washington, D.C.
http://www.epa.gov/oppts/

(4) European Commission (1997). Appendix A – Metabolism and Distribution in Plants, Document 7028/VI/95 rev. 3, 22/7/97, Directorate General for Agriculture VI B II-1. Draft Part B1, Metabolism in Plants, personal communication.

(5) 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.

(6) Canada. Pest Management Regulatory Agency (PMRA) (1998). Residue Chemistry Guidelines, Directive 98-02. http://www.pmra-arla.gc.ca/

(7) Japan Ministry of Agriculture, Forestry, and Fisheries (MAFF) (2000). Data Requirements for Supporting Registration of Pesticides, 2-4-1 Studies of metabolic fate in plants, Notification No. 12 – Noan – 8147, 24 November 2000.

(8) Australia Pesticides and Veterinary Medicines Management Agency. Residue Guidelines. http://www.apvma.gov.au/

ANNEX 1

Crops and Crop Groups for Purposes of Metabolism in Crops Studies

Metabolism in crops studies are needed for relevant crops; therefore, for each crop on which use is proposed, a metabolism study is needed from that category. A maximum of three categories is adequate, provided a consistent metabolism picture is obtained from the three studies. Additional categories may need to be studied if there are differences in metabolism across groups.

Code	Category	Crops
F	Fruit	Citrus fruit Tree nuts Pome fruit Stone fruit Berries Small fruit Grapes Fruiting vegetables Banana Persimmon
R	Root crops	Root and tuber vegetables Bulb vegetables
L	Leafy crops	Brassica vegetables Leaf vegetables Stem vegetables Hops Tobacco
C/G	Cereal/Grass crops	Cereals Grass and forage crops
P/O	Pulses and oilseeds	Legume vegetables Pulses Oilseeds Peanuts Legume fodder crops Cacao beans Coffee beans
-	Miscellaneous	In general, crops not listed above or not covered by a grouping are considered as miscellaneous and will not normally be accepted as one of the three crop groups. However, if it is proposed to use such a crop to cover one of the three crop groups due to its national/regional importance, applicants are strongly urged to consult with regulatory authorities.