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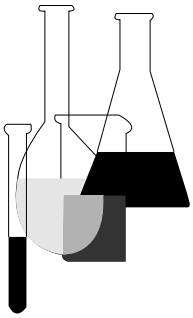
Residue Chemistry Test Guidelines

OPPTS 860.1300 Nature of the Residue—Plants, Livestock



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

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OPPTS 860.1300 Nature of the residue—plants, livestock.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 301, *et seq.*).

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are 0PP 171–4 Results of Tests on the Amount of Residue Remaining, Including A Description of the Analytical Methods Used and 171–16 Translation of Data (Pesticide Assessment Guidelines, Subdivison O: Residue Chemistry, EPA Report 540/9–82–023, October 1982). This OPPTS guideline should be used in conjunction with OPPTS 860.1000, Background.

(b) **Purpose.** The purpose for conducting metabolism studies is to determine the qualitative metabolic fate of the active ingredient, i.e. examine what happens to it when it is applied to a plant or administered to livestock. Many pesticides undergo change during or after application to the soil, water, crop, or livestock. The composition of the terminal residue must therefore be determined before complete residue detection methodology and residue quantification data can be developed. To obtain this information, the pesticide is labeled with a radioactive atom, to follow the compound to see if and where it breaks down within a plant or livestock. The determination of whether the residues have been sufficiently characterized/identified is dependent on many factors. Plant metabolism studies are usually required for a minimum of three diverse crops (unless the pesticide is to be used on only one or two crops). If the metabolism in three diverse crops is similar, then the metabolism in other crops is assumed to be similar. If the pesticide is applied to crops used for livestock feed, or if the pesticide is intended for treatment of livestock, then livestock metabolism studies are required in addition to plant metabolism data. Livestock metabolism studies are generally carried out on ruminants (cows or goats) and poultry (chickens).

(c) **Introduction**—(1) **General.** (i) While in vitro data are useful to show if the pesticide is likely to undergo hydrolysis (acid, base, or enzymatic), oxidation or reduction, photolysis, or other changes, additional data must usually be submitted to show the fate in the plants and livestock. These metabolism studies are required whenever a pesticide use is determined to be a food use. Based on the results of the characterization and/or identification studies, the chemical definition of the total toxic residue (TTR) should be proposed. The term total toxic residue is used to describe the sum of the parent pesticide and its degradation products, metabolites (free or bound), and impurities which are of toxicological concern. All components of the TTR will normally be included in the tolerance expression for the pesticide and residue analytical methods must be developed for all components of the TTR.

(ii) The identification of the components of the terminal residue and the definition of the TTR often present complex problems that must be resolved before finalizing the analytical methodology and gathering the residue quantification data. Thus, petitioners may wish to consult with the Agency's chemists and toxicologists to determine whether the residue has been sufficiently characterized and/or identified, which metabolites should be covered by the tolerances, and which components of the residue must be determined by the residue analytical methodology. The determination of whether the residue has been sufficiently characterized and/or identified will depend on the level of activity remaining unidentified, the importance of the plant or livestock commodity containing the unidentified residue as a food or feed, the chemical structure of the active ingredient and identified metabolites, and the toxicity of chemicals similar in structure to potential metabolites.

(iii) Petitioners should delineate, preferably in a flowsheet, the routes of degradation or metabolism in plants and livestock, and clearly specify the capability of the analytical methods utilized in the metabolism study to determine the components of the residue, whether free or bound. Photographs of thin-layer chromatographic (TLC) plates, paper chromatograms, radioautographs, or output from other appropriate imaging systems.

(iv) The petitioner should always be alert to the possibility of new and unexpected metabolites of the pesticide which may affect future tolerance proposals. Where the structure of a metabolite or alteration product is identical to another registered pesticide chemical, the petitioner should state this fact.

(2) **Nature of the residue in plants.** (i) The term plant metabolism is used here for convenience to describe the formation of all alteration products of the pesticide in or on plants regardless of whether they result from plant metabolic processes. Adequate plant metabolism studies fulfill at least four purposes:

(A) They provide an estimate of total residues in the treated crops.

(B) They identify the major components of the terminal residue, thus indicating the components to be looked for in residue quantification studies (i.e. the TTR).

(C) They indicate the distribution of residues, e.g. whether the pesticide is absorbed through roots or foliage, whether translocation occurs, or whether the residues are entirely surface residues.

(D) They show the efficiency of extraction procedures for various components of the residue.

(ii) A metabolism study must be submitted for each type of plant for which use is proposed. For example, metabolism studies in bean plants

would be representative of all legumes but would not be translatable to root crops such as potatoes or carrots. In general, one metabolism study will be required for each of the crop groups defined in CFR 40 180.34(f), except for herbs and spices.

(iii) If the results of three metabolism studies on dissimilar crops indicate a similar metabolic route in the three crops, then additional metabolism studies will not be required.

(3) **Nature of the residue in livestock.** (i) The purpose of these studies is to identify the nature of the residue in the edible tissue of livestock, milk, and eggs. Animal metabolism studies are required whenever a pesticide is applied directly to livestock or to crops or crop parts used for feed, or when livestock premises are to be treated. Information on whether crop byproducts are used for feed can be obtained from Table 1 of OPPTS 860.1000.

(ii) Data on the metabolism of a pesticide in laboratory animals which are required in the toxicology section of these guidelines will generally not substitute for metabolism data on livestock. Laboratory animal metabolism studies should, however, be summarized or referenced in the residue chemistry section of a petition to allow for comparisons of the metabolism in several species. In some cases laboratory animal metabolism data may be used to supplement livestock metabolism studies in which complete characterization and/or identification of the residue is not attained.

(iii) In general, separate metabolism studies are required for ruminants and poultry. The species of choice are usually goats and chickens. Non-ruminant (swine) metabolism studies may be required if the rat metabolism is significantly different than goat or chicken metabolism. Additional animal metabolism studies are required if direct dermal or inhalation application to livestock is proposed. These additional studies should reflect the proposed use so that it can be determined whether dermal or inhalation exposure results in the same metabolic patterns as oral dosing.

(iv) The minimum dosage used in livestock metabolism studies should approximate the level of exposure expected from the feeding of tolerance level residues on crops with existing, proposed, or anticipated tolerances, or the proposed use rate for direct livestock treatment. However, exaggerated dosages are usually required to obtain sufficient residue in the tissues for characterization and/or identification. Regardless, for oral studies, livestock must be dosed at least at a level of 10 ppm (i.e. 10 mg/kg feed) in the diet (see paragraph (h)(10) of this guideline). Livestock dosed orally should be dosed daily for at least 3 days. The dosing material for oral studies should not be a mixture of active ingredient and plant metabolites. In most cases this study should involve dosing with only the parent pesticide. In those cases where plant and livestock metabolites are found to differ, a separate study in which livestock are dosed with a unique plant

metabolite may be required in addition to the study with parent compound. Direct livestock treatment dosing should reflect the proposed use with regard to the dosing material and mode of application.

(v) The Agency strongly discourages predosing of livestock. Due to possible changes in the specific activity of the parent and metabolites, predosing may result in low levels of radioactivity in tissues, milk, and eggs masking both the degree of residue transfer and precluding the identification of the components of the terminal residue. Also, the resulting differences in specific activities of components of the total radioactive residue (TRR) may make the comparison of relative amounts of parent and metabolites problematical. However, the acceptability of studies employing predosing will be considered on a case-by-case basis. If the radioactivity levels in such a study low enough to preclude identification of residues, the study will need to be repeated without predosing the livestock.

(vi) Livestock should be sacrificed within 24 hours of cessation of dosing.

(vii) Milk and eggs should be collected twice daily. Tissues to be analyzed should include at least muscle, liver, kidney (ruminants only), and fat. Characterization of the residue in urine and feces frequently facilitates characterization of the lower levels of residue found in tissue, but is not required.

(viii) The livestock metabolism study should primarily identify the compounds for which analytical methods and residue data must be generated. It should also indicate the distribution of residues in tissues, eggs, and milk. The livestock metabolism study should also result in elucidation of the efficiency of extraction of the various components of the residue so that extraction/residue release procedures can be developed as part of the analysis.

(d) **Discussion of test method**—(1) **Application of radiolabeled pesticide**. (i) The first consideration in designing a metabolism study is radiolabeling. The radiolabel should be positioned in the molecule so that potentially significant toxicological moieties can be tracked. In choosing the position to be labeled, assurance is required that a labile position is not chosen. This should involve ring labeling (preferred) or even double labels, i.e. molecules containing two rings are labeled in both or each ring is labeled in separate experiments. ^{14}C is the preferred isotope, although ^{32}P, ^{35}S, or other elements may be more appropriate if no carbons, or only labile carbon side chains, exist in the molecule. The use of tritium as a label is strongly discouraged. If a potentially labile side chain or tritium labeling is chosen, a metabolism study will be considered adequate only if all significant activity in the plant or livestock 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.

(ii) Other initial considerations include the method of application and the application rate of radiolabeled pesticide to be used. Since the primary purpose of a metabolism study is to identify the chemical components of the residue, the application rate must be high enough to result in sufficiently high radioactivity levels to allow for characterization and/or identification of the residue. A rate of at least 1× (the registered application rate) should generally be used for plant metabolism or dermal livestock metabolism studies. In the case of oral livestock metabolism studies, the dose should, at a minimum, approximate the maximum anticipated dietary burden, but in no instance should the level be less than 10 ppm in the diet (i.e. 10 mg/kg of feed). However, for certain pesticides/uses it is necessary to apply radioactive material at exaggerated rates. The decision as to what rate to utilize is contingent upon several factors. For example, in the case of herbicides, phytotoxicity which may stress or even kill the plants may limit the exaggerated rate which can be used. For all pesticides, the minimum application rate required to allow adequate characterization and/or identification of residues (up to a maximum of 10× as discussed at paragraph (d)(4)(iii) of this guideline) must be utilized in plant metabolism studies unless reasons such as phytotoxicity prevent this. Safety concerns when using large amounts of radioactivity must also be considered. In addition, the following should be considered when selecting the dosing material, a method of application and an application rate or dosage for plant or livestock metabolism studies:

(A) The plant should be treated with parent only.

(B) Livestock metabolism studies should reflect feeding of one compound, usually the parent. If the plant metabolites are also found to be animal metabolites, then additional livestock metabolism experiments which involve dosing with plant metabolites will not generally be required. However, as discussed under paragraph (c) of this guideline, if a plant metabolite comprises a major portion of the TRR on a feed item or is not found to be an animal metabolite, additional livestock metabolism studies involving dosing with the plant metabolite may be required.

(C) The specific activity of the labeled material should be high enough to assure acceptable limits of detection for radioactive residues. In cases where there has been little or no characterization/identification of the residue, in crops, milk, eggs, or animal tissues because of low levels of activity, the Agency will make a determination as to the adequacy of efforts registrants have made to maximize specific activity so that application rates would yield characterizable/identifiable levels of radioactivity in edible plant parts or livestock commodities.

(D) In cases where low levels of radioactivity are observed even at exaggerated rates, utilization of adjuvants or typical inerts may enhance absorption of the active ingredient into the plant or animal (dermal).

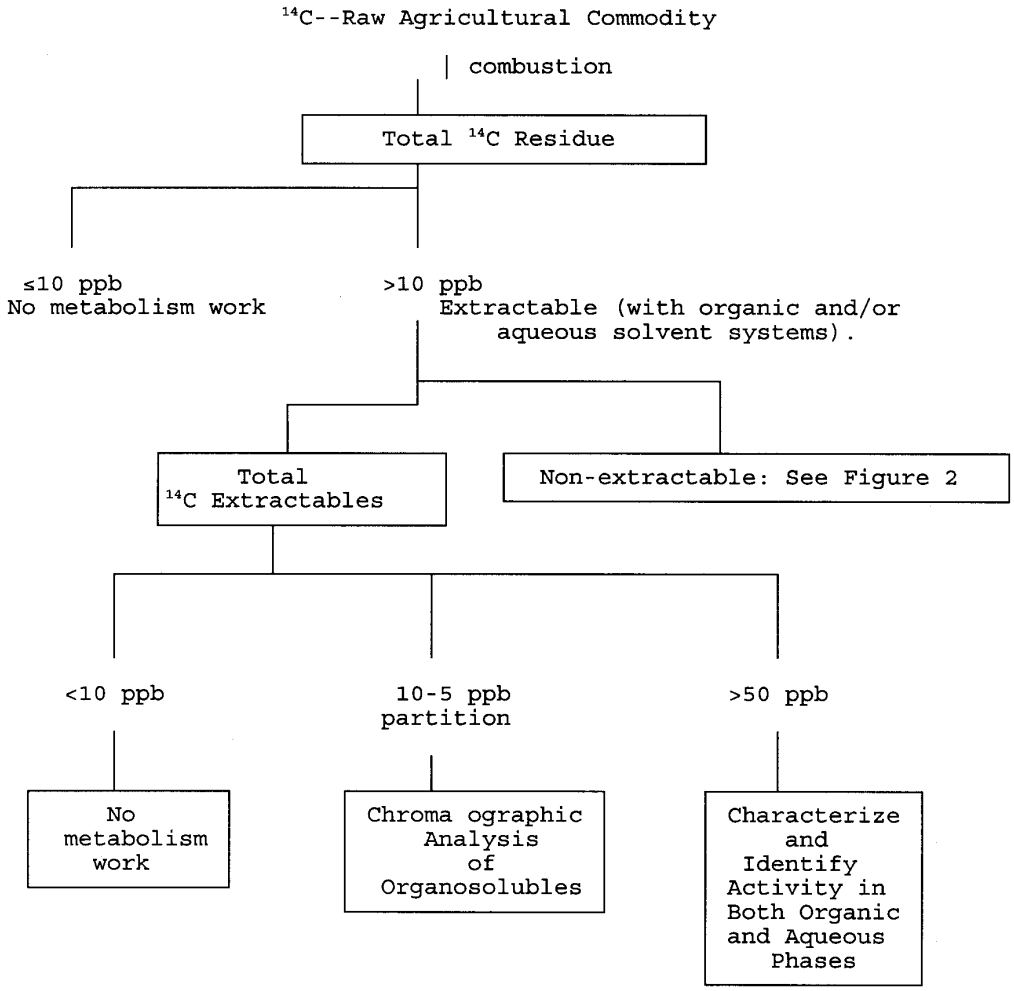
(E) Selection of specific crops and use patterns should reflect the situation where the highest amount of radioactivity would be expected in the edible portions of the plant at harvest. If a pesticide has two distinct use patterns that could lead to different metabolic situations (e.g. preplant soil application and a foliar treatment), then two metabolism studies may be required.

(F) If exaggerated application rates of a phytotoxic herbicide are necessary to achieve sufficient radioactivity for characterization and/or identification of residues, and the required rate causes phytotoxicity in the plant, metabolism information on the ‘‘sick’’ plant is preferable to having no information due to lack of sufficient radioactive residue.

(2) **Sampling of plant parts.** Samples of all raw agricultural commodities (RACs), as defined in Table 1 of OPPTS 860.1000, should be obtained for characterization and/or identification of residues. In some cases, collection of samples of immature plant parts not in Table 1 of OPPTS 860.1000 may be considered as an aid to facilitate the characterization and/or identification of residues when low residue levels are expected in the mature plants. Although collection of immature plant parts not in Table 1 of OPPTS 860.1000 is not required (note that materials such as corn forage are immature plant parts but are considered to be RACs), it may facilitate characterization and/or identification of residues in cases where the ‘‘trigger’’ values (discussed under paragraph (d)(4)(i) of this guideline) are exceeded, but residues present unusual difficulties in characterization and/or identification due to low residue levels or the nature of the metabolites. These data may provide adequate information to allow conclusions to be drawn about the identity of residue in mature parts of the plant. Registrants may also wish to use mature but inedible crop parts (e.g. apple leaves, potato foliage) to help identify residues on the mature RAC. However, if this information is to be used in support of the study, evidence of similar chromatographic profiles for mature edible and inedible plant portions is preferred.

(3) **Analytical phase.** (i) In the analytical phase of a plant/livestock metabolism study, the plant/animal parts to be analyzed are sampled, chopped or homogenized, total radioactivity is determined and the samples are extracted with a series of solvents and/or solvent systems (including aqueous) with various polarities and other characteristics depending on the nature of the expected residues. These initially obtained residues are defined as extractable residues. The required characterization and/or identification of extractable residues is summarized in the following Figure 1. (This is a diagram of ‘‘trigger’’ values described in paragraph (d)(4)(i) of this guideline.)

FIGURE 1: STRATEGY FOR IDENTIFICATION/CHARACTERIZATION OF EXTRACTABLE RESIDUES FROM PLANT AND LIVESTOCK METABOLISM STUDIES



(ii) Before discussing Figure 1 in greater detail, the terms characterization and identification of residues will be defined as follows:

(A) Identification refers to the exact structural determination of components of the TRR. Typically, this is accomplished by comparing chromatographic behavior to that of known standards and/or actual spectroscopic analyses (mass spectrometry (MS), nuclear magnetic resonance (NMR), etc.).

(B) Characterization refers to the elucidation of the general nature/characteristics of the radioactive residue short of metabolite identification. Terms used to characterize residues include organosoluble, water or aqueous soluble, neutral, acidic or basic, polar, nonpolar, nonextractable, etc.

Characterization may also involve descriptions of chemical moieties known to be present in the molecule based on conversion to a common structure or due to reactivity with particular reagents. The degree of characterization refers to how close the assignment comes to structural identification. 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, 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 (i.e. by conversion to a common moiety) but unidentified residues. (This radiovalidation of the method would be important both for future development of enforcement methodology and in a case where a significant amount of radioactivity is observed in a matrix but it consists of a large number of individual moieties at levels below ''trigger'' values but which can be converted to one or two distinct compounds by procedures such as oxidation or hydrolysis.) Therefore, the terms characterization and identification clearly have different meanings and should not be used interchangeably.

(iii) Identification of metabolites must be established using two different analytical techniques except when unambiguous identification is made using a spectroscopic method such as gas or liquid chromatography/mass spectrometry (GC/MSX or LC/MS), or the metabolite is determined to be of minimal importance due to its low absolute level (<0.05 ppm) or percentage of the TRR (<10 percent of TRR). In the second case, identification by one technique such as co-elution with standards 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 TRR represents a high absolute residue level. In general, the Agency will not consider chromatographic techniques utilizing the same stationary phase with two different solvent systems to be adequate two-method verification of metabolite identity.

(4) **Strategy for determining when identification of metabolites is needed.** (i) The strategy illustrated in Figure 1 for extractable polar and nonpolar residues was developed by Ciba-Geigy (see paragraph (h)(9) of this guideline) and initially was applied primarily to animal metabolism studies. The radioactivity trigger values shown in Figure 1 reflect the characterization and/or identification required for each RAC. If total activity in a crop/animal part is ≈ 0.01 ppm (10 ppb) or less, no differentiation of the radioactivity would be required unless there are toxicological concerns over residues occurring at lower levels. For activity greater than about 0.01 ppm, the sample should be extracted with solvents and/or solvent systems (including aqueous) of various polarities. The levels of ex-

tractable and nonextractable activity should then be quantitated to determine the degree of characterization that is needed. If the extractable activity represents about 0.01 ppm or less, it need not be examined further. For extractable activity of about 0.01-0.05 ppm, the partitioning behavior between aqueous and organic solvents should be determined, followed by chromatographic analysis (TLC, high performance liquid chromatography (HPLC)) of the organosoluble activity. The chromatographic behavior of this activity can be compared to that of the parent pesticide and likely metabolites (characterization and/or identification). When the extractable activity exceeds ≈ 0.05 ppm, complete characterization and identification should be attempted for both organic and aqueous activity. It is important that the components of the aqueous soluble portions of the radioactivity be identified since they may contain toxic compounds. For the aqueous soluble portion of the activity however, the ''trigger'' values for characterization and identification would be levels down to 0.05 ppm or 10 percent of the TRR, whichever is greater. The exception for this would be toxicology concerns over potential residues which might occur at lower levels. Identities of metabolites should be confirmed with a second technique, spectroscopic if possible.

(ii) Complete characterization and identification for extractable residues above 0.05 ppm does not necessarily mean that individual components at this level need to be identified. Low level (in terms of both parts per million and percent of total residue) individual residues do not typically need to be identified if the major components of the residue have been identified. For example, if the total activity in a crop part is 3 ppm and 75 percent of that has been firmly identified, it is unlikely that identification of a series of individual residues in the 0.05-0.1 ppm range would be required. On the other hand, extensive efforts toward identification of 0.05-0.1 ppm residues would be expected when the total activity is only 0.3 ppm.

(iii) The radioactivity levels shown in Figure 1 apply regardless of the application rate used in plant metabolism studies. However, this is not meant to discourage use of exaggerated application rates necessary to provide sufficient radioactivity for adequate delineation of the plant metabolism. If application rates are insufficient to provide adequate radioactivity for characterization and/or identification of residues, additional studies may be required at increased application rates up to the point of unacceptable plant phytotoxicity. The maximum exaggerated rate which will be required for a plant metabolism study is 10×. The use of highly exaggerated doses in livestock metabolism studies for situations where low residues are present on feed items is discussed in paragraph (e)(8) of this guideline. It is important to note that plant metabolism studies with little or no identification of residues will not normally be acceptable to support new uses which reflect different kinds of treatments, especially modes of applications that result in higher residues.

(iv) Other recent techniques which, depending on the circumstances, may be appropriate to utilize as alternate extraction procedures prior to the techniques suggested under paragraph (d)(5) of this guideline are supercritical fluid extraction and microwave extraction.

(5) **Release of nonextractable/bound residues.** (i) The remainder of this discussion will pertain to nonextractable/bound radioactive residues and will provide guidance on what steps need to be taken to provide enough information to allow the Agency to draw conclusions as to the terminal residue of concern in plants/livestock.

(ii) There are three situations in which radioactive residues are observed to be nonextractable in plants/livestock.

(A) Incorporation into biomolecules (i.e. amino acids, sugars, etc.) which occurs when the test compound is degraded into small (usually one or two) carbon units which enter the carbon pool, and which the plant/ animal uses to build new compounds.

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

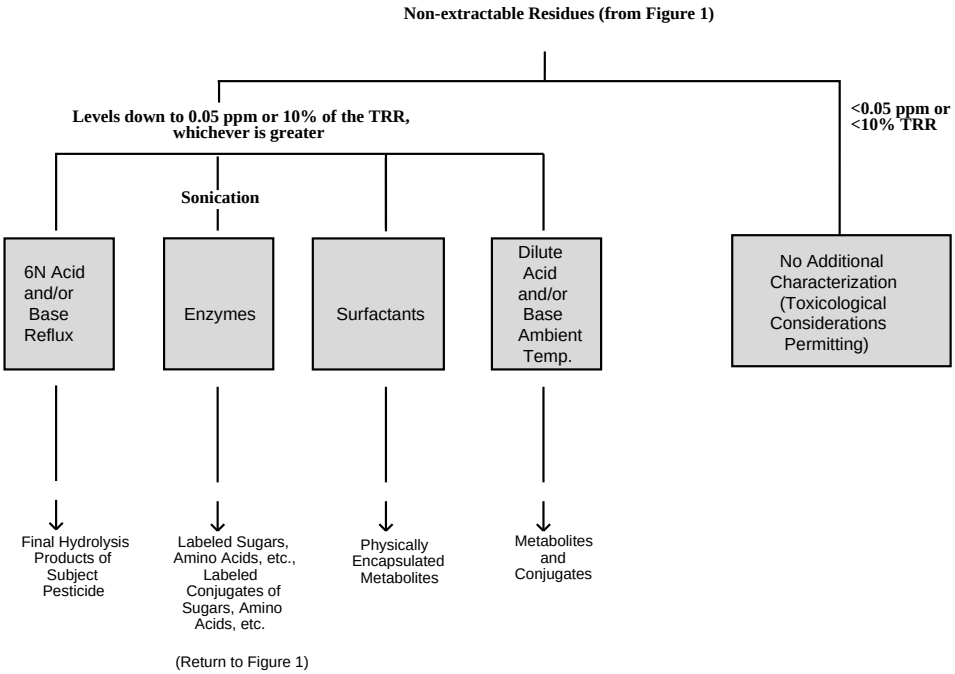
(C) Physical encapsulation or integration of radioactive residues into plant/livestock matrices (such as cellulose and lignin for plants). Release of residues in this situation may require solubilization of the tissue, usually by drastic treatment with base, although use of surfactants may allow the radioactive residue to be released under less severe conditions.

(iii) The following general road map for dealing with nonextractable/ bound residues is intended to provide clarification of Agency policy as well as more specific guidance regarding characterization and/or identification of these residues.

(iv) The extracted solid plant/animal material (as shown in Figure 1) should be assayed and, if radioactivity is present down to the trigger values of the greater of 0.05 ppm or 10 percent of the TRR, release of the activity should be attempted (see the following Figure 2). It is emphasized that, if toxicology expresses concerns over potential residues at lower levels, the trigger values will not necessarily apply. Treatments may be performed sequentially or on subsamples. The types of treatments include addition of dilute acid and/or base at ambient temperatures (note that these procedures should be employed initially for both metabolism and method development considerations), or the use of surfactants, enzymes, and 6N acid and/or 10 N base with reflux. It should be kept in mind that the milder procedures provide more accurate assignments of metabolite structures released, i.e. acid/base reflux would probably release moieties as their final hydrolysis products which could have only a minor relationship to the conjugated form of the radioactive portion. An ambient temperature

acid treatment followed by ambient temperature base treatment will provide a mild hydrolysis of conjugated moieties, and again possibly release any biomolecules containing incorporated radioactivity. The use of surfactants may release physically encapsulated or membrane-bound residues. Because membrane and/or cell wall disruption may improve substrate accessibility to the enzyme, a sonication step should be employed, followed by a carefully chosen enzymatic battery. (Note: In each case the activity of each enzyme utilized should be confirmed using standard substrates and controls. These experiments should be documented.) These steps could release chemically-bound residues including any biomolecules containing incorporated radioactivity. The final release steps would involve reflux acid and base hydrolysis which will likely solubilize the plant/animal part/tissue. Radioactivity released at this time would probably reflect amino acids, sugars, and encapsulated or conjugated compounds which may or may not have any relationship to the original bound/encapsulated structures. However, this step does provide evidence that residues of the pesticide can be released, and may provide data on incorporated radioactivity and limited information about the nature of the metabolites. In all cases, samples, homogenates, and extracts should be buffered and maintained at low temperatures except during hydrolytic steps in order to reduce degradation/artifact formation (see the discussion in paragraph (d)(7) of this guideline regarding storage stability in metabolism studies). Figure 2 provides a visual description of the steps discussed above.

Figure 2: Characterization/Identification of Unextractable/Bound Residues



(v) Comments on Figures 1 and 2. (A) At each step shown in Figure 2, the radioactivity of the released residues should be quantitated. If the

trigger values shown in Figure 1 for extractable residues are met, the activity should again be partitioned against various solvents/solvent systems and characterized and/or identified as required. With respect to characterization, it should be emphasized that the chromatographic behavior of the released activity (including water soluble materials) should be compared to that of the parent and likely metabolites which are close in structure to the parent. This will indicate whether the released activity is chemically different from the parent molecule. If the remaining nonextracted activity after a given procedure is <0.05 ppm or <10 percent of the TRR, whichever is greater, further attempted release of activity is not necessary.

(B) The trigger values shown in Figure 1 are meant to eliminate the need for characterization and/or identification of metabolites present at very low and insignificant levels. However, in many cases, a potentially important metabolite may partition into multiple fractions because of solubility characteristics, and/or because it is present in both free and conjugated forms. In order for the trigger values to apply, particularly in cases where the TRR is distributed among numerous fractions, it must be demonstrated (e.g. by HPLC analysis of each fraction) that no single metabolite is distributed among the various fractions in such amounts so that the combined level (sum) of this component significantly exceeds the trigger value.

(C) Identification of specific radiolabeled amino acids, sugars, phenolic compounds, nucleotides, etc., may alleviate the need for further characterization and/or identification of bound residues in many instances, since this usually means that the pesticide has been degraded into small carbon units which have entered the carbon pool. This conclusion does not, however, apply to tritium labeled compounds, or to pesticides in which the ^{14}C label is incorporated at a labile site in the pesticide molecule. This conclusion would also not apply in cases where a single released metabolite, which comprises a significant portion of the TRR (>10 percent of the TRR or >0.05 ppm), has not been identified.

(D) When a fraction such as lignin, cellulose, or protein contains radioactivity, the radioactivity does not necessarily consist of radioactive amino acids or sugars. The radioactivity may consist of biological macromolecules having radioactive portions of the pesticide either chemically conjugated onto them, or physically encapsulated within them. This is an important distinction from having the macromolecules constructed from low molecular weight radiolabeled building blocks. Registrants are responsible for providing such determinations in a scientifically supportable manner. The Agency will make an evaluation of the data and, if not already provided, require definitive information regarding which of the three conditions exist (i.e. incorporation, conjugation, or encapsulation).

(6) **Further comments.** (i) The pathway described above should be viewed as a broad outline of the type of information needed to determine that a plant/livestock metabolism study is acceptable. Different procedures

and methodologies may be appropriate in a given circumstance. The basic concepts regarding ‘‘trigger’’ values for identification of radioactivity, methodologies required for characterization and/or identification of radioactivity, and steps which should be taken to assure adequate release of nonextractable/bound residues must be observed to assure that the submitted study is adequate.

(ii) The following additional comments should be considered in carrying out a plant/livestock metabolism study.

(A) For a case where bound residues are present at levels down to 0.05 ppm or more than 10 percent of the TRR (whichever is greater), the Agency will require workup and attempted identification.

(B) All unsuccessful attempts at releasing nonextracted activity and characterization and/or identification of the TRR should be documented and submitted.

(C) The Agency will not accept situations where the degree of exaggeration of the application rate or livestock dietary burden is used to calculate trigger values. For example, if a crop/animal is treated/dosed with radiolabeled material at an exaggerated rate (e.g. 5×), the resulting radioactivity levels should not be divided by the degree of exaggeration (e.g. 5) to arrive at trigger values. However, when the Agency decides which identified residues are to be of regulatory concern, the degree of exaggeratiom of relevant metabolism studies will be considered.

(D) Consultation with the Agency prior to initiation and during the metabolism study is appropriate and encouraged.

(E) The discussion above is intended to provide guidance on how a plant/livestock metabolism study is to be conducted. However, plant/livestock metabolism studies are complex and defy a protocol which follows strict adherence to established criteria. The scientific techniques used to study xenobiotic metabolism and conjugate formation, isolation of plant/animal macromolecules, and procedures for generating monomers/oligomers are constantly advancing. It is, therefore, the responsibility of registrants to utilize state-of-the-art techniques and provide citations of such techniques when they are used. Plant/livestock metabolism studies will always be examined on a case-by-case basis, and will frequently require scientific judgment to come to sound conclusions and to make recommendations.

(F) The desired result of a metabolism study is identification of 90 percent of the TRR in each RAC. However, the Agency recognizes that in many cases this is not possible, especially when low total levels of residue are present and/or when the pesticide is extensively metabolized to numerous low level components. In the latter case it is important for registrants to demonstrate clearly that numerous components are present

and, as discussed above, attempt to characterize these residues by conversion to a common moiety where feasible.

(7) **Storage stability.** The Agency needs to make determinations as to whether sample integrity was maintained during collection, preparation, and storage. In light of the difficulty of spiking samples before the identity of the residue is known and the length of time needed for metabolism studies, the present Agency position is that storage stability data should not normally be required for samples analyzed within 4 to 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. In those cases where a metabolism study can not be completed within 4 to 6 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. Such analyses should show that the basic profile of radiolabeled residues has not changed during that time. 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 required. Registrants are referred to OPPTS 860.1380, Storage Stability Data, for further details.

(e) **Clarifications.** (1) With respect to the determination of total radioactivity in a plant part, it is difficult to obtain a representative subsample that will give accurate total ^{14}C by combustion for samples where the residue is not evenly distributed or which have a high water content. For these types of samples, it would be acceptable instead to use a combination of extraction and combustion in order to determine the total residue. Since the weighed subsample is extracted by maceration, and the supernatant is separated by centrifugation, there are no losses due to work-up. Radioactivity in the liquid extract is determined by liquid scintillation counting (LSC) and radioactivity in the solid residue (which will be much more evenly distributed than in the original sample) is determined by combustion and LSC.

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

(3) With regard to whether the specific activity should be reported as microcuries per milligram (μCi/mg) instead of disintegrations per minute per gram or Curies/mole, any units that would permit calculation of parts per million radioactivity using reported counts are acceptable. Sufficient information on counts should be provided so that the Agency can verify the parts per million reported for crop parts, livestock tissues, and the various chromatographic fractions thereof. Regardless of the unit used,

a sample calculation should be submitted showing how the analyst arrived at parts per million from the experimental data.

(4) Photos (or radioanalytical imaging detection) of TLC plates or autoradiograms that were critical to the identification should be provided. If HPLC coupled to a detector capable of measuring radioactivity was employed, then appropriate liquid chromatograms should be submitted. Regardless of the chromatographic technique used, chromatograms showing the behavior of the analytical standards should also be included in the report.

(5) At a minimum, registrants should report the total ppm radioactivity (usually in ppm equivalents of parent pesticide) for each crop/livestock part/tissue that could be used for food or feed. For those studies where the activity is measured in all plant/livestock parts/tissues, it would be useful to report the percent of total plant/animal activity in each part/tissue, but this is not required.

(6) The radiovalidation of analytical methods should be submitted as part of the report on the analytical method (see OPPTS 860.1340), or it may stand by itself as a report. The cover letter or summary of the full data package should indicate where it has been placed.

(7) Livestock metabolism studies are now required whenever a pesticide is to be used on a crop having a livestock feed item in Table 1 of OPPTS 860.1000.

(8) It should be noted that the above part per million trigger values are not absolute requirements, but rough guides as to how much characterization and/or identification is adequate. In the metabolism studies in which highly exaggerated feeding levels are employed and low activity results in tissues, characterization and/or identification requirements should be less stringent than when expected dietary burdens lead to significant activity in animal products. For example, if the anticipated dietary burden to livestock is about 0.01 ppm, 10 ppm radiolabeled compound is fed (1,000×), and total activity in tissues, milk, or eggs is < 0.1 ppm, minimal characterization and/or identification of residues should be adequate (unless toxicologists express a special concern with residues at this level). Such situations often arise with early season herbicides having low application rates.

(9) When activities ≥ 0.1 ppm are observed in livestock commodities from ingestion of the pesticide at levels expected on feed items, thorough identification of the residues is generally required. This is likely when pesticides are applied to foliage at high rates through the entire growing season.

(10) With respect to the need for conventional feeding studies, such data will not be required when no detectable residues are observed in feed

items from crop field trials reflecting the proposed use of the pesticide (maximum rate, minimum preharvest interval) unless the metabolism study indicates potential for significant bioaccumulation. When trace residues are detected in the field trials, the Agency will consider the anticipated dietary burdens and the results of the radiolabeled metabolism study when determining whether feeding studies are necessary. In the example cited in paragraph (e)(8) of this guideline (0.01 ppm dietary burden, 1,000× dose leading to <0.1 ppm total activity in meat/milk/eggs), a feeding study would not be necessary as expected residues in animal commodities from ingestion of 0.01 ppm would be on the order of 0.1 ppb (assuming a linear relationship between dose and residues). In this case the metabolism study also serves as a feeding study and tolerances would not be needed for meat, milk, poultry, and eggs.

(f) **Data reporting—plant studies**—(1) **Purpose.** This data reporting guidance is designed to aid petitioners/registrants in the collection and organization of data to facilitate the Agency review process. Data submitters are encouraged to submit complete reports following this guidance for efficient review by the Agency. This guidance pertains to the substance of the data report. PR Notice 86–5, effective on November 1, 1986 (available from the Registration Support and Emergency Response Branch, Office of Pesticide Programs, U.S. EPA, see paragraph (h)(7) of this guideline) pertains to the physical formatting of reports (which are referred to as studies) and submittal packages. Some of the requirements in PR Notice 86–5 are mandatory. Additional data reporting guidance is also given in OPPTS 860.1000 and in the individual topical guidelines of the 860 series.

(2) **Objective.** (i) An outline for study reports is provided and describes the topics which should be addressed such as application of radiolabeled materials, identification of residue components, degradation pathways, validation of enforcement methodology, etc. and provides guidance on the presentation of the results of the study.

(ii) Data submitters can use this guideline in preparing reports for submission to the Agency to meet 40 CFR part 158 requirements for the registration of pesticides.

(iii) Petitioner/registrant reports on plant metabolism studies should include all information necessary to provide a complete and accurate description of treatments and procedures. The information submitted in the report should include the following elements:

(A) Radiolabeling techniques to include rate, method, and time of radiolabel application in relation to the development and growth cycle of the treated RAC.

(B) Extraction, fractionation, and characterization techniques employed for the identification of residue components whether free or bound at each sampling interval.

(C) Definition of total terminal residues, to include data for all major components of the total terminal residue reflecting their distribution within the RAC expressed as both percentage of the total recovered radioactivity and concentration (in parts per million) found at time of harvest and/or when utilized for animal feed.

(D) A detailed discussion, preferably accompanied by a flowsheet, of the possible routes of degradation or pathways of metabolism observed in the subject RAC.

(E) Enforcement analytical methodology must be validated with radiolabeled samples derived from the plant metabolism study, accompanied by a statement made as to their capability to determine all components of the TTR whether free or bound/conjugated in the RAC. This informationm should be submitted in conjunction with reports on residue analytical methods (see OPPTS 860.1340).

(3) **Format of data report.** The following presents a suggested order and format for a study report item by item. However, other formats are acceptable provided the information listed in this paragraph is included.

(i) *Title/cover page.* Title page and additional documentation requirements (i.e. requirements for data submission and procedures for claims of confidentiality of data) if relevant to the study should be reported. Current mandatory requirements are described in PR Notice 86–5.

(ii) *Table of contents.* A concise listing, preceding the body of the report, of all essential elements of the study, and the page or table number where the element is located in the report.

(iii) *Summary/introduction.* This section should include appropriate background and historical information relative to the study. In addition, the purpose and summary of the study, a discussion of the results obtained, and conclusions arrived at regarding the qualitative nature of the total terminal residue in the treated crop should be included in this section. The following specific topics should be discussed briefly:

(A) Registration history and proposed use of the subject chemical.

(B) If applicable and/or available via an appropriate citation or reference, compare and contrast observed metabolic routes in the subject RAC to those observed in earlier plant metabolism studies conducted on the subject RAC or on other commodities or to those observed in animal metabolism studies conducted with the subject chemical.

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

(D) The overall experimental procedure employed, to include a discussion, if applicable, of unusual experimental problems encountered, at-

tempts 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.

(E) The modes and routes of metabolism observed including a complete description of the identity and quantity (both free and bound) of all major components of the terminal residue and their distribution within the RAC. (The foregoing information could be summarized in a narrative with or without tables and/or figures.)

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

(iv) *Materials/methods*—(A) *Test substance.* The following should be included:

(*1*) Identification of the test pesticide active ingredient (ai), including chemical name; Common name (American National Standards Institute (ANSI), British Standards Institution (BSI), or Internation Organization for Standards (ISO)); company developmental/experimental name; and Chemical Abstracts Service (CAS) number.

(*2*) Chemical structures for the parent compound and metabolites constituting the residue.

(*3*) Information on relevant formulation parameters as pertinent (e.g. nature of the solvent, carrier, bait, adjuvant, or other matrix in which the radiolabeled pesticide was applied).

(*4*) Report the purity, specific activity in Curies/mole, disintegrations per minute per gram, nature of the radiolabel and its source and the sites of labeling in the molecule for radiolabeled test material. The identity of radiolabeled impurities, if any, derived from the test material should also be reported.

(*5*) A rationale provided for selection of radiolabels other than ^{14}C and for sites of labeling in the molecule (where possible, emphasis is placed on labeling the ring position).

(*6*) Other. Any and all additional information petitioners/registrants consider appropriate and relevant to provide a complete and thorough description of the test chemical, such as physical/chemical properties (e.g. solubility, etc.).

(B) *Test site.* Provide the following information:

(*1*) 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 doc-

umentation of soil characteristics (not required for materials applied to foliage) at the testing site.

(*2*) An explanation or rationale provided by petitioners/registrants 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.

(C) *Test crop*. Include the following:

(*1*) Identification of the test crop including type/variety and crop group classification according to 40 CFR 180.41 as revised by 60 FR 26626, May 17, 1995.

(*2*) A rationale or statement provided by petitioners/registrants for selection of a test crop other than that for which use is proposed.

(*3*) Identification of specific crop parts harvested and subjected to ^{14}C residue analysis for a determination of the TRR.

(*4*) The developmental stages, general condition (immature/mature, green/ripe, fresh/dry, etc.) and sizes of the test crop at time of pesticide applications and at harvestings.

(*5*) Other. Any and all additional information petitioners/registrants consider appropriate and relevant to provide a complete and thorough description of the test crop.

(D) *Application of the pesticide.* Include the following:

(*1*) A detailed description of the type of pesticide applications to the test crop (i.e. preplant soil incorporated, over-the-top postemergent foliar application, bait application, etc.), including the formulation (i.e. solvent, carrier, bait, adjuvant, or other matrix) in which the radiolabeled pesticide was applied and the method of application (i.e. hand sprayer, topical, soil injection, etc.)

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

(*3*) Number and timing of applications, between-application intervals, and treatment to sampling intervals (also known as TSI, or PHI).

(*4*) 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), and dates of pesticide applications and harvest of mature crop).

(*5*) An explanation or rationale by petitioners/registrants for any significant deviation in either the rate or mode of application to the test crop from the intended use pattern.

(E) *Sample harvest (collection)*. Provide:

(*1*) 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/labeling). The sampling procedure used to obtain representative samples should be clearly stated.

(*2*) A detailed description of additional relevant information on the growing of the test crop, applications of the pesticide formulated products, and harvestings of samples. Refer to the data reporting guidance provided in OPPTS 860.1500, Crop Field Trials, for additional guidance on this subject area.

(F) *Sample handling and storage stability*. Provide:

(*1*) A detailed description of the handling, preshipping storage, and shipping procedures, as applicable, for harvested (collected) samples. Refer to the data reporting guidance provided in OPPTS 860.1500, Crop field trials, for additional guidance on this subject area.

(*2*) A detailed description of the conditions and length of storage of harvested (collected) samples following their receipt in the laboratory. Refer to the data reporting guidance on in OPPTS 860.1380 for additional guidance on this subject area.

(G) *Analyses of radioactive residues.* Report the following:

(*1*) Quantitation and distribution of total recovered radioactivity.

(*2*) Quantitative radioactivity data for all plant parts sampled, including fractions which maybe processed into food or feed, at time of normal harvest or at a stage of development when normally utilized for animal feed.

(*3*) A detailed description of sample preparation (i.e. dissection, grinding, lyophilization, etc.) prior to oxidative combustion/liquid scintillation analyses.

(*4*) A quantitative accountability for a majority of total radioactivity recovered from the treated crop at times of sampling or harvest as a result of aggregate sample analyses.

(*5*) Total distribution of radioactivity in the treated crop at time of sampling or harvest, provided in narrative, figure, or tabular format.

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

(*7*) Details of radioactive counting data for several selected representative samples to include counting times, total counts recorded, corrected counts, counting efficiencies, parts per million equivalents found, sensitivity, and limit of detection including representative calculations should be reported.

(*8*) For each sample analyzed (plant part or fraction) results should be reported as:

(*i*) Total radioactive counts (disintegrations per minute per gram).

(*ii*) The percentage that these radioactive counts represent of the total recovered radioactivity in the treated plant at time of sampling or harvest.

(*iii*) The parts per million equivalents (expressed as parent compound) that these radioactive counts represent of the total recovered radioactivity in the treated plant at time of sampling or harvest.

(H) *Extraction and fractionation of radioactivity.* Provide:

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

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

(*3*) 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.

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

(*5*) Petitioners/registrants should provide a quantitative estimate of residual radioactivity (i.e. nonextractable or bound) 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 parts per million (as parent equivalents) of total recovered radioactivity. Attempts at bound-residue extraction by

exotic or other procedures, or extractions following repeated treatments with concentrated acids and/or bases at elevated temperatures should also be reported by petitioners/registrants, and a rationale for their use given.

(*6*) Radiochemical extraction efficiencies calculated and reported for all harvested plant tissues.

(*7*) 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.

(*8*) 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 petitioners/registrants to minimize these losses should be discussed.

(*9*) Petitioners/registrants should report detailed procedures for the fractionation of nonextractable or bound radioactivity in plant tissues into proteins, starch, lignin, cellulose, etc.

(*10*) Following chemical analyses of the fractionated plant tissues described in paragraph (f)(3)(iv)(G)(*3*) of this guideline for amino acids, glucose, etc., petitioners/registrants should then report if significant quantities of the original radioactive residue characterized as nonextractable or bound have been incorporated into these natural products.

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

(*12*) A detailed description of the conditions and length of storage of extracts prior to identifcation of residues.

(I) *Characterization/identification of radioactivity.* Provide:

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

(*2*) 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 of 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.

(*3*) Complete details of additional confirmatory analytical procedures used to separate and characterize/identify metabolites (i.e. high voltage electrophoresis, ion-exchange, or exclusion chromatography, derivatization, etc.) and determinative methods (i.e. mass spectroscopy in electron impact (EI) and chemical ionization (CI) modes) used for ultimate identification of metabolites.

(*4*) Explanations for all lost or unaccounted-for radioactivity in each plant extract or fraction. The amount reported should be expressed as both percentage and parts per million (as parent equivalents) of total radioactivity recovered from the particular plant part or fraction analyzed and of the total plant at harvest (terminal residue) or when utilized as an animal feed.

(*5*) Individual and/or aggregate quantitative radioactive residue data for all nonidentified and/or noncharacterized discrete extractable and resolvable radioactive entities with amounts reported as in paragraph (f)(3)(iv)(H)(*5*) of this guideline.

(*6*) A report of each of the major metabolite components and, if possible, provide information on the chemical nature of discrete (minor) metabolite components. Major metabolite components should be quantified with amounts reported as in paragraph (f)(3)(iv)(G)(*8*)(*iii*) of this guideline; quantification of minor metabolite components should be attempted and the results reported, if possible.

(*7*) A report of data/information delineating attempts made to characterize/identify chemically any conjugated or complex bound chemical species originating from the parent pesticide in edible plant parts used for food or animal feed.

(*8*) Quantitative data for each minor metabolite component identified.

(*9*) 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 should be submitted. Photographs of radio-TLC plates as well as samples or reproductions of HPLC/GLC chromatograms including mass spectral scans, etc., should also be submitted.

(*10*) Any and all additional information petitioners/registrants consider appropriate and relevant to provide a complete and thorough description of the conduct of the plant metabolism study and the determination of the TTR.

(v) *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 unextractable or bound residues. It should include a discussion of the impact or effects, if any, of those deviations on the results of the study.

(B) *Metabolic pathways.* If possible, a detailed discussion, preferably accompanied by a flowsheet, 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 livestock metabolism studies conducted with the subject chemical. The comparison could also be provided in a separate summary report or overview document.

(C) *Characterization/identification and distribution of TTR.* (*1*) Use a tabular or graphic format. Identify all major components of TRR in the RAC, both free and conjugated/bound, including name, structure, and quantity (expressed both as percentage of TTR and parts per million as parent equivalents), and report their distribution within the RAC plant parts. All activity should be reported as free, conjugated, or bound metabolites or natural constituents as defined in paragraph (g)(3)(v)(D)(*3*)(*v*) of this guideline.

(*2*) If the immature RAC (including plant 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 plant development must also be reported.

(*3*) Petitioners/registrants should provide information on any properties and/or characteristics of all significant unidentifiable and/or uncharacterizable components of the terminal residue, their quantities, and their distribution within the RAC.

(D) *Statistical treatments.* Include representative examples of any statistical tests applied to the raw data obtained during sampling/analyses in the course of the plant metabolism study.

(E) *Other*. Any and all additional information petitioners/registrants consider appropriate and relevant to provide a complete and thorough description of the plant metabolism study including quality control measures/ precautions taken to ensure validity of all aspects of the study.

(vi) *Conclusions.* Discuss conclusions that may be arrived at as a result of the submitted plant metabolism study, such as:

(A) The routes or pathways, mechanisms involved and extent or degree of metabolism observed when the subject RAC is grown to maturity or harvest.

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

(vii) *Tables/figures*—(A) *Tables* (for example):

(*1*) Weather and/or environmental data.

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

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

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

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

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

(B) *Figures.* (for example): (1) Diagram of location, topography, and size of outdoor test plots.

(*2*) Photographs, figures, or diagram of greenhouse and/or plant growth chamber facilities used in study.

(*3*) Overall extraction and fractionation strategies/schemes employed for each sample matrix analyzed.

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

(*5*) Metabolism flow charts.

(viii) *Certification.* A signed and dated certification of authenticity by, and identifying information (typed name, title, affiliation, address, telephone number) on, the personnel responsible for the various phases of this report (e.g. Study Director, Field Supervisor, and Laboratory Supervisor).

(ix) *References.*

(x) *Appendixes*. (A) Representative chromatograms, spectra, etc. (as applicable).

(B) Cite or reference reprints of published and unpublished literature, company reports, letters, analytical methodology, etc., used by petitioners/ registrants (unless physically located elsewhere in the overall data report, in which case cross-referencing will suffice).

(C) Other. Any relevant material not appropriate to any of the other sections of the report.

(g) **Data reporting—livestock studies**—(1) **Purpose.** This data reporting guidance is designed to provide a data reporting format for a study of the qualitative nature of residues in food animals.

(2) **Objective.** (i) This section outlines the data needed to support a livestock metabolism study and the form in which those data are to be reported. This guidance will aid the petitioners/registrants in the collection and organization of data with the goal of developing complete data packages and facilitating the Agency's review of the study report. Additional data reporting guidance is given in OPPTS 860.1000, and in the individual topical guidelines.

(ii) This guidance is designed to aid petitioners/registrants in generating reports compatible with the Agency's review process. Data submitters are encouraged to submit complete reports for efficient review by the Agency. It pertains to organizing and presenting the substance of the data report. PR Notice 86–5, effective on November 1, 1986 and available from the Office of Pesticide Programs, U.S. EPA (see paragraph (h)(11) of this guideline), pertains to physical formatting of reports (which are referred to as studies) and submittal packages. Some of its requirements are mandatory.

(iii) Petitioner/registrant reports on livestock metabolism studies should include discussions of the following topics: The test material, experimental animals, dosing, sample collection, quantitation of activity, extraction of activity, characterization and identification of activity, conclusions, and raw data. Because data may be more clearly presented in tables or figures, data requirements that are best submitted as a table or figure are identified.

(3) **Format of the data report.** The following describes a suggested order and format for a study report item by item: However, other formats are also acceptable provided the information described in this paragraph is included.

(i) *Title/cover page.* Title page and additional documentation requirements (i.e. requirements for data submission and statement of data confidentiality claims), if relevant to the study report should precede the content of the study formatted below. These current requirements are described in PR Notice 86–5.

(ii) *Table of contents.* The table of contents should provide page numbers on which are found the essential elements of the study, to include the following: Introduction and Summary, Materials, Methods, Results and Discussion, Conclusions, Tables/Figures, Certification, References, and Appendices. The requirements of each of these sections are discussed below.

(iii) *Introduction and summary.* This section should provide background for the study and should include the proposed use of the pesticide, the purpose of the study, and a summary of the results. The summary of the experiment should include a discussion of any unusual problems encountered and how these were resolved, a discussion of any deviation from experimental protocol and the effect this may have had on the results, and a brief description of the findings of the study (identity and quantity of significant metabolites in each of the major tissues analyzed, and a proposal as to which metabolites are in need of regulation). A comparison of the results with findings of earlier plant and animal metabolism studies, if any are available, should be included here.

(iv) *Materials*—(A) *Test substance.* (*1*) The test pesticide active ingredient should be identified by chemical name, common name (ANSI, BSI, or ISO), company developmental name or number, and, if available, the Chemical Abstracts Service (CAS) number.

(*2*) If the molecule is labeled in a potentially labile portion or a radioactive atom that is subject to exchange reactions is used, a rationale should be provided. Petitioners should explain their choice for the test material.

(*3*) The impurities in the test material and the potential effect of these on the study should be discussed. The purity of the test material should be reported along with its specific activity in Curies per mole (or microCuries per mmole) and disintegrations per minute per gram (dpm/g).

(*4*) Chemical structures (these should be submitted as figures) for parent and metabolites should be provided; each should be accompanied by chemical names and, if available, company developmental name or number.

(B) *Test facilities.* Animal housing should be described. For some pesticides for which volatile metabolites are expected to predominate, it will be necessary to establish that volatilization accounts for a significant amount of activity. It will then be necessary to provide a description of the precautions taken to ensure that this activity is detected.

(C) *T*est animals. A description of the test animals should include age, weight, health status, and breed. Any health problems or unusual treatment of the animals should be reported; the effect of these on the results of the study should be discussed.

(v) *Methods*—(A) *Dosing.* (*1*) For oral metabolism studies, petitioners should describe the preparation of dose (for example, capsule, with feed, bolus, etc.) and the level, timing, and duration of dosing. If the dose is given with feed, the total feed consumed should be reported; the level of pesticide in the feed (determined by counting radioactivity) should also be reported. Doses for ruminants should be expressed on a dry matter basis.

(*2*) For a dermal metabolism study, the number, application level, and type of treatments should be described. A comparison of the treatments to those proposed for use on animals, with particular attention to and explanation of any differences in the formulation, dosing level, or other experimental parameter, should be provided.

(*3*) Petitioners/registrants should describe the precautions taken to assure that dermally applied pesticide is not orally taken up due to grooming; this is particularly important for ruminants.

(B) *Sample collection.* (*1*) Petitioners/registrants should describe the collection of milk and eggs taken, and provide an explanation if this is different from normal practice.

(*2*) The amount of milk and number of eggs, as well as a comparison of these with normal production, should be provided in tabular form.

(*3*) The interval from the last dose to sacrifice should be specified to within 1 hour. If the animals are sacrificed more than 24 hours after the final dose, an explanation should be provided along with a discussion of the effect of this on the results.

(*4*) A list of the tissues taken and their weights should be provided in a table. If samples are combined from different animals, this should be stated.

(C) *Sample handling and storage stability.* The storage and handling of samples should be described, including the conditions during any shipment and the time in transit. Petitioners should provide evidence that the length or conditions of storage have not significantly affected the results of the study. Additional details are provided in OPPTS 860.1380.

(D) *Analysis of radioactivity.* (*1*) Quantitation and distribution of total recovered radioactivity. Include:

(*i*) The preparation of the sample prior to counting of activity should be described in detail.

(*ii*) The radioactivity recovered in each tissue sampled should be reported in tabular form as total radioactive counts and in parts per million as equivalents of parent compound.

(*iii*) Counting times, total counts, corrected counts, counting efficiencies, and other raw data (sample sizes, sensitivity, limit of detection, etc.) should be submitted tabular form. Sample calculations should be reported for representative samples.

(*2*) Extraction and fractionation of radioactivity.

(*i*) The fractionation and extraction strategies for each tissue should be described by way of a flowsheet The solvents used, the order of their use, the extraction procedures (e.g. blending, maceration, Soxhlet, etc.) used, and other techniques used to effect extraction should be provided in tabular form.

(*ii*) Any efforts to release nonextractable and conjugated residues (acid, base, or enzyme hydrolysis, exhaustive extraction, etc.) should be described. The use of severe conditions (e.g. heat plus strong acid) should be justified and the possible effect of these treatments on pesticide residues should be discussed.

(*iii*) For each tissue the amount of activity that is water soluble, organosoluble, and nonextractable should be reported as a percentage of the total activity in that tissue and in parts per million (parent equivalents).

(*iv*) Detailed description of the conditions and length of storage of extracts prior to the identification of residues.

(*3*) Characterization/identification of radioactivity. (*i*) A table listing compounds that were synthesized to serve as standards for known and suspected metabolites should be provided. If TLC, GLC, HPLC, or other chromatographic techniques were used to identify metabolites, appropriate retention times should be provided.

(*ii*) Any analytical procedures used to identify metabolites should be described in detail.

(*iii*) For each tissue, any losses of activity that occur during the various procedures required for characterization and/or identification should be explained as fully as possible. This activity should be reported in parts per million (parent equivalents) and as a percentage of the TRR (these data requirements are best submitted in tabular form).

(*iv*) For each tissue, milk, or eggs, any discrete, unidentified activity (e.g. an unidentified spot on a TLC plate) should be reported in parts per million (parent equivalents) and as a percentage of the TRR. For each tissue, milk, or eggs, identified metabolites should be reported in parts per million (parent equivalents) and as a percentage of the TRR (submit these data in tabular form). All data supporting the identification (e.g. reproductions of chromatograms and spectra) should be provided. Failure to identify a metabolite should be accompanied by an explanation and a description of the attempts that were made to characterize/identify the resi-

due. Any information on the identification and characterization of minor metabolites should be reported.

(*v*) All activity should be reported as either:

(*A*) Free metabolites—normally extractable by organic solvents and do not require chemical treatment to be released.

(*B*) Conjugated metabolites—those that have been metabolized by the animal to form water soluble compounds. Conjugates are made up of two parts, one derived from the pesticide, called the exocon, and one from the animal, called the endocon. The endocon is often a sugar, but there are other possibilities (e.g. sulfates, amino acids, glutathione). Identification of the exocon is not normally possible without cleavage of the conjugate bond. This is normally done by acid, base, or enzymatic hydrolysis. After hydrolysis, the pesticide or pesticide metabolite, free of the conjugating moiety, is usually soluble in organic solvents.

(*C*) Bound metabolites—from pesticide or pesticide metabolites bonding with cellular components to yield products that cannot be removed from the matrix by exhaustive extraction with polar and nonpolar solvents. If these residues are removed chemically, e.g. by acid, base, or enzymatic hydrolysis, a subclass of bound residues must be established.

(*D*) Natural constituent—applies to a pesticide that has been degraded into small fragments that have been channeled into anabolic cycles and is incorporated into normal cell constituents. If soluble, natural constituents may be difficult to distinguish from conjugates and may be misclassified.

(*vi*) If the natural constituents are nonextractable, they are difficult to distinguish from bound metabolites. This may lead to the misclassification of these residues as bound pesticide residues, when they are not pesticide residues at all. It may be desirable to establish that radioactive residues are natural constituents, particularly if these residues are thought to comprise a large portion of the terminal activity. If needed, there are literature references that will serve as guidelines (see paragraphs (h)(6) and (h)(7) of this guideline).

(vi) *Results and discussion.* Residue characterization and/or identification: For each tissue of concern (liver, kidney, muscle, fat, milk, and eggs), petitioners/registrants should provide a flowsheet depicting the metabolites and how they were identified. Petitioners/registrants should also provide discussion of the results including the significance of activity not fully characterized and/or identified.

(vii) *Conclusions.* Petitioners/registrants should reach a tentative conclusion as to the residue in need of regulation.

(viii) *Tables and figures.* This section need only include tables or figures not included in other sections.

(A) The following data should be presented in tabular form:

(*1*) Vital statistics of the test animals including, as applicable, weight, milk production, egg production, etc.

(*2*) Level of radioactivity (ppm parent equivalents) in tissues, milk, and eggs.

(*3*) Name, structure, and purity of all model compounds used as metabolite standards.

(*4*) Retention times (in the case of GC and HPLC data) and Rf values for parent and metabolites under the solvent and stationary phase conditions used.

(*5*) For each tissue of concern (liver, kidney, muscle, and fat) and milk and eggs, the name, structure, and level of all identified metabolites.

(B) The following should be presented as figures:

(*1*) Schemes employed for extraction of each tissue.

(*2*) Clear reproductions of TLC plates, GC and HPLC spectra, mass spectra, autoradiograms, and any other graphic data essential to the conclusions of the study.

(*3*) Flowsheets showing the significant metabolites in each tissue of concern (liver, kidney, muscle and fat) and how their identity was established.

(ix) *Certification.* Certification of authenticity by the study director (including signature, typed name, title, affiliation, address, telephone number, and date).

(x) *References.* Complete citations to any references cited in the report should be included here.

(xi) *Appendices.* Tables and figures not included elsewhere should be included in the appendices. Reproductions of published reports or other materials that support the submitted study may also be included in this section if, in the registrant's opinion, it will increase the efficiency of the Agency's review of the report.

(h) **References.** The following references should be consulted for additional background material on this test guideline.

(1) Environmental Protection Agency, Pesticide Reregistration Rejection Rate Analysis—Residue Chemistry; Follow–up Guidance for: Generating Storage Stability Data; Submission of Raw Data; Maximum Theoretical Concentration Factors; Flowchart Diagrams. EPA Report 737–R–93–001, February, 1993.

(2) Environmental Protection Agency, Pesticide Reregistration Rejection Rate Analysis—Residue Chemistry; Follow–up Guidance for: Updated Livestock Feeds Tables; Aspirated Grain Fractions (Grain Dust); A Tolerance Perspective; Calculating Livestock Dietary Exposure; Number and Location of Domestic Crop Field Trials. EPA Report 737–K–94–001, June, 1994.

(3) Environmental Protection Agency, Pesticide Reregistration Rejection Rate Analysis—Residue Chemistry; EPA Report 738–R–92–001, June 1992.

(4) Environmental Protection Agency, FIFRA Accelerated Reregistration—Phase 3 Technical Guidance. EPA Report 540/09–90–078, December 1989.

(5) Environmental Protection Agency, Pesticide Assessment Guidelines, Subdivision O, Residue Chemistry, Series 171–4; Addendum No. 3 on Data Reporting, Nature of the Residue: Plants, EPA Report 540/09–87–199.

(6) Environmental Protection Agency, Pesticide Assessment Guidelines, Subdivision O, Residue Chemistry, Series 1714; Addendum No. 7 on Data Reporting, Metabolism (Qualitative Nature of the Residue: Food Animals, EPA Report 540/09–89–009.

(7) Environmental Protection Agency, Pesticide Regulation Notice PR 86–5, Standard Format for Data Submitted under the FIFRA and Certain Provisions of the Federal Food, Drug, and Cosmetic Act (FFDCA), May 3, 1986.

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

(9) Strategy for determination of extent of metabolism studies and development of residue methods based on trigger values, January 27, 1988, Dr. B. Donzel, Ciba-Geigy Corp.