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APPENDIX A

METABOLISM AND DISTRIBUTION IN PLANTS

1   Introduction

The fate of the active <u>substance</u> of a plant protection product in plants is a complex process which comprises uptake of the product mainly through leaf surfaces, fruit and roots, distribution in the xylem and phloem and evaporation from the plant surface as well as degradation, conversion and metabolism of the product in and on the plant. Active <u>substance</u>s of plant protection products are in most cases metabolized in several stages, often down to <u>non-extractable</u> residues. Complete degradation pathways are known for only a few of the active substances currently in use. Types and amounts of metabolites are influenced by the uptake, distribution and time of persistence of the product in the plant.

A product can be taken up by the leaves, fruits or roots. The roots may also take up metabolites formed in the soil by microbial or abiotic degradation. Plant roots and leaf surfaces provide quite different surfaces for the active substance to penetrate. The cuticle is a thin, lipoid membrane while the endodermis is an internal structure of the roots, which has to be overcome by an active transport mechanism before the substance can be transported on to the shoots.

A plant has two different vascular systems for the distribution of the active substance and/or metabolites. Normally, there is a passive transport in the xylem, with the transpiration stream, from the roots upwards to the top of the plant. But there is also an active transport in the phloem, towards the centres of metabolic activity (such as growth points of shoots and roots). Therefore distribution of plant protection products in plants is not uniform but depends on the kind of active <u>substance</u> and the route of penetration into the plant. Leaves and roots are centres of degradation of the active <u>substance</u>. The degradation capacity varies widely with the different ages of the individual plant parts.

2   Objectives

The objectives of these studies are:

-       to provide an estimate of total terminal residues in the relevant portion of crops at harvest following treatment as proposed;

-       to identify the major components of the total terminal residue;

-       to indicate the distribution of residues between relevant crop parts;

-       to quantify components of the residue and to establish the efficiency of extraction procedures for these components;

-       to decide on the definition and expression of a residue.

3   Plant metabolites

Metabolites of plant protection products are formed by several stages. The process includes the formation of free compounds, conjugate compounds, bound residues and natural products.

Free metabolites are formed in/on plants by hydrolysis, oxidation, reduction, enzyme-catalysed re-combination or photolysis. These reactions and the primary products formed by them are often identical to those in animals.

The second stage is normally the conjugation of these primary metabolites (also referred to as "exocon") and plant substances (also "endocon") (Dorough, 1976). Conjugated compounds are usually more polar than first stage metabolites and dissolve in water or other polar solvents. Frequent conjugates in plants are those in which glucose is the "endocon": O-glucosides, glucose esters, N-glucosides, and S-glucosides. However, conjugates can also be formed with glutathione, amino acids and others (Frear, 1976; Hutson, 1976; Iwan, 1976; Kaufmann et. al., 1976; Mumma and Hamilton, 1976; Climie and Hutson, 1978; Sonobe et al., 1981 and 1983; Shimabukuro et al., 1982; Hatzios and Penner, 1982; Harvey, 1986).

The exocon (the metabolite of the plant protection product) can also be covalently bound to insoluble plant components as a bound residue. According to a 1981 IUPAC definition,non-extractable residues (also referred to as 'bound residues' or non-extracted residues) are chemical compounds which cannot be extracted by methods which do not essentially change the chemical structure of these compounds. Under this definition, structural fragments which are transformed into natural products by metabolic processes are not included (Kovacs, 1986).

Far-reaching degradation may lead to the release of carbon dioxide or other low-molecular weight fragments (e.g. formate and acetate). In some cases, these fragments are incorporated into naturally occurring substances (Harvey, 1986).

Metabolism in plants can be slower than in animals, since plants lack detoxifying organs which could compare in performance with, for instance, the liver. Their metabolism also differs from that of animals in that polar compounds cannot be excreted as in animals. Plants generally metabolize plant protection products to water-soluble conjugate compounds and bound residues which stay in the plant. Full oxidation of organic plant protection substances to carbon dioxide and water is only of minor importance (Shimabukuro and Walsh, 1979; Shimabukuro et al., 1982; Hatzios and Penner, 1982; Harvey, 1986).

4   **Test methods**

Metabolism studies aim at clarifying the nature and amounts of the metabolites formed. The amount of active substance used should be chosen according to the proposed practical application rates. It may also be necessary to produce more residues to enable the identification of metabolic and degradation products (Harvey, 1986; Kovacs, 1986). Metabolism studies with radiolabelled active substance, carried out as field trials under practical conditions, most accurately reflect the real conditions of application. Such tests have been described in literature (Belasco et al., 1981; Chrzanowski and Leitch, 1983; Harvey, 1986).

Usually, metabolism trials use radiolabelled substances, including 14C, 35S, 32P and others. Tritium should not be used because of its uncertain localization (EPA, 1982). The substance must be labelled so that the degradation pathway can be traced as far as possible. Favourable positions are cyclic atoms in aromatic or cyclic compounds and atoms with several substituent groups in other compounds. Plant metabolism studies must state the radiolabel position, the radiolabel purity and the specific activity of the radiolabel used.

It may also be necessary to label active substances with several important structural elements in more than one place, (for instance, labelling synthetic pyrethroids on either side of the ester bond, or active substances with one phenol ring and one heterocyclic ring in both rings etc.) (Huber and Otto, 1983; Harvey, 1986; Kovacs, 1986).

Metabolism studies should be appropriate to the intended use. For example, where treatment is intended on mature crops, treatment in the plant metabolism study should be carried out in the same way so that the composition of the compounds identified is representative of the residues at harvest-time in residue studies. All plant parts suitable for food and fodder shall be included in the studies (EPA, 1982).

4.1   Whole plants in the laboratory, climate chamber, glasshouse and field

Complex results which come closest to the practical conditions of the planned application are delivered by trials with whole plants growing in non-sterile conditions. Similarity to practical conditions is achieved by application of formulated products and by allowing the influence of climate and microbial activity. Many of such metabolism trials have been carried out. Some shall be cited here: climate chamber (Stratton and Wheeler, 1983), glasshouse (Chrzanowski and Leitch, 1982; Sonobe et al., 1983; Wink et al., 1984), field/lysimeter (Führ, 1975, 1984, 1985; Führ et al. 1976; Belasco et al., 1981; Chrzanowski, 1983; Haque et al., 1983). Often, several of the methods mentioned are used to study one active substance: field and climate chamber (Harvey and Reiser, 1973; Kuck, 1987), glasshouse and climate chamber (Larson and Lamoureux, 1984), field, glasshouse and climate chamber (Harvey et al., 1978).

Trials in closed climate chambers can be used to investigate the behaviour of an active substance in the soil-plant-air-leaching water system (Schaerer, 1983; Figge et al., 1985; Schuphan, 1985).

Trying to set up detailed requirements for the execution of such trials would not make sense because of the different properties of the active substances and the complexity of the system. In planning their trials the applicant, or the person carrying out the trials, must take into consideration the above mentioned objectives.

4.2   Plant parts and plant tissues

Studies on parts of plants are common and are often chosen as a first step to quickly clarify the metabolism. In particular the first steps of degradation can be studied in this way. The procedure is to dip freshly cut plant parts into an aqueous solution of the radiolabelled substance. After the plant part has taken up enough of the solution, it is placed in fresh water. The procedure allows a relatively large amount of active substance to enter the plant, because neither plant surfaces nor root membranes must be passed. The metabolites formed are often the same as those found in complete plants, but there may be quantitative differences.

Experiments on small, cut-out leaf areas have also been successful in studying the inter-actions of various plant protection products. It was demonstrated, for instance, that some insecticides (organophosphates and carbamates) inhibit the metabolization of phenyl urea herbicides. Such results can be obtained faster and more easily with leaf segments than with whole plants (Chang et al., 1971; Shimabukuro and Walsh, 1979).

These test methods are also a simple way of producing larger amounts of metabolites. The duration of the experiment is, however, limited to approx. 48 h because of the aging of the plant parts (Shimabukuro and Walsh, 1979; Harvey, 1986; Kuck, 1987). An overview of the use of different plant parts and isolated cells and of the test methods has been given by Shimabukuro and Walsh (1979).

4.3   Whole 'sterile' plants

Trials with plants yield information on the uptake, transport and evaporation of active substance and metabolites. Such trials are carried out under sterile conditions, allowing to exclusion of influences not originating in the plant, such as powerful radiation and microorganisms. The trials can be carried out under reproducible conditions and supply helpful data on metabolism (Schuphan, 1987).

4.4   Cell cultures

While experiments with complete plants or parts of plants are suitable to provide detailed results on the complex processes of degradation and conversion, in-vitro experiments are able to give insight into specific physiological reactions in the plant.

There are a number of methods for cultivating plant cells on solid or in liquid medium. Mumma and Hamilton (1979) gave a comprehensive survey of relevant tests up to 1979. Normally, the cells are cultivated on sterile material containing growth regulators. Plant cells grow fast and homogenously with little pigmentation. These methods allow the production of large amounts of metabolites, making their isolation, purification and identification easier.

Increasingly, metabolism experiments are carried out in cell suspension cultures. The metabolites isolated in plant cell cultures are often the same as those identified in complete plants (Mumma and Hamilton, 1979; Hatzios and Penner, 1982; Larson and Lamoureux, 1984; Harvey, 1986). Comparable ways of degradation can therefore usually be assumed. Quantitative differences were observed in the degradation and metabolic products found. Conjugates like glucosides are often found in smaller amounts than in a complete plant, as would be expected with this type of study.

The metabolism in cell cultures is influenced by the part of plant where the tissue is taken, the kind of plant, the growth conditions of the cell culture, the composition of the culture medium, the age and the general state of the cell culture (cell cultures must be old enough to develop full enzymatic activity) (Mumma and Hamilton, 1979).

Experiments with cell cultures cannot completely replace experiments with whole plants. They mainly serve to produce larger quantities of metabolites.

4.5   Isolated enzyme systems

The four biochemical reactions most important for the degradation or conversion of plant protection products in plants (hydrolysis, oxidation, reduction and conjugation) have also been studied in isolated enzyme systems (aryl-acyl-amidases, esterases, peroxidases, hydroperoxidases, arylnitro-reductases, glutathion-S-transferases and various enzyme systems conjugating glucose). A relevant bibliography has been compiled by Lamoureux and Frear (1979).

The use of enzymes poses some difficulties for the execution of the experiment. Problems may arise while isolating the enzyme, testing it as to whether it is deactivated, or defining the correct enzyme system and the relevant co-factors. In spite of such problems, the experiments may supply important extra information:

- individual steps of reaction can be studied separately,
- the kinetics of the reaction can be analysed,
- intermediate products which are normally not accessible
  can be detected and isolated,
- different enzyme activities of different plants can be
  detected and studied,
- the disintegration of non-extractable residues in
  digestive tracts can be tested

This all helps to better understand the degradation processes. In addition, experiments with isolated enzyme systems allow to synthesis of special metabolites for further studies (Lamoureux and Frear, 1979; Harvey, 1986).

5 Analytical methods

5.1   Extraction

If the fresh plant samples cannot be immediately analysed, they must be frozen at -18°C or a lower temperature. The storage stability of the active substance and metabolites must be taken into account.

If the samples are to be dried before extraction, any volatile active substances and metabolites must be trapped and analysed. Normally, the drying shall not be done by freezing, air stream or heat, except when natural drying is simulated, such as hay drying or hop drying in a kiln.

The samples shall be homogenized before extraction. Extraction must be done if possible at room temperature or lower. Appropriate extraction procedures must be used. The following paragraphs give an example of a possible approach. The extraction procedures used have to be fully described and justified.

Extraction using non-polar solvents followed by solvents of increasing polarity may give a separation of the active substance and metabolites and relatively clean fractions, making the identification or characterisation of the different compounds easier.

Bound residues are commonly only isolated using methods which may change the structure of the active substance and the metabolites.Information about extraction of bound metabolites, i.e. bound to proteins, starch, lignin or cellulose is given in the literature (e.g. Huber and Otto, 1983; Kovacs, 1986; Balba et al., 1979; Khan, 1980 and 1982).

These procedures cannot always solve the problems of extracting bound residues; bound residues have to be analysed and evaluated depending on the actual situation.

5.2   Isolation and identification

The metabolites dissolved in the extracts have to be isolated and identified. Preferred techniques in metabolism studies are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC)/mass spectrometry. If nothing is known about the structure and stability of the metabolites, the analyst should try to avoid the breakdown of the compounds.

There are radioactivity detectors for all three chromatographic methods. Autoradiography and radio scanners are used with TLC plates. The detectors used with HPLC and GC are often not sufficiently sensitive. In such cases, the analyst has to collect equal fractions of the eluates and count them by scintillation counter (Harvey, 1986; Zweig and Sherma, 1986).

In identifying metabolites, mass spectrometry is the method of choice because of the small amounts of degradation and conversion products which may remain after isolation and purification. If there are metabolites found in the analysis, the structure found should be confirmed by mass spectrometry or by any other appropriate analytical procedure.

Highly polar conjugates are hard to isolate and identify. The common procedure of cleavage using beta-glucosidase or dilute acid often leads to the conclusion that the conjugate in question was a glucoside although the endocon was not charactarized. Advances in HPLC-mass spectrometry allow identification of uncleaved conjugates. (Harvey, 1986; Fenseleau et al., 1984; Capriel et al., 1986).

If analysis indicates that residues in edible portions of the crop following treatment as proposed are <0.01 mg/kg (human food) or <0.05 mg/kg (animal feed), no characterisation of terminal metabolites is generally required. Nevertheless experiments should be carried out to establish the major metabolic pathways. Studies using high application rates, analyses of immature plants and in vitro techniques may be used.

If total radioactive residues are >0.01 mg/kg (human <u>food</u>) or >0.05 mg/kg (animal <u>feed</u>) then the aim should be:

a)   to characterise and identify all single components of the residue which exceed 0.05 mg/kg or 10% of the total radioactive residue,

b)   to characterise fractions or individual components of the residue which represent 0.01 - 0.05 mg/kg to the extent that they can be placed in one of the following categories:

1) organosoluble but does not cochromatograph with postulated metabolites and cannot be converted to these compounds by chemical or enzyme hydrolysis;

2) water soluble and either cannot be converted to organosoluble material by chemical or enzyme hydrolysis or is converted to organosoluble material of nature 1) above; or

3) not extracted by solvents; (including refluxing conditions); water, acid or alkali (including refluxing conditions;) or enzyme digestion.

These trigger values of 0.05 mg/kg or 10% of total radioactive residues are only meant as guidance. In some circumstances, generally governed by toxicological concerns, it may be necessary to identify terminal metabolites, which are present at concentrations lower than 0.05 mg/kg or <10% of total radioactive residues. The trigger values only indicate that it is not generally necessary to identify metabolites which are present at very low and insignificant levels.

Characterization refers to the elucidation of the general nature/characteristics of the radioactive residue short of metabolite identification.

Identification refers to the exact structural determination of components of the total radioactive residue. Typically, this is accomplished by comparing chromatographic behaviour to that of known standards and/or actual spectroscopic analysis (MS, NMR,etc.).

Reports should include: full details of the methods of analysis <u>(including extraction scheme)</u>, recoveries of radioactivity and sufficient representative chromatograms, spectra and photographs of autoradiograms to illustrate the quality of the data and to indicate the confidence which can be attached to characterisations and identifications.

6 **Extent of data required**

Metabolism studies have to involve crops or categories of crops in which plant protection products containing the active substance in question would be used. If a wide range of uses in different crop categories or in the category fruits is envisaged, studies have to be carried out on at least three crops unless it can be justified that a different metabolism is unlikely to occur. In cases where use is envisaged in different categories of crops, the studies must be representative for the relevant categories. For this purpose crops can be considered as falling into one of five categories: root vegetables, leafy crops, fruits, pulses and oilseeds, cereals. If studies are available for crops from three of these categories and the results indicate that the route of degradation is similar in all three categories then it is unlikely that any more studies will be needed unless it could be expected that a different metabolism will occur. The metabolism studies have also to take into account the different properties of the active substance and the intended method of application.

7 Interpretation

The relevance of results should be discussed in relation to the proposed uses.

The toxicological significance of plant metabolites (including chemically unavailable or non-extractable residue) should be considered.

Where residues are found to be non-extractable under usual chemical laboratory conditions, e. g. without running the risk of changing them by applying very rigorous extraction methods, they may be termed 'bound' or 'chemically unavailable'. Any statement to the effect that residues are 'bound' (to a substrate, or matrix) must necessarily be a function of the effort undertaken to identify or to 'free' them, e. g. of the extraction procedure used, and remains meaningless unless the conditions under which such bound residues were found to be extractable are specified. It is preferable, therefore not to use the term bound residues in an analytical context but to use 'chemically unavailable' or 'non-extractable residue', each individual case being supported by a statement of the method of investigation. The question then remains as to whether such residues will be available to biological systems, e. g. the digestive tract of ruminants or other warm-blooded animals, including humans when persisting in certain constituents of feed or food. If biological unavailability of residues can be demonstrated, such residues can be considered non-significant.

It may be necessary to evaluate the bio-availability of conjugated metabolites in the same way as non-extractable residues. For example, a conjugate found in an edible plant should be considered as a residue only if it can be demonstrated, or it is considered highly likely, that the xenobiotic component of the conjugated molecule may be physiologically available to an animal via its digestive tract.

Generally, if the non-extractable residue is less than 0.05 mg/kg or 25 % of the total radioactive residue and a significant proportion of the total residue has been identified, no further work is required. A significant proportion means 75%; however this may not be possible in all cases. All deviations must be fully justified.

If non-extractable residues exceed 0.05 mg/kg or 25 % of the total radioactive residue, then depending on the absolute level of residue represented by the non-extractable residue and the toxicity of the parent molecule it may be necessary to investigate the biological availability of these residues.

The extractability of residues should be discussed in relation to the proposed analytical methodology.

A schematic diagram of the metabolic pathway with a brief explanation of the distribution and chemical changes involved should be worked out.

8. Residue definition

A proposal for the residue definition should be made. There are three general considerations which are fundamental to the decision as to whether or not specific metabolites/degradation products should be included in the definition and expression of a residue

- their basic toxicology;
- their presence in significant amounts;and
- the suitability of the analytical procedure for routine   monitoring

The following approach is recommended:

1) Expression as parent compound

- Residues are expressed as parent compound if there are no metabolites or if the metabolites are known to be of no toxicological significance.

- If metabolites are known to be present in significant amounts, and the analytical method measures the total residue as a single compound the residues should preferably be expressed as parent compound. The metabolites included in the residue should be listed.
- If the analytical method measures parent compound and metabolites separately, the compounds of the total residue may be expressed additively as parent compound with recalculation for differences in molecular weight only when the differences are substantial.

2) Expression as single metabolite

- Residues are expressed as a single metabolite or alteration product if the parent is entirely metabolised to another chemical entity.

- If metabolites of toxicological concern are known to be present in significant amounts and the analytical method measures all of the residues as one metabolite the residue may be expressed as that metabolite.

- If however the analytical method measures residue components separately the result may be expressed additively in terms of the parent or of a metabolite with recalculation for differences in molecular weight only when differences are substantial.

3) Expression as parent compound and metabolites separately

- Residues are expressed as parent and metabolites separately if the metabolites have a different toxicological profile and are known to be present in significant amounts and the analytical method measures each component of the total residue separately (JMPR 1979).

Metabolites of some pesticides are also registered pesticides. Analysing food commodities in trade for the metabolite provides no information on which compound was used. Whenever possible, the parent pesticide and its metabolite(s) used as pesticides should be subject to separate MRLs. Where it is not possible to set separate MRLs (e. g. where the parent pesticide is degraded rapidly), the MRLs applying to the pesticides concerned can only be expressed in terms of the metabolite(s).

9 Annex 1: Crop groupings for plant metabolism data requirements

Plant metabolism studies are required for relevant crops, therefore for each crop on which use is proposed a metabolism study is required from that crop group.

Group of products[1]   Category[2]

1.   Fruit
    - citrus fruit   F
    - tree nuts   F
    - pome fruit   F
    - stone fruit   F
    - berries and small fruit   F
    - miscellaneous   F

2.   Vegetables

    - root and tuber vegetables   R
    - bulb vegetables   R
    - fruiting vegetables   F
    - brassica vegetables   L
    - leaf vegetables and fresh herbs   L
    - legume vegetables   P/O
    - stem vegetables   L
    - fungi   F

3. Pulses   P/O

4. Oilseeds   P/O

5. Potatoes   R

6. Tea   L

7. Hops   L

8. Cereals   C

9. Grass and forage crops   C

10. Tobacco   L

[1] Individual crops in groups 1-8 are listed in Directives 90/642/EEC and 86/362/EEC. Sugarbeet and fodder beet, which are not mentioned in these directives, are covered by group 2, root and tuber vegetables.

[2]   R   = root vegetables
    L   = leafy crops
    P/O   = pulses and oilseeds
    F   = fruits
    C   = cereals

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