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IUPAC Reports on Pesticides (40)

BOUND XENOBIOTIC RESIDUES IN FOOD COMMODITIES OF PLANT AND ANIMAL ORIGIN

(Technical Report)

Prepared for publication by;

M. W. SKIDMORE[1], G. D. PAULSON[2], H. A. KUIPER[3], B. OHLIN[4] AND S. REYNOLDS[5]

[1] Zeneca Agrochemicals, Jealotts Hill, Bracknell, UK

[2] US Department of Agriculture, ARS, Fargo, ND 58105, USA

[3] State Institute for Quality Control of Agricultural Products, Wageningen, Netherlands

[4] National Food Administration, Uppsala, Sweden

[5] Central Science Laboratories, York, UK.

Pesticides report: bound xenobiotic residues in food commodities of plant and animal origin

Synopsis: In order to assess the dietary risk resulting from the use of pesticides or veterinary drugs the nature of the chemical residues on food commodities needs to be determined. Elucidation of the nature of the chemical residue is carried out using radiolabelled studies where the radiolabelled xenobiotic is applied or dosed in a manner which reflects use conditions. Food commodities are exhaustively extracted to remove the individual components of the residue. Once extracted the identity and toxicological significance of the components can be assessed and, where appropriate, analytical methods developed to quantitatively determine the amount of the components in food items.

Depending on the characteristics of the components of the residue, the extraction regime may not remove all the chemical residue from the sample matrix. These residues are frequently characterised as being "bound", however the amount and nature of this residue will be highly dependant on the extraction regime used. To provide guidance and standardisation a definition of the term "bound residues" is recommended. This definition builds on a previous IUPAC definition but takes account of the current availability of enzyme systems which effectively solubilise the entire matrix rather than extracting the residue. It is also recommended that where the extraction falls short of the full definition then the residues should be termed as "unextractable" and the conditions of the extraction should also be defined .

Where residues are bound the assessment of the dietary risk cannot be directly assessed thus raising issues relating to the significance of the bound residue. The overall toxicological significance of a bound residue will depend primarily on its bioavailability and the level of exposure. In order to determine the bioavailability, study design is crucial in order to perform a critical safety assessment.

THE IUPAC COMMISSION ON AGROCHEMICALS AND THE ENVIRONMENT MAKES THE FOLLOWING RECOMMENDATIONS

1) The revised definition for what constitutes a bound residue should be adopted.

> **A xenobiotic bound residue is a residue which is associated with one or more classes of endogenous macromolecules. It cannot be dis-associated from the natural macro molecule using exhaustive extraction or digestion without significantly changing the nature of either the exocon or the associated endogenous macromolecules.**

Where residues have not been fully defined as bound they should be termed as unextractable and the procedure specified. The definition does not include fragments recycled through pathways leading to natural incorporation.

2) The characterisation of residues as bound should only be derived from radiolabelled studies.

3) More investigations should be carried out to define the nature of the binding of xenobiotics in food and feed items and to develop correlations with bioavailability.

4) The use of *in-vitro* methods is recommended to develop a better understanding of the binding mechanisms and to allow inter species extrapolation.

5) When bioavailability studies are conducted appropriate consideration should be given to the choice of test species and to the design of the study.

6) The extraction procedures used in residue analytical methods should be validated using samples from radiolabelled studies where the chemical has been applied in a manner consistent with the label and to Good Agricultural Practices.

CONTENTS

1. INTRODUCTION

The use of pesticides, veterinary drugs or growth enhancing compounds is an important factor in the production of sufficient quantities of safe and affordable food. The use of these agents (xenobiotics) may however, give rise to chemical residues in food items. The characterisation and identification of these residues is essential in assessing their toxicological significance and, following quantitative analysis to determine the extent of the exposure, in carrying out subsequent dietary risk assessment (a function of the intrinsic hazard and the exposure).

To define the nature of the chemical residue in the presence of biological material, studies are carried out using radiolabelled xenobiotic. The radiolabel, usually carbon-14 or hydrogen-3, is incorporated into a metabolically stable portion of the compound, and the test system, (i.e. plants or livestock), is treated at times and at rates which broadly simulate agricultural practice. Exaggerated rates are sometimes utilised to facilitate the identification of the residues. Commodities are taken from the test system to simulate agricultural practices and the total radioactive residues are quantified. Following exhaustive extraction, components of the radioactive residue are subsequently characterised by chromatographic and spectroscopic methods.

Dorough [1] proposed that a residue resulting from the use of the xenobiotic could be fundamentally characterised into one of four categories; i) parent and free metabolites, ii) conjugated residues, iii) bound residues and iv) residues formed through natural incorporation of atoms or fragments of the xenobiotic

Extracted residues can be readily characterised and their significance assessed, based on their concentration, inherent toxicity or by structure activity relationships. Residues which are unambiguously shown to result from the incorporation of the radiolabel into naturally occurring compounds are of no toxicological concern. The significance of bound or unextracted residues has always been difficult to rationalise and for many years it was believed that because the residue was unextractable it would also be unavailable[2], i.e. no exposure - no risk. Recent investigations have however, shown that the bioavailability of a bound residue upon oral ingestion will vary depending on the compound involved and on the nature of the binding [3,4]. The most fundamental point is, however, that the significance of any bound/unextracted residue will be directly related to the methods used to extract the commodity and the investigators interpretation of the term "bound".

The objective of this paper is to address the issues by considering the following points; (i) the approaches used to extract and fractionate residues and thus highlight the dilemma in the definition of a bound residue, ii) the definitions of a bound residue used in the last 10 -15 years, (iii) to review current knowledge of the chemistry of formation of bound residues, (iv) to review existing biological models used to assess bioavailability and examine the potential use of *in-vitro* methods, (v) review methods used to detect bound residues, (vi) to compare strategies applied by international advice bodies and regulatory authorities responsible for risk assessment of xenobiotic and animal drug residues, and finally vii) to recommend an update of the 1984 IUPAC definition[5], taking into consideration advances in technologies and scientific understanding.

2. APPROACHES USED TO EXTRACT AND FRACTIONATE RESIDUES IN FOOD COMMODITIES

In the context of xenobiotic residues in food and feed commodities, extraction is the process where residues are partitioned from the sample matrices into a solvent or solution for further investigation. The procedure of extraction is usually a balance of obtaining a high level of partitioning whilst minimising the decomposition of residues and the extraction of endogenous compounds that will frequently interfere with subsequent analytical/chromatographic steps. Advances in technologies and in the understanding of the sample matrices are providing an increasing number of options to facilitate extraction, (e.g. it is possible to be either very selective, and design the optimum extraction sequence, or to completely solubilise the sample). The term "solubilisation", in this instance, should be seen as distinct from extraction.

The objective of this section of the report is to review the range of extraction procedures reported in the literature so as to highlight the issues of when a residue should be defined as bound and whether there is a distinction between the terms "bound" and "unextracted"

The preparation of a homogenous sample is paramount in achieving reproducible subsamples. In addition, the more finely divided the matrix the larger the surface area available to the solvent to facilitate an efficient partition of the chemical residue from the solid to the solvent. It is now common practice in metabolism or nature of the residue studies to physically macerate the sample in the presence of either CO_2 or liquid nitrogen to produce a finely divided sample for analysis.

Solvent extraction regimes used to extract radioactive residues from crop commodities and animal tissues vary depending on the nature of the matrix, the xenobiotic and, to a large extent, on individual preferences. Any extraction regime must, by definition, be extensive and exhaustive for the analyte or residue and, in nature of the residue studies, include a sequence of solvents of increasing polarities, including aqueous solutions. The optimisation of solvent extraction procedures involves variation of solvent polarity/mixtures, physical maceration and the use of elevated temperatures. Control of the solvent polarity can provide advantages when the objective is to extract a specific analyte from the matrix. In addition, by understanding the composition of the sample matrix endogenous components may be selectively removed providing a cleaner extract. A comprehensive investigation into the extraction efficiencies of 10 pesticides from three plant species (radish, straw and mustard greens) has been carried out using three solvents (acetonitrile, acetone and methanol)[6]. Using statistical analysis of the data it was concluded that the most consistently effective solvent for extraction of the pesticides in the study

was methanol. An interesting observation from this investigation was that the behaviour and effectiveness of acetonitrile as an extraction solvent varied with the amount of water present, a phenomenon not observed with acetone or methanol. A similar conclusion was reached by Bertuzzi *et al*[7] who showed that the addition of 35% water to acetonitrile significantly improved the extractability of a field incurred residue from low moisture crops.

The use of continuous or repeat extractions will also improve the efficiency of the extraction, a principle used in Soxhlet extraction. This technique has been widely used to provide the basis for a so-called "exhaustive extraction". In this technique the extraction is achieved through contact of the finely divided sample with warm solvent which is continuously refreshed. Increasing the temperature of the extraction solvent results in a decrease in viscocity allowing improved penetration of the sample matrix and thus improving the overall extractability and kinetics of the process. The same principle has been adopted in microwave assisted extraction and in the accelerated solvent extraction (ASE^{TM}) systems. In the former case, the sample and solvent are placed in a sealed container and the temperature and pressure of the system controlled by the use of pulsed microwave energy. The use of the sealed containers enables elevated temperatures to be attained, e.g. at 175 psi[a] methanol can reach a temperature of 151^{0}C. Nicollier and Donzel[8] reported that by using this technique up to 66% of a residue previously unextracted by Soxhlet extraction was removed from the sample matrix. The addition of acid to the solvent mixture significantly increased the extractability of residues from grain samples presumably through hydrolysis of polymeric matrices. The authors concluded that the technique was effective in facilitating extraction of residues that are encapsulated or physically bound to the sample matrix. ASE operates on a similar principle enabling pressures of 1500-2500 psi and temperatures of 100 - 200 ^{0}C to be achieved. Advantages of this latter technique include good precision, low solvent consumption, variable sample size, short extraction times and good recovery[9].

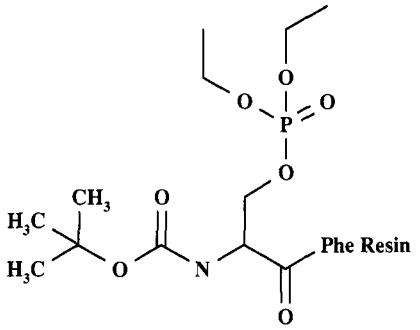
The principle of decreasing the solvent viscosity has also been utilised in supercritical fluid extraction (SFE). In this technique a solvent, usually carbon dioxide or methanol, is subject to conditions of temperature and pressure that result in a critical point where it becomes a supercritical fluid. In this state the solvent has a low viscosity and high diffusivity thus enhancing the extractability. To enhance the solvating power of the solvent, various modifiers, e.g. methanol, triethylamine, trichloroacetic acid and pyridine[10] have been used. The technique is seen as particularly useful for thermally labile compounds. Many reviews and articles have been published on this technique which often demonstrates an increase in extractability following conventional extraction regimes[11,12,13].

During enzyme hydrolyses it is frequently found that the buffer solutions are themselves eliciting the extraction of the residue and control incubations in the absence of the enzyme are recommended. Lee *et al.* [14]reported that 35% of the "unextracted tissue bound " residues of fenvalerate in wheat plants was released with pH 5 buffer at 37 °C. Incubation in the presence of β-glucosidase did not release significantly greater quantities of the residues whilst cellulases released a further 15% of the residue. None of the extracted residues could be identified due to interferences from co-extractives. Analysis of bound residues of deltamethrin in bean plants showed that incubations at pH 4.75 released 45% of the radioactivity in the sample whilst pH 7.55 buffer released 56%. The pH of the buffer can also therefore be a significant variable[15] . It can only be presumed that residues extracted in these processes represent ionic interactions with natural matrices. In this case the use of mild acids or bases may also have achieved a similar result.

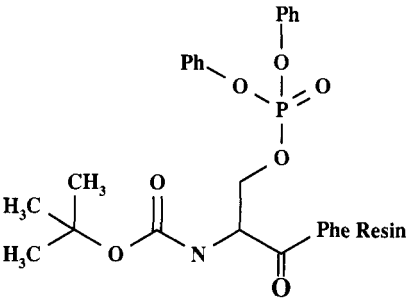
In all the above cases, excepting those which incorporate the use of acids and bases, the extraction is achieved through the solvation of residues that are either free, encapsulated or that have a weak ionic interaction with natural cell components. It would be unlikely that the conditions used would result in the cleavage of a covalent bond without some form of chemical interaction, e.g. esterification. It has however been reported[16] that metabolites of organophosphate pesticides covalently bound to serine residues in a protein chain may be released from proteins by SFE. In the study, model compounds, Figure 1, were immobilised onto a solid support (amino-methylated resin) to mimic proteins containing covalently

[a] 1 psi = 6.89 kPa

attached metabolites of organophosphorus pesticides. These were subjected to SFE using supercritical carbon dioxide containing 25% methanol.



Boc-Ser(PO_3Et_2)-Phe-Resin



Compound A

Boc-Ser(PO_3Ph_2)-Phe-Resin

Fig. 1. Model Peptides Used to Demonstrate the Release of Covalently Bound Residues[16]

The extraction was carried out over a period of 4 hours at a temperature of 200 °C and at a pressure of 355 atmos. Analysis of the extracts, for compound A, showed the presence of diphenyl phosphoric acid and of components containing the diphenylphosphoserine moieties. Peptides made by the same procedures but not immobilised onto the support were simply distilled by the conditions of the extraction and were recovered intact. It was concluded that xenobiotic residues which form a covalent bond with natural cell components may therefore be extracted using SFE. Further work in this area would help to elucidate the mechanism of cleavage of the covalent bond.

Enzyme preparations are widely used in extraction schemes and can be divided into those that cleave an exocon-endocon[i] linkage, (e.g. β-glucosidase), and those which breakdown large macromolecules into their constituent parts, examples shown in Table 1. The latter enzymes can be used to effectively solubilise the matrix which may[17] or may not release the exocon from the endogenous component.

TABLE 1. Some of the Commonly Used Enzymes Employed to Breakdown Macromolecules

Enzyme	*Activity*	*Source*	*Optimum pH*
Cellulase	Degrades cellulose into glucose	Aspergillus sp.	5
Collagenase	Degrades collagen into peptides or amino acids	Clostridium histolycum	7
Pepsin	An endopeptidase with a preferred activity at hydrophobic amino acids	Porcine stomach	2-4
Subtilisin	An endopeptidase with a preferred activity at neutral and acidic amino acids	Bacillus subtilis	6
Papain	An endopeptidase with preferred activity at arginine and lysine	Papaya	6-7
Protease K	An endopeptidase with a preferred site of activity at the hydrophobic and aromatic amino acids	Tritirachium album	7-12

Detergents are increasingly used as part of fractionation strategies based on their ability to solubilise cell membrane components including lipids and proteins and to act as decomplexing agents. It has also been reported [18] that surfactants can release encapsulated residues although no example was given. Detergents in common use can be broadly classified as ionic (e.g. sodium dodecyl sulphate) or non ionic (e.g. Tweens and Triton), each having specific properties and advantages in specific situations. Practical

[i] Exocon - refers to the part derived from a xenobiotic

Endocon - refers to the part derived from the natural product

considerations relating to the removal of the detergent to allow further characterisation of the residue should be given. The properties of the various detergents have been reviewed [19].

The use of so called "exhaustive extraction strategies" has been proposed to provide a full characterisation of the total radioactive residue in food commodites. In these schemes, cells are systematically fractionated into their component parts, (e.g. Krowke[20] and Sun and Dent[21] for animal tissues and by Langebartels and Harms[22], Hatfield [23] and Harborne[24] for plant commodities). In other cases enzyme cocktails have been employed to solubilise the cell wall material in a single incubation[25].

Langebartels and Harms[22] investigated the distribution of ^{14}C- pentachlorophenol in cell walls of plant suspension cultures. In this study, fractionation of the cell wall was carried out using a modified extraction procedure developed by Takeuchi and Komamine[26], producing 6 fractions, (i.e. starch, proteins, pectins, lignin, hemicelluloses and cellulose). The procedures used are shown in Table 2. Prior to the fractionation the cells were extracted with potassium phosphate buffer followed by a methanol/chloroform mixture.

TABLE 2. Fractionation of Plant Cell Components (Langebartels and Harms[22])

Treatment	**Fraction**	**Method**
Amylase	Starch[iii]	30 °C for 20 hours in pH 7 buffer.
Pronase E	Protein	30 °C for 16 hours in pH 7.2 buffer
EGTA[i]	Pectin	80 °C for 4-6 hours incubation in 100 ml of 50 mM EGTA in pH4.5 buffer
DMSO[ii]	Lignin	48 - 96 hours at room temperature. Followed by a second extraction carried out at 80 °C.
Potassium Hydroxide	Hemicellulose	27 °C for 24 hours in 24% KOH
Sulphuric acid	Cellulose	4 hours in 72% sulphuric acid.

i) - EGTA - Ethylene glycol bis-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

ii) - Delignification can also be achieved using sodium chlorite solution[24]

iii) - Starch extraction can be achieved with DMSO followed by precipitation with ethanol

Each extraction was carried out for sufficient time to ensure no further material could be removed. The data showed that the majority of the ^{14}C-pentachlorophenol residues were associated with proteins, lignin and hemicellulose. Residues in the hemicellulose fractions could not be extracted with solutions of urea and SDS (reported as only releasing noncovalent associations), but were released with hemicellulase. The radioactive residues released from hemicellulose were shown, based on their behaviour and characteristics, to contain covalently bound pentachlorophenol.

As shown in the above example, to unambiguously characterise the residue in terms of natural incorporation or covalently bound material, endogenous macromolecules are broken down into their component parts. Some examples of the techniques used are given in Table 3. Where radioactivity is clearly identifiable as associated with naturally occurring cellular components then natural incorporation can be assumed.

TABLE 3. Methods Used to Degrade Naturally Occuring Macromolecules into their Constituent Parts.

Macromolecule	Method of degradation	Products
Protein	6M HCl 140 °C[27] 3M Toluenesulphonic acid[28] A general review of chemical methods has been prepared[29] Proteolytic enzyme digestion (exhaustive)[30]	Amino acids
Starch	0.05M HCl at elevated temperatures Enzymes (Amylase)	Glucose
Lignin	White Rot Fungi[31] Cupric oxide at 170 °C[32]	Unclear Phenols
Hemicellulose	Hemicellulase	Galactose
Cellulose	Cellulase	Glucose

The methods described above represent a wide range of capability in terms of extraction, solubilisation of the matrices, and fragmentation of endogenous macromolecules, any one of which may or may not release the xenobiotic residue. Based on the information available it is feasible to solubilise any sample matrix and consequently the residue. It is however important not to loose sight of the overall objective of a nature of the residue study which is to characterise and identify xenobiotic residues for risk evaluation. The term "bound" serves to characterise a residue as having specific properties but whose overall toxicological significance may need to be further investigated. To reduce the uncertainty of when a residue can and should be characterised as bound, several definitions have been recommended.

3. DEFINITION OF A BOUND RESIDUE

In 1975 Kauffman[33] wrote that "like any new term the definition or interpretation of what a bound residue was, varied with each individual scientist and the extraction used". It would be difficult to dispute that this statement could still be held true twenty years later.

The first attempt at providing an international definition for bound residues in plants was developed in 1984[5] by IUPAC (International Union of Pure and Applied Chemistry).

> "Non-extractable residues in plants are defined as chemical species originating from pesticides, used according to good agricultural practice, that are unextracted by methods which do not significantly change the chemical nature of these residues. These non extractable residues are considered to exclude fragments recycled through pathways leading to natural products".

The footnotes provided with the statement are a crucial part of the definition;

i) these residues are considered to exclude fragments recycled through pathways leading to natural incorporation.

ii) methods refer to any procedures such as solvent extraction and distillation used to **exhaustively** remove chemical species from a plant matrix.

iii) the extraction procedure should be cited.

In 1986 a large co-ordinated research program was initiated by the Food and Agriculture Organisation of the United Nations (FAO) and International Atomic Energy Authority IAEA to investigate the significance of bound residues in grain following post harvest treatment with a range of ^{14}C-pesticides[34]. The definition of a bound residue used in this project was;

"Non-extractable (or bound) xenobiotic residues may be defined as chemical species, originating from the xenobiotic usage, which cannot be extracted by methods commonly used in residue analysis and metabolism studies."

The extraction method used in the program was a single 24 hr Soxhlet extraction with methanol.

In the animal drug area, Vilim[35] proposed a characterisation of bound residues based on the residue extractability:

"Exhaustive extractions are carried out employing solvents with various polarities and under conditions, such as pH levels, enzymatic or chemical hydrolyses, denaturation, and solubilisation techniques. Care should be taken when employing the various procedures to make sure that they are not destructive or result in alteration products or artifacts. Bound residues are then further classified as natural incorporated or xenobiotic conjugates originating from covalent binding of the parent drug or metabolites to biological macromolecules."

It is clear from the recent literature that these, and other, definitions have been widely used and resulted in a less disparate classification for the term "bound residue" as compared to earlier publications. There are however still differences in interpretation of the term "bound residue", many of which originate from samples which have been extracted using the solvents and techniques defined in the residue analytical method and then re-extracted with a further technique only to demonstrate the release of more of the analyte. It is doubtful whether this residue actually constituted a bound residue but simply reflects the incomplete extractability of the analytical method.

4. MECHANISMS OF THE FORMATION OF BOUND RESIDUES

The formation of bound residues can occur through a number of processes resulting in the formation of covalent bonds or through physical encapsulation within the macromolecular matrix. It has also been noted that the proportion of a bound residue may increase, above that found in the raw agricultural commodity, during processing. Matthews[36]reported that the proportion of bound residue from four organophosphorus insecticides was greater in bread and certain extrusion breakfast cereals than was originally observed in the grain. The nature of these interactions is likely to be through encapsulation and is an area for further research and definition. Associations constituting a bound residue are generally difficult to unequivocally characterise but an increased understanding of the mechanism would result in a greater appreciation of the significance of the residue and the associated risk. The majority of the mechanistic work to date has been carried out in the pharmaceutical and veterinary drug area and a number of selected examples are described below. Some of the examples are not necessarily related to the theme of the paper but nevertheless serve to exemplify some of the possible mechanisms.

The formation of covalent bonds between xenobiotic residues and macromolecules is most frequently observed when metabolic processes generate reactive metabolites which can subsequently react with endogenous macromolecules.

The most widely studied mechanism is the formation of a covalent bond from the reaction of a xenobiotic electrophile with the nucleophilic sites on proteins or nucleic acids.

During the *in-vitro* incubation of carbaryl (1-naphthyl methylcarbamate), (Figure 2), with rat liver microsomes[37] the amount of binding was markedly increased (2-3 fold) by pretreating the rats with phenobarbital, a known cytochrome P450 initiator. Binding was however decreased when inhibitors such as, cysteine or glutathione were introduced into the incubates. These data suggest the production of a reactive intermediate resulting from oxidative metabolism. By analogy with naphthalene, and the nature of the metabolism of carbaryl , the formation of epoxides were postulated.

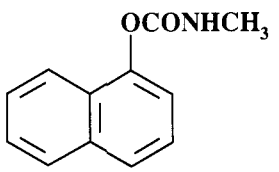
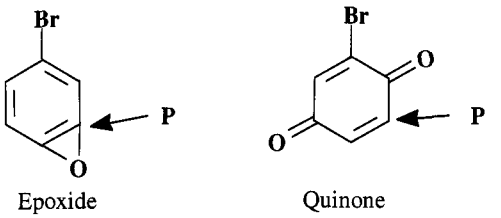


Fig. 2. Structure of Carbaryl

Although epoxides undoubtably contribute to the formation of protein bound residues, there is evidence to suggest that these reactive molecules can be further metabolised to quinones by enzymic action, e.g. dihydrodiol dehydrogenase. The mechanism has been postulated for *o*-quinones[38] as resulting from the nucleophilic attack at the 4 position, rearrangement and oxidation. The involvement of quinones in the formation of bound protein residues has also been found following an *ip* injection of bromobenzene to rats. Protein adducts were shown to be formed which resulted from both an epoxide and a quinone intermediate, Figure 3 [39]. Determination of the nature of the bound residue was achieved using an alkaline permethylation reaction[40] (16M potassium hydroxide and methyl iodide at 120°C) to cleave the xenobiotic residue from the protein. This method is particularly useful since the conditions used did not degrade the acid labile quinone derived adducts. In the case of bromobenzene, the formation of protein adducts with quinones were found to be more extensive that for epoxides



P = Protein S

Fig. 3 Epoxide and Quinone Intermediates from the Oxidation of Bromobenzene[39]

Further investigation of the nature of the bound residues of ^{14}C-carbaryl suggested the involvement of a second reactive metabolite, i.e. methyl carbamate. The methyl carbamate part of the molecule is readily cleaved and potentially bound through direct methyl carbamylation of the serine hydroxyl group or through further metabolism with the radiolabelled atom becoming part of the carbon pool.

The cephalosporin ceftiofur, used to treat respiratory diseases in livestock has been found to result in bound kidney residues, following *ip* treatment, Following precipitation of the protein with trichloroacetic acid (TCA), 62% of the total radioactive residue was shown to be associated with proteins[41]. The proposed pathway suggested the cleavage of the furoyl moiety resulting in the formation of a sulfhydryl group which subsequently reacted with protein, Figure 4.

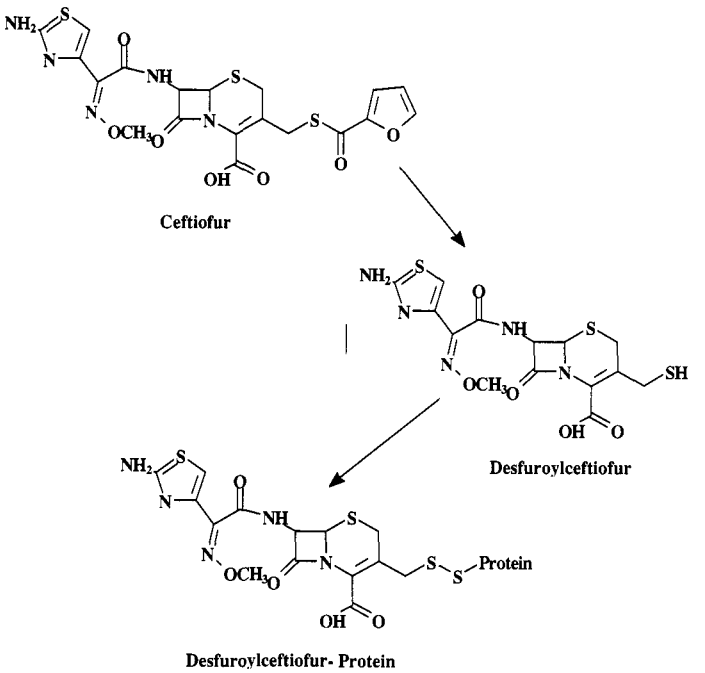
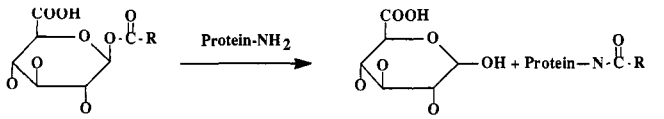
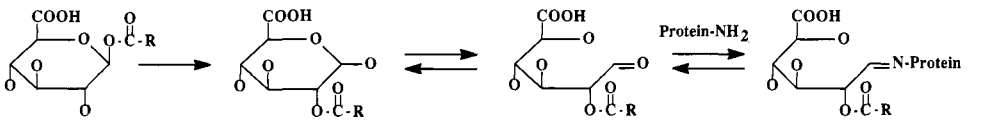


Fig. 4. Proposed Pathway for the Binding of Ceftiofur [40]

The formation of glucuronide conjugates is generally seen as a significant detoxification step leading to excretion. However, several workers have reported that certain acyl glucuronides are unstable and readily react with proteins. This protein binding has been linked to acute renal toxicity found from a group of non steroidal anti inflammatory drugs (NSAID). In the case of a typical NSAID, suprofen, Smith[42] proposed that the suprofen glucuronide undergoes acyl migration which results in the formation of an imine between the amino group on lysine and the aldehyde of the glucuronic acid, Figure 5b. Although the imine mechanism explains some of the binding the nucleophilic displacement of the glucuronic acid could not be excluded in the study, Figure 5a.



5a



5b

Fig. 5. Proposed Mechanisms for the Covalent Binding of Acyl Glucuronide Conjugates.

Ronidazole,((1-methyl-5-nitroimidazol-2-yl) methyl carbamate) (Figure 6), used for the treatment of swine dysentery and turkey blackhead, is rapidly depleted from animal tissues 2-3 days after treatment, but bound residues remain for greater periods of time. A combination of *in-vivo* and *in-vitro* studies [4,43] have provided detailed information on the nature of bound residues and the mechanisms of covalent bond formation. *In-vitro*, the formation of protein bound residue was characterised as consisting of the intact imidazole ring, loss of the carbamate group and loss of the proton located at the C-4 position. The formation of the protein adduct occurred primarily at the 2-methylene carbon position. Studies carried out in rat liver microsomes indicated that three radiolabelled hydrolysis products could be released from bound residues following incubation with ^{14}C-ronidazole, namely methylamine, carboxylmethyl cysteine and oxalic acid. These products may be expected on the basis of the presumed structure of the protein adduct. *In-vivo*, administration of ronidazole radiolabelled at different positions of the molecule to rats and swine, yielded similar results as observed in the *in-vitro* systems. In liver samples taken from rats six hours after administration of ronidazole all radioactivity in the protein bound residue fractions could be attributed to an intact imidazole structure. However, in samples collected two days after dosing, less than 20% of the protein bound residues could be correlated to ronidazole-related bound residues. These data suggest the incorporation of the radiolabel into endogenous components or adducts formed from smaller structural fragments.

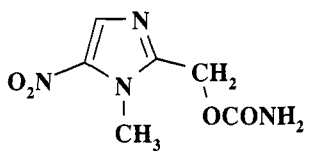


Fig. 6 Structure of Ronidazole

Furazolidone,(3-[(5-nitrofurfurylidene)amino]oxazolidin-2-one), (Figure 7) widely used for treatment of intestinal infections caused by E.coli in swine and for treatment of gastrointestinal tract and bronchial infections in poultry exhibits mutagenic and carcinogenic properties.

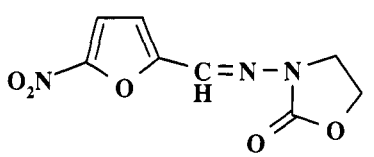


Fig. 7 Structure of Furazolidone

Oral administration of furazolidone to pigs resulted in an extensive biotransformation, leading to almost no detectable levels of the parent compound in tissues soon after last treatment[44]. However significant amounts of total radioactivity were shown to be tightly bound to tissue proteins even after a withdrawal period of 2 weeks. Formation of protein-bound residues of furazolidone could be reproduced in pig liver microsomes[45] and in primary cultures of pig hepatocytes [46]. The major degradation pathway of nitrofurans involves the reduction of the nitro group presumably resulting in the formation of reactive intermediates which bind covalently to macromolecules. *In-vitro* experiments with incubations of ^{14}C-furazolidone in pig liver hepatocytes and microsomes indicated a dose and time related increase in the formation of bound residues. It was further shown that at least 70% of the bound material still contained the intact side chain 3-amino-2-oxazolidinone (AOZ). From liver samples of piglets treated with furazolidone for 10 days, followed by withdrawal periods of 2 hours or 10 days, 23% and 15% of the bound residue respectively could be released as AOZ. These experiments prove the drug-related nature of at least part of the bound metabolites of furazolidone and that *in-vivo* more than one type of bound residue may be formed.

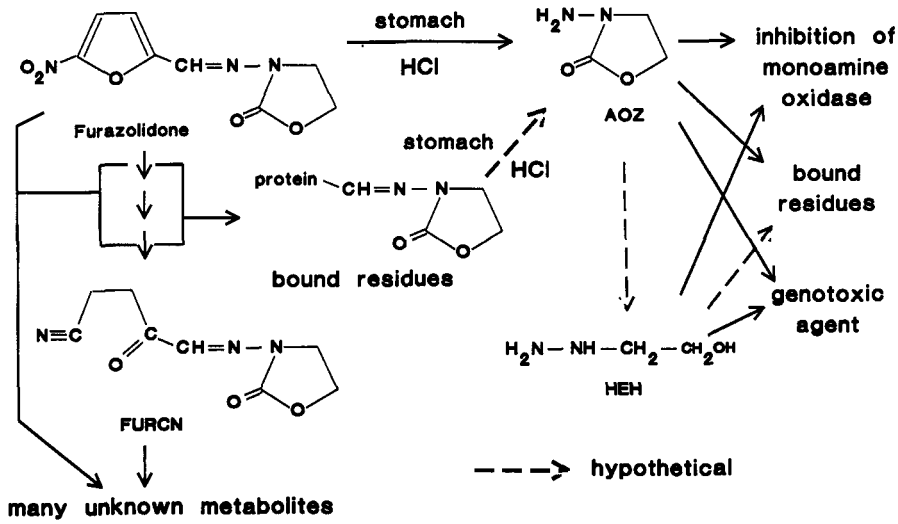
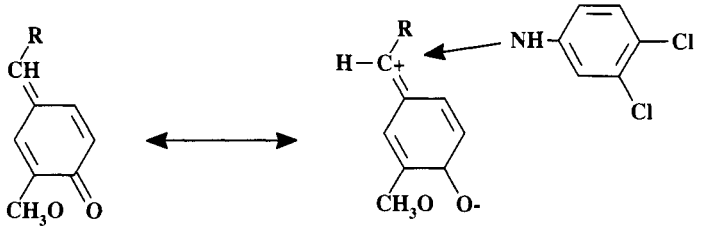


Fig. 8 Proposed Biotransformation Pathway for Furazolidone

Further investigations of the toxicological relevance of the bound residues of furazolidone have been carried out [47]. The bioavailability of bound radiolabelled metabolites of furazolidone in muscle tissue of treated piglets has been determined in rats. At least 41% of the total bound radioactivity was taken up by the animals, as determined from radioactivity levels in urine, tissues, carcass and expired air. In addition, a considerable part of the residual radioactivity in muscle and liver tissue of rats proved to be bound. Similar results have been obtained using the Gallo-Torres model [48]. The bioavailability of liver residues of furazolidone derived from treated swine decreased from 40% in samples taken at 0-day withdrawal, to 19% in samples taken at 45 days of withdrawal, while muscle residues showed no change in relative bioavailability with values of 37 and 41%. Pretreatment of tissue samples with organic solvents to generate a non-extractable pellet removed 10-20% of the initial radioactivity, but did not change the bioavailability of the remaining residues. The toxicological properties of the releasable part of furazolidone, AOZ, have further been investigated. It was shown that AOZ irreversibly inhibits the monoamine oxidase activity in primary cultures of pig hepatocytes, as well as ß-hydroxyethylhydrazine (HEH), a compound presumably formed from AOZ which possesses pronounced genotoxic potency[49]. The possible pathways involved in the degradation of furazolidone and its protein-bound metabolites[50] (Figure 8,) and the observed toxicological properties of the furazolidone metabolites indicate that protein bound residues may constitute a risk for the consumer. Furthermore these experiments have shown the usefulness *of in-vitro* systems to elucidate biotransformation pathways of xenobiotics and to characterize the structure and nature of macromolecular adducts.

In contrast to animal systems, little in the way of mechanistic work has been conducted to define the nature of the binding to endogenous macromolecules in plants. Perhaps one exception being the association to lignin which has been recognised for many years as a major binding site in plants[51] and is viewed as the plants method of excretion where residues are effectively locked away in the cell wall[52]. Lignins are somewhat unique in that unlike other macromolecules they do not have any defined structure because of the variety of monomers involved, the number of binding sites available and the degree of polymerisation[53]. In some cases the binding is thought to be via covalent binding whilst in others residues are believed to be encapsulated in the matrix of the lignin[54]. In a model system established to study the polymerisation of coniferyl alcohol, a known precursor of lignin, Freudenberg and Neish[55], found that quinone methide, Figure 9 , an intermediate in the process, can readily react with the hydroxy and acidic groups of cellulose. It is probable that a range of xenobiotic nucleophiles could potentially react with the quinone-methide. The binding of 3-chloroaniline (3-CA) and 3,4-dichloroaniline (3,4-DCA) to lignin in rice *in-vivo* has been further investigated using the polymerisation model; these studies have demonstrated that the compounds would polymerise with coniferyl alcohol through the reaction of the amino group with the electrophilic carbon. In the model system the incorporation of 3,4-DCA was higher than for 3-CA a result supported in *in-vivo* studies. The results may be due to the blocking of the para position of the aniline thus preventing other reactions of the molecule.



R= $CH(CH_2OH)OPh(OCH_3)CHCHCH_2OH$

Fig. 9. Formation of a Quinone-methide intermediate

In an investigation into the metabolism of ^{14}C-oryzalin, (4-(dipropylamino)-3,5-dinitrobenzenesulfonamide), a dinitroaniline herbicide, Figure 10, in corn a significant amount of the ^{14}C was bound to particulate matter. Further investigation demonstrated that the residues could be solubilised with mild detergents suggesting some association with cellular membranes. Further investigation suggested that the association was with lipid rather than the protein component of the cellular membrane[56].

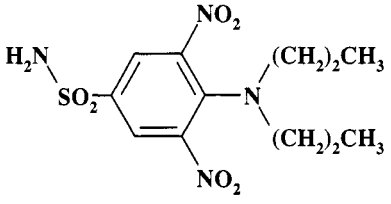


Fig. 10. Structure of Oryzalin

A comprehensive report detailing the detection and significance of reactive metabolites of agrochemicals[57] concluded that the bound residues that result from interactions of these entities with macromolecules can act as a marker for exposure. Although this would not be a measure of the toxicity it would nevertheless provide a quantitative measure for the exposure side of the risk equation The report also recommends that further study be carried out to determine the toxicological significance of bound residues derived from active metabolites. A similar conclusion was also made by Hawkins[58] in a review article illustrating the reactions of some of the more unusual or novel reactive intermediates.

5. REGULATORY APPROACHES TO BOUND RESIDUES

5.1 Joint FAO/WHO Expert Committee on Food Additives (JECFA)

Public health issues related to residues of veterinary drugs in foods of animal origin are periodically reviewed by the JECFA as recommended by a joint FAO/WHO Expert Consultation held in 1984 in Rome[59]. During the JECFA meeting, held in 1988, an approach for the evaluation of non-extractable residues of veterinary drugs with respect to human food safety was outlined[60]. The committee defined "total residues" of a drug in animal derived food as consisting of the parent compound and all metabolites/drug-based products that remain in the food after administration of the drug. "Extractable residues" are residues extracted from tissues or biological fluids by means of aqueous acidic or basic media, organic solvents and/or hydrolysis with enzymes (e.g. sulphatases or glucuronidases) to hydrolyse conjugates. Non-extractable residues are obtained by subtracting the extractable residues from the total residues and comprise residues of the drug incorporated through normal metabolic pathways into cellular constituents like amino acids, proteins or nucleic acids(which are of no toxicological concern), and chemically bound residues formed through interaction of the parent drug or its metabolites with cellular macromolecules.

The committee recommended that in the absence of other information the toxicological potency of bound residues may be considered as being equal to that of the compound for which the ADI was set. Thus when the total residues do not exceed the established MRL, bound residues do not need to be further examined. In cases where total residues exceed the MRL, the contribution of bound residues to the toxic potency of total residues should be taken into account. In this case data on chemical structure, bioavailability, biotransformation and toxic potency of the bound residues must be evaluated.

Assessment of the biological significance of bound residues depends on the extent to which these residues are absorbed by humans upon oral ingestion of foods (bioavailability), and on the toxic potency of these residues. Biological models to measure the bioavailability of bound residues and to characterise toxic potency are discussed in Section 6.

5.2 U.S. Food and Drug Administration (FDA)

The FDA has adopted an approach for safety evaluation of bound residues of veterinary drugs[61] which is similar to the one proposed by JECFA. Full structural identification of the bound residue is normally not required, and if the total residue is below the" safe concentration" at the requested withdrawal period, no additional safety testing is required. A procedure has been specified for a non-carcinogenic drug which is not bioavailable, as determined in bile cannulated rats (the Gallo-Torres model[62]). The problem of the carcinogenic potency of bound residues has been addressed specifically. One of the specific concerns is the potential gastro-intestinal tract binding and related carcinogenic effects. In these cases a safety evaluation of the bound residue derived from a carcinogenic veterinary drug, should be based on a suitable combination of *in-vivo* and *in-vitro* studies[63]. Data should be provided on the following:

- Bioavailability of the bound residue
- Toxicological potential of the bound residue
- Reversibility of adduct formation
- Mechanism of bound residue formation.

The most important approach in determining the need to assess the toxicological significance of bound residues is to evaluate their bioavailability in laboratory test animals. The outcome of these studies may or may not lead to further mechanistic studies of the toxicity of the bound residue.

It is important to note that no particular test package has been outlined, since the study design *(in-vivo* and *in-vitro)* should be based on the nature of the bound residue and the toxicological profile of the parent compound.

5.3 UNITED STATES ENVIRONMENTAL PROTECTION AGENCY (EPA)

The EPA guidelines for conducting plant and animal metabolism studies provide detailed guidance on the scientific approaches to characterisation of residues in general. The definition of a bound residue, although not explicit, is implicit in the recommended procedures which refer to a bound residue as that remaining after extraction with solvents and solvent systems (including water) of various polarities. Recommended procedures are provided to characterise the residues using methods such as extraction with acids/bases, surfactants, enzymes and finally strong acids and bases. Where there are no toxicological concerns about the parent or potential metabolites, EPA does not require characterisation of unextractable residues that are less that 0.05ppm parent equivalents or 10% of the total radioactive residue. Where toxicological concerns are evident or the unextractable residue is large and cannot be identified then bioavailability studies may be requested[64]

6 METHODS USED FOR THE DETERMINATION OF THE BIOAVAILABILITY OF BOUND PESTICIDE RESIDUES

6.1 *In-vivo* methods

Any consideration of methodology to measure bioavailability must take into account the fact that the overall disposition of an orally consumed xenobiotic related residue may range from very simple to very complex. Thus, the investigator must keep in mind that if an agrochemical residue is absorbed from the gastrointestinal tract of an animal, the final disposition of the compound may be a function of a wide variety of competing metabolic transformations, rates and routes of secretions and excretions. These include: (1) metabolism by one or more sites in the gastrointestinal tract; (2) transport of the parent compound and/or metabolites to the liver via the portal vein (i.e. without exposure to the general vascular system); (3) absorption via the lymphatic system (i.e. entering the general vascular system prior to entering liver); (4) metabolism of the parent compound and/or further metabolism of primary metabolites in the liver; (5) transfer of the parent compound and/or metabolites from the liver back into the gastrointestinal tract via the bile; (6) transfer of the parent compound and/or metabolites from the liver **into** the general vascular system; (7) transfer of parent compound and/or metabolites from the general vascular system into other organs with or without metabolism in these organs; (8) transfer of the parent compound and/or metabolites from the general vascular system into urine via the kidney; (9) transfer of the parent compound and/or metabolites from the general vascular system back into the gastrointestinal tract via the saliva or diffusion or transport across the gut wall; (10) reabsorption of the parent compound and/or metabolites which re-enter the gastrointestinal tract via the bile, etc; (11) exhalation of a volatile metabolite(s) and (12) microbial metabolism in the gastrointestinal tract[65]. Thus, the investigator must be prepared to use a variety of techniques and methods to adequately answer the question of whether or not an agrochemical related residue is bioavailable.

6.1.1 Animal selection and dosing

The rat is the species most commonly used to study bioavailability for a variety of reasons including: (1) low animal cost; (2) low animal maintenance cost; (3) availability of large numbers of animals with very similar genetic, nutrition and management background; (4) the rat is commonly used in the toxicological evaluation of the parent agrochemical; and (5) the rat is very resistant to infections and trauma associated with surgical intervention. However, the investigator needs to be aware that the rat is not always the best animal species and may be an extremely poor animal model in some instances. For example, the bioavailability of a residue bound to cellulose will most likely be different in the rat and a ruminant. Other species differences which may affect the disposition of a xenobiotic related residue include, molecular weight threshold for biliary secretion[66,67,68] and the amount of salivary secretion[69]. Because of the concern for human safety, the question of bioavailability will frequently occur for animals raised for human consumption (cattle, sheep, swine, chickens and turkeys). Therefore, this discussion will include methodology appropriate for ruminants, swine and poultry in addition to methodology used for the rat.

The most "natural way" of oral dosing is to mix the test material with feed and allow the animal to ingest the mixture. Methods to train rats[70] and swine[71] to rapidly consume "meals" containing test materials have been reported. Other procedures to incorporate test materials into semiliquid diet or ingesta from donor animals and administration to rats or swine by stomach tube have been described[72]. In principle the same procedure could be used for other animal species. Methods have been reported [73]for introducing a stomach tube into ruminants. If the amount of radiolabeled test material to be given is not too large, it is often convenient to place it in a gelatine capsule(s) and administer the dose to ruminants or swine with a "balling gun". Birds can be conveniently dosed by placing the test material into a gelatine capsule, holding the mouth open and inserting the capsule directly into the crop. This approach is especially convenient for dosing ducks, and turkeys.

6.1.2 Collection of urine and faeces

Any experiment designed to evaluate bioavailability must include methods to quantitatively collect both urine and faeces whilst preventing cross contamination of the excretion products. Metabolism cages designed to separately collect urine and faeces from rats[73], cattle[74] swine[75] and sheep and goats[76] have been described. Other animal metabolism stalls (some of which accommodate separation of faeces and urine) for laboratory and farm animals have been reviewed[77,78]. Other investigators have described a variety of devices (glued to animal or held in place by straps or harnesses) to facilitate separate collection of faeces and urine from cattle[79,80], swine [81,82]and sheep and goats[83,84]. More recently an apparatus that can be adapted for separate collection of faeces and urine from cattle, sheep, goats and swine has been used with excellent success[85]. Catheterization of the urinary bladder of large female cattle, sheep, goats and swine[86,87,88] provides for quantitative collection of urine and prevents cross contamination of faeces and urine. Catheterization of the urinary bladder of small animals such as the rat is not feasible; however, through surgical intervention the ureter and the urinary bladder can be cannulated to facilitate urine collection[86].

In chickens, turkeys, ducks and geese the faeces and urine collect in the cloaca and are voided as a mixture. Some investigations have simply attached devices (no surgical intervention)[87] to birds or surgically exteriorized the urethral opening[88,89,90]to facilitate separate collection of faeces and urine. However, colostomy provides a better way for quantitative separation and collection of the faeces and urine from chickens and other birds[91,92,93]

6.1.3 Blood collection

The presence of radioactivity in the vascular system after oral administration of ^{14}C-labelled agrochemical residues provides qualitative evidence of bioavailability and serial collection, and analysis of blood from an animal provides an estimate of the kinetics of absorption, metabolism and excretion. Comparing the amount and chemical nature of residues in blood samples collected at selected sites (e.g. portal vein and jugular vein) may provide valuable information regarding intermediary metabolism of an orally administered agrochemical residue.

Methods for collecting blood from the orbital sinus, jugular vein, heart, caudal artery, carotid artery, portal vein and tail vein of the rat are available[89]. Blood is easily obtained from the jugular vein of cattle, sheep, and goats; however, if serial samples are to be collected from these animals, it is better to install a catheter in the jugular vein[94,95]. Procedures for collecting blood from the tail vein and a wide variety of other cites in the ruminant animals have been summarized[77].

Blood samples can be collected from the orbital sinus, ear vein, tail vein, the jugular vein or other large blood vessels in the neck region and a variety of other sites in swine[96]. However, all of these procedures require that the animal be tightly restrained and under these conditions swine very quickly become excited and belligerent. This problem can be minimized by anesthetizing the animal and placing a cannula in the jugular vein (catheter passed under skin to exit at the top of the neck) or in the femoral artery or vein with catheter exited at top of back[96]. Blood samples can be collected relatively easily from a number of locations in poultry including the jugular vein[97], brachial veins[98], femoral artery and vein[99], and heart juncture[100].

6.1.4 Bile collection and perfusion

Gallo-Torres [101]reviewed and discussed many aspects of determining bioavailability in the rat including surgical procedures for installing a bile collection cannula and a bile perfusion cannula and the importance of perfusing "replacement bile," or a solution of a representative bile salt (sodium taurocholate for the rat) when bile is being collected. Other methods for cannulation of the bile duct of the rat[102,103], sheep and cattle[94], swine[104], chickens[105,106] and goat[107] have been published.

6.1.5 Saliva collection

In some instances, passage of agrochemical related residues from the vascular system into the gastrointestinal tract via the saliva is substantial[108,109,110,111,112]. For example, the concentrations of three sulfonamide drugs in the saliva were all higher than the concentrations of these drugs in the plasma after intravenous dosing of cows[109]. Thus, the potential for salivary secretion must be considered when planning and conducting bioavailability studies, especially with ruminant animals which secrete such copious amounts of saliva[69]. Methods for collection of saliva from sheep[113,114,115,116] cattle[117,118] and goat[119] have been reported.

6.1.6 Collection of respiratory products

All glass metabolism cages designed to facilitate collection of respiratory products from rats and other small animals are commercially available. The animal is placed inside of the cage and air is drawn through the cage and then through a trap(s) [filled with an appropriate trapping solution(s)] with a vacuum pump. Larger cages to facilitate collection of respiratory products from chickens[93] and dogs[120]have been described.

Although the systems described above work well for collecting respiratory products from small and medium size animals, this approach becomes very difficult and expensive for large farm animals. Therefore, most investigators have used masks (placed over only nose of animal) or hood arrangements (enclosing entire head of animal) for collection of respiratory products from sheep[121], goats[76] and cattle[122,123]

6.1.7 Whole body autoradiography

Whole body autoradiography is a procedure which involves rapid freezing of the whole animal in an aqueous gel of carboxymethyl cellulose, cutting the animal into thin sections in a cryostat microtome and exposing the sections to x-ray film. The x-ray film is developed to reveal the location of the radiolabel in the animal. Depending on how the sections are prepared, the nature of the isotope (^{14}C and ^{35}S work well), the nature of the x-ray film, etc. the autoradiograms give different levels of resolution ranging from ultrastructure level to whole body level. This procedure does not define the "chemical nature" of the radiolabeled residue(s) in the animal; however, it does provide qualitative, and with appropriate calibration, quantitative information on radioactivity in various animal body parts and, in that way, bioavailability of radiolabeled residues[124,125,126].

6.2 The use of *in-vitro* methods for safety evaluation of xenobiotics

Development and application of *in-vitro* models for studying the biotransformation and toxicity of xenobiotics have become popular over the last decades. These models provide valuable information on mechanisms of toxicity of compounds and, in principle, allow interspecies comparison. Therefore *in-vitro* bioassays may contribute substantially to a more reliable extrapolation of animal data to the human situation [127,128,129]. The use of hepatocytes of both animal and human origin in an early stage of drug development for residue toxicity and kinetic studies, is highly recommended since (i) species specific biotransformation pathways may be identified, (ii) the appropriate animal species for toxicity testing may be selected, and (iii) metabolites may be identified as marker residues in residue studies. This has successfully been demonstrated in case of the anthelmintic levamisole [130]. The observed similarity

between metabolic pathways of levamisole in hepatocytes from various animal species, and the agreement between *in-vitro* and *in-vivo* metabolism in cattle has led to a substantial reduction in animal experiments with radiolabeled material. The Joint Expert Committee on Food Additives (JECFA) has adopted this approach, which may be considered more widely[131].

The major organ involved in metabolism of a xenobiotic is the liver and not surprisingly methods for the isolation and culturing of hepatocytes from livers of small laboratory animals, humans, pigs, cows, goats and sheep[127] are well defined. The increasing use of liver slices may have distinct advantages over hepatocytes due to a less disrupted structural organization and the presence of different cell types[132]. Numerous studies in isolated hepatocytes and liver slices have shown that these models mimic *in-vivo* biotransformation of xenobiotics. Studies in pig hepatocytes of the veterinary drugs furazolidone, furaltadone and dimetridazole have shown that metabolites identified *in-vitro* were also observed *in-vivo* [133,134,135].

Metabolism of xenobiotics does not only take place in the liver but also extrahepatically and on occasions complementary *in-vitro* systems may be appropriate, e.g. gastrointestinal tract models to study mechanisms of absorption and first-pass metabolism of xenobiotics. These processes have a great impact on the systemic availability of substances, and on the so-called secondary bioavailability of residues i.e. the uptake of xenobiotic residues present in food items. Various *in-vitro* systems have been developed like isolated epithelial cells, brush border membrane vesicles of enterocytes, and isolated gut segments (everted sac model) to study transport mechanisms of xenobiotics [136,137]. Furthermore a perfused gut segment model has been developed, which mimics closer the physiological conditions than the isolated cell systems [138]. For example in isolated rat jejunum segments the bioavailabilty and biotransformation of the anthelmintic mebendazole has been studied [139]. Metabolic patterns and the distribution of the parent compound and its metabolites in the perfused gut compartments, indicating a low bioavailability, corresponded with *in-vivo* data. Furthermore the intestinal uptake of xenobiotics can be studied in these systems in particular in relation to influences on energy uptake metabolism, as was demonstrated in the case of furazolidone [140,141].

The use of data obtained from *in-vitro* systems derived from human material and of corresponding *in-vivo* data from animal experimentation, may allow a more quantitative extrapolation to the human situation, as compared to traditional toxicity testing. This so called *parallelogram approach* deserves further attention and validation[142].

7. QUANTITATIVE DETERMINATION OF BOUND RESIDUES.

In general the significance of a bound residue is determined on a case by case basis. Where exposure is considered to be significant and there are toxicological concerns, then the bound residue may be subjected to bioavailability studies to assess the level of uptake and first pass metabolism. A discussion of the conduct of these studies has been described in section 6.1.

If bioavailability of bound residues is considered to be significant and/or their toxicological potency is relevant then it may be necessary to develop methods to measure the levels of these residues in food commodities resulting from the use of the xenobiotic under good agricultural and veterinary conditions. Various methods for purification, extraction and detection of bound residues may be applied[143,144] These include simple hydrolytic techniques involving acid, base, sulfhydryl agents or Raney nickel in order to remove a metabolite or a fragment of the parent compound. Enzymatic hydrolysis of macromolecules under normal or denaturing conditions may yield adducts of the xenobiotic containing fragments of the endogenous substance. Methods of detection of bound residues or derived fragments include thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC) linked to various spectrometric detectors.

In the case of ronidazole the release of radiolabelled methylamine upon acid hydrolysis of N-$^{14}CH_3$-ronidazole protein adducts has been used to quantify the amount of ronidazole-related bound residue present in tissues, as previously discussed in section 4.

Determination of bound residues of furazolidone in pig liver hepatocytes and microsomes showed that at least 70 % of the bound residues still contained the intact side chain 3-amino-2-oxazolidinone (AOZ)

[46]. An analytical HPLC method has been developed for the detection of the AOZ- side chain which could be released under acid conditions and subsequently derivatized with 2-nitrobenzaldehyde to the UV detectable nitrophenylmethyleneamino-2-oxazolidinone (NPAOZ) (Figure 11). *In-vitro* studies with isolated pig hepatocytes have also shown that similar bound residues are formed in the case of the related nitrofuran furaltadone [145]. It was demonstrated that a proportion of furaltadone possesses the intact side-chain 5-(morpholinomethyl)-3-amino oxazolidin-2-one (AMOZ), which can be released under mild acidic conditions. Introduction of a clean-up step following the derivatisation procedure effectively eliminates or decreases interference from 2-nitrobenzaldehyde in the HPLC-UV determination of NPAOZ [146]. An LC-MS method has also been developed for the quantitative and confirmatory determination of AOZ and AMOZ, using essentially the same procedure as described above. Limits of detection for fortified control liver samples were 5 ng AOZ g^{-1} and 10 ng AMOZ g^{-1} by HPLC-UV and 10 ng AOZ or AMOZ g^{-1} by LC-MS.

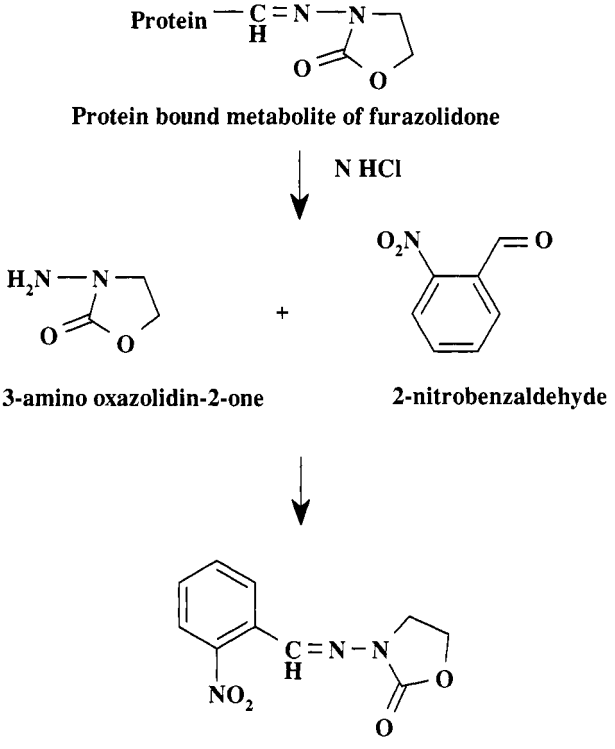


Fig. 11 Reaction of the furazolidone metabolite AOZ with 2-nitrobenzaldehyde.

Recently, immunochemical methods have been described for the detection and identification of molecular adducts[147] . Specific antibodies may be raised which recognize reactive metabolites bound to cellular proteins. To this end the structure of the reactive intermediate chemical species must be known or predicted on the basis of chemical analogy, in order to prepare a hapten carrier complex necessary to raise antibodies. The preparation of polyclonal antisera is prefered, in order to increase the cross-reactivity against multiple epitomes of the bound residue and against structurally similar complexes. Detection of the formation of antibodies may occur using ELISA-techniques, with a test antigen different from the hapten-carrier conjugate used for immunization, in order to avoid cross reactions with the carrier protein. On the basis of the structure of the hapten-protein conjugate relevant information on the specific protein targets of the reactive metabolites which form cellular adducts may be obtained. The detection of protein adducts associated with halothane hepatitis, acetaminophen hepatotoxicity, ethanol hepatotoxicity and diclophenac hepatotoxicity have already been described. This immunochemical approach is promising, and its applicability to agrochemicals bound to food constituents should be further explored.

An attempt to use a bioassay using the stored product insect pest *Tribolium castaneum* has been reported for the detection of bound residues of chlorpyrifos-methyl and malathion in stored cereal grains[148]. In the study up to 29% of the applied dose was unextractable from the grain after a prolonged period of storage. Exhaustive extraction was carried out with methanol and the remaining residue could not be extracted with a range of enzymes, e.g. amylase, protease of cellulase, or acid/base. However it was successfully extracted with methanol/water mixtures. Analysis of this residue revealed the presence of the pyridinol metabolite of clopyrifos-methyl in additional to some polar materials. Exposing the insects to the extracts did not show any acute toxic effects but a delay in reproduction did suggest chronic effects may be associated with the extracts.

8. PROPOSAL FOR A NEW DEFINITION FOR BOUND RESIDUES.

As stated earlier in this paper there remain differences in definitions of what constitutes a bound residue. Apparent anomolies to previous definitions are raised by the use of classical biochemical procedures that allow the sequential dissolution of a sample leaving behind only minimal cellular debris, e.g. Krowke[20]. From these methods it is evident that any definition of a bound residue needs to clearly differentiate between the term solubilsation and extraction. Using detergents (sodium dodecyl sulphate) it is possible effectively solubilise certain cellular macromolecules Residues associated with the protein, for example, will appear to have been extracted but have only been solubilised and can be recovered as solid material through precipitation with trichloroacetic acid or organic solvents

The increasing use of certain enzymes which fragment endogenous macromolecules into their basic subunits, presents exceptions to classical definitions since these agents can result in the solubilisation of residues even though they may still be covalently bound to the subunits. These methods are destructive to the macromolecule and serve merely as a means of characterising the association.

Taking into account new technologies and methodologies used by researchers a new definition is recommended.

> **A xenobiotic bound residue is a residue which is associated with one or more classes of endogenous macromolecules. It cannot be dis-associated from the natural macro molecule using exhaustive extraction or digestion without significantly changing the nature of either the exocon or the associated endogenous macromolecule.**

It is clear from the data presented that the existence and nature of a bound residue can only be accurately defined from radiolabelled studies. It is also however recognised that there is a need to be able to express that a residue is **unextractable** under a specified procedure. The term bound residue excludes fragments recycled through pathways leading to natural incorporation.

9. CONCLUSIONS

In assessing the dietary risk from the use of a pesticide or a veterinary drug the nature of the residues in food commodities need to be determined to allow hazard and exposure to be determined. In some cases chemical residues become associated with natural macromolecules and cannot be readily extracted from the commodity. In this instance the assessment of the dietary risk associated with the residues cannot be directly addressed and raises the question of the significance of a bound residue. To address this issue guidance is required to assist workers in the field to standardise the definition of what constitutes a bound residue and when a residue is simply unextracted because the methods used were not sufficiently rigorous. A definition has been recommended in this project which builds on the previous IUPAC definition whilst taking into account recent advances in techniques and technology available to scientists working in the field. It is also recommended that the characterisation of a bound residue can only be made from radiolabelled studies.

The overall toxicological significance of a bound residue for the consumer depends on the nature of the associated complex, the level of exposure and its bioavailability. In cases where associated residues are endogenously incorporated or not bioavailable then there is no toxicological concern.

It is clear from the data available that to further understand the significance of bound residues the mechanism and nature of the associations with macromolecules must be further investigated and correlated with bioavailability.

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