SC

BOUND (NONEXTRACTABLE) RESIDUES OF TRIAZINE HERBICIDES IN SOYBEAN AND CANOLA PLANTS

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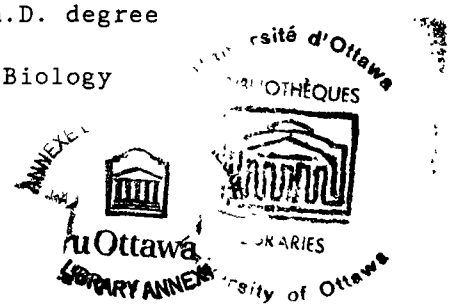

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ABSTRACT

The distribution and nature of the bound residues of metribuzin [4-amino-6-tert-butyl-3 (methylthio)-1,2,4-triazin-5(4H)-one] in soybean (Glycine max L. merr.) and atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) in canola (Brassica napus) were studied. For each species a resistant and a susceptible variety were grown in hydroponic culture and treated by addition of (5-^{14}C)-metribuzin or (ring -^{14}C)-atrazine to their nutrient solution. The metribuzin concentrations in the treatment solutions were maintained at 0.5 ppm during three days for the Maple Arrow variety of soybean (metribuzin tolerant) and 0.15 ppm during four days for the Maple Amber variety (metribuzin susceptible). The atrazine concentrations were 4.0 and 0.1 ppm for the Atr Tower (atrazine resistant) and Tower (atrazine susceptible) canola varieties, respectively. These concentrations were maintained for ten days. The plants were harvested 3, 10 and 28 (soybeans) or 25 (canola) days after the end of the treatment.

The roots, shoots and beans of each plant were exhaustively extracted with solvents. The extractable and bound fraction of each sample was radioassayed. All plants rapidly absorbed the herbicide applied. In soybeans the Maple Arrow variety accumulated in its roots proportions of bound and extractable ^{14}C-residues that were close to three times higher than in Maple Amber. The plants of the resistant variety harvested at 3 days, also displayed higher levels of bound residues in the shoots. These characteristics may have contributed to the tolerance of Maple Arrow by reducing the proportions of free residues in the shoots.

The nature of the bound residues in soybean was investigated. The ^{14}C-residues were released by high temperature distillation (HTD) or supercritical acetone extraction (SAE) and chromatographed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Preliminary recovery studies showed that metribuzin was partially decomposed during HTD. Only 28% of the metribuzin added to control plant samples was recovered intact. The deaminated (DA) and deaminated-diketo (DADK) metribuzin derivatives, as well as CO_2 and other unknown products were also recovered. Larger proportions of metribuzin were recovered by SAE, along with the DA, DADK and unknown products. The HTD and SAE of extracted soybean samples containing bound ^{14}C-residues released free metribuzin (ranging from about 3% to 12% of the ^{14}C in the sample), DA and DADK. In addition, during HTD, an important proportion of the ^{14}C-residues was thermally decomposed to $^{14}CO_2$. This decomposition was greater for Maple Arrow than for Maple Amber samples - 51% and 28%, respectively, for plants harvested at 10 days. The analysis of the supercritical acetone extracts of soybean samples harvested at 10 days showed that an important part of the radioactivity (72% for Maple Arrow and 50% for Maple Amber) was in the form of unknown polar compounds which apparently retained an unreleased metribuzin residue moiety. It is concluded that the parent herbicide is probably present in the bound residue fraction in significantly larger proportions than those observed. Furthermore, it appears that a fraction of the residues was covalently linked to polar cell wall constituents.

The distribution of the radioactivity in ^{14}C-atrazine-treated canola plants was relatively similar in both varieties with the exception that the Tower variety translocated larger proportions of ^{14}C-compounds to the

pods. Most of the radioactivity was evenly distributed between the bound and extractable fractions of the shoot. The HPLC analysis of the methanol extracts of canola shoots showed the presence of atrazine and two unidentified products. The nature of the bound ^{14}C-compounds in the canola samples was investigated by supercritical methanol extraction, TLC, HPLC and gas chromatography (GC). The supercritical methanol extract contained at least five ^{14}C-compounds most of which could not be identified due to low activity levels. Evidence for the presence of traces of atrazine was obtained by GC.

The data suggest that the widely used herbicides atrazine and metribuzin both form large fractions of bound residues in plants. In the case of metribuzin-treated soybean the parent herbicide constituted an important part of that bound residue fraction. Future studies must therefore be directed to determine whether these bound residues are bioavailable or have any toxicological significance.

The HTD and supercritical solvent extraction techniques developed show potential for the analysis of some bound pesticide residues. However these techniques for other pesticides have not been fully explored.

RÉSUMÉ

Cette étude porte sur la distribution et la nature des résidus liés de la métribuzine [4-amino-6-tert.butyl-3-(méthylthio)-1,2,4-triazin-5(4H)-one] dans le soya (Glycine max L. merr.) et de l'atrazine (2-chloro-4-éthylamino-6-isopropylamino-s-triazine) dans le colza (Brassica napus). Pour chaque espèce, deux variétés de plante (résistante et sensible) en culture hydroponique ont été traitées par addition d'herbicide marqué au ^{14}C dans la solution nutritive. Pour la variété de soya tolérante (Maple Arrow), une concentration de métribuzine dans la solution, de 0.5 ppm a été maintenue pendant 3 jours. Pour la variété sensible (Maple Amber) la concentration et la durée du traitement étaient de 0.15 ppm et 4 jours. Les concentrations d'atrazine dans le traitement du colza étaient de 4.0 ppm pour la variété Atr Tower (résistante) et de 0.1 ppm pour la variété Tower (sensible). Ces concentrations ont été maintenues pendant 10 jours. La récolte des plantes traitées eut lieu 3, 10 et 28 (soya) ou 25 (colza) jours après la fin du traitement.

Les racines, parties aériennes et gousses de chaque plante furent soumises à une extraction complète. Les mesures de niveaux de radioactivité dans les fractions extractible et liée de chaque échantillon ont montré que toutes les plantes absorbaient rapidement l'herbicide appliqué. La variété de soya Maple Arrow accumule dans ses racines des proportions de résidus liés et extractibles presque trois fois supérieures à celles de la variété Maple Amber. La partie aérienne des plantes résistantes récoltées 3 jours après le traitement contenait des niveaux de résidus liés plus élevés que ceux des plantes sensibles. Ces caractéristiques pourraient avoir contribué à la tolérance de Maple Arrow

en réduisant les proportions de résidus libres dans les parties aériennes.

La nature des résidus liés dans le soya fut étudiée. La distillation à haute température (HTD) et l'extraction à l'acétone supercritique (SAE) ont permis de libérer les résidus radioactifs liés; ceux-cis ont alors été analysés par chromatographie en couche mince (TLC) et chromatographie liquide à haute performance (HPLC). Les études préliminaires de recouvrements ont montré qu'une partie de la métribuzine utilisée pour fortifier des échantillons témoins de plantes se décomposait lors de la HTD. Les dérivés déaminé (DA) et déaminé-dicéto (DADK) de la métribuzine, le CO_2 et d'autres produits non-identifiés ont ainsi été récupérés. Seulement 28% de la métribuzine fut récupérée intacte. Un pourcentage de métribuzin plus élevé a pu être récupéré par SAE, ainsi que le DA, DADK et autres composés inconnus. La HTD et SAE d'échantillons extraits de soya contenant des résidus radioactifs liés ont libéré de la métribuzine intacte (environ 3% à 12% du ^{14}C présent dans l'échantillon), du DA et du DADK. De plus, pendant la HTD une proportion importante de résidus s'est décomposé en $^{14}CO_2$ sous l'effet de la chaleur. Cette décomposition était plus importante pour les échantillons de Maple Arrow que pour Maple Amber - 51% et 28%, respectivement, pour les plantes récoltées 10 jours aprés le traitement. Une partie importante de la radioactivité dans les extraits de soya par l'acétone supercritique était constituée de composés polaires inconnus (72% et 50% pour les échantillons de Maple Arrow et Maple Amber récoltés à 10 jours, respectivement). Ces composés semblent contenir un résidu de métribuzine non libéré. La conclusion tirée est que l'herbicide parent est probablement présent dans la fraction liée des résidus dans des proportions substantiellement supérieures à celles observées. De plus, il

semblerait qu'une fraction des résidus était liée par covalence à des constituants polaires de la paroi cellulaire.

La distribution de la radioactivité dans les plantes de colza traitées à l'atrazine était relativement similaire pour les deux variétés à l'exceptions des gousses. La variété Tower apporte dans les gousses des proportions de composés radioactifs plus grandes que la variété Atr Tower. La majeure partie des composés radioactifs était distribuée équitablement entre les fractions liée et extractible des parties aériennes. L'analyse par HPLC des extraits méthanoliques de ces parties aériennes a montré la présence d'atrazine et de deux produits non-identifiés.

La nature des composés radioactifs liés dans les échantillons de colza fut étudiée par extraction au méthanol supercritique, TLC, HPLC et chromatographie en phase gazeuse (GC). L'extrait par le méthanol supercritique contenait au moins cinq composés radioactifs dont la pluspart n'était pas identifiable dû à des niveaux de radioactivité trop bas. La présence de traces d'atrazine a été indiquée par l'analyse en GC.

Les résultats suggèrent que les herbicides atrazine et métribuzine forment d'importantes fractions de résidus liés dans les plantes. Dans le cas du soya traité avec la métribuzine, l'herbicide parent constitue une partie importante de la fraction liée des résidus. Il serait donc nécessaire de déterminer la biodisponibilité et éventuelle importance toxicologique de ces résidus liés.

Les techniques de HTD et extraction par solvants supercritiques développées ici sont potentiellement utiles pour l'étude des résidus liés de certains pesticides. Cependant leur champ d'application est encore mal connu.

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Bound (Nonextractable) Residues of Triazine Herbicides in Soybean and Canola Plants

Résidus Liés (Non-Extractibles) d'Herbicides

de Type Triazines dans le soya et le Colza

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LIST OF ABBREVIATIONS

CPM:	counts per minute
DA:	deaminated metribuzin
DADK:	deaminated diketo metribuzin
DK:	diketo metribuzin
DPM:	disintegrations per minute
GC:	gas chromatography
HPLC:	high performance liquid chromatography
HTD:	high temperature distillation
i.d.:	internal diameter
LSC:	liquid scintillation counting
mn:	minute
ppm:	parts per million
PSI:	pounds per square inch
R:	resistant
Rf:	TLC unit = ratio of the distance moved by the solute to that moved by the solvent
Ri:	percent recovery of the initial fluorescence level after 200 s
Rt:	retention time
S:	susceptible
SAE:	supercritical acetone extraction
SME:	supercritical methanol extraction
SSE:	supercritical solvent extraction
TLC:	thin layer chromatography
UV:	ultra violet

1. INTRODUCTION

The use of agrochemicals has become indispensable to the maintenance and improvement of crop production levels in modern agriculture. The possible risks that this practice poses to the consumers of the treated crops and to the environment in general must, therefore, be carefully assessed and kept to a minimum. The registration of a chemical for pest control purposes requires, among many other things, that its fate and residue levels in plants be established.

The use of radiotracers within the pesticide molecule has been shown to be the only effective means for studying pesticide metabolism in plants. This facilitates the characterization and quantitation of the radiolabeled pesticide and/or metabolites after extraction from the plant by suitable solvents. The extracted residues may be free compounds like the parent pesticide and/or its metabolites derived from phase 1 reactions (e.g.dehalogenation, hydroxylation, hydrolysis, oxidation, reduction, etc.) and/or they may also be extractable conjugates produced by metabolic reactions of phase 2 involving the conjugation of an exogenous compound (i.e. the pesticide molecule or its metabolite) with an endogenous compound (e.g. glucose, or other sugars, amino acids, glutathione, etc.) (Menn and Still, 1977; Lamoureux and Rusness, 1986). During the quantitation of radiolabeled pesticide residues it became apparent that a significant portion of certain radioactive species was not extracted from the plant matrix as had been expected (Huber and Otto, 1983). These unextracted ^{14}C-residues were quantitated by combustion to $^{14}CO_2$ followed by liquid scintillation counting. However this technique cannot be used to determine the chemical nature of these residues. In some cases, the unextractable

^{14}C was shown to be part of a natural compound. This indicated that the pesticide had been extensively metabolized to a simple natural molecule and then used by the plant as a metabolic precursor for insoluble biopolymers. Such natural compounds cannot be called pesticide residues. On the other hand, the unextractable ^{14}C in plants may often be characterized as part of the parent pesticide and/or its phase 1 or 2 metabolite. Such nonextractable residues are referred to as "bound residues". Several authors have reviewed the available literature on the subject. (Kaufman et al., 1976; Khan, 1982; Klein and Scheunert, 1982; Hubert and Otto, 1983; Pillmoor and Roberts, 1985; Khan and Dupont, 1987).

The International Union of Pure and Applied Chemistry (IUPAC) has proposed the following definition of bound pesticide residues:

"Nonextractable residues (sometimes referred to as 'bound' or 'non-extracted' 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 nonextractable residues are considered to exclude fragments recycled through metabolic pathways leading to natural products. 'Chemical species' in this context refers either to the parent material or to derivatives or fragments of it. 'Methods' in this context refer to any procedure such as solvent extraction and distillation used to exhaustively remove chemical species from a plant matrix. In each reference to a nonextractable residue, the extraction procedure must be given." (Roberts, 1984).

Bound residues remain undetected by methods employed in routine residue analyses, thus leading to an underestimation of the possible plant content of total pesticide residues. Therefore, in recent years, concern

over the biological and environmental significance of bound residues has been expressed by several workers. It is possible that these residues may become released during decomposition of the plant material in the soil, turnover of the plant cell wall components (Lamoureux and Rusness, 1986) or more importantly, digestion of the contaminated food by humans or livestock. Bound pesticide residues are considered to be "bioavailable to animals" when they are released from the plant matrix during the digestion process and absorbed into the blood stream through the gut wall. Regulatory agencies such as the Environmental Protection Agency (EPA) have recently carefully considered the bioavailability of bound residues for the registration of certain chemicals. In a recent publication Kovacs (1986) outlined and illustrated the EPA guidelines regarding bound residues. Generally, these residues are considered to be of no toxicological concern when i) the levels of bound ^{14}C are low (e.g. less than 10% of the total ^{14}C activity or 0.1 ppm), ii) chemical characterization shows the residues to be non toxic or, iii) feeding experiments with laboratory animals prove that they are not bioavailable. Otherwise, these bound residues are considered part of the total toxic residue of the plant and subject to regulation.

It is difficult to determine the level of risk that bound residues in food products present to human health. It is now common knowledge that bound residues do exist in plants, often in very large quantities. However we can seldom attest to their safety or lack thereof.

1.1 Bound residues and the insoluble plant polymers

1.1.1. Insoluble plant polymers

The bound pesticide residues are generally considered to be associated

with plant insoluble biopolymers by chemical binding (covalent bond, hydrogen bond, etc.) and/or physical entrapment. These polymers are mainly: lignin, cellulose, hemicellulose and pectic polysaccharides and, to a lesser extent, cutin and suberin.

Cutin forms an important part of the plant cuticle, it appears as a matrix of polyesters of hydroxylated fatty acids (mainly C_{16} and C_{18}). Suberin is similar to cutin except that the fatty acids are also thought to be esterified with phenolic compounds attached to cell wall polysaccharides. It is present mostly in the walls of cork cells (Kolattukudy and Espelie, 1985). Based on a model study of photoaddition of DDT and methoxychlor to methyloleate, Schwack (1988) suggested that pesticides deposited on plant surfaces may become nonextractable by photoaddition to cutin. In this context, it is interesting to note that Still et al. (1976) were able to release 15 to 25% of the rice-bound residues of ^{14}C-propanil by a catalytic reduction reaction known to remove cuticle components.

Cell wall polysaccharides are mainly formed of cellulose fibers embedded in a matrix of hemicellulose and pectic compounds. Cellulose is constituted of linear β(1-4) glucans. Several types of hemicellulose are known, the more common ones being xylans and xyloglucans. Common pectic substances are rhamnogalacturonans. The matrix may also contain a structural protein called extensin. The role of these polysaccharides is to maintain the structural integrity of the plant while permitting growth by allowing the net-like structure of the wall to be stretched and locally broken by enzymatic hydrolysis, and then reformed (Hori and Elbein, 1985). Some cell wall fractionation studies (discussed later) provide evidence for binding of pesticide residues with the insoluble polysaccharides (Huber and Otto, 1983; Pillmoor and Roberts, 1985).

Lignin is a complex polymer of coniferyl, p-coumaryl and sinapyl alcohols distributed throughout the polysaccharidic wall and in the middle lamella. Its role is to solidify the cell wall towards the end of cell elongation and "cement" cells together thus increasing the structural resistance of the plant (Higushi, 1985).

1.1.2. Bound residues in lignin

The incorporation of pesticide residues into lignin has been suggested as an important pathway of detoxification of pesticides in plants (Scheel and Sandermann, 1981; Khan, 1982; Sandermann et al., 1983). Indeed, lignification is an irreversible phenomenon in plants. Several researchers reported the release of bound pesticide residues from plants by lignin extraction procedures. Chin et al. (1964) released the radioactivity from different plants containing bound ^{14}C-Swep or ^{14}C-Solan residues with a lignin solubilization procedure using HCl-Dioxane. Further acid hydrolysis of the extracted radioactive lignin yielded intact Swep and 3,4-dichloroaniline (3,4-DCA), a Solan metabolite. Khan (1980) used a similar procedure to release bound ^{14}C-prometryn residues from oat plants. A treatment of rice plants with ^{14}C-propanil produced bound residues representing as high as 60 to 70% of the ^{14}C in the plant (Yih et al., 1968; Sutherland, 1976). A lignin extraction solubilized about 40% of the bound radioactivity which was shown to be 3,4-DCA after hydrolysis. Scheel and Sandermann (1981) utilized different methods to remove the lignin from wheat and soybean cells obtained from cell suspension cultures treated with 2,4-dichlorophenoxy acetic acid (2,4-D). They showed the bound residues to be 2,4-D and 4-hydroxy 2,5-D.

Using dimethylsulfoxide (DMSO) as a solvent for lignin, Chin et al., (1973) were able to release 74% of the bound radioactivity from ^{14}C-carboxin-treated barley as intact carboxin and its sulfoxide.

In an attempt to elucidate the binding mechanism of pesticide residues in lignin, Still et al. (1981) developed a model system in which coniferyl alcohol was polymerized in the presence of chloroanilines (CAs) to form a lignin-CA polymer. That polymer was shown to be an addition product from the nucleophilic attack of the amino group of the CAs on a quinone methide intermediate (Still et al., 1981) (Figure 1).

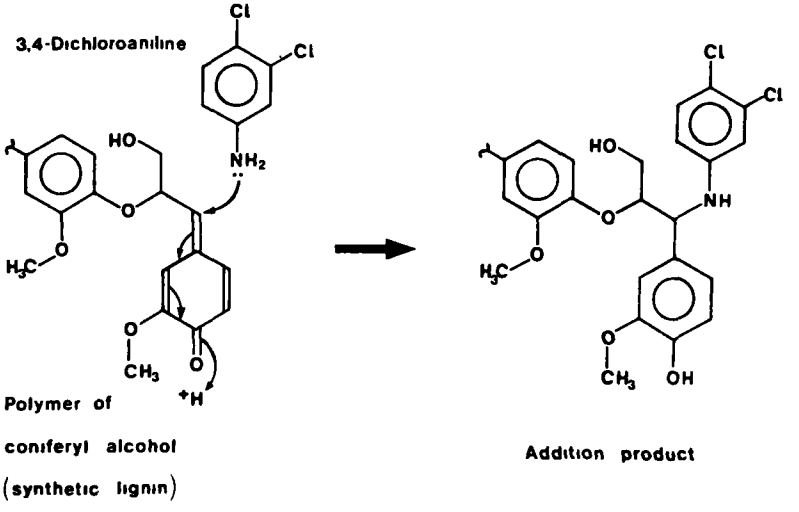


Figure 1: Proposed mechanism of addition of chloroanilines on artificial lignin.

Thus it appears that bound chloroaniline residues in plants are likely to be covalently linked to lignin. This is consistent with the fact that 3,4-DCA is often released from dissolved lignin only after hydrolysis (Chin et al., 1964; Yih et al., 1968; Sutherland, 1976).

The binding mechanism described above is also possible with other pesticide residues containing nucleophilic groups, such as -SH, -NH_2, OH,

-COOH etc. Sandermann (1983) was also able to copolymerize coniferyl alcohol and benzo(α) pyrene quinones which are common metabolites for the environmental contaminant benzo(α) pyrene. However, this does not rule out the possibility of physical entrapment into lignin, especially when the residue can be released in a free form by lignin solubilization (Chin et al., 1973).

In the environment the biodegradation of lignin proceeds slowly and is effected only by a few types of aerobic microorganisms, such as white rot fungi. Lignin also reduces the efficiency of other organisms to degrade cellulose and hemicellulose anaerobically (Kirk and Shimada, 1985).
In order to gain an insight into the long term fate of lignin-bound residues in the environment, Arjmand and Sandermann (1985) studied the degradation of the model lignin-chloroaniline copolymers by the white rot fungus Phanerochaete Chrysosporium. They found that the ^{14}C-CA residues were bioavailable to the fungus which transformed them into $^{14}CO_2$ (about 60% of the ^{14}C), and two unknown radioactive compounds. They speculated that this type of biodegradation might be the last step in the removal of chloroanilines from the environment.

1.1.3. Fractionation of the cell wall as a bound residue analysis method.

Several researchers used sequential fractionation procedures in order to solubilize the main constituents of plant cell walls one at a time.

Starch and proteins that may be precipitated with the cell wall can be first removed with mildly acidic or ionic aqueous solutions (Sutherland, 1976; Haque et al., 1976; Rouchaud et al., 1978; Pillmoor et al., 1984) or by incubation with suitable enzymes (Langebartels and Harms, 1985).

Pectic polysaccharides can be dissolved by a decomplexing agent such as ethylenediaminetetraacetic acid (EDTA). The remaining polysaccharides may be solubilized after lignin has been removed by one of the methods discussed earlier. Hemicellulose is soluble in alkaline solutions while cellulose is often hydrolyzed to glucose, usually with sulfuric acid. (Huber and Otto, 1983; Honeycutt and Adler, 1975; Sutherland, 1976; Haque et al., 1976; Rouchaud et al., 1978, 1979a, 1979b; Pillmoor et al., 1984; Langebartels and Harms, 1985).

Other methods have been used to separate the cell wall constituents. The AOAC's (Association of Official Analytical Chemists) indirect lignin analysis solubilizes the proteins and polysaccharides, thus separating the lignin from all the other constituents. Stratton and Wheeler (1983 and 1986) demonstrated that this method yielded lignin-bound radioactive fractions as well as releasing bound residues of ^{14}C-carbofuran, ^{14}C-dieldrin and ^{14}C-permethrin from extracted radishes.

When a sequential procedure is carried out, some radioactivity is usually released with every fraction. Pillmoor and Roberts (1985) attribute this to the "fact that the cell wall is an integrated structure and that the chemical solubilization methods are inevitably rather harsh and nonspecific". The use of these methods also showed in some cases that the bound radioactivity was ^{14}C (or ^{3}H) reincorporated in cell wall biopolymers such as starch and cellulose, respectively from the grains and plants of ^{14}C nitrofen-treated barley (Honeycutt and Adler, 1975; Wargo et al., 1975). Rouchaud et al., (1979a, b) treated barley plants with ^{3}H-triforine and traced the radioactivity to starch and hemicellulose from the grains and straw, respectively.

Some of the fractionation studies confirmed the importance of lignin for "residue binding" (Sutherland, 1976). Haque and coworkers (1976) fractionated extracted wheat plants containing bound ^{14}C-buturon residues. The radioactivity was released as follows: 25.5% with water-soluble polysaccharides (mostly unknown polar compounds), 9% with pectic substances, 28.4% with lignin (buturon and unknowns), 6% with hemicellulose and 11% with cellulose.

Other studies showed that cell wall polysaccharides may also play an important role in the formation of bound residues. The binding of pesticide residues with lignin is usually considered to happen as a result of the presence of the exogenous compound at the time and location of lignin synthesis. Alternatively, it has been suggested that the formation of bound residues with cell wall polysaccharides may start inside the cell with the synthesis of sugar conjugates and proceed by successive addition of monomeric units and incorporation into the wall (Pillmoor and Roberts, 1985; Lamoureux and Rusness, 1986).

A study on the metabolism of ^{14}C-oxamyl in plants showed that it proceeded through the formation of glucose and polysaccharide conjugates (Harvey et al., 1978). The authors postulated that these may be precursors for the bound residues observed in some of the plants, although some evidence for ^{14}C reincorporation was found as well.

Pillmoor and coworkers (1984) fractionated wheat straw containing bound ^{14}C residues of the herbicide MCPA. Most of the radioactivity was solubilized with the water soluble polysaccharides and hemicellulose fractions from which, intact MCPA and a metabolite could be released by acid hydrolysis.

After addition of ^{14}C-pentachlorophenol (PCP) to wheat and soybean cell suspension cultures, Langebartels and Harms (1985) found that the levels of PCP glucoside and other polar metabolites rose sharply for about 12 hours, then decreased while the proportion of bound residues kept increasing. Fractionation of the cell walls released radioactivity with each constituent but mainly with proteins, lignin and hemicellulose. The ^{14}C in the lignin was thought to be a PCP metabolite (Scheel et al., 1984) while incubation of the dissolved hemicellulose with hemicellulase released intact PCP. Therefore, it is possible that two binding mechanisms might have taken place simultaneously : i) incorporation in lignin and ii) build-up of cell wall polysaccharides from sugar conjugate of PCP.

Enzymatic methods for cell wall fractionation have been used to release plant-bound pesticide residues. Smith (1979) was able to release less than 5% of the bound radioactivity from ^{14}C 2,4-dichlorophenoxybutyric acid (2,4-DB) treatment of white clover cells by incubation with protease, pectinase, hemicellulase, cellulase and clarase. Similar results were observed by Pillmoor and Roberts (1984) in releasing bound residues of MCPA and Flamprop from wheat straw with similar enzymes before and after lignin extraction. However, Khan et al., (1984a) found that the enzymes β-glucosidase, cellulase and protease as well as their corresponding buffer solutions could partially release ^{14}C-deltamethrin bound residues from bean plants. Cellulase was also able to extract 60% of the bound residues of ^{14}C-dieldrin in radishes as shown by Stratton and Wheeler (1986), intact dieldrin represented about 18% of the ^{14}C released.

1.2 Determination of bound residues by high temperature distillation and supercritical solvent extraction.

The extraction procedures described in the previous section often resulted in a modification and/or destruction of the identity of the bound pesticide residues. A significant portion of the radiolabeled compounds solubilized and released by acid and alkaline hydrolyses often remained unidentified. This prompted researchers to investigate the applicability of other techniques to the study of bound pesticide residues.

A novel technique for identification of bound residues was reported by Balba et al. (1979). They demonstrated that for chloroaniline bound residues, a pyrolysis in the absence of oxygen released more than 80% of the rice-bound radioactivity. Gas chromatography-mass spectrometry of the solvent-trapped pyrolysates indicated that the chloroanilines represented a major portion of the ^{14}C released. A somewhat modified technique, known as high temperature distillation (HTD) has been used by Khan and Hamilton (1980) to release and identify soil-bound prometryn residues. The HTD technique involves heating the sample in a quartz tube under a flow of helium and collecting the volatile products in different solvent traps in a form suitable for identification. Some $^{14}CO_2$ is usually produced due to the thermal decomposition of the compound(s) and can be trapped with a suitable absorber. The applicability of this technique to a given type of bound residue depends upon the thermal stability and volatility of the residue as well as the nature of the bond with the cell wall matrix.

Since its development, the HTD technique has been used to analyze the bound residues of several pesticides in different soil and plant samples (Khan, 1982; Khan and Dupont, 1987). Radish samples containing bound

residues of ^{14}C-dieldrin, ^{14}C-permethrin or ^{14}C-carbofuran were analyzed by HTD (Khan et al., 1984b). The technique was very efficient with the more stable dieldrin, allowing a recovery of 90% of the pure standard from fortified samples of extracted control radishes . Only 5% of the ^{14}C residues bound to the radish tissues were decomposed to $^{14}CO_2$ during the distillation, the remaining 95% being trapped in the solvent traps where only intact dieldrin was found. However, carbofuran was less stable; about 56% remained unchanged after the preliminary recovery study. HTD of the samples yielded about 34 and 66% of the ^{14}C in the CO_2 trap and the solvent traps, respectively. The solvent traps contained two carbofuran metabolites.

In another study, bound residues of ^{14}C-deltamethrin in the shoots of bean plants were investigated (Khan et al., 1984a). About 11 to 13% of the bound ^{14}C was characterized as intact deltamethrin or known metabolites. Similarly, 65% of the bound ^{14}C in the shoots of atrazine-treated corn plants was identified as atrazine and its 7 metabolites (Khan et al., 1985a).

The efficiency of the HTD technique varies according to the thermal stability of the pesticide residues and the type of material to which they are bound. The decomposition of these residues to $^{14}CO_2$ may be an important drawback in some cases (Khan and Dupont, 1987).

Very recently, a new technique involving supercritical fluid extraction has been used to release bound pesticide residues from plant and soil samples (Capriel et al., 1986). The supercritical state describes a fluid under conditions above its critical temperature, i.e. temperature above which the fluid cannot be liquefied by compression, and critical pressure, i.e. the pressure required to bring a gas at critical temperature

to liquefaction. In supercritical state a fluid has physical properties intermediate between gas and liquid which makes it a superior solvent: a low viscosity and high diffusion coefficient allow the fluid to penetrate the micropores of solid substances while the density can vary widely depending on the pressure and temperature. For example, an increase in pressure can dramatically increase the density of the supercritical fluid which will enhance its solvent power (Wilke, 1978; Schneider, 1978; Johnston, 1985).

While the supercritical state and some of its properties have been known for several decades, it was not until the 1970's that large-scale practical applications were developed (Wilke, 1978). Currently, supercritical CO_2 is widely used to extract caffeine from coffee beans. The coal industry also uses supercritical fluid extraction (SFE) procedures to solubilize coal products (Johnston, 1985).

Spiteller (1985) used several supercritical fluids to extract soil organic matter. Supercritical acetone was shown to be much more efficient than a customary pyrolysis method for thermal degradation of cellulose. Almost 100% of the cellulose was solubilized and degraded, mostly to simple sugar derivatives (Köll and Metzger, 1978).

Capriel and coworkers (1985) applied a supercritical methanol extraction (SME) technique to soil and plant samples containing bound pesticide residues. They compared the results to those obtained by HTD of the same samples. It was found that the overall recovery of ^{14}C was often better for SME and the chemical identity and amounts of the released residues was usually very similar. Moreover, some labile compounds like 2,4-D and methylparathion which could not be detected by HTD probably due

to thermal decomposition to $^{14}CO_2$ were shown to be present by SME. However, supercritical methanol chemically reacted with some residues to yield their methoxy derivatives.

In view of the foregoing, it appears that HTD and supercritical fluid extraction techniques can be very useful in analyzing bound pesticide residues even though their potential and limitations are not yet fully determined.

1.3. Bioavailability of plant-bound pesticide residues to animals:

Akhtar (1987) reviewed the available literature on this subject. Bioavailability studies usually involve feeding extracted plant tissues containing bound ^{14}C-residues to test animals, usually rats but occasionally also sheep. The radioactivity in the feces, urine and sometimes bile and exhaled CO_2 is then monitored for several days, after which the animals are sacrificed and the amount of ^{14}C in their organs and carcass is determined. When the radioactivity is quantitatively recovered in the feces in an unextractable form, the bound residues are not considered bioavailable. However, significant proportions of ^{14}C present in the urine and/or other tissue samples would indicate some degree of bioavailability.

Few studies dealing with this area of concern have been published. In most cases, the bound radioactivity in the plant was not characterized. For example, Bakke et al. (1972) fed a sheep and two rats with extracted sorghum shoots containing an unidentified bound ^{14}C fraction from ^{14}C-atrazine treatment. The sheep and rats excreted only 7 and 2% of the radioactivity via the urine and 100 and 90% via the feces, respectively. Similar results were reported by Paulson and coworkers (1975) regarding the bioavailability to rats of bound ^{14}C-compounds obtained from a propham

treatment of alfalfa plants. The results of these two studies indicated that the bound radioactive fractions were only marginally bioavailable. Therefore, the missing information on their chemical identity may be considered non essential.

Marshall and Dorough (1977) studied the bioavailability to laboratory rats of bound ^{14}C-residues from different carbamates in bean plants. Carbaryl produced a higher proportion of bound radioactivity (57%) than carbofuran (7%) and croneton (3%). Moreover, more bound ^{14}C from carbofuran (17%) and croneton (15%) was released by acid hydrolysis than from carbaryl (3%). In this context, it is interesting to note that carbaryl-derived ^{14}C was the least bioavailable to rats with 1% of the administered dose found in urine as opposed to 11 and 16% for carbofuran- and croneton-derived ^{14}C, respectively. The authors suggested that the acid-soluble fraction was likely to be bioavailable while the remaining ^{14}C was excreted unreleased in the feces. Mostafa and coworkers (1985) found that laboratory rats excreted about 66% of the ^{14}C from bound radioactive compounds in ^{14}C-carbofuran-treated sweet potatoes via the feces while 9% was excreted with the urine and 11% exhaled as $^{14}CO_2$. These two studies showed that, under the experimental conditions applied, the bound radioactive fractions from plants treated with croneton and carbofuran were moderately bioavailable to rats. In view of this, a characterization of these fractions would have been very informative. Indeed, interpretation of the toxicological implications would vary depending on whether the bioavailable bound ^{14}C was the highly toxic carbofuran, croneton, some of their metabolites or ^{14}C recycled into natural compounds.

More comprehensive studies have also been carried out, one in particular deals with the bioavailability of bound residues of dieldrin and

carbofuran in radishes (Khan et al., 1987). In ^{14}C-dieldrin-treated radishes 26% of the radioactivity was not extractable by organic solvents. That bound residue fraction was shown by HTD and SME to contain intact dieldrin (Khan et al., 1984b; Capriel et al., 1986). Acid Hydrolysis of this fraction by the AOAC's indirect lignin analysis released 70% of the dieldrin, whereas 30% of the ^{14}C remained lignin-bound (Stratton and Wheeler, 1986). When the extracted tissues were fed to laboratory rats, 75% of the bound residues were eliminated via the feces and 16% with the urine. The remaining ^{14}C was found mostly in the liver (5.2%) and kidneys (3.3%). Intact dieldrin was present in the urine as well as in the feces from which 40% of it could be extracted. Radishes treated with ^{14}C carbofuran retained 57% of the radioactivity after extraction. About 55% of this bound fraction remained unreleased after acid hydrolysis. HTD and SME indicated that carbofuran and two metabolites were present. The test rats excreted 2% of the ^{14}C via the urine and 90% via the feces; 2.3 and 1.1% were still present in the liver and kidneys respectively. It was shown by HTD that carbofuran and its 3-hydroxy metabolite remained present in a bound form in the feces (Khan et al., 1984b; Stratton and Wheeler, 1986; Capriel et al., 1986; Khan et al., 1987). The results of this study point out that the bound carbofuran residues in radishes had a higher ratio of lignin-bound to acid-soluble material than bound dieldrin. Bound dieldrin was relatively more bioavailable, thereby indicating that its release was effected by hydrolysis at low pH in the stomach. The characterization of the material at different stages provided a better understanding of the toxicological importance of the bound residues. It is notable that the carbofuran-derived bound compounds examined in other studies showed a higher degree of bioavailability than the radish-bound

carbofuran and metabolites. This confirms the need to consider each study individually.

A similar study was carried out with bound ^{14}C-atrazine residues in corn. The rats eliminated about 10% of the ^{14}C via the urine and only trace amounts were found in their organs. However, about 88% of the ^{14}C-residues were recovered bound in the feces. HTD and GC analysis showed a pattern very similar to the initial corn-bound residues with the main compounds being the two mono N-dealkylated hydroxy metabolites. This indicates that the bound material passed through the gastrointestinal (GI) tract without significant modification (Khan et al., 1985a).

Pesticide residues have been found to become bound with different insoluble plant constituents. The digestibility of these constituents would be a major factor in determining whether or not the residues may be released and absorbed in the GI tract. Monogastric animals and humans in particular can be expected to digest only starch and other polysaccharides loosely attached to the cell wall. The remaining polysaccharides like hemicellulose and cellulose can be attacked by the rumen microorganisms in the digestive system of ruminants. Lignin is the most common residue deposition place identified so far, it is not digested by animals and bound residues associated with it are unlikely to be bioavailable to animals. This is substantiated by the observation that the bound residues with a large hydrolyzable fraction - i.e. associated with cell wall polysaccharides - are more readily bioavailable than the ones predominantly bound to lignin (Marshall and Dorough, 1977; Stratton and Wheeler, 1986; Khan et al., 1987).

1.4 Scope of the research project

1.4.1 Bound residues in soybean treated with metribuzin

Several workers studying the fate of ^{14}C-metribuzin in soybeans (Glycine max L.Merr) have reported the presence of bound radioactivity in the solvent-extracted soybean material. Metribuzin [4-amino-6-tert.butyl-3-(methylthio)-1,2,4-triazin 5(4H) one] (Figure 2) is a selective preemergence herbicide which is used against annual grasses and broadleaf weeds in soybean culture (Herbicide Handbook, 1983). It is a photosynthesis inhibitor and acts by blocking electron transport during the light reactions. There is evidence that inhibition is effected by binding of the herbicide to a protein on the reducing side of the photosystem II. This prevents plastoquinone compounds from attaching to the protein, being reduced and passing the electrons on to the photosystem I (Arntzen et al., 1983; Gressel, 1985). The major route of absorption and translocation of metribuzin in soybean is through the roots and along the xylem to the foliar tissues. The susceptibility to metribuzin of the numerous soybean varieties used in agriculture range from tolerant to very sensitive (Falb and Smith, 1984). Several studies have concentrated on the causes and mechanisms of this differential tolerance and found that absorption, translocation and metabolism are involved. Smith and Wilkinson (1974) showed that the ^{14}C-metribuzin remained largely unmodified in two susceptible varieties. These varieties also translocated the herbicide to the photosynthetic foliar tissues where it could exert its phytotoxic action. However, a resistant variety appeared to metabolize metribuzin more rapidly and restrict the radiolabeled compounds to its vascular tissues. These results were partially confirmed by Mangeot and coworkers (1979) who, along with Fedke and Schmidt (1983), established that the

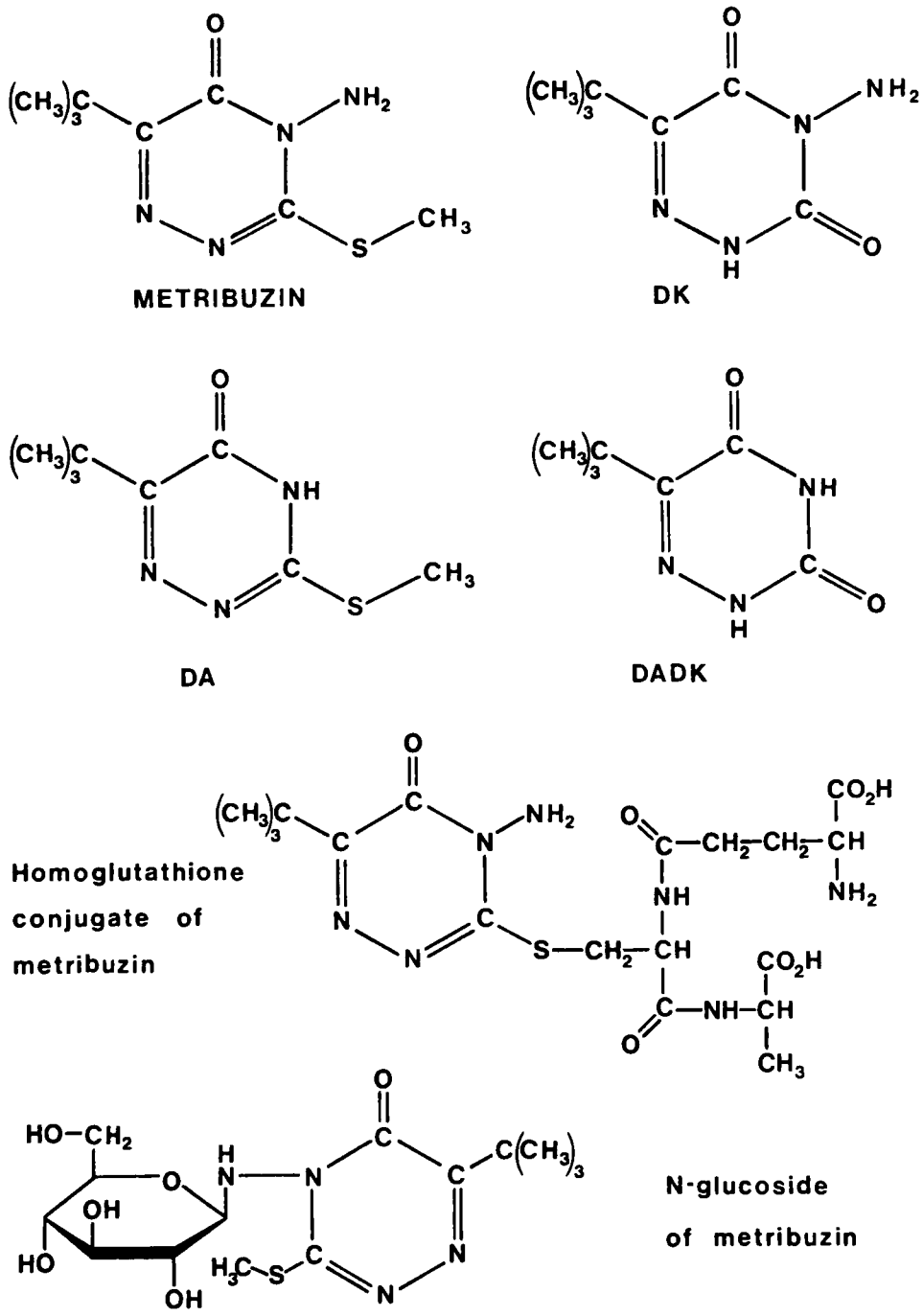


Figure 2: Chemical structures of metribuzin and its metabolites from phase 1 and 2 reactions.

metabolism of metribuzin produced mainly its deaminated metabolite (DA, see Figure 2) along with lesser amounts of diketo and deaminated-diketo metabolites (DK and DADK, see Figure 2).

Differential absorption of metribuzin by various soybean cultivars was reported by Falb and Smith (1984) and Abusteit et al. (1985). However, neither Fedtke and Schmidt (1983) nor Frear et al. (1985) found any significant difference in the rates of metribuzin absorption of their soybean varieties.

The nature of the conjugates in the extractable polar fraction was first determined by acid hydrolysis which released DADK (Method for residue analysis of Sencor in soybeans - Chemagro Ltd., 1974). Later, Abusteit et al. (1985) reported the unintentional release of intact metribuzin and, to a lesser extent, of DA from polar conjugates by addition of water to the mobile phase of the thin layer chromatographic (TLC) method used in the analysis. Finally, a more detailed study on the polar metabolites obtained from excised soybean leaves treated with metribuzin was performed. The results showed that metribuzin was conjugated mainly to homoglutathione, (Glu-Cys-Ala) (Figure 2). The N-glucoside and malonated N-glucoside conjugates of metribuzin were also found in the soybean leaves (Frear et al., 1985).

Mangeot et al. (1979) reported relatively low levels of bound radioactivity in young soybean plants treated for 48 h with ^{14}C-metribuzin. However, these levels were significantly higher in tolerant than in susceptible plants. They attributed the tolerance to the capacity of the plants to detoxify metribuzin to DA, several polar metabolites and a bound residue fraction. In another study where the plants were treated with the herbicide at a later stage and for a longer

period, Falb and Smith (1984) found that 18 to 47% of the radiolabel in roots, shoots or leaves was unextractable. The levels in the suceptible and tolerant varieties however, were not significantly different.

A review of the literature revealed that no information is available on the nature of these bound radiolabeled residues in soybean plants. The possible role of residue binding as a tolerance mechanism was also unclear. Soybean is one of the main crops worldwide and metribuzin is widely used to control weeds in this crop. Therefore, the amount as well as the nature of bound residues formed may be of concern even though metribuzin itself has a low oral acute toxicity to rats (1090 mg/kg).

1.4.2. Bound residues in Canola treated with atrazine

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) (Figure 3) is a s-triazine herbicide and its mode of action is essentially the same as metribuzin. It is used to control monocot and dicot weeds in several crops. Many crop species, however, are very sensitive to atrazine (Herbicide Handbook, 1983). This is the case of most canola (Brassica napus) species. Beversdorf and coworkers (1980) developed a triazine resistant variety by crossing the suceptible canola with a resistant biotype of wild turnip rape (Brassica campestris). This resistance is mainly due to a modified site of action on the thylakoid membranes in the chloroplasts which prevents herbicide binding (Beversdorf, personal communication). Both resistant and susceptible wild turnip rape varieties were found to metabolize atrazine to hydroxyatrazine (Figure 3) and form a bound residue fraction in very similar proportions. The levels of bound radioactivity represented up to 30% of the ^{14}C in these plants (Ali and Souza-Machado, 1983).

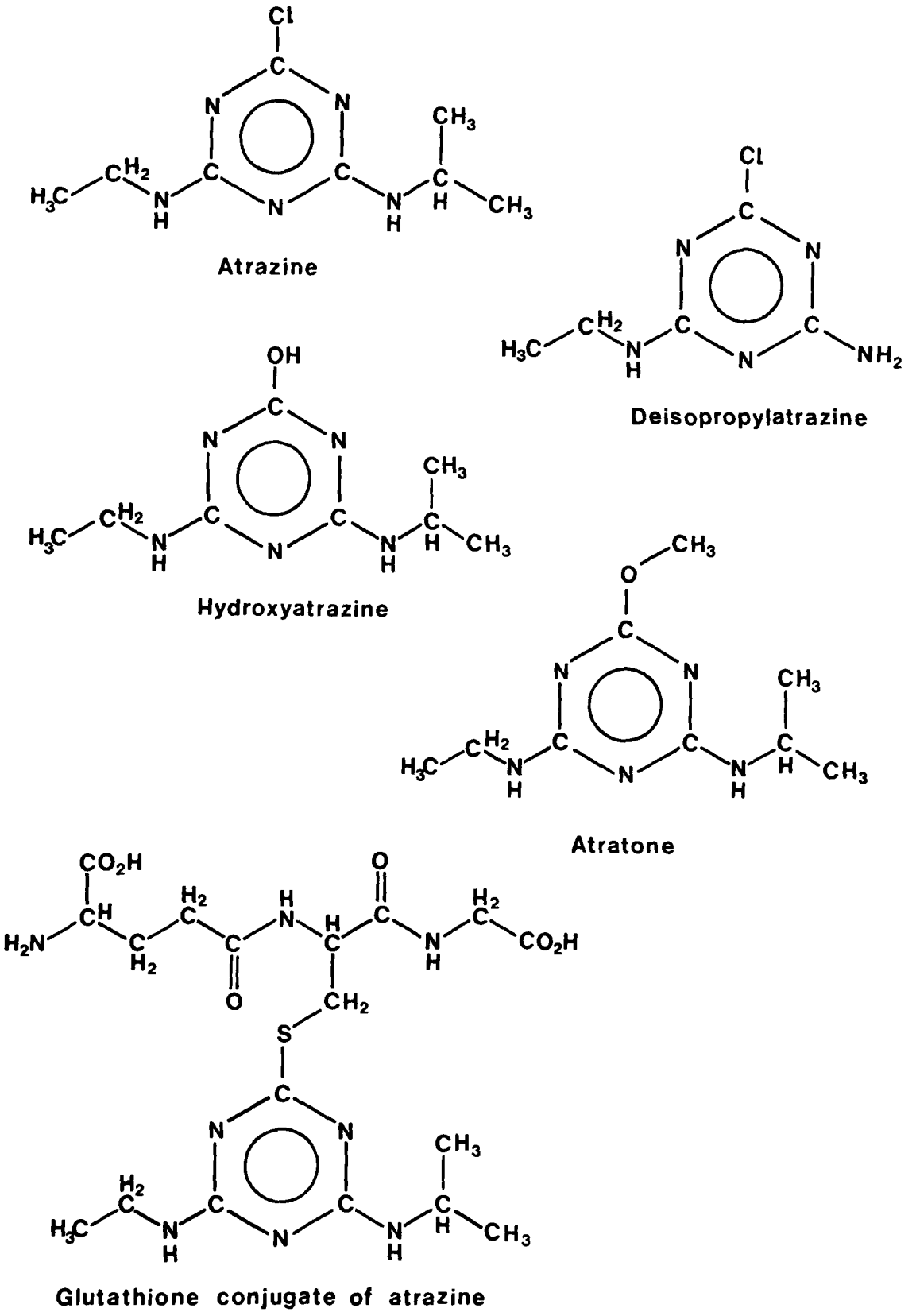


Figure 3: Chemical structures of atrazine and some of its metabolites and derivatives

In a different study the resistant biotypes of three weed species incorporated significantly higher proportions of ^{14}C-atrazine residues in the unextractable fraction than the susceptible ones (Khan et al., 1985). A literature search provided no information on the metabolism and bound residue formation of atrazine in canola.

Canola is an economically important crop, especially in Canada which is the first world producer (2.5 million tons produced in 1980).

1.4.3. Goals of the Research Project and Hypotheses

Plants generally lack the means to excrete unwanted products and toxic xenobiotics. Instead they usually have to "store" these compounds in vacuoles or specialized organs. The formation of bound residues is a mechanism by which plants may "store" as well as detoxify xenobiotics. As mentioned earlier, it has been postulated that bound residue formation may contribute, along with phase 1 and 2 metabolism to the tolerance of some plant varieties to herbicides (Mangeot et al., 1979; Khan et al., 1985b). The validity of this hypothesis was tested in this research project for two related herbicides in resistant and susceptible plant varieties: metribuzin in soybean and atrazine in canola. These two crop species display different resistance strategies: differential metabolism for soybean and a modified site of action for canola.

The present investigation was undertaken to determine the nature and distribution of the bound residues of metribuzin in soybean and atrazine in canola with respect to their role in the resistance of the varieties and their possible environmental and toxicological significance. The high temperature distillation and supercritical solvent extraction techniques have been shown to release plant bound pesticide residues in several cases,

as described earlier. These techniques were chosen for their potential to release bound metribuzin or atrazine residues in a form suitable for chemical analysis. The applicability of high temperature distillation and supercritical solvent extraction to routine analyses of bound pesticide residues was also considered.

2. MATERIALS AND METHODS

2.1 Bound residues of metribuzin in soybeans

2.1.1. Chemicals

The Mobay chemical corporation provided the analytical reference grade samples shown in Table 1.

All the organic solvents used were of pesticide analysis grade (distilled in glass).

2.1.2. Plant growth and herbicide treatment

The soybean seeds were obtained from Dr. H. Voldeng (Plant Research Centre, Agriculture Canada). The susceptible (Maple Amber) and the tolerant (Maple Arrow) varieties are known to have a short life cycle and indeterminate growth. Both are widely used in Canada and the U.S.

The procedure followed for growing and treating the soybean plants with the herbicide is shown in Figure 4. The plants were grown in a growth chamber (Controlled Environment Ltd., model PGW-1B) with a relative

Table 1: Metribuzin and metabolites used in studying the bound residues in soybean

Common Name	Chemical Name	Quantity	Purity (%)
Metribuzin	4-amino-6-tert-butyl-3(methylthio)-1,2,4-triazin-5(4H)-one	1.0 g	99.6
^{14}C-Metribuzin[1]	4-amino-6-tert-butyl-3(methylthio)-1,2,4-triazin-5-^{14}C(4H)-one	0.1 mCi	98
DA:	6-tert-butyl-3-(methylthio)-1,2,4-triazin-5(4H)-one	0.1 g	>95
DK:	4-amino-6-tert-butyl-1,2,4-triazine-3,5(2,4H)-dione	0.1 g	>95
DADK:	6-tert-butyl-1,2,4-triazin-3,5(2,4H)-dione	0.1 g	>95

[1]specific activity = 21.9 mCi/mmole

humidity of 60% and a photoperiod of 14 h. of light. A combination of incandescent and fluorescent bulbs provided a photon flux density of 250 $\mu E.m^{-2}.s^{-1}$ in the photosynthetically active radiation (P.A.R.) range. The temperatures in the chamber were maintained at 24°C and 18°C during the day and night, respectively.

2.1.2.1. Nutrient Solution

The solution used to subirrigate the plants in hydroponic culture was essentially the same as that described by Hoagland and Arnon (1950) with some minor modifications. This involved adding KCl and providing iron by EDTA Na, Fe chelate (Helson, 1967). The composition of the solution used is shown in Table 2.

2.1.2.2. Seed germination

The seeds were germinated in trays containing coarse vermiculite. The trays were placed in the controlled environment chamber and irrigated with distilled water. Within one week about 82 and 93% germinated seeds emerged for Maple Arrow and Maple Amber varieties, respectively.

Table 2. Composition of the Hoagland's nutrient solution used to subirrigate the soybean plants in hydroponic culture.

Name of the salt	Formula	Concentration x 10^{-5} mol/l
Potassium phosphate monobasic	KH_2PO_4	100
Potassium nitrate	KNO_3	500
Calcium nitrate tetrahydrate	$Ca(NO_3)_2,4H_2O$	500
Magnesium sulfate heptahydrate	$Mg\ SO_4,\ 7H_2O$	200
Boric acid	H_3BO_3	4.63
Manganese Chloride tetrahydrate	$Mn\ Cl_2,\ 4H_2O$	0.92
Zinc sulfate heptahydrate	$Zn\ SO_4,\ 7H_2O$	0.08
Copper sulfate pentahydrate	$Cu\ SO_4,\ 5H_2O$	0.03
Molybdic acid	$H_2MoO_4,\ H_2O$	0.01
Potassium Chloride	KCl	8.45
EDTA Na, Fe		0.02 (of Fe)

2.1.2.3. Hydroponic culture

At the end of the 2nd week, 12 healthy seedlings of each variety were potted in washed, coarse silica sand in plastic pots and irrigated with Hoagland's nutrient solution diluted 4 times. The concentration of the solution was increased to twice diluted during the 4th week after germination. Full strength nutrient solution was used after the 5th week.

2.1.2.4. Herbicide treatment

The herbicide treatment was started on the 7th week after germination. Stock solutions of metribuzin in methanol were prepared for each treatment. A precisely known volume of stock solution was added to the nutrient solution needed to irrigate the plant every day. That volume was calculated to produce the required concentration of metribuzin in the nutrient solution. The proportion of methanol in the nutrient solution was maintained below 1%.

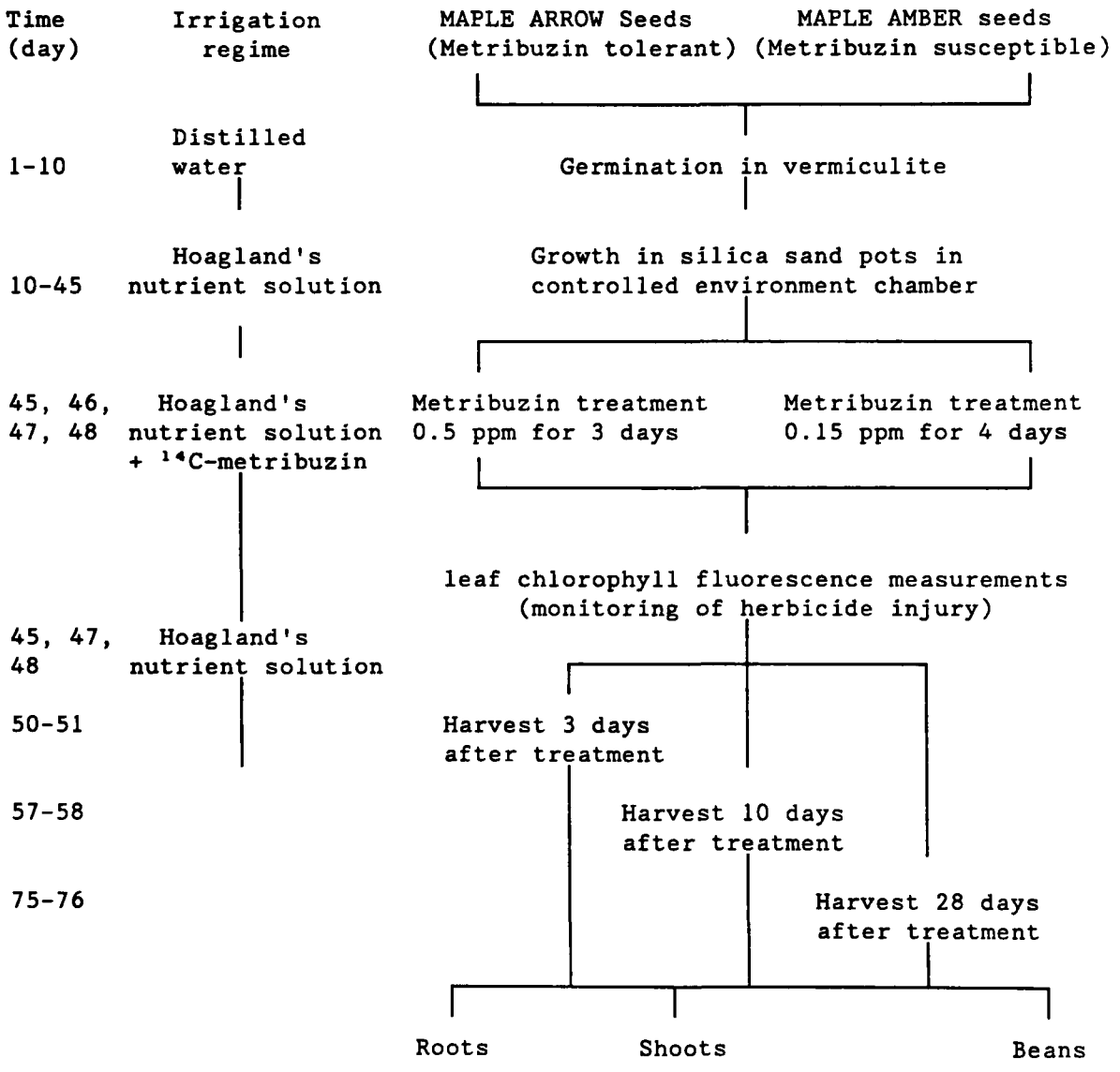


Figure 4: Flow diagram for the growth of soybean plants and their treatment with metribuzin.

i - First preliminary treatment

For each variety, the largest and smallest plants were discarded. The 10 remaining healthy plants were paired to form five groups. One group was not treated with metribuzin and was designated as the control group. The other four groups of Maple Amber plants were treated with nutrient solution having the following metribuzin concentrations: 0.10, 0.25, 0.50 and 1.00 ppm. For Maple Arrow, the metribuzin concentrations were 0.5, 1.0, 2.5 and 5.0 ppm. Two stock solutions of metribuzin in methanol containing 2.515 mg/50 ml and 24.970 mg/50 ml of the herbicide were used for the 0.10 to 0.50 ppm and 1.0 to 5.0 ppm treatments, respectively. Each plant received 100 ml/day of nutrient solution containing metribuzin and the treatment was continued for five days.

ii - Second preliminary treatment

The plants were grouped in 4 pairs for each variety: 3 Maple Amber groups were treated with 0.10, 0.15 and 0.20 ppm of metribuzin and 3 Maple Arrow groups with 0.3, 0.5 and 0.7 ppm of metribuzin. The remaining plants were not treated with the herbicide and were used as controls. The stock solution used contained 2.47 mg of metribuzin in 25 ml of methanol. Each plant received 100 ml of treatment solution per day for four days.

iii - Treatment of the plants with ^{14}C-metribuzin

Eleven healthy plants of each variety were selected for this experiment. Two plants were not treated whereas 9 plants were treated with nutrient solution containing 0.15 and 0.5 ppm of ^{14}C-labeled and non-labeled metribuzin for Maple Amber and Maple Arrow, respectively. Two stock solutions of metribuzin were prepared for this experiment (Table 3).

Table 3. Stock solutions of radiolabeled metribuzin used for the treatment of the soybean plants.

Stock solution	Variety	Concentration (µg/ml)	Radioactivity (DPM/ml)
A	Maple Amber	21.08	1222.78 x 10^3
B	Maple Arrow	66.88	1222.78 x 10^3

One Maple Amber plant received 700 ml of nutrient solution over a four day period (109.2 µg of metribuzin, 6333.98 x 10^3 DPM). Each of the remaining eight plants received 600 ml of nutrient solution over the same period (93.6 µg of metribuzin, 5429.12 x 10^3 DPM). Similarly, one Maple Arrow plant received 550 ml of nutrient solution over a three day period (198.0 µg of metribuzin, 3619.47 x 10^3 DPM) while the remaining eight plants received 600 ml (222.7 µg of metribuzin, 4071.84 x 10^3 DPM).

2.1.2.5. Determination of the phytotoxicity of the herbicide

For the 1st preliminary treatment, the phytotoxic effect of metribuzin was assessed by visual observation of herbicide burns on the leaves of the plants. An approximate estimation was made by observing the proportion of damaged area on the leaves. The phytotoxicity of the other treatments were monitored by leaf chlorophyll fluorescence measurements. When the photosynthetic electron transport pathway is blocked, most of the light collected by the chlorophyll is released as fluorescence. This can be measured to obtain qualitative information on the efficiency of photosynthesis in herbicide-treated plants (Shaw et al., 1986). The measurements were taken with a plant productivity fluorometer (model SF-20, Richard Brancker Research, Ottawa) on dark-accustomed areas of the foliage. This was done by placing the leaf between a dark surface and the

fluorometer probe while it was turned off for about 30 minutes. The fluorometer was then turned on, the probe emitted light with a wavelength of about 670 nm and measured the relative intensity of the returning fluorescence. The fluorescence measured from a healthy plant rapidly increased from a low level to an initial inflexion point (I) and the initial peak (P). It then decreased somewhat before reaching a second maximum and finally returned to a steady-state value (F). When the electron transport pathway is blocked, the fluorescence remains high after the initial peak (Figure 5). The apparatus displayed the relative fluorescence intensity of the 3 major points on the curve: the initial point (I), the peak (P) and the final value (F). These three values were used for the calculation described in section 3.1.1.1. which provided a measurement of the herbicide-induced inhibition of the photosynthesis.

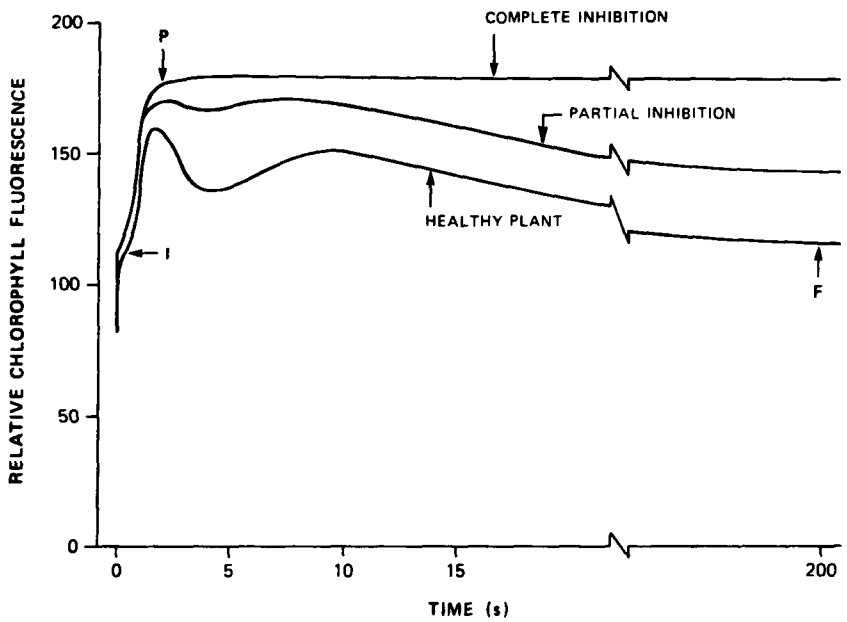


Figure 5: Typical fluorescence induction curves as measured with the plant productivity fluorometer on healthy plants and plants showing a partial or complete inhibition of photosynthesis due to herbicide treatment (after Shaw et al., 1986).

2.1.2.6. Harvest of the plant material

After the last herbicide treatment the plants were subirrigated with nutrient solution alone. The treated plants from the preliminary experiments were destroyed ten days after the end of the treatment or earlier when overexposure to the herbicide resulted in the death of the plant. The plants treated with ^{14}C-metribuzin were harvested 3, 10 and 28 days after the end of the treatment. Untreated controls were harvested on the 9th week after germination (approximately equivalent to 10 days after treatment). Each plant was harvested by collecting the beans when present, cutting the shoot and separating the root system from the silica sand under cold running water. For the plants treated with radiolabeled metribuzin, the pot, saucer and silica sand were washed with methanol and distilled water prior to removing the roots. The washing solution was collected and assayed for radioactivity as described in 2.1.3.3. The beans, shoot and root samples were placed in plastic bags, sealed and frozen separately at -18° C.

2.1.3. Determination of the nature of bound metribuzin residues in soybeans.

The methods and techniques used for the extraction of beans, shoot and root samples and analysis of the bound metribuzin residues are summarized in Figure 6.

2.1.3.1. Extraction of the plant material

Each plant sample was chopped into the blender container (Sorvall Omni-Mixer). Two replicate shoot samples of Maple Arrow plants harvested three days after treatment were extracted seven times with organic solvents

(1:5, fresh wt.:vol.). Each extraction was carried out for 5 minutes while the container was immersed in an ice bucket. The first and second extractions with acetone and chloroform, respectively, were performed following the method for residue analysis of Sencor in soybeans (Chemagro Ltd., 1974). Five additional methanol extractions were performed in order to assess the efficiency of the extraction procedure. After each extraction the samples were filtered under vacuum on Whatman #42 filter paper. The filter cake was then washed twice with approximately 1:1, wt.:vol. of the extracting solvent and it was returned to the blender for the next extraction. Each extract was concentrated to approximately 30 ml on a rotary evaporator (Büchi, Rotavapor R 110), transferred to a volumetric flask and the volume adjusted to 50 ml. Two 1.25 ml aliquots were pipetted with a Volac high precision pipetter for radioassay. The remaining extracts of each plant were combined and further concentrated. They were then transferred to 15 ml test tubes which were sealed and stored in a refrigerator. It was observed that the first three extractions with acetone, chloroform and methanol were effective in removing almost all the extractable ^{14}C. Therefore, each remaining plant sample was successively extracted first with acetone, then with chloroform and finally with methanol (1:5, fresh wt.:vol.) following a procedure similar to that described above. The three extracts and washing solutions were combined, concentrated to approximately 50 ml, transferred to a volumetric flask and finally adjusted to 100 ml. Two 2.5 ml aliquots were pipetted for radioassay. The extract was further concentrated and stored in a refrigerator as described above. The filter cake of each sample was transferred to a tared beaker, allowed to dry at room temperature with occasional mixing to remove all the solvent and then weighed.

2.1.3.2. Radioassay of the samples

i - Solid samples

Known amounts of extracted and dried plant samples containing bound ^{14}C were placed in ashless combustion cones (United Technologies Packard), together with cellulose powder (Whatman CC41). Five replicates of each samples were prepared and the cones were combusted to CO_2 in a Packard sample oxidizer model 306. The Carbosorb used as CO_2 absorber and the scintillation cocktail Permafluor V (U.T. Packard) were mixed in 20 ml polyethylene vials (New England Nuclear (NEN) Research Products).

ii - Liquid samples

The aliquots pipetted from the concentrated extracts described above were added to the cellulose powder in combustion cones and air-dried overnight at room temperature. The cones were then combusted as described for solid samples. This procedure prevented the interference of chemiluminescence and bioluminescence during scintillation counting.

For each sample containing radiolabeled compounds, the background radioactivity was obtained by combusting an equivalent amount of the corresponding control plant material. In order to determine the combustion efficiency of the sample oxidizer a known amount of a ^{14}C-metribuzin standard (20 µl = 1.34 µg and 24460 DPM) was added to the control plant samples and combusted.

iii - Liquid scintillation counting (LSC)

A Beckman scintillation counter, model LS3801 was used. The counting efficiency of the instrument was determined with a sealed vial

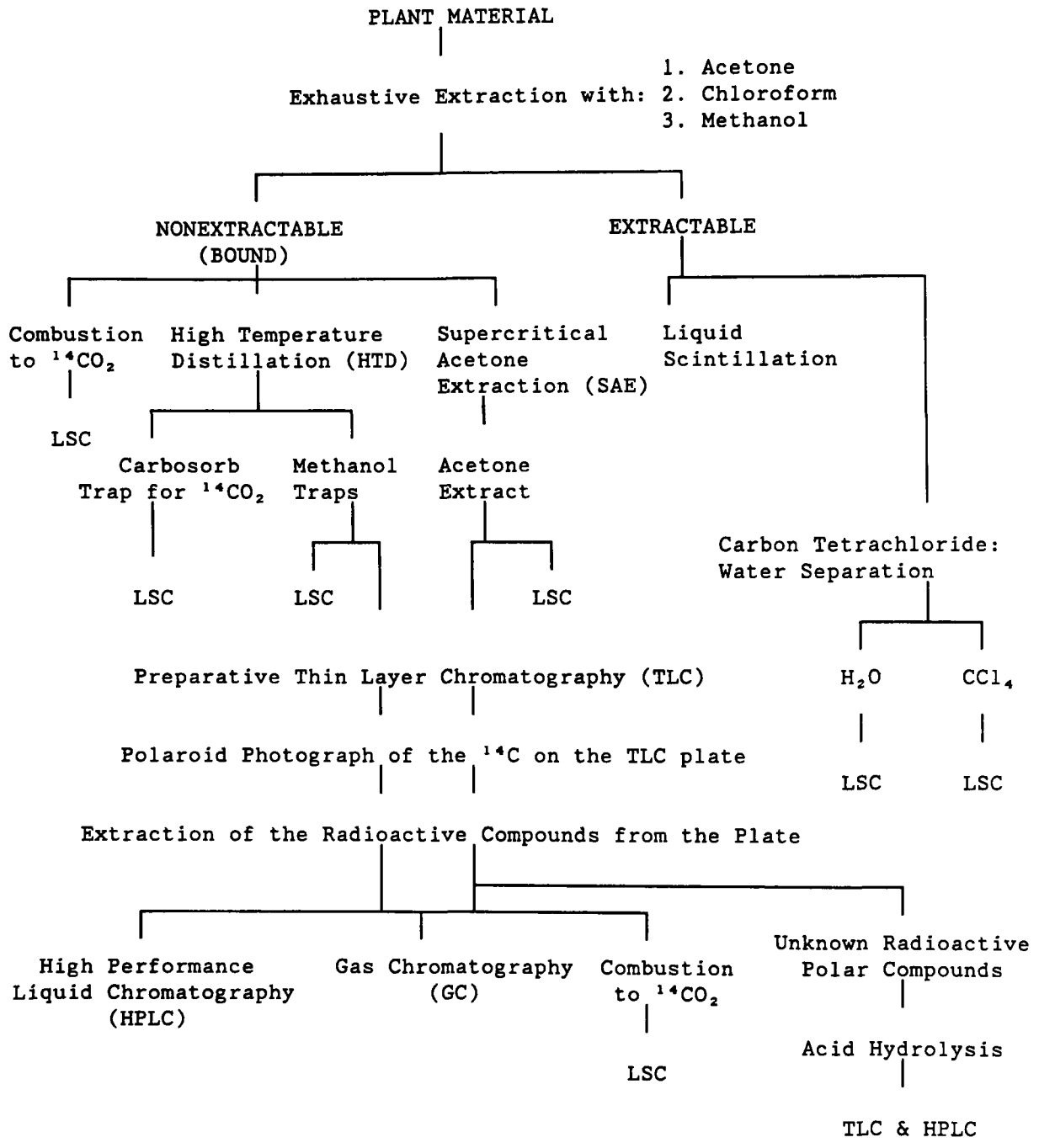


Figure 6. Flow diagram for the analysis of the bound residues of ^{14}C-metribuzin in soybeans

containing a standard solution of 52500 disintegrations per minute (DPM) activity. The background of the standard was measured with a sealed vial containing only the scintillation cocktail. Both vials were obtained from Beckman Instruments Inc. The counts were measured between a lower and higher pulse height limits of 0 and 670, respectively.

The quenching in the samples was measured by the "H number" method. The counter automatically corrected the counts per minute (CPM) values for quenching according to a predetermined quench curve. The frequency of single photon events was also monitored. This provided an indication of the extent of chemiluminescence and bioluminescence in the samples.

2.1.3.3. Distribution of the radioactivity in the extractable fraction

The extracts of the shoot and root samples harvested ten days after the end of the treatment were further separated into polar and non-polar fractions according to the procedure described in the method for residue analysis of Sencor in soybean (Chemagro Ltd., 1974). The extracts were evaporated on a rotary evaporator until only a few milliliters of aqueous solution remained in the flask. They were then redissolved in water (75 ml) and carbon tetrachloride (75 ml), transferred to a 250 ml separatory funnel and shaken for about 30 seconds. The organic phase was drained from the funnel and the aqueous phase was further extracted twice with 50 ml of carbon tetrachloride. Both phases were concentrated on a rotary evaporator, transferred to a volumetric flask and adjusted to 50 ml. Duplicate aliquots (1.25 ml) were taken from each phase for radioassay. The remaining solutions were further concentrated, transferred to 15 ml test tubes, sealed and finally stored in the refrigerator.

2.1.3.4. High Temperature Distillation (HTD) of the soybean samples

The apparatus shown in Figure 7 was used for the release of bound ^{14}C residues from plant samples. A Lindberg single zone tube furnace and a quartz tube (55 cm long, 1.0 cm internal diameter) were used. One end of the tube was tapered to approximately 0.6 cm (1/4 inch) of external diameter in order to fit a standard 1/4 in. Swagelok connection. The sample was placed in the tube, as described later. The tube was then placed in the furnace and connected with the Swagelok fitting to four successive glass traps joined by ground glass joints (Figure 7).

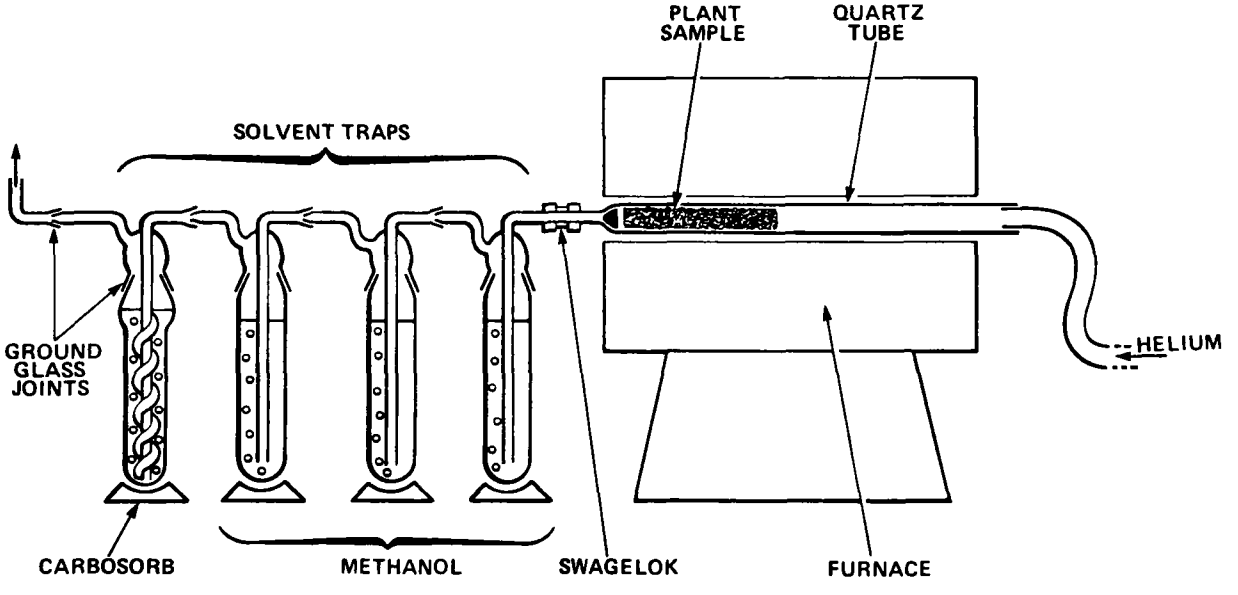


Figure 7. Schematic diagram of the high temperature distillation apparatus

Each of the first three traps contained 60 ml of methanol whereas the last trap contained 12 ml of carbosorb. The last trap was used to absorb $^{14}CO_2$ released by thermal decomposition of a portion of the bound ^{14}C-residues during distillation. Helium was used as purge gas at a rate of

50 ml/minute. The helium stream was connected to the quartz tube five minutes before starting the heating cycle in order to displace the air in the apparatus and sample. The furnace was then heated from room temperature up to a maximum of 600°C at a rate of about 30°C/minute and maintained at the maximum temperature for 10 minutes. The heating was then stopped and the system allowed to cool to room temperature. The material collected was processed as described later.

i - Recovery of pure metribuzin and its metabolites by HTD.

A known volume of standard solutions containing about 10 µg of the compound studied was pipetted into a small ceramic boat (Coors USA, # 03) and allowed to evaporate. The boat was placed inside the quartz tube for the HTD of the sample. At the end of the distillation, the quartz tube was washed with methanol. The washing was combined with the trapping methanol solutions, concentrated on a rotary evaporator, transferred to a 15 ml test tube, evaporated just to dryness under a nitrogen stream and finally redissolved in 0.5 ml of methanol. When ^{14}C-metribuzin was not used the methanol solution was analyzed by gas chromatography (GC) as described later. Two experiments were carried out with ^{14}C-metribuzin (24456 DPM). After HTD the quartz tube washing and trapping methanol were combined and concentrated to 0.5 ml as described above. Duplicate aliquots (10 µl each) were taken from the concentrated solution prior to the GC analysis and mixed with 18 ml of Aquasol-2 scintillation cocktail (NEN Research Products) in scintillation vials for radioassay. The ceramic boat was also washed with methanol. The washing was concentrated and mixed with Aquasol-2 for radioassay.

ii - Recovery of metribuzin and its metabolites from fortified, solvent extracted control plant material

The tapered end of the quartz tube was plugged with glass wool. Approximately 1 g of untreated extracted control plant material was placed in the quartz tube. A known amount of the reference standard of the compound studied was pipetted onto the plant material (Table 4). The solvent was evaporated under a slow stream of N_2. The sample was then subjected to HTD as described earlier and maintained at 600°C for 10 minutes. After the distillation the remaining solid plant material residue was washed in the quartz tube with methanol. The washing solution was combined with the methanol solutions in the traps and concentrated on a rotary evaporator. For experiments #1, 2, and 3 (Table 4) the concentrated methanol solution was transferred to a 10 ml volumetric flask and adjusted to volume. Aliquots (250 µl) were taken in duplicate for radioassay as described in 2.1.3.2. The remaining solution was transferred to a test tube and concentrated under N_2 to about 1 ml for thin layer chromatography (TLC) clean up. The carbosorb from the last trap was prepared for scintillation counting as described above. For experiments # 4, 5, and 6 (Table 4) the concentrated methanol solution was transferred directly to a test tube and concentrated for TLC clean up.

iii - HTD of control plant material

Solvent extracted, air dried, untreated root and shoot samples without fortification with metribuzin or its metabolites were processed by HTD as described above. This procedure provided control background samples for the radioassay of the carbosorb and the trapping and washing methanol solutions obtained from the HTD of the various plant samples.

Table 4: HTD recovery study using control plant material fortified with metribuzin or a metabolite

Recovery experiment #	Compound	Amount (µg)	Activity (DPM)	Control soybean material
1	Metribuzin	198.5	24456	root
2	Metribuzin	198.5	24456	shoot
3	Metribuzin	198.5	24456	root + shoot (1:1)
4	DA	205.6	0	root + shoot (1:1)
5	DK	223.2	0	root + shoot (1:1)
6	DADK	214.6	0	root + shoot (1:1)

iv - Determination of bound ^{14}C-residues in plant samples by HTD.

The solvent extracted and air dried root (≃ 1 g) or shoot (≃ 2 g) sample containing bound ^{14}C residues was placed in a tared quartz tube plugged with glass wool at the tapered end. The packed tube was then weighed again to determine the accurate weight of the sample. The sample was then processed by HTD as described in 2.1.3.4. At the end of the experiment the sample residue in the tube was washed with methanol and the washing combined with the methanol solution in the traps. After concentration of the methanol solution, duplicate aliquots were taken for radioassay. The remaining solution was further concentrated for TLC clean up. The carbosorb was radioassayed as described above.

2.1.3.5. Supercritical solvent extraction (SSE) of the soybean samples

The following components were assembled for this apparatus (Figure 8):

- Mini Pump (Milton Roy Co., model MSI 33R)
  with flow rate adjustable for 0.3 to 2.7 ml/minute
- Rheodyne pressure-release valve
- Weksler Instruments 5000 PSI gauge
- Stainless steel HPLC tubing (0.5 mm i.d.)

- GC oven (Pye Unicam)
- Sample holder: empty HPLC column (25 cm long, 10 mm i.d.)
- two regulating valves: Sno-Trik 10000 PSI valve
  Whitey 5000 PSI low volume valve

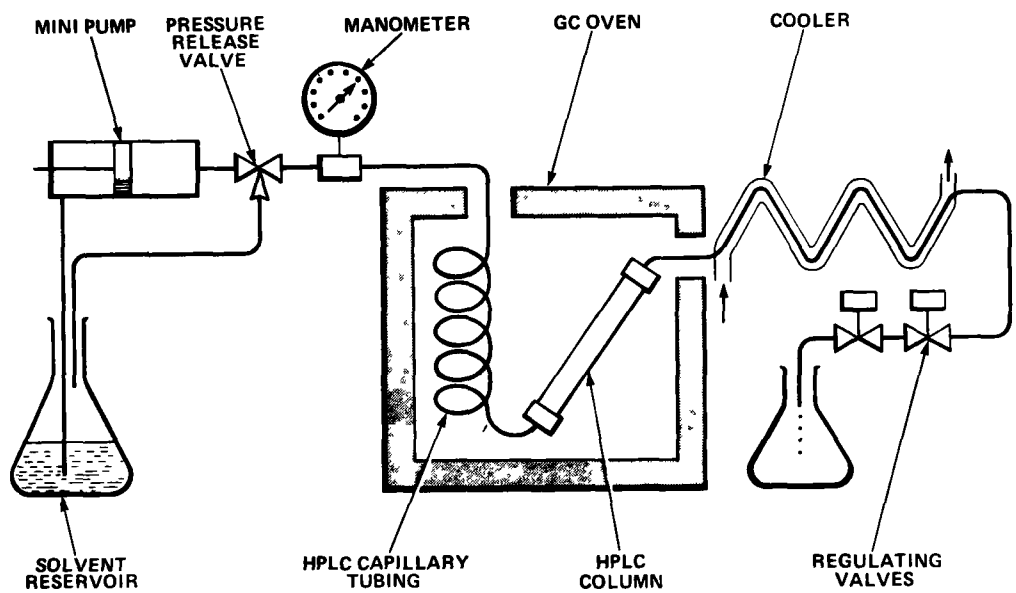


Figure 8. Schematic diagram of the supercritical solvent extraction apparatus

Two solvents, methanol and acetone, were used for SSE under the experimental conditions shown in Table 5. The critical temperature and pressure for these two solvents, as reported in the literature, are also shown in Table 5.

i - Clean-up of the SSE apparatus

The apparatus was cleaned by pumping methanol (2.7 ml/minute) at atmospheric pressure and room temperature for 2 hours. The flow rate was

Table 5: Critical temperatures and pressures of the solvents used and experimental conditions of SSE.

Solvent	Critical Temperature (°C)	Critical Pressure			Experimental Conditions		
					Temperature	Pressure	
		(Atm)	(Bar)	(PSI)	(°C)	(Bar)	(PSI)
Methanol	240.0	78.5	79.5	1154	250	152	2200
Acetone	235.5	47.0	47.6	691	250	117 or 152	1700 or 2200

then reduced to 1.5 ml/minute and the pressure increased and stabilized at about 3000 PSI by adjusting the two regulating valves. The oven was then heated to 250°C to bring the methanol to the supercritical state. The increase in temperature caused the pressure to rise to about 3200 PSI. These temperature, pressure and flow rate conditions were maintained for three hours while the cooled methanol was collected at the outlet (Figure 8). The oven and HPLC column were then allowed to cool to room temperature. The pump was stopped, allowing the methanol to return to atmospheric pressure. The methanol collected was then concentrated on a rotary evaporator, transferred to a test tube and adjusted to approximately 10 ml volume. The sample was analyzed by GC and HPLC for possible contaminants. It was then used as a control background sample in the gas chromatographic and high pressure liquid chromatographic analyses of the recovery of pure metribuzin by supercritical methanol extraction (SME). The same procedure was repeated with acetone as the supercritical solvent. The collected and concentrated acetone was used as a control background sample in the recovery study of metribuzin by supercritical acetone extraction (SAE).

ii - Recovery of pure metribuzin from supercritical solvents

The flow rate of the solvent was adjusted to 1 ml/minute. The pressure was increased and stabilized with the regulating valves at about 100 PSI below the desired experimental pressure. The oven was then heated to 250°C. This brought the pressure close to the experimental pressure which was then adjusted more precisely. Standard stock solutions of metribuzin were prepared in methanol and acetone. A known amount of metribuzin (about 800 µg) was pipetted from the standard stock solution into the solvent reservoir and mixed. The metribuzin solution was then circulated through the apparatus, brought to supercritical conditions, cooled and finally collected at the outlet. By following this procedure all the metribuzin collected was exposed to the supercritical solvent. This condition could not have been achieved by placing the metribuzin standard in the sample holder prior to starting the experiment. Stable supercritical conditions were usually attained only about 45 minutes after starting the pump. During that time the metribuzin would have been extracted by the solvent in the liquid state. The pump was stopped when about one-third (100 ml) of the metribuzin solution remained in the reservoir. The reservoir was replaced by one containing only the pure solvent. The pump was started again immediately. During this operation the pressure dropped only about 100 PSI and the original pressure was recovered within two minutes. One hour after replacing the reservoir the oven and HPLC column were allowed to cool down and the pump was stopped. The solution left in the column was combined with the solution collected at the outlet. The column was refilled with the pure solvent and a final purge of the system was achieved by starting the pump again for about 30 minutes and collecting the solution. The metribuzin solution remaining in

the first reservoir as well as the combined solution collected during SSE were concentrated on a rotary evaporator, transferred to volumetric flasks and adjusted to 10 ml volume for GC and HPLC analyses.

iii - Recovery of pure metribuzin from supercritical acetone extraction (SAE) of control plant material

A solvent extracted, air dried, untreated shoot sample (≃2 g) was placed in the column. The pump was started, slowly filling the column while tapping it to prevent air bubbles from becoming trapped in the sample. Sea sand, washed and ignited (Fischer Scientific Co.) was placed in the column, on top of the sample to keep sample particles from plugging the stainless steel filter at the column outlet. When the acetone level reached the top of the column, the pump was stopped and the column was closed tightly. The pump was started again at a flow rate of 1 ml/ minute. The pressure was then increased to about 2100 PSI and the oven heated to 250°C as described above. As soon as stable supercritical conditions were reached (2200 PSI and 250°C) an acetone solution containing 1.2694 mg of ^{14}C-metribuzin (218700 DPM) was added to the acetone reservoir and mixed. This brought the volume of acetone to about 300 ml. The supercritical acetone extract was then collected at the outlet. After four hours, about 50 ml of acetone solution remained in the reservoir. The solution was then replaced by pure acetone. The SAE was allowed to proceed for one hour after which the oven and column were cooled and the pump stopped as described above. The acetone collected was concentrated, transferred to a volumetric flask, and adjusted to 10 ml. Duplicate aliquots (250 µl each) were taken for radioassay. The remaining solution was transferred to a test tube and concentrated under a stream of nitrogen

for further TLC clean up and GC and HPLC analyses. The column was emptied and rinsed twice with acetone into a beaker. The acetone solution was then decanted from the beaker. The column was refilled with pure acetone, closed and the pump was started again for one hour (1 ml/minute, 2100 PSI). The solution collected during that time was combined with the decanted solution, concentrated, transferred to a test-tube, dried under N_2 and finally redissolved in 1 ml of acetone. Two aliquots (250 µl each) were taken for radioassay.

iv - SAE of soybean samples containing bound ^{14}C-residues

The solvent extracted, air dried root (≃ 1 g) and shoot (≃ 2 g) samples from the combined soybean plants harvested 10 days after treatment were accurately weighed, transferred to the column and topped with sea sand as described before. The column was closed, the pump was started, the pressure was adjusted and finally, the oven was heated (1 ml/minute, 2100 PSI, 250°C). The pressure was then readjusted to 2200 PSI. The extraction was carried out for three hours during which the valves were occasionally adjusted to keep a constant pressure. The acetone extract was collected in three separate fractions which corresponded to the first, second and third hour of extraction. At the end of the SAE, the oven and column were allowed to cool down and the pump was stopped as described above. Each acetone extract fraction was then concentrated and transferred to a 10 ml volumetric flask. Duplicate aliquots (250 µl ea.) were taken for radioassay. The remaining solution was transferred to a test tube and further concentrated for TLC clean up. After the extraction the column was emptied and rinsed with acetone into a beaker. The acetone solution in the beaker was decanted. The residue of plant material in the beaker was

separated from the sand by adding acetone, suspending the plant material by agitation and pouring the acetone suspension into a tared beaker. This procedure was repeated until no plant residue was left with the sand in the first beaker. The acetone was then decanted from the second beaker and combined with the acetone previously decanted from the first beaker. The solid plant residue was air dried and later weighed and radioassayed. The column was refilled with pure acetone and closed. The pump was turned on for an additional one hour and the acetone solution collected was combined with the solution decanted from the two beakers described above. This was then concentrated, transferred to a test tube, dried and redissolved in 1 ml of acetone. Two aliquots (250 µl each) were taken for radioassay.

2.1.3.6. Development of a clean-up procedure for high temperature distillates

i - Liquid - Liquid extraction

A known amount of metribuzin (197.2 µg) was pipetted and mixed with methanol distillates obtained from the HTD of 1 g of control soybean shoot. The solution was then evaporated just to dryness on a rotary evaporator. The dried residue was only marginally soluble in n-hexane, benzene or distilled water but was completely soluble in chloroform. The dried residue was redissolved in 75 ml of chloroform, transferred to a 250 ml separatory funnel containing 75 ml of distilled water and shaken for about 30 seconds. The aqueous phase did not extract the natural plant distillates from the organic phase.

ii - Column Chromatography

Various column chromatrographic methods were evaluated for the clean-up of high temperature distillates and supercritical acetone extracts of soybean samples. In a preliminary experiment the recovery of pure metribuzin and metabolites by different solvent systems on various types of column packing was determined. Three solid phases (packings) were used: acidic alumina (Fischer Scientific Co.), florisil (60-100 mesh size, Supelco Inc.) and silica gel (type Davidson 12, 80-200 mesh size, Supelco Inc.). The columns used for these preliminary experiments were 25 cm long, had an internal diameter (i.d.) of 1 cm and were fitted with teflon taps at one end and 40 ml solvent reservoirs at the other. Glass wool plugs were placed at the bottom of the columns. In each case, the solid phase was weighed and mixed with enough of the solvent system to form a slurry. It was then transferred to the column and washed with three column volumes of the same solvent system. A 2 ml solution of the solvent used for elution containing pure reference standards of metribuzin (47.7 µg), DA (39.8 µg), DK (35.8 µg) and DADK (67.6 µg) was then pipetted on the top of the column. When a single solvent system was used, the standards were eluted with four column volumes of the solvent. When chloroform - methanol mixtures were used in various proportions, each system represented two column volumes. All the experiments were repeated twice. The eluted fractions were collected separately, concentrated, transferred to test-tubes, dried under N_2, redissolved in 0.5 ml of methanol and analyzed by GC. The details of the various recovery experiments performed are shown in Table 6. The solid and liquid phases of the experiments #2 and 6 were the same as those used for the clean up of extractable metribuzin residues as described in the method for residue analysis of Sencor in soybeans

(Chemagro Ltd., 1974). The results of experiments #4, 5 and 6 showed overall recoveries of about 75%.

The efficiency of the procedures #4, 5 and 6 to clean up high temperature distillates of control soybean samples fortified with metribuzin and its metabolites was determined. The columns used were 50 cm long, 2 cm i.d. and were fitted with teflon taps. The florisil or silica gel (25 g each) were mixed with the solvent system, transferred to the column and washed as described before. A methanol solution containing the high temperature distillates of 1 g of control soybean shoot, 106.8 µg of metribuzin, 99.5 µg of DA, 89.5 µg of DK and 84.5 µg of DADK was evaporated just to dryness on a rotary evaporator. It was then redissolved in 5 ml of the solvent system used (Table 6), pipetted on the top of the column and eluted as described before. Each experiment was repeated twice. The dry control distillate residue was insoluble in benzene-acetonitrile (9:1) and had to be dissolved in 1 ml of acetonitrile prior to adding the remaining 9 ml of benzene. The cleaned fractions collected were processed as described above and analyzed by GC.

Table 6: Experimental conditions of the recovery study of pure metribuzin and metabolites by column chromatography

Experiment number	solid phase	weight (g)	solvent system used (relative proportions)
1	Acidic Alumina	15	Chloroform-Methanol (1:0)(2:1)(1:2)(0:1)
2	Florisil	10	Benzene-Acetonitrile (99:1)
3	Florisil	10	Benzene-Acetonitrile (9:1)
4	Florisil	10	Chloroform
5	Silica Gel	10	Chloroform-Methanol (1:0)(2:1)(1:2)(0:1)
6	Silica Gel	10	Benzene-Acetonitrile (9:1)

A modified column clean-up technique was also evaluated. The methanol solution of control distillates and pure standards was

concentrated to about 1 ml under N_2 and pipetted on 2 g of silica gel in a beaker. The methanol was then evaporated by placing the beaker in an oven at 40°C for one hour. The silica gel was then mixed with the solvent system and transferred to a previously washed column containing 25 g of silica gel. The solvent systems used were chloroform, benzene-acetone (80:20), (65:35), (50:50), benzene-acetonitrile (90:10) and benzene-chloroform (50:50). When an appreciable clean-up of the sample was achieved, the fractions collected were processed and analyzed by gas chromatography and high pressure liquid chromatography.

iii - Thin layer chromatogaphy (TLC)

Smith and Wilkinson (1974) reported using a TLC procedure for the analysis of extractable metribuzin residues in soybean. They used silica gel plates and a solvent system of chloroform-dioxane (9:1). The efficiency of this procedure for the clean-up of high temperature distillates of soybean plant samples was assessed. In this study, preparative silica gel plates (Whatman, PLK5F, 20 x 20 cm, 1000 µm thick) containing a fluorescent marker (inorganic phosphor) were used. In order to remove possible contaminants from the silica gel, the plates were developed in acetone and air-dried 15 minutes before use. Two plates were spotted on the celite band covering the bottom 3 cm of the plates with reference standards of metribuzin and/or its metabolites and developed in chloroform-dioxane (9:1) as described by Smith and Wilkinson (1974). After air drying the plates, the standards were located by observing the areas of quenched fluorescence under a UV light (λ = 254 nm). Using these experimental conditions the Rf values of metribuzin, DA, DK and DADK were 0.89, 0.66, 0.28 and 0.22, respectively. On two other plates, two 1.5 cm

wide vertical channels on each side of the plates were created by scraping the silica gel off of 3 mm wide grooves between the channels and the main portion of the plates (Figure 10). A mixture of metribuzin and its three metabolites was spotted on the celite of each channel. Another mixture containing 106.8 µg of metribuzin, 99.5 µg of DA, 89.5 µg of DK and 84.5 µg of DADK was applied as a streak, with a glass capillary on the celite of the main central portion of each plate. The plates were air dried for 10 minutes, developed in chloroform-dioxane (9:1) and dried again. The four compounds were observed under UV, on the two side channels while the central portion was covered with aluminum foil in order to avoid any photo-decomposition (Hatzios and Penner, 1988). The silica gel in the central part of the plate between the Rf values of 0.94 and 0.85 (metribuzin), 0.73 and 0.60 (DA) and 0.17 and 0.33 (DK and DADK) was scraped and transferred to three beakers. Approximately 20 ml of acetone was added to each beaker and the silica gel slurries were transferred to fine scintered glass funnels. The funnels were placed under suction and the silica gel was eluted with an additional 160 ml of acetone. Each acetone extract was concentrated on a rotary evaporator, transferred to a test tube, dried under N_2 and redissolved in 2 ml of methanol for GC analysis.

The recovery of metribuzin and its metabolites from the TLC plates of high temperature distillates of control plant samples was determined. The procedure is shown in Figure 9. The high temperature distillates of 2.4 g of extracted control soybean shoots were concentrated on a rotary evaporator, transferred to a graduated cylinder and adjusted to 30 ml. Three 10 ml fractions of the distillates were placed in test tubes. Two fractions were fortified with 321.5 µg of unlabeled and ^{14}C-metribuzin (26710 DPM), 298.6 µg of DA, 268.4 µg of DK and 253.3 µg of DADK. Each

fraction was concentrated to about 1 ml and applied on the central portion of the celite band of a TLC plate, in the form of a 5 mm wide streak. The test tubes were washed twice with about 0.5 ml of methanol and the washings were also applied on the plates. The plates were allowed to dry overnight. Before performing the TLC, reference standards of metribuzin and its metabolites (about 50 µg each) were applied to the side channels of each plate and allowed to dry for 10 minutes. The plates were developed in chloroform-dioxane (9:1) and allowed to air-dry. A polaroid photograph of the ^{14}C-metribuzin band on the two TLC plates of the fortified fractions was taken with the Berthold's beta camera as described later. The bands corresponding to Rf metribuzin, Rf DA and Rf DK + DADK were scraped from all three plates and extracted as described above (Figure 9). Each extract of the unfortified sample was separated in two equal subfractions, one of which was mixed with 160.2 µg of metribuzin, 149.3 µg of DA, 134.2 µg of DK and 127.5 µg of DADK. The extracts were then concentrated, transferred to test tubes, dried and redissolved as shown in Figure 9. Duplicate aliquots (25 µl each) were taken from the two fortified fractions for radioassay. The remaining solutions were analyzed by GC.

The high temperature distillates and supercritical acetone extracts of soybean samples containing bound ^{14}C-residues were subjected to clean up by TLC as described above. One plate was used for the clean up of each root sample ($\simeq$ 1 g) whereas the clean up of shoot samples ($\simeq$ 2 g) required the use of two plates. The concentrated sample was applied on the celite band of the acetone washed plate(s) as a streak and allowed to dry overnight. Reference metribuzin and metabolite standards were applied on the celite of the channels on each side of the plate(s) and allowed to dry. Each plate was developed in chloroform-dioxane (9:1), allowed to dry and photographed

with Berthold's beta camera. The ^{14}C bands were scraped and extracted with 200 ml of acetone as described above. The extracts were then concentrated, transferred to volumetric flasks and adjusted to 10 ml. Duplicate aliquots (250 µl each) were taken for radioassay. The remaining solutions were transferred to test tubes, dried and redissolved in methanol for GC and/or HPLC analysis.

2.1.3.7 Photography of TLC plates containing ^{14}C-compounds with Berthold's beta camera.

The spark chamber of Berthold's beta camera (model LB 292) operates on the principle that β-emissions trigger sparks between the grid-like anode and cathode above the point of emission. The chamber was turned on ten minutes before taking a photograph, it was flushed with P-10 gas (10% methane in argon) containing 3-5% of methylal (dimethoxymethane), at a rate of 100 ml/minute. This was achieved by bubbling the P-10 gas in about 300 ml of methylal maintained at 10°C. The voltage between the electrodes was kept between 3700 and 3900 volts. The TLC plate was wrapped in plastic foil (saran-wrap) to avoid contamination and placed in the spark chamber. The apparatus was set to record 30 000 counts (sparks) on Polaroid 667 film. The aperture of the camera lens was set to F11 in order to obtain better definition. After developing the print, its image was projected back on the TLC plate by means of the episcope of the beta camera. The areas containing radiolabeled compounds were marked on the plate and scraped for further analysis as described earlier. Figure 10 shows some typical prints obtained by this technique.

2.1.3.8. Gas chromatographic analyses (GC)

The gas chromatograph used was a Varian model 3400 equipped with a

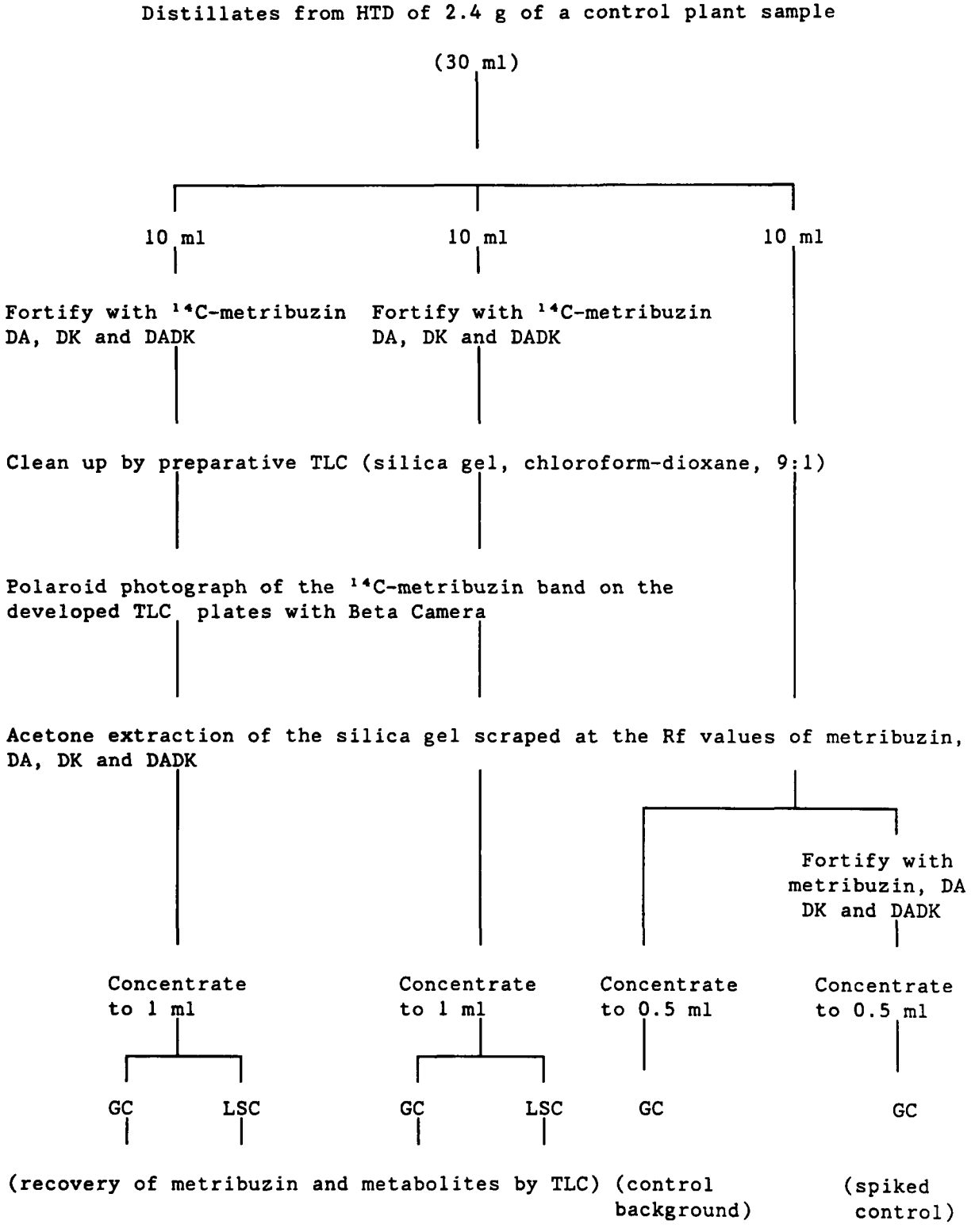


Figure 9: Procedure followed for the study of the recovery of metribuzin and its metabolites from the TLC of fortified control distillates

Varian thermoionic specific detector (TSD). The bead current of the detector varied between 3.2 and 3.8 A. A flow rate of 5.0 ml/minute of hydrogen gas was maintained through the detector. The chromatograms were obtained with either a Varian 4270 integrator or a Varian Vista 402 data station. Nitrogen was used as carrier gas in all GC analyses. Various types of columns were evaluated for the analysis of bound ^{14}C residues of metribuzin in soybeans (Table 7). The packed glass columns had an i.d. of 2.0 mm and were injected with sample volumes of 1 to 3 µl. The fused silica capillary column had an i.d. of 0.25 mm and the sample size was 1 µl. Table 7 shows the experimental details and GC conditions for the various methods evaluated. A few typical chromatograms are shown in Figure 11. The quantitation of metribuzin, DA, DK and DADK in samples analyzed by GC was carried out by comparing the integrated area of each sample peak with that of a known amount of the corresponding reference standard. The size of the standard injected was calculated to produce a peak area close to that of the sample peak.

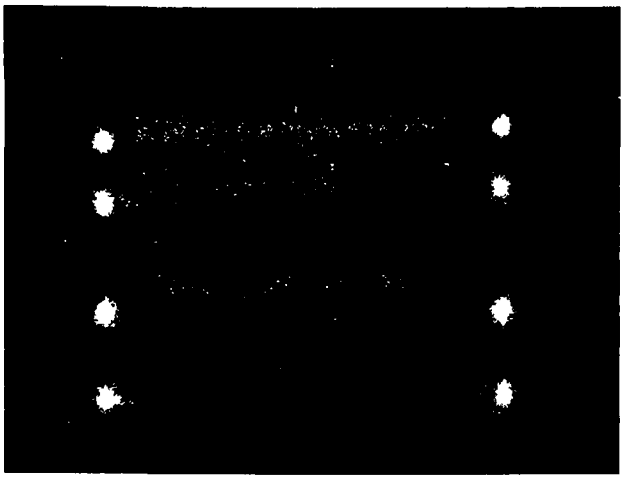
2.1.3.9. High Performance Liquid Chromatographic analyses (HPLC)

The analyses were performed on a Varian 5500 chromatograph equipped with a variable wavelength UV detector and a radioactivity monitor (Berthold, model LB 504). The ultraviolet spectra of metribuzin and its metabolites in methanol-water (1:1) were taken on a UV and visible spectrophotometer (Varian, model DMS 200). The analysis of the spectra indicated that the average wavelength for optimum absorption of the four standard reference compounds was 220 nm. The testing of the UV detector at various wavelength settings confirmed that 220 nm provided the highest overall sensitivity. The UV chromatograms were obtained on a Varian 4270 integrator whereas the ^{14}C radiograms were recorded and analyzed with a

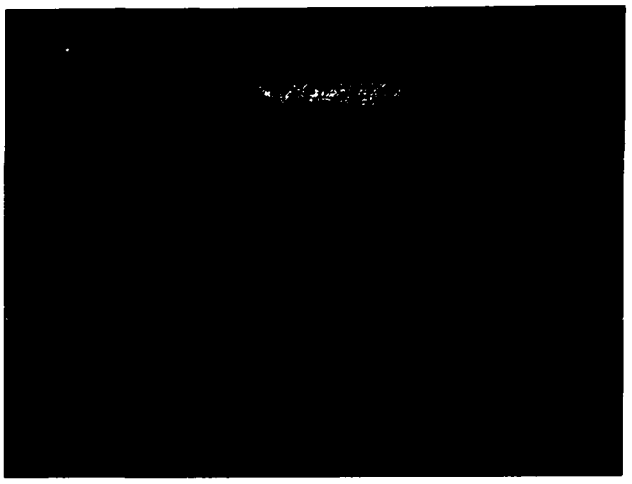
Figure 10: The photographs shown on the opposite page were taken with a Berthold beta camera. They represent the radioactive areas on TLC plates obtained during metribuzin recovery studies or analyses of extracted soybean samples containing bound ^{14}C-residues by high temperature distillation (HTD) and supercritical acetone extraction (SAE). A radioactive marker was added after developing some of the plates at the Rf values of metribuzin: 0.88 (top of the photographs), DA: 0.65, DADK: 0.29 and, for the HTD recovery study: Rf 0 (bottom of the photograph).

Recovery of metribuzin with control plant material

HTD

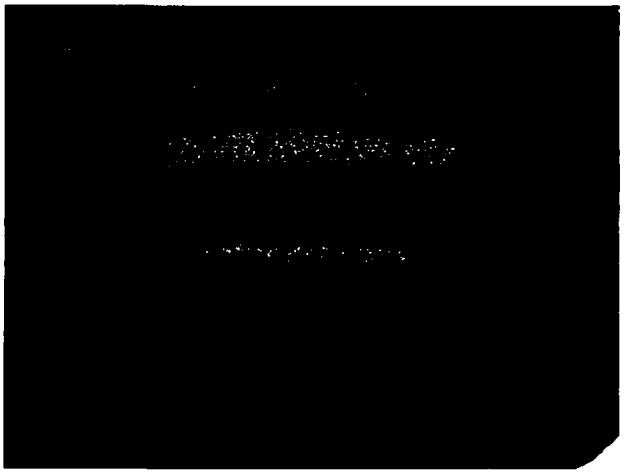


SAE

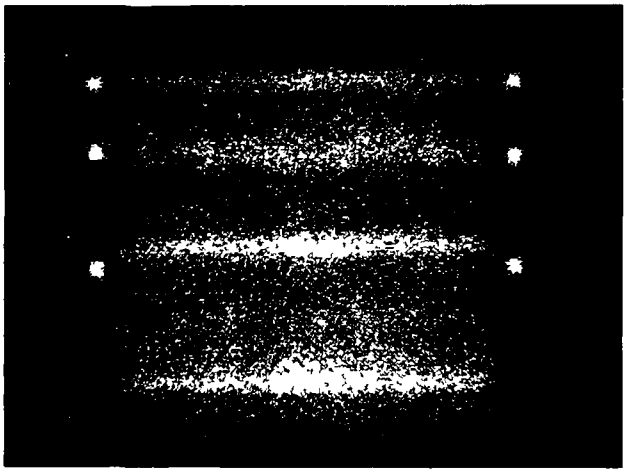


Analysis of shoot samples containing bound ^{14}C-residues

HTD

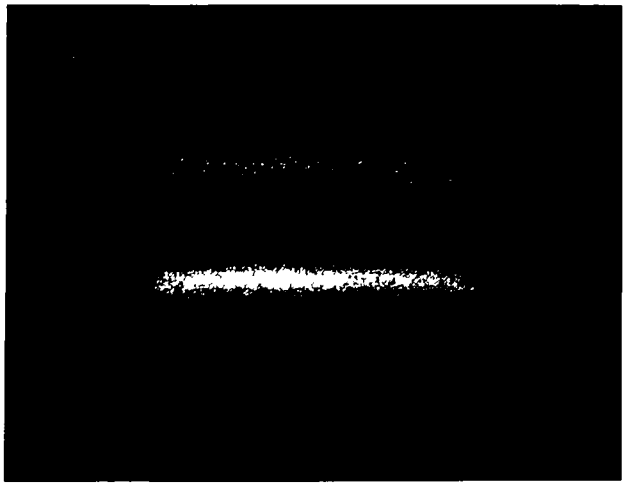


SAE



Analysis of root samples containing bound ^{14}C-residues

HTD



SAE

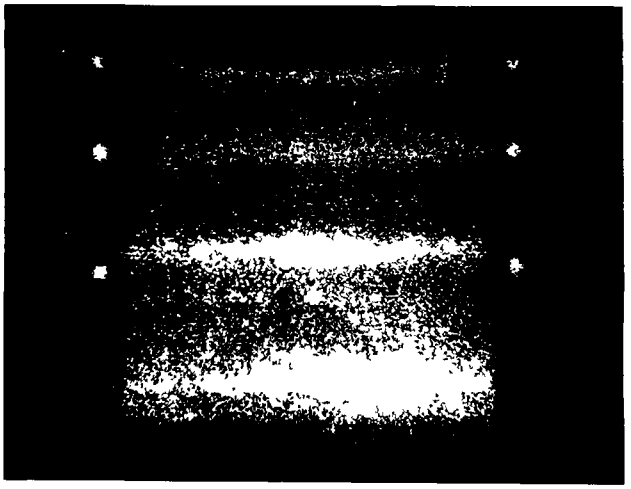


Figure 10: Typical photographs obtained from TLC plates of high temperature distillates and supercritical acetone extracts of soybean samples.

Table 7. Gas chromatographic analyses: types of columns, operating conditions and resulting retention times of metribuzin and its metabolites.

Liquid phase	Type of column	Length of column (m)	Flow rate of carrier gas (ml/mn)	Column temperature (°C)	Injector temperature (°C)	Detector temperature (°C)	Retention times (mn) Metribuzin	DA	DK	DADK
3% XE60	P[a]	1.0	30	175	190	265	Not determined(ND), column unsuitable			
3% OV-210	P	1.8	30	150 to 210[c]	180	270	9.0	ND	ND	ND
3% OV-225	P	1.2	30	230	250	300	1.2	ND	ND	ND
5% Reoplex	P	2.0	70	190	200	280	10.5	22.9	11.8	8.7
1% Reoplex	P	1.5	45	190	200	280	6.0	12.8	7.2	5.3
SE 30	C[b]	15.0	15	140 to 190[c]	190	285	7.0	10.5	5.0	3.7

[a]: packed column [b]: capillary column [c]: the column temperature was programmed at a rate of 3°C/minute

Berthold LB 512 data station. The HPLC column used was a Whatman Partisil 10 ODS-2 (25 cm long, 4.6 mm i.d.). A Whatman pellicular ODS guard column placed after the injector prevented the contamination of the column. The mobile phase was methanol water (1:1), pumped at a flow rate of 1 ml/minute. The retention times of metribuzin, DA, DK and DADK under these experimental conditions, as determined with the UV detector were 18.1, 20.9, 8.1 and 10.2 minutes, respectively. A few typical chromatograms are shown in Figure 12.

2.1.3.10. Acid hydrolysis of the unknown polar compounds.

The TLC of supercritical acetone extracts of the soybean samples showed an important and poorly defined radioactive band at Rf =0. The silica gel containing these unknown compounds was scraped and extracted with acetone. The acetone was evaporated from a 200 ml round bottom flask on a rotary evaporator. The dried residue was redissolved in 50 ml of 1N HCl in distilled water and refluxed for 45 minutes using a water-jacketed condenser. The aqueous hydrolysate was transferred to a 250 ml separatory funnel containing 75 ml of chloroform. About 1.5 g of sodium chloride (Anachemia, ACS reagent grade) was added to the aqueous phase to reduce the formation of an emulsion during the separation. The aqueous phase was washed six times with 75 ml of chloroform in order to overcome the loss of efficiency caused by emulsification. After separation, the aqueous and organic solutions were evaporated to dryness on a rotary evaporator. The residue of the aqueous phase precipitated around NaCl cristals during the evaporation thus yielding a loose powder that was transferred to a tared beaker. The powder was radioassayed as described above for solid samples. The dry residue of the chloroform solution was

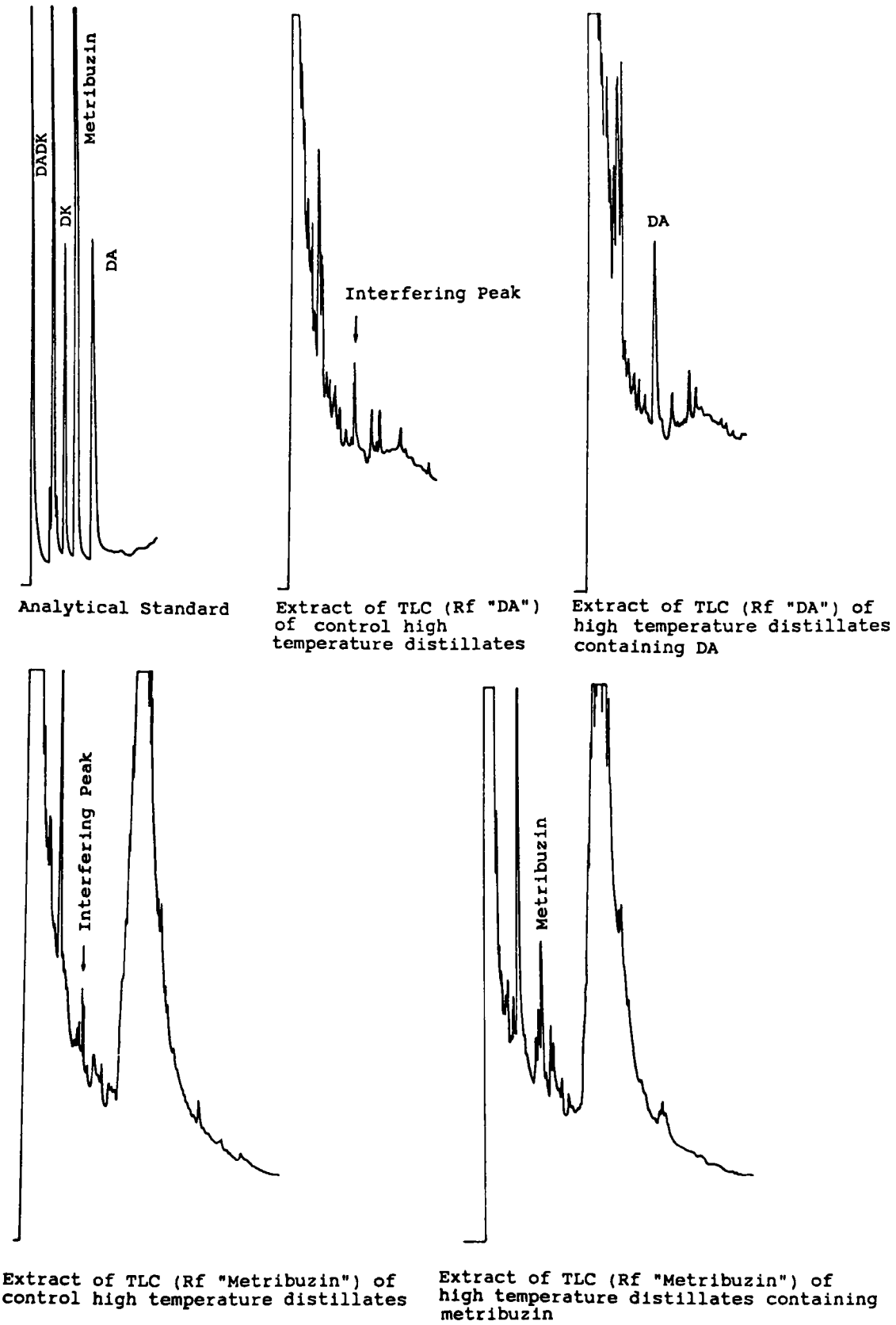


Figure 11: Typical chromatograms obtained in the study of bound metribuzin residues in soybean by gas chromatography with SE-30 capillary column.

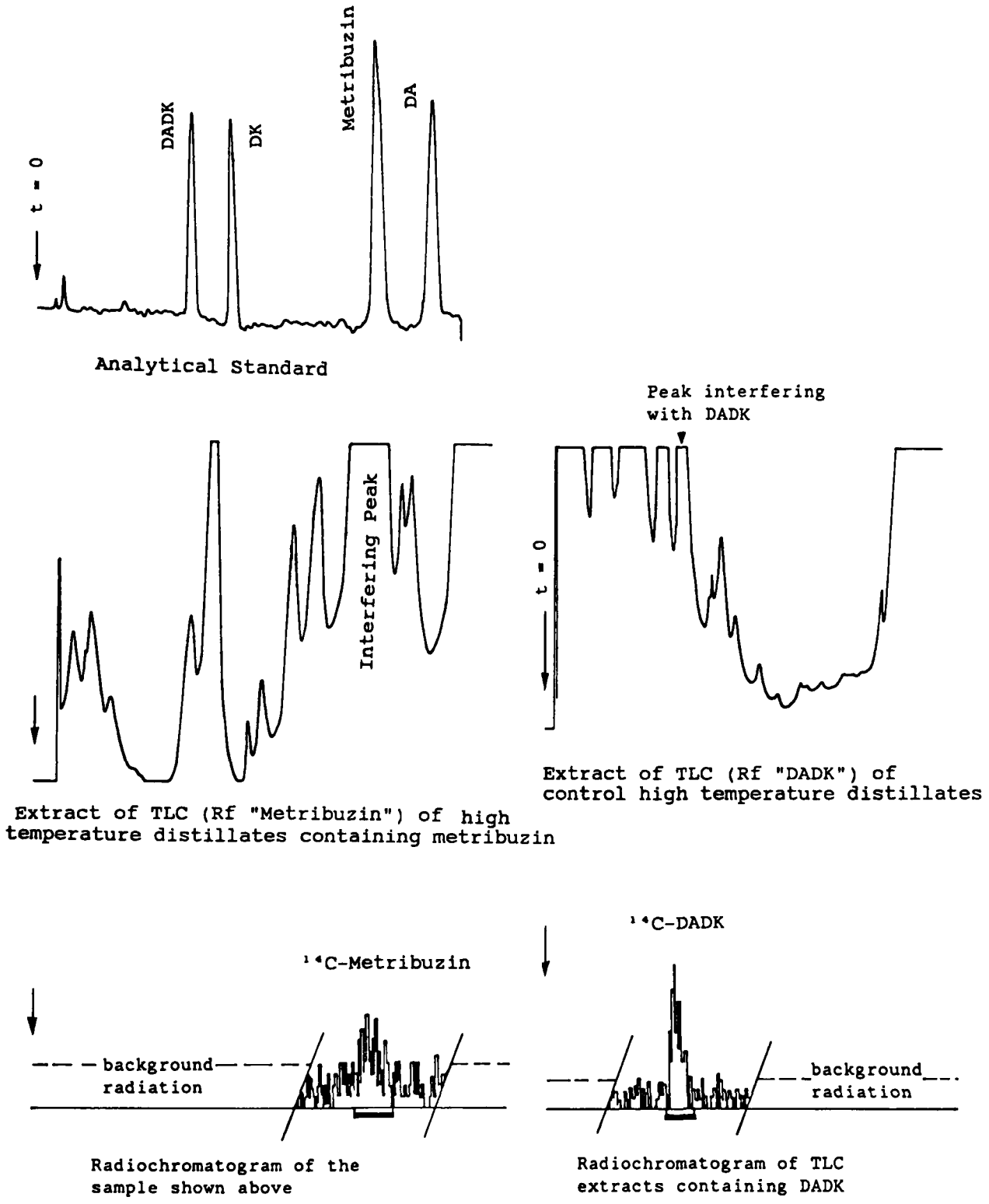


Figure 12: Typical chromatograms obtained by HPLC with UV and radioactivity detectors during the study of bound metribuzin residues in soybean.

redissolved in methanol, transferred to a volumetric flask and adjusted to 5 ml. Duplicate aliquots (100 µl each) were taken for radioassay as described above. The remaining solution was transferred to a test tube and concentrated for further TLC clean up and HPLC analysis.

2.2 Bound atrazine residues in Canola

2.2.1. Chemicals

The Analytical grade standards and radiolabeled atrazine shown in Table 8 were provided by the Ciba-Geigy corporation.

2.2.2. Plant growth, treatment and harvest

The canola seeds were provided by the Crop Science department, Ontario Agricultural College, University of Guelph. The two varieties: Atr Tower (O.A.C. Triton) and Tower were respectively resistant and suceptible to atrazine.

The procedure followed for growing and treating the canola plants with atrazine is shown in Figure 13. The plants were grown in the same controlled environment chamber used for growing soybean plants. The temperature was maintained at 22°C during the day and 18°C at night. The light intensity and photoperiod were identical to those described earlier.

2.2.2.1. Germination and hydroponic culture

The techniques used were similar to those described earlier. The seeds were germinated in vermiculite. After ten days, about 85% of the Atr Tower seeds had germinated, emerged and grown to about 10 cms (first true leaf stage). The germination of the Tower seeds occurred over a longer period of time. This resulted in 60% emergence after 10 days, with

Table 8: Atrazine and metabolites used in studying the bound residues in canola.

Common name	Chemical name	Quantity	Purity (%)
Atrazine	2-chloro-4-ethylamino-6-isopropylamino-s-triazine	1.0 g	>99
^{14}C-Atrazine[a]	2-chloro-4-ethylamino-6-isopropylamino-s-^{14}C-ring triazine	0.5 mCi	>99
Deethylatrazine	2-chloro-4-amino-6-isopropylamino-s-triazine	0.25 g	>96
Deisopropylatrazine	2-chloro-4-ethylamino-6-amino-s-triazine	0.25 g	>96
Diaminoatrazine	2-chloro-4-amino-6-amino-s-triazine	0.25 g	>96
Hydroxyatrazine	2-hydroxy-4-ethylamino-6-isopropylamino-s-triazine	0.25 g	>96
Deethylhydroxyatrazine	2-hydroxy-4-amino-6-isopropylamino-s-triazine	0.25 g	>96
Deisopropylhydroxyatrazine	2-hydroxy-4-ethylamino-6-amino-s-triazine	0.25 g	>96
Diaminohydroxyatrazine	2-hydroxy-4-amino-6-amino-s-triazine	0.25 g	>96
Atratone	2-methoxy-4-ethylamino-6-isopropylamino-s-triazine	0.25 g	>96

[a]specific activity: 26.1 mCi/mg

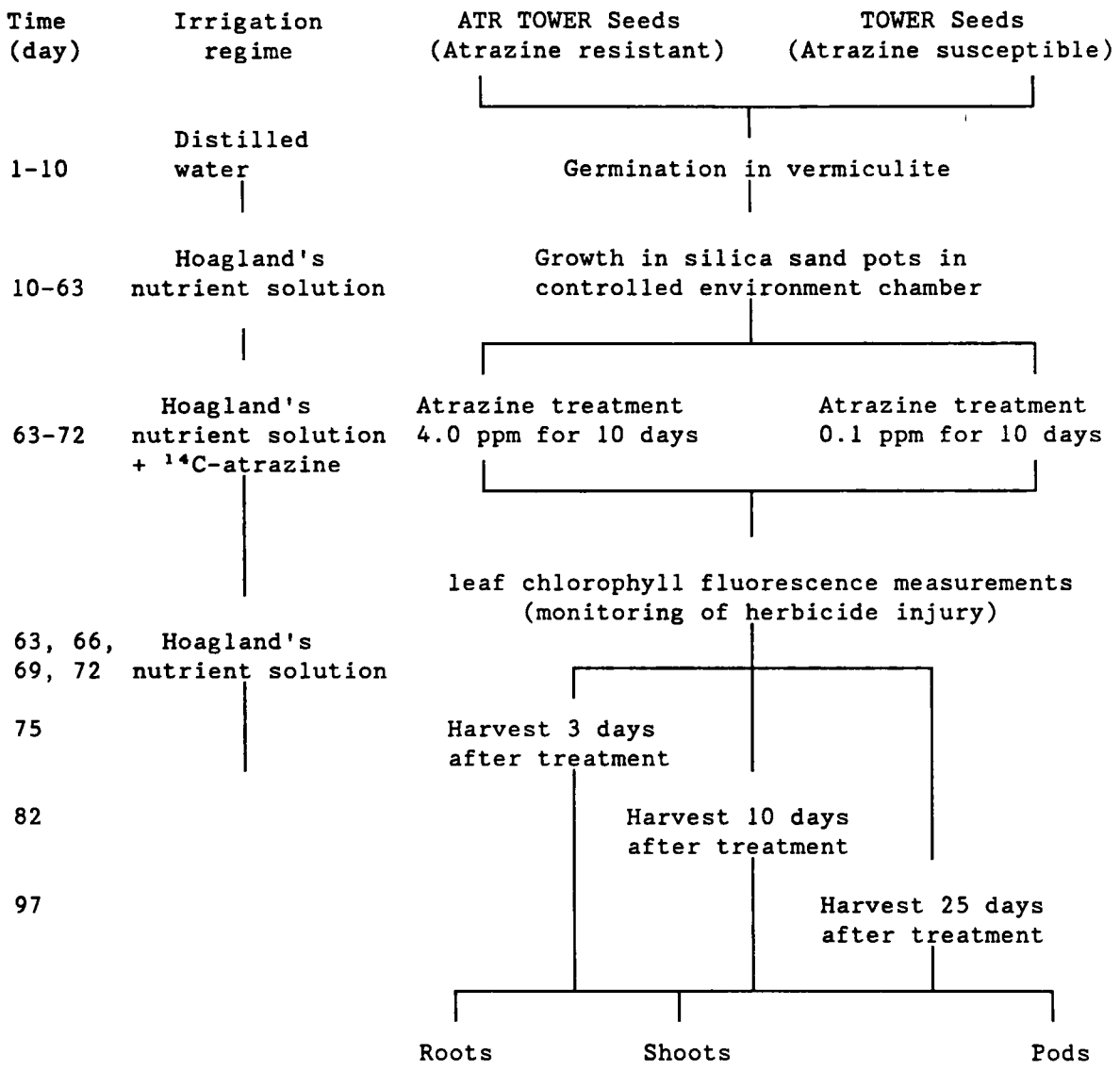


Figure 13: Flow diagram for the growth of canola plants and their treatment with atrazine.

seedling heights varying between 2 and 12 cms. The seedlings were then potted and irrigated with 1/4 strength Hoagland's nutrient solution as described earlier. The increases in nutrient solution concentration to half and full strength took place on weeks 5 to 6 and 8 to 9, respectively.

2.2.2.2. Herbicide treatments

Stock solutions of atrazine in methanol were prepared. On the 9th week following emergence, the plants were treated by irrigating them with nutrient solution containing a known amount of the herbicide stock solution, as described for soybeans.

i - First preliminary treatment:

Initially, 10 plants of each variety were potted. One was used as a control and the others were combined in 3 groups of 3 replicates each. The atrazine stock solution used for this treatment contained 50.125 mg of atrazine in 250 ml of methanol. The plants of the Tower variety were treated with nutrient solution containing 0.25, 0.50 or 1.00 ppm of atrazine. The rate of irrigation was 125 ml per day, per plant, on average, for 6 days. The atrazine concentrations for the Atr Tower plants were 0.5, 1.0 and 2.0 ppm. They received an average of 142 ml of solution per day for 10 days.

ii - Second preliminary treatment

Twelve plants of each variety were grouped in triplicates. One group was not treated and used as control. Tower plants were treated with nutrient solution containing 0.05, 0.10 or 0.15 ppm of atrazine, prepared from a stock solution containing 2.070 mg of the herbicide in 100 ml of

methanol. Each plant received an average of 134 ml/day of nutrient solution, for 10 days. Atr Tower plants were treated at concentrations of 2.0, 4.0 or 8.0 ppm. The stock solution contained 300.26 mg of atrazine in 250 ml. Each plant received an average of 151 ml/day of nutrient solution for 10 days.

iii - Treatment of the canola plants with ^{14}C-atrazine

Initially 16 potted plants of each variety were grown. On the 7th week, the 2 larger and smaller plants were discarded. The remaining Tower and Atr Tower plants were treated with nutrient solution containing, 0.1 and 4.0 ppm of ^{14}C-labeled plus non labeled atrazine, respectively. Two ^{14}C-atrazine stock solutions were prepared for this experiment (Table 9.)

Table 9: Stock solutions of radiolabeled atrazine used for the treatment of the canola plants.

Stock Solution	Variety	Concentration (µg/ml)	Radioactivity (DPM/ml)
A	Tower	88.08	2765.9×10^3
B	Atr Tower	1000.0	331.9×10^3

The plants of the Tower and Atr Tower varieties received an average of 200 and 195 ml of solution per day, respectively, over a 9 day period. This represented an averaged total of 182 µg and 7.05 mg of atrazine for each Tower and Atr Tower plant, respectively.

2.2.2.3 Determination of the phytotoxicity of atrazine to the two canola varieties.

The phytotoxic effect of atrazine on the canola plants was determined by leaf chlorophyll fluorescence measurements as described for soybeans in

section 2.1.2.5. For both preliminary experiments and the ^{14}C-atrazine treatment an initial fluorescence measurement was taken just previous to the first treatment. The plants were monitored for inhibition of photosynthesis every three days thereafter, until the end of the treatment.

2.2.2.4. Harvest of the plant material

After the preliminary treatments, the plants were subirrigated with nutrient solution for up to ten days. For the plants treated with ^{14}C-atrazine, groups of 4 plants of each variety were harvested 3, 10 and 25 days after the end of the treatment. The harvest was carried out in a manner similar to that described for soybeans. Shoots, roots and pods (fruits) were frozen separately at -18°C.

2.2.2.5. Control plants

Ten untreated control plants of each variety were grown separately. They were subirrigated with nutrient solution only and were harvested and frozen on the 11th week after emergence.

2.2.3. Determination of the nature of atrazine residues in canola.

Figure 14 summarizes the methods and techniques used to analyse the extractable and bound residues of atrazine in canola plants.

2.2.3.1. Extraction of the plant material

Due to the long extraction procedure, the four replicates of each sample could not be extracted separately. They were combined at random to obtain duplicate samples just previous to the extraction. Each combined root sample and the combined pod samples harvested ten days after treatment

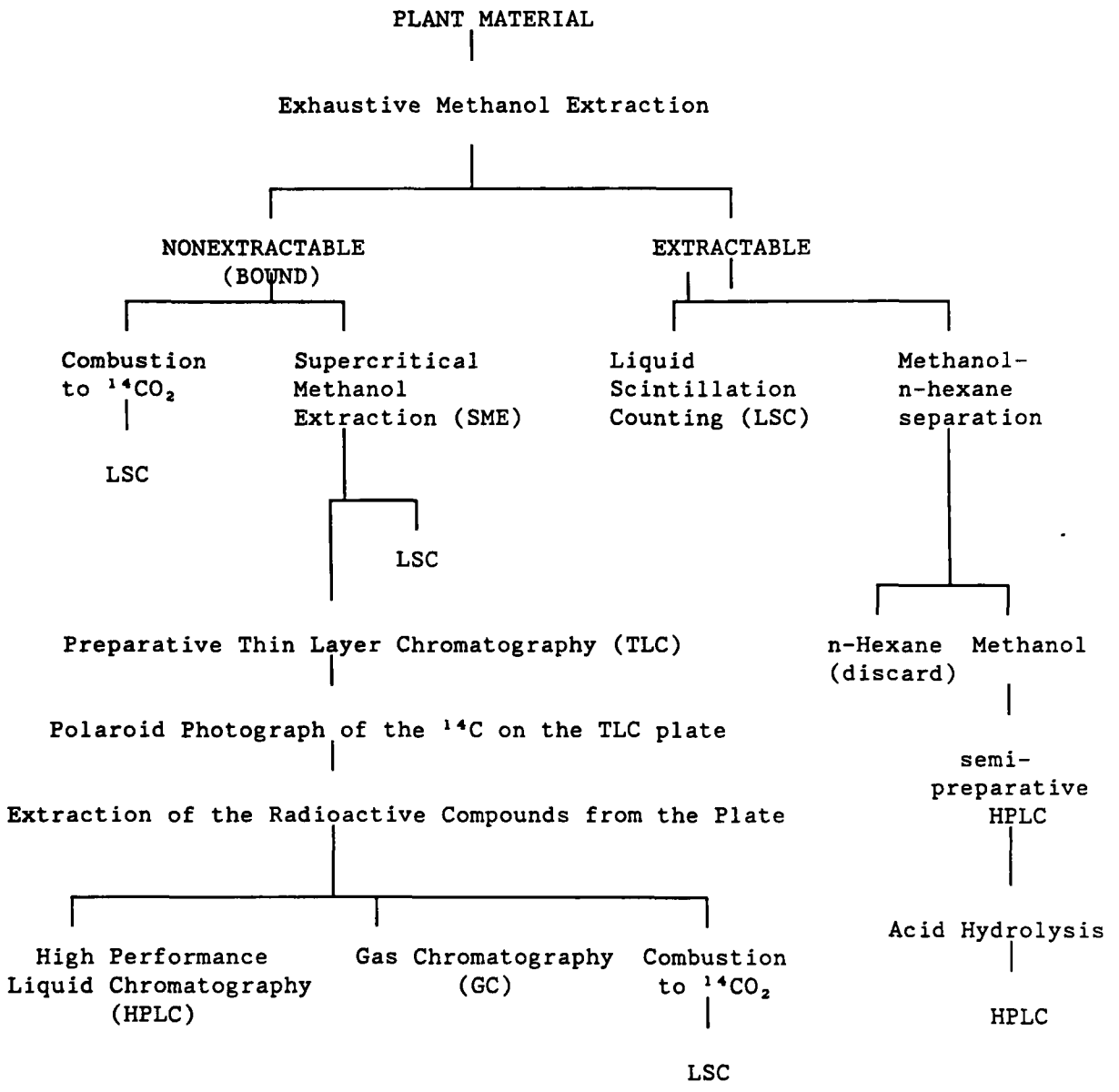


Figure 14. Flow diagram for the analysis of the extractable and bound residues of ^{14}C-atrazine in canola.

were chopped directly into the blender container (Waring Commercial Blendor). The combined shoot samples and the pod samples harvested at 25 days were too large for the blender container. Therefore, they were finely chopped and mixed while kept frozen. Subsamples (30 g) were taken from the homogeneous mixture and were transfered to the blender. The samples and subsamples were extracted with methanol (1:10, w:v), filtered and washed as described for soybeans. This procedure was repeated five times for each sample. Duplicate aliquots (2% of the total volume) were taken for radioassay from each extract of samples harvested at 10 days or from the combined extracts of samples harvested at 3 and 25 days. The combined extracts were then concentrated to about 75 ml on a rotary evaporator and added to 75 ml of n-hexane in a 250 ml separatory funnel for liquid-liquid extraction. After extraction, the methanol fraction was drained and the n-hexane fraction was rinsed twice with 50 ml of methanol. Aliquots taken from the n-hexane fraction of various samples contained no radiolabeled residues. That fraction was discarded. The combined methanol fractions were concentrated on a rotary evaporator, transferred to a 10 ml volumetric flask and adjusted to volume. The residual extracted plant samples containing bound ^{14}C-residues were transferred to tared beakers, allowed to dry and weighed.

2.2.3.2. Analyses of the extracts by gas chromatography.

i - GC Conditions.

The gas chromatograph used was a Varian model 3700 fitted with a TSD. The column was a fused silica capillary 15 m long and 0.324 mm i.d. coated with DB wax liquid phase. The injector and detector temperatures were 190 °C and 280°C respectively. The N_2 carrier gas had a flow rate of

20 ml/minute. The column temperature was programmed from 170°C to 210°C at 5°C/mn and kept at 210°C for 30 mns.

ii - Synthesis of diazomethane

A Wheaton glassware kit for diazomethane synthesis was used. 5.0 g of KOH pellets were placed in the round bottom flask and dissolved with 8 ml of water and 25 ml of methanol. The contents of the flask was stirred continually and maintained at about 45°C in a water bath while a solution of 21.5 g of diazald (Aldrich Co. Inc.) in 200 ml of ethyl ether was added a drop at a time. The diazomethane produced codistilled with ether, was cooled by a water jacketed condenser and collected in an Erlenmeyer flask. The ether solution of diazomethane was used immediately following its synthesis.

iii - Analysis of the methanol extracts

The concentrated methanol solution in the 10 ml flask was analyzed for atrazine and its 2-chloro dealkylated metabolites by direct GC injection and comparison of peak retention times with those of analytical standards as described earlier. The analysis for possible free hydroxylated metabolites of atrazine by GC required methylation of the sample (Khan et al., 1975). A 3.332 ml aliquot of the concentrated methanol extract was pipetted with two Volac precision pipetters in a 15 ml test tube. The solution was then concentrated to about 1 ml under a nitrogen stream. Approximately 13 ml of the ethereal diazomethane solution was added to the tube and mixed. The tube was then sealed and the methylation of the sample was allowed to proceed at room temperature overnight. The solution in the tube was then dried under a nitrogen

stream, redissolved in 3.332 ml of methanol and analyzed by GC. The retention times of the peaks observed on the chromatograms of methylated samples were compared to those of the methoxy derivatives of the four authentic hydroxylated atrazine metabolites. These derivatives were obtained by methylating known amounts of reference metabolite standards in methanol solution following the same procedure described above. In order to determine whether or not conjugated metabolites might be present in the extracts an additional 3.332 ml fraction was pipetted into a round bottom flask and evaporated to dryness. The dry residue was redissolved in an aqueous solution of 1N HCl, the flask was fitted with a water-jacketed condenser and the sample was refluxed for 30 minutes. The solution in the flask was then concentrated on a rotary evaporator, transferred to a test tube, dried under a nitrogen stream and redissolved in about 1 ml of methanol. The sample was methylated and analyzed by GC as described above. Typical chromatograms are shown in Figure 15.

2.2.3.3. Analysis of the methanol extracts of canola plants by high performance liquid chromatography.

The HPLC system used was essentially identical to that described earlier. The chromatograph was equipped with a semi preparative reverse phase column (Whatman Partisil 10 ODS-2, 25 cm long, 9.4 mm i.d.). The detector measured the absorbance at 240 nm. The flow rate of the methanol-water mobile phase was 4.0 ml/minute. The composition of the mobile phase was initially maintained at 50:50 for 5 minutes, then changed to 70:30 (methanol:water) for the next 13 minutes, and finally increased to 100% methanol in order to clean the column. Under these experimental conditions, atrazine had a retention time of 18.5 minutes as determined

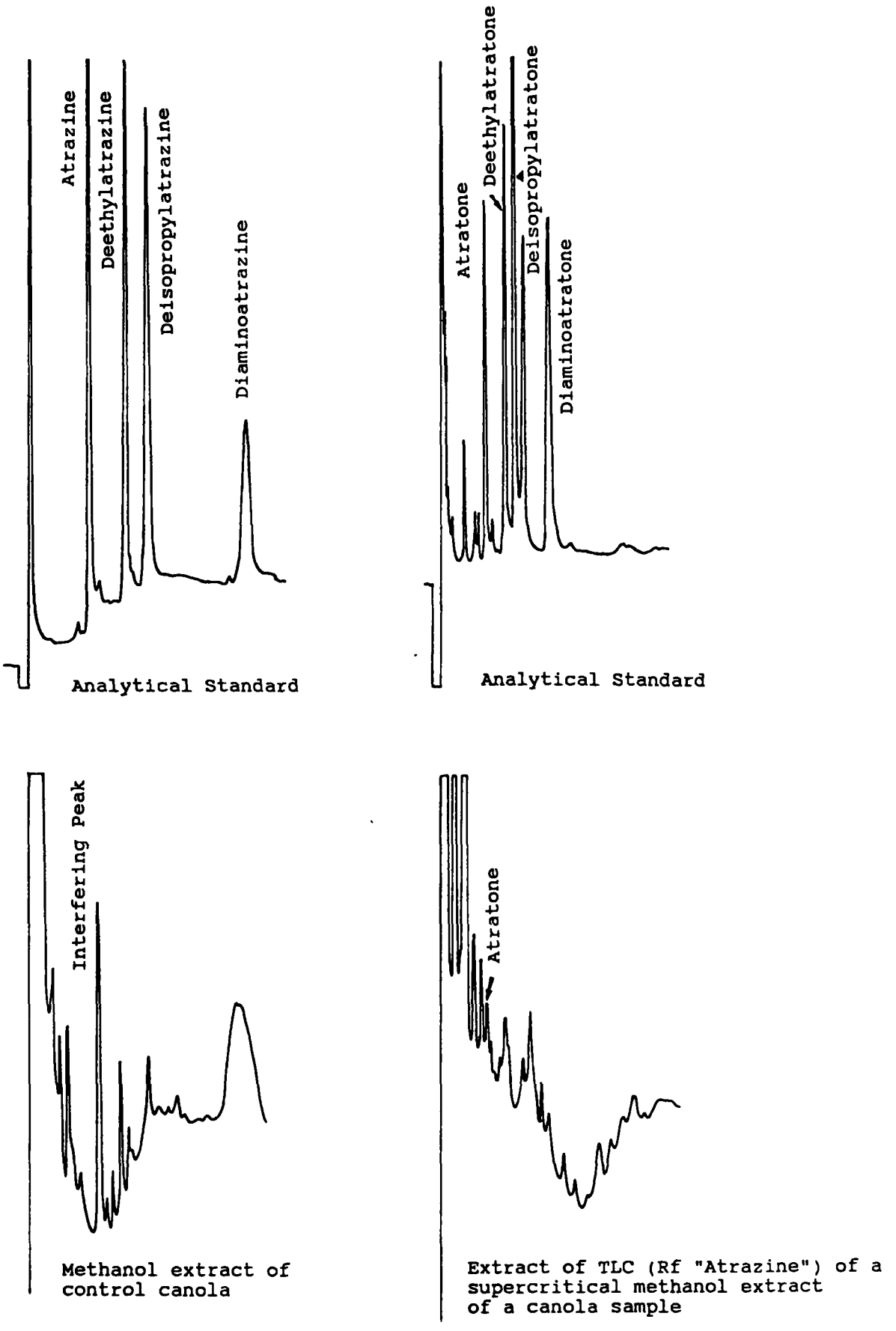


Figure 15: Typical GC chromatograms obtained during the study of atrazine metabolism in canola.

with the UV detector (Figure 17). The methanol extracts of canola shoot samples harvested at the same times were combined. The shoot extracts of Tower plants harvested 3, 10 and 25 days and of Atr Tower plants harvested on the tenth day after the treatment were analyzed by HPLC. During the chromatography of the canola extracts the fractions A and B, containing unknown radiolabeled compounds were collected at the outlet of the radioactivity monitor. Fraction B was hydrolyzed by refluxing in a 1N HCl aqueous solution for 30 minutes as described earlier. The hydrolyzed fraction was then dried, redissolved in methanol and rechromatographed by HPLC. The same procedure was used for fraction A. However, upon rechromatography, the radioactive peak appeared unchanged and was collected again. A second hydrolysis with 3N HCL refluxed for one hour was performed. The fraction was then chromatographed one more time. A few typical chromatograms are shown in Figure 17.

2.2.3.4. Recovery of atrazine from fortified control plant material by high temperature distillation.

The efficiency of the HTD technique for the study of bound residues of atrazine in canola was evaluated by determining the recovery of radiolabeled atrazine from extracted control canola shoots or roots. The plant material (2 g.) was placed in the quartz tube and fortified with 247.3 µg of ^{14}C-labeled and unlabeled atrazine (18670 DPM) as described earlier for metribuzin. The fortified material was then subjected to HTD at 600°C. The distillates recovered in the methanol traps and quartz tube were radioassayed and chromatographed by TLC and HPLC. The carbosorb used in the last trap was radioassayed for $^{14}CO_2$.

2.2.3.5. Supercritical methanol extraction (SME) of the canola samples

The supercritical solvent extraction (SSE) apparatus described earlier (Figure 8) was used in this study.

i - Recovery of pure atrazine from supercritical methanol

The SSE apparatus was cleaned with supercritical methanol (250°C, 3000 PSI) pumped at the rate of 2.0 ml/minute for two hours. The pressure was then reduced to 2200 PSI and the flow rate to 1.5 ml/minute. A mixture of ^{14}C-labeled and unlabeled atrazine in methanol solution (409.3 µg, 91115 DPM) was pipetted into the solvent reservoir and mixed. The 200 ml solution of atrazine thus obtained was allowed to circulate through the apparatus for 100 minutes. The reservoir containing the atrazine solution was then replaced by one containing pure methanol following the same procedure described earlier. After circulating the pure methanol through the apparatus for 45 minutes, the oven was allowed to return to room temperature and the pump was stopped. The methanol solution left in the apparatus was recovered as described earlier and combined with the atrazine solution previously collected. This combined solution as well as the atrazine solution left in the first reservoir and the methanol collected during the second hour of the initial washing were concentrated and analyzed by HPLC. This experiment was performed in duplicate.

ii - Recovery of atrazine from the SME of control plant material.

An extracted control canola shoot or root sample ($\simeq$ 2 g) was placed in the column as described earlier. Pure methanol was pumped through the

apparatus at the rate of 1.5 ml/minute and brought to the supercritical state (250°C and 2200 PSI). ^{14}C-Atrazine (818.5 µg, 182230 DPM) was added to the reservoir. The 200 ml atrazine solution was processed as described earlier and replaced by pure methanol after 100 minutes. The methanol was allowed to return to room temperature and atmospheric pressure 45 minutes later. The total plant extract was collected, concentrated, radioassayed and analyzed by TLC and HPLC.

iii - SME of canola samples containing bound ^{14}C-residues

The extracted, air dried and combined root, shoot or pod samples of Atr Tower and Tower plants harvested ten days after treatment were extracted by SSE. Each sample was accurately weighed (≃ 2 g) and transferred to the column. The extraction was carried out for three hours, in the same conditions described above. The extracts collected were concentrated, radioassayed and analyzed by TLC, HPLC and GC. The plant residue remaining in the column was recovered as described before, dried, weighed and radioassayed.

2.2.3.6. Thin layer chromatography clean up of high temperature distillates and supercritical methanol extracts of plant samples

Preliminary experiments were carried out on Whatman MK6F 200 µm thick silica gel plates. Tables 10 and 11 show the Rf values obtained when atrazine and its metabolites were eluted with different solvent systems.

For the preparative TLC clean up procedure, the type of plate and procedure used were the same as those described for the clean up of soybean HTD distillates or SAE extracts. The solvent system was a

chloroform- methanol mixture (95:5). Under these conditions the Rf values of atrazine, deethyl- and deisopropylatrazine, diaminoatrazine and hydroxyatrazine were 0.82, 0.60, 0.36 and 0.00, respectively.

As described earlier, plates developed with radiolabeled compounds were photographed with the beta-camera. A few typical photographs are shown in Figure 16. The silica gel corresponding to each radioactive bands was scraped and extracted 200 ml of methanol following the procedure described earlier. These methanol extracts were concentrated, radioassayed and analyzed by HPLC and/or GC. The TLC of the supercritical methanol extracts of some canola samples showed an important radioactive band at Rf 0.00. The methanol extract of this band obtained from the TLC of the Tower shoot sample was rechromatographed on the same type of plate, in methanol-acetic acid (8:2). Under these conditions, hydroxyatrazine and the two mono-N-dealkylated hydroxyatrazine metabolites migrated at Rf 0.80.

2.2.3.7. Chromatographic analyses

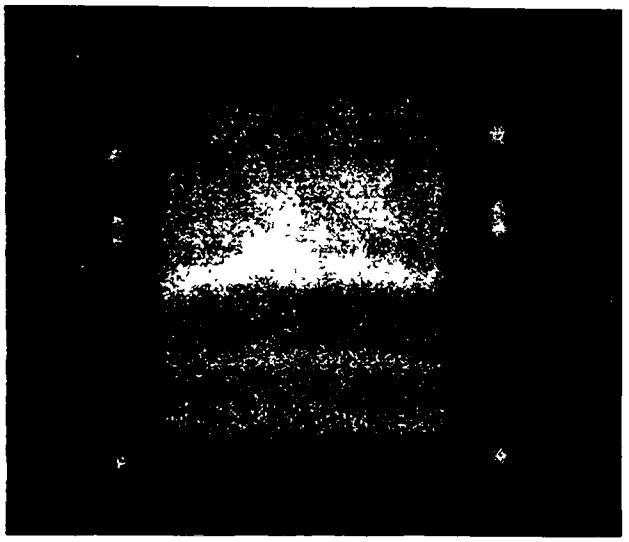
For the gas chromatography, the same apparatus and conditions described in section 2.2.3.2. were used.

The high pressure liquid chromatographic analyses were performed on the same apparatus as described earlier, using an analytical Whatman Partisil 10 ODS-2 column (25 cm long, 4.6 mm i.d.). The flow rate of the liquid phase was 1 ml/minute and its composition was programmed from 50% to 70% methanol in water over ten minutes and maintained at 70% for an additional ten minutes. A few typical chromatrograms are shown in Figure 17.

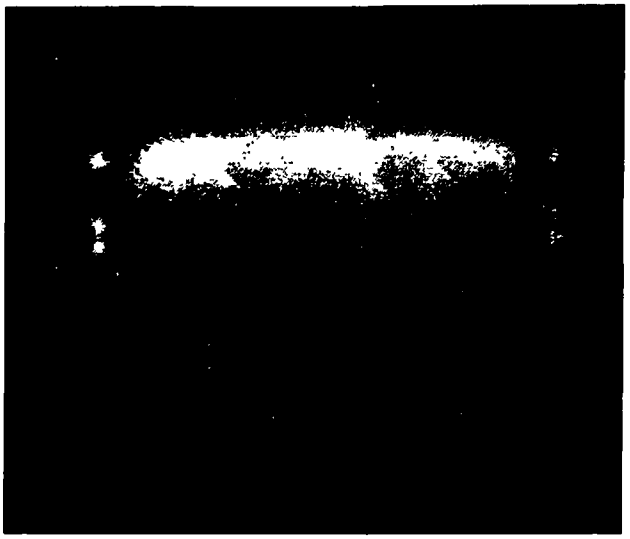
Figure 16: The photographs shown on the opposite page were taken with a Berthold beta camera. They represent the radioactive areas on TLC plates obtained during atrazine recovery studies or analyses of canola shoot samples by supercritical methanol extraction (SME). A radioactive marker was added on the plates at the Rf values of atrazine and some of its metabolites. The Rf values marked were: Rf atrazine: 0.82 (top of the photographs), deethylatrazine: 0.61, deisopropylatrazine: 0.59 and Rf 0, for the recovery of atrazine by high temperature distillation (HTD); Rf atrazine, deethyl - and deisopropyl atrazine for the recovery of atrazine by SME and Rf atrazine, deisopropyl - and diaminoatrazine: 0.36 for the canola shoot sample analyses.

Recovery of atrazine with control plant material

HTD

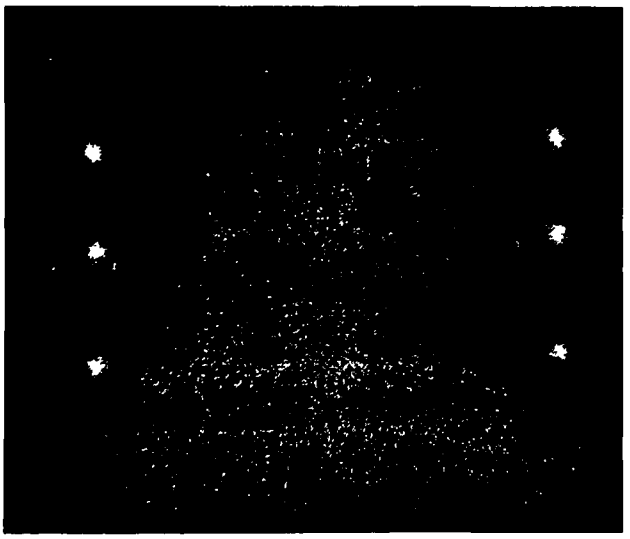


SME



Analysis of shoot samples containing bound ^{14}C-residues by SME

Variety : Atr Tower



Variety : Tower

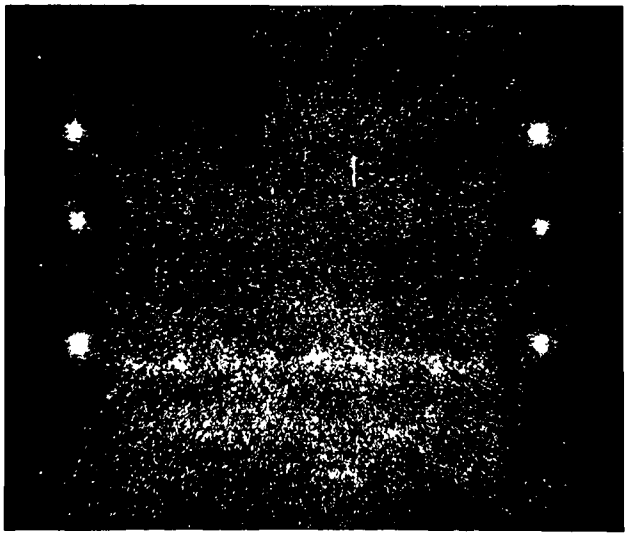


Figure 16: Typical photographs obtained from TLC plates of high temperature distillates and supercritical methanol extracts of canola samples.

Table 10: Selected preliminary TLC experiments with atrazine and its dealkylated metabolites.

Solvent system	Rf values			
	Atrazine	Deethylatr.	Deisopropylatr.	Diaminoatr.
Chloroform-Dioxane (9:1)	0.91	0.48	0.33	0.06
Ethylacetate	0.97	0.89	0.85	0.63T[a]
Chloroform-Methanol (9:1)	0.97	0.87	0.80	0.49 T
Chloroform-Methanol (95:5)	0.94	0.62	0.54	0.26

[a]compound moderately trailing

Table 11: Selected preliminary TLC experiments with hydroxyatrazine and its dealkylated derivatives.

Solvent system	Rf values			
	Hydroxy atrazine	Deethyl OHatr.	Deisopropyl OHatr.	Diamino OHatr.
Acetone-Acetic Acid (9:1)	0.61T[a]	0.38T	0.38T	0.00
Methanol-Acetic Acid (9:1)	0.84T	0.78T	0.78T	0.00
Methanol-Acetic Acid (8:2)	0.85	0.82	0.82	0.00

[a]compound strongly trailing

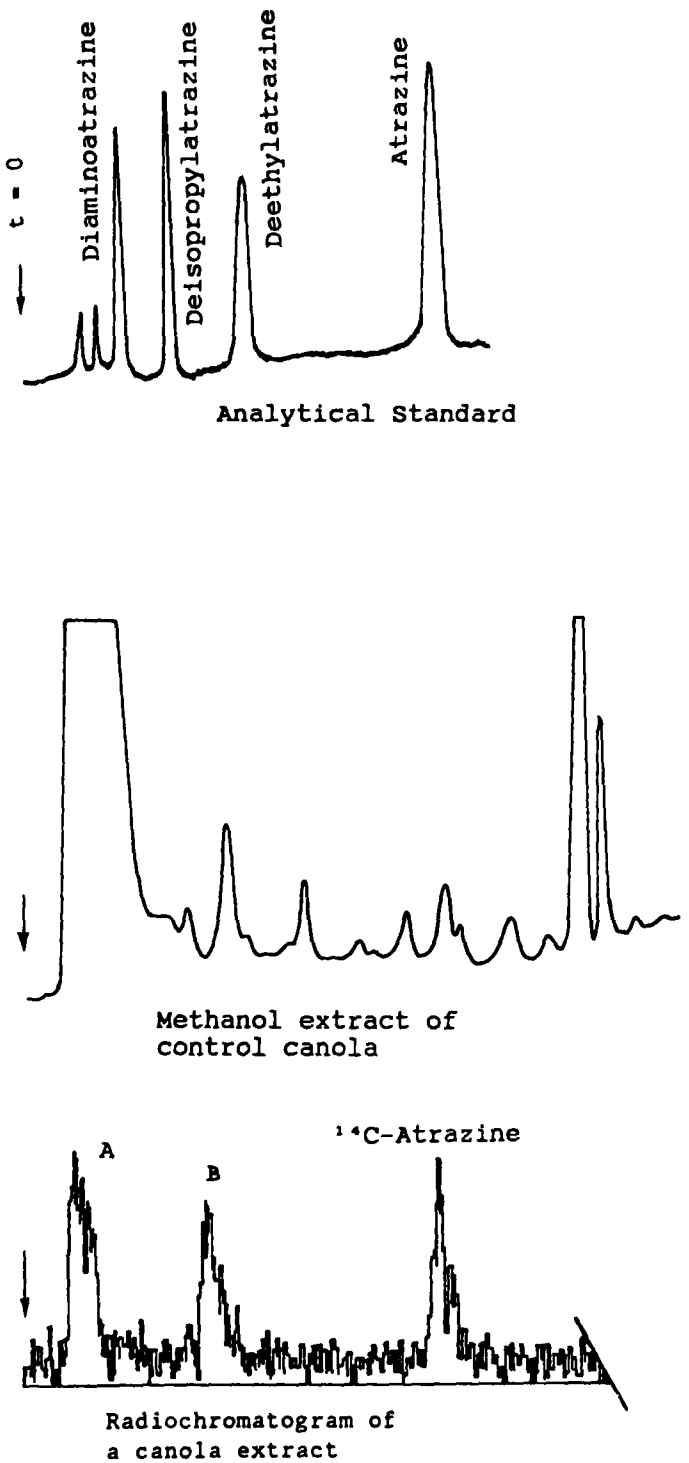


Figure 17: Typical HPLC chromatograms obtained during the study of atrazine metabolism in canola.

3. RESULTS AND DISCUSSION

3.1 Bound residues of metribuzin in soybeans

3.1.1. Plant treatments

3.1.1.1. Preliminary experiments

The determination of the chemical nature of bound pesticide residues in plants is often difficult. The low levels of bound residues present and the harshness of the techniques used to release them often hamper their characterization.

In this study the plants were grown hydroponically, the herbicide was administered in the nutrient solution and absorbed via the root system. In this manner, maximum absorption of the herbicide by the plants occurred without interferences from soil components. The herbicide treatment was started at the beginning of the flowering stage in order to investigate the possible translocation of metribuzin to the seeds.

i - first preliminary treatment

The plants were treated with various concentrations of the herbicide for five consecutive days (Table 12). After the treatment the plants were examined daily for signs of herbicide injury indicated by the chlorotic regions on the leaves. The data in Table 12 show that the upper limits of tolerance of the Maple Amber plants was between 0.10 and 0.25 ppm while the tolerance limit of the Maple Arrow plants was between 0.5 and 1.0 ppm. These results permitted the selection of the range of concentrations tested for the two varieties in the second preliminary experiment as shown in Table 13.

Table 12. Phytotoxic effect of metribuzin on soybeans as observed after the first preliminary treatment.

Soybean variety	concentration of metribuzin in the nutrient solution (ppm)	extent of chlorosis, one week after treatement (% of foliage surface)
Maple Amber (suceptible)	0.10	0
	0.25	30-40
	0.50	70-80
	1.00	80-90
Maple Arrow (resistant)	0.5	0
	1.0	70-80
	2.5	80-90
	5.0	80-90

ii - Second preliminary treatment

The plants were treated with various concentrations of the herbicide for four consecutive days. One day after the end of the treatment, leaf chlorophyll fluorescence measurements were taken from each plant.

Various researchers have suggested different mathematical equations for the quantitation of the phytotoxic effect of photosystem II inhibitors. These equations are based on the values of specific points on the fluorescence induction curves. Most of these methods require the use of a recorder in conjunction with the fluorometer. However, according to the manufacturer (Richard Brancker Co.) the values of three of these points displayed by their instruments (initial : I; Peak : P and Final: F) are sufficient to obtain an acceptable approximation of the phytotoxic effect. As shown in Figure 5 a higher F value indicates a stronger effect.

It has been suggested (S. Nelson, personal communication) that equation (1) provides a good assessment of the extent of inhibition of the photosynthesis in a plant while also allowing plant-to-plant comparisons.

$$(1) \quad Ri = \frac{P-F}{P-I} \times 100$$

Ri = percentage of recovery of the initial fluorescence level after 200 seconds.

Table 13. Phytotoxic effect of metribuzin on soybean as measured after the second preliminary treatment.

soybean variety	metribuzin concentration (ppm)	Average of Ri values (% recovery)	extent of chlorosis, one week after treatment (% of foliage surface)
Maple Amber(S)	0 (control)	81.8 (6.0)[a],4[b]	0
	0.1	78.1 (3.4),2	0
	0.15	78.6 (4.2),2	0
	0.20	51.3 (8.2),2	10-20
Maple Arrow (R)	0 (control)	90.6 (3.3),4	0
	0.3	92.4 (2.1),2	0
	0.5	87.7 (5.7),2	0
	0.7	51.7 (9.7),2	20-30

[a]standard deviation [b]number of replicates

The data shown in Table 13 demonstrated that Maple Amber and Maple Arrow plants did not show phytotoxic effects at metribuzin concentrations of up to 0.15 and 0.5 ppm, respectively. Therefore, in subsequent experiments these concentrations were used for the treatment of soybean plants with ^{14}C-metribuzin.

3.1.1.2. Treatment of soybean plants with radiolabeled metribuzin

Figure 4 outlines the methodology used in this experiment. The average Ri value for the eleven Maple Amber plants selected for this experiment measured two days before treatment was 84.9 ± 4.7% (Ri ± 95% confidence interval). After three days of treatment, the average Ri value

for four treated plants chosen at random was 75.1 ± 11.6%. This value was not significantly different from that of the healthy plants as determined by the Student's t-test. The treatment was applied for one additional day after which leaf chlorophyll fluorescence measurements were taken from each Maple Amber plant. The average Ri value of the nine treated and two untreated control plants were 43.2 ± 9.8% and 84.7%, respectively. These data show that the herbicide treatment caused a partial inhibition of photosynthesis in the Maple Amber plants. Therefore the treatment was discontinued and the plants were irrigated with nutrient solution without the herbicide.

The average Ri value of the eleven Maple Arrow plants measured two days before treatment was 90.5 ± 3.0%. After three days of treatment the mean Ri of the nine treated plants was 23.4 ± 9.2% while the two control plants remained at 85.2%. The phytotoxic effect of metribuzin was also observed on the Maple Arrow plants and their treatment was discontinued after the third day.

For both varieties, chlorotic areas appeared on some of the leaves of each plant. However, the plants continued to grow and produce beans. During this experiment, the plants absorbed nutrient solution at a faster rate than during the two earlier preliminary experiments as described above. The rate of absorption of metribuzin in plants is shown to be directly proportional to their rate of transpiration (Hatzios and Penner, 1988). Thus, it appears that the plants treated with radiolabeled metribuzin suffered herbicide injury at treatment concentrations that were shown to be safe during the preliminary studies. A different growth chamber was used for the ^{14}C-labeled and non-labeled herbicide treatments. The light intensity, humidity, temperature and photoperiod were the same in

both chambers. However, a difference in the ventilation may have induced a higher rate of transpiration in the ^{14}C-metribuzin-treated plants, thus increasing metribuzin absorption.

3.1.2. Distribution of the ^{14}C label in the soybean plants

3.1.2.1. Recovery of the ^{14}C applied in the plants and growth media.

The proportion of the total ^{14}C applied recovered in each plant was calculated by adding the DPM values obtained from the radioassay of the bound and extractable fractions of the root, shoot and bean samples (Table 14). The level of ^{14}C in the plant remained relatively constant between 3 and 28 days after treatment. This is consistent with the fact that metribuzin is readily absorbed by plants (Hatzios and Penner, 1988). However due to the daily fluctuation in the level of nutrient solution in the saucers, deposits of nutrient salts containing some ^{14}C-metribuzin appeared on the walls of the saucers and pots and on the silica sand. This ^{14}C-metribuzin was partially recovered in the methanol and distilled water washings of the saucers, pots and sand (Table 14). The proportion of radioactivity in the washings decreased with time and this accounted for the decrease of the total recovery of radioactivity between 3 and 28 days after treatment.

No evidence was found in the literature of ring cleavage of the metribuzin molecule during its metabolism by plants and its subsequent oxidation to CO_2. Therefore no attempt was made to measure any possible release of $^{14}CO_2$ by the treated plants.

Table 14: Recovery of ^{14}C in soybean plants and their growth medium (% of the total ^{14}C applied ± 95% confidence interval, average of 3 replicates).

soybean variety	time of harvest	total ^{14}C in the plant	^{14}C extracted from the medium	total ^{14}C recovered
Maple Amber (S)	3 days	80.3 ± 31.8	10.8 ± 5.5	91.1 ± 27.3
	10 days	77.8 ± 44.1	7.7 ± 15.2	85.5 ± 29.1
	28 days	78.0 ± 2.5	2.3 ± 1.7	80.3 ± 4.8
Maple Arrow (R)	3 days	63.3 ± 6.2	29.4 ± 15.6	97.7 ± 9.6
	10 days	74.2 ± 18.2	13.3 ± 10.8	87.5 ± 7.5
	28 days	71.3 ± 8.9	7.3 ± 1.8	78.6 ± 7.2

[a] includes washings from saucers, pots and silica sand

3.1.2.2. Efficiency of the extraction procedure

The shoot samples of the Maple Arrow plants harvested three days after treatment were successively extracted with acetone, chloroform and then five times with methanol. The radioassay of aliquots of each extract showed that more than 96% of the extractable ^{14}C was present in the first three fractions. Therefore the extraction procedure used for the remaining samples as described earlier (Figure 6) effectively removed the extractable residues of metribuzin.

3.1.2.3. Distribution of the ^{14}C label in soybean plants treated with ^{14}C-metribuzin

The roots of Maple Arrow plants retained more ^{14}C-residues (extractable and bound) than those of the Maple Amber variety (Table 15). Furthermore, in both varieties, the levels of bound ^{14}C-residues was two to four times higher than the levels of extractable ^{14}C-residues. These observations indicate the possible role of the root system in the tolerance of the Maple Arrow variety to metribuzin. The accumulation of metribuzin and/or metabolites in the roots, especially in a bound form could

effectively prevent them from being translocated to the photosynthetic tissues of the leaves where they would inhibit the photosynthesis. However it was also observed that the total DPM levels of root bound ^{14}C-residues of both varieties decreased between 10 and 28 days after treatment. This would seem to suggest that some ^{14}C-residues were naturally released from the root cell walls during this period. Since no parallel increase in the extractable residues in the roots was observed, the released ^{14}C-residues may have been translocated upward to the shoots.

The levels of bound ^{14}C-residues in the shoots of both varieties increased with time while the levels of extractable ^{14}C-residues decreased. However, it should be noted that during the early stages of the experiment the shoots of the susceptible Maple Amber plants contained a higher proportion of extractable residues while the shoots of the resistant Maple Arrow plants contained relatively more bound residues.

In view of the foregoing observations, it appears that the formation of bound residues of metribuzin in a soybean variety may be related to its tolerance of the herbicide. However, it is not clear how the formation of bound residues contributes to the resistance mechanism. Theoretically, when the main resistance mechanism is a phase I and/or II detoxication reaction, the binding of the metabolite produced to the cell wall matrix would not directly influence the resistance of a variety. For example, if the herbicide is detoxified by conjugation with a sugar molecule, the bound residues may originate from the incorporation of the conjugate into cell wall polysaccharides (Langebartels and Harms, 1986). A higher proportion of bound residues would be formed in a resistant variety having a fast rate of conjugation. In this case, bound residue formation would only be a consequence of the resistance mechanism. However if a plant

Table 15: Distribution of extractable and bound ^{14}C-residues in roots, shoots and beans of ^{14}C-metribuzin-treated soybean plants (% of total ^{14}C in the plant, average of 3 replicates ± 95% confidence interval)

Plant Sample	^{14}C-residues	Harvest time (days after treatment)					
		3		10		28	
		Maple Arrow (R)	Maple Amber (S)	Maple Arrow	Maple Amber	Maple Arrow	Maple Amber
Shoots	Extractable	50.4 ± 1.8	65.5 ± 2.8	35.7 ± 2.7	51.5 ± 6.7	35.0 ± 2.9	43.6 ± 0.8
	Bound	29.0 ± 6.3	24.4 ± 4.5	34.7 ± 8.4	34.7 ± 8.2	39.7 ± 4.1	42.6 ± 0.9
Roots	Extractable	6.8 ± 3.1	2.7 ± 0.6	7.2 ± 1.8	2.0 ± 1.1	7.1 ± 2.4	1.3 ± 0.6
	Bound	13.8 ± 5.3	7.4 ± 1.2	21.0 ± 5.1	9.3 ± 3.9	16.1 ± 1.3	7.4 ± 1.7
Beans	Extractable	Included with shoots		0.4 ± 0.2	1.3 ± 1.6	0.6 ± 0.3	3.2 ± 0.3
	Bound			1.0 ± 0.6	1.3 ± 1.2	1.5 ± 0.4	1.8 ± 0.1

variety inactivates a herbicide by directly incorporating the parent compound in lignin (Stratton and Wheeler, 1986; Sandermann et al. 1983), the herbicide would be prevented from reaching its sites of action in the chloroplasts. In this case, the rate and extent of bound residue build-up would have a direct impact on the tolerance of that variety to the herbicide.

3.1.2.4. Distribution of the ^{14}C-residues in beans

In order to investigate the possible presence of residues in the edible seeds, the beans of the plants harvested ten days after treatment were separated into seeds and shells (pericarps) before extraction. In both varieties, the shells contained more than 96% of the ^{14}C in the beans and the radioactivity in the seeds represented less than 0.1% of the total ^{14}C in the plants (Table 16). Therefore, it appears that soybean plants do not translocate a significant portion of the ^{14}C-residues to their seeds.

Table 16: ^{14}C distribution in the beans of ^{14}C-metribuzin-treated soybeans (average % of the total ^{14}C in 3 replicates ± 95% confidence interval)

		Maple Amber (S)	Maple Arrow (R)
Shells	Extractable	47.2 ± 16.7	28.0 ± 4.4
	Bound	49.5 ± 19.1	71.2 ± 4.2
Seeds	Extractable	2.6 ± 2.9	0.0
	Bound	0.6 ± 0.6	0.7 ± 0.4

3.1.2.5. Fractionation of the extractable residues

The metabolic pathway of metribuzin in susceptible and resistant soybean varieties has been reported earlier (Smith and Wilkinson, 1974; Mangeot et al., 1979; Fedtke and Schmidt, 1983; Falb and Smith, 1984; Abusteit et al., 1985; Frear et al., 1985). In this study, the extracts of the plants harvested 10 days after treatment were further fractionated into polar and non-polar residues by carbon tetrachloride-water partition. The separation was carried out in order to determine whether the Maple Amber and Maple Arrow varieties metabolized the herbicide in the same manner as that described in the literature. Table 17 shows the distribution in shoots and roots of ^{14}C-residues in the non polar and polar fractions which contained the parent herbicide and the conjugates, respectively. The results of similar studies reported in the literature are also presented for comparison purposes.

Table 17. Polar and non-polar extractable residues in the shoots and roots of suceptible (S) and resistant (R) varieties of soybean treated with ^{14}C-metribuzin. (% of the total ^{14}C in the shoot or the root of the soybean variety).

Plant Sample	Extractable Fraction	Soybean varieties					
		Essex[a] (R)	Cocker 102[a] (S)	Cocker 338[b] (R)	Semmes[b] (S)	Maple[c] Arrow (R)	Maple Amber (S)
Shoots	non-polar	50	90	30	70	58.6	80.1
	polar	50	10	70	30	41.4	19.9
Roots	non-polar	60	80	30	60	17.3	42.3
	polar	40	20	70	40	82.7	57.7

[a]from Mangeot et al. (1979), the values were rounded off
[b]from Falb and Smith (1984), the values were rounded off
[c]present study

Although the experimental conditions in the three studies were different, it is apparent from the data reported in Table 17 that all the resistant varieties produce more polar, non phytotoxic residues in the shoots and roots than the susceptible varieties. Thus Maple Arrow and Maple Amber were shown to metabolize metribuzin in a manner similar to that described for other varieties.

3.1.3. Analysis of bound ^{14}C-residues of metribuzin in soybean

The method used to analyze the bound ^{14}C-residues present in extracted soybean plants treated with ^{14}C-metribuzin is shown in Figure 6.

3.1.3.1. Development of a gas chromatographic method for the analysis of metribuzin and its metabolites

The GC procedures evaluated are described in Table 7. The liquid phases XE-60, OV-210 and OV-225 were found to be unsuitable since the retention times (Rt) of two or more of the four reference standards of metribuzin, DA, DK or DADK could not be determined due to the absence of an identifiable peak on the chromatograms. Glass columns filled with a packing coated with Reoplex liquid phase as recommended in the "Method for residue analysis of Sencor in soybeans" (Chemagro Ltd., 1974) were also tested. However, with this liquid phase, metribuzin, DK and DADK eluted at relatively close retention times resulting in the poor resolution of the peaks. Under the experimental conditions used, metribuzin, DK and DADK eluted at 10.5, 11.8 and 8.7 minutes, respectively for a 5% Reoplex packing and 6.0, 7.2 and 5.3 minutes, respectively, for a 1% Reoplex packing. Furthermore the column had to be operated at 190°C which is close to its maximum operating temperature (200 °C). Under these conditions, the liquid phase degraded rapidly, requiring frequent replacements.

The SE-30 capillary column provided a good resolution of metribuzin, DA, DK and DADK (Rt = 7.0, 10.5, 5.0 and 3.7 minutes, respectively) although the peaks tended to trail. The SE-30 liquid phase is stable at up to 350°C which allowed the programming of high temperatures (280°C) particularly when samples containing high concentrations of contaminants were injected. Therefore, all subsequent GC analyses were performed on the SE-30 capillary column.

The detector conditions used, including H_2 flow rate and bead current were found to produce the highest sensitivity to metribuzin.

3.1.3.2. Development of a high pressure liquid chromatographic method for the analysis of metribuzin and its metabolites.

The HPLC conditions used are described earlier (section 2.1.3.9.). The counting efficiency of the radioactivity monitor was calculated by injecting ^{14}C-labeled metribuzin alone or with control soybean high temperature distillates. The number of counts recorded for the metribuzin peak was corrected for background activity and represented an average of 34% of the DPM level of the metribuzin injected. The presence of the control distillates did not cause any significant quenching. The minimum detectable activity for a peak was 300-400 DPM. The average background radioactivity was 58 CPM. However the integration of the background levels of a large number of intervals of one minute duration each showed that their levels of activity were scattered between about 45 to 75 counts. One minute was the time required to integrate a metribuzin peak of less than 5000 DPM activity. Therefore, it is clear that for samples containing relatively low levels of ^{14}C-labeled compounds, using the radioactivity monitor to quantitate these compounds could result in large errors caused

by variations in the background activity. Consequently, in this study the radioactivity monitor was used only for the identification of the ^{14}C labeled residues while accurate quantitation was obtained by LSC.

3.1.3.3. Development of a clean-up procedure for high temperature distillates of soybean samples.

The HTD and SAE of soybean samples containing bound ^{14}C-residues released large amounts of dark coloured coextractive material from the plant matrix along with the ^{14}C-residues. The GC and HPLC analysis of these residues could not be performed without first removing the interfering compounds by a suitable clean-up procedure.

i - Liquid - liquid extraction and column chromatography clean-up procedures

The plant distillates obtained by HTD of control soybean material were soluble in methanol, acetone, ethylacetate and chloroform. However, they were only marginally soluble in water, benzene and n-hexane. This range of solubility is similar to that of metribuzin and its metabolites. This characteristic prevented the use of liquid-liquid extraction procedures for the clean-up of high temperature distillates and supercritical acetone extracts. No combination of two non-miscible solvents could be found to separate metribuzin and its metabolites from the natural distillates. The same characteristic also interfered with column clean-up procedures since a solvent system capable of dissolving the natural distillates as well as the herbicide and its metabolites would elute them in the same fractions. The results of column chromatrography clean-up experiments are shown in Table 18.

Table 18. Recovery of pure analytical standards of metribuzin and its metabolites by column chromatography

Experiment number[a]	Solid Phase	Eluent	Recovery (% of initial amount)			
			metribuzin	DA	DK	DADK
1	acidic alumina	chloroform-methanol[b]	85	8	3	4
2	florisil	benzene-acetonitrile(99:1)	51	NDC	ND	ND
3	florisil	benzene-acetonitrile(90:10)	67	15	2	6
4	florisil	chloroform	96	64	76	5
5	silica gel	chloroform-methanol[b]	100	80	97	70
6	silica gel	benzene-acetonitrile(90:10)	100	104	63	70

[a]See Table 6 for experimental details
[b]chloroform-methanol in the following proportions: 100:0, 67:33, 33:67, 0:100, successively
[c]not determined

The experiments #4, 5 and 6 were repeated with control distillates fortified with metribuzin and metabolites standards. The first two of these experiments did not result in an appreciable clean up of the samples. Experiment #6 appeared to be a somewhat more efficient clean up procedure, nevertheless the eluates were not clean enough to allow direct GC or HPLC analysis.

In another experiment several methanolic solutions of control distillates and pure analytical standards of metribuzin and metabolites were added to small amounts of silica gel, allowed to dry, transferred to the top of silica gel columns and eluted (see section 2.1.3.6. for experimental details). This allowed the use of solvent systems in which the plant distillates may not be readily soluble. Elution of the silica gel columns with chloroform did not result in a significant clean up as chloroform readily removed the coextractive materials in the distillates from the top portion of the column. The solvent systems benzene-

acetronitrile (90:10) and benzene-chloroform (50:50) appeared to effect a better clean up of the samples. However the GC and HPLC analysis of the eluates showed that the recoveries of the pure standards of metribuzin and metabolites were lower than 50%. The presence of interferring peaks from the coextractive materials observed on both GC and HPLC chromotograms prevented more accurate calculations of the recoveries. Higher recoveries (>60%) as well as efficient clean up were achieved with benzene-acetone mixtures: 80:20, 65:35 and 50:50, successively. However these preliminary observations were not reproducible in two additional experiments. In both cases only small proportions of the standards (<10%) and the control distillates could be eluted from the column with increasing proportions of acetone in benzene (up to 100% acetone). It was postulated that a slight difference in the drying of the small silica gel sample containing the control distillates and the metribuzin and metabolites standards may have resulted in their complete dessication and irreversible binding to the silica gel. This problem might have been avoided by placing the solution of fortified control distillates in celite instead of silica gel and experimenting with more thoroughly calibrated drying procedures.

It is apparent that the column chromatography procedures evaluated in this study were not suitable for the clean-up of high temperature distillates and supercritical acetone extracts of soybean samples. Consequently the thin layer chromatography (TLC) clean-up procedure described below was developed and no further column clean-up methods were attempted.

ii - Thin layer chromatography clean-up

The TLC method described in section 2.1.3.6. was evaluated for the

clean-up of high temperature distillates and supercritical acetone extracts. The recovery of pure analytical standards of metribuzin, DA, DK and DADK from the TLC plate was determined as described earlier by scraping from the glass plate the silica gel corresponding to the Rf values of the four compounds, eluting each silica gel fraction with acetone and analyzing the eluates by GC. The average recoveries obtained were 86%, 62%, 74% and 99% for metribuzin, DA, DK and DADK, respectively. When the TLC was performed on control distillates fortified with relatively large amounts of ^{14}C-metribuzin and unlabeled DA, DK and DADK the recoveries were 87%, 31%, 10% and 63% respectively, as determined by GC. These values are corrected for interfering background peaks which were present in each eluate. The recovery of metribuzin calculated by LSC was 85%. This TLC procedure removed substantial amounts of the coextractive materials, thus reducing their interference with GC and HPLC (with UV detector) analyses. However, the background peaks remained large enough to overlap and completely mask metribuzin and its metabolites when present in concentrations smaller than about 10 ppm. This effectively prevented the use of GC as well as the UV detector of HPLC for the analysis of the high temperature distillates and supercritical acetone extracts of soybean samples containing bound ^{14}C-residues. However the TLC method had the advantage of providing a visualization of the radioactive bands on the plates by photography with the beta camera. This allowed a comparison of the Rf values of the ^{14}C-labeled compounds released by HTD or SAE of the soybean samples with those of pure metribuzin, DA, DK and DADK, thus providing additional information on those radiolabeled compounds for their analysis.

3.1.3.4. Recovery of metribuzin and its metabolites by HTD

i - Recovery of pure metribuzin and metabolites by HTD

The recovery of pure analytical standards of metribuzin and its metabolites was determined by distilling the pure compounds dried on the surface of a ceramic boat and analyzing the distillates by GC and LSC. The results of this experiment are shown in Table 19.

Table 19. Recovery of pure standards of metribuzin and metabolites by HTD

original compound	compound recovered	distillation of individual compounds (% of the applied)	distillation of a mixture of the four compounds (% of the applied)
Metribuzin	Metribuzin	81.8 (5.1)[a] , 4[b]	71.2 (9.1) , 2
	$^{14}CO_2$	2.3 (0.8) , 2	
	DA	9.2 ___ , 1	
DA	DA	103.7 ___ , 1	143.7 (13.2) , 2
DK	DK	43.1 ___ , 1	39.5 (1.3) , 2
DADK	DADK	83.3 ___ , 1	110.3 (13.1) , 2

[a]: standard deviation
[b]: number of replicates

Approximately 75% of pure metribuzin was recovered intact by HTD. In the experiments where ^{14}C-metribuzin was used, a small amount of $^{14}CO_2$ originating from the thermal degradation of the parent herbicide was also observed. Less than 1% of the initial radioactivity remained in the ceramic boat. The conversion of metribuzin to DA occurred in one out of the four experiments with metribuzin alone. This type of conversion might explain the recovery of substantially more than 100% of DA when the four compounds were distilled together. The recovery of DK was low (about 40%). However, it appears that the deamination of DK to produce DADK was not a major pathway in the thermal decomposition of DK. DA was very stable, with a recovery of about 100%. The thermal stability of DADK was also high (80-100%).

ii - HTD recovery study using extracted control plant material fortified with ^{14}C-metribuzin or non-labeled metabolite standards

The recovery of metribuzin and its metabolites by HTD was also determined by distilling various samples of control plant material fortified with a pure analytical standard of either of the four compounds. When the recovery of ^{14}C-metribuzin was thus investigated, it was observed that an average of 10.9 ± 6.5% (average ± 95% confidence interval) of the ^{14}C was recovered as $^{14}CO_2$ while 84.9 ± 8.5% was recovered as methanol soluble material from the quartz tube and in the methanol traps. The methanol soluble material was chromatographed by TLC and the plate was photographed with the beta camera (Figure 10). The radiolabeled compounds were extracted from the silica gel, radioassayed and finally analyzed by GC and HPLC. The results are shown in Table 20. It will be noted that about two thirds of the radioactivity applied to the TLC plate was recovered in the three silica gel extracts. A combustion of the extracted silica gel fractions from one of the three replicate experiments in the sample oxidizer showed that no radioactivity remained in the silica gel. Therefore, the extraction method was effective in removing all ^{14}C labeled compounds. Less than one third of the metribuzin used to fortify the plant material was recovered intact. This was confirmed by GC and by HPLC using both UV detector and radioactivity monitor. However these techniques did not allow an accurate quantitation of metribuzin due to the interference of background peaks with GC and HPLC/UV and the precision of the ^{14}C monitor.

A band of radioactive material migrating at Rf 0.65 suggested the presence of DA. However only traces of DA were found in the silica gel

Table 20: Recovery of metribuzin by HTD using control plant material fortified with ^{14}C-metribuzin

Radioactive band on TLC (Rf value)	^{14}C extracted from silica gel (% of the total ^{14}C[a])	compounds observed	
		GC+HPLC/UV	HPLC/^{14}C
0.88	28.0 ± 6.7	Metribuzin (about 50µg)	Metribuzin
0.65	12.4 ± 4.5	-	DA + unknown compounds
0.29	14.3 ± 3.2	-	DADK

[a]: 95% confidence interval

extracts by HPLC with ^{14}C monitor. Other unknown compounds were also observed in the extract. Another band of ^{14}C compounds migrated at Rf 0.29 which corresponds to DK. The GC and HPLC analysis using an UV detector showed peaks at the retention times of DK and DADK that were not larger than the interferences measured at the same times on the control chromatograms. However the analysis of the silica gel extracts by HPLC with the radioactivity monitor clearly showed that only ^{14}C-DADK was present. The recoveries of DA, DK and DADK by HTD with control plant material could only be approximately determined by GC. Due to the unavailability of radiolabeled metabolites it was not possible to use the HPLC with the radioactivity monitor. The recovery of intact DA under these experimental conditions was about 15%. DK and DADK proved to be highly unstable since no appreciable amount of either of these two compounds was observed by GC. Furthermore, no DADK was produced during the HTD of DA or DK.

The data obtained showed that the presence of plant material induced a partial degradation of metribuzin and DA and a complete breakdown of DK and DADK by HTD. In determining these recoveries, the control plant

material was fortified by drying a standard of metribuzin or one of its metabolites absorbed in the plant matrix. It is difficult to postulate whether the HTD of the fortified plant sample could be considered to closely mimic the HTD of a plant sample containing the bound compound. Therefore, the recovery figures obtained from this study should not be used to extrapolate the actual amounts of bound residues in a plant sample.

3.1.3.5. Recovery of metribuzin by supercritical solvent extraction (SSE)

i - Recovery of pure metribuzin from supercritical methanol and acetone.

The pure non-labeled metribuzin standard was dissolved in the solvent at room temperature. The solvent was then brought to the supercritical state as described in section 2.1.3.5. Under these experimental conditions only 40% of metribuzin was recovered intact from the supercritical methanol, as determined by GC. No known metabolites were formed and no other significant unknown peak appeared on the chromatograms. From these observations supercritical methanol appeared to be unsuited to the extraction of bound residues of metribuzin in soybean due to the poor stability of the herbicide in the extraction fluid.

Acetone was evaluated as a possible substitute for methanol. Supercritical acetone was shown to dissolve and degrade cellulose (Köll and Metzger, 1978) and the supercritical pressure and temperature conditions used were close to those of supercritical methanol. The metribuzin recovery experiments were carried out at 1700 and 2200 PSI. In both cases the recoveries were very similar. An average of 77.8% (standard deviation

= 0.3) of metribuzin was recovered intact from the supercritical acetone as determined by HPLC using the UV detector. A small amount of DA was also recovered: 12.2% (0.8). Metribuzin was therefore relatively stable in supercritical acetone.

ii - Recovery of ^{14}C-metribuzin from supercritical acetone with control plant material

The experiment described in section 2.1.3.5. (iii) was carried out to determine the effect of extracted plant material on the recovery of metribuzin from supercritical acetone. As observed for HTD, the control plant material appeared to induce the degradation of metribuzin (Table 21). The TLC and HPLC of the recovered ^{14}C labeled compounds obtained by SAE showed a pattern similar to that observed for high temperature distillates (Figures 10 and 12). However, the proportion of ^{14}C-compounds extracted from the three TLC fractions of the supercritical acetone extracts were larger than those obtained for high temperature distillates. The fact that no breakdown of metribuzin to CO_2 occurred during SAE accounted for only part of that difference. The reason for the higher recovery of metribuzin by SAE than HTD remained unclear. It is possible that the SAE technique itself was milder than HTD. In this case potentially higher levels of metribuzin could be recovered from soybean samples containing bound residues by SAE than by HTD. It should be noted that in the case of the SAE method the fortification was achieved by adding the herbicide in the solvent after it reached the supercritical state. However for the HTD method the plant material was directly fortified with the herbicide before distillation. Thus, it appears that the difference in the recovery results could be merely due to the different procedures used to fortify the control plant material.

Table 21. Recovery of ^{14}C metribuzin from SAE with control plant material

Control plant material	Rf value by TLC	^{14}C extracted from the silica gel (% of the total ^{14}C)	Compounds identified by HPLC with ^{14}C monitor
Root (0.9g)	0.88	76.7	Metribuzin
	0.65 & 0.33	Less than 5%	-
Shoot (2.0g)	0.88	52.2	Metribuzin
	0.65	21.6	DA + Unknown compounds
	0.30	19.2	DADK

For reasons discussed earlier the recovery figures obtained in SAE were not used to correct the data obtained from the SAE of extracted soybean samples containing bound ^{14}C-residues. It appears that metribuzin is relatively unstable when submitted to a high temperature in the presence of plant material. The mechanism by which metribuzin is modified under these conditions remains unclear. However the plant cell wall matrix has a very complex molecular structure as mentioned earlier. The various structural biopolymers possess numerous alcohol, phenol, ether, ketone, carboxylic acid and ester groups which could either catalyze a thermal decomposition of metribuzin or react with the herbicide at high temperature. In this situation, an increase in the temperature applied to a metribuzin residue would likely enhance the extent of its degradation. This is consistent with the results obtained in the recovery studies. Metribuzin residues unextractable from the plant cell wall matrix may decompose to a larger extent than observed in the recovery studies when subjected to HTD or SAE due to their closer interaction with the cell wall polymers.

3.1.3.6. Analysis of soybean-bound residues of metribuzin by HTD

The recoveries of radioactivity after HTD of the various soybean

samples containing bound ^{14}C-residues were very similar, regardless of the type of sample (root or shoot), variety and harvest time. For the 22 samples analyzed the average recovery ± 95% confidence interval was 84.9 ± 2.0% of the total ^{14}C in the plant tissue. No radioactive compound remained in the pyrolyzed plant residue left in the quartz tube after distillation, as determined by combustion and radioassay. The $^{14}CO_2$ fraction collected in the carbosorb trap represented a larger part of the radioactivity recovered from Maple Arrow than from Maple Amber samples (Table 22). Within the two varieties, no significant difference between the proportions of $^{14}CO_2$ produced by shoot and root samples was observed, as determined by the Student's t-test. For both varieties, the production of $^{14}CO_2$ during HTD increased with time (Table 22). These observations may be explained by the fact that ^{14}C-residues bound for a longer period would likely be more deeply embedded in the cell wall matrix. Such residues might decompose to $^{14}CO_2$ more readily than residues bound for shorter times. This would account for the increase in $^{14}CO_2$ production in samples from plants harvested later in the experiment. Futhermore, Maple Arrow plants were shown to bind ^{14}C-residues at a faster rate than Maple Amber (see section 3.1.2.3.). Thus for a given harvest time, Maple Arrow plants will contain "older" bound residues than Maple Amber. This may account for the difference in $^{14}CO_2$ production between the two varieties. The remaining ^{14}C labeled compounds released by HTD were methanol soluble. They were present in the quartz tube and the methanol traps. The TLC of the methanol solution of these ^{14}C-residues showed the three band pattern previously seen in the metribuzin recovery study (Figure 10). The HPLC analysis of the silica gel extracts confirmed the identity of the compounds as metribuzin, DA, DADK and other unknown products. The relative proportions

of the three identified fractions are reported in Table 23. They were significantly different between roots and shoots but not between the two varieties or at the various harvest times.

^{14}C-Metribuzin was shown to decompose to $^{14}CO_2$, DA, DADK and other unknown products during HTD of plant samples. Therefore the presence of small amounts of ^{14}C-metribuzin in the high temperature distillates of soybean samples containing bound residues indicates that in fact the parent herbicide was bound to the cell wall matrix in quantities larger than those actually measured. However, due to experimental difficulties, no accurate quantitation could be performed at this time. It should be realized that the other ^{14}C-residues and $^{14}CO_2$ released by HTD also originated, at least in part, from the breakdown of ^{14}C-metribuzin.

Considering the nature of the HTD technique and the observed fact that metribuzin is unstable in the presence of plant material at high temperatures, it is unlikely that different HTD conditions could release the bound metribuzin residues while simultaneously avoiding their thermal degradation.

Table 22: $^{14}CO_2$ and methanol soluble radioactivity produced by HTD of the soybean samples (% of the total recovered ^{14}C)

Harvest time	Maple Arrow (R)			Maple Amber (S)		
	$^{14}CO_2$	Methanol		$^{14}CO_2$	Methanol	
		a	b			
3 days	47.8	57.2 (2.8)	6	18.9	81.1 (8.5)	4
10 days	51.1	48.9 (3.6)	4	27.8	72.2 (4.5)	4
28 days	57.1	41.9 (1.4)	2	31.9	68.1 (3.7)	2

[a] Standard deviation [b] number of replicates

3.1.3.7. Analysis of soybean-bound residues of ^{14}C-metribuzin by SAE

The plant samples harvested ten days after treatment were extracted by SAE as described earlier. The total acetone extract contained an average 69.2 ± 4.5% of the total radioactivity in the sample. The last acetone fraction was collected during the third hour of extraction, it contained only about 1% of the radioactivity while 6.2 ± 2.4% remained unextracted from the cell wall matrix as determined by combustion of the residual plant material and radioassay.

The TLC of acetone extracts showed the expected three band pattern as well as an important fourth band at Rf 0.0 - 0.2. The HPLC of the extracts from the three bands confirmed the identity of metribuzin, DA, DADK and some unknown products (Table 23). A comparison of the data obtained by HTD and SAE for these compounds shows that with both techniques larger amounts of metribuzin were recovered from shoots than roots. Furthermore, the relative proportions of the "DA + unknown compounds" and DADK fractions were very similar in the shoots whereas in the roots more DADK was observed. The cause of this difference between roots and shoots is not clearly understood. The recovery study did not reveal any particular

Table 23: Comparison between the three radioactive fractions obtained from the TLC analyses of high temperature distillates and supercritical acetone extracts. (% of the ^{14}C in the three fractions)

Rf value	HTD		SAE		Compounds identified by HPLC with ^{14}C monitor
	shoots[a]	roots[b]	shoots[c]	roots[c]	
0.88	12.6 (5.5)[d]	7.0 (2.7)	22.8 (5.2)	16.5 (6.7)	Metribuzin
0.65	43.2 (7.8)	25.5 (3.7)	38.8 (3.5)	25.2 (2.8)	DA + unknown
0.29	44.1 (9.1)	67.5 (5.1)	38.4 (1.7)	58.3 (9.5)	DADK

[a]10 replicates, [b]11 replicates, [c]2 replicates, [d]standard deviation

influence of the type of sample (root or shoot) on the recovery of metribuzin.

The radioactive compound(s) extracted from the band at Rf 0.0-0.2 chromatographed by HPLC as a broad and irregular peak at Rt ≃ 1 minute. The TLC and HPLC results indicated that these compounds probably had high polarities and/or molecular weights. These "unknown polar compounds" represented the only qualitative difference between the results of SAE and HTD experiments. In the latter technique the unknown polar compounds yielded $^{14}CO_2$. It was noted that Maple Arrow samples which produced more $^{14}CO_2$ than Maple Amber by HTD, also released relatively more unknown polar compounds in the proportions of 72.1% (standard deviation = 1.6) and 49.6% (9.5), respectively. Both these amounts are greater by about 20% than the corresponding $^{14}CO_2$ levels observed for HTD. This indicated that the unknown polar compounds probably contained unreleased ^{14}C-residues in the form of conjugates. These compounds were hydrolyzed with an aqueous solution of 1N HCl as described before. The solution was extracted with chloroform. The aqueous phase retained 58% of the initial radioactivity, probably in the form of unknown polar compounds. The organic phase was chromatographed by TLC and two radioactive bands were observed. The band at Rf 0.0-0.2 (unknown polar compounds) represented 26% of the initial radioactivity. However a band at Rf 0.3 representing 11% of the radioactivity was also observed. The identity of that ^{14}C-residue could not be confirmed by HPLC due to low radioactivity levels. However, the TLC analysis indicated that it was likely to be DADK. These results show that the ^{14}C label of at least some of these unknown polar compounds remains a part of a distinct herbicide residue moiety conjugated to an unidentified endocon moiety. It is not known whether that herbicide residue (exocon)

was metribuzin or another metabolite since metribuzin is known to decompose to DADK during both SAE and acid hydrolysis (Frear et al., 1985).

Further release of the ^{14}C labeled exocon from these unknown conjugates by hydrolysis may be prevented by the rearrangement and modification of the conjugate during SAE. In a study on supercritical methanol extraction of bound pesticide residues from soil and plant samples Capriel et al. (1986) suggested that "in some cases a considerable portion of the ^{14}C-residue in the methanol extract might be present as unknown metabolites or may have been included in macromolecular type structures". From the data obtained in this study it appears that supercritical fluid extraction of a plant-bound residue fraction may release some pesticide residues in the form of exocons conjugated with cell wall polymers in addition to the free parent compound and metabolites.

Unlike HTD, supercritical fluid extraction could be performed at lower temperatures in order to avoid the thermal decomposition of metribuzin residues. This would require the use of fluids with critical temperatures lower than acetone or methanol (e.g.: CO_2). Since such fluids would have a lower density at room temperature than acetone, high pressures would have to be applied in order to match the extraction efficiency of supercritical acetone. However, a more sophisticated apparatus than the one used here would clearly be necessary.

In a paper published during the course of this study, Frear et al. (1985) reported on the acid hydrolysis of the bound residue fraction of ^{14}C-metribuzin-treated excised soybean leaves. This procedure released about 40% of the bound residues as the metabolite DK. Since DK is a product of acid hydrolysis of metribuzin, it remained unclear whether the bound residue was the parent compound or its diketo metabolite.

More thorough cell wall fractionation procedures such as those reviewed earlier (Pillmoor et al., 1984; Langebartels and Harms, 1985) might have released higher proportions of bound residues. However, it is likely that the ^{14}C-residues released would, again, have been a metribuzin hydrolysis product, thereby leaving unanswered the question of the identity of the bound residue.

The data presented in this study show, however, that metribuzin is present in significant amounts in the bound residue fraction. Free DA and DADK metabolites were also released by HTD and SAE and a large fraction of the ^{14}C-residues was recovered as $^{14}CO_2$ after HTD. Both metabolites and $^{14}CO_2$ originated, at least in part, from the breakdown of metribuzin. A large fraction of ^{14}C-polar compounds extracted by SAE partially released a less polar ^{14}C-residue (probably DADK) upon acid hydrolysis. This suggested that the polar compounds may be conjugates of cell wall biopolymers with an exocon moiety (possibly metribuzin). During HTD, some of these polar compounds decomposed to $^{14}CO_2$ while others released their exocon moiety. This was due to the fact that HTD is less efficient in extracting large macromolecules than SAE. None of the techniques used to release the soybean-bound ^{14}C-residues (HTD, SAE and acid hydrolysis) allowed the characterization of the endocon moiety of the ^{14}C-polar compounds.

These results may be interpreted in various ways. The presence of DA, DK, DADK or other unknown metabolites along with metribuzin in the bound residue fraction could not be ruled out. Furthermore some of the residues may be physically trapped in the cell wall matrix while others may be covalently bound to a variety of cell wall constituents. However, it is also possible that the entire bound residue fraction was constituted of a

single type of conjugate of ^{14}C-metribuzin with a plant polymer. Such conjugate would likely be present in the cell walls in various degrees of polymerization. Furthermore, it would also be embedded at various depths in the matrix depending on when, after the herbicide treatment, it was synthesized and incorporated. HTD of the plant sample would release the metribuzin from "younger" conjugates as metribuzin, DA, DADK, other unknown compounds and $^{14}CO_2$. The "older" conjugates would more likely breakdown completely, releasing only $^{14}CO_2$. Supercritical acetone would dissolve most of the conjugates, some of them losing their ^{14}C-metribuzin in the process, others retaining it. Finally, acid hydrolysis of the bound fraction could be expected to hydrolyze the more accessible conjugates, thereby releasing DK.

A bound conjugate would be more likely to originate from the N-glucoside of metribuzin by addition of monomeric sugar units than from the homoglutathione conjugate of metribuzin. According to Frear et al. (1983 and 1985), the latter is a more important extractable conjugate in metribuzin-tolerant soybean, while the former is a major metabolite in tomato plants. Tomatoes, however, produced less bound metribuzin residues than soybeans (less than 7% of the total ^{14}C in tomato seedlings five days after treatment). This suggests the possibility that in tomatoes, slow bound residue formation may result in the accumulation of the glucose conjugate in the extractable fraction of the cells while in soybeans, this conjugate may become rapidly incorporated in the cell wall.

The presence of the parent herbicide in the bound residue fraction appears to be of little toxicological concern to humans and cattle since very low bound residue levels were found in the edible seeds. However, soybean-bound residues of metribuzin are potentially bioavailable since

they may be released upon hydrolysis. The most common reaction would probably be the acid hydrolysis of the bound residues. This could occur mainly during digestion of the plant material by animals. In this case, the released residue would probably be DK which appears to have a low toxicity to animals (Hatzios and Penner, 1988). In some cases it remains possible that a more specific enzymatic hydrolysis may release intact metribuzin from decaying soybean tissue, thus contributing to the total bioavailable metribuzin in the environment.

3.2 Extractable and bound residues of atrazine in canola

3.2.1. Herbicide treatment of the canola plants

3.2.1.1. Preliminary treatments

During the preliminary experiments several groups of Tower (atrazine susceptible) and Atr Tower (atrazine resistant) plants were treated with different herbicide concentrations in order to determine the tolerance threshold of each variety. The concentrations of atrazine used and their phytotoxic effect as measured by leaf chlorophyll fluorescence are reported in Table 24. The Atr Tower variety owes its resistance to a mutated protein on the reducing side of the photosystem II (PSII) (Beversdorff, personal communication). This protein is the target site of triazine and other herbicides. The mutation considerably reduces its affinity for these herbicides, thereby greatly increasing the tolerance of the plant. The first and second preliminary treatments clearly showed the high tolerance of the Atr Tower variety to atrazine since no inhibition of photosynthesis

was observed with the maximum concentration tested (8 ppm). The mutated protein results in a reduction of the electron transport efficiency of the PSII. The data obtained from the fluorescence measurements taken before the treatment and, later, on untreated control plants show that the Atr Tower variety had a lower Ri recovery at 200 seconds than the Tower variety: 78.1 ± 5.7% and 91.4 ± 3.4%, respectively (average ± 95% confidence interval). This was due to the reduced photosynthetic efficiency of the resistant biotype.

Although no inhibition of photosynthesis was observed on the Atr Tower plants treated with 8 ppm of atrazine, two of the three replicates showed decreased nutrient solution absorption and some loss of turgidity towards the end of the ten day treatment. Upon harvest, sections of their roots showed dark lesions on the vascular system. These symptoms appeared to

Table 24. Leaf chlorophyll fluorescence measurements of canola plants treated with various concentrations of atrazine

	Atrazine Concentration (ppm)	Ri %[a] at half time of treatment		Ri % at end of treatment		inhibition of photosynthesis
Tower plants(S)						
first preliminary	1.00	69.7 (4.6)[b]	3[c]	2.0 (2.1)	3	Yes
experiment	0.50	76.8 (13.1)	3	1.9 (1.9)	3	Yes
	0.25	87.2 (7.9)	3	11.6 (12.9)	3	Yes
second preliminary	0.15	84.2 (4.1)	3	77.5 (6.3)	3	No
experiment	0.10	87.0 (7.9)	2	83.2 (7.3)	3	No
	0.05	85.6 (4.2)	2	85.9 (6.9)	3	No
Atr Tower plants (R)						
first preliminary	0.5	74.1 (-)	1	79.8 (7.7)	3	No
treatment	1.0	73.7 (6.8)	3	71.4 (7.6)	3	No
	2.0	73.3 (6.0)	5	72.6 (9.8)	6	No
second preliminary	2.0					
treatment	4.0	82.4 (8.9)	2	68.8 (6.0)	3	No
	8.0	73.2 (4.7)	2	73.4 (3.1)	3	No

[a] see section 3.1.1.1. for details on the calculation the Ri recovery value.
[b] standard deviation [c] number of replicates

match those of the little known brown girdling root rot disease as described by Martens (1984). This disease causes the obstruction of the vascular system in the roots which results in the dessication of the plant. It was not determined whether the disease was in any way related to the high concentration of the atrazine treatment.

3.2.1.2. Radiolabeled atrazine treatment of the canola plants

The herbicide concentrations used for the ^{14}C-atrazine treatment were somewhat lower than the tolerance threshold determined in the preliminary study. This was done in order to compensate for the possible inaccuracy in the determination of the threshold, caused by the limited number of replicates used in that study. The Tower and Atr Tower varieties received ^{14}C-atrazine concentrations of 0.1 ppm and 4.0 ppm, respectively. Leaf chlorophyll fluorescence measurements were taken before and during the treatment and no inhibition of photosynthesis was observed. The last measurements, taken at the end of the treatment produced Ri values of 90.8 ± 4.9% and 76.7 ± 5.9% for the Tower and Atr Tower plants, respectively.

3.2.2. Distribution of the ^{14}C label in the canola plants

Due to time constraints and the length of the extraction procedure, the four replicates of each canola sample collected could not be extracted individually. They were combined at random to form two replicates. Therefore, the values representing a given variety and time of harvest are averages of two means of two samples each (Table 25 and 26). The standard deviation of these two means were included with the averages even though they are of limited statistical value. The combined samples were extracted five consecutive times with methanol. The last extract contained an

average of 4.1%, 4.3% and 2.8% of the total extracted radioactivity for roots, shoots and pods, respectively. It is clear that only a small fraction of the unextracted radioactivity could have been released by performing additional extractions. Thus, the number of extractions was maintained at five. The radioactivity unextracted by this procedure was reported as bound radioactivity.

3.2.2.1. Recovery of the ^{14}C applied in the plants and growth medium

As described for soybeans, the total radioactivity present in the plant was calculated by adding the DPM values of bound and extractable ^{14}C-residues in the roots, shoots and pods. The proportions of the ^{14}C applied recovered in the plants and their growth medium are shown in Table 25. As for the soybean plants, the levels of radioactivity in canola plants remained relatively constant between 3 and 25 days and the ^{14}C levels extracted from the medium decreased with time. The Atr Tower variety plants appeared to contain greater proportions of ^{14}C than Tower plants. However the data were rather scattered and the difference was not statistically significant (Student's t-test).

Table 25. Recovery of ^{14}C in canola plants and their growth medium

Canola	Time of harvest (days after treatment)	Total ^{14}C applied (average DPM ± 95% confidence interval x 10^{-3})	^{14}C recovered (% of total ^{14}C applied)		
			plants	medium	total
Tower	3	3795.0 ± 637.1	45.8(12.5)[a]	11.6(1.9)	57.4(10.6)
	10	3441.8 ± 238.5	47.9 (0.9)	6.8(1.5)	54.7 (2.4)
	25	3719.1 ± 258.1	45.1 (2.3)	4.3(0.9)	49.4 (3.2)
Atr Tower	3	2389.8 ± 225.1	57.2(21.1)	9.5(1.8)	66.7(19.3)
	10	2279.1 ± 534.7	62.1(6.2)	4.5(0.2)	66.6 (8.4)
	25	2356.6 ± 134.5	58.0(17.2)	1.6(0.4)	59.6(17.5)

[a] standard deviation of two means

The overall recoveries of radioactivity were relatively low. The canola plants absorbed large volumes of nutrient solution causing significant fluctuations of the levels of solution in the pots and saucers. This may have resulted in deposits of ^{14}C-atrazine and nutrient salts. These deposits may have been only marginally soluble in the aqueous methanol used to rinse the silica sand and saucers previous to removing the root system. Another possible reason for the low recovery levels is the loss of ^{14}C through the complete metabolism of atrazine to $^{14}CO_2$. It is known that some plants which can rapidly metabolize atrazine may also completely oxidize it to CO_2 (Montgomery and Freed, 1964). Corn seedlings grown for three days in an aqueous solution of ^{14}C-atrazine released about 17% of the ^{14}C absorbed as $^{14}CO_2$ when maintained under a normal photoperiod and 28% when maintained in darkness (Montgomery and Freed, 1961). These results indicated that the $^{14}CO_2$ released was probably reincorporated into natural products when photosynthesis was allowed to proceed. However when plants such as oats, alfalfa and cucumber were studied under similar conditions the proportions of $^{14}CO_2$ produced were approximately one-tenth of the levels in corn (Montgomery and Freed, 1964).

3.2.2.2. Distribution of the ^{14}C-residues in canola plants treated with ^{14}C-atrazine

The roots of the canola plants contained only small proportions of ^{14}C-residues (Table 26). Furthermore, the roots of the Atr Tower plants contained ^{14}C-residue proportions only marginally greater than those of the Tower plants.

The distribution of the radioactivity in the shoots of the canola plants is reminiscent of the soybean plants in that the resistant variety

initially contained higher proportions of shoot-bound ^{14}C-residues than the susceptible variety. Ten days after treatment the proportions of bound ^{14}C-residues in the shoots of both varieties were the same.

It is interesting to note that between the third and tenth day after treatment the Tower plants translocated larger proportions of ^{14}C-compounds to the pods than Atr Tower. Approximately equal proportions of ^{14}C-residues were found in the bound and extractable fractions of the Tower pods. Due to the large number of pods produced, the distribution of the ^{14}C-residues in the two constituents of the pods (seeds and shells) was not investigated. Very little additional change in the ^{14}C-residue distribution occurred between 10 and 25 days after treatment for both varieties. Therefore, it appears that after the tenth day very little translocation or bound residue formation occurred.

Bound residue formation does not seem to contribute to a significant extent to the resistance of the Atr Tower variety. This is consistent with the fact that this variety does not owe its resistance to quick detoxification of the herbicide like metribuzin-tolerant soybeans. As mentioned earlier, Atr Tower has a modified herbicide site of action with a reduced affinity for atrazine.

Fast bound residue formation may be expected to increase the herbicide tolerance of a plant variety that relies on metabolism to detoxify the xenobiotic before it can have its effect. However, for plants relying on other strategies such as a modified site of action, fast pesticide binding would not represent a genetic advantage over other resistant plants under the selective pressure of a herbicide treatment.

3.2.3. Analysis of the extractable residues of ^{14}C-atrazine in canola plants

3.2.3.1. Direct gas chromatographic analysis

Atrazine may produce several metabolites in plants (Khan et al., 1985b). These metabolites cover a wide range of polarities from intermediate (parent compound) to high (conjugate(s)). The extraction procedures described in the literature for extractable atrazine residue analyses usually involve an initial separation between chloroform soluble and water soluble residues followed by different clean-up procedures (Lamoureux et al., 1970; Ali and Souza-Machado, 1984; Khan et al., 1985b). The procedure described by Khan et al. (1985b) involved successive extraction of the plant material with chloroform, methanol and water. The chloroform extract was cleaned up by column chromatography on acidic alumina. The remaining extracts were cleaned up with an XAD-2 ion exchange resin column. The procedure was initially designed for freeze-dried weed samples, it was modified in this study for frozen fresh canola samples. The total extracts of the shoot-samples of plants harvested ten days after treatment were partitioned betwen chloroform and water (section 2.2.3.2.). However the acidic alumina column chromatography did not clean up the chloroform fraction thus obtained.

Rather than attempting to develop a complete clean up procedure more adapted to the type of samples studied, the methanol extracts of the canola samples were simply partioned with n-hexane. This effected a limited but rapid clean up since the n-hexane fraction did not contain any ^{14}C-residues and could thus be discarded. The concentrated methanol fraction was then divided into three equal parts. The first part was directly injected on GC, the second was methylated with diazomethane prior to injection and the

Table 26. Distribution of extractable and bound ^{14}C-residues in roots, shoots and pods of ^{14}C-atrazine-treated canola plants (% of total ^{14}C in the plant, average of 2 means of duplicate samples).

Plant Sample	^{14}C-residues	Harvest time (days after treatment)					
		3		10		25	
		Atr Tower(R)	Tower(S)	Atr Tower	Tower	Atr Tower	Tower
Shoots	Extractable	53.8 (9.8)a	62.7 (3.8)	39.8 (0.4)	31.5 (0.3)	38.9 (5.9)	31.6 (1.6)
	Bound	39.6 (6.0)	32.2 (2.5)	44.8 (0.8)	46.2 (2.6)	45.9 (0.5)	46.1 (1.4)
Roots	Extractable	3.3 (2.6)	2.2 (2.5)	5.8 (0.6)	3.4 (0.1)	3.7 (1.0)	1.7 (0.1)
	Bound	3.3 (1.2)	2.9 (0.8)	4.5 (0.1)	3.0 (0.1)	4.3 (0.7)	2.6 (0.1)
Pods	Extractable	included with shoots		3.5 (0.6)	8.8 (1.1)	4.0 (2.2)	8.2 (2.1)
	Bounds	" "	"	1.6 (0.2)	7.1 (2.0)	3.2 (1.6)	9.9 (0.7)

[a] standard deviation of the two means

third was subjected to acid hydrolysis and then methylated and analyzed by GC (see section 2.2.3.2.). The GC procedure was used to determine the possible presence in the extracts of atrazine and its dealkylated metabolites (part 1), hydroxy atrazine and its dealkylated derivatives (part 2) and conjugated metabolites (part 3). However, large peaks of plant coextracts and/or their methylation products interfered with the detection of atrazine, atratone (methylated hydroxyatrazine) and several other 2-chloro- and 2-methoxy- metabolites. This, in turn, prevented the identification of any known compound by GC (Figure 15).

3.2.3.2. Direct HPLC analysis of the canola extracts.

The methanol extracts of canola shoot samples were analyzed by HPLC on a semi-preparative reverse-phase column. The radiochromatograms obtained showed three major peaks of ^{14}C-labeled compounds comprising of two compounds and the parent herbicide (Table 26). The two unknown compounds were named A and B and had retention times of 2.5 and 9.0 minutes, respectively. No known atrazine metabolites were observed. The integration of the counts recorded by the radioactivity monitor and correction of the integrated value for background radiation confirmed that (A, B and atrazine) represented more than 90% of the ^{14}C in the samples.

It is clear that in the extract of the Tower shoots the relative proportion of the parent herbicide sharply decreased over the period studied while the compounds A increased and B remained relatively constant (Table 27). The shoot extract of Atr Tower plants harvested ten days after treatment contained relatively more compound A and less atrazine than that of Tower plants harvested at the same time. Although only one Atr Tower sample was analyzed it appeared that the resistant variety may have been somewhat faster in metabolizing atrazine than the susceptible.

Table 27: Relative proportions of the radioactive peaks in canola shoot extracts. (% of the total counts in the three peaks, corrected for background, average of 2 values)

Peak name	Tower			Atr Tower
	3 days	10 days	25 days	10 days
A	42.2(2.3)[a]	53.4(2.1)	65.2(8.3)	64.9(1.0)
B	23.5(0.6)	28.5(4.1)	23.8(6.7)	23.5(4.0)
Atrazine	35.3(1.7)	18.1(2.0)	11.0(2.1)	11.6(3.0)

[a] standard deviation

The possibility of A and B being conjugates was investigated by isolating the corresponding fractions and subjecting them to acid hydrolysis. A mild hydrolysis of the compound B readily yielded ^{14}C labeled compounds that chromatographed by HPLC at the same retention time as the compound A. However the compound A appeared unchanged by acid hydrolysis, even after refluxing for one hour in a 3 N aqueous solution of HCl. None of the hydrolysis experiment released hydroxyatrazine or any other known atrazine metabolite. The compound B was likely to be a condensation product of A with either itself or other unlabeled compounds. Even though the compound A was not hydrolyzed it may still be in the form of a conjugate. Another possibility is that A may be a natural polar compound containing ^{14}C recycled after the complete metabolism of atrazine to $^{14}CO_2$. However, in the event of such complete metabolism, one would expect to observe the presence of various atrazine metabolites in addition to the parent herbicide in the methanol extracts. No further attempts to determine the nature of the unknown compounds A and B were made.

3.2.4. Analysis of the bound ^{14}C-residues of atrazine in canola

3.2.4.1. Recovery of atrazine by HTD.

In order to determine the recovery of atrazine by HTD, two extracted control canola samples were fortified with ^{14}C-atrazine and subjected to HTD. The methanol solutions recovered from the traps and the quartz tube washings contained an average of 86.0% (standard deviation = 3.0) of the initial radioactivity while an additional 12.1%(2.5) was recovered as $^{14}CO_2$. The TLC analysis of the methanol solution showed six radioactive bands at Rf 0.13, 0.27, 0.45, 0.59, 0.68 and 0.82 (Figure 16). The radiolabeled compounds extracted from the bands at Rf 0.82 (Rf value of atrazine) and 0.59 (Rf deisopropylatrazine) were tentatively confirmed as atrazine and deisopropylatrazine. A positive identification was difficult due to the low radioactivity levels. The HPLC radiochromatogram of the extract of the band at Rf 0.68 (Rf deethylatrazine) showed no ^{14}C peak. The band at Rf 0.45 (Rf diaminoatrazine) was the most intense of all six. The presence of diaminoatrazine in the extract of this band was confirmed by HPLC. However diaminoatrazine represented only about 25% of the radioactivity in this extract, the large peak of an unknown compound (Rt = 13 minutes) represented the remaining 75% of the ^{14}C. Other unknown ^{14}C-compounds were observed by HPLC of the extracts of the bands at Rf 0.13 and 0.27.

These results indicate that during HTD with fortified canola material, most of the atrazine was degraded, yielding several known and unknown products. Such an extensive and complex breakdown was not consistent with the fact that the HTD technique was used before to study bound residues of atrazine in corn (Khan et al., 1985a). However the

results of the HTD recovery experiment were essentially the same whether the control canola material was a shoot or a root sample. Due to this breakdown pattern of the parent herbicide, the study of the bound ^{14}C-residues of atrazine in canola by HTD would probably yield very complex results. Therefore this study was not further attempted.

These results point out that a pesticide which has been shown to be stable enough to warrant the use of HTD on a given type of plant sample may be much less stable with a different kind of sample. This demonstrates the need for very specific recovery studies when considering the use of HTD to release a given type of bound residues.

3.2.4.2. Recovery of atrazine from supercritical methanol.

The recovery of a pure ^{14}C-atrazine standard from supercritical methanol was determined in duplicate (section 2.2.3.5). The average recovery as calculated by HPLC was 96.2% (standard deviation = 3.1).

Another recovery experiment was performed by placing a control canola root or shoot sample in the sample holder of the SSE apparatus prior to bringing the ^{14}C-atrazine solution to the supercritical state. The TLC analysis of the methanol solution collected showed a single band of ^{14}C labeled material at the Rf value of atrazine (0.80). The methanol extract of this band contained 93% of the radioactivity of the total ^{14}C-atrazine exposed to the supercritical methanol. The HPLC analysis of the methanol extract revealed that two ^{14}C-compounds were present: atrazine and atratone. The relative proportions of these compounds as determined by integration of the counts recorded for each peak were: 48% and 52% for ^{14}C-atrazine and ^{14}C-atratone, respectively.

Atratone is the 2-methoxy derivative of atrazine, its formation by derivatization of atrazine in supercritical methanol was initially reported by Capriel et al. (1986). They observed the presence of atratone in the supercritical methanol extracts of corn samples containing bound ^{14}C-atrazine residues. Based on the data shown above, it appears that the methoxylation of atrazine does not occur in pure supercritical methanol, the derivatization reaction seems to require the presence of plant material. Capriel et al. (1986) also reported that only atrazine and its dealkylated metabolites may be methoxylated in supercritical methanol while the "2-hydroxy" metabolites remain unchanged. Plants do not produce "2-methoxy" derivatives of atrazine therefore all such compounds found in the supercritical methanol extract of a plant sample will indicate the presence of their "2-chloro" equivalents in the sample. The SME technique appears to be well suited for the analysis of the bound residues of ^{14}C-atrazine in canola.

3.2.4.3. Analysis of the bound ^{14}C-residues of atrazine in canola by SME.

The shoot, root and pod samples of canola plants harvested ten days after treatment were extracted with supercritical methanol. An average of 91.3% (standard deviation = 1.1) of the radioactivity in shoot and pod samples was released in the process while 3.5% (1.9) remained unextracted. For the root samples the figures for the extracted and unextracted fraction were 80.3% (3.4) and 17.0% (4.5), respectively.

Some samples contained relatively low levels of radioactivity, these were the root and pod samples of Atr Tower plants (22010 and 21630 DPM/g, respectively) and the Tower root sample (24520 DPM/g). The supercritical

methanol extract of these samples were chromatrographed on TLC (see section 2.2.3.6.). The polaroid photograph of the TLC plates showed no radioactive band. The amount of ^{14}C on each TLC plate (about 17000 to 20000 DPM) was sufficient to produce a clear photograph if present in three or fewer well defined bands. The absence of visible bands indicated that the ^{14}C-compounds were probably distributed between several poorly defined bands. These samples were not further analyzed. The ^{14}C levels in the remaining samples were high enough to produce clearer photographs (Figure 16). The TLC of each of the three samples displayed the same five radioactive bands. Each band was scraped off from the plate and extracted with methanol. The distribution of the radioactivity in the extracts was similar for each sample: 5% of the ^{14}C applied on the plate was extracted from the band at Rf 0.80, 10% from Rf 0.55, 7% from Rf 0.30, 33% from Rf 0.20 and 45% from Rf 0.00. The extracts of the bands at Rf 0.80, 0.55, 0.30 and 0.20 were analyzed by HPLC. However, the low radioactivity levels in the extracts prevented the characterization of the ^{14}C compounds. Most of the radiochromatograms obtained showed no peak above background. Two small radioactive peaks were observed on the radiochromatogram of the extracts of the band at Rf 0.20. The peaks had retention times (Rt) similar to those of deisopropylatrazine and deisopropylatratone. However, the normal Rf value of these compounds for the experimental TLC conditions used was 0.60 and it is unlikely that they could be found at Rf 0.20.

The TLC extracts were also chromatographed by GC, along with their corresponding TLC fractions of the supercritical methanol extracts of a control canola shoot sample. No deisopropylatrazine was observed by GC and a background peak was observed on both control and sample chromatograms at the Rf of deisopropylatratone. The GC analysis of the extracts of the bands

at Rf 0.55 and 0.30 also failed to show any known compound. Both the sample and control extracts of the band at Rf 0.80 showed an interfering background peak at the Rt of atrazine. However, traces of atratone (<0.1 ppm), apparently free of interference, were detected. The compound was cochromatographed with a small amount of an atratone standard (≈0.3 ng). The possible presence of a small amount of atratone and consequently of atrazine in the TLC extracts does not necessarily indicate that the parent herbicide was a bound residue since the initial methanol extraction of the plant samples removed only about 95% of the extractable radioactivity.

The extracts of the TLC band at Rf 0.00 (bound polar fraction) contained almost one-half of the radioactivity on the plates. However these extracts also contained high concentrations of the plant coextracts which prevented their analysis by GC or HPLC. An additional TLC of the bound polar fraction of the Tower shoot sample was performed. With a methanol-acetic acid (8:2) solvent system, the radioactive compounds migrated as a broad band (Rf 0.75 - 0.90) and no clean-up was achieved. The nature of these polar compounds remained undetermined.

The results obtained provide very little insight into the nature of the bound ^{14}C residues of atrazine in canola. The analysis were hampered by the low specific activity of the extracted plant samples and the apparent diversity of the bound radiolabeled compounds present. It remains unclear whether or not the parent herbicide was present in the bound fraction. The GC results seem to indicate that only a very small amount of atrazine was bound. However, depending on the type of binding, bound atrazine may be modified upon release. The possibility raised earlier that some extractable compounds may contain ^{14}C reincorporated after complete metabolism of atrazine could also apply to some of the non-extractable radiolabeled compounds.

4. CONCLUSION

The tolerant soybean variety, Maple Arrow, contained higher proportions of ^{14}C-residues in the root samples and bound fractions than Maple Amber, especially at early stages after the treatment.

The parent herbicide metribuzin was present in significant amounts in the bound residue fraction of soybean samples. The results also suggested that at least part of the bound residues of metribuzin were covalently bound to polar cell wall constituents. The modification of metribuzin during high temperature distillation (HTD) and supercritical acetone extraction (SAE), as well as the possiblity that herbicide residues covalently bound to cell wall polymers may be released as free compounds by these techniques prevented a more accurate determination of the nature of the bound residues of metribuzin in soybeans. If metribuzin is directly incorporated in the cell wall, the greater production of bound residues by Maple Arrow would directly contribute to the tolerance of the variety. The possible incorporation of a conjugate of metribuzin may contribute indirectly to the tolerance by acting as a "sink" for the product of the conjugation reaction.

It is known that acid hydrolysis of extracted soybean material containing bound residues of metribuzin releases the metabolite DK (Frear et al., 1985). On this basis, the current pathway for metribuzin metabolism in soybean proposed by Hatzios and Penner (1988) suggests that the bound residues may arise by incorporation of DK in the cell wall. Although the results obtained here do not disprove that possibility, they clearly indicate that incorporation of free or conjugated metribuzin is likely to be an important metabolic pathway.

In canola plants, the difference in ^{14}C distribution between resistant and susceptible varieties was small. The resistant Atr Tower variety relies on its modified herbicide site of action to avoid the phytotoxic effects of atrazine. Therefore, increased bound residue production would be unlikely to increase the resistance of the Atr Tower variety. It can be speculated that differential residue binding is more likely to be found in plant varieties having tolerance strategies aimed at detoxifying the herbicide.

In both canola varieties, atrazine appeared to be extensively metabolized to a variety of unidentified compounds found in both the extractable and bound fractions.

The HTD and supercritical solvent extraction (SSE) are useful - but not universal - techniques for the study of bound pesticide residues. Their usefulness depends on the stability of the ^{14}C-residues. In determining the stability care must be taken to mimick the conditions of the sample as closely as possible. The HTD and SSE of plant samples containing a bound pesticide may yield known metabolites thereby making the interpretation of the results more complicated. The clean up of high temperature distillates and supercritical solvent extracts can be difficult. In this study this problem was avoided by relying on radiotracer detection techniques. The SSE method is milder than HTD and allows the recovery of material that would be thermally decomposed to CO_2 by HTD. Both the HTD and SSE techniques may provide a relatively fast assessment of the possible presence of the parent pesticide in a bound ^{14}C-residue fraction.

5. FUTURE WORK

As mentioned above, the acid hydrolysis of soybean-bound residues of metribuzin can release free ^{14}C-residues. This reaction would be likely to occur upon digestion of the plant material by animals, thereby raising the question of the bioavailability of these bound residues. Further investigation in this area would provide answers to this question as well as a model for future bioavailability studies of hydrolyzable bound residues.

The apparently complex metabolic pathway of atrazine in canola would also deserve further investigation. The unknown extractable compounds could be purified and characterized. This might provide some insight into the possible nature of the bound residues.

The supercritical fluid extraction technique could probably be modified and improved in the future. The use of gases with low critical temperature instead of solvents may prevent the thermal degradation of bound residues while maintaining the extraction efficiency observed for supercritical methanol and acetone. The development of more efficient clean up procedures for high temperature distillates and supercritical fluid extracts would also greatly increase the usefulness of these techniques. Such improvements may allow the establishment of standardized protocols for the analysis of bound residues of non-labelled pesticides in plants.

6. REFERENCES

Abusteit, E.O.; Corbin, F.T.; Schmitt, D.P.; Burton, J.W.; Worsham, A.D.; Thompson Jr., L. (1985). Absorption, Translocation and metabolism of metribuzin in diploid and tetraploid soybean (Glycine max) plants and cell cultures. Weed Sci., 33, 5, 618-628.

Akhtar, M.H. (1987) The disposition of bound ^{14}C agrochemical residues in plants and animal tissues when consumed by animals. In "Pesticide science and biotechnology". Sixth IUPAC congress of pesticide chemistry (Greenhalgh, R. and Roberts, T.R., eds.). Blackwell scientific publications.

Ali, A.; Souza-Machado, U. (1984). A comparative analysis of leaf chlorophyll fluorescence, Hill reaction activity and ^{14}C-atrazine tracer studies to explain differential atrazine suceptibility in wild turnip rape (B. campestris) biotypes. Can. J. Plant Sci., 64, 707-713.

Arjmand, M.; Sandermann Jr., H. (1985). Mineralization of chloroaniline/lignin conjugates and of free chloroanilines by the white-rot fungus Phanerochaete chrysosporium, J. Agric. Food Chem., 33 6, 1055-1060.

Arntzen, C.J.; Steinback, K.E.; Vermaas, W.; Onad, I. (1983). Molecular characterization of the target site(s) for herbicides which affect photosynthetic electron transport. In "Pesticide chemistry" (Miyamoto, J. and Kearney, P.C.; eds) Vol. 3, Pergamon, Oxford.

Bakke, J.E.; Shimabukuro, R.H.; Davidson, K.L.; Lamoureux A.L. (1972). Sheep and rat metabolism of the insoluble ^{14}C-residues present in ^{14}C-atrazine-treated sorghum. Chemosphere, 1, 21-24.

Balba, H.M.; Still, G.G.; Mansager, E.R. (1979). Pyrolytic method for estimation of bound residues of chloroaniline compounds in plants. J. Assoc. Off. Anal. Chem., 62, 2, 237-240.

Beversdorf, W.D.; Weiss-Lerman, J.; Erickson, L.R.; Souza-Machado, U. (1980). Transfer of cytoplasmically inherited triazine resistance from bird's rape to cultivated oilseed rape (Brassica Campestris and B. napus). Can. J. Genet. Cytol., 22, 167-177.

Capriel, P.; Haisch, A.; Khan, S.U. (1986). Supercritical methanol, an efficacious technique for the extraction of bound pesticide residues from soil and plant samples. J. Agric. Food Chem., 34, 1, 70-73.

Chin, W.T.; Stanovick, R.P.; Cullen, T.E.; Holsing, G.C. (1964). Metabolism of Swep by rice. Weeds, 12, 201.

Chin, W.T.; Kucharcsyk, N.; Smith, A.E. (1973). Nature of Carboxin (Vitavax)-derived bound residues in barley plants. J. Agric. Food Chem., 21, 3, 506-507.

Falb, L.N.; Smith Jr., A.E. (1984). Metribuzin metabolism in Soybeans. Characterization of the intraspecific differential tolerance. J. Agric. Food Chem., 32, 6, 1425-1428.

Fedtke, C.; Schmidt, R.R. (1983). Behaviour of metribuzin in tolerant and suceptible soybean varieties. In "Pesticide chemistry" (Miyamoto, J. and Kearney, P.C. eds.) vol. 3. Pergamon, Oxford.

Frear, D.S.; Swanson, H.R.; Mansager, E.R. (1985). Alternate pathways of metribuzin metabolism in soybean. Formation of N-glucoside and homoglutathione conjugates. Pestic. Biochem. Physiol., 23, 56-65.

Gressel, J. (1985). Herbicide tolerance and resistance: alteration of the site of activity. In weed physiology. Vol. II (Duke, S.O., ed), CRC press.

Haque, A; Weisberger, I; Klein, W. (1976). Buturon-^{14}C-bound residue complexes in wheat plants. Chemosphere, 3, 167-177.

Harvey Jr. J.; Han, J.C.-Y.; Reiser, R.W. (1978). Metabolism of oxamyl in plants. J. Agric. Food Chem., 26,3, 529-536.

Hatzios, K.K.; Penner, D. (1988). Metribuzin. In "Herbicides - chemistry, degradation and mode of action" (Kearney, P.C.; Kaufman, D.D. eds.) Vol. 3. Marcel Dekker.

Helson, V.A. (1967). Culture methods used in the Plant Research Institute phytotron. Greenhouse-Garden-Grass. 6. 6-16.

Herbicide Handbook of the weed Society of America (1983)

Higushi, T. (1985). Biosynthesis of lignin. In "Biosynthesis and biodegradation of wood components" (Higushi, T., ed:) Academic press.

Hoagland, D.R.; Arnon, D.I. (1950). The water culture method for growing plants without soil. Calif. Agric. Exp. Stn. Circ. 547.

Honeycutt, R.C.; Adler, I.L. (1975). Characterization of bound residues of nitrogen in wheat straw. J. Agric. Food Chem., 23, 6, 1097-1101.

Hori, H.; Elbein, A.D. (1985). Biosynthesis of plant cell wall polysaccharides. In "Biosynthesis and biodegradation of wood components" (Higushi, T. ed.) Academic press.

Huber, R.; Otto, S. (1983). Bound pesticide residues in plants. In "Pesticide chemistry" (Miyamoto, J. and Kearney, P.C. eds.) Vol. 3, Pergamon, Oxford.

Johnston, K. (1985). Supercritical fluids. In "The Kirk-Othmer concise encyclopedia of chemical technology". 1141-1142. J. Wiley and Sons.

Kaufman, D.D.; Still, G.G.; Paulson, G.D.; Bandal, S.K. (eds) (1976). "Bound and conjugated pesticide residues". ACS Symposium series 29.

Khan, S.U.; Hamilton, H.A. (1980). Extractable and bound (nonextractable) residues of prometryn and its metabolites in an organic soil. J. Agric. Food Chem., 28, 1, 126-132.

Khan, S.U. (1980). Plant uptake of unextracted (bound) residues from an organic soil treated with prometryn. J. Agric. Food Chem., 28, 6, 1096-1098.

Khan, S.U. (1982). Bound residues in soil and plants. Res. Rev. 84, 1-25.

Khan, S.U.; Zhang, L-Z.; Akhtar, M.H. (1984 a). Bound residues of deltamethrin in bean plants. J. Agric. Food Chem., 32, 5, 1141-1144.

Khan, S.U.; Stratton, G.D.; Wheeler, W.B. (1984 b). Characterization of bound (nonextractable) residues of dieldrin, permethrin and carbofuran in radishes. J. Agric. Food Chem., 32, 5, 1189-1191.

Khan, S.U.; Kacew, S.; Molnar, S.J. (1985 a). Bioavailability in rats of bound ^{14}C-atrazine. J. Agric. Food Chem., 36, 3, 645-648.

Khan, S.U.; Warwick, S.I.; Marriage, P.B. (1985 b). Atrazine metabolism in resistant and suceptible biotypes of Chenopodium album L., Chenopodium Strictum Roth. and Amaranthus powellii S. Walts. Weed Res., 25, 33-37.

Khan, S.U.; Dupont, S. (1987). Bound pesticide residues and their bioavailability. In "Pesticide science and biotechnology". Sixth IUPAC Congress of pesticide chemistry (Greenhalgh, R. and Roberts, T.R. eds.). Blackwell scientific publications.

Khan, S.U.; Kacew, S.; Dupont, S.; Stratton Jr., G.D.; Wheeler, W.B. (1987). Bioavailability in rats of bound residues from radishes treated with either radiolabeled dieldrin or carbofuran. J. Agric. Food Chem., 35, 1, 89-93.

Kirk, T.K.; Shimada, M. (1985). Lignin biodegradation: the microorganisms involved and the physiology and biochemistry of degradation by white rot fungi. In "Biosynthesis and biodegradation of wood components" (Higushi, T. ed.). Academic press.

Klein, W.; Scheunert, I. (1982). Bound pesticide residues in soil, plants and food with particular emphasis on the application of nuclear techniques. In "Agrochemicals; fate in food and the environment". Proc. Internat. Symp. IAEA. SM 263-38.

Kolattukudy, P.E.; Espelie, K.E. (1985). Biosynthesis of cutin, Suberin and associated waxes. In "Biosynthesis and biodegradation of wood components" (Higushi, T. ed). Academic press.

Köll, P.; Metzger, J. (1978). Thermal degradation of cellulose and chitin in supercritical acetone. Angew. Chemie (Intermat. ed.), 17, 10, 754-755.

Kovacs Jr., M.F. (1986). Regulatory aspects of bound residues (chemistry). Res. Rev., 97, 1-17.

Lamoureux, G.L.; Rusness, D.G. (1986). Xenobiotic metabolism in higher plants. In " Xenobiotic conjugation chemistry" (Paulson, A.D.; Caldwell, J.; Hutson, D.H.; Menn, J.J. eds.) ACS symp. ser. 299, 63-105.

Langebartels, C.; Harms, H. (1985). Analysis for nonextractable (bound) residues of pentachlorophenol in plant cells using a cell wall fractionation procedure. Ecotoxicol. environ. safety, 10, 268-279.

Mangeot, B.L.; Slife, F.E.; Rieck, C.E. (1979). Differential metabolism of metribuzin by two soybean (Glycine max) cultivars. Weed Sci., 27, 3, 267-269.

Marshall, T.C.; Dorough, H.W. (1977). Bioavailability in rats of bound and conjugated plant carbamate insecticide residues. J. Agric. Food Chem., 25, 5, 1003-1009.

Martens, J.W. (1984). "Diseases of field crops in Canada: an illustrated compendium". Canadian phytopathological society.

Menn, J.J.; Still, G.G. (1977). Metabolism of insecticides and herbicides in higher plants. In CRC critical reviews in toxicology.

Montgomery, M.L., Freed, V.H. (1961). The uptake, translocation and metabolism of of simazine and atrazine by corn plants. Weeds, 9, 231-237.

Montgomery, M.L., Freed, V.H. (1964). Metabolism of triazine herbicides by plants. J. Agric. Food Chem., 12, 1, 11-14.

Mostafa, I.Y.; Zayed, S.M.A.D.; Farghaly, M. (1986) Bioavailability to rats of methomyl and carbofuran bound residues in sweet potato. In "Quantification, nature and bioavailability of bound ^{14}C-pesticide residues in soil, plants and food". IAEA publication.

Paulson, G.D.; Jacobsen, A.M.; Still, G.G. (1975). Animal metabolism of propham (isopropyl carbanilate): the fate of residues in alfalfa when consumed by the rat and sheep. Pestic. Biochem. Physiol., 5, 523-535.

Pillmoor, J.B.; Gaunt, J.K.; Roberts, T.R. (1984). Examination of bound (non extractable) residues of MCPA and Flamprop in wheat straw. Pestic. Sci., 15, 375-381.

Pillmoor, J.B., Roberts, T.S. (1985). Approaches to the study of non extractable (bound) residues in plants. In "Progress in pesticide biochemistry and toxicology" (Huston, D.H. and Roberts, T.S. eds.) Vol. 4. J. Wiley and son.

Roberts, T.R. (1984). Non-extractable pesticide residues in soil and plants. Pure Appl. chem., 56, 7, 945-956.

Rouchaud, J.; Decallone, J.R.; Meyer, J.A. (1978). The nature of bound residues derived from triforine in barley plants. Pestic. Sci. 9, 74-78.

Rouchaud, J.; Moons, C.; Meyer, J.A. (1979 a). Characterization of bound residues of (^{3}H)triforine in the straw of barley grown in the field. Pestic. Sci., 10, 438-446.

Rouchaud, J.; Moons, C.; Meyer, J.A. (1979 b). Characterization of bound residues of (^{3}H)triforine in barley grains grown in the field. Pesti. Sci., 10, 509-518.

Sandermann Jr., H; Scheel, D.; V.d. Trenk, T. (1983). Metabolism of environmental chemicals by plants - copolymerization into lignin. J. Appl. Polymer Sci.: Appl. Polymer Symp., 32, 407-420.

Scheel, D.; Sandermann Jr., H. (1981). Metabolism of 2,4-dichlorophenoxyacetic acid in cell suspension cultures of soybean (Glycine max L.) and wheat (Triticum aestivum L.) II: Evidence for incorporation into lignin. Planta, 152, 253-258.

Scheel, D.; Schäfer, W.; Sandermann Jr., H. (1984). Metabolism of pentachlorophenol in cell suspension cultures of soybean (Glycine max L.) and wheat (Triticum aestium L.). General results and isolation of lignin metabolites. J. Agric. Food Chem., 32, 6, 1237-1241.

Schneider, G.M. (1978). Physicochemical principles of extraction with supercritical gases. Angew. Chem. (Internat. ed.), 17, 10, 716-727.

Schwack, W. (1988). Photoinduced additions of pesticides to biomolecules. 2: model reactions of DDT and methoxychlor with methyl oleate. J. Agric. Food Chem., 36, 3, 645-648.

Shaw, D.R.; Peeper, T.F.; Nofziger, D.L. (1986). Evaluation of chlorophyll fluorescence parameters for an intact plant bioassay. Crop Sci., 26, 756-760.

Smith Jr. A.E.; Wilkinson, R.E. (1974). Different absorption, translocation and metabolism of metribuzin in soybean cultivars. Physiol. Planta., 32, 252-257.

Smith, A.E. (1979). Metabolism of 2,4-DB by white clover (Trifolium repens) cell suspension cultures. Weed Sci., 27, 392-396.

Spiteller, M. (1985). Extraction of soil organic matter by supercritical fluids. Org. Geochem., 8, 1, 111-113.

Still, G.G.; Morris, F.A.; Iwan, J. (1976). Solubilization of bound residues from 3,4 dichloroaniline-^{14}C and propanil-phenyl-^{14}C treated rice root tissues. In "Bound and conjugated pesticide residues" (Kanfman, D.D.; Still, G.G., Paulson, G.D.; Bandal, S.K. eds.) ACS symp. series 29.

Still, G.G.; Balba, H.M.; Mansager, E.R. (1981). Studies on the nature and identity of bound chloroaniline residues in plants. J. Agric. Food Chem., 29, 4, 739-746.

Stratton Jr., G.D.; Wheeler, W.B. (1983). Fate of diedrin in radishes. J. Agric. Food Chem. 31, 5, 1076-1079.

Stratton Jr., G.D.; Wheeler, W.B. (1986). Characterization of bound residues in plants. In "Quantification, nature and bioavailability of bound ^{14}C-pesticide residues in soil, plants and food. IAEA publication.

Sutherland, M.L. (1976). Nature of propanil bound residues in rice plants as measured by plant fractionation and animal bioavailability experiments. In "Bound and conjugated pesticide residues" (Kaufman, D.D.; Still, G.G.; Paulson, G.D.; Bandal, S.K. eds.). ACS Symp. series 29.

Wargo, J.P.; Honeycutt, R.C., Adler, I.L. (1975). Characterization of bound residues of nitrofen in cereal grains. J. Agric. Food Chem., 23, 6, 1097-1101.

Wilke, G. (1978). Extraction with supercritical gases. Angew. Chem. (Internat. ed.), 17,10, 701-702.

Yih, R.Y.; Mc Rae, D.H.; Wilson, H.F. (1968). Metabolism fo 3'-4' dichloropropioanilide: 3,4 dichloroaniline-lignin complex in rice plants. Science, 161, 376-377.