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Application of microcolumn liquid chromatography–continuous-flow fast atom bombardment mass spectrometry in environmental studies of sulfonylurea herbicides

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ABSTRACT

The use of 0.25-mm I.D. packed capillary liquid chromatography columns coupled with continuous-flow fast atom bombardment (FAB) mass spectrometry has proven to be a very valuable technique, especially for the identification of unknown sulfonylurea herbicide metabolites. Several new and unusual heterocycle ring-opened metabolites and hydrolysis products were identified, and metabolic pathways were proposed. Typical column flow-rates are 1–2 μl/min, which allows direct coupling with no sample splitting. This is important in our metabolite identification work, since we are usually sample-limited. Techniques for increasing injection volume to allow analyses of dilute solutions and the use of polymeric packing for separation of polar metabolites are discussed. The FAB mass spectra usually provide unequivocal molecular weights and structurally useful fragments ions, which often allows structure assignments on exceedingly small quantities of isolated metabolites.

INTRODUCTION

The environmental impact of crop protection chemicals is of growing concern, and DuPont is developing many new environmentally safe agricultural products. These new products are very specific to the target organisms, have very low use rates and break down rapidly in the environment. Prime examples of these new products are the sulfonylurea herbicides, which have use rates 100-fold less than earlier herbicides and are metabolized extensively in plants, animals and soil [1]. These herbicides were first introduced by DuPont in 1982, and we now have developed and introduced nine new sulfonylureas. These compounds act on an enzyme found only in plants [1], and have no significant toxicity to animals or other non-target organisms.

The low use rate and extentisive breakdown of the sulfonylurea herbicides requires very sensitive analytical techniques for identification of their environmental metabolites, since only very small quantities of metabolites are isolated. These compounds are extremely thermally labile, precluding their analysis by gas chromatography (GC)–mass spectrometry (MS). Attempts at derivatization prior to GC–MS have

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been unsuccessful due to chemical breakdown during derivatization or thermal instability of the derivative. The method of choice for identification of sulfonylurea herbicide metabolites is liquid chromatography (LC)–MS [2]. Since these compounds are very temperature-sensitive, their mass spectra typically show very weak or no molecular ions with electron ionization, chemical ionization and thermospray ionization when the thermospray vaporizer temperature is set for optimum sensitivity [2]. A low-temperature mass spectral ionization technique, such as fast atom bombardment (FAB) or electrospray–ion spray [3], is needed to obtain prominent molecular ions for unequivocal molecular-weight assignments.

Miniaturization of the LC column gives increased mass sensitivity, since the minimum amount detectable is directly proportional to the square of the column radius [4]. Use of a 0.25 mm I.D. packed capillary column will give a 300-fold increase in sensitivity over a conventional 4.6 mm I.D. column, and the low flow-rates facilitate MS interfacing. In previous work, the use of 0.25 mm I.D. packed fused-silica LC columns allowed us to obtain improved performance in reversed-phase LC–MS analyses with a moving belt interface [5–7]. The 1–2-μl/min flow-rates used with these columns are ideally suited to interfacing with continuous-flow FAB, since no effluent splitter or make-up flow are required [7–9].

A further advantage of micro- over conventional LC columns is that the 1000-fold reduction in solvent consumption greatly reduces costs and solvent waste.

In previous work [9], the utility of microcolumn LC interfaced with continuous-flow FAB-MS for the identification of microbial metabolites of sulfonylurea herbicides was demonstrated, using the soil bacterium *Streptomyces griseolus*. Crude broth extracts were injected directly. Identification of plant, animal or soil metabolites typically requires some cleanup, since these extracts are more complex mixtures.

This paper shows the utility of LC–FAB-MS for the identification of sulfonylurea herbicide metabolites obtained in environmental studies.

EXPERIMENTAL

A Beckman (Berkeley, CA, USA) Model 114M LC pump was used in the constant pressure mode. Column pressures were typically 100–200 bar to obtain flow-rates of 1–2 μl/min. A Valco (Houston, TX, USA) Model C14W submicroliter injection valve was used with a 0.1-μl rotor. Fused-silica columns (J&W Scientific, Folsom, CA, USA), 30 cm × 0.25 mm I.D. were slurry packed as described in ref. 5 with 3-μm Zorbax ODS (DuPont, Wilmington, DE, USA), Nucleosil ODS (Macherey-Nagel, Düren, Germany), Spherisorb ODS-2 (Phase Sep, Norwalk, CT, USA) or 10- and 5-μm PRP-1 (Hamilton, Reno, NV, USA). The transfer line (epoxy glued into the column prior to packing) was 0.025 mm I.D. 0.195 mm O.D. fused-silica tubing (Polymicro Technologies, Phoenix, AZ, USA). An Isco (Lincoln, NE, USA) Model CV4 ultraviolet (UV) detector was used on-line at 238 nm wavelength. A 1-cm length of the polyimide coating on the 0.025 mm I.D. fused-silica transfer line was burned off to serve as the UV detector cell. Teflon (polytetrafluoroethylene) tubing of 0.18 mm I.D., 1.6 mm O.D. (Alltech Stock No. 35676) was cut into 5-mm lengths to serve as unions in the transfer line before and after the UV detector. The Teflon tubes were reamed out with a short length of transfer line, then cleaned with solvent prior to use. The continuous-flow FAB probe was designed and fabricated in our laboratory and is

interchangeable with a moving-belt interface, as described in ref. 7. A Finnigan MAT Model 8230 magnetic-sector mass spectrometer was used with an Ion Tech FAB gun using xenon gas and 8-kV anode voltage. The ion source temperature was 200°C, which heats the FAB target by radiation to 45–50°C. Typical MS parameters were: resolution, 1000; source ion gauge reading, $4 \cdot 10^{-4}$ mm; scan rate, 10 s/decade; mass range, 95–650.

RESULTS AND DISCUSSION

System description

Incorporation of an on-line UV detector is especially important when working with low-molecular-weight (below 500) compounds. The glycerol matrix ions are more intense at lower mass, making detection of trace level LC peaks in the reconstructed ion current chromatogram difficult. The Isco CV4 UV detector was designed for capillary zone electrophoresis, but works well for micro-LC.

The lifetime of packed capillary LC columns in our laboratory is typically 2–6 months. Since we often inject biological extracts which leave material at the head of the column that can cause a loss in performance, we periodically cut off 0.5–1 cm from the head of the column to restore performance. All analyses were carried out using isocratic conditions, with acetonitrile (0–70%)–water (pH 3, formic acid)–10% or 5% glycerol (FAB matrix). In most cases, gradient elution was not essential since the samples were cleaned up by preparative LC or solid-phase extraction. Addition of glycerol to the mobile phase has caused no problems with the chromatography or LC pump. Initial work was carried out using 10% glycerol in the mobile phase. Reduction to 5% glycerol gave better stability and chromatographic performance, likely due to obtaining a thinner film of glycerol on the FAB target.

Injection techniques for dilute solutions

Although microcolumns increase mass sensitivity, there is no increase in concentration sensitivity [3] since the injection volume must be reduced in proportion to the column diameter to avoid band broadening. In conventional LC, increased concentration sensitivity is obtained by dissolving the analyte in a weak LC solvent (high percentage of water) which allows injection of a large volume, since the analytes concentrate at the head of the column if they are non-polar to moderately polar. We have used this same technique in micro-LC to allow injection of 1–2-μl volumes. Another technique we have developed for analyses of dilute solutions for identification of small quantities is to dissolve the sample in a volatile solvent, such as acetonitrile or methanol, and coat 1–5 μl onto the groove in the Valco 0.1-μl rotor [5–7]. The coated rotor is held in the "inject" position for 10 s. While this technique worked well with the moving-belt interface (*ca.* 80% recovery of analytes), we obtained low recoveries (*ca.* 5%) using the standard W rotor (Valcon S, polyphenylene sulfide) in the FAB mode, apparantly due to the 10% glycerol in the mobile phase preventing efficient removal of analytes from the rotor. We evaluated rotors obtained from Valco made from three other materials: WF (polyether–Teflon), WT (Vespel polyimide) and WP (polyphenylene sulfide cross-linked with Teflon), and obtained good recoveries (50–70%) only with the WP rotor. Recoveries were determined by injecting ^{14}C-labelled compounds, and collecting the peaks for liquid scintillation counting.

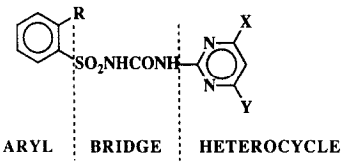

Fig. 1. General structure of sulfonylurea herbicides.

Polymeric column packings

We evaluated the use of PRP-1 (polysterene–divinylbenzene copolymer) column packing for separating polar metabolites. Initially we prepared a 30-cm column using 10-μm particles. Efficiency was poor (1800 theoretical plates), but peaks were symmetrical and selectivity was good. We were able to obtain on-column concentration of polar compounds by dissolving the sample in a weak LC solvent (90% water), allowing injection of 1–2-μl volumes. Addition of 5% glycerol to the sample solvent was necessary to maintain FAB stability during elution of the solvent. We recently obtained 5-μm PRP-1 packing, and prepared a 30-cm column which had 10 000 theoretical plates.

Chromatography of sulfonylurea herbicides

A general structure of sulfonylurea herbicides is shown in Fig. 1. The sulfonylurea bridge portion of the molecule is quite labile. The bridge cleaves thermally and hydrolytically on both sides of the carbonyl to give the aryl sulfonamide and heterocycle amine.

A separation of eight sulfonylurea herbicides is presented in Fig. 2. This sep-

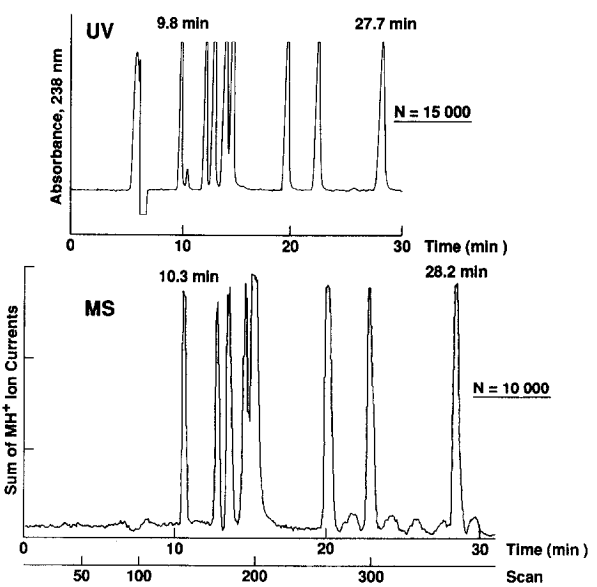

Fig. 2. Separation of eight sulfonylurea herbicides (50 ng/peak). In order of elution, the active ingredients in: Accent, Harmony, Ally, Glean, Oust, Londax, Express and Classic. Column: 27 cm × 0.25 mm I.D. Spherisorb ODS-2 (3 μm). Mobile phase: acetonitrile–water (pH 3, formic acid)–glycerol (50:40:10).

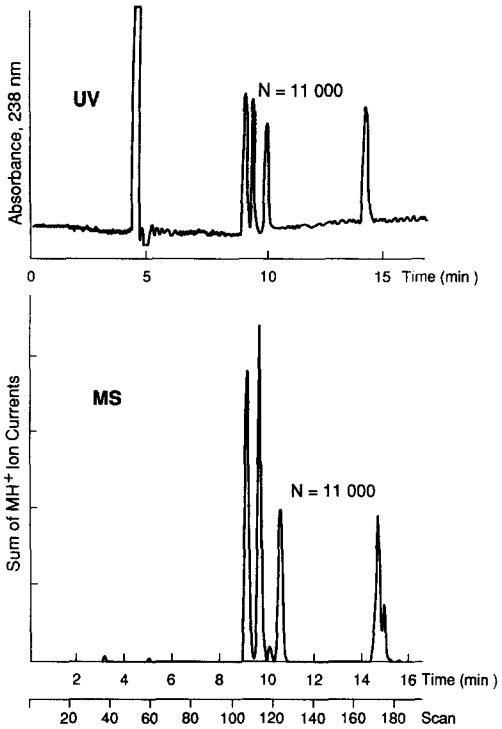

Fig. 3. Separation of four sulfonylurea herbicides (10 ng/peak). In order of elution, the active ingredients in: Harmony, Ally, Glean and Londax. Column: 20 cm × 0.25 mm I.D. Spherisorb ODS-2. Mobile phase: acetonitrile–water–glycerol (50:45:5).

aration was obtained by injection of 0.1 μl of a 0.5-μg/μl solution of each component in acetonitrile–water (50:50, 50 ng/peak), with the UV detector on-line. The LC retention times in the MS-extracted ion chromatogram (sum of protonated molecular ions) are 0.5 min later than those obtained with the UV detector, due to the 1-m length of 0.025 mm I.D. transfer line between the UV and MS (0.5-μl volume). The theoretical plate count (N) obtained on the last-eluting peak dropped from 15 000 with the UV detector to 10 000 with the MS. Most of the drop in plate count appears to be due to slow desorption off the FAB target, since when we reduced the glycerol level in the mobile phase from 10% to 5%, there was no difference in efficiency between the UV and MS chromatograms. This is illustrated in Fig. 3, a separation of four sulfonylurea herbicides at the 10 ng/peak level (we routinely obtain strong, good-quality FAB mass spectra on 10 ng/component injected into the LC) obtained using 5% glycerol in the mobile phase. N values of 11 000 were determined on the third-eluting peak on both the UV and MS chromatograms. These data also show there is no significant band spreading in the transfer line between the UV and MS.

As in GC–MS, plotting the individual mass chromatograms allows one to use the MS as a multiple-ion detector to effectively improve the chromatographic resolution.

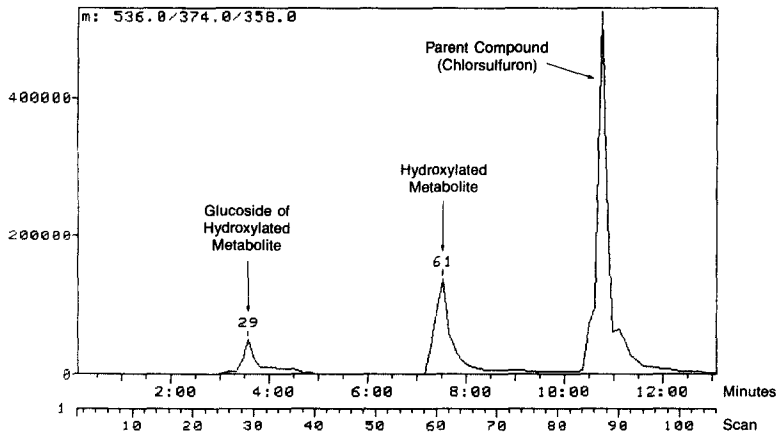

Fig. 4. Separation of chlorsulfuron and two wheat metabolites (extracted-ion current of the protonated molecular ions, *m/z* 536, 374, 358). Column as in Fig. 1. Mobile phase: acetonitrile–water–glycerol (40:50:10).

Chlorsulfuron wheat metabolites

Most sulfonylurea herbicides are selective for a particular crop, *i.e.*, they kill weeds but do not harm the crop. The basis of selectivity is mainly due to rapid conversion to inactive metabolites by the crop plant, but not by weeds [1]. For example, chlorsulfuron, the active ingredient in Glean herbicide, is used to control weeds in wheat and other cereal crops. It is rapidly metabolized in wheat plants by hydroxylation on the phenyl ring, followed by conjugation with glucose. Fig. 4 shows the extracted-ion chromatogram of the protonated molecular ions obained on a mixture of chlorsulfuron and the two wheat metabolites. Fig. 5 shows the background-subtracted FAB mass spectra obtained on chlorsulfuron and the wheat metabolites. The protonated molecular ion is the base peak in all three spectra, and all show prominent fragment ions, typical of sulfonylureas, due to the protonated triazine amine, isocyanate and urea. These fragments show there was no change in the triazine portion of the molecules, which helps elucidate their structures.

Chlorsulfuron hydrolysis products

In aqueous hydrolysis studies at pH 5 with [^{14}C]chlorsulfuron, five degradation products were formed. The products were separated and purified by preparative LC, and identified by LC–FAB-MS. Proposed hydrolysis pathways, given in Fig. 6, are based on chemically logical hydrolysis reactions involving cleavage of the sulfonylurea bridge or *O*-demethylation followed by hydrolytic cleavage of the triazine ring. Identification of the sulfonamide, triazine amine and O-desmethylchlorsulfuron products were confirmed by comparison of LC retention times and FAB mass spectra with synthetic standards, using a 27-cm Zorbax ODS column and an acetonitrile–water–glycerol (50:40:10) mobile phase. The FAB mass spectra of the triazine amine and sulfonamide showed only the protonated molecular ions and glycerol adduct ions with no significant fragment ions, while the spectrum of O-desmethylchlorsulfuron shows the fragment ions expected from demethylation on the triazine portion of the molecule.

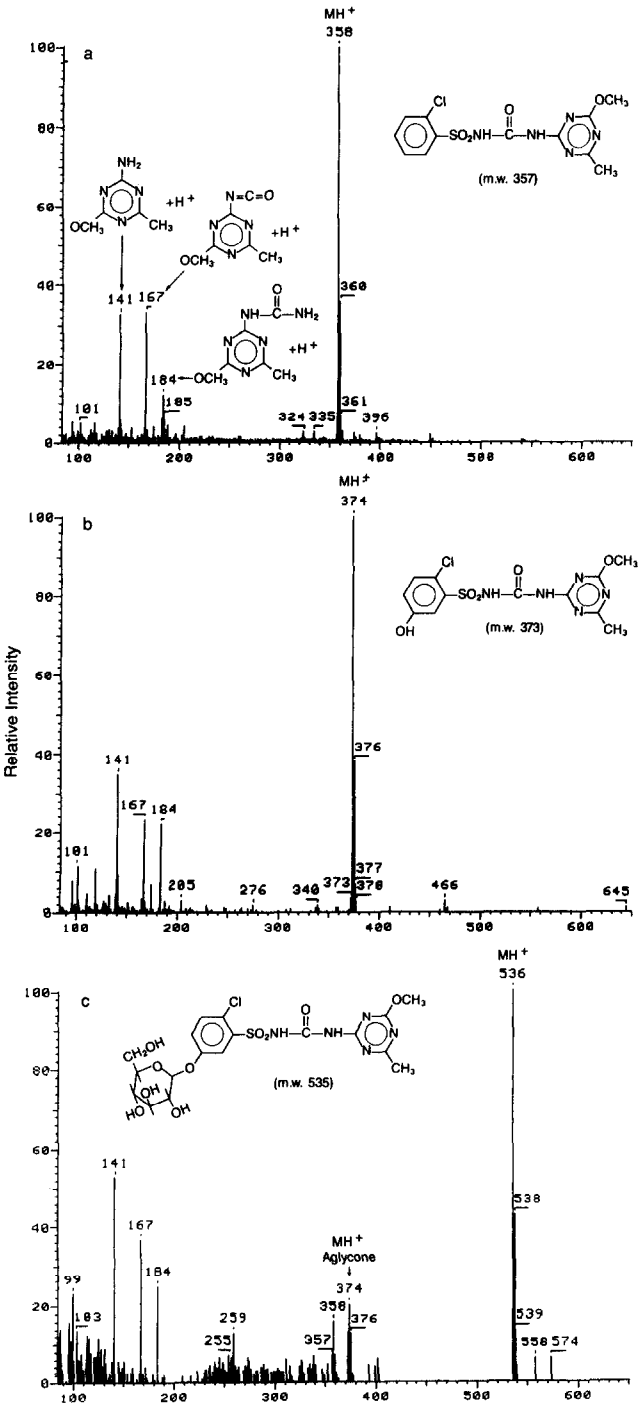

Fig. 5. FAB mass spectra of (a) chlorsulfuron; (b) hydroxylated chlorsulfuron wheat metabolite; and (c) glucoside wheat metabolite. m.w. = Molecular weight.

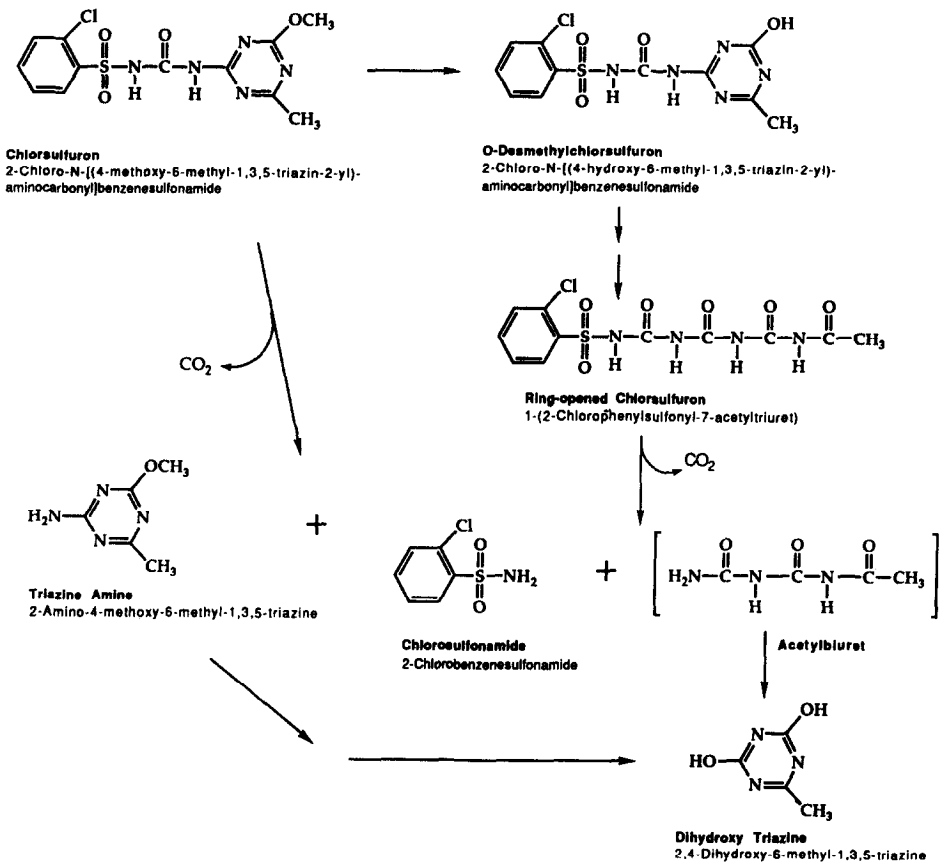

Fig. 6. Proposed hydrolytic degradation pathways for chlorsulfuron at pH 5.

The FAB mass spectrum of the triazine ring-opened product (Fig. 7) shows it is a monochloro compound of molecular weight 362, an unusual 5 mass units higher than the parent compound. Lack of heterocycle fragment ions indicates a major change in this portion of the molecule. NMR data showed four aromatic protons and an acetyl methyl, consistent with the proposed structure. This novel compound forms from O-desmethylchlorsulfuron by cleavage of the triazine ring in a two-step hydrolysis process.

Initial analyses of the most polar hydrolysis product, the dihydroxy methyl triazine, were unsuccessful since it eluted near the void volume of the ODS column and sample contaminants suppressed its ionization. Analysis of this sample on a PRP-1 column (30 cm × 0.25 mm I.D., 10 μm) resulted in a separation of the polar product from the contaminants, and its FAB mass spectrum showed a prominent protonated molecular ion at m/z 128. The structure was confirmed by electron impact probe/library search on a more purified sample.

Bensulfuron methyl poultry metabolites

Three unknown poultry metabolites of bensulfuron methyl, the active ingredient in Londax and Mariner rice herbicides, were purified by preparative LC and

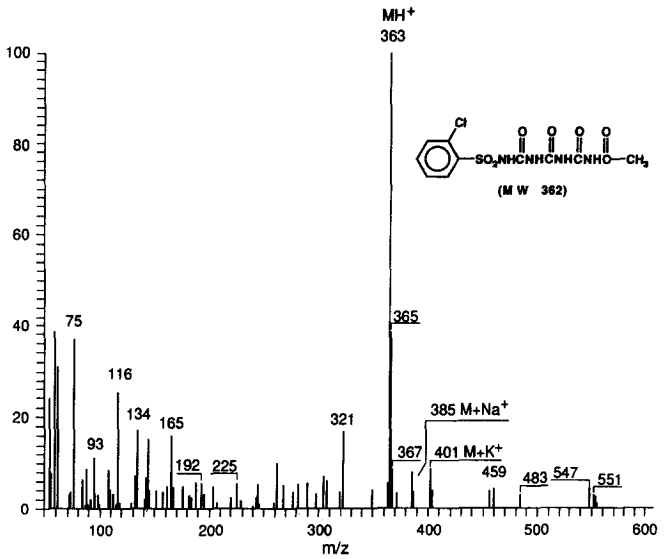

Fig. 7. FAB mass spectrum of triazine ring-opened chlorsulfuron hydrolysis product. MW = molecular weight.

identified by LC–FAB-MS. A proposed metabolic pathway is given in Fig. 8. These metabolites were obtained in separate studies using [^{14}C]phenyl-labelled bensulfuron methyl and pyrimidine-2-^{14}C-labelled bensulfuron methyl. A Spherisorb ODS-2 mi-

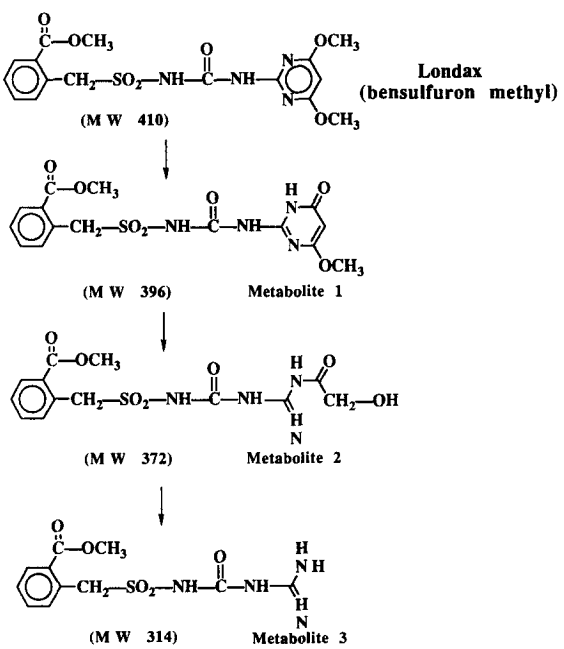

Fig. 8. Proposed metabolic pathway for bensulfuron methyl poultry metabolites.

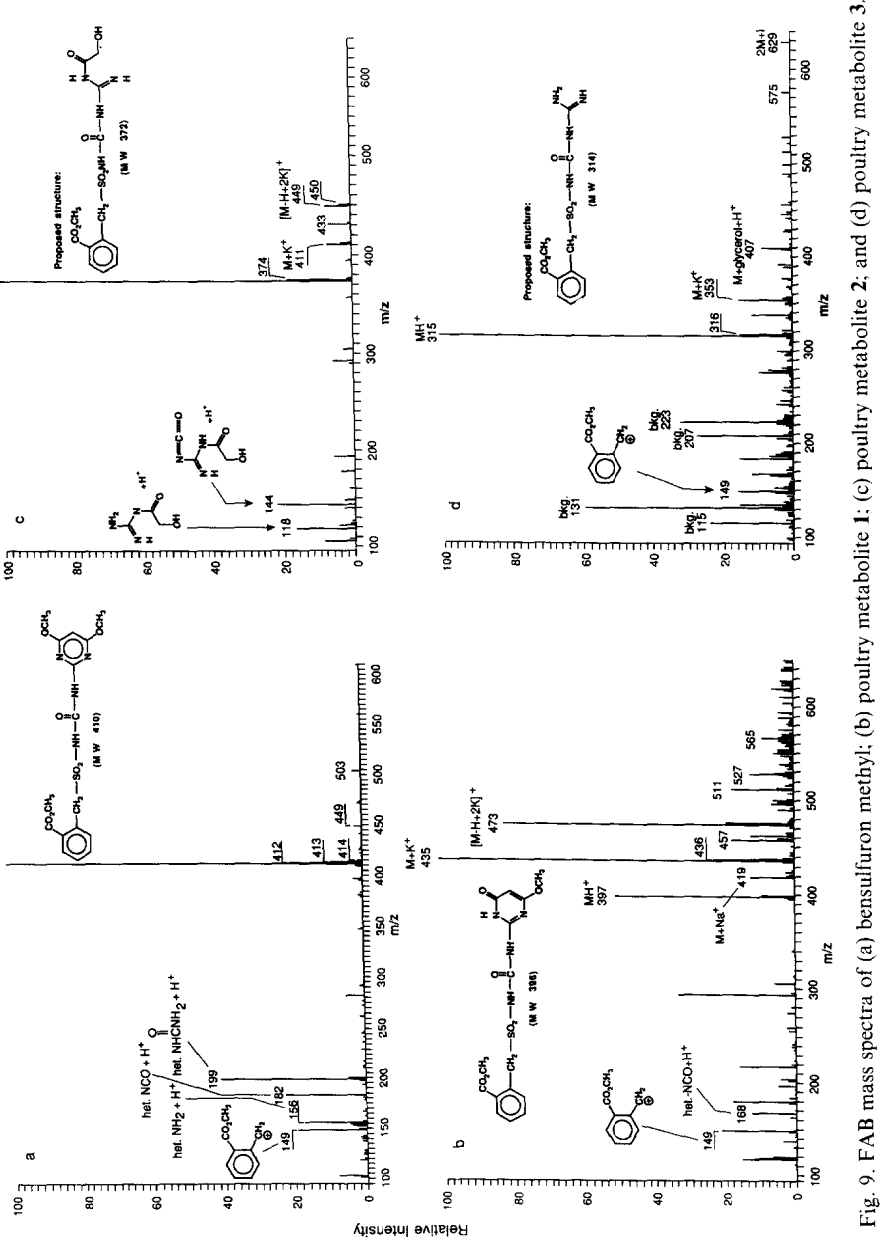

Fig. 9. FAB mass spectra of (a) bensulfuron methyl; (b) poultry metabolite **1**; (c) poultry metabolite **2**; and (d) poultry metabolite **3**.

crocolumn (27 cm × 0.25 mm I.D.) was used with an acetonitrile–water–glycerol (50:40:10) mobile phase. Bensulfuron methyl eluted at 24 min, metabolite **1** at 8.7 min and metabolite **2** at 9.2 min. Metabolite **3** eluted near the void volume (*ca.* 6 min) on the ODS columns, and did not give a spectrum due to suppression of ionization by coeluting impurities. Use of a PRP-1 column was again successful in separating the metabolite from contaminants and allowed us to obtain a FAB spectrum. The spectrum of the parent (Fig. 9a) shows the protonated molecular ion as base peak, the expected protonated heterocycle fragments and a significant aryl fragment at m/z 149. This aryl fragment, likely a tropylium ion, appears in the spectra of all three metabolites, showing there is no change in this portion of the molecule. The spectrum of metabolite **1** (Fig. 9b) shows a prominent MH^+ ion and atypically strong potassium ion adducts, indicating the sample contained potassium. The spectrum of metabolite **2** (Fig. 9c) shows the MH^+ as base peak, and protonated fragments of the ring-opened pyrimidine portion of the molecule in support of the proposed structure. The spectrum of metabolite **3** (Fig. 9d), obtained using the PRP-1 column, shows it has a molecular weight of 314. The proposed structure was confirmed by comparison of its FAB mass spectrum with that obtained on a synthetic sample.

The proposed metabolic pathway is based on a logical sequence of metabolic reactions, *i.e.*, demethylation (metabolite **1**), oxidation–hydrolytic ring-opening–decarboxylation (metabolite **2**) and hydrolysis (metabolite **3**).

CONCLUSIONS

Microcolumn LC coupled with continuous-flow FAB-MS has proven to be a practical technique for the identification of sulfonylurea herbicide metabolites and degradates. High-quality, strong-intensity FAB mass spectra are routinely obtained on 10 ng (25 Pmol) quantities injected into the LC system. Use of this technique has facilitated the identification of many intractable compounds, and has provided information that was previously impossible or very difficult to obtain.

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