# Confirmatory Assay for Ivermectin in Cattle Tissue using Chemical Ionization Mass Spectrometry/Mass Spectrometry (MS/MS)

**P. C. Tway† and G. V. Downing**
Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065, USA

**J. R. B. Slayback, G. S. Rahn and R. K. Isensee**
Oneida Research Services, Inc., New Hartford, New York 13413, USA

**A method based on direct exposure, positive ion, chemical ionization mass spectrometry/mass spectrometry (ms/ms) was developed for the confirmatory assay of the antiparasitic drug, ivermectin, in animal tissue. Following extraction, column and preparative liquid chromatography, mass spectrometric/mass spectrometric analysis of the drug in liver samples provided reliable detection limits to 8–10 parts-per-billion at a signal: noise of greater than 10:1. Blank tissue consistently displayed no chemical/matrix interference. Besides the development of a confirmatory assay, the study also demonstrates the analytical capability and the role of MS/MS *vis-a-vis* other applied mass spectrometric techniques.**

## INTRODUCTION

The avermectins are a new family of antiparasitic compounds which are broad spectrum agents active at very low doses. Ivermectin is a mixture of avermectin homologues and is comprised of not less than 80% 22,23 dihydroavermectin $B_{1a}$ and not more than 20% 22,23 dihydroavermectin $B_{1b}$. The structures of these compounds are shown in Fig. 1. Bioavailability and pharmacokinetic studies of ivermectin in animal tissues have been performed using a highly specific and sensitive chemical derivatization liquid chromatographic fluorescence assay.[1] Because of regulatory agency requirements, a confirmatory assay was sought which preferably would yield high chemical specificity concomitant with providing positive structural information for unambiguous drug residue identification.

To approach such an assay three influencing factors, *viz*, detection level, tissue/matrix chemical interference, and the inherent physiochemical properties of the drug itself were considered. Since ivermectin is an extremely potent drug, the confirmatory assay required a sensitivity of 10–25 ppb (parts per billion; $10^{-9}$ g/g) to ensure detection of non-negligible residues. Gas chromatography coupled with mass spectrometry (GC/MS) is typically the method of choice for such an assay because of its sensitivity, specificity and ability to yield information characteristic of drug structure. However, on initial examination ivermectin appeared to be a poor candidate for GC/MS or mass spectrometry. The molecule is large (mol. wt 874), exhibits a high degree of non-volatility and is thermally labile with respect to its overall structure. These factors precluded gas chromatographic/mass spectrometric analysis with or without chemical derivatization. Furthermore, the molecule was observed to have a low ionization efficiency which affects overall assay detection limits.

In the course of this work to develop a confirmatory assay for the drug in tissue, numerous analytical and mass spectrometric techniques, targeted at either the intact ivermectin molecule or a structurally significant portion of the molecule, that is the aglycone, the monosaccharide, or the sugar moieties, were attempted. The monosaccharide is the ivermectin molecule with one sugar removed, and the aglycone with both removed. Techniques, such as field desorption (FD), fast atom bombardment (FAB), and liquid chromatography/mass spectrometry on the intact molecule or on the aglycone, did not have the required sensitivity. While GC/MS of the sugar moieties afforded the necessary sensitivity, the ions were in the mass range 100 and were not sufficiently unique to form a specific analysis. An assay based on the sugar moieties was not practical as these sugars are too volatile to handle through an assay clean-up procedure. Only solid probe/EI mass spectrometric analysis on the intact molecule had sufficient sensitivity and gave sufficient structural information to meet regulatory requirements, but despite extensive sample clean-up, even high resolution solid insertion mass spectrometry (10 000 resolution at 10% valley)

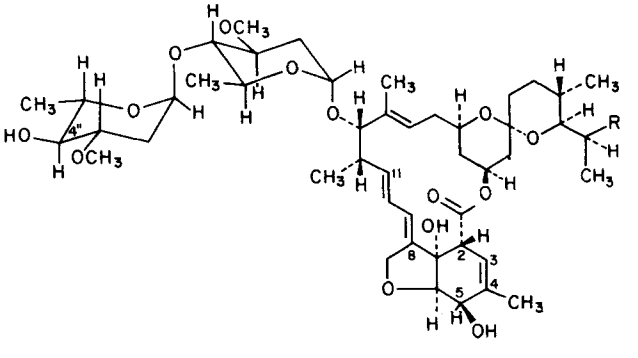

**Figure 1.** Structure of the avermectins; R = $C_2H_5$ for dihydroavermectin $B_{1a}$, R = $CH_3$ for dihydroavermectin $B_{1b}$.

† Author to whom correspondence should be addressed.

CCC-0306-042X/84/0011-0172 $02.50

did not afford the necessary specificity. Incipient material in the samples had the same exact masses as the ivermectin fragmentation ions. Moreover, efforts to form a chemical derivative to yield greater mass spectrometric sensitivity were unsuccessful at the low concentration levels necessary for this confirmatory assay.

Within the past several years, a newer form of mass spectrometry has received much attention. This technique is based on tandem mass spectrometry (MS/MS) and utilizes several stages of mass separation 'on-the-fly' to improve specificity and sensitivity of detection. Mass spectrometric equipment utilizing MS/MS technology is centered around both magnetic sector and quadrupole-based instruments.[2–6] Applications including quantitative GC/MS/MS in a pharmacokinetic assay,[7,8] the use of direct insertion MS/MS in structure analysis of synthetic medicinals,[9] and the role of MS/MS in drug metabolite screening[10] have been published. However, to date few studies have been reported specifically on the application of MS/MS to pharmaceutical-related problems.

In this paper we report on the development of a confirmatory assay for ivermectin employing direct exposure, positive ion, chemical ionization MS/MS methodology.

## EXPERIMENTAL

### General

A general flow diagram for sample work-up is shown in Fig. 2. Briefly, the drug is extracted from 5 g of tissue into iso-octane, carried through a series of liquid–liquid distributions, and then fractionated through a florisil column. The sample is evaporated to a small volume, injected onto a preparative reversed phase liquid chromatographic column, and the appropriate fraction collected for mass spectrometric analysis. A detailed procedure of the tissue extraction and liquid distributions has been published previously,[1] and the column and preparative liquid chromatography are described below in more detail.

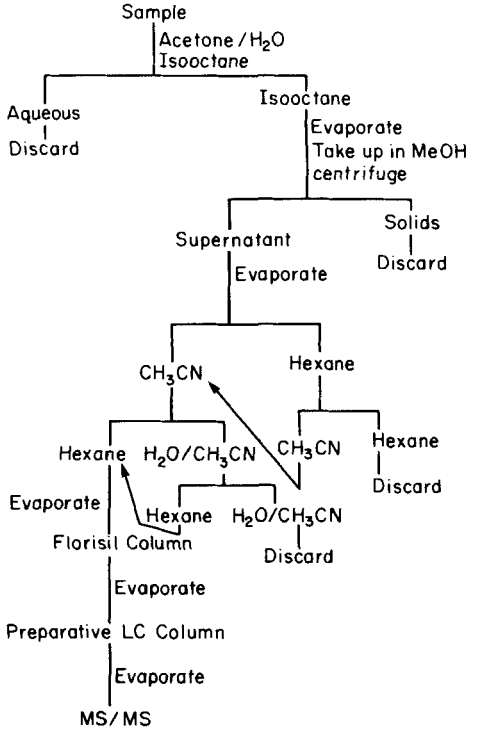


**Figure 2.** Flow diagram for the clean-up of ivermectin tissue residue samples.

### Reagents

Organic solvents were either Burdick and Jackson 'distilled in glass' or pesticide quality. All other reagents were of analytical grade purity. Aqueous solutions were prepared with water which had been doubly distilled. Water which was used in the LC mobile phase was filtered through a 5 μm Millipore filter. The florisil was 100–200 mesh (Floridin Company, Pittsburgh, PA), and was washed thoroughly with 3:1 chloroform–ethyl acetate, dried, washed with water, and heated overnight at 120 °C. It was stored in a tightly capped bottle until used.

### Column and preparative liquid chromatography

A gravity-fed florisil column (14 cm × 0.8 cm; reservoir, 7 cm) was prepared by placing a small plug of silanized glass wool in the bottom of the column and filling with 0.4 g of florisil. The column was washed with 5 ml chloroform containing 2% isobutyl alcohol immediately before use.

A Beckman Model 110 A liquid chromatographic pump, a Rheodyne sample valve with a syringe loading sample loop, and a LDC Spectromonitor III UV detector at 254 nm comprised the basic chromatographic instrumentation. The column was 50 cm × 1 cm packed with C-18 packing (12 μ, Dupont) and the precolumn was a C-18 Brownlee HPLC guard column (Rainin). The precolumn was changed whenever a significant pump pressure increase occurred, or when extraneous peaks appeared in a chromatogram of the standard. The chromatographic conditions were: mobile phase, methanol/water (90:10); flow rate, 5 ml $min^{-1}$; column temperature, 22 °C.

Because of the presence of substantial interfering material from the tissue the level of ivermectin (10–25 ppb) present in residue samples is too low to be seen by UV detection. It was, therefore, necessary to inject a large amount (4 μg) of an ivermectin standard onto the liquid chromatography system periodically to determine the retention time of a standard, and consequently the sample fraction to be collected from the preparative liquid chromatography run. The standard retention time was approximately 32 min; the sample fraction collected for MS/MS analysis was taken continuously from retention times 29.5 to 34.5 min. This large sample aliquot was taken to assure complete recovery. Following the injection of a high level standard, 40 μl of methanol was injected through the system to insure no memory effect in sample chromatograms. Figure 3 shows a typical preparative liquid chromatography run of a control sample.

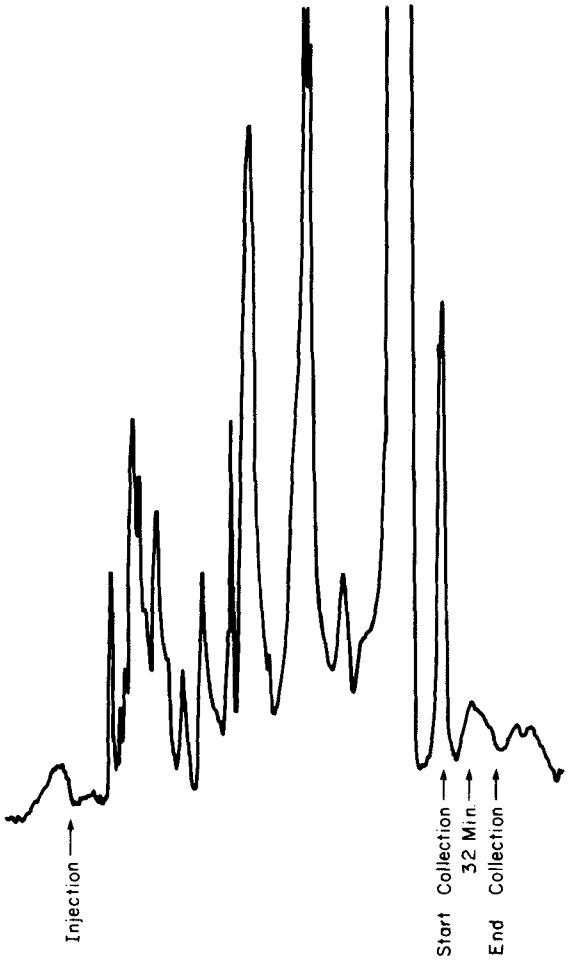


**Figure 3.** Preparative liquid chromatogram of a control liver sample showing sample fraction collected for MS/MS analysis.

## Mass spectrometry

Mass spectrometric and tandem mass spectrometric analysis of ivermectin was performed on a Finnigan MAT 4500-based triple stage quadrupole system equipped with a Finnigan MAT direct exposure probe. The ion source was run in the direct inlet, chemical ionization (CI) mode. Ion source temperature, electron energy and filament emission were set at 110 °C, 43 eV and 0.21 mA, respectively. Dynode and electron multiplier (EM) detector voltages were ±3000 V and 1200–2400 V. CI reagent gas was chemically-pure ammonia metered into the instrument through a high precision Whitey valve. An ion source pressure study using an ivermectin standard showed that the optimum ion source pressure was 37.3 ± 1.4 Pa (0.28 Torr; indicated reading). At this pressure and with an EM setting of 800 V, the CI plasma ions were $[NH_4]^+$ (3.5 V), $[N_2H_6+H]^+$ (0.15 V) and $[N_3H_9+H]^+$ (0.003 V).

In the MS/MS mode, chemically pure argon was used as the collision gas. Due to the energy dependence of MS/MS spectra in quadrupole instruments[11] a separate set of experiments were performed wherein the collision energy and pressure were varied from 10–30 V and 0.08–0.26 Pa, respectively. For ivermectin analysis in liver extracts, final optimization of these parameters with regard to specificity and sensitivity requirements occurred at −30 V (energy) and 0.13 Pa (pressure). Daughter ion experiments were run in either a quadrupole 1 (Q1) mass set or program RF (PRF) mode with Q1 resolution adjusted from 0.5–4 u depending on the experiment. Quadrupole 3 (Q3) was typically scanned from $m/z$ 100–800 in 0.3 s/scan (full scan daughter spectra), or scanned over $m/z$ 567.3 ± 0.4 u in 0.21 s/scan (PRF $892^+ \rightarrow 567^+$ monitoring mode). In all experiments, Q3 was maintained at unit mass resolution.

## Procedure

The procedure is the same as described previously through the extraction of the sample from acetonitrile/water into hexane.[1] The sample, dissolved in 2 ml of chloroform/isobutyl alcohol, was put on the previously washed florisil column. The sample tubes were washed with several small aliquots of chloroform/isobutyl alcohol and the washes put on the florisil column. After elution with 40 ml of chloroform:ethyl acetate (3:1 by volume), the eluent was transferred to a 15 ml centrifuge tube and taken to dryness. Samples were dissolved in 100 µl of methanol and injected onto the preparative liquid chromatography column. The appropriate fraction was collected, evaporated to dryness, and then dissolved in 1 ml of methanol for storage at 4 °C until tandem mass spectrometric analysis.

Aliquots (1–2 µl) of the samples were placed on a rhenium, direct exposure probe (DEP) filament, and allowed to dry. The filament/sample was then inserted into the CI plasma/ion volume and analysed directly. DEP conditions were 0–1350 mA at 200 mA $s^{-1}$ with filament bakeout at 1350 mA (*c.* 1300 °C). Under these conditions, desorption profiles showed that no significant thermal degradation products of the ivermectin molecule were produced.

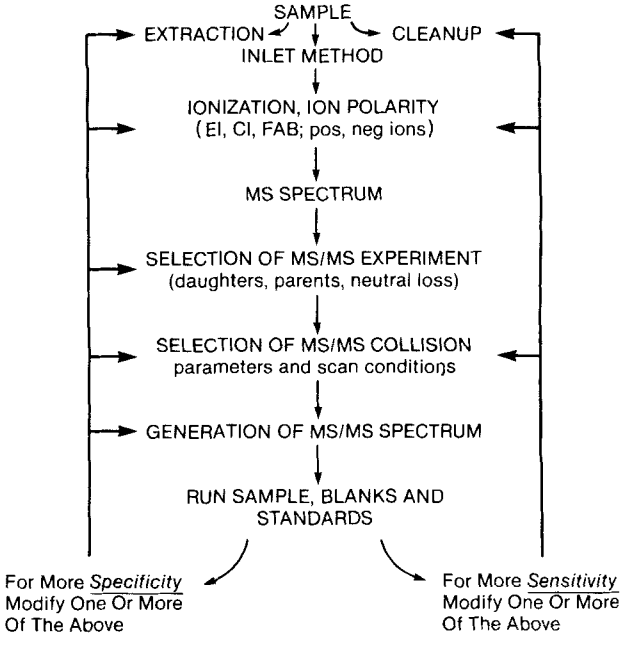


**Figure 4.** Flow diagram of the analytical approach to develop a tandem mass spectrometric assay of a complex mixture.

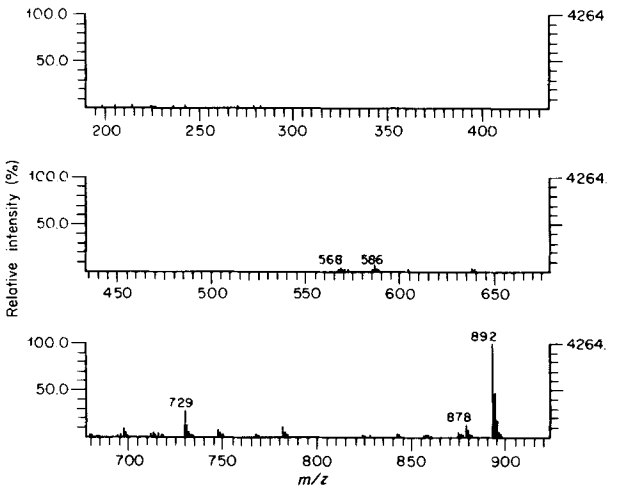


**Figure 5.** DCI spectrum of ivermectin obtained using direct exposure probe-ammonia positive ion chemical ionization.

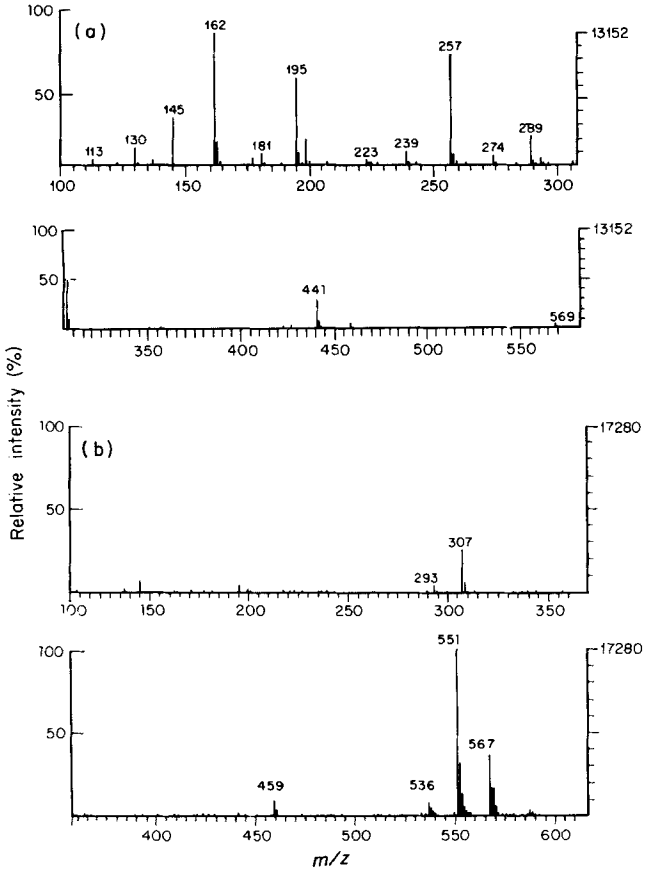


**Figure 6.** MS/MS daughter spectra (parent ion 892) of ivermectin at (a) −12 V collision energy, (b) −30 V collision energy.

## RESULTS AND DISCUSSION

The analytical approach used in the development of a final protocol in complex mixture tandem mass spectrometric assays is usually an iterative process. Figure 4 shows a flow chart of the logic used during the course of the ivermectin assay. This approach may be applied in general to virtually any complex mixture assay when

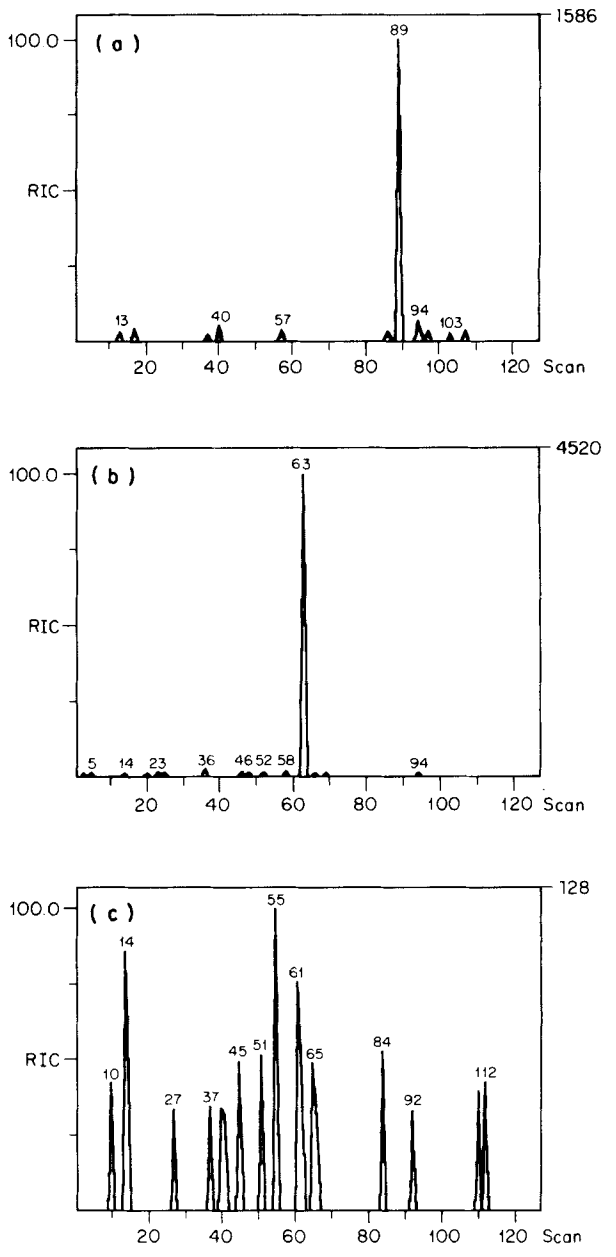


**Figure 7.** Daughter ion scans ($892^+$–$567^+$) of (a) 10 ppb ivermectin standard, (b) 38 ppb ivermectin spike in liver, (c) a control liver sample. The number in the upper right-hand corner of each figure is the number of ions to which the figure was normalized.

MS/MS is used. Key variables which affect the decision making process are the complexity of the matrix, the detection limit required, and the accuracy/precision of measurement (qualitative or quantitative).

Using this approach, Fig. 5 shows the mass spectrum of ivermectin obtained using direct exposure probe-ammonia positive ion chemical ionization. The molecular ion adduct (892) was also base peak and represents the $[M+NH_4]^+$ ion of the 22,23 dihydroavermectin $B_{1a}$. $M/z$ 878 represents the $[M+NH_4]^+$ species of the 22,23 dihydroavermectin $B_{1b}$. Under these inlet/ionization conditions, the spectrum reveals that significant fragmentation (thermal, ion-molecular) of the intact ivermectin structure did not occur.

Full scan MS/MS daughter spectra (parent ion 892; 4 u resolution in Q1) at −12 and −30 V collision energies

are shown, respectively, in Fig. 6(a) and (b). Substantial spectral differences are seen as a function of the collision energy. This observation was also noted with respect to collision chamber pressure between 0.08–0.26 Pa. Selected reaction monitoring MS/MS experiments were performed sequentially on each major daughter ion shown in these spectra; however, cleaned-up control tissue samples persistently showed chemical interference. These matrix interferences were also seen previously in electron ionization, high resolution mass spectrometric experiments.

Using the 'interative process', MS/MS instrument parameters were found which when used in conjunction with the extraction/clean-up methods afforded the required detection limit and chemical specificity. Best results were obtained with instrument parameters of 30 V collision energy, 0.13 Pa collision pressure, using a programmed RF mode and selectively monitoring the $892^{+}$–$567^{+}$ transition.

Figure 7(a), (b) and (c) show typical scans of a 10 ppb ivermectin standard, 38 ppb ivermectin in liver (S:N > 60:1) and a control liver sample, respectively. The lowest level of reliable detection was 5–10 ppb; a 10 ppb standard (Fig. 7(a)) had an ion count of 1586 with a signal to noise ratio greater than 10:1. A liver sample spiked at 38 ppb ivermectin and carried through the assay procedure (Fig. 7(b)) had an ion count of 4520 with a signal to noise ratio greater than 60:1; a 5–10 ppb spike into liver then would have an ion count of 500–1000 with signal to noise greater than 5:1. Control liver samples consistently showed no chemical interference, maximum ion count 128. Liver samples, however, which had not been taken through the preparative liquid chromatography column had large chemical interferences in the MS/MS.

This confirmational method developed for ivermectin analysis in liver tissue by combining wet chemical methods with MS/MS produced a highly sensitive and specific qualitative assay. Since virtually all other mass spectrometric techniques failed, this work clearly demonstrates the utility and the incremental power of the tandem mass spectrometric technique.

## REFERENCES


1. P. C. Tway, J. S. Wood Jr and G. V. Downing, *J. Agric. Food Chem.* **29**, 1059 (1981).
2. R. W. Kondrat and R. G. Cooks, *Anal. Chem.* **50**, 81A (1978).
3. F. W. McLafferty and F. M. Bockhoff, *Anal. Chem.* **50**, 69 (1978).
4. R. A. Yost and C. G. Enke, *Anal. Chem.* **51**, 1251A (1979).
5. D. F. Hunt, J. Shabanowitz and A. B. Giodani, *Anal. Chem.* **52**, 386 (1980).
6. J. R. B. Slayback and M. S. Story, *Ind. Res. Develop.* **23**, 129 (1981).
7. M. Senn and R. Endele, *Ann. Conf. Mass Spec. Abstr.* 45 (1982).
8. J. R. B. Slayback, R. Schubert, R. Abshagen, R. Endele and M. Senn, *Finnigan Topics* **8161**, 1 (1981).
9. W. J. Richter and W. Blum, *Ann. Conf. Mass Spec. Abstr.* 796 (1982).
10. R. J. Perchalski, R. A. Yost and B. J. Wilder, *Anal. Chem.* **54**, 1466 (1982).
11. C. A. Boitnott, J. R. B. Slayback and V. Steiner, *Finnigan Topics* **8160**, 1 (1981).


Received 9 May 1983; accepted (revised) 1 August 1983