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Montreux, October 21–22, 1982

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RNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

CHROMATOGRAPHIC REVIEWS (Vol. 27, No. 1)

edited by

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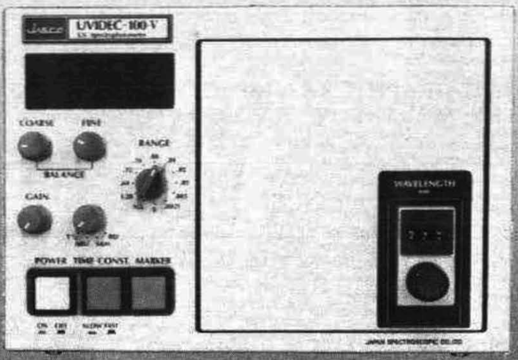
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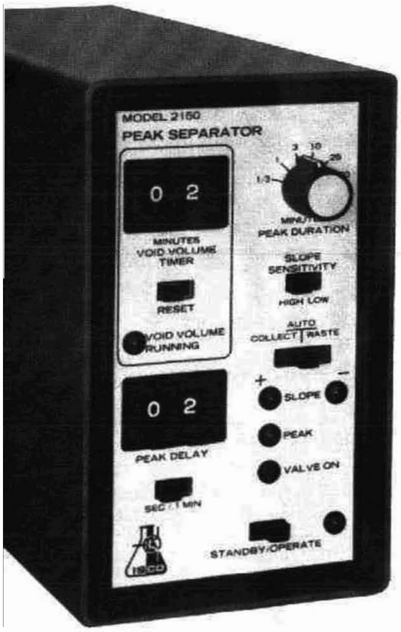
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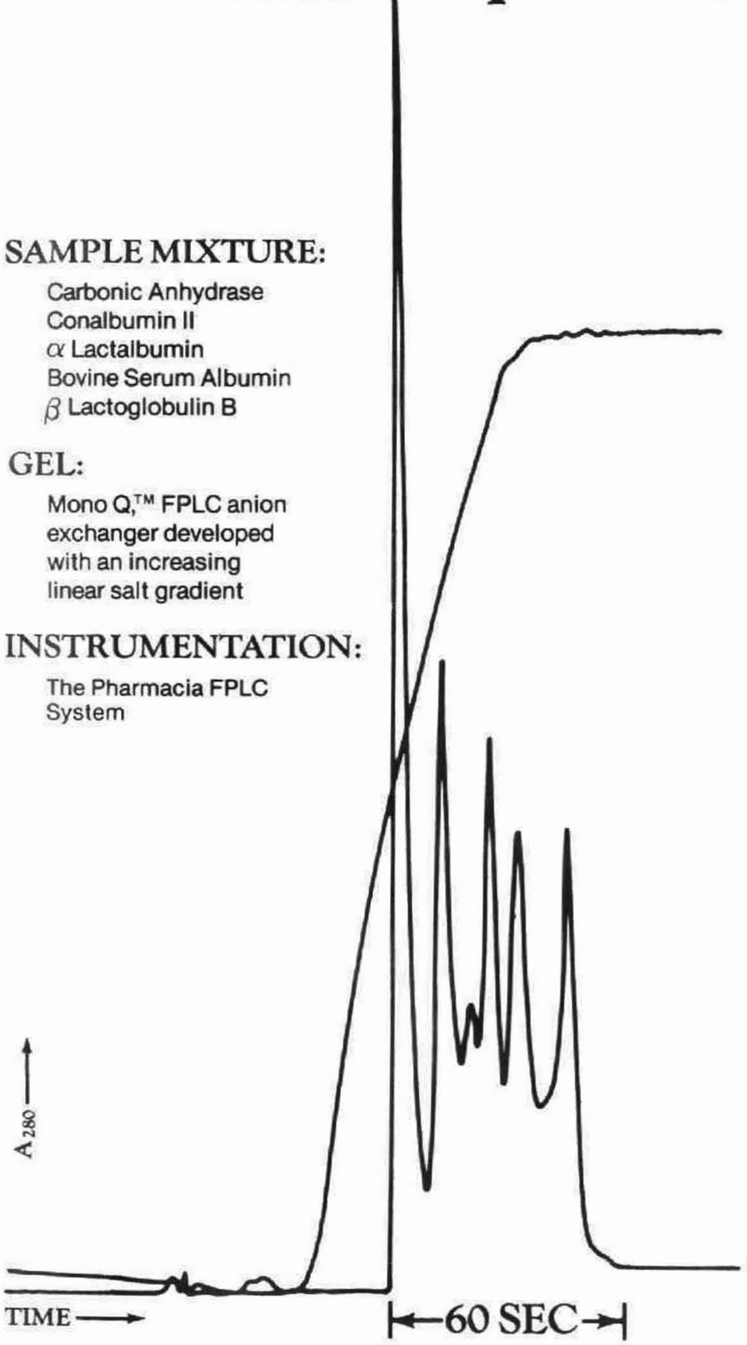

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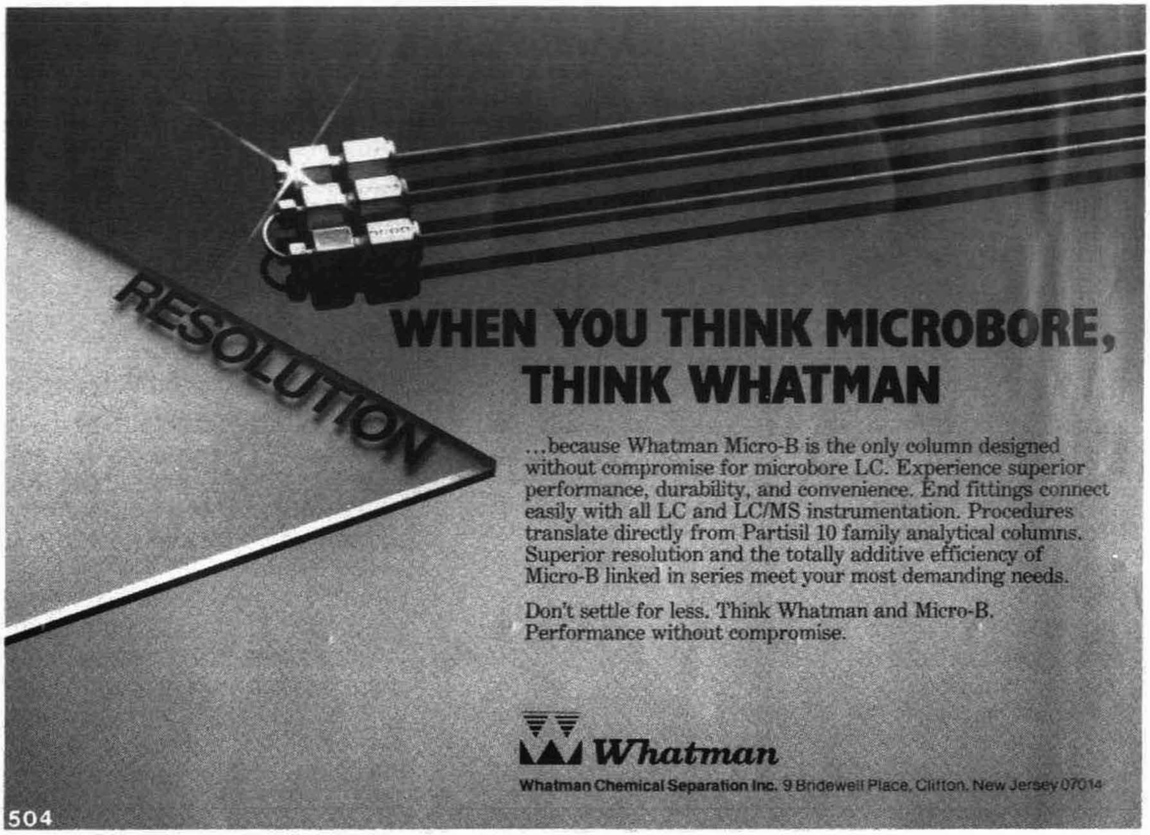

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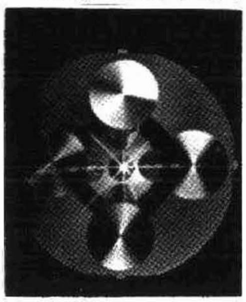

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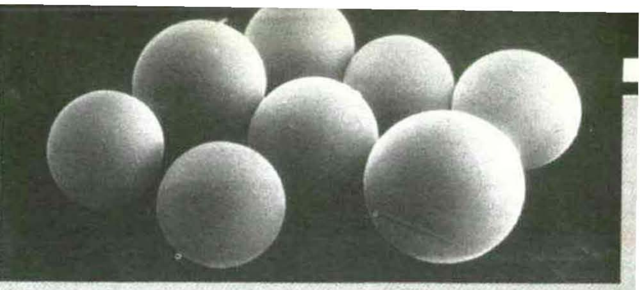

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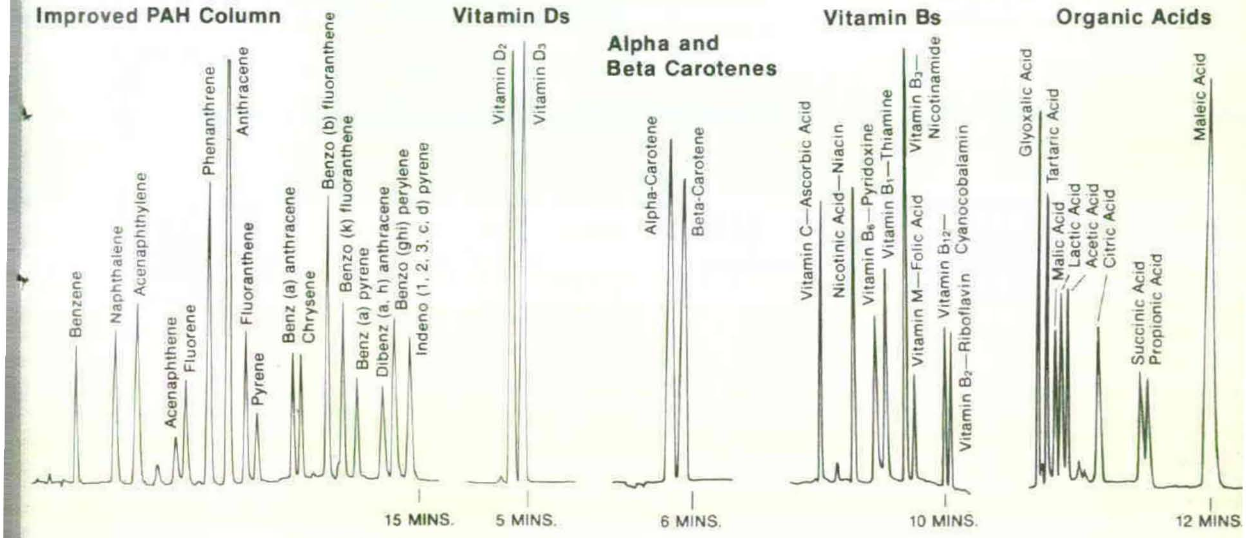

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CHROMATOGRAPHIC REVIEWS

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Michael Lederer

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SPECIAL ISSUE

SECOND WORKSHOP ON LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY AND MASS SPECTROMETRY–MASS SPECTROMETRY

Montreux, October 21–22, 1982

Edited by

R. W. FREI

(Amsterdam)

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PREFACE

For many of us this was the second time that we had gathered in Montreux to discuss the topics of liquid chromatography (LC)–mass spectrometry (MS) and MS–MS and, although some progress has been made in the past year, we can conclude that the frontier spirit that existed in 1981 was also very much alive in 1982. Much scepticism and uncertainty still prevail in both camps, the mass spectroscopists and the chromatographers, as to the real potential and future of on-line LC–MS systems. At the workshop nobody really doubted the potential need for such a combined technique; after all, the success story of GC–MS applies also to LC–MS. The problem is, however, that with LC–MS we are trying to marry two seemingly incompatible partners. Much hope was laid on new ionization techniques and on the possibilities of adapting them to the direct coupling approach, although one has to realize that some of the more publicized new developments in fast atom bombardment, secondary-ion MS or field desorption seem to be rather handicapped for on-line operation. Nevertheless, many participants believed that there is much room for optimism about on-line coupling techniques, particularly in view of the continuing miniaturization in LC. The growing trend of applying LC–MS to the solution of real problems was also manifested by the increased number of application papers submitted to the workshop and appearing in the literature.

Much controversy existed over the optimal ways of coupling an LC with an MS unit. The question of transport interface *vs.* direct inlet systems was the subject of a special panel discussion based on interlaboratory comparison data with thermolabile pharmaceuticals. Its conclusion was in favour of direct inlet systems when very thermolabile compounds are to be handled. Such a conclusion is not surprising when one considers the heating steps needed in transport systems to eliminate the solvent.

Unfortunately, off-line methods in LC–MS were neglected in this workshop. This does not mean that they were considered of lesser importance; in fact, many believe that the argument between off-line and on-line techniques, discussed extensively in 1981, is still very much alive and the organizers will have to reconsider this area seriously in the next workshop along with all the developments going on in the area of new ionization techniques.

Regarding MS–MS, one noticed at this event a rapidly growing interest in this tandem technique which often is claimed, by enthusiastic disciples, to be likely to displace chromatography in the years to come. During the presentations and discussions one indeed got the impression that multistage mass spectrometers have the power to provide highly selective and valuable information on trace components in complex matrices often comparable or superior to the chromatographic approach. However, the general consensus was along the lines of the two techniques being really more of a complementary than of a competitive nature.

From the many positive comments received, we can conclude that for most participants the 1982 workshop was a positive and informative experience. A future (3rd) workshop in this field is planned again in Montreux for October 24–26, 1984.

Amsterdam R. W. FREI
January 10, 1983

Journal of Chromatography, 271 (1983) 3–12
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CHROM. 15,583

IONIZATION OF MIDDLE MASS MOLECULES: EJECTION OF IONS FROM SOLUTION

CATHERINE FENSELAU*, R. J. COTTER, D. HELLER and J. YERGEY
Middle Atlantic Mass Spectrometry Center, The Johns Hopkins University, School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205 (U.S.A.)

SUMMARY

Attention is directed to the role of the solution in a number of liquid phase ionization techniques and to common elements among these techniques as they are used for analysis of middle molecules. One important feature is the role of the solvent in ionizing the samples. A second feature is the thermodynamic contribution of the solvent to the desorption process. A third feature is the role solution chemistry plays in determining the nature of the ions detected in the spectrum.

Middle molecule mass spectrometry (MS), the analysis of compounds with molecular weights between 1000 and 10,000 has been achieved with most of the existing ionization techniques[1]. However, those techniques which do not require vaporization of the sample into the gas phase prior to ionization appear to have the most potential. Plasma desorption[2], field desorption[3] and fast atom bombardment[4] have probably had the most success to date[5].

One of the most interesting applications of field desorption is the analysis of mixtures of industrial polymers in the middle mass range. This has been reviewed previously in this journal[1] and shown to provide assessments of the number average masses of oligomer populations comparable to those provided by vapor phase osmometry and also high-pressure liquid chromatography (HPLC). Field desorption also permits analysis of the range and relative abundances of oligomers in mixtures comparable to that provided by HPLC and which cannot be obtained with more traditional techniques such as vapor phase osmometry and gel permeation chromatography. Assuming minimal fragmentation and correcting for any mass discrimination, relative abundances of oligomers can be directly related to the relative areas of their molecular ion clusters.

A histogram is shown in Fig. 1 of the oligomer population determined by field desorption in a fraction of polyethylene glycol. This determination provided a number average mass of 1360[6]. This histogram is to be compared with that obtained from the same sample using electrohydrodynamic ionization[7] also in Fig. 1. This latter envelope has a number average mass of 1365. End-group titration measurements on this sample provided a value of 1396[7].

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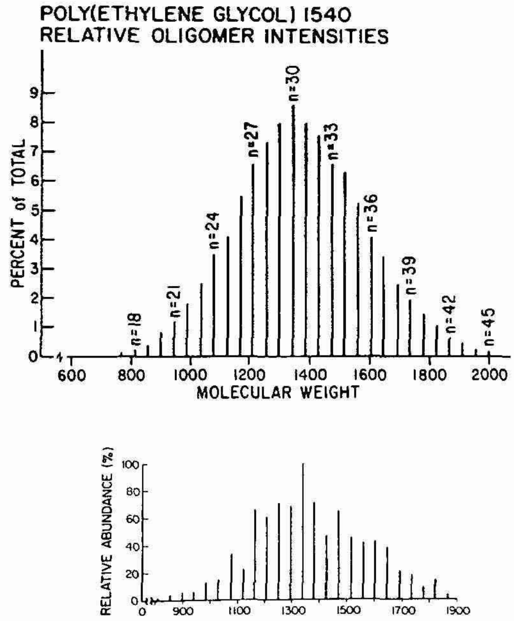
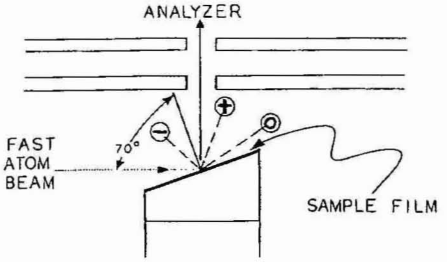


Fig. 1. Oligomer population in a polyethylene glycol fraction analyzed by field desorption (top)[6] and by electrohydrodynamic ionization (bottom)[7].

At least three different mechanisms are considered to be responsible for the field desorption of various compounds[3]. The major mechanism for desorption of involatile middle molecules is described by Giessmann and Rollgen[3] as the extraction by the field of preformed (positive or negative) ions already preformed in solution.

In electrohydrodynamic ionization the sample is introduced in an involatile conducting solution fed through a capillary into a vacuum in a high voltage field[8]. Glycerol is usually used as the involatile solvent, in which sodium iodide or another alkali halide salt is dissolved. The mechanism of desorption is very similar to that of field desorption. The field induces ion evaporation from the meniscus at the end of the capillary. This means that the ions analyzed are preformed in the solution by protonation, natriation or other cationization. The quality of spectra from both field desorption and electrohydrodynamic ionization is profoundly influenced by the presence and nature of salts. The addition of HCl is reported to increase sensitivity for production of MH$^+$ in electrohydrodynamic studies of nucleosides and nucleotides[9]. Fragment ions observed in electrohydrodynamic ionization spectra are thought to be formed by hydrolysis in solution followed by desorption.

Fast atom bombardment (FAB) is a technique in which the sample is dissolved in a drop of a non-volatile solvent on the end of a direct insertion probe, inserted into the high vacuum and bombarded with atoms or ions (Fig. 2) which have been accelerated through 1 to 10 kV[4,10]. Several proposals have been made to account for ionization and desorption[4]. One interesting model for non-volatile middle molecules requires formation of ions in solution followed by desolvation and ion ejection. The energy for ejection is vibrational energy transferred through the liquid medium from



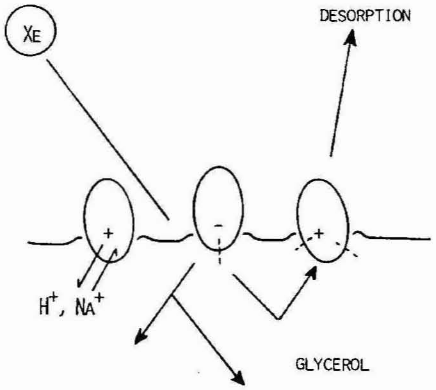


Fig. 2. Schematic representation of sample bombardment by fast atoms.

Fig. 3. Schematic representation of the liquid matrix on a FAB probe.

the point of impact of the bombarding particle. This is a localized disturbance, not an equilibrium. Neutral sample molecules are ejected, as well as cations and anions, however neutral species are not analyzed by the mass spectrometer. This requirement for preformed ions is also proposed for the antecedent technique of secondary ion MS[11]. A number of rôles for the solvent are illustrated in Fig. 3. The solution re-

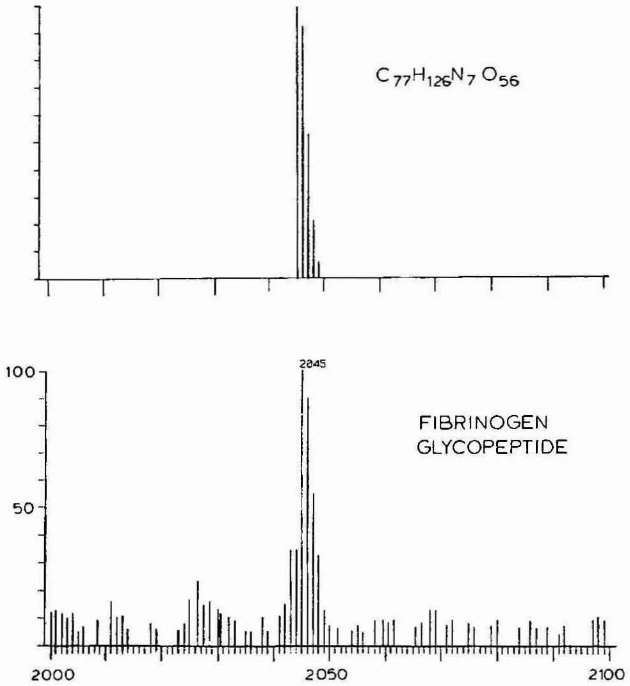
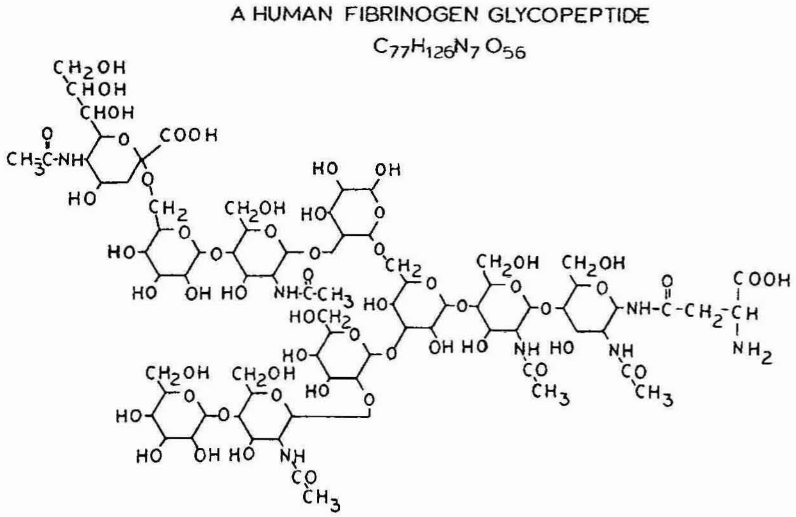


Fig. 4. Molecular ion region of a positive ion FAB spectrum of a monosialylated bidentate glycopeptide from human fibrinogen (bottom) and the computer generated theoretical protonated molecular ion cluster (top).



A HUMAN FIBRINOGEN GLYCOPEPTIDE
$C_{77}H_{126}N_7O_{56}$

Scheme 1.

plenishes sample ions at the surface throughout the bombardment. Non-ionic samples are ionized in the solution, *e.g.*, by protonation or natriation, or by removal of active protons in normal solution equilibria. Charge separation by solvation of ions also makes an important contribution to the desorption process. The heat of solution can be viewed as lowering the energy required for desorption. Once the ions are formed at the surface, only desolvation remains to be accomplished.

An example of a successful middle molecule analysis using FAB is shown in Fig. 4, the protonated molecular ion region of a monosialylated bidentate glycopeptide (Scheme 1) isolated from human fibrinogen[12]. The computer-generated theoretical cluster[13] of protonated molecular ions for the formula $C_{77}H_{126}N_7O_{56}$ may be compared (Fig. 4). FAB desorbs ionic or ionizable middle molecules more readily than non-polar compounds. Attempts to desorb heavy polystyrenes have been unsuccessful and a polypropylene glycol mixture was analyzed only after a number of different solvents had been tried. A portion of the spectrum obtained with tetraglyme heavily salted with lithium chloride is compared in Fig. 5 to the field desorption spectrum[14]. The FAB spectrum contains several molecular ion species and fragment ions from each oligomer and is generally of poorer quality than the field desorption spectrum. This illustration is not intended to detract from the very great analytical potential of FAB, but rather to indicate the continuing utility of field desorption.

The secondary ion yield from bombardment of a sample in a glycerol matrix is strong, in excess of 10^{-10} A, steady (in marked contrast to the ion current of many middle molecules generated by field desorption) and prolonged. This makes FAB an ideal desorption technique for high resolution or accurate mass studies, for computerized acquisition and manipulation, for metastable studies, for MS–MS work or any combination. Fig. 6 shows an example of the accurate measurement at resolution of 1 part in 10,000 of the $(M-H)^-$ molecular anion of a ganglioside of theoretical

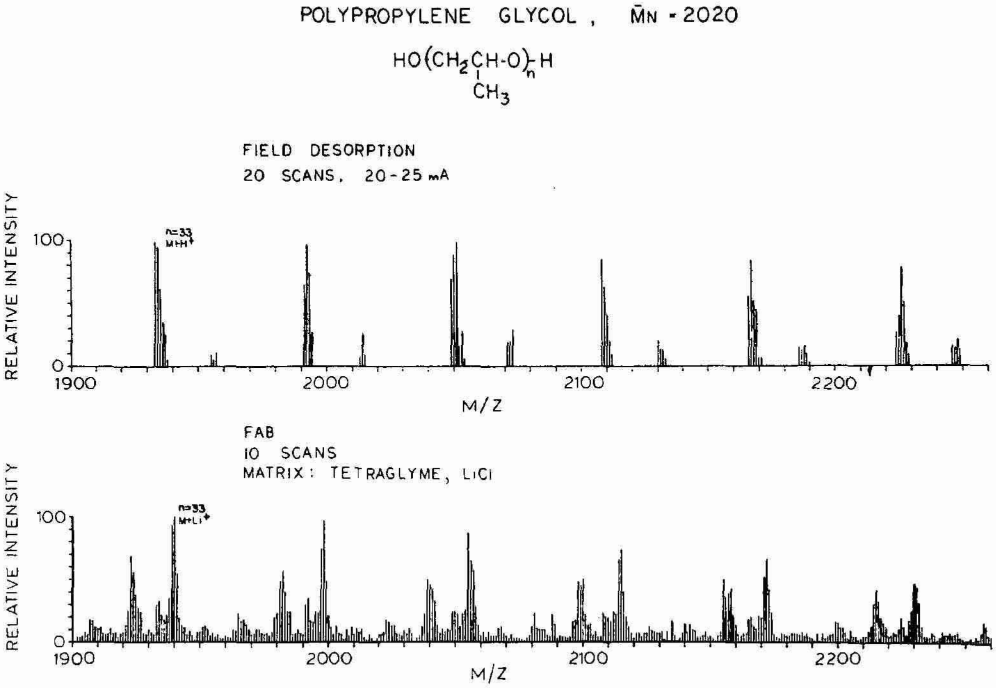


Fig. 5. Partial field desorption spectrum (top) and partial FAB spectrum (bottom) of a polypropylene glycol fraction.

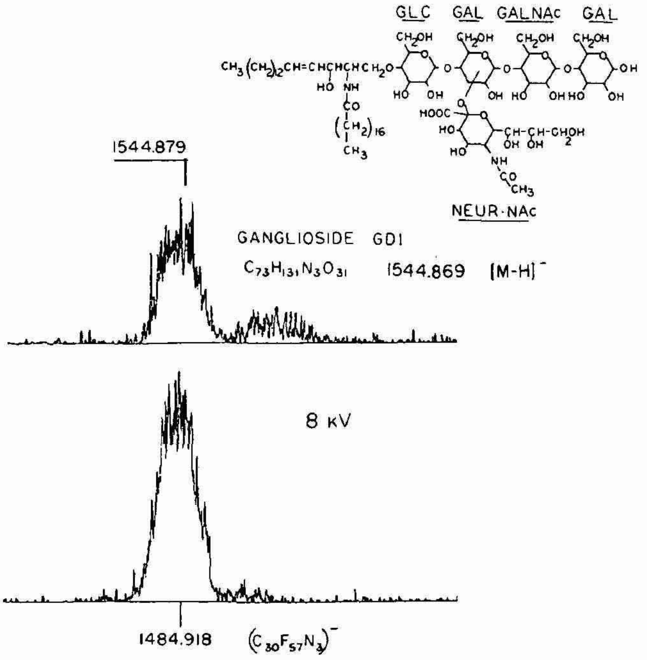


Fig. 6. Accurate mass measurement made at 10,000 resolution of a ganglioside (M − H)$^-$ anion. The reference compound is triazene 1485.

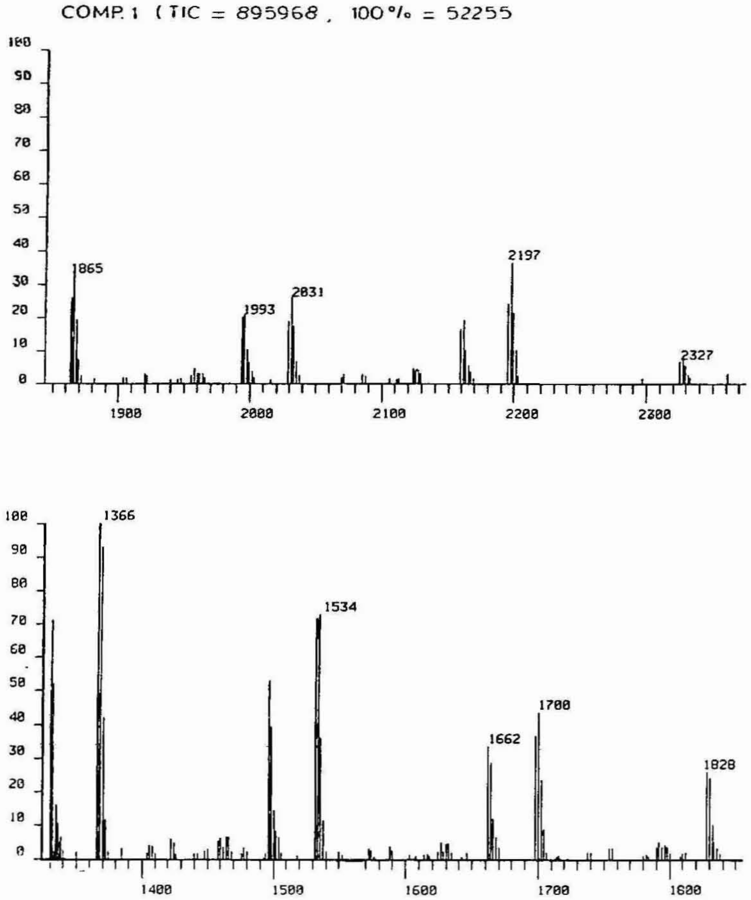


Fig. 7. Computer calibration spectrum of potassium iodide glycerol clusters used to acquire the spectrum in Fig. 4.

mass 1489.869. The experimental value agrees to 1 part in 150,000. Fig. 7 contains a partial spectrum of potassium iodide glycerol cluster ions used to calibrate the computer acquisition of the spectrum in Fig. 4. We calibrate our computer system routinely to between 2000 and 3000 a.m.u.

Many papers in the literature report the use of FAB to analyze peptides and a great deal of comment has been offered about how to employ solution chemistry to increase the abundance of M + H or M − H ions. The addition of acids to the matrix or the use of a more acidic matrix enhances formation of $(M+H)^+$ ions. Neutral or basic matrix are more favorable for analysis of $(M-H)^-$ ions. This is analogous to the effect of added acid in electrohydrodynamic ionization.

At least two laboratories have reported FAB spectra of unprotected polynucleic acids[5,15]. Spectra of samples with homogeneous counter ions (H^+) on the phosphate groups provide sequence information[15]. If care is not taken to control the phosphate counter ion, mixtures of molecular ion species are observed, which carry different numbers of protons and sodium ions (potassium, etc.)[16]. In theory nine

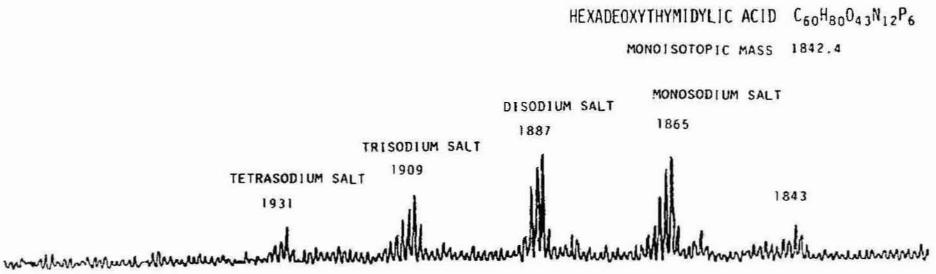


Fig. 8. Molecular cation region of the hexanucleotide hexathymidyllic acid[5]. (With permission from the American Chemical Society.)

different mixtures of protonated and natriated molecular ion species could be obtained, if a hexanucleotide is prepared as its sodium salt. In the spectrum in Fig. 8 six of these nine are detected. One might use this phenomenon to count active protons in an unknown sample[16].

A summary of the anionic molecular species determined for a series of organophosphates and sulfates is shown in Table I, along with the number of titratable/exchangeable protons in each compound. (The cationic salt is indicated beside the name of the compound.) This approach works well for compounds with as many as five titratable protons[16]. However, the proliferation of molecular ion species can also be viewed as degrading the sensitivity of any individual molecular ion, and as confounding fragmentation patterns. The preceding discussion reveals another aspect of desorption of solids from solution. Chemical equilibria within the solution can cause partial replacement of active hydrogens by alkali cations, and this most definitely affects the quality of the spectrum.

The last ionization technique included in this overview is the thermospray technique developed for use as a liquid chromatography–mass spectrometry (LC–

TABLE I

MOLECULAR ION SPECIES IN SELECTED PHOSPHATES AND SULFATES

Compound	*No. of titratable protons*	*Molecular species relative to the free acid form*	
FAD, Na^+	2	2	$(M - H)^-$, $(M + Na - 2H)$
Coenzyme A, Li^+	4	3	$(M + Li - 2H)^- \rightarrow (M + 3Li - 4H)^-$
Uridine diphosphoglucuronic acid, $NH_4{}^+$	3	3	$(M - H)^- \rightarrow (M + 2NH_4 - 3H)^-$
NADPH, Na^+	4	4	$(M + Na - 2H)^- \rightarrow (M + 4Na - 5H)^-$
ADP, Na^+	3	3	$(M - H)^- \rightarrow (M + 2Na - 3H)^-$
UTP, Na^+	4	4	$(M + Na - 2H)^- \rightarrow (M + 4Na - 5H)^-$
ATP, Na^+	4	4	$(M - H)^- \rightarrow (M + 3Na - 4H)^-$
Adenosine tetraphosphate, Na^+	5	5	$(M - H)^- \rightarrow (M + 4Na - 5H)^-$
Phytic acid, Na^+	12	5	$(M - Na)^- \rightarrow (M - 5Na + 4H)^-$
Glucuronylestradiol sulfate, K^+	2	2	$(M - H)^-$, $(M + K - 2H)^-$
Glucose-6-sulfate, K^+	1	1	$(M - H)^-$

MS) interface[17,18]. Here a solution of both the sample and a volatile salt (*e.g.*, ammonium acetate) is forced through a jet at the rate of 1 ml/min. Thermal coupling with the solution evaporates the solvent from the droplets in the jet spray. Complete desolvation can lead to ionization of volatile samples by chemical ionization processes. Involatile middle molecules, very polar compounds and organic ions are considered to be ejected as preformed ions from the increasingly small droplets[18]. Following the theory of Iribarne and Thompson[19,20], the ions in the solution will be distributed through these drops with the probability of net positive or negative charges on some drops. High local fields in these charged drops will facilitate ejection of ions, including ionized sample molecules. This ionization mechanism works best with high concentrations of buffer salts. Typically 0.1 *M* ammonium acetate is used. Generally $(M + H)^+$ and $(M - H)^-$ molecular ions are observed. As in FAB, positive and negative anions appear to be formed in about equal amounts[18]. The spectrum of a tetradecapeptide renin substrate is shown in Fig. 9[21], certifying applicability of the thermospray technique to middle molecules. Fig. 10 illustrates another aspect of this and also the FAB and electrohydrodynamic ionization solution techniques. Positive ion current is reconstructed for an equimolar mixture of three glucuronides eluting from the liquid chromatograph. Sensitivity is clearly different for each of the three compounds.

Excluding inductively coupled plasma and supercritical fluid chromatography most of the techniques for desorption of solids from solutions have many features in common. These techniques include FAB, electrohydrodynamic ionization, elec-

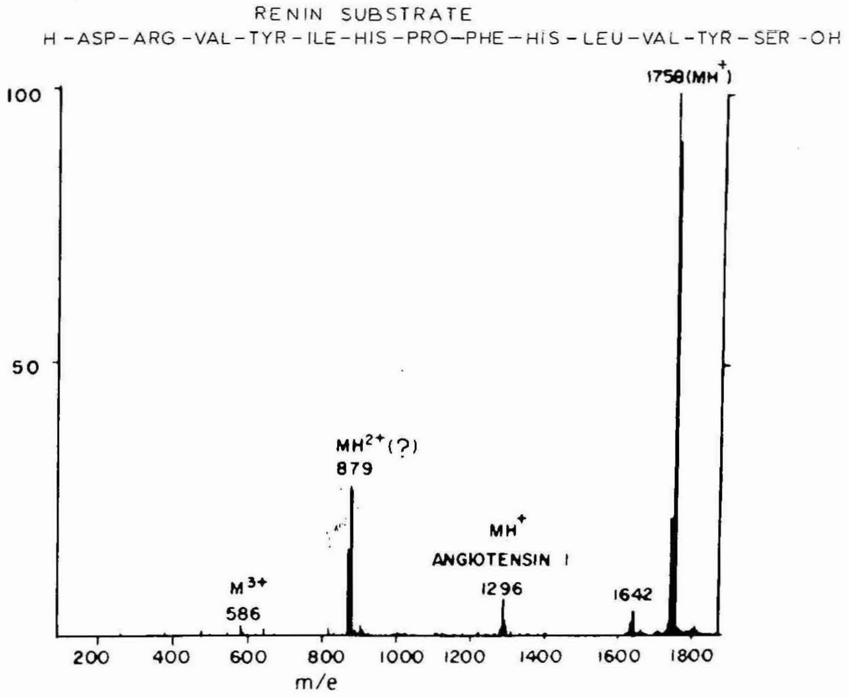


Fig. 9. Thermospray spectrum of renin[21]. (With permission from the American Chemical Society.)

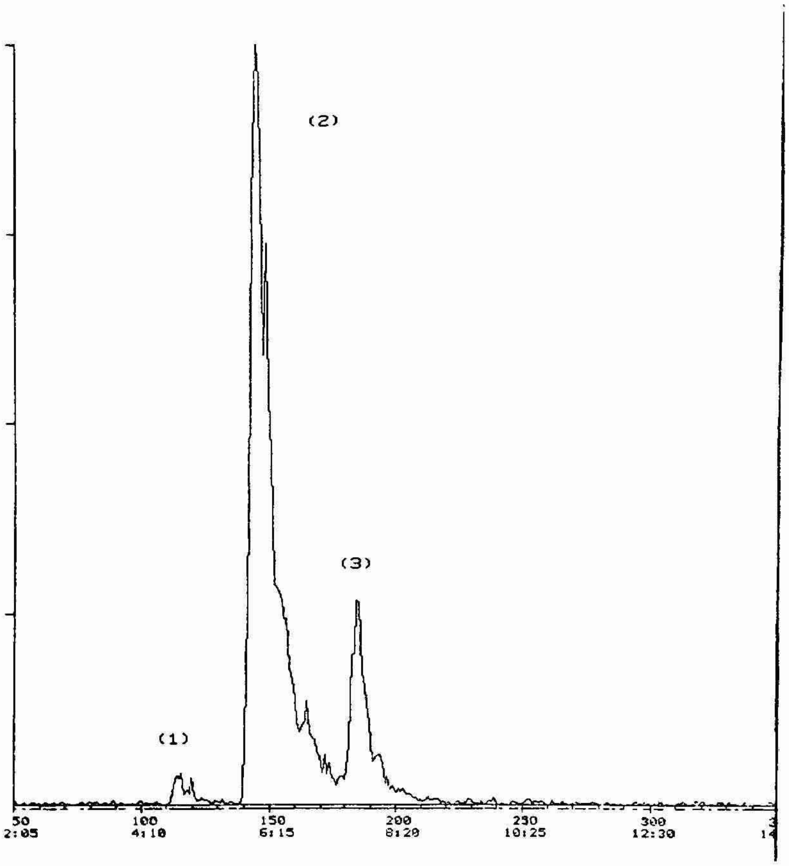


Fig. 10. Reconstructed mass chromatogram of an equimolar mixture of three glucuronides analyzed by thermospray LC–MS[22]. Column = 15 cm Ultrasphere ODS. Eluent = methanol–0.05 *M* ammonium acetate (20:80); flow-rate 1 ml/min. Peaks: 1 = *p*-nitrophenyl-β-D-glucuronide; 2 = 4-methylumbelliferyl-β-D-glucuronide; 3 = 8-hydroxyquinine-β-D-glucuronide.

trospray techniques, field desorption, thermospray and ion evaporation. First, solution chemistry strongly influences the nature of the ions formed and analyzed and thus the nature of the spectrum. Secondly, solutions assist desorption. They provide ionization of the sample in solution. They provide charge separation by solvation, lowering the energy required for desorption. Of course, the solution also plays various special roles in each of the different techniques, *e.g.*, replenishing sample at the surface in FAB, providing salts to increase the probability of charged droplets in thermospray and providing a conducting medium for electrohydrodynamic ionization.

In conclusion, there are at least three major reasons to continue to develop techniques for desorption of middle molecules from solutions. Middle molecules are easier to handle in solutions. Solution techniques are compatible with introduction of the sample by liquid chromatography. And lastly, the desorption process is facilitated by solution chemistry.

ACKNOWLEDGEMENTS

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HOW TO INTERFACE A CHROMATOGRAPHIC COLUMN TO A MASS SPECTROMETER

G. GUIOCHON* and P. J. ARPINO
Laboratoire de Chimie Analytique Physique, École Polytechnique, Route de Saclay, 91120 Palaiseau (France)

SUMMARY

A chromatographic column is a separation and a dilution unit. Normally it would be expected that each component of a mixture will elute separated from the others, each of them being about one order of magnitude more dilute in the mobile phase than it was in the original mixture. Complete separation is not always possible, especially with unknowns, with which liquid chromatography–mass spectrometry is mainly concerned. In gradient elution, the composition of the mobile phase is continuously changing, but the dilution is reduced. Some times the compounds eluted are more concentrated than they were in the original sample. Typical chromatographic zones are a few seconds to a few minutes wide and contain from 1 mg to less than 1 fg. The chromatographer wants to know the identity of all compounds eluted from the column and seeks very low detection limits (10–100 fg for most compounds would be excellent), reasonably good quantitative results (10%) and a wide range of linearity (up to 100 μg). He also desires some information regarding overlapping peaks and requires the mass spectrometer to accept the column effluent with a very simple, trouble-free interface that will have a very small transit time, so as to contribute as little as possible to the remixing of zones separated by the column. With the present equipment the main problems are probably the sensitivity and linear dynamic range.

INTRODUCTION

The problems of interfacing a liquid chromatograph to a mass spectrometer (LC–MS) stem from the relative incompatibility between the dilute solution eluted from the chromatographic column and the low-pressure gas plasma inside the source of the mass spectrometer, which makes this coupling much more difficult than that between a gas chromatograph and a mass spectrometer, which is now quite conventional[1–3]. These problems have often been discussed, but most reviews have so far focused on the interface itself, whereas here we want to consider the main constraints introduced by the chromatographic process and the minimum requirements that any interface should meet to have a chance of being competitive and to suggest the main compromises acceptable from the chromatographic point of view.

Chromatography is a separation process; the components of the sample are

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eluted as bands having a profile more or less similar to a Gaussian curve, provided that the column is not overloaded, and which are disengaged from each other with a certain degree of resolution. In chromatography the resolution is the ratio of the distance between band maxima and the half-sum of the bandwidth at their base (*i.e.*, four times the standard deviation of the Gaussian curve).

As this process occurs in spontaneous, *i.e.*, irreversible, conditions from a thermodynamic point of view, the decrease in entropy arising from this separation is more than compensated for by an increase in entropy due to dilution in the mobile phase. Accordingly, elution chromatography is also a dilution process. Possible exceptions may occur with temperature programming [in gas chromatography (GC)] or in gradient elution [in liquid chromatography (LC)], provided that the gradient is steep, the retention of the corresponding compound is very large under the initial conditions and a large enough sample is injected, so the entire cross-section of the column is loaded with sample[4]. These conditions are relatively rare and are encountered mainly in the analysis of trace impurities in a simple matrix, such as in air or water pollution analysis[4].

The main problems to be encountered will thus be the compatibility of flow-rates, the resolution and the contribution of the mass spectrometer to band broadening and the detection limits and the dynamic linear range.

NATURE AND FLOW VELOCITY OF THE MOBILE PHASE

The number of solvents currently used in liquid chromatography is relatively small (Table I), although in most instances mixtures of these solvents are used and a large number of possible additives can be incorportated. From our point of view, only

TABLE I

GASEOUS VOLUME AND VAPORIZATION ENTHALPIES OF COMMON LC SOLVENTS

V_g = Volume of vapour at boiling point (1 atm) or at 20°C (P^0) obtained by vapourization of 1 ml of liquid solvent; T_b = boiling point. ΔH = vaporization enthalpy.

Chromatography	*Solvent*	ρ *(g/cm³)*	V_g		T_b*(°C)*	P^0 *(mbar)*	ΔH*(cal/g)*
			at 25°C	*at* T_b			
Normal-phase	*n*-Pentane	0.626	407	220	36	512	87
	n-Heptane	0.684	3630	208	98.4	46	87
	Benzene	0.879	2900	326	80.1	95	104
	Toluene	0.867	8200	296	111	28	99
	Methylene chloride	1.335	870	404	40	436	80.5
	Chloroform	1.492	1550	342	61.2	197	62
Reversed-phase	Water	1.0	$56 \cdot 10^3$	1700	100	23.8	583
	Acetonitrile	0.786	5270	554	80	84	204
	Ethanol	0.789		493	78.5	na	
	Methanol	0.791		685	65	na	
	Propanol-2	0.785		380	82	na	na
	Tetrahydrofuran	0.888		341	64	na	na
	Dioxane	1.034		360	101	na	na

non-volatile additives need be considered. These are mainly salts: buffers used to adjust the pH of the mobile phase or ions used in ion-pair chromatography, tetraalkylammonium, alkylsulphates or alkylbenzenesulphonates, with Cl^- or Na^+ as counter ions[5].

Whatever the LC–MS interface selected, the use of a non-volatile buffer seems prone to generate considerable troubles. The liquid–liquid extraction scheme studied by Karger *et al.*[6] is a possible solution, although its contribution to band broadening is significant even with 4 mm I.D. columns[7]. Ammonium acetate, trifluoroacetate, formate and chloride can be used as buffers to some extent and a number of volatile acids or bases are available (Table II), but it is nearly impossible to do without the traditional compounds used in ion-pair chromatography. In view of the increasing importance of this method in biochemical analysis, some investigation of this problem is clearly necessary. A solution could come from the use of organic ions that would decompose rapidly in the gas phase. As pre-formed ions seem to be transferred easily through some interfaces[8–10] from the introduced solution to the source, the ideal solution would be for the mass spectrometrist to use the conventional LC ion-pair reagents in the source to perform some useful reaction.

TABLE II

pK_a VALUES OF SOME VOLATILE ACIDS AND BASES

Compound	pK_a	*Compound*	pK_a
Ammonia	9.25	Acetic acid	4.75
Aniline	4.58	Benzoic acid	4.2
Diethylamine	11	Chloroacetic acid	2.85
Diisobutylamine	10.7	Cyanoacetic acid	2.45
Hydrazine	8.5	Dichloroacetic acid	1.48
Hydroxylamine	6	Formic acid	3.75
Pyridine	5.3	Phenol	9.9
Quinoline	4.8	Picric acid	0.40
Hydrocyanic acid	9.3	Thioacetic acid	3.33
Hydrogen sulphide	7.0		

Although gradient elution is much talked about in liquid chromatography, it is more rarely used. In many instances it does not provide a significant reduction in analysis time compared with isocratic elution[11]. When it does, a step gradient or a small number of successive analyses carried out under different conditions can replace it, so it is not really necessary that an LC–MS interface can accommodate a rapid change in solvent composition, which with most interfaces would certainly be the source of serious difficulties.

The flow-rate of solvent through a chromatographic column is proportional to the product of the column cross-section and the flow velocity. This velocity is chosen as an optimum compromise permitting the achievement of a reasonable efficiency and an acceptable analysis time[12]. The column diameter depends on the technology available, as both very narrow and very large columns are difficult to pack and operate. At present, columns between 1 and 10 mm I.D. are available with comparable performances. The choice is a matter of convenience: if one wants to use a large sample or to reduce the importance of equipment contributions to band broadening, a wide column is preferred, whereas when a low flow-rate is desired or only a small sample is available, a narrow column is preferred, although the importance of equipment contributions to band broadening may then be a source of major difficulties.

The column efficiency is independent of the volumn cross-section over a reasonable range, provided that a good packing technique is used[13]. It depends only on ν, the reduced velocity of the solvent:

$$\nu = \frac{u\, d_p}{D_m} \tag{1}$$

where u is the actual solvent velocity, d_p the average particle size and D_m the diffusion coefficient of the solute analyzed in the mobile phase.

For well packed columns, the column efficiency is usually a maximum for a value ν_0 of the reduced velocity around 3. It decreases only slowly with increasing velocity[12]. The decrease in efficiency is approximately 10% at twice the optimum velocity, $2\nu_0$, 25% at $4\nu_0$ and 50% at $10\nu_0$. Because of this slow trend, most analysts tend to operate the columns at relatively high values of the reduced velocity, *e.g.*, around 8. Larger values are precluded in most practical cases because the inlet pressure increases in proportion to the velocity and pressures much higher than 100 atm are avoided, and because a rapid analysis reduces the actual time bandwidth and makes accurate recording of chromatograms and peak integration impossible when the bandwidth becomes smaller than about 10 sec without the use of a microcomputer. Accordingly, the flow-rate, F, across the column is

$$F = \frac{\nu D_m}{d_p} \cdot \varepsilon\pi \cdot \frac{d_c^2}{4} \tag{2}$$

where ε is the porosity of the packing, usually around 0.7–0.8 (ref. 5), and d_c is the column diameter. We can rewrite eqn. 2, using reasonable values for the constants ($\nu = 8$, $\varepsilon = 0.75$), as

$$\mathrm{F} \approx 4.7 \cdot \frac{D_m}{d_p} \cdot d_c^2 \tag{3}$$

The recent trend towards the use of small particles (7, then 5, now 3 μm) results in an increase in the flow velocity in proportion to the inverse of the particle size, but it is important to realize that the analysis of high-molecular-weight compounds and the use of viscous eluents (necessary for the separation of many large peptides which lose bioactivity in water–methanol or water–acetonitrile mixtures but not in water–isopropanol mixtures) also result in a marked decrease in the flow velocity. The diffusion coefficient can be estimated from the Wilke and Chang[14] equation:

$$D_m = 7.4 \cdot 10^{-10} \cdot \frac{T\sqrt{\varphi M}}{\eta_1 V_2^{0.6}} \tag{4}$$

where T is the temperature (°K), η_1 the viscosity of the solvent, M its molecular weight, φ an association constant (2.4 for water, 1.7 for methanol and 1 for non-associated solvents) and V_2 the molar volume of the solute. Eqn. 4 is only approximate.

In methanol–water mixtures light compounds such as phenol and polymethylphenols, often used to calibrate columns and measure their efficiency, have diffusion coefficients around $1 \cdot 10^{-5}$ cm^2/sec, whereas a compound with a molecular weight of 2000 has a diffusion coefficient between $1 \cdot 10^{-6}$ and $2 \cdot 10^{-6}$ cm^2/sec. To achieve the same efficiency, the flow velocity should be reduced by a factor of about 6. A compromise involving the use of a larger velocity to achieve a shorter analysis time and some loss of efficiency will usually be found[12]. Some numerical data are given in Table III.

TABLE III

TYPICAL FLOW-RATES IN LC COLUMNS

d_p (μm)	D_m (cm^2/sec)	d_c (mm)	F (μl/min)* (eqn. 3)	u (cm/sec)
5	$1 \cdot 10^{-5}$	4	900	0.16
		2	225	
		1	56	
	$5 \cdot 10^{-6}$	4	450	0.08
		2	112	
		1	28	
	$2 \cdot 10^{-6}$	4	180	0.032
		2	45	
		1	11	
3	$5 \cdot 10^{-6}$	4	750	0.13
		2	188	
		1	47	

* $\nu = 8$.

There is enough flexibility in the parameters of LC columns to optimize separately the flow velocity for maximum efficiency, or for any separation/analysis time compromise, and the volume flow-rate to accomodate the MS requirements. The remaining constraint depends on whether the sample size available is very small, in which event the column used must be narrow. This is often the case in clinical analysis.

RESOLUTION AND EQUIPMENT CONTRIBUTION

The separation of the components of an unknown mixture is a difficult operation. The remixing of the bands at the column exit should be carefully limited. It may occur as a result of axial diffusion or convexive mixing or simply because of the parabolic flow profile in empty tubes used for connections.

Although the volume of the ionization source of the MS is extremely large compared with the cell volume of any other LC detector, and the diffusion coefficients under reduced pressure are very large, the residence time in the source is very short and this, more than the volume, is the critical parameter controlling zone remixing. As is well demonstrated in GC–MS, the ion source itself contributes negligibly to band broadening. It is essential, however, that the volume of the connecting tubes be kept to the minimum, that if the effluent is nebulized there is no turbulence inside the droplet cloud to mix them and that if the effluent is placed on a belt prior to solvent vaporization it does not flow on that belt. Convective mixing may be promoted in the last instance by too rapid a vaporization, leading to various forms of instability of the liquid film.

It does not seem too difficult to design and build direct liquid interfaces whose contribution to band broadening is negligible[1,15]. With belts this contribution is also small with the condition that vaporization of the solvent proceeds smoothly, but liquid–liquid extraction is very difficult to miniaturize[7]. A more important contribution arises from the response time.

The mere direct comparison of chromatograms obtained for a given mixture on the same column, using MS and a conventional chromatographic detector, shows considerable decrease in resolution for the MS trace. This is because usually only one spectrum is recorded every few seconds and mass chromatograms are recalculated from these stored spectra.

This long time between two successive mass spectra stored during a chromatographic analysis stems from two reasons, both of which have now become obsolete. First, the cost of a computer memory was large: the typical 3–5 sec interval is a compromise between measurement frequency, memory size and the time during which spectra corresponding to one analysis can be stored. The recent development of cheap, 10–60 Mbyte disks and of rapid microprocessors has made possible both on-line data reduction and large-scale storage. Second, magnetic instruments cannot be scanned very rapidly, because of the important self-induction of magnetic coils. Thus a significant time is required to scan the spectrum by exponential decay of the current and then to restore the magnetic field to the starting conditions. The fastest scanning speed was about 1–2 sec per mass decase, and still is on many instruments used for LC–MS coupling: this means that it is difficult to store more than one spectrum every 4–5 sec. Advanced magnet technology has now made it possible to record one mass spectrum every 1 sec (for one mass decade; faster speeds are possible for narrower ranges).

Increasingly often quadrupole instruments are being used, however, and these can be scanned much faster. It is possible and useful, however, to spend a longer time on each mass and to jump from mass to mass unit, assuming the analyst knows the exact masses of the ions (within *ca.* 0.1 dalton) and the decimal position is the same for all the ions he is looking for. Current value acquisition for a few milliseconds on each mass requires about 1 sec to scan a range of 500–1000 daltons. On the other hand, a negligible time is necessary for restarting. Thus magnetic and quadrupole instruments offer comparable performances from the scanning time point of view. The quadrupole permits shorter scan times, however, if necessary by reducing the time spent on each mass, with a correlative decrease in the signal-to-noise ratio. It would be possible to store one spectrum every 0.1 sec, which is the requirement for the accurate analysis of a typical LC band as discussed below.

Finally, magnetic instruments offer a relatively wide scan range, exceeding several thousand daltons on many current instruments, while mass spectrometers with a capability considerably exceeding 10,000 daltons are under development Although obvious problems of scan range frequency and sensitivity have to be solved, these instruments will be timely for the generation of LC–MS systems devoted to protein and polynucleotide analysis. On the other hand, it does not seem that the mass range available to quadrupole instruments will significantly exceed 2000 in the near future, barring a possible breakthrough in the design of high-frequency, high-voltage power supplies. The time, t, during which ions of a given mass are collected with a magnetic instrument (continuous scan with exponential decay) is

$$t = \frac{t_{10}}{R \ln 10} \approx 0.43 \cdot \frac{t_{10}}{R} \tag{5}$$

where t_{10} is the scan time for one mass decade and R the resolution (MS definition). For $t_{10} = 1$ sec and $R = 1000$, this time is 0.43 msec, while it can be 2.6 times longer with a quadrupole scanning masses of 100–1000 in 1 sec with the same resolution. This advantage in terms of sensitivity may not be very significant, apart from offsetting the discrimination of the quadrupole against ions of larger masses.

It is important to increase the frequency of data acquisition above 1 Hz, especially for the early peaks of the chromatogram, because it has been shown that in order to observe a decrease in column efficiency smaller than 10% (and hence a decrease in band resolution smaller than 5%) due to data acquisition speed, it is necessary to have a detector with a time constant smaller than one fifth of the time standard deviation of the peak. This standard deviation, σ, is related to the analysis time, t_R and the column efficiency, N, by the conventional equation

$$\sigma = \frac{t_R}{\sqrt{N}} \tag{6}$$

Most LC analyses are now carried out using 10–25 cm long columns with an efficiency between $1 \cdot 10^4$ and $2.5 \cdot 10^4$ plates. With a velocity of 0.05 cm/sec, the elution time of the first components is between 200 and 600 sec, corresponding to standard deviations between 2 and 4 sec. As can be seen in Table III, this is a low velocity and most often the first component has a width of 1 sec. Less drastic specifications can be accepted for the first compounds, which are rarely the most interesting, but for most compounds the peak width will be between 4 and 20 sec. Consequently, it seems necessary to store more than one spectrum every 1 sec, preferably one every 0.1–0.5 sec. Otherwise, resolution is lost, and quantitative analysis and identification made more difficult because cross-contamination between the spectra of separated compounds is created by the data system (Fig. 1)*. A considerable loss of valuable information results. At a distance from peak maximum of 2 standard deviations (Fig. 2), the signal height is about 15% of the maximum, so in many instances the signal is still large enough to be used. If two compounds of similar concentration are separated by 0.1 standard deviation (resolution 0.025), the ratio of their relative concentrations on the tailing and leading edges of their common peak, at a distance from the maximum of $+2\sigma$ and -2σ, will be 0.80 and 1.20, respectively, which should result in an observable difference between the two mass spectra. At a distance of $\pm\sigma$ these ratios are still 0.90 and 1.11, respectively. On the other hand, to obtain a complete separation of these two compounds ($R = 1$) a column 1600 times longer should be used, as the

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* See "Note added in proof" on p. 25.

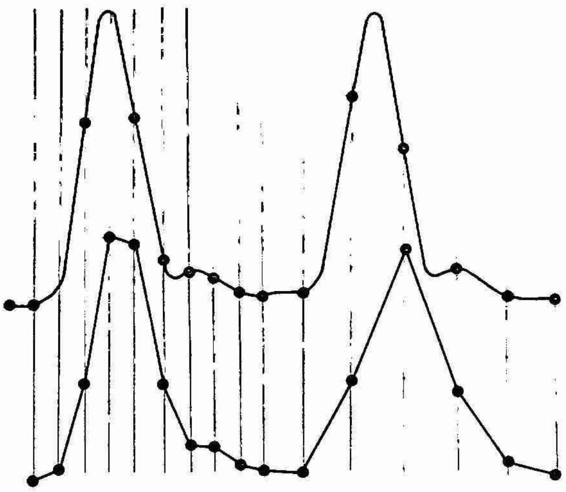

Fig. 1. Upper trace: concentration profile at column outlet; two successive identical injections. Lower trace: reconstructed mass fragmentogram derived from mass spectra recorded at a frequency of one per standard deviation (left) and one per 2 standard deviations (right).

resolution increases only as the square root of the column length. To observe significant band broadening by coelution with an authentic compound, the bandwidth at half-height must increase by at least 10 %, which requires a resolution of about 0.40 between two bands of equal size, 16 times more than the resolution at which a significant difference between the mass spectra of the two wings can be observed.

This illustrates the kind of sensitivity at which the purity of a band could be checked, at least if the signal-to-noise ratio is large enough, provided that spectra can

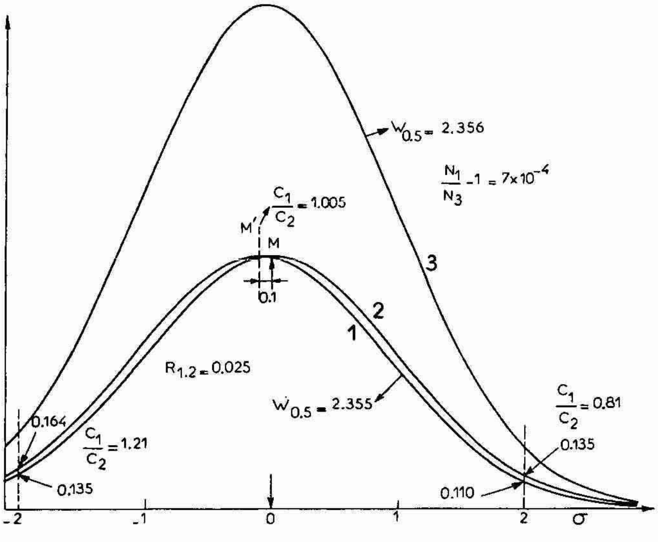

Fig. 2. Concentration profiles of two solutes (1 and 2) at column exit. Resolution: 0.025. The chromatogram recorded is profile 3, slightly wider than profiles 1 and 2 which are identical but shifted by 0.1 × σ.

be recorded with a small enough time constant. Admittedly, one data point per standard deviation would be sufficient for this application.

DETECTION LIMITS

It is usual to consider two classes of chromatographic detectors, those which respond to changes in concentration of solutes in the eluent, such as optical detectors (UV photometers, refractive index detectors, etc.), and those which respond to changes in the mass flow of the solute, such as destructive detectors[16]. The mass spectrometer belongs to the latter class. The main properties are as follows: (i) if the eluent stream is switched off the signal falls to zero exponentially; (ii) if the eluent is diluted in a stream of scavenger, the signal is unchanged, as the solute mass flow rate remains constant; (iii) if the velocity of eluent through the column increases, the maximum peak height increases constantly; and (iv) the peak area remains independent of the flow-rate. These properties assume that within the range of solvent velocities considered, the response factor, *i.e.*, in this instance the ionization efficiency, remains independent of the eluent flow-rate. In GC–MS this seems to be reasonable assumption over a sufficiently large range to be practical. In LC–MS this is more questionable. In interfaces where the solvent is eliminated before the solute enters the ionization source, the response factor remains constant provided that the solvent flow-rate does not overload the interface. When the whole column effluent, or a constant aliquot of it, is injected into the source, the density of vapour in the source will be a function of the flow-rate, whose changes may affect the response. In such a case the total flow-rate of solvent and/or reagent gas or vapour to the source must be optimized separately. For this reason, the use of ammonia or another chemical ionization reagent in the DLI has also the advantage of eliminating the influence of flow-rate oscillations due to pulsations of the pump[17]. It also makes the response factor independent of solvent flow-rate, within some limits, and permits the use of properties (ii) above, which is interesting when using very narrow bore packed columns or capillary columns, and (iv), which is important because it provides for good quantitative results.

The detection limit of a chromatographic detector is defined as the mass of compound that generates a signal equal to twice the noise. This definition can be extended straightforwardly to the mass spectrometer working with single ion monitoring, as the signal obtained is identical with a classical chromatogram. We note in passing that in such a mode hardly any problem arises because of too large a time constant, even with magnetic instruments. In good conditions the detection limit is of the order of a few picograms, unless the corresponding compound has an unusually large ionization yield, as happens, for example, to haloaromatics in electron-capture ionization and negative-ion detection, a case in which the detection limit can be several orders of magnitude smaller[18].

The chromatographer is always surprised by the low ionization yield of the mass spectrometer, *E*, the number of ions collected on the MS detector per molecule introduced into the source. For this reason, it is worth reviewing briefly the various sources of losses[19]:

(i) To detect a signal on a given mass and calculate the coordinates of its maximum, *i.e.*, the corresponding molecular weight, we need about 100 ions at the detector entrance slit; practically all ions reaching this slit are detected.

(ii) The object and image slits are rectangular. Because of the scanning the convolution product of these two slits, assumed to be identical, is a triangle and we require 200 ions to enter the analyser. Losses in the analyser are assumed to be negligible.

(iii) The extraction yield of ions from the source to the analyser across ion optics (focusing of ions) is about 10%. We need to make 2000 ions in the source.

(iv) To obtain a spectrum useful for identification purposes, the previous figure must be applied to ions accounting only for small peaks in the mass spectrum. Peaks that are 10% of the base peak should be detectable as described in (i) above. We need to make at least $2 \cdot 10^4$ molecular ions during the time when the corresponding mass is scanned.

(v) The ionization yield varies widely with the ionization method used and the particular compound being analysed. Although it can be close to 1 for electron capture by haloaromatics, it can also be as low as 10^{-4} for electron impact. Assuming an average value of 10^{-3} means that $2 \cdot 10^7$ molecules should be present during the scan.

(vi) The scan of one mass lasts about 1 msec. The introduction of sample molecules into the source must proceed at a speed of $2 \cdot 10^{10}$ molecules/sec.

The maximum concentration of the Gaussian band of a solute of retention volume V_R and efficiency N is

$$C_M = \frac{m\sqrt{N}}{V_R\sqrt{2\pi}} \tag{7}$$

where m is the sample mass. If the column capacity factor is k' and the liquid cross-section of the column is s, we have

$$C_M = \frac{m}{s} \cdot \frac{\sqrt{N}}{L(1 + k')\sqrt{2\pi}} \tag{8}$$

where L and u are the column length and the solvent velocity, respectively. The mass flow-rate of sample to the MS source is then the product $C_M F$, where $F(= Su)$ is the solvent flow-rate. With a splitting ratio r, the mass flow-rate of sample to the source is

$$\frac{\mathrm{d}m}{\mathrm{d}t} = C_M Fr = \frac{m\,u\,r\sqrt{N}}{L(1 + k')\sqrt{2\pi}} \tag{9}$$

Comparing eqn. 9 with the condition (vi) above, we must have

$$\frac{m\,u\,r\sqrt{N}}{ML(1 + k')\sqrt{2\pi}} \cdot N' = 2 \cdot 10^{10} \tag{10}$$

where M is the molecular weight of the solute and N' is Avogadro's number. With $L = 15$ cm, $N = 1.5 \cdot 10^4$ plates, $u = 0.05$ cm/sec, $r = 1$, $M = 500$ and $k' = 1$, we have $m = 2 \cdot 10^{-10} = 0.2$ ng. This is in agreement with the specifications of modern instruments[20], which give a detection limit of 100 pg of methyl stearate ($M = 298$, hence $m = 120$ pg), although the specifications may not have been calculated with the rather favourable chromatographic conditions selected above: narrow peaks with small retention give large maximum concentration.

The sensitivity in this scanning mode is diffucult to define as we are not looking for a threshold above which the detector signal corresponds to the elution of a band, but for a more complex set of information. "Chemical noise" resulting from column bleeding, minor sample constituents or other sources of eluent pollution contributes significantly to the detection limit, and in some instances makes sample "clean-up" mandatory. The definition of this sensitivity and its measurement are clearly the mass spectrometrist's problem. In most instances it does not seem that the detection limit is below 1 ng.

If there is no scanning but MS is working in a true single-ion monitoring (SIM) mode, a smaller amount of sample is necessary: with a 1 sec time constant, 1000 times less, around 1 pg. The values are similar for a magnetic instrument or a quadrupole. The only possibility of improving them markedly is to use a very efficient ionization technique, which explains why haloaromatics such as polychlorodioxins can be detected at the femtogram level in GC–MS with negative ions.

To be meaningful these figures must be compared with the sample size that can be accomodated by the column. With a 4 mm I.D. column it rarely exceeds a few milligrams. In other words, the current state of the art permits in most instances the identification of impurities at the ppm level and their detection at the ppb level, assuming the total effluent would be injected into the ion source. With 1 mm I.D. columns these figures become 16 times larger, *i.e.*, identification of compounds above 10–20 ppm and a detection limit in SIM above 20 ppb.

These figures must be reduced further by up to one order of magnitude, sometimes more, because the column does not always accept such a large sample. The solubility of some compounds in the eluent is very low and the solutions injected must be more dilute than a saturated solution if the equilibrium isotherm corresponding to the chromatographic mechanism used is to be linear. Otherwise, the band profile is not Gaussian, but unsymmetrical and broader and the resolution is poor, although sometimes the profile of trace component bands which are well resolved from the main compounds is still acceptable.

For all of the above reasons, it is hoped that mass spectrometrists will find ionization mechanisms permitting a reduction in these detection limits by one or, better, two orders of magnitude, unless specialists in ion optics find a more efficient design for the extraction and focusing of the ions that are formed but escape collection.

If these figures seem demanding, they can be compared with the specifications that we can draft for a mass spectrometer to be coupled to an LC capillary column. It is easy to calculate that in order to be competitive with present packed LC columns, such capillary columns should have an inner diameter smaller than 10 μm[21]. The resolving power of these columns, which will probably be used in some advanced laboratories within a few years, would be tremendous, as are the equipment problems which they produce. The maximum sample size is of the order of 10 ng at most (volume flow-rate *ca.* $7 \cdot 10^{-3}$ μl/min). To detect an impurity at the ppb level, we have to be able to obtain a signal with fewer than 12,000 molecules. This is a challenge and probably requires new approaches, such as the use of cross-collision molecular beams.

The linearity is the last important property of the MS detector to be discussed. It is usually represented by the dynamic linear range, which is the ratio of the sample size for which the deviation from a linear response is 10 % to the detection limit[16]. A large dynamic linear range is required in chromatography as the concentration of a given compound in a series of samples can vary over several (3–4) orders of magnitude, while the range of concentration investigated during one analysis can vary from almost 1 % for the main component (column overloaded) to less than 10^{-10} %. This quality is required from the mass spectrometer especially when it is used in single-ion monitoring. In many instances it permits the quantitative analysis of incompletely resolved or non-resolved compounds. The degree of resolution necessary for the use of another, non-selective, detector would be such that the analysis time would be so long and the dilution so great that the analysis would become impossible. Alternatively, selective extraction and enrichment must be used, which are tedious, time consuming and increase the risk of sample pollution, alteration and errors. The use of a detector with a dynamic linear range of 100–1000 is still possible if it is sensitive enough, using dilution and an internal standard, although tedious. The narrow dynamic linear range of the mass spectrometer, although smaller than that of other LC detectors, is the last complaint of chromatographers.

CONCLUSION

Up to now most work on LC–MS coupling has been instrumental and has focused on interfaces that permit the transformation of the sample solution into a vapour mixture at a pressure low enough for the proper functioning of more or less conventional ion sources[1–3]. Some work has been done to adapt LC instruments with this aim, but little to modify MS instruments, although advantage has been taken of the recent progress in ionization methods and instrumentation.

Now that the feasibility of LC–MS coupling has been amply demonstrated, it may be time to develop an LC–MS instrument which would be integrated.

Among other features it is important that this instrument should permit the scanning of mass spectra up to large masses (several thousands), as the elution of large peptides, small proteins, polynucleotide sequences, etc., is now possible, if not always easy, easy adjustment of this mass range and the scanning frequency up to at least 10 Hz, true single-ion monitoring on a number of masses simultaneously, flexible adjustment of the composition of gases and vapours making up to source plasma and an improved ionization yield.

The choice of interface is difficult. A direct liquid interface must be used, because it permits the ionization of heavy, complex, sensitive molecules, the direct transfer of pre-formed ions and the use of complex reactions leading to ionization. It gives spectra with few characteristic features, however. Often only the quasi-molecular ion and a few aggregate ions with solvent molecules, the composition of which is not easily predictable, are observed[22]. With large molecules some fragments are also recorded. This does not permit easy identification, assuming that for molecules of that size there is an easy way. Possibly for smaller molecules, in the 100–300 to 500 dalton range, electron impact spectra may provide enough useful information to warrant the design and use of a multi-interface instrument. Electron impact spectra of large molecules are often too complex to be useful, however.

At any rate the development of digital electronics will permit the design of more flexible, easier to use instruments.

NOTE ADDED IN PROOF

In a discussion at the meeting, Dr. Henneberg pointed out that we look upon the mass spectrometer as an LC detector and demand from it the same performance as that given by other LC detectors, in spite of its cost and complexity. Dr. Henneberg showed that with a frequency of one data point per standard deviation, sufficient information is obtained to decide whether a band is pure or not—the essential use of the mass spectrometer. This is true, but then we need another detector to obtain the chromatogram and to see for which bands the purity should be checked. This does not make the design of the interface easier. Perhaps we need to be able to use the MS instrument for performing both tasks and to choose a compromise for each analysis between the scanning rate (*i.e.*, response time) and the signal-to-noise ratio.

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MECHANIZED OFF-LINE COMBINATION OF MICROBORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND LASER MASS SPECTROMETRY

JOSEF F. K. HUBER*, TADEUSZ DZIDO* and FRANZ HERESCH
Institute of Analytical Chemistry, University of Vienna, Waehringerstrasse 38, A-1090 Vienna (Austria)

SUMMARY

Laser desorption mass spectrometry is shown to be very suitable for a mechanized off-line combination with liquid chromatography. In the procedure described, a simple mechanical device provides fractionwise collection of the chromatographic effluent at flow-rates up to 0.5 ml/min. The peak broadening effect of this module is very low because of the particular design of the sample holder used. Up to 30 chromatographic fractions have been monitored by mass spectrometry, the fraction sizes being in the order of one half of the peak standard deviation.

INTRODUCTION

The combination of different analytical unit operations requires, in general, a mutual adjustment of their individual operating conditions and this compromise can lead to a loss in performance. For optimum results the integrity of the unit operations involved should be maintained as far as possible. The question of whether or not to couple different unit operations in real-time (provided modes are technically feasible) is dependent on their time compatibility which should be evaluated before any experimental approach to coupling.

As to the combination of chromatography and mass spectrometry (MS), it is necessary not only to minimize extra-column sample dispersion effects, restrictions in the choice of phase systems and ionization modes, etc., but also to maintain the full range of tractable compounds. With liquid chromatography (LC)–MS, in contrast to gas chromatography (GC)–MS, the limiting factor is the mass spectrometry because of its low (or even zero) sensitivity for many substances that can be handled by LC, especially macromolecules. In order to cope with non-volatile and thermolabile compounds and, in consequence, with low ion currents (signal/noise ratios), large mass ranges, etc., the MS measurement should not be limited in time, particularly when not only structural confirmation is required and more sophisticated

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* Permanent address: Department of Inorganic and Analytical Chemistry, Medical Academy, Lublin, Poland.

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and time-consuming techniques such as high-resolution scanning and MS–MS have to be used. In this analytical situation, which may be classified as "non-routine", the MS measurement should be independent of the chromatographic speed.

Based on such considerations, an off-line LC–MS system is proposed in which the linking steps, namely (i) collection of the LC effluent and (ii) MS measurement, are mechanized. A multi-sample holder specially designed for quantitative laser desorption mass spectrometry[1] is used for localized storage of the LC fractions. In this paper the performance of the set-up is reported with emphasis on its extra-column peak broadening effect using a microbore column. Microbore packed columns are increasingly applied for on-line LC–MS coupling[2], especially with direct liquid introduction[3–7], and offer several advantages such as low flow-rate, low sample dilution, etc.

As a model problem for the evaluation of the proposed off-line LC–MS coupling, the separation and detection of L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine (DA) was investigated mainly because of the possibility of using electrochemical detection as a reference[8,9] which is known to give negligible peak broadening[10].

EXPERIMENTAL

Microbore high-performance liquid chromatography (HPLC)

The chromatographic conditions for the liquid chromatographic separation of L-DOPA and DA were as follows: high-performance liquid chromatograph (Model S-101; Siemens, Karlsruhe, G.F.R.); stainless-steel column (200 × 1 mm), packed with 10-μm octyl silica (LiChrosorb RP-8; E. Merck, Darmstadt, G.F.R.); mobile phase, 0.1 N aqueous HNO_3 + NaOH to pH 2.8, flow-rate 5–30 μl/min; room temperature; injection device, pneumatic sampling valve (Model 60 AH; Valco Instruments, Houston, U.S.A.) with 0.5-μl loop, injection solvent 0.1 N aqueous trichloroacetic acid; reference detector, modified electrochemical detector with glassy carbon electrode (Model LC-2A; Bioanalytical Systems, West Lafayette, U.S.A), as described[11].

Laser MS

Mass spectrometric detection was carried out on a magnetic sector instrument (MS 902; AEI-Kratos, Manchester, Great Britain) adapted for pulsed laser desorption[12,13]. The measurement conditions and procedures for quantitative work were as described earlier[1]. The indented stainless-steel rod used as sample holder is shown in Fig. 1. Each indentation is loaded with a fraction of the chromatographic effluent. The indentations were designed for two particular tasks; (i) to allow laser desorption or related surface ionization techniques; (ii) to enable localized deposition of liquids. Depending on the surface tension of the liquid it is possible, for instance, to overload an indentation having a volume of 1 mm^3 as listed in Table I.

The laser desorption spectra of L-DOPA and DA exhibit mainly pseudomolecular signals such as $[M + \text{alkali}]^+$, $[M - H + \text{alkali}_2]^+$ and $[M + H]^+$. The intensity ratio of protonated *vs.* cationized species is highly dependent on the chemical environment and pretreatment of the sample. Calibration experiments were made with compounds dissolved in the LC eluent. Due to the acidic pH of 2.8, a $[M + H]^+/[M + Na]^+$ ratio of about 10 was observed in this case.

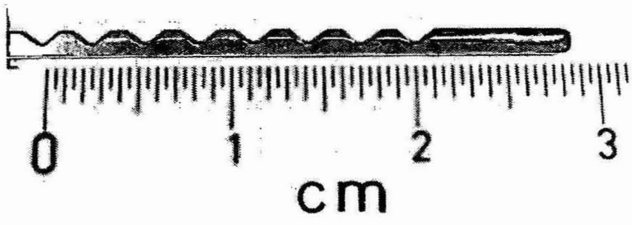

Fig. 1. Indented sample holder.

For detection, measurements were performed in scanning and in selected ion monitoring modes (using accelerating voltage alternation). Since a pulsed ion source is involved, both types of operation are equivalent and have to be pulse-synchronized. For details see ref. 13.

Mechanized fractionation

The mechanized discontinuous (fractionwise) collection of LC effluent requires two simultaneous, but independent, operations; (i) shift of the storage device; (ii) actual sampling. In the mechanized apparatus shown in Fig. 2 these operations are both made by means of a screw rod (R1)driven by a motor (M). The periodic deposition of effluent is effected by the helical motion of rods R1 and R2 (onto which the sample holder S is mounted excentrically). Each turn thus brings a new indentation into the chromatographic outlet where the liquid (droplet accumulated since the previous indentation) is collected. The appropriate shift along direction Z is determined by the indentation of the sample holder (see Fig. 1) to which thread T corresponds. The time interval, Δt_f, between collections is independently regulated only via the speed of the motor.

TABLE I

CORRELATION BETWEEN SURFACE TENSION, γMM), AND MAXIMUM VOLUME, ΔV_{max}, ACCOMMODATED PER INDENTATION FOR DIFFERENT SOLVENTS

(a) Manual loading (from syringe); (b) mechanized loading (from chromatographic outlet) at sampling rate of 20 min^{-1}.

Solvent	$\gamma^{20°C}$ *(dyn/cm)* *(ref. 14)*	ΔV_{max}*(μl)*	
		(a)	*(b)*
Water	73	13	5.8
Water–methanol (1:1, v/v)	35	8	4.5
Water–acetonitrile (1:1, v/v)	31	7	3.8
Acetonitrile	29	5	2.9
Methanol	23	1.5	1.4

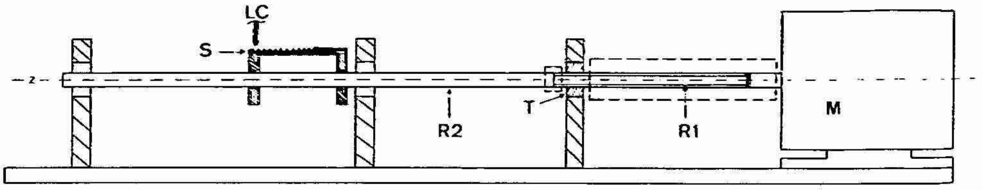

Fig. 2 Apparatus for automated storage of LC fractions: M = motor; R1 = screw rod; R2 = rod connected to R1; T = thread; S = sample holder; LC = chromatographic outlet; Z = rotation axis.

In this way the chromatographic effluent is gradually stored in the indentations, *i.e.*, in one sample holder or in a number of them placed in series (on R2), which can then be transferred to the mass spectrometer. The solvent is usually removed just after deposition. If necessary, *e.g.*, in case of labile compounds, the fractions may be kept in solution until insertion into the spectrometer.

For measurement, the sample holder(s) were attached to the direct probe and manually inserted into the mass spectrometer as described[1].

RESULTS AND DISCUSSION

Fraction size

The size of the effluent fractions collected, *i.e.*, their volume, ΔV_f, or duration, Δt_f, has to be optimized with respect to the chromatographic and MS integrity as mentioned earlier. In this context it is useful to relate these parameters to the chromatographic scale, namely to the volume or time standard deviations, σ_V or σ_t, of a peak, and to employ a relative fraction size, $\alpha = \Delta V_f/\sigma_V = \Delta t_f/\sigma_t$. It was shown that α has to be in the order of 0.5 if no information is to be lost[15], which will happen if α, is larger, whereas in the case of smaller values only redundant information may be gained. The MS information content, on the other hand, is mainly affected via the signal/noise ratio which depends on the sample level and therefore increases with relative fraction size, α. Due to the nature of the chromatographic process, σ_V and σ_t are functions of retention volume and retention time, respectively. In order to keep α constant, the fraction size (ΔV_f or Δt_f) has to be increased in the course of a chromatogram. This can be done by programming the motor of the mechanized fraction collector shown in Fig. 2. If only a limited number of chromatographic fractions are to be investigated (see Fig. 3), the size of the fractions may remain unchanged.

The working conditions for mechanized fractionation have to be adjusted to the chromatographic flow-rate, w, in such a way that the sampling rate, $1/\Delta t_f$ = motor revolutions per minute, is greater or equal to $w/\Delta V_{max}$. The values of ΔV_{max} were determined for various liquids and are given in Table I. So far, sampling rates up to 60 min^{-1} have been used which corresponds to a maximum flow-rate of almost 0.5 ml/min for aqueous effluent (see Table I).

In Fig. 3 the results of electrochemical and MS detection are contrasted. The selected mass profiles (histograms) refer to the $[M + H]^+$ ions of L-DOPA and DA (m/z 198 and 154, respectively). As can be seen from the mass profile, L-DOPA also shows a weak signal at m/z 154 resulting from the loss of CO_2.

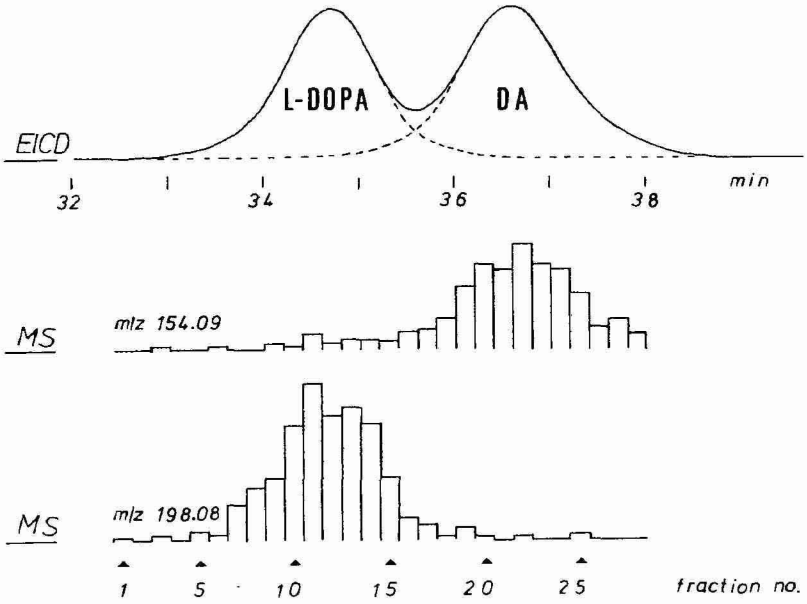

Fig. 3. Electrochemical (EICD) *versus* MS peak profiles for L-DOPA and DA (100 ng each).

Peak broadening

As with any other part of a chromatograph, the evaluation of the mixing characteristics of the MS detector has to be based on an analysis of the peak variance. The variance, σ^2, of a recorded peak is the sum of different contributions

$$\sigma^2 = \sigma_S^2 + \sigma_C^2 + \sigma_D^2 \qquad (1)$$

where σ_S^2 = variance of the sample volume and sampling device, σ_C^2 = variance of the chromatographic column and σ_{VC}^2 = variance of the detection system.

The peak variance, σ_{VC}^2 in volume units, caused by the chromatographic column is given by[16]

$$\sigma_{VC}^2 = \varepsilon_m AHL(1 + \kappa)^2 \qquad (2)$$

in which ε_m = fraction of the column volume filled by the mobile phase, m, A = cross-sectional area of the column, H = theoretical plate height of the column describing its mixing characteristics, L = length of the column and κ = capacity factor describing the retardation of the analyte. It can be seen from this equation that the contribution of the chromatographic column to the peak variance decreases proportionally with the cross-sectional area of the column. Therefore the extra-column contributions, σ_{VS}^2 and σ_{VD}^2, to the peak variance are crucial for the use of microbore columns.

On the other hand, microbore columns exhibit a high mass sensitivity since the peak height is given by[17]

$$c_{i,\max} = Q/(2\pi)^{\frac{1}{2}}\sigma_V \tag{3}$$

where $c_{i,\max}$ = concentration maximum of the peak in the detector, Q = amount of analyte and σ_V is described by eqns. 1 and 2. In addition, microbore columns have a low flow-rate which is favourable for MS.

In LC–MS the detection system consists of the interface and the spectrometer. The contribution of the described off-line interface plus the mass spectrometer to the peak variance can be calculated by means of eqn. 1.

Due to the extremely low dead-volume of the electrochemical detector and its low time constant it may be assumed that its contribution, σ_D^2, to the total peak variance is negligible. Assuming that the contribution of sampling, σ_S^2, is constant, it follows that the volume standard deviation for MS detection, $(\sigma_{VD})_{MS}$, is given by

$$(\sigma_{VD})_{MS} \approx \sqrt{(\sigma_V^2)_{MS} - (\sigma_V^2)_{EICD}} \tag{4}$$

where $(\sigma_V^2)_{MS}$ and $(\sigma_V^2)_{EICD}$ are the total volume variances of the peaks for MS and electrochemical detection, respectively.

It is evident from eqn. 1 that σ_{VC}^2 and σ_{VS}^2 should be kept as small as possible when determining $(\sigma_{VD})_{MS}$. This implies high chromatographic performance (low theoretical plate height, low sample volume, low dead-volumes of injector and connecting lines) and low κ values of the investigated peaks. The σ_V (µl) data given in Table II were obtained for the L-DOPA peak separated at two different flow-rates with κ = 2.15. For MS detection the $[M + H]^+$ ion (m/z 198) was monitored.

The results listed in Table II indicate negligible peak broadening for the off-line MS detection. Independently of the experimental conditions (flow-rate, fraction size), the observed peak standard deviation only increases by 0.1–0.2 µl when applying off-line MS instead of electrochemical detection. The greater extra-column effect at low sample level (200 ng) clearly results from the reduced precision of quantitation (standard deviation *ca.* 25%). The actual detector contribution, $(\sigma_{VD})_{MS}$ calculated according to eqn. 1, is given in column 6 of Table II and amounts to about 1.5 µl.

TABLE II

PEAK WIDTH OBTAINED WITH ELECTROCHEMICAL (EICD) OR OFF-LINE MASS SPECTROMETRIC (MS) DETECTION UNDER DIFFERENT CONDITIONS

Theoretical plate height: 34 and 62 µm at flow velocities of 0.27 and 0.83 mm/sec. Precision of the mean value of the peak standard deviation by off-line MS calculated from five measurements: 0.2 µl for 1 µg; 0.5 µl for 0.2 µg.

Flow-rate (µl/min)	*Flow velocity (mm/sec)*	*Amount injected (µg)*	*α*	*Mean value of peak standard deviation (µl)*		*Contribution of MS detection* $(\sigma_{VD})_{MS}$ *(µl)*
				$(\bar{\sigma}_V)_{EICD}$	$(\bar{\sigma}_V)_{MS}$	
8.7	0.27	1.0	0.24	4.6	4.7	0.9
			0.48	4.6	4.8	1.4
		0.2	0.48	4.6	5.7	3.3
28.0	0.83	1.0	0.31	6.1	6.3	1.7

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CHROM. 15,499

FRACTIONAL SAMPLING INTERFACE FOR COMBINED LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY WITH ^{252}Cf FISSION FRAGMENT-INDUCED IONIZATION

HARTMUT JUNGCLAS*, HARALD DANIGEL and LOTHAR SCHMIDT
Kernchemie, FB 14, Philipps-Universität, D-3550 Marburg (G.F.R.)

SUMMARY

Time-of-flight mass spectrometry (MS) utilizing ^{252}Cf fission fragment-induced ionization and desorption of non-volatile compounds is suitable as a universal detector in high-performance liquid chromatography (HPLC). The LC–MS combination is achieved by nebulizing the unsplit effluent (*ca.* 0.5 cm^3 min^{-1}) into a vacuum chamber (*ca.* 10^{-4} bar) and vacuum-drying the non-volatile compounds on collector foils. The permanent storage of the separated compounds on twelve discrete sample foils decouples the LC and MS operations. Stepping the disk interface periodically, each sample is transported into the ion source for the non-destructive MS analysis. It can be repeated afterwards and samples may be recovered for other analyses.

INTRODUCTION

The capabilities of high-performance liquid chromatography (HPLC) in the quantitative analysis of therapeutic agents[1] (actually all organic compounds with low volatility) are generally recognized. However, the use of mass spectrometry (MS) as a universal detection method[2] should overcome some problems of sensitivity, selectivity and reproducibility. Chemical ionization, field desorption and laser- or keV–MeV particle-induced desorption are soft ionization techniques[3] suitable for organic solids[4].

Common to all LC–MS combinations is the interface problem, *i.e.*, the need to separate the eluent from the dissolved compounds which must be transferred into the ion source. Commercial systems offering chemical ionization and induced desorption (fast atom bombardment) utilize a moving belt interface[5]. This performs continuous sampling of the effluent at atmospheric pressure, assists evaporation of the mobile phase using an infrared heater and overcomes the pressure decrease towards the ion source by three differentially pumped vacuum stages. The semi-permanent storage of the chromatographically separated compound on the belt decouples the LC and MS operations.

In order to use ^{252}Cf fission fragment-induced desorption[6] we have modified these interface solutions to obtain a stepping disk interface, which performs fractional sampling in a vacuum drying process. Again the retention time scale is transformed into a spatial distribution. As the ^{252}Cf fission fragment-induced ionization is virtually non-destructive, this sampling procedure results in a permanent storage of the collected compounds. Thus the mass spectrometry can be performed both on-line and off-line with respect to the LC sampling.

EQUIPMENT

The linkage of the LC–MS system is shown in Fig. 1. The dissolved compound is purified (when necessary extracted from the matrix) in a preparative procedure. The modular liquid chromatograph is run in the reversed-phase mode with isocratic elution. The effluent is fed unsplit, in some instances by-passing the UV detector, into the interface, which, together with the time-of-flight mass spectrometer (TOF MS), was designed and assembled at our laboratory.

The LC–MS interface

The polar effluent, typically methanol–water at a flow-rate of 0.5 cm^3 min^{-1}, emerges from the stainless-steel LC capillary (0.2 mm I.D.) directly into the evacuated sampling chamber (Fig. 2). The last section of the capillary (*ca.* 2 cm) is heated to prevent plugging by freezing and to achieve proper nebulization of the effluent. When the heating power is properly adjusted, the expansion process results in a hardly visible mist formation. The resulting aerosol impinges on a 2 μm thick foil, where the non-volatile compounds are collected in a vacuum-drying process. A very thin layer of frozen effluent (water) indicates proper collection by freeze-drying.

A PTFE disk (Fig. 3) sandwiched between two flanges serves as a sample carrousel and spans the pressure difference between the sampling chamber (*ca.* 10^{-4} bar) and the ion source (*ca.* 10^{-9} bar).The fractional sampling of the chromatogram in up to twelve fractions, due to twelve discrete sample positions, is sufficient for

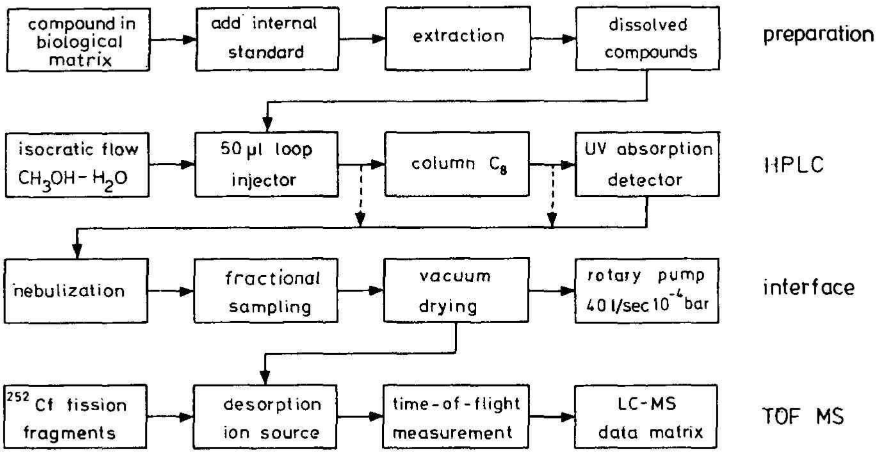

Fig. 1. Flow chart of the combined LC–MS system.

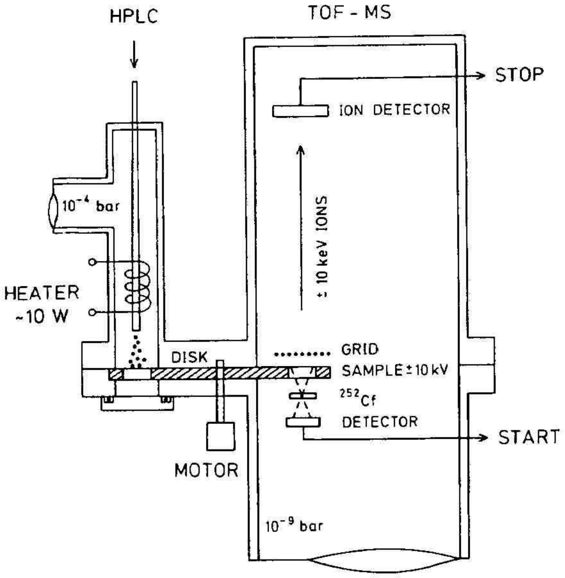

Fig. 2. Time-of-flight mass spectrometer with disk interface for HPLC effluent (cut vertically along B–C in Fig. 3).

most LC–MS applications. In many instances (routine analyses) it is even possible to cut a single fraction rather than monitoring the complete chromatogram.

Fractionation can be periodic (typically 1 min^{-1}) or adjusted to the chromatographic separation. The first sample foil may be analysed in the ion source (cut C in Fig. 3) while the fifth foil is collected in the sampling chamber (cut B in Fig. 3) and so on. In this instance the sampling time per fraction limitis the data acquisition time. To avoid this limitation, the mass spectrometric analysis can be performed after the sampling procedure (off-line).

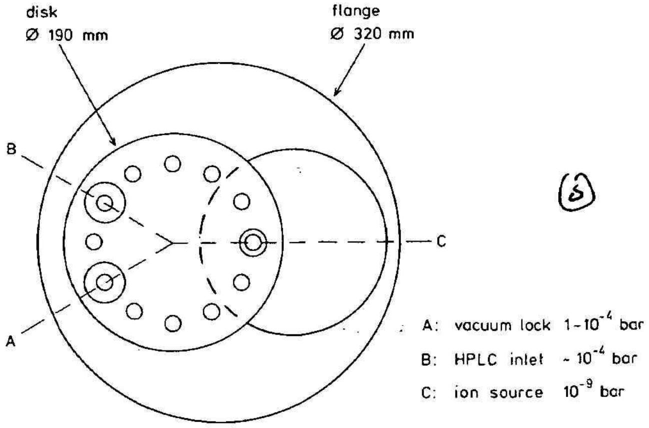

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Fig. 3. Top view of the sample changing disk indicating the typical pressures at the three main sections.

The time-of-flight mass spectrometer

Each sample is transported by the PTFE insulating disk into the ion source, where it is set at the acceleration potential (±10 kV) and irradiated with fission fragments (Fig. 2). The sudden (< 1 nsec) perturbation in the sample caused by each penetrating fission fragment produces quasimolecular and fragment ions of the compound. As all ions are accelerated to the same kinetic energy-to-charge ratio, E/z, their mass-to-charge ratio m/z can be determined from a time-of-flight t measurement ($m/z = 2E/zv^2 = \alpha t^2$, with a calibration constant α, velocity v).

The acquisition of each time-of-flight spectrum in a multi-channel-analyser lasts 1–10 min (depending on the sample coverage of 0.1–1 μg cm^{-2})[7,8], but it results in a complete mass spectrum (*e.g.*, m/z 1–1000) without scanning. Further, it can be repeated to obtain results on positive and negative ions from the same sample (or to obtain better statistics), as the analysis is non-destructive.

EXPERIMENTAL

The LC–MS analysis as displayed in Fig. 1 is utilized routinely to investigate the pharmacokinetics of the anti-neoplastic agent Etoposide (VP16-213) using the homologous compound Teniposide (VM26) as an internal standard[9]. For example, a 1 cm^3 serum sample is spiked with 20 μg of VM 26 and a chloroform extraction is performed. The effluent (methanol–acetonitrile–water, 2:1:1; 500 μl min^{-1}) is sampled on up to twelve foils, typically 0.5–1 min per fraction. The resulting data of matrix retention time *versus* ion mass (Fig. 4) shows the quasimolecular ions $[M + H]^+$ and $[M + Na]^+$ at m/z 589 and 611 for VP16-213 (mol. wt. 588) and at m/z 657 and

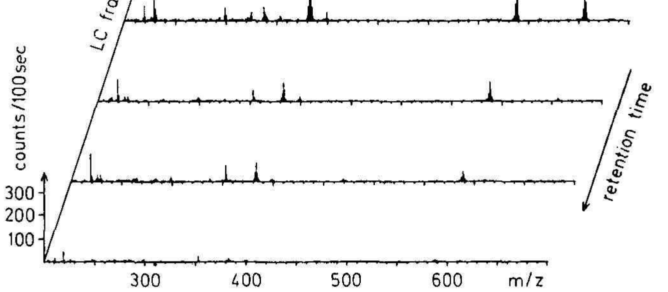

Fig. 4. An LC MS data matrix given by seven spectra of seven consecutive fractions. The sample was taken from a patient 2 h after a 250-mg infusion of VP16-213 (mol. wt. 588) and spiked with 20 μg/cm^3 of VM26 (mol. wt. 656).

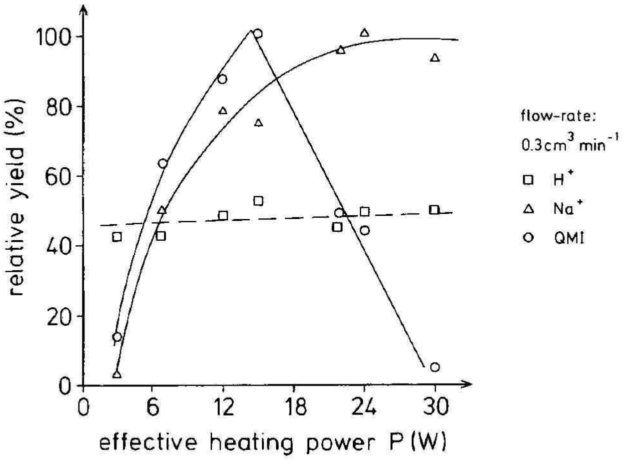

Fig. 5. Relative yields of quasimolecular ions (QMI) from Verapamil (*m/z* 453–456), Na^+ and H^+ at a flow-rate of 0.3 cm^3 min^{-1} (methanol–water) as a function of the effective heating power deposited at the end of the LC capillary.

679 for VM 26 (mol. wt. 656). The retention time for both compounds differs only *ca.* 0.5 min. The concentration of VP16-213 is calculated from the quasimolecular ion intensity using a calibration graph[9].

The performance of the disk interface, *i.e.*, the collection efficiency of the non-volatile compounds and their mass spectrometric detection, depends on the following parameters: the flow-rate and composition of the polar eluent (*i.e.*, proportion of water), the heat deposited at the end of the LC capillary and the capillary nozzle–collector foil spacing. A systematic investigation of these parameters was performed by eluting the antiarrhythmic drug Verapamil (mol. wt. 454) with a polar eluent (methanol–water, 3:1).

A typical result is shown in Fig. 5, obtained by using a constant flow-rate and varying the heating power, *P*. The unaffected intensity of H^+ ions demonstrates stable conditions of the MS operation. Hence the fluctuations of the yields of the other ions resemble the variations in collection efficiency. The different behaviour between the quasimolecular ions (*m/z* 453–456) and Na^+ ions indicates differences in volatility and probability that the sample sticks on the collector surface.

The complex situation for the collection of Verapamil molecules is displayed in Fig. 6 as a function of flow-rate, *F*, and effective heating power, *P*. Optimal collection efficiency is achieved at about $P/F = 35$ W min cm^{-3}, resulting in perfect nebulization of the effluent. The effluent splashes at lower and vaporizes at higher heating power.

At high power ($P/F \approx 60$ W min cm^{-3}), the resulting vapour jet not only prevents the collection of Verapamil molecules but even removes a pre-collected sample. This washing effect is proved by the following experiment. At 0.3 cm^3 min^{-1} and 12 W, a perfect sample (*ca.* 10 μg cm^{-2}) of Verapamil and its homologous compound D600 (mol. wt. 484) is collected during 1 min. The resulting mass spectrum (Fig. 7a) yields the quasimolecular ions and typical fragments of *m/z* 303 and 333. Now the effective heating power is increased to 30 W, resulting in a vapour jet of the

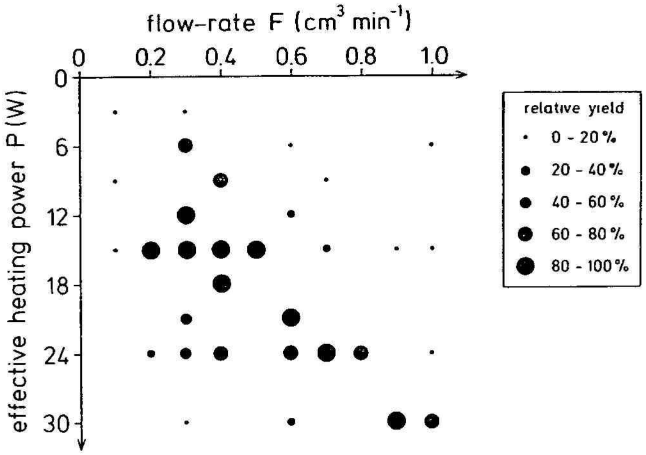

Fig. 6. Relative yields of quasimolecular ions from Verapamil as a function of flow-rate, *F*, and effective heating power, *P*. Optimal efficiency is achieved at $P/F \approx 35$ W min cm^{-3}.

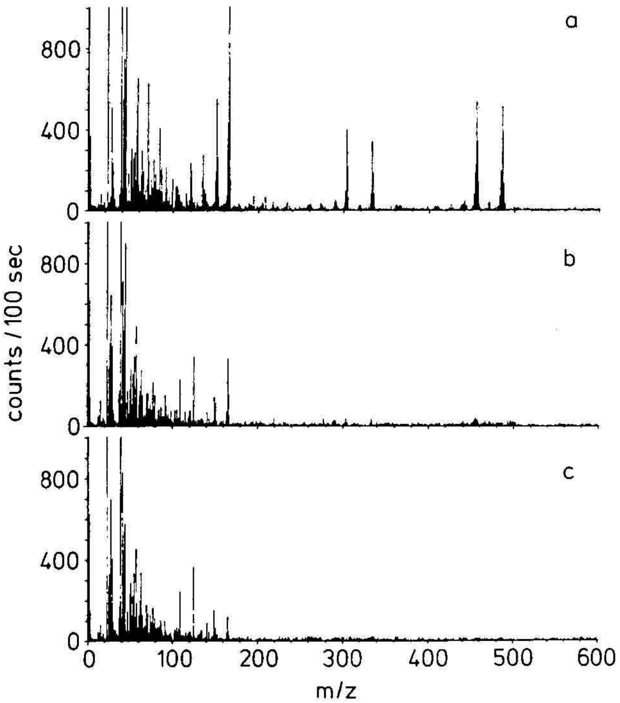

Fig. 7. Mass spectra of Verapamil (fragment *m/z* 303 and QMI *m/z* 453–456) and its homologous compound D600 (fragment *m/z* 333 and QMI *m/z* 483–486) at three stages: (a) after collection under proper nebulization conditions; (b) after cleaning for 10 sec with vapour jet; and (c) after additional cleaning for 20 sec.

pure eluent. The sample is turned back into the sampling chamber, first for 10 sec, analysed (Fig. 7b) and then for a further 20 sec, and analysed again (Fig. 7c). Obviously the pre-collected sample is washed off by the vapour jet within 10–30 sec.

Under optimal nebulization conditions the divergence of the aerosol jet is so small that the diameter of the collected sample can be adjusted in the range 3–10 mm by varying the capillary nozzle–collector foil distance from 2 to 8 cm.

DISCUSSION

The LC–MS interface described here assists nebulization of the unsplit effluent by heating the end of the LC capillary, similarly to the LC–MS thermospray[10], but using approximately ten times less heating power, and collects the aerosol under medium-high vacuum conditions. This deposition and collection process involving vacuum drying is unique by different to all other storage methods, as no infrared heater is involved, which could cause the sample to volatilize or even to pyrolyse.

As the fission fragment-induced ionization is virtually non-destructive (fast ion bombardment to some extent also), our LC–MS system and an HPLC–secondary-ion MS combination[11] operate with permanent storage of the collected chromatographic distribution. As the latter LC–MS technique uses an endless belt (ribbon), which can be prepared for the next sampling cycle by a silver deposition procedure[11], this system allows continuous and unlimited monitoring. The limitation in our method due to the number of collector foils (twelve at present) could be overcome by cleaning the foils after the analysis with a hot vapour jet (as described above). However, any cleaning procedure restricts the re-analysis capability of the LC–MS system.

ACKNOWLEDGEMENT

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OPTIMIZATION OF THE INSTRUMENTAL PARAMETERS OF A COMBINED LIQUID CHROMATOGRAPH–MASS SPECTROMETER, COUPLED BY AN INTERFACE FOR DIRECT LIQUID INTRODUCTION

IV*. A NEW DESOLVATION CHAMBER FOR DROPLET FOCUSING OR TOWNSEND DISCHARGE IONIZATION

P. J. ARPINO*
École Polytechnique, Laboratoire de Chimie Analytique Physique, 91128 Palaiseau (France)
J. P. BOUNINE
Rhône-Poulenc Recherches, Centre Nicolas Grillet, 94400 Vitry (France)
M. DEDIEU**
S.N.G. Nermag, 92500 Rueil-Malmaison (France)
and
G. GUIOCHON
École polytechnique, Laboratoire de Chimie Analytique Physique, 91128 Palaiseau (France)

SUMMARY

A new desolvation chamber has been coupled to a liquid chromatograph–mass spectrometer interface. The shape of the drift tube was designed to focus the electrically charged droplets from the diaphragm nebulizer into the chemical ionization source. A voltage applied to the desolvation chamber influenced both the total ion current and the relative abundance of the ions when constant flow-rates of acetonitrile were nebulized. A stable but unsustained Townsend discharge occurred when the voltage on the desolvation chamber was above 140 V.

INTRODUCTION

Recent work has emphasized the importance of a droplet drift zone placed between the liquid nebulizer of a direct liquid inlet (DLI) interface and the chemical ionization (CI) source block of the mass spectrometer for liquid chromatographic–mass spectrometric operations[1-4]. The drift zone is believed to play several roles, although explanations in different reports often appear contradictory. Liquid droplets of analyte solutions must be injected under a vacuum in order to sample non-volatile molecules to the mass spectrometer ion source[5]. Droplet injection is com-

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[*] For Part III, see ref. 25.

[**] Present address: St. Mary's Hospital Medical School, Department of Pharmacology, London W2 1PG, Great Britain.

patible with liquid chromatographic requirements as it does not broaden chromatographic peaks. As a consequence, several ingenious devices, including pneumatic nebulizers[6], ultrasonic nebulizers[7], diaphragms[4,8] and heated tubes[9,10], have been used to produce stable and reproducible droplet sprays under a vacuum. The phenomena and the parameters affecting the production of solute ions from these solution droplets are less clear.

Rapid desolvation of the solution aerosol may leave neutral evaporated sample molecules which are subsequently ionized in the gas phase by ion–molecule reactions with the reactive species of a plasma[11]. Alternatively, it has been demonstrated that preformed ions (*e.g.*, carbocations, quaternary ammonium ions, protonated molecules, ionic clusters) can cross the surface of liquid droplets charged at high potentials and be isolated in the gas phase, either intact or clustered with solvent molecules; thus solution parameters and electrical charges would appear to be more important[12,13]. The direct isolation of solute-derived ions from electrolytic solutions in the absence of any external ionization process confirms the validity of the second hypothesis[9,10]. However, it was verified experimentally that heat must be applied to the droplets prior to their introduction into the ion source, and that an optimum temperature exists for different analytes[2,3]; thermal conditions for a desolvation process must therefore also be considered.

Although the exact mechanisms involved in the ionization of samples in solution cannot yet be established, the above work has oriented research towards the design of simple devices that may assist the desolvation and/or the ionization of solute molecules, and are currently referred to as desolvation chambers[1–4]. We described one such device in previous papers[1,2] that proved to be very effective in the determination of series of non-volatile molecules. The shape of the inner drift tube was designed so as to accelerate the speed of the droplets and minimize possible collisions with the walls of the desolvation chamber. This device is still in use in our laboratories and will not be discussed here. However, a different model has been developed for the purpose of comparison, and utilizes a slightly different concept. Preliminary work had shown that the liquid droplets are highly positively charged during the nebulization and that contacts with the walls of the desolvation chamber should in general be avoided; thus a positive potential on the walls could repel droplets. By adopting an appropriate geometry, the droplets could be focused along the drift tube axis, or converge to a favourable location. The design of this desolvation chamber and results of a series of experiments at different voltages are reported here; applications to the determination of quaternary ammonium salts will be presented in the next paper in the series.

EXPERIMENTAL

General equipment

The DLI probe, the quadrupole mass spectrometer and the cryopump were as described previously[8,14], apart from recent modifications and the new desolvation chamber, which are described below.

A Gilson (Villiers-le-Bel, France) Model 302/5S solvent delivery system and a Rheodyne (Berkeley, CA, U.S.A.) Model 7120 loop valve injector equipped with a 10-μl loop were directly connected to a modified DLI probe, and acetonitrile was pumped at a constant flow-rate in the range 20–50 μl/min through the entire system, from the solvent reservoir to the vacuum space of the mass spectrometer. A thin capillary Monel tube, 0.2 mm O.D. × 0.1 mm I.D. (Le Guellec, Douarnenez, France) was connected at one end to the loop valve injector, and the other end was inserted inside the shaft of the DLI probe as far as the nickel diaphragm nebulizer. The rest of the DLI assembly was as described elsewhere[8]. The interface was kept at constant temperature by circulating water at 15°C, and the cryopump attached to the mass spectrometer envelope was chilled with liquid nitrogen[14]. Regeneration of the cryopump was effected overnight after turning off the liquid nitrogen supply and the diffusion pumps, and blowing 5 cm^3 (STP)/min of helium through the CI source block to avoid source contamination by materials desorbed from the trap. This procedure is completely safe and reproducible.

Desolvation chamber

The new droplet transfer line (Figs. 1–3) is permanently attached to the CI source block (6). It consists of a heated drift tube held at different voltages and temperatures, and a Macor (Corning machinable ceramic) insulator.

The drift tube (4) is a copper cylinder with an internal conically shaped tube. The apex of the cone is located inside the ion source on the trajectory of the electron beam emitted by a heated rhenium ribbon (8) (Fig. 3). Under normal LC–MS experiments, the Vespel (DuPont polyimide) nut at the end of the DLI probe is sealed tightly against the cone entrance.

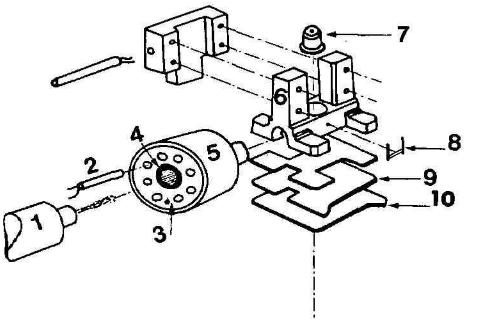
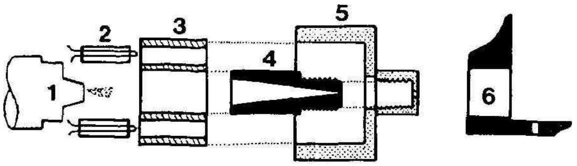


Fig. 1. Exploded view of the LC–MS interface. 1 = DLI probe end (Vespel) with 2-μm nickel diaphragm; 2 = cartridge heater; 3 = copper heater housing; 4 = copper desolvation chamber; 5 = Macor insulator; 6 = ion source block; 7 = repeller assembly; 8 = rhenium filament; 9 = focusing lens; 10 = ion energy lens.



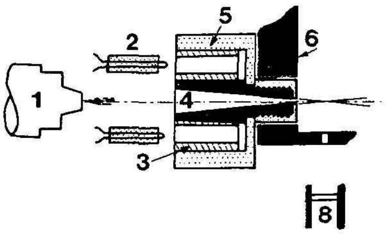


Fig. 2. Section of an exploded representation of the LC–MS interface. Components as in Fig. 1.

Fig. 3. Section of the assembled interface showing the intersection of droplet flights with the electron beam from the rhenium filament. Components as in Fig. 1.

The copper cylinder (3) that surrounds the drift tube was machined to accommodate several cartridge heaters, an iron-Constantan thermocouple and a connection to a voltage supply taken from the ion optic power supply (consequently this voltage could only be varied between -200 V and $+200$ V). A 1 mΩ (2 W) resistor was added between the voltage supply and the copper cylinder for current limiting.

The Macor insulator (5) provides thermal and electrical insulation, so the desolvation chamber can be set at any voltage or temperature within given limits.

The vacuum conductance of the ion source could be easily varied by changing a plug pierced by a small hole fitting to the repeller block (7). One was selected such that an inflow of 30 μl/min of acetonitrile in the ion source at *ca.* 100°C produced a vapour pressure of 0.3 Torr as monitored by an MKS (Burlington, MA, U.S.A.) Model 221 capacitance manometer. The ionization gauges indicated the pressure to be $1 \cdot 10^{-4}$ Torr in the source housing and $2 \cdot 10^{-5}$ Torr in the analyser housing. Acetonitrile was obtained from Carlo Erba (Milan, Italy) and contained 1% of water; it was not further purified but was filtered through 0.2-μm Millipore filters.

Voltage experiments

The influence of the voltage, V_d, applied to the desolvation chamber was evaluated under the following conditions: both the desolvation chamber and the ion source were set at 120°C and 30 μl/min of pure acetonitrile were nebulized. The CI source block, the repeller (7), the focusing plate (9) and the ion energy plate (10) were set at 0 V. Optimum focusing for the next three lenses in the Riber (Rueil-Malmaison, France) Model SQ 156 quadrupole analyser was determined and was not varied during the experiments. Mass spectra were acquired every 1 sec over the mass range 20–200 a.m.u., using a Nermag Model Sidar 5A data system for different values of V_d.

RESULTS

The plot of the total ion current for ions in the mass range 20–200 a.m.u. expressed in arbitrary units *versus* the voltage applied to the desolvation chamber shows two maxima (Fig. 4).

Very weak currents were recorded for V_d values between -200 V and $+60$ V, then the current started to increase to a first maximum at *ca.* 100 V. The value of V_d for this maximum depends on the acetonitrile flow-rate and the source block pressure

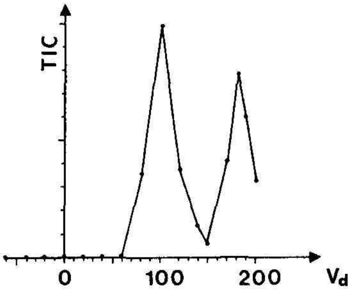
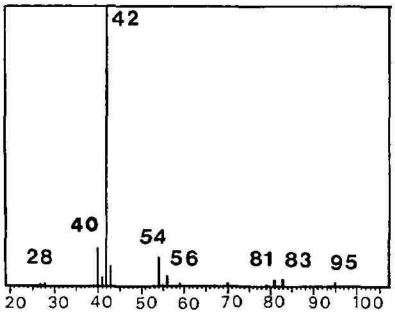


Fig. 4. Plot of the total ion current for ions in mass range 20–200 (arbitrary units) *versus* the potential, V_d, applied to the desolvation chamber.

and temperature. For flow-rates between 20 and 50 μl/min and temperatures between 100 and 150°C, the maximum was at 90 ± 20 V. The total ionization current for acetonitrile depended on the energy of the ionizing electrons as under conventional chemical ionization conditions with the plasma initiated by a beam of electrons. The signal was reduced to zero when the voltage difference between the filament and the ion source was 0 V, independent of the current through the rhenium ribbon.

A second regime was observed for values of V_d above 140 V. The total ion current increased again and reached a second maximum at *ca.* 180 V, then it decreased for higher V_d values. Experiments above 200 V were not pursued. The second region of the plot for $140 < V_d < 200$ is a discharge mode. Once the discharge had been established, the voltage on the rhenium filament could be reduced to zero with no visible effect on the total ion current or the mass spectra. However, the current through the filament could not be turned off completely, otherwise the discharge disappeared. In that event, it could be re-established by increasing the current through the filament to 50 μA and the electron voltage to *ca.* 30 V before setting this voltage back to *ca.* 0–3 V. The discharge was stable and reproducible.

The relative abundances of the ions in the mass spectrum of acetonitrile were affected when V_d was changed (Figs. 5–7). Plots of the percentage of the total additive ionization for major ions *versus* V_d were prepared (Fig. 7). The plots corresponding to ions at m/z = 27 and 26 have not been included for reasons of simplification, but they follow the same pattern as the plot for m/z = 28 ions.



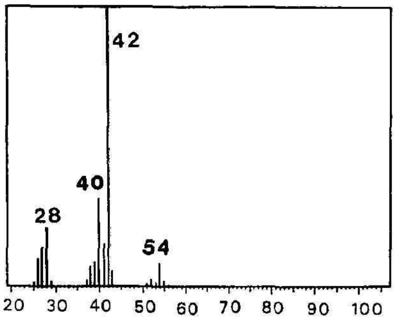


Fig. 5. Mass spectrum of acetonitrile for V_d = 80 V and 70-eV electrons.

Fig. 6. Mass spectrum of acetonitrile for V_d = 180 V and 3-eV electrons.

The major ions appear at same m/z values previously observed by Voyksner *et al.*[15], who identified ion structures using collision-induced dissociation of the parent ions in tandem mass spectrometers. The decrease in the abundance of cluster ions [m/z 83 = M_2H^+; 81 = $(M_2H - H_2)^+$; 56 = MCH_3^+; 54 = $(MCH_3 - H_2)^+$; M = CH_3CN] and protonated molecules (m/z 42 = MH^+), and the increase in the abundance of fragment ions [m/z 40 = $(MH - H_2)^+$; 28 = CH_2N^+; 27 = $CHN^{+\cdot}$; 26 = CN^+], is consistent with the expected high energy content of the plasma in the discharge mode. The transition at V_d = 140 V is self-evident.

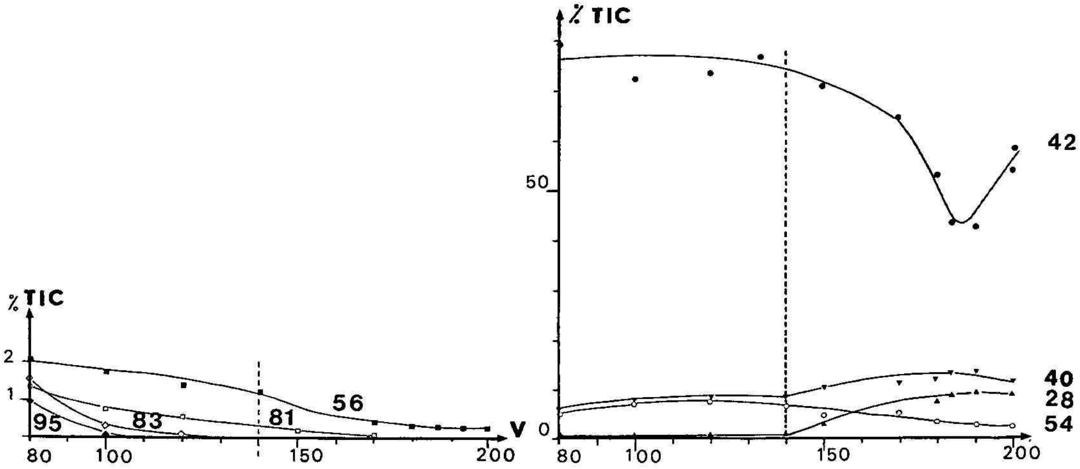


Fig. 7. Plots of percentages of total additive ionization for major ions in mass spectra of acetonitrile *versus* V_d.

DISCUSSION

Until now, generation of mass spectra under DLI LC–MS conditions was accomplished by bombarding the ion source with conventional electron beams from heated filaments. However, since the report by Hunt *et al.*[16] on a Townsend discharge ion source for CI operations and other application work that has demonstrated the success and the simplicity of this ionization method[17], possible adaptation to LC–MS operations should have been investigated, as LC–MS also suffers severe problems of filament burn-outs when injecting strongly oxidizing vapours, and is the case with aqueous solutions. In fact, preliminary work on electrical discharge experiments in LC–MS had been carried out in the laboratory of Professor McLafferty at Cornell University, but had not been extended[11]. On the other hand, simple filament modifications that extend significantly the life-time of a rhenium filament were found[1,14], which may explain the temporary lack of interest in discharge ion sources in LC–MS.

The results presented in this paper are very preliminary and will be completed by further work using different solvents and solutions, but some observations are already noteworthy.

The first region in the plot of the total ion current *versus* the potential on the desolvation chamber is assumed to correspond to conditions focusing the droplets: either they are repelled from the walls and are less decomposed, or they are better focused to the location where they intersect the beam of electrons from the filament. It should be pointed out that a small discharge current of *ca.* 100 nA throught the 1 mΩ resistor could be measured and probably corresponds to the collection of charged liquid droplets on the walls on the desolvation chamber.

The second region corresponds to a non-self-sustained but stable Townsend discharge. Electrons from the heated rhenium filament are still required. Although both the filament and the ion source walls were at 0 V, the voltage of *ca.* 180 V on the desolvation chamber could attract enough electrons from the filament to sustain the discharge.

When attempting to operate the interface in the anodic mode of operation (using the same nomenclature adopted by Hunt *et al.*[16], *i.e.*, the CI source block being the anode and the desolvation chamber being the cathode), no discharge occurred for biasing voltages down to −200 V, which is consistent with results from other experimental set-ups in which the discharge onset was at *ca.* −1200 ± 200 V[16,17]. In contrast, the relatively low breakdown voltage of *ca.* 140 V for the discharge in the cathodic mode of operation (the desolvation chamber being the anode and the CI source block being the cathode) should be compared with values of *ca.* +1200 V observed for a Townsend discharge through reagent gases in conventional CI operations[16,17]. It is recalled that the mechanical disintegration of the liquid effluent through the diaphragm nebulizer into an aerosol generates highly electrically charged droplets[18]. Thus a possible explanation for the lower breakdown voltage in this work is the increase in the space charge resulting from the drifting of the charged droplets through the desolvation chamber; however, the reason why the anodic mode of operation is less favoured is not yet understood.

As already observed by Hunt *et al.*[16], the discharge in the cathodic mode affords spectra in which the relative abundance of the low-molecular-weight ions is enhanced at the expense of the cluster ions at higher mass. The results for acetonitrile show that this is also the case for protonated molecules, however, a loss of ions because of a reduced transmission through the quadrupole analyser of high kinetic energy ions from the Townsend discharge cannot be totally excluded. If the relative abundance change results mainly from the ionization mode, then the variation of V_d appears as a very simple method for forcing the decomposition of a protonated molecule with good yields, and may serve for fingerprinting purposes, as is currently done using the alternative decomposition method of colliding charged precursors with inert gas molecule in a tight cell[19] or in a radiofrequency quadrupole field[20].

CONCLUSION

The new source is currently being used with the LC–MS prototype LUCIE[14], and has also recently been implemented on the quadrupole mass spectrometer in the Rhône-Poulenc laboratory. Applications to the determination of quaternary ammonium salts have already been achieved.

To date, several LC–MS interfacing methods have proved to be effective in handling non-volatile molecules, *e.g.*, thermospray ionization[9,10], liquid ionization[21], nebulization into an atmospheric pressure ionization source[12,13,22] or nebulization into a CI source under a vacuum[1–8,23,24]. Although the experimental set-ups differ, they share in common charged liquid droplets, from spray electrification or static charging, thus indicating that a common mechanism may operate in these methods. In any case, these experimental results confirm our previous assumption that the first objective in an LC–MS experiment should not be to attempt the complete removal of the liquid solvent[25].

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DETERMINATION OF PHENYLUREAS BY ON-LINE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

K. LEVSEN* and K. H. SCHÄFER
Institut für Physikalische Chemie, Wegelerstrasse 12, D-5300 Bonn (G.F.R.)
and
J. FREUDENTHAL
Rijks Instituut voor de Volksgezondheid, P.O. Box 1, 3720 BA Bilthoven (The Netherlands)

SUMMARY

The performance of an interface for direct introduction of the total effluent of a micro high-performance liquid chromatograph into a mass spectrometer is described. The mass spectrometer is operated under chemical ionization (CI) conditions were the solvent acts as CI reagent gas. The separation and identification of various phenylureas was optimized. It is demonstrated that this approach can be used as a prescreening of surface water for phenylureas.

INTRODUCTION

At present, gas chromatography–mass spectrometry (GC–MS) represents the method of choice for the identification of organic pollutants in water as it combines the high separation efficiency of capillary GC with the specificity and high sensitivity of MS. Unfortunately, only relatively volatile compounds are amenable to this technique; these comprise only 20–30% of the total organics in water. Thus considerable efforts have been made to separate and identify the less volatile compounds by liquid chromatography (LC). For these studies, on-line LC–MS is desirable for two reasons: (a) the commercially available LC detectors are neither very specific nor as sensitive as GC detectors; (b) it would allow a rapid, and ideally, an unequivocal identification of less volatile organics in water. To this end, a variety of LC–MS interfaces have been developed[1–7], among which the moving belt system[4] and the direct liquid introduction (DLI) system[5] have been used most frequently. With the latter interface the LC effluent is usually split prior to introduction into the mass spectrometer where the solvent acts as chemical ionization (CI) reagent gas. The coupling is facilitated if micro LC is used. In this case the total effluent can be introduced into the mass spectrometer as first demonstrated by Henion and Maylin[6]. Our approach is based on their concept. The application of this system to the analysis of polycyclic aromatic compounds, phenols and aliphatic acids has been reported previously[7,8].

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EXPERIMENTAL

Interface and apparatus

The interface is strikingly simple. Basically it consists of a stainless-steel capillary (30 cm × 0.1 mm I.D.) which acts as a flow restriction between the micro liquid chromatograph and the ion source of the mass spectrometer. Technical details of this interface have been published previously[7]. It can be introduced into the mass spectrometer via a sliding rod and connected rapidly to the chromatograph using PTFE tubing.

For this study a JASCO Familic-100 N micro high-performance liquid chromatograph was employed with acetonitrile–water solvents, flow-rates of 10 μl/min, pressure *ca.* 100 bar and a 20-cm reversed-phase RP-18 micro column. The sample was injected onto the column via a loop injector (capacity 0.3 μl). In a first step the separation was optimized using a UV detector (UVIDEC 100 II). The UV detector was then disconnected and the components identified mass spectrometrically. For this purpose the mass spectrometer (Finnigan 44 S) was operated in the CI mode (both positive and negative CI) where the LC solvent acted as reagent gas (source temperature 250°C, source pressure 150–500 μbar).

Performance of the interface

The interface has minimal dead volume. It can be operated reliably over extended periods of time. In particular, plugging of the capillary rarely occurs. Depending on the compound to be studied, a lower detection limit of 30 pg may be possible if the mass spectrometer is operated in the single ion monitoring mode. During operation the end of the interface capillary is heated by the hot ion source. This appears to be a prerequisite for proper performance of the interface, but precludes the analysis of thermally very labile compounds which at least in part can still be handled if a diaphragm is used as flow restrictor[9].

It is important to note that the ease of coupling of the chromatograph to the mass spectrometer is achieved at the expense of chromatographic resolution which is poorer with micro LC as compared with standard LC.

As the mass spectrometer is operated in the CI mode (see above) the mass spectra of thermally stable compounds exhibit mainly quasimolecular ions, *i.e.*, $[M + H]^+$ or $[M - H]^-$, but hardly any fragments. Thus the MS information is reduced to determination of the molecular weight. However, in general, the quasimolecular ion and the chromatographic retention time should allow identification of "targeted compounds".

RESULTS AND DISCUSSION

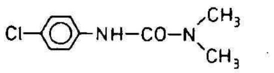
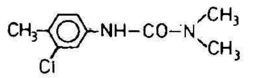
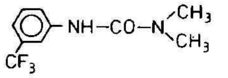
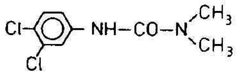
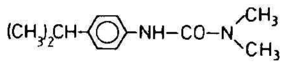
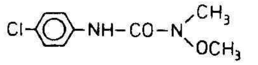
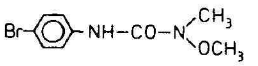
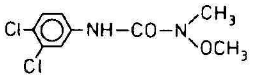
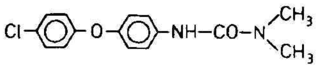
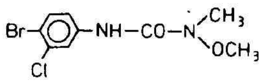
Analysis of pure phenylureas

Phenylureas are widely used as herbicides and are released as such or as metabolites into the environment[10,11]. Gas–liquid chromatography (GLC) has been applied to the separation and identification of these compounds, but partial thermal decomposition during GLC analysis may cause erratic results[11]. Thus the application of LC to determination of phenylureas has been reported[12,13] and the potential of an off-line LC–MS coupling, in which several preseparation and prepurification steps were necessary, has been explored[14]. We have used on-line LC–MS coupling to separate and identify phenylureas.

First, the separation of these compounds was optimized using a mixture of ten commercially available phenylureas, Table I. Fig. 1a shows the total ion chromatogram (mass range 140–300) obtained under positive chemical ionization (PCI) conditions at a concentration of 30 ng for each component injected onto the column. As a result of the limited chromatographic resolution, some components are not separated, *i.e.*, chlorotoluron and fluomethuron, chloroxuron and chlorbromuron. Note that a complete separation is possible with conventional LC[14]. The mass chromatograms of the protonated molecules reconstructed from the complete spectra are show in Fig. 1b and c. It is apparent that components having identical retention times can still be distinguished on the basis of their molecular weights. The complete mass spectra of these phenylureas are, as expected, dominated by the quasimolecular ions.

TABLE I

INVESTIGATED PHENYLUREAS

Common name	*Formula*	*Molecular weight*
(1) Monuron		198
(2) Chlorotoluron		212
(3) Fluomethuron		232
(4) Diuron		232
(5) Isoproturon		206
(6) Monolinuron		214
(7) Metobromuron		258
(8) Linuron		248
(9) Chloroxuron		290
(10) Chlorbromuron		292

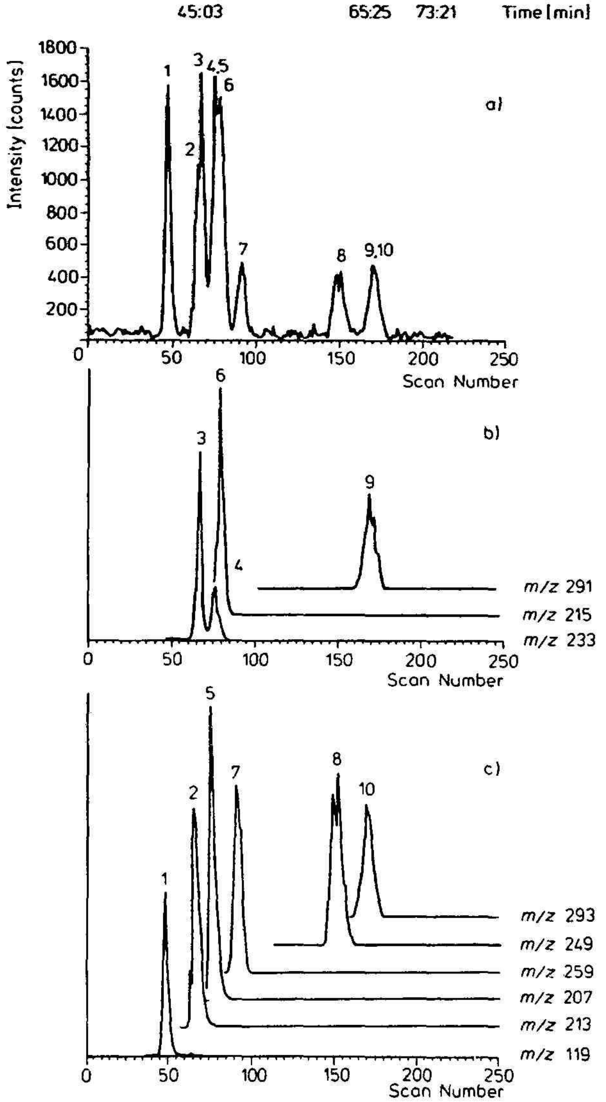


Fig. 1. LC–MS analysis of a mixture of ten phenylureas, 30 ng per compound injected onto the column (*m/z* values of protonated molecules in parentheses): 1 = monuron (199); 2 = chlorotoluron (213); 3 = fluomethuron (233); 4 = diuron (233); 5 = isoproturon (207); 6 = monolinuron (215); 7 = methobromuron (259); 8 = linuron (249); 9 = chloroxuron (291); 10 = chlorbromuron (293). Experimental conditions: acetonitrile–water (40:60); 19-cm RP-18 column, 5-μm particles; flow-rate 10 μl/min; LC pressure 105 bar; CI pressure 230 μbar; source temperature 290°C. a, Total ion chromatogram; b, c, reconstructed mass chromatograms of the protonated molecules.

This is demonstrated in Fig. 2 for linuron and in Fig. 3 for metobromuron. The mass spectrum does not only give information on the molecular weight. In addition, the cluster of isotope peaks reveals the presence of two chlorine atoms in the case of linuron and a bromine atom in the case of metobromuron, while structure specific fragments are missing. Fig. 4 shows the mass chromatogram of monuron (protonated

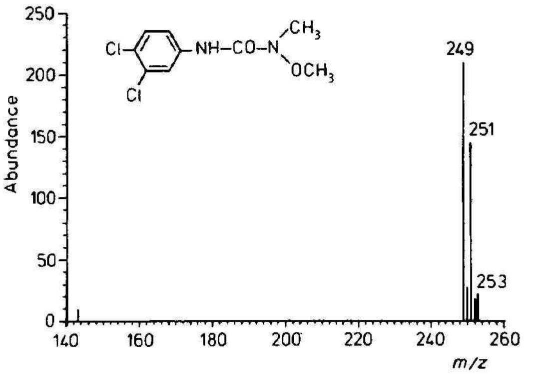


Fig. 2. Mass spectrum corresponding to peak 8 in Fig. 1 (linuron).

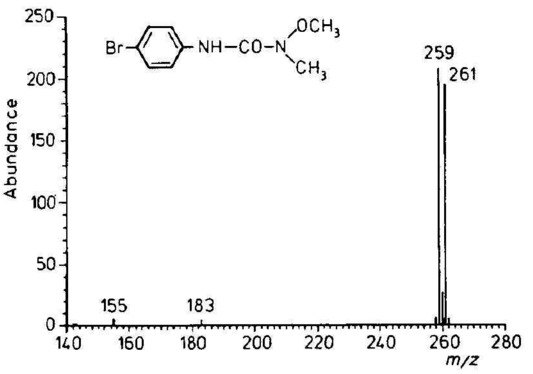


Fig. 3. Mass spectrum corresponding to peak 7 in Fig. 1 (metobromuron).

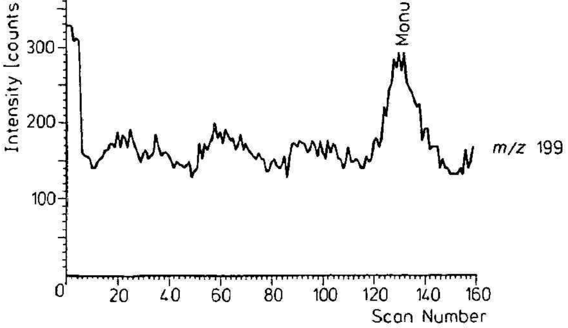


Fig. 4. Reconstructed mass chromatogram of *m/z* 199 (monuron); 30 pg injected onto the column (conditions as in Fig. 1).

molecule) obtained with 30 pg injected onto the column, demonstrating that at present about 30 pg represents the lower detection limit of the system.

Alternatively the mass spectrometer can be operated under negative chemical ionization (NCI) conditions. If acetonitrile–water is used as solvent, the dominant $C_2H_2N^-$ anion abstracts a proton from the sample molecules thus giving rise to the formation of $[M - H]^-$ quasimolecular ions. The NCI mass chromatogram of the mixture of ten phenylureas at a concentration level of 30 ng per component is shown in Fig. 5; the $[M - H]^-$ ions were monitored.

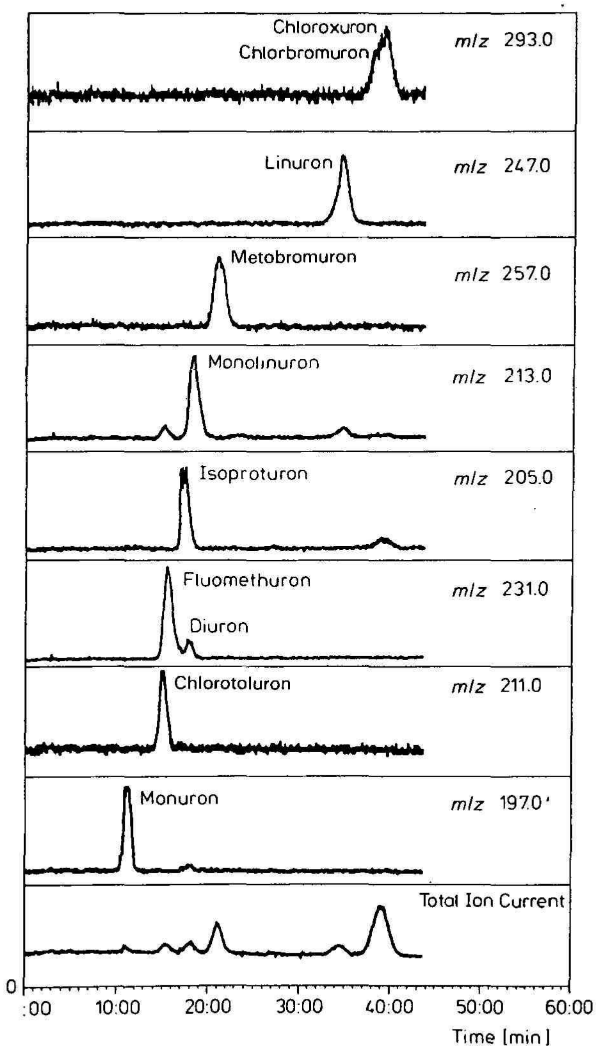


Fig. 5. Mass chromatogram (obtained in the multiple ion selection mode under negative chemical ionization conditions) of a mixture of ten phenylureas, 30 ng per compound injected onto the column. The $[M - H]^-$ ions were monitored (conditions as in Fig. 1).

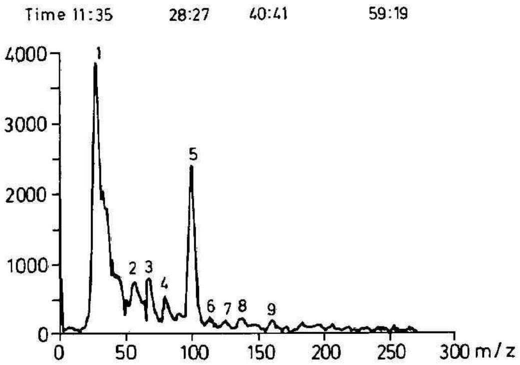


Fig. 6. Total ion chromatogram of a Rhine water extract (*m/z* 140–300) spiked with the ten phenylureas listed in Fig. 1 at the concentration level of 6 ng. Conditions as in Fig. 1.

Identification of phenylureas in surface water

While separation of a mixture of pure phenylureas and their MS identification is readily achievable, the analysis is expected to be much more complicated if these compounds are to be identified from complex matrices such as water samples. To test the potential of our method for the analysis of organic pollutants in water we spiked a water extract from the river Rhine with the ten phenylureas listed in Table I such that the injected sample contained 6 ng of each herbicide. For the analysis only 1 % (0.3 μl) of the total water extract (30 μl) was injected. Thus the 6 ng of herbicide injected onto the column correspond to 600 ng in the original sample. Fig. 6 shows the total ion chromatogram (*m/z* 140–300) of the water extract. MS analysis demonstrates that the two major peaks 1 and 5 do not correspond to any phenylurea. Although the retention times of some of the smaller peaks discernible in the total ion chromatogram correspond to those of the phenylureas, an unambiguous identification based on the total ion chromatogram alone is not possible.

An unequivocal detection of the "targeted" compounds is, however, possible, if the information from the mass spectra is used. Fig. 7 shows the mass spectrum of peak 2. The quasimolecular ions of chlorotoluron and fluomethuron with the correct isotopic pattern can readily be detected. These results demontrate that even in com-

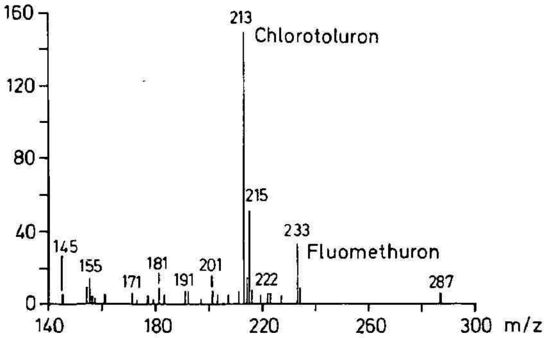


Fig. 7. Mass spectrum of peak 2 in Fig. 6. Quasimolecular ions of chlorotoluron (*m/z* 213 and 215) and fluomethuron (*m/z* 233).

plex matrices phenylureas can be detected by this method at the lower nanogram level.

In a second step a freeze-dried sample (corresponding to 5 l water) from the river Main was screened for the presence of phenylureas. The sample was extracted with chloroform, concentrated to dryness and dissolved in 30 μl acetonitrile. 0.3 μl from this extract were injected onto the column. In order to obtain optimum sensitivity the mass spectrometer was repeatedly scanned first over a mass range from m/z 195 to 240 then from m/z 240 to 300. (The molecular weights of all phenylureas should fall within these mass ranges.) The total ion chromatograms for these two mass ranges are shown in Fig. 8a and b. From the mass spectra, the mass chromatograms of the protonated molecules and those of the corresponding isotope peaks (if the suspected phenylurea contains halogen atoms) were reconstructed as shown in Fig. 9 for m/z 207, 249 and 251. Comparison with the test mixture run on the same day

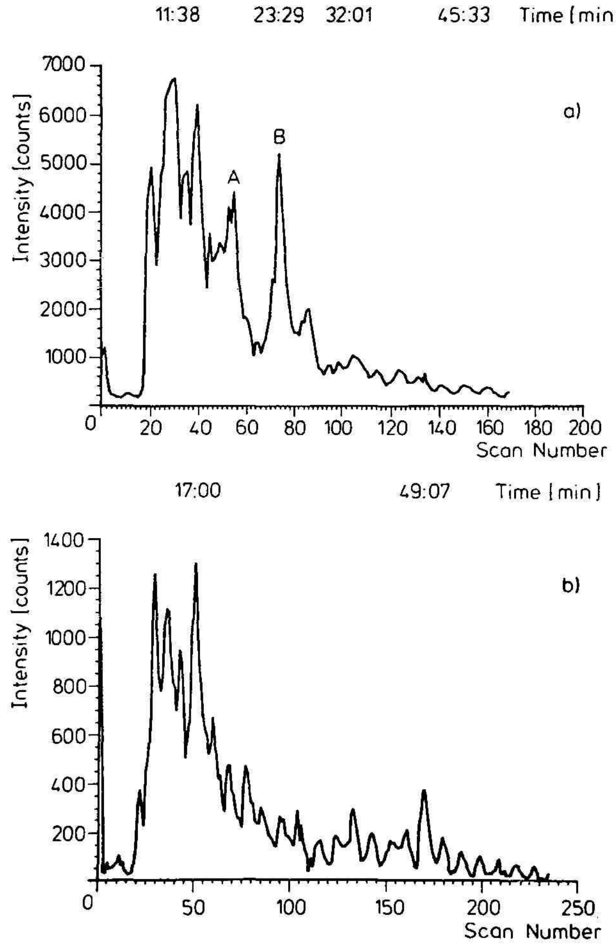


Fig. 8. Total ion chromatogram of a water extract from the river Main (conditions as in Fig. 1): a, mass range from m/z 195 to 240 (peak B = isoproturon, see Fig. 9a); b, mass range from m/z 240 to 300 (conditions as in Fig. 1).

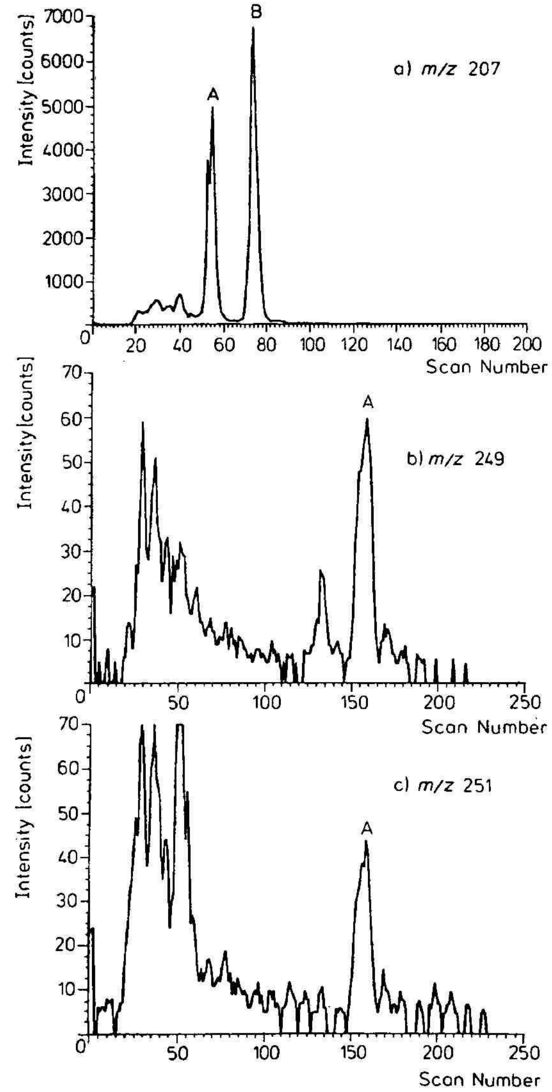


Fig. 9. Reconstructed mass chromatogram of: a, *m/z* 207 (peak B: isoproturon, peak maximum at scan number 76 in test mixture); b, *m/z* 249; c, *m/z* 251 (peak A: linuron, peak maximum at scan number 161 in test mixture).

allowed tentative assignment of peak B in Fig. 9a as isoproturon, while peak A in Fig. 9b is probably due to linuron. The latter assignment does not only rest on the correct retention time and mass of the protonated molecule, but also on the mass and intensity of the isotope peak at *m/z* 251 (Fig. 9c) which is due to the two chlorine atoms. (The observed intensity ratio *m/z* 251:249 = 1.42 is close to the theoretical ratio of 1.54.) Furthermore it is very likely that the sample contained also minor amounts of monolinuron and chlorotoluron as supported by the correct retention time and isotope pattern. Owing to the large number of organic compounds in surface water, the interference from other compounds of the same mass and retention time cannot be excluded. Thus it is desirable to confirm the proposed constituents by other methods, *e.g.*, high-resolution measurement.

CONCLUSIONS

The described method of on-line LC–MS allows the rapid prescreening of surface water samples for "targeted" compound classes provided that these compounds are thermally stable. However, as a result of the micro columns employed, the chromatographic resolution is poorer than with standard LC equipment. The identification of the compounds rests on the determination of the retention time and the molecular weight, and on the interpretation of the isotope pattern if halogen atoms are present in the sample molecules. If complex matrices such as water samples are analyzed this information may not be sufficient for an unequivocal identification of a given compound. Thus the results should be confirmed by methods such as high-resolution measurements or tandem MS[15]. One disadvantage is that the sample volume injected onto the column is only 0.3 μl, as it is difficult to concentrate a water extract to this size. Thus although targeted compounds at a concentration level below 10 ng injected onto the column can be identified from complex matrices, this detection limit can in practice only be realized if the above mentioned sampling method is improved.

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QUANTITATIVE TRACE ANALYSIS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

R. G. CHRISTENSEN*, E. WHITE V, S. MEISELMAN and H. S. HERTZ
Organic Analytical Research Division, Center for Analytical Chemistry, U.S. Department of Commerce, National Bureau of Standards, Washington, DC 20234 (U.S.A.)

SUMMARY

In order to overcome difficulties in spraying aqueous solvents into the vacuum of a mass spectrometer, an ultrasonic spraying device has been constructed. The vibration is achieved by means of magnetostriction in the nickel inlet tube itself. Applications to aliphatic acid determination in a shale oil process water and to determination of valproic acid, an anti-convulsant, in human serum (SRM 1599) are presented.

INTRODUCTION

The development of an on-line combination of liquid chromatography (LC) and mass spectrometry (MS) is being pursued in a number of laboratories, spurred on by the demonstrated great effectiveness of gas chromatography (GC)–MS. A number of LC–MS interfaces have resulted. Several reviews describe the operating principles, construction and performance of these devices[1–6]. Three methods, direct liquid injection (DLI), evaporation onto a moving belt, and DLI with sample pre-concentration, are now offered commercially.

Although DLI alone is intrinsically simple to implement, a large fraction of the sample usually must be discarded. (Exceptions are interfaces with micro-LC instruments, *e.g.* that of Henion[7], and the more complex, rapid evaporation approach of Blakely *et al.*[8].) The moving belt has the advantage of concentrating the solute, thereby allowing a greater proportion of the sample to be introduced into the ion source of the mass spectrometer.

A system which pre-concentrates the liquid stream and introduces the concentrate by DLI conceivably combines many of the advantages of ordinary DLI and the moving-belt techniques. We have described an interface which was designed and built with this aim in mind[9]. The interface device concentrates a liquid stream by allowing it to flow down a resistance-heated stationary wire. Some of the residual liquid is drawn into the mass spectrometer through a metal capillary tube with a needle valve at the ion source end (see Fig. 1). The liquid sprays from the needle valve into the ion source of a conventional, differentially-pumped, quadrupole mass spectrometer.

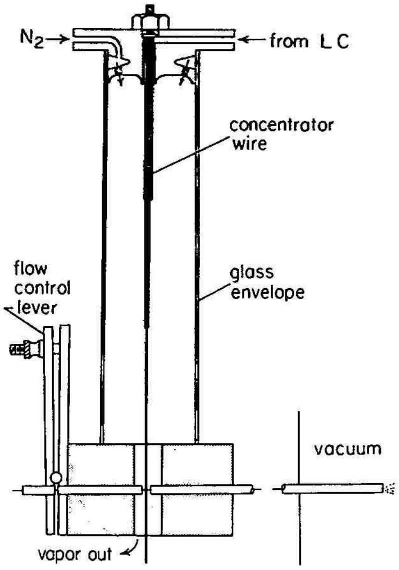

Fig. 1. Concentrator wire and inlet probe for normal-phase LC–MS.

This system operated satisfactorily with typical solvents used in normal-phase LC, but difficulties were encountered with the aqueous solvents used in reversed-phase LC. The liquid which issued from the tip did not spray well, accumulating in a drop at the orifice, and often freezing owing to evaporative cooling. The trouble apparently arises from the large surface tension and high heat of vaporization of water solutions. Conductive and radiative heating were tried in attempts to get a dispersal of liquid from the tip, as was the introduction of an atomizing gas (see Fig. 2). After these approaches failed, ultrasonic vibration of the probe was attempted. The use of ultrasonics in LC–MS interfaces has been reported by other researchers[10–12]. The vibration which we employ is a longitudinal one and is produced by using the nickel inlet tube itself as a magnetostrictive oscillator, details of which are given below.

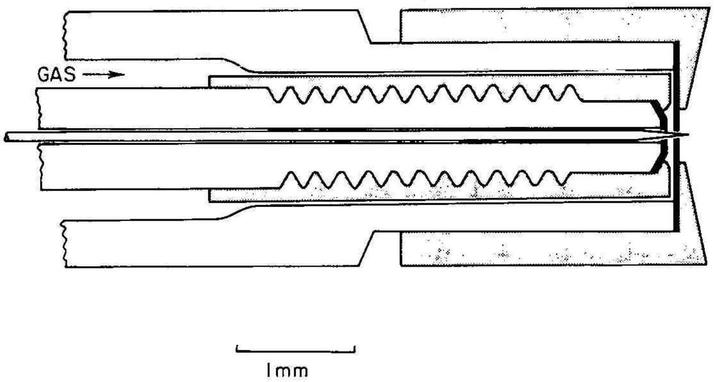

Fig. 2. Cross section of probe tip with provision for concentric admission of atomizing gas.

EXPERIMENTAL

Concentrator wire

The construction of the concentrator used is substantially similar to that previously described[9]. The wire is composed of three segments, a large diameter piece at the top with a high electrical resistance, an intermediate diameter piece in the center with intermediate electrical resistance, and a small diameter piece at the bottom with low electrical resistance. This provides for a high rate of evaporation where the flow-rate is high, and a low rate of evaporation at the bottom where the flow-rate is low. The feedback circuit for controlling the current input to the wire has been eliminated, and an adjustable constant-current supply is now being used.

DLI probe

A sectional drawing of the ultrasonic DLI is shown in Fig. 3. The inlet tube is 15 cm long, and the resonant frequencies are 16,000 Hz for vibration as 1/2 wavelength, and 50,000 Hz for vibration as 3/2 wavelength. A discussion of magnetostriction can be found in the textbook by Bozorth[13]. Dead volume in the inlet tube was minimized by re-drawing the nickel tubing. Annealed nickel tubing (1.5 mm O.D., 0.25 mm I.D.) was reduced to 1.25 mm O.D., 0.15 mm I.D. in several steps, using simple steel dies with lanolin or soap as the lubricant. The tip is formed into a needle valve in order to control the rate of flow of liquid into the mass spectrometer vacuum. The stem of the valve is a 0.10-mm tungsten wire ground to a sharp point. The seat was made by swaging the nickel inlet tube down upon the pointed tungsten wire, then withdrawing the wire. This leaves a durable tapered seat that closely matches the wire (see Fig. 4).

Amplitude gain is provided by having the driving section *ca.* 4 mm in diameter, while the driven portion is *ca.* 1.2 mm in diameter. The probe is supported on a diaphragm of phosphor bronze soldered at the node. This also serves as the vacuum seal.

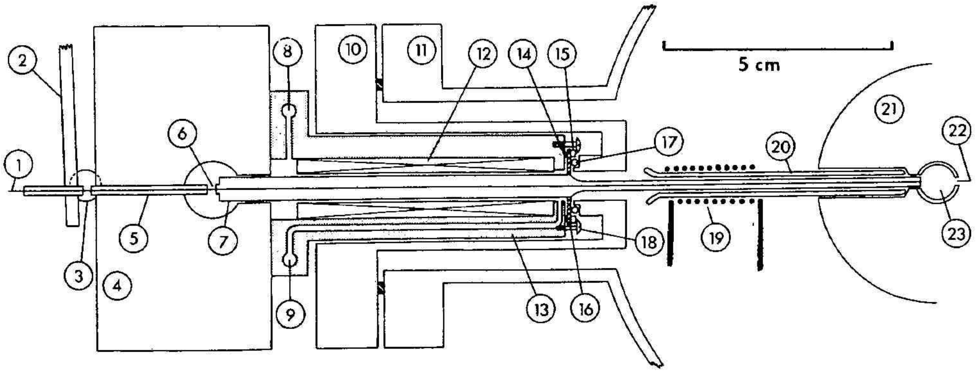

Fig. 3. Cross section of the ultrasonic DLI probe assembly. 1 = Flow control wire; 2 = flow control lever; 3 = fulcrum balls; 4 = brass support block; 5 = guide tube; 6 = liquid entrance; 7 = inlet tube; 8 = cooling-water outlet; 9 = cooling-water inlet; 10 = re-entrant flange; 11 = instrument flange; 12 = driving solenoid; 13 = nylon support; 14 = gasket; 15 = support ring; 16 = phosphor-bronze diaphragm; 17 = O-ring; 18 = retaining screw; 19 = pick-up coil; 20 = glass support sleeve; 21 = ion-source block; 22 = viewing port; 23 = ion-source cavity.

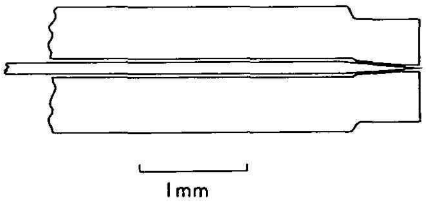

Fig. 4. Cross section of needle valve with swaged seat.

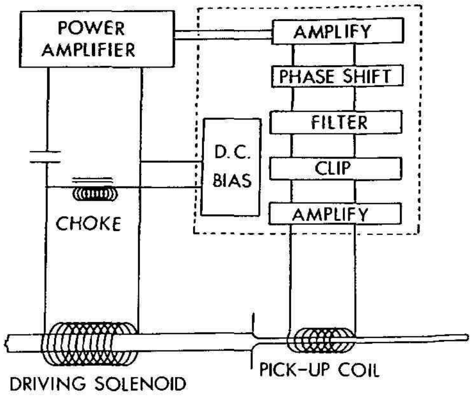

Fig. 5. Feedback circuit for ultrasonic DLI probe.

The principles of operation of the driving/feedback electronics are shown in Fig. 5. The signal from the pick-up coil is amplified and clipped to constant amplitude. It is then filtered with a variable-bandwidth, variable-frequency filter, providing rejection of unwanted modes of vibration. The phase of the signal is adjusted and fed to a power amplifier, the output of which is variable up to 75 W. An adjustable direct current is supplied separately to the driving solenoid, with the power amplifier and direct current supply being isolated from each other by a capacitor and a choke. Water cooling is provided for the driving section and solenoid to carry away the heat which is produced in operation.

Mass spectrometer

The mass spectrometer used is a variation of the Extranuclear Laboratories SpectrEL*, equipped for differential pumping and fitted with an electron-impact ion source. The source was usually operated in the temperature range 150–180°C. Liquid influx rates were in the range 5–20 μl/min. A flange opposite the DLI inlet flange, previously occupied by a solids probe inlet, was fitted with a viewing window through which the tip of the probe could be examined with a telescope during operation.

The source volume is pumped by a 450-l/sec turbomolecular pump and the quadrupole volume is pumped by a 280-l/sec diffusion pump. This combination allows the vacuum system to be opened rapidly for repairs and modifications.

Liquid chromatography

The liquid chromatograph used was a conventional commercial instrument equipped with a loop injector and an octadecylsilane column. Injections of 20 μl were used for the examples presented. Solvents used were spectro- or HPLC-grade, although solvent quality was not found to be critical for the analyses discussed below.

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* Certain commercial equipment, instruments or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

All solvents used in these analyses contained 0.25% (v/v) of acetic acid for suppression of ionization. The flow-rate was 1 ml/min through the chromatographic column for all work. This system was used for both the quantitative analysis of aliphatic acids in a shale oil process water and the analysis of an anti-convulsant, valproic acid, in serum.

The chromatography of the aliphatic acids was performed by isocratic or gradient runs of 35–80% methanol in water as shown in Table I. Determinations of valproic acid in serum were performed on NBS Standard Reference Material 1599 by elution with 100% water for 5 min, followed by a linear 3-min 0–80% methanol gradient. While the water and gradient were being run, a stream of 80% methanol in water was run on the concentrator wire, and at the end of the gradient the analytical stream was switched to the concentrator wire. This procedure avoided contaminating the mass spectrometer with the salts and proteins present in the serum sample. The LC arrangement for this analysis is shown in Fig. 6.

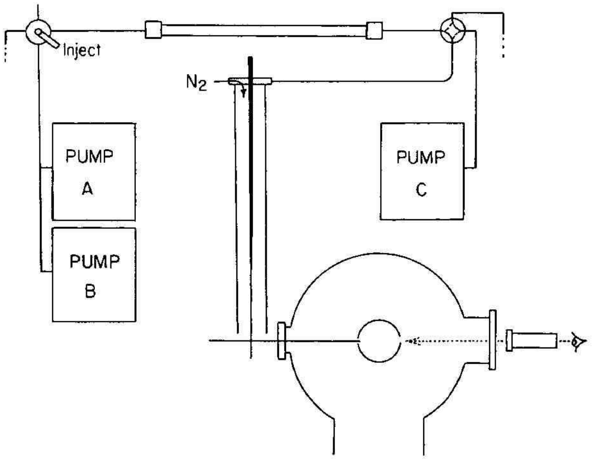

Fig. 6. Connections of liquid chromatograph and mass spectrometer with provision for auxiliary stream, used in analysis of serum samples.

RESULTS AND DISCUSSION

Concentrator performance

The same concentrator which was previously used for normal-phase solvents was used for the water–methanol solvents of the present study. About 95% evaporation of the solvent could be obtained with a 1-ml/min flow-rate for solvents containing 10–50% water, 90–50% methanol. Because of concern that there would be insufficient methanol to prevent freezing at the tip of the probe, measurements were made of the methanol concentration in the residual liquid. Samples were collected and the methanol content inferred from refractive index measurements. With a starting solution of 80% methanol, the residual liquid contained 25% methanol at about 95% evaporation; with a starting solution of 60% methanol, the residual liquid contained about 10% methanol at about 95% evaporation. This methanol content proved to be high enough for the probe to function without plugging by freezing and without generating large ice crystals within the ion source of the mass spectrometer.

DLI probe performance

An early model of the ultrasonic probe had the needle valve seat formed from a 0.05-mm thickness film of poly(vinylidene fluoride) as shown in Fig. 7. This soft seat gave good flow control and a tight shut-off seal. It operated well with the conventional (non-ultrasonic) interface used with normal-phase solvents, but was subject to rapid wear when used with the oscillating probe. It was therefore replaced with the needle valve described in the experimental section, which had a hard seat formed integrally with the tip (Fig. 4).

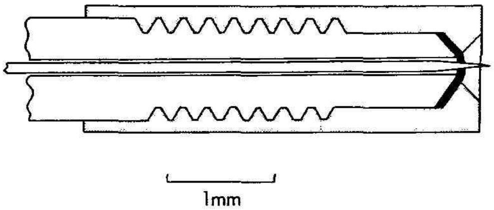

Fig. 7. Cross section of probe tip with soft needle valve seat made of poly(vinylidene fluoride) film.

Trials of the hard seat ultrasonic probe in a vacuum test chamber showed that the tip vibrated with an amplitude of 20–60 μm. When liquid was admitted, the tip of the probe became covered with a liquid film from which a fog of droplets 20–25 μm in diameter emanated. In the mass spectrometer, pure water could be sprayed without freezing at the tip at a source block temperature of 200°C, and 20% methanol in water could be sprayed at 100°C. Trials with 5% (w/v) salt solutions showed no tendency for evaporative clogging, but viscous solutions, *e.g.*, above 15% (w/v) glycerol in water, could not be sprayed. The diameter of the droplets was estimated by allowing them to strike a microscope slide. The size of the wetted spots and an estimate of the wetting angle allowed calculation of the droplet volume.

A number of experiments were made in attempts to improve the performance of the DLI probe. It is believed that smaller droplets would improve the sensitivity of the mass spectrometer to larger, less volatile, molecules, and that a well-directed stream of droplets, rather than a diffuse fog, is desirable[14]. Both increasing the frequency and increasing the vibrational amplitude were tried in order to obtain smaller droplets. Raising the frequency to *ca.* 50,000 Hz, where the inlet tube resonates as 3/2 wavelength, gave smaller amplitude but about the same spraying characteristics as vibration at 16,000 Hz. Since the amount of useful input power is limited by the magnetic saturation of the driving section, attempts to increase vibrational amplitude were focused on changes in the geometry of the inlet tube and investigations into sources of damping. The amplitude proved to be less sensitive to the geometry of construction than had been expected. The taper at the node and the location of the supporting diaphragm did not seem to be particularly critical, for example. One model was constructed where the taper from the node to the spray tip was exponential, but no improvement in action was seen. Observable damping occurs when the flow control wire is adjusted so that it strikes against the seat of the needle valve, with a 20–30% diminution in vibrational amplitude.

An attempt to obtain a directed stream of small droplets was made by constructing a tip for the probe in which the diaphragm in the probe was allowed to vibrate against a flat-ended flow control wire as shown in Fig. 8. It was hoped that the impact would generate a pumping action, where localized high pressure would cause a spurt of liquid with enough momentum to tear itself free of the diaphragm. The first model was constructed with a poly(vinylidene fluoride) film, through which a hole was made with pulses from a focused laser. A second was built with a stainless steel diaphragm from a commercial supplier. Both of these probes failed immediately from particles plugging the 10-μm orifices. This appears to be due to the considerable liquid surface which is exposed, and to the moving parts employed in the interface, making it difficult to exclude contamination from microscopic particles.

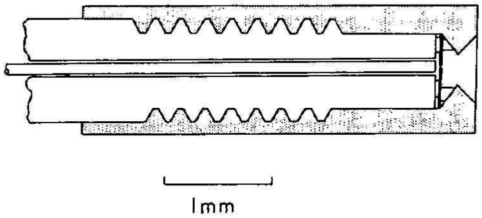

Fig. 8. Cross section of probe tip with 10-μm orifice and flat-ended flow-control wire.

Quantitative determinations

The rapid analysis of industrial and energy-related effluents to determine concentrations of possibly toxic and/or hazardous organic constituents presents a formidable task. Determination of individual trace organic compounds in complex matrices generally involve extraction of the compounds from the matrix, possibly column chromatographic clean-up and derivatization of the analytes, followed by quantitation using GC, LC, or GC–MS with internal or external standards. The results presented below show two examples of the use of LC–MS system described in this paper for the direct quantitative analysis of trace organic compounds by injection of a complex mixture onto the reversed-phase LC column without any prior extraction or derivatization.

The first such analysis is the examination of the acidic components in a shale oil process water. Using the present LC–MS system the process water was injected directly onto the column and a chromatogram was obtained in 15 min, from which the peak heights of analytes of interest could be measured and compared to peak heights for the same compounds from an external standard solution. Four aliphatic acids, butanoic (C_4) through heptanoic (C_7), were determined. Fig. 9 contains the chromatograms for the shale oil process water (top) and the external standard solution (bottom) obtained using the single ion records at m/z 131 [$(M + H)^+$] to quantitate heptanoic acid. The results for all four acids examined are summarized in Table I. Included in the data presented are the results of the LC–MS analyses, the chromatographic conditions under which the compound was eluted and the preconcentration achieved on the interface concentrator wire. For comparison purposes, results obtained by the much more time-consuming approach of solvent extraction and GC–MS analysis are also presented. In general, the results agree well, with the exception of butanoic acid. The GC–MS measurements are only for the n-acids, whereas branched acids with the same molecular weight were not separated in the LC separation used. GC–MS

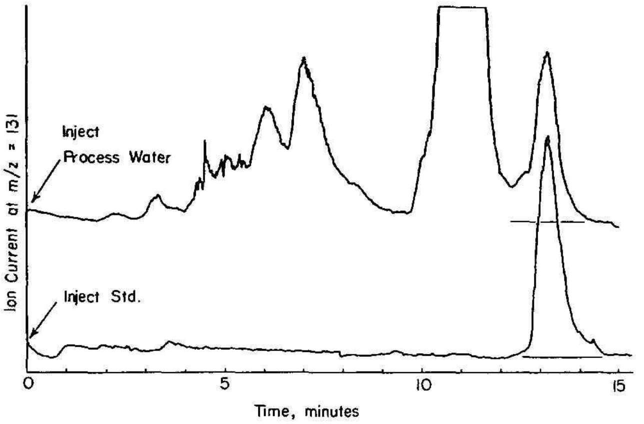

Fig. 9. Single-ion record at m/z = 131 from determination of heptanoic acid in a shale oil process water. Upper trace, process water; lower trace, standard solution of *n*-heptanoic acid.

examination of the C_4 acids indicated the presence of sufficient 2-methylpropanoic acid to account for the difference in LC–MS and GC–MS results.

In a second example, valproic acid (an anticonvulsant drug) was determined in NBS Standard Reference Material (SRM) 1599, Anticonvulsants in Human Serum. Two independent methods were used in the certification analyses of this SRM[15]. The first involved extraction from serum and GC analysis and the second, extraction, derivatization and LC analysis. Using the present LC–MS system in the reversed-phase mode, serum was directly injected onto the LC column and analysis, including comparison with an external standard, was ompleted within 20 min. Quantitation was achieved by comparison of peak areas of the valproic acid from serum to peak areas of standards injected shortly after the sample. A typical chromatogram, with single-ion monitoring at m/z 145 $[(M + H)^+]$, is depicted in Fig. 10. Note that the valproic acid peak is broadened somewhat by the buffering effect of the serum. Table

TABLE I

DETERMINATION OF ALIPHATIC ACIDS IN A SHALE OIL PROCESS WATER

Acid	*Level by LC–MS ($\mu g/ml \pm \sigma$)*	*Level by GC–MS* ($\mu g/ml \pm \sigma$)*	*Approx. concentration factor obtained*	*Chromatographic conditions*
Butanoic	91 ± 10**	49.6 ± 7.6	1	35% Methanol, isocratic
Pentanoic	84 ± 10	71.9 ± 8.9	3	40–60% Methanol gradient in 5 min
Hexanoic	87 ± 13	87.8 ± 4.4	6	50–75% Methanol gradient in 10 min
Heptanoic	136***	144.5 ± 9.2	8	50–80% Methanol gradient in 10 min

* Specific for straight-chain acids.
** Includes 2-methylpropanoic acid.
*** Average of only 2 determinations, 133 and 139 μg/ml.

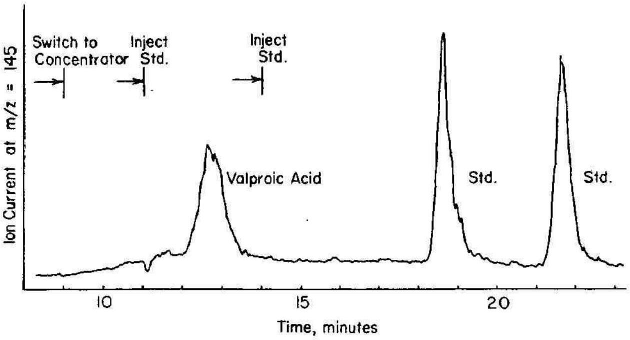

Fig. 10. Single-ion record at $m/z = 145$ from determination of vaproic acid in serum, showing valproic acid peak followed by peaks from two subsequently injected standards.

TABLE II

DETERMINATION OF VALPROIC ACID IN HUMAN SERUM (SRM 1599)

Level	*LC–MS**	*Certified***
Low	13.5 ± 1.4	14.5 ± 0.3
Medium	65.4 ± 10	69 ± 1.0
High	139 ± 12	143 ± 1.0

* Mean ± standard deviation of a single measurement.
** Mean ± standard error of the mean.

II contains the results obtained by LC–MS analysis of the serum, as well as the certified concentrations for the SRM.

CONCLUSIONS

The LC–MS interface described in this paper has been successfully applied, in the reversed-phase LC mode, to the direct quantitative analysis of individual organic compounds in complex matrices. The system permits preconcentration of LC effluents and their introduction into the mass spectrometer when operated with solvent mixtures up to 65% water, and, under some conditions, 100% water.

The precision of analyses reported in this paper are better for the other, more time-consuming methods than for the LC–MS analyses, but the other methods involved a greater number of analyses, bracketing internal standards or standard addition experiments in many cases, and more sophisticated data handling hardware than were available for the LC–MS analyses. While rapid quantitative analyses of organic compounds in complex samples do not yet involve a routine transfer of methodology from a stand-alone LC to the LC–MS system, we belive that the examples described in this paper present adequate evidence for the utility and potential of this technique for achieving accurate and rapid trace organic analyses.

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CHROM. 15,497

EXPERIMENTS WITH THE COMBINATION OF A MICRO LIQUID CHROMATOGRAPH AND A CHEMICAL IONIZATION QUADRUPOLE MASS SPECTROMETER, USING A CAPILLARY INTERFACE FOR DIRECT LIQUID INTRODUCTION

SOME THEORETICAL CONSIDERATIONS CONCERNING THE EVAPORATION OF LIQUIDS FROM CAPILLARIES INTO VACUUM

A. P. BRUINS* and B. F. H. DRENTH
State University, Department of Pharmacy, A. Deusinglaan 2, 9713 AW Groningen (The Netherlands)

SUMMARY

The total effluent from a micro liquid chromatograph, operating at a flow-rate of 8 μl/min, has been introduced into the ion source of a quadrupole mass spectrometer, which is used in the chemical ionization mode. The solvent vapour serves as the reactant gas. The interface is constructed from flexible fused-silica capillary tubing. The capillary extends into the ion source and the tip of the capillary is in thermal contact with the source via a copper block. Transfer of heat is necessary to support evaporation of the liquid. Calculations on the rate of evaporation indicate that the liquid vaporizes inside the capillary. It is shown that the rate of evaporation and the distance between the liquid front and the end of the capillary are strongly dependent on temperature.

INTRODUCTION

When we investigated the possibility of on-line combined liquid chromatography–mass spectrometry (LC–MS), the Finnigan transport system[1] was ruled out by its price, which could not be accommodated in our budget. Because other interface systems are not commercially available for the Finnigan 3300, our attention was directed to home-made interfaces.

Direct liquid introduction systems for quadrupole mass spectrometers using a glass capillary and a splitter have been published[2,3]. The prime advantage is the very low cost of the interface. The major disadvantage lies in the use of only 1–2% of the eluate for mass spectrometric identification.

If the standard liquid chromatograph is replaced by micro high-performance liquid chromatographic (HPLC) equipment operating at a much lower flow-rate (5–15 μl/min)[4] the total effluent can be fed into the chemical ionization (CI) source, giving a dramatically increased sensitivity. This approach has been pioneered by

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Rottschaefer *et al.*[5] and Henion and Maylin[6], and has also been adopted by Schäfer and Levsen[7].

The use of a pneumatic nebulizer[8] was not considered because of the constraints imposed by the small diameter of the solids probe introduction port of the Finnigan 3300 mass spectrometer.

The various types of small-bore HPLC columns may be classified as open-tubular (capillary), packed capillary and microbore columns[9]. At present, the last category is beyond the development stage. Columns, pumps and UV detectors can be purchased from an increasing number of suppliers.

This paper describes the development of a direct liquid introduction interface for the connection of an unmodified micro-HPLC instrument with a standard quadrupole mass spectrometer, operating in the CI mode.

EXPERIMENTAL

A Finnigan 3300 GC–MS instrument equipped with the standard CI source was used. Data were processed with a Finnigan 6110 computer system. Full-scan spectra were recorded under the following conditions: mass range, 150–350; integration time, 12 msec; seconds per scan, 3. As will be shown below, it is imperative that the source temperature be kept constant, which was effected with a CRL 405 digital temperature controller (CRL, Worthing, Great Britain), using the original Finnigan thermocouple as sensor. The thermocouple was repositioned and clamped inside a hole drilled in the source block close to the solids probe inlet port. The temperature readout was calibrated against a platinum resistance thermometer, inserted into the source via the solids probe inlet. The calibrated source temperature was varied between 200 and 265°C.

The original Varian thermocouple vacuum gauge is not well suited for recording the source pressure. It is located upstream in the flow of vapours and gases towards the ion volume and it has a fairly slow response. We have used an IM 10 high-pressure ion gauge (Leybold-Hereaus, Cologn, G.F.R.), which has a measuring range of $1 \cdot 10^{-6}$ to 1.0 mbar, to monitor the pressure in the pumping line to the source diffussion pump. A value of approximately $2 \cdot 10^{-3}$ mbar (1 mbar = 0.75 Torr) was measured if acetonitrile–water (70:30) was introduced at 10 μl/min. The magnitude of the pressure fluctuations was recorded by connecting the output of the IM 10 to a pen recorder. The reactant ion spectrum was also recorded, using the hardware ion current integrator of the Quadrupole Electronics Module (mass range, 10–100; response, fast; electron multiplier at 1000 V) connected to the second channel of the two-pen recorder. When ammonia gas was added to modify the reactant ion spectrum[10] it was admitted via the solids probe solenoid valve, to ensure that solvent vapour is swept efficiently towards the ion volume.

The micro liquid chromatograph was a Jasco Familic 100N (Jasco, Hachioji, Japan), equipped with a Jasco ML 422 micro loop injector (0.3 μl internal loop), a Jasco pressure monitor with changeover valve and a Jasco Uvidec 100-III spectrophotometer detector (cell volume 0.3 μl). The home-made PTFE columns (about 150 mm long × 0.5 mm I.D. × 1.8 mm O.D.) were packed with Nucleosil C_{18} (5 μm particles) (Machery, Nagel & Co., Düren, G.F.R.) using a tetrabromoethane–*n*-butanol (1:1) slurry. The columns had a plate count of 2000–2500 for the compounds

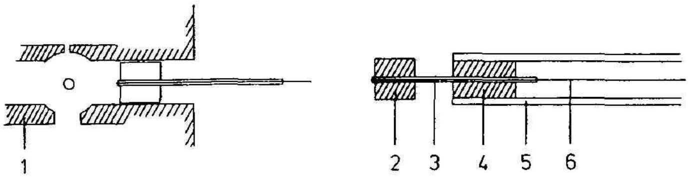

Fig. 1. Schematic diagram of the LC–MS interface probe. 1 = Source block; 2 = copper cylinder, 4.9 mm O.D. × 5 mm long; 3 = stainless-steel tube, 0.50 mm O.D. × 0.25 mm I.D.; 4 = PTFE insulator; 5 = stainless-steel tube, 6.4 mm O.D. × 4.6 mm I.D.; 6 = 50 μm I.D. flexible fused-silica capillary.

shown below. It is expected that better columns can be made by following the directions given by Westwood *et al.*[11].

The LC–MS interface probe is shown in Fig. 1. The essential part is an approximately 70 cm–1 m long × 50 μm I.D. flexible fused-silica capillary tube (SGE, Melbourne, Australia). One end of the capillary protrudes into the source and is heated by heat transfer from the source via a copper block (4.9 mm O.D.) located approximately half way inside the inlet port of the source block (5.1 mm I.D.). The fused-silica capillary and the copper cylinder are mounted in a 1/4 in. O.D. stainless-steel tube. The entire system can be inserted and removed via the solids probe insertion lock in the same manner as the standard solids probe. The other end of the capillary is connected to the outlet of the UV detector.

RESULTS

During preliminary experiments with standard HPLC equipment only a very small portion of the eluate (1–2%) was actually used for mass spectrometry. The interface was a 0.1 mm I.D. × 4.0 mm O.D. glass capillary tube with a narrow restriction on the side which protrudes into the ion source[12,13]. Attempts to create a jet of droplets[10,14] were successful but a fairly high liquid pressure was required. A stable source pressure could not be maintained, however. To investigate the cause of this problem, we observed the jet inside a glass envelope. It appeared that a jet which was straight in air started to bend when a vacuum was applied. Our explanation is that both large and small droplets are formed and the small droplets evaporate more rapidly than the larger droplets. If the small droplets are predominantly formed on one side of the jet, owing to the irregular shape of the orifice, the expanding vapours from the small droplets push the larger droplets away from the axis of the jet when a vacuum is applied. This phenomenon is not unique to the simple capillary system: the occasional deviation of the jet emitted from a pinhole orifice has been reported[10]. Because we had no rigid control over the shape and diameter of the restriction in the glass capillary, we abandoned the principle of jetting the liquid into the source.

For the next series of experiments the standard HPLC equipment was replaced by the Jasco micro-LC system[5–7], and the restriction made to the glass capillary was not as narrow. The solvent and solute now have to evaporate at the restricted end of the capillary. This, of course, limits the LC–MS system to samples that can be run off the normal direct insertion probe at reasonable temperatures (probably < 300°C). Nevertheless, there are enough problems that can be solved in this way[2,3,5–7]. In our hands success was variable, but the time spent on this approach was rather short.

Severe source pressure fluctuations were sometimes observed. Making connections to the glass capillary was also inconvenient.

A major step forward was the advent of narrow-bore flexible fused-silica capillary tubing. A length of 70 cm is sufficient to achieve transfer from the UV detector into the ion source without making further connections, and still allows adequate flexibility in the positioning of the chromatograph relative to the mass spectrometer. Capillaries with inner diameters of 25 and 50 μm have been tried so far. The 25-μm capillary required a pressure drop of 20 bar at a flow-rate of 10 μl/min (acetonitrile–water, 70:30), whereas the 50-μm capillary showed a pressure drop of only a few bars. All further experiments were carried out with the 50-μm I.D. capillary, which was considered more suitable in view of the pressure that can be delivered by the syringe pump of the Jasco micro-LC system.

The main problem with a simple capillary direct liquid introduction system is to obtain a stable ion source pressure. Because no restriction is made to the side of the capillary that is located in the source, the liquid may be under reduced pressure. Moreover, the last 20 cm of the capillary, located inside the solids probe insertion lock, is at a slightly elevated temperature (30–35°C) by the convection of warm air circulating around the diffusion pumps and rotary pumps. Gases dissolved in the mobile phase may easily form bubbles that expand and make the liquid being transferred into the source in the form of a series of short plugs. Severe pressure fluctuations are the result. This problem has been overcome by thoroughly degassing the mobile phase using an ultrasonic bath, followed by extensive flushing of all connecting tubing, the column and the detector cell.

A second problem is that the mobile phase mixture may freeze during the evaporation process if insufficient heat is transferred to the tip of the fused-silica capillary. This phenomenon can easily be recognized if the fused-silica capillary is connected directly to the micro loop injector valve, omitting the column and UV detector. As soon as a plug of ice is formed inside the capillary, the Jasco pressure monitor indicates a pressure build-up, which is accompanied by a pressure drop in the source and source pumping line. After some time the plug of ice is forced out of the capillary and the source pressure shows a peak, while the liquid pressure drops. A nude fused-silica capillary is showed this effect very markedlyu. The thermal mass of the tip and the transfer of heat to the tip are insufficient to support steady evaporation. The situation was improved by sliding a 50 mm long piece of 0.5 mm O.D. × 0.25 mm I.D. stainless-steel tubing over the fused-silica capillary. To have better control over the temperature the interface was further modified as shown in Fig. 1. The fused-silica capillary is fed through so far that its end is just observed under a magnifying glass. All further experiments were performed with this interface.

The Finnigan 3300 CI source has been designed to accept 20 ml (STP) methane per minute from a packed GC column, and reach a pressure reading of 1.0 Torr at this flow-rate (0.5 Torr on a McLeod gauge). A flow of 10 μl/min of water will produce 12 ml (STP) of water vapour per minute. A 10-μl volume of liquid acetonitrile will produce 4.2 ml of vapour, so the CI source can easily accept the total effluent from the micro-LC system at 10 μl/min, and there is enough pumping capacity to modify the reactant ion spectrum by bleeding ammonia gas into the source[10].

Fig. 2 shows the reactant ion spectrum. Sample molecules are ionized by pro-

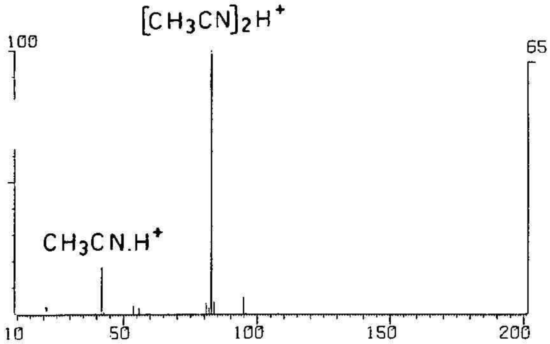

Fig. 2. Reactant ion spectrum of acetonitrile-water (70:30). Source temperature, 250°C.

ton transfer from the $[M + H]^+$ and $[2M + H]^+$ ions of acetonitrile at *m/z* 42 and 83. The reactant ion spectrum modified by ammonia is shown in Fig. 3. Ammonia has a higher proton affinity than acetonitrile so that the ions at *m/z* 42 and 83 have disappeared, while *m/z* 18 and solvated ammonium ions are generated instead. It may be advantageous to use a modified reactant gas because it will result in a softer proton transer to the sample, or in the formation of $[M + NH_4]^+$ ions[10]. Owing to our limited experience we cannot yet judge whether the performance of the interface is also influenced by the addition of ammonia gas.

Fig. 4 shows the liquid chromatogram of a mixture of four components. Full details regarding the analytical chemical aspects of the samples have been published elsewhere[15]. Fig. 5 presents the reconstructed (total ion current) liquid chromatogram for the same run, and indicates that 10 ng per component is just sufficient in the scanning mode (*m/z* 150–350). The signal-to-noise ratio of the total ion current profile can be improved by using only *m/z* 200–350 from the same data file for calculation of the reconstructed chromatogram. Injection of 50 ng per component of the same mixture resulted in the total ion current profile (*m/z* 150–350) in Fig. 6. The extracted ion current profile of *m/z* 266, corresponding to components 1 and 2, is also shown. The separation efficiency has deteriorated compared with the UV trace.

During these experiments it appeared to be necessary to keep the source tem-

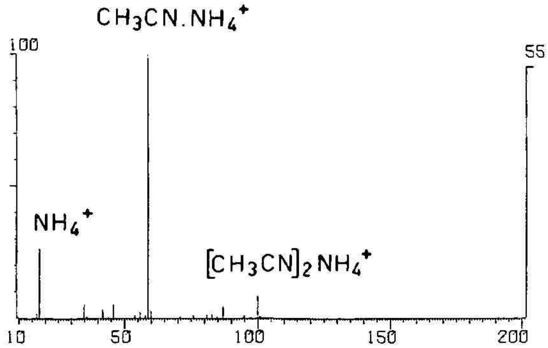

Fig. 3. Reactant ion spectrum of acetonitrile-water (70:30), modified with ammonia gas. Source temperature, 250°C.

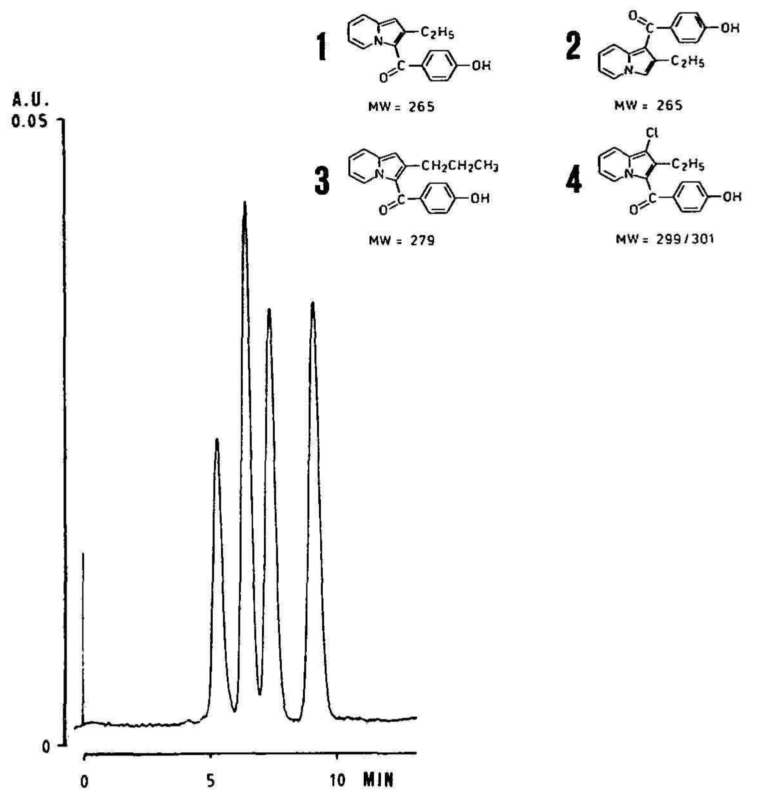

Fig. 4. Liquid chromatogram of a mixture of four components, 10 ng per component. The numbers indicate the order of elution. Mobile phase, acetonitrile-water (70:30); flow-rate, 8 µl/min; column, 150 × 0.5 mm I.D. Nucleosil $5C_{18}$; UV detection at 390 nm.

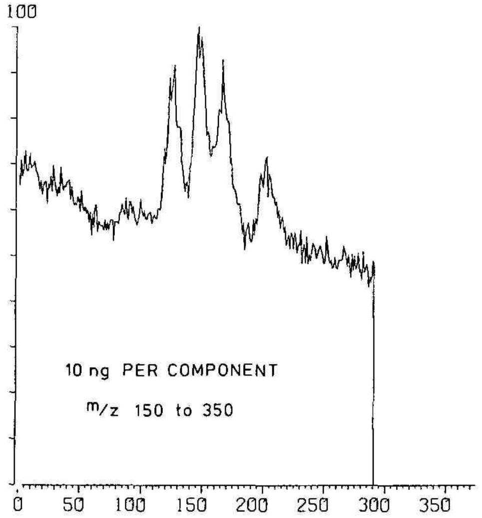

Fig. 5. Total ion current profile (*m/z* 150-350) of the liquid chromatogram shown in Fig. 4. Source temperature, 250°C.

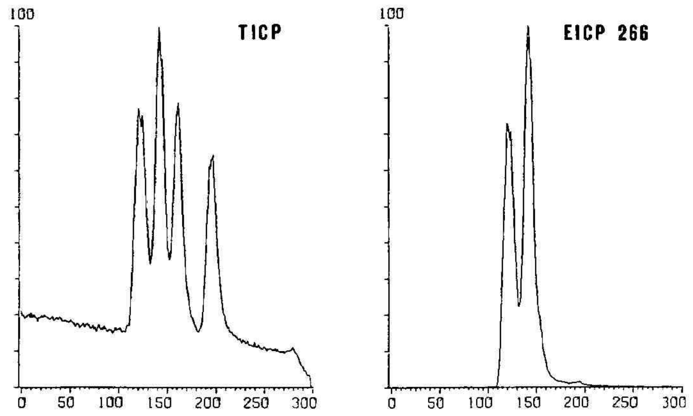

Fig. 6. Left: total ion current profile of the same mixture as in Figs. 4 and 5 (*m*/*z* 150–350); 50 ng per component injected. Right: extracted ion current profile (mass chromatogram) of *m*/*z* 266, corresponding to components 1 and 2. Ammonia gas added; source temperature, 250°C.

perature at at least 250°C, which is in accordance with the observations made by Schäfer and Levsen[7]. Ammonia gas was added to the source in the experiment shown in Fig. 6. It did not cause a change in the observed mass spectra of the compounds under investigation. Fig. 7 presents the mass spectrum of component 4.

DISCUSSION

The results show how we arrived at a reasonable system in a purely empirical way. The diameter of the fused-silica capillary was dictated by the problems that we expected to occur when the syringe pump had to deliver a high pressure. The length of the copper block which transfers heat to the tip of the interface was arbitrarily chosen as 5 mm.

The major theoretical question concerning a capillary system, where liquid jet formation does not occur, is whether evaporation of the mobile phase takes place

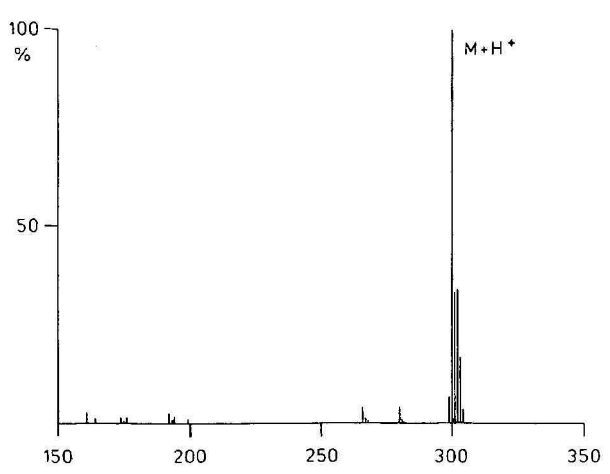

Fig. 7. Mass spectrum of component 4 from Figs. 4 and 6.

inside or outside the capillary. Talroze *et al.*[16] have made calculations, which have been summarized by Arpino *et al.*[10]. Using the Poiseuille equations for flow of an incompressible liquid and a compressible, ideal gas, the lengths of the liquid column L and gas column l were calculated:

$$\frac{dn}{dt} = \frac{\pi r^4 \rho \left(P_i - P + \dfrac{2\sigma}{r}\right)}{8\,\eta_l L M} \text{ mol/sec (liquid)} \tag{1}$$

$$\frac{dn}{dt} = \frac{\pi r^4 (P^2 - P_s^2)}{16\,\eta_g l R T} \text{ mol/sec (gas)} \tag{2}$$

The liquid flow entering the capillary equals the gas flow leaving the capillary, so by elimination of $\frac{dn}{dt}$, the ratio

$$\frac{l}{L} = \frac{\text{length of gas column}}{\text{length of liquid column}}$$

can be calculated. It was concluded that evaporation always takes place inside the capillary.

This conclusion is correct, as long as the maximum possible speed of evaporation at the liquid–vapour phase transition is high compared with the mass flow-rates of liquid and gas in the capillary. In a more recent paper, Zolotoi *et al.*[17] have shown that if this condition is not fulfilled, *i.e.*, if on the contrary the evaporation rate is smaller than the flow-rate of the liquid, it is indeed possible to make the liquid flow out of the capillary into the vacuum. Their calculations were confirmed by experiments. Fig. 8 gives a schematic representation of the evaporation of the liquid. Situation a corresponds to eqns. 1 and 2, and the maximum evaporation rate from the area of the meniscus is high compared with the liquid flow-rate. In case b the evaporation rate is equal to the liquid flow-rate. In case c, the liquid flow-rate is higher than the evaporation rate from the area of a meniscus just inside the capillary. The

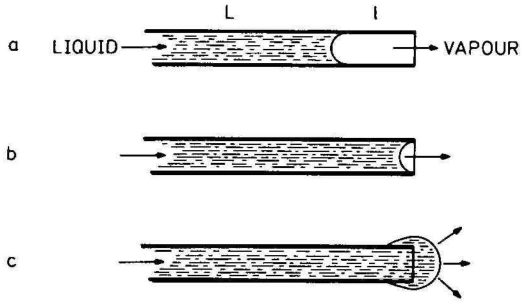

Fig. 8. Three possible situations during evaporation of a liquid from a capillary into a vacuum.

liquid now flows out of the capillary, the evaporation area is enlarged and equilibrium between liquid flow-rate and evaporation area is established.

The rate of evaporation, Z, of a liquid at a liquid-vacuum interface, from an area A at temperature T, can be calculated from the kinetic gas theory[18]:

$$Z = P_0 A \sqrt{\frac{1}{2\,\pi\, mkT}}\ \text{molecules/sec} \tag{3}$$

or in SI units:

$$G = P_0 A \sqrt{\frac{10^{-3} M}{2\pi RT}}\ \text{kg/sec} \tag{4}$$

The area A is assumed to be a hemisphere with radius r (radius of the capillary). P_0, the saturated vapour pressure of the liquid, can be taken from tables (water, methanol) or can be calculated (acetonitrile) from critical temperature and pressure using the Van der Waals expression[19]:

$$\log \frac{P_0}{P_c} = \text{constant}\left(1 - \frac{T_c}{T}\right) \tag{5}$$

Fig. 9 shows the calculated evaporation rates from an area equal to a hemisphere of radius 25 μm as a function of temperature for water, methanol and acetonitrile. At a liquid flow-rate of 10 mg/min (10 μl/min for water, 12 μl/min for

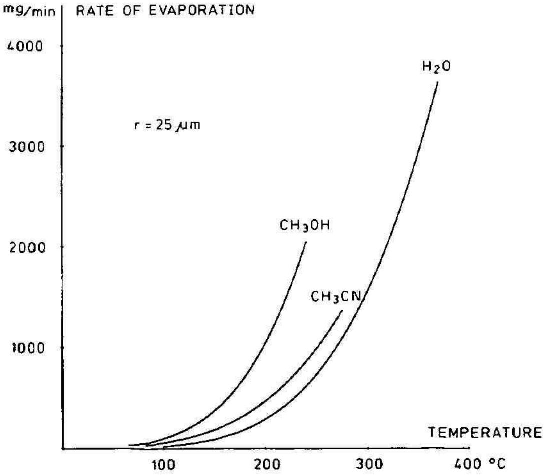

Fig. 9. Rate of evaporation of three liquids from a capillary of radius 25 μm as a function of temperature between the boiling point and the critical point. The evaporation area is assumed to be a hemisphere, see Fig. 8b.

methanol and acetonitrile), situation b in Fig. 8 is met at 76, 36 and 44°C with water, methanol and acetonitrile, respectively. A higher temperature results in situation a and a lower temperature gives situation c.

Experimentally, a stable source pressure is achieved above about 250°C, as demonstrated by our experiments and literature data[3,6,7]. Schäfer and Levsen[7] have reported that cooling the capillary interface results in strong source pressure fluctuations. In case c, the evaporation area is not fixed, but is variable, which explains the uneven evaporation of the liquid phase. The experimental data, combined with the theory presented above, suggest that it is necessary to work in situation a in Fig. 8.

Further calculations can be made to obtain the length, l, of the gas column in case a. When the meniscus has retracted inside the capillary, the vapour pressure above the liquid rises. The pressure required to force a gas flow, dn/dt, through a capillary is calculated from eqn. 2. If the assumption is made that the source pressure P_s is very small compared with P, eqn. 2 is reduced to

$$G = \frac{\pi r^4 \cdot 10^{-3}\, MP^2}{16\, \eta_g l RT} \text{ kg/sec} \tag{6}$$

or

$$P = \sqrt{\frac{16\, \eta_g l RTG}{\pi r^4 \cdot 10^{-3}\, M}} \text{ Pa} \tag{7}$$

Using this equation, the pressure, P, necessary to force 10 mg/min of water vapour into the ion source through a capillary of length l was calculated and is shown in Fig. 10.

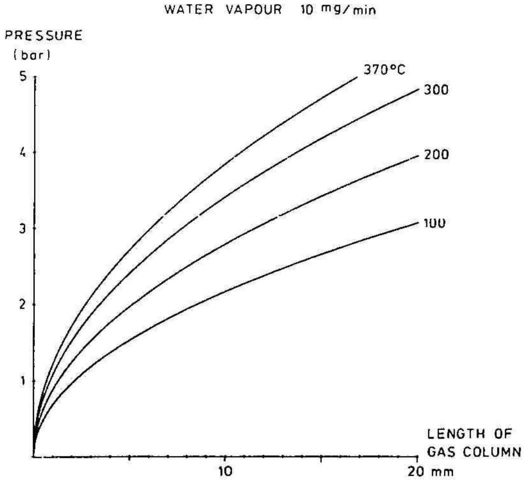

Fig. 10. Pressure build-up inside a capillary (radius 25 μm) when water vapour is transferred to a vacuum at a flow-rate of 10 mg/min as a function of the distance from the outlet of the capillary.

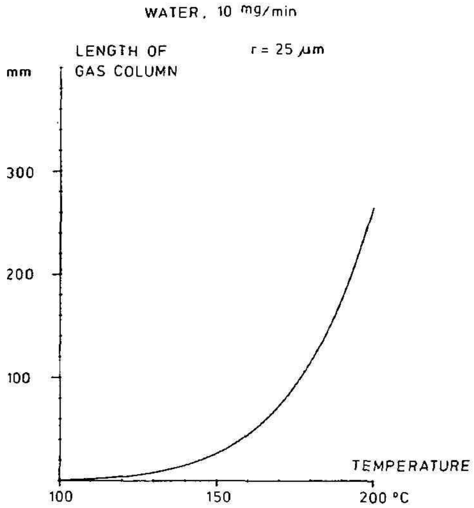

Fig. 11. Distance between the liquid level and the outlet of a capillary (radius 25 μm) as a function of temperature for water at 10 mg/min.

If evaporation does not take place into a vacuum, but into a vessel with pressure P, the evaporation rate has to be calculated from[18]

$$G = (P_0 - P)\,A\,\sqrt{\frac{10^{-3}\,M}{2\pi RT}}\ \text{kg/sec} \qquad (8)$$

After substitution of eqn. 7 into eqn. 8 and choosing G = 10 mg/min, the relationship between l and T is obtained, which is shown in Fig. 11. It appears that at 122°C the liquid level of water has retracted 5 mm inside the capillary, which is the length of the copper block (Fig. 1).

The calculations show that evaporation inside the capillary takes place at moderate temperatures. The experimental data seem to indicate that the temperature of the liquid at the meniscus is much lower than the source temperature, in spite of the heat transferred by the copper block.

CONCLUSION

A simple LC–MS interface can be constructed from fused-silica capillary tubing, provided that care is taken to transfer enough heat to the tip of the capillary inside the ion source. The experimental data and theoretical considerations show that evaporation of the liquid takes place inside the capillary. Temperature plays a very important role, so that a constant source temperature is required to achieve a stable evaporation rate and a stable source pressure.

A disadvantage of the present system, compared with direct liquid introduction systems using liquid jet formation, is that non-volatile samples and impurities will tend to block the capillary. On the other hand, fused silica tubing can be purchased by the metre, and is easily replaced, if necessary.

SYMBOLS

STP	standard temperature and pressure
$\mathrm{d}n/\mathrm{d}t$	molar flow-rate (mol/sec);
r	radius of capillary tube (m);
σ	solvent surface tension;
P_i	liquid pressure at inlet of capillary (Pa);
P	vapour pressure at liquid–vapour phase transition (Pa);
P_0	saturated vapour pressure of liquid (Pa);
P_s	ion source pressure (Pa);
P_c	critical pressure (Pa);
η_l	viscosity of liquid (Pa sec);
η_g	viscosity of gas (Pa sec);
R	gas constant, 8.3 (Joule/°K · mol);
M	molecular weight;
$10^{-3}\,M$	mass of 1 mol (kg);
l	length of gas column inside a capillary (m);
L	length of liquid column inside a capillary (m);
G	mass flow-rate of evaporation rate (kg/sec);
T	temperature (°K);
T_c	critical temperature (°K);
A	area of surface from which evaporation takes place (m^2);
m	mass of one molecule;
k	Bolzmann constant.

Conversion factors: 1 bar = 100 kPa; 1 mbar = 0.75 Torr.

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MASS SPECTROMETRY OF BIOGENIC CATECHOLAMINE ION PAIRS BY DIRECT LIQUID INTRODUCTION

H. MILON* and H. BUR
Nestlé Research Department, CH-1814 La Tour-de-Peilz (Switzerland)

SUMMARY

The mass spectrometry of ion pairs of dopamine, noradrenaline and 3,4-dihydroxyphenylacetic acid with *n*-octylsulphonate has been investigated by direct liquid introduction in an acidic buffer currently used for their high-performance liquid chromatographic separation. In order to follow the fate of the ion pairs, they were first studied by desorption/chemical ionization as dichloromethane extracts or in the buffer.

The results show that characteristic spectra of the solutes can be obtained by direct liquid introduction provided that the interface between the chromatographic system and the mass spectrometer permits the desolvation of the ion pairs from the cluster of solvent molecules.

INTRODUCTION

Biogenic catecholamines dopamine (DA) and noradrenaline (NA) and one of their metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), are currently determined in biological samples by reversed-phase high-performance liquid chromatography (HPLC) and electrochemical detection as ion pairs[1]. However, the specificity of this method is not absolute. Extra peaks can interfere with those of the compounds under analysis, arising either from endogenous substances when analysing samples of different biological origin or from exogenous substances (or their metabolites) when applying pharmacological treatments, for example. There are several ways of confirming the identity and purity of an HPLC peak.

We were interested in investigating the possibility of using direct liquid introduction (DLI) into a mass spectrometer, taking advantage of the Nermag R10–10C instrument facility[2], to solve this type of problem. Such small molecules might not represent the best example of the application of coupling of liquid chromatography (LC) and mass spectrometry (MS). They have low molecular weights and other solutions could be envisaged. However, from the chromatographic point of view, this system works routinely in our laboratory and one of our aims was to become familiar with LC–MS coupling. Moreover, we believe that in many circumstances LC–MS coupling will be useful only if one can couple the separation system one is currently using with other types of detectors to a mass spectrometer without needing to adapt

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it. In our case, this results in two major difficulties: first, to introduce a mobile phase buffered at low pH values (*ca.* 3) and containing dissolved salts, and second to be able to ionize ion pairs with the production of spectra that are characteristic of the solute.

After a first negative attempt, we undertook a systematic study of the ionization of ion pairs when introduced directly in the ion source, taking advantage of a recently developed interface. Our results are compared to those obtained by Vouros and co-workers[3,4], who used a moving belt interface and a continuous dichloromethane extraction device[5].

EXPERIMENTAL

Chemicals

DA and NA hydrochloride salts were purchased from Fluka (Switzerland), DOPAC from Sigma (U.S.A.) and sodium *n*-octylsulphonate from Eastman-Kodak (U.S.A.), and were used as received. Water was doubly distilled. Methanol (Merck, G.F.R.) and other solutes were of analytical-reagent grade and were used without further purification.

Chromatographic conditions

The liquid chromatographic system consisted of a Waters (U.S.A.) 6000 pump, an Altex 210 (U.S.A.) injector equipped with a 100-μl loop and a TL-5 glassy carbon electrochemical detector (ElCD) (BAS, U.S.A.) operated at a potential of 0.9 V *versus* a silver–silverchloride reference electrode. The column (25 × 4.6 mm I.D.) was packed with 7-μm RP-18 material (Knauer, G.F.R.). For the DLI or LC–MS experiments, the mobile phase consisted of either methanol-water (1:4) or buffer (2 vols. of 0.02 *M* citric acid and 1 vol. of 0.02 *M* Na_2HPO_4)–methanol (80:20), made 2.5 m*M* with sodium *n*-octylsulphonate and 0.05 m*M* with Na_2EDTA. The pH was then adjusted to 3.2 with phosphoric acid. The flow-rate was 1 ml/min and the splitting ratio to the mass spectrometer was 1% to achieve an optimal flow-rate of 10 μl/min in the ion source.

Mass spectrometry

A Nermag R10–10C system monitored by a PDP 11/23 computer was used. The running conditions of the mass spectrometer and the interface between the chromatographic system are described below.

RESULTS

The three compounds (solutes) were first introduced into the ion source by DLI in the methanol–water mobile phase in order to obtain their chemical ionization-positive mode (CI^+) spectra, which are shown in Fig. 1. They exhibit a base peak corresponding to the $(M + H)^+$ ion (except for NA) and at least one important fragment corresponding to the basic structure. In addition, the catechol acid DOPAC produced an ion corresponding to the addition of one molecule of methanol to the pseudo-molecular ion.

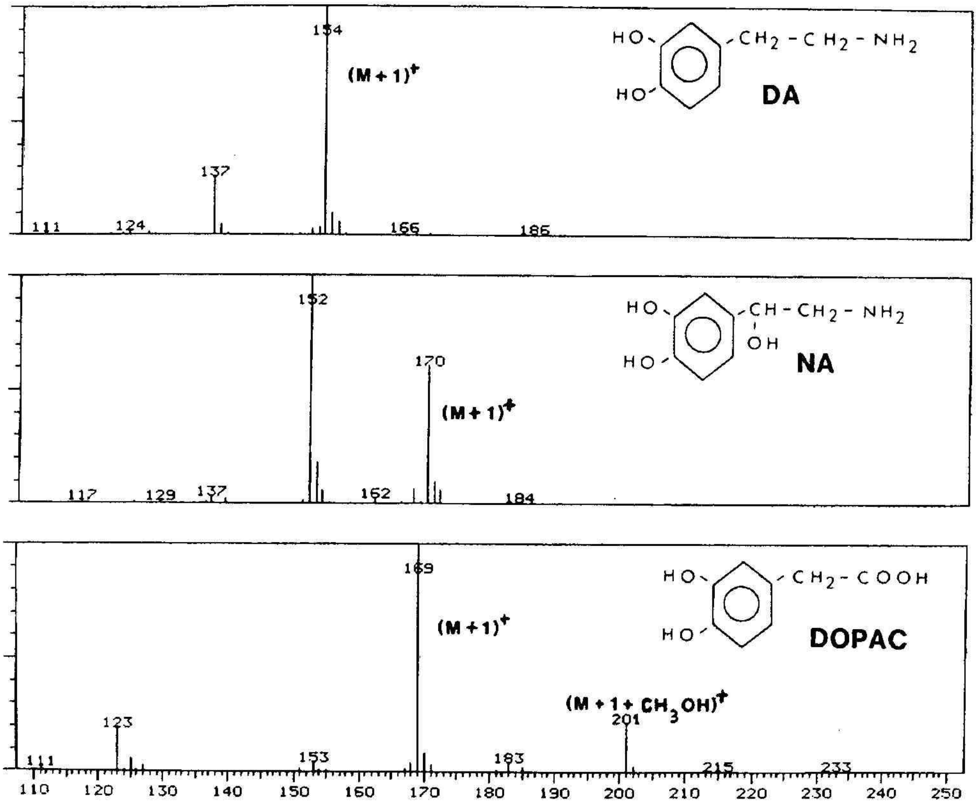

Fig. 1. Mass spectra of DA, NA and DOPAC by DLI (CI^+) in methanol-water (1:4) of the pure compounds.

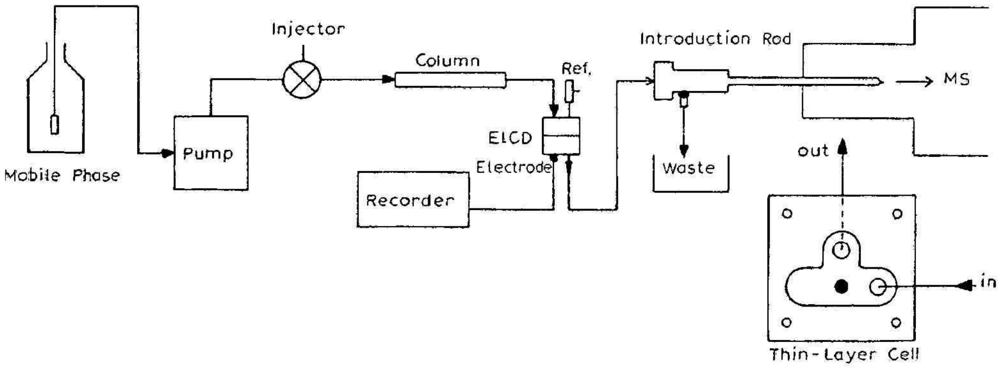

Fig. 2. Schematic diagram of the HPLC-ElCD-MS system. The modification of the thin-layer is shown on the lower right.

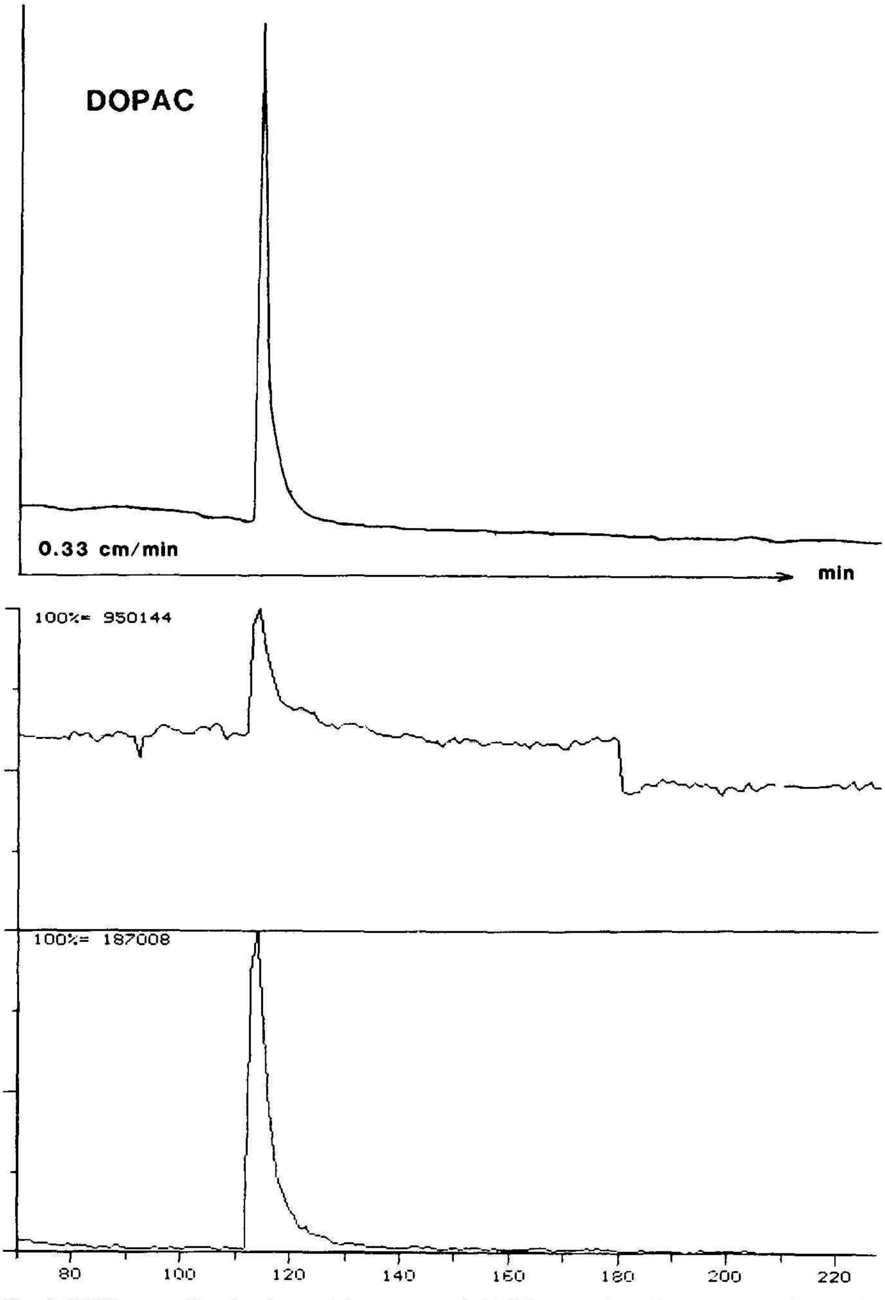

Fig. 3. ElCD recording (top), total ion current (middle) and m/z 169 ion current (bottom) traces for DOPAÇ-*n*-octylsulphonate ion pair. DOPAC was injected onto the RP-18 column in a methanol solution. The buffer (pH 3.2) mobile phase contained *n*-octylsulphonate (2.5 m*M*) (for details, see text). CI^+.

LC–MS of the ion pairs

We then directly attempted to couple our chromatographic system to the mass spectrometer, keeping the EICD in series. For this purpose, we had to modify the thin-layer cell of the EICD: in order not to lose resolution, the thin plastic film forming the cell was enlarged to expose the small dead volume exit from the detector (initially the mobile phase left the detector through the large-volume reference electrode assembly), as shown in Fig. 2. The only substance that produced ions in this configuration was DOPAC (Fig. 3). In fact, this compound is an acidic metabolite of deaminated dopamine and probably does not form an ion pair.

Ion pairs by desorption/chemical ionization-positive mode (D/CI$^+$) in dichloromethane extracts and buffer

In order to follow more closely the fate of the ion pairs, we followed an approach parallel to that of Kirby *et al.*[3], who first extracted the ion pairs by with dichloromethane and studied them by direct EI–MS and LC–MS using a moving-belt interface.

In our case, as expected, the dichloromethane extract of pure *n*-octylsulphonate (counter ion) or solutes did not produce ions. However, when the dichloromethane extracts of the ion pairs were introduced, we obtained spectra corresponding to the sum of the spectra of both components (Fig. 4).

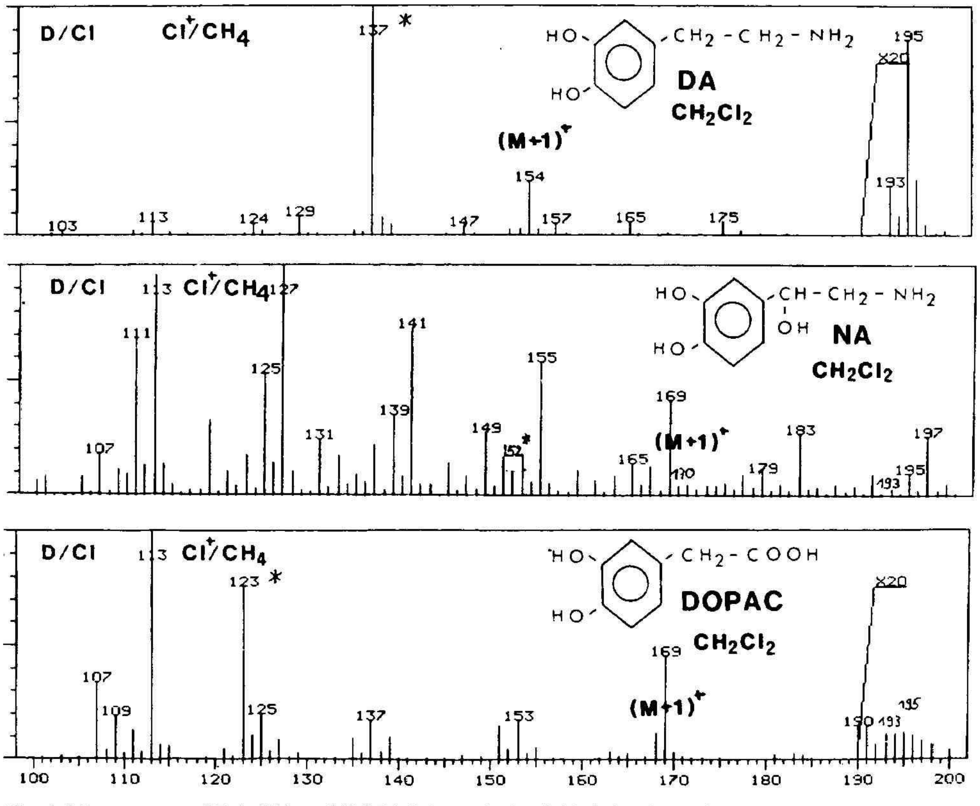

Fig. 4. Mass spectra of DA, NA and DOPAC ion pairs by D/CI$^+$ (methane) in dichloromethane extracts.

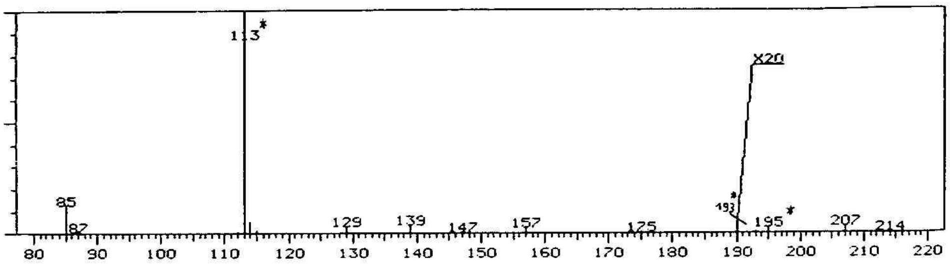

Fig. 5. Mass spectrum of sodium *n*-octylsulphonate by D/CI$^+$ (methane) in buffer (pH 3.2).* Characteristics fragment ions.

When the buffer solution was deposited on the desorption/chemical ionization (D/CI) filament without any solute, a spectrum of the counter ion was obtained (Fig. 5) in which the base-peak was at *m/z* 113 and two major fragments at *m/z* 195 and 193.

Finally, the buffer solution containing the solutes was deposited on the D/CI filament and spectra containing characteristic ions from both the solutes and the counter ion were observed (Fig. 6).

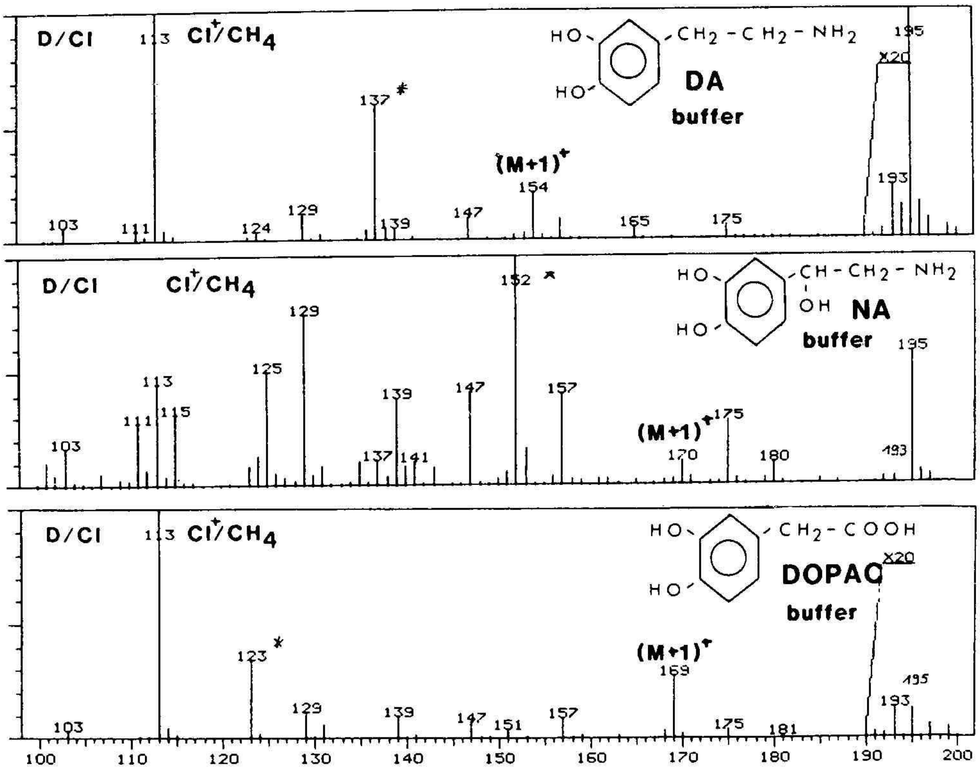

Fig. 6. Mass spectra of DA, NA and DOPAC ion pairs by D/CI$^+$ (methane) in buffer.* Characteristic fragment ions of the solutes.

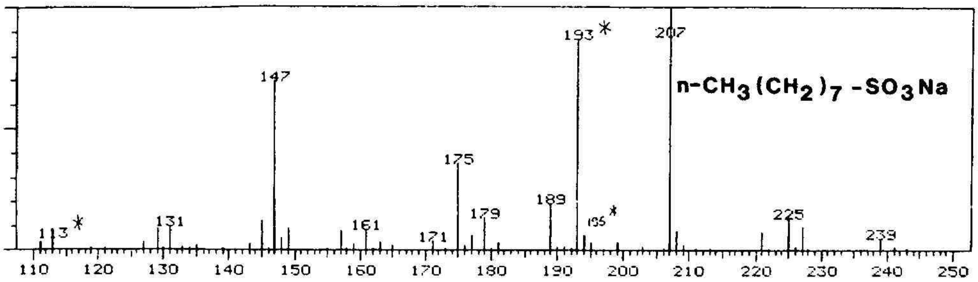

Fig. 7. Mass spectrum of sodium *n*-octylsulphonate by DLI (CI^+) in methanol-water (1:4) mobile phase with desolvation chamber (operated at 250°C).

Ion pairs by DLI in methanol-water and buffer mobile phases

In order to facilitate the ionization of the ion pairs, we then tentatively used the desolvation chamber described by Dedieu *et al.*[6]. An exploratory series of experiments showed that the optimal temperature of the desolvation chamber depends on the compound under study. Therefore, we chose a compromise temperature of 250°C for all the subsequent experiments.

When injected in methanol-water as the mobile phase, the counter ion exhibited a spectrum containing fragments at *m/z* 195, 193 and 113 but, in contrast to the spectrum obtained by D/CI, the base peak was at *m/z* 193 (Fig. 7). When the ion

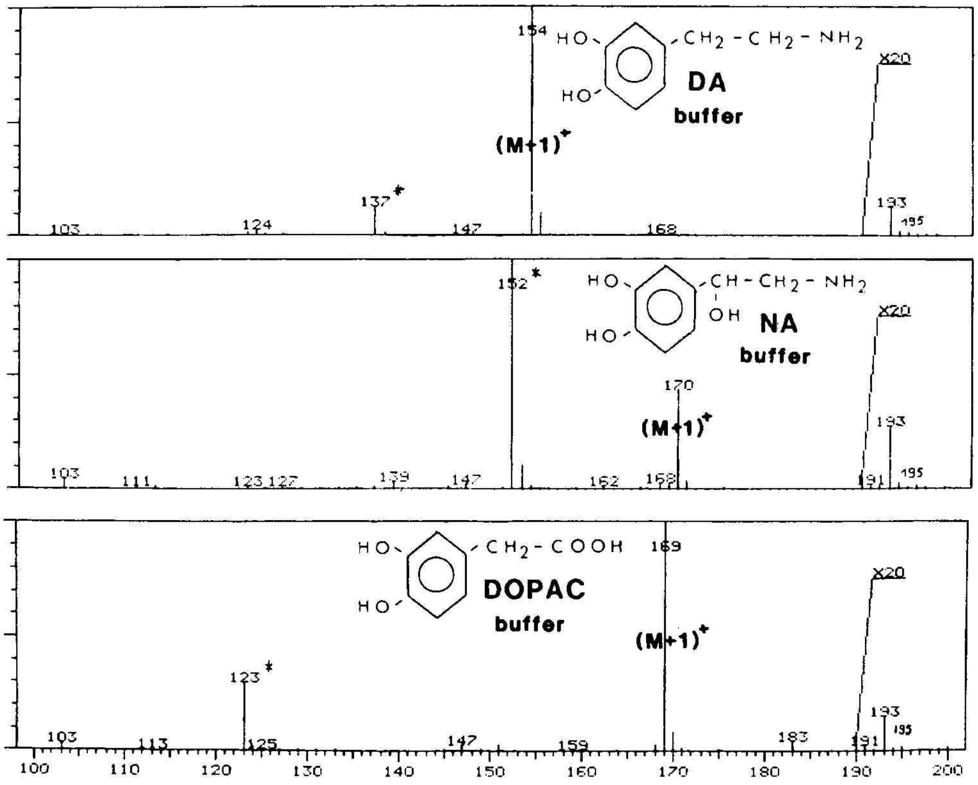

Fig. 8. Mass spectra of DA, NA and DOPAC ion pairs by DLI (CI^+) in methanol-water (1:4) mobile phase with desolvation chamber (250°C).

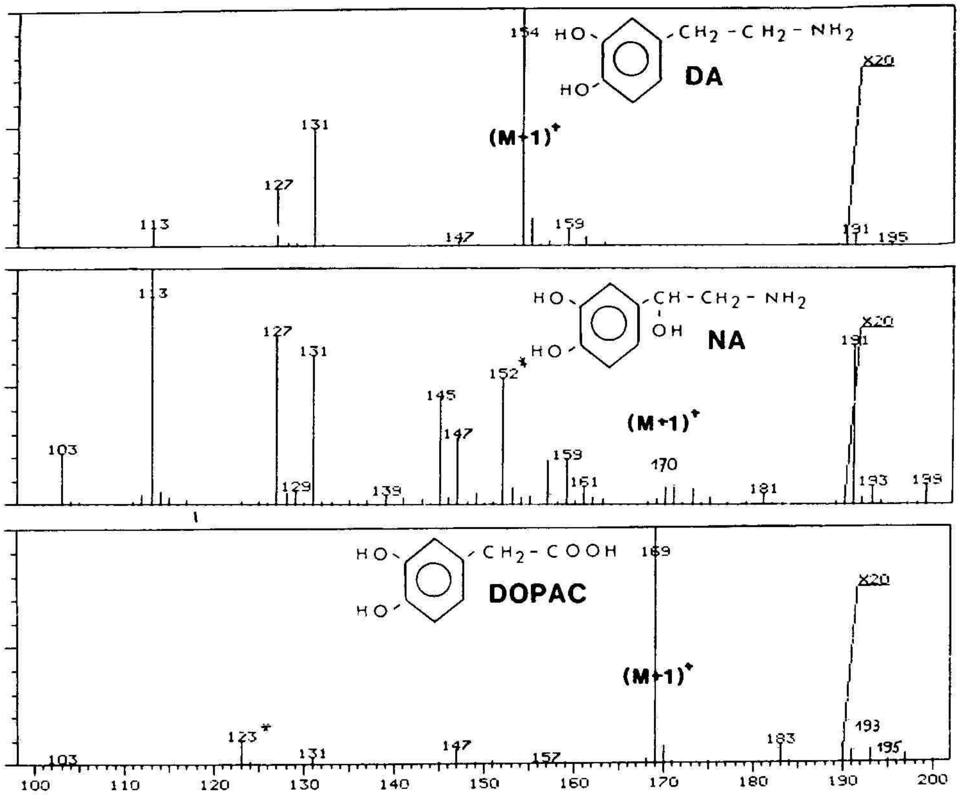

Fig. 9. Mass spectra of DA, NA and DOPAC by DLI (Cl^+) in buffer (pH 3.2) mobile phase containing *n*-octylsulphonate (2.5 m*M*) with desolvation chamber (250°C).

pairs were injected (they were formed by dissolving the solutes in the buffer solution), characteristic fragments from both the counter ion and the solutes were observed (Fig. 8).

We then used the buffer solution containing the counter ion (concentration 2.5 m*M*) as the mobile phase and injected the solutes dissolved in methanol. The same ions as above were observed but those originating from the fragmentation of the solutes were weaker (Fig. 9).

DISCUSSION

These experiments show that the ionization of ion pairs introduced by DLI is possible. In fact, similar results have also been obtained with the biogenic indole derivatives 5-hydroxytryptamine (serotonin) and 5-hydroxyindoleacetic acid. However, the interface system has to be more sophisticated than the straightforward introduction of the liquid effluent into the ion source. This is probably due to the difficulty of removing the cluster of solvent molecules surrounding the ion pairs. In our case, the desolvation has been achieved by using a heated interface, leading at the same time to an acceleration of the molecules at a lower pressure[6]. As the effect is more pronounced with effluents of low molecular weight, the situation of an aqueous buffer could be favourable. Moreover, this type of interface permits the ion source to be run at lower temperatures (*ca.* 125°C) which also favours better ionization.

We shall now discuss some problems encountered in this work, and also those which might be foreseen in future work.

Buffer: the introduction of this type of mobile phase into the ion source first causes problems due to its acidic pH. All the tubing must be made from stainless steel containing no nickel or from platinum. Further, in our instrument, the diaphragms are normally made from pure nickel, the only metal that allows the manufacture of 2–3-μm holes. Such diaphragms are attacked by the buffer solution and permit work for only a few minutes. To protect the nickel, they must be gold plated (*ca.* 1 μm thick). Second, the dissolved salts introduced into the ion source contaminate it very quickly. It appeared that the contamination can cause changes in the relative intensities of the fragment ions. It is therefore of prime importance to keep the source very clean. The dichloromethane extraction device of Vouros and coworkers could overcome this drawback. However, the conditions of the extraction should be very carefully optimized as it appeared to us that the extraction yield could lower the sensitivity considerably.

Sensitivity: all our experiments were conducted by introducing large amounts of compounds (hundreds of nanograms) in the source. Such large amounts are never found in practice, as indicated in the Introduction. Several solutions now exist for increasing the splitting ratio before the introduction into the source. One can either use a microbore column, although at present there is not a wide choice on the market and a danger of overloading the column with the injection volume or the amount of solute exists, or one can use a small-bore column (*ca.* 2 mm I.D.) combined with a larger diaphragm hole and therefore increase the splitting ratio to 1:10 or even 1:5.

As mentioned above, comparable experiments have been performed by Kirby *et al.* using a moving-belt interface[3]. Some differences were observed in the spectra of the counter ion. Whereas they observed only a single ion when using *n*-octylsulphonate, corresponding to the protonated sulphonic acid of the salt, we observed a fragmentation pattern corresponding to that observed by Kirby *et al.* for the *n*-octylsulphate salt. However, in our case, it must be noted that the intensity of the fragments depended on the introduction conditions: in the D/CI experiments, the base peak corresponded to the protonated alkene ion (m/z 113) but in the DLI experiments the base peak corresponded to an ion at m/z 193. As far as the spectra of the ion pairs are concerned, no essential differences were observed from those of Kirby *et al.* It must also be pointed out that the differences observed between the results of Kirby *et al.* and our D/CI results could have been caused by the different reagent gases used (isobutane by Kirby *et al.* and methane by us), and second between our D/CI and DLI experiments because in the latter no reagent gas was used. We tentatively add methane and ammonia in the DLI experiments, but we observed no effects on the fragmentation pattern. It can be said that DLI does not necessitate an extraction procedure with all its drawbacks and ensures that all the compounds separated by the HPLC column are introduced into the ion source.

Fig. 10 shows a comparison of the spectra obtained in the different modes for DA as a representative example of the other compounds studied. In all modes, characteristic ions from both the counter ion and the solute are present, the $(M + H)^+$ ion of the solute being present in every instance. However, it is obvious that concerning the solute, the relative intensities of the $(M + H)^+$ ion and the fragments depend on the mode of introduction. As expected, DLI appears to produce a softer ionization than D/CI. If this could lead to a loss of specificity, it might increase the sensitivity of detection.

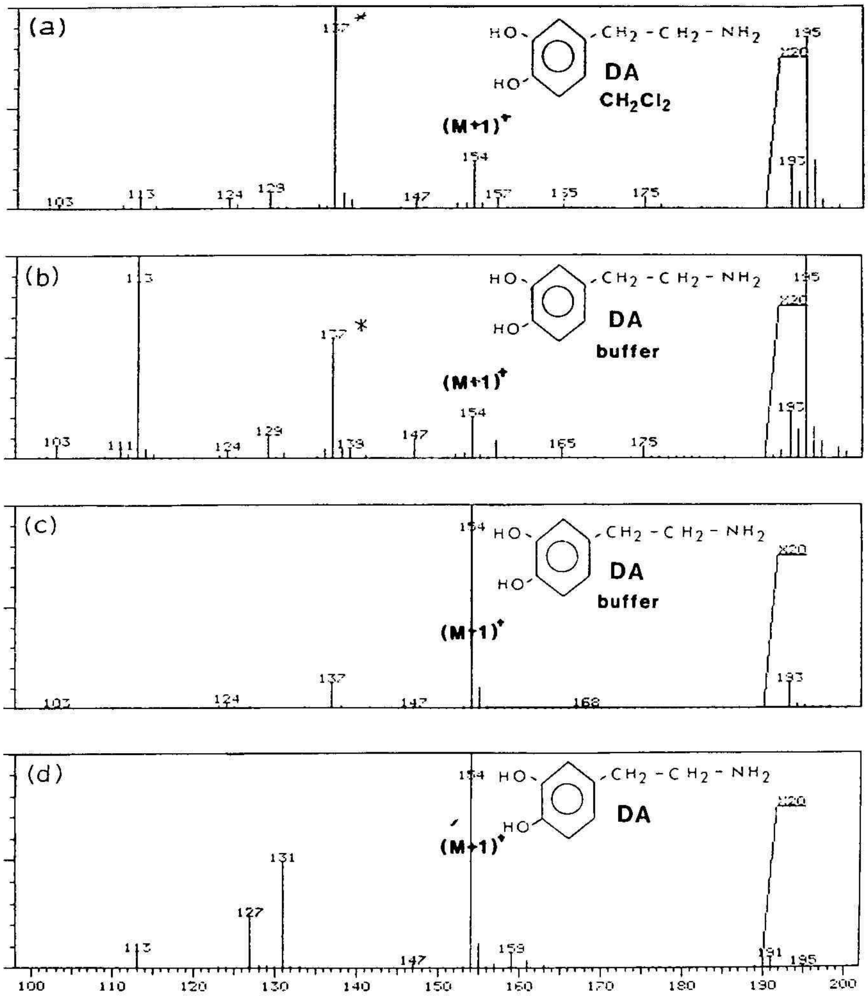

Fig. 10. Mass spectra of DA in different modes showing the effect on the relative intensities of the fragment ion of DA and counter ion. a and b, D/CI$^+$ (methane); c, DLI (CI$^+$), methanol–water (1:4); d, CI$^+$, buffer pH 3.2.

In conclusion, we have shown that spectra of ion pairs can be obtained when injected by DLI, provided that the interface allows the desolvation of the ion pairs from the cluster of solvent molecules.

Studies are under way to achieve the sensitivity required for biological work and to analyse other types of compounds necessitating the use of MS.

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ANALYSIS OF ORGANICS IN THE ENVIRONMENT BY FUNCTIONAL GROUP USING A TRIPLE QUADRUPOLE MASS SPECTROMETER

DONALD F. HUNT*, JEFFREY SHABANOWITZ, T. MICHAEL HARVEY and MILDRED L. COATES
Department of Chemistry, University of Virginia, Charlottesville, VA 22901 (U.S.A.)

SUMMARY

A comprehensive scheme for the direct analysis of organics in the environment is described. The technique of collision-activated dissociation on a triple quadrupole mass spectrometer is employed for the characterization of both knowns and unknowns by both molecular weight and functional group. Neutral loss scans are used for the analysis of carboxylic acids, phenols, polynuclear aromatic hydrocarbons, amines and chlorocarbons. Parent ion scans are used for detection of aldehydes, ketones and phthalates. The total analysis time per sample is typically 25–30 min by this approach.

INTRODUCTION

Analysis of water samples for the 114 toxic organic chemicals on the U.S. Environmental Protection Agency (EPA) priority pollutant list presently involves separation of the organics from the matrix using a combination of wet chemical extraction steps, sample concentration and clean-up by various types of chromatography, and final analysis by a gas chromatograph–mass spectrometer–data system[1]. This approach has proved to be highly reliable and applicable to a variety of different matrices. Efforts to extend the methodology and develop a master analytical scheme for the analysis of all organics in water that can be made to pass through a gas chromatograph are presently in progress[2].

Disadvantages of the above approach include: (a) the inability to detect highly polar compounds too involatile or thermally labile to pass through the gas chromatograph, (b) the high labor costs dictated by the need for extensive sample clean-up and preseparation prior to analysis, and (c) the large amount of time required to perform even a single analysis on a relatively expensive gas chromatograph–mass spectrometer–data system.

To overcome these problems, we have initiated research to develop an alternate comprehensive analytical scheme that uses the technique of collision-activated dissociation on a triple quadrupole mass spectrometer for the direct, rapid, qualitative–semiquantitative analysis of organics in liquid and solid environmental matrices. Elimination of all or most wet chemical and chromatographic separation steps,

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characterization of both knowns and unknowns by molecular weight and functional group at the 0.1–1.0-ppm level, and a total analysis time per sample of under 30 min are additional objectives of this effort.

Here we describe the instrumentation employed in this analytical scheme and outline the approach used for the direct analysis of phthalates, chlorocarbons, polycyclic aromatic hydrocarbons, phenols, amines, carboxylic acids, ketones, and aldehydes in environmental matrices.

Additional examples of mixture analysis by tandem mass spectrometry can be found in two excellent reviews[3,4].

EXPERIMENTAL

All experiments were performed on a Finnigan triple stage quadrupole mass spectrometer–data system. This instrument consists of a conventional electron ionization/chemical ionization ion source, three quadrupole filters, Q1, Q2, and Q3, and a conversion dynode electron multiplier detector (Fig. 1)[5]. Mixture analysis on this instrumentation can be accomplished with or without prior chromatographic separation of the components.

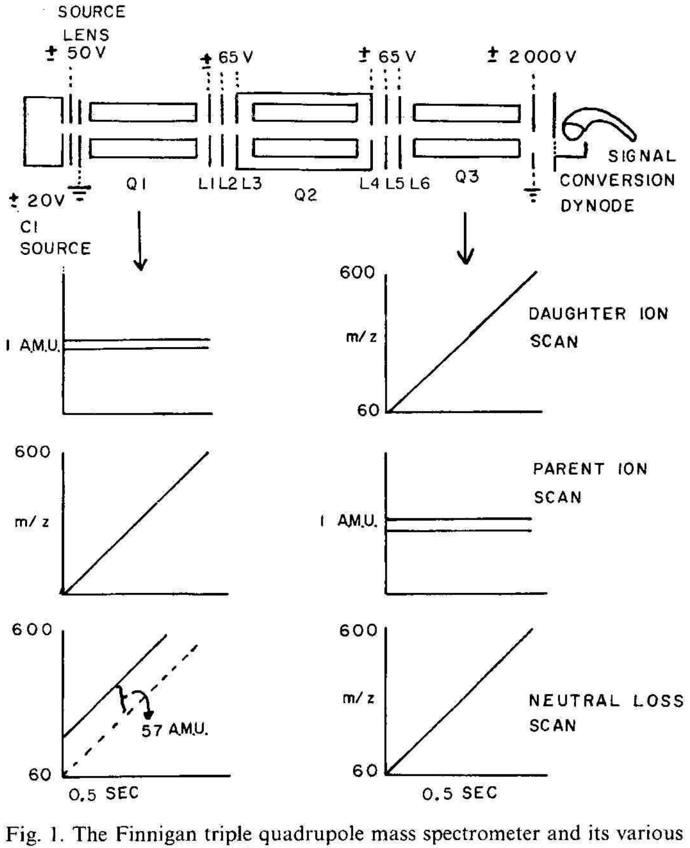

Fig. 1. The Finnigan triple quadrupole mass spectrometer and its various modes of operation.

Operation of the triple quadrupole mass spectrometer as a conventional single analyzer instrument

When a quadrupole mass filter operates with only low radiofrequency (r.f.) potential on the rods, it functions as an ion-focusing device and transmits ions of all m/z ratios. Accordingly, if Q1 and Q2 function in the r.f. only mode, and Q3 is operated with both r.f. and d.c. potentials on the rods, the triple quadrupole mass spectrometer behaves like a conventional single analyzer instrument. All ions exiting the ion source are transmitted through Q1 and Q2 and are mass analyzed in Q3. Standard electron ionization or chemical ionization mass spectra of each component eluting from the gas chromatograph result.

Operation of the triple quadrupole mass spectrometer in the daughter ion scan mode

Daughter ion scans (Fig. 1) are particularly useful when a complex matrix is to be analyzed for the presence or absence of a small number of known compounds. To obtain collission-activated dissociation mass spectra, matrix components are volatilized into the ion source and Q2 is filled with argon gas to a pressure of 1–4 mTorr and operated in the r.f. only mode. The r.f. and d.c. potentials on Q1 are then set to pass ions with a particular m/z value (Fig. 1). When the selected ions enter Q2, they suffer collisions with argon atoms, become vibrationally excited, and dissociate to produce ions characteristic of their structure. In the presence of a weak r.f. field, all of these fragment ions are transmitted to Q3 where they undergo mass analysis. A mass spectrum of the fragment ions derived from each ion entering Q2 results. Other matrix components will usually contribute to the total signal at the particular m/z value selected by Q1 and the resulting collision activated dissociation mass spectrum will contain fragment ions derived from several components. All ions in the collision-activated dissociation mass spectrum of the pure analyte should be present in the mixture spectrum if the known compound is present in the matrix.

Previous reports from this laboratory have described the use of this approach for the detection of nitrophenols and phthalates in industrial sludge at the 100-ppb* level[6] and for sequence analysis of polypeptides in mixtures[7].

Operation of the triple quadrupole mass spectrometer in the parent ion scan mode

If Q3 is set at a particular m/z value, Q2 is operated as a collision cell, and Q1 is scanned over the desired mass range, the resulting spectra contain all of the parent ions that afford a particular fragment in the collision-activated dissociation process. This is referred to as a parent ion scan and is particularly useful for the analysis of either a homologous series of compounds or a class of compounds containing the same functional group. In the present work, phthalates are analyzed using this scan mode.

Operation of the triple quadrupole mass spectrometer in the neutral loss scan mode

Neutral loss scans are employed for the rapid analysis of complex mixtures for members of a class of compounds that undergo the same type of fragmentation, loss of the same neutral moiety, under collision-activated dissociation conditions. Q2 is employed as a collision chamber and both Q1 and Q3 are set to scan repetitively at a fixed mass separation over the desired mass range (Fig. 1). Mixture components are

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* Throughout this article, the American billion (10^9) is meant.

volatilized into the ion source and converted into ions characteristic of sample molecular weight under either positive or negative chemical ionization conditions. At any point in the scan of Q1, all ions of a particular *m/z* value will be transmitted to Q2. Only those ions that lose a particular neutral of specified mass will have the right *m/z* ratio for transmission through Q3. Nitroaromatic compounds, for example, form abundant $(M + H)^+$ ions under methane positive ion chemical ionization conditions and then suffer loss of 17 a.m.u. $OH^{\bullet}$, on collision-activated dissociation in Q2. Since loss of 17 a.m.u. is highly characteristic of nitroaromatic compounds, the neutral loss scan with Q1 and Q3 separated by 17 a.m.u. facilitates detection of these compounds and is transparent to most other matrix components. Fragment ions produced by loss of neutrals having masses other than 17 a.m.u. fail to pass through Q3 and are never detected. Spectra recorded in the neutral loss scan mode yield both the molecular weight and relative abundance of all members of a particular class of compounds in the matrix.

Recently this approach has been used for the analysis of heterocyclic organosulfur compounds in hydrocarbon matrices[8] and·carboxylic acids in urine[9]. Here, neutral loss scans are employed for the analysis of chlorocarbons, polycyclic aromatic hydrocarbons, amines and phenols.

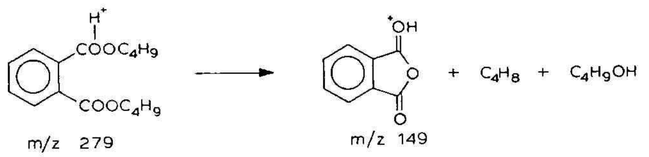
Analysis of phthalates, polycyclic aromatic hydrocarbons, chlorocarbons, phenols and amines

Solid matrices (5–10 mg) and residues from lyophilized aqueous samples are placed on or between glass-wool plugs in a 4.5-cm piece of 4 mm O.D. × 2.5 mm I.D. glass tubing. Amines and phenols in the matrix are converted into ureas and carbamates, respectively, by saturating the sample with 10 μl of ethyl acetate–methyl isocyanate (4:1) containing 100 ppm of triethylamine. After 5 min at room temperature, excess reagents are removed by purging the sample with a stream of nitrogen for 10–15 sec at a flow-rate of 20 ml/min. Polycyclic aromatic hydrocarbons are then converted into nitroaromatic compounds by exposing the sample matrix to gaseous dinitrogen tetroxide for 3–5 sec[10]. A stream of nitrogen is employed to remove excess reagent and the sample is then placed on the end of a modified solids probe and inserted into the removable ion source volume on the Finnigan triple stage quadrupole mass spectrometer. A mixture of methane and nitrous oxide sufficient to maintain a pressure of 500 microns in the ion source is employed as both the carrier gas and to generate the chemical ionization reactants, CH_5^+ and OH^-. Gas flows through the sample tube into the ion source at all times. Slow thermal volatilization of organics from the matrix is facilitated by heating the sample from 25 to 360°C over a period of 5–9 min. During this period the instrument, under computer control, is cycled repetitively through a series of six 0.5-sec experiments, one parent ion scan at *m/z* 149 for phthalates and five neutral loss scans for the other four classes of compounds studied. At the end of the heating period all data acquired from each type of scan is summed together and printed out in conventional bar graph format. The result is a plot of molecular weight *vs.* relative abundance for all mixture components containing the particular functional group being detected. Total time for sample derivatization, data acquisition, and data processing in the above procedure is typically 25 min.

RESULTS AND DISCUSSION

Success of the functional group analysis approach depends on the assumption that ions characteristic of the molecular weight of all members of a particular class of organic compounds will suffer collision-activated dissociation with high efficiency and either lose a highly characteristic neutral or form a highly characteristic charged fragment. Unfortunately this is not the case for several types of organic compound under the low-energy conditions employed in the collision cell of the triple quadrupole instrument (10–20 eV). Those compounds that fail to meet the above criteria must be derivatized in order to promote the desired behavior in the collision-activated dissociation process. Ideally the derivatization reaction should (a) employ volatile reagents that can be removed easily from the matrix, (b) proceed in high yield under mild conditions, and (c) introduce a functional group that is sufficiently basic or acidic to localize proton addition or abstraction at the site of the newly introduced substituent.

Phthalates



Collision-activated dissociation of $(M + H)^+$ ions from all phthalates except dimethylphthalate affords the protonated anhydride, *m/z* 149, in high yield. Since this fragment is highly characteristic of phthalate esters, members of this class of compounds can be monitored in mixtures using parent ion scans on the triple quadrupole instrument. Results from the analysis of an uncharacterized sample of industrial sludge are shown in Fig. 2. Signals at *m/z* 223, 279, 313, and 391 correspond to $(M + H)^+$ ions of diethyl-, dibutyl-, butylbenzyl-, and either di-2-ethylhexyl- or di-*n*-octylphthalate, respectively. Fragment ions in the main beam chemical ionization (CH_4) spectrum of these four phthalates at *m/z* 167, 177, and 205 are also observed in the parent ion scan because they too undergo further dissociation in the collision chamber to produce *m/z* 149. The signal at *m/z* 149 represents that fraction of the protonated anhydride species generated in the ion source that passes through the collision chamber intact. An unknown, perhaps the phthalate ester of propyleneglycol, ap-

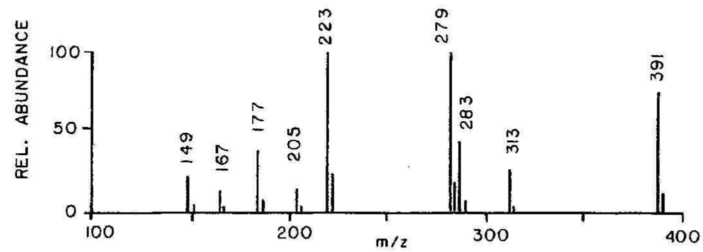
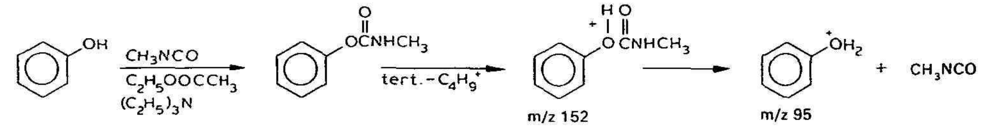


Fig. 2. Results of a *m/z* 149 parent ion scan for detection of phthalates in an uncharacterized sample of industrial sludge.

pears in the spectrum at *m/z* 283. Comparison of spectra recorded on spiked and unspiked samples indicated that the phthalates in this particular sludge matrix are present at the 1–5-ppm level.

Phenols



Collision-activated dissociation of either M^+ or $(M + H)^+$ ions from phenols is highly inefficient under the low-energy conditions employed in the triple quadrupole instrument. Accordingly, members of this class of compounds are derivatized prior to analysis. Treatment of the lyophilized sludge with methyl isocyanate converts phenols into carbamates. This derivative is highly basic and readily forms $(M + H)^+$ ions that in turn suffer facile dissociation with loss of methyl isocyanate (57 a.m.u.) in the collision cell. Analysis of phenols in mixtures is accomplished, therefore, using 57 a.m.u. neutral loss scans on the carbamate derivatives. Results obtained on sludge spiked at the 1-ppm level with seven phenols from the EPA priority pollutant list are shown in Fig. 3. The signals observed correspond to $(M + H)^+$ ions of the parent phenols since these are the ions transmitted through the second mass analyzer. Ions at *m/z* 95, 123, 129, 143, 163, 197, and 265 are assigned to phenol, dimethylphenol, chlorophenol, chloromethylphenol, dichlorophenol, trichlorophenol and pentachlorophenol, respectively. Phenols of unknown structure are also detected at *m/z* 134, 140, 157, 171, and 205.

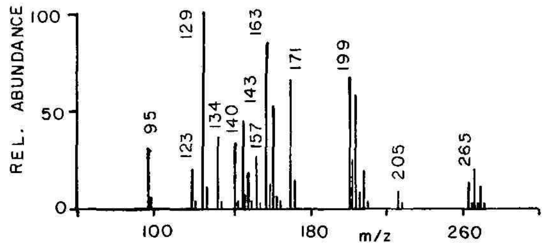


Fig. 3. Results of a 57 a.m.u. neutral loss scan for detection of phenols in an uncharacterized sample of industrial sludge spiked with seven phenols from the U.S. EPA priority pollutant list.

Polynuclear aromatic hydrocarbons

Like the situation with phenols, M^+ and $(M + H)^+$ ions from polynuclear aromatic hydrocarbons also dissociate with low efficiency during collision experiments conducted with ion energies in the range 10–20 eV. Derivatization of this class of compounds for analysis on the triple quadrupole is accomplished by exposing the sample matrix to gaseous N_2O_4 for 3–5 sec. Under these conditions mono- and/or dinitro derivatives are formed in good yield from all-polynuclear aromatic hydrocarbons on the EPA priority pollutant list. Carbamate derivatives of phenols on the same list are not nitrated in the above procedure.

Nitroaromatic compounds make excellent derivatives for analysis by the tandem mass spectrometry approach since the nitro group is highly basic and easily protonated when methane is used as the chemical ionization reagent gas. The resulting $(M + H)^+$ ions dissociate readily in the collision cell with loss of one or more of the neutrals, OH, NO, NO_2 and HNO_2 (Fig. 4). Loss of OH (17 a.m.u.), NO (30 a.m.u.), NO_2 (46 a.m.u.) and HNO_2 (47 a.m.u.) is highly characteristic of the nitro functional group and one or more of these pathways is observed for all of the polynuclear aromatic hydrocarbons on the priority pollutant list. Analysis of this class of compounds can be carried out, therefore, using neutral loss scans for one of the above losses. If it is necessary to distinguish between nitro polynuclear aromatic hydrocarbons already in the environmental matrix from those produced in the derivatization step, a second sample of the untreated matrix must be examined directly.

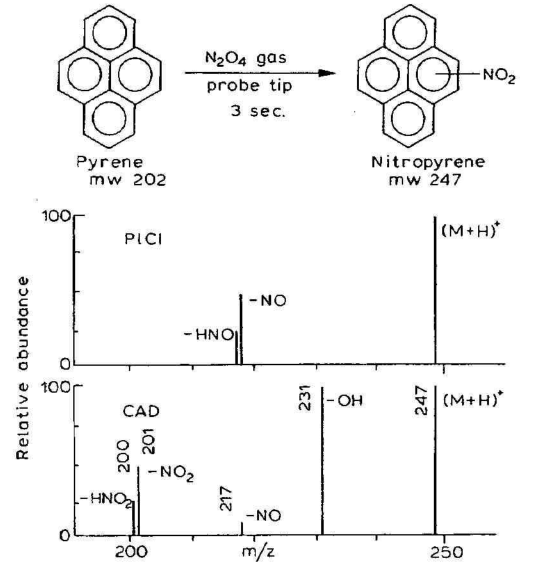


Fig. 4. (Top) Main beam positive-ion chemical ionization methane mass spectrum of nitropyrene. (Bottom) Collision-activated dissociation (CAD) mass spectrum of the $(M + H)^+$ ion from nitropyrene.

Results of 17 a.m.u. neutral loss scans on a sample of lyophilized industrial sludge spiked with a standard mixture of nine polynuclear aromatic hydrocarbons are presented in Fig. 5. Most of the ions in the spectrum occur at *m*/*z* values 16 a.m.u. $(M + H - OH)^+$ below the molecular weight of the nitro derivative and therefore 29 a.m.u. above the molecular weight of the parent aromatic hydrocarbon. Signals at even *m*/*z* values contain an even number of nitrogen atoms and are formed by loss of OH from either $(M + H)^+$ ions or $(M + H - NO)^+$ fragment ions in the main beam chemical ionization spectrum of the corresponding dinitro derivatives. Dinitration is observed for most polynuclear aromatic hydrocarbons and is particularly facile for fluorene, pyrene, fluoranthene and benzopyrene.

All components of the standard mixture are readily identified in Fig. 5. Signals at *m*/*z* 157, 181, 183, 195, 207, 231, 257 and 281 correspond to $(M + H - OH)^+$ fragments from mononitro derivatives of naphthalene, acenaphthalene, acenaphthene, fluorene, anthracene-phenanthrene, pyrene-fluoranthene, chrysene and

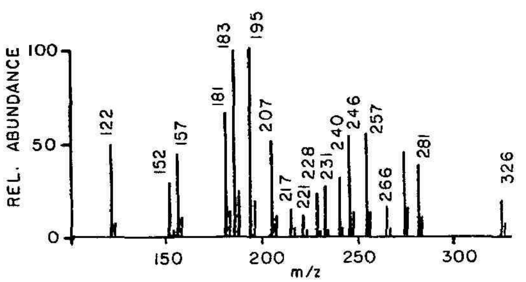


Fig. 5. Results of a 17 a.m.u. neutral loss scan for detection of nitro derivatives of polynuclear aromatic hydrocarbons in an uncharacterized sample of industrial sludge spiked with nine standards from the U.S. EPA priority pollutant list.

benzopyrene, respectively. Dinitro derivatives of fluorene, pyrene-fluoranthene, and benzopyrene give rise to the signals at *m*/*z* 240, 246–276, and 326, respectively. Signals due to unknowns at *m*/*z* 122, 152, 221–266, and 245 can be assigned to nitroaniline, dinitrobenzene, methylphenanthrene, and methylpyrene. Comparison of spectra obtained from spiked and unspiked samples indicates that acenaphthalene, fluorene, phenanthrene-anthracene, pyrene-fluoranthene, chrysene, and benzopyrene are all present in this particular sludge matrix at the 100–500-ppb level.

Amines

Derivatization of phenols with methyl isocyanate as described above also converts amines into N-methylureas. These latter derivatives form abundant $(M + H)^+$ and $(M - H)^-$ ions under chemical ionization conditions when CH_5^+ and OH^- are employed as the reactant ions. Pathways observed for collision-activated dissociation of both these types of ion are shown in Fig. 6. Elimination of the neutral, CH_3NCO (57 a.m.u.) is essentially the only pathway observed for dissociation of $(M + H)^+$ and $(M - H)^-$ ions from primary and secondary amines in which the nitrogen atom is attached directly to an aromatic ring. Loss of CH_3NCO from the $(M - H)^-$ ion is also a major pathway for collision-activated dissociation of aliphatic secondary amines. For urea derivatives of primary amines, formation of the $NHCONHCH_3$ anion at *m*/*z* 73 competes favorably with elimination of CH_3NCO from the $(M - H)^-$ ion since the latter pathway is considerably more endothermic when the product ion is stabilized by one rather than two alkyl groups.

In the positive-ion mode, loss of olefin rather than CH_3NCO frequently dominates the dissociation process for $(M + H)^+$ ions of secondary amines. Elimination of CH_3NCO is the preferred pathway for dissociation of $(M + H)^+$ ions derived from primary amine derivatives.

Based on a study of more than 100 compounds, we conclude that most amines can be analyzed effectively under tandem mass spectrometry conditions using 57 a.m.u. neutral loss scans to monitor collision-activated dissociation of $(M - H)^-$ ions from N-methyl urea derivatives. Results of such an experiment on a standard mixture of amine derivatives are shown in Fig. 7. Spectra from neutral loss scans of 57 a.m.u. in both the positive- and negative-ion modes are displayed. The ions that exit the collision cell and reach the detector correspond to the $(M + H)^+$ and $(M - H)^-$ species from the underivatized amines. Carbamate derivatives of the phenols ex-

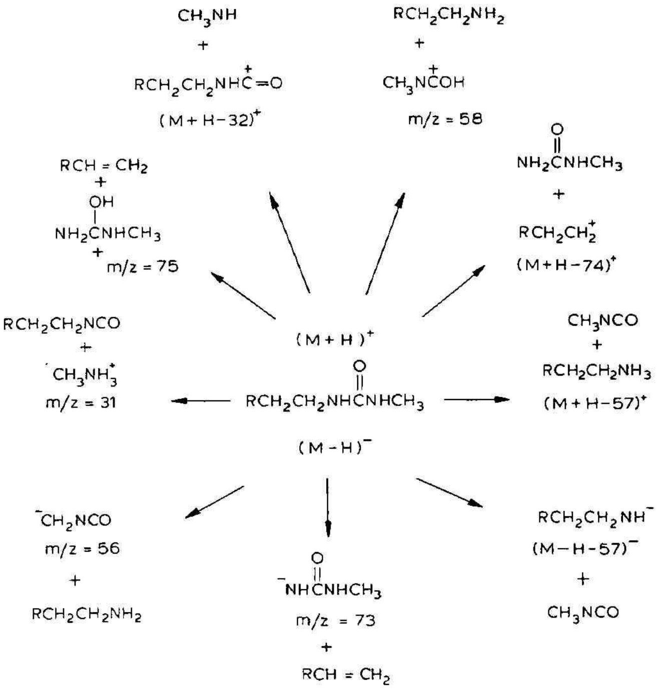


Fig. 6. Pathways observed for fragmentation of $(M + H)^+$ and $(M - H)^-$ ions from N-methylurea derivatives of amines under collision-activated dissociation conditions on the triple quadrupole mass spectrometer.

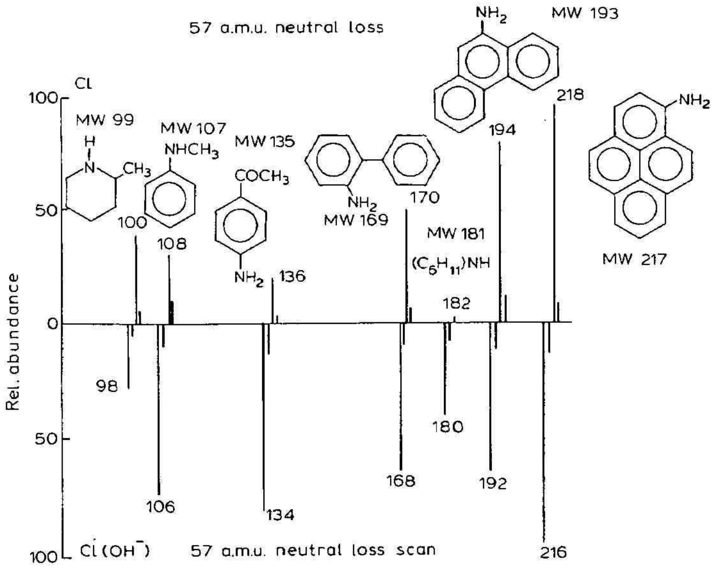


Fig. 7. Results of a 57 a.m.u. neutral loss scans in both the positive (top) and negative (bottom) mode on a standard mixture of N-methylurea derivatives of amines.

amined to date dissociate completely with loss of CH_3NCO (57 a.m.u.) in the ion source rather than in the collision cell and therefore, do not interfere with the above analysis for amines using negative ions. Signals due to both phenols and amines appear on the positive-ion 57 a.m.u. neutral loss scan, but only the amine affords signals on the negative-ion trace at a position 2 a.m.u. lower than the corresponding signal on the positive-ion trace.

Chlorocarbons

The EPA priority pollutant list contains *ca.* 40 chlorocarbon compounds and/or preparations. We find that these can be analyzed under positive-ion chemical

TABLE I

CHLORINE-CONTAINING COMPOUNDS ANALYZED BY NEUTRAL LOSS SCANS OF 35 a.m.u. AND 36 a.m.u., RESPECTIVELY

Compound	*Neutral loss scan*	
	35 a.m.u.	*36 a.m.u.*
Hexachlorobenzene	Yes	No
Hexachlorobutadiene	Yes	No
Hexachloroethane	Yes	No
Heptachlor	Yes	Yes
Chloronaphthalene	Yes	Yes
3,3′-Dichlorobenzidine	Yes	Yes
1,2,4-Trichlorobenzene	Yes	Yes
Arochlor 1242, 1254	Yes	Yes
2-Chlorophenol	Yes	Yes
4-Chloro-3-methylphenol	Yes	Yes
2,4-Dichlorophenol	Yes	Yes
2,4,6-Trichlorophenol	Yes	Yes
Pentachlorophenol	Yes	Yes
DDT	Yes	Yes
DDE	Yes	Yes
DDD	Yes	Yes
Chlorobenzene	No	Yes
1,2-Dichlorobenzene	No	Yes
1,3-Dichlorobenzene	No	Yes
1,4-Dichlorobenzene	No	Yes
trans-1,2-Dichloroethylene	No	Yes
1,1-Dichloroethylene	No	Yes
Trichloroethylene	No	Yes
Tetrachloroethylene	No	Yes
1,1-Dichloroethane	No	Yes
1,2-Dichloroethane	No	Yes
1,1,2-Trichloroethane	No	Yes
1,1,1-Trichloroethane	No	Yes
1,1,2,2-Tetrachloroethane	No	Yes
1,2-Dichloropropane	No	Yes
Chlordane	Yes	Yes
Aldrin	Yes	Yes
Endrin	Yes	Yes
Dieldrin	Yes	Yes
Heptachlorepoxide	Yes	Yes

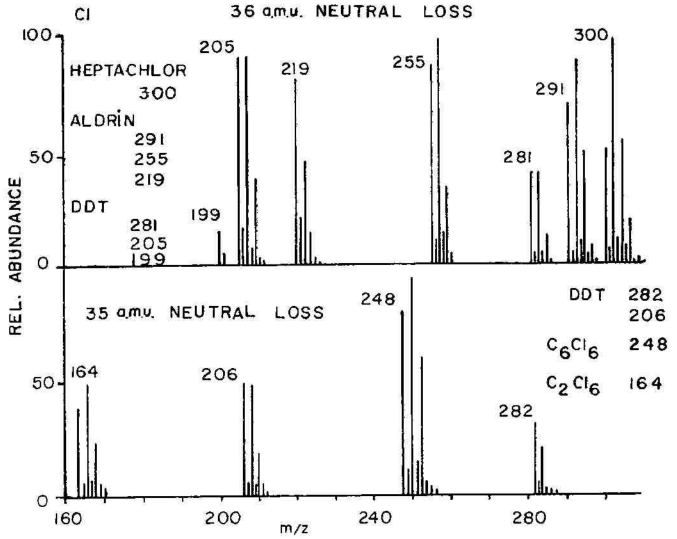


Fig. 8. Results of 36 a.m.u. and 35 a.m.u. neutral loss scans on standard mixtures of chlorocarbons containing heptachlor, aldrin, and DDT in one case and DDT, hexachlorobenzene, and hexachloroethane in the other.

ionization conditions using neutral loss scans that monitor for the loss of either Cl (35 a.m.u.) or HCl (36 a.m.u.) (Table I). Results obtained using 35 and 36 a.m.u. neutral loss scans to analyze a standard mixture of halogenated compounds are presented in Fig. 8. In the 36 a.m.u. neutral loss spectrum, ions due to aldrin appear at *m/z* 291, 255, and 219. Loss of HCl during collision-activated dissociation of the $(M + H - HCl)^+$, $(M + H - 2HCl)^+$, and $(M + H - 3HCl)^+$ ions in the main beam spectrum account for the observed pattern of signals.

The main beam chemical ionization spectrum of heptachlor is considerably less

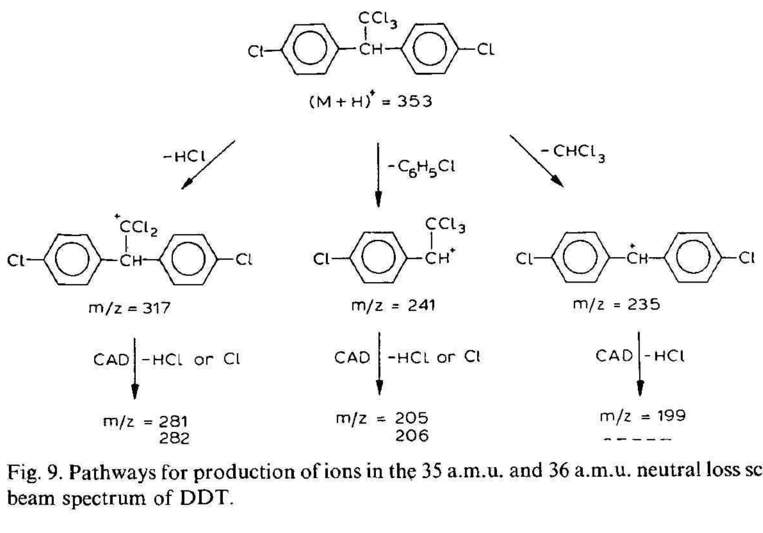


Fig. 9. Pathways for production of ions in the 35 a.m.u. and 36 a.m.u. neutral loss scans on ions in the main beam spectrum of DDT.

complicated and accordingly only one group of peaks appears in the 36 a.m.u. neutral loss scan. $(M + H - HCl)^+$ dissociates to $(M + H - 2HCl)^+$. The three ions observed in the DDT spectrum result from loss of HCl from the $(M + H - HCl)^+$, $(M + H - C_6H_5Cl)^+$ and $(M + H - CHCl_3)^+$ ions in the main beam spectrum.

DDT is one of the compounds in Table I that can be monitored by either 35 or 36 a.m.u. neutral loss scans (Fig. 9). The two signals at *m/z* 282 and 206 in Fig. 8 result from loss of Cl from the $(M + H - HCl)^+$ and $(M + H - C_6H_5Cl)^+$ ions and therefore appear at a position 1 a.m.u. higher than the DDT signals in the top half of Fig. 8. Ions characteristic of hexachlorobenzene and hexachloroethane in Fig. 8 are found at *m/z* 248 and 264 and are generated by loss of Cl from the $(M + H)^+$ and $(M + H - HCl)^+$ ions, respectively, in the main beam spectrum of the sample mixture.

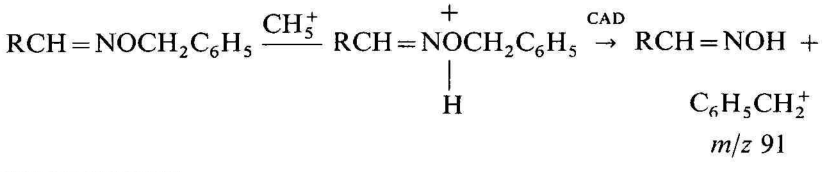
Carboxylic acids

Negative-ion collision-activated dissociation mass spectra have been recorded for more than 90 carboxylic acids. Abundant fragments corresponding to loss of CO_2 (44 a.m.u.) from the $(M - H)^-$ ion are observed for most of these acids. Hydroxy acids and dicarboxyl acids lose both CO_2 and H_2O to form $(M - H - 62)^-$ ions. The utility of 44 and 62 a.m.u. neutral loss scans for monitoring carboxylic acids in urine has already been demonstrated[9]. Experiments to evaluate the procedure on environmental matrices are in progress.

Aldehydes and ketones

One approach to the analysis of aldehydes and ketones by triple quadrupole mass spectrometry is outlined below. Treatment of aldehydes and ketones with benzyloxylamine affords derivatives whose $(M + H)^+$ ions readily dissociate to the benzyl cation (*m/z* 91) under collision-activated dissociation conditions. Formation of *m/z* 91 is highly characteristic of the benzyloxylamine derivatives but is also observed in the mass spectra of many other molecules containing the benzyl moiety. An increase in the specificity of the above reaction pathway can be achieved by using either $[^2H_5]$benzyloxylamine or pentafluorobenzyloxylamine in the derivatization step. This would shift the mass of the benzyl cation from *m/z* 91 to *m/z* 96 and *m/z* 181, respectively, and remove any ambiguity about the origin of the characteristic fragment. Use of *m/z* 96 or *m/z* 181 parent ion scans on benzyloxylamine derivatives should facilitate the analysis of aldehydes and ketones in a variety of environmental matrices.

$$RCHO + C_6H_5CH_2ONH_2 \rightarrow RCH{=}NOCH_2C_6H_5$$



$$RCH{=}NOCH_2C_6H_5 \xrightarrow{CH_5^+} RCH{=}\underset{H}{\overset{+}{N}}OCH_2C_6H_5 \xrightarrow{CAD} RCH{=}NOH + \underset{m/z\ 91}{C_6H_5CH_2^+}$$

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DETERMINATION OF DRUGS IN BIOLOGICAL SAMPLES BY THIN-LAYER CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

JACK HENION* and GEORGE A. MAYLIN
New York State College of Veterinary Medicine, Cornell University, Ithaca, NY 14853 (U.S.A.)
and
BRUCE A. THOMSON
Sciex, 55 Glen Cameron Road, Unit 202, Thornhill, Ontario L3T 1P2 (Canada)

SUMMARY

In this work thin-layer chromatography–tandem mass spectrometry (TLC–MS–MS) allowed detection and confirmation of caffeine and nicotine in human urine and of butorphanol, betamethasone, and clenbuterol in equine urine. In most cases of trace analysis of labile compounds the drugs could not be identified unless they were developed on a TLC plate, scraped from the plate and the TLC scrape eluted with a suitable organic solvent prior to MS–MS. Usually a sample prepared in this way still had several components in it, but was sufficiently cleaned up to allow collision-induced dissociation (CID) experiments to unequivocally identify the drug. In contrast, trace levels of labile drugs could not be identified by CID experiments either directly from the raw urine extracts or by thermally desorbing them from the TLC scrape.

INTRODUCTION

Tandem mass spectrometry (MS–MS) has been shown to be a highly specific and sensitive technique in targeted compound analysis[1–3]. The ability to analyze relatively complex samples for trace species offers real savings in both manpower and time over more conventional techniques of gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), and liquid chromatography–mass spectrometry (LC–MS). Examples have been published showing that direct probe analysis with an MS–MS instrument could allow many samples to be rapidly screened for the presence of specific compounds or compound classes with confirmation of the few positive samples by GC–MS or GC–MS–MS[2,3]. There are other situations, however, in which it would be more efficient to screen the high volume of samples with an inexpensive technique and use the high specificity of MS–MS to provide confirmation of those samples believed to contain drug related substances. In a previous paper we reported on the use of LC–MS–MS for the detection of administered drugs in equine plasma and urine extracts[4]. This earlier work demonstrated that chromatographic separation and on-line MS–MS analysis could provide clean collision-induced-dis-

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sociation (CID) mass spectra "unequivocally" identifying the compound in question, even in the presence of high levels of co-eluting material which might otherwise constitute an interference. We are now investigating the use of MS–MS for the confirmation of drugs in plasma and urine samples which have been screened by thin-layer chromatography (TLC). The goal of the work is to explore whether this combination can provide a rapid assay capable of detecting and identifying the presence of administered drugs in urine and blood with a high degree of confidence. This is of current interest to drug-testing laboratories as well as the clinical chemistry community.

Various embodiments of the combination of TLC and MS–MS can be envisaged. In all cases, it is probably most efficient to use TLC alone to screen all of the samples (raw extracts), and proceed with MS–MS analysis only if a foreign substance is observed. It is desirable, once a positive is observed by TLC, to perform the analysis as quickly and as simply as possible. This might be done by introducing the sample into the ion source directly at any of several stages of cleanup ranging from the raw urine to the eluted TLC scrape. While the direct analysis of raw urine would be attractive from the point of view of simplicity, its success is unlikely in most situations due to low solute levels, the presence of the compound in conjugated form and high levels of other endogenous material. The remaining options available are direct analysis of raw urine extract, direct thermal desorption of sample from the TLC silica, or elution of the TLC spot with an organic solvent followed by direct analysis of the extract. In each case the intact molecules must be vaporized and ionized, and the MS–MS must provide clean CID spectra of the foreign substances in order to allow identification. Each type of sample analysis has advantages. In this paper results are presented from a study in which a systematic comparison of each method was made for four different administered drugs. Samples were screened by TLC and introduced at each stage of work-up by direct insertion probe into an atmospheric pressure ionization (API) source coupled to a triple quadrupole MS–MS instrument.

EXPERIMENTAL

Materials

Authentic standards of each drug were obtained from commercially available sources. Caffeine was purchased from Eastman (Rochester, NY, U.S.A.) and used without further purification. Butorphanol was isolated from injectable Stadol tartrate (Bristol Labs., Syracuse, NY, U.S.A.) by adjusting the pH to 10 and extraction (twice) with light petroleum. Clenbuterol was isolated from NAB 365-CL (Boehringer Ingelheim, Ridgefield, CT, U.S.A.) by adjusting the pH to 12 and extraction twice with light petroleum (b.p. 30–60°C). Preparative TLC using silica gel 60 G_{254} TLC plates (E. Merck, Darmstadt, G.F.R.) produced a material that produced one spot. Authentic betamethasone was obtained from Steraloids (Wilton, NJ, U.S.A.) and used without further purification.

The TLC plates were stored in a desiccator and used without prior activation. Sample application to the TLC plates was accomplished utilizing disposable home-made glass capillary applicators which had been drawn out in a flame. The applied sample was restricted to a spot with a maximum diameter of 2 mm by directing a gentle stream of warm air at the point of sample application. Development of the

TABLE I

TLC ANALYSIS OF THE DRUGS STUDIED

Solvent systems: A = ethyl acetate–methanol–ammonium hydroxide (85:10:5); B = chloroform–methanol–propionic acid (72:18:10); C = ethyl acetate–acetic acid (39:1); D = ethyl acetate–methanol–ammonium hydroxide (85:10:65).

Compound	*Solvent system*	*Visualization*	R_F
Caffeine	A	quench	0.62
Butorphanol	B	quench, Dragendorff	0.36
Betamethasone	C	quench	0.70
Clenbuterol	D	quench	0.71

respective drugs was conducted in a covered, pre-equilibrated glass developing tank containing a developing solvent mixture best suited for optimum chromatographic separation for the drug from interfering endogenous substances. Visualization of the components of interest was accomplished either under UV light or by an appropriate chromogenic spray reagent. Table I summarizes the pertinent details of the TLC analysis of the four drugs under investigation in this study.

Preparative TLC involved the selective removal of the distinct, intact "spot" of each drug in question at its corresponding R_F value. This differs from conventional preparative TLC wherein an entire streaked band of the drug may be scraped from the R_F region corresponding to that known for the substance in question. The latter technique appears less desirable due to poorer resolution from potential overloading of the plate. In addition, excessive dissolved silica interferes when the chemical substance is eluted from the silica scrape in a subsequent step. We prefer the careful application of the biological extract at the origin as concise spots[5] which are scraped as a spot after development.

Spot removal is accomplished with a glass micro preparative TLC probe fabricated by drawing out the large diameter end of a 15-cm Pasteur pipette so it is symmetrical about the center. Porous polypropylene plugs were cut from No. W-11 polypropylene wicks (Schleicher and Schuell, Keene, NH, U.S.A.) and gently tapped into the Pasteur pipette constriction prior to drawing out the opposite end. This device was used to scrape off gently the TLC spot of interest. A vacuum created at the opposite end of this micro preparative TLC probe sucks the silica particles from the TLC plate into the central, larger diameter region of this device. When the spot has been removed from the TLC plate the micro preparative TLC probe may be charged with a suitable solvent to elute the preparatively collected organic substance from the silica for subsequent analysis. This technique is particularly well suited for recovering trace levels of drugs which may be susceptible to adsorptive losses[6].

The disposition of each drug into the urine resulted from normal excretion of administered drugs. Caffeine was extracted from the urine of a human volunteer who is a moderate coffee drinker and cigarette smoker. Butorphanol was recovered from equine urine subsequent to the administration of Stadol to a standardbred horse, betamethasone was isolated from equine urine subsequent to the administration of betamethasone and clenbuterol was isolated from equine urine subsequent to the administration of NAB-365CL. In the latter three instances control urines were collected prior to the administration of the respective drugs and analyzed in parallel with the post administration samples.

Equipment

The mass spectrometer used in this study was a TAGA® 6000 triple quadrupole mass spectrometer system equipped with an API source[7]. All samples were introduced into the API source on glass surfaces in a direct insertion probe. In the case of liquid extracts, 2-μl aliquots were carefully deposited from a syringe onto a glass surface in which a small heating coil is inbedded. The silica was introduced by placing a few of the particles in a 2-mm glass tube with a closed end, and heating this tube in the direct insertion probe using another type plug-in heating tip. The probe was situated in a stream of carrier gas (Zero Air, Specialty Gases, Toronto, Canada) which flows into the ion source at 3 l/min. After the few seconds required for the solvent to evaporate, the heating current was slowly raised until a stable ion signal from the compound was obtained. Full-scan mass spectra and CID spectra of the parent MH^+ ions were obtained [except for betamethasone, where the CID spectrum of the $(MH^+ - 60)^+$ was obtained]. Each sample was cleaned from the probe by raising the current to bake the probe for a few seconds. No memory or sample carryover was observed, and each analysis required approximately 2 min.

RESULTS

The choice of the drugs studied in this work, caffeine, butorphanol, betamethasone and clenbuterol, was dictated by a combination of their relative importance in horse racing and the degree of difficulty in their detection and confirmation by conventional methods. In particular, the recovery of caffeine by preparative TLC can be very low especially at trace levels[8]. Although caffeine can be determined by GC–MS, it exhibits considerable chromatographic peak tailing and is interfered with by endogenous components. Butorphanol may also be determined by GC–MS if a suitable derivative is prepared, but its low urinary levels and thermal instability causes difficulties with its GC–MS determination. The confirmation of betamethasone in equine urine is difficult because it too must be detected at low ng/ml levels and distinguished from many endogenous steroids and other interfering substances. It is not easily amenable to GC–MS at the low levels found in equine urine. Clenbuterol is a potent respiratory stimulant that is administered at approximately 200-μg doses to horses so it is present in biological fluids at extremely low levels. Although clenbuterol may be determined by GC–MS, the extensive sample cleanup necessary to provide good GC–MS results often results in unavoidable sample loss such that GC–MS detection limits are not adequate for the trace amount of material available. Thus, reduced sample handling and direct insertion of the sample into the MS should preclude some of these difficulties.

Caffeine

The API mass spectrum of authentic caffeine is shown in Fig. 1A. The protonated molecular ion at *m/z* 195 is the base peak in this spectrum with only minor fragment ions at lower mass. This is a desirable situation for MS–MS as reported by

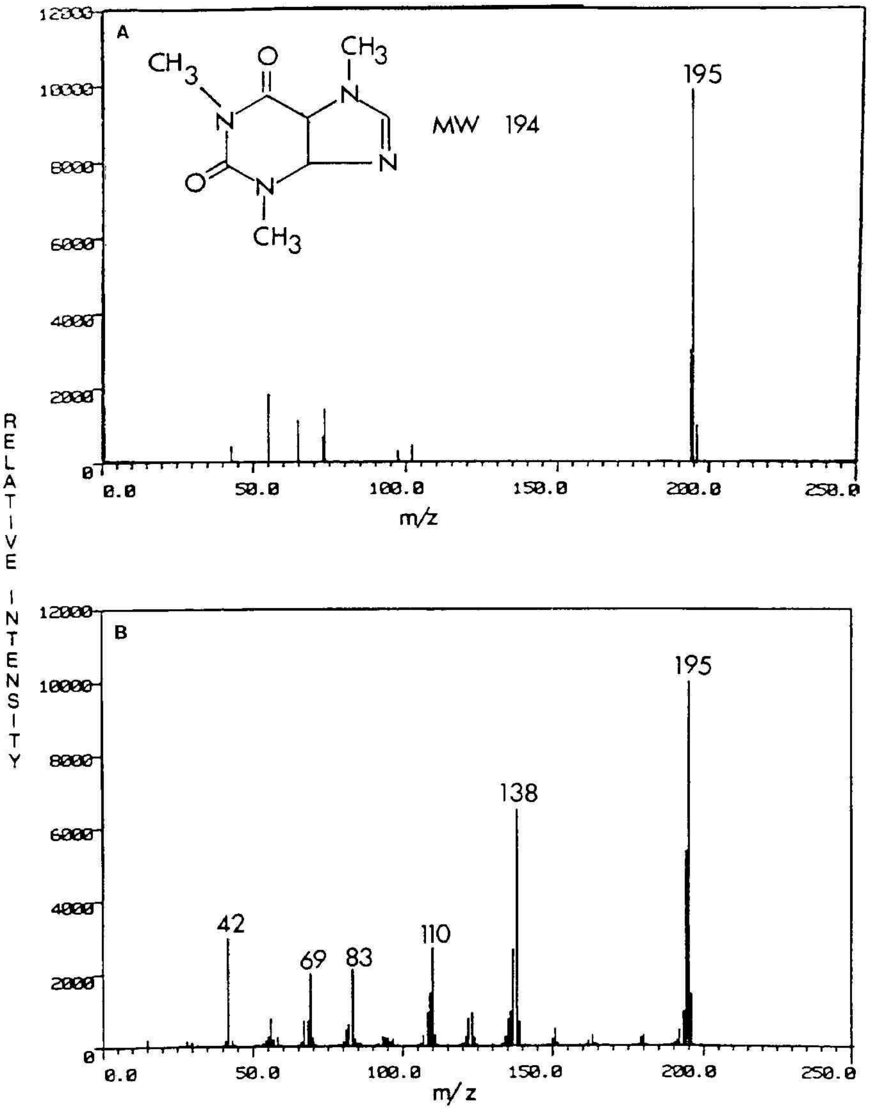

Fig. 1. (A) Full-scan direct insertion probe API mass spectrum of standard caffeine. (B) API CID mass spectrum of standard caffeine (M + 1)$^+$ ion (*m*/*z* 195).

others since it provides a unique ion characteristic of the molecular weight of the compound, which may be dissociated by collision with neutral gas molecules thus providing a CID mass spectrum of the (M + 1)$^+$ ion. Fig. 1B shows the CID mass spectrum for the (M + 1)$^+$ ion of caffeine using argon collision gas with a laboratory collision energy of 44 V. The daughter ion spectrum under these conditions includes abundant fragment ions at *m*/*z* 138, 110, 83, 69, 56 and 42 characteristic of the structure of caffeine.

The full-scan API mass spectrum of a 2-μl aliquot of a human urine extract (no

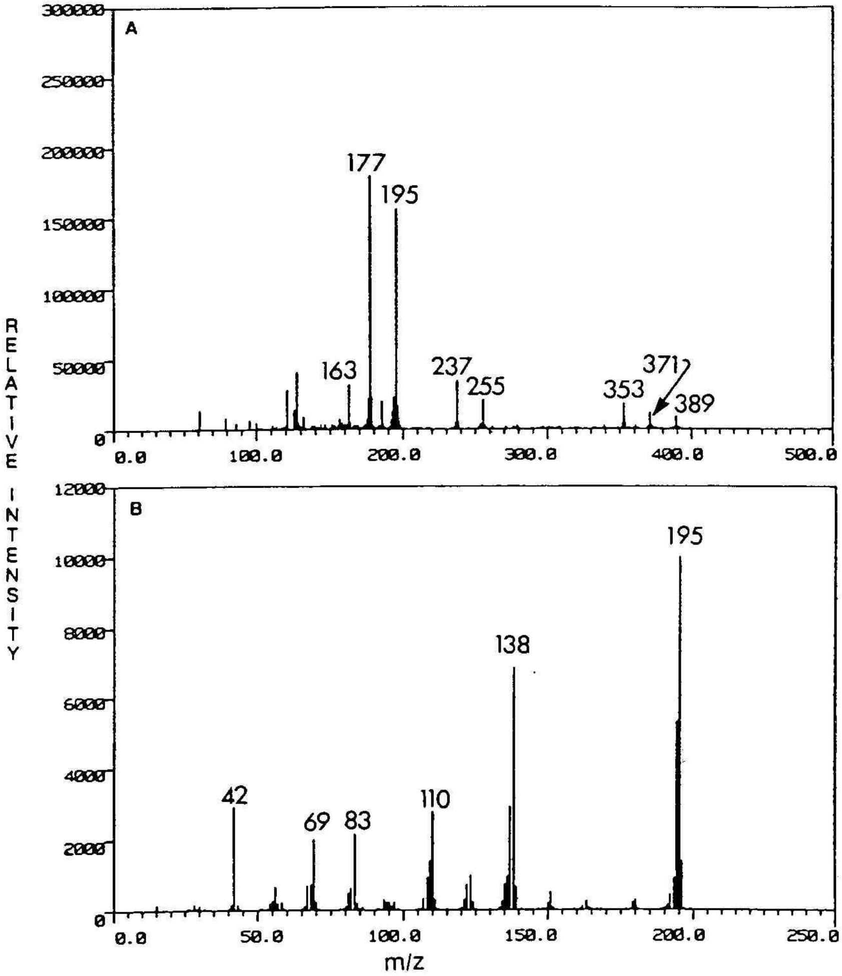

Fig. 2. (A) Full-scan direct insertion probe API mass spectrum of an organic solvent extract of human urine obtained from a person who was known to drink coffee and smoke cigarettes. (B) API CID mass spectrum of the human raw urine extract *m/z* 195 ion observed in A.

TLC clean up) is shown in Fig. 2A. There are several abundant ions in the molecular weight region of caffeine in addition to ions at *m/z* 237, 255, 353, 371 and 389. Clearly this is a "mixed" API mass spectrum, presumably indicating the protonated molecular ions of numerous organic components in this raw urine extract. However, if either TLC screening results or other information has suggested that caffeine may be in this urine extract, one can perform CID experiments on the $(M + 1)^+$ ion, or *m/z* 195, observed in Fig. 2A. This experiment provides the CID spectrum shown in Fig. 2B which compares very well with that for standard caffeine shown in Fig. 1B and verifies the identity of caffeine in the human urine extract.

The effects of some sample clean up by preparative TLC are shown in Fig. 3A. The components observed at higher masses in the full-scan API mass spectrum of the

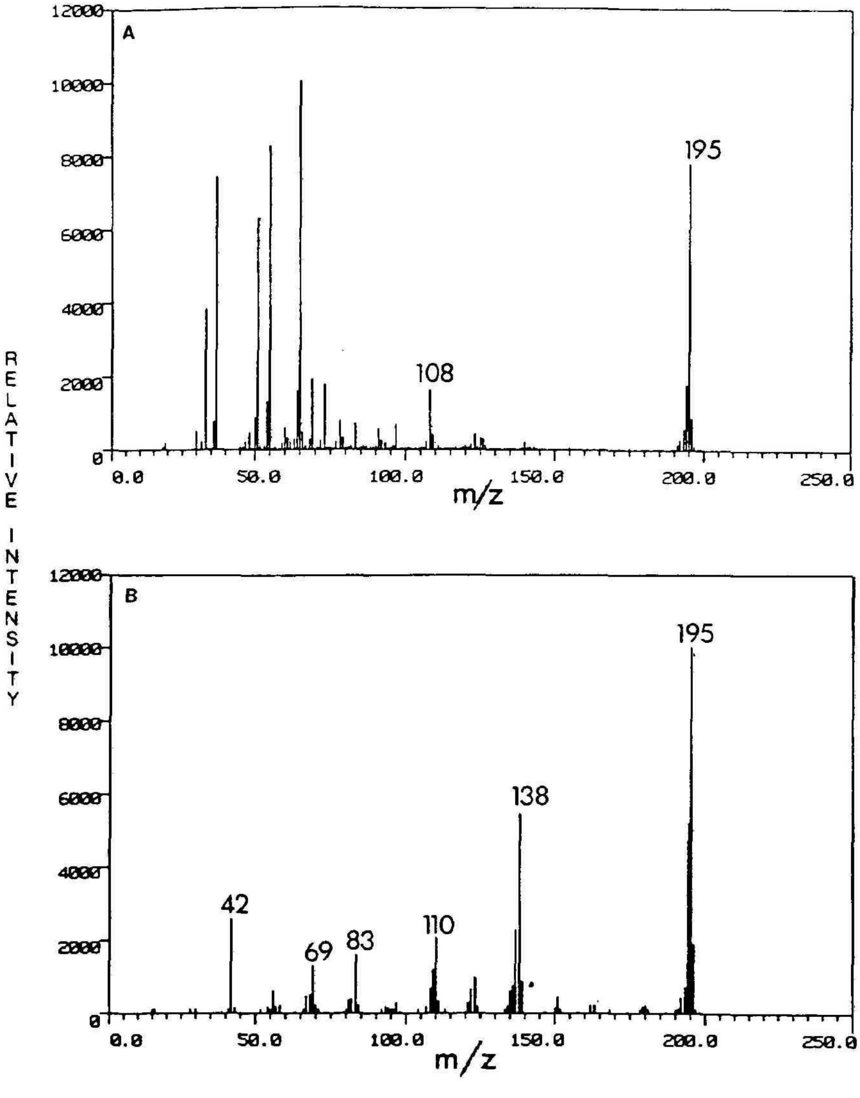

Fig. 3. (A) Full-scan direct insertion probe API mass spectrum of an eluted TLC scrape obtained from the human raw urine extract described in Fig. 2A. (B) API CID mass spectrum of the *m/z* 195 ion observed in A.

raw urine extract are not observed when a TLC spot of the same R_F as standard caffeine is analyzed. The full-scan API results on such an "eluted TLC scrape" are shown in Fig. 3A. An abundant ion at *m/z* 195 is observed in addition to numerous lower mass ions which are probably $(M + 1)^+$ ions of other components present in this sample. This "mixed" mass spectrum is not sufficient for identifying caffeine in this instance so CID was carried out on the *m/z* 195 ion for caffeine whose presence had been suggested by TLC screening results. The CID mass spectrum for the *m/z* 195 ion observed in Fig. 3A is shown in Fig. 3B. This mass spectrum is identical to that

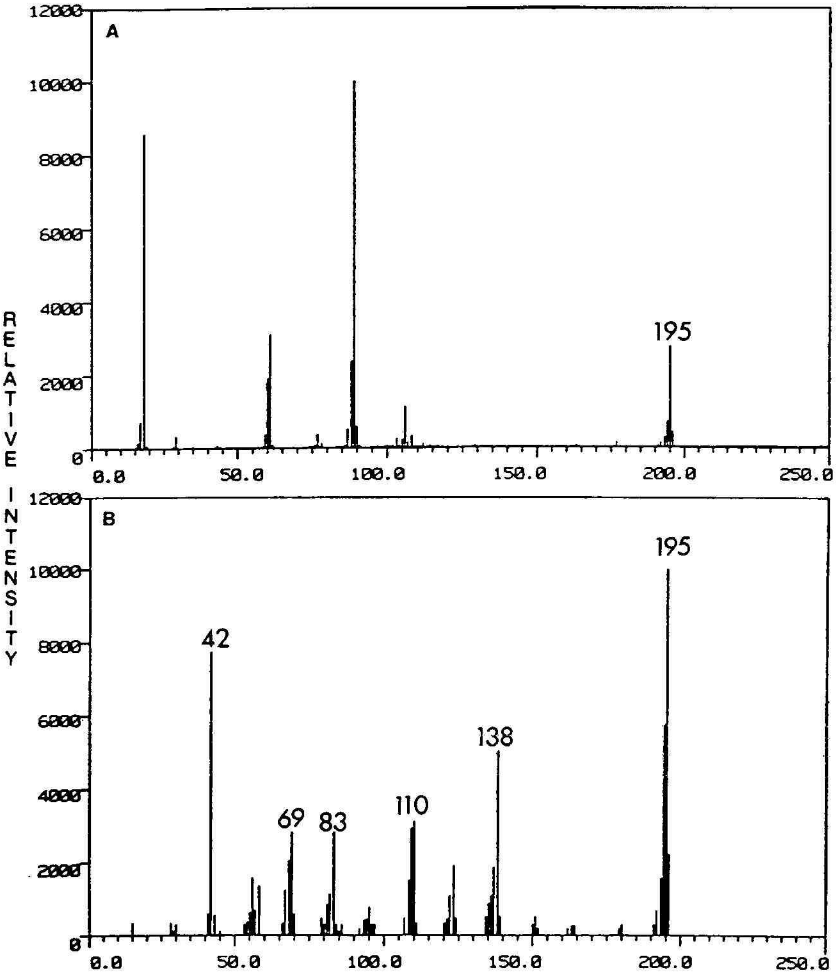

Fig. 4. (A) Full-scan direct insertion probe API mass spectrum of the TLC scraped spot not eluted with solvent. The silica particles from the scrape were placed directly on the direct insertion probe and the sample thermally desorbed. (B) API CID mass spectrum of the *m/z* 195 ion observed in A.

obtained similarly for standard caffeine (Fig. 1B) and thus identifies caffeine in this eluted TLC scrape.

The direct thermal desorption of organic substances from silica isolated from a TLC scrape can be accomplished[9]. This technique does not require elution of a drug, for example, from the silica surface subsequent to the preparative TLC step. The problem, however, is that certain substances that are thermally labile and present at very low levels may be thermally degraded by heating them on the silica surface. This technique, therefore, is useful only when the organic substance is present at high levels and/or is not thermally labile.

Fig. 4A shows the full-scan API mass spectrum of a TLC scraped spot which had been observed at an R_F identical to that of standard caffeine. The silica recovered from the surface of the TLC plate was placed directly on the probe tip, inserted into

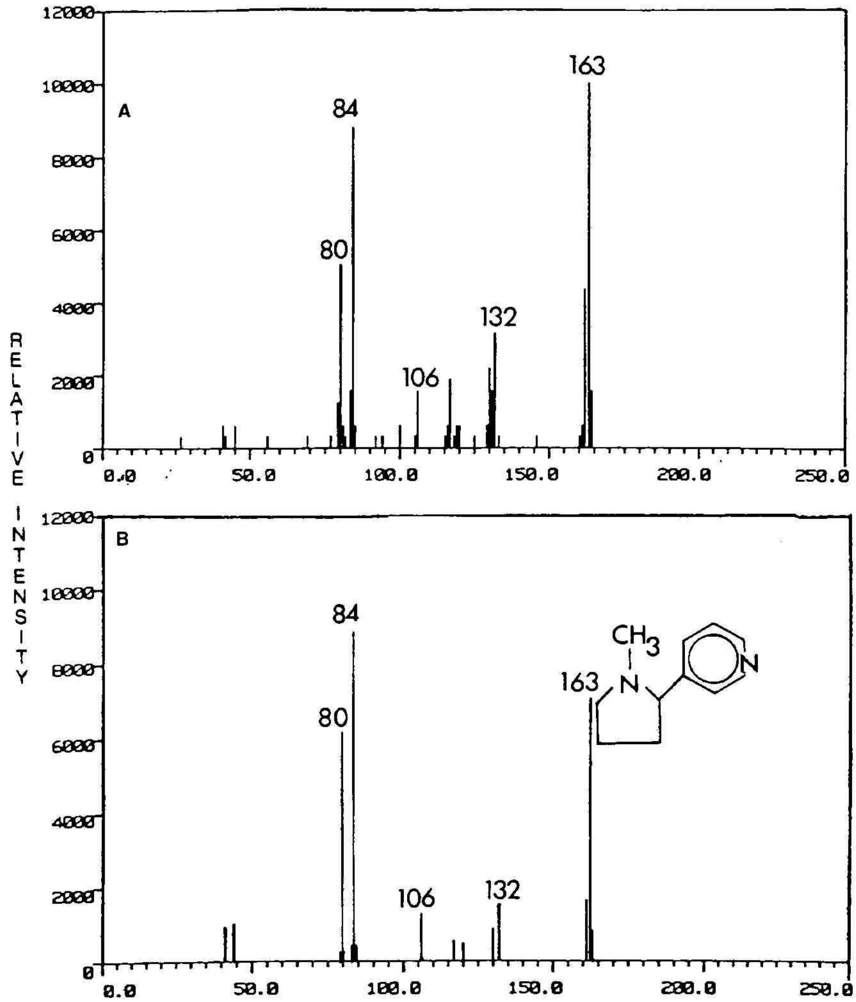

Fig. 5. (A) API CID mass spectrum of the *m*/*z* 163 ion observed in Fig. 2A. (B) API CID mass spectrum of standard nicotine $(M + 1)^+$ ion (*m*/*z* 163).

the API source and heated to desorb any volatile substances from the surface of the silica. Fig. 4A shows several abundant ions, including *m*/*z* 195. It is interesting to note, however, that there is little similarity between the mass spectra shown in Figs. 3A and 4A although they are both derived from preparative TLC scrapes of the same sample. The differences observed are presumably due to the different effects caused by eluting the sample from the silica *versus* thermally desorbing the sample from the silica. The CID mass spectrum shown in Fig. 4B, however, is the same as that observed for standard caffeine and thus demonstrates that caffeine at these rather high levels can be thermally desorbed from the TLC scrape and identified by TLC–MS–MS.

The *m*/*z* 163 ion observed in the full-scan API mass spectrum of the human urine raw extract shown in Fig. 2A is also of possible interest. The TLC screening re-

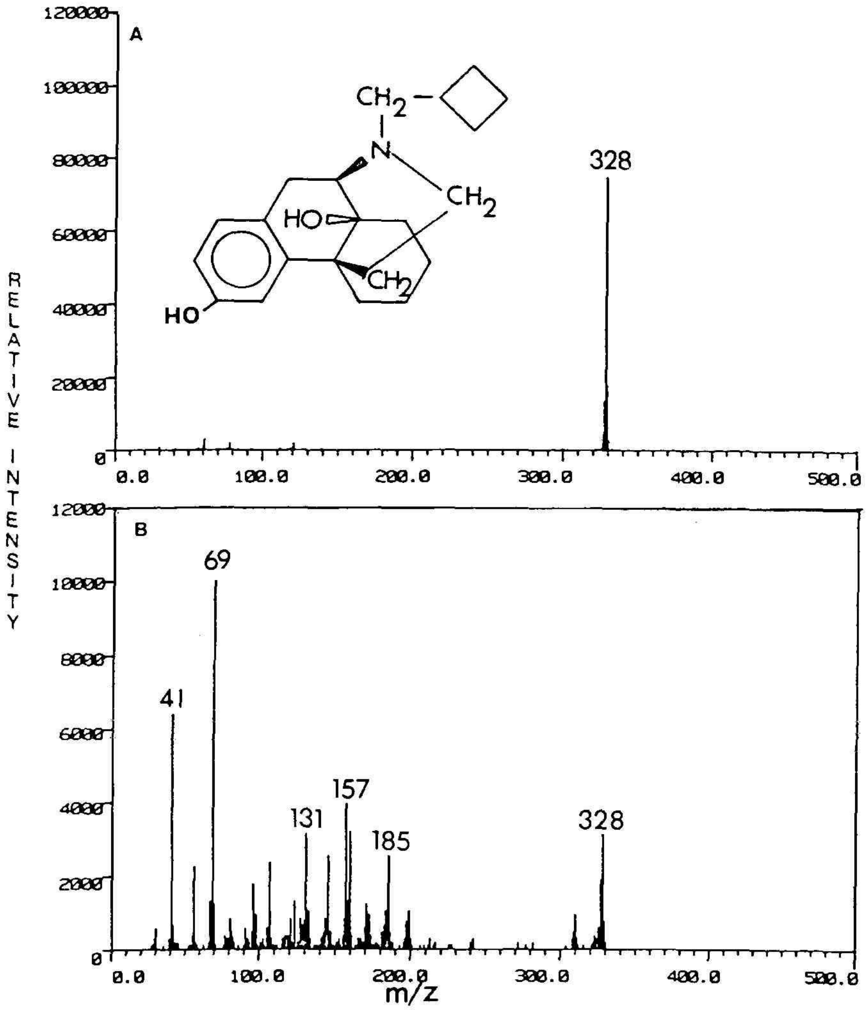

Fig. 6. (A) Full-scan direct insertion probe API mass spectrum of standard butorphanol. (B) API CID mass spectrum of standard butorphanol $(M + 1)^+$ ion (m/z 328).

sults described above suggested the presence of nicotine in this sample so a CID experiment was conducted on the m/z 163 ion. This produced the CID mass spectrum shown in Fig. 5A which compares favorably with that of a nicotine standard shown in Fig. 5B. Thus the combination of TLC screening followed by MS–MS allows the rapid identification of organic substances in a raw organic extract of urine when the concentrations of these substances are rather high.

In this human urine example caffeine and nicotine were identified in the raw extract of the urine. Although many potentially interfering substances were present, CID on the $(M + 1)^+$ ions of each component gave satisfactory results from the raw extract, the eluted TLC scrape, and the TLC scrape. Clearly one does not have to bother with the latter two experiments if a high level of a targeted compound is present. However, as we shall see in later examples, one does not always enjoy this luxury.

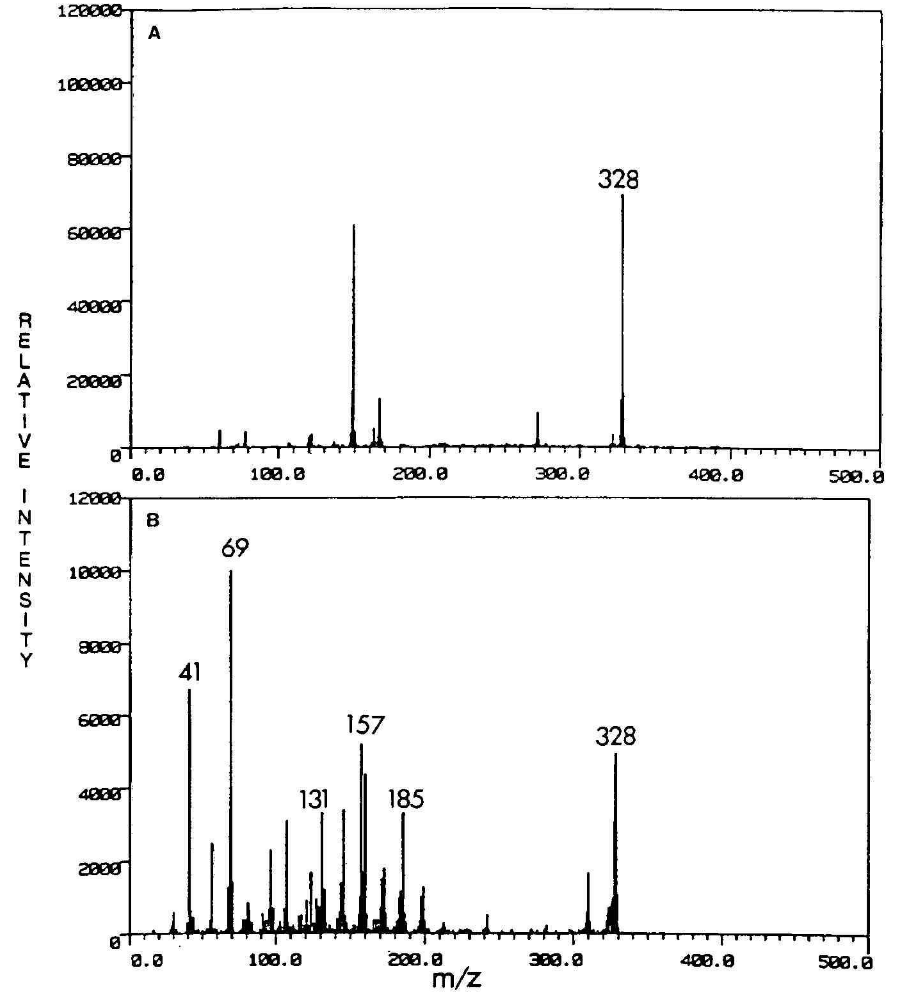

Fig. 7. (A) Full-scan direct insertion probe API mass spectrum of an eluted TLC scrape obtained from an organic extract of an equine urine subsequent to the administration of butorphanol. (B) API CID mass spectrum of the *m/z* 328 ion observed in A.

Butorphanol

The full-scan API mass spectrum from a standard sample of the analgesic, butorphanol, shows only the protonated molecular ion at *m/z* 328 in Fig. 6A. This extremely simple mass spectrum easily verifies the molecular weight of the drug, but provides no structural specificity that is essential for "unequivocal" identification. However, the CID mass spectrum of the *m/z* 328 ion of standard butorphanol shown in Fig. 6B provides a wealth of fragment ions. The base peak at *m/z* 69 is accompanied by other abundant ions which include *m/z* 328, 185, 157, 131 and 41 to mention a few.

In contrast to the caffeine experiments described above, neither a full-scan API nor a CID mass spectrum of a crude extract of an equine urine containing administered butorphanol revealed any information allowing the identification of this drug in the crude urine extract. This appeared to be due to intolerable matrix effects in the extremely complex equine urine extract and ng/ml levels of the drug in the urine. The complexity of equine urines compared to human urine is a well known fact and has been addressed previously[4,6].

When an eluted TLC scrape of butorphanol was analyzed by TLC–MS–MS the full-scan API mass spectrum shown in Fig. 7A was observed. An abundant *m/z* 328 ion was seen in addition to several other ions that are probably the $(M + 1)^+$ ions of other organic substances eluted from the TLC spot. The CID mass spectrum for the *m/z* 328 ion in Fig. 7A is shown in Fig. 7B. This spectrum compares favorably with the corresponding CID spectrum for the *m/z* 328 of standard butorphanol and thus satisfactorily identifies this drug in the eluted TLC spot.

Fig. 8 shows the full-scan API mass spectrum obtained by placing the TLC scrape of silica containing butorphanol from the equine urine extract into the API source. The spot was isolated from an equine urine extract containing the administered drug. Thus the isolated drug present at about 50 ng/ml in equine urine was thermally desorbed from the silica which had been placed on the direct insertion probe. The full-scan API mass spectrum shown in Fig. 8 reveals no significant *m/z* 328 ion as was observed in Fig. 7A. Similarly, CID experiments on *m/z* 328 in this instance provided no useful CID data. Thus it appears that butorphanol cannot be thermally desorbed from a silica surface for MS analysis at these levels but satisfactory results may be obtained if the spot is first eluted with organic solvent. This is perhaps not surprising in view of the structure of this drug.

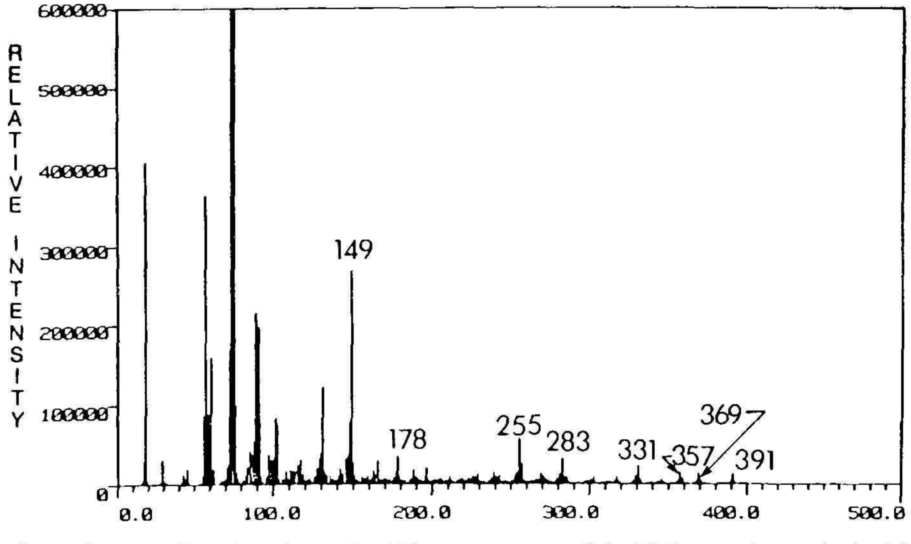

Fig. 8. Full-scan direct insertion probe API mass spectrum of the TLC scraped spot obtained from an organic extract of an equine urine subsequent to the administration of butorphanol. The silica particles were not eluted with solvent, but placed directly on the direct insertion probe and the volatile organics thermally desorbed from the silica.

Betamethasone

The administration of the corticosteroid, betamethasone, to a horse is followed by rapid metabolism and excretion of the parent drug. Thus plasma and urinary levels are routinely quite low in spite of the fact that routine administration may be given. The detection and identification of betamethasone in the presence of many other endogenous steroid related substances poses a challenging analytical problem[10].

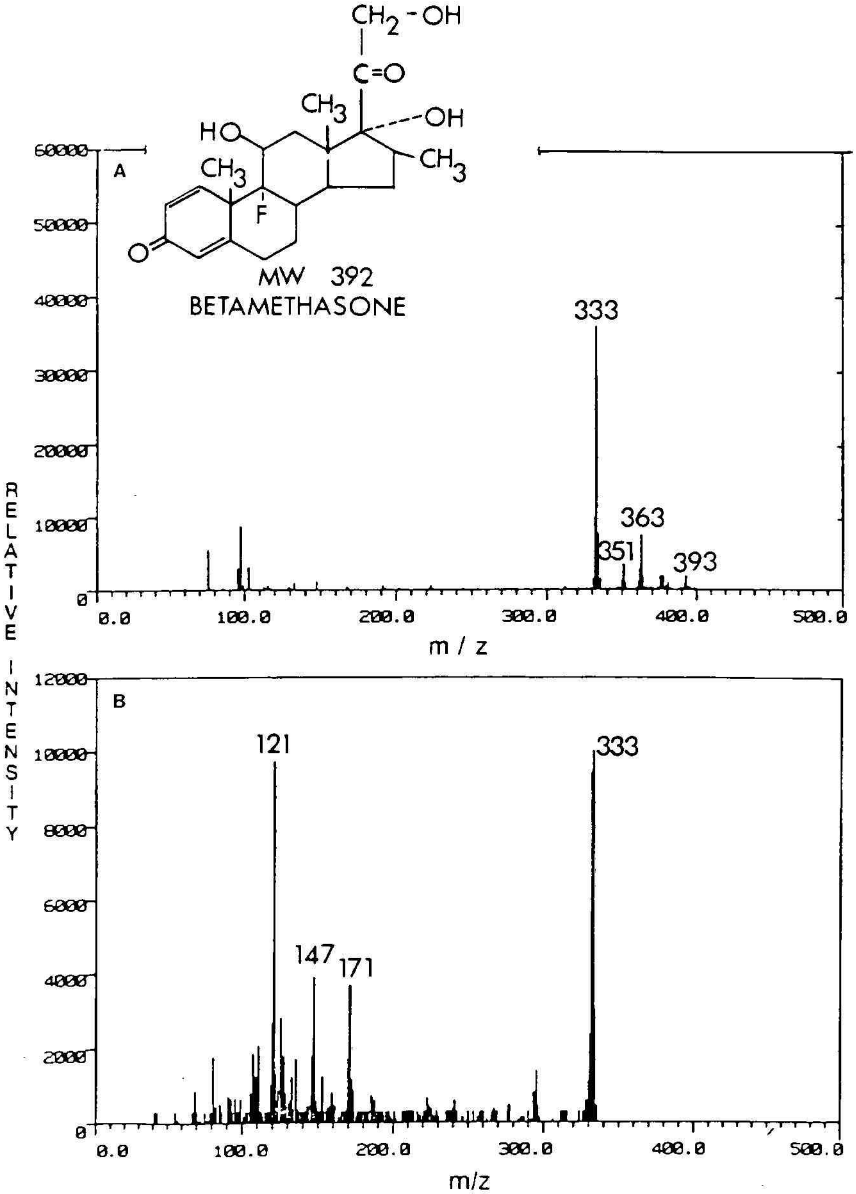

Fig. 9. (A) Full-scan direct insertion probe mass spectrum of standard betamethasone. (B) API CID mass spectrum of standard betamethasone $(M + 1 - 60)^+$ ion (*m/z* 333).

We have developed TLC screening methods for detecting betamethasone and its metabolites in equine urine and plasma[9], but confirmation is best accomplished by micro LC–MS[10]. Although this method provides satisfactory results, considerable sample clean up and instrumental set up is required.

We have investigated the possibility of identifying betamethasone by TLC–MS–MS. Fig. 9A shows the full-scan API mass spectrum of standard betamethasone. Unfortunately, the facile loss of $C_2H_4O_2$ from the D ring[11] yields a relatively weak $(M + 1)^+$ ion. However, the structurally significant *m/z* 333 ion resulting from this fragmentation is the base peak and a convenient parent ion for CID experiments (Fig. 9B). This CID daughter ion mass spectrum provides sufficient specificity for identifying betamethasone in mixtures.

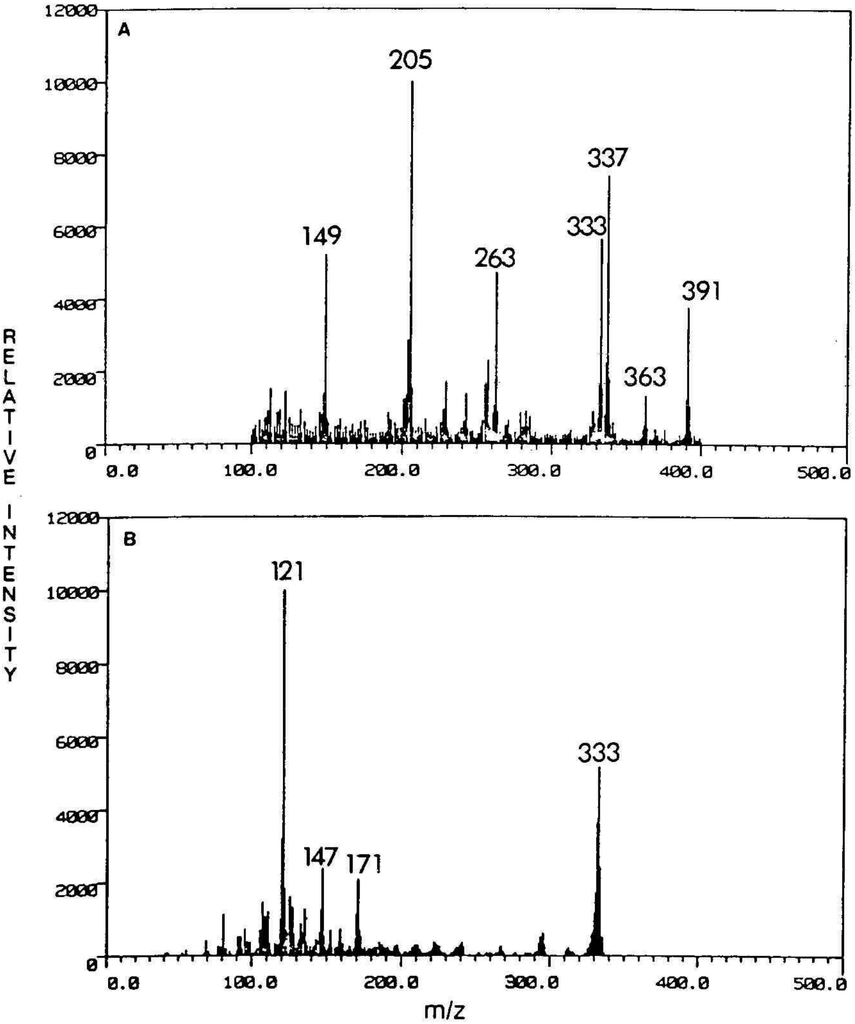

Fig. 10. (A) Full-scan direct insertion probe API mass spectrum of an eluted TLC scrape obtained from an organic extract of equine urine subsequent to the administration of betamethasone. (B) API CID mass spectrum of the *m/z* 333 ion observed in A.

The full-scan API mass spectrum shown in Fig. 10A was obtained from an eluted TLC scrape of an equine urine obtained after a betamethasone administration. The eluted spot was removed from a TLC plate at an R_F that was the same as that of standard betamethasone. As can be seen from Fig. 10A there are numerous ions present in addition to those expected from betamethasone. However, if a CID experiment is performed on the *m/z* 333 ion the daughter ion spectrum shown in Fig. 10B is observed. This spectrum compares favorably with the CID mass spectrum from standard betamethasone shown in Fig. 9A and thus identifies betamethasone in this eluted TLC scrape. Comparable experiments from the raw urine extract and the TLC scrape of the betamethasone urine did not provide any useful results. In this case, too, the matrix effects in the raw extract and the thermal decomposition on the heated silica preclude satisfactory results from these experiments. However, the simple two-step procedure of urine extraction followed by TLC–MS–MS provides rapid, "unequivocal" identification of this important corticosteroid in equine urine.

Clenbuterol

Clenbuterol is a potent respiratory stimulant administered to horses in doses ranging from 100 to 200 μg. The rapid onset of pharmacological effects coupled with a short duration are manifested by low levels of the parent drug in the plasma and urine. The detection of clenbuterol in equine urine and its confirmation by GC–MS can be accomplished by scrupulous preparative TLC procedures and derivatization prior to GC–MS analysis on well conditioned GC columns[8]. A simplified confirmation step requiring less sample cleanup is clearly desirable due to the sample losses resulting from multistep clean up.

Fig. 11A shows the full-scan API mass spectrum of standard clenbuterol. The base peak at *m/z* 277 readily documents this drug's molecular weight of 276 with a few fragment ions characteristic of the benzylic secondary amine side chain moiety appearing at lower masses. The CID daughter ion spectrum for *m/z* 277 shown in Fig. 11B displays fragment ions at *m/z* 259, 203, 168, 132 and 57.

The full-scan API mass spectrum shown in Fig. 12A was obtained from an eluted TLC scrape of an equine urine extract obtained after intravenous administration of 200 μg of clenbuterol. The developed spot was removed from the TLC plate at the R_F equal to that observed for standard clenbuterol using the micro spot scraper described above. This small amount of silica was eluted with methanol to recover the organic material adsorbed to its surface. The resulting methanol solution was concentrated to about 10 μl and a 2-μl aliquot was placed on the direct insertion probe. Fig. 12A shows the full-scan API mass spectrum of this eluted TLC scrape. Although there is a relatively small *m/z* 277 ion observed, there are many other abundant ions present that preclude identification of clenbuterol from this mixed mass spectrum. However, if CID is performed on the *m/z* 277 ion, the daughter ion mass spectrum shown in Fig. 12B is obtained, which agrees well with the CID mass spectrum obtained from standard clenbuterol shown in Fig. 11B. This TLC–MS–MS experiment allows the "unequivocal" identification of clenbuterol from the eluted TLC screening plate following minimal but essential sample cleanup.

Similar TLC–MS and TLC–MS–MS experiments on the raw urine extract and the TLC scrape analyzed directly on the direct insertion probe without elution by methanol failed to provide useful MS data for the identification of clenbuterol in the

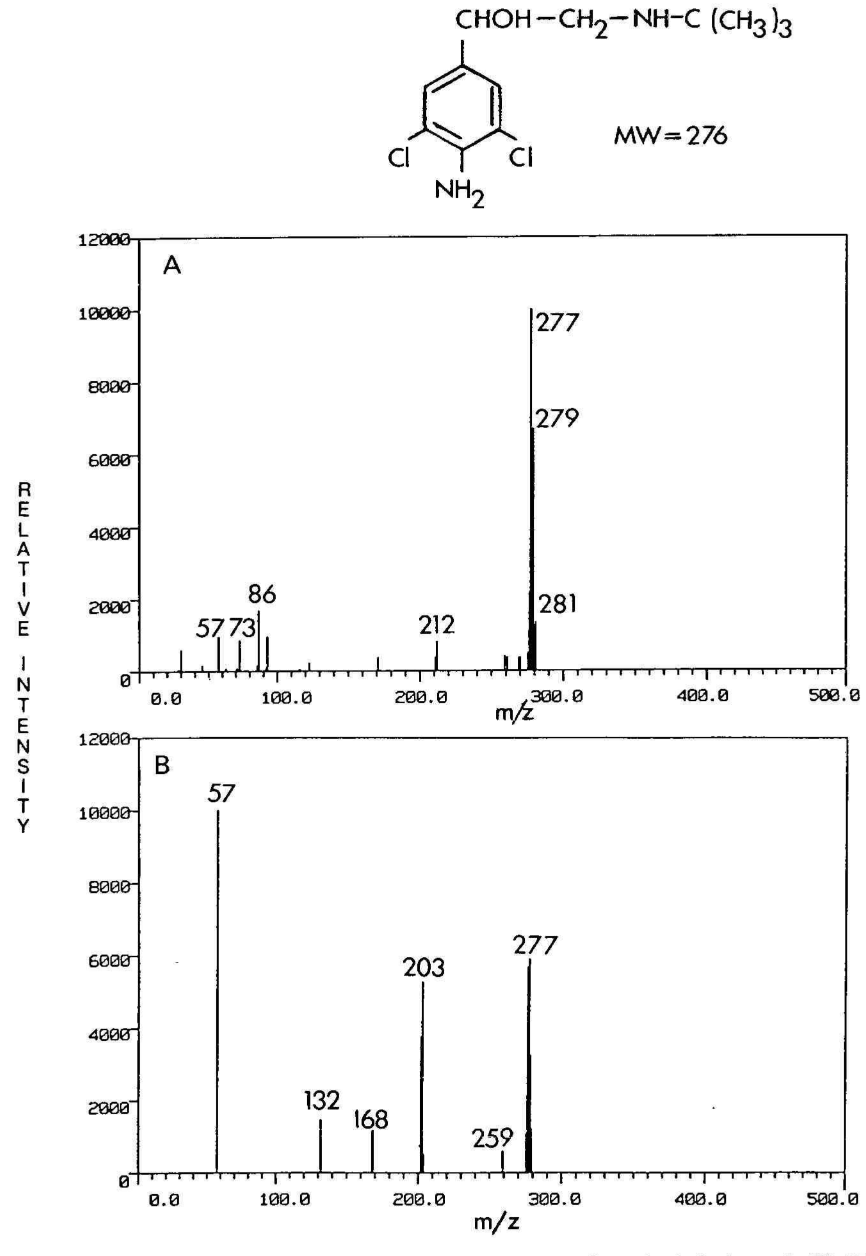

Fig. 11. (A) Full-scan direct insertion probe mass spectrum of standard clenbuterol. (B) API CID mass spectrum of standard clenbuterol $(M + 1)^+$ ion (m/z 277).

equine urine extract. In the former case the matrix effects resulting from the complexity of the urine extract precluded useful MS data. In the latter case thermal desorption of the labile drug from the surface of the silica particles produced no $(M + 1)^+$ ions for clenbuterol presumably due to decomposition of the drug on the silica prior

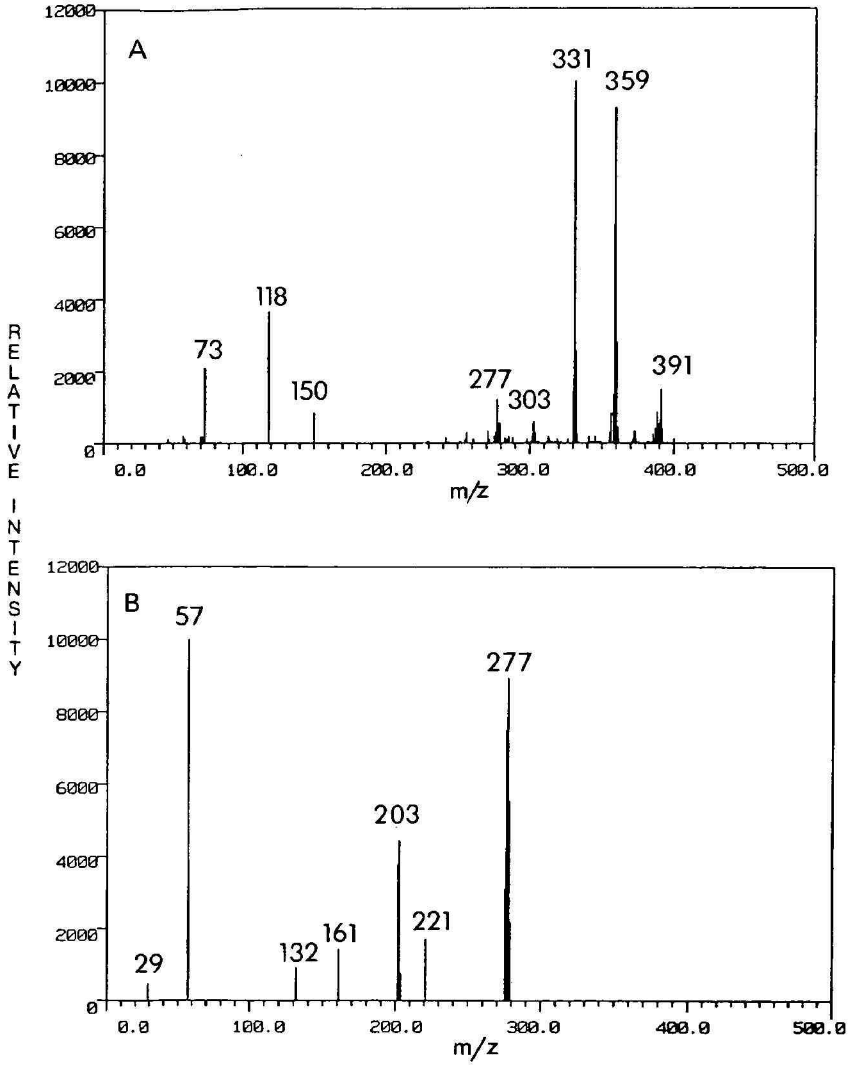

Fig. 12. (A) Full-scan direct insertion probe API mass spectrum of an eluted TLC scrape obtained from an organic extract of equine urine subsequent to the administration of clenbuterol, (B) API CID mass spectrum of the *m/z* 277 ion observed in A.

to chemical ionization. Thus, as was observed above for both butorphanol and betamethasone, the technique of TLC–MS–MS is well suited for rapid identification of clenbuterol after it has been isolated from the major endogenous components present in the organic extract of the equine urine by solvent elution from the TLC silica. However, both matrix effects and catalytic effects appear to preclude successful identification of these drugs at trace levels by the method described in this work.

DISCUSSION

The four drugs chosen for this work vary in the degree of difficulty for their identification in biological fluids. In this work caffeine and nicotine in the human urine of a chronic coffee drinker and cigarette smoker were relatively easy to detect and identify. Thus, these drugs were easily determined in the raw extract, the eluted TLC scrape and the TLC scrape itself. This was the only case in the examples cited where this was possible. We feel this was due primarily to the μg/ml concentrations of these substances. It is known, for example, that caffeine is very difficult to elute from a TLC spot when it is present at low ng/ml levels[8].

The other drugs in this study enjoyed a similarity with respect to their rather low urine concentrations and thermal instability. Each of these factors contribute to analytical difficulties when it comes to their detection and identification in biological fluids. As is often the case, the more the analyst handles a sample during workup the more likely sample loss will occur with additional sample purification steps. We have endeavored, therefore, to combine the highly successful TLC screening technique with the relatively new MS–MS technique to provide high volume screening capability with unmatched high specificity mixture analysis. We introduced a fast TLC sample clean up step that has been shown to be very effective. This is demonstrated by the lack of success with CID of raw urine extracts and TLC scrapes of butorphanol, betamethasone and clenbuterol, but favorable results from the eluted TLC scrapes for these drugs. This viewpoint is in contrast to earlier reports of MS–MS experiments on untreated samples[1–3], but in line with a more practical concensus developing[4,12].

ACKNOWLEDGEMENTS

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Part A – Fundamentals and Techniques – presents an introduction to the field, including the development and theory of chromatography and electrophoresis, and then gives the theoretical and instrumental basis of each technique.
Part B – Applications – is a critical review of the isolation and quantification methods in current use for various classes of substances.

Both parts of this work belong in all libraries serving chromatographers at technical colleges, research institutes, universities, pharmaceutical companies and instrument manufacturers. Practising chromatographers and analysts will refer to it often when faced with new or unusual analytical problems and situations.

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