Journal of Chromatography, 271 (1983) 3–12
Chromatographic Reviews
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

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 spectiometry (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]

0378-4355 83/$03 00

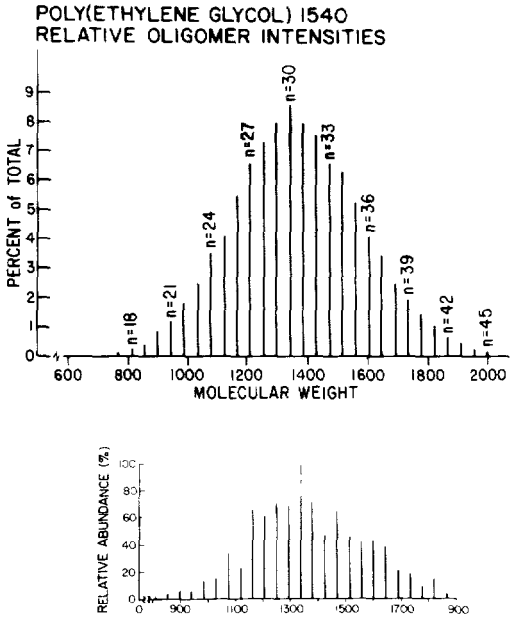
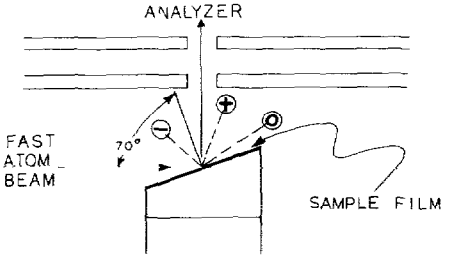


Fig 1 Oligomer population in a polyethylene glycol fraction analyzed by field desorption (top)[6] and by electrohydrodynamic ionization (bottom)[7]

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



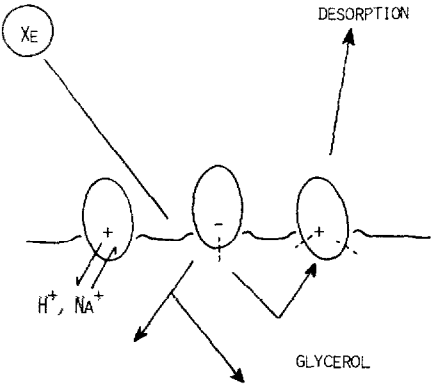


Fig. 2 Schematic representation of sample bombardment by fast atoms.

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-

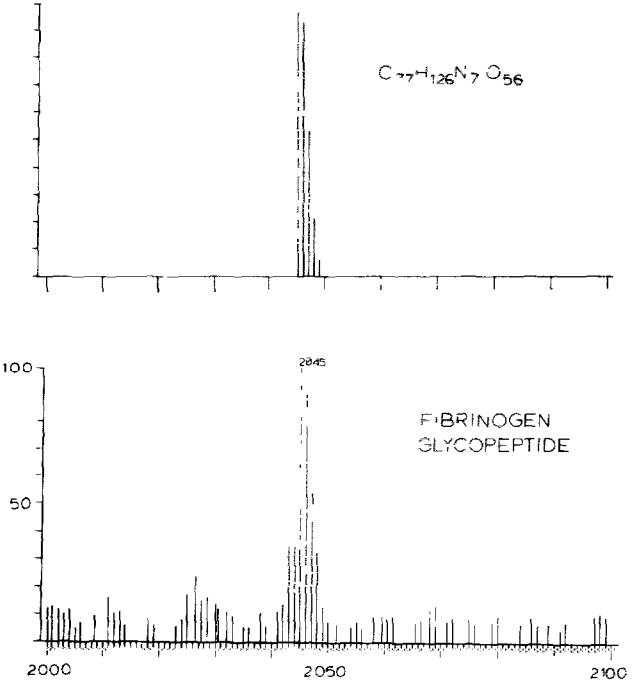
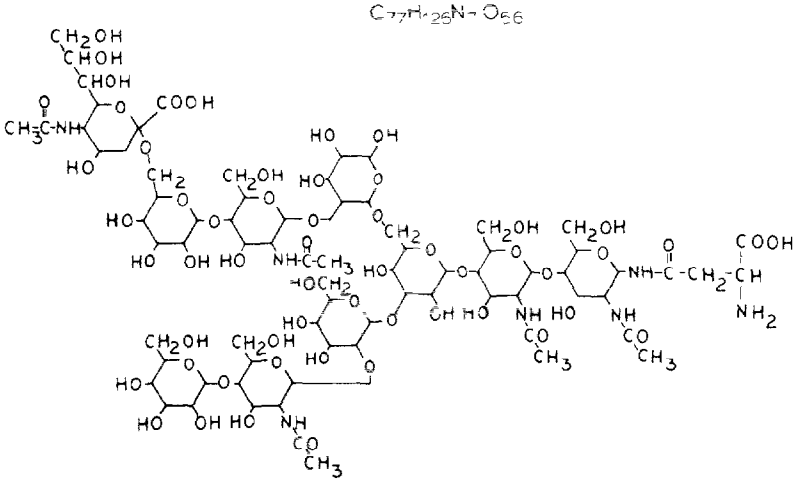


Fig 4 Molecular ion region of a positive ion FAB spectrum of a monosialylated bidentate glycopeptide from human fibrinogen (bottom) and the computer generated theoretical protonated molecular ion cluster (top)

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

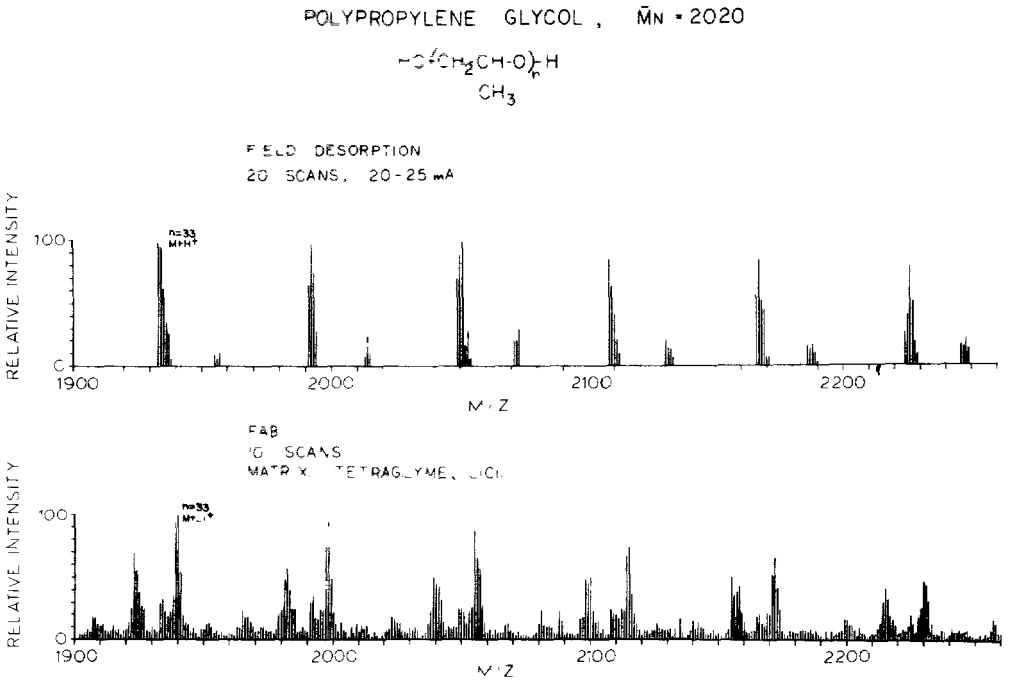


Fig 5 Partial field desorption spectrum (top) and partial FAB spectrum (bottom) of a polypropylene glycol fraction

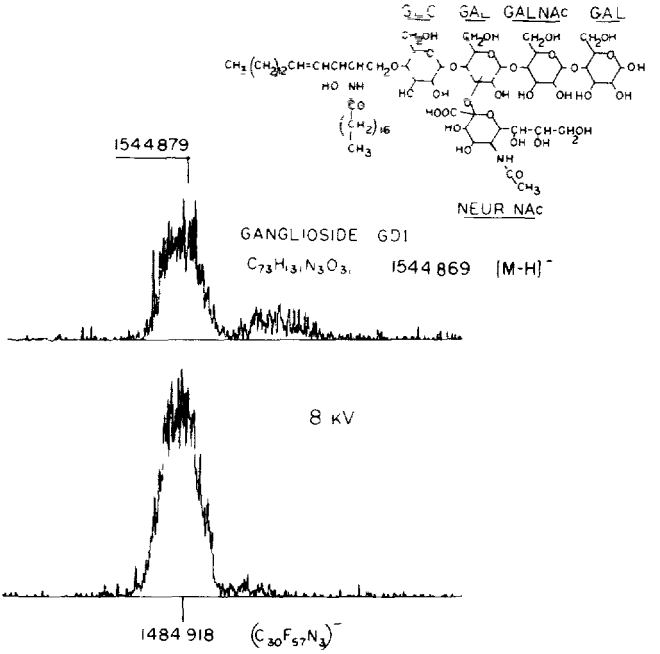


Fig 6 Accurate mass measurement made at 10.000 resolution of a ganglioside $(M - H)^-$ anion. The reference compound is triazene 1485

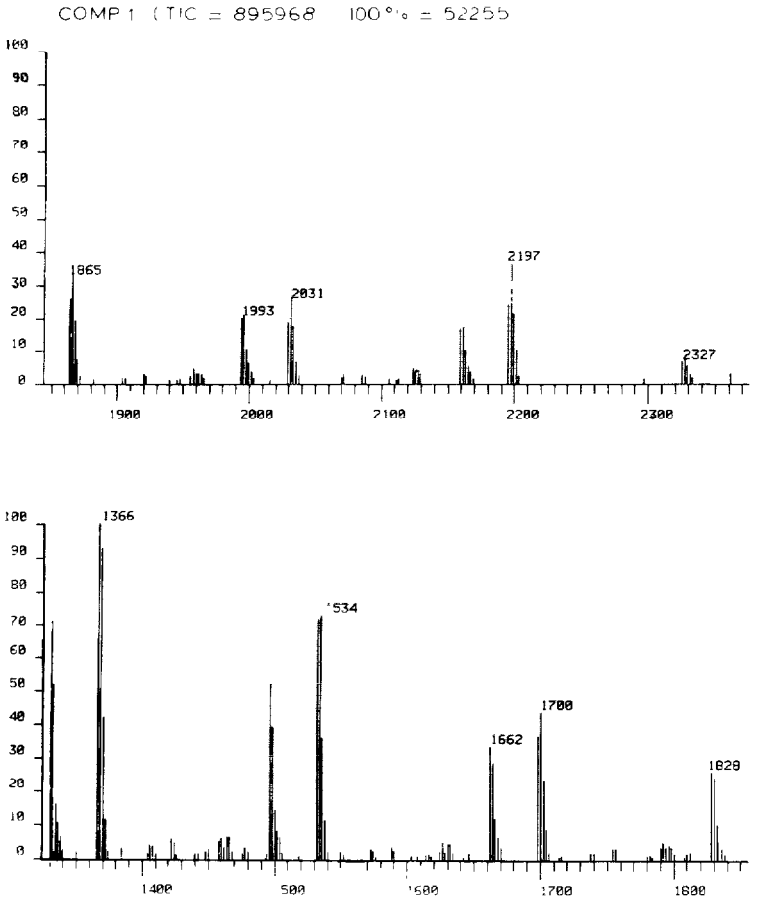


Fig 7 Computer calibration spectrum of potassium iodide glycerol clusters used to acquire the spectrum in Fig. 4

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

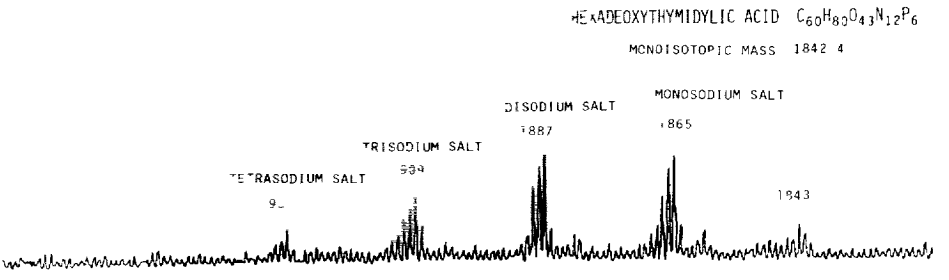


Fig 8 Molecular cation region of the hexanucleotide hexathymidylic acid[5] (With permission from the American Chemical Society.)

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-

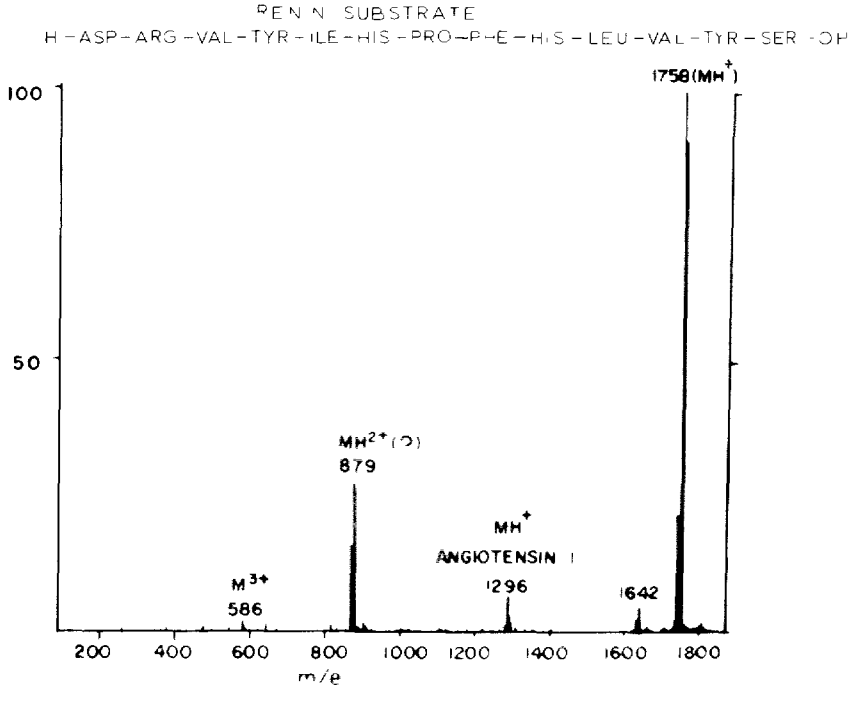


Fig 9 Thermospray spectrum of renin[21] (With permission from the American Chemical Society)

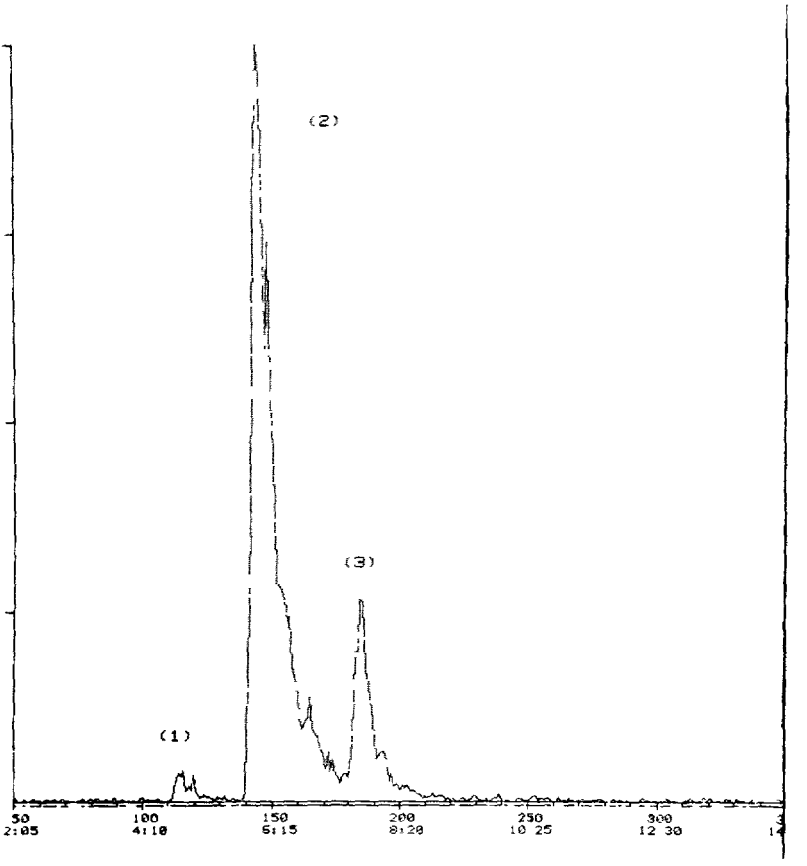


Fig 10 Reconstructed mass chromatogram of an equimolar mixture of three glucuronides analyzed by thermospray LC–MS[22] Column = 15 cm Ultrasphere ODS Eluent = methanol–0.05 *M* ammonium acetate (20 80), flow-rate 1 ml min Peaks 1 = *p*-nitrophenyl-β-D-glucuronide, 2 = 4-methylumbelliferyl-β-D-glucuronide,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

This work was supported by grants from the National Science Foundation, CHE 7818396 and PCM 8209954.

REFERENCES

1 C Fenselau, R. Cotter, G Hansen, T Chen and D Heller, *J Chromatogr*, 218 (1981) 21
2 P Hakansson, I Kamensky, B Sundqvist, J Fohlman, P Peterson, D J. McNeal and R D MacFarlane, *J Amer Chem Soc*, 104 (1982) 2948
3 U. Giessmann and F W Rollgen, *Int J Mass Spectrom. Ion Phys*, 30 (1981) 267
4 C Fenselau, in A Benninghoven (Editor), *Proceedings of the Second Workshop on Ion Formation from Organic Solids— IFOS II*, Springer Berlin, Heidelberg, New York, 1983
5 C Fenselau, *Anal Chem.*, 54 (1982) 105A
6 R P Lattimer and G E Hansen, *Macromolecules*, 14 (1981) 776
7 S T. F Lai, K W Chan and K D Cook, *Macromolecules*, 13 (1980) 953.
8 D S Simons, B N Colby and C H Evans, *Int J Mass Spectrom Ion Phys*, 15 (1974) 291
9 S T F Lai and C. A Evans, *Biomed Mass Spectrom*, 6 (1979) 10
10 M Barber, R S Bordoli, G J Elliott, R. D Sedgwick and A N Tyler, *Anal Chem*, 54 (1982) 645A
11 A. Benninghoven, *Trends Anal Chem*, 1 (1982) 311
12 R R Townsend, E Milliker, Y T Li, R A Laine, W R Bell and Y C Lee, *J. Biol Chem*, 257 (1982) 9704
13 J Yergey, D Heller, G Hansen, R J Cotter and C Fenselau, *Anal Chem*, 55 (1983) 353
14 G Hansen, D Heller, J Yergey, R J Cotter and C Fenselau, *Chem Biomed Environ Instr*, in press
15 L Grotjahn, R Frank and H. Blocker, *Nucleic Acids Res*, 10 (1982) 4671
16 C Fenselau, V T Vu, R J Cotter, G Hansen, D Heller, T Chen and O M Colvin, *Spectros Int. J*, 1 (1982) 132.
17 C R Blakley, J J Carmody and M L Vestal, *J Amer Chem Soc*, 102 (1980) 3931
18 M L Vestal, in Benninghoven (Editor), *Proceedings of the Second Workshop on Ion Formation from Organic Solids— IFOS II*, Springer, Berlin, Heidelberg, New York, 1983
19 J V Iribarne and B A Thomson, *J Chem Phys.*, 64 (1976) 2287
20 B A Thomson and J V Iribarne, *J. Chem Phys*, 71 (1979) 4451
21 C R Blakley and M L. Vestal, *Anal Chem* 55 (1983) in press
22 D J Liberato, A L Yergey and C Fenselau, unpublished results