Journal of Chromatography, 394 (1987) 35–49
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 454

MICRO LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY WITH LOW FLOW GRADIENT ELUTION. STUDIES OF ELECTROSTATIC NEBULIZATION AND FUSED-SILICA COLUMN DESIGN

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SUMMARY

Fused-silica columns (I.D. 0.22 mm), packed with ordinary small-particle HPLC packing material, were directly connected with an electron-impact ion source. A system for micro flow gradient (1–5 μl/min) has been developed. The electrostatic field between the column end and the ion source is the major factor for nebulization. The electrostatic effect was studied with regard to different column tip designs. Experiments with focusing of the solvent spray to increase the sensitivity are also presented. Applications to plant extracts, phenolic acids and other polar compounds are reported.

INTRODUCTION

In our laboratory, the main interest concerns plant allelochemicals. Capillary gas chromatography (GC) and liquid chromatography (LC) are the most important analytical techniques in this field. For those substances which are thermally labile or non-volatile, LC is a useful method. Identification of the separated components on-line by mass spectrometry (MS) will be of great importance if a good LC–MS connection can be achieved. If this is possible, LC has the ability to deliver samples into the MS ion source with low or no thermal decomposition. Desirable qualities of an LC–MS system suitable for analytical work with natural products are a good chromatographic separation (compounds not degraded in the interface or the ion source), the possibility of operating with different packing materials and solvents, gradient elution, useful for thermally labile and non-volatile compounds, the possiblitity of using a mass spectral reference library, a high sensitivity and linear dynamic range, a rapid analysis and reliable and easy operation.

Most mass spectrometers can tolerate a solvent flow-rate into the vacuum system of 5–10 μl/min, which suggests the use of packed fused-silica columns operating at this low flow-rate. A micro column with a length of 20–50 cm and an inner diameter (I.D.) of 0.2 mm is used in our system, which we first presented at the 3rd Montreux meeting in 1984[1].

Well packed fused-silica columns have a high separation efficiency. They also give low sample dilution and a low pressure drop compared with 4–5 mm I.D. stain-

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less-steel columns packed with the same stationary phase. They are also easy and inexpensive to manufacture. For solvent delivery, a standard HPLC pump operating under constant pressure can be used.

In this paper, we describe three current developments of interfacing this type of column to a mass spectrometer: The invention of a low-flow gradient elution system, the design and preparation of the column, and the use of an extraction–focusing plate in front of the column tip. We also report experimental results obtained with compounds of allelochemical interest.

EXPERIMENTAL

Chromatographic system

An HPLC pump (Spectra-Physics 8700) was used in the constant-pressure mode and flow-rates of 1–5 μl/min were obtained without any modification of the pump. A 0.5-μl syringe-loaded micro injector (Rheodyne 7520) was used for sample injection. To minimize the dead volume to less than 0.02 μl, the fused-silica column was connected directly to the injector block.

Interface design

The column was led into the ion source through a ball valve and a 0.5 mm I.D. stainless-steel tube. The tube terminates about 15 mm from the ion chamber entrance of a standard electron-impact ion source. The high vacuum seal is a Vespel (DuPont, Wilmington, DE, U.S.A.) ferrule mounted on the ball valve. A change of LC columns or a change-over to the GC system can be effected in a few minutes.

Nebulization and vaporization are governed by several factors. The most important factor is the electrostatic field between the column tip and the ion source block. Owing to this, the eluate from the column leaves the tip in the form of invisible droplets. If no electrostatic field is applied, large drops will form at the tip. To prevent electrical breakdown, the end part of the column must be isolated from ground. Another factor for nebulization is the configuration of the column tip. The pressure in the ion source housing and the ambient temperature affect the vaporization.

The electrospray ionization process[2] produces charged droplets which evaporate to charged molecules. This occurs, with the help of a heated inert gas, just below atmospheric pressure. The electrospray produces molecular ions and no fragmentation occurs.

The electrostatic spray process occurs in high vacuum. In this process, the electric field is used for nebulization and no or very few charged droplets are produced. The field causes a distribution of charges on the surface of the droplets, needed for focusing the eluent spray into the ion source. The ionization of the molecules occurs in the electron-impact source.

Mass spectrometer

The mass spectrometer is a large-radius (300 mm) magnetic sector instrument, built in our laboratory. It has a high-vacuum system similar to ordinary mass spectrometers adapted to chemical ionization. For the chromatographic registration a system that discriminates low-mass ions was used[1]. Only mass spectra of positive ions could be obtained with the electron multiplier used.

Columns

Fused-silica tubing (20–50 cm × 0.22 mm I.D.) was packed with a high-performance liquid chromatography (HPLC) packing material (3–5-μm particles) using a high-pressure slurry packing technique described previously[1]. The use of anhydrous slurry and pumping solvents is important in order to obtain a homogeneous packing.

In order to effect a simple and efficient coupling of the column to the MS ion source, the column must end with a fine tip. The design of the column tip is important in order obtain a low dead volume, a high mechanical strength (must withstand freezing of the solvent), a low back-pressure, a small tip area (gives a high electrostatic field), easy manufacture and high reproducibility.

Several column designs (Fig. 1) were tested as follows.

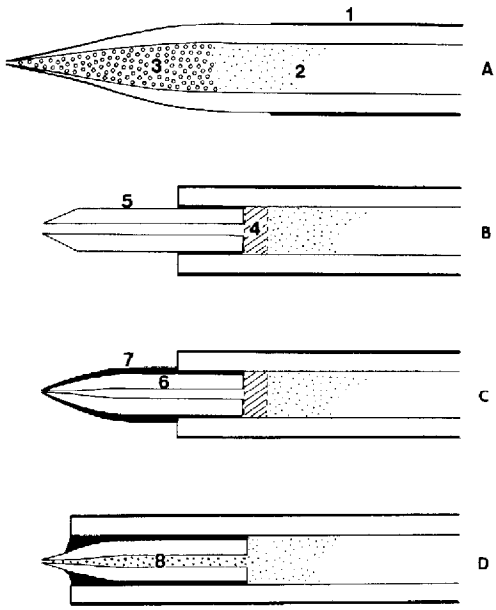

Fig. 1. Schematic diagram of column tip designs A–D. 1, Fused-silica column (I.D. 0.22 μm); 2, chromatographic bed; 3, coarse packing material (40 μm); 4, glass-fibre frit; 5, fused-silica tube (I.D. 50 μm); 6, drawn-out 50-μm fused-silica tube; 7, high-temperature epoxy coating; 8, 10-μm packing material.

(A) The column tube end is drawn to a fine tip. To prevent fouling of the ion source with packing material, a small amount of a coarse (40-μm) HPLC material is placed inside the tip. This was the first tip design, and good chromatographic results were obtained. Columns, 30 cm long, packed with 3-μm Spherisorb ODS particles gave almost 50000 theoretical plates. The main disadvantage is the low mechanical strength. LC solvents with a high water content can cause freezing and the tip is easily split by the frozen eluate.

(B) Inside the column, at the end, a 50-μm I.D. fused-silica tube is cemented with a high-temperature epoxy glue. A small glass-fibre frit in front of the small tube holds the chromatographic bed. The advantage is a higher mechanical strenght than

in the previous design. The signal-to-noise ratio is highly dependent on the temperature of the ion source, probably owing to the lack of a distinct evaporation point. With a smaller inner diameter of the end tube this effect decreases, but is still troublesome.

(C) This design is a development of design B. After packing, the end of the tube is drawn to a fine tip and protected with epoxy glue. A high mechanical strength of the tip is obtained. The signal-to-noise ratio is larger than with design B but smaller than with A. The noise is due to fluctuations of the evaporation and is dependent on the solvent composition.

(D) A narrow tube (I.D. 50 μm) is drawn to a tip, fixed to the column with an epoxy glue and filled with 10-μm particles. The column is then packed in the normal way. This design is a combination of A and C and seems to have the advantages of both. It gives a signal-to-noise ratio equal to that of A, but the column, in contrast to A, can withstand freezing of the solvent.

The importance of a tip filled with packing material is illustrated in Fig. 2. The signal-to-noise ratio of the LC registration is very much increased.

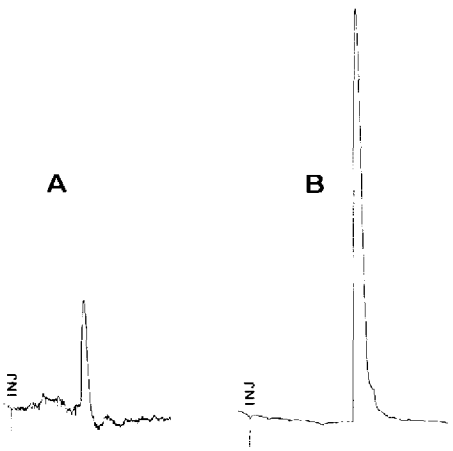

Fig. 2. Illustration of the importance of the column tip design. Chromatograms of raffinose (1 μg injected) obtained by a column (A) *without* and (B) a column of type D *with* packing material inside the tip. Detection: total ion current (TIC) (ion of $m/z < 40$ suppressed). Ion source temperature: 210°C.

Extraction–focusing plate

In order to study and improve the effect of the electrostatic field between the column tip and the ion source, which is the major factor for nebulization, an extra plate was mounted in front of the column tip. The distance between the plate and the ion source block is 3 mm (Fig. 3). By the use of a separate high-voltage power supply the electric potential of the plate can be varied between -6 and $+6$ kV. The tip terminates in the centre of a 2.5-mm diameter hole with sharp edges. To achieve an optimal signal-to-noise ratio, the distance between the tip and the hole can easily be adjusted during operation. In all the experiments a $+5$ kV acceleration voltage was applied to the ion source, giving a $+5$ kV electrostatic potential relative to ground.

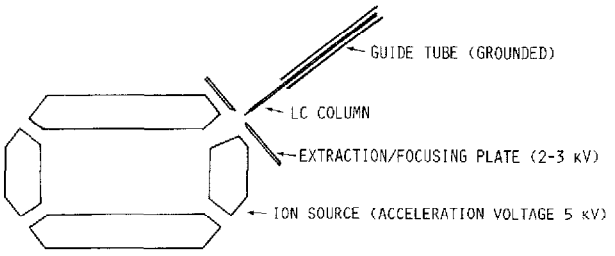

Fig. 3. Ion source–LC column connection with an extraction–focusing plate.

RESULTS AND DISCUSSION

Experiments with a negative focusing plate potential to ground showed a deleterious effect on the solvent spray. The solvent seemed to be spread out and very small amounts reached the ionization area. The electrostatic phenomenon explains the negative result. When a droplet is pulled out of the column tip by the negative electric field, positive charges on the surface of the droplet move to the front. After passing the plate, the polarized droplet enters an electric field of opposite polarity, becomes repelled and changes direction.

If the plate is grounded, the column tip must pass through the hole in the plate to obtain nebulization without visible drops. Therefore, no extra advantage is obtained.

A positive potential, however, has a positive effect on the vaporization of the solvent and the focusing of the spray. Fig. 4 shows two chromatograms run (A) with and (B) without a focusing plate. In A, the column tip was in the plane of the focusing plate and located 18 mm from the electron beam. The potential of the plate was +2 kV. In B, the position of the tip was placed close to the ion source block inlet hole at a distance of 15 mm from the electron beam.

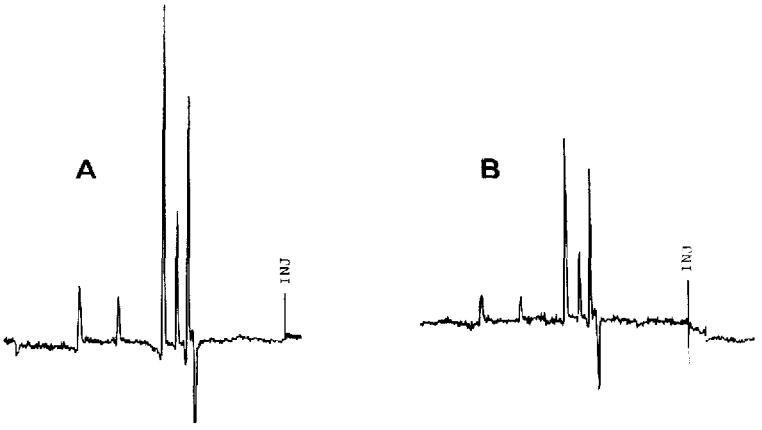

Fig. 4. Illustration of the effect of the extraction–focusing plate. Chromatograms of aromatics obtained (A) with the focusing plate and (B) without the plate. The plate potential was 2.5 kV. Optimal signal-to-noise ratio, the same sample amount, flow-rate, column and ion source temperature were used in both instances. Detection: TIC (ion of $m/z < 40$ suppressed). Ion source temperature: 210°C.

For different columns the optimal potential varies between 2 and 3 kV. An example of an optimization of the plate potential is illustrated in Fig. 5. Raffinose was used as a test substance. When a column of the latest design (D) is used, the optimal potential is the same for different solvent composition (0–100% methanol in water).

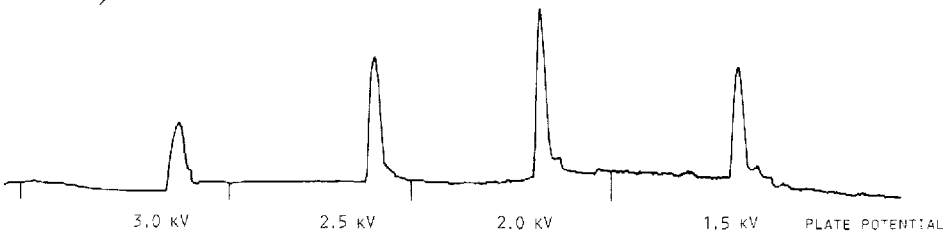

Fig. 5. Determination of the optimal extraction–focusing potential by four 1-μg injections of raffinose at different potentials. Detection: TIC (ion of m/z < 40 suppressed). Ion source temperature: 220°C.

By the use of a focusing plate, the signal-to-noise ratio was increased by at a factor of at least two. No heating is needed for nebulization and the column tip is protected from the ion source heat radiation.

Gradient elution

Practicable gradient elution with a solvent flow of just a few microlitres per minute is a delicate problem. Two principles are common in normal flow HPLC (0.1–5 ml/min), as follows.

The first principle is pre-pump mixing with one pump. The volume in the flow controller, the mixing chamber and the pump will limit the lowest practical flow-rate. However, this volume has been used to pre-form and store a gradient with a flow-rate down to 50 μl/min[3].

The second principle is mixing of the solvents, delivered from two pumps, on the high-pressure side. The most advanced pumps available can create gradients down to a minimum of 10 μl/min without flow-splitting arrangements.

We decided to test other principles in order to achieve a gradient for a micro flow system that would have the same accuracy and reliability as normal flow systems. One idea was to use temperature to alter the flow properties of a liquid. When a liquid is pumped through a tube the Hagen–Poiseuilles equation[4] applies:

$$V = \frac{R^4 \Delta P \pi}{8 L \mu} \tag{1}$$

where V = volume flow-rate; R = radius of tube; ΔP = pressure difference (pressure drop); μ = viscosity of liquid; L = length of tube.

With an apparatus consisting of two parallel tubes (Fig. 6), the pressure drop is the same for both sides and the flow distribution can be controled by changing the viscosity of the flowing media.

The viscosity of liquids is dependent on temperature and is determined[3] by

$$\mu = C\, e^{E/RT} \tag{2}$$

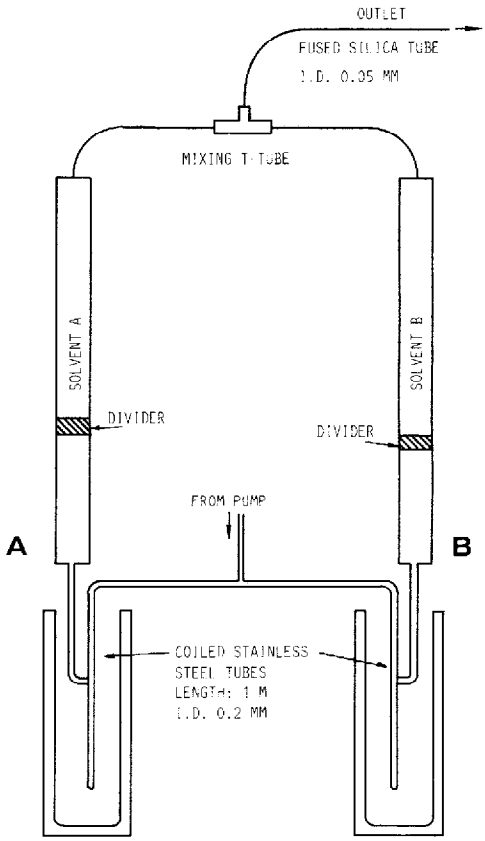

Fig. 6. Schematic diagram of the gradient system.

where T = temperature; E = activation energy of flow (constant); C = constant; R = constant.

The combined equations (1 and 2) for parallel tubes (a and b) can be written as

$$\ln(V_a/V_b) = E/R(T_b - T_a)$$

Hence the distribution of flow (V_a/V_b) in the two tubes is dependent only on the temperature difference ($T_b - T_a$) between the tubes. Therefore, by temperature programming it is possible to obtain a solvent gradient. A prototype of such a gradient system is shown scematically in Fig. 6. It is assembled from standard chromatographic components.

The flow out of the pump is split into two temperature-regulated coiled 1-m tubes. While waiting for accurate temperature programmers to be constructed, water-baths were used as a substitute.

After passing through the temperature-regulated coiled tubes, the pumping medium enters the solvent containers. Inside each container, a dividing bellow or a membrane prevents the LC solvent from being contaminated with the pumping medium or *vice versa*.

Different types of dividers, including different types of materials, have been tested, *e.g.*, pistons of PTFE, bellows of stainless steel and membranes of silicone rubber. However, an ideal (flexible, inert and non-penetrable) divider has not yet been developed.

The two solvents from the containers are mixed in a low-volume tee-tube and led through a 50-μm I.D. tube of length 50 cm to the injector. After the mixing of the solvents, it is important to use tubing with the smallest internal volume possible.

The calculations and the first experiments were performed with glycerol as the pumping medium. Glycerol was dissolvable in the mobile phase used and, because of its tendency to contaminate the solvents, it was replaced with liquid paraffin. The viscosities of liquid paraffin and glycerol are temperature dependent in the range 0–100°C.

The results of the theoretical calculations and of the experiments are plotted in Fig. 7. If glycerol is used as the pumping medium (curve 1) and the temperature difference of the tubes is changed from −60 to +60°C, the solvent gradient ranges from 2 to 98%. If the pumping medium is changed to a mixture of glycerol and water (50:50), an almost linear function is obtained in the composition range between 25 and 75% (curve 2).

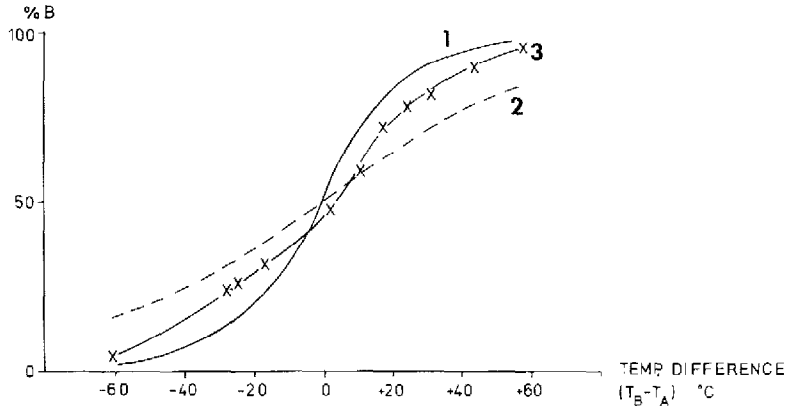

Fig. 7. Solvent composition as a function of the tube temperature difference. Curves 1 and 2 show theoretical calculations for two different pumping media: (1) 100% glycerol and (2) glycerol–water (50:50). Curve 3 shows the experimental results with liquid paraffin as the pumping medium.

Curve 3 in Fig. 7 shows a measurement of the solvent composition as a function of the temperature difference of the coiled tubes. Liquid paraffin was used as the pumping medium. Trace amounts of acetone in one of the solvents were detected by UV absorption. The curve shows a good correlation with the theoretical expectation.

The time delay to change solvent composition has been measured and the result is shown in Fig. 8. A time delay of 1 min with a normal flow-rate (approximately 1 μl/min) and a total dead volume of *ca.* 2 μl have been achieved.

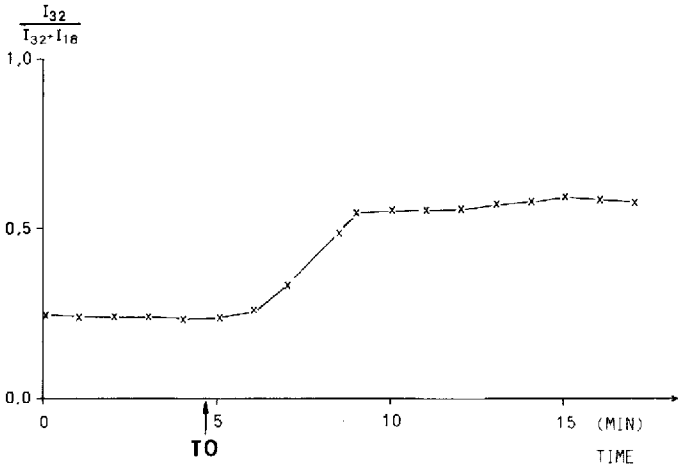

Fig. 8. Time diagram for rapid change of solvent composition. By fast heating of one of the coiled tubes (at time zero) the eluate was changed. The composition was calculated as the molecular ion intensity ratio of the eluate. The time T_0 (4.7 min) marks the elution time of unretained samples. The new solvent composition reaches the column after 1.3 min. The volume in the connection line was 1 μl.

Detection limits

The LC detection limits of, *e.g.*, ferulic acid and raffinose were a few nanograms. The amount of sample needed to obtain useful mass spectra varied from a few nanograms to a few micrograms, depending on the fragmentation ability of the substances.

Phenolic acids

Phenolic acids are often found in plant tissues and have been implicated in many cases of allelopathy[5]. Fig. 9 shows a separation of three free phenolic acids and Fig. 10 shows the mass spectra of these compounds. The results were obtained with a column of type C and no extraction plate was used[6]. The mass spectra give both molecular weights and structural information.

Phenolic acids are easily thermally decarboxylated. The intensity of the molecular ion peaks varies considerably owing to the ion source temperature. A mass spectrum of free ferulic acid is shown in Fig. 11. The intensity ratios of the molecular ion (*m/z* 194) to the molecular ion of the decarboxylated ferulic acid (*m/z* 150) obtained at different ion source temperatures are also shown in Fig. 11. The fragmentation patterns are almost unchanged up to 180°C. Higher temperatures cause a dramatic increase in thermal decarboxylation.

Chlorogenic acid

Chlorogenic acid, the most widespread depside in the plant kingdom, has been associated with the resistance of plants to fungal attack[7]. A mass spectrum of chlorogenic acid is shown in Fig. 12. The peak at *m/z* 354 is the molecular ion peak and only weak peaks (*e.g.*, M − 18 at *m/z* 336) are present in the high-mass region. The

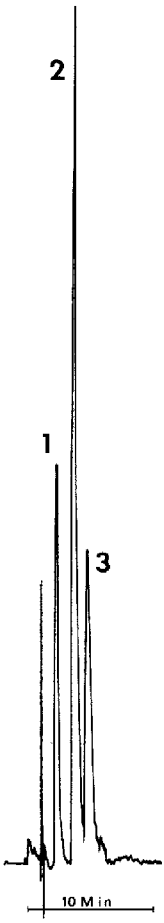

Fig. 9. Separation of free phenolic acids. 1, Caffeic acid; 2, *p*-coumaric acid; 3, sinapic acid. Column: 18 cm × 0.22 mm I.D. 3-μm Spherisorb ODS. Mobile phase: methanol–water–acetic acid (20:75:5). Detection: TIC (ions of $m/z < 60$ suppressed). Ion source temperature: 210°C. From ref. 6.

high-intensity peaks at m/z 180 and 163 are related to the aromatic (caffeic acid) part of the structure and only weak fragment peaks from the quinic acid part are present.

Gradient elution chromatograms

Fig. 13 shows gradient elution chromatograms (total ion current detection with ions of $m/z < 40$ suppressed) of a plant extract. The extract was purified from phenolic compounds and consists mainly of cardenolodides. Fig. 13A shows a gradient elution from aproximately 0 to 80% of methanol in water. All cardenolides elute between *ca.* 30 and 50% of methanol. In Fig. 13B the solvent in tube B was changed from 100% methanol to 50% of methanol in water, to obtain a gradient from *ca.* 20 to 45% of methanol. The separation is better but the peaks shows tailing.

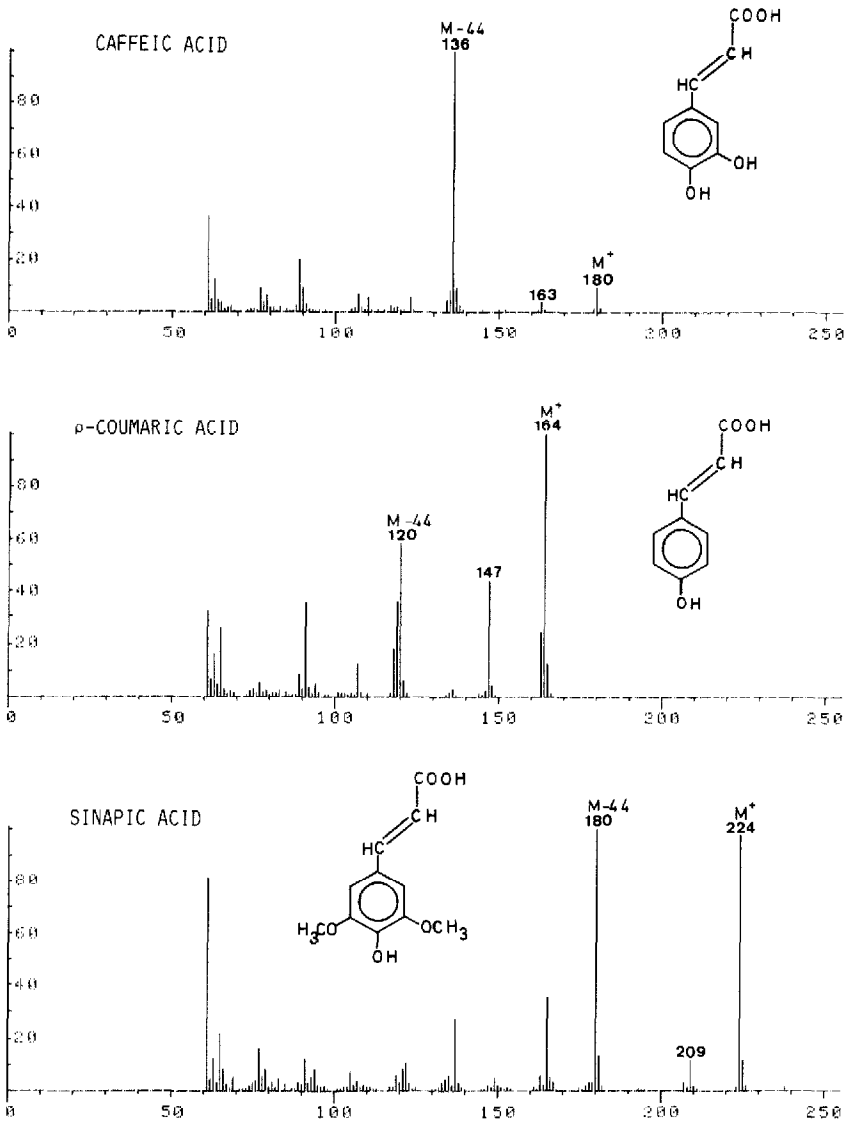

Fig. 10. LC–mass spectra of phenolic acids. Conditions as in Fig. 9. From ref. 4.

Cardenolides

A mass spectrum of one of the separated cardenolides is shown in Fig. 14 and compared with a reference cardenolide mass spectrum obtained with the same system. No molecular weight-related ions were detected in the mass spectrum of the unknown, but the ions related to the genin (strophanthidin) part are similar. NMR spectroscopy confirmed the results and the genin part was shown to have two sugars attached[7,8].

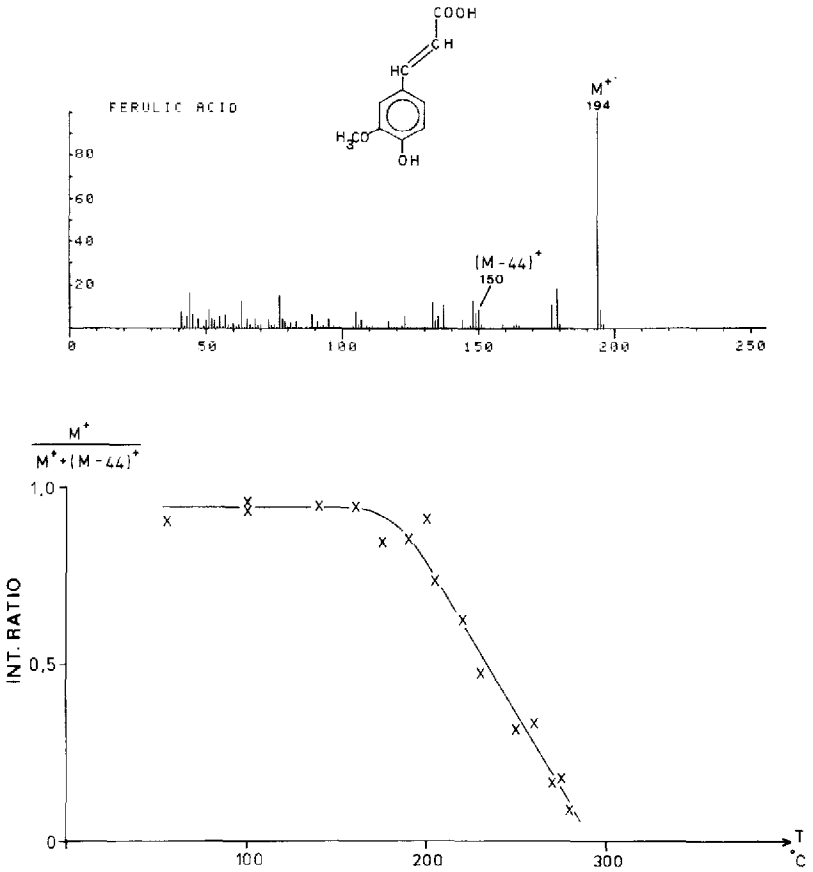

Fig. 11. LC–mass spectral data for free ferulic acid. Above, mass spectrum obtained at 150°C; below, ratios between the intensity of *m/z* 194 (the molecular ion) and the total intensity of *m/z* 150 (molecular ion of decarboxylated ferulic acid) and *m/z* 194 measured at different ion source temperatures.

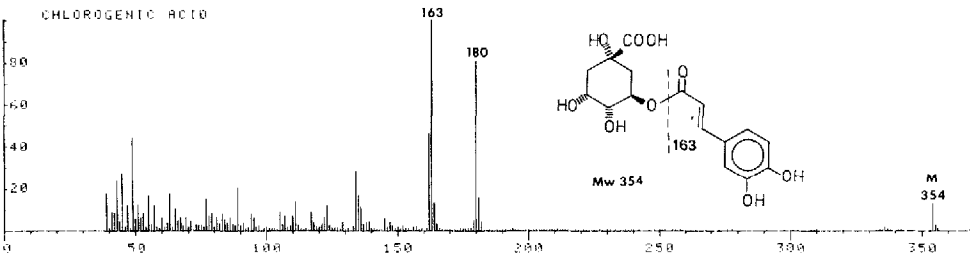

Fig. 12. LC–mass spectrum of free chlorogenic acid. Ion source temperature: 200°C. LC mobile phase: methanol–water (80:20). From ref. 6.

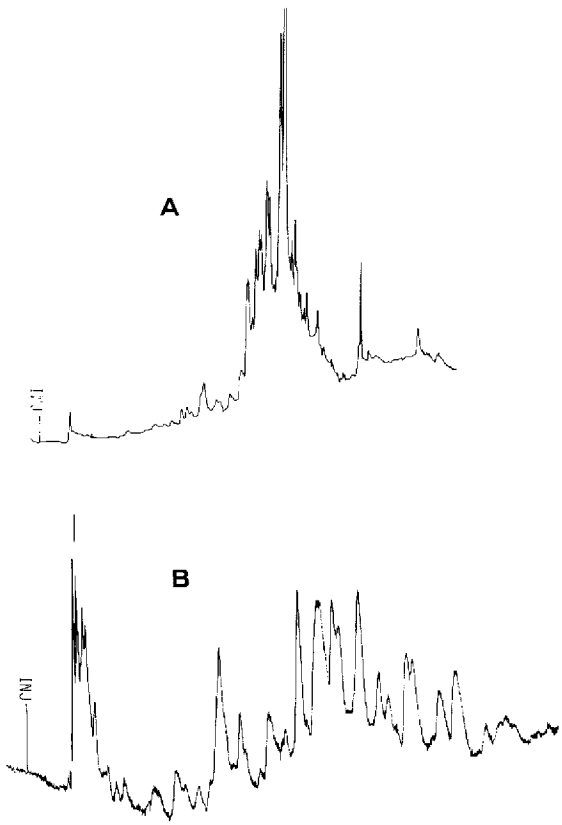

Fig. 13. Gradient elution of a plant (leaf) extract (0.5 μl injected). The extract was purified from phenolic compounds and consists mainly of cardenolides. A gradient from *ca.* 0 to 80% of methanol in water was used in A. The cardenolides elute between 35 and 50%. A slow gradient elution of the cardenolide extract is shown in B. The slow gradient is started at *ca.* 20% and is increased to 45% in 45 min. Detection: TIC (ions of $m/z < 40$ suppressed). Extraction plate potential: 2.5 kV.

CONCLUSIONS

Good chromatographic separations and informative mass spectra are of equal importance in our work with plant defence substances. The mass spectrometer, when used as a detector for LC, is a universal detector. LC mass spectra obtained from volatile and low-volatile compounds are similar to ordinary electron-impact spectra and may be interpreted by comparison with reference library spectra collections[9]. Useful mass spectra can also be otained from non-volatile compounds.

Heating of the column tip to nebulize the solvent has been shown, by experiments with the extraction plate, to be of less importance than thought. In the present design of the ion source, the eluate spray hits the hot metal walls of the inlet hole (see Fig. 3). Thermal decomposition occurs, which ruins the fragmentation pattern of thermally labile compounds. If the vaporization area is situated much closer to the ionization area and contact is avoided, we believe that the mass spectra will show

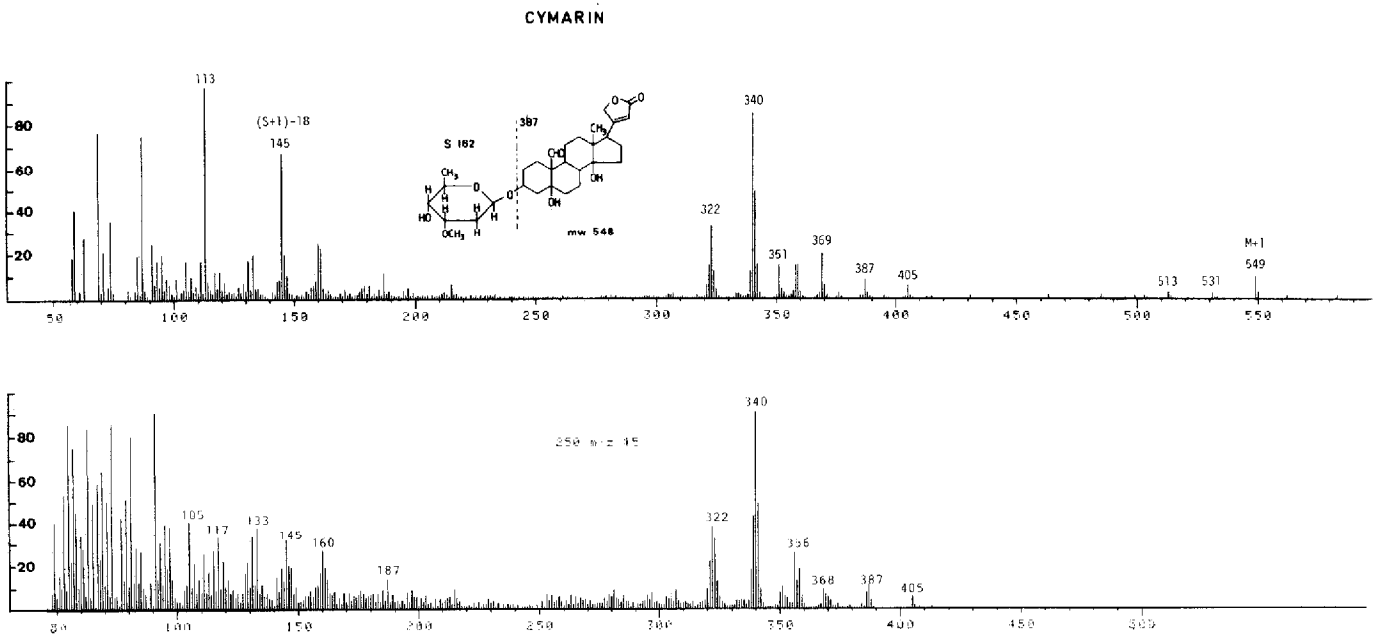

Fig. 14. Mass spectrum of the main cardenolide of the plant (leaf) extract in comparison with a mass spectrum of cymarin obtained with the same system. No molecular weight information is obtained, but the peaks in the high-mass region can be related to the genin part and some high-intensity peaks in the low-mass region to the sugar part of the molecule. Ion source temperature: 260°C.

more high-mass ions. Adsorption on the walls of the inlet port is probably the main reason for tailing of the cardenolides. The sensitivity will probably increase as the thermal decomposition decreases.

The new column tip design, in combination with the reduced heating of the tip, has made the system much more reliable. The LC column can operate for several weeks without any loss of separation efficiency. For our applications, such as plant substances, the access to gradient elution will be of great importance.

This LC–MS system can easily be connected to most modern magnetic sector mass spectrometers with only a few modifications. An extraction–focusing plate can be used in combination with a quadrupole instrument to obtain an electrostatic spray.

The system can tolerate a large amount of water in the eluate and also organic acids or ammonia for pH adjustment. Buffered solvents will increase the noise level and buffering agents can only be used at low concentrations. During normal conditions the flow-rate of the solvent into the mass spectrometer is 1–5 μl/min. The pressures in the ion source housing and in the analyser are 10^{-3} and 10^{-4} Pa, respectively. No deterioration of the vacuum system during 3 years of operation has been observed.

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