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DIRECT COUPLING OF PACKED FUSED-SILICA LIQUID CHROMATOGRAPHIC COLUMNS TO A MAGNETIC SECTOR MASS SPECTROMETER AND APPLICATION TO POLAR THERMOLABILE COMPOUNDS

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SUMMARY

Fused-silica columns of 0.22 mm I.D. packed with normal small-particle high-performance liquid chromatography packing material were studied. The shape of the column end allows direct connection to a combined electron impact chemical ionization (EI–CI) ion source. To inject small volumes a syringe-loaded micro-sample injector is used with the time splitting technique. Mass spectra obtained from easily vaporized compounds are similar to, but display less thermal decomposition than, normal EI spectra. Mass spectra of underivatized native mono- and disaccharides give useful structural information. Results from the analysis of cardiac glucosides are also shown. The system provides high-efficiency chromatography with a universal detector, suitable for the analysis of non-volatile and labile compounds.

INTRODUCTION

Many non-volatile and thermally labile compounds can be well separated by liquid chromatography. Identification of the separated components on-line by mass spectrometry (MS) is of great value and great efforts have been made in recent years to combine these methods. The most widely used techniques are direct liquid inlet, moving belt and thermospray[1], all of which are complicated. Good mass spectra can often be obtained, but the interface often has a deleterious effect on the chromatographic separation. A mass spectrometer adapted for chemical ionization (CI) can tolerate a solvent flow-rate into the vacuum system of 10 μl/min. This suggested the use of a liquid chromatography (LC) column operating at this low flow-rate. A micropacked fused-silica column is well suited for this application and it has been used successfully for high-resolution[2,3], normal-resolution[4,5] and, recently, for high-speed LC[6]. This type of column can be very simply interfaced to a mass spectrometer with a combined electron impact (EI)–CI ion source.

In this paper our procedure for preparing the column and the chromatographic and spectrometric system is described and we also report experimental results obtained with volatile, low-volatile and non-volatile compounds.

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EXPERIMENTAL

Micropacked fused-silica column preparation

Fused-silica tubing (20–50 cm × 0.22 mm I.D.) was packed with 3-, 5- and 10-μm high-performance liquid chromatography (HPLC) packing material, using a high-pressure slurry packing technique. It was found necessary to bake the fused-silica tubing at 300°C for 1 h, after which it would withstand the high packing pressure.

In order to effect a simple and efficient coupling of the column to the ion source of the mass spectrometer, the column end was drawn to a fine tip (Fig. 1), designed to hold the chromatographic bed. Inside the tip was placed a small amount of coarser HPLC material (30–50 μm) of the same type as that in the bed. This coarse material was transferred dry into the column. The column was then connected to a slurry bomb (Fig. 2) filled with acetonitrile. Solvent was first pumped through the column for a few minutes at about 100 bar with a simple one-piston pump (Beckman ACCO-FLO) and methanol as pumping media. The chromatographic bed was then packed in the following way.

The packing material, 20 mg for a 30-cm column, was dispersed in 0.8 ml of acetonitrile. The same volume of the solvent in the packing bomb was sucked up

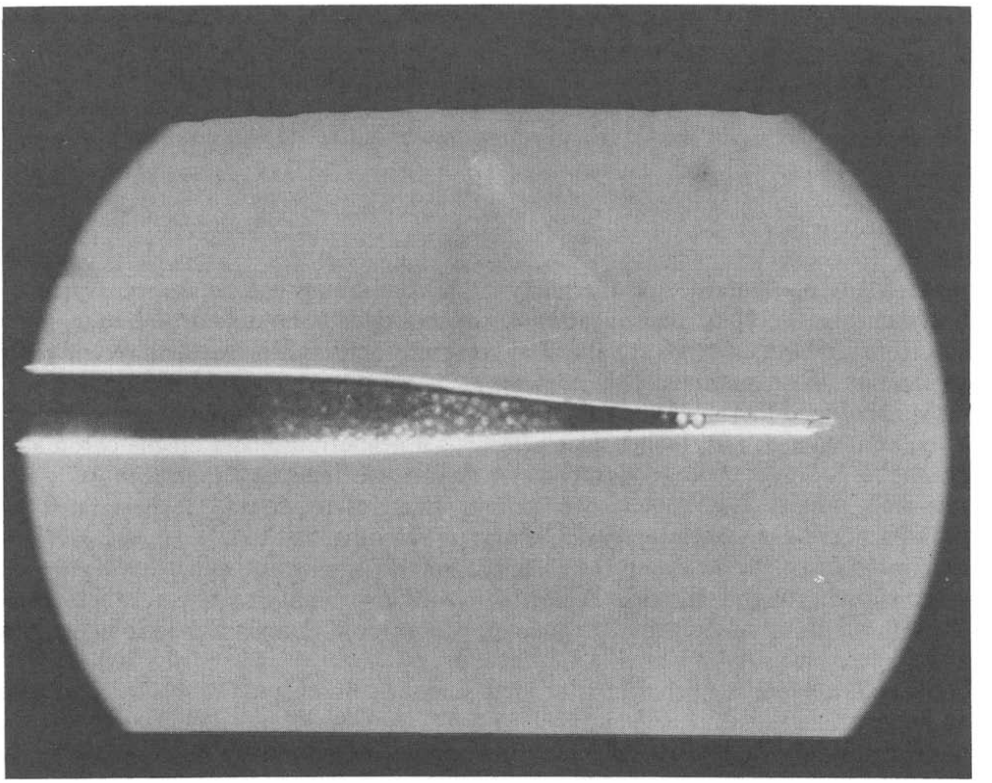

Fig. 1. Column tip. Column I.D., 0.22 mm; outlet I.D., 40 μm. Approximately 1 mm of the column constriction is filled with rougher packing material (30 50 μm).

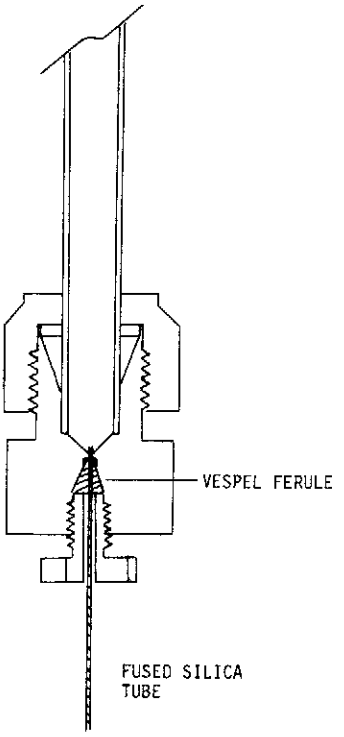

Fig. 2. Connection of the column tube to the slurry bomb. The conical internal shape of the reducer fitting is necessary for homogeneous packing of the column. Vespel is a trademark of DuPont.

with a hypodermic syringe through the top connection. The slurry was then transferred to the bomb. The pressure was increased to 650 bar in 2 min and held at this level. The growth of the column bed can be followed visually. The column was filled in 20 min, then the pressure was allowed to decrease slowly to zero before disconnection.

Chromatography system

An SP 8700 solvent delivery system was used in the constant pressure (purge) mode. The mobile phase was placed in a stainless-steel container under helium at a few bars pressure to maintain the solvent composition and eliminate the problem caused by air bubbles. The normal flow-rate was 1–5 μl/min and was obtained without any modification of the pump.

A 0.5-μl Reodyne 7520 syringe-loaded micro-sample injector was used for sample injection. The fused-silica column was connected directly to the injector (Fig. 3). The dead volume in the injector was about 30 nl. It is important to minimize this volume, because even a slight increase could ruin the chromatographic separation (Fig. 4). To inject small volumes (< 0.5 μl) on the column, a time splitting technique was used. The sample loop was in the inject position for only a few seconds. The straight flow configuration of the injector gave sharp "cuts" of the injected volume.

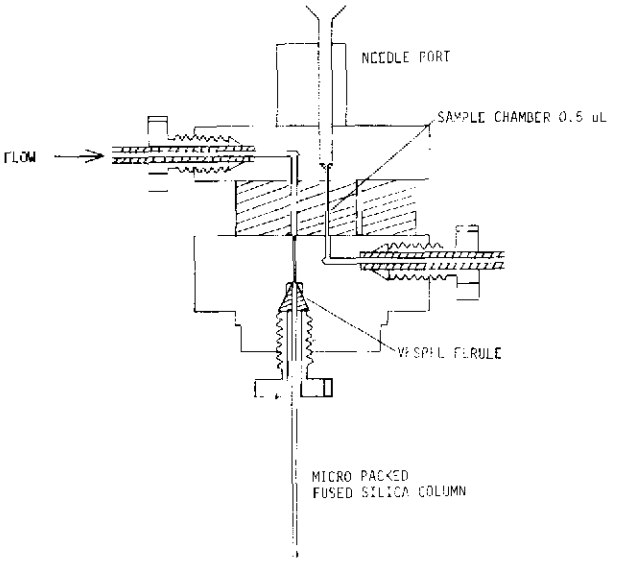

Fig. 3. Schematic diagram of the injector column connection, showing the straight flow configuration of the injector and the simple column connection.

The injected volume can be calculated from the equation

$$v_{inj} = t_{inj} F_c$$

where

$$t_{inj} < \frac{F_c}{v_{loop}}$$

$$F_c = \frac{\pi d^2 L \varepsilon}{4 t_0}$$

F_c = flow-rate (μl/sec); t_0 = unretained sample retention time (sec); d = column I.D. (mm); L = column length (mm); t_{inj} = injection time (sec); ε = pore volume of packing material; v_{loop} = injector loop volume (μl).

Fig. 5 shows that it is possible to inject sample volumes less than the injector chamber volumes. A 10-fold sample reduction is easily achieved. A reduction in sample volume is important if the sample is dissolved in a solvent with a higher or the same solvent strength compared with the mobile phase. For a large sample volume it is necessary to use a solute on-column focusing technique. With this technique a weak solvent modifier should either be present in the sample solution or be introduced just before the injection of the sample[5].

Coupling to the ion source

The column is mounted on a support and is passed through a ball valve (Whi-

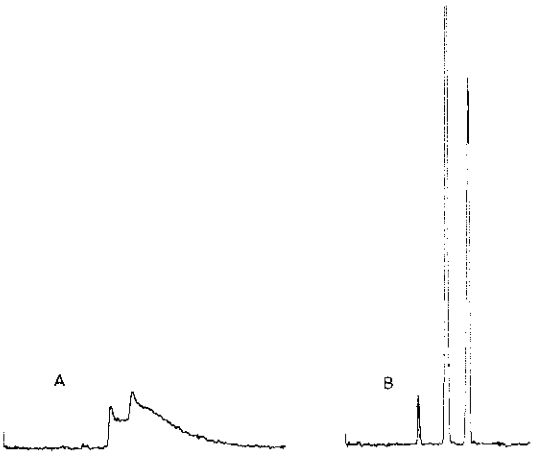

Fig. 4. Illustration of the importance of a low dead volume in the injector–column connection: (A) dead volume *ca.* 2 μl in the connection; (B) minimum dead volume *ca.* 30 nl. Sample: benzene–toluene. Column: 25 cm × 0.22 mm I.D. 5 μm. Nucleosil C_{18}. Mobile phase: methanol water (80:20). Pressure: 60 bar. Detection, total ion current (TIC) (ions of $m/z < 40$ suppressed).

tey 42F2), and is then led through a 0.5-mm I.D. stainless-steel tube of length 100 mm. The tube terminates about 15 mm from one of the four sample ports in the ion source block (Fig. 6). The vacuum seal consists of a ferrule (Vespel) with a precise hole for the column. It is possible to adjust the end position of the column tip simply

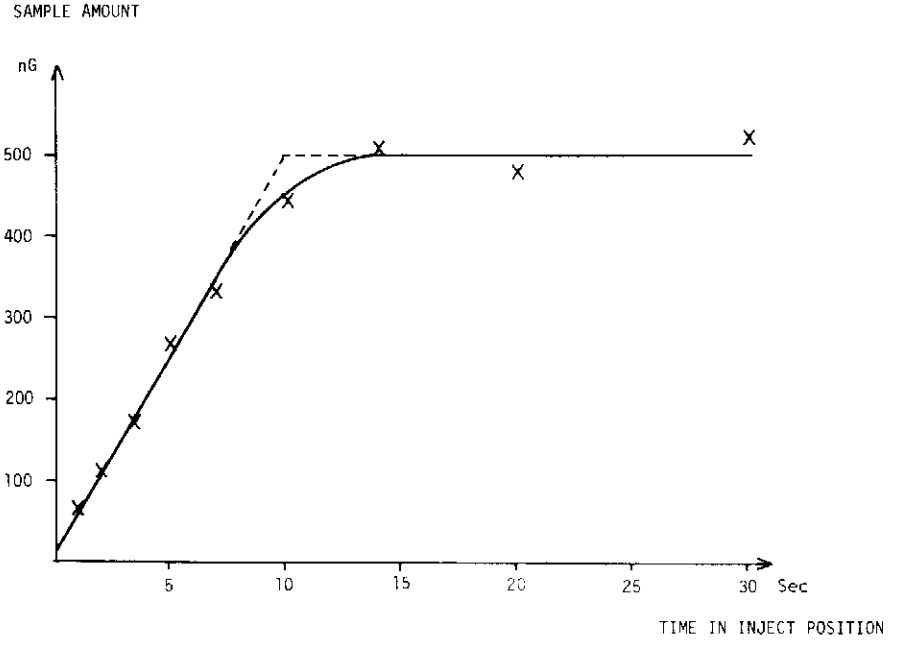

Fig. 5. Sample size reduction with time splitting technique. A flow-rate of 3 μl/min gives full sample injection after 10 sec.

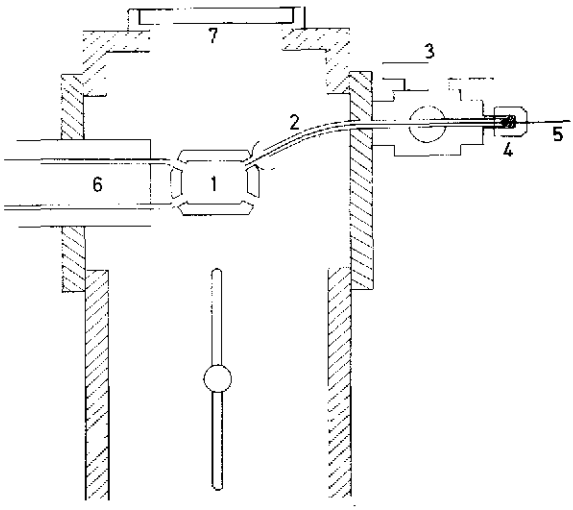

Fig. 6. Schematic diagram of the ion source column connection. 1, Ion source; 2, stainless-steel tube, O.D. 1.6 mm, I.D. 0.5 mm; 3, ball valve; 4, ferrule; 5, LC column; 6, GC column inlet; 7, window.

by moving the column in or out. The ion source (VG 70/70) is a standard EI–CI source, the only modification necessary being to uncover the inlet hole.

Vaporization is governed by four factors, first the configuration of the column tip, second the electrostatic field between the column tip and the ion source block, third the low pressure in the ion source housing and fourth the surrounding temperature. Owing to the electrostatic field, the eluate from the column leaves the tip in form of very small droplets. A finer column tip will give a stronger effect because of the higher field between the column and the ion source block.

The mass spectrometer

The mass spectrometer is a large radius (300 mm) magnetic sector (90°) instrument. Originally it was built by one of the authors (G.S.) for high sensitive GC–MS work. It consists of equipment from several manufacturers, but the high-vacuum system and the analyser were constructed in our laboratory. The ion source housing is pumped by a diffusion pump (Edwards Diffstak 160-700) and is largely isolated from the analyser vacuum system by the ion source exit slit. The analyser is pumped with a second diffusion pump (Edwards EO4) and the detector by a turbomolecular pump (Pfeiffer 250). The forevacuum system consists of two independent forevacuum pumps (Edwards 112). The first pump backs the ion source diffusion pump and is used with gas ballast to prevent contamination of the pump oil with LC solvent. The second forevacuum pump backs the analyser and the detector.

Recording of the chromatogram

To obtain a sensitive recording of the chromatogram, even though relatively large amounts of evaporated eluent are present in the ion source, a special method[7] is used. At a certain strength of the magnetic field, small ions move off in a curve with a small radius and do not hit the electrode (Fig. 7), whereas larger ions hit the electrode as their paths do not curve so much. It is possible to decide the limit of the

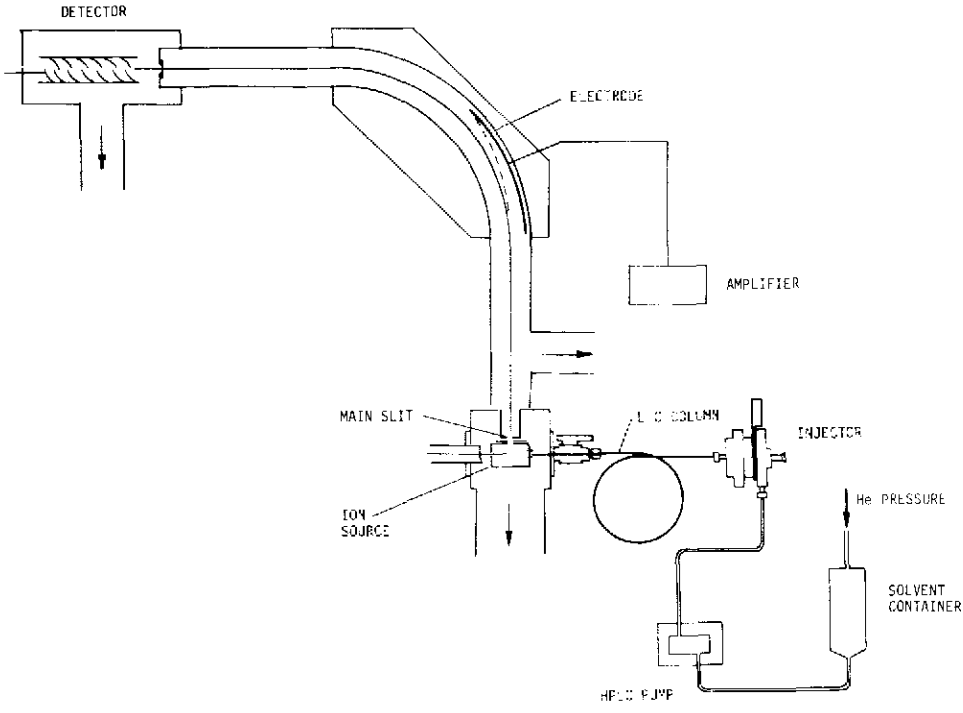

Fig. 7. Schematic diagram of the LC–MS system.

heavy ions that will be discriminated by simple adjustment of the magnet current. The advantage with the described arrangement is that ions derived from the LC eluent are not registered. A very sensitive amplifier (Keithly 614) and a plotting integrator (Perkin-Elmer LCI 100) are used.

RESULTS

Column testing

The column performance was determined by injection of easily vaporized samples. Fig. 8 shows a typical test chromatogram. The columns are characterized by a high number of theoretical plates, N (165 000 plates per metre for this column), good peak symmetry and a low pressure drop. The height equivalent to a theoretical plate (HETP) is about twice the particle size for 3- and 5-μm material. The number of theoretical plates obtained depends on the injection technique but with good injection the HETP value is constant for K values greater than 0.3. The HETP values for these types of columns are less dependent on the flow-rate than glass or PTFE columns[4], which makes it possible to choose a suitable flow-rate for fast or sensitive chromatography.

LC detection

A great advantage of the micro LC system is its ability to handle both small and relatively large amounts of substance (several micrograms). Micro systems are

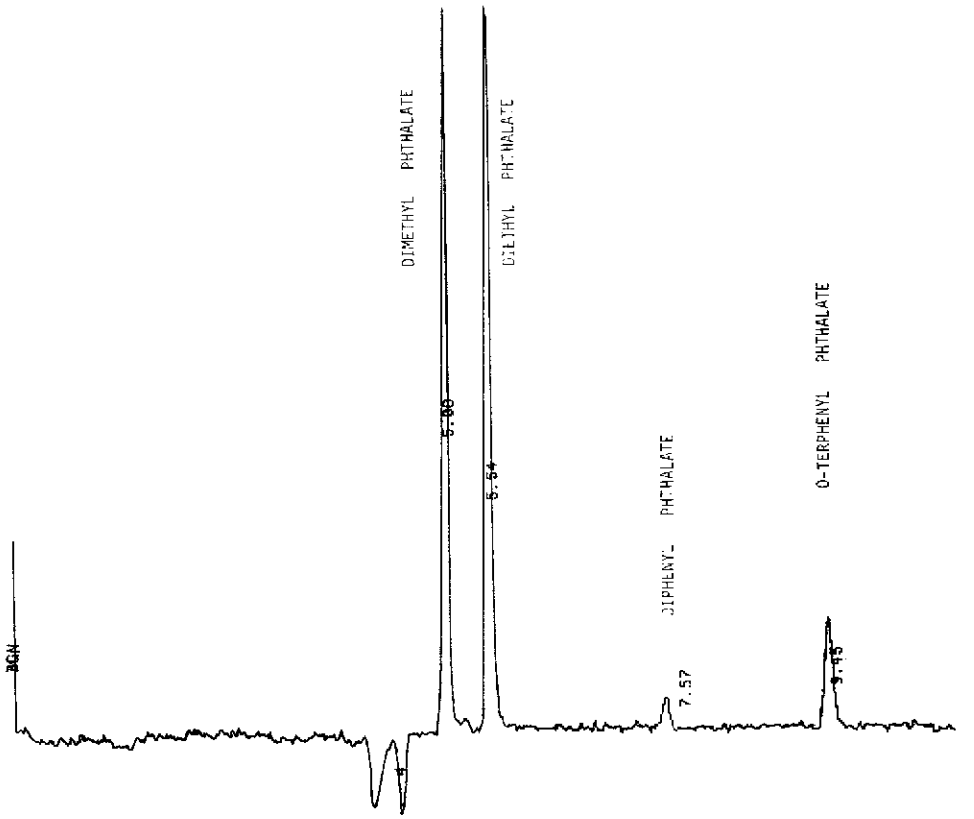

Fig. 8. Column test chromatogram. Sample: HP isocratic test solution. Column: 30 cm × 0.22 mm I.D. 3 μm Spherisorb ODS. Mobile phase: methanol–water (80:20). Pressure: 60 bar. Detection: TIC (ions of m/z <40 suppressed). Number of theoretical plates, N = 49,500 (for *o*-terphenyl phthalate k' = 1.4).

often used when the amount of sample is limited and a sensitive detector is therefore desirable.

Our mass spectrometer, used as a detector for liquid chromatography, is a universal detector and in most instances has no influence on the chromatographic separation. The LC sensitivity for easily vaporized compounds (*e.g.*, xylene) is in the low nanogram range (Fig. 9).

Mass spectra

The mass range for the spectrometer is m/z 0–1500 at an acceleration voltage of 4 kV and 0–1200 at 5 kV. A resolving power ($m/\Delta m$) of over 2000 can be achieved but for higher sensitivity a lower resolution (*ca.* 1200) is often chosen.

Most spectra from the LC–MS system were obtained under the following conditions: electron energy, 70 eV; acceleration voltage, 5 kV; distance between column tip and electron beam, *ca.* 10 mm; ion repeller voltage, 0 V; and ion source temperature, 230°C.

Good mass spectra of easily vaporized compounds eluted from the LC column (*e.g.*, xylene) can be obtained from a few nanograms. If we compare the sensitivity

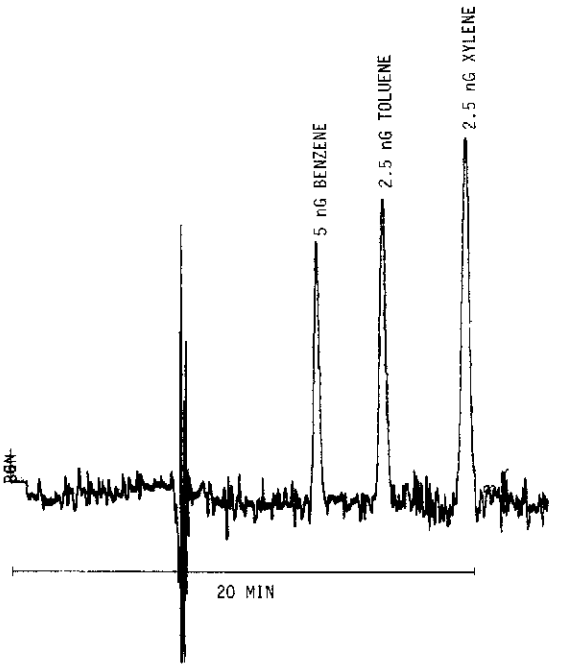

Fig. 9. Separation of nanogram levels of hydrocarbons. Column: 25 cm × 0.22 mm I.D. 3 μm Nucleosil. Mobile phase: methanol–water (80:20). Pressure: 90 bar. Detection: TIC (ions of m/z <40 suppressed).

(10 pg) for the same compound run in the GC–MS mode on the same spectrometer, there seems to be possibilities for improvements.

APPLICATIONS

The main advantage of LC–MS over GC–MS is the ability of LC to deliver samples into the ion source with considerably reduced or no thermal decomposition. The following results from labile compounds demonstrate this ability.

Volatile and low-volatile compounds

Sinapic acid. Fig. 10 shows the mass spectra of free sinapic acid obtained with three different chromatographic systems. The top spectrum (A) was obtained from a GC system with a split–splitless injector (Grob type) working at 230°C. The GC column was a 1 m × 0.22 mm I.D. deactivated fused-silica column coated with SE-54. The column temperature was increased from 30 to 200°C in 5 min. To obtain the mass spectrum in Fig. 10B, the same column was used but with an on-column injector (Schomburg type) working at 30°C. The bottom spectrum was obtained with the LC system under the same conditions as in Fig. 8. The ion source temperature was 230°C in all three instances.

In Fig. 10A there are no peaks above m/z 181. The eluate from the GC column was not sinapic acid but thermally decarboxylated sinapic acid. The decarboxylation occurs in the injector and the substance formed gives a sharp GC peak. Fig. 10B

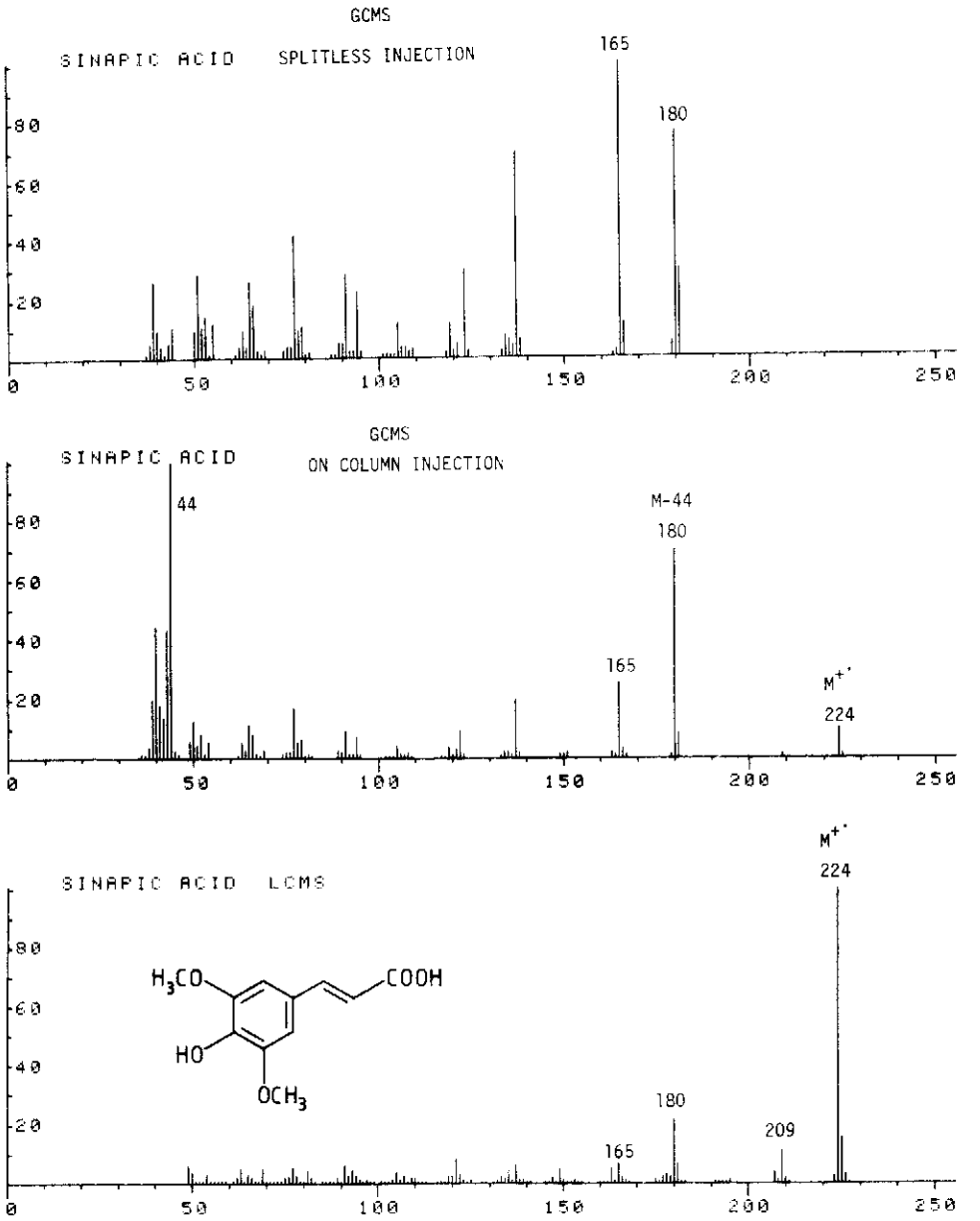

Fig. 10. Mass spectra of free sinapic acid obtained with three different chromatographic systems: GC–MS with splittless injection; GC–MS with on-column injection; LC–MS.

shows an M^+ peak at m/z 224 and a base peak at 180 m/z $(M - CO_2)^+$. A prominent peak at m/z 44 indicates that decarboxylation occurs in the ion source or in the end of the GC column. The LC–MS spectrum of free sinapic acid (Fig. 10C) shows that the M^+ peak at m/z 224 is the base peak. Other prominent ions are (M − 15) at m/z 209 and (M − 44) at m/z 180.

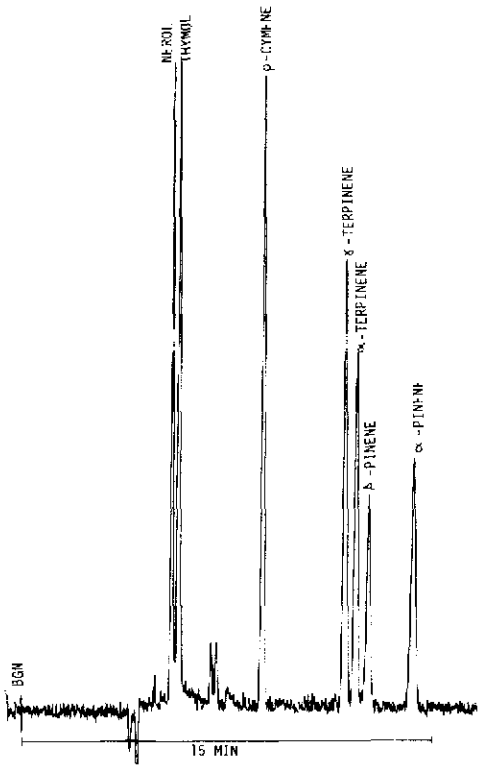

Fig. 11. Separation of a terpene mixture. LC conditions as in Fig. 8.

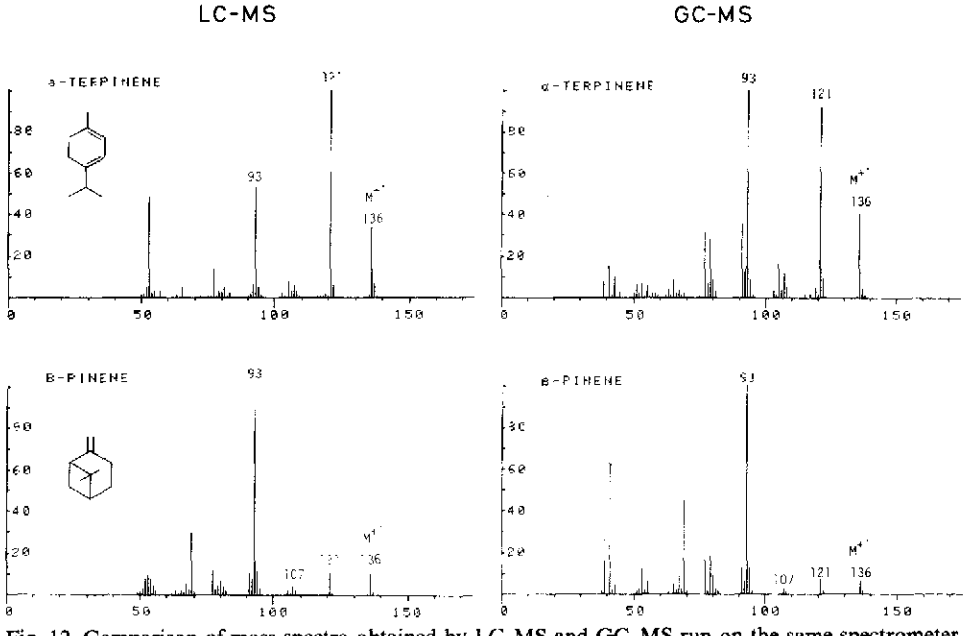

Fig. 12. Comparison of mass spectra obtained by LC–MS and GC–MS run on the same spectrometer.

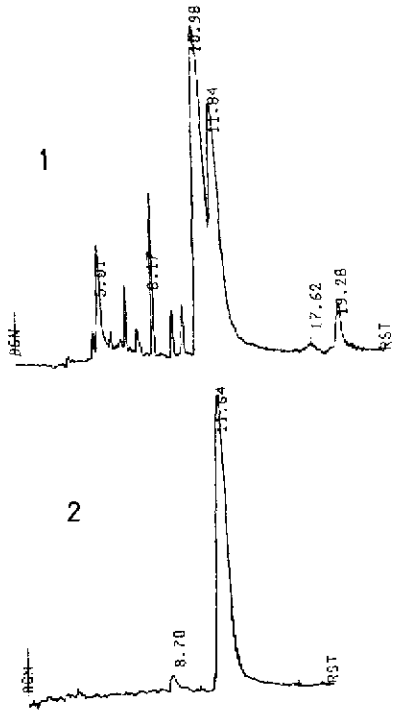

Fig. 13. Liquid chromatogram of (1) crude pine resin in comparison with (2) reference sample (abietic acid). Column: 30 cm × 0.22 mm I.D. 3 μm Spherisorb ODS. Pressure: 150 bar. Mobile phase: methanol–water (90:10) adjusted to pH 4 with formic acid. Detection: TIC (ions of $m/z < 40$ suppressed). Source temperature: 230°C.

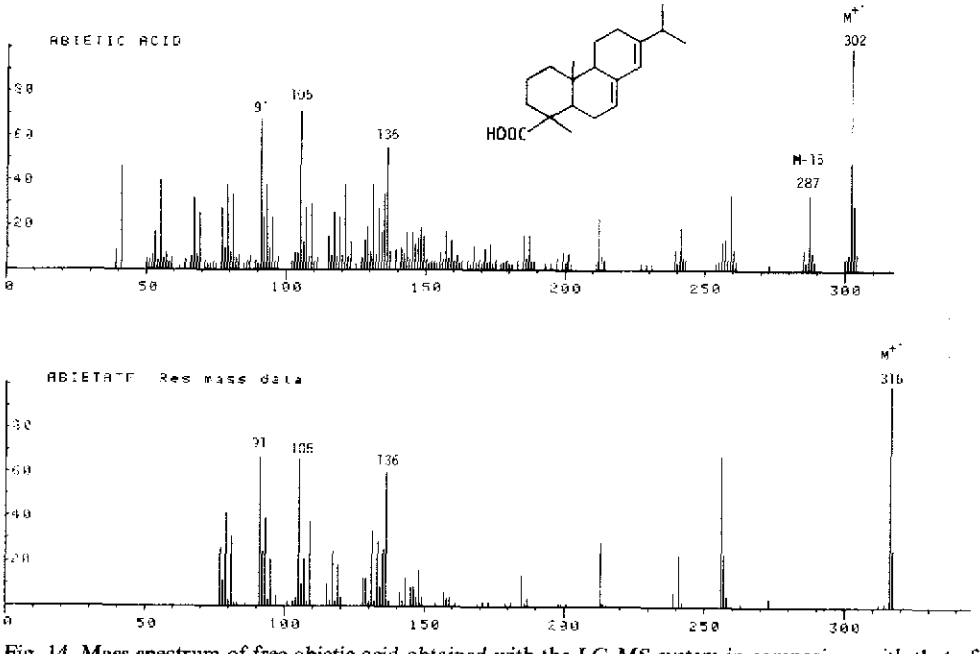

Fig. 14. Mass spectrum of free abietic acid obtained with the LC–MS system in comparison with that of methyl abietate obtained from ref. 9.

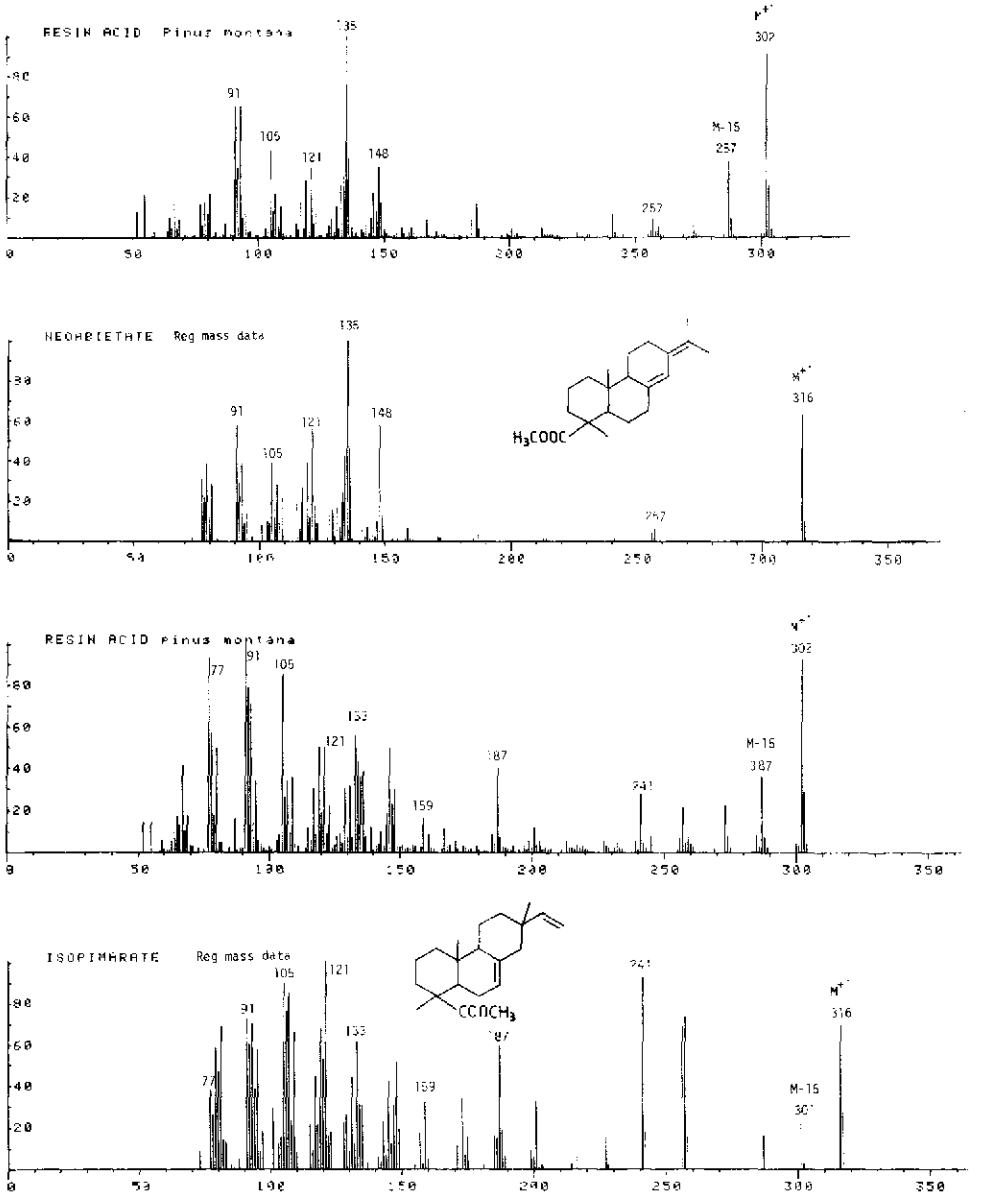

Fig. 15. Mass spectra of the two main components of *Pinus montana* resin in comparison with reference spectra from ref. 9.

Monoterpenes. Monoterpenes are often well separated by capillary GC[8]. LC–MS offers a new method for the separation and identification of this class of compound. The separation occurs in a cold liquid phase, which decreases the risk of decomposition. The increased possibility of influencing the selectivity is another advantage of LC. Fig. 11 shows the separation of a terpene mixture and in Fig. 12 LC–MS mass spectra from two monoterpenes are shown in comparison with the

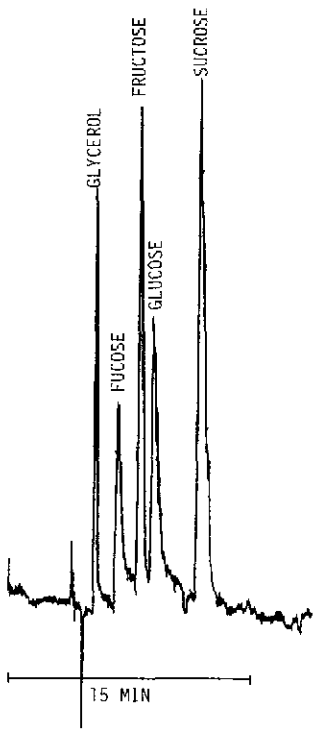

Fig. 16. Separation of a sugar mixture. Column: 26 cm × 0.22 mm I.D. 5 μm Nucleosil-NH_2. Pressure: 50 bar. Mobile phase: acetonitrile–methanol water (60:30:10). Detection: TIC (ions of m/z < 50 suppressed).

corresponding GC–MS spectra. These comparisons show that spectra obtained from the two systems are very similar. Mass spectra from the LC–MS system can thus be interpreted by comparison with spectra from reference collections.

Resin acids. The best known of the diterpenes is abietic acid, the major constituent of pine rosin. To obtain good mass spectra of resin acids it has previously been necessary to esterify the acid. Free resin acid can be analysed with the LC–MS system. Fig. 13 shows the chromatogram of a crude sample (*Pinus montana*) in comparison with a reference compound (abietic acid). The small peaks at the beginning of the chromatogram are monoterpenes. LC–MS spectra of abietic acid and of abietate from a reference library[9] are compared in Fig. 14. The molecular ions are prominent in both spectra. Note the great similarity in the lower mass region. Abietic acid was not observed in the *Pinus montana* resin. Mass spectra (Fig. 15) indicate two related compounds.

Non-volatile compounds

Monosaccharides. A common HPLC method for the separation of monosaccharides is the use of an amino column and a mobile phase consisting of 60–80% acetonitrile in water. To suit our LC–MS system we had to modify the mobile phase because of freezing at the column tip owing to the high heat of vaporization, by adding a small amount of methanol to the acetonitrile.

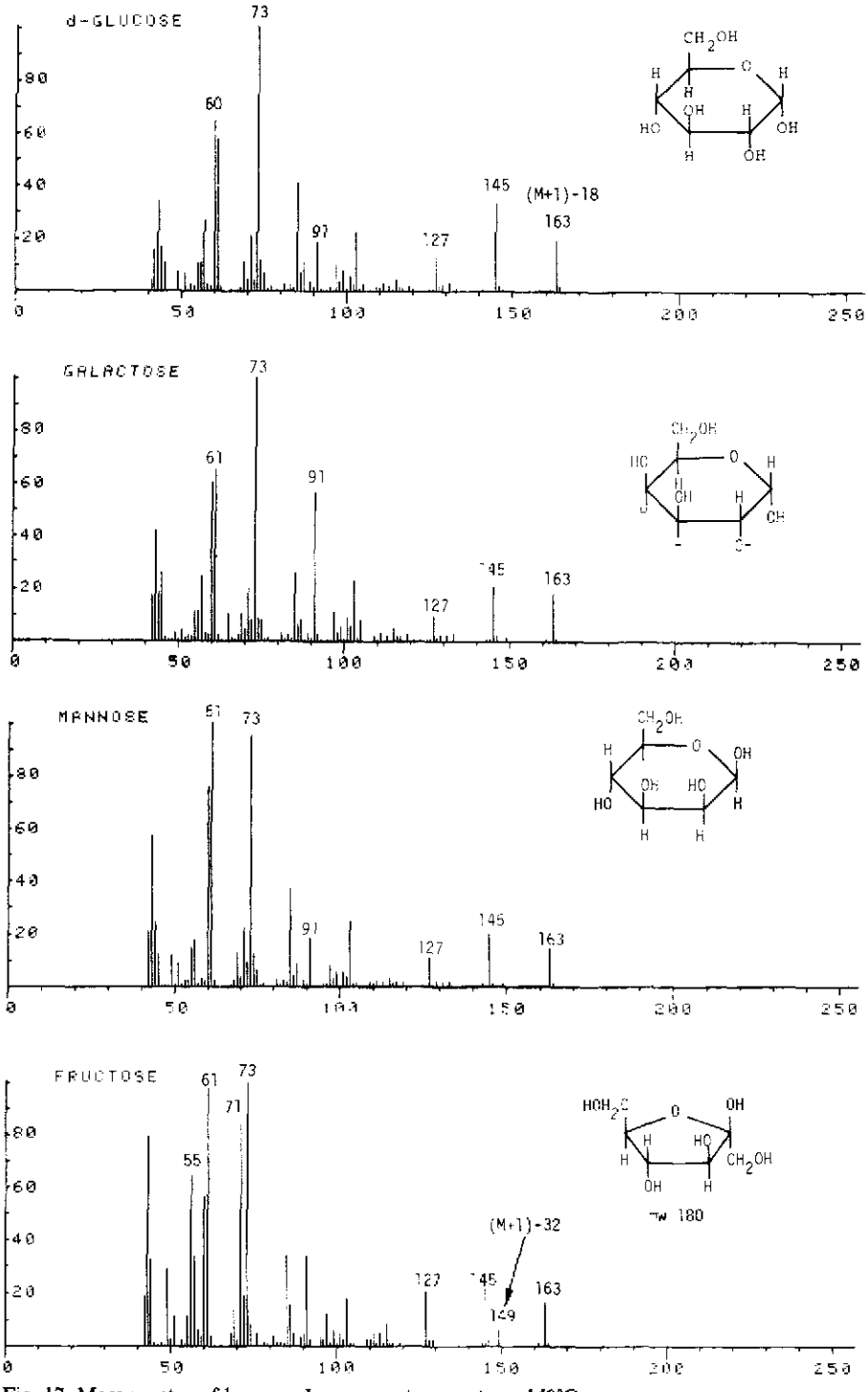

Fig. 17. Mass spectra of hexoses. Ion source temperature: 140°C.

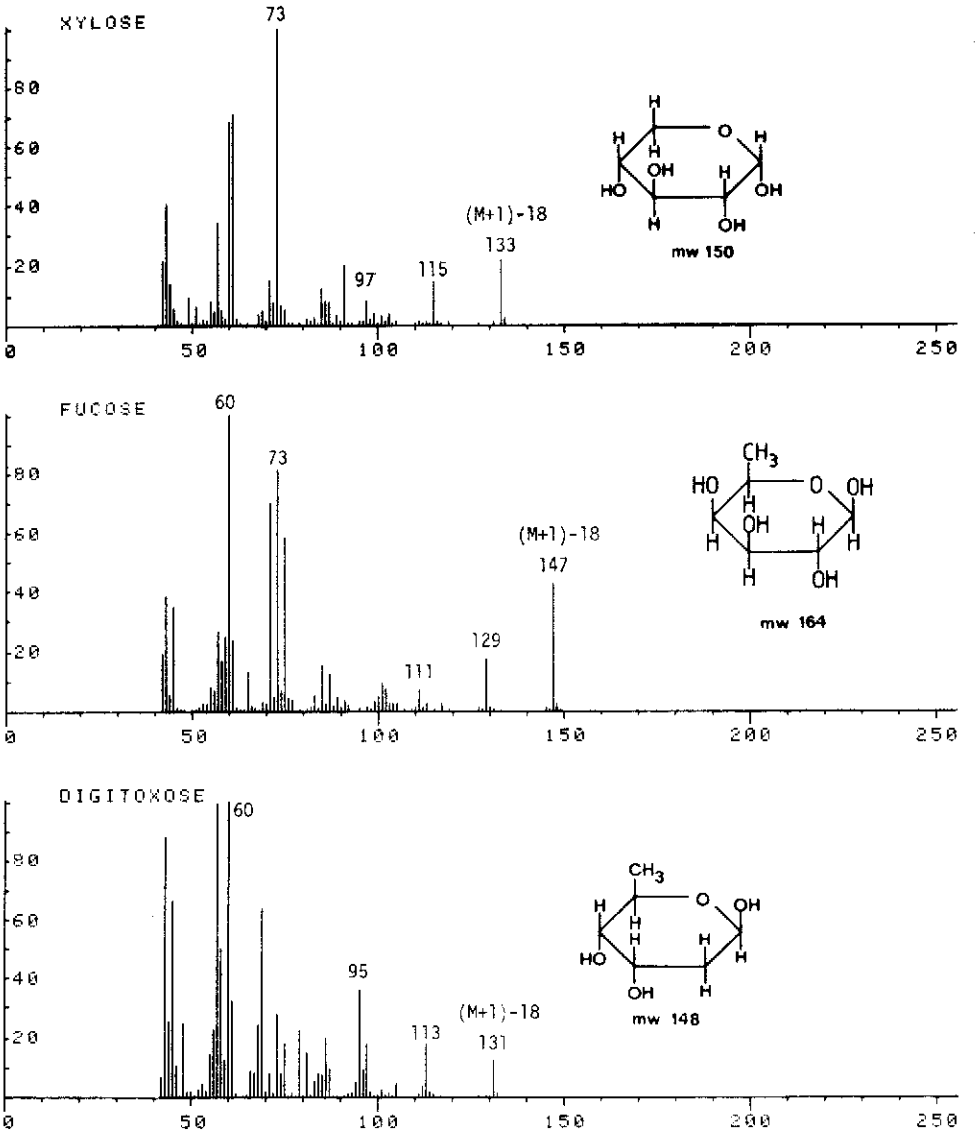

Fig. 18. Mass spectra of xylose, fucose and digitoxose. Conditions as in Fig. 17.

An example of the separation of a sugar mixture is shown in Fig. 16. Mass spectra of hexoses (Fig. 17) show no molecular peak but a relatively strong $(M + H - H_2O)^+$ peak. Other prominent peaks are due to loss of water, *m/z* 145 and 127. The spectra of glucose, galactose and mannose are similar but the intensities of the peaks at *m/z* 60, 61 and 91 are different. The fructose spectrum shows a peak at *m/z* 149 [probably $(M + H - CH_3OH)^+$]. Other differences are the high-intensity peaks at *m/z* 55 and 71. The LC–MS spectra of xylose, fucose and digitoxose (Fig. 18) do not show any peak above the $(M + H - H_2O)^+$ peak. The fragmentation pattern can be interpreted in the same way as in the previous example.

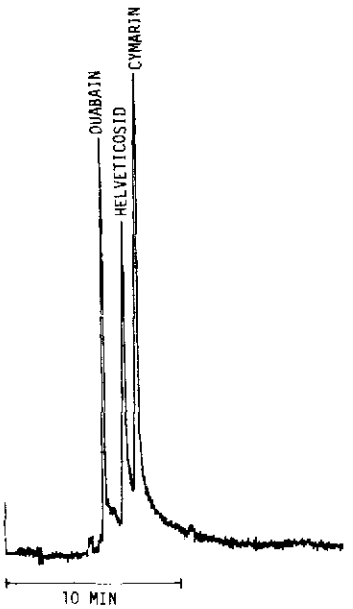

Fig. 19. Separation of three cardiac glucosides. Column: 26 cm × 0.22 mm I.D. 3 μm Spherisorb ODS. Pressure: 150 bar. Mobile phase: methanol–water (80:20). Detection: TIC (ions of m/z <40 suppressed). Ion source temperature: 280°C.

Cardiac glucosides. The primary reason for starting LC–MS work in our laboratory was a structural investigation of cardiac glucosides, which have high biological activity not only medically but also as defence substances in both plants and insects. A separation of three cardiac glucosides is illustrated in Fig. 19. These substances are inclined to stick to the column tip and to overcome this effect the ion source temperature and solvent flow-rate were increased. Complete separation is achieved although there is still a tendency for peak tailing to occur. A narrower bore tip gives the same positive effect but increases the back-pressure.

Mass spectra obtained from cymarin and ouabain are shown in Fig. 20. The ion source temperature was 280°C. Weak but measurable peaks at m/z 549 and 585 are the $(M + H)^+$ peaks. Most of the peaks in the high mass region are related to the successive elimination of water. The middle region of the mass spectra is related to the genin part of the substances. In the low mass region some high-intensity peaks can be related to the sugar part (*e.g.*, m/z 145 for cymarin and m/z 147, 129 and 111 for ouabain).

Cationization

In fast atom bombardment, laser desorption and field desorption mass spectra of glycosides, a peak corresponding to $(M + Na)^+$ is often observed[10,11]. This peak can be of high intensity and is generated by cationization of the sample molecule by sodium ions. Sodium salts are difficult to avoid in water extracts. The sodium ions in a crude natural extract will pass unretained in a reversed-phase LC system. If the sample elutes at the same time, cationization can also occur in our LC–MS system.

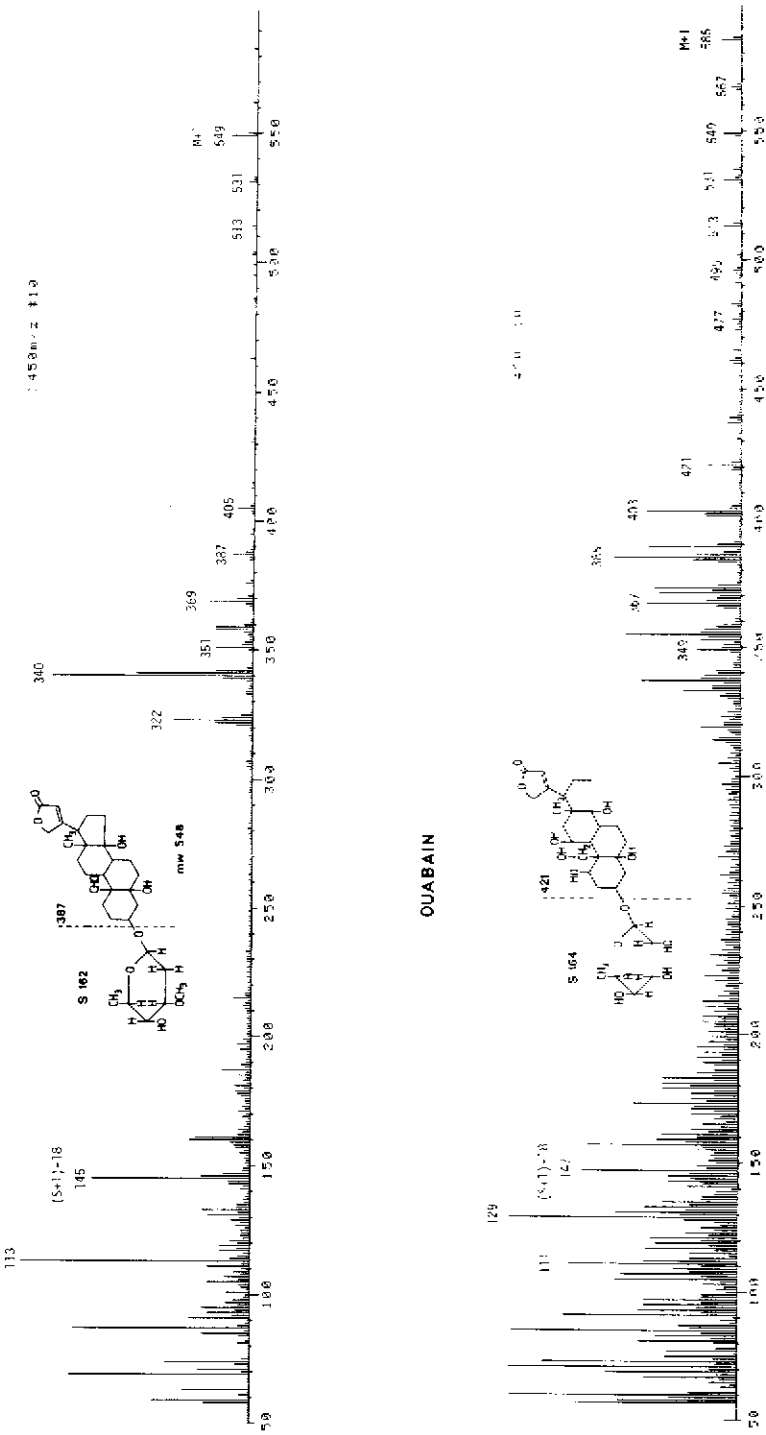

Fig. 20. Mass spectra of cymarin and ouabain.

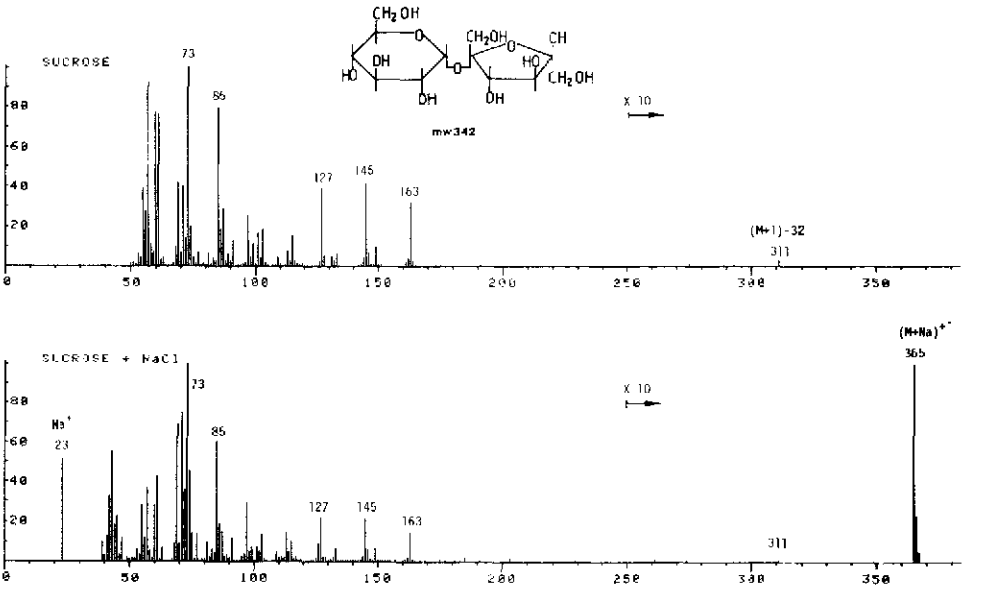

Fig. 21. Mass spectra of sucrose with and without a small addition of sodium chloride to the sample solution.

Fig. 21 shows mass spectra of unretained sucrose. The spectrum looks like a combination of a glucose and a fructose spectrum. A small $(M + 1 - 32)^+$ ion at m/z 311 is present. By adding a small amount of NaCl to the sample solution we obtain the mass spectrum shown in Fig. 21. $(M + Na)^+$ ions give an intense peak at m/z 365. The presence of sodium is also indicated by the peak at m/z 23. With the same method cationization also occurs with potassium chloride.

DISCUSSION

We have reported both LC and MS results for terpenes, phenolic acid, terpene acid, mono- and disaccharides and cardiac glycosides. Only two types of LC columns have so far been tested, but we see no reason why other types of columns and mobile phases cannot be used with this system. Interpretable mass spectra can also be obtained from non-volatile compounds. Owing to the low solvent flow-rate, CI reactant gases can also be added to the ion source to obtain more molecular weight related ions. LC–MS spectra obtained from volatile and low-volatile compounds are similar to ordinary EI spectra and may be interpreted by comparison with normal reference spectra collections. We believe that the sensitivity could be improved with only small modifications to the system. Future tests will also be made with gradient elution.

ACKNOWLEDGEMENTS

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