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DEVELOPMENTS OF MICRO LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY WITH GRADIENT ELUTION

IMPROVEMENTS TO OBTAIN LESS THERMAL DECOMPOSITION OF LABILE COMPOUNDS

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SUMMARY

Fused-silica columns (I.D. 0.22 mm) packed with ordinary small particle liquid chromatographic material were used in direct connection to an electron impact ion source. The electrostatic field between the column end and an extraction–focusing plate, located close to the ion source inlet, was used for nebulization of the solvent. The ion source was modified to obtain higher efficiency and to reduce the thermal decomposition of labile compounds. A system for micro flow gradients (less than 2 μl/min) has been developed. The flow of a pumping medium (glycerol) is divided into two parallel streams and the flow distribution is controlled using the temperature dependence of the viscosity. The glycerol flow is changed by two "media converters" to the chromatographic solvents. Applications of micro liquid chromatography–mass spectrometry are shown for a plant allelochemical, dhurrin, obtained from Sorghum leaf extract. Mass spectra of glucose, sucrose, neral, geranial, chlorsulphuron, myoinositol, 3,5-dinitrobenzoic acid, amitriptyline, 10-hydroxyamitriptyline and omeprazole after liquid chromatography are shown.

INTRODUCTION

The main difficulties involved in coupling liquid chromatography (LC) with mass spectrometry (MS) are much the same as the difficulties of combining packed gas chromatography (GC) with MS: the two techniques operate at pressures which differ by several orders of magnitude. In LC–MS, the problem is even more complicated, since the chromatograph works in the liquid state and the mass spectrometer in the vapour state.

An important consideration in an LC–MS interface is the maintenance of high chromatographic performance. From a chromatographic point of view, freedom to select mobile phases and additives, possibility of gradient elution, no loss of resolution caused by the interface, high linear dynamic range, low detection limits, and high-molecular-weight detection capabilities are important factors for an effective LC–MS system. The LC–MS system is a particularly useful tool for the identification

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of thermolabile allelochemicals, especially when informative mass spectra of the separated components can also be obtained. The implications of narrow-bore packed columns (*e.g.*, micro-packed fused-silica columns) for LC–MS become apparent when one considers the low flow-rate relative to normal-bore columns. This is because, for the same velocity, the flow-rate is proportional to the square of the column diameter. A 0.2 mm I.D. column operates at liquid flow-rates approaching 1 μl/min, corresponding to gas flows of *ca.* 1 ml/min. This gas flow-rate is equivalent to that found in capillary GC–MS and, as a result, the fused-silica micro-packed columns can be attached directly into the mass spectrometer ion source for electron impact (EI) ionization.

The LC–MS system discussed here was first presented at the Montreux meeting in 1984[1], and the first version of the gradient system was presented at the following Montreux meeting in 1986[2].

EXPERIMENTAL

The micro-packed fused-silica LC column

The column which is used in the LC–MS system is prepared by packing fused-silica tubing of 200–500 mm length and 0.2 mm I.D. with 3- or 5-μm liquid chromatography (HPLC) packing material[1]. Special attention has to be paid to the design of the column end[2]. The best performance, so far, is obtained when a small silica tube is placed at the end of the column. This tube is filled with coarser packing material (10–50 μm) and its purpose is to support the column bed and to provide an effective spray of the effluent into the ion source via a small and distinct nebulization point.

Coupling to the ion source

The column, which is mounted on a support for easy handling, is simply led (through a valve) into the ion source. The tip of the column terminates just in front of a 1-mm diameter hole in an extraction–focusing plate (EF plate) which is placed 1.5 mm in front of the inlet hole in the ion source block (Fig. 1). The inlet hole is 10 mm wide except for the entrance, where the diameter is 4 mm. Nebulization is governed mainly by the electrostatic field between the column tip and the EF plate, causing the

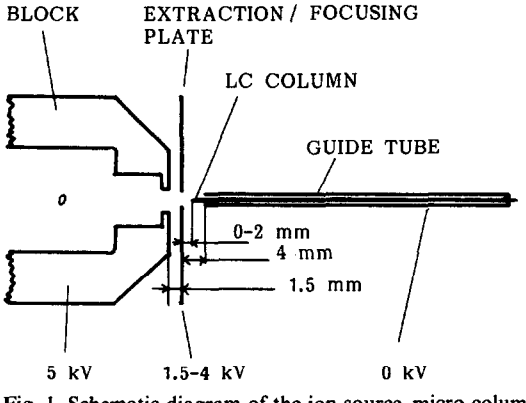

Fig. 1. Schematic diagram of the ion source–micro column connection.

eluate from the column to leave the tip as very small droplets. This is due to two main effects caused by the electrostatic field: (1) the surface tension of the solvent is decreased, and (2) the formed droplets are polarized as the distribution of charges at their surface is changed. The polarized droplets are drawn out by the field between the tip and the EF plate and focused by the field between the plate and the ion source block.

The distance between the column tip and the electron beam inside the ionization chamber of the source has to be short, otherwise the sample molecule may possibly hit the hot walls of the ion source block on its way into the ionization chamber. To avoid this, the EF plate was included between the tip and the block. The distance between the tip and the plate is critical, as shown by Fig. 2. The same sample amount was injected and all other conditions were the same except for the distances, which were 0.0 mm and 1.0 mm, respectively. In the latter case the signal-to-noise ratio was increased by a factor of at least five.

Fig. 3 shows the effect of the EF plate potential on peak height. The same amounts of sucrose were injected at different potentials between 1200 and 3100 V on a reversed-phase system with the mobile phase consisting of methanol–water (80:20). The best response was obtained at 1800 V.

Another important observation was that no heating is needed for the nebulizaiton of the solvent. With the new design of the ion source inlet, the nebulization of the solvent and the ionization of the molecules are processes independent of each other.

Injection of samples

The injection of sample on to the column is made with a syringe-loaded microsample injector (Rheodyne 7520) utilizing a pulsatile injection method for each sam-

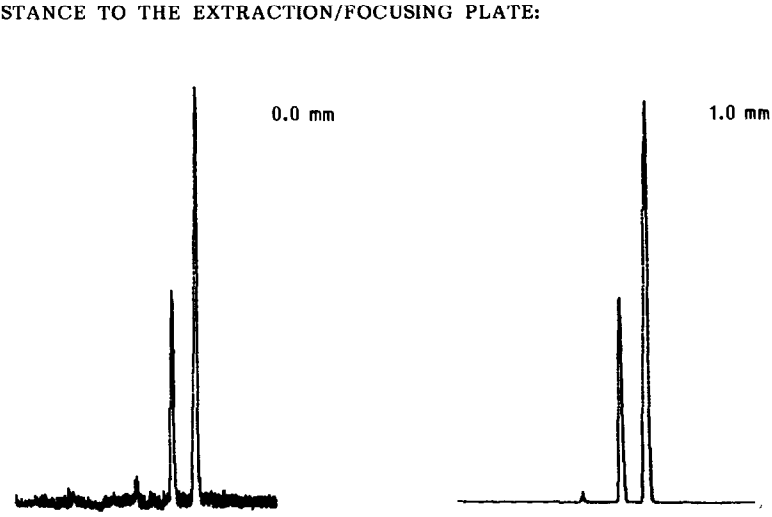

Fig. 2. Effect of the distance between the column tip and the extraction–focusing plate. Column: 25 cm × 0.22 mm I.D., 3-μm Nucleosil C_{18}. Mobile phase: methanol–water (80:20). Injection: 2 ng toluene and 4 ng *m*-xylene.

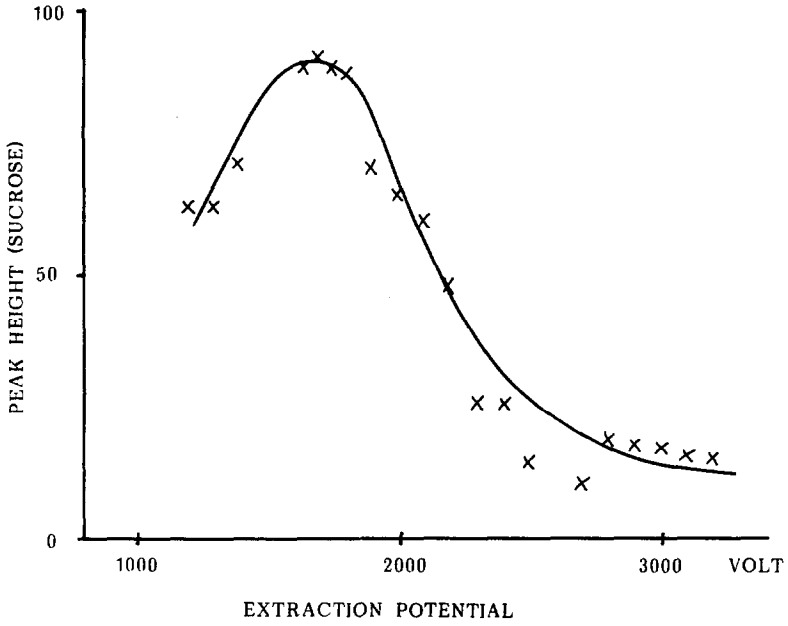

Fig. 3. Effect of the extraction–focusing plate potential on peak height. Peak height (mm) of sucrose (100 ng injections) at different plate potentials.

ple[1]. The sample chamber of the rotor is turned to the inject position for only a few seconds. The configuration of the rotor gives sharp "cuts" of the injected volume, and this is important for the utilization of the high separation efficiencies of the micro-packed columns. An important consideration, when a micro system is used, is to avoid the extra band broadening effects of, *e.g.*, "dead volumes" in the connections of the column. Therefore the column is connected directly to the injector and the ion source block.

Solvent delivery system

An ordinary LC solvent pump is used in the system, and has the ability to work in a constant pressure mode. However, it would be easier to obtain the optimal flow-rate and to predict the retention if a constant flow pump was available for these particular flow-rates, around 1 μl/min.

Gradient system

With a gradient system utilizing the flow properties of a liquid, a practical gradient elution with such low flow-rates (1–2 μl/min) is possible (Fig. 4). If the flow is divided into two parallel tubes which are connected at the ends, the pressure drop is the same for both tubes and the flow distribution between the tubes can be controlled by changing the viscosity of the flowing media. The viscosity of, *e.g.*, glycerol is highly dependent on temperature and, therefore, by temperature programming of the tubes it is possible to obtain a solvent gradient.

The greatest difficulty in the construction of the gradient system has been the

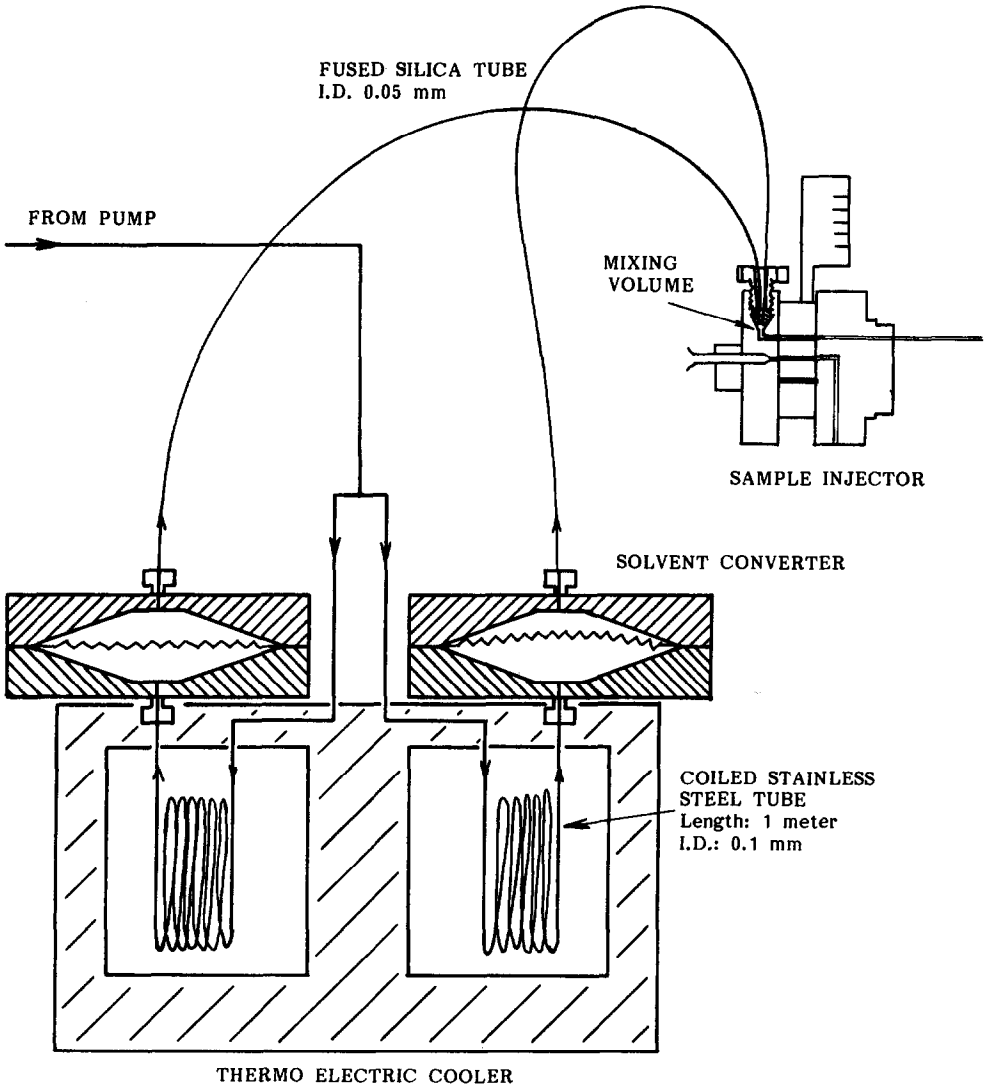

Fig. 4. Schematic diagram of the gradient system.

design of the device which converts the flow of glycerol to the same flow of LC solvent. The divider which separates the LC solvents from the pumping media must be flexible, inert and non-penetrable. After testing several designs and materials for the "solvent converter", we finally chose a divider made of thin, checkered, soft copper sheet. This membrane is placed between strong flanges of stainless steel. The solvent volume of each "converter" is 17 ml, which is sufficient for more than 200 hours of operation. The solvents are led through 50 μm I.D. fused-silica tubes of 50 cm length to a small mixing volume (1 μl) at the LC injector inlet.

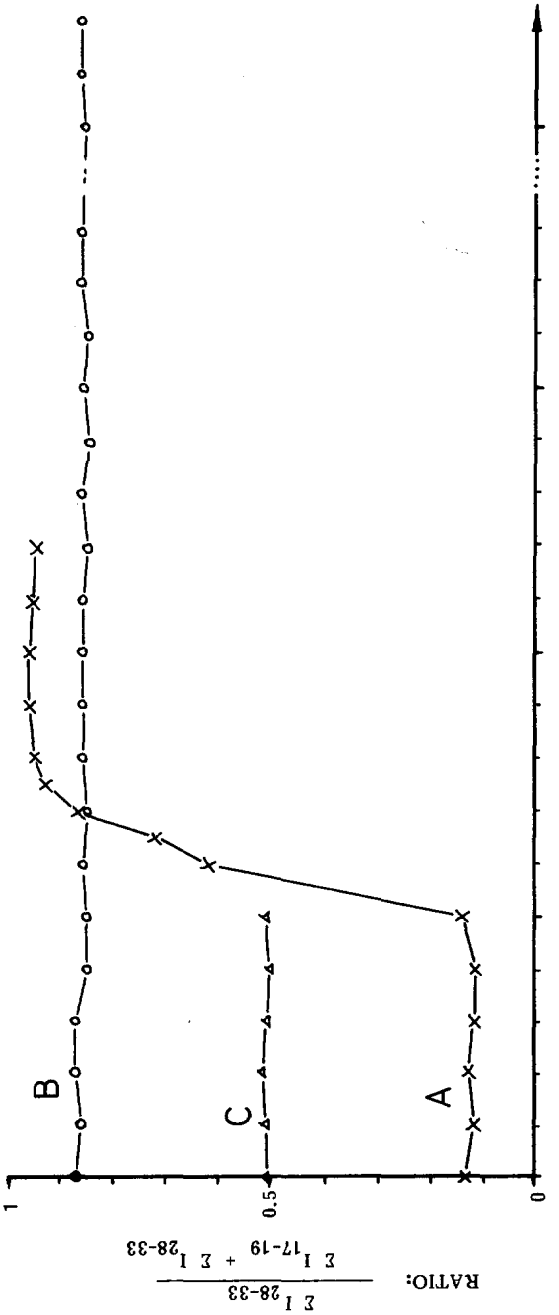

Fig. 5. Time diagram of solvent composition. Curve A: a rapid change from 14 to 95% of methanol in water. Curve B: constant setting at 86% during a period of 1 h. Curve C: constant setting at 50%. For method of obtaining solvent ratio see text.

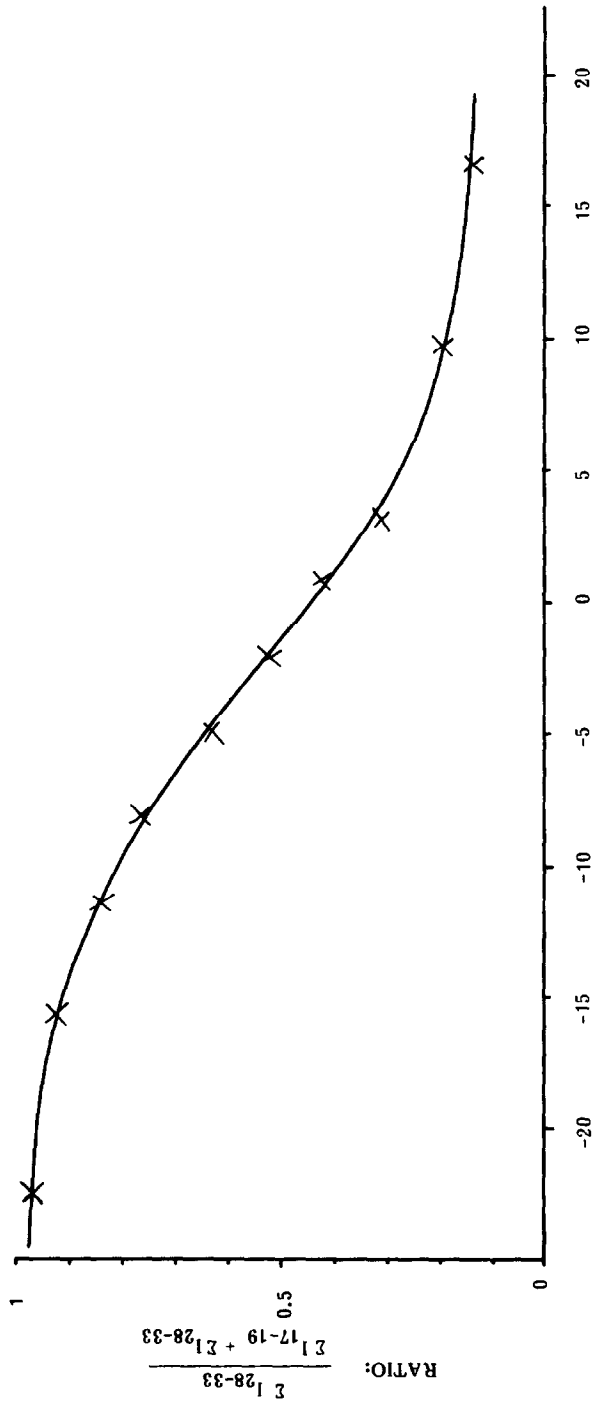

Fig. 6. Solvent composition (methanol–water) as a function of the tube temperature difference. For explanation of y axis see text.

The viscosity of glycerol is highly dependent on the temperature, especially in the lower temperature range. If the temperature is changed from −8 to +8°C the viscosity changes from 4500 to 18 000 cP. To be able to work with temperatures below ambient, this being needed to obtain a wide composition range, a thermoelectric cooler is used. The tubes inside the cooler can be heated by an electric current led directly through each tube. The electric power input for heating of the tubes matches the heat pumping of the cooler, and that power can be directed to the tubes as desired. Because the tubes are air-cooled their temperatures can be changed rapidly.

Curve A in Fig. 5 shows the result of a rapid change from 14% to 95% methanol in water. The composition of the solvent is measured by its mass spectrum and calculated in the following way: the sum of the peak intensity values for methanol (*m/z* 28–33) is devided by the sum of the intensities for water (*m/z* 17–19) plus the sum for methanol. The elution time for an unretained sample (t_0) is 4.0 min, and the new solvent composition reaches the column after 1 min. The final composition (95%) elutes from the column after less than 8 min. Measurements at a constant setting of

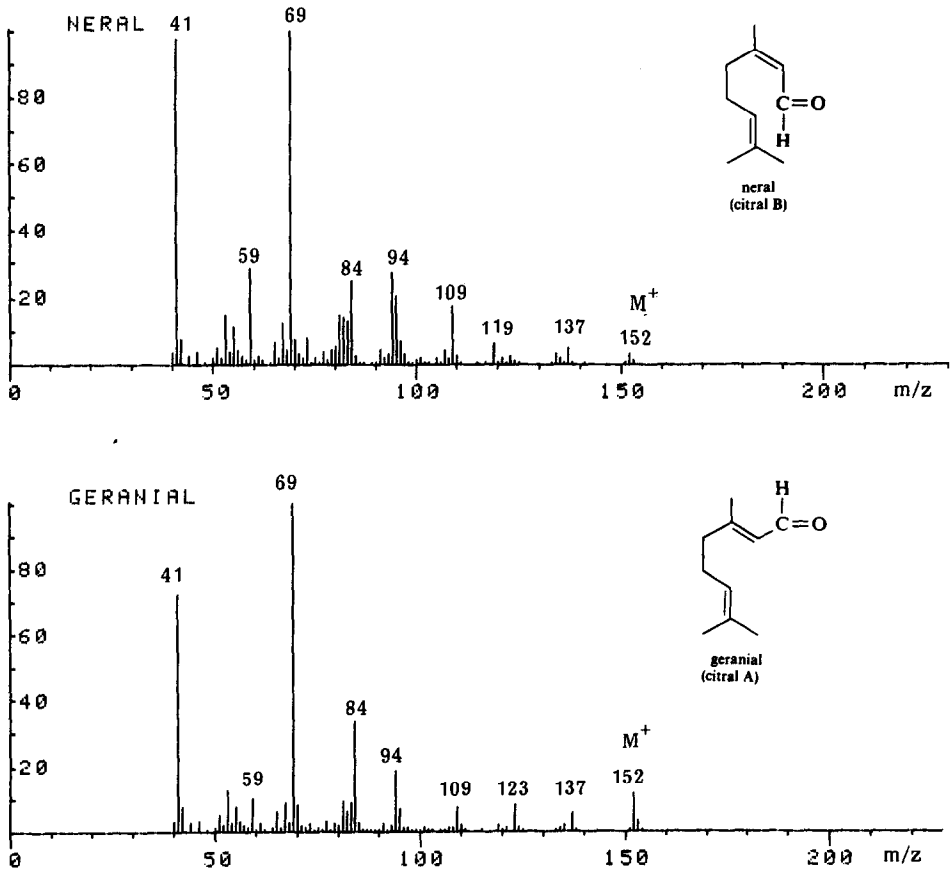

Fig. 7. Mass spectra of neral (above) and geranial. Ion source temperature 110°C.

methanol–water (86:14) during operation for 1 h are shown in Fig. 5, curve B, providing a standard deviation of 1%. The same tendency is shown for the 50% setting, curve C. Fig. 6 shows the solvent composition as a function of tube temperature difference. The differential temperature measurements were made with a digital thermometer (Fluke 52) using thermocouples attached to the tubes. The solvent composition was calculated by its mass spectrum, as above. The mean value of three spectra for each temperature difference are plotted. The curves can be used to calculate the solvent composition when the temperatures of the tubes are known. However, a more accurate determination of the solvent composition can be made by using its actual mass spectrum.

Measurements of the capacity ratio (k') at constant solvent composition for the injections of a solution of aromatics during a period of 3 h gave the following results: for benzene, mean k' 0.54 with standard deviation (S.D.) of 0.002; for toluene, mean k' 0.572, S.D. 0.004; and for *m*-xylene, mean k' 0.612 with S.D. 0.006.

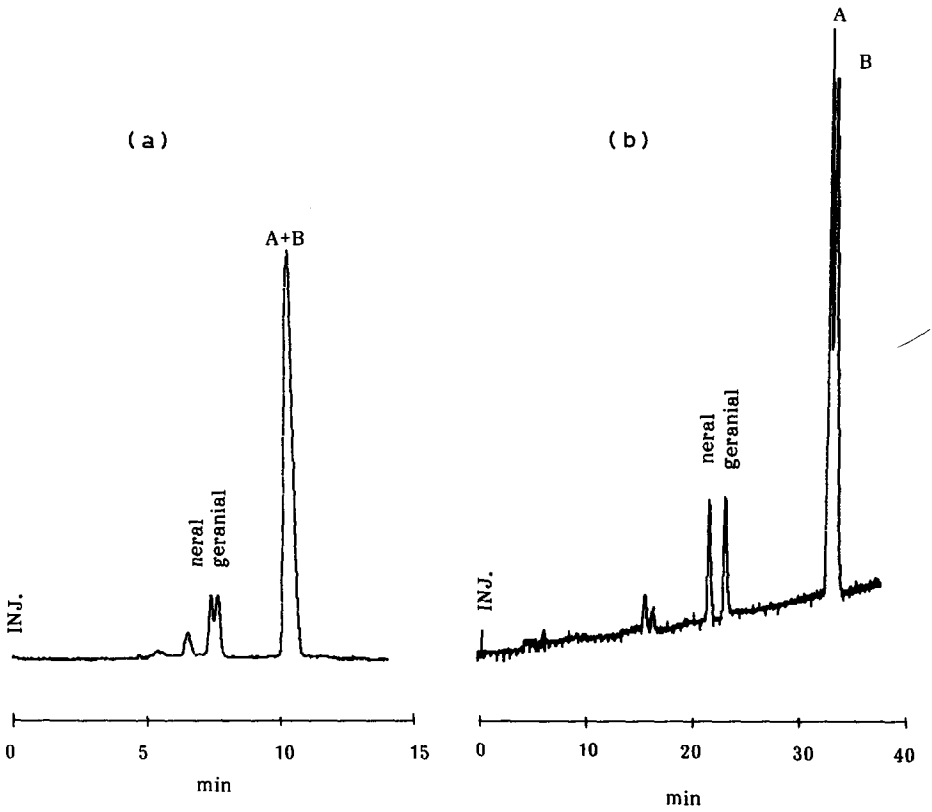

Fig. 8. Liquid chromatograms of a methanol–water (50:50) solution of citral. (a) Isocratic, methanol–water (85:15). (b) Gradient of methanol–water from 60:40 to 90:10 in 40 min. Column: 25 cm × 0.22 mm I.D., 3-μm Nucleosil C_{18}. Detection: total ion current (ions of m/z <40 suppressed).

RESULTS

LC–MS analysis of citral

Citral is a mixture of two components, neral (3,7-dimethyl-2-*trans*, 6-octadien-1-al) and geranial (3,7-dimethyl-2-*cis*, 6-octadien-1-al), which were once difficult to separate. Citral has been found to be a widespread trigger of behaviour in insects. Fig. 7 shows mass spectra of neral and geranial after LC. Like other mass spectra obtained from volatile and less volatile compounds, they are similar to ordinary EI spectra and therefore, may be interpreted by comparison with normal reference spectra.

Repeated injections of citral dissolved in methanol–water (50:50) showed that the amounts of neral and geranial decrease gradually and new peaks appear in the chromatograms. As shown by Fig. 8, the components were difficult to separate with an isocratic reversed-phase system (C_{18}) and, therefore, were used as test substances for the gradient system. With a methanol–water gradient composition from 60:40 to 90:10, the two compounds are separated, not completely, but sufficiently to give mass spectra of the individual compounds, as shown in Fig. 9. The peak at *m/z* 198 is the molecular ion peak; the strong *m/z* 69 peak indicates an acyclic monoterpenoid and arises by loss of the terminal isopentenyl group. The identification has not yet been

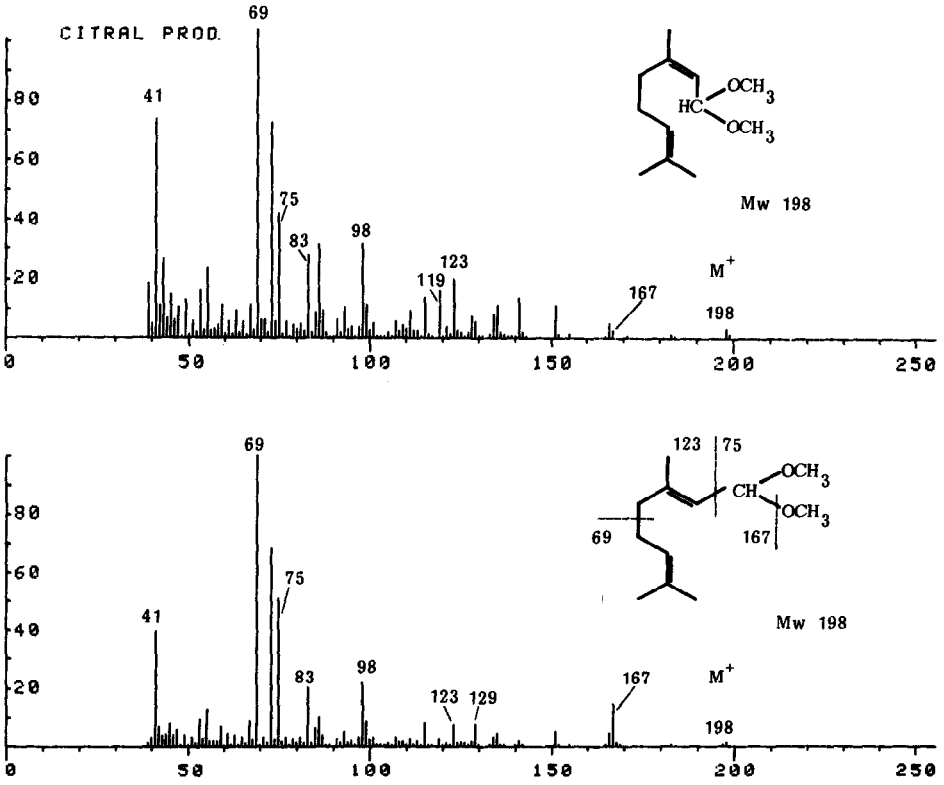

Fig. 9. Mass spectra of nerylacetal (above) and geranyl-acetal. Ion source temperature 110°C.

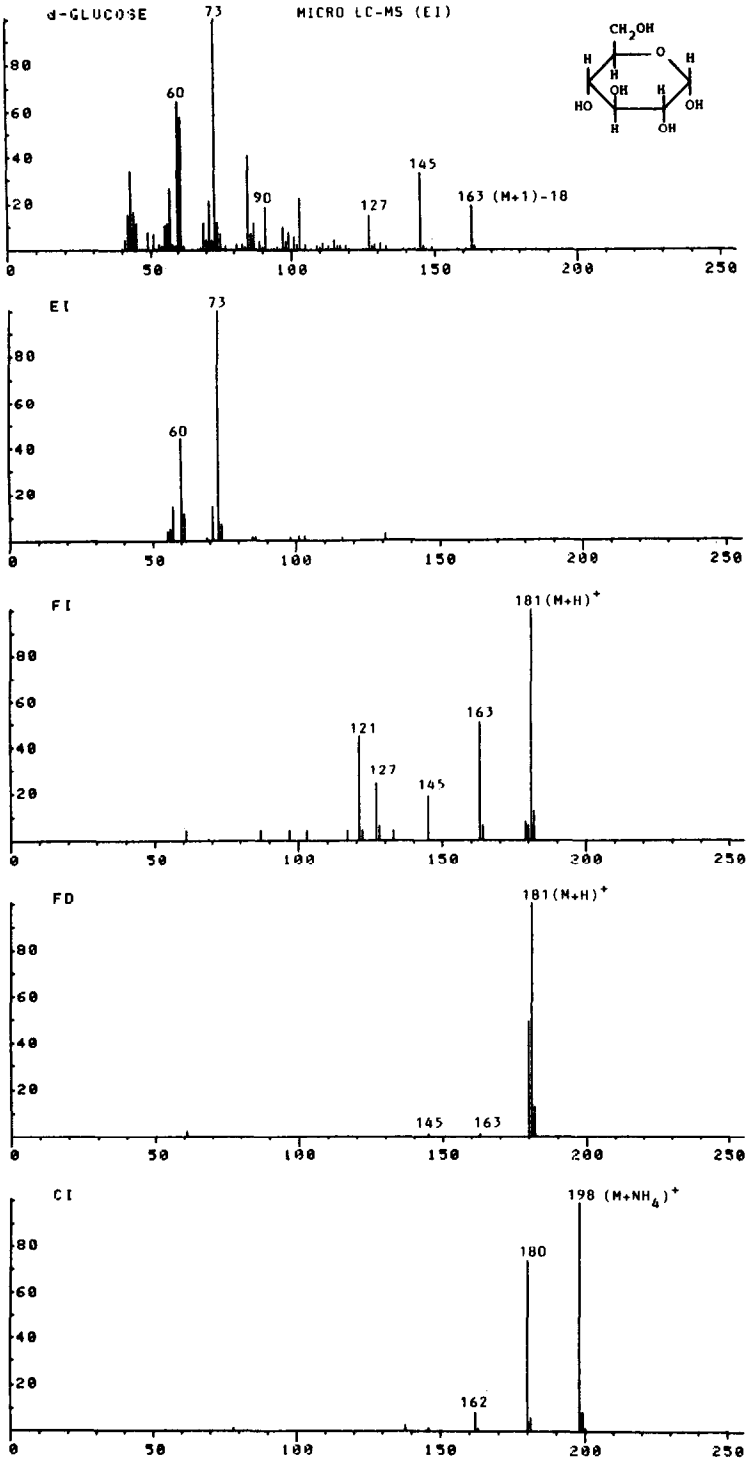

Fig. 10. Micro LC–MS, electron impact (70 eV), field ionization (FI), field desorption (FD) and ammonia chemical ionization (CI NH_4) spectra of D-glucose.

confirmed, but the mass spectra give strong evidence of acetal formation of the two components, *e.g.*, as shown by the peak at *m/z* 75.

Saccharides

A comparison can be made in Fig. 10 between the mass spectra of glucose obtained by using five different MS techniques: micro LC–MS, EI, field ionization (FI), field desorption (FD) and chemical ionization (CI)[3]. Although the spectrum from micro LC–MS shows no molecular ions, it gives structural information from the fragmentation pattern.

The sucrose spectrum obtained after modification of the LC column coupling to the ion source is shown in Fig. 11 (B). It is similar to the spectrum of Fig. 1(A) except for the peak at *m/z* 311 [(M + 1) − 32], which is about three times as intense owing to the decreased thermal degradation.

LC mass spectrum of dhurrin

One major peak in the chromatogram of a leaf extract (from sorghum cultivars susceptible to insect pests) was elucidated by the LC–MS system. The fraction was collected from an analytical LC system and injected on to the micro-LC column, giving a sharp peak. By its mass spectrum, which is shown in Fig. 12, the fraction was identified as dhurrin, a cyanogenic glucoside previously found in sorghum. The peak at *m/z* 311 is the molecular ion. The mass spectrum also shows typical glucose peaks at *m/z* 60, 61, 73, 91, 127, 145 and 163. There are two alternative ways of breaking the glucosidic bond, which give the peaks at *m/z* 149 and 132. In the spectrum, the *m/z* 132 peak dominates that at *m/z* 149, probably because of the nitrile group attached to the same carbon atom as the sugar.

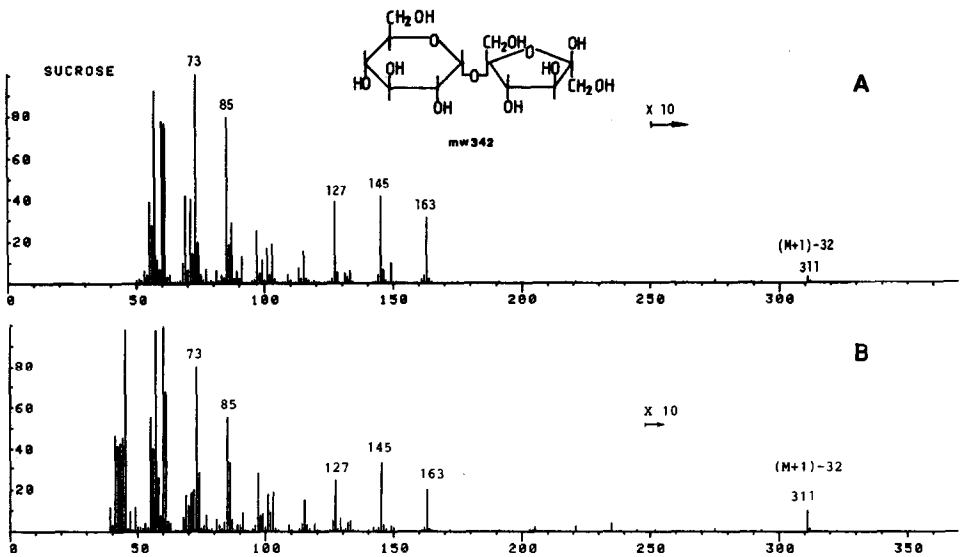

Fig. 11. Mass spectra of sucrose before (A) and after (B) the modification. Ion source temperature: 180°C.

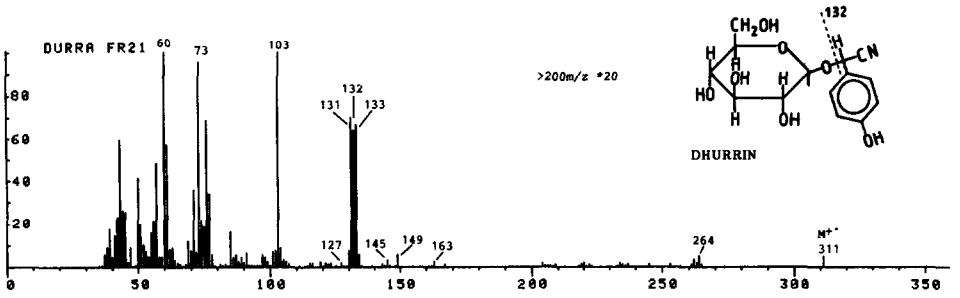

Fig. 12. Mass spectrum of dhurrin obtained from Sorghum leaf extract. Ion source temperature: 140°C.

Mass spectra of some other thermolabile compounds after LC

Chlorsulphuron, a sulphonylurea herbicide, gave at 110°C ion source temperature the mass spectrum shown in Fig. 13. The molecular ion at *m/z* 357 is present. A peak at *m/z* 321, due to loss of hydrogen chloride, and a number of other structurally useful fragments of the molecule are observed. At ion source temperatures above 200°C all information in the high mass region is lost. A chemical ionization mass spectrum of chlorsulphuron is given in ref. 6.

The mass spectrum of myoinositol can be seen in Fig. 14. No molecular ion peak is present, but a small M + 1 peak exists at *m/z* 181. The peak at *m/z* 163 is formed by the loss of water from the protonated molecular ion.

The mass spectrum of 3,5-dinitrobenzoic acid is shown in Fig. 15. The molecular ion peak (*m/z* 212) is the most abundant in the spectrum. The peak at *m/z* 182 (M − 30) corresponds to the loss of nitric oxide, and other large peaks are due to elimination of the nitro groups, *viz.* *m/z* 155 and 120.

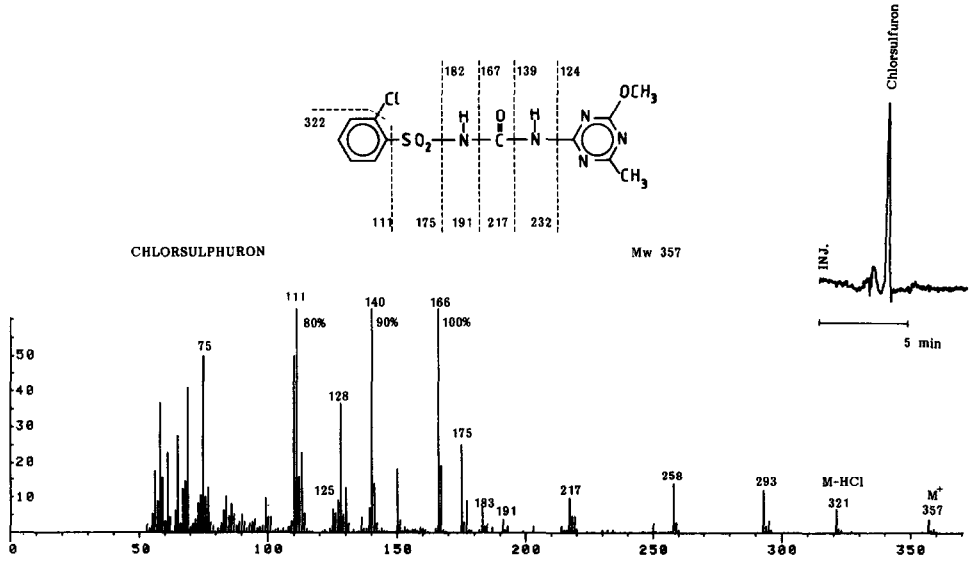

Fig. 13. Mass spectrum of chlorsulphuron. Ion source temperature: 110°C. The total ion current trace (ions of *m/z* <40 suppressed) of 50 ng chlorsulphuron injected on column is shown to the right.

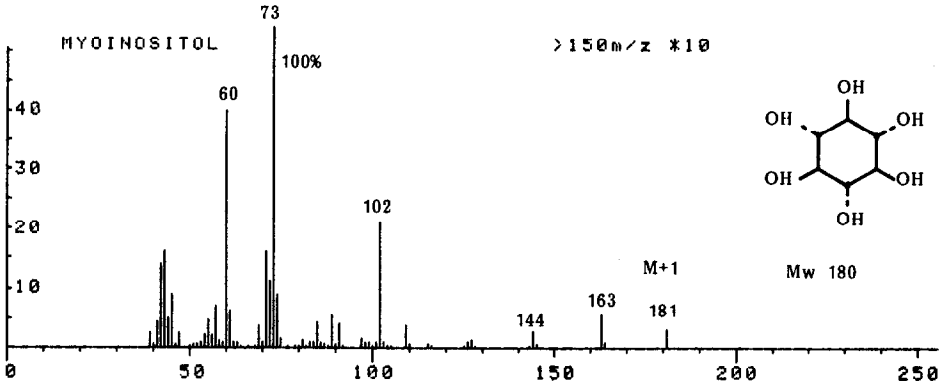

Fig. 14. Mass spectrum of myoinositol. Ion source temperature: 165°C.

The mass spectra in Fig. 16 of amitriptyline (an antidepressive drug) and 10-hydroxyamitriptyline (a metabolite) are both dominated by a very intense immonium ion peak at *m/z* 58. The spectra show molecular ion peaks at *m/z* 277 and 293. Hydroxyamitriptyline gives a peak at *m/z* 276 probably due to loss of water from the protonated molecular ion ($M + 1 - H_2O$). The fragmentation patterns in the middle region, which are almost the same for the two compounds, are not yet fully interpreted. However, the LC–MS method could give structurally important information in addition to the acetylation or chemical ionization MS techniques used[7].

Fig. 17 shows a mass spectrum of omeprazole in comparison with a direct (in-beam) EI spectrum. The molecular ion peak is present at *m/z* 347 in both spectra. The *m/z* 329 peak in the mass spectrum is due to the loss of oxygen. The fragment at *m/z* 150 corresponds to the protonated fragment at *m/z* 151, which is the base peak in the mass spectrum.

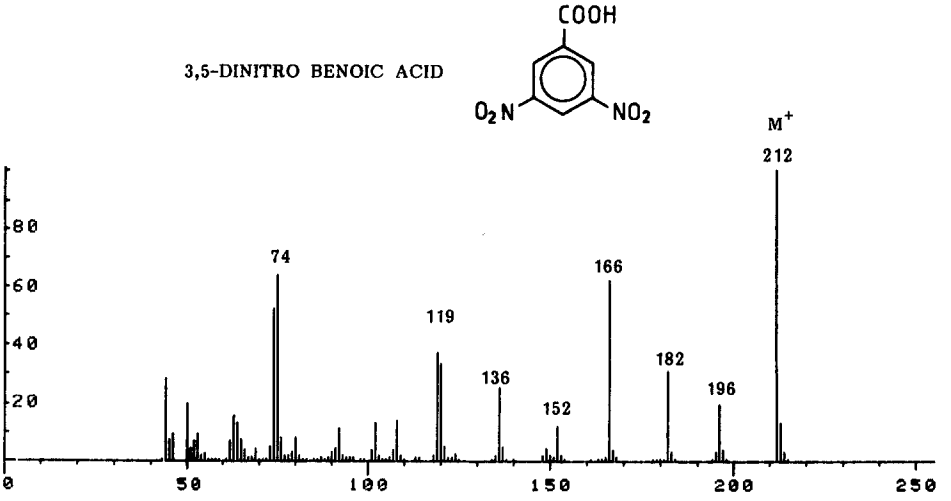

Fig. 15. Mass spectrum of 3,5-dinitrobenzoic acid. Ion source temperature: 210°C.

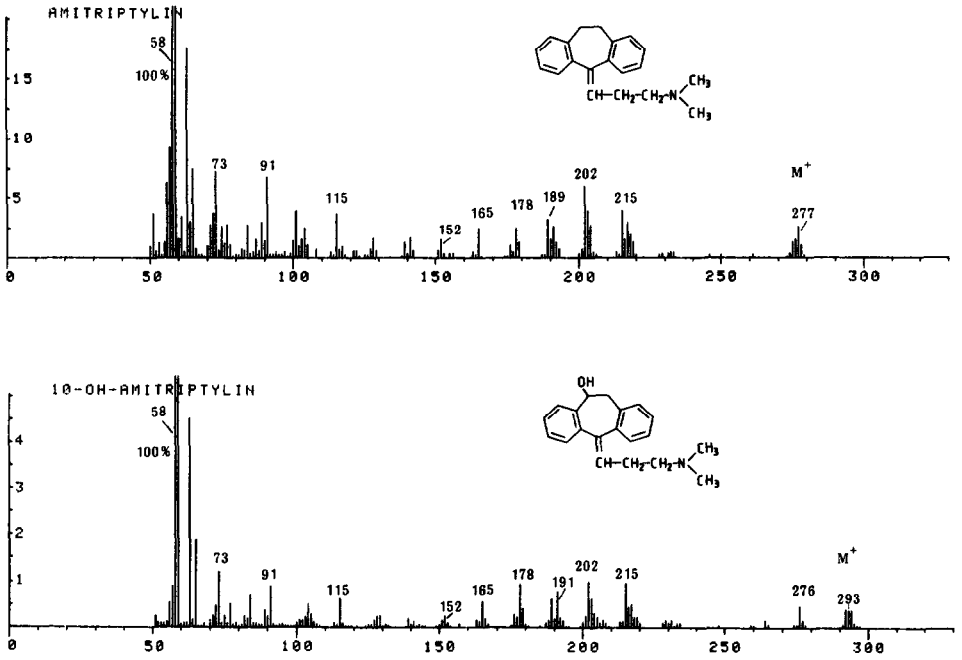

Fig. 16. Mass spectra of amitriptyline (above) and 10-hydroxyamitriptyline. Ion source temperature: 150°C.

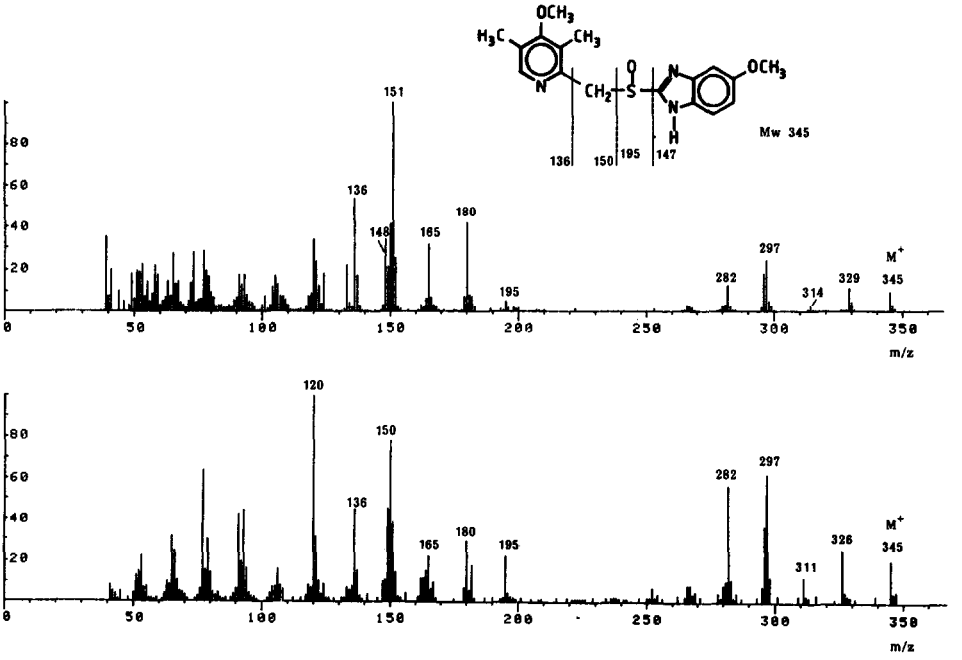

Fig. 17. Mass spectra of omeprazole. Above: mass spectrum at 165°C ion source temperature. Below: direct in-beam EI spectrum (70 eV) at 200°C.

CONCLUSION

The key point in the connection of the micro LC column to the electron impact ion source of the mass spectrometer is the behaviour of the spray into the source. With careful design of the column tip, the correct geometric distances between the ion source inlet parts and the column tip, and good control of the electrical potential between the focusing plate and ion source, it is possible to obtain stable nebulization of the effluent, even at low ion source temperature. Thus advantage can be taken of both the high performance[8,9] of the packed capillary LC column and the possibility of obtaining informative mass spectra. The gradient gives the micro LC technique the same ability for separation of complex samples as ordinary LC methods. The combination of micro LC with the mass spectrometer also gives a powerful tool for the identification of the separated substances.

The LC–MS system has already proved its usefulness in our work with biologically active compounds, *e.g.*, with allelochemicals. As the specific plant allelochemicals and their actions are identified, it will be easier to incorporate the ability to produce the desired chemicals into the plant of interest. Furthermore, suspected new components emerging from the consumed parts of the plant during the plant breeding programme will be more quickly recognized and identified.

REFERENCES

1 H. Alborn and G. Stenhagen, *J. Chromatogr.*, 323 (1985) 47–66.
2 H. Alborn and G. Stenhagen, *J. Chromatogr.*, 394 (1987) 35–49.
3 L. Lundgren, G. Norelius and G. Stenhagen, *Hereditas*, 97 (1982) 115–122.
4 H. Alborn, G. Stenhagen and K. Leuschner, in press.
5 T. Radford and D. C. Dejonh, in G. R. Waller and O. C. Dermer (Editors), *Biochemical Applications of Mass Spectrometry*, Vol. 2, Wiley, New York, 1980, pp. 266–272.
6 A. C. Barefoot and R. W. Reiser, *J. Chromatogr.*, 398 (1987) 217–226.
7 R. Ishida, T. Ozaki, H. Uchida and T. Irikura, *J. Chromatogr.*, 305 (1984) 73–82.
8 G. Crescentini, F. Bruner, F. Mangani and G. Yafeng, *Anal. Chem.*, 60 (1988) 1659–1662.
9 K.-E. Karlsson and M. Novotny, *Anal. Chem.*, 60 (1988) 1662–1665.