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Liquid chromatography–mass spectrometry system using column-switching techniques

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ABSTRACT

A liquid chromatography–mass spectrometry (LC–MS) system, was developed to overcome the problems imposed by the use of non-volatile mobile phases, such as buffers. The peak of interest was heart-cut from the effluent from the analytical column, passed into sampling loops and adsorbed on a trapping column after dilution with the analytical mobile phase. The buffer constituents were washed out and the compounds of interest were eluted from the trapping column and re-chromatographed with a suitable mobile phase for LC–MS. The potential of this system was explored in the LC–frit-fast atom bombardment-MS determination of tocopherol and riboflavin. The system can provide high sensitivity.

INTRODUCTION

Combined liquid chromatography–mass spectrometry (LC–MS) has become one of the most useful and powerful techniques for the analysis of non-volatile and thermolabile organic compounds, which cannot be analysed by gas chromatography–mass spectrometry. Although LC–MS has been applied to the characterization of molecular species, the identification of metabolites and the determination of drugs, the interface remains a major problem. Most LC–MS interfaces which have been developed do not allow the use of a mobile phase containing the non-volatile buffers which are usually used in routine LC, because in LC–MS the use of these buffers causes serious problems such as inhibition of ionization or precipitation at a heated nebulizer in the thermospray method and at atmospheric pressure ionization interfaces. Therefore, the optimum mobile phase for separation of compounds of interest often cannot be utilized.

In recent papers [1–4], an approach based on the use of valve-switching techniques has been discussed as a means of overcoming some of these problems. In particular, Verheij *et al.* [5] have described the use of coupled column chromatography, called phase-system switching (PSS), which has been developed to solve problems of mobile phase incompatibility in LC–MS target compound analysis.

We have developed a new column-switching LC system, consisting of the

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analytical LC column (LC-1), the introduction LC column (LC-3) for passing materials to the mass spectrometer and the trapping LC column (LC-2) which is installed between LC-1 and LC-3. Each LC column is run independently. The mobile phase in LC-1 moves through LC-2 and the components of the mobile phase are exchanged for the most favourable mobile phase for MS analysis.

In order to explore the potential of this LC system combined with frit fast atom bombardment MS (LC–frit-FAB-MS), which was developed by Ishii and co-workers [6,7], we have attempted to optimize the LC system (reversed-phase mode) to be combined with MS by using tocopherol as a test sample, and applied it to the determination of riboflavin with phosphoric acid as a non-volatile buffer.

EXPERIMENTAL

Materials and reagents

Methanol of HPLC grade and phosphoric acid, ammonium acetate, trifluoroacetic acid and glycerol of analytical-reagent grade were purchased from Wako (Osaka, Japan). α-, β- and δ-tocopherol were synthesized in our laboratories. Riboflavin was obtained from Sigma (St. Louis, MO, U.S.A.).

Samples

Stock solutions of α-, β- and δ-tocopherol (100 ng/ml) were each prepared in volumetric flasks by dissolution in and dilution to volume with methanol. A stock solution of riboflavin (100 μg/ml) was prepared in a volumetric flask by dissolution in and dilution to volume with methanol–water (1:1, v/v).

Chromatographic system

A schematic diagram of the system consisting of three HPLC instruments is illustrated in Fig. 1. All the LC equipment was obtained from Shimadzu (Kyoto, Japan).

In LC-1, a pump (P1) (Model LC-6AD), which was controlled by a gradient controller (Model SCL-6B), delivered the mobile phase (M1). The effluent was monitored with monitored with a variable-wavelength UV detector (D1) (Model SPD-6A). Injection was performed using a manual injector (I) [Rheodyne (Berkeley, CA, U.S.A.) Model 7125] with a 500-μl loop.

In LC-2, a pump (P2) (Model LC-9A) delivered the mobile phase (M2) for adsorption of the compounds of interest on the trapping column (TC).

In LC-3, a pump (P3) (Model LC-9A) delivered the mobile phase (M3) at a flow-rate of 20 μl/min for elution of the compounds of interest from the trapping column. Before being passed to the LC–MS interface, the compounds of interest were re-chromatographed on the microcolumn (C2), monitoring with a UV detector (D2) (Model SPD-6A) equipped with a micro-cell (cell volume 0.6 μl).

Switching was done with Rheodyne 7000 (V1, V4) and Rheodyne 7060 (V2, V3, R) switching valves.

The introduction to the frit-FAB-MS system was performed by using a pneumatic splitter [8] (S) (Model MS-PNS; JEOL, Tokyo, Japan) in order to reduce the flow-rate from 20 to 1 μl/min.

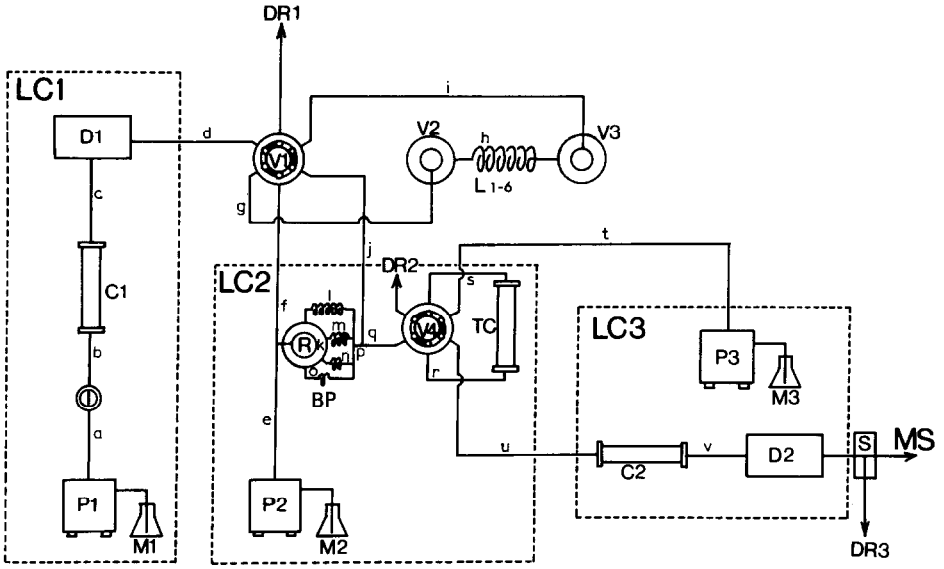

Fig. 1. Schematic diagram of the column switching system for LC–MS. P1 = solvent pump; M1 = mobile phase for separation; C1 = analytical column; D1 = UV detector for monitoring separation with the analytical column; P2 = solvent pump for trapping; M2 = mobile phase for trapping on the trapping column (TC); BP = by-pass line; L = sampling loops; P3 = solvent pump for flow to mass spectrometer; C2 = micro-column for re-chromatography; D2 = UV detector with micro-cell for monitoring the flow to the mass spectrometer; S = splitter; V1, V4 = six-port switching valves for changing the flow direction; V2, V3 = six-port switching valves for selection of sampling loops: R = six-port switching valve for changing the dilution ratio; DR1, DR2 and DR3 = drain. Sizes of connecting tubes: (a) 400 mm × 0.25 mm; (b) 930 mm × 0.25 mm I.D.; (c) 200 mm × 0.20 mm I.D.; (d) 310 mm × 0.25 mm I.D.; (e) 480 mm × 0.25 mm I.D.; (f) 60 mm × 0.25 mm I.D.; (g) 100 mm × 0.50 mm I.D.; (h) 500 mm × 0.80 mm I.D.; (i) 200 mm × 0.25 mm I.D.; (j) 430 mm × 0.50 mm I.D.; (k) 360 mm × 0.50 mm I.D.; (l) 280 mm × 0.25 mm I.D.; (m) 140 mm × 0.25 mm I.D.; (n) 90 mm × 0.25 mm I.D.; (o) 70 mm × 0.25 mm I.D.; (p) 720 mm × 0.50 mm I.D.; (q) 320 mm × 0.25 mm I.D.; (r) 100 mm × 0.10 mm; (s) 200 mm × 0.10 mm I.D.; (t) 350 mm × 0.25 mm I.D.; (u) 200 mm × 0.10 mm I.D.; (v) 100 mm × 0.20 mm.

Column

C1 (the analytical column) was Inertsil ODS-2 (150 mm × 4.6 mm I.D., particle size 5 μm; Gaskuro Kogyo). TC (the trapping column) was a reversed-phase Inertsil ODS-2 cartridge precolumn (10 mm × 4 mm I.D., particle size 5 μm). The separation using C2 (microcolumn) was performed on Inertsil ODS-2 (150 mm × 0.7 mm I.D., particle size 5 μm).

Mass spectrometry (MS)

The combined LC–MS system with a frit-FAB interface was performed on a JEOL JMS-HX100 apparatus. MS conditions in the FAB mode included a xenon atom beam from a saddle field gun operated at 8 kV and 1.0 mA; the scan range was 100–1100 dalton at a scan speed of 3.1 per decade. The FAB mass spectra were recorded with a normal resolving power of 1000. The source operating pressure was typically 10^{-5} Torr.

Column-switching procedure

As shown in Fig. 1, this system consisted of three HPLC instruments. LC-1 was a conventional HPLC set-up with C1 and M1 (the optimum mobile phase) to separate the compounds of interest. LC-2 served to trap the compounds on the trapping column (TC) and LC-3 to pass the compounds to the frit-FAB-MS system after re-chromatography. The three instruments were connected with V1, V2, V3, V4 and R.

The valve-switching procedure is illustrated in Fig. 2. In the first stage, the three columns were run independently so the flow pattern through the apparatus (Fig. 1) is as shown in Fig. 2a. The sample solution was injected at I and eluted from C1, peaks being detected at D1. In the second stage, when a peak was detected at D1 the valve V1 was switched so the flow pattern changed to that shown in Fig. 2b. The valves V2 and V3 were adjusted so that the sample-containing eluate flowed into one of six 1-ml loops connecting the two, where it was stored. When the detector D1 showed that the sample was no longer leaving the column, valve V1 was returned to its original position (*i.e.*, the flow pattern returned to that in Fig. 2a) until the next peak was detected. Thus, by repeating the process of switching V1 and adjusting V2 and V3, samples of up to six different peaks could be stored in the 1-ml loops. In the third stage, the compounds in the loops (L) were pushed out with M2 by P2 and sent to TC while simultaneously being diluted with M2 via the by-pass (BP) in order to improve the adsorption of the compounds of interest on TC. A valve (R) was fitted to BP so that the dilution ratio could be adjusted by varying the resistance to the flow of M2 in the apparatus. This was

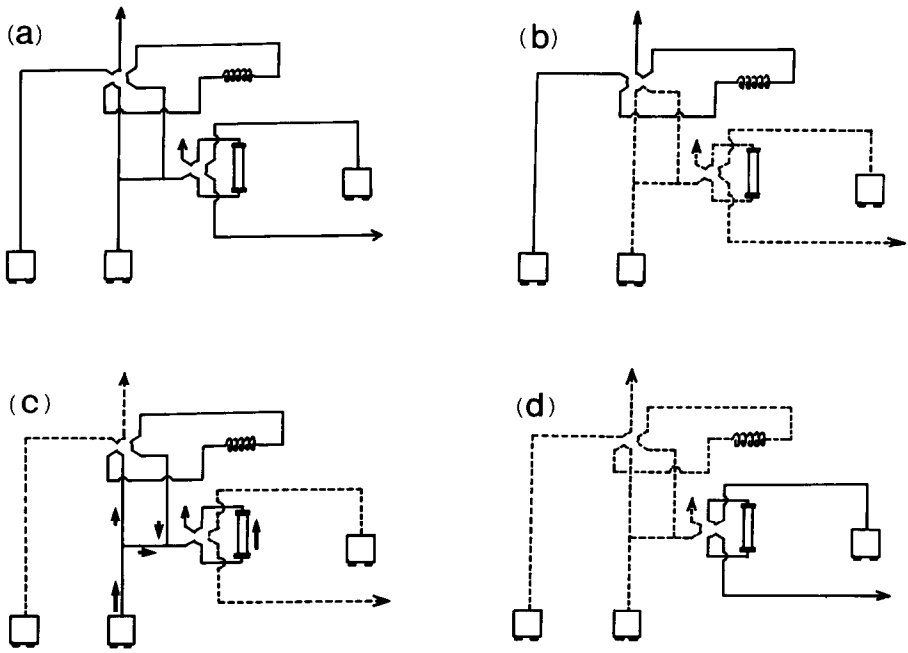

Fig. 2. Column-switching procedure showing (a) separation configuration, (b) sampling configuration, (c) sample washing configuration (the arrows show the relative flow-rates using one tube at BP in Fig. 1 when the dilution ratio was 2) and (d) flow to mass spectrometer and re-chromatography configuration.

done by using different lengths of stainless-steel tubing connecting R and V4 (see Fig. 1, l–o). When the flow-rate of M2 (using water) was 1 ml/min, the flow-rate at BP for dilution ratios of 2, 3, 4 and 5 were 0.48, 0.68, 0.73 and 0.81 ml/min, respectively. M1 in L was drained through TC together with M2. The compounds were adsorbed on TC and M1 was exchanged with M2 (see Fig. 2c). In the fourth stage, the adsorbed compounds were eluted from TC with M3 by P3, sent to C2 for re-chromatography and introduced into the Frit FAB MS system (see Fig. 2d). After the completion of these four stages, the procedure could be repeated for another sample.

RESULTS AND DISCUSSION

We attempted to optimize the LC system to be combined with MS by using tocopherols as test samples. It has been shown that the frit-FAB interface can be used effectively in an on-line LC–MS system.

Liquid chromatography of tocopherols

HPLC was performed according to the procedure described under Experimental. α-Tocopherol was dissolved in methanol and adjusted to 1 mg/ml.

In LC-1, 1 μl of α-tocopherol solution (1 μg as α-tocopherol) was injected into C1. M1 was methanol at a flow-rate of 1 ml/min. The peak of tocopherol was monitored by D1 and D2 at 282 nm.

In LC-2, M2 for adsorption on TC was glycerol–methanol–water (0.8:30:70, v/v/v) at a flow-rate of 1 ml/min.

In LC-3, α-tocopherol was passed to C2 and was eluted with glycerol–methanol (0.8:100, v/v) at a flow-rate of 20 μl/min.

α-Tocopherol could not be adsorbed on TC without BP in Fig. 1, because M1 in the 1-ml L made α-tocopherol elute from TC. In order to adsorb α-tocopherol on TC, M1 in L had to be sufficiently diluted with M2 before reaching TC. Therefore, BP was attached between L and the TC. This is a critical feature of the system.

If more hydrophilic compounds are applied, it is necessary to increase the dilution ratio or to change M2 to a more aqueous mobile phase in order to allow the compounds to be adsorbed on TC. α-Tocopherol could be adsorbed on TC by setting the dilution ratio at 2.

The diffusion of α-tocopherol in this LC system was investigated with C2 connected (Fig. 3b) and without C2 (Fig. 3c), monitoring with D2. The results are shown in Fig. 3. The chromatographic efficiency of α-tocopherol was very high in Fig. 3b but low in Figs. 3c. Hence, it was possible to decrease the diffusion by connecting C2[a]. This is very important in order to increase the sensitivity in MS analysis. In addition, the separation of tocopherol analogues was investigated by using only C2. In LC-1 α-, β- and δ-tocopherol were simultaneously injected into C1 and sampled in L with monitoring by D1. After adsorption of the tocopherol analogues on TC, the chromatogram obtained with D2 is shown in Fig. 4. C2 clearly has the ability to separate α-, β- and δ-tocopherol. Hence, if the separation in C1 is poor, it can be improved by using C2 to obtain a sufficient separation for the MS analysis. When

[a] A reference pointed out that this implies that the connecting tubing had too large an I.D. We used the narrowest gauge of tubing available, which was 200 mm × 0.1 mm I.D. stainless steel.

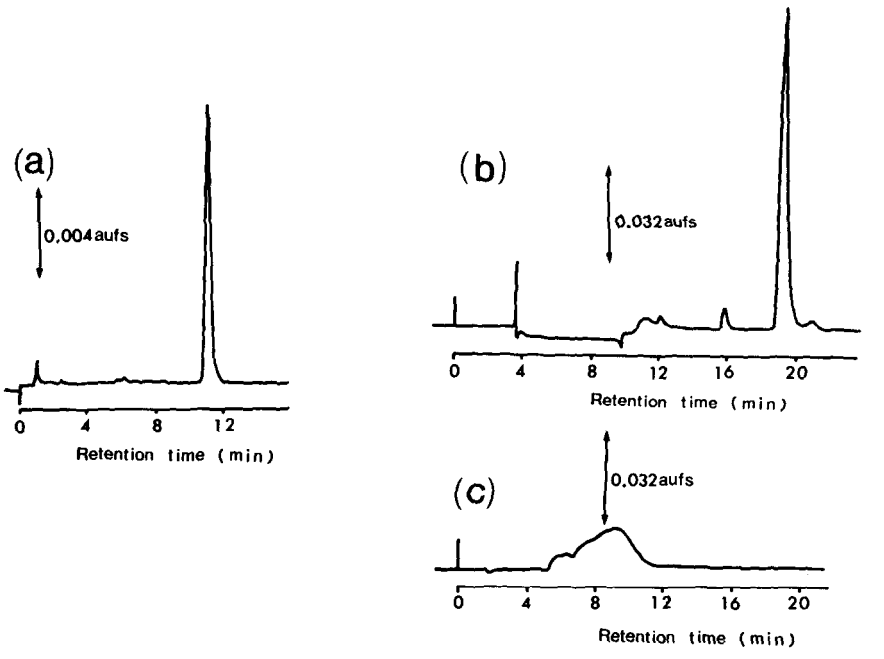

Fig. 3. Liquid chromatograms for a 1-μg injection of α-tocopherol: (a) detection at D1 (282 nm); (b) detection at D2 (282 nm) with C2; (c) detection at D2 (282 nm) without C2.

LC–frit-FAB-MS is used, not more than a few microlitres per minute can be passed to the FAB-MS system [6,7]. Therefore, if an LC microcolumn is directly coupled to a FAB-MS or frit-FAB-MS system, the injection volume into the microcolumn must usually be less than 1 μl. This is unsuitable for microanalysis. In our LC system using the microcolumn, the same volume could be injected as in the usual LC.

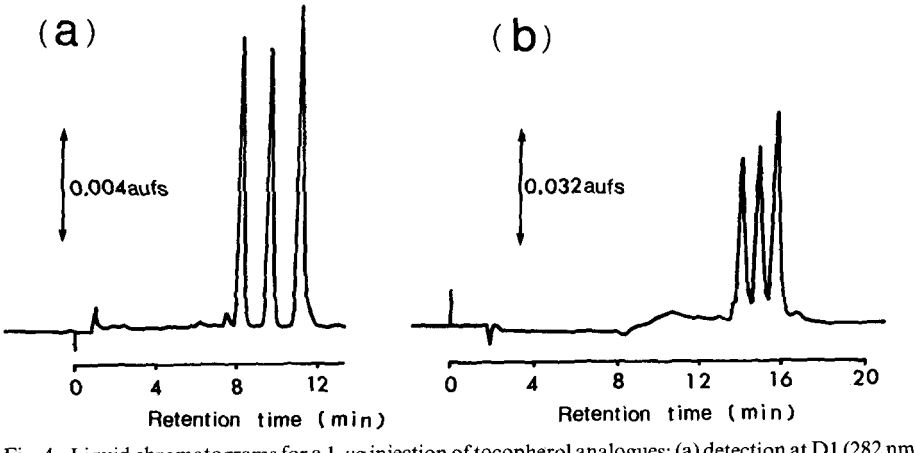

Fig. 4. Liquid chromatograms for a 1-μg injection of tocopherol analogues: (a) detection at D1 (282 nm); (b) detection at D2 (282 nm) with C2.

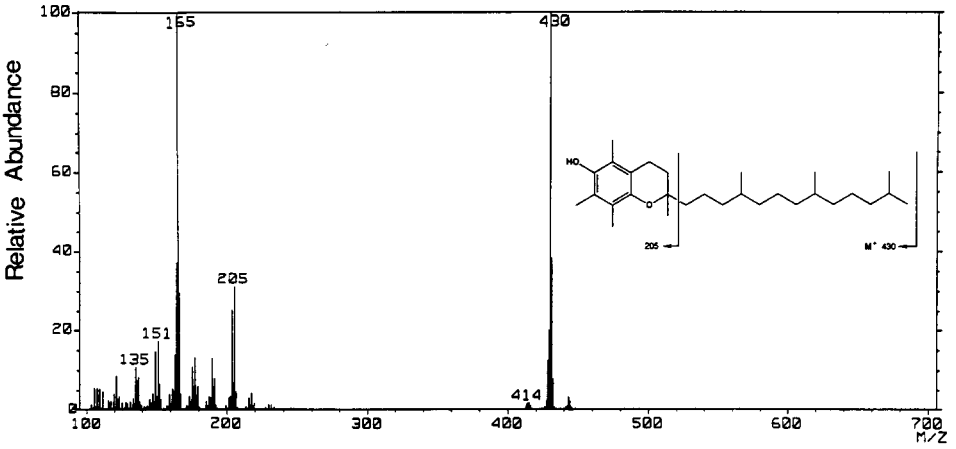

Fig. 5. FAB glycerol mass spectrum of about 1 μg of α-tocopherol using the direct insertion probe.

FAB-MS of α-tocopherol from a glycerol matrix

Initially, about 1 μg of α-tocopherol was analysed by FAB-MS using a direct insertion probe and a glycerol matrix. The spectrum and fragmentation pattern of α-tocopherol are shown in Fig. 5. The FAB-MS of α-tocopherol showed the molecular ion ($M^{+\bullet}$) at *m/z* 430.

FAB-MS of α-tacopherol obtained with the LC–frit-FAB-MS system

The flow-rate of the mobile phase passed to the FAB-MS system (JEOL JMS-HX 100) was restricted to 1 μl/min. In LC-1, 100 ng of α-tocopherol were injected into C1. The mass spectrum of α-tocopherol is shown in Fig. 6a and the mass chromatogram of the molecular ion ($M^{+\bullet}$) at *m/z* 430 in Fig. 6b. The molecular ion at *m/z* 430 was clearly observed and the spectrum was the same as that shown in Fig. 5. The protonated molecular ion was still observable when 500 pg were injected.

FAB-MS of riboflavin from a glycerol matrix

Initially, about 1 μg of riboflavin was analysed by FAB-MS using a direct insertion probe and a glycerol matrix. The spectrum and fragmentation pattern of riboflavin are shown in Fig. 7. The FAB mass spectrum of riboflavin showed a protonated molecule [$(M + 2H)^{+\bullet}$] at *m/z* 378.

When a mixture of riboflavin, glycerol and phosphoric acid was used, it was impossible to obtain the spectrum owing to the inhibition of ionization by the phosphoric acid, a so-called non-volatile buffer.

Liquid chromatography of riboflavin

HPLC was performed according to the procedure described under Experimental. In LC-1 1 μl of riboflavin solution (1 μg as riboflavin) was injected into C1. M1 was phosphoric acid–methanol–water (0.5:30:70, v/v/v) at a flow-rate of 1 ml/min. The peak of riboflavin was monitored by D1 and D2 at 254 nm. In LC-2, M2 for adsorption on TC was glycerol–0.5% aqueous ammonium acetate (0.8:100, v/v) at a flow-rate of

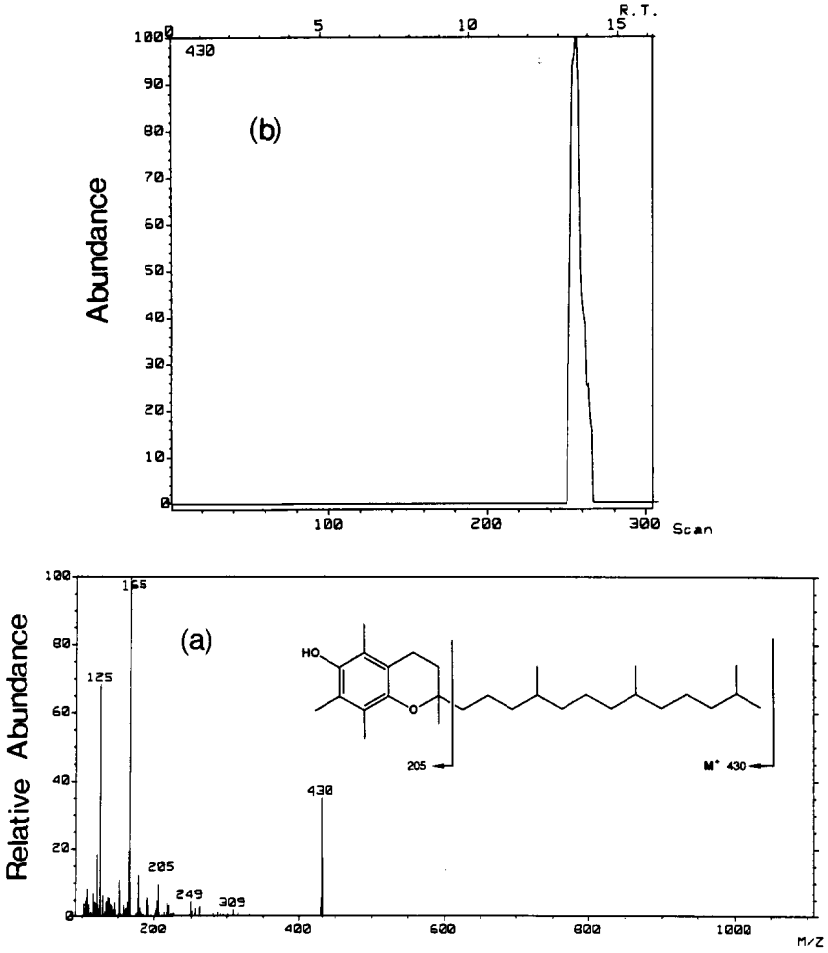

Fig. 6. (a) FAB mass spectrum of 100 ng of α-tocopherol obtained with LC–frit-FAB-MS; (b) mass chromatogram at *m/z* 430. R.T. is scanning time in min.

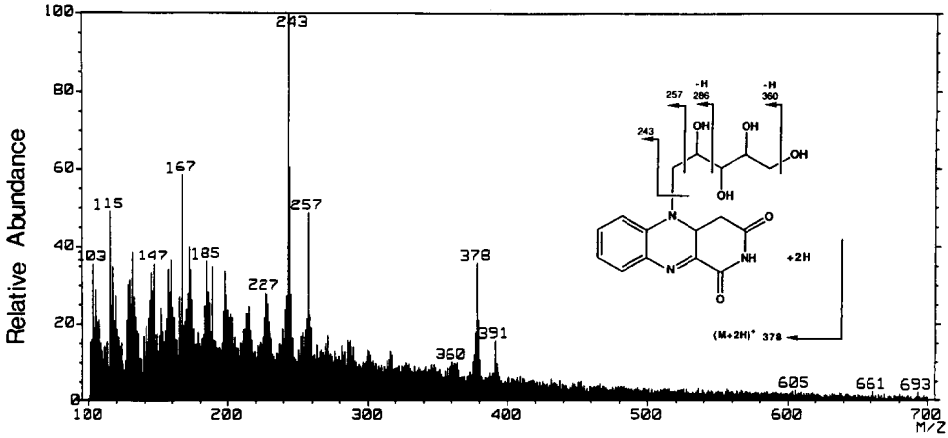

Fig. 7. FAB glycerol mass spectrum of about 1 μg of riboflavin using the direct insertion probe.

1 ml/min. In LC-3, riboflavin was passed into C2 and M3 was glycerol–trifluoroacetic acid–methanol–water (0.8:0.2:30:70, v/v) at a flow-rate of 20 μl/min.

The adsorption of riboflavin on TC was investigated, and chromatograms showing the effect of dilution ratio at D2 are presented in Fig. 8. The efficiency of the chromatography of riboflavin increased with increasing dilution ratio. These phenomena may be due to the diffusion of riboflavin in TC. In order to minimize the diffusion in TC and to obtain a high sensitivity, a high dilution ratio had to be used. Hence, the attachment of the sampling loop L to this system could change the dilution ratio.

FAB-MS of riboflavin obtained with the LC–frit-FAB-MS system

The mass chromatogram and the mass spectrum of riboflavin after injection of 1 μg into C1 are shown in Fig. 9. The spectrum showed a protonated molecule

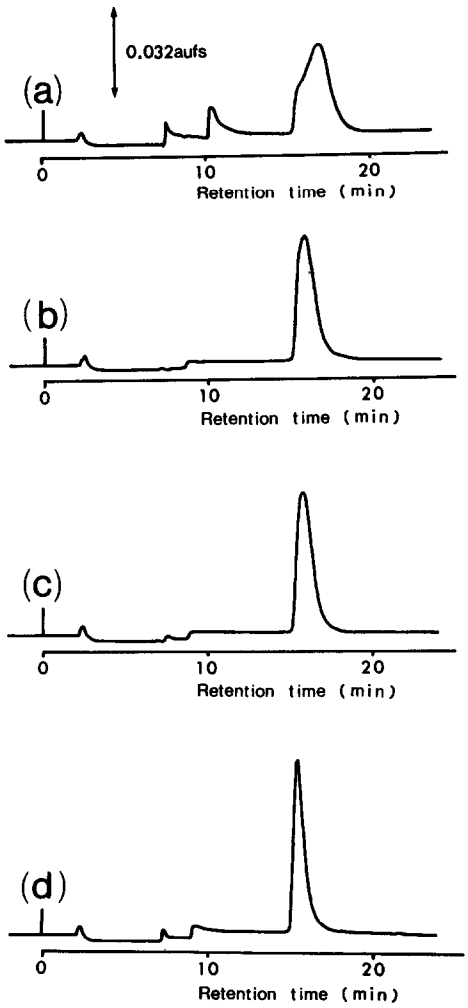

Fig. 8. Effect of dilution ratio on LC for a 1-μg injection of riboflavin with D2 at 254 nm. Dilution ratio: (a) 2; (b) 3; (c) 4; (d) 5.

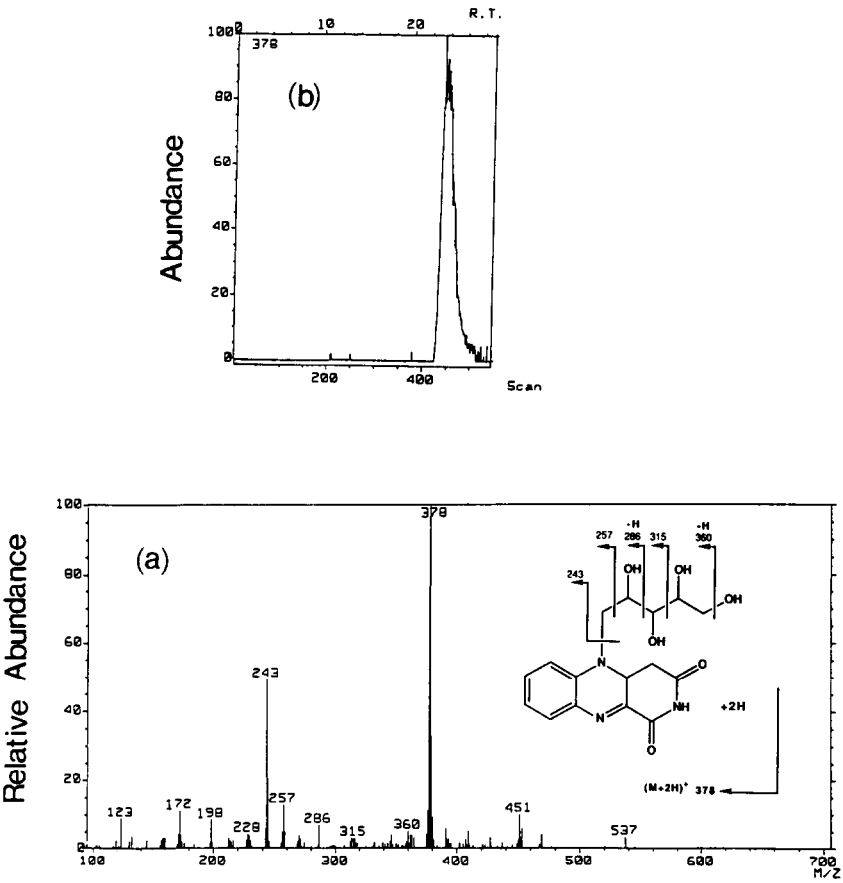

Fig. 9. (a) FAB mass spectrum of 1 μg of riboflavin obtained with LC–frit–FAB-MS; (b) mass chromatogram at *m/z* 378. R.T. is scanning time in min.

$[(M + 2H)^{+\cdot}]$ at *m/z* 378. It was still observable when 300 ng were injected. The spectrum was the same as that obtained with the direct insertion probe in the absence of phosphoric acid (see in Fig. 7). When phosphoric acid was added to M3, the mass spectrum of riboflavin could not be obtained. Hence, a non-volatile buffer could be used as the mobile phase for optimum separation in this LC–frit-FAB-MS system without incurring the problem of inhibition of ionization. This system is very powerful and offers high sensitivity.

CONCLUSIONS

It has been shown that a LC–frit-FAB-MS interface can be used effectively for on-line LC–MS. The application of a valve-switching technique in a new LC system allowed us to overcome previous limitations of LC–MS interfaces so that optimum mobile phases (containing non-volatile buffers) could be used to separate the

compounds of interest. The method offers high sensitivity. Investigations aimed at improving the sensitivity further are in progress.

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