Journal of Chromatography, 363 (1986) 147–158
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 730

SUPERCRITICAL FLUID CHROMATOGRAPHIC AND SUPERCRITICAL FLUID CHROMATOGRAPHIC–MASS SPECTROMETRIC STUDIES OF SOME POLAR COMPOUNDS

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(First received February 27th, 1986; revised manuscript April 16th, 1986)

SUMMARY

Silica and amino-bonded silica columns (4.6 mm I.D.), with carbon dioxide modified with methanol or methoxyethanol, have been used to effect supercritical fluid chromatographic separations of mixtures of xanthines, carbamates, sulphonamides, steroids and ergot alkaloids. Combined supercritical fluid chromatography–mass spectrometry has been accomplished using a moving belt high-performance liquid chromatographic–mass spectrometric interface with a modified thermospray deposition device. The applicability of the technique is illustrated with mixtures of xanthines, sulphonamides and an extract from *Claviceps purpurea*.

INTRODUCTION

Supercritical fluid chromatography (SFC) is complementary to gas chromatography (GC) and high-performance liquid chromatography (HPLC) for the analysis of organic compounds[1–8]. Unlike GC, it is not restricted by compound volatility or lability and if capillary columns are used chromatographic efficiencies approaching those obtained by capillary GC are possible. SFC is more efficient than HPLC, even if packed columns are used and thus the technique offers advantages in terms of speed of analysis and rapidity of method development. In addition, flame-ionization detectors can be used, thus offering universal detection capabilities. Capillary SFC offers optimal chromatographic efficiency[4,5,7,8], whereas the packed column approach enables a wide range of modifiers to be used[9] and can be adapted for preparative work[6]. Thus it is our belief that the two approaches to SFC are complementary in nature and we are pursuing studies with both approaches.

A large portion of the literature on SFC is devoted to the analysis of relatively non-polar materials, such as polymers and natural fuel samples. The potential of the technique for the analysis of polar materials was shown at an early stage[1], and recently studies of carbamate pesticides[10], steroids[9], anthraquinones[9], xanthines[9], sucrose polyesters[11], nitro-polynuclear aromatic hydrocarbons[12], paprika oleoresins[13], ubiquinones[14], polar drugs[15] and trichothecene mycotoxins[16] have been reported.

A further attraction of SFC is that interfacing of the technique to a mass

0021-9673/86/$03.50

spectrometer should be easier than interfacing a high-performance liquid chromatograph to a mass spectrometer, since the gas volumes generated from the mobile phase are lower. A number of SFC–MS systems have been reported. For packed-column studies, a molecular beam interface has been constructed[17]. A simpler approach involved use of a capillary inlet system, through which a portion of the eluent from the chromatograph was fed into the ion source of a mass spectrometer operating in the electron impact (EI) mode[18]. Recently use of a modified direct liquid introduction HPLC–MS interface has been used for SFC–MS and impressive data were obtained from a range of polar drugs[15]. With this approach a diaphragm with a 3-μm diameter hole was used and *ca.* one-twelfth of the eluent from the chromatograph could be handled by the mass spectrometer operating in the chemical ionization (CI) mode, when 2.1 mm I.D. columns were used. Use of capillary SFC for SFC–MS enables all of the eluent from the chromatograph to be handled by the mass spectrometer, and the system can be operated in the EI[19] or CI[20,21] mode, in the latter case with conventional CI reagent gases such as ammonia and isobutane. Capillary SFC–MS has been used for studies of a range of sample types, which include marine diesel fuel[22], lipids[23], carbamate pesticides[24] and trichothecene mycotoxins[16].

We report here on our studies of mixtures of a range of polar compounds using a Hewlett-Packard supercritical fluid chromatograph with conventional HPLC columns. The technique has also been interfaced to a mass spectrometer operating in the EI and CI modes, using a moving belt HPLC-MS interface with a thermospray deposition device.

EXPERIMENTAL

For SFC and SFC–MS a Hewlett-Packard 1084B liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) modified for SFC operation[25] was used. The chromatograph was coupled to a Finnigan 4000 mass spectrometer (Finnigan MAT, San Jose, CA, U.S.A.) which was equipped with a 4500 EI/CI source and a moving belt HPLC–MS interface. Interfacing of the chromatograph with the moving belt interface was effected using a Finnigan MAT thermospray deposition device. The spray deposition device was connected in-line via a "T" piece between the UV detector exit and the outlet back-pressure regulator of the chromatograph, thus effecting a split of the eluent. In order to use the full density range for SFC, the end of the stainless-steel tubing used in the spray deposition device was slightly crimped. The spray deposition device was connected to a 6 V power supply which was adjusted through a rheostat to prevent freezing of the mobile phase at the tip. The infrared heater on the HPLC–MS interface was used to assist in vaporization of the solvent and was maintained at its lowest setting.

Instrument grade liquid carbon dioxide supplied in cylinders with a dip tube (BOC, London, U.K.) and glass redistilled methanol and methoxyethanol were used for mobile phases. The carbon dioxide was introduced directly into the "A" pump of the 1084B, and methanol, or methoxyethanol, was placed in the "B" pump, which was operated in the HPLC mode. This approach works well if percentages of methanol and methoxyethanol are in excess of 10% and providing the total flow is in excess of 1 $cm^3\ min^{-1}$. The liquid carbon dioxide and the pump heads of the chromatograph were cooled to -25°C using a Neslab RTE-4Z refrigerated bath (Neslab Instruments, NH, U.S.A.).

For SFC, 100 × 4.6 mm I.D. columns packed with 5 μm amino-bonded Spherisorb (Phase Separations, Queensferry, Clwyd, U.K.) and 5 μm LiChrosorb (E. Merck, Darmstadt, F.R.G.) were used. Detection was by a UV detector.

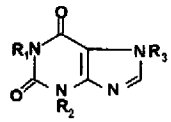
SFC–MS measurements in the EI mode were performed at 70 eV with a source temperature of 150°C (indicated), and the source pressure was 7×10^{-6} Torr. For CI the source temperature was 100°C and the pressure 0.6 Torr. The belt vaporizer temperature was at 200°C (indicated) and the clean-up heater was at 200°C (indicated). Kapton® belts were used.

RESULTS AND DISCUSSION

Our objectives in these studies were to evaluate the utility of conventional column SFC with carbon dioxide and modifiers for the analysis of a variety of polar compounds and to investigate the possibility of effecting SFC–MS using a moving belt interface. Use of a moving belt interface for conventional column SFC–MS would enable a higher proportion of the eluent from the chromatograph to be handled than using the direct liquid introduction approach[15]. A further advantage of this approach would be the availability of both EI and CI mass spectral data.

The methodology for SFC which we have used is essentially the same as that adopted by other groups using conventional HPLC columns[6,9,13–15,25]. The importance of maintaining supercritical fluid conditions has been emphasized[15] and critical pressures and temperatures were calculated as reported in that study.

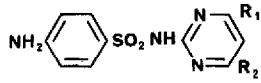
SFC studies of xanthines have been reported previously[9,26]. In order to evaluate our instrumentation, SFC of a mixture of caffeine (1a), theophylline (1b) and theobromine (1c) was studied. Good separation was achieved using a mixture of 12% methanol in carbon dioxide and a column packed with 5 μm LiChrosorb. The UV trace obtained from this mixture is shown in Fig. 1, together with the chromatographic conditions used. We noted that because of the speed of analysis and the rapidity of equilibration of columns with SFC, method development was much faster than has been our experience with HPLC. Encouraged by these results, we studied SFC of a mixture of five sulphonamide drugs. Modification of conditions enabled all five components to be resolved and the UV trace obtained from this mixture is shown in Fig. 2. Separation of a mixture of six carbamate pesticides (Fig. 3) was also effected with the same column using a lower pressure and initial flow-rate than for the sulphonamides. The data obtained from the carbamate mixture compare favourably with that recently reported by fast capillary SFC[10,24]. In our study, faster analysis (half the time) was effected by use of a higher column pressure and higher flow-rate of the mobile phase. However, under these conditions the pirimicarb and



1a R_1=R_2=R_3=CH_3

1b R_1=R_2=CH_3; R_3=H

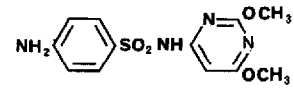
1c R_1=H; R_2=R_3=CH_3



2a R_1=R_2=CH_3

2b R_1=Me; R_2=H

2c R_1=R_2=H



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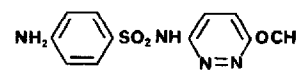


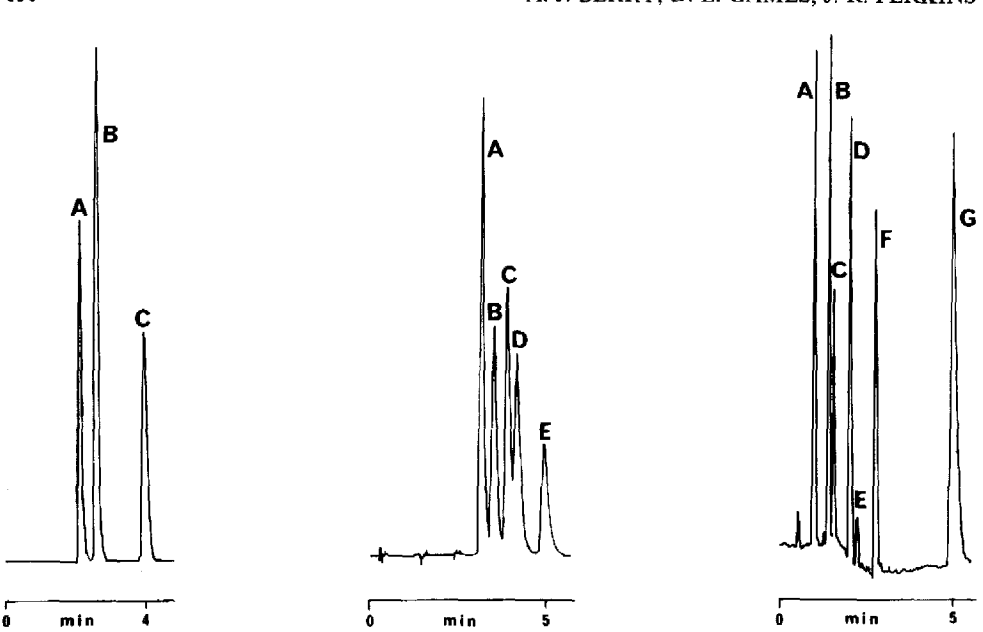


Fig. 1. UV trace (270 nm) obtained from SFC of a mixture of (A) caffeine (1a), (B) theophylline (1b) and (C) theobromine (1c), on a 100 × 4.6 mm I.D. column packed with 5 μm LiChrosorb. The mobile phase was carbon dioxide modified with 12% methanol at a flow-rate of 2.5 cm^3 min^{-1}; column pressure, 385 bar; temperature, 70°C.

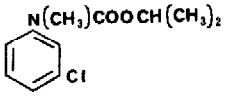
Fig. 2. UV trace (270 nm) obtained from SFC of a mixture of (A) sulphadimethoxime (3), (B) sulphamethazine B (2a), (C) sulphametheoxypyridazine (4), (D) sulphamerazine (2b) and (E) sulphadiazine E (2c). Column as in Fig. 1, with carbon dioxide modified with 15% methanol at a flow-rate of 4 cm^3 min^{-1}; column pressure, 271 bar; temperature, 70°C.

Fig. 3. UV trace (254 nm) obtained from SFC of a mixture of (A) chloropropham (5), (B) pirimcarb (6), (C) methiocarb (7), (D) carbaryl (8), (E) impurity in phenmedipham, (F) phenmedipham (9) and (G) asulam (10). Column and mobile phase as in Fig. 2 with an initial flow-rate of 2 cm^3 min^{-1}, changed to 4 cm^3 min^{-1} after 1.9 min; column pressure, 179 bar; temperature, 70°C.

methiocarb peaks were not resolved. Use of methanol as a modifier failed to effect complete resolution of a mixture of eight steroids. This problem was overcome by the use of methoxyethanol which enabled all eight steroids to be resolved, Fig. 4.

Problems were encountered with the use of silica columns in studies of ergot alkaloids of the clavine type. Use of a basic modifier with carbon dioxide is not possible and so we investigated the use of an amino-bonded column. Studies with a range of standard alkaloids, which were available from our previous studies in this area[27], gave good rapid chromatographic resolution. Fig. 5 shows the UV trace obtained by SFC from a crude extract of *Claviceps purpurea*. The peak assignment given are on the basis of comparison of retention times with standard compounds. SFC of ergot alkaloids of the peptide type was also investigated and Fig. 6 shows the UV trace obtained from a mixture of bromocryptine mesilate and ergocryptine.

Having established the applicability of SFC to a range of compound types of interest to us, we proceeded to investigate interfacing of the technique to a mass spectrometer using a moving belt HPLC–MS interface[28]. Initially the supercritical



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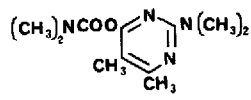

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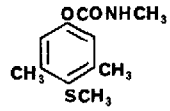

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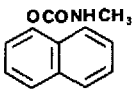

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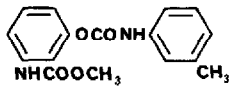

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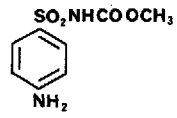

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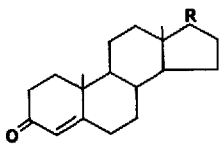
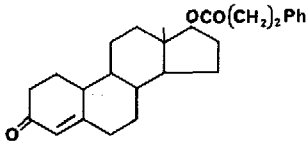


11a R=OH

11b R=$COCH_3$



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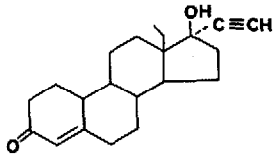

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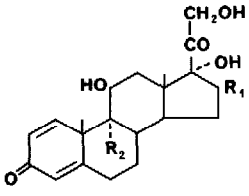
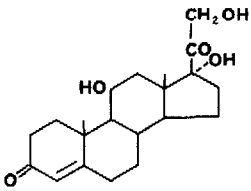


14a R_1=R_2=H

14b R_1=CH_3, R_2=F



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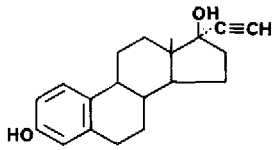

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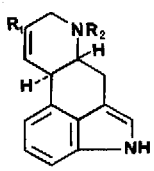
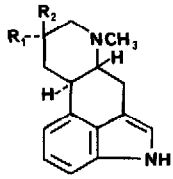


17a R_1=R_2=CH_3

17b R_1=CH_3, R_2=H

17c R_1=CH_2OH, R_2=CH_3



18a R_1=CH_3, R_2=H

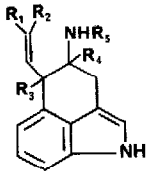
18b R_1=H, R_2=CH_3

19a R_1=CH_2OH, R_2=OH, R_3=CH_3

19b R_1=CH_3, R_2=OH, R_3=CH_3

19c R_1=OH, R_2=CH_3, R_3=CH_3

19d R_1=OH, R_2=CH_3, R_3=H

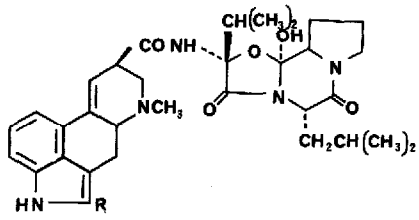


20a R_1=CH_2OH, R_2=CH_3, R_3=R_4=H(trans), R_5=CH_3

20b R_1=CH_2OH, R_2=CH_3, R_3=R_4=H(cis), R_5=CH_3

20c R_1=CH_3, R_2=CH_2OH, R_3=R_4=H(trans), R_5=CH_3

20d R_1=CH_2OH, R_3=CH_3, R_3=R_4=H(cis), R_5=H



21a R=Br

21b R=H

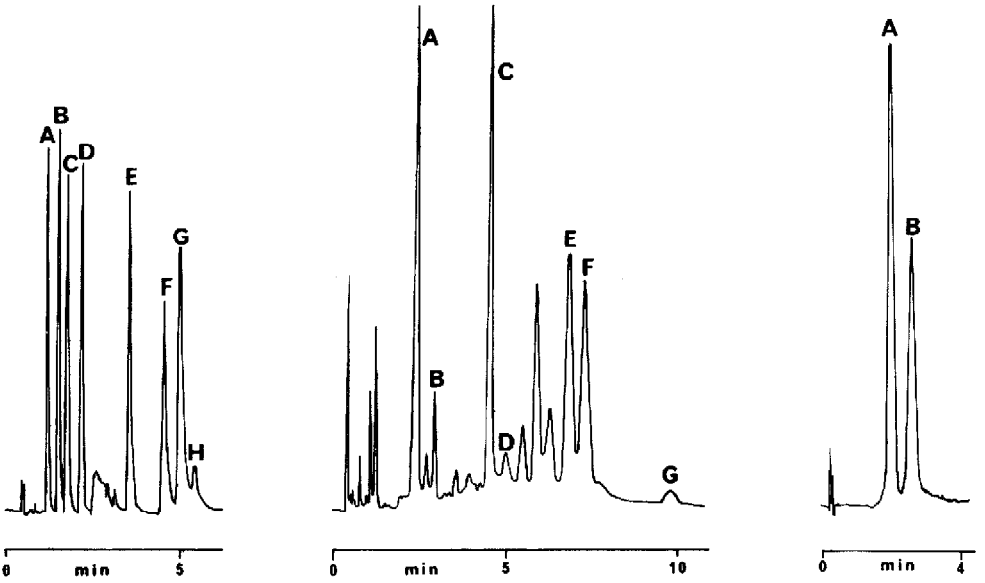


Fig. 4. UV trace (254 nm) obtained from SFC of a mixture of (A) testosterone (11a), (B) progesterone (11b), (C) nandrolone phenylpropionate (12), (D) norgestrel (13), (E) prednisolone (14a), (F) hydrocortisone (15), (G) dexamethasone (14b), and (H) ethynylestradiol (16). Column as in Fig. 1, with carbon dioxide modified with 20% methoxyethanol at an initial flow-rate of 2 cm^3 min^{-1} changed to 5 cm^3 min^{-1} after 2.1 min; column pressure, 195 bar; temperature, 75°C.

Fig. 5. UV trace (280 nm) obtained by SFC of an extract of *Claviceps purpurea*. Identifications based on comparison of retention times; (A) agroclavine (17a); (B) festuclavine (18a); (C) elymoclavine (17c); (D) noragroclavine (17b); (E) chanoclavine I (20a); (F) chanoclavine II (20b); and (G) norchanoclavine II (20d). Column, 100 × 4.6 mm I.D., packed with 5 μm amino-bonded Spherisorb. The mobile phase was initially carbon dioxide modified with 10% methanol at a flow-rate of 3 cm^3 min^{-1}. After 2.5 min the flow-rate was increased to 5 cm^3 min^{-1}, and the percentage of methanol was increased to 15% after 2.8 min and to 20% after 5 min. Column pressure, 365 bar; temperature, 75°C.

Fig. 6. UV trace (280 nm) obtained by SFC of a mixture of (A) bromocryptine mesilate (21a) and (B) ergocryptine (21b). Column as in Fig. 5, with carbon dioxide modified with 20% methoxyethanol as mobile phase at a flow-rate of 4 cm^3 min^{-1}; column pressure, 380 bar; temperature, 75°C.

fluid chromatograph was connected directly to the moving belt interface using 0.006 in. I.D. stainless-steel tubing, connection being made via a "T" piece between the UV detector and the back-pressure regulator of the chromatograph. With this arrangement the UV trace from the SFC could be monitored in series with the computer reconstructed total ion current trace (TIC) from the mass spectrometer. Although on-line HPLC–MS data were obtained from a mixture of polynuclear hydrocarbons, freezing occurred at the tip of the tubing resulting in a loss of chromatographic resolution, and the full pressure range of the chromatograph could not be maintained. It was decided that some form of heating of the column effluent was necessary and that a restriction was necessary to maintain SFC conditions in the connection to the interface. These problems were overcome by use of a Finnigan MAT thermospray deposition device. The end of the stainless-steel tubing in the device was crimped to maintain appropriate pressures and when in use the temperature of the spray de-

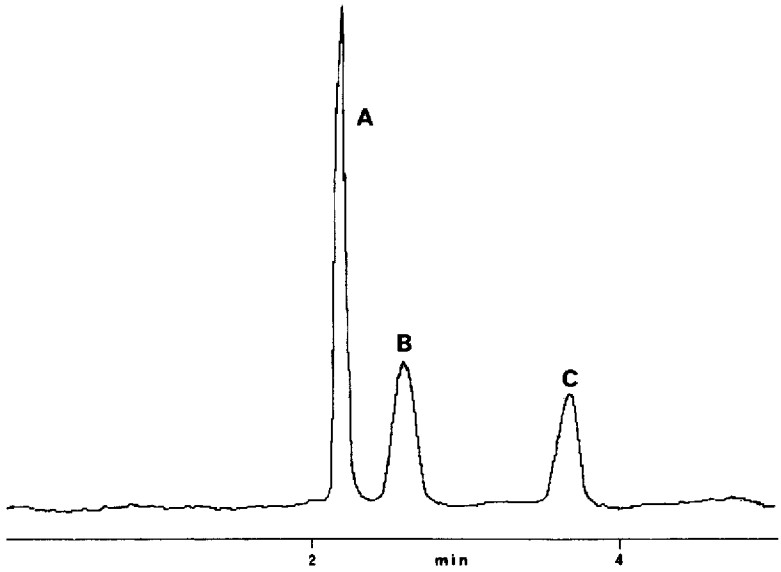


Fig. 7. Computer reconstructed TIC trace obtained by EI SFC–MS of a mixture of (A) caffeine (1a), (B) theophylline (1b) and (C) theobromine (1c). SFC conditions as in Fig. 1.

position device was optimized to ensure that no freezing of the mobile phase occurred at its tip. In all other respects the moving belt interface was used as for HPLC–MS.

Fig. 7 shows the TIC trace obtained by SFC–MS under EI conditions of a mixture of caffeine (1a), theophylline (1b), and theobromine (1c). The relative amount of caffeine present in the mixture had been increased over that present in the mixture

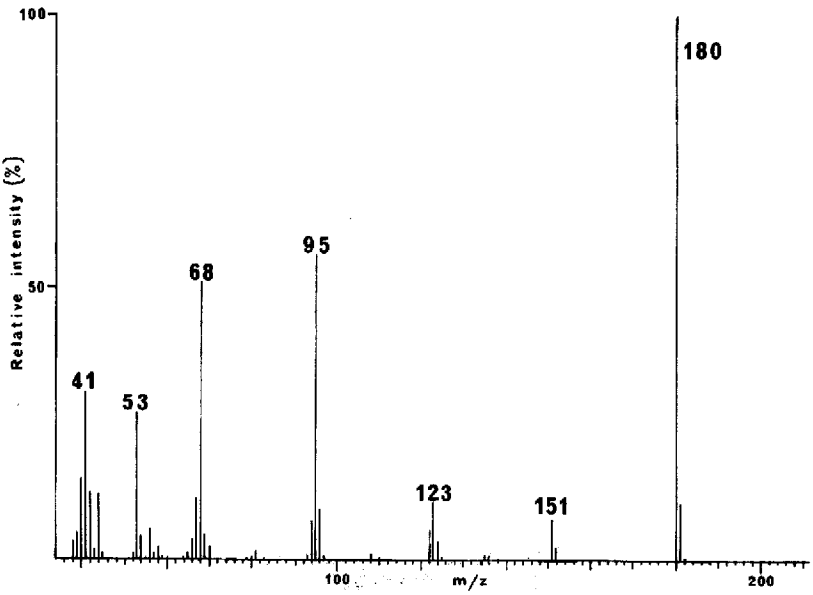


Fig. 8. EI mass spectrum of theophylline obtained by SFC–MS.

TABLE I

EI AND CI (METHANE) MASS SPECTRA OF XANTHINES AND SULPHONAMIDES OBTAINED BY SFC–MS

Compound	*MW*	*Ionization mode*	*m/z (% rel.int.)*
Caffeine (1a)	194	EI	194(100), 165(5), 136(4), 109(50), 82(23), 67(35), 55(36), 42(15)
Theobromine (1c)	180	EI	180(100), 137(7), 109(23), 95(11), 82(22), 67(37), 55(36), 42(16)
Sulphamethazine (2a)	278	EI*	279(1), 215(16), 214(100), 213(84), 156(5), 123(16), 108(29), 107(21)
		CI	307(5), 279(100), 214(9), 152(6), 124(66)
Sulphamerazine (2b)	264	EI*	265(0.5), 201(10), 200(77), 199(100), 184(7), 140(5), 109(17), 108(26)
		CI	293(4), 279(10), 265(100), 200(8), 138(6), 126(10), 110(60)
Sulphadiazine (2c)	250	EI*	214(10), 199(9), 186(83), 185(100), 170(5), 156(7), 140(9), 108(32)
		CI	279(28), 251(100), 186(7), 124(20), 96(47)

* These spectra are of the eight significant ions above *m/z* 100.

in Fig. 1. The elution times are shorter than in Fig. 1 and this is attributed to deterioration in the performance of the column. The low background present from the interface and mobile phase enabled spectra to be acquired from *m/z* 40. The EI mass spectrum obtained for theophylline is shown in Fig. 8 and those obtained for caffeine and theobromine are summarized in Table I. These spectra gave excellent matches

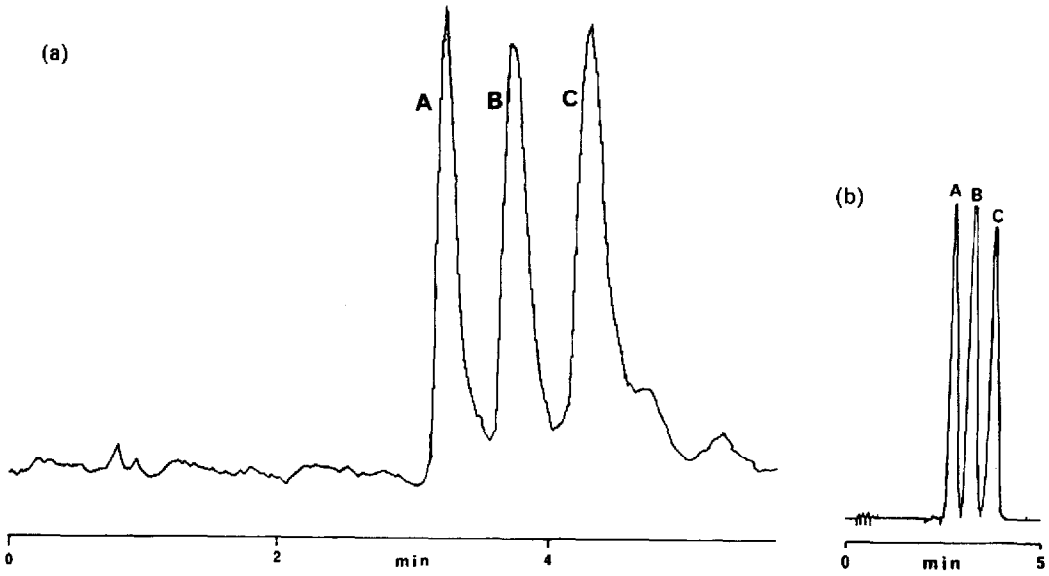


Fig. 9. (a) Computer reconstructed TIC trace obtained by methane CI SFC–MS of a mixture of (A) sulphamethazine (2a), (B) sulphamerazine (2b) and (C) sulphadiazine (2c). SFC conditions as in Fig. 2, except a pressure of 288 bar was used. (b) UV trace obtained in series with the CI SFC–MS TIC trace in (a).

with the library spectra of these compounds, and theophylline and theobromine are readily distinguished on the basis of their EI fragmentation pattern[29].

A mixture of three sulphonamides, sulphamethazine (2a), sulphamerazine (2b) and sulphadiazine (2c), were next studied by SFC–MS. An excellent TIC trace was obtained under EI conditions and the mass spectra obtained are summarized in Table I. All three compounds failed to exhibit molecular ions in this ionization mode, although weak $(M+1)^+$ ions were present for sulphamethazine and sulphamerazine. Use of methane CI SFC–MS gave abundant protonated molecules for all three compounds (Table I) and the TIC and UV traces obtained during the same SFC–MS run are shown in Fig. 9. As can be seen chromatographic performance has been reasonably maintained. There is some evidence of peak broadening, which we attribute to the dead volumes in the connection between the supercritical fluid chromatograph and the moving belt. An improved lower dead volume connection is under construction to obviate this problem. The slightly longer retention times of the three components in the TIC trace are due to the cycle time of the belt on the SFC–MS interface. There is also deterioration in chromatographic performance compared with the trace shown in Fig. 2. This we attribute to deterioration in the chromatography efficiency of the column used.

We have found that deterioration of column performance is comparable with that observed in running similar samples by HPLC, for a similar number of samples run. However, it is more rapid with SFC, because a higher sample throughput is achievable. As yet, we have found no evidence for deterioration of column performance due to adsorption of more polar compounds.

In order to assess the utility of SFC–MS for the study of a complex mixture, the ergot extract discussed earlier (Fig. 5) was examined by EI SFC–MS. The TIC

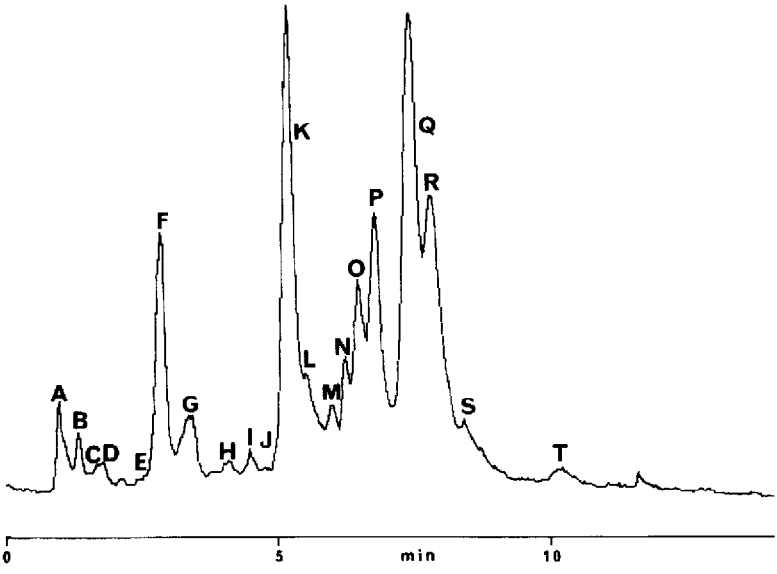


Fig. 10. Computer reconstructed TIC trace obtained by EI SFC–MS of an extract of *Claviceps purpurea*. SFC conditions as Fig. 5, except a column pressure of 396 bar was used.

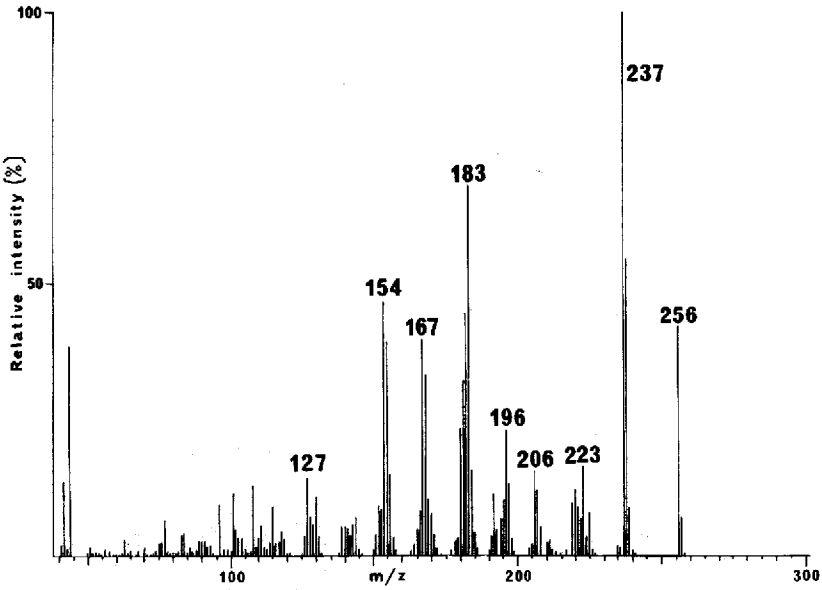


Fig. 11. EI mass spectrum of peak O in Fig. 10, isochanoclavine I.

trace obtained is shown in Fig. 10 and the EI mass spectrum of peak O is shown in Fig. 11. On the basis of its mass spectrum and comparison of retention times with an authentic sample, it was identified as isochanoclavine I. Comparison of the TIC trace and the UV trace in Fig. 5 shows some loss of resolution; this was because the SFC–MS was performed at a later date and the column performance had deteriorated. Good chromatographic integrity was observed with the UV trace obtained at the same time as the SFC–MS data and the data obtained compared favourably with that obtained by HPLC–MS on the same sample[27]. In this paper we are unable to report data on a number of new compounds which were located because of the collaborative nature of the project. Full details of the HPLC–MS studies are available[30]. Structural assignments for the various components are given in Table II and are in agreement with the assignments given in Fig. 5, which were based on comparative retention times. Mass spectra are not reported, since they are similar to those reported previously[27,30]. Whereas the HPLC–MS analyses required a run time of over 1 h, SFC–MS analysis was accomplished in 10 min. Differing selectivities were observed resulting in the location of some components not found by HPLC–MS and a failure to locate the palliclavine isomers found by HPLC–MS (see Table II).

A major advantage of combined chromatographic–mass spectrometric studies for the analysis of complex mixtures is an ability to enable multicomponent peaks to be located and often resolved. This is illustrated in peak G which was shown to consist of four components, two with MW 258 and 240 and an isomeric pair of MW 254.

TABLE II

IDENTIFICATIONS FROM SFC–MS OF EXTRACT FROM *CLAVICEPS PURPUREA* (FIG. 10)

Peak	*Identification*	*MW*	*Found in previous study ref.*
A	Dioctylphthalate	390	
B	Unknown	242	30
C	Unknown	268	
D	Unknown	268	
E	Pyroclavine (18b)	240	
F	Argroclavine (17a)	238	27
G	Unknown	254	
	Unknown	258	30
	Festuclavine (18a)	240	27
	Unknown	254	
H	Isosetoclavine (19c)*	254	30
I	Setoclavine (19b)*	254	27
J	Unknown	242	
K	Elymoclavine (17c)	254	27
L	Penniclavine (19a)	270	27
M	Noragroclavine (17b)	224	27
N	Unknown	256	
O	Isochanoclavine (20c)	256	27
P	N-norisosetoclavine (19d)	240	30
Q	Chanoclavine I (20a)	256	27
R	Chanoclavine II (20b)	256	27
S	Unknown	254	
T	N-norchanoclavine II (20d)	242	27

* Positions may be reversed: authentic samples were not available for comparison of retention times.

CONCLUSIONS

SFC has been shown to be effective in providing rapid chromatographic separations of a range of polar compounds, many of which are not directly amenable to GC study. Use of conventional column SFC enables more rapid analysis to be achieved than by HPLC and method development is quicker.

Combined SFC–MS has been accomplished using a moving belt HPLC–MS interface with a modified thermospray deposition device. This approach enables both EI and CI mass spectra to be obtained and allows a high proportion of the eluent from the chromatograph to be handled. The utility of the combination has been shown with mixtures of xanthines, sulphonamides and ergot alkaloids.

We believe from these preliminary investigations that SFC and SFC–MS will prove to be invaluable complementary techniques to GC and HPLC and their combination with mass spectrometry for many of our studies.

ACKNOWLEDGEMENTS

A.J.B. and J.R.P. thank the SERC and The Ministry of Agriculture Fisheries and Food, respectively, for financial support. We are deeply indebted to the AFRC

for provision of funds for the purchase of the SFC equipment and to Finnigan MAT for the thermospray deposition device. The SERC are thanked for assistance in the purchase of mass spectral equipment. We are grateful to Dr. E. C. Kornfeld (Lilly Research Laboratories, Indianapolis) for the provision of standard alkaloid samples and for the crude fermentation extract and to Dr. F. Erni (Sandoz, Basle) for the samples of bromocryptine and ergocryptine. Finally, we thank Drs. D. R. Gere and L.- G. Randall (Hewlett-Packard, Avondale, Penn.) and Dr. J. D. Henion (Cornell University) for stimulating and helpful discussions on SFC and SFC–MS.

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