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ANALYSIS OF DRUG RESIDUES IN TISSUE BY COMBINED SUPERCRITICAL-FLUID EXTRACTION–SUPERCRITICAL-FLUID CHROMATOGRAPHY–MASS SPECTROMETRY–MASS SPECTROMETRY

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SUMMARY

The combination of supercritical-fluid extraction–supercritical-fluid chromatography–tandem mass spectrometry has been evaluated for the detection of residues of a small group of veterinary drugs in freeze-dried pig's kidney. During extraction with supercritical CO_2 the drugs were retained by the column while non-polar endogenous material was not retained and thus passed to waste. Subsequent changes to the mobile phase composition eluted the drugs which were detected with high specificity by tandem mass spectrometry. Although the sensitivity in this preliminary study was not adequate for surveillance or enforcement, there is potential for further development of the approach.

INTRODUCTION

Supercritical-fluid extraction (SFE) has been used for the industrial-scale separation and isolation of a variety of compounds[1,2]. The relatively low critical temperatures of fluids such as CO_2 (T_c 31.1°C) enable extractions to be performed under mild conditions and this, coupled with low cost, miscibility, non-toxicity, non-flammability and ease of removal of the solvent has made SFE with this fluid especially attractive[3]. Many aspects of SFE have been reviewed in detail[4].

On the analytical scale SFE has also attracted attention[5,6] and a variety of techniques have been used for on-line analyses. Unger and Roumeliotis[7] have described the combination of SFE with high-performance liquid chromatography (HPLC) applied to the analysis of valtrate and didrovaltrate from *Radix valerianae*. The combination of SFE with gas chromatography (GC) has been applied to the analysis of polycyclic aromatic compounds in model matrices and air particulates[8], and also to the analysis of flavour and fragrance compounds in a variety of matrices[9]. The latter work also included the use of SFE–GC on-line to mass spectrometry (MS).

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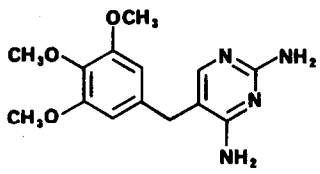
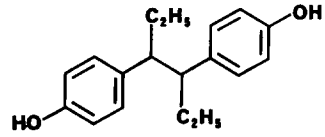
Direct fluid injection of supercritical-fluid extracts into a mass spectrometer has been explored by Kalinoski *et al.*[10]. In this work, dealing with the determination of trichothecene mycotoxins in wheat, tandem mass spectrometry (MS–MS) was employed to achieve adequate specificity.

Supercritical-fluid chromatography (SFC) has also been coupled to SFE[11–13]. Sugiyama *et al.*[11] have demonstrated this approach in the analysis of caffeine in coffee beans, and present a detailed exploration of the effects of varying the SFE conditions. Engelhart and Gross[12] have used the combination with flame ionization detection for the study of plant materials. McNally and Wheeler[13] have used SFE–SFC with ultraviolet (UV) detection for the analysis of sulphonylurea herbicides in various matrices including soil and plant material. Although these workers all used packed column SFC, capillary column SFC can also be used on-line with SFE and a prototype apparatus has recently been described[14].

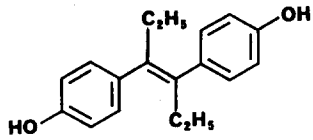
In this laboratory we have paid great attention to the potential of modern instrumental methods for rapid trace analysis, and especially to the use of automated methods or on-line combinations of techniques. We have previously reported the use of MS–MS[15] and HPLC–MS–MS[16] for the detection of veterinary drug residues in animal tissues following simple extraction methods and minimal clean-up. We now report a preliminary investigation of SFE–SFC coupled to MS–MS for the detection, in pig's kidney, of a small group of veterinary drugs including trimethoprim (molecular weight, MW 290), hexestrol (MW 270), diethylstilbestrol (MW 268) and dienestrol (MW 266).



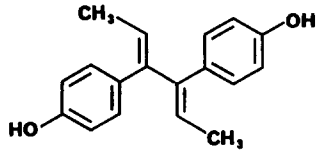
Trimethoprim



Hexestrol



Diethylstilbestrol



Dienestrol

EXPERIMENTAL

Materials

HPLC-grade methanol was obtained from Rathburn (Walkerburn, U.K.). Instrument-grade liquid carbon dioxide supplied in a cylinder with a syphon tube was obtained from British Oxygen (London, U.K.). Trimethoprim, hexestrol, diethylstilbestrol, dienestrol and sulphamethazine were obtained from Sigma (Poole, U.K.).

Sample preparation

A whole pig kidney, purchased from a local supplier, was blended to a smooth paste in a food processor. The paste (167 g) was spread onto trays and freeze dried using an Edwards EF6(S) shelf freeze drier (Edwards High Vacuum, Crawley, U.K.). The dried sample (43.4 g) was sieved through a 0.710-mm aperture sieve (Endecotts, London, U.K.). Portions of the powdered kidney (1 g) were mixed with methanol (10 ml), spiked with the appropriate quantity of each drug, allowed to equilibrate for one hour whilst being sonicated and then evaporated to dryness in a nitrogen stream. All samples were thoroughly mixed prior to being loaded into the extraction cell.

Supercritical-fluid chromatography

Throughout these studies supercritical fluid was supplied by a Hewlett-Packard packed column SFC apparatus based on a 1084B liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.). The liquid carbon dioxide supply line and the pump heads were cooled to −25°C using a Neslab RTE-42 refrigerated bath. Carbon dioxide–methanol was used as mobile phase, and was supplied at a flow-rate of 4 ml/min. The mobile phase composition was initially 100% carbon dioxide for 8 min and was then programmed linearly to carbon dioxide–methanol (80:20) in 0.5 min. The final composition was maintained for 10 min. The 100 × 4.6 mm I.D. column used in these studies was packed in our own laboratory with amino-bonded Spherisorb 5 (Phase Separations, Queensferry, U.K.). The column pressure was 302 bar and oven temperature was 75°C. For sample introduction a Rheodyne 7125 valve was used with a 20-μl loop.

Supercritical-fluid extraction

A schematic of the SFE–SFC system is shown in Fig. 1. An Uptight 20 × 2 mm I.D. HPLC precolumn (Anachem, Luton, U.K.) was used as the extraction cell and was packed with freeze-dried material retained by 2 μm frits. The cell was connected in place of the fixed volume loop of the Rheodyne valve. The inject and load positions of the valve were used to allow the contents of the cell to be extracted or isolated as the elutropic strength of the mobile phase was varied during the SFC gradient programme. Samples were extracted for the first 8.5 min of the SFC gradient programme. Simple SFE experiments were conducted with a length of 0.15 mm bore stainless-steel tubing replacing the analytical column. For SFE–SFC the column and chromatographic conditions were as above.

Mass spectrometry

All MS studies were carried out using a VG 7070EQ tandem mass spectrometer (VG Analytical, Wythenshawe, U.K.) equipped with a standard VG water cooled

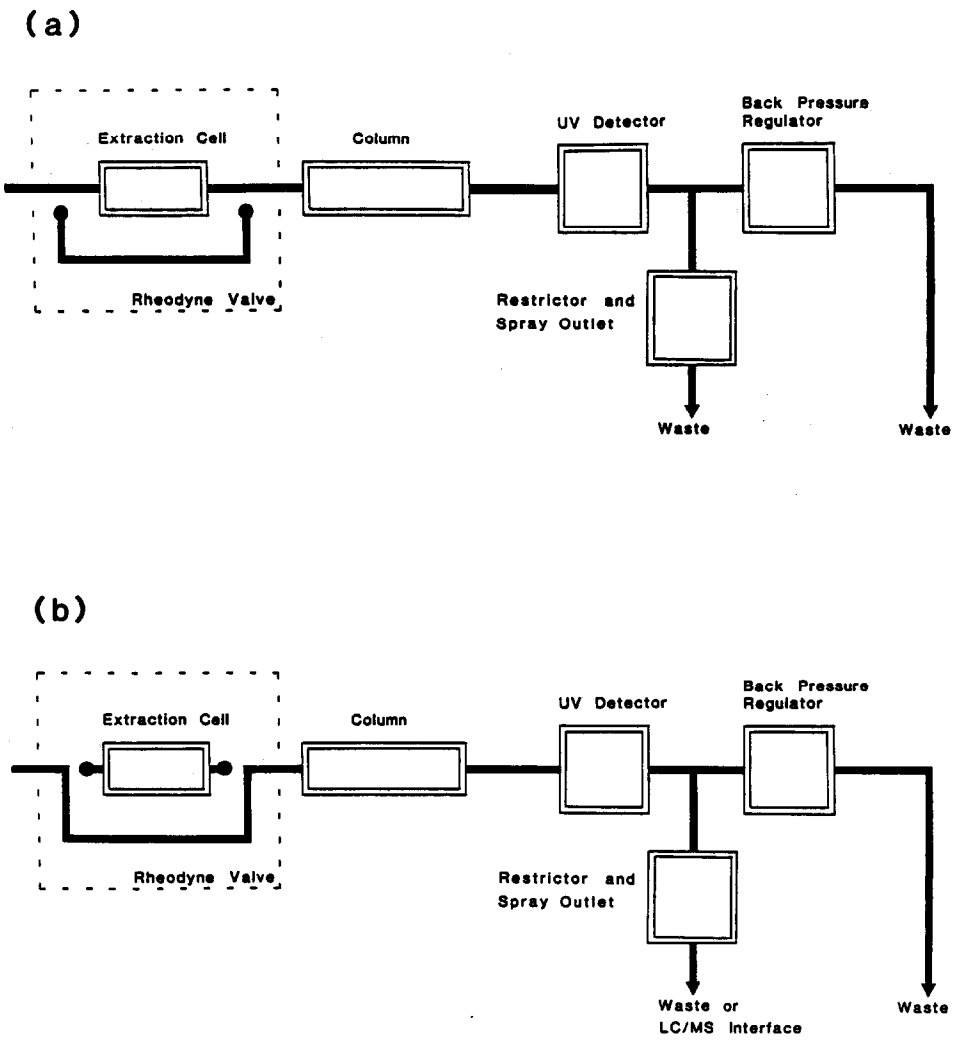


Fig. 1. Schematic of SFE–SFC–MS–MS system (a) during extraction, (b) during post extraction chromatography.

temperature-programmable probe and moving belt HPLC interface. A VG 11-250 datasystem was used for instrument control, and for data acquisition and analysis.

Electron ionization mass spectra were recorded at a source temperature of 240°C with an electron energy of 70 eV and a trap current of 200 μA. For normal mass spectra the double focussing part of the instrument was set to operate at a resolution of 1000 (10% valley definition) and ions were detected at the intermediate collector. For MS–MS experiments the parent ion resolution was set to 500 and the daughter ion resolution was unit mass. The collision gas was argon and the collision gas pressure was adjusted to give a housing pressure indication of $8 \cdot 10^{-6}$ mbar. The collision energy was set to 15 eV. Reference mass spectra were obtained using the direct insertion probe with a temperature programme of 40 to 350°C at 2°C/s.

Supercritical fluid chromatography–mass spectrometry

Combined SFC–MS was carried out using the moving belt interface in the manner described by Berry[17]. The eluent was applied to the belt with a modified Finnigan MAT (San Jose, CA, U.S.A.) thermospray deposition device. The spray deposition device was connected by a 60-cm length of 0.15 mm bore stainless-steel tubing to a tee-piece situated between the UV detector exit and the back-pressure regulator of the chromatograph. In order to maintain SFC conditions to the point at which the eluent left the system, the end of the thermospray jet was slightly crimped. This arrangement resulted in a 1:1 split of the column eluent between UV and MS detection and also facilitated use of the full density range of supercritical carbon dioxide for SFC. A 6-V power supply and rheostat adjustment was used to provide direct heating of the thermospray jet in order to prevent solute precipitation and freezing of the eluent at the tip. The belt speed was 1.2 cm/s resulting in a 40-s lag between responses at the UV detector and the mass spectrometer. The sample evaporator was set at 240. During SFE–SFC–MS and SFE–SFC–MS–MS experiments the eluent was directed to the mass spectrometer only during the elution period corresponding to the compounds of interest (approximately 10–12 min).

During MS–MS experiments the datasystem was used to control the instrument so that the quadrupole comprising MS-2 was scanned repetitively about once per second while MS-1 was used, by accelerating voltage switching at constant magnet current, to select the desired parent for each scan.

RESULTS AND DISCUSSION

In order to establish the feasibility of direct SFE–MS or SFE–MS–MS analyses, initial experiments were performed to determine the quantity of endogenous material extracted from the loaded extraction cell. The SFC gradient programme described above was used during these preliminary investigations and the amino bonded column was replaced with a length of 0.15 mm bore stainless-steel tubing. The average cell loading was 28.4 mg and these preliminary experiments showed that approximately 20% of this material was removed during the initial 8.5 min extraction period.

Even if MS detection were able to provide sufficient specificity, the use of direct SFE–MS or SFE–MS–MS would have been impractical due to the rapid contamination of the mass spectrometer which would have occurred. To avoid this problem an SFE–SFC procedure was developed to provide a degree of clean-up. This approach was designed to exploit the fact that most drugs are rather polar while the major part of the co-extracted endogenous material is relatively non-polar, and involved the use of a polar amino phase SFC column. During the period of extraction with unmodified CO_2, the extracted drugs were strongly retained at the inlet of the column whilst the majority of extracted endogenous components were eluted rapidly and directed to waste. The drugs and more polar endogenous components were then eluted by adding modifier to the mobile phase. The mass of extracted endogenous material present in the same elution window as the drugs was estimated by collecting and weighing this fraction. This indicated that approximately 100–200 μg of material was deposited onto the moving belt interface per analysis.

The SFC–UV chromatogram of standards shown in Fig. 2 was obtained by replacing the extraction cell with a 20-μl valve loop prior to analysing a standard

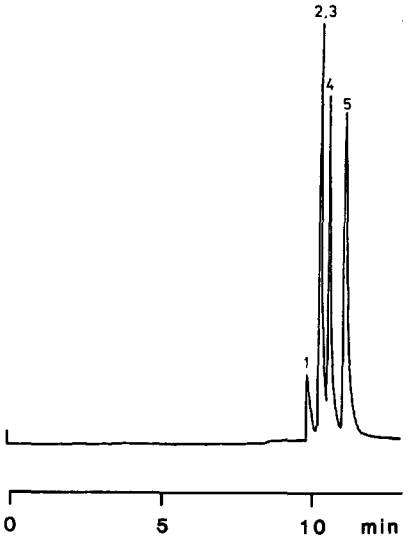


Fig. 2. SFC analysis of a standard drug mixture: 1 = trimethoprim; 2 = hexestrol; 3 = diethylstilbestrol; 4 = dienestrol; and 5 = sulphamethazine. Of each drug 20 μg was injected on column. UV detection at 254 nm. Range 0.4 a.u.f.s.

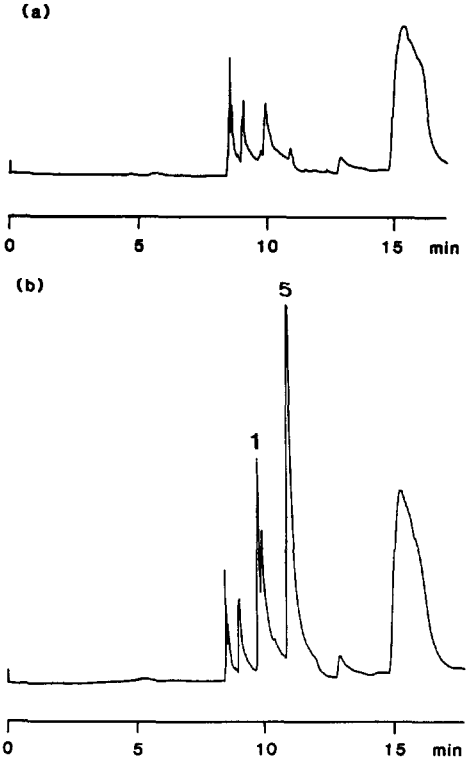


Fig. 3. SFE–SFC analysis (UV detection at 254 nm, range 0.4 a.u.f.s.) of (a) blank kidney sample (b) kidney sample spiked with 150 mg/kg each of the marker drugs trimethoprim (1) and sulphamethazine (5).

solution of the drugs and demonstrates the chromatographic separation obtained with the SFE–SFC gradient programme. As shown in Figs. 3 and 4, SFE–SFC with UV detection at either 254 or 215 nm did not afford sufficient resolution and specificity to allow detection of the drugs spiked at the 10 mg/kg level. Although our ultimate aim was to develop an approach using MS–MS to provide specificity, and in which a high degree of chromatographic separation was not required, the initial attempts to develop an SFE–SFC programme were hampered by the high background of endogenous components. This problem was overcome by the preparation and subsequent analysis of a kidney sample spiked with high levels of trimethoprim (the earliest eluting drug) and sulphamethazine (eluting after the last drug of interest). Sulphamethazine was introduced as a retention range marker because it exhibited a slightly longer retention time that dienestrol, but gave a sharp chromatographic peak in contrast to the tailing obtained with dienestrol. The SFE–SFC chromatogram obtained for a kidney sample spiked with 150 mg/kg of the marker drugs is shown in Fig. 3.

Although precise extraction efficiencies for the drugs have not been established during these preliminary investigations, re-analysis by SFE–SFC–MS–MS of the dried residue of a sample initially analysed after spiking at 10 mg/kg suggested that all extractable drugs had been recovered in the first cycle.

We have previously discussed[15] some of the mass spectral properties desirable for the development of an MS–MS procedure for target compound detection. Direct probe introduction of standards was used during preliminary mass spectral studies since this permitted control of the evaporation rate to give moderately persistent spectra and thus facilitated adjustment of the mass spectrometer tuning. The electron impact (EI) mass spectra obtained for trimethoprim, hexestrol, diethylstilbestrol and

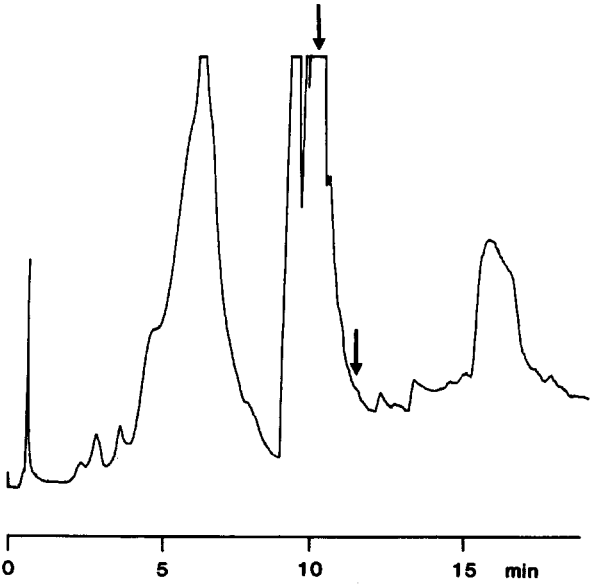


Fig. 4. SFE–SFC analysis of a blank kidney sample. UV detection at 215 nm. Range 1.6 a.u.f.s. Elution range of drugs indicated by arrows.

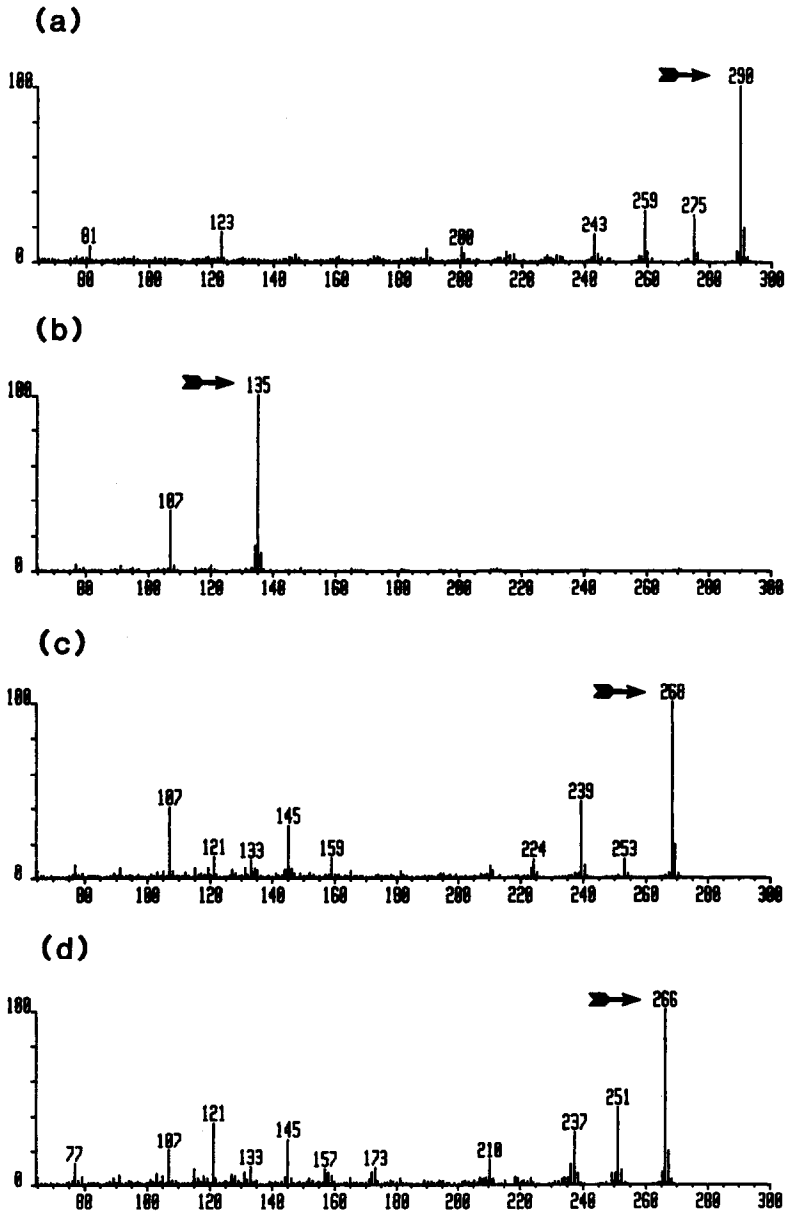


Fig. 5. Direct insertion probe EI mass spectra obtained for (a) trimethoprim, (b) hexestrol, (c) diethylstilbestrol, (d) dienestrol.

dienestrol are shown in Fig. 5. With the exception of hexestrol, the molecular ion of each drug is the most abundant species in the mass spectrum and was selected as the parent ion for collision-induced dissociation (CID) MS–MS experiments. In the case

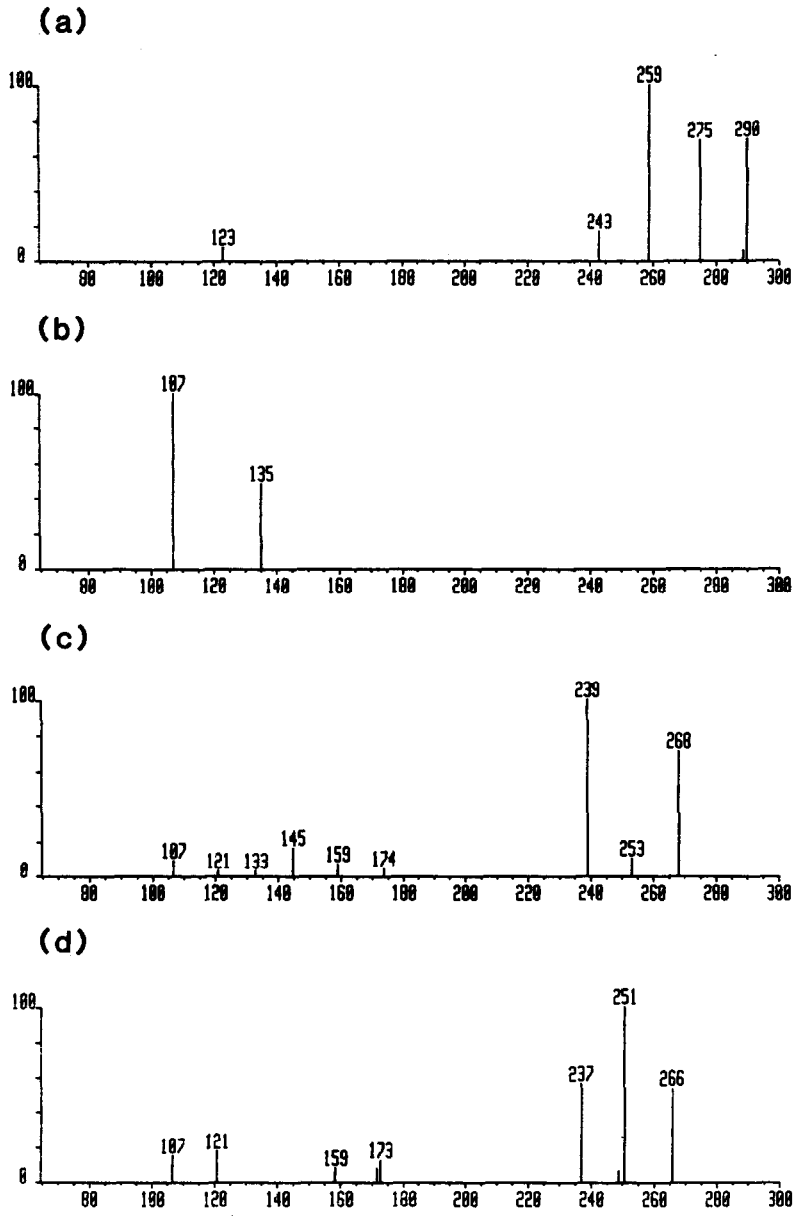


Fig. 6. Direct insertion probe CID daughter ion mass spectra obtained for (a) $M^{+\cdot}$ (m/z 290) trimethoprim, (b) m/z 135 hexestrol, (c) $M^{+\cdot}$ (m/z 268) diethylstilbestrol, (d) $M^{+\cdot}$ (m/z 266) dienestrol.

of hexestrol the fragment ion at m/z 135 was selected. The CID daughter ion mass spectrum of each drug is shown in Fig. 6.

In experiments using SFE–SFC–MS the intense background mass spectra of the

co-chromatographing endogenous components prevented the analysis of the drugs. The mass spectrum obtained at the retention time corresponding to trimethoprim is shown in Fig. 7, where the mass spectrum of the drug is completely obscured. SFE–SFC–MS–MS analysis provided daughter ion spectra which were virtually free of interferences (Fig. 8) and permitted the unambiguous detection of the drugs at the 10-mg/kg level.

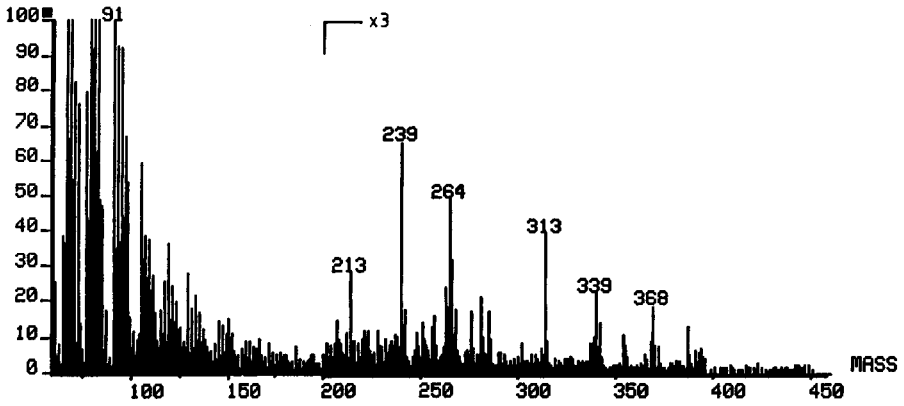


Fig. 7. EI mass spectrum obtained at retention time of trimethoprim. Result from SFE–SFC–MS analysis of kidney sample spiked with 10 mg/kg of each drug.

The technique of selected-reaction monitoring (SRM) seeks to extend the sensitivity of MS–MS in an analogous manner to selected-ion monitoring in conventional MS, and records information about selected daughter ions formed from selected parents. Although we have previously reported[16] that the neutral noise effect sometimes observed on our instrument has precluded SRM analyses of some matrices, in the present study SRM was found to be a useful means of extending the sensitivity of SFE–SFC–MS–MS and analysis of blank kidney samples did not indicate problems associated with neutral noise effects or chemical interference. The results of an SRM SFE–SFC–MS–MS analysis, using two reactions per drug, for a kidney sample spiked with 1 mg/kg of each of the three drugs trimethoprim, dienestrol and diethylstilbestrol are shown in Fig. 9. Hexestrol could also be detected at the 1-mg/kg level with this approach but the simplicity of the CID daughter ion spectrum under the specified collision conditions, meant that only one reaction was suitable for monitoring.

CONCLUSIONS

This preliminary study demonstrates considerable potential for the combination of SFE–SFC–MS–MS in the detection of trace levels of contaminants in foods and other matrices. Although in the current study the detection limits are not sufficiently low to meet the stringent controls on drug residues in meat for human consumption, we believe that there is considerable scope for further optimisation in this respect.

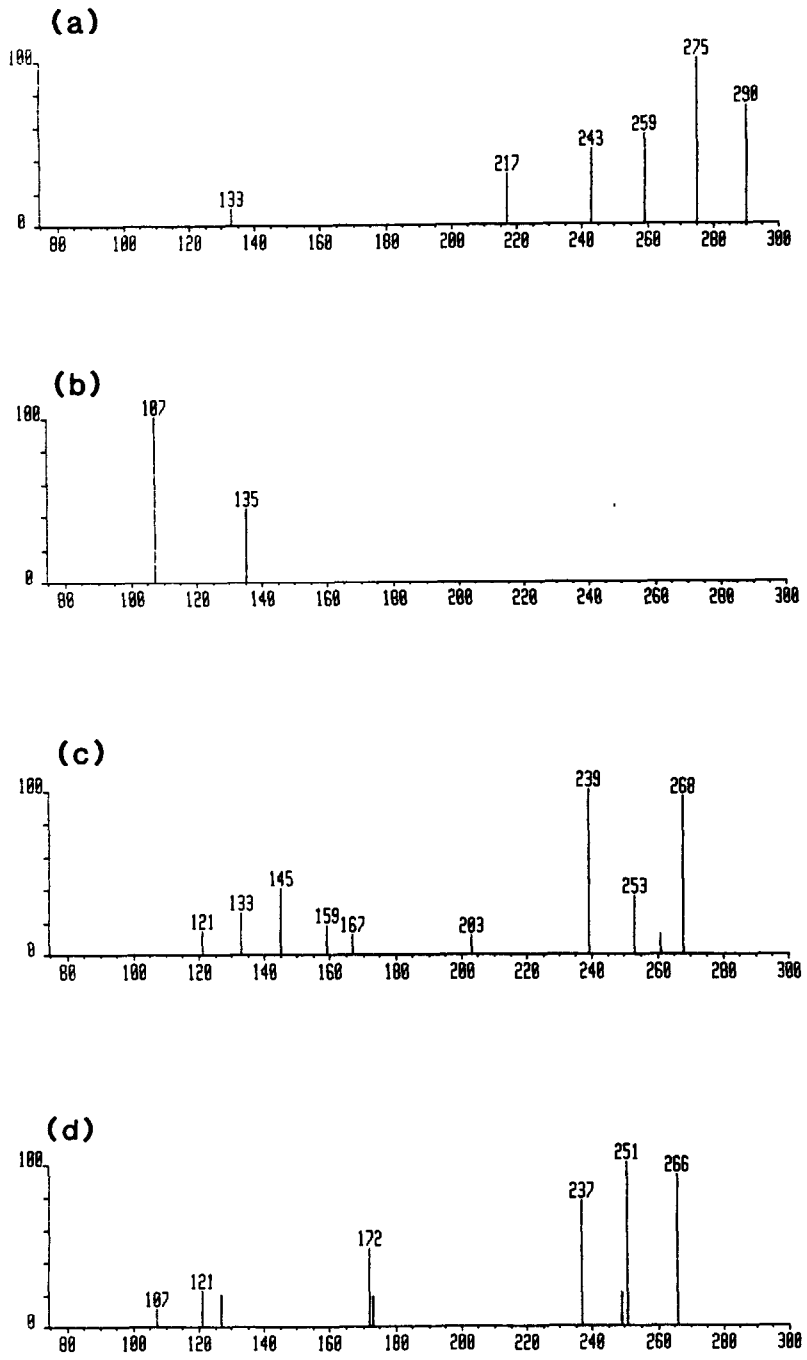


Fig. 8. CID daughter ion mass spectra obtained for (a) trimethoprim, (b) hexestrol, (c) diethylstilbestrol, (d) dienestrol. Results from SFE–SFC–MS–MS analysis of kidney sample spiked with 10 mg/kg of each drug.

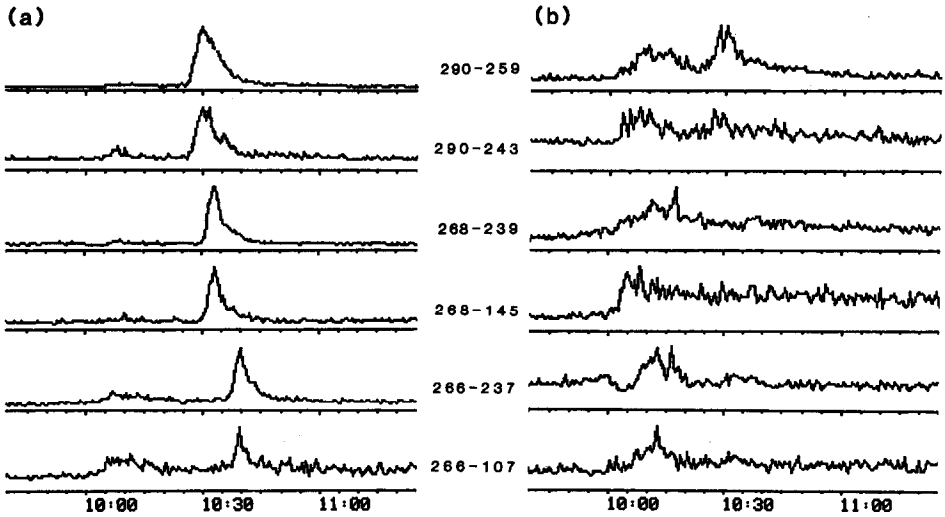


Fig. 9. SRM chromatograms obtained from SFE–SFC–MS–MS analysis of (a) kidney spiked with 1 mg/kg each of trimethoprim (*m/z* 290–259; *m/z* 290–243), diethylstilbestrol (*m/z* 268–239; *m/z* 268–145) and dienestrol (*m/z* 266–237; *m/z* 266–107), (b) blank kidney.

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