Supercritical Fluid Extraction of Organophosphate and Carbamate Insecticides in Honeybees

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Supercritical fluid extraction (SFE) was evaluated for the extraction of organophosphate and carbamate insecticides in honeybees. Extracts were analyzed by gas chromatography (GC) with nitrogen−phosphorus detection or flame-photometric detection with confirmation by GC with ion-trap mass spectrometry. Samples were mixed with diatomaceous earth and optimal extraction conditions were: (1) CO_2 density of 0.7 g/mL, (2) 60 °C, (3) 2 min equilibration time, (4) 40 mL of CO_2, and (5) 1.6 mL/min flow rate. Analytes were trapped on octadecyl silane (ODS) and eluted with acetonitrile. No further cleanup was necessary and chromatographic interferences in SFE extracts were comparable to those in solvent extracts that had undergone extensive cleanup procedures. Recoveries from honeybees fortified at 1 and 0.1 μg/g were greater than 75% for all pesticides studied except omethoate. Samples containing residues were also extracted by SFE, and there was good quantitative agreement with results from analyses based on solvent extractions.

Keywords: *Supercritical fluid extraction (SFE); residue determination; toxicology; organophosphate insecticides; carbamate insecticides; honeybees*

INTRODUCTION

Honeybees play an important ecological role due to their involvement in pollination of plants. They are also commonly kept commercially for the production of honey. Their ecological and economic importance is reflected in the European Union's regulations for registration of plant protection products which require honeybee toxicity data. Of the pesticides registered for use in the United Kingdom a number of insecticides are classified as dangerous or harmful to honeybees. To monitor possible adverse effects of pesticides on honeybees, sensitive and reliable methods for determining residues are required.

The determination of pesticide residues in honeybees poses challenging analytical problems. Honeybees contain large amounts of beeswax which is readily extracted by solvents typically used in residue analysis. As well as causing chromatographic interferences this wax is particularly effective at blocking active sites in gas chromatography injection ports and columns, resulting in severe quantitation problems. Sample cleanup procedures to remove as much wax as possible from solvent extracts are essential for sensitive and reliable determination.

Supercritical fluid extraction (SFE) is emerging as an alternative to solvent-based extraction techniques traditionally used in residue analysis. SFE has recently been shown to be applicable to the extraction of a range of pesticides in various sample matrices such as soil (Snyder *et al.*, 1993), plant material (King *et al.*, 1993; Skopec *et al.*, 1993; Howard *et al.*, 1993; Lehotay and Eller, 1995) and meat (France and King, 1991). In addition to reducing extraction time, solvent consumption and waste generated SFE can also give rise to more selective extraction of analytes such as pesticides. Lehotay and Eller (1995) showed that a range of pesticides could be determined in SFE extracts of fruit and vegetables by gas chromatography−mass spectrometry (GC−MS) without the need for additional cleanup. The possibility of more selective extraction of pesticides from honeybees was an attractive prospect because of the problems encountered with co-extractives in solvent extracts. It was therefore decided to evaluate SFE for extraction of a range of organophosphate and carbamate insecticides from honeybees. These two groups of pesticides, when sprayed onto agricultural crops, have caused the great majority of incidents of pesticide poisoning of honeybees in the U.K. over recent years (Fletcher *et al.*, 1995).

EXPERIMENTAL PROCEDURES

Gas Chromatography−Flame Photometric Detection (GC−FPD). A model Ai93 GC was used (Analytical Instruments, Cambridge, U.K.), equipped with a J+W model 210−1063 splitless injection port (Jones Chromatography, Hengoed, U.K.), Tracor flame photometric detector and a Hewlett Packard model 7673 autosampler. Operating conditions were as follows: 2 μL injection into injection port at 220 °C; initial oven temperature 140 °C for 0.5 min, then linearly increased to 180 °C at 10 °C/min, then to 270 °C at 6 °C/min, held for 10.5 min; 4.5 mL/min flow of N_2 through column plus 60 mL/min N_2 as make-up gas. The column was 15 m × 0.53 mm i.d. coated with DB17 at a film thickness of 1 μm.

Gas Chromatography−Nitrogen Phosphorus Detection (GC−NPD). A Hewlett Packard model 5890 with a model 7673 autosampler, on-column temperature programmable injector and nitrogen phosphorus detector was used. Operating conditions were: 1 μL injection; initial oven temperature 50 °C held for 0.5 min, then linearly increased at 20 °C/min to 235 °C, held for 4 min; initial injector temperature 53 °C, then programmed to be 3 °C above the oven temperature at all times; column was 15 m × 0.53 mm i.d. coated with DB1 at a film thickness of 1.5 μm; 10 mL/min flow of He through column plus 15 mL/min N_2 as make-up gas.

Gas Chromatography−Mass Spectrometry (GC−MS). Confirmation of residues from incurred samples was performed with a Hewlett Packard model 5890 GC equipped with a split/splitless injector connected to a Finnegan model ITD800 ion-trap mass spectrometer (Finnegan MAT, Hemel Hempstead, U.K.). One-microliter injections were made into the injector operated in splitless mode at 150 °C; initial oven temperature was 60 °C held for 1 min, then linearly increased at 25 °C/min to 260 °C, held for 3 min; column was 25 m × 0.25 mm i.d. coated with BPX5 at 0.25 μm film thickness, He head

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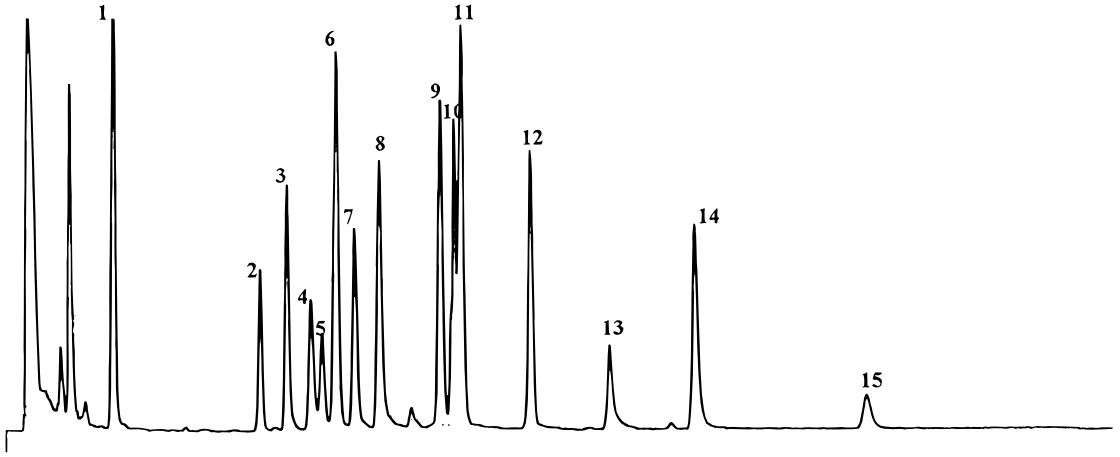

Figure 1. GC−FPD chromatogram of honeybee sample spiked with 1 μg/g of 15 organophosphates. Peaks: 1 = dichlorvos, 2 = heptenophos, 3 = demeton-*S*-methyl, 4 = omethoate, 5 = thiometon, 6 = diazinon, 7 = disulfoton, 8 = dimethoate, 9 = pirimiphos-methyl, 10 = chlorpyrifos, 11 = fenitrothion, 12 = quinalphos, 13 = vamidothion, 14 = triazophos, 15 = azinphos-methyl.

pressure 15 psi. Typical mass spectrometer operating conditions were: full-scan acquisition mode from *m/z* 50−400, electron impact ionization at 70 eV, 1700 V multiplier tube voltage.

Chemicals. CO_2 used for SFE extractions was instrument grade with dip tube, and CO_2 used for cryogenic cooling of the SFE apparatus was of standard grade (Air Products, Basingstoke, U.K.). Hydromatrix was used to absorb water from samples (Varian Ltd., Walton-on-Thames, U.K.). All solvents were of glass-distilled or HPLC grade (Rathburn Ltd., Walkerburn, U.K.).

Pesticide Standards. Chlorpyrifos, vamidothion, and carbaryl were obtained from Rhone-Poulenc (Ongar, U.K.) and were >98% purity. Disulfoton (99% purity) was from Bayer (Bury St. Edmunds, U.K.). Heptenophos (98% purity) was from Hoechst (Kings Lynn, U.K.). Pirimiphos-methyl (99%) was from Zeneca (Jealott's Hill, U.K.). All other pesticide standards were from Greyhound Chemicals (Birkenhead, U.K.) and were >98% purity except demeton-*S*-methyl (95%), omethoate (93%), thiometon (75%), and triazophos (93%).

Sample Preparation. A 1 g subsample of honeybees was thoroughly homogenized with 2 g of Hydromatrix diatomaceous earth by grinding in a glass pestle and mortar. After 15 min had been allowed for all water to be absorbed this mixture was packed into a 7 mL extraction vessel and any remaining space filled with Hydromatrix. Samples that were spiked had 25 μL of a pesticide solution added, and solvent was allowed to evaporate for 15 min prior to adding Hydromatrix. All bee samples used in recovery experiments had previously been shown to contain none of the pesticides of interest in this study.

Extraction. SFE apparatus used was a model 7680T (Hewlett-Packard, Bracknell, U.K.) with automated restrictor and solid sorbent collection system. Optimal extraction parameters were as follows: 187 bar pressure and 60 °C (CO_2 density, 0.7 g/mL); 2 min equilbration time followed by 40 mL of CO_2 at 1.6 mL/min; 50 °C restrictor temperature, collection on an octadecylsilica (ODS) trap at 25 °C; elution with 1.5 mL of acetonitrile at 0.5 mL/min into 2 mL sealed vials; trap rinsed to waste with 4 mL of ethyl acetate followed by 2 mL of acetonitrile. All vials were weighed before and after collection of eluate to ensure that the volume of eluate was accurately known.

Solvent Extraction and Gel Permeation Chromatography (GPC) Cleanup Method. Honeybee samples containing residues were analyzed by this method, which has been in use for some years in this laboratory, to compare with results from SFE extracts. In the method a 5 g sub sample of honeybees was ground in a pestle and mortar with 25 g of anhydrous sodium sulfate. The mixture was extracted in a Soxhlet apparatus with 100 mL diethyl ether for 16 h. This extract was evaporated to dryness and reconstituted in 10 mL of hexane:ethyl acetate (50:50). Five milliliters of this was injected into a glass column of 400 mm × 25 mm i.d. packed with Bio-Beads SX3 (Bio-Rad, Hemel Hempstead, U.K.). The elution solvent was hexane:ethyl acetate (50:50) at 5 mL/min. The first 90 mL of eluent was discarded, and the second 90 mL collected, evaporated to dryness and reconstituted in 5 mL of hexane.

Analysis. Recoveries of organophosphates from spiked bee samples were measured using GC−FPD by comparing peak areas with matrix-matched calibration solutions. These calibration solutions were prepared by diluting the spiking solution into a nonfortified extract of the same bee sample used for spiking. Recoveries of the carbamate insecticides bendiocarb, carbaryl, and propoxur, from spiked samples were measured by GC−NPD. Sample extracts were evaporated to dryness under a stream of N_2, reconstituted in 2 mL of ethyl acetate:hexane (50:50), and measured by comparing peak areas with calibration standards prepared from spiking solution diluted into ethyl acetate:hexane (50:50). All residues obtained from SFE extracts were measured using GC−NPD by evaporating to dryness, reconstituting in ethyl acetate:hexane (50:50), and comparing with peak areas of external calibration standards in the same solvent. Solvent extracts were initially screened using GC−FPD after GPC cleanup. Once a residue had been tentatively identified, the extract was further cleaned, if required, on a Sep-Pak silica solid-phase extraction (SPE) column (Millipore, Watford, U.K.). For dimethoate, a volume equivalent to 0.5 g of tissue was evaporated to dryness and reconstituted in 1 mL of diethyl ether:hexane (5:95). This was applied to a silica Sep-Pak column (pre-washed with 10 mL of diethyl ether and 10 mL of hexane). The column was washed with 10 mL of diethyl ether:hexane (50:50), and the dimethoate was eluted with 10 mL of acetone:diethyl ether (5:95). This was evaporated to dryness and reconstituted in 1 mL of acetone:diethyl ether (5:95). For bendiocarb, a volume equivalent to 2 g of tissue was evaporated to dryness and reconstituted in 1 mL of diethyl ether:hexane (5:95). This was applied to a silica Sep-Pak column (pre-washed with 10 mL of diethyl ether and 10 mL of hexane). The column was washed with 10 mL of diethyl ether:hexane (10:90), and the bendiocarb was eluted with 10 mL of diethyl ether:hexane (65:35). This was evaporated to dryness and reconstituted in 2 mL of hexane. No further cleanup was required for chlorpyrifos. Residue concentrations were then measured on GC−NPD by comparison of peak areas with external standards in hexane.

RESULTS AND DISCUSSION

Development of SFE Procedure. The starting point for the development of suitable extraction conditions was a previous study by Lehotay and Eller (1995). They used CO_2 at 0.85 g/mL with a temperature of 60 °C and a 2 min static extraction followed by 42 mL at a flow rate of 1.6 mL/min. These conditions were suitable

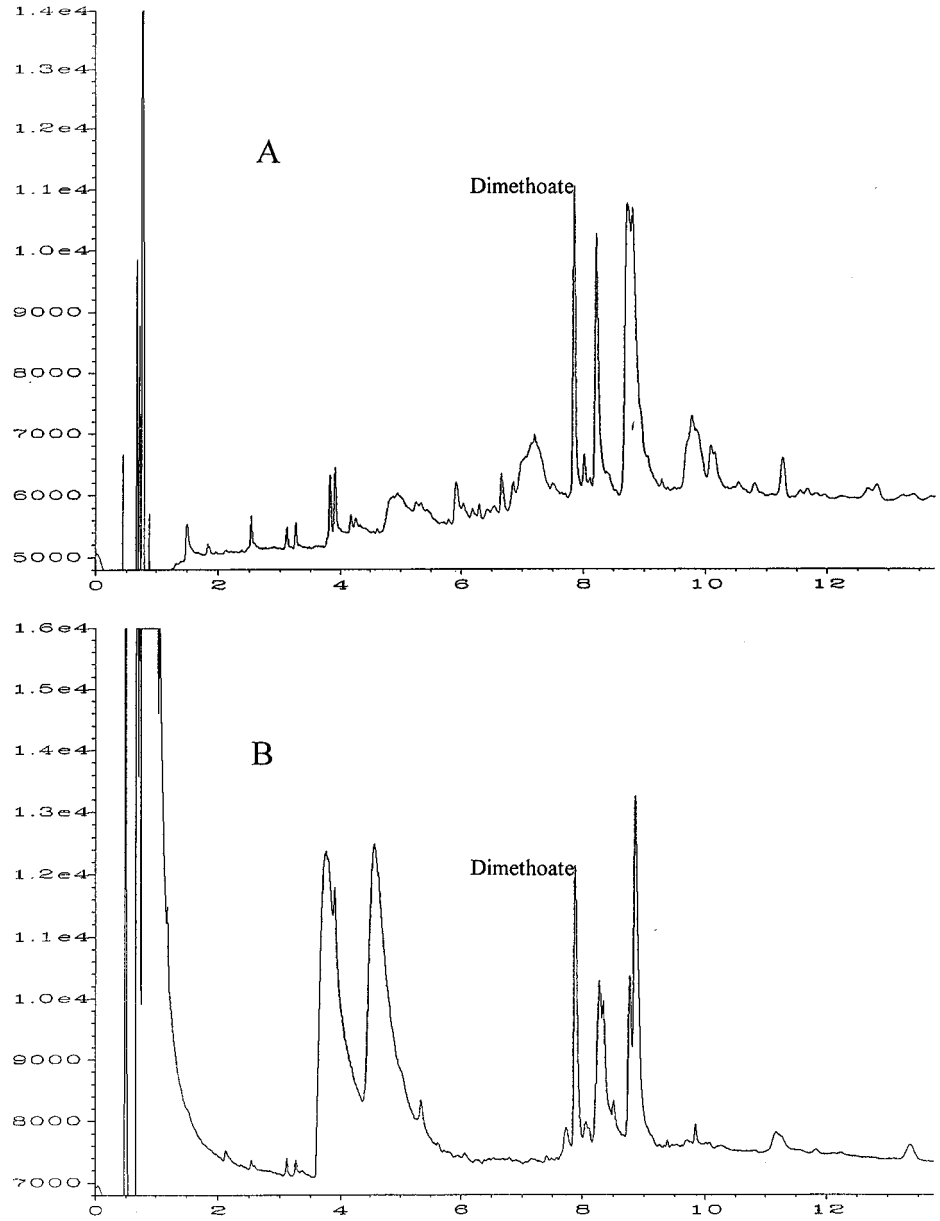

Figure 2. GC−NPD chromatograms of honeybee sample containing dimethoate. Chromatogram A: Solvent extract after GPC and SPE cleanup. Chromatogram B: SFE extract without additonal cleanup.

for a range of organophosphate and carbamate insecticides. The results showed that such analytes could be trapped on an ODS trap and that a temperature of 25 °C was adequate for most purposes. These conditions proved suitable for the extraction from honey bees of the majority of compounds of interest in this study. Only omethoate showed inadequate recoveries at these conditions. Although it was obvious that the extracts contained much less co-extractives than solvent extracts it was apparent that there was sufficient co-extracted material to cause significant chromatographic interferences when analyzed by GC−NPD or GC−MS. Additionally experiments in which blank, nonfortified bee extracts, obtained under these conditions, were fortified after extraction and compared with standard solutions in acetonitrile showed that a number of pesticides exhibited greatly enhanced responses when dissolved in sample extracts, especially when splitless injection was used. Lehotay and Eller (1995) reported similar effects and attributed them to matrix components in the extracts filling active sites on the glass injection liner. We therefore investigated the effect of lowering the CO_2 density to reduce the amount of co-extractive material in the extracts and hence reduce this enhancement effect. The best compromise between adequate recoveries and reduced co-extractives was found to be at 0.7 g/mL.

GC Determination. Extracts analyzed by GC−FPD with splitless injection were largely free of chromatographic interferences (Figure 1) and compared well with extracts analyzed by GC−FPD after the traditional solvent extraction and GPC cleanup method. However, even at the optimum extraction conditions the organophosphate pesticides azinphos-methyl, diazinon, dimethoate, pirimiphos-methyl, and vamidothion exhibited enhanced responses in sample extracts when compared with solutions in pure solvent. Lehotay and Eller (1995) virtually eliminated the problem by preparing standard solutions in blank sample extracts which was found to be very successful. As the responses of some organophosphates varied according to which sample extract was used it was necessary to prepare the standard solution in an extract from the same bee sample as was used for fortification. The GC−NPD was

Table 1. SFE Recoveries of Pesticides from Spiked Honeybee Samples[a]

	recovery, %	
	0.1 mg/kg	1.0 mg/kg
azinphos-methyl	*b*	98 ± 25
bendiocarb	*b*	77 ± 26
carbaryl	*b*	101 ± 27
chlorpyrifos	98 ± 18	80 ± 11
demeton-*S*-methyl	89 ± 21	95 ± 6
diazinon	96 ± 16	86 ± 11
dichlorvos	68 ± 29	81 ± 6
dimethoate	78 ± 28	89 ± 13
disulfoton	88 ± 16	88 ± 6
fenitrothion	95 ± 17	87 ± 11
heptenophos	90 ± 23	84 ± 5
omethoate	27 ± 16	54 ± 22
pirimiphos-methyl	102 ± 20	84 ± 11
propoxur	*b*	81 ± 29
quinalphos	97 ± 18	88 ± 10
thiometon	81 ± 18	86 ± 10
triazophos	96 ± 27	95 ± 10
vamidothion	*b*	76 ± 39

[a] Data are means ± standard deviations of four determinations on separate bee samples. [b] Below limit of reliable determination (signal/noise < 5).

equipped with on-column injection and problems of quantitation were less severe. Chromatographic interferences were more extensive than with GC−FPD but were no worse than those experienced by the solvent extraction/GPC cleanup method with the additional (SPE) cleanup (Figure 2). Due to its selectivity, the GC−FPD system was used to measure recoveries from samples spiked with organophosphates. Carbamate insecticides could not be determined by GC−FPD so recoveries of these compounds were measured using GC−NPD by comparison to standard solutions in pure solvent. Residues obtained were measured using GC−NPD.

Analysis of extracts by GC−MS showed that, in most cases, the total-ion chromatogram (TIC) contained many chromatographic interferences. Indeed the chromatographic background was such that peaks due to pesticides could not be detected at all. It was possible to confirm residues by monitoring selected ions for the pesticides of interest. It was not therefore possible to use GC−MS to screen for these compounds using the TIC in the same way as Lehotay and Eller.

Recovery Experiments. Table 1 presents the recoveries for 18 pesticides at 1.0 mg/kg and for the 13 pesticides which could be reliably detected at 0.1 mg/kg. It should be stressed that these results reflect the variation between different samples, containing differing amounts of wax and moisture and extracted on different days. They are not simply a measure of the repeatability of multiple determinations of the same sample. At the higher level acceptable recoveries of >80% were obtained for 17 pesticides with only omethoate giving an unacceptable recovery that was low and highly variable. Recoveries were also highly variable for the organophosphates azinphos-methyl and vamidothion and for all three of the carbamates studied. For both organophosphates this probably reflects the fact that the sensitivity of the GC−FPD system is lower for these compounds than for the other organophosphates so that the spiking level is closer to the limit of detection. For the carbamates the variability is probably due to matrix effects causing variable responses as these compounds were measured against standard solutions in pure solvents. At the 0.1 mg/kg level, recoveries were greater than 80% for 11 pesticides with only omethoate giving recoveries of <65%. In all cases variability was much higher than at the 1.0 mg/kg level.

Table 2. Comparison of Chlorpyrifos Residue Obtained in Samples Using SFE and Solvent-Based Methods and Evaluation of Repeatability of SFE Method

pesticide	SFE residue[a] (μg/g)	solvent extraction/GPC cleanup residue (μg/g)
chlorpyrifos	4.73 ± 0.64	4.24

[a] Mean ± standard deviation of five replicate determinations.

Table 3. Comparison of Residues Obtained in Honeybee Samples Using SFE and Solvent-Based Methods

pesticide	SFE residue[a] (μg/g)	solvent extraction/GPC cleanup residue (μg/g)
dimethoate	1.34, 1.28	1.48
dimethoate	0.38, 0.59	0.49
bendiocarb	5.38, 33.93	4.68

[a] Results from duplicate determinations.

Incurred Residues. Extraction methodologies cannot be fully validated with spiked samples as they may not accurately reflect the degree of interaction between the analyte(s) and the matrix that is found in actual samples containing incurred residues (Burford *et al.*, 1993). Once extraction conditions have been evaluated with spiked samples it is preferable to perform further evaluation with samples that have previously been analyzed by fully validated methods. In the case of pesticide analysis of honeybees no methods exist which have been subjected to interlaboratory evaluation. Nevertheless it was felt that comparison of results obtained by SFE extraction with results from the traditional solvent extraction and GPC cleanup method used in this laboratory was essential. Four separate samples, which had previously been analyzed using the solvent based method, were used in this comparison. Two samples contained residues of dimethoate, one with chlorpyrifos and one with bendiocarb. Five replicate determinations using the SFE method were performed on the chlorpyrifos sample to asses the repeatability of the SFE extraction procedure. The other samples were extracted in duplicate by SFE and analyzed. Results are shown in Tables 2 and 3. The results for the chlorpyrifos sample show that SFE gives comparable results to the traditional method. The repeatability is within acceptable limits for toxicological investigations. These results also suggested that 1 g subsamples (as compared to the 5 g used in the traditional method) are sufficiently representative to be taken for analysis. The results in Table 3 confirm that SFE gives comparable results to the traditional method for dimethoate. For bendiocarb, one of the duplicate determinations gave a result comparable to the traditional method but the other determination gave a residue over 7 times higher. The large difference between the two replicates suggests that a 1 g subsample is not sufficiently representative in all cases. This appears to contradict the results for the chlorpyrifos sample which suggested that subsamples of 1 g could be taken without homogenization prior to subsampling. One solution to this would be to homogenize the whole sample of bees received, which may consist of thousands of individuals with a total weight of hundreds of grams. This is a time-consuming and messy operation. It would be simpler to take a 5 g subsample and thoroughly homogenize with 10 g of Hydromatrix before taking 3 g of this mixture for SFE extraction.

Conclusion. This study demonstrates that SFE is suitable for the routine extraction of organophosphate

and carbamate insecticides from honeybees. The most striking advantage of SFE over solvent-based extractions was in the selectivity of the extraction. SFE extracts were much cleaner and, without further cleanup, gave rise to chromatograms that were comparable with solvent extracts that had undergone extensive cleanup procedures.

The SFE equipment proved reliable from day-to-day. The major problem encountered was a gradual drop-off in optimal performance over time as evidenced by a decline in recoveries from spiked samples. Is is believed that this was due to the ODS trap becoming coated with co-extracted wax over time. Thorough rinsing of the trap with approximately 50 mL of hexane restored performance to acceptable levels, although it remains to be seen how many times this can be repeated before the trap will need to be replaced.

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