Integrated SFE/SFC/MS System for the Analysis of Pesticides in Animal Tissues

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The development of an integrated system for an on-line supercritical fluid extraction/cleanup/supercritical fluid chromatography/mass spectrometry analysis (SFE/SFC/MS) of thermally labile carbamate pesticides from beef and chicken meat samples is discussed. A key to the system is the mixed stationary phase (7% diol in C_{18}) used in the cleanup column to separate target analytes from the extracted lipid matrix interferences. The detection limits of the system at 2:1 signal to noise ratio were 200 and 175 ppb for bendiocarb and carbaryl, respectively. The reproducibility of the present system was not totally favorable due to trapping problems, resulting from frequent clogging of either the cryogenic retention gap between the extraction cell and the SFC or the actual restrictor in the retention gap. Operating in an integrated mode, 53% of the experiments were completed without complications connected to extracting and detecting the analyte.

Keywords: *Carbamates; pesticides; SFE; SFC; integrated system; on-line cleanup; animal tissues*

INTRODUCTION

Trace analysis of analytes in complex sample matrices such as animal fat and meat tissues requires three major steps: extraction of the analytes from the sample matrix, cleanup and preconcentration steps, and chromatographic separation and detection of the individual analytes. Extraction of the analytes from fatty matrices is usually accompanied by simultaneous extraction of significant amounts of lipid material. This necessitates the need for additional cleanup and preconcentration steps to reduce interference and to prevent deterioration of the analytical chromatographic column. The cleanup procedures are often the most painstaking and can be the limiting step in the analysis. The final step in the analytical scheme is the chromatographic separation of the target analytes. Conventional methods of analysis such as gas chromatography (GC) cannot be used for nonvolatile or thermally labile compounds; in the past, lack of sensitive and selective detectors has limited the utility of liquid chromatography (LC). These problems have led to a multitude of single-residue methods for separating and analyzing compounds of the same chemical class.

Carbamate pesticides are a group of polar, thermally labile compounds that are not amenable to GC without derivatization. The methods approved by the Environmental Protection Agency (EPA) for the analysis of such compounds (U.S. EPA, 1988) require extraction with methylene chloride, cleanup using GPC and solid phase extraction (SPE), and HPLC analysis involving post-column derivatization.

The unique properties of supercritical fluids make them attractive media for extraction and chromatography of compounds such as the carbamate pesticides. Supercritical fluid extraction (SFE) and chromatography of pesticides with pure or modified supercritical CO_2 from a variety of sample matrices have been reported in the literature (Wheeler and McNally, 1989; France et al., 1991; Hopper and King, 1991; Murugaverl and Voorhees, 1991; Nam et al., 1991; Howard and Taylor, 1992; Nishikawa, 1992a,b; Snyder et al., 1992; Howard et al., 1993; Lopez-Avila et al., 1993; Rochette et al., 1993; Wigfield and Lanouette, 1993; Berger et al., 1994; Nam and King, 1994; Robertson and Lester, 1994; Argauer et al., 1995; Lehotay, 1997).

Two basic approaches have been used in the application of SFE as a sample preparation technique for lipid-containing samples. In the first approach, the target analytes are selectively extracted with a minimum of matrix coextraction by controlling the solvent strength of the supercritical fluid (Nam, 1989; Nam et al., 1990). Negligible amounts of the lipid matter were extracted with low target analyte recoveries at low pressures up to 95 atm. When the pressure is increased to 204 atm, 75% recovery of the analyte was obtained with substantial amounts of lipid coextractives (King, 1989). In the second approach, the solvent strength of the supercritical fluid is increased with a modifier to maximize the recovery of the analytes. These conditions lead to the inevitable coextraction of significant amounts of the lipid matrix, which requires an additional cleanup step (Hopper and King, 1991; Murugaverl and Voorhees, 1991; Nam et al., 1990) before chromatographic separation.

SFE cleanup procedures for pesticides have been reported. Fish tissues fortified with chlorinated pesticides were equilibrated in the extraction vessel with supercritical CO_2 for 1 h. Ten microliter aliquots of the extract were then subjected to on-line cleanup on a octadecylsiloxane (C_{18}) packed column and analyzed by SFC (Nam et al., 1991). These researchers also reported an off-line cleanup on deactivated alumina or silica columns using either pure or methanol-modified supercritical CO_2 (France et al., 1991) and an on-line cleanup using a column packed with octylsiloxane (C_8) stationary phase (Nam and King, 1994). Methods to extract organic chlorine pesticides from rendered chicken fats without removing significant quantities of fats using supercritical fluoroform were discussed (Ashraf-Khorassani and Taylor, 1996). The supercritical fluoroform

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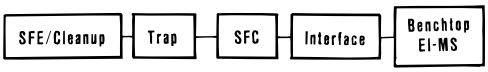

Figure 1. Block diagram of integrated extraction/cleanup/SCF/MS system.

was selective and did not extract fatty materials. Off-line SFE/solid phase cleanup of pesticides in produce (Lehotay and Eller, 1995; Lehotay et al., 1995) and mussel samples using Florisil (Ling and Teng, 1997) was recently reported.

On-line systems with an SFE/cleanup apparatus and a chromatograph require an interface between the two components. Numerous researchers have reported the coupling of SFE with GC (Wright et al., 1987; Hawthorne et al., 1989, 1990; Raymer and Velez, 1991) or with SFC (Nam et al., 1991; Murugaverl and Voorhees, 1991; Sugiyama et al., 1985; Xie et al., 1989). The cryofocusing technique using a liquid carbon dioxide-cooled retention gap is the most commonly used interfacing technique for trapping of the analytes in these studies.

The main objective of this research project was to develop an integrated on-line SFE/cleanup/supercritical fluid chromatography/mass spectrometry system (SFE/cleanup/SFC/MS) for the analysis of carbamate pesticide residues in animal tissues. The system reported by Sugiyama et al. (1985) served as a model for the project. The integration of several analytical steps into one system offers the advantage of faster analysis and the possibility of automation of the extraction and analysis process to reduce operator intervention. Although numerous studies of on-line SFE/GC or SFE/SFC have been cited in the literature, their applications were limited to sample matrices devoid of interfering lipid coextractives. Since the carbamate pesticides are thermally labile and are not amenable to GC, SFC was chosen as the separation technique for our system. The detection system selected was an electron ionization (EI) bench-top mass spectrometer that had been previously utilized with SFC (Murugaverl et al., 1993a).

EXPERIMENTAL PROCEDURES

A block diagram of the integrated on-line SFE/cleanup/SFC/MS system is shown in Figure 1. This system consists of a one-step extraction and cleanup apparatus using a packed column interfaced to a capillary SFC by means of a cryofocusing retention gap, followed by an MS detector. Supercritical CO_2 was delivered to the various components by a Lee Scientific series 600 syringe pump (Lee Scientific, a former division of Dionex Corp., Sunnyvale, CA) with a dedicated controller. An HP 5720A GC oven (Hewlett-Packard, Avondale, PA) was used to maintain constant temperatures for extraction and chromatography. A schematic diagram of the cryogenic retention gap employed in this study is shown in Figure 2. This trap consists of a $^1/_{16}$ in. Swagelock tee, a 30 cm long, 10 or 15 μm i.d. fused silica-restrictor, a $^1/_{16}$ in. zero dead volume Swagelock union, and a 500 μm i.d. × 6 cm long stainless steel expansion volume or retention gap. The retention gap was enclosed in a cooling jacket fabricated from a $^1/_8$ in. Swagelock union modified with two holes in the body. The retention gap was cooled with commercial grade liquid CO_2 introduced through one of the holes in the jacket body; the other hole served as a vent.

The analytical capillary column (Lee Scientific) used in this study was an SB-Biphenyl-30, 1.5 m long, 50 or 100 μm i.d., 200 μm o.d., and 0.25 μm film thickness. A capillary frit restrictor 50 cm × 50 μm i.d. (Dionex Corp.) was connected to the MS end of the analytical column to maintain supercritical fluid conditions inside the chromatographic system. The restrictors used in this study were purchased with a linear

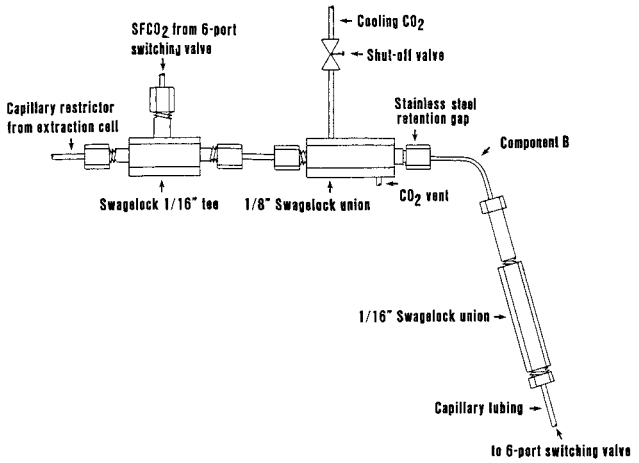

Figure 2. Cryogenic trap (retention gap) assembly.

velocity of 5 mm/s at 100 atm of CO_2 head pressure, which was adjusted by trimming the frit end to obtain a mass spectrometer manifold pressure of 2×10^{-5} Torr at a CO_2 head pressure of 90 atm. A modified Perkin-Elmer Q-Mass 910 (Perkin-Elmer, Norwalk, CT) bench-top mass spectrometer (Murugaverl et al., 1993a) was used as the detector.

The carbamate pesticides bendiocarb, carbaryl, and methiocarb were obtained from Ultra Scientific, North Kingstown, RI. The liquid chromatography stationary phases were purchased from Keystone Scientific, Bellefonte, PA. Diatomaceous earth (Hydromatrix; Varian, Harbor City, CA) was used as a dehydrating agent. The stainless steel cleanup column blanks (Keystone Scientific) used in this study were of three different sizes: 2 mm i.d. × 5 cm length, 3 mm × 4 cm, and 3 mm × 5 cm with 2 μm frit filters. SFE/SFC grade CO_2 (Air Products and Chemicals, Allentown, PA) was used in this study. A variety of high-pressure valves including a six-port switching valve (Rheodyne, Cotati, CA), a 0.06 μL injection valve (Valco Instruments, Houston, TX), SnoTrix shut-off valves, and Swagelock fittings (Denver Valve and Fittings, Lakewood, CO) were utilized in the system. The fat and meat samples were purchased from a local supermarket.

The animal tissue samples were chopped and ground into a paste and then spiked with a standard solution containing ~1.5 μg/g levels of bendiocarb, methiocarb, or carbaryl in methanol. The ground tissue samples (typically 5 g) were then mixed using a mortar and pestle with ~30% by weight of Hydromatrix (Hopper et al., 1991) to a uniform consistency. The spike was allowed to interact with the matrix for a minimum of 3 h before the samples were subjected to extraction.

The empty cleanup column blanks were cleaned by sonication for ~10 min in a warm detergent solution and rinsed with large quantities of hot tap water and then deionized water. Finally they were sonicated with HPLC grade methanol and oven-dried at 110 °C for 20 min. The column blanks were dry packed with ~100 mg of Soxhlet-extracted 7% diol in C_{18} mixed stationary phase (Murugaverl et al., 1993b) by the tap and fill method. The column was then flushed with supercritical CO_2 at 219 atm and 90 °C for ~40 min to aid in further cleaning and packing of the sorbent. The pesticide-spiked tissue sample (up to 100 mg) was placed directly on top of the stationary phase of the cleanup column. The column was then attached to the supercritical CO_2 line inside the SFC oven and allowed to equilibrate to the set oven temperature.

The extraction was initiated by opening valve B with the six-port switching valve in the extraction position as shown in Figure 3. The extraction was usually conducted for ~30 min using ~4 mL of CO_2 (measured at the pump) at 90 °C and 219 atm. During extraction, the retention gap assembly was placed outside the oven and cooled with commercial grade liquid CO_2 to enhance the trapping effficiency of the analytes. Valve A was kept open to keep the analytical column purged with CO_2 during the extraction cycle.

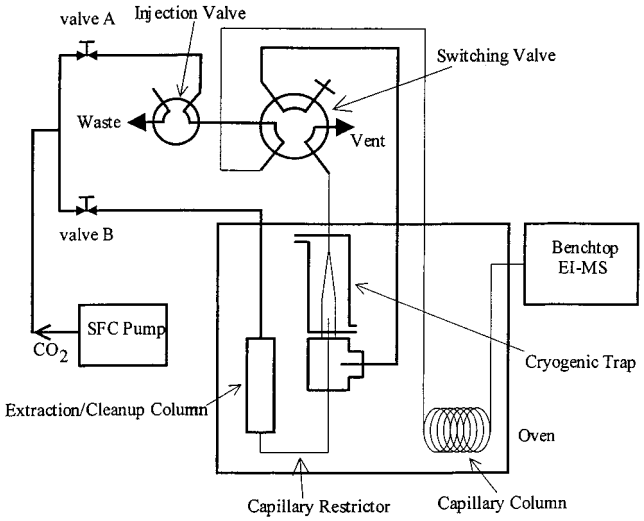

Figure 3. Schematic diagram of the on-line SFE/cleanup/SFC/MS system. The switching valve is in the extraction mode.

After extraction was completed, the retention gap assembly was lowered inside the oven and the deposited analytes were swept from the retention gap by switching the six-port valve in Figure 3 to divert the supercritical fluid mobile phase through the retention gap into the capillary column. SFC analysis was performed at an oven temperature of 70 °C and density programming of the supercritical mobile phase at 0.2−0.7 g/mL at a rate of 0.015 g/(mL·min). The SFC/MS interface main heater was maintained at the oven temperature while the interface tip heater was set at 155−165 °C and the ion volume temperature at 280 °C. The MS was scanned in the mass-to-charge (*m*/*z*) range of 75−250.

RESULTS AND DISCUSSION

There are >750 pesticides, herbicides, and related compounds that require analytical detection. Among these, 250 are done by GC/MS; the majority of the remaining compounds are analyzed by single-residue methods. The single-residue methods are time-consuming and provide a wide variation in sensitivity. In addition, each single residue method adds to the training required of the technical staff. The major goal of this project has been the integration of the extraction and analysis of pesticides, herbicides, and related materials in animal tissues with the secondary goal of simplifying the existing methodology that involves multistep extraction and cleanup procedures.

Initial SFE experiments were conducted off-line on rendered beef fat spiked with methiocarb and carbaryl. Small samples (10−15 mg) of pesticide-spiked beef fat were extracted through C_{18} packed columns, and the extracts collected off-line in a small flask containing ∼5 mL of ice-cooled methanol. After concentration, these extracts were analyzed by SFC/MS. The selected ion monitoring (SIM) chromatograms for the ions of *m*/*z* 168 and 153 (the major fragments of methiocarb) shown in Figures 4 and 5 were obtained from two consecutive 30 min off-line extractions of a 11.28 mg sample of rendered beef fat spiked with 15 ppm of methiocarb that had been passed through a C_{18} cleanup column. Similarly, Figures 6 and 7 show the SIM chromatograms of the ions *m*/*z* 144 and 115 (the major fragments of carbaryl) of two consecutive off-line extractions of 10.93 mg of rendered beef fat spiked with 30 ppm of carbaryl. In both cases, no pesticides were detected in the second extraction, which indicated that 30 min of extraction

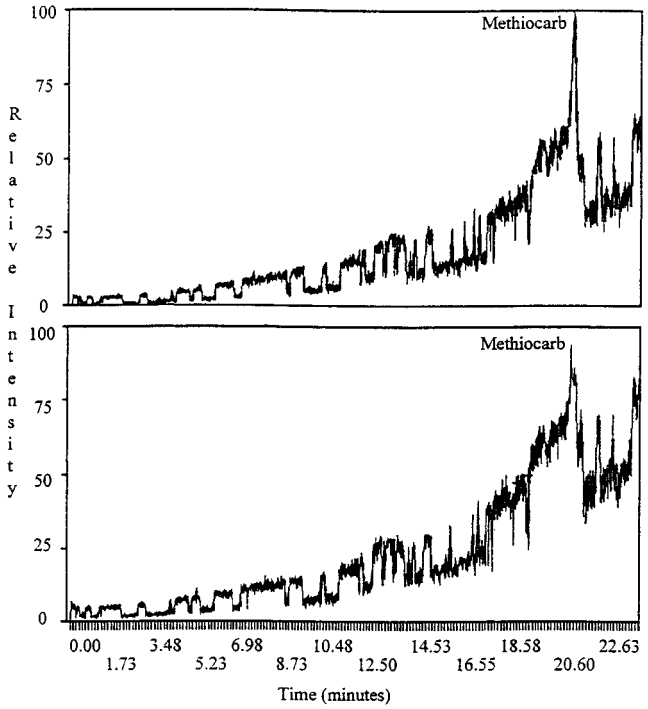

Figure 4. SFC/MS of the off-line SFE of 11.28 mg of rendered beef fat spiked with 15 ppm of methiocarb in the SIM mode (*m*/*z* 168 and 153).

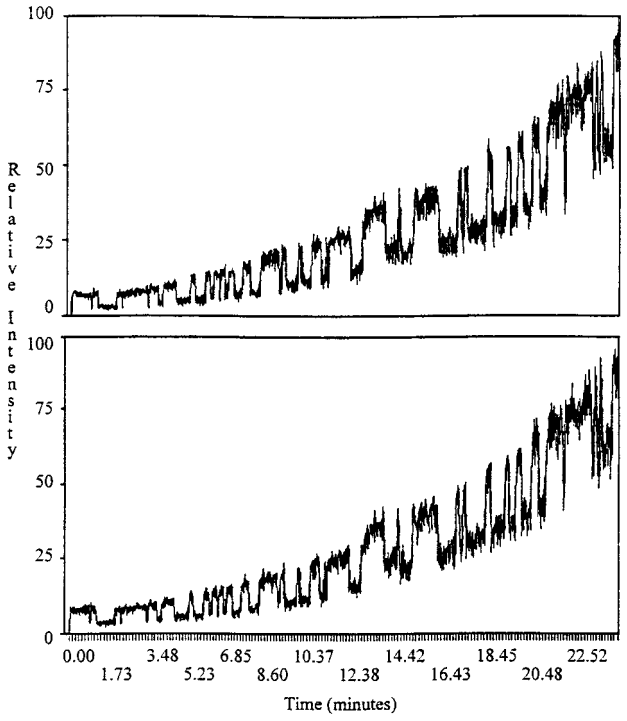

Figure 5. SFC/MS of the second off-line extraction of 11.28 mg of rendered beef fat in Figure 4 in the SIM mode (*m*/*z* 168 and 153).

was adequate for the elution of the analytes through the cleanup column. This experimental condition was then adapted for the on-line experiments.

For the on-line system, small sample sizes (10−15 mg) gave results similar to those obtained in the off-line mode. However, when the sample size was increased to ∼100 mg, the cryogenic retention gap restrictor clogged and a rapid deterioration of the analytical capillary column was experienced due to the increased

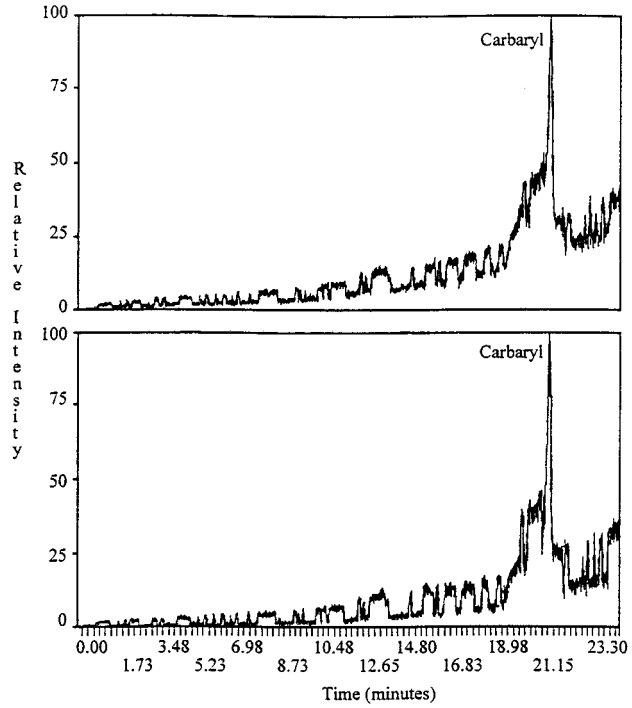

Figure 6. SFC/MS of the off-line SFE of 10.93 mg of rendered beef fat spiked with 30 ppm of carbaryl in the SIM mode (*m*/*z* 144 and 115).

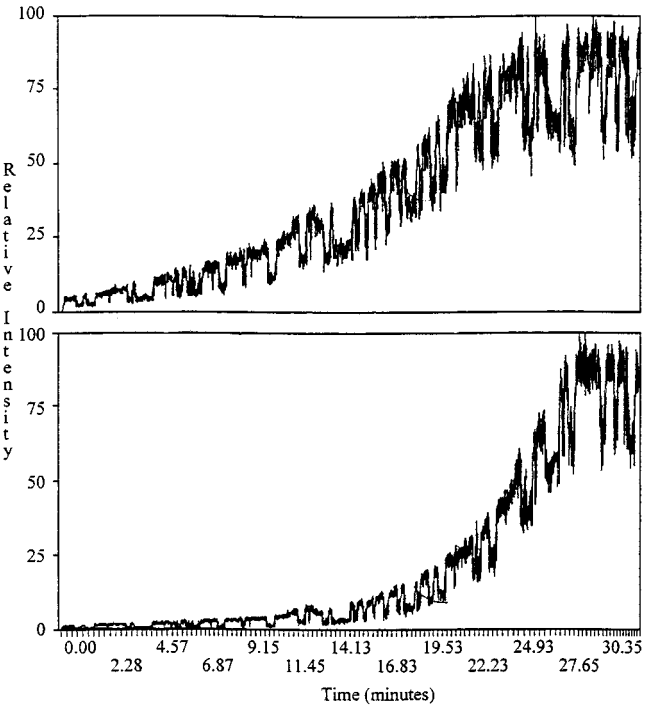

Figure 7. SFC/MS of the second off-line extraction of 10.93 mg of rendered beef fat in Figure 6 in the SIM mode (*m*/*z* 144 and 115).

amounts of coextracted endogenous lipid matter. Due to these difficulties, fat samples were replaced by muscle samples, which contain smaller quantities of lipid matter.

Preliminary on-line experiments on muscle samples were carried out using C_{18} as the cleanup stationary phase. The total ion chromatogram of the on-line extraction of 55 mg of beef muscle spiked with 5.5 ppm of bendiocarb is shown in Figure 8. The chromatogram shows a relatively small bendiocarb peak (at ~26 min

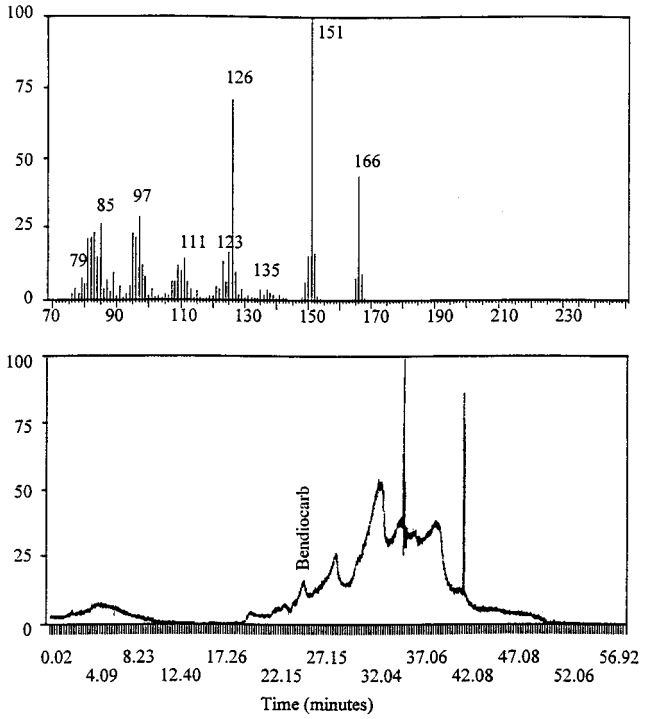

Figure 8. Total ion chromatogram of the on-line SFE/cleanup/SFC/MS of 55 mg of beef muscle spiked with 5.5 ppm of bendiocarb through a C_{18} column. The mass spectrum is for the bendiocarb peak at 26.19 min.

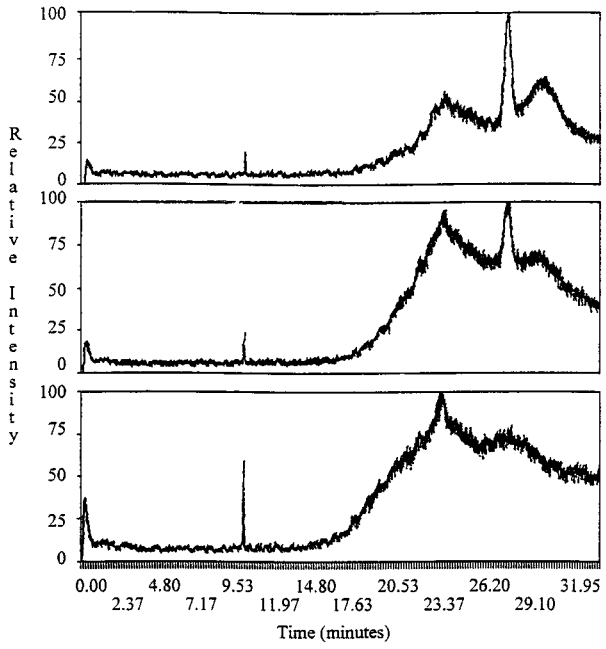

Figure 9. SIM chromatogram (*m*/*z* 166, 151, and 126) of the on-line SFE/cleanup/SFC/MS of 45 mg of beef muscle spiked with 4 ppm of bendiocarb through a C_{18} column.

retention time) with overwhelming interferences (background) due to coextracted endogenous matter. The SIM chromatogram in Figure 9 shows extraction and cleanup of 45 mg of beef muscle spiked with 4 ppm of bendiocarb. Despite monitoring of a selected ion of the analyte, no reduction in the background signal was observed. Analysis of the mass spectra of the background peaks showed fragment ions at every mass unit in the scan range. Analysis of these spectra (library search using the National Institute of Standards and

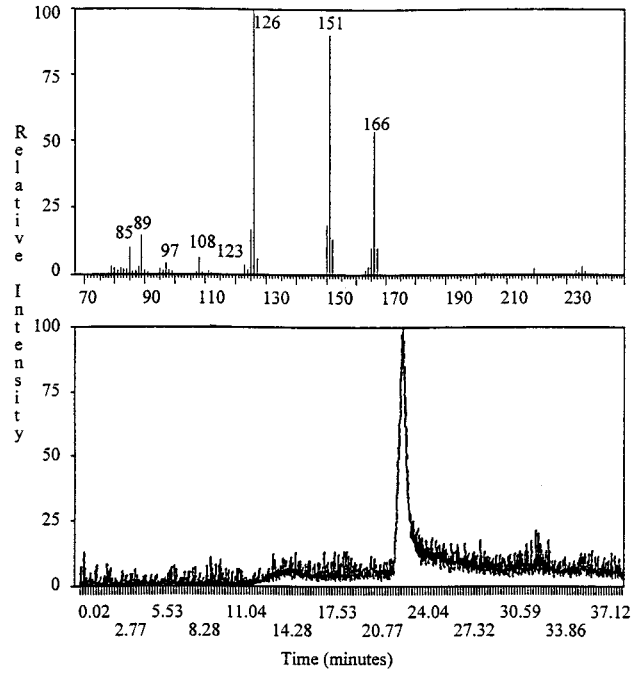

Figure 10. Total ion chromatogram of the on-line SFE/cleanup/SFC/MS of 50 mg of beef meat spiked with 4.4 ppm of bendiocarb through the diol/C_{18} mixed cleanup phase.

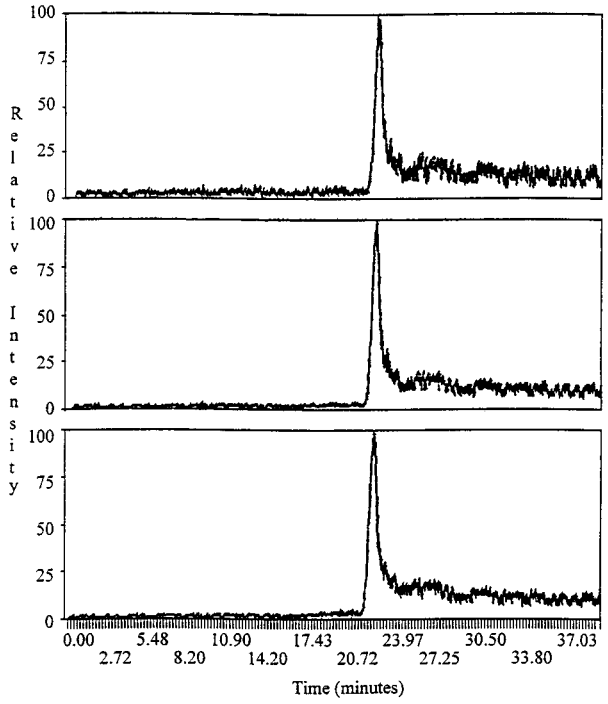

Figure 11. SIM chromatogram of the on-line SFE/cleanup/SFC/MS of 75 mg of beef meat spiked with 1.5 ppm of bendiocarb through the diol/C_{18} mixed cleanup phase (*m/z* 166, 151, and 126).

Technology mass spectral library) revealed that they consist mainly of lipid matter.

Several chromatographic stationary phases including octadecylsiloxane (C_{18}), propylsiloxane (propyl), aminopropylsiloxane (amino), cyanopropylsiloxane (cyano), and diol were tested as potential packings for the cleanup column. In a 30 min extraction time, C_{18}, cyano, and propyl stationary phases allowed both the analytes and the coextractives to pass through, while amino and diol held both moieties. Therefore, it was thought a mixture of amino or diol with one of the other stationary phases might provide the desired retention properties. Due to availability, C_{18} and diol were chosen. After numerous experiments using different ratios of these two stationary phases, a mixture of 7% diol and 93% C_{18} exhibited the best retention characteristics. This mixture produced longer retention of the lipid matter than the analytes.

Figure 10 shows the total ion chromatogram of the SFE/cleanup/SFC/MS of 4.4 ppm of bendiocarb in 50 mg of beef muscle using the 7% diol/C_{18} stationary phase. This chromatogram demonstrates the effectiveness of the mixed stationary phase in holding the endogenous lipid matter yet allowing the analyte to elute. The chromatogram in Figure 11 shows the detection of 1.5 ppm of bendiocarb in 75 mg of beef muscle sample in the SIM mode.

The data set presented in Table 1 shows the short-term reproducibility of the integrated system at the 1 ppm level of bendiocarb in beef muscle. An average analyte recovery of 55% was obtained with 8% relative standard deviation (%RSD). The results in Table 2 (bendiocarb) and Table 3 (carbaryl) were collected over longer periods of time using both beef and chicken muscle samples. The day-to-day results presented in these tables showed a greater scattering when compared to the results taken in the same day (Table 1). The average recovery for bendiocarb (Table 2) was calculated to be 46% with 85% RSD, while that of carbaryl (Table 3) was 40% with 24% RSD. The recovery and %RSD

Table 1. Short-Term Reproducibility at 1 ppm Levels of Bendiocarb in Beef Muscle Samples Using the On-line SFE/Cleanup/SFC/MS System

wt of sorbent (mg)	wt of sample (mg)	wt of bendiocarb (ng)	bendiocarb % recovery
95	105	110	57
95	131	110	58
74	134	110	56
130	133	110	48

Table 2. Long-Term Reproducibility of Bendiocarb Recoveries from Beef and Chicken Muscle Samples Using the On-line SFE/Cleanup/SFC/MS System

muscle type	wt of muscle (mg)	wt of bendiocarb (ng)	bendiocarb % recovery
beef	96	90	46
beef	110	110	43
beef	77	150	19
beef	93	400	146
beef	50	110	30
beef	106	110	16
chicken	86	90	40
chicken	76	220	44
chicken	65	220	30

were found to be influenced by the sample, the retention gap dimensions, the trap temperature, and the flow rate through the restrictor. These factors were found to have major effects on the performance of the system, where the extraction, trapping, and analysis were interrupted. The number of experiments in which the target analyte was actually extracted and detected was 53% of the 70 experiments performed. The average trapping efficiency was 55% in the above experiments. At this trapping effficiency, analytes were detected at levels as low as 1 ppm for carbaryl and 0.5 ppm for bendiocarb in the MS full scan mode, and 200 ppb for bendiocarb and 175 ppb for carbaryl in the SIM mode.

The performance of the system is affected primarily by the sample composition and the performance of the

Table 3. Long-Term Reproducibility of Carbaryl Recoveries from Beef and Chicken Muscle Samples Using the On-line SFE/Cleanup/SFC/MS system

muscle type	wt of muscle (mg)	wt of bendiocarb (ng)	bendiocarb % recovery
beef	131	100	50
beef	113	100	55
beef	71	55	30
beef	133	55	31
chicken	82	100	42
chicken	123	100	48
chicken	111	95	41
chicken	139	95	39
chicken	85	55	28

cryogenic retention gap assembly. High water content in the tissue samples was found to interfere with the extraction process. Although the samples were ground with Hydromatrix to absorb most of the water present, some water was extracted due to "solvent drag" and transferred through the restrictor to the trap assembly. The extracted water caused a serious clogging problem in the restrictor or the trap due to the Joule−Thompson cooling effect. Another sample-related problem encountered was the deterioration of these samples when not stored in the freezer. Extracts from deteriorated samples showed significant amounts of interferences in the SFC/MS spectra. Besides overloading the analytical column, these interferences were observed to greatly shorten the lifetime of the column.

Trapping effficiency and chromatographic peak shapes were significantly affected by the dimensions of the retention gap. Retention gaps with larger internal volume were found to enhance the trapping effficiency, but at the expense of chromatographic peak shape due to the increased dead volume. Furthermore, retention gaps that were coiled or had sharp bends seemed to improve the trap performance over a straight tube design (see Figure 2, component B). The coil shape or bends in the trap body helped disturb the flow of the supercritical fluid and aided in the deposition of the analyte. Retention gaps up to 500 μm i.d. and lengths up to 15 cm have been used in this study without compromising the chromatographic peak shape.

The temperature of the retention gap was found to have a large effect on the trapping efficiency. Collection of the analyte was improved by reducing the trap temperature using external cooling with liquid carbon dioxide. However, freezing of any extracted water led to restrictor clogging. Elimination of the clogging problem required restrictor heating and at the same time cooling of the trap. A compromise between these two opposing effects had to be achieved to improve the performance of the system.

The SFC pump used in this study did not have a flow control capability; therefore, the flow rate of the supercritical fluid through the cleanup column was maintained by a linear restrictor. The use of the restrictor to control the flow rate rather than the pump does not allow for fine tuning of the flow through the cleanup column. This affects the elusion profile through the cleanup column and, hence, the separation and cleanup of the analyte. The added lipid material reaching the trap caused restrictor clogging leading to incomplete recoveries and poor reproducibility. When the restrictor became clogged, the flow through the cleanup column was stopped. To resume the extraction, either the cooling CO_2 was shut off or the trap assembly was lowered inside the heated chromatographic oven. Unclogging the restrictor usually resulted in a sudden pressure burst due to the pressure buildup inside the cleanup column. These pressure bursts caused disturbance of the chromatographic process and mixing of the cleanup column stationary phase, which reduced the effficiency of the cleanup process. The trapping efficiency was also affected by these pressure bursts because the deposited analytes were postulated to have been blown out of the cryogenic retention gap.

CONCLUSIONS

Encouraging results have been achieved in the use of an on-line system for the extraction and analysis of pesticide residues from "difficult" sample matrices. Since the target analytes in the study were thermally labile, SFC was chosen as the analysis technique and MS as the detection system. This necessitated the development of two interfaces, one between the extraction and chromatography systems and the other between the chromatography and detection systems.

The first important achievement during the course of this project was the development of a simple SFC/MS interface from a standard commericially available GC/MS interface. The second major development was the cleanup stationary phase, capable of retaining the SFE fat extracts from tissues. This is the first time an effective material for this cleanup has been reported, and it should aid in both on-line and off-line cleanup systems of SFE involving fat samples.

Extraction and analysis using this system conducted on 1 ppm of bendiocarb spiked on beef muscle showed that good reproducibility could be achieved on a short-term basis, whereas on the long-term basis the reproducibility was poorer. The average efficiency for analyte recovery and the success rate achieved on this system was ~53%. The two major problems identified with the integrated system were the clogging of the cryogenic interface restrictor and the temperature control of the retention gap. With innovative trap design, we are optimistic that an automated on-line SFE/SFC/MS system is technically feasible and that such systems will offer rapid extraction, cleanup, and detection for thermally labile analytes without taxing the environment. Although this work has not met all of the initial goals, it does demonstrate that an integrated on-line supercritical fluid based system is possible.

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