In-line and Off-line Trapping of Lipids Extracted from Chicken Liver by Supercritical Carbon Dioxide

Shufen Li[1], Robert J. Maxwell*, and Alan R. Lightfield

ERRC, ARS, USDA, Wyndmoor, Pennsylvania 19038

ABSTRACT: This supercritical fluid extraction study determined the retentive properties of neutral alumina sorbent as an in-line trap for lipids in the dynamic state over a pressure range of 490–680 bar and temperatures of 40 and 80°C. Lipids were extracted from a chicken liver matrix using supercritical carbon dioxide over a 40-min period at a flow rate of 3 L/min (expanded gas), then were quantified by high-performance liquid chromatography using an evaporative light-scattering detector. Approximately 30 and 18%, respectively, of the total extracted lipids were trapped on the in-line alumina sorbent bed at 40°C as the operating pressure increased from 490 to 680 bar, while the remaining lipids were trapped off-line after CO_2 decompression. The major lipid classes trapped in-line were fatty acids and cholesterol, whereas only minor amounts of the less polar lipid classes such as sterol esters and triacylglycerols were retained. At 80°C and 680 bar, less than 1.5% of the extracted total lipids was trapped in-line, indicating the lack of adsorptive selectivity for lipids by alumina under these conditions.

Paper no. J8817 in *JAOCS 76,* 523–528 (April 1999).

KEY WORDS: Alumina, chicken liver, cholesterol adsorption, fatty acids, lipids, sterol esters, supercritical carbon dioxide, triacylglycerols.

A current major public health concern is the presence of trace levels of drug and pesticide residues in animal tissues destined for human consumption. In the United States, federal regulations have established maximal tolerance levels for these residues in animal food products (1). Traditional analytical methods used to monitor for such residues in tissues generally use organic solvents. However, because of the increasing awareness of the health hazards associated with organic solvents by regulatory agencies and the cost of their disposal (2), supercritical fluid extraction (SFE) has been proposed as an alternative approach for many analytical extraction methods (3,4). Supercritical carbon dioxide ($SC\text{-}CO_2$) is the supercritical fluid most widely used to devise such analytical methods, due to its easily attainable critical parameters, low cost, ready availability, minimal toxicity, and chemical inertness.

Trace level (ppb to ppm) veterinary pharmaceuticals found in animal tissues vary widely in polarity, which necessitates that SFE methods for these compounds be tested over a wide range of pressures (300–680 bar) and temperatures (40–100°C) (5,6). When these analytes are isolated from animal tissues by SFE, lipids are often co-extracted along with the target analytes. Then the lipids must be removed from the extract since their presence would interfere with subsequent chromatographic analysis (4). Two analyte collection techniques, off-line and in-line sorbent trapping, are currently under investigation in this laboratory and have the potential to reduce or eliminate the co-extracted lipids from the SFE extract (7,8). In the first example, all of the SFE extract is trapped off-line after CO_2 decompression on a sorbent contained in a solid phase extraction (SPE) column. In the second example, extracted analytes are trapped in the dynamic supercritical state on an in-line sorbent bed contained in the extraction vessel together with the sample matrix (Fig. 1). Our earlier investigations demonstrated that quantitative recoveries of some drug classes are not possible by the off-line method without additional clean-up procedures (5,6). On the other hand, the in-line trapping technique often yields a significantly cleaner

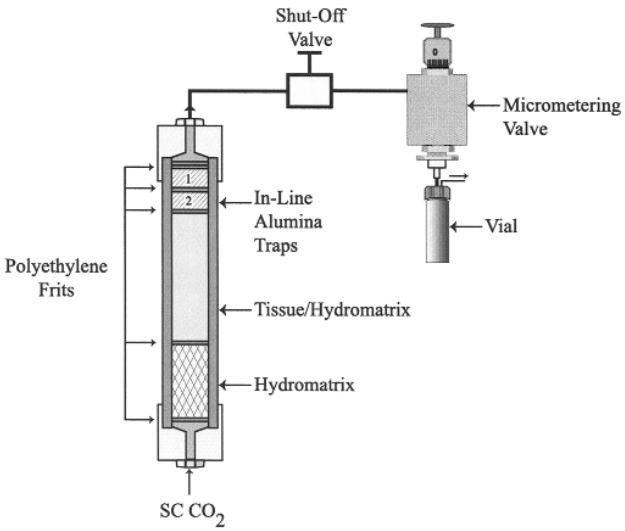

FIG. 1. Diagram of a supercritical fluid extraction vessel containing both the two-level in-line alumina sorbent bed and the sample matrix, which in turn is connected to an off-line collection vial through the micrometering valve.

[1]Permanent address: Department of Chemical Engineering, Tianjin University, 300072, People's Republic of China.

*To whom correspondence should be addressed at ERRC, ARS, USDA, 600 East Mermaid Lane, Wyndmoor, PA 19038.
E-mail: rmaxwell@arserrc.gov

extract than can be obtained using off-line sorbent collection (5,8), although lipids retained in-line may sometimes complicate the analysis. Because of this complication, it was necessary to determine which lipid classes were retained on in-line sorbent beds in order to devise successful strategies for their removal both during and after SFE. In this study, chicken liver, the target tissue for many veterinary drug classes, was selected as the experimental matrix. Neutral alumina was used as the in-line sorbent to determine the nature of the lipid classes retained during the dynamic SC-CO_2 extraction process.

EXPERIMENTAL PROCEDURES

Materials. Hydromatrix (Celite 566) was obtained from Varian Sample Preparation Products (Harbor City, CA). Neutral alumina (Brockman #1, 80–200 mesh) was purchased from Fisher Scientific (Malvern, PA). Supercritical fluid chromatography (SFC)-grade CO_2 was from Scott Specialty Gas (Plumsteadville, PA). The neutral lipid standard, labeled 178-1, was a product of Sigma (St. Louis, MO) and contained the following lipids in equal amounts by weight: cholesterol, cholesterol oleate, oleic acid, and triolein. Acetic acid, hexane, methanol, methylene chloride, and 2-propanol [high-performance liquid chromatography (HPLC) grade] were purchased from Baxter Health Care (Muskegon, MI). All reagents and solvents were used as received. Chicken liver was purchased locally, homogenized, and stored in packets at −20°C until needed.

Experimental apparatus and SFE procedure. The SFE instrumentation and procedure used in the present study employed the same overall experimental design as that previously described for the isolation of veterinary drugs from animal tissues using in-line and off-line sorbent trapping (6–8). Therefore, only a brief description of the apparatus and method is given here. Extractions were performed on an Applied Separations Spe-ed SFE instrument (Allentown, PA), which could extract four samples simultaneously and which was comprised of three modules: a pump cooled to −10°C, an oven, and four micrometering valves. Extraction vessels (24 mL, Keystone Scientific, Bellefonte, PA) were capped and then packed tightly with a tamping rod in the following sequence relative to the exit of the SC-CO_2 from the vessel: polyethylene frit; 2 g of neutral alumina (divided into two 1-g layers separated by a polyethylene frit); polypropylene frit; chicken liver matrix composed of 1.0 g of chicken liver blended with 2.0 g of Hydromatrix; polyethylene frit; 3.5 g of Hydromatrix; polyethylene frit. The alumina trap closest to the end cap was labeled as trap 1, while the alumina bed adjacent to the sample matrix was labeled as trap 2 (Fig. 1). The packed extraction vessel containing the in-line alumina trap was connected through the fluid lines of the SFE to the micrometering valve through which the fluid was decompressed into a capped 9-mL vial fitted with a gas discharge vent (Fig. 1).

The micrometering valves were heated to 110°C prior to installation of the extraction vessels in the oven. The oven then was heated to the desired temperature, followed by pressurization of the system with carbon dioxide (SFE grade with no helium headspace; Scott Specialty Gases). A 10-min static hold was employed during each experiment to equilibrate the system. The SC-CO_2 flow rate, controlled by the micrometering valve, was measured using a flow meter–gas totalizer (Floline SEF-51; Horriba, Sunnydale, CA). The run time for each extraction was 40 min at a flow rate of 3 L/min for a total volume of 120 L of CO_2 (expanded gas).

At the end of the SFE, lipids remaining in the fluid transfer lines were washed into the off-line collection vial with hexane and then transferred into a volumetric flask. The lipids trapped on the two in-line sorbent layers were recovered by first transferring each sorbent layer separately from the extraction vessel to an empty 6-mL solid phase extraction (SPE) column. The lipids were eluted from the SPE columns with CH_2Cl_2/MeOH (2:1). The collected eluates were reduced to dryness, taken up in hexane, and transferred to volumetric flasks for HPLC analysis and quantitation.

HPLC lipid analysis. An HPLC technique developed by Moreau *et al.* (9) was used for separating and quantifying the lipid classes extracted from chicken liver. Analyses were performed on a Beckman 334 HPLC system consisting of two model 110A pumps and a model 421 controller, which in turn was connected to a Sedex 55 Evaporative Light Scattering Detector (ELSD: Richard Scientific, Novato, CA). The ELSD was operated at 40°C with a nitrogen nebulizing gas pressure of 1.5 bar. The HPLC column was a Chromsep glass cartridge, LiChrosorb Diol, 5 µm, 3 × 100 mm (Chrompack, Raritan, NJ). The mobile phase binary gradient was formed from solution A, hexane/acetic acid (1000:1,vol/vol), and solution B, hexane/2-propanol (100:1 vol/vol) at a flow rate of 0.5 mL/min. The gradient was programmed as 100% A from 0 to 8 min and stepped to 99.1% A/0.9% B at 25 min at which time it was returned to 100% A over a 1 min period. Chromatograms were recorded on a Hewlett-Packard Model 3396A integrator (Avondale, PA). The standard curves for the four lipid standards (cholesterol oleate, triolein, oleic acid, and cholesterol) were calculated over two concentration ranges (3.1–31.25 µg/mL with a SD of 0.998, and 7.8–250 µg/mL with a SD of 0.996) from data obtained at two ELSD gain settings. The identities of the individual lipids were confirmed by comparing HPLC/ELSD peaks from the liver extracts with authentic samples and by peak identification using liquid chromatography–mass spectrometry (Hewlett-Packard 1050 HPLC coupled to an HP 5989A spectrophotometer).

RESULTS AND DISCUSSION

In our previous SFE investigations, we found it necessary to use pressures at or near the limits of the SFE instrumentation (450–690 bar) in order to successfully isolate trace levels (1ppb—1 ppm) of certain polar veterinary pharmaceuticals such as sulfonamides and nitrobenzamides from chicken liver (6,8). For that reason, the experiments carried out in the present study were performed at or above 490 bar. The extracted lipids were trapped either in-line on sorbent beds or off-line

in vials after decompression of the supercritical fluid (Fig. 1). The two in-line traps contained in the extraction vessel were packed with neutral alumina, a sorbent found in our earlier work to trap polar drug residues more efficiently in the dynamic state than other materials tested (5–8). In actual experiments using fortified or incurred tissues only a single layer of alumina is used. In the present study, we separated the alumina bed into two equal layers in order to obtain a better understanding of how lipids migrate through or are retained by the alumina during the dynamic extraction process. In addition to pressure as a variable, we conducted these experiments at 40 and 80°C, two temperatures commonly used in this laboratory to isolate chemical residues from tissues.

In most of our earlier studies, flow rates of 3.0 L/min and a total of 120 L of CO_2 (expanded gas) were needed to achieve high recoveries of the target analytes (6,8). Accordingly, the same conditions were used in this study on co-extraction of lipids from liver tissue. It was not expected that the lipids in the chicken liver samples in the present study would be exhaustively extracted. Instead, the lipid profiles of the extracted material in the in-line and off-line traps would be those of lipids co-extracted with the target analytes. The recoveries of lipids from chicken liver at 40 and 80°C using the two trapping techniques are shown in Tables 1 and 2. They were composed of four major classes: sterol esters, triacylglycerols, fatty acids, and cholesterol. The HPLC method used in this study can separate classes such as fatty acids into their molecular species. However, they were not so listed in the tables, since the purpose of this investigation was only to compare major changes in the lipid classes with variations in the experimental extraction and trapping parameters. Qualitative analysis of the lipid classes was accomplished by comparison of peak areas of lipids with external standards. Each of the in-line and off-line extracts was analyzed at least twice, and the results are presented in the tables both as the mean (mg) for each lipid class and as the percentage (%) of the total lipid recovered from each in-line and off-line trap.

In the experiments carried out on chicken livers at 490 bar and 40°C (Table 1), about 7.18 mg of lipids was extracted, of which 5.05 mg (70%) was collected in the off-line vial and 2.14 mg (30%) from the 2 g of in-line neutral alumina. In all, about 30% of lipids extracted by SC-CO_2 was adsorbed on the in-line alumina sorbent bed. At 680 bar and 40°C, 7.74 mg of total lipid was extracted, of which 6.36 g (82%) was collected in the vial and only 1.34 mg (18%) was retained in the two in-line traps. The results indicate that the adsorption of total lipid in-line was reduced with the increased pressure, even though a somewhat larger amount of lipid was extracted at the higher pressure.

The experimental results presented in Table 2 demonstrate the influence of a modest pressure increase at a more elevated temperature on the in-line retention of lipids compared to those reported in Table 1. At 600 bar and 80°C, 8.00 mg of total lipid was extracted, of which 7.88 mg was collected in

TABLE 1
Recoveries of Lipids from Chicken Liver by In-line and Off-line Trapping at 40°C Using SC-CO_2

	Lipid classes				
Lipid collection technique	Sterol esters (mg) %	Triacyl-glycerols (mg) %	Fatty acids (mg) %	Cholesterol (mg) %	Total lipids (mg)
			490 bar 40 °C		
Off-line	0.833 16.52	1.592 31.55	0.371 7.36	2.249 44.57	5.045
In-line (trap 2)	0.023 1.44	0.116 7.28	1.123 70.77	0.325 20.50	1.587
In-line (trap 1)	0.000 0.00	0.099 17.90	0.453 82.10	0.000 0.00	0.552
In-line total	0.023 1.07	0.214 10.02	1.576 73.70	0.325 15.21	2.139
In- + off-line total	0.856 11.92	1.806 25.14	1.948 27.11	2.574 35.83	7.184
			680 bar 40°C		
Off-line	0.557 8.76	0.979 15.39	1.942 30.52	2.884 45.33	6.362
In-line (trap 2)	0.071 6.84	0.063 6.02	0.728 69.87	0.180 17.27	1.041
In-line (trap 1)	0.000 0.00	0.000 0.00	0.292 86.51	0.046 13.49	0.337
In-line total	0.071 5.17	0.063 4.54	1.020 73.94	0.225 16.34	1.379
In- + off-line total	0.628 8.12	1.042 13.46	2.961 38.26	3.109 40.17	7.741

TABLE 2
Recoveries of Lipids from Chicken Liver by In-line and Off-line Trapping at 80°C Using SC-CO_2

Lipid Collection Technique	Lipid Classes: Sterol esters (mg) %	Triacyl-glycerols (mg) %	Fatty acids (mg) %	Cholesterol (mg) %	Total lipids (mg)
			600 bar 80 °C		
Off-line	0.991	1.394	2.969	2.528	7.882
	12.57	17.68	37.67	32.08	
In-line	0.000	0.036	0.000	0.045	0.080
(trap 2)	0.00	44.49	0.00	55.51	
In-line	0.000	0.035	0.000	0.000	0.035
(trap 1)	0.00	100.00	0.00	55.51	
In-line	0.000	0.071	0.000	0.045	0.116
Total	0.00	61.48	0.00	38.52	
In- + off-line	0.991	1.465	2.969	2.573	7.998
Total	12.39	18.32	37.12	32.17	
			680 bar 80°C		
Off-line	0.796	1.354	4.129	2.354	8.633
	9.22	15.68	47.83	27.27	
In-line	0.000	0.050	0.000	0.043	0.093
(trap 2)	0.00	53.78	0.00	46.22	
In-line	0.000	0.003	0.000	0.000	0.003
(trap 1)	0.00	100.00	0.00	0.00	
In-line	0.000	0.053	0.000	0.043	0.096
Total	0.00	55.45	0.00	44.55	
In- + off-line	0.796	1.407	4.129	2.397	8.729
Total	9.12	16.12	47.30	27.46	

the off-line vial and 0.12 mg was trapped on the in-line alumina sorbent beds. An even larger amount of lipid was recovered (8.73 mg) at 680 bar and 80°C, with 8.63 mg of lipid collected in the off-line vial and a mere 0.10 mg trapped in-line. Comparison of the data listed in Table 1 at 40°C with that found in Table 2 at 80°C illustrates that the affinity of lipids for the in-line sorbent in the dynamic state at the same flow rate diminished with increasing temperature, whereas the total amount of extracted lipid increased with increasing temperature and pressure (or density).

The process of in-line trapping is expected to be dynamic in that retained lipids and target analytes may migrate on sorbent beds in an adsorption–desorption process as the flow of the supercritical fluid continues. Other parameters such as sorbent type, particle size, and the amount (length of sorbent bed) in the extraction vessel will influence the extent to which each of the lipid classes is retained at the end of the experiment, as will the amount of the supercritical fluid passing through the sorbent bed (length of experiment). Tables 1 and 2 also list the distribution of various lipid classes on the two layers of alumina sorbent at the pressures and temperatures used. It can be seen that at the temperatures and pressures tested greater amounts of lipid were trapped in the lower layer (trap 2), close to the sample matrix, than in upper layer (trap 1). These data indicate that the retention of lipids by alumina decreased across the length of the sorbent bed, although it is not clear why the majority of the lipids were concentrated in trap 2 since it was expected that the lipids would be distributed more uniformly along the length of the bed.

The adsorptive selectivity of alumina differed for each of the four lipid classes and varied with changes in pressure and temperature. Retention of the individual classes on the in-line traps followed in the increasing order of fatty acids, cholesterol, triacylglycerols, and sterol esters at 490 and 680 bar and 40°C, which might be expected on the basis of their relative polarities. However, when the temperature was increased to 80°C both at 600 and 680 bar, nearly all the extracted fatty acids passed through the in-line sorbent bed, which retained only small amounts of the triacylglycerols and cholesterol. The differences in the lipid classes retained by in-line sorbents and off-line collection traps can be seen from the HPLC chromatograms illustrated in Figure 2. In those chromatograms the designations for the four major lipid classes are listed on the chromatogram shown in Figure 2A for the lipids trapped off-line at 80°C and 680 bar. Three of the lipid classes in that chromatogram are represented as single peaks, whereas the fatty acids are separated into four peaks, which are considered collectively as a single component in the mixture for the purposes of this discussion. In Figure 2B note that under the same experimental conditions (80°C, 680 bar), only triglycerides and cholesterol appeared in the chromatogram for the lipids isolated from the in-line sorbent bed. However, a surprising shift in composition for the lipids trapped in-line occurred at the reduced temperature of 40°C at 680 bar. Under

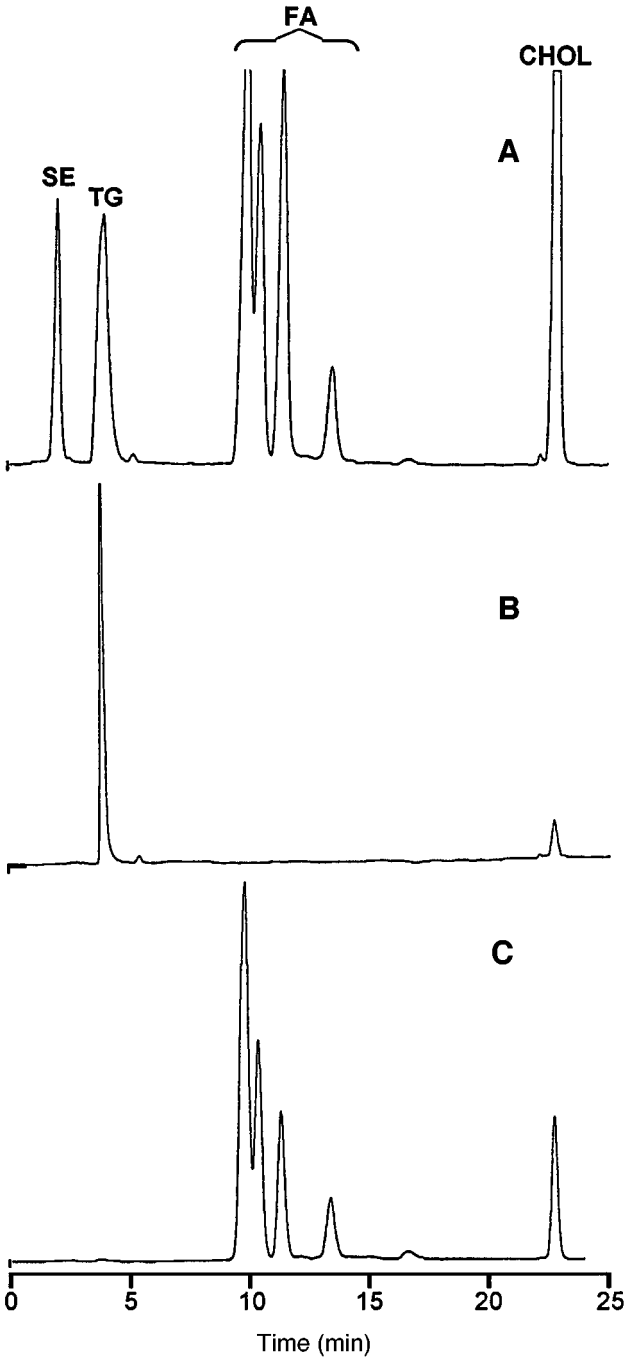

FIG. 2. High-performance liquid chromatography/evaporative light scattering detection chromatograms of lipids (SE, sterol esters; TG, triglycerides; FA, fatty acids; and CHOL, cholesterol) from supercritical fluid extracts collected (A) at 80°C and 680 bar from the off-line collection vial, (B) at 80°C and 680 bar from the in-line alumina sorbent (trap 2), and (C) at 40°C and 680 bar from the in-line alumina sorbent (trap 2).

those conditions no triglycerides were found in the mixture, instead the lipid mixture consisted of only fatty acids and cholesterol (Fig. 2C).

In general, adsorption depends primarily on differences in the affinity of the analyte for the adsorbing species. Affinity is mainly determined by polar interactions, with nonpolar van der Waals forces being of minor importance (10). Among the four groups of lipid classes, fatty acids have the highest polarity followed in order by cholesterol, triacylglycerols, and sterol esters, where the latter two are regarded as nonpolar. It can be seen therefore that there exists a greater affinity of alumina for fatty acids than for the other three classes in the pressure range of 490 to 680 bar at 40°C. However, the adsorptive selectivity of alumina for fatty acids disappeared when the temperature was increased from 40 to 80°C, at which temperature lesser amounts of other lipid classes were retained on the alumina sorbent.

We observed that larger amounts of lipids were extracted with SC-CO_2 at 80°C and 600 and 680 bar than at 40°C at 490 and 680 bar, but that smaller amounts were trapped in-line by alumina at the higher temperature, demonstrating that the extraction temperature had a greater effect than pressure (or density) for extracting the lipids from chicken liver. These results are similar to those observed by Stahl and Quirin for the behavior of soybean oil in SC-CO_2 (11). They observed that, although the solubility of soybean triglycerides in SC-CO_2 increases with increasing pressure at constant temperature, each solubility isotherm has a maximum which is more pronounced at 60 than at 40°C. They also reported that above 60°C and 550 bar the lipid solubility in SC-CO_2 rises sharply, and that complete miscibility occurs at 80°C and about 700 bar.

The nature of the matrix and the manner in which analytes are incorporated in the matrix influence SFE extraction kinetics (4). Although the solubility of many drugs investigated is in the ppb range in pure SC-CO_2, the interactions between a drug and a tissue substrate lower the solubility of the drug in SC-CO_2 so that it generally does not attain its neat solubility level (12). The ability of SC-CO_2 to overcome analyte–matrix bonding is in many cases of greater importance than high analyte solubility for obtaining efficient SFE recoveries. Increasing the temperature and pressure (density) can enhance the extractability of some analytes by overcoming analyte–matrix interactions and thereby releasing the analyte for more efficient partitioning into the supercritical fluid. However, these effects also increase the amount of unwanted co-extracted lipids along with the target analyte from a biological matrix, complicating post-SFE analysis of the analyte. This problem may be obviated when an in-line trapping technique is employed, since increasing pressure and temperature may not only increase the concentration of the desired analyte collected on the sorbent but also reduce the amount of retained lipids, thus producing a cleaner analyte extract. Our previous results with analytes in biological matrices suggest that the in-line trapping technique may be attractive for recovering polar analytes in a cleaner state than is possible for those collected by using off-line recovery techniques (6–8). For certain drug classes that are extractable at much lower pressures such as steroids, the interference of lipids trapped in-line could pose significant problems in their post-SFE analysis (5). In this case, determination of specific temperature and pressure conditions on sorbent activity to optimize in-line trapping efficiency requires further investigation.

ACKNOWLEDGMENT

We gratefully acknowledge funding support for this project provided by USDA/FAS/ICD/RSED.

REFERENCES

1. Code of Federal Regulations 21, *Tolerances for Residues of New Animal Drugs in Food,* 556–591, 1989.
2. U.S. EPA Pollution Prevention Strategies, Washington, DC, *Federal Register 56,* 7849–7864 (1991).
3. Hawthorne, S.B., Analytical-Scale Supercritical Fluid Extraction, *Anal. Chem. 62:*633A–644A (1990).
4. King, J.W., Fundamentals and Applications of Supercritical Fluid Extraction in Chromatographic Science, *J. Chromatogr. Sci. 27:*352–364 (1989).
5. Stolker, A.A.M., L.A. van Ginkel, R.A. Stephany, R.J. Maxwell, O.W. Parks, and A.R. Lightfield, Supercritical Fluid Extraction of Methyltestosterone, Nortestosterone and Testosterone at Low ppb Levels from Fortified Bovine Urine, *J. Chromatogr.,* in press.
6. Parks, O.W., and R.J. Maxwell, Isolation of Sulfonamides from Fortified Chicken Tissues with Supercritical CO_2 and In-line Adsorption, *J. Chromatogr. Sci. 32:*290–293 (1994).
7. Maxwell, R.J., A.R. Lightfield, and A.A.M. Stolker, An SPE Column-Teflon Sleeve Assembly for In-line Retention During Supercritical Fluid Extraction of Analytes from Biological Matrices, *J. High Resolut. Chromatogr. 18:*231–234 (1995).
8. Parks O.W., R.J. Shadwell, A.R. Lightfield, and R.J. Maxwell, Determination of Melengestrol Acetate in Supercritical Fluid-Solid Phase Extracts of Bovine Fat Tissue by HPLC–UV and GC–MS, *J. Chromatogr. Sci. 34:*353–357 (1996).
9. Moreau, R.A., M.J. Powell, and K.B. Hicks, Extraction and Quantitative Analysis of Oil from Commercial Corn Fiber, *J. Agric. Food Chem. 44:*2149–2154 (1996)
10. Lim, S., and S.S.H. Rizvi, Adsorption and Desorption of Cholesterol in Continuous Supercritical Fluid Processing of Anhydrous Milk Fat, *J. Food Sci. 61:*817–820 (1996).
11. Stahl, E., and W. Quirin, Dense Gas Extraction on a Laboratory Scale: A Survey of Some Recent Results, *Fluid Phase Equilibria 10:*269–278 (1983).
12. McHugh, M.A., and V.J. Krukonis, *Supercritical Fluid Extraction: Principles and Practice,* Butterworth, Boston, 1993.

[Received March 10, 1998; accepted January 14, 1999]