Concentration of Phytosterols for Analysis by Supercritical Fluid Extraction

Janet M. Snyder*, Jerry W. King, Scott L. Taylor, and Angela L. Neese
Food Quality and Safety Research Unit, NCAUR, ARS, USDA, Peoria, Illinois 61604

ABSTRACT: Fractionation of sterols from plant lipid mixtures was accomplished using a multistep supercritical fluid extraction (SFE) procedure. Samples of seed oils, margarine, corn germ oil, and corn fiber oil were extracted to yield enriched phytosterol fractions. Supercritical fluid chromatography (SFC) was utilized to separate and determine the concentration of the plant sterols in the extracts from the various samples. The sterol concentration in the original samples varied from 2.2 mg/g in soybean oil to 13.2 mg/g in oil extracted from corn fiber. After the SFE-based fractionation of the samples, the sterol concentration was increased to 64.4 mg/g in the extract from soybean oil and 166.2 mg/g in the extract from corn fiber oil. Oil extracted from corn bran, which measured 8.6 mg/g in the original oil, increased to 322.2 mg/g using the fractionation process. The benign conditions utilized by SFE and SFC proved to be effective for the analyses of these compounds without inducing degradation of the analytes.

Paper no. J8991 in *JAOCS 76*, 717–721 (June 1999).

KEY WORDS: Canola, carbon dioxide, chromatography, corn bran, corn fiber, cottonseed, extraction, margarine, phytosterol, rice bran, soybean, supercritical fluid.

Extraction of the seed oils with supercritical carbon dioxide (SC-CO_2) followed by selective fractionation has the potential of offering industry an analytical method to determine the sterol content in these matrices. The extraction of rice bran and corn oil (1,2) and the refining of rice bran by supercritical fluid extraction (SFE) have indicated that supercritical fluids are effective as processing solvents for this purpose (3). In addition, SFE coupled with a variety of sorbents can selectively fractionate targeted natural products from lipid matrices. Fractionation of rice bran oil using SC-CO_2 and a silica-$AgNO_3$ column was achieved by Saito *et al.* (4). Separation of cholesterol from dried egg yolk has been achieved by SFE followed by solid-phase extraction (5), and cholesterol has been removed from butterfat by selectively adsorbing the sterol onto a sorbent during SFE (6). Previously it has been shown that spina sterol and stigmasterol recoveries could be increased during the selective extraction of shea butter using SC-CO_2 at 45°C and 150 bar (7).

*To whom correspondence should be addressed at Food Quality and Safety Research Unit, NCAUR, ARS, USDA, 1815 N. University St., Peoria, IL 61604. E-mail: snyderjm@mail.ncaur.usda.gov

An amino propyl sorbent has been reported to effectively separate individual lipid classes by utilizing various organic solvents with different polarities (8). The addition of organic modifiers to supercritical carbon dioxide can increase the polarity of the supercritical fluid so as to extract more polar compounds (9). Therefore, a supercritical fluid with a minimal amount of cosolvent can replace the larger amount of solvent needed for efficient elution of the compounds from the amino propyl sorbent.

Supercritical fluid chromatography (SFC) has a number of significant benefits including speed of analysis when compared to separations using liquid mobile phases. Methods utilizing SFC as an effective technique to analyze different lipid classes, without resorting to the derivatization of various analytes, have been reported in the literature (10–12). Moreover, SFC data have also been shown to be quantitative and equivalent to gas chromatography data when determining the concentration of cholesterol in milk (13) or the tocopherol content in antioxidant samples (14). In addition, reaction products from glycerolysis of soybean oil to monoglyceride have been measured by SFC (15).

In this study, the authors have incorporated some of the above principles and observations to develop an analytical method including SFE and SFC for the analysis of sterols in various lipid-laden matrices. Using principles demonstrated with solid-phase cartridges and organic solvents, the authors have developed a method predominantly using SC-CO_2 in place of organic solvents, for the enrichment of sterol moieties in various oil- and fat-containing matrices. This enrichment facilitates more accurate determination of the sterols as opposed to trying to determine them in low levels in the native matrix. The reported SFE and SFC methods save considerable time for sample preparation/cleanup and reduce costs with respect to solvent usage.

EXPERIMENTAL PROCEDURES

Pioneer Hybrid soybeans were flaked and extracted at our laboratory by Soxhlet extraction with hexane for 5 h (hexane-soy) and with supercritical carbon dioxide (SC-CO_2) at 8000 psi and 50°C (SFE-soy) (16) to produce two crude soybean oil samples. Canola, corn, and cottonseed oils were obtained from Archer Daniels Midland (Decatur, IL).

Corn germ oil was extracted with hexane at our laboratory using a procedure developed by Norton (17). Corn oil was extracted from corn fiber and rice oil from rice bran using a Isco SFX 2-10 SFE unit (Isco Inc., Lincoln, NE) with SC-CO_2 at 8000 psi and 80°C to extract the total lipid content. Extractions from both the corn fiber and the rice bran were accomplished using 10-g samples; a total of five extractions from the corn fiber and four from the rice bran were needed to obtain enough sample for the sterol separation analyses. Benecol margarine samples with 40% fat and 80% fat were obtained from Raisio Group (Raisio, Finland) (18). Lauric acid and sterol standard compounds were obtained from Sigma-Aldrich (St. Louis, MO). Stigmastanol was obtained from TCI-EP (Tokyo, Japan) The cosolvents included methanol (Fisher Scientific, Fairlawn, NJ) and methyl *tert*-butyl ether (MTBE) (Sigma-Aldrich).

Method development utilized a refined, bleached, deodorized (RBD) soybean oil containing known concentrations (0.1–1.0 wt%.) of stigmasterol to determine the efficiency of the extraction method. Recovery of the stigmasterol in each fraction was monitored by SFC.

Oil samples (0.3 g) were mixed with 0.15 g Hydromatrix (Analytichem International, Harbor City, CA) and added into a 2.5 mL extraction vessel. Glass wool was then inserted into the cell and 0.7 g NH_2-Mega Bond Elut (Varian, Harbor City, CA) coated sorbent was added (8). Margarine samples (0.3 g) were measured directly into the extraction cell and 0.7 g NH_2-bonded sorbent added. Extractions were accomplished using an Isco SFX 2-10 SFE unit. The extraction cell was positioned in the oven with the sample on top; the flow of the SC-CO_2 was from top to the bottom of the cell. The conditions for the initial SFE step were 30 mL SC-CO_2 at 8000 psi and 80°C. The second extraction sequence on the same sample matrix was accomplished using 30 mL SC-CO_2/10% modifier at 2000 psi and 80°C; then the pressure was raised to 4000 psi and an additional 30 mL SC-CO_2/10% modifier utilized. The pressure was increased to 6000 psi using 80 mL SC-CO_2/10% modifier to remove any remaining lipids from the sorbent after the sterol-enriched fraction had been isolated. Each sample, using either methanol or MTBE as cosolvents, was extracted three times using the above four-step procedure.

Each of the resultant fractions was analyzed using a Dionex Series 600 supercritical fluid chromatograph (Dionex, Inc., Salt Lake City, UT), with a Dionex SB-Octyl-50 capillary column (10 m × 100 μm × 0.5 μm film thickness). The analyses were conducted isothermally at 100°C using the following pressure gradient program: 100 atm for 5 min to 150 atm at 5 atm/min, then to 180 atm at 2 atm/min, and finally to 280 atm at 5 atm/min for a total run time of 52 min. A time/split automatic injection using a Valco valve (Valco, Inc., Houston, TX) was used for 1.8 s to inject the sample from a 200 nL internal injection loop. A flame-ionization detector (FID) was used as the detector utilizing a temperature of 350°C (9). Lauric acid was added as an internal standard.

RESULTS AND DISCUSSION

The SFC method was developed to separate the lipid classes for the determination of the sterol concentration. The FID response was linear for stigmasterol over the range of 0.0002–1 mg/mL with correlation coefficient $r^2 = 0.99894$. Additional correlation coefficients for the response curves for campesterol, β-sitosterol, and sitostanol were 0.9951, 0.9932, and 0.9998, respectively.

The initial SF extracted soybean oil contained 0.05% brassicasterol, 0.11% β-sitosterol, 0.06% stigmasterol and 0.04% campesterol. The chromatograms in Figure 1 show the effect of the four steps in the separation process. The first extraction of the soybean oil using only SC-CO_2 at 8000 psi and 80°C removed 95% of the triglycerides and only trace amounts of sterol as shown in Figure 1A. There were also only trace amounts of sterol in the second extraction step at 2000 psi and 80°C using SC-CO_2/10% MTBE (Fig. 1B). The effect of the third extraction sequence is evident in the chromatogram (Fig. 1C) of the extract produced at 4000 psi and 80°C using SC-CO_2/10% MTBE. Here, the concentration of sterols increased from 0.2 wt% in the initial oil to 40 wt% after completion of the third SFE-fractionation. The fraction extracted at 6000 psi and 80°C with SC-CO_2/10% MTBE also had a 27% sterol content (Fig. 1D).

While investigating different solvents to extract the sterols from the NH_2-bonded sorbent, methanol seemed to be an attractive solvent for sterols; however, not all the sterols were removed from the NH_2-bonded sorbent, even after the last step conducted at 6000 psi and 80°C. An exhaustive extraction of the sorbent with chloroform/methanol indicated that some sterol remained on the sorbent. After further investigation using other solvents, MTBE proved to be a better modifier than methanol (Figs. 2–4). Four individual sterol compounds were identified in the chromatograms (Fig. 1), and the sterol content was determined from the total of the four compounds. In this study, brassicasterol was only measured in small quantities in SFE-soy fractions.

The highest sterol concentration from the seed oils was found to be in the second step from all seed oils except corn oil when methanol was used (Fig. 2). Fatty acids present in the cottonseed oil and the hexane-extracted soybean oil were also eluted from the NH_2-bonded sorbent in the second step using SC-CO_2/10% MeOH at 2000 psi and 80°C which lowered the sterol weight percentage in the second fractions. The results from the seed oil extractions using MTBE show that the largest quantity of sterol elutes from the sorbent in the third step for SFE-soybean oil and corn oil and in the fourth step for hexane-extracted soybean oil, cottonseed oil, and canola oil. Only trace amounts of sterols were measured in the second fraction using MTBE.

Recovery of the sterol from the oils that were extracted from corn bran and fiber and oils from rice bran was also better using MTBE (Fig. 3). However, the fractionation pattern for each oil was different. Sterol concentration in corn bran oil was high in three fractions with the highest content in the

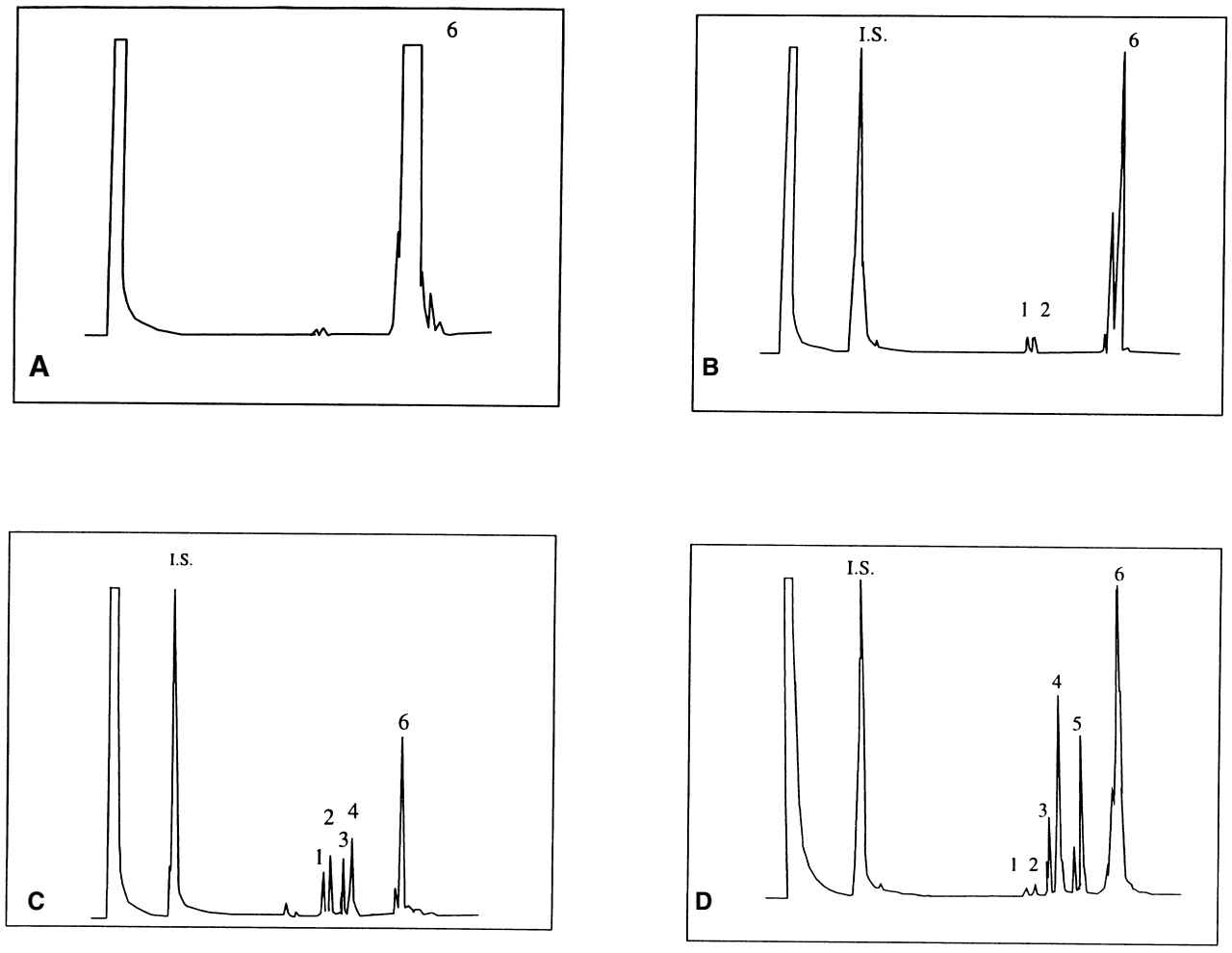

FIG. 1. Chromatograms for fractionation of supercritical fluid extraction (SFE) soybean oil. (A) First extraction with supercritical carbon dioxide (SC-CO_2) at 8000 psi and 80°C; (B) extraction products after SC-CO_2/10% methyl *tert*-butyl ether (MTBE) at 2000 psi and 80°C; (C) extraction products after SC-CO_2/10% MTBE at 4000 psi and 80°C; (D) extraction products after SC-CO_2/10% MTBE at 6000 psi and 80°C; 1—brassicasterol, 2—stigmasterol, 3—campesterol, 4—β-sitosterol, 5—diglycerides, 6—triglycerides. I.S. internal standard (lauric acid).

third fraction using MTBE while little was extracted using methanol as cosolvent. Oil from corn fiber oil was extracted from the NH_2-bonded sorbent in the same manner as the majority of seed oils using MTBE; the highest concentration was in the fourth fraction. Rice bran oil was the only sample where the largest amount of sterol was extracted by SC-CO_2 in the first step.

MBTE was also the best cosolvent to separate the sterols in both margarine samples which were enriched with sitostanol. The amount of sterols in the margarine sample that contained 80% fat yielded the highest sterol concentration in the second extraction, with small amounts of sterols in the third and fourth fractions (Fig. 4). The total amount of sterol was less in the 40% fat margarine; also, the moisture content in that sample appeared to decrease the extraction of the sterol from the sorbent.

Initial concentration of sterols and concentration after fractionation using MTBE as the cosolvent, listed in Table 1, are calculated from the SFC data using response curves derived from the sterol standards. The initial concentrations vary from 2.2 mg/g in SFE-soybean oil to the higher concentration of 15.4 mg/g in cottonseed oil and 15.4 mg/g in the initial rice bran oil. The analytical method effectively increased the sterol concentration of every oil sample in the study. The values that are listed under the column labeled "SFE fraction" are from the step of the fractionation process that yielded the highest sterol content. The two enriched margarine samples (16) had 24.5 mg/g in the initial 40% fat margarine and 30 mg/g sterol in the 80% fat margarine. After the supercritical fractionation process, the sterol content increased to 116.5 mg/g and 219 mg/g, respectively, in these two samples.

The above results show that by combining SFE, using SC-CO_2 and select cosolvents, with an adsorbent, a significant enrichment with respect to analytical detectability can be realized. By varying the extraction/fractionation conditions enacted upon the sample matrix, one can remove most of the in-

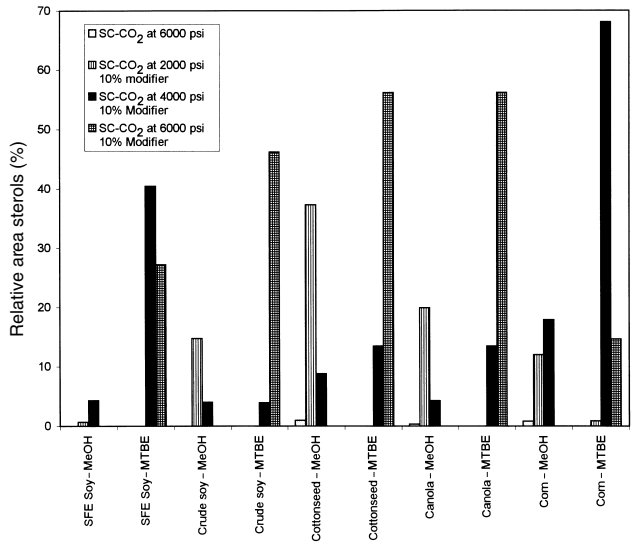

FIG. 2. Fractionation results for sterols from seed oils. Comparison of SFE data using MeOH and MTBE as cosolvents. For abbreviations, see Figure 1.

terfering background triglyceride components and then sequentially extract and elute the sterol-containing fraction for further analysis. Postextraction analysis can be conveniently handled by using the described capillary SFC assay.

These steps and methods reduce analysis cost and analysis time, as well as minimize the use of organic solvents involved in the sample preparation and final analysis. The solute selectivity exhibited in this scheme suggest some interesting options that could be scaled up to effect the isolation of the sterol components from these natural oil- and fat-containing matrices.

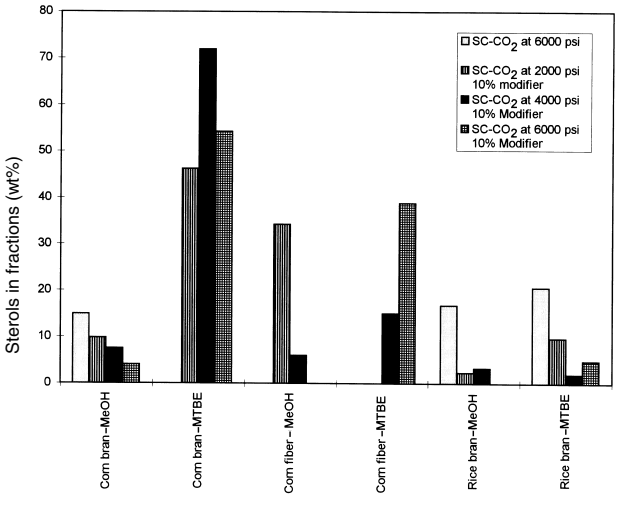

FIG. 3. Fractionation results for sterols from oils extracted from bran and fiber. Comparison of the SFE data using MeOH and MTBE as cosolvents. For abbreviations see Figure 1.

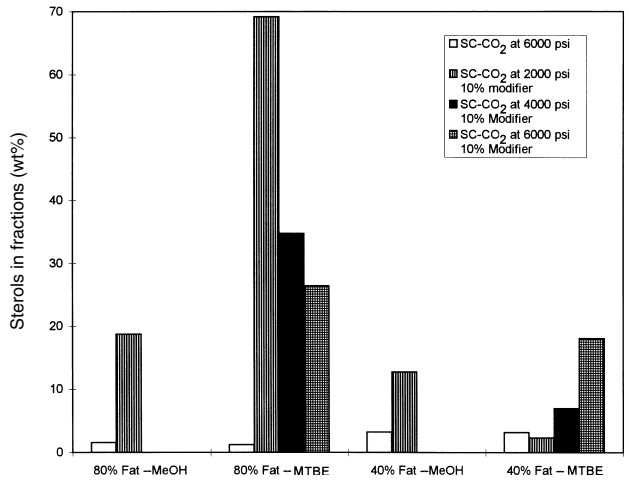

FIG. 4. Fractionation of sterols in margarine samples. Comparison of the SFE data using MeOH and MTBE as cosolvents. For abbreviations see Figure 1.

ACKNOWLEDGMENTS

The authors thank Robert Norton of National Center for Agricultural Utilization Research for providing some of the standard compounds and for his helpful advice on sterol analysis. In addition, the authors thank Eila Jarvenpaa of the University of Turku, Turku, Finland, for providing the Benecol margarine samples.

REFERENCES

1. Shen, Z., M.V. Palmer, S.S.T. Ting, and R.J. Fairclough, Pilot Scale Extraction of Rice Bran Oil with Dense Carbon Dioxide, *J. Agric. Food Chem. 44*:3033–3039 (1996).
2. Moreau, R.A., M.J. Powell, and K.B. Hicks, Extraction and Quantitative Analysis of Oil from Commercial Corn Fiber, *Ibid. 44*:2149–2154 (1996).
3. Ramsay, M.E., J.T. Hsu, R.A. Novak, and W.J. Reightler, Pro-

TABLE 1
Concentration of Sterols in Lipid-Containing Samples After Supercritical Fractionation with MTBE

	Concentration (mg/g)	
Lipid sample	Initial	SFE fraction[a]
Corn oil	6.8	153.5 ± 10[b]
Canola oil	11.4	104.3 ± 12[c]
Cottonseed oil	15.7	131.0 ± 23[c]
Hexane-soy oil	8.9	51.6 ± 2[c]
SFE-soy oil	2.2	64.4 ± 2[b]
Corn bran oil	8.6	322.1 ± 53[b]
Corn fiber oil	13.2	166.2 ± 39[c]
Rice bran oil	15.4	58.0 ± 2[d]
Margarine (80% fat)	30.0	219.0 ± 44[e]
Margarine (40% fat)	24.5	116.5 ± 6[c]

[a]Extractions from the fraction with the best sterol concentration; $n = 3$.
[b]Values from the third fraction at 4000 psi and 80°C using SC-CO_2/10% MTBE.
[c]Values from the fourth fraction at 6000 psi and 80°C using SC-CO_2/10% MTBE.
[d]Values from the first fraction at 8000 psi and 80°C using SC-CO_2.
[e]Values from the second fraction at 2000 psi and 80°C using SC-CO_2/10% MTBE. Abbreviations: MTBE, methyl *tert*-butyl ether; SFE, supercritical fluid extraction; SC-CO_2, supercritical carbon dioxide.

cessing Rice Bran by Supercritical Fluid Extraction, *Food Technol. 45*:98–104 (1991).
4. Saito, N., Y, Ikushima, K. Hatakeda, S. Ito, and T. Goto, Fractional Extraction of Rice Bran Oil and Their Ester with Supercritical Carbon Dioxide, *J. Agric. Chem. Soc. Jpn. 65*:153–161 (1991).
5. Boselli, E., M.F. Caboni, and G. Lercker, Determination of Free Cholesterol from Dried Egg Yolk by On-Line Coupling of Supercritical Fluid Extraction with Solid-Phase Extraction, *Z. Lebens. Unters. Forsch. A205*:356–359 (1997).
6. McLachlan, C.N.S., O.J. Catchpole, and B.H. Hamilton, Separation of Sterols from Lipids, U.S. Patent 5,024,846 (Jun 18, 1991).
7. Montanari, L., A. Sensidoni, and P. Fantozzi, A New Approach to Shea Butter Extraction Technology, Advances in Oils and Fats, Antioxidants, and Oilseed By-Products, in *Proceedings of the World Conference on Oilseed and Edible Oils Processing*, Vol. 2, edited by S.S. Koseoglu, K.C. Rhee, and R.F. Wilson, AOCS Press, Champaign, IL, 1998, pp. 20–24.
8. Kaluzny, M.A., L.A. Duncan, M.V. Merritt, and D.E. Epps, Rapid Separation of Lipid Classes in High Yield and Purity Using Bonded Phase Columns, *J. Lipid Res. 26*:135–140 (1985).
9. Hawthorne, S.B., and D.J. Miller, Quantitative Extraction of Linear Alkylbenzenesulfonates Using Supercritical Carbon Dioxide and a Simple Device for Adding Modifiers *J. Chromatogr. 541*:186–194 (1991).
10. King, J.W., and J.M. Snyder, Supercritcal Fluid Chromatography: A Shortcut in Lipid Analysis, in *New Techniques and Applications in Lipid Analysis*, edited by R.E. McDonald and M.M. Mossoba, AOCS Press, Champaign, IL, 1997 pp. 139–162.
11. Laakso, P., Supercritical Fluid Chromatography of Lipids, in *Advances in Lipid Methodology*, Vol. 1, edited by W.W. Christie, The Oily Press, Alloway, Scotland, 1992, pp. 81–118.
12. Sandra, P., and F. David, Basic Principles and the Role of Supercritical Fluid Chromatography in Lipid Analysis, in *Supercritical Fluid Technology in Oil and Lipid Chemistry*, edited by J.W. King and G.L. List, AOCS Press, Champaign, 1996, pp. 321–347.
13. Huber, W., A. Molero, C. Pereyra, and E. De la Ossa, Determination of Cholesterol in Milk Fat by Supercritical Fluid Chromatography, *J. Chromatogr. A 715*:333–336 (1995).
14. Snyder, J.M., S.L. Taylor, and J.W. King, Analysis of Tocopherols by Capillary Supercritical Fluid Chromatography and Mass Spectrometry, *J. Am. Oil Chem. Soc. 70*:349–364 (1993).
15. Temelli, F., J.W. King, and G.R. List, Conversion of Oils to Monoglycerides by Glycerolysis in Supercritical Carbon Dioxide Media, *Ibid. 70*:349–364 (1993).
16. List, G.R., J.P. Friedrich, and J.W. King, Supercritical CO_2, Extraction and Processing of Oilseeds, *Oil Mill Gazetteer 95*:28–34 (1989).
17. Norton, R.A., Quantitation of Steryl Ferulate and *p*-Coumarate Esters from Corn and Rice, *Lipids 30*:269–274 (1995).
18. Miettinen, T.A., and H. Gylling, Sitostanol-Ester Margarine in *New Technologies for Healthy Foods and Nutraceuticals*, edited by M. Yalpani, ATL Press, Shrewsbury, 1997, pp. 71–83.

[Received August 26, 1998; accepted February 18, 1999]