An Improved Micro-Extraction Cell for Supercritical Fluid Extraction and Chromatography of Fatty Acids

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An improved supercritical fluid micro-extraction cell of increased reliability was designed for on-line supercritical fluid extraction and chromatography (SFE/SFC) of food and other lipid-related samples. The key components in the modified cell include a Swagelok stainless steel reducing union with a dual ferrule as the cell, with polyetheretherketone (PEEK) ferrules and nuts to connect the cell to the control valve. The new cell did not leak under all conditions examined (100–500 atmospheres, 40–80°C), even after numerous extractions (>250). The quantitative performance of the cell was evaluated with fatty acid standard solutions, technical grade fatty acid sources and wheat flour. The percent relative error (%RE) for the fatty acid standards and technical-grade fatty acid samples was ≤6.0% for oleic, linoleic and linolenic acid. The %RE for oleic and linoleic acid in the whole-wheat samples was ≤10%. The results demonstrate that the new extraction cell can be used for quantitative extractions and that the sensitivity of the SFE/SFC technique is excellent. Similar SFE/SFC methods could prove useful in studying the interaction of free fatty acids with various food components such as enzymes, amylose and proteins.

KEY WORDS: Fatty acids, supercritical fluid chromatography, supercritical fluid extraction.

Application development for analytical supercritical fluid extraction (SFE) recently has received substantial attention. Qualitative and quantitative analytical SFE of environmental solids (1–5), coal tars (6), foods and pharmaceuticals (7–12) have been successfully coupled with both gas chromatography (GC) and supercritical fluid chromatography (SFC). The SFE and SFE/SFC analysis of food, flavor- and lipid-related samples recently has been reviewed in depth (13). Due to the solubility of lipids and their derivatives in supercritical CO_2 under relatively moderate conditions, lipid extraction and analysis with SFE/SFC has been of particular interest. Gmuer *et al.* (7) extracted and separated free fatty acids from cheese and butter by SFE/SFC and suggested that this method could be used to evaluate the ripening of cheese, flavor defects in butter, and hydrolytic rancidity of dairy products in general. Free fatty acid separations have often been applied to the evaluation of new analytical methods and instrumentation (13). Variations in molecular size, degree of unsaturation, the presence of a variety of functional groups, their solubility in supercritical CO_2, as well as their ubiquity in nature, make free fatty acids excellent test models for new SFE/SFC techniques and instrumentation.

Due to the lack of a reliable, leak-resistant, micro-analytical extraction cell, application development in on-line micro-analytical SFE has been restrained. Leaking of previous cell designs was especially problematic at the tubing connections of the cell body and switching valve ports. Many investigators were required to develop their own extraction devices for supercritical applications (2–5,10,14) to overcome these problems. A new design is reported here, which is inexpensive and resistant to leakage, even after numerous extractions.

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EXPERIMENTAL PROCEDURES

Cell design. An extraction cell, based on concepts published by Hawthorne and Miller (2–4) and Hawthorne *et al.* (5,10), was prepared and installed on a Lee Scientific Model 501 capillary SFC with the on-line SFE option (Lee part #012970) (Lee Scientific, Salt Lake City, UT). The key component of the extraction cell was a Swagelok dual ferrule stainless steel reducer/reducing union connector (Peoria-Decatur Valve and Fitting Co., Morton, IL). The connecting lines from the cell to the Valco 10-port valve (VICI, Houston, TX) require disassembly and reconnection for each extraction. To insure leak-free connections to the 10-port valve, high-performance polyetheretherketone (PEEK) nuts and ferrules (Alltech Associates, Inc., Deerfield, IL) were used. Specifically, the new design (Fig. 1) consisted of: (B) a 0.159-cm (1/16″) PEEK double-sided ferrule (DSF); (C) a 10/32 PEEK nut; (D–G) a Swagelok stainless steel (SS) reducer and (H–M) reducing union, which includes (D) a 0.159-cm (1/16″) SS female nut, (E) a 0.159-cm (1/16″) SS dual ferrule, (F) a 0.794-cm (5/16″) SS nut, (G) a 0.318-cm (1/8″) o.d. SS tubing, (H) a 0.318-cm (1/8″) SS female nut, (I) a 0.318-cm (1/8″) SS dual ferrule, (J) a 0.5-μm SS frit for the 0.318-cm (1/8″) i.d. tubing, (K) a 0.318-cm × 0.159-cm (1/8″ × 1/16″) SS reducing union, (L) a 0.159-cm (1/16″) SS dual ferrule, (M) a 0.159-cm (1/16″) female nut; (N) 0.013-cm × 0.157-cm (0.005″ × 0.062″, i.d. × o.d.) Valco SS tubing. The Valco ten-port valve (A), heat sink and insulated cover from the Lee Scientific extraction unit were used. The cell was insulated with 1.27 cm of fiberglass batting. The dual Swagelok ferrules eliminated leaking at the cell connections to the transfer lines (D–E & L–M). The 0.318-cm (1/8″) SS dual ferrule (I), used to seal the cell body prior to extraction, was much better suited to repeated use than the single ferrule used in the original cell design. An extraction could be continued for an hour at approximately 500 atm at 80°C with no evidence of leaking, even after 200 extractions. The stainless steel nuts and ferrules normally used to connect the cell tubing to the Valco valve ports were replaced with PEEK (Alltech Associates, Inc.) nuts and ferrules to prevent leaking at the valve connection. This also reduced the maintenance costs because the SS tubing connecting the extraction and the Valco 10-port valve (Fig. 1, N) need not be replaced after leakage occurred, which occurred typically after 25–50 extractions with SS ferrules and nuts. However, the PEEK ferrules and nuts do require replacement after approximately 30–40 extractions, due to the physical deterioration that occurred during repeated reassembly. Replacement of the PEEK ferrules and nuts is facile and the cost is minimal.

Fatty acid analysis. Oleic, linoleic and linolenic (99, 99

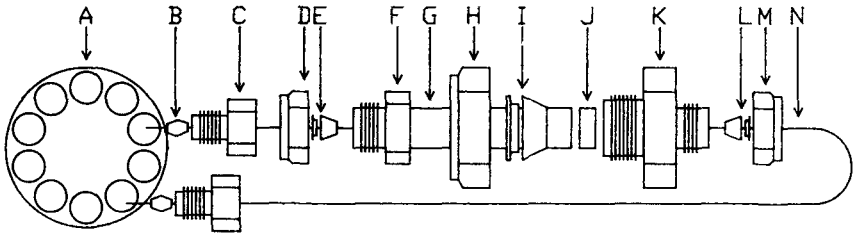

FIG. 1. Modified supercritical fluid extraction cell. (A) Valco ten-port valve; (B) 0.159-cm (1/16″) polyether ether ketone (PEEK) double-sided ferrules, (C) 10/32 PEEK nut; (D–G) Swagelok SS reducer and (H–M) reducing union, (N) 0.013-cm × 0.167-cm (0.005″ × 0.062″, i.d. × o.d.) Valco SS tubing. CO_2 flow is from left to right, top to bottom.

and 98% pure by GC, respectively) fatty acid standards were purchased from Sigma Chemical Co. (St. Louis, MO). Fatty acid standards were prepared as a mixture of oleic, linoleic and linolenic acids at a concentration of approximately 5.0×10^{-4} g/mL per fatty acid in pentane (American Burdick and Jackson, Muskegon, MI). Serial dilutions to concentrations of 2.0×10^{-5} to 2.6×10^{-4} g/mL of each fatty acid were prepared from the 5.0×10^{-4} g/mL fatty acid mixture. All reagents were reagent grade unless stated otherwise.

The reliability of the system for the quantitative analysis of liquid samples was evaluated with technical-grade oleic, linoleic and linolenic fatty acids (Kodak Co., Rochester, NY). Technical-grade oleic, linoleic and linolenic fatty acid was 98, 96, and 96% pure by titration, respectively, according to the manufacturer. The specific fatty acid content of each source of technical-grade fatty acid was analyzed at two concentrations with 3–4 replicates.

Flour sample analysis. The oleic/linoleic/linolenic acid content of Ceresota Unbleached Whole Wheat Flour (Uhlman Co., Kansas City, MO) was determined with SFE/SFC. Samples were stored at −20°C over anhydrous $CaSO_4$ (W.A. Hammond Drierite Co., Xenia, OH), under nitrogen in polyethylene desiccators.

Supercritical fluid extraction and chromatography. A Lee Scientific 501 supercritical fluid chromatograph and extraction system (Lee Scientific) equipped with the on-line supercritical fluid extraction option and a flame-ionization detector (FID) was used. Two columns (SB-cyano-50, 20 m × 100 μm i.d., 0.25 μm film thickness, Lee Scientific), connected in series, were used. Chromatograms were recorded and integrated on an HP3390A integrator (Hewlett-Packard, Avondale, PA). A 0.35-m × 15-μm deactivated fused silica restrictor was used to achieve the required back pressure during extraction. Deactivated fused silica restrictors provided increased extraction rates and reduced cleanup relative to restrictors that had not been deactivated. The cryofocusing unit was cooled with industrial-grade CO_2 (Depke, Inc., Urbana, IL), so that a light frost appeared just on either side of the intersection in the cryofocusing T located inside the oven. If the T was heavily frosted or frosted along the entire length of the cryofocusing T, it resulted in a substantial reduction in the extracting CO_2 mobile phase flow. To achieve the appropriate level of cooling in the cryofocusing T, a two-stage, high-pressure regulator can be used. The second stage was set at 700 psi. Even with heavy frosting, quantitative collection of volatile compounds (*e.g.*, cinnamonaldehyde) at the cryofocusing T can be less than 40% (W.E. Artz and R.M. Sauer, Jr., unpublished data).

For liquid samples, one μL of fatty acid standard or sample was placed onto a small triangular (0.3 cm × 1.0 cm, base × height) piece of #541 filter paper (Whatman International Ltd., Maidstone, U.K.), which was positioned in the center of the extraction cell. Preliminary investigations indicated that placement of the liquid sample on a filter paper support improved the reproducibility of the extraction. A short extraction (5 min, 500 atm) was required between each sample to remove the small percentage (<3%) of sample remaining in the lines, valves, cell, etc. After the cleanup step, further extractions indicated no remaining sample.

The standard solutions, technical-grade fatty acid mixtures and the flour samples were extracted at approximately 500 atm at 80°C for 15.0 min. The column oven was maintained at 40°C during the extraction. At the end of the extraction period, the pressure was reduced to 140 atm over a 2.5-min period, during which the cryofocusing was continued. When the pressure had been reduced to 140 atm, a control valve was switched from extraction to column to direct the extracted analytes onto the capillary column. Immediately after the depressurization step following the extraction, the oven temperature was rapidly increased to 100°C (70° C/min) and then maintained at 100°C. A linear pressure ramp was initiated at 17.5 min into the extraction/separation procedure, which was immediately after the pressure had been reduced to 140 atm. The pressure ramp program was: 10 atm/min to 150 atm (1 min); +0.5 atm/min to 152 atm (4 min); hold at 152 atm (52 min); +0.2 atm/min to 155 atm (15 min); +0.7 atm/min to 170 atm (21 min); hold at 170 atm (19 min) for a total of 112 min.

RESULTS AND DISCUSSION

Standard curves of integrator area *vs* the quantity of oleic, linoleic and linolenic acid are shown in Figure 2. The correlation coefficient (R^2) for each standard curve was ⩾0.989. The standard deviation of each set of replicates at each concentration is shown as error bars (Fig. 2). Gross outliers were rejected with the Dixon Q-test (15). Representative separations of the extracted fatty acid standards are shown in Figure 3. Oleic acid eluted at approximately 57.5 min, linoleic at approximately 66.5 min and linolenic at approximately 76.5 min. Retention times varied slightly (±1.5 min) per fatty acid among the samples used for the

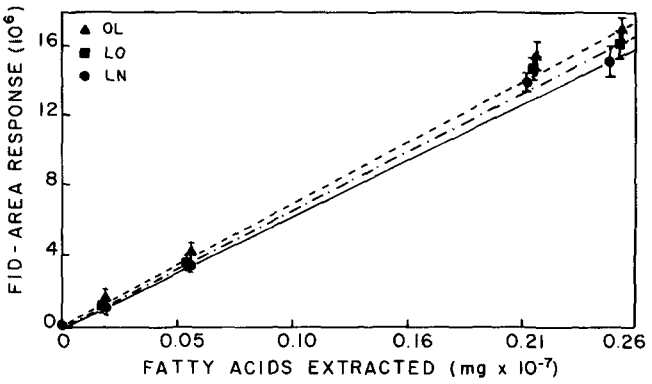

FIG. 2. Standard curves for SFE/SFC analysis of oleic, linoleic and linolenic acid. Y-axis = FID peak area (μvolts-sec) ($\times 10^6$). X-axis = mass, grams of fatty acid ($\times 10^{-7}$). Sample volume = 1 μL. Oleic ($R^2 = 0.992$); linoleic ($R^2 = 0.990$); linolenic ($R^2 = 0.989$). Error bars = standard deviation about the mean. Oleic is represented by a dotted line and triangles; linoleic by a dashed line and diamonds; linolenic by a solid line and circles.

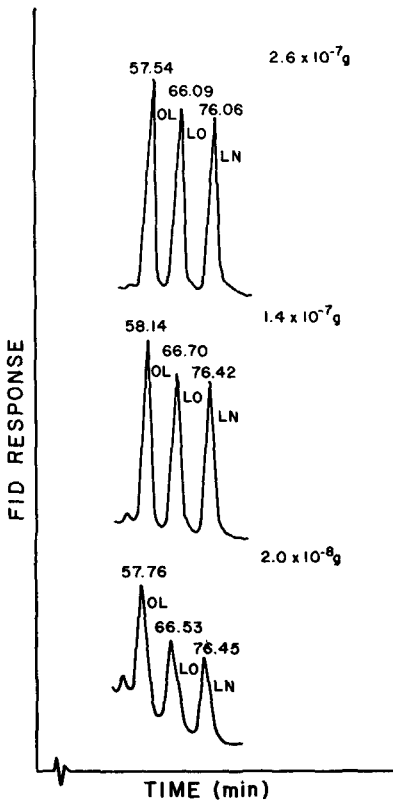

FIG. 3. SFE/SFC of 1 μL of fatty acid standards at 3 concentrations; 2×10^{-5} g/mL, 1.4×10^{-4} g/mL and 2.6×10^{-4} g/mL. OL = oleic, LO = linoleic and LN = linolenic acid. Extraction: 500 atm at 80°C for 15.0 min. Chromatography: two (20 m × 100 μm) SB-cyanopropyl-50 columns; mobile phase pressure program 140 atm to 170 atm at 100°C.

standard curve. Resolution was excellent throughout the entire concentration range. At the smallest concentration (2.0×10^{-5} g/mL) of fatty acid standard mixture analyzed, there was some downward baseline drift during the separation, which resulted in a systematic increase for the

TABLE 1

Percent Relative Error (%RE) for the SFE/SFC Analysis of Oleic, Linoleic and Linolenic Acid in Both Standards and Samples

Sample (g/mL)	Percent relative error, (n) = number of repetitions		
	Oleic	Linoleic	Linolenic
Std. solutions[a]			
2.0×10^{-5}	5.0% (3)	4.1% (3)	3.6% (3)
6.0×10^{-5}	0.9% (3)	0.8% (3)	1.6% (3)
2.2×10^{-4}	5.2% (4)	4.5% (4)	3.5% (4)
2.6×10^{-4}	4.3% (4)	5.0% (4)	6.0% (4)
TG oleic acid[b]			
1.6×10^{-4}	5.3% (3)	ND[c]	ND
2.4×10^{-4}	1.5% (3)	ND	ND
TG linoleic			
2.0×10^{-4}	7.7% (3)	2.3% (3)	4.3% (2)
3.0×10^{-4}	4.7% (3)	4.9% (3)	4.3% (2)
TG linolenic			
3.0×10^{-4}	4.7% (3)	4.4% (3)	1.7% (3)
4.0×10^{-4}	4.9% (3)	2.9% (3)	2.6% (3)
Whole wheat flour (0.3 mg)	9.2% (3)	10.0% (3)	ND

[a]Each fatty acid (oleic, linoleic and linolenic acid) was prepared in the same series of concentrations (2.0-26.0 × 10^{-5} g/mL).

[b]TG refers to technical grade.

[c]ND, not determined.

integrated peak area of oleic acid. Baseline drift only occurred at the smallest concentration. Reproducibility was good at most sample concentrations (Table 1). Following the first extraction, a second extraction of the standard solutions was performed to determine the extraction efficiency for each fatty acid. Extraction efficiencies were determined by dividing the peak area of each fatty acid from the first extraction by the sum of the peak areas from both the first and second extraction and multiplying the ratio by 100. Extraction efficiency was greater than 99% for the initial extraction of each fatty acid at each concentration.

Individual fatty acid composition of the technical-grade fatty acids is reported in Table 2. The free oleic, linoleic and linolenic fatty acid composition of technical-grade fatty acid and flour samples was determined from the standard curves for each fatty acid. The separation of technical-grade oleic acid (TG OL), technical-grade linoleic acid (TG LO) and technical-grade linolenic acid (TG LN) is presented in Figure 4. The oleic acid content of technical-grade oleic acid sample was approximately 64-67.5%. Linoleic and linolenic fatty acids were not present in sufficient quantity to accurately determine their concentrations. The linoleic acid content of the technical-grade linoleic acid sample was approximately 57-58%. A significant amount of oleic (*ca.* 27.5%) was found in the technical-grade linoleic acid sample. Linolenic acid was clearly present in the linoleic acid samples (*ca.* 5%), but could not be accurately determined because the concentration was below that of the smallest concentration on the standard curve. Technical-grade linolenic acid contained approximately 47% of the reported concentration. Both oleic and linoleic acids were found at approximately 15% in the technical-grade linolenic acid sample. The linolenic acid content determined by SFE/SFC was calculated as percent 9,12,15,-octadecatrienoic acid. However, the actual

TABLE 2

Fatty Acid Composition of Technical-Grade (TG) Fatty Acid Sources As Determined by SFE and Capillary SFC

Sample (g/mL)	Average ± std. dev. (mg fatty acid/g sample)		
	Oleic	Linoleic	Linolenic
TG oleic acid			
1.6×10^{-4}	639.8 ± 34.2	ND[a]	ND
2.4×10^{-4}	674.5 ± 10.4	ND	ND
TG linoleic			
2.0×10^{-4}	275.0 ± 21.2	580.4 ± 13.6	51.6 ± 1.8
3.0×10^{-4}	278.2 ± 13.1	573.1 ± 28.2	55.1 ± 2.4
TG linolenic			
3.0×10^{-4}	137.8 ± 6.5	153.9 ± 6.7	462.3 ± 7.8
4.0×10^{-4}	198.7 ± 9.7	141.3 ± 4.9	440.2 ± 11.3

[a]ND, not determined.

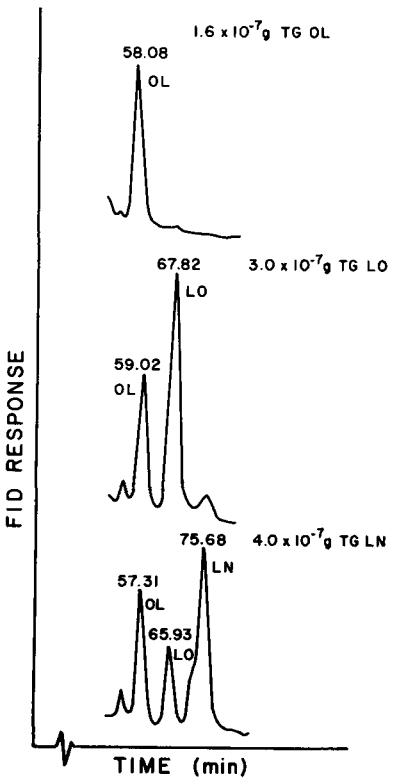

FIG. 4. SFE/SFC of 1 μL of technical-grade oleic acid (TG OL), TG LO and TG LN at 1.6×10^{-4} g/mL, 3.0×10^{-4} g/mL and 4.0×10^{-4} g/mL, respectively. OL = oleic, LO = linoleic and LN = linolenic acid. Extraction: 500 atm at 80°C for 15.0 min. Chromatography: two (20 m × 100 μm) SB-cyanopropyl-50 columns; mobile phase pressure program 140 atm to 170 atm at 100°C.

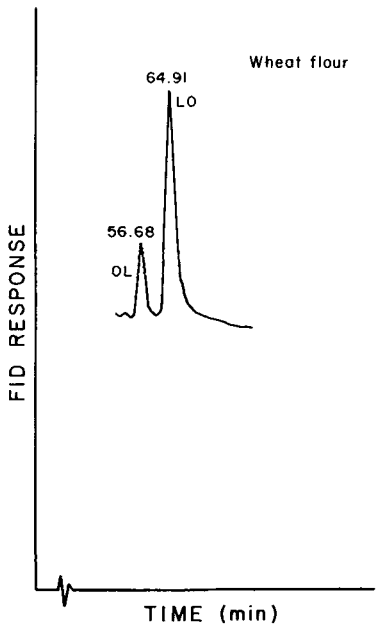

FIG. 5. SFE/SFC of wheat flour sample (0.30 mg sample). OL = oleic and LO = linoleic. Extraction: 500 atm at 80°C for 15.0 min. Chromatography: two (20 m × 100 μm) SB-cyanopropyl-50 columns in series; mobile phase pressure program 140 atm to 170 atm at 100°C.

percentage of this linolenic acid isomer may be even lower than the calculated value. The shoulder on the linolenic acid peak was integrated with the main peak as 9,12,15-octadecatrienoic acid, but it may actually represent the γ-linolenic (6,9,12-octadecatrienoic) acid isomer. The extraction efficiency for oleic, linoleic and linolenic acid in the technical-grade fatty acids was greater than 99%.

The SFE/SFC chromatogram of freeze-dried whole wheat is shown in Figure 5. The free oleic content for the wheat flour samples was 163.6 ± 12.3 μg/g, while the free linoleic acid content was 605.0 ± 49.5 μg/g. Free linolenic fatty acid was not present in sufficient amount to be quantitated accurately. The reproducibility (percent relative error (%RE), Table 1) achieved from the SFE/SFC analysis of the wheat flour samples was ≤10.0%. Additional work is needed to improve the %RE of the wheat flour samples to an acceptable value. Extraction efficiency was greater than 99% for the flour samples. The low %RE observed for both the free oleic and linoleic fatty acid concentrations of the wheat flour sample and the sensitivity achieved in this sample demonstrates the potential of SFE/capillary SFC for the analysis of free fatty acid and related compounds.

The results indicate that the new extraction cell can reproducibly maintain supercritical conditions after numerous extractions for quantitative analysis of liquid and solid samples. The cell is very resistant to leaking and is easy to use. Most of the cells developed for SFE/GC have overlooked the utility of the double ferrule for the cell body connection. Frequent leaking of these commercial cells seemed to be caused by the repeated stress of tightening and untightening the cell body connection. The cell reported here should be easily adaptable for SFE/GC with the attachment of a polyimide ferrule in the exit nut (M in Fig. 1) to allow the insertion of a capillary restrictor.

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REFERENCES

1. Engelhardt, H., and A. Gross, *J. High Res. Chrom. Chrom. Com. 11*:726 (1988).
2. Hawthorne, S.B., and D.J. Miller, *J. Chrom. Sci. 24*:258 (1986).
3. Hawthorne, S.B., and D.J. Miller, *J. Chrom. 403*:63 (1987).
4. Hawthorne, S.B., and D.J. Miller, *Anal. Chem. 59*:1705 (1987).
5. Hawthorne, S.B., D.J. Miller, M.S. Krieger and Z. Fresenius, *Anal. Chem. 330*:211 (1988).
6. Raynor, M.W., I.L. Davies, K.D. Bartle, A.A. Clifford, A. Williams, J.M. Chalmers and B.W. Cook, *J. High Res. Chrom. Chrom. Com. 11*:766 (1988).
7. Gmuer, W., J.O. Bosset and E. Plattner, *J. Chrom. 388*:335 (1987).
8. Flament, I., C. Chevallier and U. Keller, in *Flavour Science and Technology*, edited by M. Martens, G.A. Dalen and H. Russwurm, Jr., John Wiley & Sons, Ltd., London, England, 1987, p. 151.
9. Favati, F., J.W. King, J.P. Friedrich and K. Eskins, *J. Food Sci. 53*:1532 (1988).
10. Hawthorne, S.B., M.S. Krieger and D.J. Miller, *Anal. Chem. 60*:472 (1988).
11. Hawthorne, S.B., D.J. Miller and M.S. Krieger, *J. Chrom. Sci. 27*:347 (1989).
12. Schmitz, H.H., W.E. Artz, C.L. Poor, J.M. Dietz and J.W. Erdman, Jr., *J. Chrom. 479*:261 (1989).
13. Lee, M.L., and K.E. Markides, in *Analytical Supercritical Fluid Chromatography and Extraction*, Chromatography Conferences, Inc., Provo, UT, 1990, p. 141.
14. Onuska, F.I., and K.A. Terry, *J. High Res. Chrom. Chrom. Com. 12*:357 (1988).
15. Dean, R.B., and W.J. Dixon, *Anal. Chem. 23*:636 (1951).

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