Analytical Supercritical Fluid Extraction with Lipase Catalysis: Conversion of Different Lipids to Methyl Esters and Effect of Moisture

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ABSTRACT: The fat content of lipid-containing samples has been determined by extraction of the fat with supercritical carbon dioxide, followed by enzyme-catalyzed methylation of the fat under supercritical conditions, prior to gas chromatography (GC) analysis. This study was initiated to determine the effect of moisture content on the extraction and conversion of lipids in oilseed and meat samples to their fatty acid methyl ester (FAME) derivatives. These samples were freeze-dried or mixed with Hydromatrix and compared with untreated control samples by employing the above-described supercritical fluid extraction-reaction sequence. Particular attention was focused on minor constituents, such as phospholipids and cholesteryl esters, to see if they could be extracted and derivatized by the above technique. Recoveries and reaction conversions of the lipid species were determined with the aid of GC, high-performance liquid chromatography, and supercritical fluid chromatography for analyses of the extracted lipids. Total fat values were higher from the freeze-dried meat and oilseed samples than from samples mixed with Hydromatrix or left untreated. Extraction of cholesteryl esters was better than 90%, and conversion of the cholesteryl esters to FAME was 93% or higher. Extraction of phosphatidic acid was only 88% compared to more than 90% recoveries for the other phospholipid species. FAME conversion was better than 96% for all phospholipid samples in the study. *JAOCS 74*, 585–588 (1997).

KEY WORDS: Cholesteryl esters, fatty acid methyl esters, lipase, phospholipids, reaction, supercritical fluid extraction.

The use of lipase-catalyzed reactions of lipids under supercritical fluid conditions (1,2) has considerable promise in the analysis of food products and biological samples. Such techniques with supercritical carbon dioxide (SC-CO_2) are important options for minimizing the expanded use of solvent in the food analysis laboratory, thereby ensuring compliance with new environmental regulations (3) that are designed to eliminate the use of flammable and carcinogenic solvents. Jackson and King (4) have shown that fatty acid methyl esters (FAME) may be synthesized by using sequential supercritical fluid extraction and enzyme reaction (SFE/SFR) of soybean flakes. This lipase-catalyzed SFE/SFR method was further modified for the purpose of analyzing specifically the total nutritional fat content of meat samples (5). Good agreement was obtained for the nutritional fat content of more than nine different meat samples between the SFE/SFR procedure and an established procedure employing organic solvent extraction (6).

Nutritional fat as established by the Nutritional Labeling and Education Act (NLEA) is currently defined as the sum of fatty acids from major lipid constituents, such as mono-, di-, and triglycerides, as well as minor lipid species, phospholipids and sterols, expressed as triglycerides (7). Unfortunately, the presence of moisture in foods can have an adverse effect on the quantitative extraction of fat from foods by SFE (8,9). Moisture can also affect the efficiency of enzymatic reactions in SC-CO_2 (10), leading to incomplete ester conversion and promotion of hydrolysis. In this study, we have determined the effect of moisture on the extraction of lipids and the lipase-catalyzed reaction as employed in the described SFE/SFR technique. In addition, we determined if the other lipid components, besides glycerides, which are commonly found in food samples could be successfully derivatized to FAME by the enzyme-catalyzed SFE/SFR method. Such conversions are critical under the NLEA protocol to ensure full accountability of the lipid content of foodstuffs and biological tissues.

MATERIALS AND METHODS

Phospholipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and cholesteryl ester standards from Nu-Chek-Prep, Inc. (Elysian, MN). Meat samples were prepared by the University of Illinois Department of Animal Science as previously described (5,11); oilseed samples were obtained from commercial markets. Novozym 435 enzyme (*Candida antarctica*), adsorbed on a polyacrylamide resin, was purchased from Novo Nordisk (Franklinton, NC); methanol, chloroform, and ammonium hydroxide were from Fisher Scientific (Pittsburgh, PA). Triundecanoin and the FAME standards were obtained from Nu-Chek-Prep, Inc. SFE-grade CO_2 was obtained from Air Products (Allentown, PA).

Canola, soybean, sunflower, and wheat germ were the oilseeds used in this study. The approximate moisture content of each sam-

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ple was: canola, 15%; soybean, 11%; sunflower, 15%, and wheat germ, 8%. The moisture content of the meat samples was: ground beef, 54%; sausage, 54%; and bacon, 42%. The samples were treated in three different ways prior to SFE/SFR: (i) as control samples with no pre-treatment; (ii) as samples mixed with 500 mg Hydromatrix (Chen-Tube Hydrometrix; Analytichem International, Harbor City, CA) per 500-mg sample; and (iii) as samples lyophilized for 30 min in an FTS Systems Model FD-1-54A lyophilizer (Stone Ridge, NY) to approximate moisture contents of 3.5% for canola; 2.8%, soybean; 2.9%, sunflowers; 6.3%, wheat germ; 19%, beef; 25%, sausage; and 11%, bacon.

The sequential SFE and methanolysis of the extracted lipids were accomplished with a Hewlett-Packard Model 7680T SFE unit (Hewlett-Packard, Wilmington, DE), interfaced with a Hewlett-Packard 5890 II gas chromatograph, and a Hewlett-Packard 'bridge' software program. Meat and oilseed samples (500 mg) with 1.25 mg undecanoin as an internal standard were placed into a 7-mL extraction cell, partitioned upstream from the 2 g of Novozym 435 by a glass wool plug. When the cholesteryl ester or phospholipid standards were extracted, reacted, and analyzed, only 25-mg samples were used. These standard compounds were dissolved in $CHCl_3$ prior to SFE and then added to glass wool to permit more efficient extraction *via* SFE. Chloroform was then allowed to evaporate before inserting the sample-laden vessel in the extractor. SFE/SFR conditions were 2500 psi and 50°C for 80 min at a CO_2 flow rate of 1 mL/min as provided by the liquid pump. Methanol was metered into the SC-CO_2 prior to the sample cell at a flow rate of 5 µL/min. The synthesized FAME were collected in a 1.8-mL GC injection vial after SFE/SFR as described previously (5). The robotic arm of the GC then transferred the vial with the derivatized extract to the GC.

The SFE-extracted matrix was also emptied from the extraction cell and extracted for residual lipid content by the Bligh and Dyer solvent extraction procedure (12). These extracted lipids were then reacted with BF_3/CH_3OH to form FAME (6) for an off-line GC analysis.

Analysis of the total fat content from the resulting FAME distribution was accomplished in a Hewlett-Packard 5890 Series II GC with a Supelco SP-2340 column (60 m × 0.25 mm × 0.2 µm film thickness) (Supelco, Inc., Bellefonte, PA). The injector temperature and flame-ionization detector (FID) temperature were 235 and 250°C, respectively. The GC oven temperature was held at 100°C for 5 min and then programmed to 200°C at 3°C/min. Helium was used as the carrier gas at a flow rate of 1 mL/min. Column head pressure was held constant at 20 psi.

The completeness of methanolysis reaction of the meat samples, oilseed samples, and cholesteryl ester standards was determined with a Lee Scientific Series 600 SFC (Dionex, Inc., Salt Lake City, UT) and a Dionex SB-Octyl-50 capillary column (10 m × 100 µm × 0.5 µm film thickness). The pressure gradient program was as follows: 120 atm for 5 min, then ramped to 300 atm at 8 atm/min. The corresponding simultaneous temperature program consisted of the following: The column temperature was initially held at 100°C for 5 min, then programmed to 190°C at 8°C/min. A time/split automatic injection of the samples was accomplished through a Valco valve (Valco, Inc., Houston, TX) for 1.8 s with a 200-nL loop. Detection was accomplished by a FID at 350°C.

The polar phospholipid samples were analyzed by high-performance liquid chromatography (HPLC) with a Spectra Physics Model SP8800 liquid chromatograph (San Jose, CA) interfaced with a Varex evaporative light-scattering detector (ELSD) Model Mark III (Alltech, Deerfield, IL). The column was a LiChrospher Si-60 column (5 µm, 250 mm × 4.6 mm) (Alltech), held isothermally at 30°C. A linear-gradient elution profile was used from 40% of solvent A, consisting of $CHCl_3/(CH_3OH/H_2O/NH_4OH$, (60:34:5.5:0.5, by vol), plus 60% of solvent B consisting of $CHCl_3/CH_3OH/NH_4OH$ (80:19.5:0.5, by vol), to 100% solvent B over a 12-min run (13). The column flow rate was 1 mL/min.

All extractions and analyses were performed in triplicate. Statistical analysis of data was accomplished with SAS/STAT software (14).

RESULTS AND DISCUSSION

In previous work, we have shown the enzyme-catalyzed SFE/SFR method to be comparable to a hydrolysis solvent extraction method (6) for the analysis of total fat content for nine meat samples (5). This is reconfirmed for most of the extracted samples by comparing the values obtained from a Soxhlet method with hexane with those from the SFE/SFR method for total fat (Table 1). For most samples, the SFE/SFR values are slightly higher than the Soxhlet values; however, there is a significant difference between methods for canola and wheat germ values.

The effect of eliminating moisture *via* lyophilization is apparent upon analyzing the total fat content of all four oilseeds by the SFE/SFR method (Fig. 1). All fat contents are greater for the individual oilseeds after freeze-drying compared to the samples mixed with Hydromatrix and the samples with no pretreatment. Wheat germ, with 8% moisture in the original sample and 6.3% after drying, showed the smallest effect on fat determination by SFE/SFR after lyophilization. The addition of Hydromatrix aids in extraction of the lipids; however, conversion of the triglycerides to FAME, as determined by SFC, is not complete and lowers the fat content calculated from the FAME. Ap-

TABLE 1
Comparison of Soxhlet and SFE/SFR

	Soxhlet[a]	SFE/SFR[b]
Canola	39.1% (2.9)[c]	45.5% (1.1)
Soybean	22.1% (1.2)	22.6% (2.2)
Sunflower	35.4% (3.5)	34.7% (2.2)
Wheat germ	9.3% (2.7)	12.9% (3.4)
Bacon	40.9% (1.5)	41.4% (2.8)
Beef	28.6% (3.1)	29.7% (3.4)
Sausage	20.4% (2.3)	21.9% (1.7)

[a]Soxhlet extraction with hexane.
[b]Lipase-catalyzed supercritical fluid extraction/supercritical fluid enzyme reaction (SFE/SFR).
[c]RSD, relative standard deviation, $n = 3$.

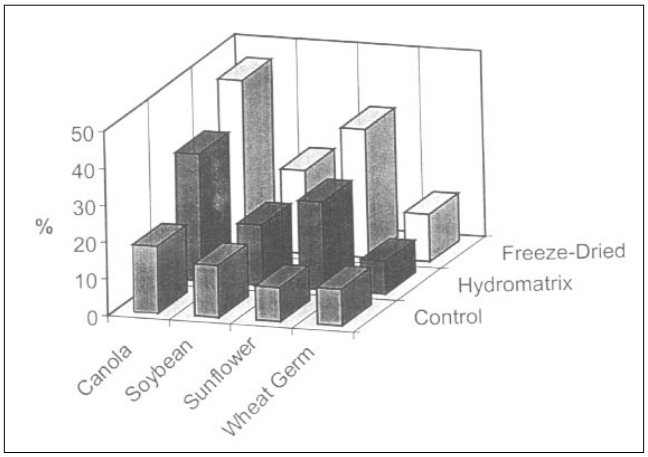

FIG. 1. Supercritical fluid extraction/supercritical fluid enzyme reaction of four oilseed samples. Effect of moisture content on total fat content.

parently, the presence of Hydromatrix in the extraction cell does not moderate the effect of water on the reaction conversion. Moisture affects both the total extraction and conversion to FAME, as shown by analysis of the control samples without drying. The effect of moisture on analysis of the meat samples (Fig. 2) follows the same trends as the total fat determination with the oilseeds samples (Fig. 1).

GC analysis indicates that the moisture content in the samples had little effect on the determined fatty acid composition of each oilseed; analysis of variance was run for each FAME from each oilseed. Different letters in each row represent differences within the treatment methods at $P < 0.05$ by *t*-tests of least squares' means (Table 2).

There are significant differences in the area percentage data of $C_{16:0}$, $C_{18:1}$, and $C_{18:2}$ FAME from the canola extractions and $C_{16:0}$ and $C_{18:0}$ FAME from soybean extractions. The area percentage values of the four FAME from sunflower extractions also tend to be different. However, the differences found in the three oilseeds are random and do not appear to be related to the method of treatment. Therefore, the effect of water in the sample appears to be one of inhibiting quantitative extraction/conversion of the lipid moieties, not their qualitative conversion.

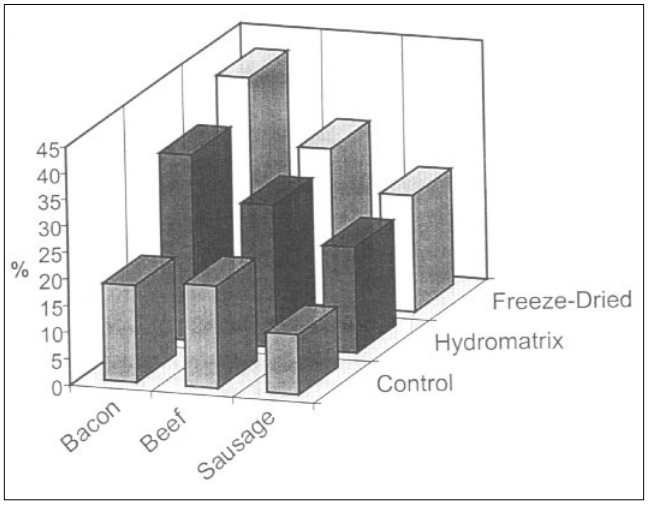

FIG. 2. Supercritical fluid extraction/supercritical fluid enzyme reaction of three meat samples. Effect of moisture content on total fat content.

TABLE 2
Fatty Acid Composition of Seed Oils—Effect of Moisture

	FAME (%)		
	Freeze-dried[a]	Hydromatrix[b]	Control
Canola			
$C_{16:0}$	5.86a	5.70a	4.65b
$C_{18:0}$	1.84a	1.94a	2.03a
$C_{18:1}$	61.71a	58.19b	59.54c
$C_{18:2}$	21.61a	25.25b	24.66b
$C_{18:3}$	8.98a	8.92a	9.12a
Soybean			
$C_{16:0}$	12.72a	12.51b	12.14c
$C_{18:0}$	3.69a	4.36b	4.43b
$C_{18:1}$	20.68a	20.76a	20.57a
$C_{18:2}$	55.32a	55.02a	54.58a
$C_{18:3}$	7.59a	7.35a	7.28a
Sunflower			
$C_{16:0}$	5.83a	6.39b	6.40b
$C_{18:0}$	5.11a	5.36a	6.63b
$C_{18:1}$	21.83a	21.38b	20.57c
$C_{18:2}$	67.23a	66.87a	66.40b
Wheat germ			
$C_{16:0}$	18.69a	18.90a	18.68a
$C_{18:0}$	0.74a	0.73a	0.73a
$C_{18:1}$	15.05a	14.91a	15.04a
$C_{18:2}$	58.45a	58.30a	58.46a
$C_{18:3}$	7.17a	7.17a	7.09a

[a]Samples were lyophilized before SFE/SFR.
[b]500 mg Hydromatrix was added to samples before SFE/SFR.
[c]Values with different letters in each row are significantly different at $P > 0.05$ level. FAME, fatty acid methyl esters. See Table 1 for other abbreviation.

To further evaluate if the method was a valid technique, the three meat and four oilseed samples were extracted and analyzed by the SFE/SFR method. The remaining cell contents were then extracted with chloroform/methanol (12), and the FAME were prepared and analyzed, allowing calculation of the total fat content as shown in Table 3. Solvent extraction showed that the residual total fat was less than 1% for all samples. This indicates that the SFE/SFR scheme was a fairly exhaustive extraction.

TABLE 3
Results for the Determination of Extent of Total Extraction and Reaction

	SFE/SFR[a]	Solvent extraction[b]
Canola	45.5% (1.3)[c]	0.5% (2.2)
Soybean flakes	21.6% (2.1)	0.9% (1.9)
Sunflower	34.7% (2.0)	0.9% (0.0)
Wheat germ	12.9% (2.9)	0.3% (3.6)
Bacon	41.4% (2.8)	0.9% (2.5)
Beef	29.7% (2.4)	0.5% (0.0)
Sausage	21.9% (1.7)	0.2% (3.5)

[a]Determined from the FAME analysis after SFE/SFR.
[b]Determined from the second extraction (Bligh/Dyer; Ref. 12) of the remaining sample and FAME analysis from BF_3/CH_3OH.
[c](RSD), relative standard deviation, $n = 3$. See Tables 1 and 2 for abbreviations.

TABLE 4
Results for the SFE/SFR of Cholesteryl Esters (CE) to FAME

Cholesteryl ester	SFC analysis (%)			Recovery[a] (%)	Conversion[b] (%)
	CE[c]	Cholesterol	FAME		
$C_{16:0}$	0.1	46.2	43.7	96	99
$C_{17:0}$	3.3	40.7	46.0	99	97
$C_{18:0}$	2.1	44.0	43.9	96	98
$C_{20:0}$	2.9	40.6	47.5	95	97

[a]Determined from remaining weight after extraction and SFC analysis.
[b]Determined from SFC analysis.
[c]CE remaining after SFE/SFR. See Tables 1 and 2 for other abbreviations.

In addition, cholesteryl esters were successfully extracted and converted *via* SFE/SFR, as indicated in Table 4. Few cholesteryl esters were left after SFE/SFR, as indicated by SFC analysis. Conversion to approximately equal amounts of cholesterol and FAME was better than 97% for all cholesteryl ester samples.

SFE/SFR results on the neat phospholipid samples are given in Table 5. Extraction recovery ranged from 99% for phosphatidylcholine to 88% of phosphatidic acid with the SFE stage. Conversion to methyl esters was better than 96% for all phospholipid standards.

In conclusion, moisture in the oilseed and meat samples affects the extraction of lipids and their subsequent conversion to FAME. Total fat content is highest when the samples are lyophilized, and lowest with most untreated samples. Further, total fat content of the freeze-dried samples is comparable to values derived from Soxhlet-extracted samples. The fatty acid composition of the derived extracts appears not to be affected by the moisture content in the oilseed and meat samples.

The successful extraction and conversion of cholesteryl esters to FAME indicate that the SFE/SFR method does not discriminate against lipid moieties in the total fat analysis of the meat samples. Extraction and conversion of phospholipids to FAME by the SFE/SFR method also indicated that the phospholipids in oilseeds and meat samples can be derivatized sufficiently to be included in the total fat assay.

An additional investigation was also conducted by using a Bligh/Dyer extraction method (12) after SFE/SFR to determine residual lipid content in the extracted material. Only small amounts of lipid materials were found in the residual material, indicating that the described enzyme-catalyzed SFE/SFR method can be used as a rapid, automated method to analyze for total fat content in foods.

Table 5
Results for the SFE/SFR of Phospholipids

Sample	Extraction[a] (%)	Conversion[b] (%)
Phosphatidylcholine	99	99
Phosphatidylethanolamine	92	96
Phosphatidylinositol	96	97
Phosphatidic acid	88	98

[a]Determined from gravimetry and high-performance liquid chromatography (HPLC) analysis.
[b]Determined from HPLC analysis. See Table 1 for other abbreviations.

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REFERENCES

1. Shishikura, A., K. Fujimoto, T. Suzuki, and K. Arai, Lipase-Catalyzed Incorporation of Long-Chain Fatty Acids into Medium-Chain Triglycerides Assisted by Supercritical Carbon Dioxide Extraction, *J. Am. Oil Chem. Soc. 71*:961–967 (1994).
2. Pasta, P., G. Mazzola, G. Carrel, and G. Rica, Subtilisin-Catalyzed Transesterification in Supercritical Carbon Dioxide, *Biotechnol. Lett. 2*:643–648 (1989).
3. *Federal Register,* U.S. Government Printing Office, Washington, D.C., Vol. 58, 1993, p. 41983.
4. Jackson, M.A., and J.W. King, Methanolysis of Seed Oils in Flowing Supercritical Carbon Dioxide, *J. Am. Oil Chem. Soc. 73*:353–356 (1996).
5. Snyder, J.M., J.W. King, and M.A. Jackson, Fat Content for Nutritional Labeling by Supercritical Fluid Extraction and an On-Line Lipase Catalyzed Reaction *J. Chromatogr. A. 750*:201–207 (1996).
6. House, S.D., P.A. Larson, R.R. Johnson, J.W. DeVries, and D.L. Martin, Gas Chromatographic Determination of Total Fat Extracted from Food Samples Using Hydrolysis in the Presence of Antioxidant, *J. Assoc. Off. Anal. Chem. 77*:960–965 (1994).
7. *Federal Register,* U.S. Government Printing Office, Washington, D.C., Vol. 58, 1993, pp. 631–2964.
8. King, J.W., J.J. Johnson, and J.P Friedrich, Extraction of Fat Tissue from Meat Products with Supercritical Carbon Dioxide *J. Agric. Food Chem. 36*:951–954 (1989).
9. Hopper, M.L., J.W. King, J.J. Johnson, A.A Serino, and R.J. Butler, Multivessel Supercritical Fluid Extraction of Food Items in Total Diet Study *J. Assoc. Off. Anal. Chem. 78*:1072–1079 (1995).
10. Marty, A., W. Chulalanksananukul, R.M. Willemot, and J.S. Condoret, Kinetics of Lipase-Catalyzed Esterification in Supercritical CO_2, *Biotech. Bioeng. 39*:273–280 (1992).
11. King, J.W., F.J. Eller, J.M. Snyder, J.H. Johnson, F.K. McKeith, and C.R. Stites, Extraction of Fat from Ground Beef for Nutrient Analysis Using Analytical Supercritical Fluid Extraction, *J. Agric. Food Chem. 44*:2700–2704 (1996).
12. Bligh, E.G., and W.J. Dyer, A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol. 37*:911–917 (1959).
13. Becart, J., C. Chevalier, and J.P. Biesse, Quantitative Analysis of Phospholipids by HPLC with a Light-Scattering Evaporating Detector—Application to Raw Materials for Cosmetic Use, *J. High Resolut. Chromatogr. 13*:126–130 (1990).
14. SAS Institute, Inc., *SAS/STAT User's Guide*, Version 6, 6th edn., Vol. 1, Cary, NC, 1989.

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