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Total milk fat extraction and quantification of polar and neutral lipids of cow, goat, and ewe milk by using a pressurized liquid system and chromatographic techniques

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ABSTRACT

Although milk polar lipids such as phospholipids and sphingolipids located in the milk fat globule membrane constitute 0.1 to 1% of the total milk fat, those lipid fractions are gaining increasing interest because of their potential beneficial effects on human health and technological properties. In this context, the accurate quantification of the milk polar lipids is crucial for comparison of different milk species, products, or dairy treatments. Although the official International Organization for Standardization-International Dairy Federation method for milk lipid extraction gives satisfactory results for neutral lipids, it has important disadvantages in terms of polar lipid losses. Other methods using mixtures of solvents such as chloroform:methanol are highly efficient for extracting polar lipids but are also associated with low sample throughput, long time, and large solvent consumption. As an alternative, we have optimized the milk fat extraction yield by using a pressurized liquid extraction (PLE) method at different temperatures and times in comparison with those traditional lipid extraction procedures using 2:1 chloroform:methanol as a mixture of solvents. Comparison of classical extraction methods with the developed PLE procedure were carried out using raw whole milk from different species (cows, ewes, and goats) and considering fat yield, fatty acid methyl ester composition, triacylglyceride species, cholesterol content, and lipid class compositions, with special attention to polar lipids such as phospholipids and sphingolipids. The developed PLE procedure was validated for milk fat extraction and the results show that this method performs a complete or close to complete extraction of all lipid classes and in less time than the official and Folch methods. In conclusion, the PLE method optimized in this study could be an alternative to carry out milk fat extraction as a routine method.

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INTRODUCTION

Milk lipid analysis is an important area of research and the field has experienced a new renaissance in the last decades. Although some concern exists about the high amount of saturated fat present in whole milk, the latest advances indicate the presence of bioactive FA, such as short-chain FA and CLA, and other minor components, such as polar lipids (phospholipids and sphingolipids), which may have favorable effects on human blood lipids and other cardiometabolic risk factors (Hilmarsson et al., 2006; Heinze and Actis, 2012; Küllenberg et al., 2012). Polar lipids in milk are the main constituents of the milk fat globule membrane, mainly constituted of phosphatidylcholine (**PC**), phosphatidylethanolamine (**PE**), phosphatidylinositol (**PI**), phosphatidylserine (**PS**) and sphingomyelin (**SM**; Singh, 2006). The interest in these molecules is high due to the potential positive effects on human health of dietary phospholipids (Küllenberg et al., 2012).

For an analysis of the total milk lipid composition, it is necessary to select the appropriate method of lipid extraction for preventing either the loss of some of these components or their chemical changes. The standard milk fat extraction methods, such as the Röse-Gottlieb (ISO, 2001), using a mixture of diethyl ether and *n*-pentane, as well as the method based on extraction with a mixture of hexane:isopropanol proposed by Hara and Radin (1978), give satisfactory results for neutral lipid extraction but they present important disadvantages due to losses of some phospholipids and sphingolipids (Feng et al., 2004; Avalli and Contarini, 2005). In addition, they are often performed manually, involving exhaustive and time-consuming steps and hazardous solvents at the large amounts required to remove the fat from the sample matrix. Moreover, these methods either are incompatible with the extraction of lipids with a wide range of hydrophobicity as phospholipids

or result in lower recoveries (Avalli and Contarini, 2005; Gallier et al., 2010).

One of the most commonly used methods for extracting and purifying lipids is the Folch procedure (Folch et al., 1957). Even though this method is highly efficient for extracting polar lipids, it is also associated with low sample throughput, long time, and large solvent consumption.

All these classical extraction schemes for fat extraction have meanwhile been outperformed by pressurized liquid extraction (**PLE**). Pressurized liquid extraction has developed into the most powerful extraction approach in routine analysis of lipids/FA in biological matrices as well as foods (Schäfer, 1998; Herrero et al., 2005; Señoráns and Luna, 2012). By means of a proper combination of temperature, pressure, time, and number of cycles of extraction, a reduction both in solvent consumption and in the extraction time per sample could be achieved, using the same mixture of solvents as in the traditional methods and offering as an additional advantage the possibility of process automatization (Conte et al., 1997; Macnaughton et al., 1997; Jansen et al., 2006). The aim of this study was to compare the classical extraction methods with a PLE procedure and to validate the procedure for milk fat extraction. Fat yield, FAME composition, triacylglyceride species, cholesterol (**CHOL**) content, and lipid class compositions, with special attention to polar lipids such as phospholipids and sphingolipids, were determined in raw whole cow, ewe, and goat milk.

MATERIALS AND METHODS

Samples

Raw whole milk from 3 different ruminant species (cows, ewes, and goats) was obtained from different farms of Castilla-La Mancha, Spain (10 samples for each species), and analyzed for composition in milk fat and protein by the Interprofessional Dairy Laboratory of Castilla-La Mancha (LILCAM, Castilla-La Mancha, Spain). One hundred milliliters of drawn milk was rapidly frozen and shipped to our laboratory in isothermal containers and then freeze-dried and stored at −35°C until use. A commercial powder skim milk with maximum 1% fat content [Corporación Alimentaria Peñasanta S.A. (CAPSA), Granda-Siero, Asturias, Spain] was used to optimize the lipid extraction conditions by the PLE method.

Reagents

All solvents (dichloromethane, chloroform, hexane, methanol, isooctane, and isopropanol) were HPLC grade and purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland; Labscan brand). Sodium carbonate and sea sand were obtained from Panreac Química S.A. (Barcelona, Spain). Formic acid (98%), trifluoroacetic acid, triethylamine (99.5%), trinonanoin, tritridecanoin, pelargonic acid (C9), tridecanoic acid (C13), myristic acid (C14), palmitic acid (C16), estearic acid (C18), arachidonic acid (20:4), eicosapentaenoic acid (20:5), docosahexapentaenoic acid (22:6), monostearin, diolein, PI, PS, PE, SM, PC, and *N*-oleoylethanolamine were purchased from Sigma (Bellefonte, PA). Reference butterfat BCR-164 and BCR-519 (EU Commission, Brussels, Belgium) were purchased from Fedelco Inc. (Madrid, Spain).

Fat Extraction

First, total milk fat amount was determined in the Interprofessional Dairy Laboratory (LILCAM) by either the Röse-Gottlieb method based on solvent extraction according to the official reference procedure (ISO, 2001) and by using an infrared spectrophotometer (MilkoScan; Foss Electric España S.A., Barcelona, Spain) as fat total content determination method.

Milk fat was extracted in our laboratory from each of the 30 stored freeze-dried milk samples (cow, ewe, and goat milk; n = 10) using the following 2 methods:

1) Folch method according to Iverson et al. (2001), modified as follows: from a well-mixed freeze-dried milk sample, a 2-g aliquot was placed in 50-mL centrifuge tubes with 1 mg of previously added trinonanoin as internal standard. Fifteen milliliters of a dichloromethane-methanol solution (2:1, vol/vol) was then added to each tube. The mixture was shaken mechanically for 30 min and centrifuged at 6,600 × *g* for 5 min at 4°C. As much of the upper organic solvent fraction as possible was carefully removed with a pipette. The sediment was washed with 12 mL of a dichloromethane-methanol solution (2:1, vol/vol) and, after shaking for 1 min, the sample was, again centrifuged at 6,600 × *g* for 5 min at 4°C. The removed organic solvent was combined with that previously collected and 3 mL of a 0.9% solution of sodium chloride was added and mixed mechanically for 1 min before the tubes were stored overnight at 4°C. Afterward, they were again centrifuged at 6,600 × *g* for 5 min at 4°C and the bottom dichloromethane layer was collected and filtered through a Whatman 1-phase separator filter paper (Whatman, Maidstone, UK) containing approximately 3 g of anhydrous sodium sulfate. Finally, the extract

was concentrated by removing dichloromethane in a rotatory evaporator and dried under a gentle stream of nitrogen. The extracted fat was weighted before and after in amber vials flushed with nitrogen and stored at −35°C until chromatographic analysis.

2) PLE method: extractions were carried out with an Accelerated Solid Extraction ASE-200 extractor (Dionex Corp., Sunnyvale, CA) using 2 g of freeze-dried milk sample that was well mixed with 2 g of sea sand and loaded into a stainless steel extraction cell covered with filters on both sides. For the maximum milk fat yield, the extraction included the use of dichloromethane-methanol solution (2:1, vol/vol) as solvent mixture and 10.3 MPa of pressure as fixed conditions. The extraction time assayed was either 1 or 2 static cycles of 5 min each and temperatures of 60, 80, or 100°C, using a commercial powder skim milk (see samples above) for optimization. The combined solvent extracts (approximately 11 mL from each cycle) were gently evaporated in a vacuum rotary evaporator (Strike 202 model; Steroglass S.r.l., Perugia, Italy) and the lipid extract was weighed and stored in amber vials, exposed to a stream of nitrogen, and frozen at −35°C until analysis.

FA Determination and Quantification

Fatty acid methyl esters were prepared by base-catalyzed methanolysis of the extracted FA fraction using 2 *N* KOH in methanol as described by the International Organization for Standardization (**ISO**; ISO, 2002). Fatty acid methyl esters were separated using a CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2-μm film thickness; Agilent Technologies Inc., Palo Alto, CA) in an Agilent chromatograph (model 6890N; Agilent Technologies Inc.) equipped with a mass spectrometry detector. The column was temperature programmed as in Castro-Gómez et al. (2014) at 7°C/min to 170°C, held at 170°C for 55 min, and then temperature programmed at 10°C/min to 230°C and held at 230°C for 33 min. The injector temperature was set at 250°C. Helium was the carrier gas with a column inlet pressure of 206.9 kPa. The mass spectrometry detector conditions were as follows: transfer line temperature: 250°C, source temperature: 230°C, quad temperature: 150°C, electron impact ionization: 70eV, and the range from 50 to 500 m/z was scanned. For identification of the peaks, the National Institute of Standards and Technology (NIST, Gaithersburg, MD) library and mass spectra of the standards used in our laboratory were used. The injection volume was 1 μL and the split ratio used was 1:25. Response factors were calculated using an anhydrous milk fat (reference butterfat BCR-164) and tritridecanoin as internal standard (200 μL; 1.3 mg/mL) was used.

Triacylglycerides and CHOL Determination

Triacylglycerides (**TAG**) and CHOL analysis of milk fat was performed following Fontecha et al. (2005), on a Clarus 400 gas chromatograph (PerkinElmer Ltd., Beaconsfield, UK) equipped with an automatic split/splitless injector and a flame ionization detector. An Rtx-65TAG fused-silica capillary column (30 m × 0.25 mm i.d. × 0.1-μm film thickness; Restek Corp., Bellefonte, PA) was used. Experimental chromatographic conditions were carried out with a temperature program as follows: 120°C held for 30 s, 10°C/min to 220°C and held for 30 s, and 6°C/min to 350°C and held for 30 min. Injector and flame ionization detector temperatures were 355 and 370°C, respectively. Helium was used as carrier gas (172 kPa) and the injection volume was 0.5 μL of dilutions of milk fat (30 mg/mL) in hexane. For TAG and CHOL determination and quantification, the reference butterfat BCR-519 of known TAG and CHOL composition and glyceryl trinanoate as internal standard (100 μL; 1 mg/mL) were used.

Lipid Class Compositions by HPLC-Evaporative Light Scattering Detection

Separation of lipid classes was accomplished in an HPLC system (model 1260; Agilent Technologies Inc.) coupled with an evaporative light scattering detector (SEDEX 85 model; Sedere SAS, Alfortville Cedex, France) using prefiltered compressed air as the nebulizing gas at a pressure of 350 kPa at 60°C; the gain was set at 3. Two columns in series (250 × 4.5 mm Zorbax Rx-SIL column with 5-μm particle diameter; Agilent Technologies Inc.) and a precolumn with the same packing were used. Before analysis, samples were dissolved in CH_2Cl_2 (at 5 to 30 mg/mL) and 50 μL was injected after column equilibration at 40°C. The solvent gradient was as detailed in Rodríguez-Alcalá and Fontecha (2010), with slight modifications shown in Table 1.

Statistical Analysis

All results are expressed as means and standard deviations (n = 10 for each group). An exploratory analysis of data was performed to test normal distribution and homogeneity of variance. Thus, during the optimization of PLE conditions, the results where compared using the Kruskal-Wallis test.

Table 1. Solvent gradient required for the HPLC-evaporative light scattering detector elution of lipid classes and reactivation of the column

Time (min)	Solvent[1] (%)				Flow (mL/min)
	A	B	C	D	
0.00	0	0	100	0	1.4
5.00	0	0	100	0	1.4
5.10	5	0	95	0	0.5
9.50	9.2	0	90.8	0	0.5
13.50	85	0	15	0	0.5
19.49	100	0	0	0	0.5
19.50	100	0	0	0	1.0
25.00	75	25	0	0	1.0
35.00	0	100	0	0	1.0
45.50	0	0	0	100	1.0
46.00	0	0	0	100	1.0
46.50	0	0	100	0	1.0
47.00	0	0	100	0	1.4
53.00	0	0	100	0	1.4

[1]A = chloroform:methanol:water [87.5:12:0.5 (vol/vol/vol); 1 *M* formic acid; triethylamine; pH 3]; B = chloroform:methanol:water [28:60:12 (vol/vol/vol); 1 *M* formic acid; triethylamine; pH 3); C = isooctane:tetrahydrofuran [99:1 (vol/vol)]; D = 2-propanol.

The proposed PLE method was assayed on cow, ewe, and goat milk and results for lipid classes, FAME, TAG species, and CHOL contents were compared versus isolation using the Folch method. For such comparisons, the Mann-Whitney test was carried out.

All analyses were performed using the SPSS Statistics software (v19.0 for Windows; IBM Corp., Armonk, NY). The level of significance was fixed at $P < 0.05$.

RESULTS AND DISCUSSION

Yield of Milk Lipid Extraction

As is well known, the lipid extraction efficiency by PLE is dependent on the following factors: extraction time, extraction temperature, solvent composition, and number of extraction cycles. Extraction pressure usually does not have an important effect of the extraction yield, given it is kept high enough to maintain the solvent in the liquid state during the extraction; therefore, a pressure of 10.3 MPa was selected. In this study, a commercial skim milk powder with less than 1% fat content and the same sample spiked with 200 mg of anhydrous milk fat were used for optimization of the PLE conditions.

To maximize the yield of milk fat extraction, the effects of 2 factors: extraction temperature (at 60, 80, and 100°C) and extraction time (1, 2, or 3 cycles of 5 min each) were studied. The solvent selected was chloroform:methanol (2:1, vol/vol) because of its ability to extract total polar lipids.

The results of the effect of the extraction temperature are summarized in Table 2. The efficiency of milk fat extraction (% yield) from commercial skim milk powder and from the same milk spiked with 200 mg of milk fat was not significantly different among the temperatures tested, with values close to 100% in all cases. However, when the obtained lipid extract was analyzed to identify and quantify the lipid class contents, the total concentration of the polar lipid fraction at 100°C tended to decrease as the effect of temperature, especially due to the PE and SM, decreased. At the same time, a significant increase ($P < 0.05$) in phosphatidic acid concentration was observed and was related to the degradation of polar compounds. Other phospholipids, such as PI, PS, and PC, appeared to increase slightly but not significantly. Also, a light brown color of the lipid extracts was observed when the extraction temperature was 100°C, which was correlated with the progression of the Maillard reaction. The same trend was observed when the PLE method was used at 80°C, although at a lesser extent than at 100°C. Therefore, 60°C was the temperature selected to be used for the rest of the experiments. On the other hand, the extraction time was set at 2 cycles of 5 min each due to the fact that the extract weight was constant after 10 min.

To attain the maximum fat recovery, the PLE procedure was used applying the optimized parameters described above with 30 samples of freeze-dried raw whole milk from cows, ewes, and goats (n = 10 of each). The results were compared with the values obtained with 2 other extraction techniques: namely, the Röse-Gottlieb method [which is the official extraction procedure for milk fat (ISO, 2001)] and the Folch procedure, modified by Iverson et al. (2001). To accurately know how much fat was originally in the milk samples, infrared spectroscopy by MilkoScan analysis was also used as a quantitative fat-detection technique.

Table 2. Effect of the pressurized liquid extraction (PLE) temperature on the efficiency of milk fat extraction (% yield; mean ± SD) from commercial skim milk powder (SMP) and the same sample spiked with 200 mg of anhydrous milk fat (SSMP) and on the lipid class compositions

	Temperature (°C)			
Item[1]	60	80	100	*P*-value
Yield (%)				
SMP	98 ± 0.3	102 ± 0.7	99 ± 0.5	0.11
SSMP	98 ± 0.8	100 ± 0.5	101 ± 1.1	0.06
Lipid class (%)				
CE	0.17 ± 0.01	0.14 ± 0.02	0.14 ± 0.01	0.11
TAG	87.32 ± 0.49	88.59 ± 0.36	88.82 ± 0.81	0.06
DAG	1.24 ± 0.08	1.18 ± 0.01	1.09 ± 0.04	0.06
CHOL + FFA	1.51 ± 0.13	1.37 ± 0.03	1.23 ± 0.09	0.05
MAG	0.20 ± 0.01	0.24 ± 0.03	0.28 ± 0.03	0.05
GLUCER	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.20
LACCER	0.02 ± 0.01	0.03 ± 0.02	0.06 ± 0.02	0.25
PL	9.46 ± 0.64	8.36 ± 0.36	8.30 ± 0.76	0.11
Lipid compound (% of PL)				
PA	1.01 ± 0.21[a]	1.18 ± 0.10[a]	2.20 ± 0.64[b]	<0.05
PE	19.57 ± 2.31	17.88 ± 1.51	13.33 ± 4.82	0.33
PI	8.30 ± 0.53	8.65 ± 0.80	10.32 ± 1.40	0.19
PS	9.40 ± 1.61	8.88 ± 2.33	11.55 ± 0.79	0.11
PC	36.25 ± 0.46	39.73 ± 1.56	39.29 ± 2.61	0.12
SM	25.48 ± 1.68	23.67 ± 0.41	23.31 ± 2.16	0.43

[a,b]Means with different superscript letters within a same row are significantly different ($P < 0.05$).

[1]SMP = skim milk powder; SSMP = SMP spiked with 200 mg of anhydrous milk fat; CE = cholesterol esters; TAG = triacylglycerides; DAG = diacylglycerides; CHOL = cholesterol; MAG = monoglycerides; GLUCER = glucosylceramides; LACCER = lactosylceramides; PL = polar lipids; PA = phosphatidic acid; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylserine; SM = sphingomyelin.

Figure 1 shows that the detected amount of fat in cow milk by the infrared spectroscopy method was 29.5%, which matched exactly the amount of fat extracted by the ISO and PLE methods. For ewe and goat milk, the amount of fat extracted by the PLE method was slightly lower (but not significantly at $P < 0.05$) than that indicated by infrared spectroscopic determination and that obtained with the ISO method. However, the recovery of milk fat when the Folch method was used was significantly lower ($P > 0.05$). The Folch method was the least efficient method of all the tested procedures; this fact was particularly remarkable for cow milk, providing total milk fat yield that was less than half of the other methods (13.87 vs. 29%). These results are in agreement with other studies by Mulbry et al. (2009) and Boselli et al. (2001), who described higher fat extractions using the PLE procedure (50–75% higher) than using the Folch method for algae and egg noodles, respectively.

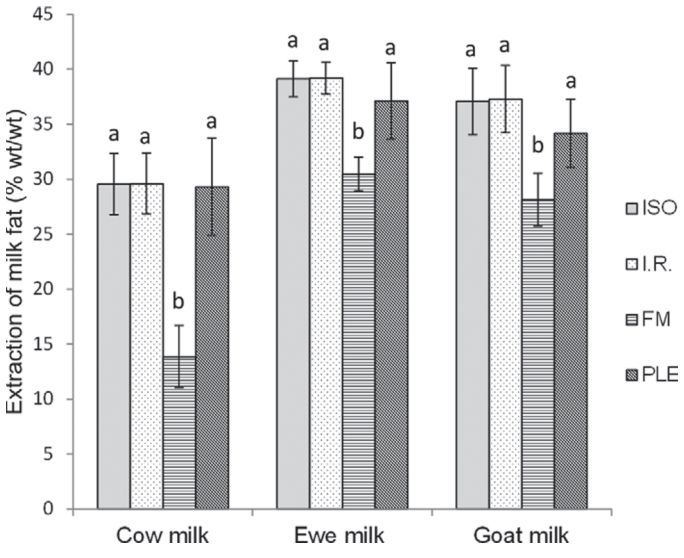

Figure 1. Yield of milk fat extracted (g of fat/100 g of milk powder) using different extraction procedures from cow, ewe, and goat milk powder. ISO = International Organization for Standardization Röse-Gottlieb method, which is the official extraction procedure for milk fat (ISO, 2001); I.R. = infrared spectroscopy method by MilkoScan Foss Electric España S.A., Barcelona, Spain) analysis; FM = Folch method (Folch et al., 1957), modified by Iverson et al. (2001); PLE = pressurized liquid extraction with optimized conditions. Different letters (a and b) show the significant differences between procedures ($P < 0.05$). Error bars represent SD values (n = 10).

Lipid Class Compositions

Comparison of the lipid class compositions of the milk fat from cows, ewes, and goats extracted by the PLE and Folch methods are shown in Table 3. Analyses were done by HPLC-evaporative light scattering detection as in Rodríguez-Alcalá and Fontecha (2010), with

Table 3. Lipid class analysis (mean ± SD) by HPLC-evaporative light scattering detection of the fat from cow, ewe, and goat milk powder extracted with the pressurized liquid extraction (PLE) and Folch (Folch et al., 1957) methods

	Cow milk		Ewe milk		Goat milk	
Lipid class[1] (%)	PLE	FM[2]	PLE	FM	PLE	FM
CE	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
TAG	99.08 ± 0.20	99.04 ± 0.19	99.64 ± 0.18	99.53 ± 0.21	99.62 ± 0.06	99.60 ± 0.04
DAG	0.76 ± 0.21	0.80 ± 0.18	0.25 ± 0.13	0.30 ± 0.30	0.26 ± 0.04	0.25 ± 0.02
CHOL + FFA	0.09 ± 0.02	0.09 ± 0.03	0.07 ± 0.04	0.11 ± 0.11	0.08 ± 0.02	0.08 ± 0.01
MAG	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
GLUCER	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
LACCER	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
PL	0.07 ± 0.03	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.02	0.07 ± 0.01
g/100 g of PL						
PA	0.19 ± 0.18	<0.01	ND[3]	ND	ND	ND
PE	42.00 ± 4.60	46.73 ± 1.65	40.01 ± 3.57	43.04 ± 2.09	41.40 ± 2.43	46.08 ± 2.74
PI	3.98 ± 0.65[a]	0.10 ± 0.28[b]	4.43 ± 0.78[a]	1.53 ± 0.69[b]	5.88 ± 0.98[a]	2.21 ± 0.43[b]
PS	3.45 ± 0.57[a]	0.12 ± 0.34[b]	6.50 ± 1.08[a]	1.61 ± 0.76[b]	9.25 ± 3.73[a]	2.41 ± 0.78[b]
PC	29.30 ± 2.94	33.22 ± 1.88	26.43 ± 3.18	30.54 ± 2.45	27.37 ± 2.66	31.47 ± 1.02
SM	21.07 ± 4.45	19.82 ± 1.06	22.63 ± 4.10	22.28 ± 1.41	16.11 ± 2.25	17.84 ± 2.67

[a,b]Means with different superscript letters within a row and between extraction methods are significantly different ($P < 0.05$).

[1]CE = cholesterol esters; TAG = triacylglycerides; DAG = diacylglycerides; CHOL = cholesterol; MAG = monoglycerides; GLUCER = glucosylceramides; LACCER = lactosylceramides; PL = polar lipids; PA = phosphatidic acid; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylserine; SM = sphingomyelin.

[2]FM = Folch method modified by Iverson et al. (2001).

[3]ND = not detected.

some modifications described previously in the Materials and Methods section. This method permits not only the separation of lipid classes but also a further separation of phospholipids in the same run without prior lipid fractionation, thus allowing their qualitative and quantitative characterization.

The lipid extracts obtained by the ISO method were not analyzed because of its incomplete and minor ex-

Table 4. Comparison of FAME composition (%; mean ± SD) of milk fat obtained from cows, ewes, and goats determined by GC-MS and extracted with the pressurized liquid extraction (PLE) or Folch (Folch et al., 1957) method

	Cow milk		Ewe milk		Goat milk	
FAME (%)	PLE	FM[1]	PLE	FM	PLE	FM
C4:0	3.02 ± 0.25	3.20 ± 0.23	2.37 ± 0.40	2.47 ± 0.30	1.86 ± 0.36	2.01 ± 0.40
C6:0	1.97 ± 0.21	2.08 ± 0.17	1.96 ± 0.16	1.97 ± 0.19	2.32 ± 0.38	2.39 ± 0.47
C8:0	1.15 ± 0.15	1.21 ± 0.14	1.82 ± 0.05	1.78 ± 0.23	2.69 ± 0.44	2.70 ± 0.47
C10:0	2.91 ± 0.47	3.05 ± 0.47	6.07 ± 0.14	6.00 ± 0.99	9.64 ± 1.60	9.71 ± 1.48
C10:1	0.79 ± 0.16	0.84 ± 0.16	0.58 ± 0.09	0.52 ± 0.09	0.68 ± 0.14	0.69 ± 0.14
C12:0	3.16 ± 0.51	3.28 ± 0.53	3.15 ± 0.15	3.15 ± 0.53	4.22 ± 0.52	4.26 ± 0.63
C14:0	11.88 ± 0.96	12.15 ± 0.99	11.76 ± 0.56	11.21 ± 1.00	9.56 ± 0.74	9.63 ± 0.72
C14:1	0.99 ± 0.15	1.02 ± 0.15	0.16 ± 0.01	0.17 ± 0.03	0.14 ± 0.03	0.14 ± 0.02
C15:0	1.05 ± 0.17	1.09 ± 0.17	0.81 ± 0.07	0.85 ± 0.11	0.66 ± 0.09	0.67 ± 0.10
C16:0	32.12 ± 1.68	32.19 ± 1.58	29.69 ± 3.34	29.32 ± 2.02	27.70 ± 2.45	27.69 ± 1.76
C16:1	1.37 ± 0.14	1.38 ± 0.15	1.09 ± 0.09	1.04 ± 0.07	0.70 ± 0.08	0.70 ± 0.08
C17:0	0.56 ± 0.07	0.57 ± 0.06	0.51 ± 0.07	0.55 ± 0.20	0.50 ± 0.04	0.51 ± 0.07
C17:1	0.30 ± 0.06	0.32 ± 0.05	0.24 ± 0.04	0.31 ± 0.07	0.26 ± 0.05	0.27 ± 0.05
C18:0	9.14 ± 1.74	8.93 ± 1.81	11.04 ± 0.68	11.75 ± 1.20	9.88 ± 1.67	9.66 ± 1.94
Total *trans* C18:1	2.65 ± 0.80	2.59 ± 0.81	2.36 ± 0.02	2.39 ± 0.55	2.62 ± 0.57	2.57 ± 0.43
Total *cis* C18:1	22.93 ± 2.05	22.21 ± 1.91	22.28 ± 1.52	22.45 ± 2.57	21.87 ± 2.45	21.70 ± 2.32
cis-9,*cis*-12 C18:2	3.25 ± 0.35	3.00 ± 0.91	2.85 ± 0.01	2.73 ± 0.39	3.71 ± 0.73	3.74 ± 0.70
cis-9,*trans*-11 C18:2 (CLA)	0.38 ± 0.16	0.38 ± 0.18	0.48 ± 0.10	0.52 ± 0.17	0.53 ± 0.15	0.51 ± 0.13
αC18:3	0.25 ± 0.04	0.24 ± 0.09	0.51 ± 0.38	0.53 ± 0.41	0.28 ± 0.05	0.28 ± 0.06
C20:0	0.11 ± 0.02	0.27 ± 0.49	0.29 ± 0.08	0.29 ± 0.09	0.18 ± 0.04	0.17 ± 0.04
Σ SFA	67.09 ± 2.79	68.02 ± 2.56	69.47 ± 2.76	69.35 ± 2.87	69.21 ± 3.04	69.40 ± 2.60
Σ MUFA	29.03 ± 2.56	28.36 ± 2.34	26.70 ± 2.38	26.87 ± 2.53	26.27 ± 2.63	26.07 ± 2.36
Σ PUFA	3.89 ± 0.36	3.61 ± 0.98	3.84 ± 0.38	3.78 ± 0.76	4.52 ± 0.85	4.53 ± 0.76
SFA/UFA[2]	2.06 ± 0.27	2.15 ± 0.26	2.27 ± 0.35	2.29 ± 0.32	2.28 ± 0.33	2.29 ± 0.28

[1]FM = Folch method modified by Iverson et al. (2001).

[2]UFA = unsaturated FA.

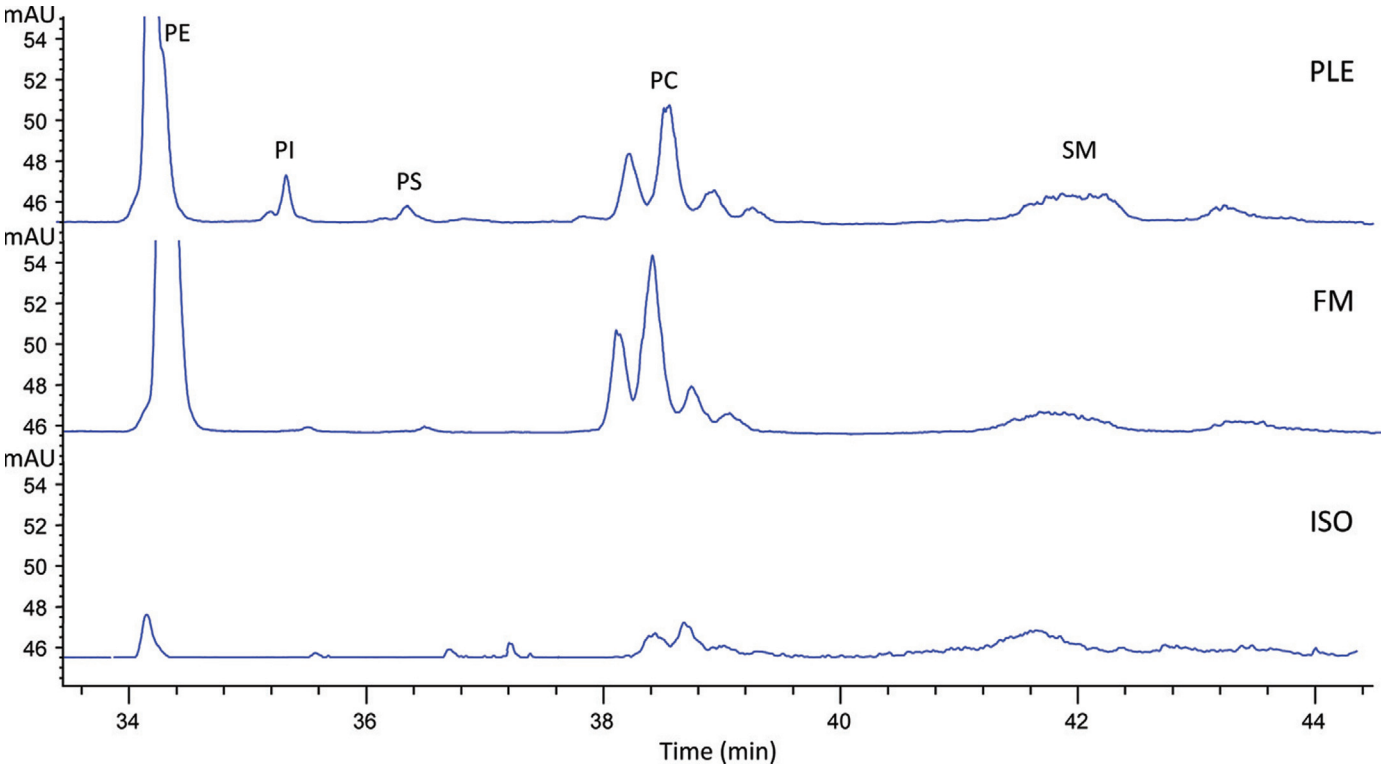

Figure 2. Chromatogram profile area of phospholipids and sphingomyelin from milk fat extracted by the pressurized liquid extraction (PLE), Folch (Folch et al., 1957), and International Organization for Standardization (ISO) methods. ISO = Röse-Gottlieb method, which is the official extraction procedure for milk fat (ISO, 2001); FM = Folch method, modified by Iverson et al. (2001); PLE = PLE with optimized conditions; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylserine; SM = sphingomyelin. Color version available in the online PDF.

traction of polar lipids (Figure 2). This is in agreement with a previous statement that this method does not extract all components and the same amount of polar lipids as some phospholipids (Avalli and Contarini, 2005).

In milk and dairy products, total lipids are dominated by TAG (~98%), whereas polar lipids are only found in trace amounts (0.1%), in agreement with Lopez et al. (2008), who observed less than 0.3% in cow milk fat. As expected, the neutral lipids were the major fraction (at more than 99%) in all samples. Triacylglycerides were the main components, followed by diacylglycerides and CHOL plus FFA. On the other hand, cholesterol esters, monoacylglycerides, glucosylceramides, and lactosylceramides were present in minor amounts. All these components did not show significant differences in their compositions when they were extracted either by the PLE or Folch method. This is in agreement with the results reported by Cescut et al. (2011) for yeast lipids isolated using both a chloroform:methanol extraction method (Bligh and Dyer, 1959) and a PLE method with the same solvents.

The phospholipid concentration was very low in milk (Table 3) and dairy products, except for some by-products of the butter process, such as dried butter serum and buttermilk and also in skim milk powder. Rombaut et al. (2006) obtained a polar lipids amount of 33.05 and 29.06% from fat in buttermilk and butter serum and 19.06% in skim milk, whereas less than 1% was obtained from milk.

As can be seen in Table 3, although the total amount of the polar lipid fraction did not present significant differences among extraction methods used, some of the individual species of phospholipids contained in this fraction, such as PI and PS, were significantly higher when using PLE extraction than with the Folch method. No significant differences were found in the other phospholipids PE, PC, and SM, which were not affected by the extraction method. Zhou et al. (2010) observed similar results after lipid extraction by the Folch method of soybeans, egg yolk, calf brain, and ox liver and reported a recovery of less than 78% of total PI, whereas PE and PC were recovered at more than 90%. Moreover, Cescut et al. (2011) observed higher

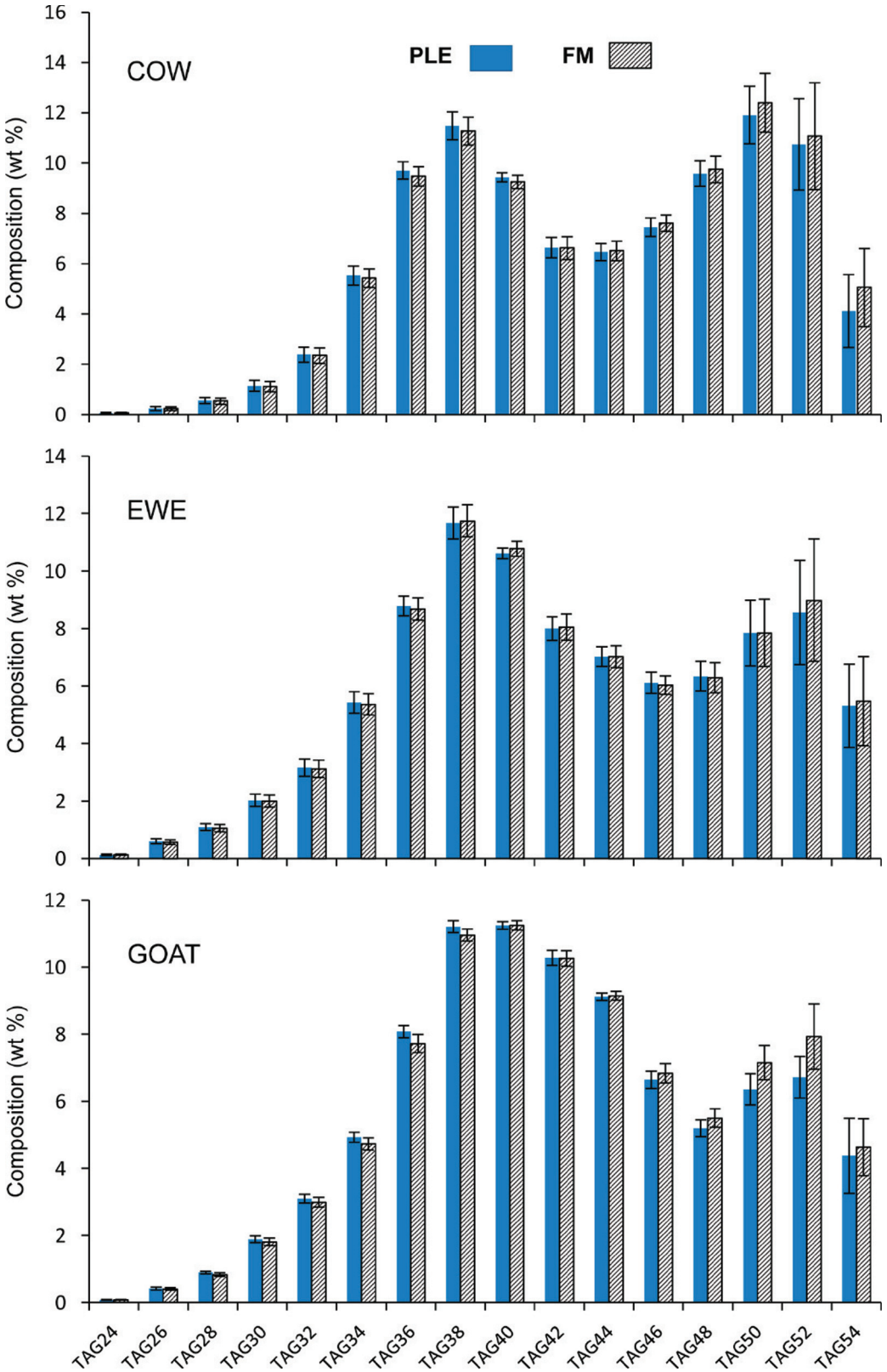

Figure 3. Effect of milk fat extraction by pressurized liquid extraction (PLE) and Folch (Folch et al., 1957) procedures on triacylglyceride (TAG) composition (wt %) from carbon number 24 to 54 (cow, ewe, and goat milk). FM = Folch method, modified by Iverson et al. (2001). Error bars represent SD values (n = 10). Color version available in the online PDF.

PI extraction from yeast when using the PLE method than the Bligh and Dyer (1959) method.

FA Analysis (FAME)

The FAME composition of the milk fat samples extracted with the PLE and Folch methods is shown in Table 4. With both methods, the major FAME in cow, ewe, and goat milk were C16:0, total *cis* C18:1, C14:0, and C18:0, showing values between 27.7 to 32.2%, 21.7 to 22.9%, 9.5 to 12.1%, and 8.9 to 11.7%, respectively. Although short-chain FA (C4–C10) had a slight trend to increase with milk extracted with the Folch method modified by Iverson et al. (2001), neither of the FAME displayed significant differences ($P < 0.05$) among the extraction methods. This is in agreement with Toschi et al. (2003), Mulbry et al. (2009), and Cescut et al. (2011), who did not find differences in FAME composition in poultry meat, algae, and yeast, respectively, when comparing a similar Folch method with PLE extraction with chloroform:methanol.

It might be reasonable to expect differences in the FAME profile, given that the PLE method extracted higher amounts of some phospholipids, such as PI and PS, than the Folch method. However, being minor compounds, this fact did not significantly affect to the total FAME profile.

TAG and CHOL Composition

A great deal of interest exists in the determination of TAG and CHOL composition of dairy fats because of their influence on technological properties (melting point and crystallization behavior, among others), but especially because of their role in nutrition and cardiovascular diseases. Figure 3 shows the profiles of TAG from cow, ewe, and goat milk fat, considering the quantification of 16 groups, corresponding to TAG of 24 to 54 carbon atoms. The TAG composition presented the same distribution as those in previous studies of TAG of milk fat from different species reported under similar experimental conditions (Fontecha et al., 2005). Low-molecular-weight TAG (C24–C40) are related to those containing short-chain FA, (e.g., C4, C6, or C8), and the medium-chain TAG (C42–C46), which include most C10 and C12, are TAG mainly present in dairy fats. These TAG (short chain and medium chain) are generally considered a good biologically inert source of energy because they diffuse from the gastrointestinal tract to the portal system without requirement for modification and, therefore, can help in the process of excess calorie burning and weight loss (Tsuji et al., 2001; Marten et al., 2006). The rest of TAG with high molecular weight (C48–C54) contain the 3 long-chain FA. This distribution is also related to the need for maintaining the fluid condition of the fat at physiological temperatures.

Although a tendency exists for slightly higher extraction of higher-molecular-weight TAG than the lower-molecular-weight TAG by the Folch method compared with the PLE method, none of the TAG groups described showed significant differences ($P < 0.05$) between the extraction methods tested. With regard to CHOL, also no significant differences in the amount of CHOL from different milk fat species were observed when using the PLE or Folch extraction methods. Cholesterol values (in g/100 g of total fat) were 0.43 ± 0.08 and 0.43 ± 0.05 in cows, 0.35 ± 0.04 and 0.37 ± 0.04 in ewes, and 0.37 ± 0.02 and 0.39 ± 0.02 in goats for the PLE and Folch methods, respectively.

CONCLUSIONS

The optimized PLE method proved to be capable of extracting milk lipids efficiently. Dichloromethane:methanol extracts gave the best results in terms of fat yield and 60°C did not alter the milk fat chemical composition. Furthermore, although TAG, FAME, and CHOL extraction did not present differences between methods, the PLE method extracted the phospholipids PI and PS much better and faster (only 10 min) than the official ISO method and most of the methods usually used to extract lipids from milk. In conclusion, the PLE method could be a valuable alternative to extract milk fat as a routine method. The PLE method used less than the half of the solvent mixture necessary to carry out the milk fat extraction compared with other methods, and the time applied per sample was significantly lower and offers the possibility of automation.

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REFERENCES

Avalli, A., and G. Contarini. 2005. Determination of phospholipids in dairy products by SPE/HPLC/ELSD. J. Chromatogr. A 1071:185–190.

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911–917.

Boselli, E., V. Velazco, M. F. Caboni, and G. Lercker. 2001. Pressurized liquid extraction of lipids for the determination of oxysterols in egg-containing food. J. Chromatogr. A 917:239–244.

Castro-Gómez, P., J. Fontecha, and L. M. Rodríguez-Alcalá. 2014. A high-performance direct transmethylation method for total fatty

acids assessment in biological and foodstuff samples. Talanta 128:518–523.
Cescut, J., E. Severac, C. Molina-Jouve, and J. L. Uribelarrea. 2011. Optimizing pressurized liquid extraction of microbial lipids using the response surface method. J. Chromatogr. A 1218:373–379.
Conte, E., R. Milani, G. Morali, and F. Abballe. 1997. Comparison between accelerated solvent extraction and traditional extraction methods for the analysis of the herbicide diflufenican in soil. J. Chromatogr. A 765:121–125.
Feng, S., A. L. Lock, and P. C. Garnsworthy. 2004. A rapid method for determining fatty acid composition of milk. J. Dairy Sci. 87:3785–3788.
Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497–509.
Fontecha, J., H. Goudjil, J. J. Ríos, M. J. Fraga, and M. Juárez. 2005. Identity of the major triacylglycerols in ovine milk fat. Int. Dairy J. 15:1217–1224.
Gallier, S., D. Gragson, C. Cabral, R. Jiménez-Flores, and D. W. Everett. 2010. Composition and fatty acid distribution of bovine milk phospholipids from processed milk products. J. Agric. Food Chem. 58:10503–10511.
Hara, A., and N. S. Radin. 1978. Lipid extraction of tissues with a low-toxicity solvent. Anal. Biochem. 90:420–426.
Heinze, V. M., and A. B. Actis. 2012. Dietary conjugated linoleic acid and long-chain n-3 fatty acids in mammary and prostate cancer protection: A review. Int. J. Food Sci. Nutr. 63:66–78.
Herrero, M., P. J. Martín-Álvarez, F. J. Señoráns, A. Cifuentes, and E. Ibáñez. 2005. Optimization of accelerated solvent extraction of antioxidants from Spirulina platensis microalga. Food Chem. 93:417–423.
Hilmarsson, H., L. V. Larusson, and H. Thormar. 2006. Virucidal effect of lipids on visna virus, a lentivirus related to HIV. Arch. Virol. 151:1217–1224.
ISO (International Organization for Standardization). 2001. Milk and milk products—Extraction methods for lipids and liposoluble compounds. ISO 14156:2001-IDF 172:2001. ISO, Geneva, Switzerland.
ISO (International Organization for Standardization). 2002. Milk fat—Preparation of fatty acid methyl esters. ISO 15884-IDF 182:2002. ISO, Geneva, Switzerland.
Iverson, S. J., S. L. C. Lang, and M. H. Cooper. 2001. Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. Lipids 36:1283–1287.
Jansen, B., K. G. J. Nierop, M. C. Kotte, P. de Voogt, and J. M. Verstraten. 2006. The applicability of accelerated solvent extraction (ASE) to extract lipid biomarkers from soils. Appl. Geochem. 21:1006–1015.
Küllenberg, D., L. A. Taylor, M. Schneider, and U. Massing. 2012. Health effects of dietary phospholipids. Lipids Health Dis. 11:3.
Lopez, C., V. Briard-Bion, O. Menard, F. Rousseau, P. Pradel, and J.-M. Besle. 2008. Phospholipid, sphingolipid, and fatty acid compositions of the milk fat globule membrane are modified by diet. J. Agric. Food Chem. 56:5226–5236.
Macnaughton, S. J., T. L. Jenkins, M. H. Wimpee, M. R. Cormiér, and D. C. White. 1997. Rapid extraction of lipid biomarkers from pure culture and environmental samples using pressurized accelerated hot solvent extraction. J. Microbiol. Methods 31:19–27.
Marten, B., M. Pfeuffer, and J. Schrezenmeir. 2006. Medium-chain triglycerides. Int. Dairy J. 16:1374–1382.
Mulbry, W., S. Kondrad, J. Buyer, and D. L. Luthria. 2009. Optimization of an oil extraction process for algae from the treatment of manure effluent. J. Am. Oil Chem. Soc. 86:909–915.
Rodríguez-Alcalá, L. M., and J. Fontecha. 2010. Major lipid classes separation of buttermilk, and cows, goats and ewes milk by high performance liquid chromatography with an evaporative light scattering detector focused on the phospholipid fraction. J. Chromatogr. A 1217:3063–3066.
Rombaut, R., J. V. Camp, and K. Dewettinck. 2006. Phospho- and sphingolipid distribution during processing of milk, butter and whey. J. Food Technol. 41:435–443.
Schäfer, K. 1998. Accelerated solvent extraction of lipids for determining the fatty acid composition of biological material. Anal. Chim. Acta 358:69–77.
Señoráns, F. J., and P. Luna. 2012. Sample preparation techniques for the determination of fats in food. Pages 203–211 in Comprehensive Sampling and Sample Preparation. J. Pawliszyn, ed. Academic Press, Oxford, UK.
Singh, H. 2006. The milk fat globule membrane—A biophysical system for food applications. Curr. Opin. Colloid Interface Sci. 11:154–163.
Toschi, T. G., A. Bendini, A. Ricci, and G. Lercker. 2003. Pressurized solvent extraction of total lipids in poultry meat. Food Chem. 83:551–555.
Tsuji, H., M. Kasai, H. Takeuchi, M. Nakamura, M. Okazaki, and K. Kondo. 2001. Dietary medium-chain triacylglycerols suppress accumulation of body fat in a double-blind, controlled trial in healthy men and women. J. Nutr. 131:2853–2859.
Zhou, L., J. Le Grandois, E. Marchioni, M. Zhao, S. Ennahar, and F. Bindler. 2010. Improvement of total lipid and glycerophospholipid recoveries from various food matrices using pressurized liquid extraction. J. Agric. Food Chem. 58:9912–9917.