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Improved Solvent Extraction Procedure and High-Performance Liquid Chromatography−Evaporative Light-Scattering Detector Method for Analysis of Polar Lipids from Dairy Materials

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Supporting Information

ABSTRACT: A normal-phase high-performance liquid chromatography−evaporative light-scattering detector method employing dichloromethane, methanol, and acetic acid/triethylamine buffer as the mobile phase was developed for analysis of polar lipids (PLs). This method was applicable for analysis of PLs from both dairy materials and soy lecithin. All of the PLs of interest such as glycolipids, phospholipids, and sphingomyelin were well separated with a total run time of 22.5 min and without necessitating the removal of neutral lipids beforehand. Peak retention times were stable, and the method was reproducible. In this study, a modified method of using solvents for extraction of PLs from dairy matrices was also investigated. The modified method offered higher extraction efficiency, consumed less time, and in some cases saved solvent use.

KEYWORDS: Polar lipids, phospholipids, HPLC, ELSD, extraction, MFGM

INTRODUCTION

Milk polar lipids (PLs) consist of glycerophospholipids; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS); and sphingolipids, sphingomyelin (SM), glucosylceramide (GluCer), lactosylceramide (LacCer), and a trace amount of gangliosides (Gang).[1] Lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and phosphatidic acid (PA) are sometimes reported in limited amounts. The presence of these components could be the result of careless sample preparation, long storage times of samples, or phospholipase activity.[2,3]

Applications of phospholipids from oil-bearing seeds have a long tradition and are expanding, such as in food processing, pharmaceutics, paintings, and cosmetics. Today, the PLs from dairy sources are gaining great attention due to their nutritional and technological functionalities.[4] Accompanying this trend is the increasing need to analyze PLs in either conventional dairy products or PL-enriched materials.[5]

The total content of PLs in food can be determined by first analyzing its total phosphorus content and relating this to the amount of PLs using a conversion factor.[6] Individual PLs can be analyzed using thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and ^{31}P nuclear magnetic resonance (NMR) spectroscopy.[1] The last method is the most recent and advanced, which can provide an absolute composition of PLs directly. Its major disadvantages, however, are the high instrument investments and the need for skilled persons. Consequently, presently, ^{31}P NMR spectroscopy is only used for reference validation and certification and not for routine analysis in the quality control of PLs.[1] Mass spectrometry is another approach to analyze PL extracts from biological materials.[7] This sensitive method also requires sophisticated and expensive instruments. Consequently, HPLC is still widely applied in laboratories. There are several HPLC methods developed for the separation of major phospholipids from soy lecithin.[8−10] However, the application of these methods to dairy products is complicated by the fact that dairy products besides major phospholipids (PE, PI, and PC) also contain sphingolipids such as SM, GluCer, and LacCer.[3]

Prior to HPLC analysis, the PLs need to be extracted quantitatively from the material matrix. This is a challenging step. The PLs in milk interact strongly with membrane-specific proteins, which makes it difficult to extract the PLs completely from dairy products. Among the liquid−liquid extraction methods, a combination of chloroform and methanol at various ratios, derived from the methods of Folch et al.[11] and Bligh and Dyer,[12] is considered as the most efficient for the extraction of PLs. For Folch-based methods, for example, a mixture of chloroform and methanol was added at a certain ratio to an aqueous sample in a separatory funnel. Two phases are formed, and the lower phase of chloroform contains the PLs. Depending on the samples, the phase separation does not always appear readily as observed from laboratory experience. Allowing the mixture to stand undisturbed in a fridge overnight[13] or centrifugation of the mixture can accelerate

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Table 1. Gross Composition of the Experimented Materials, Extracted Concentrations of PLs Obtained with the Three Extraction Methods, and Evaluation of the Modified Method[a]

Samples (sampling weight)		BMP (0.25 g)						MFGM material (0.8 g)	
Gross composition on dry matter basis (% w/w)[b]	Total proteins	33.1 ± 0.01						61.7 ± 0.04	
	Total lipids	10.4 ± 0.13						24.7 ± 0.71	
	Ash	7.7 ± 0.03						3.0 ± 0.45	
	Lactose	48.8 ± 0.10						10.6 ± 1.21	
Extracted PL concentrations according to different extraction methods (mg/g sample)	Extraction method	Rombaut et al. (2005) (mg/g)	B&D (mg/g)	Modified method[c] (mg/g)	Batch RSD (%)	Long-term RSD (%)	Recovery (%)	Rombaut et al. (2005) (mg/g)	Modified method (mg/g)
	Total PLs	29.75 ± 1.53[a]	31.43 ± 0.03[ab]	32.90 ± 0.38[b]	3.00	3.55	103.4 ± 8.9	8.95 ± 0.57[a]	9.95 ± 0.23[b]
	GluCer	0.53 ± 0.02[a]	0.53 ± 0.04[a]	0.60 ± 0.01[b]	3.80	4.90	103.1 ± 5.2	0.17 ± 0.01[a]	0.20 ± 0.00[b]
	LacCer	2.30 ± 0.03[a]	2.31 ± 0.04[a]	2.34 ± 0.01[a]	1.98	4.06	99.6 ± 6.0	0.67 ± 0.03[a]	0.67 ± 0.02[a]
	PI	3.15 ± 0.33[a]	2.98 ± 0.33[a]	3.50 ± 0.03[a]	1.92	2.52	95.4 ± 1.8	0.70 ± 0.02[a]	0.63 ± 0.05[b]
	PE	5.09 ± 0.01[a]	6.33 ± 0.11[b]	6.24 ± 0.21[b]	3.79	6.95	104.1 ± 4.3	1.89 ± 0.20[a]	2.39 ± 0.05[b]
	PS	2.84 ± 0.22[a]	2.96 ± 0.08[a]	3.31 ± 0.03[b]	2.52	4.07	101.2 ± 3.3	0.80 ± 0.07[a]	0.87 ± 0.09[a]
	PC	8.18 ± 0.28[a]	8.86 ± 0.41[ab]	8.95 ± 0.05[b]	2.34	4.35	101.3 ± 6.3	2.48 ± 0.14[a]	2.88 ± 0.05[b]
	SM	7.68 ± 0.68[a]	7.60 ± 0.17[a]	7.96 ± 0.10[a]	1.20	4.00	100.4 ± 1.9	2.24 ± 0.13[a]	2.31 ± 0.06[a]

[a] Data are expressed as the average ± standard deviation of three repeats. [b] Gross composition of BMP and the MFGM material or the microfiltered BM is obtained from a previous report.[25] Comparing the extracted concentrations of PLs of the two samples with different extraction methods, values in the same column that share a common letter were not significantly different. [c] Reproducibility of the analysis procedure (the modified extraction method and the new HPLC-ELSD method) is indicated by relative standard deviation (RSD). Batch reproducibility was determined from six repeats in the same day. Long-term reproducibility was determined from this batch and two other batches (three repeats for each batch) on different dates within 2 months. Recovery was determined from spiking the BMP with a known concentration of the mix standard of milk PLs before carrying out the extraction.

the separation. In such cases, the analysis consumes more time, and loss of samples due to exchanges of containers (in the case of centrifugation) is difficult to avoid. Supercritical liquid extraction can be used to extract PLs from a biological matrix.[14] However, there is still a need to optimize this technique for dairy materials.

Most dairy products contain low concentrations of PLs and a high amount of proteins. Proteins have the capacity to stabilize emulsions in which PLs are involved since they are amphiphilic.[15] This causes difficulty in having a complete phase separation and/or an efficient extraction of PLs. The four step extraction method, by Rombaut et al.,[16] using chloroform and methanol was reported to be more efficient in extracting PLs from acid buttermilk whey than other methods that were based on Folch as well as Bligh and Dyer (B&D).[17] However, we observed that the phase separation was slow when the method was applied on other dairy products such as raw milk and horse milk, which readily form an emulsion. The speed of phase separation was found to decrease with an increasing initial amount of sample while maintaining the same volume of solvents.

In this current study, a new HPLC-ELSD (evaporative light-scattering detector) method based on the method of Rombaut et al.[16] was developed for the analysis of PLs. The new method used dichloromethane instead of chloroform. The former is less toxic than the latter whose use is discouraged in many laboratories. As another part of this study, a solvent extraction method was improved to increase the extraction efficiency of PLs from dairy products prior to HPLC analysis.

MATERIALS AND METHODS

Chemicals and Materials. *Chemicals.* Ultrapure formic acid was bought from Chem-lab (Zedelgem, Belgium). Triethylamine was obtained from Sigma-Aldrich NV (Bornem, Belgium), and acetic acid was from Acros Organics (Geel, Belgium). Three main solvents, HPLC dichloromethane (stabilized with 0.1% ethanol), HPLC supra-gradient methanol, and HPLC water, were delivered from Biosolve (Valkenswaard, The Netherlands).

For identification purposes, pure PLs such as GluCer, LacCer, PI, PA, PE, PS, PC, SM, LPC (lysoPC), LPE (lysoPE), PG (phosphatidylglycerol), and cholesterol were purchased from Sigma-Aldrich NV. Standards of several mono-, di-, and triacylglycerides were obtained from NU-CHEK PREP Inc. (Elysian, MN). For quantitative determination, mixed PL standards isolated from milk and soy lecithin were obtained from Spectral Service GmbH (Koln, Germany).

Samples. The samples used for extraction experiments were raw milk from cows (delivered from a local dairy farm), buttermilk powder (BMP) (FrieslandCampina Professional, Lummen, Belgium), and microfiltered buttermilk (BM), which was produced in the laboratory from the BMP.[18] The microfiltered BM (12.24% in dry matter content), as compared to the BMP (96.04% in dry matter content), was several times higher in concentration of PLs and much lesser in concentration of minerals and lactose on dry matter basis (Table 1). The microfiltered

BM was enriched with milk fat globule membrane fragments; hence, it was called the milk fat globule membrane (MFGM) material.

Instruments. The HPLC system (Shimadzu, Tokyo, Japan) consisted of a controller (CBM-20A), an online degassor (DGU-20A_5), a solvent delivery module (LC-20AT), an autosampler (SIL-20AT), and a column oven (CTO-20AC). The HPLC system was connected to an ELSD (ELSD model 3300, Alltech Associates Inc., Lokeren, Belgium). A precolumn (7.5 mm × 3.0 mm, Prevail silica 5 μm) and separation column (150 mm × 3.0 mm, Prevail silica 3 μm) from Alltech Associates Inc. were used.

Extraction Methods. *B&D Method.* The three-step B&D method[12] was one of the three methods considered for comparison in this study. A suitable amount of sample (0.25 g for BM, 10 g for raw milk) was weighed in a beaker, and deionized water was added to obtain 20 mL. The mixture was then transferred to a separatory funnel along with 50 mL of methanol and 25 mL of chloroform. The funnel was capped tightly and shaken for 2 min. Twenty-five milliliters of chloroform and 25 mL water were added one after the other with 2 min of shaking after each addition. The mixture was then allowed to stand undisturbed. This step led to a separation of phases. The lower phase, which contains lipids, was slowly collected in a flask through a paper filter prewetted with some drops of chloroform. Afterward, 38 mL of chloroform was added to the upper phase, and the mixture was again shaken and left for separation. The lower phase was again collected in the same flask. Washing with 38 mL of chloroform was carried out once more. The pooled lower phase of the three extraction steps was evaporated at 36 °C using a rotary evaporator. The dry lipid extract was redissolved immediately in exactly 10 mL of HPLC chloroform:methanol 88:12 (v/v) mixture. This extract solution was transferred to a test tube, which was then capped tightly (to avoid evaporation) and stored at −26 °C until HPLC analysis.

Extraction Method of Rombaut et al.[16]. This method[16] was adapted from Shaikh,[19] which in turn was adapted from the Folch method.[11] The method includes four extraction steps. First, the sample was diluted with water to 20 mL and transferred to a separatory funnel along with 80 mL of 2:1 (v/v) chloroform:methanol mixture. The mixture was shaken and left undisturbed to allow phase separation. After the phase separation was completed, the clear lower phase (chloroform containing the extracted lipids) was slowly collected in a flask through a paper filter prewetted with some drops of chloroform. The extraction was done two more times with 40 mL of 20:1 (v/v) chloroform:methanol mixture added to the upper phase followed by shaking, equilibrating, and withdrawal of the lower phase. Finally, the extraction was repeated again by adding to the upper phase 40 mL of 86:14:1 (v/v/v) chloroform: methanol:water mixture. The water used contained 1 N HCl and 0.9% NaCl. The lower phase resulting from this step was collected and washed with 0.9% NaCl solution until neutral pH was reached. It was pooled with those collected in the previous extraction steps and evaporated as described in the last method. The lipid extract was prepared for HPLC analysis as described in the previous extraction method.

Proposed Modified Extraction Method. A third method, a modified method from Rombaut et al.,[16] was proposed. In this method, the sample (0.25 g of BMP, 10 g of raw milk, and 4 g of soft cheese) was first added with 3 mL of a 10% (w/v) salt solution (see the section Experiments) before it was diluted with deionized water to 20 mL. The three subsequent steps of extraction were just the same as in the method of Rombaut et al.,[16] while the fourth step, the most time-consuming, was omitted.

HPLC-ELSD Analysis. *Two Solvent Lines Were Used as Follows.* Line A contained dichloromethane, and line B contained a mixture of methanol and acetic acid/triethylamine buffer, pH 4.5, in 500:21 (v/v) ratio. The buffer was prepared by adding 7.2 mL of acetic acid and 8.0 mL of triethylamine to 118 mL of HPLC water. The mobile phase pumping was performed in linear gradient with the ratio in volume of A to B as follows: 96:4 at t = 0 min to 88:12 at t = 4 to 6:94 at t = 12 min, and back to 96:4 at t = 17 min. The pumping was maintained at this ratio until t = 22.5 min before a new injection. The total flow rate of the mobile phase was 0.5 mL/min. The column oven temperature was maintained at 40 °C, and the sample chamber temperature of the autosampler was set at 20 °C. The ELSD settings were adjusted to 65 °C for the tube temperature, 2.1 L/min for the nebulizer gas (nitrogen), and 1 for acquisition gain. The injection volume was 10 μL. Two runs were performed for each extract. PL standard solutions, which were used for identification and absolute concentration calculation, were also injected in duplicates at the same injection volume.

Experiments. *Validation of the HPLC-ELSD Method.* The detection limit (peak signal-to-noise ratio higher than 3) of the individual PLs from milk was determined by injecting a standard mix of milk PLs at decreasing concentrations. The loading capacity of the column was checked by injecting a standard mix or a BMP lipid extract. Both were dissolved in a series of increasing concentrations of a "nonpolar lipid matrix". This nonpolar lipid matrix was prepared by dissolving palmito−palmito−olein, palmito−oleo−palmitin, stearo−oleo−stearin, distearin, and cholesterol at a 125:125:99.7:1.06:1.06 ratio in the HPLC chloroform:methanol 88:12 solvent mixture. It is noted that the composition of the nonpolar lipid matrix is far from being similar to that of milk nonpolar lipid fraction. The concentrations of the added nonpolar lipids for the injection varied from 12.5 to 750 mg/mL. The maximum concentration of the added nonpolar lipids (750 mg/mL) was 6400 times of total PLs of the first (lowest) point of the standard curves (Annexes 1 and 2 in the Supporting Information). This tested range is expected to cover most of the dairy products.

Determination of a Suitable Salt To Be Used in the Modified Extraction Method. A test was performed to find a suitable salt to use during extraction of PLs for the modified extraction method. Two salts, $CaCl_2$ and trisodium citrate, which have opposite influences on protein solubility, were compared. For $CaCl_2$, 3 mL of 10% (w/v) solution and, for the citrate salt, 3 and 6 mL of 10% (w/v) solution, were tested. The MFGM material (microfiltered BM) was used as the sample for this test.

Comparison of the Three Extraction Methods. Next, the three extraction methods were compared based on the extracted amounts of PLs from raw milk, BMP, and MFGM material. Two levels of initial sample weight were used. For "level 1", the amount of sample was determined in such a way that the final extracted lipid solution would be sufficient (the output should drop within the calibration curve range) for injecting 10 μL in the HPLC without dilution or concentration (e.g., 0.25 g of BMP, 0.8 g of MFGM material). The initial sample weight for "level 2" was three times higher than that of level 1. This means that it was necessary to dilute the resulting extract solution three times before injecting. The test was carried out in triplicate for each level.

Validation of the Analysis Procedure. This part of the study covers the evaluation of analysis reproducibility, extraction recovery, and a trial analysis of several dairy products. The extraction method, which gave the best result (highest extracted amounts of PLs) among the three, was selected to extract PLs to be analyzed using the new HPLC method. The batch reproducibility of the result was assessed using the value of relative standard deviation (RSD) from six replicates of extraction on the BMP sample. Two other batches of analysis (three replicates for each batch) performed on different dates within 2 months were carried out using the same BMP. The results of the three batches were used to determine long-term reproducibility.

The recovery of the extraction was evaluated by spiking the standard mix of milk PLs into the BMP before carrying out the extraction. The recovery test was performed in triplicate.

Finally, a trial using the analysis procedure was carried out to determine the PLs in raw milk and in a commercial soft cheese. The new HPLC method was also tested for analyzing PLs in a commercial butter. For this sample, the extraction of the PLs was not carried out using the methods described above. Rather, crude lipids were just

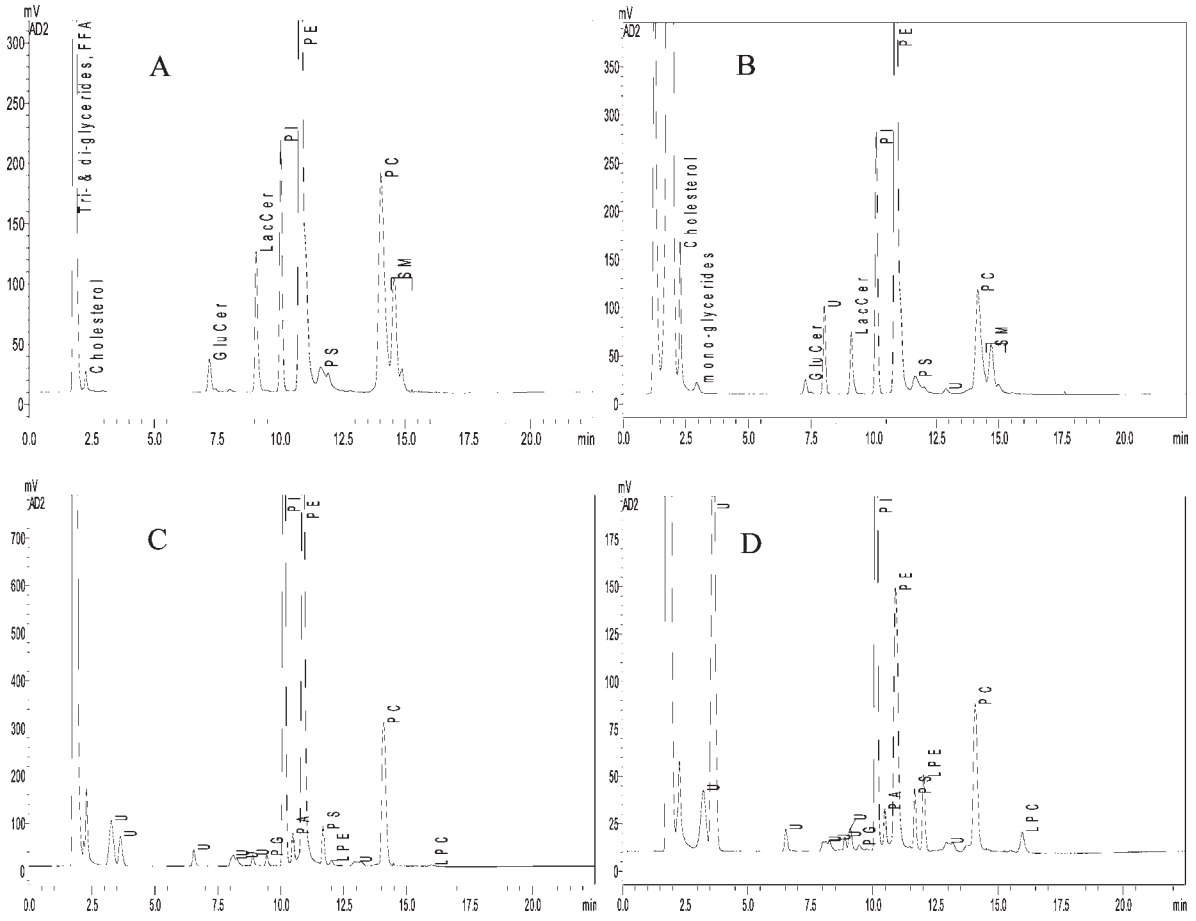

Figure 1. HPLC-ELSD chromatograms of PLs from various materials: (A) The milk PL standard, (B) BMP, (C) the soy lecithin standard, and (D) a commercial hydrolyzed soy lecithin. DGDG, digalactosyl diacylglycerol. "U" indicates unknown or unidentified peaks. Peak identification was based on retention match with standards. Many peaks were unidentified due to lack of standard.

collected by filtering out the precipitated proteins after dissolving the butter sample in 2:1 (v/v) chloroform:methanol mixture before performing HPLC analysis. The trial analysis was performed in duplicate.

Statistical Analysis. One-way analysis of variance (ANOVA) tests were performed using S-Plus 8.0 package for Windows (S-Plus, Tibco Software, Palo Alto, CA) to find the difference ($P \leq 0.05$) among samples. Paired comparisons between means were carried out using the Tukey's test when a significant difference was observed.

RESULTS AND DISCUSSION

HPLC-ELSD Analysis. Examples of chromatograms obtained from HPLC-ELSD analysis are shown in Figure 1. It is shown that, with a total run time of 22.5 min (including regeneration time), all of the peaks of interest were eluted and well-separated. The calibration curves made by using the mixed PL standard isolated from milk obtained from Spectral Service and fitted with both power law and second-order polynomial regression are shown in the Supporting Information, Annex 1. Power law regression offered satisfactory results (R^2 values >0.99). However, the use of second-order polynomial models was slightly better than that of power law models. The detection limits of GluCer, LacCer, PI, PE, PS, PC, and SM were 0.021, 0.018, 0.011, 0.009, 0.028, 0.029, and 0.024 μg, respectively. The detection limits were in agreement with the old method.[16] Using the standard mix of soy lecithin (since the mix standard of milk did not contain lysophospholipids, as described by the supplier), the detection limits of PA and LPC were found to be 0.020 and 0.028 μg, respectively.

The old method developed by Rombaut et al.[16] and adapted by Le et al.[20] has been successfully applied to analyze PLs from dairy products. However, the instability in peak retention time (RT) of duplicate injection, especially for PE, PI, and PS where the RTs could vary and reach up to 0.5 min, makes the postrun analysis difficult/time consuming. Because of this instability, uncertainties are introduced in the automatic postrun processing of data, in which analysis of one reference elution is applied to other runs. The new method presented in the current study offered very stable RTs and was reproducible in duplicate runs, which consequently reduced postrun analysis time. The variation in RT of all of the peaks of duplicate injection was in the magnitude of 0.01 min. The RSD values of the peak areas of duplicate injection of a BM sample or a standard solution were smaller than 3% for all peaks of interest while RSD values of the peak areas of PI, PE, and PS obtained with the old method were often higher than 5%. In the old method, the mobile phase was pumped from separate lines of chloroform, methanol, and formic acid/triethylamide buffer 3.0. The buffer (aqueous) was not well miscible with chloroform, and this created the instability of peak RTs in duplicate runs. In the new method, the acetic acid/triethylamide buffer was premixed with methanol. The percentage of the buffer was smaller than that in the old method, and the

solvent composition was changed. All of these led to a better miscibility between the solvents in the new method, and as a result, an improved stability in RTs and peak areas was obtained. In long-term analysis, that is, for analysis batches on different days or months, RTs of peaks could vary up to 0.3 min (Supporting Information, Annex 3). This was possibly due to the difference in solvent quality and/or the slight difference in buffer pH values of each preparation. Despite the variation in RTs, the separation of peaks was distinct even though the column was used quite intensively for more than a year already (Supporting Information, Annex 3). It is noted that the solvent quality, the sensitivity of the optic system, and the strength of laser light in the ELSD may not be maintained over time. Therefore, for accurate quantification, standard solutions should be injected in every analysis.

Glycolipids such as GluCer were eluted far from the neutral lipids (Figure 1) so the GluCer peak was not overlapping with the peak of neutral lipids as it occurred with the old method when analyzing samples containing a high concentration of neutral lipids. Therefore, it was not necessary to remove the neutral lipids before analyzing the phospholipids in HPLC. In some samples (with low neutral lipids), cholesterol and monoglycerides could be separated (Figure 1A,B). In laboratory practice, a deoiling step using a solvent or solid-phase extraction (SPE) column (e.g., silica) could be applied to preliminarily separate neutral lipids and PLs. However, there is a chance that a certain percentage of PLs might be lost.[8,21] GluCer and LacCer might also be lost after the SPE.[21]

Upon injection of the milk PL standard mix prepared with the nonpolar lipid matrix at concentrations above 350 mg/mL (or the injected amount of nonpolar lipids was above 3.5 mg), it was observed that noise peaks appeared after the peak of neutral lipids until the ninth minute of the elution (see the Supporting Information, Annex 2). These noise peaks were only in the magnitude of 2 mV and did not increase with further increase in the total added nonpolar lipids up to the maximum tested concentration (750 mg/mL or 7.5 mg). These noise peaks did not significantly affect the detection limit of the PLs in the milk standard mix. However, during injection of the lipid extracts from the soft cheese, the noise peaks were up to 5 mV (Supporting Information, Annex 4) although the load of total lipids was, based on calculation, only about 72 mg/mL. The noise peaks sometimes affected the GluCer peak. In such a case, the amount of GluCer should be higher than 0.06 μg to result in a peak signal-to-noise ratio higher than 6 (quantification limit).

The new method does not use chloroform, which is under strict rules for use at some laboratories. Dichloromethane is less toxic than chloroform and, as well, the least toxic among the simple chlorohydrocarbons.[22] However, it is noted that dichloromethane is more volatile than chloroform. There is still a need to search for less toxic alternatives.

When water in the mobile phase was removed, the peaks of PC and SM were not well-separated. So for the analysis of soy lecithin, water is not obligatory, but for milk samples, the use of water in the mobile phase was necessary. The use of acetic acid instead of formic acid to prepare the buffer gave narrow and symmetric peaks as shown in Figure 1. When formic acid was used (tested at various pH values and buffer concentrations), the eluted peaks were broad and not symmetric (e.g., tailing peaks) and had some degree of overlap. Also, the peak corresponding to PS was eluted very near to PE and was not well-separated from the latter. No difference in baseline is observed, although this was expected taking into account the difference in boiling points of the two acids.

Figure 2. Phase separation of the first separation step during extraction of PLs from BMP. Left, method of Rombaut et al.;[16] middle, own modified method with addition of $CaCl_2$; and right, B&D method.

Results of the Extraction Experiments. *Suitable Salt To Be Used as a Demulsifier for the Modified Method.* The liquid microfiltered BM was used for this preliminary test of which the procedure is described in the subsection Experiments in the Materials and Methods. The sample weight was 2.7 g, which was in accordance with level 2 of fixing initial sample weights. With this amount, it was impossible to complete the extraction with the method of B&D or Rombaut et al.[16] due to incomplete phase separation, especially for the second and the third steps. Microfiltered BM had a low mineral content, which resulted in a low ionic strength of the mixture and a poor phase separation during the extraction.

$CaCl_2$ and trisodium citrate have opposite effects on the stability of an emulsion system. Ca^{2+} ions cause aggregation of proteins and decrease their emulsifying capacity. Citrate may quench cations in the continuous phase and hence increase the emulsion stabilizing effect of the proteins. It was observed that the addition of both $CaCl_2$ and trisodium citrate accelerated the phase separation. The upper phase, after adding trisodium citrate, was white and opaque due to the presence of the emulsion. The upper phase, after adding $CaCl_2$, was transparent with a layer of precipitated proteins at the bottom (Figure 2). The addition of 3 and 6 mL of citrate salt solution could extract only 58.73 and 57.14% of the total PLs, respectively, as compared to the addition of 3 mL of $CaCl_2$ solution. Extracted concentrations of individual PL components were all reduced significantly when citrate was used as compared to $CaCl_2$.

Albalá-Hurtado et al. reported that the use of a demulsifying solution containing sodium citrate and sodium salicylate resulted in an increase of the total lipid extraction yield.[23] However, salicylate was soluble in chloroform, the lower phase during extraction in the case of the current study. The presence of salicylate in the injected solutions may be harmful for the silica packing material of the columns. On the contrary, trisodium citrate and $CaCl_2$ were not soluble in chloroform so it was expected that they would not enter the lipid phase (lower phase) during the extraction.

Table 2. Total PL Concentrations and PL Composition of Several Dairy Samples[a]

	total PLs % (w/w)		composition (% of total PLs)						
samples[b]	in sample	in total lipids	GluCer	LacCer	PI	PE	PS	PC	SM
commercial soft cheese	0.08 ± 0.002	0.41 ± 0.011	2.18 ± 0.18	6.10 ± 0.22	5.47 ± 0.17	22.55 ± 0.36	8.49 ± 0.34	30.01 ± 0.36	25.20 ± 0.10
commercial butter	0.24 ± 0.003	0.30 ± 0.003	1.97 ± 0.05	5.37 ± 0.17	5.32 ± 0.05	33.83 ± 0.06	7.05 ± 0.29	24.88 ± 0.11	21.59 ± 0.51
raw milk	0.03 ± 0.001	0.69 ± 0.019	1.95 ± 0.15	5.28 ± 0.01	5.69 ± 0.43	34.21 ± 0.50	5.83 ± 0.30	25.04 ± 0.12	22.01 ± 0.20

[a] Data are expressed as averages ± standard deviations of duplicate analysis. Total PLs included GluCer, LacCer, PI, PE, PS, PC, and SM. [b] Small amounts of PA, LPE, and LPC were detected (see chromatograms in the Supporting Information, Annex 4). However, these were not included in calculation of total PLS due to lack of standard. The total lipid content of the butter and the cheese was taken from the labels of the products. The total lipid content of raw milk was assumed to be 3.9%, an average value from the past analyses on raw milk from the delivery dairy farm.

Comparison of Different Extraction Methods. The results obtained for sample weights taken according to level 1 are given in Table 1. On the basis of total PLs, the extraction efficiency of the modified method was about 10% higher as compared to the method of Rombaut et al.[16] Extracted PL concentrations of the raw milk sample were not significantly different between the three extraction methods (values not shown). Raw milk contained much lower amounts of PLs as compared to the other two dairy samples. On a dry matter basis, raw milk contains on average 25, 31, 0.25, 5.4, and 36% total proteins, total lipids, PLs, ash, and lactose, respectively.[24]

Extracted concentrations of individual PLs from BMP and microfiltered BM are also shown in Table 1. For both materials, the application of the modified method extracted more ($p < 0.05$) GluCer, PE, and PC than that of the old method of Rombaut et al.[16] For the BMP sample, the modified method was also better for the extraction of PS. Although the difference in total PLs between the modified method and the B&D method was insignificant according to the ANOVA output (the two-sample t test, however, showed a significant difference at $p = 0.0026$), it was observed that the modified method improved significantly the extraction yield of GluCer and PS as compared to the B&D method (Table 1).

The modified method offered an immediate phase separation. For the extraction of raw milk samples, the methods of Rombaut et al.[16] and B&D showed a very slow phase separation, especially for the second and the third steps. It was even necessary to leave the separatory funnels overnight in a cooling chamber to obtain complete phase separation. The phase separation during extraction of PLs from BMP is illustrated in Figure 2. The upper phase during extraction with the modified method was transparent and had a bottom layer of precipitated proteins. While with the other two methods, the upper phases were opaque, indicating the presence of an emulsion or suspension.

To verify whether the addition of $CaCl_2$ indeed helped to extract more PLs instead of just being a result of PLs becoming more sensitive to detection by ELSD due to its possible interaction with $CaCl_2$, the modified extraction method was carried out with a milk PL standard solution with and without (the addition of) $CaCl_2$. No difference in PL concentration was found with and without the use of salt. Therefore, it could be concluded that the addition of $CaCl_2$ salt indeed improved the extraction efficiency of PLs from dairy materials.

For sample weight taken according to level 2, it was impossible (no phase separation) to apply the method of Rombaut et al.[16] for microfiltered BM without an increase in volume of the solvents. Therefore, only BMP was used as a sample for extraction as compared with the modified method. With level 2 (0.75 g), it was possible to extract BMP for PLs with the method of Rombaut et al.[16] (complete phase separation), but the efficiency was lower. More than 25% of the total PLs remained not extracted as compared to the modified method in level 1 (23.95 ± 0.92 as compared to 32.90 ± 0.38 mg/g sample). For the modified method with the addition of $CaCl_2$, no significant difference in the total extracted PLs between two levels of sample weight was noticed (32.20 ± 0.20 as compared to 32.90 ± 0.38 mg/g sample). Detailed data are in Annex 5 of the Supporting Information. Rose−Gottlieb, a recognized method for determination of total lipids from dairy ingredients, was reported to be an insufficient method for extraction of PLs because of the low extraction efficiency for PI and PS.[21] It is worth noticing that when the Rose−Gottlieb method is used for analyzing total lipids of microfiltered and ultrafiltered dairy products, which are characterized by low mineral content,[25] the addition of $CaCl_2$ is also necessary to improve the phase separation and hence the extraction efficiency.

The addition of $CaCl_2$, as a demulsifying agent and as a means to increase the ionic strength, maximized the extraction efficiency of the PLs. This modification also allows the use of a wider range (or higher level) of sample weights without the necessity to increase the volumes of organic solvents. The modified method was also one step shorter than the old method. The modified extraction method was selected for further evaluation.

Validation of the Analysis Procedure. The reproducibility of the analysis procedure (extraction and HPLC analysis) is presented in Table 1. For all PL components, batch RSD values were below 5%. For long-term reproducibility, only PS had RSD larger than 5%. This could be related to the quite broad peak of PS. The extraction recovery determined from the spiking experiment is shown in Table 1. Only the extraction of PE was incomplete. The analysis procedure was tested with several commercial dairy products, and the results are given in Table 2. These data were not standardized from the incomplete extraction of PE. It may need long-term repeated analysis to have data representative of these categories of dairy products. The data here just serve as a trial application of the suggested analysis procedure presented in this study. It was successful to analyze the concentration of total PLs as well as the relative composition among PL species. The total PLs/total lipids of the raw milk was within the range 0.56 −1.11% during lactation as reported by Bitman and Wood.[26] They also reported the changes of PL composition during lactation. During the churning process in making butter, part of the fat globule membranes is broken and shredded into the serum phase, namely, BM. The PL fraction, which does not associate with the membranes surrounding fat globules, for example, PLs of the dispersed broken membrane fragments in milk serum, is also streamed to the BM. These two reasons could explain for the lower PLs/total lipids of the butter as compared to that of raw milk. The ratio of PLs/total lipids of

the cheese was also smaller than that of raw milk. For the production of pizza cheese, Govindasamy-Lucey et al.[27] reported that the recovery of PLs was about 41%, while that of total lipids was 88%. Physical impact during processing such as agitation, cutting, stirring of the curd, and washing contribute to the lower recovery of PLs in the cheese matrix.[27] The total PL content as well as PL composition of some other dairy samples such as butter serum, BM whey, and microfiltered BM whey derived from the analysis procedure described in this study can be seen in another report.[25]

■ ASSOCIATED CONTENT

Supporting Information. Calibration curves, chromatograms of milk PL standard prepared in a nonpolar lipid matrix, and chromatograms of several dairy products. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; ELSD, evaporative light-scattering detector; PL(s), polar lipid(s); PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; LPC, lysophosphotidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; LacCer, lactosylceramide; GluCer, glucosylceramide; PG, phosphatidylglycerol; B&D, Bligh and Dyer; BM(P), buttermilk (powder); MFGM, milk fat globule membrane; RT(s), retention time(s)

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