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Comparison of dairy phospholipid preparative extraction protocols in combination with analysis by high performance liquid chromatography coupled to a charged aerosol detector

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ABSTRACT

Different fat extraction methodologies, Röse Gottlieb (RG), Folch with $CaCl_2$ (F1) and Folch without $CaCl_2$ (F2) were evaluated alongside a high performance liquid chromatography (HPLC)-based analytical protocol involving a charged aerosol detector (CAD) to optimise the separation and quantification of dairy phospholipids (PLs). Total PL recovery by F2 proved to be 1.8- and 2.5-fold greater than RG and F1, respectively. Alteration of the HPLC elution program maximised peak separation and reduced co-elution of individual PLs detected by CAD. Two-fold reduction in retention time variability was achieved, <0.15 min, compared with the original method. Improved PL recovery was reflected in the higher PL values obtained from different dairy streams using the optimised protocol, 35.32 ± 0.01% and 46.09 ± 0.01% total PL, for buttermilk and butter serum respectively. Phosphatidylinositol and phosphatidylserine, in particular, demonstrated increases of between 2% and 7%, of total PL, compared with previous studies.

1. Introduction

Phospholipids (PLs) are a class of complex polar lipids with an inherent amphiphilic nature due to the presence of a hydrophobic fatty acid tail and a hydrophilic head (Contarini & Povolo, 2013; Donato et al., 2011; Kielbowicz, Micek, & Wawrzenczyk, 2013; Rombaut & Dewettinck, 2006; Rombaut, van Camp, & Dewettinck, 2006). PLs are subdivided into glycerophospholipids and sphingophospholipids (Avalli & Contarini, 2005; Contarini & Povolo, 2013; Donato et al., 2011; Kielbowicz et al., 2013; Rombaut, van Camp, & Dewettinck, 2005; Rombaut et al., 2006). Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are the major glycerophospholipids, while phosphatidylserine (PS), phosphatidylinositol (PI) represent the minor glycerophospholipids. Phosphatidic acid (PA) is rarely reported in dairy products and its presence is usually attributed to poor sample preparation/storage or phospholipase activity. Sphingomyelin (SM) is the dominant species of sphingophospholipid (Avalli et al., 2005; Contarini et al., 2013; Le et al., 2011; Rombaut et al., 2005; Rombaut, Dewettinck, & Van Camp, 2007).

PLs are composed of a glycerol backbone to which two fatty acids, mainly unsaturated in nature, are esterified at the Sn-1 and Sn-2 positions. A phosphate group is attached at the Sn-3 position, to which a polar head group may be linked (choline, ethanolamine, serine). In general, the fatty acid attached at the Sn-1 position is more saturated than that found at the Sn-2 position (Christie, 1994, 2003; Donato et al., 2011; Rombaut et al., 2006). The sphingoid base, e.g., sphingosine, a long chain aliphatic amine containing two hydroxyls, is the characteristic subunit of sphingolipids. Attachment of a fatty acid, usually a saturated fatty acid, to the amino group of the sphingoid base results in the formation of a ceramide unit, to which an organophosphate group can be attached forming a sphingophospholipid. In the case of SM, phosphocholine represents the organophosphate group (Avalli et al., 2005; Contarini et al., 2013; Dewettinck et al., 2008; Donato et al., 2011).

PLs may represent only 1–5% of total milk lipids, but are distinctive because of their polar nature that underpins their structural and functional role in the formation of the natural emulsifying layer surrounding fat globules in milk, i.e., the milk fat globule membrane (MFGM; Contarini & Povolo, 2013; Sánchez-

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Juanes, Alonso, Zancada, & Hueso, 2009). The MFGM has a tripartite structure composed of an inner monolayer of proteins and a polar lipid, followed by a 'true' outer bilayer, and originates from the apical plasma membrane of the mammary gland secretory cells (Dewettinck et al., 2008; Keenan, 2001). The amphiphilic nature of PLs facilitates the formation of bilayers and, thus, aids in the emulsification of fat in milk (Contarini et al., 2013; Deeth, 1997; Dewettinck et al., 2008; Evers, 2004; Heid & Keenan, 2005; Rombaut & Dewettinck, 2006; Rombaut et al., 2006).

In recent decades, PLs have gained considerable interest due to their nutritional and technological functionalities which have been extensively studied and reviewed (Contarini et al., 2013; Dewettinck et al., 2008). Dairy PLs are of particular interest as milk contains a higher content of SM and PS compared with other sources (Burling & Graverholt, 2008). SM, through its bioactive metabolites ceramide and sphingosine, plays important roles in cell regulation and is referred to as a tumour suppressor (Contarini et al., 2013; Dewettinck et al., 2008; Parodi, 1997; Rombaut et al., 2006). Reports on cognitive performance improvement, with particular significance to Alzheimer's Disease treatment have been attributed to the biological activity of PS (Burling et al., 2008; Contarini et al., 2013; Dewettinck et al., 2008; Pepeu, Pepeu, & Amanducci, 1996; Rombaut & Dewettinck, 2006). Other positive biological effects associated with PLs include reduced incidence of cardiovascular disease, cholesterol absorption, antioxidative properties, stress and depression tolerance and also suppression of multiple sclerosis (Contarini et al., 2013; Dewettinck et al., 2008; Rombaut & Dewettinck, 2006). MFGM is also implicated in the physiochemical functionalities of dairy products such as emulsification, wettability, heat stability and viscosity (Contarini et al., 2013; Corredig & Dalgleish, 1998a, 1998b; Dewettinck et al., 2008; Rombaut & Dewettinck, 2006; Sodini, Morin, Olabi, & Jiménez-Flores, 2006). Milk is natural and abundant source of these highly bioactive compounds and thus there is a growing need to establish reliable methodologies for their compositional analyses.

With laboratory techniques for the measurement of PLs in milk evolving rapidly in recent years, it was opportune to reappraise the lipid extraction procedures typically used for food analyses as well as that specifically designated in international standards for milk. The most popular methods for lipid extraction include the IDF standard-based Röse Gottlieb (RG) (IDF Standard 9C; IDF, 1987), the Folch method (Folch, Lees, & Stanley, 1957) or that of Bligh and Dryer (1959) (B&D). RG exploits the use of ammonia to break lipo-protein bonds, thus allowing the lipid to be dissolved in the ether. Folch and B&D are techniques that utilise a chloroform/methanol-based solvent system to solubilise the lipids thus allowing extraction from the milk matrix.

PLs can then be analysed via thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and ^{31}P nuclear magnetic resonance spectroscopy (^{31}P NMR), all of which provide varying degrees of PL spectra. HPLC is the preferred method due to low cost compared with NMR and is widely used in laboratories (Rombaut et al., 2005). HPLC coupled to an evaporative light scattering detector (ELSD) is a regularly-featured method of PL determination and quantification (Le et al., 2011; Rombaut et al., 2007).

More recently, a detector based on charging aerosol particles by corona discharge, i.e., charged aerosol detector (CAD), has been introduced and proven to have greater sensitivity and better precision than that of the ELSD (Contarini et al., 2013; Hazotte, Libong, Matoga, & Chaminade, 2007; Kielbowicz et al., 2013; Ramos et al., 2008).

This study set out to investigate and update current methodology with regards to lipid extraction and PL analysis via HPLC-CAD. Three lipid extraction methods were compared to determine the method that offered the greatest extraction efficiency of PLs from an unpasteurised milk sample. The HPLC method of Le et al. (2011) for PL analysis was optimised for use in combination with CAD. The more efficient extraction protocol was paired with the modified HPLC method to analyse PLs in dairy streams generated during simulated processing of unpasteurised raw milk substrate.

2. Materials and methods

2.1. Materials

All chemicals were procured from Sigma Aldrich (Arklow, Ireland) unless otherwise stated. The main solvents used, dichloromethane, chloroform and methanol were Chromasolv® Plus, of HPLC grade, >99.9% (all except methanol contained amylene as a stabiliser). For identification purposes, pure (≥99.9%) phospholipid standards, sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were purchased from both Sigma Aldrich and INstruchemie BV (Avanti Polar Lipids Inc., Delfzijl, The Netherlands). Fresh, unpasteurised bovine milk was sourced from Teagasc Dairy Farm, Moorepark, Fermoy, Ireland. All subsequent dairy streams were produced from the original sample.

2.2. Generation of different dairy streams

Skim milk and cream were produced from the original unpasteurised milk sample (Teagasc Dairy Farm) through centrifugation using a bench top Armfield disc bowl centrifuge (Armfield, Ringwood, UK). Briefly the milk was heated in a water bath to 54 °C for approximately 1 h where after it was applied to the feed reservoir and subjected to centrifugation to separate the heavy phase (skim milk) from the lighter phase (cream). The cream was standardised to 45.43 ± 0.04% fat through determination of fat content by Röse Gottlieb with subsequent addition of skim milk as required.

Cream was kept refrigerated at 4 °C overnight to allow the fat globules to crystallise. The cream was then subjected to severe agitation with a Kenwood food mixer (Kenwood, Havant, UK) until butter grains were formed during emulsion inversion and the serum phase (buttermilk) was expelled. No washing of the butter grains was carried out during the subsequent working.

Butter was kept refrigerated for 2 d after which it was heated to 73 °C in a water bath for 1 h. The heated butter was then subjected to centrifugation with an Armfield bench top disc bowl centrifuge (Armfield) to separate the lighter triglyceride phase, butter oil, from the heavier serum phase, butter serum.

2.3. Lipid extractions

Milk lipids were isolated as a crude lipid fraction according to three different lipid extraction methodologies; Röse Gottlieb as per IDF Standard 9C (IDF, 1987), Folch modified by Rombaut et al. (2005) and Folch according to Rodriguez-Alcala and Fontecha (2010). In each case the lipid extracts were dried to complete dryness under N_2 and stored in 20 mL of HPLC grade $CHCl_3$/MeOH (88:12, v/v) at −20 °C until HPLC analysis.

For fat extraction according to Röse Gottlieb (IDF, 1987) (RG), 10 mL samples were weighed into a Mojonnier flask, to which 2 mL of ammonia solution (2%) was added and the sample was vortexed. To this, 10 mL of absolute ethanol was added, followed by addition of 25 mL diethyl ether and 25 mL petroleum ether, with vortexing following each subsequent addition. The samples were subjected to centrifugation at 81 × *g* for 5 min, where after the etheral layer was collected. A second wash was carried out on the lower phase with addition of 5 mL ethanol, 15 mL diethyl ether and 15 mL petroleum

ether. The etheral layers were pooled and allowed to evaporate at 105 °C in a ventilated fume hood.

The Folch method for fat extraction according to Rombaut et al. (2005) (F1) involved addition of 3 mL 10% (w/v) $CaCl_2$ to 10 mL of sample made up to 20 mL final volume with dH_2O. Sample was transferred to a separation funnel with 80 mL $CHCl_3$/MeOH (2:1, v/v) with vigorous agitation. Following phase separation, the lower $CHCl_3$ phase was collected and the upper phase was subjected to two more washes with 40 mL $CHCl_3$/MeOH (20:1, v/v). The $CHCl_3$ layers were pooled and evaporated at 43 °C in a Büchi rotary evaporator (BÜCHI, Flawil, Switzerland).

For fat extraction according to the Folch method of Rodriguez-Alcala and Fontecha (2010) (F2), 10 mL of sample was mixed with 75 mL $CHCl_3$:MeOH (2:1, v/v) and agitated at 4 °C for 1 h. Sample was subjected to centrifugation of 2330 × *g* for 5 min at 4 °C, after which the lower $CHCl_3$ phase was collected. The upper phase was mixed with 50 mL $CHCl_3$ and incubated at 4 °C for 30 min under agitation. The $CHCl_3$ layers were pooled and evaporated at 43 °C in a Buchi rotary evaporator (BÜCHI).

All extractions were performed in triplicate.

2.4. Compositional analysis

Compositional analysis was carried out on all samples and included the following: % protein via Kjeldahl (ISO, 2014) with 6.38 conversion factor, % total solids/moisture and ash via oven test (ISO, 2010), and lactose was determined polarimetrically (AOAC, 2012).

2.5. Chromatographic analysis

Separation and identification of the polar lipids was accomplished with a Waters 2595 HPLC system (Waters Ireland, Dublin, Ireland) coupled to CAD (Thermo Scientific, Hemel Hempstead, UK) with N_2 as the nebulising gas with a flow rate of 2.1 L min^{-1}. A 3 μm particle diameter Prevail Silica column (Grace Sciences, Labquip, Dublin, Ireland), 150 × 3 mm, with a guard column of the same packing and internal diameter, was utilised for the separation. The method was adapted and optimised from Le et al. (2011), and involved a linear gradient elution of two mobile phases; A: 100% dichloromethane, B: MeOH:triethylamine/acetic acid buffer, pH 3.5 (500:21, v/v). The ratio in volume of solvent A to B was as follows and was maintained until time T = 27 min; 96:4 at T = 0, 25:75 at T = 20, 6:94 at T = 21 and 96:4 at T = 22. The temperature of the column oven was set at 40 °C, while the sample chamber of the autosampler was maintained at a lower temperature of 15 °C. An injection volume of 25 μL was used, with each injection performed in duplicate. The parameters set for the CAD were as follows; filter time constant: none, scale output range: 500 pA, and the operating pressure was maintained at 241.32 kPa. Samples were dissolved in 20 mL HPLC $CHCl_3$:MeOH prior to HPLC-CAD analysis. For identification and quantification purposes, pure PL standards were used to determine the relative retention times of each PL species, the detection limit of the detector and to generate calibration curves.

All PL standards were dissolved in HPLC $CHCl_3$:MeOH to concentrations of 100 μg mL^{-1} from which serial dilutions were made down to a concentration of 5 μg mL^{-1}. Standards were injected in duplicate, and a calibration curve was generated for each PL through determination of the area under the elution peak for each standard and plotting the area (μV × s × 10^5) against concentration (μg mL^{-1}) to obtain a second order polynomial regression.

2.6. Accuracy and limits of HPLC detection system

The accuracy of the HPLC method was determined via standard addition. The responses of anhydrous milk fat (AMF) alone and AMF spiked with two known concentrations, 8 μg mL^{-1} and 80 μg mL^{-1}, of each individual PL standard and a mix of PL standards was determined, with % recovery calculated using the following equation (Vervoort, Daemen, & Török, 2008);

$$\%\text{Recovery} = \frac{A_{\text{measured}}}{A_{\text{theoretical}}} \times 100 \quad (1)$$

where, A_{measured} was the value obtained for the response of the spiked AMF at either 8 μg mL^{-1} and 80 μg mL^{-1} and $A_{\text{theoretical}}$ was the theoretical value determined for the response.

The % recovery for all PLs is represented in Table 1 and all lie within the acceptance criteria range of 98% and 102% for method validation. The PL response in the un-spiked AMF was below the limits of detection for the detector.

The limit of detection (LOD) and limit of quantification (LOQ) was determined for the detector for each individual PL. A summary of the LODs and LOQs for the detector are represented in Table 1. The LODs and LOQs were estimated using the following equations (Patel, Patel, & Gajra, 2011);

$$LOD = \frac{3.3\delta}{S} \quad (2)$$

$$LOQ = \frac{10\delta}{S} \quad (3)$$

where, δ is the standard deviation of the response and S is the slope of the calibration curve. The S was determined via calculation of the first derivative of the second order polynomial equation for each PL calibration curve.

2.7. Statistical analysis

Statistical analysis was performed with Minitab, version 15 (Minitab Ltd, Coventry, UK), with one-way ANOVAs and Tukey Tests for both intra- and inter-sample variance.

3. Results and discussion

3.1. HPLC-CAD analysis

To evaluate the linearity of the CAD system, the individual PL standards were analysed at concentrations ranging from 5 μg mL^{-1} to 100 μg mL^{-1}. The performance of CAD is known to generate a

Table 1
Validation of the HPLC-CAD system in terms of accuracy, limit detection and limit of quantification.[a]

Validation parameter	PA	PI	PE	PS	PC	SM
Recovery (%) as individual PLs						
8 μg mL^{-1}	100.49	98.82	99.50	100.15	100.16	99.08
80 μg mL^{-1}	99.96	100.22	100.06	99.95	99.99	99.85
Recovery (%) in a PL mix						
8 μg mL^{-1}	99.51	101.18	100.46	99.84	99.84	100.92
80 μg mL^{-1}	98.05	100.22	99.94	100.05	100.01	100.15
LOD (ng)	13.74	28.57	8.90	2.06	9.50	60.34
LOQ (ng)	41.66	86.58	27.03	6.25	29.03	182.86

[a] Accuracy is represented as % recovery for both individual and a mixture of phospholipid (PL) standards at two concentrations of spiking into anhydrous milkfat (8 μg mL^{-1} and 80 μg mL^{-1}); abbreviations are: PA, phosphatidic acid; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin. Limit of detection (LOD) and limit of quantification (LOQ), represented in nanogram for each PL, were determined by the standard deviation of the response based on the slope of the calibration curve with respect to the amount of PL injected onto the system.

non-linear response (Davies, Bailey, Plante, & Acworth, 2014; Liu, Fang, Cauchon, & Zhou, 2008; Nair & Werling, 2009) and so a second order polynomial relationship was fitted to obtain calibration curves with R_2 values ranging from 1.00 to 0.99 (Supplementary Fig. S1A).

To test the elution capability of the original method published by Le et al. (2011), a mix of the PL standards was injected onto the HPLC-CAD system. At pH 4.5 and a run time of 22.5 min (as per the original method), co-elution of PL species PA and PI, made it difficult to determine clear peaks for the individual PL species. Altering the pH to 5.0, 4.0, 3.5 and 3.0 led to pH 3.5 being identified as enabling the best separation of peaks of interest (Fig. 1). To further optimise separation of the individual PL peaks, the elution program was altered for the HPLC-CAD run using a 75% gradient. It was determined that by extending the run time to T = 27, optimum separation of the peaks was obtained.

PI and PE eluted as sharp defined peaks while PS eluted as a broad peak (Fig. 2c). PC and SM also eluted as broad peaks but each were characterised by two sub-peaks, PC 1, PC 2 and SM 1, SM 2 respectively (Fig. 2c). These elution patterns are in agreement with those described by Deschamps et al. (2001) and Fagan and Wijesundera (2004). Sub-peaks for both bovine PC and milk fat SM standards were observed due to the large number of molecular species that exist of which lipid chemists have been aware of for many years (Christie, Noble, & Davies, 1987; Karlsson, Michelsen, & Odham, 1998; Morrison, Jack, & Smith, 1965).

Examples of chromatograms obtained from the HPLC-CAD comparing the three lipid extraction methodologies are given in Fig. 2. The chromatograms demonstrated peaks that were well defined and separated for the individual PLs. From the chromatograms it was determined that F2 (Fig. 2c) offered the greatest

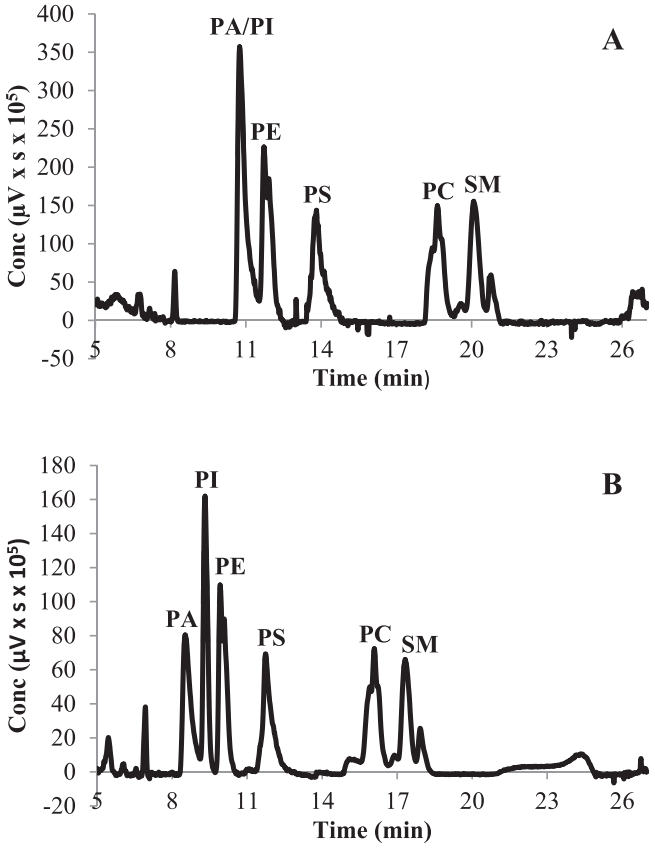

Fig. 1. Chromatograms of the phospholipid standard mix at (A) pH 4.5, and (B) pH 3.5.

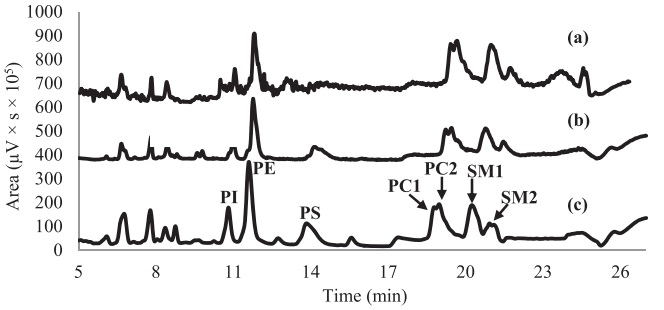

Fig. 2. HPLC-CAD chromatogram of lipid extractions, Röse Gottlieb (RG), Folch with $CaCl_2$ (F1; Rombaut et al., 2005) and Folch without $CaCl_2$ (F2; Rodriguez-Alcala & Fontecha, 2010), from raw milk sample where (a); RG, (b); F1 and (c); F2. Peak identification determined based on matching with relative standard retention times for each PL.

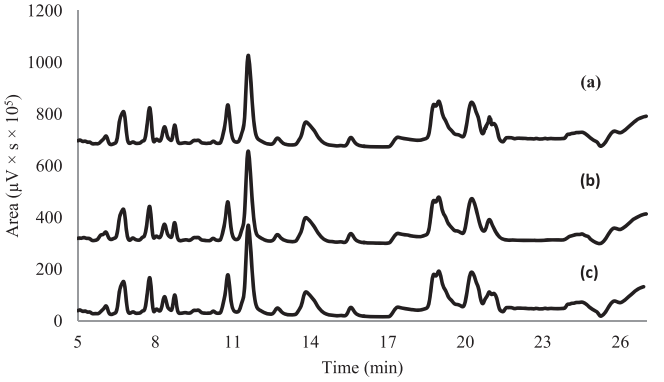

Fig. 3. Chromatogram overlay of the same crude raw milk sample injected on three different days to determine HPLC-CAD method reproducibility: a, day 1; b, day 2; c, day 3.

amount of total PL recovery by quantifying with respect to the calibration curves. Complete absence of the peak representing PS was shown in the chromatogram for the RG extract (Fig. 2a), with a low response in the peak representing PI. It has been noted in the literature that RG has significant losses of the more acidic PLs, i.e., PI and PS. This reduced efficiency in PL extraction has been attributed to the presence of ammonia in the RG extraction that may increase the water solubility of PI and PS and thus lead to losses of these PLs in the final crude extract (Avalli & Contarini, 2005; Le et al., 2011). Reproducibility of the HPLC-CAD method was determined through duplicate injections of each sample on the same day for three different days (i.e., 18 injections per day; Fig. 3.). The reproducibility was indicated by RSDs <0.95% for each individual PL species (Table 2). Variation in peak RTs was found to be minimal, no more than 0.15 min, for each injection on each of the three different d (Fig. 3). This variation may be due to solvent quality or slight differences in the pH of the buffer phase, however, it is half that described by the authors of the original method (Le et al., 2011). Le et al. (2011) also noted that variation in peak RT may be introduced through sensitivity of the optic system, an error which does not arise with charged aerosol detection thus making the CAD a more attractive detection system not only for reproducibility but also for low maintenance purposes.

3.2. Comparison of the lipid extraction methods

Comparison of the three extraction methods was based on the amount of total PL present in the crude lipid extract from the raw

Table 2
Comparison of the three extraction methods, Röse Gottlieb, Folch with $CaCl_2$ and Folch without $CaCl_2$, based on total phospholipid (PL) recovery.[a]

Extraction method	Fat extracted (%)	Total PL (% fat)	PI (% total PL)	PE (% total PL)	PS (% total PL)	PC (% total PL)	SM (% total PL)
Röse Gottlieb	4.17 ± 0.23	1.20 ± 0.11	3.35 ± 0.73	21.34 ± 0.35	–	41.76 ± 0.20	33.53 ± 0.34
Folch with $CaCl_2$	4.13 ± 0.24	0.89 ± 0.22	7.88 ± 0.95	36.37 ± 0.20	11.53 ± 0.60	23.07 ± 0.35	21.13 ± 0.13
Folch without CaCl	4.08 ± 0.22	2.30 ± 0.03	7.79 ± 0.58	23.04 ± 0.07	9.60 ± 0.29	36.02 ± 0.25	23.35 ± 0.13

[a] Data for fat are the average % fat (±% RSD) of triplicate extractions for each extraction method (RG, F1 and F2) on three different days (9 extractions per extraction method total). Data for PLs are the average % total PL (±% RSD) determined from duplicate injections per sample for three different days.

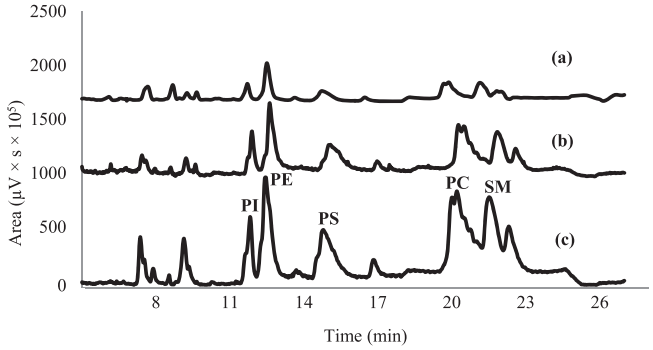

Fig. 4. Chromatogram overlay of lipid extractions from (a) milk, (b) buttermilk and (c) butter serum, with sample comparison based on total phospholipid determined via quantification with calibration equations.

milk sample. Both the Röse Gottlieb (RG) and Folch with $CaCl_2$ (F1) (Rombaut et al., 2005), generated the greater recovery of fat (expressed as % fat extracted), i.e., 4.17 ± 0.23% and 4.13 ± 0.24%, respectively, compared with 4.08 ± 0.22% fat for the Folch without $CaCl_2$ (F2) method (Rodriguez-Alcala & Fontecha, 2010) (Table 2). Each extraction method was performed in triplicate on three different d to determine the reproducibility of the method and to rule out any external factors that may interfere with the extraction.

With the focus of method selection based on PL recovery, statistical analysis (one way ANOVAs) determined that the three extraction methods were significantly different, $F(5,13)$, $P < 0.01$. Milk extracted with the RG and F1 methods had significantly lower total PL recovery, 1.20 ± 0.11% and 0.89 ± 0.22%, respectively, compared with the recovery from the F2 method, 2.30 ± 0.03% total PL, as a % of total lipid extracted (Table 2). Complete loss of PS and significant reduction in the presence of PI was noted in the RG extract. As mentioned (Section 3.1), this absence has been noted by other authors and has been associated with the presence of ammonia in the extraction procedure. The use of heat to evaporate the solvents also contributes to hydrolysis and oxidation of the PLs (Gallier, Gragson, Cabral, Jimenez-Flores, & Everett, 2010a). Hence, a cold extraction such as that provided by the Folch methodologies is preferred.

The F1 method calls for the addition of a 10% $CaCl_2$ solution to create sufficient phase separation during the solvent extraction procedure. While the addition of a salt may be successful in effecting 'salting out' and, thus, formation of a two-phase system, as mentioned by Le et al., (2011), the upper phase may retain the more polar lipids such as some PLs (Akoh & Min, 2008; Christie, 2003). It was also noted that residual salt in the crude lipid extract impacted negatively on the CAD detector. CAD measures analyte particles for its mode of detection and so requires volatile solvents and buffers to ensure optimum sensitivity. Thus the addition of residual ions from the $CaCl_2$ in the extraction will interfere with the CADs sensitivity and detection capability thereby inhibiting accurate measurement of the PLs.

Overall the F2 method yielded the greatest amount of total PL, 2.30 ± 0.03% (as a % of total fat extracted), 1.9 times that recovered in RG and 2.5 times that recovered in F1 (Table 2). Total PLs was determined by the sum of PE, PC, PI, PS and SM and the results were expressed as a % of the total fat extracted (Table 2). The absence of PA was noted in all chromatograms (Figs. 2–4). PA is usually not reported in milk products as it is a PL breakdown product. Its presence would lead to the indication of improper sample preparation/handling or lipolysis (Le et al., 2011; Rombaut et al., 2005). It was noted that the percentages of PC and SM represented in Table 2 were higher in the RG extract when compared with that of F1 and F2. This increased % was due to the fact that PC and SM represent two of four PLs recovered in the RG extract while they represent two of five PLs recovered in the F1 and F2 fractions thus leading to a higher calculated % with respect to the total PL due to the lesser amount of PL species recovered in the RG extract.

As mentioned, the reproducibility of the method was determined by performing the extraction three times for three different days (total of 9 extractions per sample) with a relative standard deviation of 0.22% achieved (Table 2).

3.3. Phospholipid partitioning within different dairy streams

The partitioning of PLs between different dairy streams originating during simulated processing of unpasteurised milk was studied using the adapted lipid extraction and HPLC protocol.

It has been reviewed extensively in the literature that PLs have a stronger affinity for the serum phases, i.e., buttermilk and butter serum. Surface-bound MFGM material present in the milk is

Table 3
Compositional analysis of all dairy streams during laboratory scale preparation of butter from milk with respect to protein (Kjeldahl), lactose (polarimetric), ash and total solids (oven tests).[a]

Dairy stream	Fat (%)	Protein (%)	Lactose (%)	Ash (%)	Total solids (%)
Milk	4.08 ± 0.22	3.12 ± 0.32	4.79 ± 0.12	0.68 ± 0.29	13.71 ± 0.07
Skim milk	0.74 ± 0.94	3.43 ± 0.29	4.36 ± 0.18	0.74 ± 0.41	9.27 ± 0.22
Cream	31.53 ± 0.94	2.15 ± 0.46	3.10 ± 0.38	0.63 ± 0.32	37.41 ± 0.08
Butter	78.17 ± 0.01	0.93 ± 0.12	0.72 ± 0.07	0.15 ± 0.13	79.97 ± 0.12
Buttermilk	2.80 ± 0.54	3.36 ± 0.29	4.23 ± 0.31	0.72 ± 0.14	11.11 ± 0.14
Butter oil	99.82 ± 0.01	0.05 ± 0.40	0.00 ± 0.00	0.00 ± 0.00	99.87 ± 0.01
Butter serum	2.15 ± 0.93	3.54 ± 0.85	4.15 ± 0.14	0.72 ± 0.13	10.56 ± 0.85

[a] Data are expressed as the average (±% RSD) of three repeats for fat, ash and total solids and two repeats for protein and lactose.

Table 4
Phospholipid (PL) composition and relative concentrations with respect to fat extracted from different dairy streams.[a]

Sample	Fat (%)	Total PL (%)	PI (%)	PE (%)	PS (%)	PC (%)	SM (%)
Milk	4.08 ± 0.22	2.30 ± 0.03	7.79 ± 0.58	23.24 ± 0.07	9.60 ± 0.29	36.02 ± 0.25	23.35 ± 0.13
Skim milk	0.74 ± 0.94	11.07 ± 0.15	8.45 ± 0.89	26.70 ± 0.10	8.41 ± 0.07	35.22 ± 0.06	21.22 ± 0.30
Cream	31.53 ± 0.94	0.37 ± 0.10	11.77 ± 0.29	30.15 ± 0.08	12.77 ± 0.17	24.43 ± 0.07	20.86 ± 0.17
Butter	78.17 ± 0.01	0.09 ± 0.10	9.49 ± 0.46	43.28 ± 0.14	8.19 ± 0.43	22.41 ± 0.18	16.62 ± 0.17
Buttermilk	2.80 ± 0.54	35.32 ± 0.01	9.63 ± 0.08	27.62 ± 0.04	10.10 ± 0.02	31.50 ± 0.03	21.49 ± 0.06
Butter oil	99.82 ± 0.01	0.01 ± 0.74	–	20.35 ± 0.62	–	53.70 ± 0.03	25.95 ± 0.03
Butter serum	2.15 ± 0.93	46.09 ± 0.001	9.27 ± 0.03	27.69 ± 0.02	6.93 ± 0.09	27.18 ± 0.01	28.92 ± 0.01

[a] Data for fat are the average % fat (±% RSD) of three replicates for each sample on three different days; data for % total PL are the average (±% RSD) of duplicate injections of each sample for three different days and for individual PLs are % total PL (summation of individual PL species).

released into the serum or skimmed milk phase upon centrifugation (Gallier et al., 2010a; Gallier, Gragson, Jiménez-Flores, & Everett, 2010b; Plantz, Patton, & Keenan, 1973; Rombaut et al., 2006). Aeration or agitation, such as that which occurs during the butter-making process, leads to emulsion inversion following complete disruption of MFGM. This creates a phase separation of lipids, butter, and water soluble components of the MFGM, which includes PLs, being recovered in the buttermilk (Gallier et al., 2010a, 2010b; Rombaut et al., 2006). Cold storage of cream (fat crystallisation) prior to agitation may also contribute to specific migration of PLs to the serum phase, buttermilk (Dewettinck et al., 2008). HPLC-CAD analysis of F2 crude extracts from all dairy streams demonstrated this PL affinity for the serum (liquid) streams. The compositional analysis of all dairy fractions analysed is represented in Table 3, where % fat was determined using the F2 method.

The concentrations of PLs in the dairy streams analysed (Table 4) were in line with those in the current literature (Dewettinck et al., 2008; Rombaut et al., 2006). A PL concentration effect was observed in the serum streams, with total PL content (represented as a % of total fat extracted) increasing to 35.32 ± 0.0% and 46.09 ± 0.01% for the buttermilk and butter serum fractions respectively (Table 4). Total PLs recovered from the different streams using the optimised protocol are greater than that previously published (Avalli et al., 2005; Rombaut et al., 2005), in particular with respect to the values obtained for PI and PS, which are higher in all fractions. For example, in buttermilk analysed using the optimised method, the values for PI and PS are 9.63 ± 0.08% and 10.10 ± 0.02% as a % of total PL, respectively, which is an increase of between 2% and 7% compared with published data (Avalli et al., 2005; Le et al., 2011; Rombaut et al., 2005). This increase in PL recovery is attributed to both the F2 extraction method and also the detection sensitivity and precision offered by the CAD (Contarini et al., 2013; Hazotte et al., 2007; Kielbowicz et al., 2013; Ramos et al., 2008). An overlay of the chromatograms representing milk, buttermilk and butter serum is shown in Fig. 4. Well-defined peaks represent each of the individual PL species with the characteristic PC and SM sub-peaks clearly noticeable. The relative concentration of the individual PL species in each dairy fraction is represented in Table 4. From both the chromatograms (Fig. 4) and the results displayed in Table 4, butter serum (Fig. 4c) with the highest concentration of total PLs (46.09 ± 0.01%) would appear to be an excellent source of PL recovery except that it is not as freely available as buttermilk. Thus, buttermilk (Fig. 4b) with its high PL content (35.32 ± 0.01%) is a very attractive source for PL analysis, i.e., extraction and enrichment.

4. Conclusions

This study demonstrated a number of key observations with the aim of the work to compare three different fat extraction methodologies in order to determine the method which offered the greatest recovery of dairy PLs and to optimise a HPLC method to achieve adequate separation and quantification of the PLs.

Firstly, the Folch, F2, method was the optimum method for lipid extraction in order to obtain the greatest recovery of PLs as alcohol allowed complete solubilisation of the MFGM polar lipids. A two-fold increase in total PL recovery was obtained in this study using the F2 lipid extraction coupled with the more sensitive CAD detection system when compared with that originally outlined by Le et al., (2011). Values obtained for total PL recovery, 2.30 ± 0.03% in milk, are higher compared with previously published works. In particular a greater recovery of the more acidic PLs, PI and PS, was achieved in all dairy fractions, with values increasing by 2% and 7% for both PLs as represented as a percentage of the total PLs.

Fine tuning of pH and run time produced chromatograms that demonstrated stable retention times and were reproducible in duplicate runs on different d thus creating a robust methodology for PL analysis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.idairyj.2016.01.022.

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