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Pilot scale production of a phospholipid-enriched dairy ingredient by means of an optimised integrated process employing enzymatic hydrolysis, ultrafiltration and super-critical fluid extraction.

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Abstract

Pilot scale production of a dairy ingredient enriched in phospholipids (PLs) was generated from a buttermilk powder (BMP) substrate utilising a combined process of targeted enzymatic hydrolysis of the innate milk proteins followed by ultrafiltration with a 50 kDa membrane. An 8.5 fold increase in PL was achieved in the 50 kDa retentate (50 R) compared to the BMP, 11.05 ± 0.02 % and 1.30 ± 0.00 % total PL, respectively. Simultaneously, total lipid content in the retentate increased 8.7 fold with reference to the BMP, 60.07± 0.54 % and 6.84 ± 0.17 % total lipid respectively. Protein reduced to 10.58 ± 0.09 % (50 R) from 31.40± 0.57 % in BMP. Supercritical CO_2 fluid extraction (SFE) was employed to generate a purified lipid fraction. SFE with ethanol as a co-solvent yielded a purified lipid extract with enriched PLs level of 56.24 ± 0.07 % on a dry matter basis.

Industrial relevance

PLs have many associated health and nutritional benefits including those related to cognitive development and repair. Generation of an ingredient enriched in dairy PLs would be advantageous from an industrial view to allow fortification of nutritionals, both infant and geriatric, in promoting brain health. The present work demonstrates a novel pilot scale process for the generation of a PL enriched ingredient from a BMP substrate. Utilising a combined process of targeted protein hydrolysis followed by selective removal by ultrafiltration of the smaller molecular weight peptide material, an ingredient with 8.5 fold increase in PL material was achieved. SFE technology was utilised to generate a purified lipid extract with greater PL levels for future applications in biological assays to determine these pathways. The need to investigation and further develop the knowledge relating to the modes of action of these bioactive compounds would be beneficial from a nutritional perspective.

Key words;

Pilot-scale, buttermilk powder (BMP), phospholipids (PLs), hydrolysis, ultrafiltration, supercritical CO_2 fluid extraction (SFE).

1. Introduction

The milk fat globule membrane (MFGM) is a unique biophysical system (Lopez, 2011; Singh, 2006) surrounding milk fat globules that facilitates their dispersal in milk. This surface active multi-layered membrane is derived from the apical membrane of the secretary cell in the mammary gland (Heid & Keenan, 2005) and is composed of numerous bioactive molecules of which 90 % of MFGM dry weight is represented by proteins and polar lipids (Lopez, 2011). Phospholipids (PLs), a complex class of polar lipids, whose structure comprises of a hydrophilic head group and a hydrophobic fatty acid tail (Contarini & Povolo, 2013), are amphiphilic in nature. This distinctive polar property is responsible for the structural architecture of MFGM which, in turn, aids in the emulsification of fat in milk (Barry, Dinan, Murray, & Kelly, 2016; Contarini & Povolo, 2013; Sánchez-Juanes, Alonso, Zancada, & Hueso, 2009).

Buttermilk, an aqueous serum phase released during cream de-emulsification/churning, possesses PL concentrations approx. 15x that of whole milk (Barry et al., 2016; Rombaut, van Camp, & Dewettinck, 2005) and, hence, is a preferred substrate for the PL enrichment. Dairy PLs are of considerable interest due to their particular prominence of sphingomyelin (SM) and phosphatidylserine (PS) (Burling & Graverholt, 2008). The technological and nutritional functionalities of PLs have been extensively studied (Contarini & Povolo, 2013; Dewettinck, Rombaut, Thienpont, Le, Messens, & van Camp, 2008). PLs, due to their emulsification and stabilisation properties afforded by their inherent amphiphilic nature are widely utilised by the food and dairy industry for their associated

physiochemical roles in dairy products that include emulsification, heat stability, wettability and viscosity (Contarini & Povolo 2013; Corredig & Dalgleish 1997; Corredig & Dalgleish 1998; Dewettinck et al.,, 2008).

Likewise, there have been numerous health and nutritional benefits associated with PLs e.g. reduction in the incidence of cardiovascular disease, tolerance of depression and stress and myelination of the central nervous system (Contarini & Povolo 2013; Dewettinck et al.,, 2008; Oshida, Shimizu, Takase, Tamura, Shimizu & Yamashiro, 2003). Sphingomyelin (SM), mainly through its sphingosine and ceramide metabolites, has been shown to affect anticancer activity notably in the suppression of colon tumours (Berra, Colombo, Scottocotnola, & Giacose, 2002; Hertevig, Nilsson, Cheng, & Duan, 2003; Kuchta-Noctor, Murray, Stanton, Devery, & Kelly, 2016; Spitsberg, 2005). The biological activity of PLs has also been associated with cognitive development and repair. Phosphatidylserine, pre-eminently, has been implicated in the treatment of Alzheimer's disease (Burling & Graverholt, 2008; Contarini & Povolo, 2013; Dewettick et al.,, 2008). In more recent years, studies on infant milk formula fortified with PLs have proved positive in terms of immunological defences (Timby, Hernell, Vaarala, Melin, Lönnerdal, & Domellöf, 2015), and also infant neurodevelopment (Timby, Domellöf, Hernell, Lönnerdal, & Domellöf, 2014). Thus, there is considerable interest and demand for the generation of PL concentrates from dairy sources.

Technological approaches utilised to concentrate PLs in buttermilk (BM) have been primarily membrane filtration based, particularly microfiltration (MF) (Astaire, Ward, German, & Jiménez- Flores, 2003; Corredig, Roesch, & Dalgleish, 2003; Holzmüller & Kulozik, 2016; Morin, Pouliot, & Jiménez-Flores, 2006; Morin, Britten, Jiménez-Flores, & Pouliot, 2007) due to its greater porosity. However, similarity in the size of the MFGM components and casein (Morin et al.,, 2007) limited the effectiveness of MF as a selective

separation process. Complementary measures adopted to enhance MF include: cream washing prior to ultrafiltration (UF) (Morin et al.,, 2007), rennet induced coagulation of the caseins prior to MF (Sachdeva & Buchheim, 1997), and disruption of the casein micelle with citrate prior to MF (Corredig et al., 2003; Roesch, Rincon, & Corredig, 2004; Rombaut, Dejonckheere, & Dewettinck, 2006). A more recent avenue of investigation employed the use of enzymes to pre-digest the milk proteins prior to UF (Barry, Dinan, & Kelly, 2017). Hydrolysis of milk proteins prior to UF enables selective transmission of smaller peptides through the membrane, thus, selectively concentrating PLs in the retentate. Barry et al., (2017) reported that PLs losses through the UF membrane using this novel process were non-detectable.

A more advanced separation technology in the form of supercritical fluid extraction (SFE) has been periodically investigated to selectively extract unique higher value lipids, in particular from primary food substrates including BM sources (Astaire et al., 2003; Costa, Elias-Argote, & Jiménez-Flores, 2010; Spence, Jiménez- Flores, Qian, & Goddik, 2009). Astaire et al., (2003) were able to selectively remove non-polar lipids from the buttermilk by coupling MF with SFC, thus, allowing concentration of the polar lipids in the buttermilk. Spence et al., (2009) concentrated dairy PLs 5-fold when applying SFC to MF pre-processed samples.

The objective of the present study was to apply SFE while upscaling a laboratory-based enzymatic hydrolysis/ultrafiltration process previously outlined by the authors (Barry et al.,, 2017), in order to produce a purified milk lipid extract with elevated levels of PLs relative to non-polar milk lipids.

2. Materials and methods

2.1. Materials

Spray dried buttermilk powder (BMP) was sourced from Tipperary Co-operative (Tipperary, Ireland) with compositional analysis by standard IDF protocols determining 6.84 % fat, 31.40 % protein, 48.00 % lactose, and 7.40 % minerals. The digestive enzyme Alcalase (E.E. 3.4.21.62) was procured from Novozymes (Bagsværd, Denmark). Alcalase is a serine type endoprotease with esterase activity enabling it to catalyse the stereoselective hydrolysis of amino esters and selective esters. Alcalase has a minimum activity of 2.4 Anson units per gram of composition (2.4 AU-A g^{-1}) at pH 7.50, with optimal conditions for alcalase activity within 35- 60°C and pH 7-9. Synder PES spiral wound membranes with a nominal molecular weight cut off (MWCO) of 50 kDa were purchased from David Kellett & Partners Ltd. (Hereford, UK). Acetonitrile was procured from ThermoFisher Scientific (Waltham, MA, USA) and all other chemicals were HPLC grade, > 99.9 %, and purchased from Sigma Aldrich (Arklow, Ireland).

2.2. Enzymatic hydrolysis of BMP substrate protein fraction

The degree of hydrolysis (% DH) is interpreted as the number of peptide bonds (*h*) that are cleaved as a percentage of the total peptide bonds (*htot*) all of which is related to the consumption of base as a result of proton release during the hydrolysis reaction according Adler-Nissen's (1986) equation (Eq. 1).

$$\% DH = 100 \; x \; B \; x \; N_B \; x \; \frac{1}{\alpha} \; x \; \frac{1}{MP} \; x \; \frac{1}{h_{tot}} \qquad (1)$$

Where, B is the volume of base in mL, N_B is the normality of the base, 1/α is the average degree of dissociation of the α-NH groups (=1.13 at pH 8.00, 50°C), MP is the molecular

weight of the protein and *htot* is the number of peptide bonds in the protein substance (=8.2 for casein/whey mix).

For all hydrolysis reactions, BMP was reconstituted to 10 % total solids in reverse osmosis (RO) water, 360 Kg total weight (37.29 Kg BMP and 322.7005 Kg RO water), and allowed to hydrate over night at 4 °C with gentle agitation. The BMP dispersions were heated gently to 50 °C with a water-jacket (to avoid burn on of the BMP to the walls of the tank) and subsequently pH corrected with 4 M NaOH to pH 8.00 (original pH 6.45). The hydrolysis reaction was initiated by addition of alcalase dissolved in RO water at an enzyme to substrate ratio (E:S) of 1: 100, w w^{-1}, (1 %) on a protein equivalent basis with the temperature, 50 °C, and pH, pH 8.00, maintained throughout the reaction. The pH was controlled by addition of 4M NaOH and the reaction was agitated throughout utilizing the inherent tank impeller. The hydrolysis reaction was terminated by means of pH inactivation with 2 M citric acid until a pH of 6.5 was achieved. All hydrolysis reactions were performed in duplicate.

Determination of the end-point DH was measured by modification of the trinitrobenzenesulfonic acid (TNBS) reaction outlined by Adler-Nissen (1979) which involves measuring the reaction of liberated α-amino groups (α-NH_2) with 2, 4, 6-trinitrobenzene 1- sulfonic acid. Briefly, samples with a protein concentration of 3 mg mL^{-1} were prepared in 1 % SDS, 1 % SDS alone serving as a blank, and 0.2125 M phosphate buffer was added. 2 mL of TNBS was added to both the samples and blank with light omitted, and the reaction was incubated for 1 hr at 50 °C. 4 mL of 0.1 M HCl was added to terminate the reaction and the samples were cooled for 30 min prior to absorbance at 314 nm being read using a Varian Cary 1 dual beam UV- visible spectrometer (Varian Ltd., Walton-on-Thames, U.K). UV readings were determined against a standard curve generated using L-

leucine at concentrations ranging from 20-250 mg L^{-1}. DH values were calculated using the following equation (Spadaro, Draghetta, del Lama, Camargo, & Greene, 1979) (Eqn. 2)

$$\% DH = \frac{AN_2 - AN_1}{N_{pb}} x\ 100 \quad (2)$$

Where, AN_1 represents the amino nitrogen content of the protein substrate (unhydrolysed) (mg g^{-1} protein), AN_2 represents the amino nitrogen content of the hydrolysed protein substrate and Npb represents the nitrogen content of the peptide bonds on the protein substrate (mg g^{-1} protein).

2.3. Ultrafiltration of the BMP hydrolysate

The BMP hydrolysate produced was subjected to membrane ultrafiltration utilising a GEA Model F unit (GEA Process Engineering A/S, Skanderborg, Denmark). UF was accomplished with two Synder PES spiral wound membranes (David Kellet & Partners, Ltd.) having a nominal molecular weight cut off (MWCO) of 50 kDa and a total membrane area of 6.69 m^2. UF was carried out at 50 °C to a volume concentration factor of 11 by addition of 4 x diafiltration steps (3x 200Kg RO water and 1 x 100Kg RO water) during the membrane process. A feed recirculation rate of 100 L h^{-1} at 1 bar and a membrane inlet pressure of 4.2 bar were maintained throughout processing. UF was carried out in duplicate. All 50 kDa retentates (50 R) and 50 kDa permeates (50 P) were evaporated to 40 % total solids on a Tetra Scheffers ™ falling film single- stage evaporator (Tetra Pak, Gorredijk, The Netherlands) and subsequently spray-dried on a pilot scale Anhydro Lab 3 spray drier (SPX Flow Technology A/S, Soeborg, Denmark) with an inlet temperature ranging from 185 to 190 °C and an outlet temperature ranging from 85 to 90 °C during spray drying. UF was carried out in duplicate.

2.4. Chromatographic characterisation of the BMP hydrolysate and UF fractions protein.

Observation of the loss of native protein and molecular weight characterisation of the BMP hydrolysate and UF fractions (50 R and 50 P), size exclusion chromatography (SEC), according to the method of O'Loughlin et al., (2013), was carried out using a Waters 2695 separation module coupled with a Waters 2487 dual wavelength absorbance detector with Waters Empower software (Milford, MA, USA). All column eluates were monitored at 214 nm unless otherwise stated. Briefly, samples with a 2.5 mg mL^{-1} protein concentration were injected, 20 µL, onto a TSK Gel G2000SW, 7.8 mm x 600 mm column (Tosoh Bioscience GmbH, Stuttgart, Germany). An isocratic gradient of 30 % acetonitrile with 0.1 % TFA was maintained for a run time of 60 min. Sample chromatograms were compared with a molecular weight curve generated from standards, 2.5 mg mL^{-1} protein, of known molecular weights; α-lactalbumin, β-lactoglobulin A and B, Bovine serum albumin (BSA), lactoferrin and CMP procured from Sigma Aldrich and Ribonuclease A, Cytochrome C, Aprotinin, Bacitracing, His- Pro- Arg- Tyr, Leu- Tyr,- Met- Arg, Bradykinan, Leu- Phe and Tyr- Glu procured from Bachem AG (Bubendorf, Switzerland).

2.5. Chromatographic quantification of BMP phospholipids.

Extraction of the crude lipid from the BMP hydrolysate and UF fractions (50 R and 50 P) was carried out according to the method of Folch (Folch, Lees, & Stanley, 1957). Briefly, 4 g of powder was dissolved in 75 mL chloroform: methanol (2:1, v v^{-1}), and refrigerated at 4 °C with agitation for 1 hr. The sample was then subjected to centrifugation at 4000 x g for 5 min following which the bottom $CHCl_3$ layer was collected and the top MeOH layer was washed with 50 mL CHCl3 and refrigerated with agitation for another hour. After centrifugation of

the sample both $CHCl_3$ layers were pooled together and evaporated using a Buechi rotary evaporator (BUCHI, Flawil, Switzerland), and dried to dryness under N_2.

Separation and quantification of the BMP hydrolysate and UF fraction PLs was carried out according to the method of Barry et al., (2016). Briefly, the crude lipid extract was re-solved in $CHCl_3$: MeOH (88:12, v v^{-1}). 25 µL of the sample was applied to 3 µm particle diameter Prevail Silica column (Labquib Dublin, Ireland) with separation carried using a Waters 2695 separation module (Waters) coupled to a Corona Charged Aerosol Detector (CAD) (Thermo Scientific, Hemel Hempstead, U.K) under the following linear gradient elution conditions with a flow rate of 0.50 mL min^{-1} and ratio in volume of solvent A to B; 96:4 at T0, 25:75 at T20, 6:94 at T21 and 96:4 at T22 and maintained until T27. All lipid extractions were performed in triplicate and were pre- filtered through 0.20 µm Hydrophobic Fluropore ™ (PTFE) membrane filters (Merek Millipore Ltd., Cork, Ireland) prior to HPLC. PL recovery was calculated by quantification with calibration curves that had been generated for each individual PL species.

2.5. Supercritical carbon dioxide fluid extraction (SFE) of 50 kDa retentate.

SFE was carried out on the 50 R that was generated from the combined hydrolysis and 50 kDa membrane ultrafiltation process. SFE was carried out at The Biocomposites Centre in Bangor University (Wales, UK) on a Thar laboratory rig, Thar SFC-1000 (Thar Energy LLC, Pittsburg, PA, USA), with a 1000 mL extractor vessel, with the following extraction parameters; 40 °C extraction temperature and automated back pressure at 300 bar. The system was controlled manually with a computer-based Windows software. The initial defatting stage involved subjecting the 50 R sample to supercritical CO_2 only, to remove non-polar lipids. The subsequent secondary PL purification stage utilised CO_2 with ethanol as a co-solvent at different percentages, 10 % and 20 %, to increase the polarity of the extraction

solvent to enable maximum separation of the PLs from the 50 R. The entire extraction took approximately 13 h and the resulting PL extract was concentrated using a Buechi rotary evaporator and dried to dryness under N_2 prior to analysis by HPLC as mentioned above (Section 2.4). SFE was carried out in duplicate.

2.6. Compositional analysis

Compositional analysis was performed on all samples using the standard IDF/ISO or AOAC methods for determination of % protein (ISO 8968-1, 2014), % total solids/moisture and ash (ISO 6731, 2010) and lactose (AOAC 896.01, 1896). % fat was determined using the Folch method described by Folch et al., (1957).

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. Enzymatic hydrolysis of BMP substrate protein fraction

A previous study carried out by the authors determined that a 19 % degree of hydrolysis (%DH) sufficiently digested milk proteins in order to enable permeation through a 50 kDa ultrafiltration (UF) membrane. In this study, the authors additionally highlight the importance of mode of inactivation utilised to terminate the hydrolytic reaction at pilot scale level i.e., thermal versus pH inactivation. Furthermore, it was also necessary to consider the implications that the selected inactivation step may have on the final hydrolysate e.g. risk of aggregate formation upon thermal treatment (80°C for 20 min) of the hydrolysate. Hence, pH-based inactivation was adopted as the method for termination of the enzymatic reaction.

Hydrolysis to 19 % DH of the BMP starting material resulted in a shift in the molecular mass distribution of the starting material (Table 1). Hydrolysis resulted in a higher percentage of smaller molecular weight material, > 50 kDa in size, compared with that of the BMP, 0.05 ± 0.03% and 99.95 % (summation of M.W > 50 kDa) respectively (Table 1). Size exclusion chromatography (SEC) chromatograms (Fig. 1) illustrate the larger molecular weight material present in the starting BMP material (Fig. 1a) and the presence of a large body of smaller molecular weight peptides following extensive digestion (Fig. 1b). These results are in agreement with those obtained previously at the laboratory scale (Barry et al.,, 2017).

3.2. Ultrafiltration of the BMP hydrolysate

Previous work carried out by the authors (Barry et al., 2017) determined that laboratory-based ultrafiltration (UF) using a cartridge-type membrane with a nominal molecular weight cut off of 50 kDa permitted maximum permeation of the digested proteinaceous material. In this pilot scale study, UF was carried out on the BMP hydrolysate utilising a GEA Model F unit fitted with two Synder PES 50 kDa spiral wound membranes in series. To ensure optimum enrichment of PLs in the retentate, 4 diafiltration steps were carried out during the 3h membrane process to allow maximum washing of peptide material in the retentate through the membrane, thus enabling a volume concentration factor of 11 to be achieved. A feed recirculation rate of 100 L h^{-1} at 1 bar was maintained throughout the membrane process with steady stage permeate flux, this indicating that there was no evidence of membrane fouling during 3h filtration (supplementary fig. 1).

An 8.7-fold increase in lipid content of the 50 kDa retentate (50 R), 60.07 ± 0.54 % total lipid on a dry matter basis (DM), with reference to the starting BMP, 6.84 ± 0.17 % total lipid (DM) (Table 2.) was achieved. Concurrent with the increase in lipid concentration, a 3-fold protein decrease in the starting material relative to 50 R was achieved, 31.40 ± 0.57 % and

10.58 ± 0.09 % respectively (Table 2.) due to permeation of peptide material < 50 kDa (Table 1.) through the UF membrane. The deproteinising effect of the combined enzymatic hydrolysis and UF process was also confirmed by SEC molecular size distribution profiling of the 50 R and the 50 kDa permeate (50 P) (Fig. 2) e.g. a maximum absorbance of 0.15 mV for 50 R (Fig. 2a) was recorded compared to an absorbance of 2.81 mV for 50 P (Fig. 2b). UF was carried out in duplicate with no significant difference between each run ($p < 0.01$, one-way ANOVA).

3.3 Phospholipid (PL) enrichment of the 50 kDa retentate via enzymatic hydrolysis and UF.

Determination of the degree of PL enrichment achieved utilising the combined enzymatic hydrolysis and UF process was confirmed through quantification (Barry et al., 2016) of the PL chromatographic profiles generated for the BMP starting material, the 50 R and the 50 P. The crude lipid fractions of each substrate were extracted via Folch (Folch et al., 1957) with, as mentioned in section 3.2., an 8.7-fold increase in the total lipid content on a dry matter basis between the 50 R and the original BMP, 60.07 ± 0.54 % total lipid (DM) and 6.84 ± 0.17 % total lipid (DM) respectively, was achieved (Table 2.). Subsequent to the crude lipid extraction, the extracts were analysed to determine their relative PL contents by HPLC with acharged aerosol detection (CAD) system. Due to permeation of the smaller molecular weight peptides (<50 kDa) and the concurrent increase in lipid content, a 8.5 fold increase in PLs was achieved in the 50 R, 11.05 ± 0.02 % total PL (summation of PI, PE, GluCer PS, PC and SM), compared to the starting BMP, 1.30 ± 0.00 % total PL (Table 2.). Thus, the 8.7-fold increase achieved at pilot scale level exceeds the 7.8-fold increase that the authors previously achieved during the development phase on a laboratory scale where a 50 kDa UF membrane cassette was employed to process the enzymatically hydrolysed buttermilk (Barry et al.,

2017). This enrichment is also greater than that reported in other work utilising similar hydrolysis and/or filtration technologies. Morin et al., (2006) achieved a 3-fold enrichment of PL during MF. However, lower filtration temperatures appear to have negatively impacted separation performance (Astaire et al., 2003; Morin et al., 2006). A 2.8-fold increase in PL was obtained by Morin at al., (2007) during combined MF and DF of washed cream buttermilk. A 2.5 % increase in PL was achieved by Konrad et al., (2013) following peptic hydrolysis and UF.

The higher sensitivity and precision of the CAD detector system (Barry et al., 2016) is largely responsible for the higher levels of the more acidic PLs, PI and PS, than those previously reported, 10.84 ± 0.04 % PI and 9.54 ± 0.01 % PS in the BMP and 10.42 ± 0.04 % and 9.88 ± 0.07 % in the 50 R. The previously reported improvements to the Folch crude lipid extraction protocol (Barry et al., 2016) is also likely to be a contributory factor.

Well defined peaks representing the five major PLs, namely phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC) and sphingomyelin (SM) along with glucosylceramide (GluCer) were demonstrated in the HPLC-CAD chromatograms generated for the BMP and 50 R fractions (Fig. 3). Also present are the sub-peaks of both PC (PC 1, PC 2) and SM (SM 1, SM 2), which represent different molecular subspecies of the PLs as previously described (Barry et al., 2016, Fagan & Wijesundera, 2004).

The PL profile representing the 50 R (Fig. 3b) demonstrates the higher concentration of PLs when compared to the PL profile of the BMP (Fig. 3a), 11.05 ± 0.02 % total PL and 1.30 ± 0.00 % total PL, respectively (Table 2.). While there was some permeation of lipid material during the membrane filtration, 0.29 ± 0.07 % total lipid in the 50 P, losses of PL during the combined hydrolysis and UF process were non-detectable, 0.00 ± 0.09 % total PL

in the 50 P (Table 2.). This is in general agreement with that previously reported by the authors (Barry et al., 2017).

3.4 Supercritical CO_2 Extraction (SFE)

After UF of the BMP hydrolysate, the 50 R was evaporated to 40 % solids and dried on a pilot scale Anhydro Lab 3 spray drier. In order to maximise PL enrichment further than that previously achieved at the laboratory scale by the authors (Barry et al., 2017), the 50 R powder obtained was subjected to further 2-stage separation utilising SFE- An initial defatting stage that removed non-polar lipid material utilising SFE alone, followed by a secondary purified PL extraction stage using CO_2 with ethanol (EtOH) as a co-solvent to create a more polar environment for extraction of PLs. Duplicate SFE runs involving 20% EtOH as co-solvent increased PL content to 56.24 ± 0.07 % (Table 2.) compared to 11.05 ± 0.02 % for the original 50 R powder. From the chromatogram obtained for the SFE sample (Fig. 4), it is noted that only the peaks representing PE, PC and SM are present. This may be due to the more polar nature of the PI and PS and, hence, it may be necessary to increase the polarity of the extraction solvent further with supercritical H_2O. A 10 % ratio of EtOH was also investigated; however there were non-detectable levels of PLs in this fraction (data not shown). While the authors were unsuccessful in extracting all of the PL species, they were successful in generating a fraction where more than half the weight, 56.24 ± 0.07 %, on a dry matter basis is represented by PLs. To our knowledge, the use of SFE technology in this manner to generate a purified PL fraction free of protein or lactose has not been previously accomplished. This purified fraction is a useful substrate with which to undertake various bioassay studies in order to properly investigate the numerous effects of PLs and the pathways by which their effects are believed to be implicated. Spence et al., (2009) achieved a PL concentration factor of between 3.49 and 4.24 when performing SFE on sweet and whey cream buttermilks following MF (Spence et al., 2009). A 4-fold increase in PLs was achieved

by Costa et al., (2010) in a whey buttermilk source treated with SFE after UF (Costa et al., 2010). However, in both cases the enrichment was carried out by reducing the non-polar lipid content, thereby, increasing both PL and residual protein content, simultaneously.

4. Conclusion

This study demonstrated a number of key observations with the aim of the work to generate a novel ingredient enriched in dairy PLs. Firstly, successful scalability of a laboratory based process was achieved. With some adaptation of the original combined enzymatic hydrolysis and UF process, the authors succeeded in producing a PL enriched 50 kDa retentate (50 R) at pilot scale that surpassed their previously reported laboratory-based results (Barry et al.,, 2017). An 8.7-fold increase in lipid content achieved in the 50 R following the combined pilot scale process compared to the starting BMP(60.07 ± 0.54 % v. 6.84 ± 0.17 %) using spiral-wound UF membranes is very favourable for industrial scale exploitation at this of enrichment. Parallel to this increase in lipid content, an 8.5-fold increase in PL material resulted comparing the BMP starting substrate and the 50 R, 1.30 ± 0.00 % and 11.05 ± 0.02 %, respectively. Concurrent with the increases in both total lipid content and PL content, a 3-fold decrease in proteinaceous material was observed, 31.40 ± 0.57 % in the BMP and 10.58 ± 0.09 % in the 50 R. The increase in PLs was greater than that previously reported using similar hydrolytic and separation technologies. The association of PLs in cognitive development and repair in the case of Alzheimer's disease could open up avenues for utilisation of this enriched PL fraction to fortify infant or geriatric nutritionals with PLs.

Secondly, to complement the PL enrichment mentioned above, generation of a purified PL fraction was achieved through the utilisation of SFE. Following SFE, a fraction with a 56.24 ± 0.07 % total PL content was achieved, with PLs representing more than half the dry matter. Generation of a purified PL fraction, depleted of proteinaceous and lactose

material, has to the best of our knowledge not been accomplished to this extent in previous published work and, hence, presents an excellent opportunity with which to pursue further *invitro* and *in vivo* studies on the putative health benefits associated with PLs.

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Figure Legends

Figure 1. SEC overlay chromatogram representing the size distribution profile of the BMP powders where A; BMP and B; 19 % DH BMP hydrolysate.

Figure 2. SEC overlay chromatogram representing molecular weight distribution profiles of the ultrafiltration fractions where A; 50 kDa retentate and B; 50 kDa permeate.

Figure 3. HPLC- CAD chromatogram representing phospholipid composition of the BMPs where A; BMP and B; 50 kDa retentate.

Figure 4. HPLC- CAD chromatogram representing phospholipid profiles of the supercritical fluid treated samples where A; CO_2 extraction only and B; CO_2: 20% Ethanol.

Tables and Figures

Table 1. Molecular weight (M.W.) distributions of BMP and BMP hydrolysate. M. W. distribution quantified with respect to calibration with known M.W. standards.

	M.W. Distributions					
Enzyme	**>100 kDa (%)**	**100-75 kDa (%)**	**75-50 kDa (%)**	**50-30 kDa (%)**	**30-10 kDa (%)**	**< 10 kDa (%)**
BMP	92.10 ± 0.03	7.30 ± 0.11	0.55 ± 0.11	0.05 ± 0.03	-	-
19 % DH	-	-		20.66 ± 0.07	42.51 ± 0.12	36.83 ± 0.07

Data are expressed as the average (+ % RSD) of two replicate hydrolysis reactions of the % of total area under the curve.

Table 2. Gross compositional analysis of Buttermilk powder (BMP), 50 kDa retentate (50 kDa R) and 50 kDa permeate (50 kDa P) with respect to % Fat (Folch, Barry et al., 2016), % protein (Kjeldahl ISO 8968-1, 2014), and % total phospholipid (PL) determined by summation of each individual PL with respect to that extracted from the sample..

	Compositional analysis		
	Fat (%)[a]	**Protein (%)[b]**	**Total Phospholipid (PL) (% of sample[a]**
BMP	6.84 ± 0.17	31.40 ± 0.57	1.30 ± 0.00
BMP hydrolysate 50 kDa retentate (50 kDa R)	60.07 ± 0.54	10.58 ± 0.09	11.05 ± 0.02
BMP hydrolysate 50 kDa permeate (50 kDa P)	0.29 ± 0.07	52.06 ± 0.16	0.00 ± 0.09
CO2 sample	72.66 ± 0.51	-	0.00 ± 0.03
CO2: 20 % EtOH extract	99.3 ± 0.01	-	56.24 ± 0.07

	Individual PL (% of total PL)		
PL Species	**BMP**	**50 kDa R**	**CO2: EtOH treated sample**
Phosphatidylinositol (PI)	10.84 ± 0.04	10.42 ± 0.04	
Phosphatidylethanolamine (PE)	21.66 ± 0.07	20.48 ± 0.01	11.26 ± 0.07
Glucosylceramide (GluCer)	0.07 ± 0.32	0.08 ± 0.11	
Phosphatidylserine (PS)	9.54 ± 0.01	9.88 ± 0.07	
Phosphatidylcholine (PC)	31.31 ± 0.01	31.75 ± 0.12	82.22 ± 0.16
Sphingomyelin (SM)	26.57 ± 0.04	27.39 ± 0.17	6.52 ± 0.02

[a]Data are expressed as the average (± % RSD) of three repeats.
[b]Data are expressed as the average (± % RSD) of two repeats.
[c]Data are expressed as % of total PL, average (± % RSD) of duplicate injections of each sample in triplicate.
Compositional data for BMP previously recorded in Barry et al., 2017.

Figure 1.

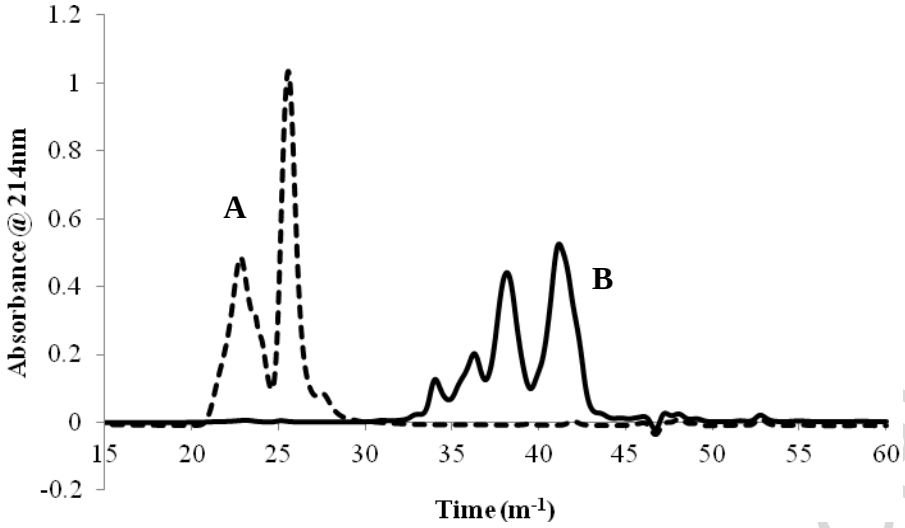


Figure 2.

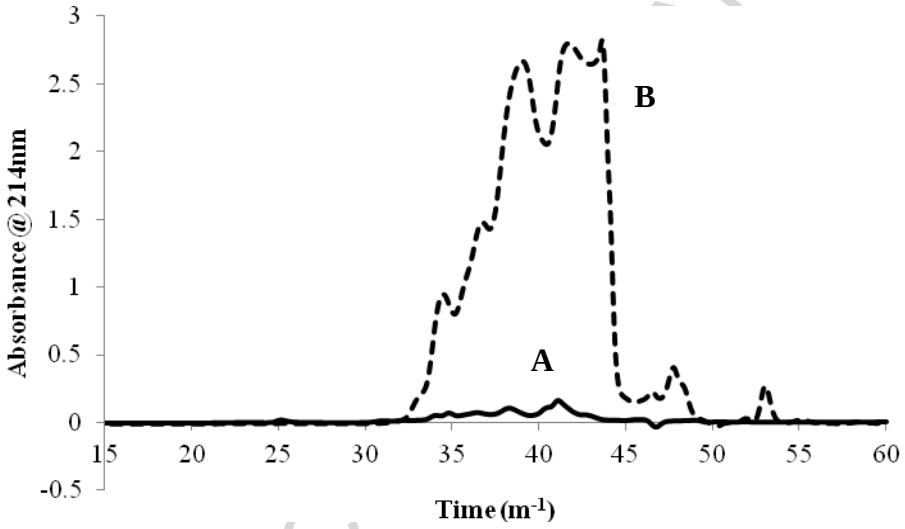


Figure 3.

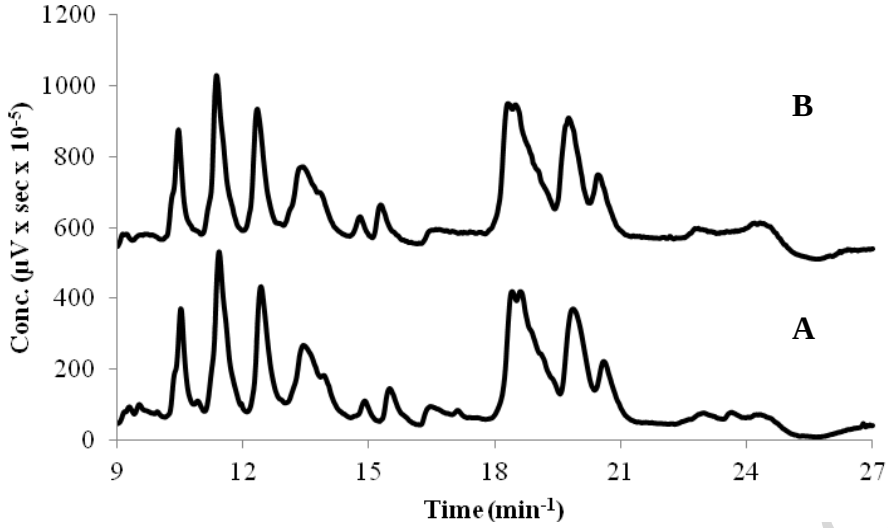
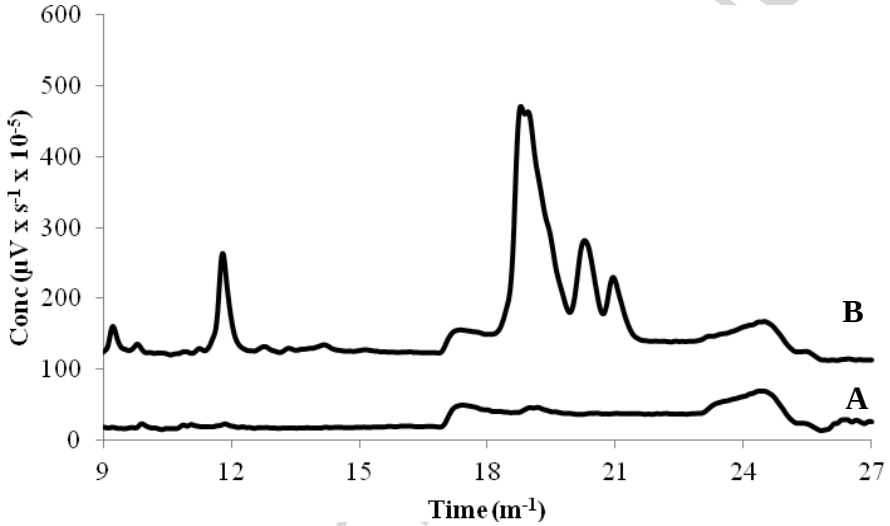


Figure 4.



Highlights

- A novel pilot scale process generates high levels of buttermilk phospholipids (PLs)
- Targeted protein hydrolysis reduces co-separation with lipids at ultrafiltration
- Features a non-thermal method of enzyme inactivation to counter membrane fouling
- An 8.7-fold PL enrichment is highest known to date for membrane separation
- Supercritical CO_2 extraction increases PL concentration further to 56.24 %