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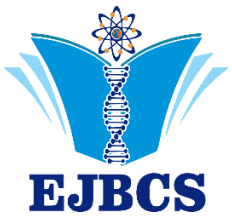

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Major Phospholipids of Selected Dairy Products as Determined by the HPLC-UVvis and ^{31}P-NMR Methods

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Abstract: This study was carried out to determine the major phospholipids in selected dairy products (Beta Serum, Procream and Phospholipids-Rich Dairy products), evaluate the accuracy of the developed fat extraction method for liquid dairy samples and to compare the major phospholipids of different dairy samples obtained using the HPLC-UVvis and ^{31}P-NMR methods. It was found that the developed fat extraction method can be used to estimate the lipid content of liquid dairy samples were still a bit satisfactory. Using the HPLC-UVvis method, it was found that the sphingomyelin (SM) consists of 2 curves in Beta Serum, Procream and Phospholipid-Rich products. The phosphatidylethanolamine (PE) separated ahead of phosphatidylcholine (PC) and then PC ahead of SM1 and SM2. The results showed that the data of the major phospholipids (PC, PE and SM) in Butter Serum, Procream and Phospholipids-Rich products as determined by the HPLC-UVvis method compared well with those of the ^{31}P-NMR method It must be noted that the analysis temperature for the HPLC-UVvis method was at 40^{o}C while the ^{31}P-NMR method was at 30^{o}C, and this might have also contributed to the slight variation of the results. Furthermore, the HPLC-UVvis method is rapid and with cheaper analysis cost compared with the ^{31}P-NMR method.

Keywords: Phospholipids, Phosphatidylcholine, Phosphatidylethanolamine, Sphingomyelin, HPLC-UVvis, ^{31}P-NMR

1. Introduction

Phospholipids 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 and Povolo, 2013; Donato et al., 2011; Kielbowicz et al., 2013). Phospholipids are subdivided into glycerophospholipids and sphingophospholipids (Donato et al., 2011). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major glycerophospholipids, while the Sphingomyelin (SM) is the dominant species of sphingophospholipids (Le et al., 2011). Phospholipids 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 and Povolo, 2013). The MFGM has a tripartite structure composed of an inner monolayer of proteins and 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). The amphiphilic nature of phospholipids facilitates the formation of bilayers and, thus, aids in the emulsification of fat in milk (Rombaut and Dewettinck, 2006).

Phospholipids were recently taken more into consideration because of their nutritional and technological characteristics (Dewettinck et al., 2008). Their inhibitory effect on some types of cancer (Kuchta et al., 2012; Castro-Gomez et al., 2016; Verardo et al., 2017), their ability to reduce blood cholesterol levels (Verardo et al., 2017; Duivenvoorden et al., 2006) and enhance brain functioning (Verardo et al., 2017; McDaniel et al., 2003), their anti-bacterial and anti-inflammatory activity (Verardo et al., 2017; Vesper et al., 1999) and their protective effect on gastric mucosa (Kivinen et al., 1992) have been studied. Additionally, their emulsifying properties can be used in several applications in the food, pharmaceutical and cosmetic industry (Lesser et al., 2006).

Dairy products are a good source of these phospholipids (Vesper et al., 1999). The biological membrane of native milk fat globules consists of about one-third phospho- and sphingo-lipids, stabilizing the milk fat globules in the serum phase of milk. Analysis of these lipids can be accomplished by means of ^{31}P-Nuclear Magnetic Resonance (NMR), High Performance Liquid Chromatography (HPLC), Thin Layer

Chromatography (TLC), Fourier Transform Infrared, and by measuring the total phosphorus content (Vanhoutte et al., 2004). Over the course of the past few decades, HPLC has become the preferred method for the determination of phospholipids, as quantitative and qualitative analysis can readily be obtained at a relatively low cost compared to ^{31}P-NMR (Rombaut et al., 2005). For the chromatographic analysis of fats and oils, the use of evaporative light scattering detection is generally preferred (Rombaut et al., 2005; Le et al., 2011), however the detector is very expensive. Rehman et al. (2017) reported a simple and rapid separation and determination of phospholipids by HPLC-UV system and obtained satisfactory results for different phospholipids standards.

Critical points in the analysis of phospholipids in food products are the method of fat extraction, separation, and detection. Often, little attention is given to the first of these. The majority of phospholipids in food products are present in membranous structures, interacting with compounds of a complex food matrix, making them difficult to extract (Rombaut et al., 2005). In order to avoid these problems, a cold-extraction procedures like those of Folch et al. (1957) and Bligh and Dyer (1959) are recommended. Lee et al. (1996) developed a simple and rapid solvent fat extraction for fish tissue based on the two methods mentioned previously and found to have satisfactory results for cod and mackerel samples compared with the Bligh and Dyer method.

There are many commercial dairy products that are obtained from milk processing including Beta Serum and Procream. Beta Serum or Buttermilk is the aqueous phase removed from pasteurized dairy cream after phase inversion during the process of Anhydrous Milk Fat production. Procream or High Fat Retentate is obtained from the microfiltration of whey retentate from the ultrafiltration of cheese whey which is a co-product obtained during manufacture of whey protein isolate (Tetra Pak, 2015).

This study was carried to determine the major phospholipids in selected dairy products (Beta Serum, Procream and Phospholipids-Rich Dairy Product), evaluate the accuracy of the developed extraction method for lipids in dairy samples and to compare the major phospholipids obtained using the HPLC-UVvis and ^{31}P-NMR methods.

2. Material and Method

2.1. Materials

The HPLC grade chemicals (99.9%) like Acetonitrile, Chloroform and Methanol were procured from Fisher Chemical (Loughborough, UK) while the reagent grade Phosphoric Acid (85%) was obtained from Scharlau Laboratory (Sentminat, Spain). The Bovine Phospholipids standards such as Phosphatidylcholine, Phosphatidylethanolamine and Sphingomyelin were procured from Larodan (Solna, Sweden). The Beta Serum powder was obtained from Tatua Dairy Company (Morrinsville, New Zealand) while the Procream powder from Mullins Whey (Mosinee, Wisconsin, USA). The Phospholipids-Rich Products were processed from Procream using a proprietary process at the Pilot Plant of the Institute for Dairy Processing, South Dakota State University, Brookings, South Dakota, USA.

2.2. Solvent Extraction of the Dairy Products Lipids

The solvent extraction method for determining the total lipids in fish tissue of Lee et al. (1996) was adapted for liquid dairy samples and modified as follows: a) weigh out the liquid dairy sample (7.0±0.1 g; record the exact amount) and place into a 100-mL Volumetric Flask with a press fit cover; b) add 70 mL of solvent (2:1 chloroform-methanol if the expected lipid content of the sample will be greater than 6%); c) stir the mixture using a magnetic stirrer set at 750 rpm for 2 minutes; d) filter the homogenate through a coarse filter paper and funnel into a 100 mL glass stoppered graduated cylinder, toward the end of draining, press the filtrate with the round tip of a spatula to moderately squeeze out the remaining solvent. Do not attempt to press all solvent out since there is no need to measure the chloroform layer; e) add 28 mL 0.5% NaCl solution (to prevent emulsion formation) and gently shake by tilting the cylinder 4 times, allow mixture to stand until visible separation occurs (takes about 30 minutes more or less); f) using a glass 10 mL pipet, remove an aliquot (about 9 mL) of the chloroform layer and transfer an exact 7.0 mL into a pre-weighed (to the nearest 1 mg) 40-mL beaker; g) evaporate the solvent using a hot plate set at low setting. Avoid excessive heating and drying (this step requires about 30 minutes); h) reweigh the beaker (to the nearest 1 mg) and calculate the total lipid content using equation 1 as shown below,

$$\text{Lipid content (\%)} = \frac{\text{Lipid extracted (g)}}{\text{Sample weight (g)}} \times \frac{\text{(Chloroform layer+Amounts lost)*}}{7.0\ \text{mL}} \times 100 \quad (1)$$

* Use a theoretical calculated volume of 46 mL

i) if the expected lipid content of the sample will be between 2 and 6%, then use 70 mL of solvent (1:1 chloroform-methanol) and then use 35 mL as the theoretical calculated volume in the Lipid Content calculation; j) if the expected lipid content of the sample will be less than 2%, then use 70 mL of solvent (1:2 chloroform-methanol) and then use 23 mL as the theoretical calculated volume in the Lipid Content calculation; and k) transfer the remaining extracted lipid to a dessicator to cool down to room temperature, then close the lid and store until use. Use the extracted lipid for the HPLC-UVvis analysis of the major phospholipids.

2.3. Determination of the Major Phospholipids by the HPLC-UVvis Method

The high-performance liquid chromatography (HPLC) Ultraviolet visible (UVvis) detector method for determining phospholipids of Rehman et al. (2017) was adapted with some modifications as follows. A Shimadzu HPLC with system controller (SCL-10A VP) equipped with a pump system (LC-10 AD VP and FCV-AL) with degasser and auto-injector (SIL-10AD VP) and a UV-vis detector (SPD-10AV) was used in the determination of the major phospholipids (Phospatidylcholine (PC), Phosphatidylethanolamine (PE) and Sphingomyelin (SM)) in the phospholipids standards and dairy samples. The chromatographic column was a Luna 5 µm Silica (2) 100 A, 150 mm x 4.6 mm with a security guard. The detector signal was analysed using the LC Solution software to obtain the

integrated area of the peaks and retention times from the chromatogram. The mobile phase was a mixture of Acetonitrile-Methanol-Phosphoric Acid (85%) (100:10:1.8 v:v:v) in isochratic pump mode with a flowrate of 0.75 mL/min. The mobile phase was degassed by magnetic stirring at 500 rpm under vacuum for about 30 minutes prior to use in the HPLC-UVvis system. The HPLC-UVvis system determination was done at 40°C by using a ThermaSphere column heater. The temperature of 40°C was found to give the satisfactory results compared with the 30oC temperature. The detector was set with a wavelength of 203 nm.

The HPLC-UVvis determination were limited to the major phospholipids (PC, PE and SM). A set of stocks solutions of PC, PE and SM standards with a concentration of 2000 µg/mL of solvent were made by oscillating the mixture for 30 seconds at 2000 rpm. The solvent used was a Chloroform-Methanol mixture (1:1 v:v) because the solvent used by Rehman et al. (2017) of n-Hexane:2-Propanol (3:1 v:v) did not fully dissolved the dairy lipids. The phospholipids standards were successively diluted with the solvent to get samples with different concentration from 164 to 1101 µg /mL for PC, from 198 to 1325 µg /mL for PE and from 177 to 600 µg /mL for SM. The samples are filtered through a 0.45 µm PTFE filter and into 1.5-mL amber vials and then loaded into the auto-injector sample holder. A 20 µL of the filtered sample was injected into the HPLC-UVvis System using the auto-injector to obtain the peak area and retention time of the sample. Calibration curves for the different phospholipids standards were obtained and used these in converting the peak areas into the different phospholipids (PC, PE and SM) concentration. For the analysis of liquid dairy samples, first the sample is passed through a 0.45 µm PTFE filter and into 1.5-mL amber vials and then loaded into the auto-injector sample holder.

2.4. Determination of the Major Phospholipids by the ^{31}P-NMR Method

The ^{31}P-NMR method of Mackenzie et al. (2009) was used in the analysis of the different phospholipids in the lipids of the dairy samples. The samples were sent for analysis to the Callaghan Innovation, Lower Hutt, New Zealand where Mackenzie and colleagues are employed.

3. Results and Discussion

3.1. Lipid Contents of the Beta Serum and Procream Products

Table 1 shows the comparison of the calculated and analyzed lipid contents of reconstituted butter serum and Procream with different dilution. The results show that the mean percentage difference between the analyzed and calculated lipid contents had a mean value of 8% for the Beta Serum products and 11% for the Procream products. The results obtained for the reconstituted Procream could probably be improved if samples analyzed were limited to 10% dilution only, since it was observed to have higher percentage difference at 15% dilution. In addition, the higher fat content of the powder (20.55% from Table 1) probably also contributed to the variability of results. This suggests that the developed extraction method for lipids can be used to estimate the lipid content of dairy samples. Considering that the lipid extraction method used was developed for fish tissue the results obtained were still a bit satisfactory.

3.2. Calibration Curves Determination for PC, PE and SM Concentration

Figures 1 to 3 show the calibration curves for PC, PE and SM with the phospholipid concentration on the x-axis and the HPLC-UVvis area under the curve on the y-axis. The separation times for the PC ranged from 7.5 to 9.7 minutes, for the PE ranged from 5.7 to 6.5 minutes and for SM curve 1 (SM1) ranged from 9.7 to 10.8 minutes while SM curve 2 (SM2) ranged from 10.5 to 11.9 minutes. The coefficient of determination (r^2) values of the calibration curves ranged from 0.991 to 0.998 indicating excellent fit of the experimental data from the curve fit. Regression equations to predict the individual phospholipid concentration for the obtained HPLC-UVvis area under the curve were derived from the given calibration curves as shown in Figures 1 to 3 and are shown as equations 2, 3 and 4 below,

PC Concentration = (70.706 x HPLC-UVvis Area) + 172.70 (µg/mL) (r^2 = 0.991) (2)

PE Concentration = (49.611 x HPLC-UVvis Area) + 114.83 (µg/mL) (r^2 = 0.996) (3)

SM Concentration = (50.647 x HPLC-UVvis Area) + 153.52 (µg/mL) (r^2 = 0.998) (4)

3.3. Chromatograms of the Major Phospholipids in Beta Serum and Procream Products

Figures 4 and 5 show the chromatograms of the major phospholipids in Beta Serum and Procream products. The separation times for PC is 8.241 mins, for PE is 5.663 mins, for SM1 is 10.801 mins and for SM2 is 11.982 mins in Beta Serum. While the separation time for PC is 8.213 mins, for PE is 5.632 mins, for SM1 is 10.841 mins and for SM2 is 11.884 mins in Procream. It is expected to have some slight variations in the separation times of the major phospholipids in Beta Serum and Procream because of the difference in their composition and processes undergone. SM usually consists of 2 curves in dairy products as shown by Ferreiro et al. (2017) for milk and Rombaut et al. (2005) for various dairy products. Rehman et al. (2017) in their HPLC-UV determination of various phospholipids standards and Rombaut et al. (2005) in their HPLC-ELSD determination of various dairy products also observed that the PE separated ahead of PC and then PC ahead of SM1 and SM2. The chromatograms also shows that all the major phospholipids can all be obtained within 12 minutes.

Table 1. Calculated and analyzed lipid contents of reconstituted Beta Serum and Procream products with different dilution.

Sample	Calculated Lipid Content*	Analyzed Lipid Content		Percentage Difference
Reconstituted Beta Serum (15% Powder) R1	2.20%	2.25%		2.27%
Reconstituted Beta Serum (15% Powder) R2	2.20%	1.93%		12.27%
Reconstituted Beta Serum (10% Powder) R1	1.47%	1.55%		5.44%
Reconstituted Beta Serum (10% Powder) R2	1.47%	1.41%		4.08%
Reconstituted Beta Serum (10% Powder) R3	1.47%	1.83%		24.49%
Reconstituted Beta Serum (10% Powder) R4	1.47%	1.50%		2.04%
Reconstituted Beta Serum (10% Powder) R5	1.47%	1.50%		2.04%
			Mean	7.52%
Reconstituted Procream (15% Powder) R1	3.08%	2.50%		18.83%
Reconstituted Procream (15% Powder) R2	3.08%	2.14%		30.52%
Reconstituted Procream (9% Powder) R1	1.85%	1.83%		1.08%
Reconstituted Procream (6.5% Powder) R1	1.34%	1.41%		5.22%
Reconstituted Procream (6.5% Powder) R2	1.34%	1.36%		1.49%
			Mean	11.43%

* - calculated lipid content based on the certificate of analysis of the Beta Serum (fat content=14.70%) and Procream (fat content=20.55%) powders

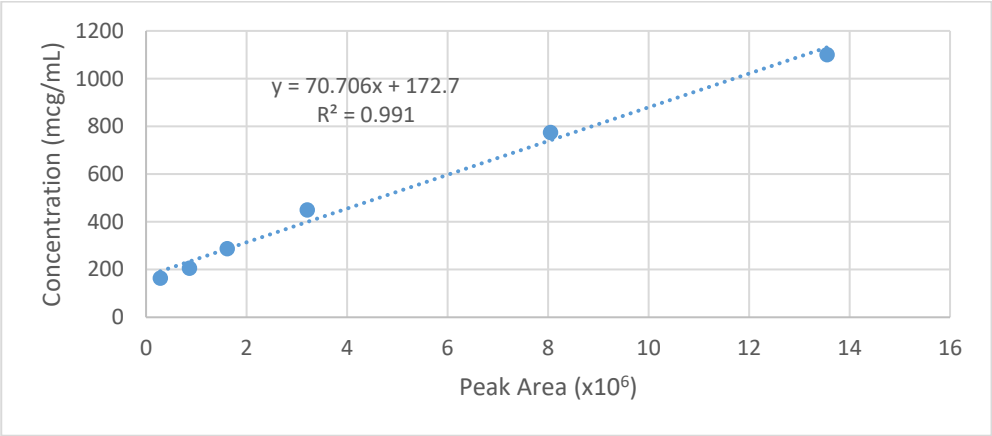

Figure 1. Plot of phosphatidylcholine (PC) concentration against HPLC-UVvis area under the curve (mcg = µg).

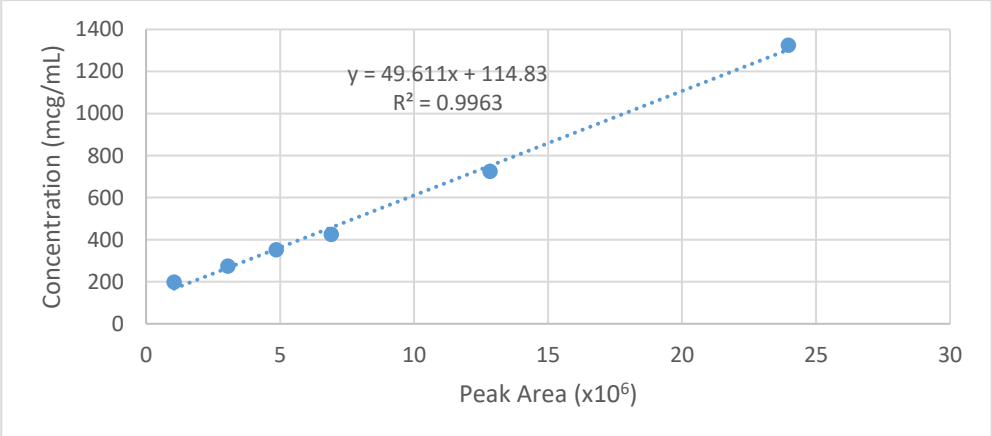

Figure 2. Plot of phosphatidylethanolamine (PE) concentration against HPLC-UVvis area under the curve (mcg = µg).

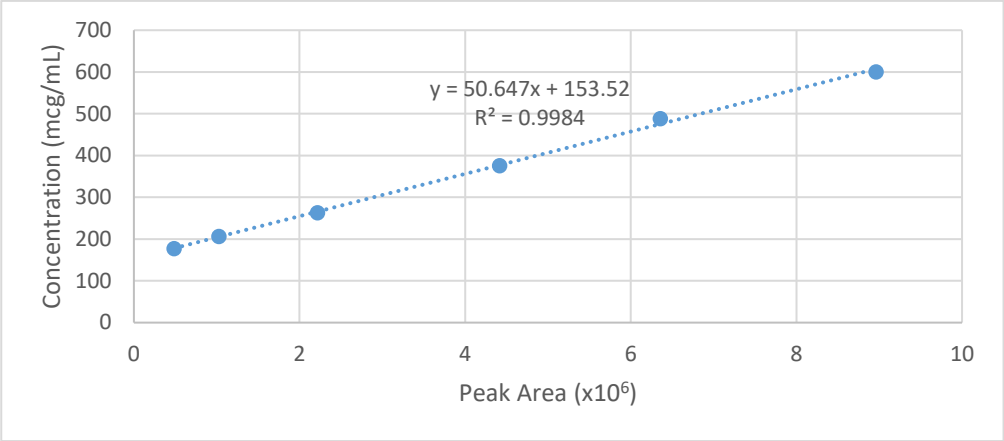

Figure 3. Plot of sphingomyelin (SM) concentration against HPLC-UVvis area under the curve (mcg = µg).

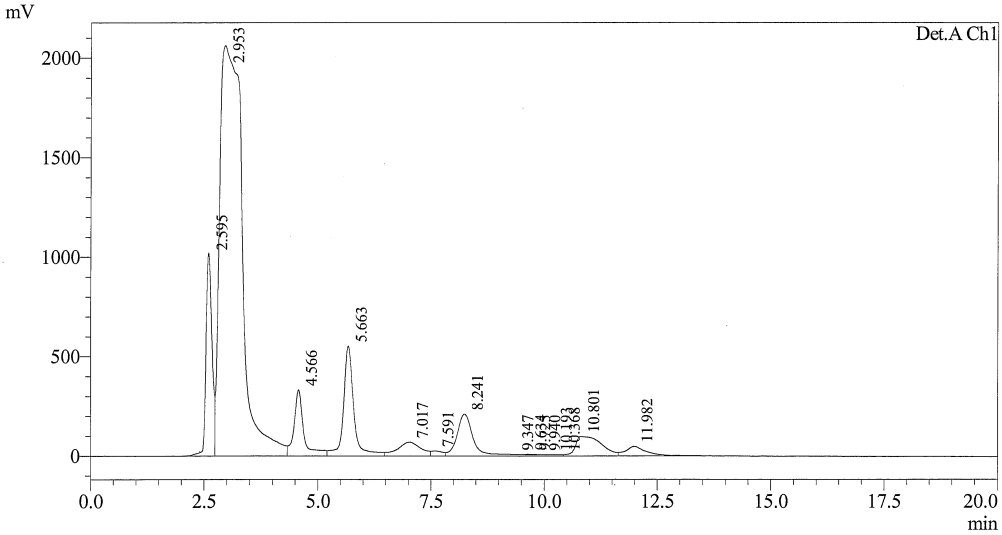

Figure 4. Chromatogram of the major phospholipids in Butter Serum (Separation times: PE= 5.66min; PC=8.24min; SM1=10.80min; and SM2=11.98min).

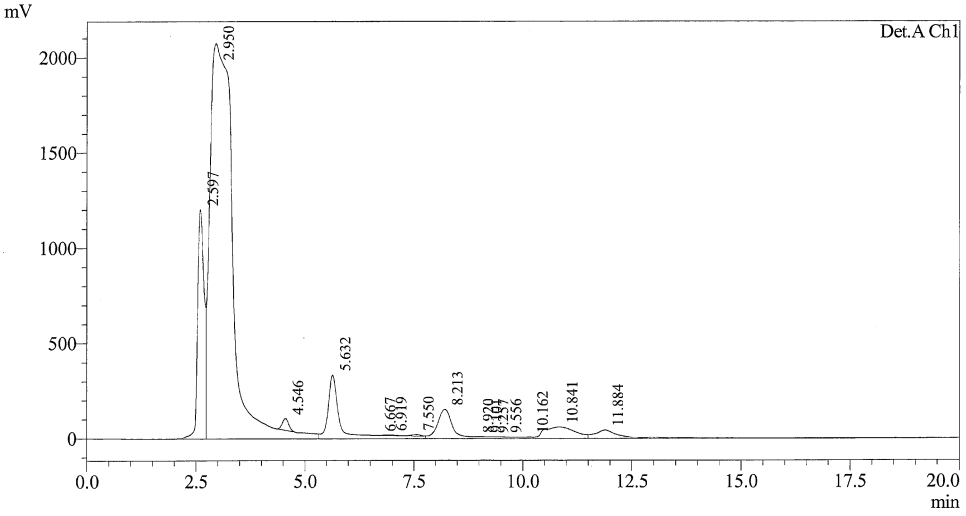

Figure 5. Chromatogram of the major phospholipids in Procream (Separation times: PE=5.63min; PC=8.21min; SM1=10.84min; and SM2=11.88min).

3.4. Amounts of Phospholipids in Beta Serum and Procream Products

Table 2 shows the amounts of PC, PE and SM from the different Beta Serum and Procream products obtained using ^{31}P-NMR and HPLC-UVvis methods. The calculated concentration of the individual phospholipids was determined using equations 2 to 4 from as shown earlier. The results show that for the Reconstituted Beta Serum (15% Powder) gave percentage difference values of 3.3%, 21.0%, 2.2% and 10.0% for PC, PE, SM and Total (PC+PE+SM), respectively between the ^{31}P-NMR and HPLC-UVvis. Only one percentage difference value was above 10% which suggests satisfactory results. On the other hand, the results show that for the Reconstituted Procream (15% Powder) gave percentage difference values of 17.0%, 6.3%, 7.4% and 5.6% for PC, PE, SM and Total (PC+PE+SM), respectively between the ^{31}P-NMR and HPLC-UVvis. Again, only one percentage difference value was above 10% which suggests satisfactory results.

It must be noted that the analysis temperature for the HPLC-UVvis method was at 40^{o}C while the ^{31}P-NMR method was at 30^{o}C, and this might have also contributed to the slight variation of the results. Furthermore, the HPLC-UVvis method is rapid and with cheaper analysis cost compared with the ^{31}P-NMR method.

3.5. Chromatogram of the Major Phospholipids in a Phospholipid-Rich Product (PU 307)

Figure 6 shows the chromatogram of the major phospholipids in a phospholipid-rich product (PU 307). The chromatograms of the PU 305 and PU 306 products were very similar in appearance as the PU 307 product and hence no longer shown. The separation times for PC is 7.538 mins, for PE is 5.131 mins, for SM1 is 10.103 mins and for SM2 is 10.916 mins in the PU 307 product. It is expected to have some slight variations in the separation times of the major phospholipids in the phospholipid-rich products as compared with Beta Serum and Procream products because of the difference in their composition and processes undergone. The chromatogram also shows that all the major phospholipids can all be obtained within 12 minutes.

3.6. Amounts of Phospholipids in Phospholipids-Rich Products

Table 3 shows the amounts of PC, PE and SM from the different phospholipids-rich products (PU305, PU306 and PU307) using ^{31}P-NMR and HPLC-UVvis methods. The calculated concentration of the individual phospholipids was again determined using equations 2 to 4 as before. The results show that for the reconstituted PU 305 product (5% Powder) gave percentage difference values of 3.5%, 7.1%, 9.1% and 4.0% for PC, PE, SM and Total (PC+PE+SM), respectively between the ^{31}P-NMR and HPLC-UVvis. These results are very satisfactory since all the percentage difference values were all below 10%. For the reconstituted PU 306 product (5% Powder) gave percentage difference values of 7.0%, 7.3%, 0% and 0% for PC, PE, SM and Total (PC+PE+SM), respectively between the ^{31}P-NMR and HPLC-UVvis. Lastly, for the reconstituted PU 307 product (5% Powder) gave percentage difference values of 3.3-6.9%, 13.6-20.5%, 3.4-12.5% and 6.8-13.2% for PC, PE, SM and Total (PC+PE+SM), respectively between the ^{31}P-NMR and HPLC-UVvis. The HPLC-UVvis results were closer to the values obtained from direct method of ^{31}P-NMR measurements.

Table 2. Comparison of the amounts of the major phospholipids in the Butter Serum and Procream products using ^{31}P-NMR and HPLC-UVvis methods.

Sample Analysed	^{31}P-NMR Analysis at 30^{o}C (g/100g lipid)				HPLC-UVvis Analysis at 40^{o}C (g/100g lipid)			
	PC	PE	SM	Total*	PC	PE	SM	Total*
(BS) Powder via Lipid	12.1	13.8	9.1	35.0				
Reconst. BS (15% Powder) via Lipid					11.7	10.9	8.9	31.5
Percentage Difference **					3.3%	21.0%	2.2%	10.0%
Procream (PRC) Powder via Lipid	8.8	7.9	8.1	24.8				
Reconst. PRC1 (15% Powder) via Lipid					9.4	9.0	7.6	26.0
Reconst. PRC2 (15% Powder) via Lipid					10.2	8.1	7.9	26.2
Reconst. PRC3 (15% Powder) via Lipid					11.3	8.0	7.0	26.3
Mean of Reconst. PRC (15% Powder) via Lipid					10.3	8.4	7.5	26.2
Percentage Difference **					17.0%	6.3%	7.4%	5.6%

Reconst. – Reconstituted * Total (PC+PE+SM) ** Percent Diff = 100 x |^{31}P-NMR – HPLC-UVvis|/^{31}P-NMR

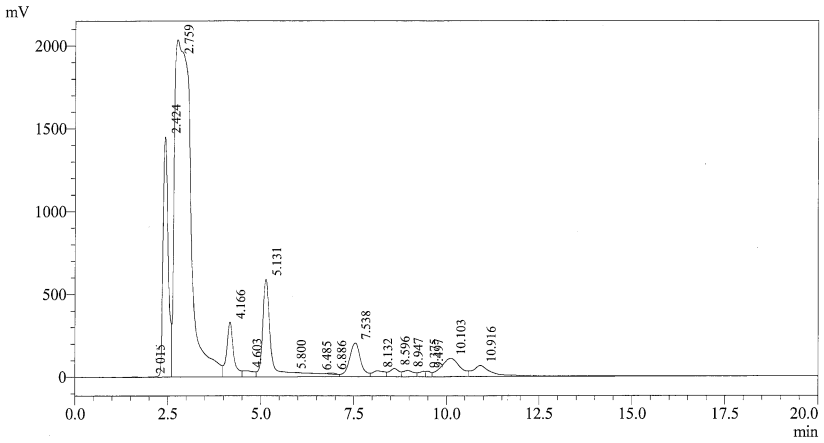

Figure 6. Chromatogram of the major phospholipids in a phospholipid-rich product (PU 307) (Separation times: PE=5.13min; PC=7.54min; SM1=10.10min; and SM2=10.92min).

Table 3. Comparison of the amounts of the major phospholipids in different phospholipids-rich products (PU 305, PU 306 and PU 307) using ^{31}P-NMR and HPLC-UVvis methods.

Sample Analysed	^{31}P-NMR Analysis at 30°C (g/100g lipid)				HPLC-UVvis Analysis at 40°C (g/100g lipid)			
	PC	PE	SM	Total*	PC	PE	SM	Total*
PU 305								
Powder via Lipid 8.6	8.4	7.7	24.7					
Reconst. PU 305 (5% Powder) via Lipid					8.3	9.0	8.4	25.7
Percentage Difference**					3.5%	7.1%	9.1%	4.0%
PU 306 Powder via Lipid	8.6	8.2	8.0	24.8				
Reconst. PU 306 (5% Powder) via Lipid					8.0	8.8	8.0	24.8
Percentage Difference**					7.0%	7.3%	0%	0%
PU 307 Powder via Lipid (A)	8.7	8.3	8.0	25.0				
PU 307 Powder Direct Method (B)	9.0	8.8	8.7	26.5				
Reconst. PU 307 (5% Powder) via Lipid					9.3	10.0	9.0	28.3
Percentage Difference (A)**					6.9%	20.5%	12.5%	13.2%
Percentage Difference (B)**					3.3%	13.6%	3.4%	6.8%

Reconst. – Reconstituted * Total (PC+PE+SM) ** Percent Diff = 100 x |^{31}P-NMR – HPLC-UVvis|/^{31}P-NMR

4. Conclusion

A new solvent extraction method for lipids from liquid dairy samples was developed with acceptable results.

Sphingomyelin (SM) consists of 2 curves in Beta Serum, Procream and phospholipid-tich products. The phosphatidylethanolamine (PE) separated ahead of phosphatidylcholine (PC) and then PC ahead of SM1 and SM2.

The developed HPLC-UVvis method can be used for determining the major phospholipids (PC, PE and SM) in dairy products with comparable results with the ^{31}P-NMR method. It must be noted that the analysis temperature for the HPLC-UVvis method was at 40°C while the ^{31}P-NMR method was at 30°C, and this might have also contributed to the slight variation of the results. Furthermore, the HPLC-UVvis method is rapid and with cheaper analysis cost compared with the ^{31}P-NMR method.

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Authors' contributions:

The article was written by LMD, as well as the data analysis.

Conflict of interest disclosure:

The authors declare no conflict of interest on the written article.

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