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ORIGINAL PAPER

Quantitative Analysis of Dairy Phospholipids by ^{31}P NMR

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Abstract ^{31}P NMR analysis of samples prepared in a sodium cholate detergent system was assessed as a method for the quantitative measurement of dairy phospholipids. Major phospholipid (PL) classes measured included: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SM) and dihydrosphingomyelin (DHSM). The ^{31}P NMR method was validated by comparison with a quantitative two-dimensional thin-layer chromatography (2D-TLC) technique. The 2D-TLC system was more sensitive, able to detect some minor compounds not observed by ^{31}P NMR. However, ^{31}P NMR is more suited to routine analysis, with sample analysis taking 36 min. The method was also more versatile and sample analysis was possible on high phospholipid containing materials without prior lipid extraction (e.g. buttermilk protein concentrate, beta serum liquid).

Keywords ^{31}P NMR · TLC · Dairy phospholipids

Abbreviations

DHSM	Dihydrosphingomyelin
EDTA	Ethylenediaminetetraacetic acid
Lac-PE	Amadori product of lactose and PE
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PL	Phospholipid
PS	Phosphatidylserine
PMG	Phosphonomethylglycine
SM	Sphingomyelin
SPE	Solid-phase extraction

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Introduction

Phospholipid (PL) preparations from dairy sources differ from the more common vegetable lecithins. Dairy phospholipids contain high levels of sphingomyelin (SM) and phosphatidylserine (PS) as well as exhibiting different fatty acid profiles. Official recommended methods for phospholipid analysis are based on quantitative two-dimensional thin-layer chromatography (2D-TLC) [1] or high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [2]. The TLC method quantitatively measures phosphorus content and so may be applied to any phospholipid extract as long as average molecular weights for each phospholipid class are known. However, the method is time consuming and is not suitable for high-throughput analysis; also resolution of the major dairy phospholipids, phosphatidylinositol (PI) and PS, and SM and phosphatidylcholine (PC) is poor using the solvent systems described in this method. HPLC with UV detection requires the use of suitable calibration standards. Since degree of unsaturation of the fatty acids in the phospholipids influences peak response it would be inappropriate to use soy lecithin phospholipid standards for calibration of analysis for dairy phospholipids. Apart from dairy sphingomyelin (Avanti Polar Lipids Inc.), commercial dairy phospholipid standards are not available. The International Lecithin and Phospholipid Society (ILPS) has conducted trials to establish HPLC methods for phospholipid analysis and recommended light-scattering detection (LSD). This

method was useful for soy lecithin but failed in cases of more complex lecithin samples (including milk) [3]. HPLC-LSD has recently been adopted as an AOCS official method for the analysis of lecithin [4].

^{31}P NMR has become an important technique for the analysis of phosphorus-containing compounds in foods. In the analysis of milk the technique has been used to investigate inorganic phosphate, casein-bonded phosphoserine, glycerophosphocholine and phospholipids [5]. The method is non-destructive and specific standards are not necessary for quantification. To obtain good resolution of phospholipid class peaks in the analysis of lipid extracts various solvent systems have been employed (e.g. chloroform/methanol/EDTA [6]; triethylamine/dimethylformamide/guanidinium chloride [7]). Detergents (e.g. sodium cholate [8]) have also been used to solubilise the phospholipids for ^{31}P NMR analysis. An advantage of using a detergent system is the option of analysing material without prior extraction of the lipids (i.e. non-lipids are also soluble). In this paper we present the quantitative analysis of dairy phospholipids by ^{31}P NMR using a sodium cholate detergent system with validation by comparison with a quantitative 2D-TLC method.

Materials and Methods

Fresh cream was purchased from a supermarket. Other dairy products [fresh beta serum, buttermilk protein concentrate 60 powder (BPC60) and PC700 powder] were supplied by Fonterra Co-operative Group. Beta serum powder was prepared from liquid beta serum by freeze-drying. The soy lecithin standard (ILPS crude lecithin standard, Lot ILPS-E03) was from Avanti Polar Lipids Inc.

Total lipid was extracted from cream (50 g) using the method of Folch et al. [9] with a yield of 19.4 g. The lipid was redissolved in chloroform (50 ml) and loaded onto a column of silica gel (Silica gel 60, 0.04–0.06 mm, 10 g) in 2.5 ml aliquots with 25 ml washes of chloroform between each aliquot. A final chloroform wash of 150 ml was used to remove neutral lipids from the column. Phospholipids were collected from the column with methanol (150 ml) followed by chloroform–methanol–water (3:5:2 v/v/v, 150 ml) [10]. Solvent was removed by rotary evaporation (yield 100.2 mg polar lipid).

Total lipid was extracted from BPC60 powder using the method of Dieffenbacher and Bracco [11] giving a yield of 17.7% w/w.

TLC Analysis

An aliquot of a sample solution was spotted on a TLC plate (10 × 10 cm, silica gel 60, Merck). The lipids were separated by 2D-TLC in the following solvent systems: (1) chloroform–methanol–28% aq. NH_3–benzene (65:30:6:10, by vol.) [12] in the first direction twice; (2) chloroform–ethyl acetate–acetone–2–propanol–absolute ethanol–methanol–water–acetic acid (30:6:6:6:16:28:6:2, by vol.) [13] in the second direction. Aminophospholipids were detected with 0.5% ninhydrin in acetone. Phospholipids were detected with the molybdate spray reagent [14]. All organic compounds were detected with 10% (by vol.) sulphuric acid in methanol followed by charring. Phospholipid classes were identified on the basis of their relative mobility on a TLC plate and comparison with standard phospholipids.

Phospholipid content and total phosphorus of lipid extracts were determined by the micro-procedure described by Vaskovsky et al. [14]. After lipid separation by 2D-TLC and their detection with sulphuric acid as described above the zones of silica gel containing phospholipids were scraped off into Pyrex test-tubes (7 ml). The samples were digested with 72% perchloric acid (0.05 ml) by heating in a sand bath at 180−200°C for 20 min. After cooling, working reagent (0.45 ml) was added to each test-tube. The working reagent was prepared by addition of 1 N sulphuric acid (26 ml) to the molybdate stock reagent (5.5 ml) and adjusting the volume to 100 ml with water. The mixtures were mixed thoroughly with a Vortex mixer and heated in a boiling water bath for 15 min. After cooling, the test-tubes were centrifuged at 3,500 rpm (Megafuge 1.0, Heraeus Sepatech) for 5 min. Absorbance was measured at 815 nm against a blank using a UV–Vis spectrometer (Unicam Helios Beta, Spectronic Unicam, UK). The analysis of each lipid sample was performed at least in triplicate. Total phospholipids were determined as a sum of individual phospholipids collected from the TLC plates.

For determination of total phosphorus of lipid extracts aliquots of their solutions with known concentration were transferred into test-tubes. The solvents were evaporated to dryness under vacuum in a SpeedVac evaporator. As a variant of this procedure aliquots of the solutions were spotted on a TLC plate. The lipids were separated in the solvent system hexane–diethyl ether–acetic acid (70:30:1, by vol.) followed by charring with sulphuric acid. The zones of silica gel on the start position containing all phospholipids were scraped off into Pyrex test-tubes. The further procedure of phosphorus determination was as described above.

The phosphorus content was calculated using a linear calibration curve determined for sodium dihydrophosphate as standard. A calibration curve was determined for each batch of samples.

^{31}P NMR Analysis

^{31}P NMR analysis was performed in triplicate and was based on the method of Lehnhardt et al. [8]. A detergent

solution was prepared containing: sodium cholate (10% w/w), EDTA (1% w/w) and phosphonomethylglycine (PMG) as an internal standard for quantification (0.3 g/l); pH was adjusted to 7.1 using sodium hydroxide. The detergent solution was an aqueous solution containing 20% D_2O for deuterium field-frequency lock capability. Sample was mixed with detergent solution (750 µl) by vortexing, then dispersed by ultrasonication with occasional shaking at 60°C for up to 10 min. The amount of sample used depended on its phospholipid content (lecithin standard 15 mg, cream polar lipid 15 mg, BPC60 powder 50 mg, BPC60 lipid 15 mg, lipid-depleted BPC60 residue powder 70 mg, PC700 20 mg, beta serum powder 60 mg, liquid beta serum 200 µl in 500 µl detergent). When required, pH adjustment was made with aqueous NaOH after the sample was fully dispersed in the detergent. The solution was then transferred to a 5 mm NMR tube for analysis.

Quantitative phosphorus NMR spectra using inverse gate proton decoupling for suppression of nuclear Overhauser effect were recorded on the two-channel Bruker Avance300 with the following instrument settings: temperature 30°C, spectrometer frequency for ^{31}P 121.498 MHz, sweep width 6,067 Hz, 65,536 data points, 90° excitation pulse. One hundred ninety-two transients were normally taken, each with a 3.5 s delay time and free induction decay acquisition time of 5.4 s. Spectra were processed with a standard exponential weighting function with 0.2 Hz line broadening before Fourier transformation.

Chemical shifts were measured relative to the PMG internal standard, and also relative to SM (−7.53 ppm relative to PMG at pH 7.1). Molecular weights were calculated from average fatty acid chain lengths for dairy phospholipids [15], and from values reported in the Avanti Polar Lipids Inc. catalogue for soy lecithin phospholipids and dairy SM. Peak identification was confirmed with pure standards. The dihydrosphingomyelin (DHSM) peak was confirmed after hydrogenation of SM [16]. A standard of Amadori product of lactose and PE (Lac-PE) was produced using the method of Utzmann and Lederer [17].

Results and Discussion

Analysis of Soy Lecithin Standard

Results for the analysis of the ILPS crude soy lecithin standard by ^{31}P NMR, TLC, and those from the certificate of analysis (from HPLC and ^{31}P NMR measurements) are presented in Table 1. Only values for the major phospholipids reported in the certificate of analysis are given in the table, and the results from the ^{31}P NMR analysis closely match those from the certificate of analysis. Other phospholipids measured in this sample include: PS, N-acyl phosphatidylethanolamine, LPE and phosphatidylglycerol, all at levels of less than 1% w/w of sample. Phospholipid levels (as wt% of sample) as measured by TLC are lower for total phospholipid and each of the major phospholipid classes given in the certificate of analysis. When the phospholipid class profile is presented as mol% (not shown) the numbers from TLC more closely match the ^{31}P NMR values and values calculated from the Avanti certificate of analysis. A direct analysis of the total phosphorus content in the soy lecithin standard using the molybdate reagent (i.e. without TLC) gave a total phospholipid content of 38.4 ± 1.4%, which is more similar to the certificate of analysis (Table 1).

Table 1 Phospholipid profile (wt% of sample) of Avanti soy lecithin standard as measured by 2D-TLC and ^{31}P NMR

	TLC		^{31}P NMR		Avanti certificate of analysis (wt%)	
	wt%	sd	wt%	sd	^{31}P NMR	HPLC
PC	14.4	1.0	15.3	0.4	15.7	15.4
PI	7.3	0.2	8.0	0.2	7.6	7.3
LPC	0.4	0.1	0.4	0.04	<0.5	<0.5
PE	12.5	0.5	13.8	0.3	13.2	13.7
PA	1.6	0.1	2.0	0.1	2.4	2.7
Total PL	36.3	1.7	39.0	0.8	38.9	39.1

Analysis of Cream Phospholipids

The 2D-TLC method provided good resolution of phospholipids in complex lipid mixtures using the solvent systems described in the "Materials and Methods" section. The acidic phospholipids (PI, PS, Lac-PE), which can be difficult to separate by TLC, were well resolved and were collected individually for further analysis (Fig. 1). However, 2D-TLC analysis revealed some minor molybdate positive spots which are yet to be identified. There were three unidentified phospholipids observed in bovine cream and assigned X1, X2 and X3 (Fig. 1). While the spot X1 was also observed in other dairy extracts analysed (PC700 and BPC60), two other spots (X2 and X3) were not detected in PC700 and BPC60.

A ^{31}P NMR spectrum of cream polar lipids (Fig. 2) shows baseline peak resolution of most of the major phospholipids (PC, PI, PS and DHSM) using the sodium cholate detergent system at pH 7.1. PE and SM are not completely resolved at this pH. The separate measurement of SM and DHSM is an advantage of this method compared with chromatographic methods (TLC, HPLC). ^{31}P NMR analysis of milk phospholipids has been conducted previously using a monophasic dimethylformamide/triethylamine/guanidinium hydrochloride solvent system [18]. This system, however, did not achieve a well-resolved PS peak, adducts

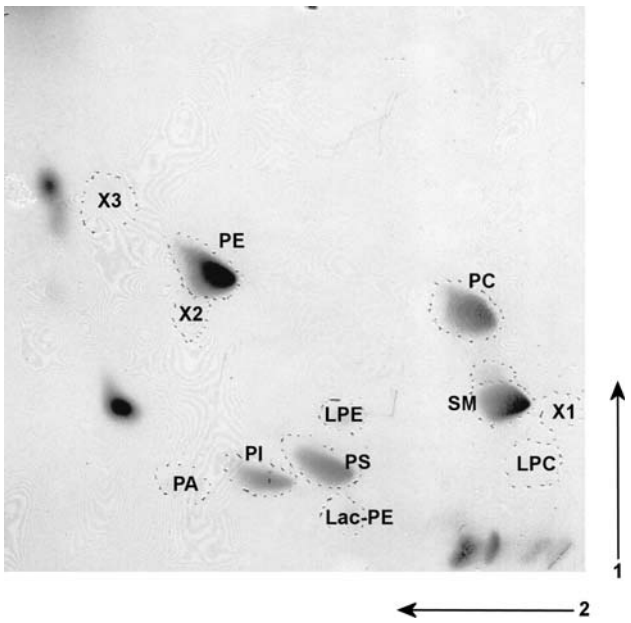

Fig. 1 2D-TLC of cream polar lipids. Molybdate positive spots are outlined and labelled. Solvent systems, 1st direction: chloroform–methanol–28% aq. NH_3–benzene (65:30:6:10, by vol.), 2nd direction: chloroform–ethyl acetate–acetone–2-propanol–absolute ethanol–methanol–water–acetic acid (30:6:6:6:16:28:6:2, by vol.)

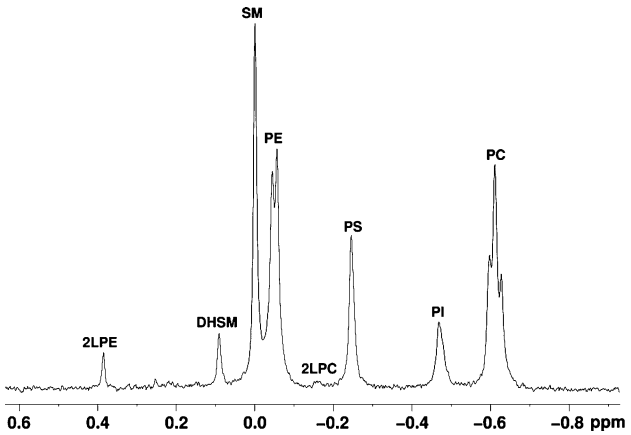

Fig. 2 ^{31}P NMR spectrum of cream polar lipids in the sodium cholate detergent system (pH 7.1)

of PE and triethylamine gave additional peaks, and DHSM was not observed at all (hidden under PI).

Quantitative results for major phospholipid classes (as mol% of total PL) exhibit some significant differences between the TLC and ^{31}P NMR methods (Table 2). This can be attributed in part to a number of TLC spots arising from minor unidentified molybdate positive compounds which were not observed with ^{31}P NMR. The sum of the minor phospholipid classes including PA, X1, X2 and X3 constituted 2.8 mol% of total phospholipid. It appears that TLC is the more sensitive method.

Table 2 Phospholipid profiles (mol% of total PL) of cream as measured by 2D-TLC and ^{31}P NMR (at pH 7.1 and pH 9.5)

	TLC[a]		^{31}P NMR		pH 9.5
	mol%	sd	mol%	sd	mol%
PC	28.1	0.3	26.5	0.3	26.0
PI	7.0	0.2	7.5	0.1	6.9
PS	10.4	0.3	11.7	0.2	10.7
LPC	1.2	0.0	1.1	0.1	1.4
PE	22.1	0.3	26.7	0.1	25.6
SM			20.8	0.5	20.1
DHSM			3.9	0.2	4.2
DHSM + SM	25.7	0.4	24.7	0.7	24.3
Lac-PE	0.7	0.1			0.9
LPE	1.9	0.2	1.8	0.4	3.1
PL in polar lipid (% w/w)	70.3	2.3	78.6	1.3	76.7
PL in total lipid (% w/w)	0.37	0.012	0.42	0.007	0.41
PL in cream (% w/w)	0.14	0.005	0.16	0.003	0.16

[a] Other molybdate positive spots on the TLC as indicated in Fig. 1 were also measured: X1 0.4 ± 0.1 mol%, X2 1.0 ± 0.5 mol%, X3 0.8 ± 0.1 mol%, PA 0.6 ± 0.1 mol%

The phospholipid class exhibiting the greatest difference between the two methods was PE (26.7 mol% by NMR, 22.1 mol% by TLC). To discount the difference in PE values being due to the partial overlap of the PE and SM NMR peaks, the pH of the detergent system was raised to 9.5 and the sample reanalysed. This gave a well-resolved PE peak, but the quantitative results (when accounting for increased levels of LPE due to base-catalysed hydrolysis) were still significantly higher than the TLC results. Major phospholipid classes for both methods are in similar proportions to those described previously in a review of numerous studies on the analysis of milk phospholipids by HPLC-ELSD or TLC densitometry [19, 20]. The proportions of DHSM and SM (15.8% and 84.2%, respectively) measured by ^{31}P NMR are similar to those previously reported by Byrdwell and Perry [21] (16.2 and 83.8%).

Total phospholipid levels measured by quantitative TLC (70.3 ± 2.3% w/w) were significantly lower than the ^{31}P NMR measurement (78.6 ± 1.3% w/w). When a cream polar lipid sample was measured directly using the molybdate spectrophotometric method (average phospholipid MW of 750), a higher value was obtained (74.6 ± 2.4% w/w). This suggests that a significant amount of phosphorus is not recovered from the TLC plates.

Ether lipids (e.g. ethanolamine plasmalogen and alkyl-acyl-phosphatidylcholine) were not detected by either ^{31}P NMR or 2D-TLC. Ethanolamine plasmalogen would appear between SM and PE on the ^{31}P NMR spectrum and is unlikely to be well resolved. Quantitative analyses of ether lipids may require additional analytical steps.

Previous analysis of deacylated phospholipids has shown ethanolamine plasmalogen to be a minor component of bovine milk phospholipids (2% of total PL) [22].

Analysis of PC700 Phospholipids

PC700 is a commercial product from Fonterra Co-operative Group Limited with high levels of phospholipids. The product is soluble in chloroform/methanol (2:1 v/v) so could be analysed directly by quantitative 2D-TLC, and could also be dispersed in the sodium cholate NMR detergent for ^{31}P NMR analysis (Table 3).

^{31}P NMR analysis showed the presence of the non-lipid phosphorus-containing compounds glycerophosphocholine (GPC) (0.03 ppm relative to SM) and glycerophosphoethanolamine (GPE) (0.58 ppm relative to SM). These compounds could be quantitatively measured but are not included in the phospholipid profiles in Table 3. Lactosylated phosphatidylethanolamine (Lac-PE) has a similar chemical shift to PE at pH 7, but could be measured by raising the pH of the sodium cholate detergent to 8.5 (i.e. two measurements were required for a complete phospholipid profile).

Analysis of BPC60 Phospholipids

BPC60 is a neutral lipid-depleted dairy powder produced by Fonterra Co-operative Group Limited. The powder contains a high phospholipid content which enabled direct analysis in the ^{31}P NMR detergent system. A comparison of ^{31}P NMR analysis of the total lipid extract with direct analysis of the powder showed some significant differences in both individual phospholipid profiles and total phospholipid levels (Table 4). Analysis of the residue powder from the lipid extraction showed significant levels of phospholipid had not been extracted. The extraction method [11] had not extracted PS and PI as effectively as the other phospholipids. A composite calculation of results from the residue and extract resulted in a similar phospholipid profile to that of the direct BPC60 powder analysis (Table 4).

There were many non-lipid phosphorus-containing compounds observed in the ^{31}P NMR direct analysis of BPC60 powder. As well as GPC and GPE, there was a large peak due to inorganic phosphate and peaks resulting from phosphoserine residues in casein. Most of these peaks were outside of the phospholipid region of the spectrum (−0.6 to 0.4 ppm relative to SM). Quantitative analysis of these compounds by ^{31}P NMR is possible and has been investigated previously [23, 24]. The TLC analysis of BPC60 lipid extract gave mol% values of phospholipid classes close to that of ^{31}P NMR analysis but with a lower total phospholipid (% w/w) measurement (Table 4).

Table 3 Phospholipid profiles (mol% of total PL) of PC700 as measured by 2D-TLC and ^{31}P NMR

	TLC[a]		^{31}P NMR	
	mol%	sd	mol%	sd
PC	31.1	0.9	30.6	0.1
PI	2.9	0.2	3.2	0.2
PS	3.7	0.4	4.0	0.1
LPC[b]	1.6	0.3	1.8	0.1
PE	28.0	0.6	27.9	0.2
SM			21.0	0.1
DHSM			5.3	0.2
DHSM + SM	28.5	0.3	26.2	0.1
Lac-PE	1.7	0.3	3.4	0.2
LPE[b]	1.8	0.3	2.9	0.2
PL in sample (% w/w)	53.2	1.1	60.6	1.5

[a] The unknown spot X1 was also measured by TLC 0.7 ± 0.4 mol% (see Fig. 1 for position of unknown spots on TLC)

[b] Sum of 1- and 2-lyso forms (both isomers were observed by ^{31}P NMR)

Analysis of Beta Serum Phospholipids

Having demonstrated that ^{31}P NMR could be used to analyse the phospholipids contained in a powder without performing a total lipid extraction, the method was applied to an aqueous by-product of the dairy industry. Beta serum is a high phospholipid content emulsion derived from the processing of milk. As with the analysis of powders, direct TLC of this aqueous by-product is not possible. A comparison of the ^{31}P NMR analysis of the liquid beta serum and freeze-dried beta serum powder gave very similar results (Table 5).

Optimal pH Conditions for ^{31}P NMR Analysis

The chemical shifts of some phospholipid classes (PE, PS, Lac-PE, PA and the corresponding lysolipids) are influenced by pH and temperature [25]. Avanti Polar Lipids Inc. [26] has described a method of analysing soy lecithin in a sodium deoxycholate detergent system at pH 8.6. The major phospholipids in soy lecithin are well resolved at this pH. However, under these conditions PE is not resolved from SM, and 2-lysophosphatidylcholine (LPC) and PS are poorly resolved so the method is unsuitable for dairy phospholipid analysis. The effect of pH on the chemical shifts of the major dairy phospholipids in the sodium cholate detergent at 30°C (Fig. 3) shows that between pH 7.1 and 9.4 there is no condition that allows for good resolution of every phospholipid class. At higher pH (>9) the PMG internal standard peak shape broadens

Table 4 Phospholipid profiles (mol% of total PL) of BPC60 as measured by 2D-TLC and ^{31}P NMR of total lipid extract, and ^{31}P NMR direct measurement of powders

	TLC		^{31}P NMR		Residue		Comp.[a]	BPC60 powder	
	Lipid extract		Lipid extract						
	mol%	sd	mol%	sd	mol%	sd	mol%	mol%	sd
PC	26.4	1.8	27.0	0.1	15.2	1.5	25.7	26.4	0.6
PI	5.4	0.9	5.8	0.2	20.6	0.7	7.5	7.6	0.5
PS	9.5	1.3	9.7	0.1	23.9	0.7	11.3	11.5	0.2
LPC[b]	0.5	0.2	0.7	0.1	n.d.		0.6	0.7	0.8
PE	26.9	2.0	25.7	0.7	19.2	2.5	24.9	25.8	1.3
SM			20.4	0.2	10.6	1.0	19.2	16.9	0.5
DHSM			4.6	0.2	4.9	1.6	4.6	4.6	0.6
DHSM + SM	26.3	1.9	25.0	0.4	15.5	2.6	23.9	21.5	0.6
Lac-PE	4.5	1.1	5.1	0.5	5.6	2.4	5.2	5.4	0.4
LPE[b]	1.1	0.1	1.0	0.1	n.d.		0.9	1.0	0.2
PL in sample (% w/w)	72.9	2.9	75.8	1.0	1.8	0.2	15.3	15.1	0.6

[a] Composite calculation of PL in the powder based on ^{31}P NMR and yield results for lipid extract and residual powder

[b] Sum of 1- and 2-lyso forms (both isomers were observed by ^{31}P NMR)

Table 5 Phospholipid profiles (mol% of total PL) of beta serum as measured by ^{31}P NMR of the liquid and freeze-dried powder

	Liquid		Powder	
	mol%	sd	mol%	sd
PC	28.3	1.4	28.0	0.5
PI	7.5	0.6	7.8	0.1
PS	11.1	0.5	11.8	0.1
LPC	1.2	0.1	1.2	0.1
PE	29.9	0.5	30.6	0.5
SM	18.8	1.0	17.8	1.2
DHSM	2.7	0.4	2.8	0.3
DHSM + SM	21.5	1.0	20.6	1.2
Lac-PE	n.d.		n.d.	
LPE	1.3	0.3	1.3	0.2
PL in liquid beta serum (% w/w)	1.07	0.14	0.89[a]	0.04

[a] Calculated from total PL measured in the powder (9.0% wt/wt) and the solids content of the liquid (9.9% w/w)

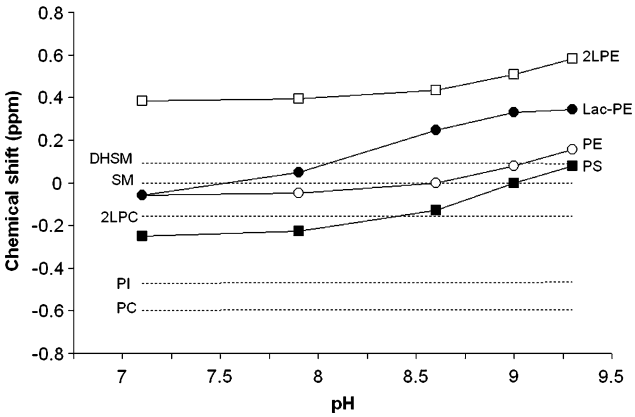

Fig. 3 The effect of pH on the chemical shifts of major phospholipid classes observed by ^{31}P NMR in the sodium cholate detergent. *Dotted lines* represent PL classes with no observed change in chemical shift over the pH range. PL classes exhibiting changes in chemical shifts are represented by *solid lines* and *filled square* PS, *open square* 2LPE, *open circle* PE, *filled circle* Lac-PE

considerably and an unacceptable level of base-catalysed hydrolysis is encountered. At pH lower than 7, EDTA (important for the scrubbing of divalent cations) precipitates out of solution. For routine analysis of dairy phospholipids it is recommended that a pH of 7–7.3 be used. If the presence of Lac-PE needs to be monitored, this can be conducted in a separate measurement at pH 8.5. The chemical shifts of the major dairy phospholipids relative to SM at pH 7.1 are: PC −0.61 ppm, PI −0.47 ppm, PS −0.25 ppm, 2LPC −0.16 ppm, PE + Lac-PE −0.05 ppm, DHSM 0.09 ppm, 2LPE 0.38 ppm. Minor lipids 1LPC 0.34 ppm, 1LPE 0.25 ppm and 2LPS 0.14 ppm may also be observed. At pH 8.5 the chemical shift of Lac-PE relative to SM is 0.24 ppm.

The effect of temperature on chemical shifts was not investigated in this study and all NMR measurements were conducted at 30°C. A previous study on phospholipid standard mixtures [25] has shown that at pH 7.6 and 10°C PI is poorly resolved from PC, and at 50°C SM, PE and PS are not well resolved. Further work could be performed to determine optimal temperature for the analysis of milk phospholipids.

Conclusion

^{31}P NMR is a versatile method for the quantitative analysis of dairy phospholipids. Major phospholipids PC, PE, SM, DHSM, PI and PS were measured. Lac-PE could also be determined by altering pH and conducting a second analysis. The method is rapid (36 min per analysis on a 300 MHz instrument with autosampler) and so is more suited to routine analysis than 2D-TLC. Lipid extracts, powders and aqueous dairy emulsions were all able to be analysed with this method. However, the TLC method was more sensitive and detected a number of compounds not seen by NMR. It is unlikely that samples containing lower levels of phospholipids (<1% w/w) could be reliably analysed by NMR without prior polar lipid extraction or employing longer analysis times and/or a higher-field instrument. The method is recommended for the routine analysis of dairy samples containing high levels of phospholipids (e.g. beta serum).

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