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Two-Dimensional $^{31}P,^{1}H$ NMR Spectroscopic Profiling of Phospholipids in Cheese and Fish

Stefanie Kaffarnik,[†] Ina Ehlers,[§] Gerhard Gröbner,[⊗] Jürgen Schleucher,*[,§] and Walter Vetter*[,†]

[†]Department of Food Chemistry, Institute of Food Chemistry, University of Hohenheim, Garbenstrasse 28, D-70599 Stuttgart, Germany

[§]Department of Medical Biochemistry and Biophysics and [⊗]Department of Chemistry, Umeå University, S-90187 Umeå, Sweden

Supporting Information

ABSTRACT: Phospholipids (PLs) comprise an important lipid class in food because of their technological use as emulsifiers and their nutritional value. This study used one-dimensional ^{31}P NMR and two-dimensional (2D) $^{31}P,^{1}H$ COSY NMR spectroscopy for the determination of the PL composition of cheese and fish after liquid−liquid enrichment. This extraction step enabled the identification of 10 PLs in cheese and 9 PLs in fish by 2D $^{31}P,^{1}H$ NMR. Variations in the ^{31}P shifts indicated differences in the fatty acids attached to the individual PLs. The total PL content in cheese fat and fish oil ranged from 0.3 to 0.4% and from 5 to 12%, respectively. Phosphatidylcholine was the most prominent PL in both matrices (up to 65%). Minor PLs (limit of detection = 4 nmol, i.e. 500 μL of an 8 μM solution) were identified in forms of phosphatidic acid, lysophosphatidic acid, and phosphatidylglycerol. Specific cross couplings and ^{1}H fine structures in the 2D $^{31}P,^{1}H$ NMR spectra proved to be valuable for the assignment and verification of known and uncommon PLs in the samples.

KEYWORDS: *phospholipids, nuclear magnetic resonance spectroscopy, ^{31}P NMR, food, milk fat, fish oil*

INTRODUCTION

Phospholipids (PLs) are a group of polar lipids widely found as minor compounds in food lipids. The most common PLs, that is, glycerophospholipids and sphingolipids, are essential constituents in the cellular membranes of nearly all organisms.[1] Due to their specific molecular geometry and amphiphilic features, PLs form spontaneous two-dimensional membrane leaflets with a unique structural and functional organization.[2−4] The properties of the hydrophilic membrane surface, for example, its charge, are governed by different headgroup moieties on the PL. Therefore, the headgroup composition of PLs is crucial for the function of biomembranes, and it directs the binding of ions, proteins, nutrients, and chemical signals.[2−4] In food, PLs serve as valuable ingredients because of their nutritional value and their technological properties (e.g., as emulsifier).[5] Likewise, the PL composition of fish has been suggested to be a valuable indicator of freshness due to enzyme-catalyzed post-mortem lipolysis,[6,7] a process accelerated by inadequate storage conditions.[6−9]

PLs in food are frequently analyzed by liquid chromatographic enrichment[10,11] followed by determination of the polar head groups by high-performance liquid chromatography (HPLC) in combination with evaporative light scattering detection (ELSD)[12,13] or electrospray ionization mass spectrometry (ESI/MS).[9,14−17] Other analysis protocols described direct analysis of PL by tandem mass spectrometry in positive and negative electrospray ionization without chromatographic separation[18] or matrix-assisted laser desorption/ionization in combination with time-of-flight mass spectrometry (MALDI/TOF-MS).[19,20] Moreover, the fatty acid composition of separated PLs was determined by gas chromatography coupled with mass spectrometry (GC-MS).[21,22] Finally, 31-phosphorus nuclear magnetic resonance spectroscopy (^{31}P NMR) has been used to determine the headgroup composition of PLs in complex lipid mixtures.[23−26] However, chemical shifts of PL headgroups in ^{31}P NMR spectra are distributed over a rather small range of only about 2 ppm.[24] As a consequence, ^{31}P resonances from different PL head groups often overlap. Although this drawback can be overcome by 1D NMR in combination with chemometrics,[28,29] these efforts do not allow for an unambiguous identification of the individual PL classes.[27] Such problems can be solved by means of two-dimensional (2D) NMR approaches.[27,30−32] 2D NMR techniques such as 2D semiconstant-time-correlated spectroscopy (COSY) $^{31}P,^{1}H$ NMR (2D $^{31}P,^{1}H$ NMR) provide not only improved ^{31}P resolution but also a good sensitivity (limit of detection, LOD, ~3 μg[27] corresponding to 500 μL of an 8 μM solution). This gain in sensitivity is achieved by correlating the headgroup phosphorus nucleus with its proton environment separated by up to three bonds.[27] Protons on both the glycerol backbone and the side chain of the polar headgroup generate distinct $^{31}P-^{1}H$ cross peaks because of their unequal chemical environment.[33] Moreover, the 2D $^{31}P,^{1}H$ NMR spectra display antiphase J_{HP} couplings and in-phase J_{HH} couplings, which result in a characteristic "fingerprint" for each PL, and both features facilitate the structure verification.[27]

The goal of the present study was the application of 2D $^{31}P,^{1}H$ NMR to the analysis of PLs in food, namely, fish and cheese samples. Because PLs represent only a small share of food lipids (e.g., 0.3−1.9% PLs in cheese fat and 6−20% PLs in

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fish lipids),[21] we initially enriched PLs by means of liquid–liquid extraction (LLE). Individual PLs were identified by 2D ^{31}P,^{1}H NMR and 1D ^{31}P NMR in the polar LLE extract.

MATERIALS AND METHODS

Materials, Standards, and Food Samples. Methanol and *n*-hexane (both of HPLC gradient grade) were from Th. Geyer (Renningen, Germany). A mixture of ethyl acetate and cyclohexane (both purest, Sigma-Aldrich, Taufkirchen, Germany) was distilled before use to give the azeotropic mixture (54:46, w/w). Chloroform, triphenylphosphate, and CsOH were from Sigma-Aldrich (Steinheim, Germany), and ethylenediaminetetraacetic acid (EDTA) was from Merck (Darmstadt, Germany). NMR measurements were performed in a 2:1 solution of chloroform and deuterated methanol (Cambrigde Isotope Laboratories, Andover, MA, USA) including 10 mM Cs-EDTA.[24] Sodium chloride was from Carl Roth (Karlsruhe, Germany). Glyceryl tricaprylate (CCC) and glyceryl tristearate (SSS) were from Fluka (Buchs, Switzerland). 1,2-Dioleoylphosphatidylethanolamine (DOPE) was from Sigma-Aldrich (Steinheim, Germany), whereas 1,2-dilaurylphosphatidylcholine (DLPC), 1,2-dimyristoylphosphatidylethanolamine (DMPE), and 1,2-dipalmitoylphosphatidylcholine (DPPC) were from Larodan (Malmö, Sweden).

Three fish (salmon fillet, trout rainbow, and gilthead seabream) and four cheese (butter cheese, organic brie, cow's milk mozzarella, and Edam cheese) samples were analyzed. The samples were chosen because all but the Edam cheese sample had been previously analyzed by Hauff and Vetter for their PL content (cheeses, 0.5–0.7%; fish, 6–11%) by GC-MS.[21] Accordingly, the PL content of NMR measurements could be compared to the previous results. However, the samples had been stored in a freezer (−20 °C) for 2 years prior to the NMR measurements, with the possible effect of partial lipolysis.[21]

Accelerated Solvent Extraction (ASE) and Sample Preparation for NMR Analyses. About 2 g of the homogenized and lyophilized cheese and fish samples was weighed in 11 mL cells, and ASE was performed by means of an ASE 200 system (Dionex, Idstein, Germany) according to the method of Weichbrodt et al.[34] Lipids were first extracted with ethyl acetate/cyclohexane (54:46, w/w) followed by ethyl acetate/methanol (1:1, v/v).[21,34] Both extracts were combined, the solvent was evaporated, and the sample was made up with 5 mL of ethyl acetate/cyclohexane (total ASE extract).

LLE of Lipid Standards and Samples. DLPC, DPPC, DMPE, and DOPE (∼100 μg per compound in 0.1 mL of methanol) as well as CCC and SSS (∼100 μg per compound in 0.1 mL of *n*-hexane) were placed in 10 mL culture tubes with screw caps. The volume of the liquid phase was increased to 8 mL by means of the two-phase solvent system of *n*-hexane/methanol with 3% water (2:1, v/v). After phase separation, 2 mL of each phase was transferred into 10 mL culture tubes for transesterification (see next section).

In a second experiment, the standards (see above) were dissolved in 5.3 mL of *n*-hexane/2.7 mL of methanol with 3% water. After thorough shaking, the upper, nonpolar phase (n) was separated from the polar phase (p) and placed in a second 10 mL culture tube (Figure 1). To the nonpolar phase (n) was added 2.7 mL of methanol solution, the mixture was shaken, and the lower polar phase was removed to give the re-extracted nonpolar phase (n-n). Likewise, 2.7 mL of *n*-hexane was added to the initial polar phase (p). The mixture was thoroughly shaken, and the upper *n*-hexane phase was removed to give the re-extracted polar phase (p-p) (Figure 1). The corresponding treatments were repeated with n-n (addition of 5.4 mL of methanol solution) and p-p (addition of 1.3 mL of *n*-hexane) to give 2-fold re-extracted fractions n-nn and p-pp. The solvents were removed from the six final fractions (Figure 1), and the corresponding samples were stored until transesterification was performed.

LLE of the total sample ASE extract was performed according to the standard distribution in 10 mL culture tubes with 8 mL of *n*-hexane/methanol with 3% water (2:1, v/v) (Figure 1). About 1 g of dried cheese fat or about 0.3 g of fish oil was redissolved in 5.3 mL of *n*-hexane and 2.7 mL of methanol (3% H_2O). LLE was performed according to Figure 1 (see above). Thereafter, the solvents of the six fractions per sample were removed, and the samples were stored until the NMR analysis.

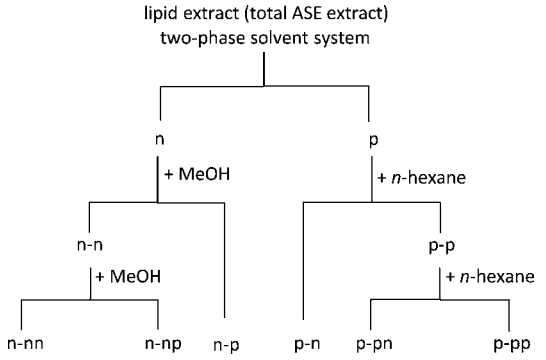

Figure 1. Scheme of liquid–liquid extraction (LLE) with *n*-hexane/methanol (3% water) for phospholipid (PL) enrichment. n, nonpolar fraction; p, polar fraction; n-nn and p-pp, 2-fold re-extracted LLE fractions, respectively.

Transesterification of Standard Lipids. Transesterification of the six dry lipid standard extracts (see previous section) was performed with 1 mL of methanol solution containing 1% sulfuric acid.[35] The resulting solutions were heated for 1 h at 80 °C and then cooled on ice. After the addition of 1 mL of *n*-hexane, 1 mL of sodium chloride solution, and 1 mL of distilled water, the mixtures were shaken and the organic phase was separated. The fatty acid methyl esters were analyzed by GC-MS in the selected ion monitoring (SIM) mode.

GC-MS. GC-MS analyses were performed in electron ionization (70 eV) mode by means of a 5890/5971 system (Hewlett-Packard, Waldbronn, Germany). The GC-MS system was equipped with a 7673 autosampler and a split/splitless injector. Injections (1 μL) were carried out in splitless mode. The injector and transfer line temperatures were set to 250 and 280 °C, respectively, whereas the detector temperature was maintained at 156 °C. Helium (purity = 5.0, Westfalen, Münster, Germany) was used as carrier gas (1.2 mL/min, constant flow). A 60 m length × 0.25 mm internal diameter × 0.1 μm film thickness Rtx-2330 column (Restek, Bellefonte, PA, USA) was installed in the GC oven. The SIM ions used for fatty acid methyl ester detection were *m*/*z* 74, 87, 81, 79, 88, and 101.[36] The oven program started at 60 °C (hold time = 1 min), followed by ramps at 6 °C/min to 150 °C, at 4 °C/min to 190 °C, and finally at 7 °C/min to 250 °C (hold time = 7 min).

One-Dimensional ^{31}P NMR Spectroscopy. Fractionated cheese and fish oil samples (p-pp, see above) were analyzed by 1D ^{31}P NMR spectroscopy. Selected total ASE extracts and the remaining five subfractions (p-n, p-pn, n-nn, n-p, n-np) of three samples (two cheese fat samples and one fish oil sample) were analyzed as well (Table S1, Supporting Information).

Lipid samples (maximum, 30 mg) were dissolved in a 2:1 $CHCl_3$/CD_3OD solution (600 μL); 0.8 μmol of triphenylphosphate as internal standard and 100 μL of buffer solution (0.4 M Cs-EDTA in D_2O[24]) were added to remove paramagnetic ions. Measurements were performed at 25 °C on a DRX 600 spectrometer equipped with a ^{1}H, ^{13}C, ^{31}P cryo-probe optimized for ^{1}H detection, a DRX 500 spectrometer equipped with a ^{1}H, ^{13}C, broadband probe tuned to ^{31}P, or a DRX 400 with a ^{1}H, broadband "smartprobe" tuned to ^{31}P (all Bruker, Fällanden, Switzerland). Spectra were recorded with 1500–11000 scans (1.25–9 h experiment time) depending on PL amount, and the spectral width was 40 ppm. ^{31}P pulse lengths corresponding to a 50° pulse were chosen, and proton decoupling was applied during the acquisition time of 1 s. A repetition time of 3 s was chosen to enable quantification of NMR spectra. Spectra were processed using TopSpin software 3.1; 1.0 Hz line broadening was applied. All peaks in the NMR spectra were integrated and converted into molar quantities by comparison with the internal standard triphenylphosphate, and the whole spectrum was integrated to obtain the total quantity of PLs. Phosphatidylcholine, the most common and highest concentrated PL,

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Table 1. Distribution of Standard Phospholipids (PLs) and Triacylglycerides (TAG) in the Two-Phase Solvent System *n*-Hexane/Methanol (3% Water), 2:1 (v/v)[a]

lipid class	lipid	fatty acid	polar fraction[b] (%)	nonpolar fraction[b] (%)
PL	1,2-dilaurylphosphatidylcholine (DLPC)	12:0	96	4
PL	1,2-dipalmitoylphosphatidylcholine (DPPC)	16:0	90	10
PL	1,2-dimyristoylphosphatidylethanolamine (DMPE)	14:0	94	6
PL	1,2-dioleoylphosphatidylethanolamine (DOPE)	18:1	89	11
TAG	glyceryl tricaprylate (CCC)	8:0	38	62
TAG	glyceryl tristearate (SSS)	18:0	11	89

[a] Individual PL standards contained one defined fatty acid species at both esterified glycerol positions to differ from neutral lipid standards glyceryl tricaprylate and glyceryl tristearate. [b] $n = 2$.

Table 2. Chemical Shifts and Their Variation for Phospholipids Identified in Fish and Cheese Samples after Liquid−Liquid Extraction

		^{1}H shifts[a]					
PL		$H_{backbone}$	$H_{headgroup}$	^{31}P shift[b], av (min; max)	^{31}P shift, fish,[c] av (min; max)	^{31}P shift, cheese,[c] av (min; max)	difference, fish − cheese
phosphatidylcholine	PC	4.00	4.27	−0.84	−0.865 (−0.898; −0.827)	−0.839 (−0.898; −0.805)	−0.026
phosphatidylinositol	PI	4.04	3.90	−0.363 (−0.391; −0.335)	−0.398 (−0.432; −0.368)	−0.358 (−0.449; −0.300)	−0.041
lysophosphatidylcholine	LPC	3.95 3.88	4.28	−0.277 (−0.313; −0.237)	−0.294 (−0.333; −0.260)	−0.281 (−0.371; −0.209)	−0.013
lysophosphatidylcholine plasmalogen	LPCp	3.94 3.83	4.28	−0.204 (−0.212; −0.195)	−0.232 (−0.266; −0.200)		
sphingomyelin	SM	4.13 3.92	4.27	−0.066 (−0.104 to −0.043)	−0.089 (−0.126; −0.053)	−0.067 (−0.162; −0.029)	−0.022
phosphatidylethanolamine	PE	4.00	4.06	0.037 (−0.063; 0.128)		0.026 (−0.121; 0.128)	
alkyl ether-linked phosphatidylethanolamine	PEe	3.91	3.97	0.061 (0.022; 0.089)		0.071 (0.004; 0.124)	
dihydrosphingomyelin	DHSM	4.14 3.90	4.26	0.142 (0.110; 0.163)		0.128 (0.052; 0.191)	
phosphatidic acid	PA	3.98		0.230 0.171; 0.247	0.202 (0.135; 0.257)	0.246 (0.173; 0.302)	−0.044
phosphatidylglycerol	PG	3.98	3.90	0.475 (0.464 to 0.489)	0.432 (0.432; 0.432)	0.469	−0.037
lysophosphatidylethanolamine	LPE	3.95 3.88	4.08	0.462 (0.450; 0.483)	0.447	0.463 (0.488; 0.438)	−0.017
lysophosphatidic acid	LPA	3.95 3.88		0.843 (0.800; 0.893)	0.811 (0.784; 0.873)	0.870 (0.783; 0.897)	−0.059
cardiolipin	CL				0.135 (0.130; 0.140)	0.194	−0.059
unknown	u					0.263 (0.243; 0.284)	

[a] ^{1}H chemical shift variation is generally below 0.01 ppm. [b] ^{31}P chemical shifts are calibrated relative to PC = −0.84 ppm. [c] ^{31}P chemical shifts are calibrated relative to triphenylphosphate = −17.95 ppm.

was used to calibrate the ppm scale (−0.84 ppm). Triplicate NMR measurements of the same sample indicated that the quantification error spanned from 1.6% (for the most abundant PL phosphatidylcholine) to 4.4% for a minor PL with a signal-to-noise ratio of 8:1.

2D Semiconstant Time COSY ^{31}P,^{1}H NMR (2D ^{31}P,^{1}H NMR). 2D ^{31}P,^{1}H NMR spectra were recorded at 25 °C on DRX 600 and DRX 500 NMR spectrometers (see above) according to the method of Petzold et al.[27] We measured 144 complex data points in the ^{31}P dimension and acquired 32 scans, which resulted in experimental times of 8 h. Spectra were processed (TopSpin software 3.1) using shifted sine and shifted squared sine bell apodization in the ^{1}H and ^{31}P dimensions, respectively. The spectral sizes were 8192 and 512 data points in the ^{1}H and ^{31}P dimensions, respectively. 2D ^{31}P,^{1}H NMR spectra were used for the identification of PLs, to determine ^{31}P and ^{1}H chemical shifts, and to extract multiplet structures from ^{1}H traces of 2D ^{31}P,^{1}H NMR spectra.

RESULTS AND DISCUSSION

LLE of Lipid Standards. Each of the four PLs (DMPE, DOPE, DLPC, DPPC) and the two triacylglyceride (CCC and SSS) standards contained one defined fatty acid species that was not found on the other standards (Table 1). Accordingly, the efficiency of the LLE could be studied by GC-MS after transesterification of the fractions. The fatty acids present in the polar phosphatidylcholine standards (12:0 and 16:0) and also the less polar phosphatidylethanolamine standards (14:0, 18:1n-9, Table 1) were effectively separated from SSS (18:0),

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that is, the triacylglycerides with long-chain fatty acids (polar phase, PLs >89%; neutral lipids, 11%; Table 1). However, 8:0 (from CCC, that is, the triacylglyceride with a short-chain fatty acid) was abundant in both phases (polar phase 38% vs nonpolar phase 62%, Table 1). Lipids of dairy products are known to contain high amounts of triacylglycerides with short-chain fatty acids,[37] whereas PLs are only minor components.[21] The expected presence of higher amounts of triacylglycerides with short-chain fatty acids and mono- and diacylglycerides necessitated that the LLE procedure of the separated phases be repeated three times (Figure 1). With a loss of ∼10% of the PLs in each step, the overall procedure of LLE provided ∼70% of each PL in the methanol phase of the third enrichment (p-pp). Discrimination among the PL standards was not observed.

Discrimination Effects of the LLE of Cheese and Fish Samples As Controlled by NMR. Partial loss of PLs from the food samples by the LLE method was in the same range as observed with the standards. 1D ^{31}P NMR measurements (spectra not shown) of the LLE fractions p-pp, p-n, p-pn, n-nn, n-p, and n-np verified that the polar fraction (p-pp) contained >60% of the total PL content of the sample. For instance, LLE of 1 g of cheese fat provided ∼30 mg of lipids in the final polar extract (p-pp). This amount was 4−6-fold higher than the typical PL content of cheese (Table S2, Supporting Information). The surplus (∼25 mg or ∼80% of the sample) most likely originated from triacylglycerides with short-chain fatty acids and/or other polar compounds still present in the sample after LLE. However, the simple LLE procedure proved to be effective for the enrichment of the PLs from a sub-% range in the (unfractionated) milk fat (total ASE extract) to major compounds suitable for NMR analysis. Accordingly, fraction p-pp was primarily used for NMR analysis. Contrary to the procedure with standards, LLE was especially successful for the polar PLs, whereas partial discrimination was observed for the less polar PL structures. For instance, lysoPL and phosphatidylinositol (both fish oil and cheese fat) were more strongly enriched in fraction p-pp compared to phosphatidylethanolamine (cheese fat). In addition, cardiolipin (Table 2; Figure S1, Supporting Information) was mainly detected in the nonpolar fraction (n-nn) of organic brie cheese fat, whereas only a small portion was found in the polar extract (p-pp). Despite these alterations of the PL composition by LLE, this enrichment of PLs was found to be essential for the determination of minor PLs due to the limited sample weight (∼30 mg), which can be dissolved in the NMR tube without negatively affecting the solvent conditions and ^{31}P chemical shifts. This limitation in sample weight reduces the total amount of PL available for the NMR measurement, and this in turn reduces sensitivity. For instance, the direct NMR analysis of non-enriched total ASE extract enabled only the detection of the most abundant PLs (e.g., phosphatidylcholine, sphingomyelin, phosphatidylethanolamine in organic brie and only phosphatidylcholine in mozzarella cheese) because of the presence of excess triacylglycerides (>250-fold) and other fat-soluble compounds (total PL fraction < 0.4%). These sample matrix compounds were accompanied with reduced PL concentrations below the detection limit of approximately 4 nmol (500 μL of 8 μM solution, 2D experiment of 8 h duration, see Materials and Methods).

In agreement with previous determinations,[21] the amount of PLs of fish oil in the polar extract was 3−60 times higher than in cheese. Therefore, the total ASE extract of fish already contained a high percentage (13% in the applied aliquot) of PLs, and most of the PL species in fish were already detected in the unfractionated total sample extract. Although the LLE procedure was not essential for the analysis of fish (21.3% PLs in the fractionated sample extract), all NMR evaluations were based on fraction p-pp.

1D ^{31}P NMR Spectroscopy of the PLs in Fish and Cheese Samples. The 1D ^{31}P NMR spectra of fish and cheese showed numerous signals, but both sample types were easy to distinguish due to their characteristic signal patterns (Figure 2).

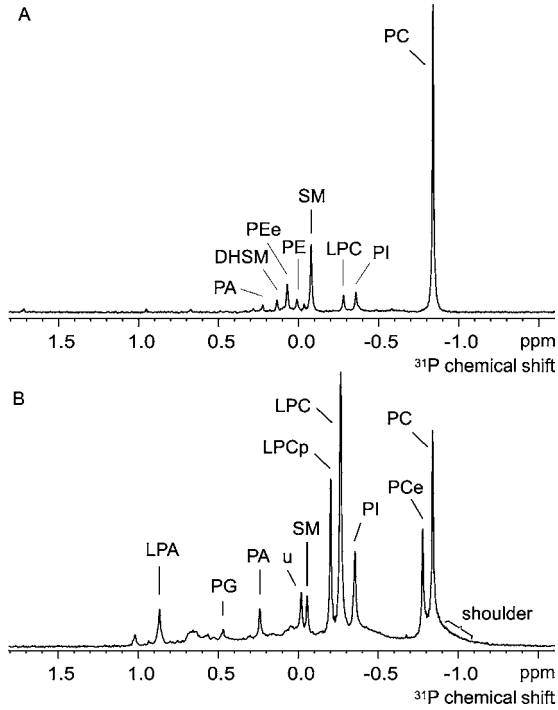

Figure 2. One-dimensional ^{31}P NMR spectra of phospholipids in the 2-fold re-extracted polar liquid−liquid extraction fraction p-pp of (A) cheese (organic brie) and (B) fish (salmon). PC, phosphatidylcholine; PCe, alkyl ether-linked phosphatidylcholine; LPC, lysophosphatidylcholine; LPCp, lysophosphatidylcholine plasmalogen; PE, phosphatidylethanolamine; PEe, alkyl ether-linked phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; DHSM, dihydrosphingomyelin; PA, phosphatidic acid; PG, phosphatidylglycerol; LPA, lysophosphatidic acid; u, unknown.

For instance, higher portions of lysolipids (especially lysophosphatidylcholine) and alkyl ether-linked phosphatidylcholine were present in the fish samples. Despite narrow line widths, the PL region of ^{31}P NMR spectra was quite crowded (Figure 2). Moreover, ^{31}P shifts of PLs depend not only on temperature and pH but also on solvent, sample matrix, and the fatty acid chains attached to the PL.[38−42] Because the latter parameters cannot be fully controlled, even common PL species were not found at one well-defined chemical shift, but showed sample-dependent variations (Table 2). For instance, ^{31}P signals of PLs with unsaturated fatty acids are shifted upfield compared to PLs with saturated fatty acids.[41] For this reason, ^{31}P resonances of phosphatidylcholine in fish samples (due to a higher content of polyunsaturated fatty acids in the PL) were shifted upfield by −0.026 ppm relative to triphenylphosphate (Table 2) compared to the respective phosphatidylcholine in cheese (higher content of saturated fatty acids). Noteworthy as well, the phosphatidylcholine signal in fish showed a broad shoulder at higher field, which was not found in the cheese

samples (Figure 2). This shoulder peak in the phosphatidylcholine signal in fish samples (Figure 2B, labeled with parentheses) was no artifact but most likely orginated from phosphatidylcholine species with multiple or polyunsaturated fatty acid composition. This effect was not restricted to phosphatidylcholine but was visible at all signals in the ^{31}P NMR spectra of fish (Figure 2B). For this reason the 1D ^{31}P NMR signals of fish were not well resolved.

Variations in the ^{31}P shifts were also observed in the four cheese samples analyzed in this work. For instance, the ^{31}P shifts of phosphatidylethanolamine in cheese varied by almost 0.2 ppm. However, not only did the ^{31}P shift of PL species vary from sample to sample (Figure 3A) but individual PL signals

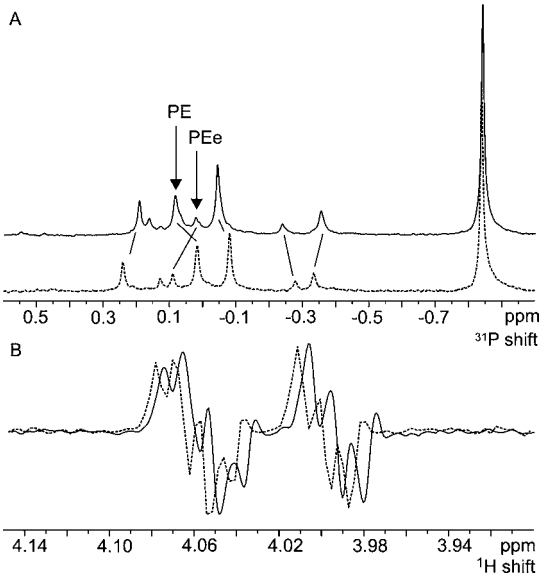

Figure 3. (A) One-dimensional ^{31}P NMR spectra of two extracts of organic brie with the 2-fold re-extracted polar liquid–liquid extraction fraction p-pp (full line) and total polar ASE extract (dotted line). Both samples contain the same PL, but the resonances were shifted (vertical lines). Note that phosphatidylethanolamine (PE) and alkyl ether-linked phosphatidylethanolamine (PEe) changed their positions. (B) ^{1}H traces for phosphatidylethanolamine (PE). Traces were identical except for a small shift.

could even switch their places in the ^{31}P spectra as was observed for phosphatidylethanolamine and alkyl ether-linked phosphatidylethanolamine (see below) in two cheese extracts (Figure 3A). As the extracts differed only in their preparation, the chemical shift variation must be due to an altered matrix, because the unfractionated total sample extract contains large amounts of triacylglycerols, in contrast to the second sample. Because such matrix effects differ between PLs (Table 2), they cannot be compensated by referencing ^{31}P chemical shifts to phosphatidylcholine at −0.84 ppm. However, the LLE procedure reduced the magnitude of the effect (Figure 3).

Although the integral and width of the peak shoulders (in fish) and the positions of the ^{31}P shifts may provide useful information on the composition of the fatty acids on PL, these inevitable variations in the ^{31}P chemical shifts impede the unequivocal peak assignment in 1D ^{31}P NMR spectra. This drawback was abolished by switching to 2D ^{31}P,^{1}H NMR, which proved to be essential for the correct assignment of structures to PLs.

Peak Assignments in the 2D ^{31}P,^{1}H NMR Spectra of the PLs in Fish and Cheese Samples. Identification of PLs in the 2D ^{31}P,^{1}H NMR spectra was based on both ^{31}P and ^{1}H chemical shifts along with the characteristic ^{31}P,^{1}H couplings via three bonds visible in the ^{1}H dimension (Figure 4). For

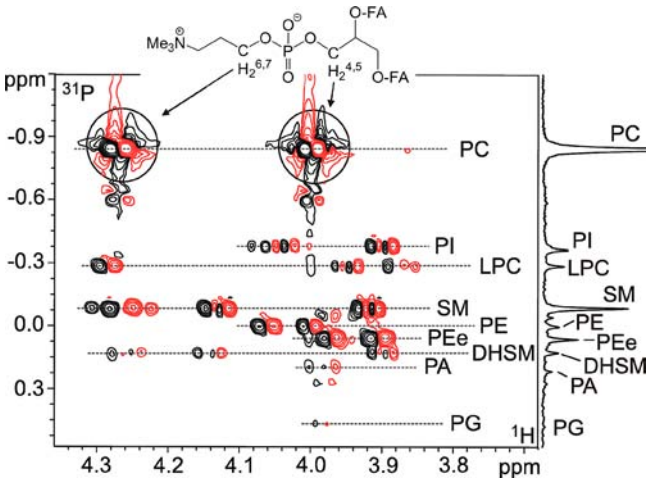

Figure 4. Two-dimensional semiconstant time ^{31}P,^{1}H COSY NMR spectrum of phospholipids (PL) in cheese fat (polar liquid–liquid extraction fraction p-pp) and the corresponding 1D ^{31}P NMR spectrum (right). Backbone and headgroup protons giving rise to cross peaks shown in a phosphatidylcholine structure are labeled. For highly abundant PLs, weak cross peaks due to four-bond couplings can sometimes be observed (not shown). PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PEe, alkyl ether-linked phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; DHSM, dihydrosphingomyelin; PA, phosphatidic acid; PG, phosphatidylglycerol.

instance, the observed protons of phosphatidylcholine were $H_{4/5}$ on the glycerol backbone and $H_{6/7}$ on the headgroup (Figure 4). The protons on the headgroup ($H_{headgroup}$) of phosphatidylcholine are shifted downfield compared to the glycerol protons $H_{backbone}$.[33] This order is valid for most known PLs except phosphatidylinositol and phosphatidylglycerol, where the shifts are reversed (Table 2).[33] For phosphatidylcholine and phosphatidylethanolamine, the protons $H_{4/5}$ are equivalent, and the same is valid for $H_{6/7}$.[33] Hence, these PL species are characterized by only one cross peak for each methylene group (Table 2; Figure 4). By contrast, the chemically unequal[33] methylene protons on the sphingosine backbone of sphingomyelin generated cross peaks with different chemical shifts (Table 2; Figure 4). Furthermore, lysolipids (i.e., PLs without a fatty acid in *sn*-2-position) generated two cross peaks (Table 2; Figure 4)[43] because the hydroxy group in the *sn*-2-position formed a hydrogen bond to the phosphate group. As a result, the $H_{backbone}$ protons on the glycerol backbone are chemically unequal, leading to two cross peaks (Table 2; Figure 4).[43] Noteworthy as well, ^{1}H shifts and fine structures are less dependent on sample composition than ^{31}P shifts (Figures 3B and 5).[27] For example, the strong shift in the ^{31}P signals of phosphatidylethanolamine between the cheese samples (Figure 3A) was not valid for ^{1}H shifts (variations in ^{1}H shifts generally < 0.01 ppm) and signal fine structures (Figure 3B). Accordingly, these characteristic features of the 2D ^{31}P,^{1}H NMR spectra were especially valuable for peak identification in the polar LLE fraction (p-pp). All together, the presence of 12 known PLs could be unequivocally

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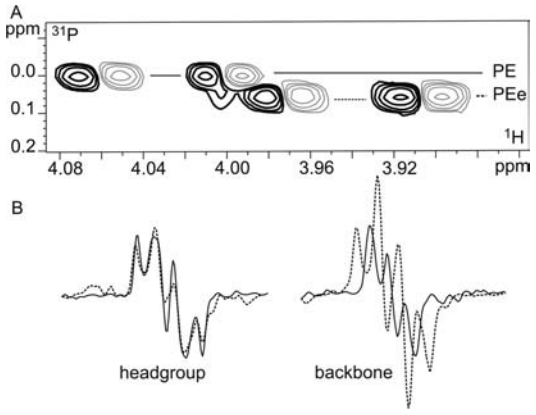

Figure 5. Comparison of (A) 2D NMR cross couplings and (B) ^{1}H traces of phosphatidylethanolamine (PE) and alkyl ether-linked phosphatidylethanolamine (PEe).

verified by 2D ^{31}P,^{1}H NMR (Table 2; Figure 6). By contrast, the unambiguous identification of uncommon PLs detected in the 2D ^{31}P,^{1}H NMR spectra proved to be more difficult because their ^{31}P-correlated ^{1}H signals are sparsely reported in the literature. No such information was available for the PL labeled "PEe", which shifted place with phosphatidylethanolamine in the ^{31}P spectra of two cheeses (Figure 3A). This PL, which represented ~5% of the PL content in cheese, could be unequivocally distinguished from phosphatidylethanolamine by means of the different ^{1}H shifts (Figure 5). Phosphatidylethanolamine plasmalogen could also be excluded because the ^{1}H chemical shifts (3.97 and 3.91 ppm) for this component in the 2D spectrum (Figure 4) deviated from phosphatidylethanolamine plasmalogen by about 0.1 ppm, which was more than 10-fold the normal variation (see above).[30] However, the similar ^{1}H signal fine structure suggested that this compound was structurally related to phosphatidylethanolamine (Figure 5). Evaluation of known PL modifications indicated that PEe most likely originated from an alkyl ether-linked phosphatidylethanolamine (Figures 3A and 5). Although the exact structure could not be verified due to the lack of authentic reference standards, the 2D ^{31}P,^{1}H NMR approach of evaluating ^{1}H chemical shifts and fine structures added valuable information and could be used to rule out possible false identifications.

As stated above, the LLE procedure helped to enrich the polar PLs, whereas nonpolar species were partly discriminated. As a consequence, cardiolipin was detected only in fraction n-nn (see above). A further phosphorus compound (labeled u_n) was detected in this nonpolar fraction (n-nn, Figure S1, Supporting Information) of two cheeses, that is, organic brie and mozzarella cheese. In the 2D ^{31}P,^{1}H NMR spectra, compound u_n did not show a signal originating from headgroup protons (Figure S1, Supporting Information). This case occurs for compounds without headgroup protons that are coupled to the phosphate moiety, as would be the case for phosphatidic acid. However, phosphatidic acid is highly polar and was found in fraction p-pp (Figures 2 and 4). Similar coupling patterns could be expected for bis(diacylglycero)phosphate, but this compound could also be ruled out because the ^{31}P shift of this phosphate diester should not be pH-dependent, but the signal shifted in pH titration experiments (data not shown). Thus, the identity of compound u_n in the nonpolar fraction n-nn could not be established.

1D ^{31}P NMR and 2D ^{31}P,^{1}H NMR Determination of the PL Distribution in the Polar Fraction (p-pp) of Fish and Cheese. Even in the case of complete overlap of PLs in ^{31}P NMR spectra, it is possible to quantify the overlapping PLs in 2D NMR spectra, as long as one cross peak of each compound is resolved in the ^{1}H dimension. Unfortunately, peak intensities in 2D ^{31}P,^{1}H NMR spectra are not equal for identical

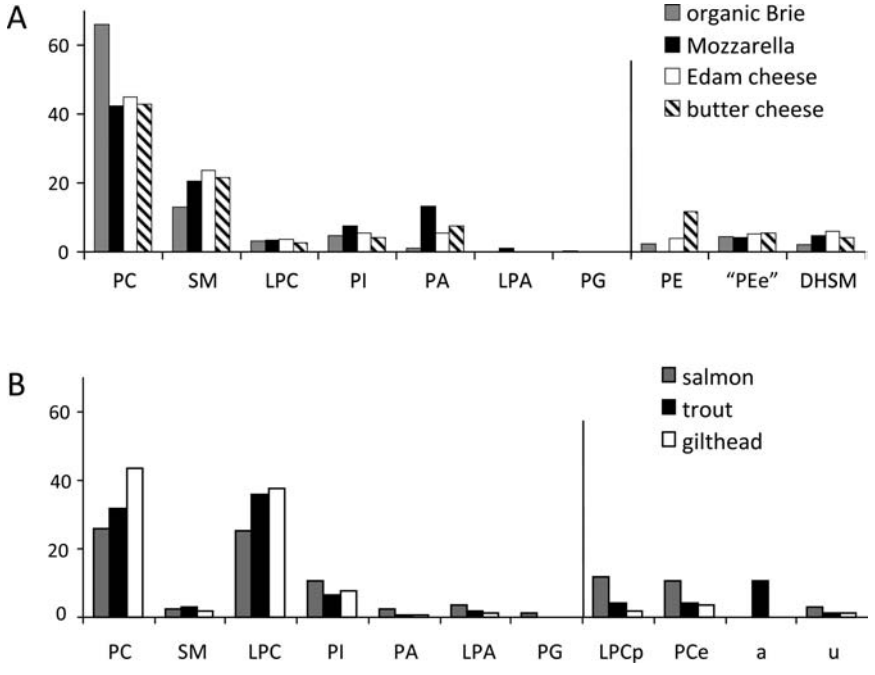

Figure 6. Phospholipid content (% of total PL) in (A) cheese fat and (B) fish oil. The vertical line separates the PLs, which are abundant in both cheese and fish (left side) and the PLs specific in this sample type (right side). Compound a was ambiguous, phosphatidylcholine with a high degree of unsaturated fatty acids bonded or phosphatidylcholine plasmalogen; the structure of compound u could not be determined. All abbreviations except PCe, alkyl ether-linked phosphatidylcholine, are explained in Table 2.

concentrations of different PLs. This effect is typically of the order of ±10% and limits the accuracy of the quantification of 2D signals.[27] However, if more accurate quantification is required, transfer factors can be applied to adjust for differences in detection efficiency between different PLs.[27] Because transfer factors reflect the coupling constants around the ^{31}P nucleus, they are molecular properties that are not influenced by variable sample conditions. In this study all signals were sufficiently resolved in the 1D ^{31}P NMR spectra. For this reason, 2D ^{31}P,^{1}H NMR was used for PL identification, whereas 1D ^{31}P spectra were used to determine the relative abundances of individual PLs (Figure 6; Table S2, Supporting Information).

Usually, 10 PLs were detected in cheese, whereas 11 PLs (including one unknown (u) and one ambiguous (a) species) were found in fish (Figure 6). Noteworthy, only 7 PLs were detected in both matrices, so that 14 PLs in total were found by 2D ^{31}P,^{1}H NMR in the polar fraction p-pp in the samples (Figure 4). In both matrices phosphatidylcholine was usually the most prominent PL (up to 65%, Figure 6). Minor PLs (contribution of <4% to the PL content) were identified in the form of phosphatidic acid, lysophosphatidic acid, and phosphatidylglycerol (Figure 6). In addition to the different PLs in both matrices, the relative abundances of the seven PLs found in both fish and cheese lipids were also varied. For instance, sphingomyelin was the second most abundant PL of cheese (>20%), whereas it was only a minor PL of fish (Figure 6). Sphingomyelin and dihydrosphingomyelin are known major PL components in bovine milk along with phosphatidylserine (~3% in bovine milk[37] and 6−9% in cheese[5]) and phosphatidylinositol (~5% in bovine milk[37] and 4−8% in cheese[5]).[44,45] The relative abundances of phosphatidylinositol and sphingomyelin in cheese compared well with those reported by Jensen in bovine milk.[37] However, phosphatidylserine was not detected in any of our samples. Noteworthy, the lower contribution of phosphatidylcholine to the PL content in fish was compensated by a higher percentage of lysophosphatidylcholine content. Phosphatidylcholine and lysophosphatidylcholine together represented ~50% of the total PL content in fish (Figure 6; Table S2, Supporting Information), which is in the reported range of fish and other marine organisms.[46−49] However, lysophosphatidylcholine only amounts to <4% in fresh fish.[47−49] For this reason, lysophosphatidylcholine was most likely formed post-mortem by lypolysis in fish muscle during the long storage of the samples (see Materials and Methods). In accordance with that, literature reports indicated the formation of 32% lysophosphatidylcholine from phosphatidylcholine 2 months after the death of fish.[8,49,50]

Remarkably as well, our samples contained only <15% (cheese) or no (fish) phosphatidylethanolamine (Figure 6). At the chemical shift typical for phosphatidylethanolamine, a peak (labeled "u" in Figure 2B) was observable in the 1D ^{31}P NMR spectrum of the fish oil fraction. However, the 2D ^{31}P,^{1}H NMR spectrum lacked the characteristic cross couplings and ^{1}H fine structure of phosphatidylethanolamine. Likewise, tests of our LLE procedure with the DOPE and DMPE standards indicated only a slight discrimination of phosphatidylethanolamine in fraction p-pp (see above). Therefore, the presence of phosphatidylethanolamine in our fish samples could not be verified (Figure S2, Supporting Information). By contrast, Rombaut et al. reported 28−45% phosphatidylethanolamine in milk and cheese, whereas phosphatidylcholine contributed 16−24% to the PL content.[5] Both PLs amounted to 50−60%.[5] In addition, phosphatidylethanolamine was reported to contribute 8−11% to the PL content of fillet of haddock and cod and 22% to the PL content of trout liver.[46] Noteworthy, Polvi et al. found that the amounts of phosphatidylethanolamine and phosphatidylcholine decreased with the storage of fish (phosphatidylethanolamine ~5% and phosphatidylcholine ~4% after 3 months at −12 °C) in favor of the corresponding lysoPLs.[50] Hence, the long sample storage time was most likely the reason for the absence of phosphatidylethanolamine in our fish samples. Therefore, the PL distribution found in our samples should not be considered as typical for fresh fish samples. However, the current NMR method may also be used to identify (partial) spoilage of fish. Note that the samples analyzed in this study were chosen because the PL content had been determined previously with an alternative method.[21] The total PL content in the cheese from 0.3 to 0.4% (calculation shown in the Supporting Information) compared well with the amounts previously determined in these samples (0.5−0.7%[21]). A very good agreement was also found for the PL content of fish (5−12% with the present NMR method compared to 6−11%[21]). Accordingly, the 2D ^{31}P,^{1}H NMR is suited for the determination of both the PL pattern (Figure 4) and the PL content of food samples. Samples rich in PLs can be directly analyzed by 2D ^{31}P,^{1}H NMR, whereas samples low in PLs need to be enriched in samples such as milk fat. This measure expends efforts in sample preparation, and it may also be accompanied with fractionation effects as observed during the LLE method used in this study. Despite these drawbacks, the strengths of the 2D ^{31}P,^{1}H NMR method are manifested in the unambiguous identification of individual PLs along with structural information that may lead to the identification of uncommon PLs which will hardly be detected with other methods.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as noted in the text (Figures S1 and S2 and Tables S1 and S2). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(W.V.) E-mail: walter.vetter@uni-hohenheim.de. Phone: +49 711 459 24016. (J.S.) E-mail: jurgen.schleucher@chem.umu.se. Phone: +46 90 786 5388.

Notes

The authors declare no competing financial interest.

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