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Comparison of Methods for the Quantitative Determination of Phospholipids in Lecithins and Flour Improvers

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Phospholipid classes were determined qualitatively and quantitatively in eight commercial lecithins and three flour improvers by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and ^{31}P nuclear magnetic resonance spectroscopy (^{31}P NMR). The total amounts of phospholipids as well as the amounts of phospholipid classes in the samples were comparable but depended on the method used for quantification. Highest selectivity was provided by ^{31}P NMR as all phospholipids and lysophospholipids could easily be quantified. By TLC only lysophosphatidylcholine could not be quantified, whereas HPLC was the method with the lowest selectivity, because lysophospholipids, except lysophosphatidylethanolamine, could not be determined. Sensitivity was best for HPLC and TLC with detection limits of 20−170 μg/mL. By means of ^{31}P NMR these figures increased by a factor of 10−70. The coefficients of variation were 5.5, 6.8, and 12.8% for quantification by TLC, HPLC, and ^{31}P NMR, respectively, showing that TLC was the method with the best reproducibility. Altogether, ^{31}P NMR can be recommended for the quantification of phospholipids, because it is easy to perform and results can be obtained quickly. As it requires minimum instrumental equipment, TLC is a good alternative to ^{31}P NMR. If high sensitivity is required, HPLC is the best method.

KEYWORDS: Lecithin; phospholipids; lysophospholipids; quantification; TLC; HPLC; ^{31}P NMR; flour improvers

INTRODUCTION

Because of common structural elements phospholipids can act as emulsifiers and can influence the baking performance of wheat doughs. Therefore, those polar lipids, for example, lecithin, that can be isolated on an industrial scale from plant sources (soybean, rapeseed, and sunflower) are used as components of improvers for breadmaking. Lecithin improves the fermentation behavior of yeasted doughs, the loaf volume of bread, and the structure of the bread crumb. Commercially available lecithin contains phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylserine (PS), and the corresponding lysophosphatidyl derivatives (LPC, LPE, LPI, LPA, and LPS). Furthermore, neutral lipids, free fatty acids, carbohydrates, sphingolipids (ceramides, cerebrosides, gangliosides, and sphingomyelin), glycolipids (sulfatides), and other derivatives [*N*-acylphosphatidylethanolamine (NAPE)] are present.

Among the various methods for the analysis of phospholipids (*1*−*6*), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and ^{31}P nuclear magnetic resonance spectroscopy (^{31}P NMR) are the most important. Silica gel is typically used as stationary phase for TLC to separate phospholipid classes (*1*, *7*). By using HPTLC plates with a concentrating zone, the resolution can be improved. Mostly a ternary mixture of chloroform, methanol, and water in various ratios (*1*, *7*) has been used as solvent. For the detection of the phospholipids, oxidizing reagents such as molybdatophosphoric acid or phosphoric acid/copper sulfate were used (*1*, *8*, *9*). Quantification of the spots can be achieved by densitometry or image analysis (*10*).

For the separation of phospholipids by HPLC, several stationary phases were used. Silica gel (*11*−*14*) and reversed phases (*15*) as well as amino phases (*16*) and diol phases (*17*) have been described previously. The eluent can be monitored by using a variety of detectors with different selectivities. Despite serious disadvantages, UV detectors have been used in most of the applications because they are present in almost every laboratory. Due to the fact that phospholipids have no chromophoric groups absorbing light in the higher UV or visible range, UV detection has to be carried out at 203−210 nm. However, many solvents suited for phospholipid separation cannot be used at these wavelengths because their UV cutoff is >210 nm (*18*). This problem has been solved by the use of the evaporative light scattering detector (ELSD), which can be used with all solvents and does not show any selectivity against the analyte (*18*). Refractive index (RI) and flame ionization detectors (FID) have also been used (*2*, *3*). A forthcoming method is detection of phospholipid classes by mass spectrometry (MS) (*20*−*23*). Different elution systems have been used for phos-

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pholipid separation. Commonly used solvent mixtures were 2-propanol/*n*-hexane/water (*14*, *21*, *25*), chloroform/methanol/water (*16*, *26*, *27*), and acetonitrile/methanol/water (*28*−*31*), most including different buffers in various ratios.

Today the analysis of phospholipid-containing material by ^{31}P NMR is a widespread method. The most important fact that has to be considered in ^{31}P NMR spectroscopy is its susceptibility to diamagnetic and paramagnetic polyvalent cations, which are able to coordinate with the phosphodiester functional group. Lecithins contain cations such as calcium, magnesium, aluminum, iron, and zinc in total amounts between 10 and 70 mg/kg (*32*). These concentrations of polyvalent cations are sufficient to interfere with the sample measurements because they lead to intramolecular interactions (*33*). As a consequence, these cations lead to broadened ^{31}P resonances (*33*−*36*). This effect induces poorly resolved ^{31}P NMR spectra, as the phosphorus compounds appear in a very narrow shift range. Excessive amounts of these cations will obliterate these signals completely. ^{31}P resonances can be sharpened by converting the phospholipids to salts with a common monovalent cation. Normally, weak EDTA complexes using potassium, sodium, or cesium as counterions are used to avoid a competing reaction between polyvalent and monovalent cations (*35*).

The results described in this paper are part of a study aiming to establish correlations between the phosphoric acid derivative of phospholipids and the activity in breadmaking. The aim of the first part of the study was the development of reliable methods for the qualitative and quantitative determination of phospholipids in commercial lecithin samples. To check the selectivity and the limitations of these methods, commercial phospholipid-containing flour improvers with ingredients that might disturb phospholipid analysis (ascorbic acid, enzymes, wheat gluten, malt flour, cysteine, and other emulsifiers) (*37*−*39*) were investigated.

MATERIALS AND METHODS

Chemicals. Phospholipids (PA, PC, PE, PI, and PS) and lysophospholipids (LPA, LPC, LPE, LPI, and LPS), NAPE, sphingomyelin, gangliosides, sulfatides, and ceramides with a purity grade of 98% were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Purity was checked by both HPLC and TLC. 1-Propanol, 2-propanol, *n*-hexane, methyl acetate, tetrahydrofuran, and methanol of HPLC grade, deuterated chloroform of spetroscopy grade, sodium acetate, potassium chloride, copper sulfate pentahydrate, and formic acid of analysis grade were obtained from Merck Eurolab GmbH (Ismaning, Germany). Cesium hydroxide, triethyl phosphate, and 85% (w/w) orthophosphoric acid of analysis grade were purchased from Aldrich (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Ethylenediaminetetraacetic acid (EDTA) of analysis grade was obtained from Fluka (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany).

Lecithins and Flour Improvers. Crude and deoiled lecithins from sunflower, rapeseed, soybean, and hen egg lecithin were provided by Degussa Texturant Systems (Hamburg, Germany). Three industrial flour improvers were provided by BakeMark Deutschland (Bingen, Germany).

Quantification of Phospholipid Classes by TLC. The separation of phospholipids by TLC was performed on HPTLC silica plates (silica G 60, Merck, 200 × 100 × 0.25 mm) with a concentrating zone (*40*). According to the method of Vourela et al. (*8*), sample (5−15 mg) was dissolved in tetrahydrofuran/water (1 mL; 3+1, v+v). An aliquot of the sample (5 μL) was loaded onto the silica plate, and, after separation of polar lipid classes with methyl acetate/chloroform/1-propanol/methanol/0.25% aqueous potassium chloride (25+25+25+10+9, v+v+v+v+v), the spots were detected by means of an acidic aqueous solution of copper sulfate. The detection reagent was prepared by dissolving copper sulfate pentahydrate (25.8 g) in water (400 mL) and addition of orthophosphoric acid (80.4 g, 47 mL) under continuous stirring. Finally, the mixture was adjusted to 500 mL with water. The TLC plate was impregnated with the detection reagent using a small painter roll and heated for 10 min at 180 °C. Quantification was performed according to the method of Stroka et al. (*10*) with some modifications. The TLC plate showing brown spots with different color densities was converted to a bitmap file (tif-format, 8 bit grayscale, 300 dpi) by means of a flatbed scanner. Each lane of the image of the TLC plate was processed with a freely available picture evaluation program (Image J: http://rsb.info.nih.gov/ij/) to obtain *x*/*y* data blocks. Loading the data file into an integration program (YASI: http://www.leb.chemie.tu-muenchen.de/YASI) gave a chromatogram trace of each lane, and the areas of the resulting peaks were determined. Calibration was performed by using standard solutions of the corresponding commercially available phospholipid classes (0.1−5 mg/mL). Three determinations were made for each sample.

Quantification of Phospholipid Classes by HPLC. A Kontron HPLC system D450 MT1 (Eching, Germany) consisting of two high-pressure pumps model 420, a UV detector 432, and a dynamic mixer M800 was used. The following conditions were used for separation: column, Nucleosil Silica, 250 × 4.6 mm, particle size = 5 μm, pore size = 10 nm (Phenomenex, Aschaffenburg, Germany); mobile phase, 2-propanol/*n*-hexane/0.1% aqueous formic acid, 8+8+1 (v+v+v) (*14*); flow rate, 0.8 mL/min; detection, UV absorbance at 205 nm; injection volume, 5−50 μL; sample, lecithin (5−25 mg) or flour improver (45−60 mg) dissolved in mobile phase (1 mL). Calibration was carried out on the basis of the peak areas by using standard solutions of the corresponding commercially available phospholipid classes (0.2−2.0 mg/mL). Three determinations were made for each sample. For the detection of phospholipid classes by MS, the eluent of the HPLC was transferred into an electrospray ionization MS (LCQ, Finnigan MAT, Egelsbach, Germany) running in the atmospheric pressure chemical ionization (APCI) mode and analyzing negatively charged ions. The settings were as follows: vaporization temperature, 450 °C; sheath gas, 70 arbitrary units; auxiliary gas, 10 arbitrary units; ionization current, 500 μA; capillary voltage, 4 kV; heated capillary temperature, 150 °C. All commercial lecithin samples and all reference compounds were analyzed.

Quantification of Phospholipid Classes by ^{31}P NMR. For the analytical sample preparation, lecithin (25−75 mg) or industrial flour improver (150−270 mg) was dissolved in deuteriochloroform/methanol 2+1 (v+v; 2 mL). An aqueous cesium EDTA solution was prepared by adjusting an aqueous suspension of EDTA (0.2 mol/L) with an aqueous cesium hydroxide solution (0.2 mol/L) to a pH of 10.5. Then, the EDTA solution (1 mL) was added to the sample solution, and the mixture was shaken vigorously. The emulsion was stored overnight at room temperature until phase separation was complete. From the two liquid phases, an aliquot (560 μL) of the lower phase was transferred into an NMR sample tube (178 × 5 mm), and calibration standard (2 μL; triethyl phosphate) was added. ^{31}P NMR spectra were recorded at 250 MHz on a Bruker AC 250 spectrometer. ^{31}P NMR spectra were acquired unlocked at 297 K with a 90° pulse of 10 μs, 16K data points, a 1.5 s pulse repetition time, and composite ^{1}H decoupling. The chemical shifts and line widths were measured digitally and referenced to triethyl phosphate. The chemical shift of triethyl phosphate was also measured with respect to the external 85% orthophosphoric acid standard ($\delta = -1.00 \pm 0.01$ ppm). The quantitative assay was performed by using calibration curves. These were recorded by using the corresponding commercially available phospholipid classes as calibration standards (5−30 mg). All reference compounds were treated and measured as described above. Three determinations were made for each sample.

Determination of the Total Phospholipid Content of Sunflower Lecithin. Crude and deoiled sunflower lecithins (0.5−2.0 g) were ashed according to the method of Rauscher et al. (*41*). Phosphorus was quantified spectrophotometrically according to the method of Köberlein and Mair-Waldburg (*42*). Phosphorus was converted to the total phospholipid content by multiplication with a factor of 24, which was obtained on the basis of the percentage of the most abundant phospholipids (PC + PE + PI + PA = 100%).

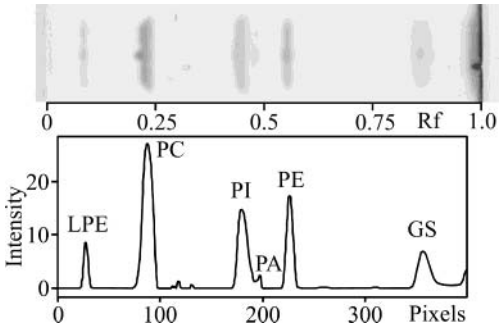

Figure 1. Separation of deoiled rapeseed lecithin by TLC and subsequent conversion to an *x*/*y* chromatogram. LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; PE, phosphatidylethanolamine; GS, gangliosides.

Table 1. R_f Values, Retention Times, and Chemical Shifts (δ) of Lipid Classes Analyzed by TLC, HPLC, and ^{31}P NMR

lipid class[a]	TLC R_f value	HPLC time (min:s)	^{31}P NMR (δ)
PA	0.59	11:38	+2.70 to +3.80
PC	0.44	39:58	–0.70 to –0.60
PE	0.63	5:43	±0.00 to +0.20
PI	0.54	18:25	–0.40 to –0.20
PS	0.50	12:23	–0.20 to –0.05
NAPE	0.61	9:51	
LPA	0.32		+3.70 to +4.30
LPC	0.24		+0.15 to +0.25
LPE	0.35	8:28	+0.30 to +0.50
LPI	0.28		+0.55 to +0.60
sphingomyelin	0.39	45:31	–0.10 to ±0.00
sulfolipids	0.68		
ceramides	0.76	4:11	
gangliosides	0.83		
triethyl phosphate			–1.0
nonpolar lipids	0.90	3:35	

[a] PA, phosphatidic acid; PC, phosphatidylcholinel; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; NAPE, *N*-acetylphosphatidylcholine; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol.

RESULTS

TLC. HPTLC silica plates with a concentration zone were shown to give the best separation of phospholipids. To achieve reproducible separation, it was essential to maintain a constant sample volume. Standardized conditions were used for detection. Application of the detection reagent by means of a small painter roll was found to be the best way to obtain reproducible results. Immersing the TLC plate into the detection reagent according to the method of ref *11* resulted in irregular distribution of the reagent. Heating the TLC plates for color development was done in this way that the plates did not contact the hot inner surface of the oven. For quantification the TLC plates were digitalized as tif files by means of a flatbed scanner. Freely available software packages were used to convert gray values into intensities and to obtain peak areas from the resulting diagrams. As an example, the spots obtained after separation of a rapeseed lecithin sample and the chromatogram obtained after processing are shown in **Figure 1**. The R_f values of the spots are given in **Table 1**. Among the different phospholipid classes PA, PC, PE, PI, and PS as well as LPA, LPE, and LPI were well separated. In addition, sphingoglycolipids and sulfolipids were resolved. LPC could not be quantified due to extreme band broadening. The detection limits (**Table 2**) were 40−100 μg/mL for phospholipids and 60−280 μg/mL for lysophospholipids. The coefficient of variation ranged from 5.2 to 6.1% ($n = 3$).

Table 2. Detection Limits and Coefficients of Variation for the Determination of Phospholipids by TLC, HPLC, and ^{31}P NMR

phospholipid class[a]	detection limit (μg/mL)			coefficient of variation (%)		
	TLC	HPLC	^{31}P NMR	TLC	HPLC	^{31}P NMR
PA	80	140	1100	6.0	6.5	21.1
PC	100	20	1400	5.3	5.8	8.2
PE	80	60	1000	5.3	6.6	12.3
PI	40	170	1500	5.2	6.0	11.4
PS	100	40	3300	5.3	4.2	18.9
LPA	280		5000	nd[b]		nd
LPC	150		2500	nd		nd
LPE	60	10	3300	6.1	4.8	4.6
LPI	260		3300	nd		nd

[a] See footnote *a* of **Table 1**. [b] nd, not determined.

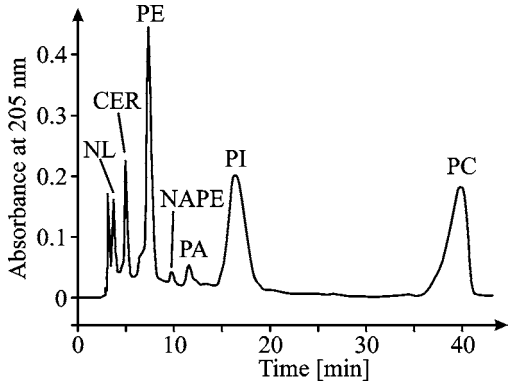

Figure 2. Separation of deoiled sunflower lecithin by HPLC. NL, nonpolar lipids; CER, ceramides; PE, phosphatidylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine.

HPLC. Initially a solvent mixture containing 2-propanol, *n*-hexane, and sodium acetate buffer following the method of Nasner and Kraus (*17*) was used for the separation of phospholipids. The solvent had to fulfill two main criteria. On the one hand, it had to be compatible with MS detection; on the other hand, it should be suited for semipreparative purposes to isolate individual phospholipid classes. Therefore, a readily volatile buffer was a prerequisite, and the sodium acetate buffer was replaced by 0.1% (v/v) aqueous formic acid. As shown in **Figure 2**, PA, PC, PE, and PI were resolved. However, of the lysophospholipids only LPE could be determined under the given (isocratic) conditions. Lysophospholipids would have been resolved by using a gradient system, but as HPLC was thought to serve as a semipreparative tool, only the isocratic system was used. To confirm the identities, eluted peaks were subjected to MS detection. As an example, the mass spectrum of PE is shown in **Figure 3**, and the negatively charged ions, which have been detected, are listed in **Table 3**. The retention times for the phospholipid classes are shown in **Table 1**. Quantification of the phospholipid classes was done by external calibration. The detection limits were 20−170 μg/mL, and the coefficient of variation ranged from 4.2 to 6.6% ($n = 3$).

^{31}P NMR. Phospholipid classes were identified by their chemical shifts in relation to the internal standard triethyl phosphate, which was set to −1.0 ppm. A pH value of the washing solution of 10.5 instead of 6.0 as described by Meneses and Glonek (*35*) was essential for the complete separation of all phospholipid classes. Quantification was done on the basis of the peak areas of the phospholipid classes in relation to the peak area of the internal standard. The assignment of the signals to phospholipid classes was carried out by measurement of

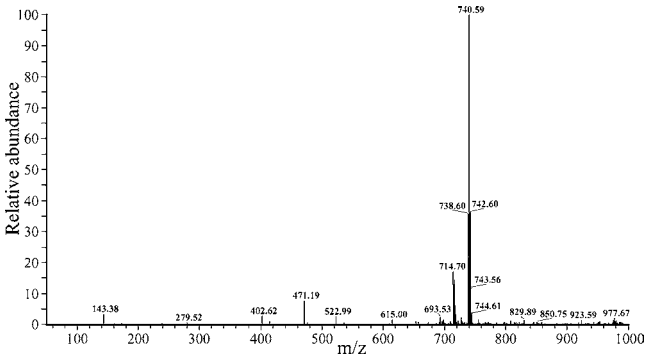

Figure 3. Mass spectrum of phosphatidylethanolamine from crude sunflower lecithin obtained after HPLC separation and atmospheric pressure chemical ionization (negatively charged ions).

Table 3. Negatively Charged Ions Recorded for Phosphatidylethanolamine from Crude Sunflower Lecithin by MS Detection in the APCI Mode after HPLC Separation

signal (*m*/*z*)	assignment to ion[a]
467.1	[PE(8:0–8:0) – H]$^-$
580.0	[PE(12:0–12:0) – H]$^-$
714.6	[PE(16:0–18:2) – H]$^-$
715.8	[PE(16:0–18:1) – H]$^-$
738.6	[PE(18:2–18:2) – H]$^-$
740.6	[PE(16:0–20:4) – H]$^-$
742.6	[PE(18:0–18:2) – H]$^-$
	[PE(18:1–18:1) – H]$^-$
743.6	[PE(18:0–18:1) – H]$^-$
766.6	[PE(18:0–20:4) – H]$^-$
790.5	[PE(18:0–22:6) – H]$^-$

[a] PE, phosphatidylethanolamine, 8:0, 12:0, 16:0, 18:0, 18:1, 18:2, 20:4, 22:6: fatty acids of PE esterified with the primary and secondary hydroxyl group of glycerol.

reference compounds. The chemical shifts of the phospholipid classes are shown in **Table 1**. All phospholipids and lysophospholipids that had been applied could be resolved as shown in **Figure 4**. For the lysophospholipids a minimum amount of 10 mg instead of 5 mg had to be present because a substantial portion of the material was lost in the washing step. Derivatives of PE (*N*-acyl-, *N*′-monomethyl-, and *N*,*N*′-dimethyl-PE) had the same chemical shift as PE and could therefore not be quantified by ^{31}P NMR. The presence of these compounds would increase the amount of PE. The detection limits were 1.0−3.3 mg/mL for phospholipids and 2.5−5.0 mg/mL for lysophospholipids. The coefficient of variation was 4.6−21.1% depending on the type and amount of phospholipid.

Analysis of Lecithin Samples. Seven commercial lecithin samples were investigated. The data obtained by analysis as well as the reference data supplied by the manufacturer are given in **Table 4**. Quantification by ^{31}P NMR was in best accordance with the reference data because this method was used by the manufacturer. The amount of phospholipid classes in the samples depended on the method. However, the ratio of the phospholipid classes was comparable for each of the three methods. In most of the samples PC was the major phospholipid followed by PI and PE with concentrations that were in the same range. Within the phospholipids PA had the lowest amount. Lysophospholipids and PE derivatives were only minor components. The methods that corresponded best were ^{31}P NMR and TLC, whereas HPLC quantification gave substantially higher amounts of PC. This might be due to the broad HPLC signal including possibly further compounds (**Figure 2**). The total amount of phospholipids differed from method to method, with TLC giving the highest and ^{31}P NMR giving the lowest amount. Calculation of the total phospholipid content on the basis of a spectrophotometric determination of total phosphorus in the sunflower lecithin samples yielded 48−58% phospholipids in the crude and 72−79% in the deoiled sample, which was in accordance with the results obtained by the other methods. For the three analytical methods used in this study calibration curves with known concentrations of phospholipid classes as well as recovery experiments after the addition of defined amounts of individual phospholipid classes to lecithin samples have been determined. The results are presented in **Table 5**. All correlation coefficients (r^2) were >0.99. The recoveries were highest for the ^{31}P NMR method; with HPLC PI and PA had recoveries of only 77 and 73%, respectively, and the lowest recoveries have been found for the determination of PE and PI by TLC (60 and 63%, respectively).

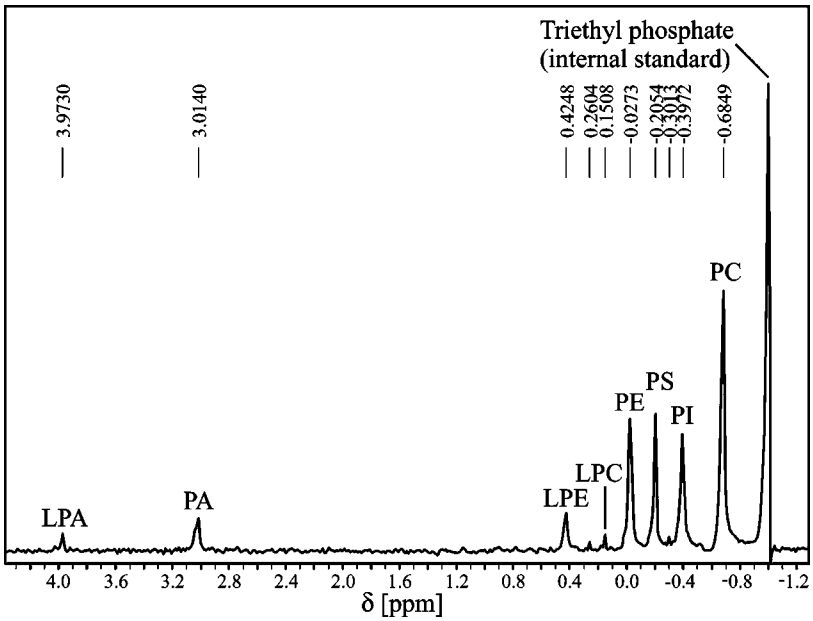

Figure 4. ^{31}P NMR spectrum of crude rapeseed lecithin. LPA, lysophosphatidic acid; PA, phosphatidic acid; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine.

Table 4. Phospholipid Content (Percent, w/w) of Commercial Lecithin Samples As Determined by TLC, HPLC, and ^{31}P NMR[a]

	soybean lecithin, crude				soybean lecithin, deoiled				sunflower lecithin, crude				sunflower lecithin, deoiled			
	TLC	HPLC	^{31}P NMR	ref[b]	TLC	HPLC	^{31}P NMR	ref	TLC	HPLC	^{31}P NMR	ref	TLC	HPLC	^{31}P NMR	ref
PA	4.9 ± 0.3	3.7 ± 0.2	6.6 ± 1.4		11.4 ± 0.7	4.9 ± 0.3	6.7 ± 1.5	5.5	9.7 ± 0.6	2.8 ± 0.2	6.6 ± 1.4	3.2	9.4 ± 0.6	4.5 ± 0.3	7.8 ± 1.6	4.8
PC	17.5 ± 0.9	32.4 ± 1.9	23.0 ± 1.9		24.0 ± 1.3	43.1± 2.5	23.2 ± 1.9	22.0	19.6 ± 1.0	27.5 ± 1.6	18.1 ± 1.5	15.9	28.9 ± 1.5	41.1 ± 2.4	30.2± 2.5	26.0
PE	17.3 ± 0.9	7.9 ± 0.5	12.3 ± 1.5		26.7 ± 1.4	11.8 ± 0.8	14.1 ± 1.7	20.0	10.5 ± 0.5	3.5 ± 0.2	7.6 ± 0.9	6.0	15.3 ± 0.8	5.3 ± 0.3	11.3± 1.4	8.9
PI	16.0 ± 0.8	13.8 ± 0.8	11.4 ± 1.3		20.3 ± 1.0	19.6 ± 1.2	13.4 ± 1.5	13.5	18.1 ± 0.9	15.4 ± 0.9	12.6 ± 1.4	16.1	25.4 ± 1.3	21.5 ± 1.3	20.9± 2.4	22.3
PS	<0.8	<0.2	1.8 ± 0.3		<1.7	<0.2	1.9 ± 0.3		<0.7	<0.2	<1.3		<2.0	<0.2	<1.3	
LPA	<2.3		<4.0		<4.7		<3.5		<1.9		<3.5		<5.7		<2.0	
LPC	<1.0		<2.0		<2.0		2.0		<0.8		<2.0		<2.4		<2.4	
LPE	<0.5	<0.1	4.6±0.2		<1.0	<0.1	4.9±0.2		0.9	<0.1	<2.3		<1.2	<0.1	<2.4	
LPI	<2.2		<2.6		<4.4		<2.3		<1.8		<2.3		<5.3		<3.6	
total	55.7	57.8	57.9		82.4	79.4	66.2	61.0	58.8	49.2	44.9	41.2	79.0	72.4	70.2	62.0

	rapeseed lecithin, crude				rapeseed lecithin, deoiled				hen egg lecithin			
	TLC	HPLC	^{31}P NMR	ref	TLC	HPLC	^{31}P NMR	ref	TLC	HPLC	^{31}P NMR	ref
PA	4.0 ± 0.2	3.2 ± 0.2	5.6 ± 1.2	1.8	6.7 ± 0.4	3.9 ± 0.2	7.2 ± 1.5	4.0	<1.6	1.5 ± 0.1	<0.7	
PC	19.5 ± 1.0	15.6 ± 0.9	17.0 ± 1.4	14.8	22.1 ± 1.2	19.8 ± 1.1	24.5 ± 2.0	32.5	42.6 ± 2.2	40.0 ± 2.3	25.7 ± 2.1	
PE	9.2 ± 0.5	3.5 ± 0.2	8.4 ± 1.0	7.8	10.8 ± 0.6	5.8 ± 0.4	12.2 ± 1.5	6.3	10.2 ± 0.5	5.7 ± 0.4	7.3 ± 0.9	
PI	14.4 ± 0.7	10.7 ± 0.6	10.6 ± 1.2	8.1	28.7 ± 1.4	11.8± 0.7	12.5 ± 1.4	12.6	<0.8	<0.6	4.9 ± 0.5	
PS	1.3 ± 2.2	0.1 ± 3.0	<1.2		<1.4	0.3 ± 3.7	2.9 ± 0.5		<2.0	1.2 ± 2.1	<1.1	
LPA	<2.2		<3.3		<4.2		<3.7		<5.6		<2.0	
LPC	<0.9		<2.0		<1.8		<2.0		<2.4		<2.1	
LPE	2.6	1.7	5.7±0.3		<0.9	1.2	11.8±0.5		<1.2	<0.1	<2.1	
LPI	<2.0		<2.2		<3.9		<2.4		<5.2		<3.1	
total	51.0	34.8	47.3	32.5	68.3	42.8	71.1	55.4	52.8	48.4	37.9	

[a] See footnote *a* of **Table 1**. [b] Ref, phospholipid content supplied by the manufacturer.

Table 5. Examples for Correlation Coefficients r^2 of Calibration Curves and Recovery for Major Phospholipid Classes, Determined with Authentic Mixtures of Phospholipid References

	TLC		HPLC		^{31}P NMR	
phospholipid class[a]	r^2	recovery (%)	r^2	recovery (%)	r^2	recovery (%)
PC	0.9960	102	0.9928	80	0.9986	100
PE	0.9975	60	0.9985	95	0.9999	90
PI	0.9999	63	0.9907	73	0.9990	95
PA	0.9987	100	0.9999	77	0.9946	98
PS	0.9978	100	0.9977	98	0.9999	105

[a] See footnote *a* of **Table 1**.

Table 6. Phospholipid Content (Percent, w/w) of Industrial Flour Improvers As Determined by TLC, HPLC, and ^{31}P NMR

phospho-	sample 1			sample 2			sample 3		
lipid class[a]	TLC	HPLC	^{31}P NMR	TLC	HPLC	^{31}P NMR	TLC	HPLC	^{31}P NMR
PC	<0.7	1.6	3.9	<0.8	0.9	2.9	<0.6	0.4	<1.7
PE	<0.5	3.0	2.5	<0.6	2.7	2.0	<0.5	<1.1	<1.3
PI	<0.3	5.4	2.8	<0.3	0.2	<2.1	<0.2	0.1	<1.8
PA	<0.5	2.3	1.6	<0.6	0.2	<1.5	<0.5	<2.6	<1.3
PS	<0.7	<0.6	0.5	<0.8	<0.5	<0.5	<0.6	<0.7	<4.0
total		12.3	11.3		4.0	4.9		0.5	

[a] See footnote *a* of **Table 1**.

Analysis of Flour Improvers. The results of the quantification of phospholipids in three industrial flour improvers are shown in **Table 6**. By TLC none of the samples gave results. For samples with a total phospholipid content of 4−12% (samples 1 and 2) HPLC and ^{31}P NMR were suited for quantification, whereas in sample 3 with the lowest phospholipid level (<4%) HPLC was required for quantification due to its superior sensitivity.

DISCUSSION

In some cases high standard deviations were obtained. However, relative standard deviations surpassing 10% occurred only when minor components were quantified. Concerning detection limits, HPLC provided the best results, followed by TLC and ^{31}P NMR, which had the highest selectivity. The reason for this assertion is justified with the detection of the phospholipids. The detection of phospholipids by nonselective detection reagents (TLC) or by nonselective UV wavelength ranges (HPLC) leads to a quantitative assay depending on many factors except the phosphorus nucleus. In the case of ^{31}P NMR, detection is more specific because the phosphorus nucleus is measured selectively. However, as already shown, ^{31}P NMR shows clear disadvantages because of its poor sensitivity. This has already been found by other authors (*7*, *33*). For sample preparation, the bulk of the solution needed 2 mL of solvent and 1 mL of washing solution, leading to an increase of the detection limits for phospholipids by a factor of 10−80 in

comparison to the other methods. The low selectivity of the HPLC method is due to the UV detection at wavelengths between 203 and 210 nm. In this spectral band, a number of different functional groups, such as amino groups (PE), quaternary ammonium salts (PC), carboxylic acid esters (all phospholipids), hydroxylic groups (phosphate group), carboxyl groups (PS), and double bonds (mono- and polyunsaturated fatty acids) are detected. Unfortunately, this poor selectivity may disturb the quantitative assay, leading to differences of the amounts of particular phospholipids in comparison to those obtained by the other methods (**Table 4**). The same is true for the specificity of the spot detection for TLC. Comparable to UV detection in HPLC, spot development by acidic copper sulfate or by the molybdatic acid reagent also shows low selectivity. This is due to the fact that both are strong oxidizing agents leading to nondefined oxidation products. Unfortunately, no specific phosphorus detecting reagent has been found until now.

Concluding Remarks. The results show that a qualitative analysis of phospholipid classes can be performed by all three analytical methods, even if the samples contain other polar components. If the identification of lysophospholipids is additionally needed, the separation should be performed by means of ^{31}P NMR or TLC, because by isocratic HPLC lysophospholipids are not eluted from the column. Altogether, ^{31}P NMR can be recommended for the quantification of phospholipids because it is easy to perform and results can be obtained quickly. Because it requires minimum instrumental equipment, TLC is a good alternative to ^{31}P NMR.

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