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Separation and quantification by high-performance liquid chromatography with light scattering detection of the main wheat flour phospholipids during dough mixing in the presence of phospholipase

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Abstract

Phospholipids (PL) are minor components of wheat flour involved in baking quality and exogenous phospholipids are used as emulsifiers giving better loaf volume and crumb grain. Few biochemical data are available on the phospholipid evolution during mixing, probably because of the time-consuming methods proposed for their extraction, separation and quantification. In the present study, the extraction, separation and quantification of the main wheat flour phospholipids were carried out. Total lipids (2% dry mass of wheat flour) were extracted from flour or dough by a mixture of chloroform–methanol–water (1:1:1 (v/v)). The phospholipids were separated from the lipid extract on silica cartridge by solid-phase extraction (SPE) procedure under a 1.5–4 mmHg vacuum, at a 0.8 mL min^{-1} flow rate (1 mmHg = 133.322 Pa). The recovery of the lipid extract was 100%, whereas the SPE yield for the PLs was 50%. The resulting fraction was then submitted to HPLC with evaporative light scattering detection on a Diol stationary phase allowing the separation and quantification of each class of phospholipids, in less than 16 min. The developed method allowed to quantify the phospholipid amounts from eight wheat flours as well as their evolution during mixing in the presence of phospholipase.

Keywords: Wheat flour; Food analysis; Phospholipids; Phospholipase; Lipids

1. Introduction

During the last 15 years, various kinds of additives have been used to improve the quality of many processed foods. However, consumers do not like the chemical additives for food ingredients, and "natural additives", such as enzymes, are being increasingly used as improvers in food processing.

Several studies have been made on the effects of wheat lipids on baking performance. They all agreed in showing polar lipids to be favourable and the non-polar fraction to be detrimental to the baking performance [1,2]. Phospholipids (PLs) are minor components (0.5% dry mass) of wheat flour. Even if PL are major components of the starch lipids [3,4], only the PL of the non-starch fraction, such as phosphatidylcholine (PC), lysophosphatidylcholine (LPC), *N*-acyllysophosphatidylethanolamine (NALPE) and *N*-acylphosphatidylethanolamine (NAPE) which are predominant among the non-starch phospholipid fraction, are involved in dough quality. During dough mixing, the increase of non-starch lipid binding, which is related to dough development [5,6], is mainly due to specific interactions between lipids and proteins in the gluten network [7]. Technological properties of PL during mixing and fermentation might be due to their ability to modify the physico-chemical properties of a liquid film wrapping gas bubbles [8]. Moreover, the physical properties of dough for bread are modified after addition of phospholipases A in the wheat flour [9]. This enzyme provides the dough with a suitable degree of elasticity and extensibility, and decreases its stickiness. As the

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result for the final product, an increased volume and an adequate degree of softness were obtained with a well-stretched structure in film form for the crumb. Nevertheless, only few biochemical data are available on the phospholipids evolution (concentration changes) during mixing probably because of the time-consuming methods proposed for their extraction, separation and quantification.

The analysis of wheat lipids presents particular problems due to the presence of appreciable amounts of glycolipids (GLs) in addition to PLs normally encountered in plant and animal tissues. Few high-performance liquid chromatography (HPLC) methods for the analysis of wheat flour PLs have been described using evaporative light scattering detection (ELSD) [10–12]. The first method [10] failed to separate all of the lipid classes present in the flour and some difficulty was encountered for the separation of GLs from PLs. This made necessary to extract out the GL fraction prior to HPLC analysis. The second one [11] showed the separation of non-starch and starch lipids in soft wheat flour on a Lichrosorb Si 60 silica column by HPLC–ELSD. The ternary phase (hexane, isopropanol and water) system gradient used for lipid classes separation was time-consuming (80 min) and did not allow an efficient separation of NAPE and NALPE. The third method [12] used for the separation of a total lipid extract was performed on a LiChrospher 100 Diol column with a mixture of heptane–isopropanol–butanol–tetrahydrofurane–isooctane and water as mobile phase. This method can separate and quantify both GL and PL. Moreover, even if the separation was performed in 25 min, there was a failure for the NALPE identification (as it is the case for methods 1 and 2).

The objectives of our work were (1) to develop an HPLC procedure using ELSD which would successfully separate all the PL classes present in soft wheat flour and corresponding dough with prior fractionation of the total lipids (TLs) by a solid-phase extraction (SPE) procedure in non-polar lipids (NLs), GL and PL; (2) to identify each PL class present and (3) to quantify each lipid present in several soft wheat flours. Then, this method was applied to the study of the effects of phospholipase during dough mixing.

2. Experimental

2.1. Solvents and standards

All chemicals were of reagent grade from Acros Organics (Noisy-le-grand, France) or VWR (Fontenay-sous-bois, France). PL standards were from Sigma (St. Quentin-Fallavier, France) for PC, LPC and NAPE and from Avanti Polar Lipids (Alabaster, USA) for NALPE.

2.2. Flours and enzymes

The flours (A–G) used were straight-grade and improver-free. They were obtained from French pure wheat varieties by Les Moulins Soufflet (Nogent-sur-Seine, France). The flour H used for these experiments was an untreated, improver-free, straight-grade commercially milled by Les Moulins Soufflet.

Phospholipase A_2 Lecitase 10 L, from porcine pancreas, was purchased from Novo Nordisk (Bagsvaerd, Denmark) and had an activity of 12 000 phospholipase units (PLU) per mL. According to the Novo data sheet, 1 PLU corresponds to the amount of enzyme that releases 1 μmol of free fatty acid per minute using homogenized egg yolk (6.7% (w/v)) in the presence of calcium chloride (6 mM) and sodium deoxycholate (3.2 mM) at pH 8.0 and 40 °C, as substrate.

2.3. Mixing conditions

Dough was prepared by adding 150 mL of distilled water to 250 g of flour in a micromixer of a Chopin (Villeneuve-la-Garenne, France) consistograph equipped with a heat-transfer system and a speed regulator. The mixing parameters were set at 75 rpm and 25 °C. Exogenous phospholipase was dissolved in the water added to the dough.

2.4. Lipid extraction

The extraction procedure was adapted from methods described previously [13,14]. Fresh dough samples (16 g) were collected directly in centrifuged tubes. Ionic complexes and lipid–protein associations are disrupted by adding 200 μL of acetic acid and 20 mL of methanol. Extraction was performed by successively adding 10 mL of chloroform containing butyl-hydroxy-toluene (0.01%), 10 mL of chloroform and 12.8 mL of NaCl (1.56%). After each new addition, the mixture was homogenized by a 20 s blending period (Ultra-turrax, IKA, Staufen, Germany). The mixture was centrifuged for 10 min at 6000 × *g*. Total lipids (TL) were obtained by collecting an aliquot (10 mL) of the chloroform phase. During the extraction procedure, tubes were kept at 0–4 °C.

2.5. Solid-phase extraction (SPE)

After evaporation of the chloroform, the TL extract was diluted in 1.5 mL of chloroform–methanol (2/1 (v/v)) and was purified by using normal-phase (silica) SPE cartridges (Sep-Pak plus Waters, Saint-Quentin-en-Yvelines, France). After the cartridge was conditioned with 5 mL of chloroform, the TL extract was applied. The NLs, GLs and PLs were separated by successive elution with 20 mL of chloroform, 20 mL of acetone and 30 mL of methanol, respectively. The flow rate of the SPE was adjusted to 0.8 mL min^{-1} by applying a 1.5–4 mmHg vacuum using a vacuum manifold. After the methanol was evaporated, the PL fraction was diluted in 2 mL *n*-hexane–isopropanol solution (80:20 (v/v)).

Table 1
Gradient elution system used for the separation of the wheat flour phospholipids

Time (min)	A^a (%)	B^a (%)	Flow rate (mL min^{-1})
0	95	5	1
5	80	20	1
8.5	60	40	0.8
13	40	60	0.8
14.20	40	60	1
15.20	40	60	1
23	95	5	1
24	95	5	1.5
27	95	5	1.5
29	95	5	1

[a] *A*, *n*-hexane–2-propanol–acetic acid–triethylamine (81.42:17.00:1.50:0.08 (v/v)) and *B*, 2-propanol–water–acetic acid–triethylamine (84.42:14.00:1.50:0.08 (v/v)).

2.6. HPLC of the PL fraction

A HPLC system (Varian Workstation 9012, Les-Ulis, France) was used with a Diol-modified silica analytical column (Merck LiChrospher 100 Diol, 5 μm, 100 mm × 4 mm i.d.) thermostated in an oven (Jones Chromatography) at 50 °C. The PL fraction (20 μL) were injected. The gradient system used for the separation of the major PL classes is given in Table 1. All solvents were degassed and filtered prior to analysis. The ELSD apparatus was obtained from Sedere (Alfortville, France). The drift tube temperature and the air inlet pressure were set at 50 °C and 2.5 bar, respectively.

2.7. Lipid fractionation by thin-layer chromatography (TLC)

TLs [triacylglycerol (TAG); 1,3-diacylglycerol ($DAG_{1,3}$); 1,2 diacylglycerol ($DAG_{1,2}$); monoacylglycerol (MAG); free fatty acids (FFAs) and polar lipids (PoLs = GLs + PLs)] were also fractionated by TLC with silica gel plates (Macherey-Nagel, Hoerdt, France) using the development system proposed by [15]. A 0.01% primulin solution was sprayed on the plates, and the lipid fractions were identified under UV light by comparing the R_f values with those of pure standard compounds.

A similar TLC procedure was applied to the NL, GL and PL fractions after SPE. After the solvents were evaporated to ≈ 2 mL, the SPE separated lipid fractions were dried under a nitrogen stream and dissolved in 5 mL of chloroform. Volumes of 0.25, 0.5 and 0.5 mL of the latter solutions were separated by TLC for NLs, GLs and PLs, respectively.

2.8. Fatty acids analyses by gas chromatography

After scrapping, 20 μg of heptadecanoic acid (internal standard) was added to each of the FFA, MAG and DAG fractions, 80 and 60 μg were added to the TAG and PoL (GLs + PLs) fractions, respectively. Fatty acids of each fraction were released and methylated with 5 mL of boron trifluoride methanol (14%) by incubation at 65 °C for 15 min. Fatty acids methyl esters (FAMEs) were extracted from methanol by adding 2 mL of water and 4 mL of pentane. After collecting the pentane fraction, the solvent was evaporated under a nitrogen stream and FAMEs were solubilized in 100 μL of heptane.

FAMEs were separated and quantified with a gas chromatograph (Agilent 4890D, Les Ulis, France) equipped with a split 6890 series injector, a 30 m × 0.25 mm DB-Wax (JW Scientific) capillary column and a flame ionisation detector. The injector, oven and detector temperatures were set at 250, 195 and 270 °C, respectively. The flow rate of the carrier gas (hydrogen) was set at 2 mL min^{-1}. Analyses of the chromatogram were performed with the HPCHEM software and the data analysed as described by [16].

FAME quantification was also carried out on the TL (just after extraction), NL, GL and PL (after SPE) extracts dissolved in chloroform not submitted to TLC. In these cases, 200, 150, 50 and 50 μg of heptadecanoic acid were added to 250, 250, 500 and 500 μL of the TL, NL, GL and PL extracts, respectively.

All extracts and all the TLC for each extract have been carried out in duplicate at least.

3. Results and discussion

Extraction of TL was performed by the method of Folch et al. [13], which allows a complete extraction of the non-starch PL [16,17]. These types of components are the most difficult to extract due to the fact that they are membrane constituents and are amphipathic molecules.

3.1. Solid-phase extraction efficiency

One of our aims was to obtain a PL fraction without any contaminant of the two others fractions (NLs and GLs). When the SPE procedure was carried out at atmospheric pressure and without controlled flow, the presence of contaminants in the PL fraction was detected by TLC for NL components and by HPLC for the GL compounds. Moreover, this method carried under air may induce lipid oxidation. To decrease lipid oxidation and enhance interactions between the stationary phase and the lipid fractions, the SPE procedure was carried out under controlled vacuum and controlled flow as described in Section 2.5.

Firstly, the SPE yield for PLs was checked by loading on the SPE cartridge the PL standards. Based on the FAME analysis, the SPE yield was closed to 100% (results not shown). Then, a TL extract from wheat flour (commercial flour H) was loaded on the SPE cartridge and the SPE yield was estimated by the comparison of the total fatty acids (FAME analysis) amount before SPE and the total fatty acids amounts in the three fractions (NLs, GLs and PLs) after SPE. No statistically significant differences were found between the two mean values (Table 2) meaning that the SPE yield was closed to 100%

Table 2
FA contents in the TL extract from wheat flour before SPE and in the NL, GL and PL fractions after SPE (mean ± standard deviation, $n = 4$)

μmol g^{-1} dm	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
TL	7.0 ± 0.1	0.34 ± 0.00	3.7 ± 0.1	21.9 ± 0.6	1.56 ± 0.05	34.4[a] ± 0.8
NL	5.8 ± 0.2	0.27 ± 0.01	3.3 ± 0.1	18.6 ± 0.7	1.36 ± 0.05	29.4 ± 1.0
GL	0.5 ± 0.1	0.03 ± 0.01	0.18 ± 0.02	1.9 ± 0.1	0.14 ± 0.01	2.7 ± 0.1
PL	1.0 ± 0.1	0.04 ± 0.01	0.27 ± 0.01	1.6 ± 0.2	0.10 ± 0.05	3.00 ± 0.3
NL + GL + PL	7.2 ± 0.3	0.34 ± 0.01	3.8 ± 0.1	22.1 ± 0.9	1.6 ± 0.1	35.0[a] ± 1.3

[a] These values are not significantly different ($P > 0.05$, ANOVA test).

for the TL extract from wheat flour. In addition, no oxidation has occurred in our experimental conditions since no loss was observed on the polyunsaturated fatty acids.

In a second step and in order to follow the behaviours of the different classes of lipids during the SPE procedure, the TL (before SPE) and the NL, GL and PL fractions (after SPE) were submitted to a TLC procedure. In addition to the expected spots (TAG, FFA, $DAG_{1,3}$, $DAG_{1,2}$ and MAG), the NL fraction exhibited a spot of PoLs whereas the GL and PL fractions did not show spots of non-polar lipids. The PoL spots of the TL, NL, GL and PL fractions were scrapped and submitted to HPLC. Both GLs and PLs were present in the TL and NL fractions. Conversely, the PoL spot of the GLs fraction contained only GLs, whereas, the PoL spot of PL fraction contained only PLs. Therefore, one can concluded that after SPE, the NL fraction was contaminated by PoLs (GLs and PLs) whereas the GL and PL fractions were devoid of non-polar lipids and were not cross contaminated. Moreover, the PLs chromatograms obtained after injection of the PoL spots scrapped from the TL and the PL extracts were very close both in the nature of the peaks and the peak areas. This means that 50% of the PLs present in the TL extract were eluted by chloroform in the NL fraction and the other 50% were eluted by methanol in the PL fraction.

In a third step, the fatty acid contents of each TLC spot obtained from the TL extract (before SPE) and from the NL, GL and PL extracts (after SPE) were determined (Tables 3–5). The comparison of the total amounts of fatty acids in the TL and NL fractions before TLC, 34.4 and 29.39 μmol g^{-1} dm (Table 2) and after TLC, 29.8 and 24.07 μmol g^{-1} dm (Tables 3 and 4), showed similar differences close to 5 μmol g^{-1} dm. These differences may be attributable, on the one hand, to unavoidable losses during the evaporation, concentration and dilution steps carried out on the lipid samples before TLC. On the other hand, small amounts of fatty acids such as these contained in steryl esters were not present in the scrapped spots. According to Morrison [3], the steryl esters represent 6–7% of the NL fraction in wheat lipids. The differences between GL and PL fractions before TLC, 2.68 and 2.96 μmol g^{-1} dm (Table 2), and after TLC, 2.45 and 2.73 μmol g^{-1} dm (Table 5) were much less important, close to 0.2 μmol g^{-1} dm. These differences are only due the evaporation, concentration and dilution steps before TLC. Since only 50% of polar lipids were present in the GL and PL extracts, the total amounts of fatty acids (FAs) contained in the glycolipids and phospholipids of the flour H were close to 5.3 and 6 μmol FA g^{-1} dm, respectively.

Table 3
FA contents in the TL extract after TLC (mean ± standard deviation, $n = 4$).

μmol g^{-1} dm	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
TAG	2.4 ± 0.1	0.16 ± 0.04	1.8 ± 0.1	7.6 ± 0.5	0.58 ± 0.03	12.5 ± 0.8
FFA	1.1 ± 0.2	0.08 ± 0.02	0.5 ± 0.1	2.7 ± 0.5	0.16 ± 0.02	4.6 ± 0.7
$DAG_{1,3}$	0.2 ± 0.1	Traces	0.09 ± 0.05	0.5 ± 0.1	Traces	0.8 ± 0.2
$DAG_{1,2}$	0.2 ± 0.1	Traces	0.19 ± 0.04	0.8 ± 0.1	0.02 ± 0.01	1.3 ± 0.2
MAG	0.08 ± 0.04	Traces	0.07 ± 0.03	0.29 ± 0.05	Traces	0.4 ± 0.1
PoL	2.1 ± 0.1	0.06 ± 0.03	0.06 ± 0.04	7.4 ± 0.4	0.51 ± 0.05	10.2 ± 0.5
Total	6.1 ± 0.2	0.3 ± 0.1	2.7 ± 0.3	19.4 ± 0.5	1.3 ± 0.1	29.8 ± 0.9

Table 4
FA contents in the NL extract after TLC (mean ± standard deviation, $n = 4$)

μmol g^{-1} dm	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
TAG	2.5 ± 0.4	0.15 ± 0.02	1.8 ± 0.1	7.5 ± 0.4	0.57 ± 0.04	12.4 ± 0.8
FFA	1.1 ± 0.1	0.08 ± 0.01	0.46 ± 0.04	2.4 ± 0.4	0.1 ± 0.1	4.1 ± 0.6
$DAG_{1,3}$	0.17 ± 0.04	Traces	0.10 ± 0.03	0.5 ± 0.1	0.04 ± 0.01	0.8 ± 0.1
$DAG_{1,2}$	0.23 ± 0.04	0.02 ± 0.01	0.17 ± 0.03	0.7 ± 0.1	0.06 ± 0.02	1.2 ± 0.2
MAG	0.09 ± 0.02	0.02 ± 0.01	0.06 ± 0.01	0.23 ± 0.02	0.01 ± 0.01	0.41 ± 0.04
PoL	0.9 ± 0.1	0.07 ± 0.01	0.3 ± 0.2	3.7 ± 0.3	0.25 ± 0.02	5.2 ± 0.5
Total	4.95 ± 0.3	0.34 ± 0.03	2.8 ± 0.1	14.9 ± 0.7	1.0 ± 0.1	24.1 ± 1.0

Table 5
FA contents in the GL and PL extracts after TLC (mean ± standard deviation, $n = 4$)

μmol g^{-1} dm	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
GL	0.41 ± 0.04	0.02 ± 0.01	0.17 ± 0.03	1.7 ± 0.1	0.14 ± 0.02	2.4 ± 0.2
PL	0.5 ± 0.1	0.03 ± 0.01	0.24 ± 0.03	1.8 ± 0.2	0.10 ± 0.04	2.7 ± 0.3
GL + PL	0.9 ± 0.1	0.05 ± 0.02	0.41 ± 0.04	3.5 ± 0.3	0.24 ± 0.05	5.2 ± 0.4

In conclusion of this study on the SPE efficiency, we succeed in obtaining a PL fraction without any contaminants of the NL and GL fractions (as well as a GL fraction devoid of NL and PL). However, based on the FAME analysis, half of the PoL fraction (GLs + PLs) was eluted by chloroform with the NL fraction. The presence of methanol in the concentrated TL extract may explain this partial elution by chloroform. In addition, according to different authors [17–20], the separation of polar lipids classes is hard to achieve. Thus, Caboni et al. [17] showed that regarding the silica cartridges, the results varied greatly, depending on the PL content of the sample. For high PL content (close to 20% of lipids), the recovery was 70%, while for lower amounts of PLs (1% of lipids) the recovery was about 60%. These data correspond to an experiment in which a 5% acetic acid solution in methanol was used as third elution solvent, the authors indicated that lower recoveries were obtained with a lower percentage of acetic acid in methanol [17]. Moreover, Ohm and Chung [20] indicated that the separation of NL wheat flour by chloroform alone using silicic acid open column chromatography has been ineffective. In addition, these authors showed that a chloroform–acetone (4:1) mixture allowed a much clearer separation of NL than chloroform.

3.2. HPLC procedure for the separation and quantification of phospholipids in the PL fraction

We firstly selected a Diol-bonded silica stationary phase since according to [12,21], this phase allows an efficient separation of polar lipids, within a relatively short time.

Based on the results obtained by Balazs et al. [22], we developed a method which allows the separation of the main phospholipids of wheat flour in less than 16 min (Fig. 1). However, at the end of the gradient, 12 min (duration of the return to initial conditions and re-equilibration time) are necessary to maintain a good reproducibility in the retention times for NAPE, NALPE, PC and LPC (Table 6). Using spherical silica as stationary phase, Buenger and Pison [23] obtained a R.S.D. higher than 1% for the PC retention time compared to 0.2% by our method.

In order to use this HPLC method for quantification, it was necessary to prepare different calibration curves for each component in the sample. Standards were injected individually as well as in a mixture to determine the retention times and the peak areas. Calibration curves for NAPE, NALPE, PC and LPC are shown in Fig. 2. As it has been already shown both experimentally and theoretically [24], the calibration curves obtained are always sigmoid (Fig. 2). Nevertheless, these curves can be linearised in a definite range of concentration which is different from one PL to another. In our chromatographic conditions, these ranges varied from 0.01 to 0.06, 0.10, 0.09 and 0.19 μmol injected for NAPE, NALPE, PC and LPC, respectively. In these ranges of concentration, the peak areas (arbitrary units) are related to the amount X of PL injected (μmol) by the following equations:

$$\text{Area} = 3.48 \times 10^8 \quad X_{\text{NAPE}} + 2.09 \times 10^6 (R^2 = 0.999)$$

for NAPE

$$\text{Area} = 1.90 \times 10^8 \quad X_{\text{NALPE}} + 1.02 \times 10^6 (R^2 = 0.974)$$

for NALPE

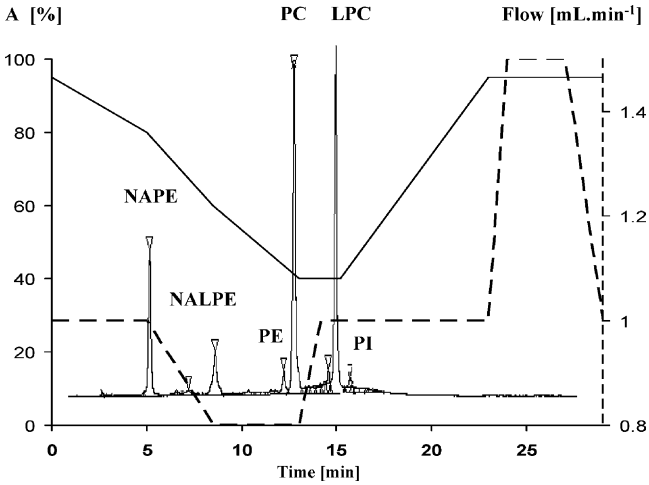

Fig. 1. HPLC chromatogram, gradient and flow used for the PL separation (A) *n*-hexane–2-propanol–acetic acid–triethylamine (81.42:17.00:1.50:0.08 (v/v)) and (B) 2-propanol–water–acetic acid–triethylamine (84.42:14.00:1.50:0.08 (v/v)). The amounts injected are 8, 4, 21 and 14 μg for NAPE, NALPE, PC and LPC, respectively.

Table 6
Retention times of NALPE, NAPE, PC, LPC, phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylinositol (PI) (mean ± standard deviation, $n = 8$)

	Retention time (min)
NAPE	4.7 ± 0.1
NALPE	7.4 ± 0.1
PE	12.2 ± 0.1
PC	13.2 ± 0.03
PG	14.8 ± 0.1
LPC	15.0 ± 0.1
PI	15.9 ± 0.1

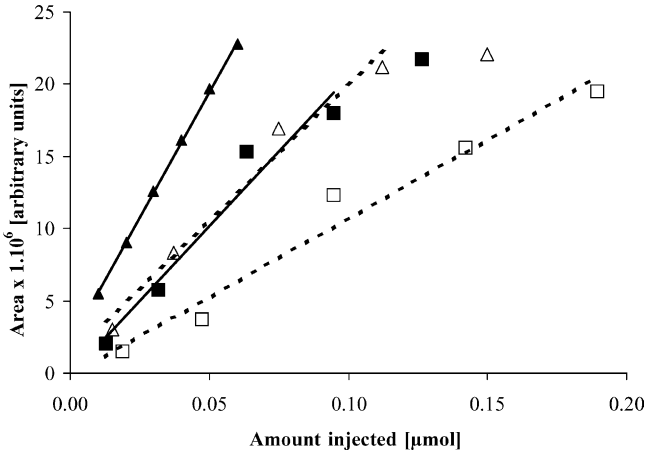

Fig. 2. Calibration curves for the quantification of (▲) NAPE; (△) NALPE; (■) PC and (□) LPC by HPLC–ELSD. The linear parts of the calibration curves are between 0.01 and 0.06 μmol injected for NAPE, between 0.01 and 0.11 μmol injected for NALPE, between 0.01 and 0.09 μmol injected for PC, and between 0.01 and 0.19 μmol injected for LPC.

$$\text{Area} = 2.05 \times 10^8 \quad X_{\text{PC}} - 9.76 \times 10^4 (R^2 = 0.952)$$

for PC

$$\text{Area} = 1.09 \times 10^8 \quad X_{\text{LPC}} - 2.33 \times 10^5 (R^2 = 0.969)$$

for LPC

3.3. Phospholipids content in eight wheat flours

From the calibration curves, the amounts of each phospholipid in the PL extract can be given in μmol. These values can be also given in μmol of FAs since for 1 mol of NAPE, NALPE, PC and LPC, there was 3, 2, 2 and 1 mol of FAs, respectively. Then, to obtain the total amount of phospholipids in the PL extract, we have to multiply by 1.2 since according to Morrison [4], NAPE + NALPE + PC + LPC represent 83% of the wheat phospholipids. Lastly, to obtain the total amount of phospholipids in the TL extract, we have to multiply by two since only 50% of the phospholipids are present in the PL fraction.

The phospholipids amount (in μmol g^{-1} dm and in μmol FA g^{-1} dm) of seven different flours obtained from French pure wheat varieties (A–G) and one commercial flour (H) is reported in Table 7. For the flour H, the total amount of FAs in PLs calculated from the HPLC analysis (6.18 μmol FA g^{-1} dm) is close to the value found by the FAME analysis in Table 2 (2 × 2.96 = 5.92 μmol FA g^{-1} dm). For the eight flours, the quantities of PLs (in μmol g^{-1} dm) vary by a factor of 1 to 1.9, between E and H. In almost all cases, LPC was the main phospholipid (29–48%) followed by PC (26–37%), NAPE (4.0–10.7%) and NALPE (4.2–8.7%) in decreasing order. This distribution is in agreement with [4] and [25].

Table 7
PL amount of eight wheat flours (A–G are flours from French pure wheat varieties and H is a commercial flour)

	A	B	C	D	E	F	G	H
μmol g^{-1} dm								
NAPE	0.12	0.16	0.18	0.25	0.19	0.34	0.37	0.34
NALPE	0.17	0.13	0.17	0.16	0.14	0.25	0.30	0.27
PC	0.79	1.04	1.14	1.05	0.65	0.99	1.21	1.20
LPC	1.43	1.25	1.10	1.13	0.61	1.31	1.01	1.20
Sum	2.51	2.58	2.58	2.59	1.59	2.89	2.89	3.01
PL total[a]	3.01	3.09	3.09	3.11	1.90	3.46	3.46	3.61
μmol FA g^{-1} dm								
NAPE	0.37	0.48	0.53	0.75	0.56	1.02	1.11	1.01
NALPE	0.33	0.25	0.34	0.31	0.27	0.49	0.60	0.54
PC	1.58	2.08	2.27	2.10	1.31	1.99	2.41	2.41
LPC	1.43	1.25	1.10	1.13	0.61	1.31	1.44	1.20
Sum	3.71	4.06	4.23	4.29	2.75	4.81	5.13	5.15
PL total[a]	4.46	4.88	5.08	5.15	3.30	5.77	6.16	6.18

Results are expressed in μmol g^{-1} dm and μmol FA g^{-1} dm.

[a] The total amount of phospholipids in the PL extract is obtained by multiplying by 1.2 the sum (NAPE + NALPE + PC + LPC) according to Morrison [4].

3.4. Effect of the addition of phospholipase during dough mixing

Model doughs composed of water and wheat flour were mixed with and without phospholipase. Aliquots of dough

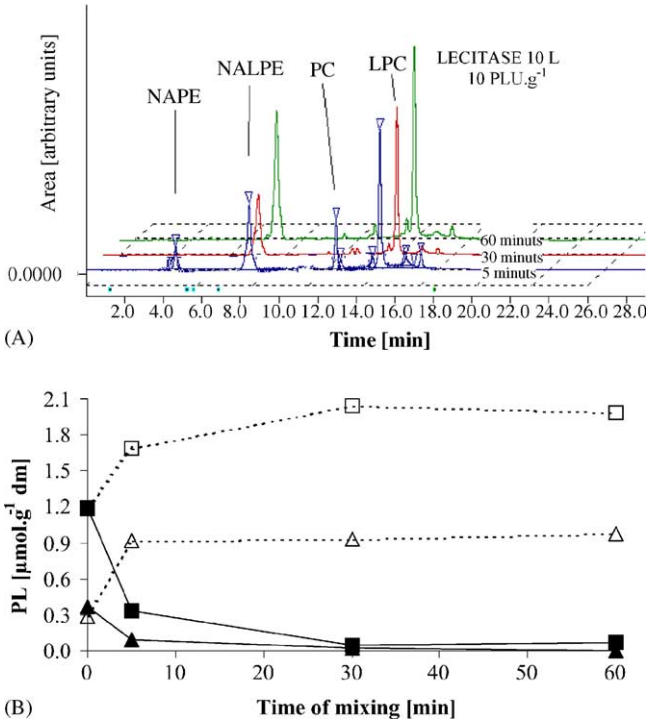

Fig. 3. Chromatograms (A) and PL evolutions (B) during mixing (75 rpm) with Lecitase 10 L (10 PLU g^{-1}). (▲) NAPE; (△) NALPE; (■) PC and (□) LPC. The amounts injected are 1, 10, 4 and 14 μg at 5 min; 0, 9, 1 and 16 μg at 30 min; 0, 13, 1 and 21 μg at 60 min for NAPE, NALPE, PC and LPC, respectively.

were collected after 5, 15 and 60 min of mixing, and their phospholipids content was analysed. The amount of polar lipids extracted remained constant during mixing without phospholipase (data not shown) in agreement with the results obtained by Castello et al. [16] on the polar lipids.

The evolution of the main phospholipids during mixing of wheat flour dough with the Lecitase 10 L is shown in Fig. 3A. When 10 PLU g^{-1} of Lecitase 10 L were added, 95% of NAPE and PC were hydrolysed after 30 min, and only traces of these phospholipids were present after 60 min. Concomitantly, an increase in the lyso forms of PLs (NALPE and LPC) was observed (Fig. 3B). A similar mixing experiment was carried out during 30 min with 5 PLU g^{-1} of Lecitase 10 L added to the dough. In this case, only 30% of PC was hydrolysed after 30 min of mixing with a concomitant increase of LPC, whereas the amount of NAPE remained almost constant during mixing (less than 10% hydrolysed). Therefore, Lecitase 10 L is more efficient on PC than on NAPE.

4. Conclusions

After fractionation of a TL extract by SPE, the presented HPLC method with Diol column in conjunction with evaporative light scattering detection allowed an efficient separation in less than 16 min of all the phospholipid classes of soft wheat flour and corresponding dough without contamination by glycolipids. Using this method, we are now able to quantify the phospholipid evolution during mixing. According to French regulation, phospholipases might be used as additional ingredients to obtain bread dough. Thanks to an air-tight mixer developed in our laboratory [26], we plan to follow the oxygen consumption and the rheological properties of dough during mixing influenced by the addition of different phospholipases. These evolutions will be compared to the effects of these enzymes on the dough phospholipids.

Fast analysis and good reproducibility are achieved with the described method. This method has been applied to the gluten network and to commercial lecithins (data not shown) and can probably be applied to the starch, resulting in good recovery of PL and their degradation products. Nevertheless, for the starch lipids, the TL extraction method remains to be optimised.

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