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ARTICLE

Improvement of Total Lipid and Glycerophospholipid Recoveries from Various Food Matrices Using Pressurized Liquid Extraction

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The extraction of three major phospholipid (PL) classes contained in soybean, egg yolk, calf brain, and ox liver was investigated by means of two methods. The PL amounts were evaluated. A new method, based on pressurized liquid extraction (PLE), was applied for total lipids (TL), including PL, extraction and compared with a standard liquid extraction method, a modified Folch method. The three PL classes (phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC)) that were recovered in the obtained TL extracts were quantified using HPLC with an evaporative light-scattering detector (ELSD). Using the PLE method, a single extraction allowed a recovery of more than 94% of TL and 96% of each PL class. Two successive extractions could achieve a total recovery of the three studied PL classes. With the modified Folch method, 77−83% of TL, 80−91% of PE, 82−94% of PC, and no more than 78% of PI could be achieved from various food matrices after one extraction. Four successive extractions were necessary to recover the whole TL content and each PL class. Results indicate that PLE is a rapid and efficient lipid extraction system for the broad range of plant and animal tissues.

KEYWORDS: Phospholipids; total lipids; PLE; modified Folch method; HPLC-ELSD

INTRODUCTION

The term "phospholipid" is used to describe all lipids containing phosphorus. These phospholipids (PL) play very important roles in many aspects of human health, in particular in protecting the liver (*1*), in improving memory and learning (*2*), and in protecting the cardiovascular system (*3*, *4*). PL are divided into two groups: glycerophospholipids and sphingolipids. Glycerophospholipids are made of a glycerol backbone esterified with two fatty acids and a polar headgroup that defines the class they belong to. The main glycerophospholipid classes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). Sphingolipids are characterized by a sphingosine backbone esterified with a single fatty acid. Sphingomyelin (SM) is the main member of this group.

Data regarding PL content are highly important when it comes to the nutritional value of foods. Incomplete extraction of total lipids (TL) can cause serious errors in PL determinations. Gregor (*5*) reported lower TL contents and a different relative distribution of the PL in carrot root than Soimajarvi and Linko (*6*), who ascribed the difference to the incomplete extraction of TL. Therefore, effective extraction methods need to be developed for various food matrices to investigate their TL and PL contents. Various procedures have been employed to quantitatively extract lipids, such as the Soxhlet method (*7*) and ultrasound-assisted extraction (UAE) (*8*), as well as the Folch method (*9*), based on chloroform/methanol extraction, which was later modified by Bligh and Dyer (*10*). An investigation by Iverson et al. (*11*) showed that Folch's method was more efficient in extracting large amounts of TL when compared with the Bligh and Dyer method. However, these extraction methods often rely on the use of high amounts of organic solvents, most of which are hazardous or expensive and must be properly handled and disposed of, once the extraction is finished (*12*). In addition, these extractions are often time-consuming, which in turn may lead to degradation of active ingredients (*13*). Among new alternative techniques that are available, there is pressurized liquid extraction (PLE), which has received particular attention recently and gained wide acceptance for the extraction of organic contaminants (*14*). It has been used to extract compounds from various biological materials (*15*−*19*). This method uses small amounts of conventional solvents at elevated temperatures and pressures to achieve quantitative extraction from solid and semisolid samples in a short time (*20*−*22*). However, available data regarding the extraction of lipids by the PLE method are scarce, especially when it comes to food matrices, and is often limited to the study of fatty acid compositions (*23*). To our knowledge, the complete extraction of TL, including PL, from food matrices by the PLE method has not been reported to date.

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PL separations have been studied by many research groups who used numerous separation methods. Commonly, quantitation and separation of PL have been performed using thin-layer chromatography (TLC) (*24*−*26*). This method has several disadvantages, including poor chromatographic resolution and difficulties in getting a good quantitative assessment. Recently, several papers describing successful separations of individual PL by HPLC have been published. Indeed, this method offers better reproducibility and higher resolution than TLC. The most common HPLC detection system is absorption spectroscopy in the ultraviolet (UV) range. This method is sensitive to gradient conditions and results in baseline drift and high background noise (*27*). Moreover, PL do not have any chromophores and are quite transparent with this detection method. A differential refractometric detection method is also widely used in HPLC. For the detection of non-UV-absorbing compounds, this method is nevertheless not usable with a gradient elution because of considerable fluctuations of refractive indices as a function of the composition of the mobile phase and other HPLC parameters (*28*). However, another type of detector gaining interests is the evaporative light-scattering detector (ELSD) (*25*). An advantage to this detector is that it is not much affected by gradient conditions as soon as the mobile phase is properly evaporated before the detection step (*28*).

In this paper, three PL classes (PE, PI, and PC) were chosen to be examined. The extraction of TL, including PL, from various food matrices (plant and animal) by the PLE method was studied and compared to the most widely used traditional technique (modified Folch method). Samples were prepared by cryogenic grinding and freeze-drying. The presence of PL and their contents were determined by high-performance liquid chromatography (HPLC-ELSD), whereas the TL content was determined by gravimetry.

MATERIALS AND METHODS

Materials. Solvents used for HPLC analyses were of HPLC grade, and those used for extraction were of analytical grade. Chloroform was purchased from VWR (Strasbourg, France), and methanol was purchased from Carlo Erba (Val de Reuil, France). Washing solution was prepared from analytical grade sodium chloride (1%, w/v; VWR) and ultrapure water (Synergy, Millipore S.A.S, Molsheim, France). Sand (Fontainebleau) was provided by VWR.

The food matrices investigated could be categorized into three groups according to their fat content: a low-fat group with ox liver and calf brain (< 10%, w/w of fresh food), an intermediate group with soybean (10−30%, w/w of fresh food), and a high-fat group with egg yolk (> 30%, w/w of fresh food). All products were purchased from a local retailer.

Standard solutions of soy lecithin mix standard (certified: 14.41% PC, 12.06% PE, and 9.64% PI) from Spectral Service GmbH (Cologne, Germany) were prepared at six concentrations between 0.1 and 1.1 mg/mL.

Sample Preparation. Preparation was different for each sample depending upon its physical state. With the exception of egg yolk, other samples were ground under cryogenic conditions (liquid nitrogen, three steps of 5 min each) using a 6870 freezer/mill (Spex CertiPrep, Stanmore, U.K.). The obtained frozen powder was freeze-dried (Alpha 2-4, Bioblock Scientific, Illkirch, France). Egg yolk powder was obtained by removing egg white from fresh eggs, homogenizing, and freeze-drying. All of the food samples were stored at −20 °C until use (*29*).

Modified Folch Method. TL was extracted according to the method of Folch et al. (*9*), with minor modifications (*10*). A total of 1 g of the prepared food sample was suspended in 30 mL of a $CHCl_3/CH_3OH$ (2:1, v/v) mixture and shaken mechanically for 20 min. The suspension was centrifuged at 8500 rpm for 10 min. The supernatant was removed, and the pellet was re-extracted four times (total of five extractions) following the same procedure until total exhaustion of the food matrix from lipids. Supernatant of each extract were washed with 5 mL of sodium chloride aqueous solution (1%, w/v). The organic phase containing the lipid fraction was collected, evaporated under vacuum using a rotary evaporator (40 °C, 30 kPa), and dried under a gentle stream of N_2. The TL extract was weighed and dissolved in 1 mL of $CHCl_3$. The TL content was expressed in milligrams per gram of fresh food.

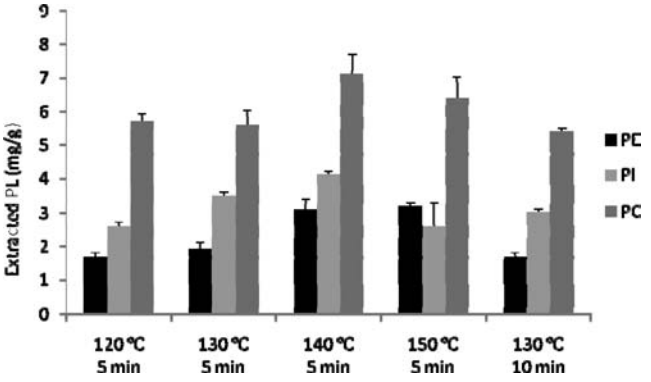

Figure 1. Amounts of PL recovered from soybean after one extraction by PLE method. Standard deviation (n = 3) is shown as error bars.

Pressurized Liquid Extraction. Pressurized liquid extractions were performed on a Dionex PLE 350 (Dionex, Sunnyvale, CA) system. Sample powder, 1.0 g, was mixed homogenously with sand so as to fill the 10 mL stainless steel extraction cell (L × Ø = 52 mm × 15 mm). The use of a dispersion agent, such as sand used for the extraction, is recommended to reduce the solvent volume by filling the empty part of the cell (*18*). The solvent mixture employed was the same as used for the modified Folch procedure, $CHCl_3/CH_3OH$ (2:1, v/v). Different extraction temperatures (120−150 °C) were investigated. The extraction time and pressure were set at 5 min and 10.34 MPa, respectively. One to three extractions (successive extractions of the same sample and collection in three independent vials) were performed on each food matrix. Each extract (17 mL) was collected in a 60 mL glass vial and washed with 5 mL of sodium chloride aqueous solution (1%, w/v). The organic phase containing the lipid fraction was collected, dried, and redissolved as previously described.

Determination of PL Classes by HPLC-ELSD. A chromatographic system, made of a 616 controller, a 2424 ELS detector, and a 717 Plus autosampler (Waters, Saint-Quentin-Fallavier, France) and controlled with Empower 2 software (Waters), was used to analyze PL classes. High-purity nitrogen from a nitrogen generator (Domnik Hunter, Villefranche-sur-Saône, France) was used as a nebulizing gas at a pressure of 310 kPa. The drift tube temperature was set at 45 °C. PL were separated into their classes using a 150 × 3 mm, 3 μm, Luna normal phase column (Phenomenex, Le Pecq, France). The flow rate of the mobile phase was 0.5 mL/min, and separations were performed at room temperature using a 20 min linear gradient ranging from $CHCl_3/CH_3OH$ (88:12, v/v) to $CHCl_3/CH_3OH$/1 M aqueous formic acid (adjusted to pH 3 with triethylamine) (28:60:12, v/v/v) (*30*). Each extract was dissolved in a mixture of chloroform/methanol (2:1, v/v), filtered through a 0.45 μm filter (Macherey-Nagel, Hoerdt, France) to eliminate particles, and injected (20 μL) in the chromatographic system. PL classes were identified by comparison of their retention times with those obtained under the same analytical conditions with standards. Quantification of each PL class was performed on the basis of a quadratic model of external calibration obtained using standard solutions. PL contents were expressed in milligrams per gram of fresh food.

Statistical Analysis. Three determinations were performed for each food matrix (n = 3). All results are given as mean ± standard deviation (SD). The Student t test was used to determine significant differences between extraction efficiencies. Significance level was considered at $P < 0.05$.

RESULTS AND DISCUSSION

Determination of Optimal Extraction Conditions for PL Included in the TL Extract by PLE. To achieve total extraction, conditions, such as temperature, time, and number of steps, are important parameters (*31*). First, the effects of extraction temperature and duration on the extraction efficiency were investigated. Soybean was chosen as a model food matrix. As shown in **Figure 1**, the recovery of PL gradually increased with the increase of temperature from 120 to 140 °C. When the temperature was

Table 1. Total Amounts of TL Extracted from Egg Yolk, Calf Brain, Soybean, and Ox Liver[a]

extraction method	egg yolk (mg/g)[b]	ox liver (mg/g)[b]	calf brain (mg/g)[b]	soybean (mg/g)[b]
PLE (two successive extractions)	331 ± 3	72 ± 4	102 ± 3	255 ± 10
modified Folch (four successive extractions)	329 ± 7	72 ± 7	101 ± 11	250 ± 21

[a] Results (n = 3) are expressed as mean ± SD. [b] TL contents are expressed in mg/g of fresh food.

Table 2. Recovery Rates of a Single Extraction of TL and PL from Egg Yolk, Calf Brain, Soybean, and Ox Liver[a]

food matrix	extraction method	TL (%)[b]	PE (%)[b]	PI (%)[b]	PC (%)[b]
egg yolk	PLE	98.88 ± 0.02	98.96 ± 0.01	96.4 ± 0.5	99.92 ± 0.02
	modified Folch	80 ± 1	91.3 ± 0.9	75.8 ± 0.9	92 ± 2
calf brain	PLE	96.1 ± 0.2	98.6 ± 0.3	97.33 ± 0.06	98 ± 1
	modified Folch	82.0 ± 0.4	90.3 ± 1.0	78.3 ± 0.8	88 ± 2
soybean	PLE	98.4 ± 0.6	97.2 ± 1.0	98 ± 1	97.6 ± 0.6
	modified Folch	83 ± 1	80 ± 1	77 ± 1	81.7 ± 0.8
ox liver	PLE	94 ± 1	99 ± 1	98 ± 1	99.0 ± 0.8
	modified Folch	77 ± 2	88 ± 2	71 ± 2	93.9 ± 0.9

[a] Results (n = 3) are expressed as mean ± SD. [b] Percentage of recovery of one extraction of TL and PL (mg/g, in fresh food).

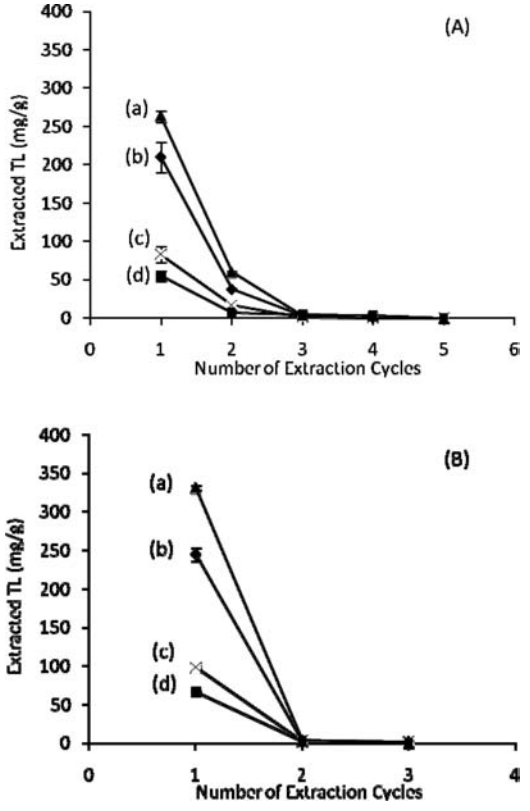

Figure 2. Amounts of TL recovered in successive extractions: (**A**) modified Folch method (a, egg yolk; b, soybean; c, calf brain; d, ox liver); (**B**) PLE method (a, egg yolk; b, soybean; c, calf brain; d, ox liver).

raised to 150 °C, the recovery of PL slightly decreased, which is in line with the results obtained by Boselli on the extraction of oxysterols from egg-containing foods (*12*). This decrease can be explained by an oxidative loss that occurs at higher temperatures (*32*). On the other hand, no significant difference ($P > 0.05$) in extraction efficiency was observed with extraction at 130 °C for 5 or 10 min (**Figure 1**). This showed that the partition equilibrium between solvent and food matrix was achieved within the first 5 min during a static extraction, which is consistent with previous results (*16*).

Table 3. Coefficients a, b, and c of the Quadratic Standard Curves Obtained for Each PL Class

	equation: $ax^2 + bx + c$			
PL class	a	b	c	R^2
PE	3.0×10^8	-3.0×10^7	4.0×10^6	1.000
PI	2.0×10^8	-2.0×10^7	2.0×10^6	1.000
PC	2.0×10^8	-2.0×10^7	3.0×10^6	1.000

Therefore, the optimal extraction temperature and time for each extraction by PLE method were set at 140 °C and 5 min, respectively.

Total Lipid Content. Soybean, egg yolk, ox liver, and calf brain were chosen as model food matrices, because they represent various levels of fat content. Using PLE and the modified Folch method, the amounts of TL (mg/g of fresh food) recovered from each food matrix were similar (**Table 1**). Egg yolk contained the highest amount of lipids (331 mg/g), which was in accordance with food composition tables (310−330 mg/g) (*33*), whereas ox liver and calf brain contained the lowest amounts, but these amounts were higher than values reported in food composition tables (37 and 76 mg/g, respectively) (*33*). Soybean, nevertheless, contained about 255 mg/g TL, which was also higher than values reported in food composition tables (180 mg/g) (*33*). The method used for sample preparation, which consisted of cryogenic grinding, probably allowed higher TL recovery for soybean, ox liver, and calf brain, matrices that are very hard to deal with using conventional methods. In our study, tests performed with soybean using an Ultraturrax grinder resulted in a content of 93 mg/g compared with 255 mg/g using cryogenic grinding.

The total amounts of TL could be recovered after four extractions using the modified Folch method (no recovery of TL in the fifth extraction) and two extractions using PLE (**Table 1**). A single extraction, however, resulted in TL recoveries of 77−83 and 94% using these two methods, respectively (**Table 2**).

The amount of extracted TL was therefore monitored according to the number of successive extractions for each selected food matrix using the modified Folch and PLE methods (**Figure 2**). With the modified Folch method, three successive extractions could recover 98% of TL, and a fourth extraction was necessary to achieve total recovery. On the other hand, the PLE method was more efficient in that only two successive extractions were needed to recover the whole TL content of each food matrix.

Table 4. Total Amounts of Extracted PL Classes from Food Matrices in Milligrams per Gram of Fresh Food[a]

food matrix	extraction method	PE (mg/g)[b]	PI (mg/g)[b]	PC (mg/g)[b]	sum of PE + PI + PC (mg/g)[b]
egg yolk	PLE	24 ± 1	2.6 ± 0.4	59 ± 3	86 ± 3
	modified Folch	24 ± 1	2.6 ± 0.1	57 ± 2	84 ± 4
calf brain	PLE	14.0 ± 0.1	3.4 ± 0.1	8.6 ± 0.2	26.1 ± 0.1
	modified Folch	13 ± 2	3.3 ± 0.3	9 ± 1	25 ± 2
soybean	PLE	3.2 ± 0.3	4.7 ± 0.5	6.6 ± 0.5	14.6 ± 0.4
	modified Folch	3 ± 1	4.8 ± 0.6	6.7 ± 0.5	15 ± 1
ox liver	PLE	8.1 ± 0.4	3.8 ± 0.4	17 ± 2	29 ± 1
	modified Folch	8 ± 1	3.8 ± 0.4	17 ± 1	29 ± 2

[a] Results (n = 3) are expressed as mean ± SD. [b] Total quantity of PE, PI, and PC.

Separation and Quantification of PL Classes. To investigate the efficiency of the PLE method in extracting PL, TL extracts were investigated for the presence of various PL classes. Using normal phase chromatography, PL peaks presented good chromatographic resolution, not only with standard mixtures but also with TL extracts ($R_{PE\text{-}PI}$ = 3.69, $R_{PI\text{-}PC}$ = 3.56). To quantify PL, calibration curves were drawn by applying equations of the quadratic model. The coefficients a, b, and c of these equations ($y = ax^2 + bx + c$) are given in **Table 3** for each PL class.

The most abundant classes of PL (PE, PI, and PC) were identified in the four food matrices studied. The total quantity of these PL classes (mg/g of fresh food) within the error range was almost identical whatever the extraction method used (**Table 4**). Egg yolk was the food matrix that contained the highest quantity of PL (sum of the three PL classes) per gram (86 ± 3 mg/g), with PC (59 ± 3 mg/g) and PE (24 ± 1 mg/g) being the main classes. PI was also identified, but as a minor class (2.6 ± 0.4 mg/g). Similar patterns for egg yolk have also been reported in previous studies (*33*−*35*). As far as soybean is concerned, the sum of the three examined PL classes was 14.6 ± 0.4 mg/g, which is higher than contents found in the literature (*35*). PC was the main PL class, with 6.6 ± 0.5 mg/g. It has been reported that PL composition of soybean depends on seed maturity (*35*). The more mature the seeds are, the higher are the amounts of PE, PI, and PC, compared with other lipids. In this study, the seeds used were intended for human consumption and, therefore, highly mature. Unsurprisingly, our results are in agreement with a high-maturity pattern of seeds (*35*).

Organ meats, particularly brain and liver, are among the major sources (such as meat and milk products) of dietary PL. In terms of PL class diversity, these two matrices are the most complex among the foods investigated in this study. They contained not only PE, PI, and PC but also PS and SM, which were not quantified in this paper. In ox liver, the PL content was 29 ± 1 mg/g, with PC (17 ± 2 mg/g) and PE (8.1 ± 0.4 mg/g) being the two predominant classes, followed by PI (3.8 ± 0.4 mg/g) as a minor class (**Table 4**). No previous data are available for ox liver PL classes in food composition tables. However, when compared to data on pork liver, our results showed a similar pattern (*33*). As far as calf brain is concerned, the sum of the three PL classes was 26.1 ± 0.1 mg/g. As opposed to the other foods investigated, the major class was not PC (8.6 ± 0.2 mg/g), but PE (14.0 ± 0.1 mg/g) instead. PI (3.4 ± 0.1 mg/g) was also present.

Table 2 shows the recovery of TL and PL of each food matrix with a single extraction using PLE and the modified Folch method. A comparison between the two methods showed that there was a significant difference ($P < 0.05$) in extraction efficiency. With the latter, one extraction resulted in the recovery of >80% of PE and PC (PE, from 80 to 91%; PC, from 82 to 94%), but not more than 78% of PI (PI, from 71 to 78%). The quantity extracted was then plotted against the number of extractions, which showed that four successive extractions were

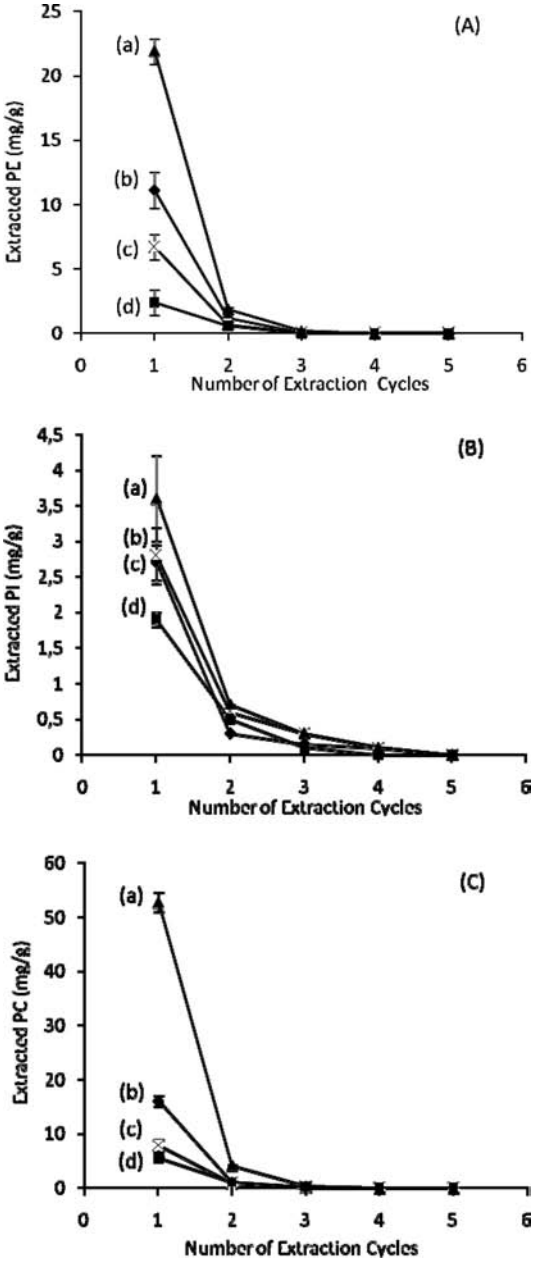

Figure 3. Amounts of PL recovered in successive extractions using modified Folch method: (**A**) PE (a, egg yolk; b, calf brain; c, ox liver; d, soybean); (**B**) PI (a, soybean; b, calf brain; c, ox liver; d, egg yolk); (**C**) PC (a, egg yolk; b, ox liver; c, calf brain; d, soybean).

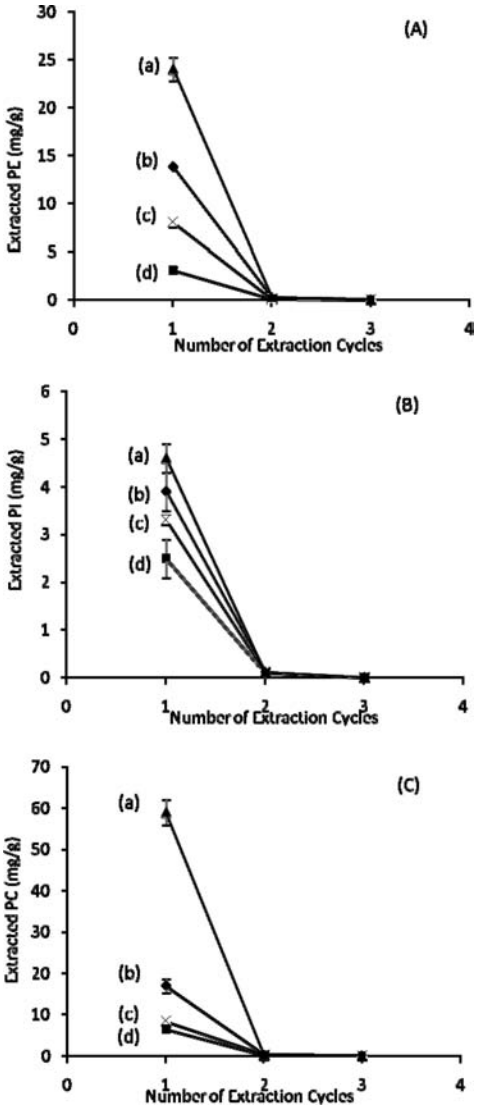

Figure 4. Amounts of PL recovered in successive extractions using PLE method: (**A**) PE (a, egg yolk; b, calf brain; c, ox liver; d, soybean); (**B**) PI (a, soybean; b, ox liver; c, calf brain; d, egg yolk); (**C**) PC (a, egg yolk; b, ox liver; c, calf brain; d, soybean).

necessary to recover all of the PL content (**Figure 3**). However, by PLE, a single extraction was sufficient to extract >96% of PL (PE, from 97 to 99%; PI, from 96 to 98%; PC, from 98 to 100%), and two successive extractions could achieve total recovery of the three studied PL classes (**Figure 4**).

This showed that, compared to the modified Folch method, PLE is a more efficient method for TL and PL extraction.

In this study, an effective method to extract TL, including PL, was reported. The PLE method offers the advantages of reducing the organic solvent consumption and the extraction time. The volume of solvent and the extraction time necessary to extract all of the TL content were, respectively, 44 mL and 15 min for the PLE method and 175 mL and 150 min for the modified Folch method. Moreover, there are health and environmental risks due to the continuous exposure of operators to hydrocarbons and chlorinated solvents when using the modified Folch method. Moreover, the PLE procedure can be partly automated.

The PLE method provides a simple, fast, and effective extraction method to analyze TL and PL from various food matrices. The results of this study demonstrate that the PLE method can be a better alternative to the modified Folch method.

ABBREVIATIONS USED

TL, total lipids; PL, phospholipids; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingolipids; PLE, pressurized liquid extraction; ELSD, evaporative light-scattering detector.

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