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Improvement of a solid phase extraction method for separation of animal muscle phospholipid classes

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Abstract

The solid phase extraction (SPE) method used by Banni et al. (2001) [Banni, S., Carta, G., Angioni, E., Muru, E., Scanu, P., Melis, M. P., et al. (2001). Distribution of conjugated linoleic acid and metabolites in different lipid fraction in the rat liver. *Journal of Lipid Research, 42*, 1056–1061] for fractionation of liver phospholipids (PLs) into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) was modified to improve its separation efficiency. A mixture of PL standards, containing PC, PE, PS and PI, was separated into individual classes by using aminopropyl minicolumns, following the method of Banni et al. (2001). The obtained eluted fractions were further analysed by thin layer chromatography (TLC) and PL classes were identified. TLC revealed the co-elution of PC and PE, that of PE with PS and no elution of PI. The SPE method was subsequently modified in order to obtain a correct PL fractionation into PC, PE, PS and PI. The principal modifications consisted of increased solvent volumes for PC, PE and PS elution, and a different solvent mixture to allow PI elution. The effectiveness of the modified SPE method was checked by TLC, using both standards and muscle samples, showing a correct elution of PC, PE, PS and PI.

Keywords: SPE; Phospholipid classes; Muscle

1. Introduction

The more representative PLs in mammal muscle are PC, PE, PS and PI (Olsson & Salem, 1997). PC is usually the major PL in animal tissues, often amounting to almost 50% of the total (Christie, 2005). PE is generally the second most abundant PL in animals, while PS usually constitutes less than 10% of the total PLs (Christie, 2005) and the proportion of PI is often below 4% in animal tissues (Genge, Licia, Wuthier, & Wutier, 2003). PC and PE are neutral PLs, while PS is a weakly acidic one and PI is strongly acidic (Christie, 2005). Due to this feature the correct separation of PS and PI is difficult and, thus, these two PLs are usually isolated together (Alasnier & Gandemer, 1998; Sánchez & Lutz, 1998; Tejeda, 1999). Fractionation of muscle PLs into major PL classes is becoming a common analytical procedure for meat scientists because it allows the analysis of individual PL classes and therefore, yields important information for each one (Kim & Salem, 1990). In this sense, variations in the fatty acid composition of individual PL classes may alter their oxidative stability and their functional properties (Olsson & Salem, 1997).

Separation of PL classes has conventionally been performed by TLC (Christie, 1982). High-performance liquid chromatography (HPLC) is increasingly used for this type of analysis due to its excellent quantification and resolution (Hemming & Hawthorne, 1999). Solid phase extraction (SPE) is also a widespread technique for separation of PL classes (Christie, 1982; Hemming & Hawthorne, 1999). This technique has been widely used, due to its easy and fast sample preparation.

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Pietsch and Reinhard (1993) developed a SPE method for the separation of individual PL classes (PC, PE, PS and PI) using single aminopropyl SPE cartridges and standards of PLs. Later, Banni et al. (2001) separated liver PL classes according to the method described by Pietsch and Reinhard (1993) with minor modifications. We have checked the effectiveness of these methods using pure PL standards and co-elutions of PC, PE and PS and no elution of PI was found. Therefore, our objective was to optimize a SPE method for separation of PL classes using standards of PLs and subsequently to check its effectiveness using muscle samples.

2. Materials and methods

2.1. Materials

PL standards were obtained from Sigma–Aldrich (St Louis, MO, CA): synthetic L-α-phosphatidylcholine, L-α-phosphatidylethanolamine from *Escherichia coli*, 3-*sn*-phosphatidyl-L-serine from bovine brain and L-α-phosphatidylinositol from bovine liver. Aminopropyl minicolumns (500 mg) were supplied by Varian (Harbor City, CA). A "Vac Elut" apparatus from Varian (Harbor City, CA) was used for flushing the cartridges. TLC plates of silica gel 60 G/UV_{254} (20 × 20 cm; 0.25 mm thickness) were from Macherey-Nagel (Düren, Germany). All solvents used in this study were obtained from Scharlau (Barcelona, Spain) or Panreac (Barcelona, Spain).

2.2. Preparation of biological samples

Adult rats, fed a mixed feed (AO4 from Panlab) were decapitated and their *Longissimus dorsi* muscle was dissected and stored at −80 °C prior to analysis. *Pectoralis major* muscle from chicken was obtained in a local supermarket. Samples were ground using a commercial grinder immediately before fat extraction. Intramuscular total lipids were extracted with chloroform:methanol (1:2; v/v) according to the method described by Bligh and Dyer (1959).

2.3. Fractionation of phospholipid classes by the Banni et al. (2001) method

Briefly, 50 μl of a mixture of PL standards (PC, PE, PS and PI) at 5 μg μl^{-1} were added to a 500 mg aminopropyl minicolumn, which had been previously activated with 3 ml of hexane. PC, PE, PS and PI were sequentially eluted with 20 ml of acetonitrile:*n*-propanol (2:1; v/v), 7 ml of methanol, 7.4 ml of isopropanol:methanolic 3 N HCl (4:1; v/v) and 10 ml of methanol:methanolic 3 N HCl (9:1; v/v), respectively. The vacuum was adjusted to generate a flow of 1 ml min^{-1} through the minicolumn. The composition and volume of eluting solvents for this SPE method are shown in Table 1.

2.4. Fractionation of phospholipid classes by the Banni et al. (2001) modified method

Briefly, 50 μl of a mixture of PL standards (PC, PE, PS and PI) at 5 μg μl^{-1} were added to a 500 mg aminopropyl minicolumn (Varian, Harbor City, USA), which had been previously activated with 7.5 ml of hexane. PC, PE, PS and PI were sequentially eluted with 30 ml of acetonitrile:*n*-propanol (2:1; v/v), 10 ml of methanol, 7.5 ml of isopropanol:methanolic 3 N HCl (4:1; v/v) and 17.5 ml of chloroform:methanol:HCl 37% (200:100:1; v/v/v), respectively. Compositions and volumes of solvents used and eluted in this SPE method are shown in Table 1.

2.5. Thin layer chromatography

The effectiveness of the SPE methods studied to separate PL classes was visually checked by following the TLC method described by Ramadan and Mörsel (2003). After evaporation of solvents under nitrogen, each SPE eluted fraction was dissolved in 50 μl of chloroform:methanol (2:1). TLC plates were divided into eight lanes and were activated at 120 °C for 2 h immediately before use. Each PL standard (15 μl) dissolved in chloroform:methanol (2:1) at 5 μg μl^{-1} was loaded onto lanes 1–4 (PC, PE, PS and PI, respectively). Sequentially eluted SPE fractions

Table 1
Solvents, eluent proportions, eluent volumes and phospholipids eluted by Banni et al. (2001) method and by Banni et al. (2001) modified method

		Solvent	Proportion	Volume (ml)	Eluted PLs
Fraction 1	BM[a]	Acetonitrile:*n*-propanol	2:1	20	PC
	MM[b]	Acetonitrile:*n*-propanol	2:1	30	PC
Fraction 2	BM[a]	Methanol	Net	7	PE + PC
	MM[b]	Methanol	Net	10	PE
Fraction 3	BM[a]	Isopropanol:methanolic 3 N HCl	4:1	7.4	PS + PE
	MM[b]	Isopropanol:methanolic 3 N HCl	4:1	7.5	PS
Fraction 4	BM[a]	Methanol:methanolic 3 N HCl	9:1	10	–
	MM[b]	Chloroform:methanol:3 N HCl	(200:100:1)	17.5	PI

[a] BM = Banni et al. (2001) method.
[b] MM = Modified method.

were loaded onto lanes 5–8. Plates were developed with chloroform:methanol:25% ammonia solution (65/25/4; v/v/v), air-dried and visualised by spraying the plates with molybdenum blue spray (1.3% molybdenum oxide in 4.2 M sulfuric acid) from Sigma (St Louis, USA). The R_f values of the eluted fractions were compared with those of PL standards.

2.6. Isolation of phospholipids from biological samples

Lipid extracts from rat and chicken muscle were separated into lipid classes using aminopropyl minicolumns (500 mg), following the method described by Ruiz, Antequera, Andres, Petron, and Muriel (2004). Briefly, minicolumns were activated with 7.5 ml of hexane. Total lipids (20 mg), dissolved in 150 μl of hexane:chloroform:methanol (95:3:2; v/v/v), were loaded onto the column. Neutral lipids were eluted with 5 ml of chloroform and free fatty acids with 5 ml of diethyl ether:acetic acid (98:2; v/v). In this way, PLs were kept on the aminopropyl cartridge. Then, individual PL classes (PC, PE, PS and PI) were sequentially eluted using the improved Banni et al. (2001) method to check its effectiveness. Thereafter, PL classes were identified using the previously described TLC method.

3. Results and discussion

We have carried out an optimization of the SPE method of Banni et al. (2001) using a mixture of PL standards (PC, PE, PS and PI). Examination of TLC plates of eluted fractions obtained with the original SPE method for separating individual PL classes (Fig. 1) showed that the first eluted fraction (fraction 1) was constituted only of PC. However, the second eluted fraction obtained (fraction 2) was constituted of both PE and PC, as can be observed in lane 6 of the TLC plate (Fig. 1). This indicates the co-elution of these two PLs. Similarly, lane 7 of the TLC plate showed two spots with R_f values corresponding to PS and PE, indicating the cross-contamination of these two PLs in the third eluted fraction obtained (fraction 3). Finally, lane 8 of the TLC, in which the fourth eluted fraction (fraction 4) was loaded, did not show any spot, indicating that the solvent used for eluting the PI in the Banni et al. (2001) method (methanol:methanolic 3 N HCl 9:1; v/v) was not able to elute such a PL class.

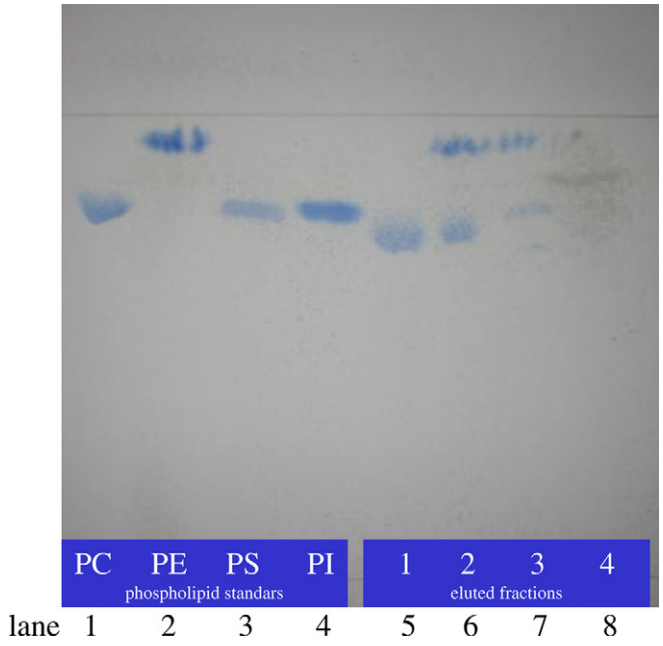

Fig. 1. TLC plate of eluted PL fractions from the mixture of PL obtained following the Banni et al. (2001) method. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI).

Due to the problems found in analysing PL classes by the Banni et al. (2001) method, summarised in the co-elution of PE and PC, and that of PS and PE and the retention of PI in the aminopropyl minicolumn, several modifications were carried out in order to obtain a correct PL classes fractionation.

Using the Banni et al. (2001) method, part of the PC was not eluted in the fraction 1, and was thereafter eluted in the second fraction, which should contain only PE. For the elution step, the objective is to elute the analytes in as small as possible volume. However, enough volume of solvent for eluting the whole amount of the analyte retained on the column is needed. Thus, when analytes are co-eluted, a higher volume of solvent might be necessary. Taking this into consideration, we tried to correctly separate PC and PE from the aminopropyl minicolumn by increasing the volume of the eluting solvent. The efficacy of increased volumes (22.5, 25, 27.5, 30 ml) of the solvent, used in the Banni et al. (2001) method (acetonitrile:*n*-propanol 2:1; v/v), in eluting the whole of the PC, were checked. Finally, by using 30 ml of the proposed solvent mixture, no PC spot was evidenced in lane 6 of the TLC plate (Fig. 2), corresponding to the second fraction eluted by the modified SPE method, and only one spot corresponding to the PE was observed from such fraction.

The third eluted fraction, using the Banni et al. (2001) method, showed a co-elution of PE and PS, indicating that the solvent (7 ml of methanol) used for PE elution (fraction 2) was not able to elute all of the PE retained in the aminopropyl cartridge. Different volumes of methanol (10 and 12.5 ml) were tested and with both the PE spot was not present in the third fraction obtained (that should only contain PS), indicating that the lower volume (10 ml) was enough for eluting all the PE. The volume of isopropanol:methanolic 3 N HCl (4:1; v/v) used for eluting PS was adjusted to 7.5 ml for practical purposes, with no effect on the subsequent elution of PI.

Pietsch and Reinhard (1993) and Banni et al. (2001) were able to correctly elute PI. However, using the same mixture of solvents (methanol:methanolic 3 N HCl 9:1; v/v) as did these authors, PI was not present in the fourth fraction of our study. Difficulties in the elution of acidic PLs such as PI have also been reported by other authors (Kim & Salem, 1990; Rizov & Doulis, 2001). In fact, Bateman and Jenkins

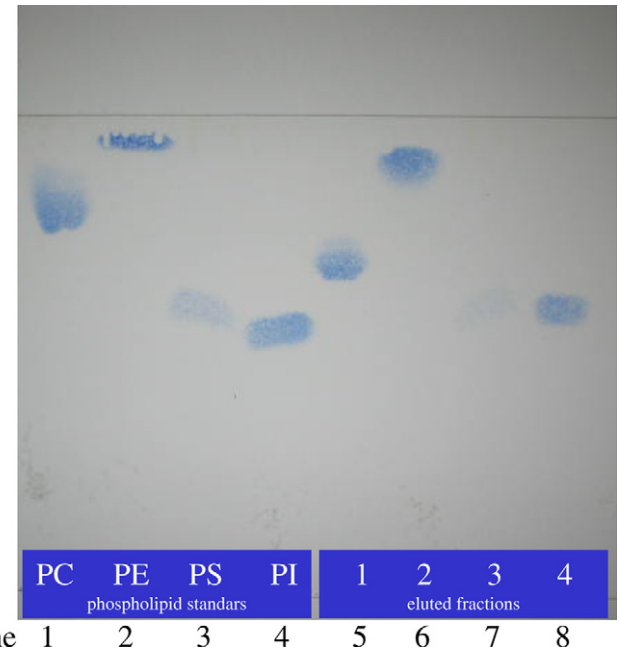

Fig. 2. TLC plate of eluted PL fractions from the mixture of PLs obtained following the Banni et al. (2001) modified method. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI).

(1997) reported only 39% of theoretical recovery of PLs when eluting with methanol, while the rest remained attached to the column, most likely due to their high acidity.

In order to extract PI from minicolumns, we tried first with increasing volumes of the proposed mixture of solvents (methanol:methanolic 3 N HCl 9:1; v/v) (12.5, 15, 17.5 and 20 ml), but none led to the elution of PI (TLC plates not shown).

The cartridges used in this study, NH_2-aminopropyl, are weak anion-exchange phases. Their primary retention mechanism is mainly based on the electrostatic attraction, retaining negatively charged or anionic compounds. Secondary interaction depends on the polarity of the compounds, those compounds showing higher polarity being more strongly retained than less polar ones. These secondary interactions can cause incomplete elution. Therefore, the more polar the functional groups contained in the PL, the more strongly is the PL adsorbed into the stationary phase (Christie, 1982). PI contains many hydroxyl groups (Lehninger, 1995), and this could lead to a strong retention by the aminopropyl minicolumns. Thus, both the acidity and the polarity of the eluting mixture of solvents influence the correct extraction of the PL molecules from the stationary phase.

The acidity of the solvent used by Banni et al. (2001) might be insufficient to break the interactions between PI and the stationary phase. The elution strategy usually consists in incorporating an acid or a base in the eluting solution to convert the analytes to their molecular, nonionic form (Fritz, 1999). In fact, Kim and Salem (1990) observed that the elution of PI could be achieved by adding phosphoric acid (5%) to a mixture of solvents, and did not show elution of such acidic PL without this acid in the mixture of the mobile phase. Previous investigations (Suzuki, Sano, Kuriki, & Miki, 1997) have shown that the elution of acidic glycerophospholipids from the NH_2-phase cartridges might be affected by the pH and salt concentration of the eluent. These authors tested a wide range of pH and obtained a correct elution of phosphatidylglycerol, PS and cardiolipin, but PI was always retained in the cartridge. Sufficient volume of the HCl in methanol solution must be used in order to neutralize all the hydroxide ions on the ion exchange SPE (Fritz, 1999). If insufficient HCl is used, the remaining active groups will retain the PLs ions farther down the column. In fact, Pietsch and Reinhard (1993) found that PI could be eluted from the aminopropyl matrix with methanol/methanolic HCl (9:1, v/v).

On the other hand, the polarity of the solvent used in the Banni et al. (2001) method (methanol:methanolic 3 N HCl 9:1; v/v), might also be inadequate for elution of PI from the NH_2-phase cartridge. In this sense, several researchers have found inadequate elution of the acidic PLs with methanol (Bateman & Jenkins, 1997; Kim & Salem, 1990; Pinkart, Devereux, & Chapman, 1998). By using chloroform:methanol (1:6), followed by an eluting solvent of 0.05 M sodium acetate in chloroform:methanol (1:6), the average recovery of PC and PE increased with respect to that obtained using methanol (Pinkart et al., 1998).

Taking the previous discussion into consideration, we tried to correctly elute the PI from the aminopropyl minicolumn with a more acid and less polar mixture of solvents than that used in the Banni et al. (2001). Such a mixture was constituted of chloroform:methanol:37% HCl and it was tested for different proportions of solvents and different volumes. The mixture of solvents in the proportion (200:100:5) produced degradation of the stationary phase, which was collected with the eluting solvents and with elution products in the test tubes. This type of silica-based phase has a stable pH range of 2–7.5. At pH levels above and below this range, the bonded phase can be hydrolyzed and cleaved off the silica surface, or the silica itself can dissolve. The pH of the solution tested was below 2, explaining the instability of the cartridge. TLC lanes where this eluted fraction was loaded onto plates did not show any PL, but a light blue stain was observed in the place where this fraction was applied. It might be that the high acidity of the mixture of solvents employed (chloroform:methanol:37% HCl, 200:100:5) could hydrolyze the inositol or the phosphate group from the phosphatidylinositol molecule. The hydrolyzed products are different from PI, and thus, their R_f should also be different. The other possible reason could be that the degraded stationary phase might interfere with the normal run of PI in the TLC plates. Anyway, due to these results, the acidity of this mixture of solvents was reduced by using less HCl. Thus, different volumes of a mixture of chloroform:methanol:37% HCl (200:100:1) were tested. By using 10 and 15 ml of this mixture PI was not eluted (TLC plates not shown). Use of

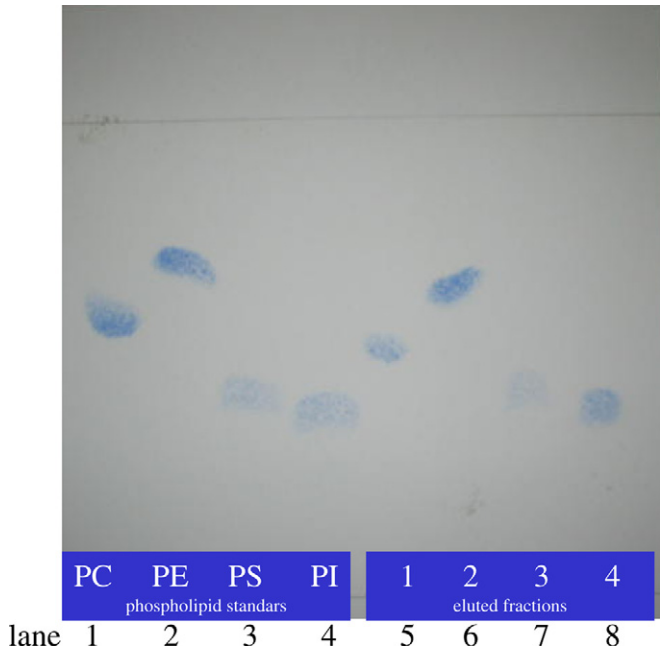

Fig. 3. TLC plate of eluted PL fractions from the *Longissimus dorsi* muscle of rats obtained following the Banni et al. (2001) modified method. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI).

20 ml of this mixture also caused the degradation of the stationary phase, and TLC lanes in which this eluted fraction was loaded also showed a blue stain with a R_f lower than that of PI. Both the high volume and the low pH of the mixture of solvents might lead to the stationary phase degradation, which could again interfere with PI run on TLC plates.

A lower volume of chloroform:methanol:37% HCl (200:100:1) (17.5 ml) was tested and PI was correctly eluted. Fig. 2 shows TLC plates in which fractions eluted using the modified SPE method of Banni et al. (2001) were loaded. As can be observed, all PLs included in the mixture (PC, PE, PS and PI) were separately eluted in their respective fractions. Validation of this SPE method was carried out using biological samples (*Longissimus dorsi* from rat and *Pectoralis major* from chicken). The eluted fractions were also analysed by TLC, showing that fractions 1, 2, 3 and 4 were constituted of PC, PE, PS and PI, respectively. TLC plates from rat and chicken muscle were similar, and only that from rat muscle is shown in Fig. 3.

It can be concluded that the modifications proposed for the Banni et al. (2001) SPE method for separation of PL classes allow the correct elution of PC, PE, PS and PI using both standards of PLs and muscle samples.

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