Journal of Chromatography A, 1218 (2011) 3453–3458

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

A new high-performance liquid chromatographic method with evaporative light scattering detector for the analysis of phospholipids. Application to Iberian pig subcutaneous fat

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ARTICLE INFO

Article history:
Received 17 January 2011
Received in revised form 24 March 2011
Accepted 27 March 2011
Available online 4 April 2011

Keywords:
Iberian pig subcutaneous fat
Phospholipids
SPE
HPLC
Evaporative light scattering detector
HPLC–MS

ABSTRACT

A new method for the analysis of phospholipids by normal-phase HPLC is described using a silica column. Addition of ammonia and triethylamine to a gradient based on chloroform/methanol/water promoted a good and rapid separation of phospholipid classes (20 min run). The use of an evaporative light scattering detector permitted an accurate analysis of a mixture of phospholipids. Calibration curves were linear within different range for each phospholipid class. The LOD and LOQ obtained were below 0.03 and 0.05 mg kg^{-1} for all cases, respectively. Besides, a new method for the separation of phospholipids from total lipids before HPLC analysis by a solid-phase extraction (SPE) with Si cartridges has been developed. This methodology gave a good recovery ranging from 97 to 117%. The method was validated with a standard mixture of phospholipids. This method has been applied to characterize the phospholipid fraction of subcutaneous fat from Iberian pig. Cardiolipin, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and sphingomyelin have been described for first time in these samples. The fatty acid composition of the different phospholipid classes and their HPLC electrospray ionization mass spectrometry have been used for characterizing the molecular species present in each one.

1. Introduction

The lipid composition of pig is very important due to the dry-cured products from these animals have a great economic importance in many countries since they are highly appreciate by consumers. Phospholipids have a significant role in the development of the flavor of meat products, like in the case of the Iberian dry-cured ham, since they are considered some of the main precursors of lipid-oxidation products [1,2] due to their fatty acids have a high degree of unsaturation [2,3]. Besides, some of them that contain nitrogenous in their molecules, such as phosphatidylethanolamine, give products of Strecker degradations and Maillard reactions [4], which are very significant for the acceptance of the product by consumers. It has been demonstrated that total phospholipids content and fatty acid content of the individual phospholipids are significantly correlated with differences in the flavor characteristics [5].

Several studies about phospholipids of pigs have been done and interesting results have been found. Cava et al. [6] investigated the effects of the finishing diet on the fatty acid profile of the intramuscular phospholipids fraction of Iberian pig muscle, finding that phospholipids from RE (fed on acorns and a commercial diet) and CE (fed on a commercial diet) pigs contained more C18:2 and C20:4 and less C18:1 than MO (fed on acorns) pigs. During the processing of French dry-cured hams, Buscailhon et al. [7] observed that phospholipids amount decreased while free fatty acid amount increased and that the fatty acid composition of both fraction were similar, which supports the hypothesis that free fatty acids come mostly from phospholipids hydrolysis. In addition, the results of their study indicate that phospholipids degradation during this process is mainly to hydrolysis and that direct oxidation of phospholipids fatty acids without previous hydrolysis is probably a minor phenomenon. The decrease of the amount of intramuscular PLs during the dry-cured process of Teruel ham has been also observed [3]. On the other hand, Muriel et al. [8] concluded that Iberian pig muscle phospholipids show different compositions in the sn-1 and the sn-2 position, the first one preferentially occupied by fatty alcohols and saturated fatty acids and the second one by polyunsaturated fatty acids.

Nowadays, the method chosen for the separation and determination of phospholipids classes is the high performance liquid chromatography (HPLC). Silica columns are the most commonly used for this purposed since silica is relatively inexpensive and robust, and it can give excellent results. However, amino

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doi:10.1016/j.chroma.2011.03.067

columns have been used in some works [9,10], but they are not widely used because they need to be periodically regenerated with ammonia solutions. The UV and the evaporative light scattering (ELSD) detectors are the most used in the study of phospholipids classes. Nevertheless, the quantification is difficult using the UV detector due to the response of this is dependent on the degree of unsaturation in the molecule. ELSD gave a stable flat baseline, is very sensitive [11] and its response, in contrast to a UV detector, is independent of the number of double bonds in the molecule. Respect to the mobile phases used, many of them have been used with silica columns. An isocratic eluent has been rarely used [12,13]. The following binary gradient has been employed by several authors [14,15]: (A) chloroform–methanol–ammonium hydroxide (80:19.5:0.5, v/v) and (B) chloroform–methanol–water–ammonium hydroxide (60:34:5.5:0.5, v/v). Boselli et al. [16] used this same gradient but with other proportions, (70:25:1, v/v) and (60:40:5.5:0.5, v/v) respectively. The elution carried out by Brouwers et al. [17] to separate the phospholipids classes, was performed by a gradient of hexane–isopropanol–dichloromethane (40:48:12, v/v) to hexane–isopropanol–dichloromethane–water (40:42:8:8, v/v). The optimum conditions for Melton [18] to resolve phospholipids of soybean and beef, were from isooctane–tetrahydrofuran–isopropanol–chloroform–water (41.5:0.5:44.6:10.4:3, v/v) to (21.6:0.4:54.6:15.4:8, v/v). Gradients of elution more complicated with three or four eluents were carried out by Wang et al. [19] and Descalzo et al. [20], respectively.

In the literature mentioned above, it can be observed that different mobile phases have been used to separate the different phospholipid classes. Nevertheless, unfortunately not all phases have the same effectiveness for the different types of samples and it can observe that the composition of the mobile phase can become critical for each type of matrix. The runtime in these methods are between 15 and 70 min. For runtimes less than 20 min, lysophosphatidylcholine (LPC) is not eluted [14,15], which makes the method invalid for samples that contain this phospholipid class. Besides, the recovery can be low according to the phase used in solid-phase extraction (SPE) [14] to isolate the PLs and the best results are obtained when amino phase columns are employed [15]. However, cardiolipin (CL) is not eluted in these techniques [14,15]. Concerning to quantification, only a few authors find lineal response [14,18,20], with a range of 5–40 μg [20] and values up to 250 μg depending on PL class [18]. Others authors apply a quadratic or power equation for calibration arguing a better correlation for low concentrations [14,15].

The aim of this work is to develop an analytical method for isolation, resolution and quantification of phospholipid classes, applicable to different types of matrices, which shows a total recovery of all PL classes, a good linear correlation for low levels of concentration and an adequate resolution for a short runtime that allows the recovery of the different PL classes for a subsequent study of molecular species of each one of them. This method has been applied to characterize the phospholipids from Iberian pig subcutaneous fat.

2. Experimental

2.1. Samples

Raw samples of Iberian pig subcutaneous fat were used. The lipids were obtained from 1.5 grams of each sample by extraction with chloroform–methanol (2:1, v/v) according to the procedure described by Folch et al. [21]. The samples were cut up into small pieces and homogenized before extraction with 3 × 10 mL of solvent using an agitator. The solution was filtered and evaporated to dryness in a rotary evaporator at 30 °C under reduced pressure.

2.2. Reagents and standards

Hexane, fraction from petroleum, Multisolvent TM HPLC ACS grade supplied by Scharlau (Barcelona, Spain) was distilled through a fractionation column. Chloroform, methanol, diethyl ether, ammonia solution and 2-propanol for analysis grade were provided by Merck (Darmstadt, Germany). Phospholipids standard solution (ref: P3817-1VL) containing phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine and lysophosphatidylcholine was purchased from Supelco (Bellefonte, PA, USA). Triethylamine, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CL) and sphingomyelin (SPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were used as standard. Chloroform and methanol, both LiChrosolv grade, were supplied by Merck (Darmstadt, Germany) and were used as HPLC solvents. All other materials were analytical grade.

2.3. Phospholipids purification

The phospholipids fraction was separated from subcutaneous lipids by means of Solid Phase Extraction (SPE). 200 mg of the lipid extract was dissolved in 1.0 mL of hexane and was transferred into a silica gel bonded column, Supelclean LC-Si, 6 mL volume; 1 g sorbent (Supelco Bellefonte, PA, USA) which was conditioned with 10 mL n-hexane previously. The column was washed with 10 mL n-hexane:diethyl ether (4:1, v/v), to remove free fatty acids, and then, hydrocarbons, cholesterol esters and triacylglycerols were eluted with 18 mL chloroform: 2-propanol (2:1, v/v). Finally, phospholipids were recovered with 10 mL of methanol and afterwards 10 mL of methanol:chloroform:water (5:3:2, v/v/v). The recovered fraction was evaporated to dryness in a rotary evaporator at 30 °C under reduced pressure and the residue was re-dissolved in 0.2 mL of chloroform for HPLC analysis.

2.4. HPLC instrumental analysis

The different phospholipid classes were analyzed by HPLC. The HPLC system consisted of an Agilent (Palo Alto, CA, USA) 1100 liquid chromatograph, with a quaternary pump, an autosampler with variable injection volume (0–100 μL), a Peltier furnace and an evaporative light scattering detector Alltech ELSD 2000 (Deerfield, IL, USA). A Chemical station HP was used for controlling and monitoring the system. The separation was performed in a 100 mm × 4.6 mm macropores size 2.1 μm and mesopores size 13 nm Chromolith Performance Si column Merck (Darmstadt, Germany). The temperature of the column was held at 25 °C. A gradient elution was carried out using different ratios of solutions A (chloroform:methanol:ammonia solution, 80:19.5:0.5, v/v/v) and B (chloroform:methanol:triethylamine:water, 69.53:25.58:0.49:4.40, v/v/v/v). A better separation was obtained using the following gradient: from 0 to 5 min, B was increased from 0% to 40%; from 5 to 7 min B was kept constant at 40%; from 7 to 13 min B was increased from 40% to 100%; from 13 to 20 min B was kept constant at 100%; from 20 to 25 min B was decreased from 100% to 0%; a time post-run of 5 min was done to equilibrate the column before the next injection. The flow rate was supported at 1 mL min^{-1} during 25 min and the injection volume was 50 μL. The evaporative light scattering detector used nitrogen as nebulising gas. The detector conditions were optimized by triplicate injection of 5 μL of Phospholipids standard solution from Supelco (ref: P3817-1VL). The maximum value of area mean of the tree injections was used for the combination of five different

gas flow (1.4, 1.5, 1.7, 1.8 and 2.0 L min^{-1}) with different drift tube temperatures (60–120 °C in 10 °C steps). The optimal conditions were at 1.5 L min^{-1} and 110 °C.

The peak area of each phospholipid was used as analytical signal. The quantification for each different phospholipid classes was carried out by a calibration curve for each phospholipid class injecting standard solutions of the different species identified.

A Valco Instruments Co. Inc. (Bandera, TX, USA) valve model VT-E90 was installed before the input of the detector to recovery the different phospholipid classes.

2.5. HPLC–MS instrumental analysis

The HPLC analyses of total phospholipids extracts were performed in a Beckman Gold system using a 126 pump with a 168 diode array detector (Beckman, Inc., USA) on-line with a MAT95ïs magnetic sector mass spectrometer (Finnigan Mat, Bremen, Germany) equipped with an ESI ionization interface. A 4.6 mm i.d. × 250 mm; particle size 5 μm Lichrospher Si 60 column (Merck, Darmstat, Germany), maintained at 25 °C, and a Rheodyne injection valve (50 μL loop) were used. Elution was performed at flow rate of 1.0 mL min^{-1}, using a gradient elution as described in HPLC instrumental analysis section. A split postcolumn of a 25% of the column flow was introduced in the ESI interface.

The ESI mass spectra, in the negative-ion mode, were obtained under the following conditions: capillary temperature of 220 °C; lens, skimmer, and octapole voltages were set to get optimal response for a pattern solution of sodium trifluoroacetate. Sprayer voltage used was 4 kV. Nitrogen at 150 kPa was used as sheath gas. Mass range was set from 500 to 2000 amu at scan range of 5 s/decade.

The use of a high concentration of chloroform on mobile phase makes to obtain a stable electrospray in the negative ion mode in the first part of our analysis difficult due to his volatility. Quality is highly dependent on the bulk properties of the solution that is analyzed, of which, the surface tension and conductivity are the most important [22,23]. A poor quality electrospray may content larger droplets or none dispersed droplet stream and, as it is well known, chloroform is a non adequate solvent to being used in electrospray. The result was an unstable electrospray with no signal in the first part of chromatogram until a higher concentration of water in mobile phase reaches the electrospray sprayer.

In order to provide a good electrical contact and an adequate surface tension from the beginning of analysis, an additional more polar solvent was added across the sheath liquid input of the electrospray interface. A solution of 0.1% formic acid in water was introduced at a flow rate of 40 μL min^{-1} and maintained throughout the analysis time. In this way, addition of sheath liquid to the column effluent allowed the detection of phospholipids by negative electrospray ionization mass spectrometry using full-scan data acquisition with sensitivity comparable to light scattering detection.

2.6. Fatty acids analysis

Fatty acid methyl esters (FAMEs) of total and phospholipids fraction were analyzed by GC. FAMEs were extracted with n-hexane after cold methylation with 2 M KOH in methanol, following a method previously described [24]. GC was performed with a Varian 3900 apparatus (Varian Co, Palo Alto, CA, USA) equipped with a split/splitless injector and a flame ionisation detector; a fused silica capillary HP 88 column (100 m × 0.25 mm, 0.25 μm film thickness) and a VARIAN 8100 automatic injector were used. The oven temperature was kept at 175 °C for 13 min and was then raised to 205 °C at a rate of 3.0 °C/min and held isothermally for 5.0 min. The injector temperature was kept at 240 °C, while the detector temperature was 250 °C. Hydrogen was used as carrier gas. A constant head pressure of 0.172 MPa and a split ratio of 1:60 were used for total FAMEs analysis and 1:5 for phospholipids fraction FAMEs. Air and hydrogen with flow rates of 300 and 30 mL min^{-1}, respectively, were employed for the detector, which had an auxiliary flow of 25 mL min^{-1} of nitrogen.

2.7. Quantitative and recovery analysis

Aliquots of a blank subcutaneous fat sample of Iberian pig, free of phospholipids, were spiked at ten different concentration levels to obtain the matrix matched calibration curves, which have been used for phospholipids quantification.

In order to study the recovery and reproducibility (R) of the present method, a complementary experiment has been carried out. Recovery data were calculated comparing the results of phospholipids determination added on a blank subcutaneous fat matrix with those obtained from the HPLC direct analysis of phospholipid standard, both being analyzed in the same way. Six replicates were determined in each case. For the determination of repeatability, the replicates were done on different days and in the same laboratory.

The limit of detection (LOD) of the method was determined considering a signal to noise ratio of 3 with reference to the background noise obtained from blank sample; limit of quantification (LOQ) was determined considering a signal to noise ratio of 10 [25]. Six independent determinations of phospholipids were made using subcutaneous fat of Iberian pig blank samples.

3. Results and discussion

3.1. Identification of components

A HPLC-ELSD chromatogram of phospholipid standards solution is showed in Fig. 1A. The relative retention times (Trr) referred to phosphatidylinositol are show in Table 1. Fig. 1B shows the HPLC-ELSD profiles of the phospholipid fractions purified by the SPE Si columns from subcutaneous fat of Iberian pig samples. As it can be observed in this figure, a good separation of the different classes of phospholipids is obtained. In relation to mobile phases, both the component B of the binary mixture and the gradient used are totally new. The use of this new gradient with the column mentioned in HPLC instrumental analysis section (length of 100 mm) allows obtaining a total and excellent separation in a runtime less than 18 min, since the wide of peaks is smaller than that indicated in references [14,15], in which 1 or 2 columns of 250 mm are used. This permits the use of our method as separation technique for a subsequent study of molecular species of each one of the PL classes or their fatty acid composition.

At the beginning of the phospholipid chromatogram (Fig. 1B), there is a high peak corresponding to free fatty acids according to previously described in literature [16]. As it can be appreciate in Fig. 1B, the peak corresponding to the PE shows a broadening due to an incipient separation of other phospholipid class or different molecular species of PE. However, we do not believe this last probably, since such separation is not observed in the standard chromatogram (Fig. 1A). On the contrary, the existence of other class of phospholipids, plasmalogen-PE (pPE) has been confirmed by other authors [16] in pork meat phospholipid fraction.

The main phospholipid classes in subcutaneous fat from Iberian pig are CL, PC and PE + pPE, as has been described in the intramuscular fat of pigs [26]. Also a clear separation of two molecular species of SPH was obtained, which is observed in both chromatograms (Fig. 1A and B). It can be remarked that LPC does not exist in phospholipid fraction from subcutaneous fat of

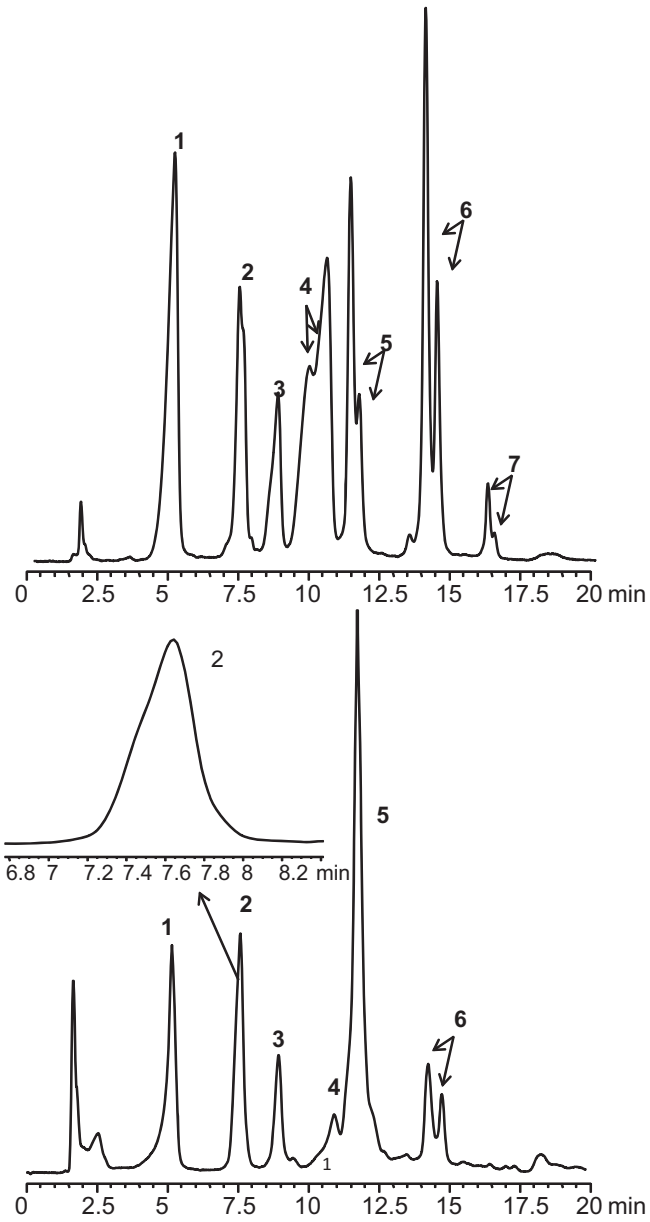

Fig. 1. HPLC chromatogram of phospholipids fraction (A) from standard soluction; (B) from subcutaneous fat of Iberian pig. 1: Cardiolipin; 2: phosphatidylethanolamine; 3: phosphatidylinositol; 4: phosphatidylserine; 5: phosphatidylcholine; 6: sphingomyelin; 7: lysophosphatidylcholine.

Table 1
Molecular species of phospholipids in Iberian pig subcutaneous fat.

Trr	Compound	$[M-H]^-$	Fatty acids[a]
0.58	Cardiolipin	1424	C16:0/3C18:2
		1446	3C18:2/C18:3
		1448	4 C18:2
		1450	3C18:3/C18:1
		1452	3C18:2/C18:0
0.84	Phosphatidylethanolamine	717	C16:0/C18:1
		719	C16:0/C18:0
		745	C18:0/C18:1
		743	C18:1/C18:1
		767	C16:0/C22:6
		791	C18:2/C22:6
		793	C18:1C22:6
1.00	Phosphatidylinositol	835	C16:0/C18:1
		837	C16:0/C18:0
		857	C18:2/C18:2
		859	C18:1/C18:2
		861	C18:1/C18:1
		863	C18:0/C18:1
		885	C16:0/C22:6
1.13–1.20	Phosphatidylserine	761	C16:0/C18:1
		763	C16:0/C18:0
		785	C18:1/C18:2
		787	C18:1/C18:1 and/or C18:0/C18:2
		789	C18:0/C18:1
		791	C18:0/C18:0
		821	C18:0/C22:6
1.30–1.33	Phosphatidylcholine	761	C16:0/C18:0
		785	C18:1/C18:1 and/or C18:0/C18:2
		787	C18:0/C18:1
		789	C18:0/C18:0
		833	C18:0/C22:6
1.60–1.65	Sphingomyelin	699	C16:1
		701	C16:0
		725	C18:2
		727	C18:1
		729	C18:0
		773	C22:6
1.86–1.89	Lysophosphatidylcholine		

[a] Tentative.

Iberian pig, although other authors have detected this in pork meat [16].

In order to confirm the different molecular species, each phospholipid class was recovered through VALCO valve as it has been described in HPLC instrumental analysis section and dried in the rotary evaporator at 30 °C under reduced pressure. A FAME analysis by GC of each phospholipid class was done. The recovery times were: from the minute 3.0 to 5.0 for CL; from the minute 6.0 to 8.0 for PE; from the minute 8.0 to 9.0 for PI; from the minute 9.0 to 10.5 for PS; from the minute 10.5 to 12.5 for PC; from the minute 13.5 to 15.0 for SPH.

In Table 2, the composition of fatty acids for the total fraction, phospholipids fraction and each phospholipid class is shown. In this table, it can be observed that the main fatty acids present in total lipid and phospholipid fraction are C16:0, C18:0, C18:1 and C18:2, which are in accordance with the literature [6]. However, MUFAs show a higher level in total lipid fraction (particularly C18:1) in comparison with phospholipids. On the other hand, SFA level in phospholipids (47.89%) is higher than in total lipid fraction (29.74%), being this difference due to the high percentage of C18:0 (33.33%). In the case of PUFAs, this is the minority fraction in the total lipids (10.13%). Nevertheless, this fraction (26.12%) shows a value slightly higher than MUFA in phospholipids (25.99%). In this group of fatty acids, C18:2 is the most abundant in both lipid fractions (8.54% for total and 18.24% for phospholipids), being the others fatty acids at much less level in total lipids, which is opposite to what is observed in PUFA from phospholipid fraction where C22:6 (n-6) acid shows a value of 6.49%.

In the case of the different phospholipid classes (see Table 2), the SFA group is the most abundant in all of them (PE, 39.59%; PI, 54.04%; PS, 61.21%; PC, 48.25%; SPH, 50.38%) except in the case of CL, in which MUFAs present the highest level (58.93%). The majority acid for PE, PI, PS and PC is C18:0 (21.46, 37.65, 39.23 and 32.70%, respectively), whereas for CL and SPH is the C18:1 acid (55.40 and 36.18%). Noteworthy that the majority group for SPH is SFA (50.38%) although the fatty acid with the highest percentage in this phospholipid class is C18:1 (36.18%).

Table 2
Fatty acid compositions (in percentage) for the total fraction, phospholipids fraction and each phospholipid class from subcutaneous fat of Iberian pig samples.

Fatty acid	Total	PLs	CL	PE	PI	PS	PC	SPH
C14:0	1.16	0.50	1.16	1.92	1.19	1.72	0.92	3.82
C16:0	19.45	13.27	24.52	16.21	15.21	20.26	14.20	24.74
C16:1	1.86	0.96	1.60	5.53	2.02	1.59	1.22	3.85
C17:0	0.29	0.46	0.39	0.00	0.00	0.00	0.43	1.25
C17:1	0.26	0.11	0.28	0.00	0.00	0.00	0.00	0.00
C18:0	8.68	33.33	12.72	21.46	37.65	39.23	32.70	20.57
C18:1	56.49	24.12	55.40	20.54	18.19	22.51	23.47	36.18
C18:2	8.54	18.24	1.58	12.42	9.98	8.91	22.44	4.55
C20:0	0.15	0.34	0.25	0.00	0.00	0.00	0.00	0.00
C18:3	0.64	0.26	0.03	0.00	0.00	0.00	0.22	0.00
C20:1	1.53	0.80	1.66	0.00	0.00	0.00	0.53	1.37
C20:4	0.63	0.66	0.11	0.00	0.00	0.00	0.53	0.00
C22:5(n-5)	0.20	0.10	0.01	0.00	0.00	0.00	0.00	0.00
C22:6(n-6)	0.11	6.49	0.06	9.76	13.60	3.09	2.63	0.00
C22:6(n-3)	0.01	0.38	0.23	12.16	2.16	2.69	0.71	3.66
SFA	29.74	47.89	39.05	39.59	54.04	61.21	48.25	50.38
MUFA	60.14	25.99	58.93	26.07	20.21	24.10	25.22	41.40
PUFA	10.13	26.12	2.02	34.34	25.75	14.70	26.53	8.21

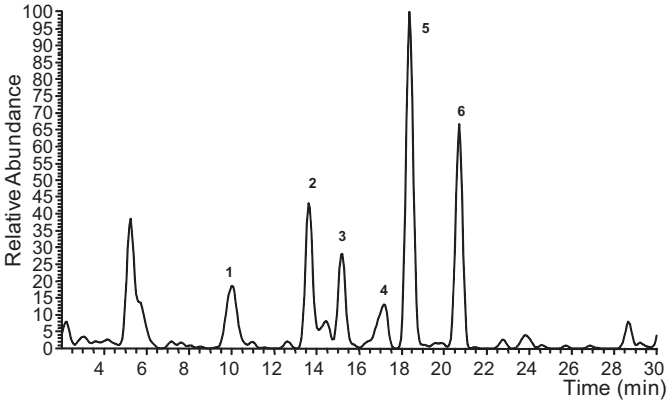

Fig. 2. Reconstructed on-line HPLC–ESI–MS chromatogram of total phospholipids fraction present in subcutaneous fat of Iberian pig: 1: cardiolipin; 2: phosphatidylethanolamine + phosphatidylethanolamine plasmalogen; 3: phosphatidylinositol; 4: phosphatidylserine; 5: phosphatidylcholine; 6: sphingomyelin.

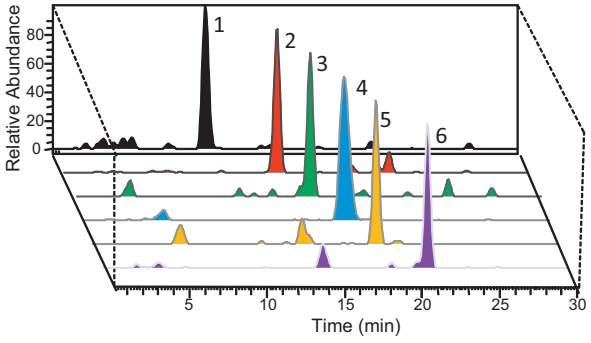

Fig. 3. Overlay (3D) representation of detected molecular species on a sample of total phospholipid fraction from subcutaneous fat of Iberian pig. 1: cardiolipin; 2: phosphatidylethanolamine + phosphatidylethanolamine plasmalogen; 3: phosphatidylinositol; 4: phosphatidylserine; 5: phosphatidylcholine; 6: sphingomyelin.

Table 3
Different calculated coefficients and correlations (R^2) for the linear equation area = slope × [mg kg^{-1}] + intercept corresponding to calibration curves for each phospholipids class.

Phospholipid	Slope	Intercept	R^2
CL	50,902	−382.31	0.9997
PE	66,038	−440.08	0.9990
PI	62,990	−490.19	0.9980
PS	64,236	−1023.1	0.9992
PC	64,610	−316.89	0.9995
SPH	56,818	−390.65	0.9992
LPC	54,920	−236.61	0.996

It can be observed that CL and SPH present a small percentage in PUFA compared to other phospholipid classes. The C22:6 acids are the ones that influence the PUFA percentage for PE and PI, being the (n-3) and (n-6) the most influential respectively. However, in the case of CL, PS, PC and SPH, the C18:2 acid is the polyunsaturated fatty acid most abundant. The facts that the C22:5(n-5) acid is nonexistent or almost nonexistent and that the C22:6(n-6) acid is present in all phospholipid classes except in SPH, should be noted.

The C17:0 and C22:6(n-6) acids have been detected in phospholipid fraction from Iberian pig subcutaneous fat, but these have not been found in the phospholipid fraction of pork meat and muscles [6,7,16]. Nevertheless, the C17:0 acid has been described in fresh loin from Iberian pig, which could be due to it contains fat [8]. Other thing that can be observed in Table 2 is that the C17:1 and C20:0 acids are only presents in CL, being besides the C22:5 (n-5) acid in traces.

On the other hand, the HPLC–MS technique has been also used to complement the previous fatty acid study to identify the possible different molecular species present in each phospholipid class. In the conditions described before, a HPLC–MS chromatogram report of phospholipid fraction from subcutaneous fat of Iberian pig in TIC (total ion current) mode is obtained as it is shown in Fig. 2.

Table 4
Evaluation of the recovery with the SPE-HPLC method. HPLC peak areas of different phospholipids obtained by direct injection and by isolation from a mixture with Iberian pig subcutaneous fat.

(n = 6)	(SPE-HPLC analysis)						(Direct analysis)					
	CL	PE	PI	PC	SPH	LPC	CL	PE	PI	PC	SPH	LPC
Mean	266,53	173,83	123,89	410,96	264,79	2945	275,73	163,71	106,16	391,13	234,09	2916
S.D.	2755	1056	641	2207	2093	147	218	290	207	695	508	15
R.S.D. (%)	10.34	6.08	5.18	5.37	7.91	5.00	0.79	1.77	1.95	1.78	2.17	0.52
Recovery	97%	106%	117%	105%	113%	101%						

Table 5
Repeatability, LOD and LOQ data of phospholipids analysis by SPE-HPLC of an Iberian pig subcutaneous fat sample using a new column each time.

(n = 6)	(SPE-HPLC analysis)						
	CL	PE	PI	PS	PC	SPH	LPC
Mean	0.493	0.255	0.187	0.641	0.339	0.531	0.060
S.D.	0.051	0.015	0.010	0.034	0.027	0.039	0.003
R.S.D. (%)	10.34	6.08	5.18	5.37	7.91	7.26	5.00
LOD[a]	0.017	0.016	0.019	0.039	0.040	0.021	0.009
LOQ[a]	0.031	0.030	0.035	0.074	0.075	0.043	0.017

[a] mg kg^{-1}.

The phospholipid species were tentatively confirmed based on their characteristic m/z value group for each molecular species based on fatty acid composition obtained by previously individual hydrolysis of them and retention time of reference compounds previously injected.

A phospholipid fraction was injected and the ion chromatograms reconstructed were obtained based on molecular m/z value for each phospholipid molecular species.

Fig. 3 shows the chromatogram reconstructed containing traces of deprotonated ions ($[M-H]^-$) of each molecular specie (Table 1) using the mass range for the possible fatty acid composition that is shown in Table 2.

The analysis of molecular species was carried out doing a separation by HPLC of the different phospholipids. Subsequent, the different peaks were collected and their corresponding methyl esters were analyzed by GC, as previously indicated in Section 2.6.

The molecular species present in each class of phospholipid were tentatively identified based on the presence of the corresponding ions $[M-H]^-$ (See Table 1).

3.2. Validation of the method

For each phospholipids class, the calibration curves has been done and the corresponding range of linearity. Each curve was prepared and injected in triplicate. The different calculated equations (area = slope × [mg kg^{-1}] + intercept) are presented in Table 3, and it can be observed that a good correlation ($R^2 > 0.99$) was obtained in all cases for a linear fit. The respective peak areas fitted a linear model within the indicate range. Linearity was observed for CL from 0.025 to 0.816 mg kg^{-1}, for PE from 0.024 to 0.384 mg kg^{-1}, for PI from 0.032 to 0.256 mg kg^{-1}, for PS from 0.061 to 0.976 mg kg^{-1}, for PC from 0.030 to 0.360 mg kg^{-1}, for SPH from 0.035 to 0.680 mg kg^{-1} and for LPC from 0.010 to 0.060 mg kg^{-1}. The method shows an excellent linear correlation for a concentration range of 10^3 times lower than those indicated in some works [14,15]. Although the slope was similar for all phospholipid classes, the highest value of this was for PC and the lowest for CL. This is not in accordance with other authors [20] who reported that the slope for PE was approximately three times higher than the others.

The trueness was assessed based on recovery assays. In order to evaluate the recovery of SPE-HPLC method proposed mean area values of different phospholipids peak obtained by direct injection and by isolation from a mixture of subcutaneous fat of Iberian pig free of phospholipids with 0.510, 0.240, 0.160, 0.610, 0.300 and 0.486 mg kg^{-1} of CL, PE, PI, PS, PC and SPH respectively. Six replicates were performed and their corresponding recoveries which are shown in Table 4. It can be seen that in all cases the recoveries lie within the range of acceptable values according the analyte concentration [27] and accordingly, trueness is significant. This new SPE method presents a total recovery of all PL classes in contradistinction to the others methods described in literature [14,15]. The analytical technique has a good repeatability and the recovery is complete. This demonstrates that the separation obtained with SPE silica columns is adequate.

The repeatability of the method was studied using a subcutaneous fat of Iberian pig sample as described in Sections 2.6 and 3.2 and a new SPE column for each replicate. The results are shown in Table 4, there are not references in Literature. However, theses values of relative standard deviation are minor than the reference value derived from Horwitz equation [28] ($R.S.D._H = 16.4\%$). Therefore, the results for different phospholipid class indicate a good repeatability for the assay. The LOD and LOQ obtained are also shown in Table 5. The LOD obtained were between 0.010 and 0.026 mg kg^{-1} and LOQ ranged from 0.022 and 0.048 mg kg^{-1}. The lowest LOD and LOQ were for SPH and the highest for CL.

4. Conclusions

The new HPLC/ELSD method detailed in this report allows a rapid, sensitive and highly reproducible separation and quantification of the different classes of PLs, which have been described for the first time in subcutaneous fat from Iberian pigs. It would be good to stand out that the response of ELSD is linear for a wide range of concentrations. The new SPE methodology developed here permitted the isolation of the PLs present in this matrix from other lipids, with the advantage of a high recovery.

This method showed that the main PLs in these samples were CL, PC and PE+pPE. Besides a study of fatty acids of the each PL class has been done using FAMEs analysis and HPLC–MS technique.

Acknowledgement

The authors are grateful to the Designation of Origin "Los Pedroches" for the collaboration and given help. This study was supported by projects PET 2007_0015 and P08-AGR-03498.

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