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An LC method for the analysis of phosphatidylcholine hydrolysis products and its application to the monitoring of the acyl migration process

Grzegorz Kiełbowicz, Damian Smuga, Witold Gładkowski, Anna Chojnacka, Czesław Wawrzeńczyk*

Department of Chemistry, Wrocław University of Environmental and Life Sciences, Norwida 25 50-375 Wrocław, Poland

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ABSTRACT

An assay for quantitative analysis of phosphatidylcholine (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) and its hydrolysis products: 1-hydroxy-2-palmitoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, *sn*-glycero-3-phosphocholine and palmitic acid using high-performance liquid chromatography with charge aerosol detector (CAD) was developed. The separation of the compounds of interest was achieved on a reversed-phase/hydrophilic interaction stationary phase with a mobile phase consisting of acetonitrile:methanol:10 mM ammonium acetate solution. The method was applied to control the acyl migration process of LPC regioisomers in the most common solvents used in the synthesis or modification of PC.

1. Introduction

1,2-Diacyl-*sn*-glycero-3-phosphocholine (PC) is a major constituent of all biological membranes and plays a crucial role in the biochemistry of cells. It is used as a component of special dietetic products, food supplements [1,2] and, because of its high entrapment efficiency it is widely applied in the pharmaceutical and liposome industries. Properties of the PC depend mainly on the fatty acid (FA) composition. An increasing demand for PC with defined fatty acids composition motivated researchers to develop different synthesis or modification methods resulting in compounds with modified physicochemical and physiological properties [3,4].

Hydrolysis product formation *i.e.* 1-acyl-2-hydroxy-*sn*-glycero-3-phosphocholine (1-acyl LPC), 2-acyl-1-hydroxy-*sn*-glycero-3-phosphocholine (2-acyl LPC), *sn*-glycero-3-phosphocholine (GPC) and free fatty acids (FA) is a serious problem especially in the enzyme-catalyzed acyl exchange of PC. This process was also observed in an aqueous phospholipid liposome dispersion and limits the shelf life of liposome-based pharmaceuticals [5]. Determination of the hydrolysis products especially lysophospholipid regioisomers represents a long-standing problem in phospholipids chemistry. These compounds are formed due to parallel hydrolysis processes and acyl migration between 1-acyl LPC and 2-acyl LPC in the reaction system and leads to a decrease of process effectiveness [6,7].

In order to optimize the reaction conditions a methodology giving a complete picture of the modification or synthesis of the PC is very important. Only a few analytical methods in this area were presented. One of them is the NMR spectroscopy that has made a detailed analysis of acyl migration in lysophospholipids and quantitative determination of the hydrolysis products possible [7]. The main drawback of this method is that NMR methodology precludes its routine use in analyses of lysolipid isomers and therefore LC methods are most suitable. There have been numerous articles detailing methods of PC and LPC analysis with LC [8–11] but only a few of them present the resolution of the regioisomers of LPC [5,6,12–16].

The majority of published methods for separation of phospholipids use normal-phase LC (NP-LC) and a silica column. NP-LC provides separation of phospholipids only by class and is useless for regioisomers determination [9–11]. Monolithic silica gel columns are increasingly used; however only one application for the lipid separation has been reported [17]. The reversed-phase LC is mostly used for the resolution of the molecular species of a particular PL class [10] and some papers also present the possibility of the determination of LPC regioisomers [13]. The main problem concerning the separation of phospholipids using reversed-phase chromatography is their high hydrophobicity and the necessity of using highly non-polar mobile phases. The hydrophobic interaction can be so strong that this phenomenon was applied to modify a reversed-phase (RP8) column. The phospholipids from the liposomes adsorb

* Corresponding author. Tel.: +48 713205211; fax: +48 713284124.
E-mail address: czeslaw.wawrzenczyk@up.wroc.pl (C. Wawrzeńczyk).

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onto the octyl chain of the stationary phase, thus altering the nature of the stationary phase and of the chromatographic interactions [18]. To reduce retention of PLs a perfluorinated stationary bonded phase was used [12]. Hydrophilic interaction chromatography (HILIC) became increasingly popular for the separation of phospholipids. HILIC can be performed on a variety of silica-based or polymer stationary phases [19]. Separation of the phospholipid classes has been achieved on a cyano phase [20], diol phase [21] and amino phase [20]. From all of these phases the latter proved to be the most useful for LPC regioisomers [6] and other PC hydrolysis products separation [5].

A highly important aspect of phospholipids analysis is their detection. There are various detectors available for use in conjunction with LC [9,10]. Most commonly, natural phospholipids are monitored by low-wave-length UV detection [20,22,23]. Unfortunately conventional UV detection is often inadequate and limited to chromophores, and therefore saturated phospholipids yield low responses and may be under represented in the samples [10,24]. Mass spectrometry [13–15,25], refractive index (RID) [5,6] and evaporative light-scattering (ELSD) detectors [8,12,16] could be used. Mass spectrometry is considered to be a selective and universal detection method but, as the response depends on the ionization process, quantitative analysis using MS coupled with liquid chromatography is currently less robust [26,27]. The refractive index detector (RID) is the least sensitive of all the commonly used detectors. It is very sensitive to changes in ambient temperature, pressure changes and flow-rate changes; furthermore, it cannot be used for gradient elution [28]. Recently, a new alternative detection method based upon aerosol charging (charge aerosol detector – CAD) has been introduced [29]. It has been proven that CAD can provide greater sensitivity and better precision than other aerosol-based detectors such as ELSD [30]. Moreover, CAD is quite user-friendly since it does not require any optimization of operating parameters and therefore can be operated by any chromatographer without significant additional training [31].

Although numerous methodologies of the analysis of PC and LPC using different stationary phases and detection methods were elaborated, only one of them presented a full profile of the migration process and provides the possibility to analyze in one run not only regioisomers of LPC but also the products of the hydrolysis process in the *sn*-1 or *sn*-2 position of PC [5]. The separation of the phospholipids of interest was achieved on an amino phase column and a refractive index detector was used. Unfortunately the amino-phase columns belong to so-called bleeding columns. The self-decomposition of the stationary phase by amino groups in aqueous eluents was shown [32–34]. Due to the self-decomposition reaction, the use of amino-phase in HILIC–MS causes higher backgrounds [33,35]. The use of a bleeding column is also not recommended in the case of some detectors *i.e.* charge aerosol detector (CAD). Therefore it was decided to elaborate a new analytical methodology in this very demanding and important area.

The aim of the present study was to develop a new chromatographic method for LC-CAD, enabling the analysis of phosphatidylcholine and its hydrolysis products in a single run. The method was applied to control the acyl migration process between LPC regioisomers in the most common solvents used in PC synthesis or modification reactions. However, to our knowledge this is the first paper describing the use of CAD and a mixed-mode column in such an analysis and the first paper presenting the stability of LPC regioisomers in such numerous reaction mixtures.

2. Experimental

2.1. Materials

All solvents for liquid chromatography were freshly opened bottles of Merck LiChrosolv® Reag. obtained from Merck. Ammonium acetate (for LC), Lipozyme® (immobilized lipase from *Mucor miehei* 86.8 U/g), boron trifluoride ethyl etherate (48% $BF_3/(C_2H_5)_2O$) were bought in Fluka®. Palmitic acid, docusate sodium salt (AOT), Trizma® Hydrochloride, Tris–Base and egg yolk phosphatidylcholine were purchased from Sigma–Aldrich®. *sn*-Glycero-3-phosphocholine (GPC) was purchased from Bachem. Phospholipase A_2 (Lecitase 10L) was a gift from Novozymes. All other solvents were of analytical grade. Thin-layer chromatography (analysis) was carried out on pre-coated aluminum plates (0.2 mm silica gel with fluorescent UV254) purchased from Merck. After elution, the plates were developed using the 0.05% primuline solution (acetone:water, 8:2, v/v) and spots were detected under an ultraviolet (UV) lamp (λ = 365 nm). Silica gel (0.040–0.063 mm, 230–400 mesh ASTM) column chromatography was run under gravity.

2.2. Instrumentation

Electrospray ionization-mass spectrometry (ESI-MS) was measured on a Bruker micrOTOF-Q.

All nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance II 600 MHz. Chemical shifts (^{1}H and ^{13}C) (δ) are given in parts per million (ppm) downfield from tetramethylsilane (TMS) as the internal standard. In ^{31}P NMR, chemical shifts were referenced to $(PhO)_3PO$ as an internal standard. Coupling constant (J) values are in Hz.

The palmitic acid ethyl ester (PAEE) was analyzed by gas chromatography (GC) on A Varian Chrompack CP-3380 apparatus with a flame ionization detector (FID).

GC conditions. The separation of palmitic acid ethyl ester (PAEE) was achieved using a 70% cyanopropyl polysilphenylene-siloxane column (TR FAME, 30 m × 0.25 mm × 0.25 μm). The oven temperature was 140 °C, held for 3 min, raised to 220 °C at a rate of 5 °C/min and then to 260 °C at a rate of 30 °C/min and held for 3 min while the injector temperature was 250 °C and the FID temperature was set at 280 °C. Hydrogen was used as the carrier gas.

The LC was performed on an Ultimate 3000 from DIONEX chromatograph equipped with a DGP-3600A dual-pump fluid control module, a TCC-3200 thermostated column compartment and an WPS-3000 autosampler. The system was controlled and data acquisition was carried out using Chromeleon 6.80 software (Dionex Corporation). The Corona™ Charged Aerosol Detector (CAD) was from ESA Biosciences. The following parameters were used: acquisition range 100 pA, digital filter set to none, N_2 pressure 35 psi. Data acquisition for CAD was carried out using the Chromeleon 6.80 software.

LC conditions. The analysis was carried out using an Acclaim® Mixed-Mode HILIC-1 5 μm (4.6 mm × 150 mm) column equipped with a precolumn containing the same sorbents. The injection volume was 10 μL in all experiments and the cooling temperature for the samples was 20 °C. The column temperature was maintained at 30 °C. The gradient had a constant flow rate of 0.8 mL/min, with solvent A = 0.01 M NH_4OAc, B = acetonitrile, and C = methanol. Gradient time table: at 0 min, 1/30/44 (%A/%B/%C), at 15 min, 1/20/79, and at 22 min, 1/20/79. The total analysis time was 47 min, including 25 min for column re-equilibration. Every new sequence of analysis was preceded by two blank gradient runs for column equilibration and the CAD response stabilization. Identification of analyzed compounds was carried out by comparison with the retention time of standards.

2.3. Synthesis of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)

A detailed procedure of DPPC synthesis has been already presented by Smuga et al. [36].

2.3.1. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)
White solid
TLC R_f: 0.42 ($CHCl_3$–MeOH–H_2O, 65:25:4, v/v/v)
HRMS *m/z* calculated for $[M+H]^+$ $C_{24}H_{51}NO_7P$ 734,5694, found 734,5705
The ^{31}P NMR, ^{13}C NMR and ^1H NMR data were in accordance with literature values [37–39]

2.4. Procedure of enzymatic ethanolysis of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) to 1-hydroxy-2-palmitoyl-sn-glycero-3-phosphocholine (2-palmitoyl LPC)

Hydrolysis of DPPC was carried out according to the procedure described by Adlercreutz and Wehtje [6]. To DPPC (50 mg, 0.07 mmol) dissolved in 2.5 mL of ethanol (96%) 40 U of Lipozyme, immobilized from *M. miehei* was added and the mixture was shaken vigorously at 25 °C. The progress of reaction was monitored by TLC ($CHCl_3$:MeOH:H_2O, 65:25:4, v/v/v) and LC. 2-Palmitoyl LPC (R_f: 0.17), DPPC (R_f: 0.44), palmitic acid (R_f: 0.88) and palmitic acid ethyl ester (R_f: 0.88) spots were detected. The reaction was completed after about 12 h. The enzyme was filtered off and washed with 10 mL of methanol. Solvents were removed at 45 °C on a rotary evaporator *in vacuo*. The residue was diluted in 0.3 mL of chloroform. The mixture was placed in an ice-bath and 5 mL of chilled aceton (−20 °C) was added causing 2-palmitoyl LPC precipitation. After 1 min of stirring and 5 min of decantation of the product in the ice-bath, supernatant was carefully removed. The procedure of precipitation was repeated six times. Chloroform was added only before first precipitation. The purification steps were monitored by TLC (hexane:diethyl ether, 7:1, v/v) with primuline test (2-palmitoyl LPC (R_f: 0.00), palmitic acid (R_f: 0.09) and palmitic acid ethyl ester (R_f: 0.80)) and by LC. The acetone was removed at 45 °C on a rotary evaporator *in vacuo* to give 32 mg of precipitation mixture (3% GPC, 6% 1-palmitoyl LPC, 91% 2-palmitoyl by LC).

2.4.1. 1-Hydroxy-2-palmitoyl-sn-glycero-3-phosphocholine (2-palmitoyl LPC)
Yield: 94.5%, white solid
TLC R_f: 0.17 ($CHCl_3$:MeOH:H_2O, 65:25:4, v/v/v), LC R_t = 9.592 min
HRMS *m/z* calculated for $[M+H]^+$ $C_{24}H_{51}NO_7P$ 496,3398, found 496,3417
The ^{31}P NMR, ^{13}C NMR and ^1H NMR data were in accordance with literature values [37–39]

2.5. Procedure of enzymatic hydrolysis of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) to 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (1-palmitoyl LPC)

The enzymatic hydrolysis reaction in the *sn*-2 position of DPPC was carried out as described for natural phosphatidylcholine by Morgado et al. [40]. The hydrolysis of DPPC catalyzed by phospholipase A_2 (PLA_2) in reversed micelles was carried out in a thermostated (40 °C) batch with magnetic stirring. A reversed micellar solution containing phospholipase A_2 was prepared by injecting, with strong magnetic stirring, 17.6 μL of Tris–HCl buffer (pH 8.5, 0.1 M) with $CaCl_2$ (0.75 M), and 26.3 μL (263 U) aqueous solution of the enzyme to pre-incubated flasks (40 °C for 0.5 h) containing 14.0 mg AOT dissolved in 1.1 mL of isooctane. The reaction was then started by the addition of a pre-incubated (40 °C for 0.5 h) mixture of 0.041 g DPPC (0.05 mmol) dissolved in 1.1 mL of isooctane. The progress of enzymatic reactions was monitored by TLC (chloroform:methanol:water, 65:25:4, v/v/v). The reaction was completed after about 10 min. 1-Palmitoyl LPC was purified on the silica gel column chromatography (eluent:chloroform:methanol:water, 65:25:4, v/v/v). The fractions containing 1-palmitoyl LPC were collected and evaporated to dryness at 45 °C *in vacuo* to give 24.5 mg of the products mixture (1.9% GPC, 1.3% 2-palmitoyl LPC, 96.8% 1-palmitoyl LPC by LC).

2.5.1. 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (1-palmitoyl LPC)
Yield: 98.8%, white solid
TLC R_f: 0.17 ($CHCl_3$:MeOH:H_2O, 65:25:4, v/v/v), LC R_t = 10.257 min
HRMS *m/z* calculated for $[M+H]^+$ $C_{24}H_{51}NO_7P$ 496,3398, found 496,3435
The ^{31}P NMR, ^{13}C NMR and ^1H NMR data were in accordance with literature values [37–39]

2.6. Acyl migration study

To study the internal transesterification between the regioisomers of LPC, 19 different solvents were used. LPC (0.002 mmol; 1 mg) was dissolved or suspended in 0.5 mL of the appropriate solvent kept at 25 °C. Samples (10 μL) were collected at constant time intervals, and 90 μL of eluent ($CHCl_3$:MeOH, 2:1, v/v) was added. The LC analysis was performed immediately after sample preparation and the injection volume was 10 μL.

3. Results and discussion

3.1. LC method development

The LC method presented in this paper enabled the separation of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and its hydrolysis products *i.e.* 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (1-palmitoyl LPC), 1-hydroxy-2-palmitoyl-*sn*-glycero-3-phosphocholine (2-palmitoyl LPC), *sn*-glycero-3-phosphocholine (GPC) and palmitic acid. Furthermore the separation of natural origin phosphatidylcholine from egg yolk and its hydrolysis products is also presented. In the experiments, the synthetic mono acyl phosphatidylcholine was chosen as a model compound to simplify the method development and to make chromatogram interpretation more reliable. It was highly important to observe even small changes in the examined mixtures. Furthermore the synthetic mono acyl PCs *i.e.* DPPC are the main components of liposomes [12] and their degradation products can destabilize their structures [5]. The natural origin PC which is a mixture of molecules with different acyl chain lengths can give a number of peaks [41] which may overlap in the complex mixture [6]. The separation of egg yolk PC, 1-acyl and 2-acyl LPC into a number of baseline resolved peaks was observed. The comparison between chromatograms obtained from natural egg yolk PC and DPPC and its regiospecific hydrolysis products is presented in Fig. 1.

The main chromatographic problems were the high polarity differences of the analyzed compounds (especially DPPC and GPC). Therefore finding an appropriate stationary phase to separate all compounds was demanding. The recently developed stationary phase was used [42]. The phase is based on spherical silica gel functionalized with a silyl ligand consisting of both hydrophilic interaction (high organic in the mobile phase) and reversed-phase (low organic content) characteristics. These properties are caused by combining hydrophobic alkyl chains with a glycol terminus. Compared to traditional HILIC diol stationary phases (usually with three carbon) the longer alkyl chain in the presented phase provides hydrophobic retention in addition to HILIC properties and demonstrates great potential for the separation of a wide range of both highly polar and non-polar molecules. Its hydrophobicity is lower than RP C8 analogues and higher than conventional diol

Table 1
HPLC/CAD calibration curves.

Compound	Range (μg)	Power model ($y = ax^b$)		Linear model ($y = a + bx$)	
		Equation	R^2	Equation	R^2
GPC	0.019–6.17	$12.876(\pm0.13)x^{0.84(\pm0.00)}$	0.9958	$9.476\ (\pm0.03)x + 1.9(\pm0.05)$	0.9893
Palmitic acid	0.19–7.70	$7.098(\pm0.08)x^{1.0552(\pm0.02)}$	0.9949	$7.650\ (\pm0.10)x - 0.345(\pm0.09)$	0.9962
1-Palmitoyl LPC	0.03–5.00	$13.184(\pm0.13)x^{0.966(\pm0.01)}$	0.9985	$12.082\ (\pm0.05)x - 0.817(\pm0.05)$	0.9948
2-Palmitoyl LPC	0.03–5.00	$13.999(\pm0.12)x^{0.9652(\pm0.01)}$	0.9994	$12.595\ (\pm0.15)x - 1.119(\pm0.03)$	0.9931
DPPC	0.05–8.80	$43.844(\pm0.12)x^{0.8055(\pm0.00)}$	0.9952	$26.599\ (\pm0.08)x + 12.884(\pm0.18)$	0.9840

phases [42]. The separation of all compounds is determined using two different modes. First, GPC and palmitic acid are separated in lower organic solvent concentrations which provide RP characteristic of the column stationary phase. Then the organic concentration increase and 1- and 2-palmitoyl LPC are separated. The highest organic concentration provides HILIC properties of the stationary phase and DPPC elution. However the hydrophobic interactions of phosphatidylcholine acyl chains with acyl chains of the stationary phase and separation of the egg yolk PC into molecular species were observed. This was probably caused by lack of equilibration time before changing from RP to HILIC mode (from water-rich to high organic concentration mobile phase) and the RP retention mechanism during the separation was dominant. Detailed aspects of a dual retention mechanism for 2-D HILIC-RP separations on single column were recently presented [19]. The ammonium acetate buffer was used to improve peak-shape. The 100 mM buffer was tried first and caused significant shape improvement especially for DPPC. Asymmetry of the peak changed from about 2.0 to 1.52. Unfortunately the high buffer concentration generated about four times higher CAD background compared to water. The phenomenon of CAD system sensitivity to contaminants or additives to the mobile phase was also observed by other authors [43,44]. Therefore ten times lower buffer concentration (10 mM) was used improving the peak-shape (1.60) and decreasing the negative effect of buffer addition to the mobile phase.

3.2. Response model

Calibration curves for each compound were calculated from the area values obtained by injecting 10 μL of chloroform–methanol (2:1, v/v) solutions of GPC (0.019–6.17 μg), palmitic acid (0.19–7.70 μg), 1-palmitoyl LPC (0.03–5.00 μg), 2-palmitoyl LPC (0.03–5.00 μg) and DPPC (0.05–8.80 μg). At least six standard concentrations of every compound were prepared. Injection was performed by triplicate of every standard mixture dissolved in chloroform–methanol (2:1, v/v) mixture. This solvent: (1) provided solubility of all compounds, (2) proved to be an acceptable solvent for all analytes and (3) exhibited minimal interference of the solvent front with the relevant compound peaks in the chromatogram. To study the relationship between the individual compound amount and the CAD response (peak area), linear ($y = a + bx$) and power models ($y = ax^b$) were evaluated to find which better describes the detector response. Some papers also present the use of log–log transformation of x and y of the latter equation to describe the relationship between signal and amount of analyte [45]. This transformation is used to linearize the response function but distorts the experimental error [30]. Table 1 presents the linear and power model for CAD. The relation of peak area to concentration of analyte resulted in good linearity with high correlation coefficients between 0.9949 and 0.9994 by applying the power model. Although the response of the detection method can be fitted to a power function, the response of palmitic acid, 1-palmitoyl and 2-palmitoyl can also be described by a linear model with a correlation coefficient between 0.9931 and 0.9962. The linearity was also reported by other authors [30,41]. The response of the 1-acyl and 2-acyl is almost identical. The variations are caused by the fact that CAD system is an aerosol-based detector and its response varies as a function of the mobile phase composition [26,27]. Therefore analytes reached the detector at somewhat different mobile phase composition and differences in the response of same mass compounds were observed.

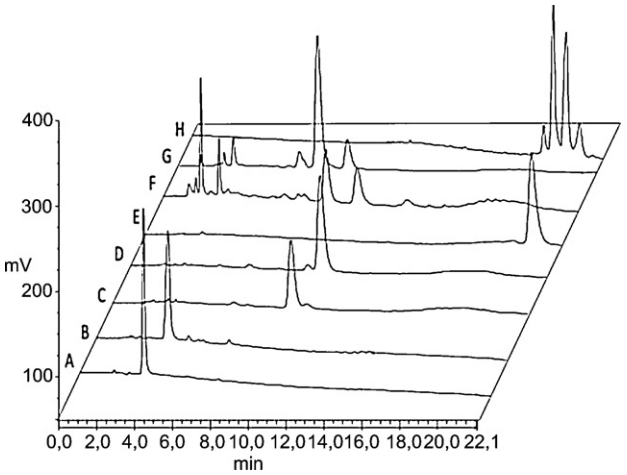

Fig. 1. CAD–HPLC chromatograms of (A) GPC, (B) palmitic acid, (C) 2-palmitoyl LPC, (D) 1-palmitoyl LPC, (E) DPPC, (F) 1-acyl LPC from egg yolk (directly from the reaction mixture – enzymatic hydrolysis by PLA_2), (G) 2-acyl LPC from egg yolk (directly from the reaction mixture – ethanolysis) and (H) egg yolk PC.

3.3. Sensitivity, limit of detection (LOD) and limit of quantitation (LOQ)

The sensitivity was calculated as the derivative of the power model ($S = Abx^{b-1}$) for the lowest concentration used in the study. The sensitivity values are presented in Table 2. An increase in the organic content of the mobile phase leads to an increase in the transport efficiency of the nebulizer which results in a greater number of particles reaching the detector, in a higher signal [27] and in higher sensitivity than can be observed especially for DPPC. The low sensitivity for palmitic acid is probably caused by its semi-volatile properties. Such compounds show a diminished response with CAD. We also tried to analyze the more volatile palmitic acid ethyl ester (PAEE) of which presence was observed (using TLC) during the ethanolysis reaction. A 3 mM PAEE standard solution was prepared, 10 μL was injected but no response of the CAD was observed. Table 2 also summarized the LOD and LOQ values for each compound. As can be seen the lowest values of LOD and LOQ parameters correspond to DPPC while the palmitic acid presented the highest values.

3.4. Precision

The precision, or more specifically the instrument precision and injection repeatability was studied by 10 injections of one sample solution. The same molar concentration (0.3 mM) of all standards

Table 2
Sensitivity (S), limit of detection (LOD) and limit of quantification (LOQ) for standard compounds with CAD detection.

Compound	Injected amount (μg)	S (mV/μg) (Abx^{b-1})[a]	LOD (μg/mL) ($3.3 \times \sigma/S$)[b]	LOQ (μg/mL) ($10 \times \sigma/S$)[b]
GPC	0.019	20.4	2.1	6.4
Palmitic acid	0. 19	6.8	4.0	12.2
1-Palmitoyl LPC	0.03	14.3	3.1	9.5
2-Palmitoyl LPC	0.03	15.2	2.7	8.3
DPPC	0.05	63	0.7	2.0

[a] A and b – terms obtained from the power model; x – concentration of the injected amount.
[b] σ – standard deviation of the response (intercept).

was used. As evaluation data for the repeatability of the method, both the retention time and the area under peaks, were evaluated (Table 3). The relative standard deviation (RSD) was criterion for the repeatability of the measurements. Precision criteria for an assay method is that the instrument precision (RSD) will be ≤1% [46]. The RSD% values indicate this method is sufficiently stable. For the mean retention time all values of RSD were lower than 0.28%. Considering the peak area, the RSD was slightly higher than 1% for palmitic acid and DPPC. The response of CAD was increased with a decrease of buffer content in moving-phase. The low response for palmitic acid (compared to GPC) is caused by its semi-volatile properties. On the other hand very high CAD response for DPPC was associated with the high organic eluent (99%) used for its elution.

3.5. Enzymatic hydrolysis of DPPC and the reactions products purification methods

Two different methods of enzymatic hydrolysis of DPPC are presented. The first method is based on enzymatic ethanolysis of DPPC in the *sn*-1 position. 2-Palmitoyl LPC was purified from palmitic acid and its ethyl esters by precipitation from chilled acetone. The process was repeated 6 times to give a 97% pure LPC regioisomers mixture (91% of 2-palmitoyl LPC and 6% of 1-palmitoyl LPC) with 94.5% recovery. To our knowledge, this is the first purification methodology presented obtaining high purity grade 2-acyl LPC regioisomer. Unfortunately GPC could not be removed from the mixture by this technique because of its insolubility in acetone.

1-Palmitoyl LPC was obtained by hydrolysis of DPPC by PLA_2 in reversed micelles. The quantitative hydrolysis of DPPC to 1-palmitoyl LPC took place after 10 min. No 2-palmitoyl LPC and no GPC in the above reaction mixture was detected. Therefore we concluded that no non-enzymatic hydrolysis or migration processes took place. The complexity of the reaction mixture compared to the mixture used during ethanolysis makes precipitation with cold acetone less attractive. The higher stability of the obtained LPC regioisomer provides the opportunity of using silica gel column chromatography as a purification technique. The mixture of both compounds with about 3% of the 2-palmitoyl and 97% of 1-palmitoyl LPC was obtained. Such a proportion is also present in the commercial 1-acyl LPC preparation [6].

3.6. The stability of the positional isomers of LPC

The LC methodology presented in this paper was applied to examine the stability of the LPC isomers in 20 different solvents usually used in enzymatic or chemical PC synthesis or modification reactions [3,4] and for PL samples preparation [12,46]. Most papers only present the possibility of slow acyl migration in organic solvents [7]. To our knowledge, there is no paper comparing the stability of LPC regioisomers in different organic solvents. The main problem during the experiment was the difference in solubility of the analyzed molecules. Very good solubility of GPC was observed for water, buffers and alcohols. However solubility of GPC decreased with increasing alcohol alkyl chain length and buffer concentration. Insolubility of GPC was observed for all organic solvents and therefore the hydrolysis process in these solvents could not be observed. The LPC was not soluble in isooctane, ethyl acetate or hexane and these eluents were rejected. In some organic solvents (toluene, diethyl ether, acetonitrile and acetone) only partial solubility of LPC occurred. Precipitation of the examined molecules from acetone and dichloromethane was also observed when the temperature was under 20 °C. Therefore special attention was paid to those aspects during the experiment.

Solutions in solvents with a given amount of appropriate LPC regioisomers were mixed and analyzed directly after mixing and at specified and constant time intervals thereafter. These results are presented in Tables 4 and 5.

3.7. 1-Palmitoyl LPC

The high stability of the 1-palmitoyl LPC isomer was confirmed for almost all organic solvents. Slow migration occurred in the chloroform mixture. This process was slightly faster when methanol was added. An increase (about 1.5%) of GPC concentration in 96 h was also observed in the latter solvent. However 1-palmitoyl LPC in the chloroform/methanol mixture was stable for at least 24 h. This was important information for us because this mixture was used to prepare the standard solutions in the analytical methodology described above. To generate reliable results, the stability of standards must be determined prior to initiating the method validation studies. It is essential that the solution should be stable enough to allow for delays such as overnight analysis using autosamplers [46]. A highly interesting aspect is that acetonitrile favors acyl-migration and after 96 h the equilibrium mixture contained about 92% of the

Table 3
Repeatability of CAD response and retention times of standard compounds.

Compound	Concentration (mM)	Peak area (mV/min)			Retention time (min)		
		Mean	SD	RSD%	Mean	SD	RSD%
GPC	0.3	10.947	0.02	0.22	3.573	0.01	0.28
Palmitic acid	0.3	5.679	0.088	1.56	3.977	0.008	0.20
1-Palmitoyl LPC	0.3	16.922	0.0407	0.14	10.257	0.022	0.21
2-Palmitoyl LPC	0.3	18.047	0.2511	0.95	9.592	0.016	0.17
DPPC	0.3	121.120	0.1129	1.68	20.513	0.038	0.18

Table 4
Stability of 1-palmitoyl LPC regioisomer in 16 different mixtures in time.

Time (h)	Compound (%)	Solvent								
		Buffer pH 8.0 (0.3 M)	Buffer pH 8.0 (2.0 M)	Buffer pH 7.4 (0.3 M)	Buffer pH 5.6 (0.3 M)	Buffer pH 5.6 (2.0 M)	D.I. water	Methanol	Ethanol anhydrous	96% ethanol
0	1-Palmitoyl LPC	97.3 ± 0.7	97.5 ± 0.7	96.2 ± 0.9	97.2 ± 1.0	97.2 ± 0.9	96.7 ± 1.2	96.8 ± 0.3	97.0 ± 0.7	96.3 ± 0.5
	2-Palmitoyl LPC	1.4 ± 0.2	1.6 ± 0.2	2.2 ± 0.4	1.4 ± 0.3	1.8 ± 0.5	1.5 ± 0.1	1.3 ± 0.1	1.6 ± 0.5	2.2 ± 0.3
	GPC	1.2 ± 0.1	0.9 ± 0.1	1.6 ± 0.2	1.4 ± 0.3	0.9 ± 0.1	1.8 ± 0.2	1.9 ± 0.3	1.4 ± 0.2	1.5 ± 0.3
24	1-Palmitoyl LPC	92.5 ± 1.0	91.7 ± 0.6	92.8 ± 0.7	96.9 ± 0.8	96.4 ± 1.0	96.8 ± 1.3	96.5 ± 0.4	97.0 ± 0.9	96.6 ± 0.8
	2-Palmitoyl LPC	2.7 ± 0.4	7.1 ± 0.5	5.2 ± 0.3	1.8 ± 0.4	2.4 ± 0.4	2.9 ± 0.2	1.8 ± 0.1	1.5 ± 0.4	2.1 ± 0.2
	GPC	1.8 ± 0.4	1.3 ± 0.2	1.9 ± 0.3	1.3 ± 0.2	1.2 ± 0.7	0.3 ± 0.07	1.6 ± 0.3	1.5 ± 0.1	1.3 ± 0.1
48	1-Palmitoyl LPC	92.5 ± 1.1	91.9 ± 0.8	92.6 ± 1.0	96.3 ± 0.9	96.6 ± 0.8	94.9 ± 1.0	95.8 ± 0.6	96.6 ± 0.3	96.6 ± 0.8
	2-Palmitoyl LPC	6.0 ± 0.2	6.6 ± 0.3	5.6 ± 0.2	2.1 ± 0.2	2.7 ± 0.2	3.5 ± 0.3	2.1 ± 0.2	1.9 ± 0.2	2.1 ± 0.3
	GPC	1.8 ± 0.3	1.4 ± 0.2	1.8 ± 0.1	1.6 ± 0.6	0.6 ± 0.1	1.7 ± 0.7	2.0 ± 0.2	1.5 ± 0.1	1.3 ± 0.08
72	1-Palmitoyl LPC	91.8 ± 0.9	91.1 ± 1.1	92.2 ± 0.9	96.0 ± 0.6	95.8 ± 0.9	95.4 ± 1.1	95.9 ± 0.3	96.7 ± 0.5	95.8 ± 0.3
	2-Palmitoyl LPC	6.4 ± 0.1	6.7 ± 0.3	6.1 ± 0.4	2.3 ± 0.4	3.3 ± 0.1	4.1 ± 0.3	2.1 ± 0.1	1.8 ± 0.2	2.5 ± 0.1
	GPC	1.7 ± 0.2	2.2 ± 0.4	1.7 ± 0.2	1.7 ± 0.4	0.8 ± 0.09	0.5 ± 0.1	1.9 ± 0.4	1.5 ± 0.09	1.7 ± 0.1
96	1-Palmitoyl LPC	89.3 ± 1.4	91.1 ± 0.9	92.1 ± 0.7	94.9 ± 1.1	95.9 ± 1.0	94.4 ± 0.9	96.3 ± 0.2	96.2 ± 0.2	95.2 ± 0.5
	2-Palmitoyl LPC	7.5 ± 0.2	6.7 ± 0.4	6.0 ± 0.5	2.9 ± 0.3	3.7 ± 0.1	5.0 ± 0.2	1.8 ± 0.2	2.2 ± 0.4	2.5 ± 0.2
	GPC	3.2 ± 0.4	2.2 ± 0.3	1.9 ± 0.3	2.2 ± 0.2	0.4 ± 0.0	0.6 ± 0.1	1.8 ± 0.4	1.6 ± 0.2	2.3 ± 0.3

Time (h)	Compound (%)	Solvent							
		2-Propamol	Chloroform	Chloroform: methanol (2:1, v/v)	Toluene	Diethyl ether	Dichloromethane	Acetonitrile	Acetone
0	1-Palmitoyl LPC	96.8 ± 0.6	98.4 ± 0.8	96.4 ± 0.7	98.2 ± 0.6	98.5 ± 0.4	97.8 ± 0.5	98.0 ± 0.7	96.9 ± 1.0
	2-Palmitoyl LPC	1.6 ± 0.2	1.6 ± 0.3	2.4 ± 0.3	1.8 ± 0.4	1.5 ± 0.2	2.2 ± 0.2	2.0 ± 0.3	3.1 ± 0.7
	GPC	1.5 ± 0.3	Ins[a]	1.2 ± 0.1	Ins	Ins	Ins	Ins	Ins
24	1-Palmitoyl LPC	96.6 ± 0.7	97.6 ± 0.7	96.3 ± 0.6	98.1 ± 0.7	98.4 ± 0.7	97.8 ± 0.9	98.0 ± 0.8	96.9 ± 0.9
	2-Palmitoyl LPC	1.9 ± 0.1	2.4 ± 0.2	2.2 ± 0.2	1.9 ± 0.5	1.6 ± 0.1	2.2 ± 0.1	2.0 ± 0.3	3.1 ± 0.8
	GPC	1.4 ± 0.2	Ins	1.5 ± 0.3	Ins	Ins	Ins	Ins	Ins
48	1-Palmitoyl LPC	97.0 ± 0.5	97.5 ± 0.6	96.0 ± 0.7	98.5 ± 0.9	98.5 ± 0.9	97.6 ± 0.7	96.8 ± 0.9	96.9 ± 1.1
	2-Palmitoyl LPC	1.6 ± 0.1	2.5 ± 0.2	2.4 ± 0.1	1.5 ± 0.4	1.5 ± 0.3	2.4 ± 0.3	3.2 ± 0.5	3.1 ± 0.7
	GPC	1.4 ± 0.2	Ins	1.6 ± 0.09	Ins	Ins	ins	Ins	Ins
72	1-Palmitoyl LPC	96.7 ± 0.7	97.4 ± 0.3	95.8 ± 0.5	98.l ± 0.9	98.2 ± 0.7	97.4 ± 0.9	93.2 ± 0.8	96.8 ± 0.8
	2-Palmitoyl LPC	1.9 ± 0.2	2.6 ± 0.1	2.4 ± 0.2	1.9 ± 0.3	1.8 ± 0.4	2.6 ± 0.4	6.8 ± 0.6	3.2 ± 0.5
	GPC	1.4 ± 0.1	Ins	1.8 ± 0.2	Ins	Ins	Ins	Ins	Ins
96	1-Palmitoyl LPC	96.1 ± 0.8	97.4 ± 0.3	94.6 ± 0.6	98.2 ± 0.8	98.3 ± 0.9	97.6 ± 0.7	91.7 ± 1.0	96.6 ± 0.9
	2-Palmitoyl LPC	2.4 ± 0.2	2.6 ± 0.1	2.7 ± 0.2	1.8 ± 0.4	1.7 ± 0.3	2.4 ± 0.4	8.3 ± 0.8	3.4 ± 0.6
	GPC	1.5 ± 0.08	Ins	2.7 ± 0.4	Ins	Ins	Ins	Ins	Ins

[a] Insoluble ($n = 3$).

Table 5
Stability of 2-palmitoyl LPC regioisomer in 16 different mixtures in time.

Time (h)	Compound (%)	Solvent							
		Buffer pH 8.0 (0.3 M)	Buffer pH 8.0 (2.0 M)	Buffer pH 7.4 (0.3 M)	Buffer pH 5.6 (0.3 M)	Buffer pH 5.6 (2.0 M)	D.I. water	Methanol	Ethanol anhydrous
0	2-Palmitoyl LPC	90.7 ± 0.3	91.3 ± 0.5	88.6 ± 0.3	94.2 ± 0.3	93.7 ± 1.0	96.3 ± 0.6	89.9 ± 0.6	90.4 ± 0.8
	1-Palmitoyllpc	6.0 ± 0.2	7.0 ± 0.6	8.3 ± 0.4	2.6 ± 0.2	4.7 ± 0.1	5.0 ± 0.5	6.8 ± 0.2	7.1 ± 0.4
	GPC	3.3 ± 0.09	1.7 ± 0.1	3.1 ± 0.2	3.2 ± 0.3	1.6 ± 0.2	0.7 ± 0.1	3.3 ± 0.2	2.5 ± 0.3
24	2-Palmitoyl LPC	7.9 ± 0.3	7.0 ± 0.5	13.7 ± 0.7	85.7 ± 1.0	81.1 ± 1.6	63.6 ± 1.2	88.6 ± 0.9	88.7 ± 0.8
	1-Palmitoyllpc	88.5 ± 0.8	91.1 ± 0.8	82.8 ± 1.4	11.0 ± 0.7	18.2 ± 1.0	35.6 ± 0.6	7.5 ± 0.5	8.3 ± 0.2
	GPC	3.6 ± 0.1	1.9 ± 0.2	3.5 ± 0.3	3.3 ± 0.2	1.7 ± 0.5	0.8 ± 0.2	3.9 ± 0.4	3.0 ± 0.1
48	2-Palmitoyl LPC	8.0 ± 0.2	6.0 ± 0.6	8.0 ± 0.6	81.1 ± 2.0	75.2 ± 0.9	56.4 ± 1.4	82.6 ± 1.1	88.0 ± 1.0
	1-Palmitoyllpc	87.6 ± 1.1	91.9 ± 1.1	88.3 ± 0.8	15.7 ± 1.2	22.5 ± 0.7	42.9 ± 1.6	13.4 ± 0.4	8.6 ± 0.2
	GPC	4.5 ± 0.08	2.1 ± 0.4	3.7 ± 0.4	3.1 ± 0.3	2.2 ± 0.2	0.8 ± 0.1	4.0 ± 0.3	3.4 ± 0.1
72	2-Palmitoyl LPC	6.6 ± 0.3	6.3 ± 0.7	4.2 ± 0.3	77.6 ± 1.1	67.7 ± 2.2	27.7 ± 1.0	82.4 ± 1.2	87.6 ± 0.6
	1-Palmitoyllpc	89.6 ± 0.9	91.4 ± 1.0	91.6 ± 0.8	18.7 ± 0.6	30.0 ± 1.3	71.6 ± 2.0	14.7 ± 0.7	8.8 ± 0.3
	GPC	3.8 ± 0.2	2.2 ± 0.08	4.2 ± 0.2	3.7 ± 0.0	2.3 ± 0.6	0.8 ± 0.1	4.1 ± 0.5	3.6 ± 0.2
96	2-Palmitoyl LPC	6.4 ± 0.3	6.1 ± 0.5	4.0 ± 0.1	72.1 ± 2.0	67.1 ± 1.4	18.6 ± 0.7	76.3 ± 1.8	87.1 ± 0.3
	1-Palmitoyllpc	89.7 ± 1.0	91.5 ± 0.9	91.5 ± 1.2	23.4 ± 1.0	30.2 ± 1.0	80.5 ± 1.9	19.2 ± 1.1	9.4 ± 0.2
	GPC	3.9 ± 0.1	2.3 ± 0.1	4.5 ± 0.09	4.5 ± 0.4	2.6 ± 0.3	0.9 ± 0.2	4.5 ± 0.1	3.5 ± 0.2

Time (h)	Compound (%)	Solvent								
		96% ethanol	2-Propamol	Chloroform	Chloroform: methanol (2:1)	Toluene	Diethyl ether	Dichloromethane	Acetonitrile	Acetone
0	2-Palmitoyl LPC	90.7 ± 1.0	90.2 ± 0.5	93.7 ± 1.0	89.1 ± 0.3	92.9 ± 2.0	89.6 ± 1.9	92.5 ± 1.7	92.2 ± 1.5	92.4 ± 2.3
	1-Palmitoyllpc	7.1 ± 0.7	7.3 ± 0.2	6.3 ± 0.8	8.4 ± 0.2	7.1 ± 1.1	10.4 ± 1.3	7.5 ± 0.6	7.8 ± 0.7	7.6 ± 1.2
	GPC	2.2 ± 0.1	2.5 ± 0.3	Ins[a]	3.7 ± 0.1	Ins	ins	Ins	Ins	Ins
24	2-Palmitoyl LPC	87.7 ± 0.9	89.4 ± 0.7	91.7 ± 1.4	87.5 ± 0.2	89.9 ± 1.9	86.0 ± 2.1	90.5 ± 1.5	91.8 ± 1.3	90.4 ± 2.0
	1-Palmitoyllpc	8.7 ± 0.8	7.8 ± 0.5	8.3 ± 0.6	8.7 ± 0.1	10.1 ± 1.2	14.0 ± 13	9.5 ± 0.4	8.2 ± 0.4	9.6 ± 1.3
	GPC	3.6 ± 0.3	2.8 ± 0.1	Ins	3.7 ± 0.1	Ins	Ins	Ins	Ins	Ins
48	2-Palmitoyl LPC	83.7 ± 1.1	88.4 ± 1.1	90.6 ± 0.6	84.9 ± 0.6	91.4 ± 1.1	82.3 ± 1.7	87.1 ± 1.8	89.6 ± 1.1	89.1 ± 2.2
	1-Palmitoyllpc	13.5 ± 0.6	8.6 ± 0.3	9.4 ± 0.2	11.3 ± 0.3	8.6 ± 0.8	17.7 ± 1.1	12.9 ± 0.8	10.4 ± 0.5	10.9 ± 1.5
	GPC	2.7 ± 0.3	3.0 ± 0.1	Ins	3.8 ± 0.2	Ins	Ins	Ins	Ins	Ins
72	2-Palmitoyl LPC	79.4 ± 1.2	88.5 ± 1.2	89.5 ± 0.8	81.6 ± 0.9	85.5 ± 2.1	73.5 ± 1.5	84.4 ± 2.0	89.3 ± 1.2	88.0 ± 1.9
	1-Palmitoyllpc	16.7 ± 0.5	8.4 ± 0.5	10.5 ± 0.4	14.6 ± 0.5	14.5 ± 0.9	26.5 ± 1.2	15.6 ± 1.2	10.7 ± 0.8	12.0 ± 0.9
	GPC	3.9 ± 0.3	3.1 ± 0.2	Ins	3.8 ± 0.1	Ins	Ins	Ins	Ins	Ins
96	2-Palmitoyl LPC	76.0 ± 1.4	88.2 ± 0.9	86.5 ± 1.0	77.6 ± 1.0	80.6 ± 2.2	60.5 ± 2.2	83.8 ± 1.4	89.1 ± 0.9	87.2 ± 2.0
	1-Palmitoyllpc	20.5 ± 0.7	8.6 ± 0.1	13.5 ± 0.3	18.5 ± 0.6	19.3 ± 1.0	39.5 ± 0.9	16.2 ± 0.9	10.9 ± 0.2	12 8 ± 1 0
	GPC	3.5 ± 0.1	3.2 ± 0.2	Ins	3.9 ± 0.4	Ins	Ins	Ins	Ins	Ins

[a] Insoluble ($n = 3$).

1-palmitoyl LPC and about 8% of 2-palmitoyl LPC. In the case of alcohol solvents less than 1% change of 1-palmitoyl LPC concentration and no hydrolysis processes as shown by the constant amount of GPC was seen. Such stability was also observed for 96% ethanol but with slightly increasing amounts of GPC. The differences in buffer concentration did not cause any significant changes in the acyl-migration process. However, both acid and base catalyze the acyl migration. This is consistent with data presented by other authors [7]. The acyl migration and hydrolysis processes were preferred at alkaline pH.

3.8. 2-Palmitoyl LPC

Very interesting data were obtained from the analysis of less stable 2-palmitoyl LPC. None of the solvents provided stability of the compound during the experiment. Surprisingly significant differences were observed even between organic solvents. Firstly, unlike in the case of 1-palmitoyl LPC, acetonitrile was the best solvent (from all organic solvents) to provide isomer stability. The amount of the main isomer decreased by only 3% in 96 h. The fastest rearrangement in this group of solvents was observed for diethyl ether. The other solvents provide more or less similar stability of the molecule. Particular attention was given to the chloroform/methanol mixture. Additional analyses after 8 and 13 h were performed to check the stability of 2-palmitoyl LPC over time, which was necessary to provide reliable results during LC method validation. The stability of the compound was observed for 13 h. This time was sufficient to carry out all analysis. The increase of GPC amount over time was observed for all other solvents (alcohols, water and buffers). The changes after 96 h were less than 1.4%. From all examined solvents the best stability of 2-palmitoyl LPC provides 2-propanol and anhydrous ethanol. The changes in the amount of the main isomer are less than 2% and 3% in 96 h, respectively. However large differences between particular alcohol-mixtures were not observed until 48 h. For 24 h all examined alcohols (except 96% ethanol) provide a similar stability of 2-palmitoyl LPC. At alkaline pH the fastest migration was observed. However, differences between alkaline buffers occurred. The equilibrium mixture containing about 90% of the more stable 1-palmitoyl LPC isomer was obtained after 24 h for pH 8.0 and after 72 h for pH 7.4. At acidic pH the acyl migration was about two times slower than in deionized water and no equilibrium mixture was present after 96 h of experiment.

4. Conclusions

The results presented herein demonstrate a suitable LC-CAD method used to study spontaneous acyl migration of the two isomers of lysophosphatidylcholine in the most common solvents used in phosphatidylcholine synthesis reactions. The procedure was also employed to analyze and quantify the hydrolysis products of phosphatidylcholine in a single run. It was shown that this method is reproducible for the most important evaluation parameters such as linearity and precision.

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References

[1] G. Helmerich, P. Koehler, Journal of Cereal Science 42 (2005) 233–241.
[2] M. Oke, J.K. Jacob, G. Paliyath, Food Research International 43 (2010) 232–240.
[3] D. Adlercreutz, H. Budde, E. Wehtje, Biotechnology and Bioengineering 78 (2002) 403–411.
[4] A. Chojnacka, W. Gładkowski, G. Kiełbowicz, C. Wawrzeńczyk, Biotechnology Letters 31 (2009) 705–709.
[5] M. Grit, D.J.A. Crommelin, J. Lang, Journal of Chromatography A 585 (1991) 239–246.
[6] D. Adlercreutz, E. Wehtje, Journal of the American Oil Chemists' Society 78 (2001) 1007–1011.
[7] A. Plueckthun, E.A. Dennis, Biochemistry 21 (1982) 1743–1750.
[8] A. Descalzo, E. Insani, N. Pensel, Lipids 38 (2003) 999–1003.
[9] J.J. Myher, A. Kuksis, Journal of Chromatography B: Biomedical Sciences and Applications 671 (1995) 3–33.
[10] B.L. Peterson, B.S. Cummings, Biomedical Chromatography 20 (2006) 227–243.
[11] R. Rombaut, J.V. Camp, K. Dewettinck, Journal of Dairy Science 88 (2005) 482–488.
[12] I. De Miguel, A. Roueche, D. Betbeder, Journal of Chromatography A 840 (1999) 31–38.
[13] J. Dong, X. Cai, L. Zhao, X. Xue, L. Zou, X. Zhang, X. Liang, Metabolomics 6 (2010) 478–488.
[14] X. Han, R.W. Gross, Journal of the American Chemical Society 118 (1996) 451–457.
[15] H. Nakanishi, Y. Iida, T. Shimizu, R. Taguchi, Journal of Biochemistry 147 (2010) 245–256.
[16] M. Narváez-Rivas, E. Gallardo, J.J. Ríos, M. León-Camacho, Journal of Chromatography A 1218 (2011) 3453–3458.
[17] M. Graeve, D. Janssen, Journal of Chromatography B 877 (2009) 1815–1819.
[18] I. Tsirkin, E. Grushka, Journal of Chromatography A 919 (2001) 245–254.
[19] P. Jandera, Journal of Separation Science 31 (2008) 1421–1437.
[20] T.H. Yoon, I.H. Kim, Journal of Chromatography A 949 (2002) 209–216.
[21] B. Sas, E. Peys, M. Helsen, Journal of Chromatography A 864 (1999) 179–182.
[22] J.-T. Lin, T.A. McKeon, C.L. Woodruff, J.A. Singleton, Journal of Chromatography A 824 (1998) 169–174.
[23] A. Nicholas, L. Khouri, J. Ellington, N. Porter, Lipids 18 (1983) 434–438.
[24] H. Bünger, U. Pison, Journal of Chromatography B: Biomedical Sciences and Applications 672 (1995) 25–31.
[25] G. Liebisch, W. Drobnik, B. Lieser, G. Schmitz, Clinical Chemistry 48 (2002) 2217–2224.
[26] T. Górecki, F. Lynen, R. Szucs, P. Sandra, Analytical Chemistry 78 (2006) 3186–3192.
[27] T. Vehovec, A. Obreza, Journal of Chromatography A 1217 (2010) 1549–1556.
[28] B. Zhang, X. Li, B. Yan, Analytical and Bioanalytical Chemistry 390 (2008) 299–301.
[29] R.W. Dixon, D.S. Peterson, Analytical Chemistry 74 (2002) 2930–2937.
[30] R.G. Ramos, D. Libong, M. Rakotomanga, K. Gaudin, P.M. Loiseau, P. Chaminade, Journal of Chromatography A 1209 (2008) 88–94.
[31] Z. Huang, M.A. Richards, Y. Zha, R. Francis, R. Lozano, J. Ruan, Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 809–814.
[32] L.N.H. Arakaki, A.P.M. Alves, E.C.D. Silva Filho, M.G. Fonseca, S.F. Oliveira, J.G.P. Espínola, C. Airoldi, Thermochimica Acta 453 (2007) 72–74.
[33] M. Lafosse, B. Herbreteau, M. Dreux, L. Morin-Allory, Journal of Chromatography A 472 (1989) 209–218.
[34] B. Porsch, J. Krátká, Journal of Chromatography A 543 (1991) 1–7.
[35] T. Ikegami, K. Tomomatsu, H. Takubo, K. Horie, N. Tanaka, Journal of Chromatography A 1184 (2008) 474–503.
[36] D.A. Smuga, M. Smuga, A. Swizdor, A. Panek, C. Wawrzenczyk, Steroids 75 (2010) 1146–1152.
[37] K. Bruzik, R.T. Jiang, M.D. Tsai, Biochemistry 22 (1983) 2478–2486.
[38] S. Lin, R.I. Duclos Jr., A. Makriyannis, Chemistry and Physics of Lipids 86 (1997) 171–181.
[39] K. Bruzik, M.D. Tsai, Biochemistry 23 (1984) 1656–1661.
[40] M.A.P. Morgado, J.M.S. Cabral, D.M.F. Prazeres, Journal of Chemical Technology & Biotechnology 63 (1995) 181–189.
[41] C. Schönherr, S. Touchene, G. Wilser, R. Peschka-Süss, G. Francese, Journal of Chromatography A 1216 (2009) 781–786.
[42] X. Liu, C. Pohl, Journal of Chromatography A 1191 (2008) 83–89.
[43] L. Nováková, S.A. Lopéz, D. Solichová, D. Satínský, B. Kulichová, A. Horna, P. Solich, Talanta 78 (2009) 834–839.
[44] N. Vervoort, D. Daemen, G. Török, Journal of Chromatography A 1189 (2008) 92–100.
[45] R. Díaz-López, D. Libong, N. Tsapis, E. Fattal, P. Chaminade, Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 702–707.
[46] G.A. Shabir, Journal of Chromatography A 987 (2003) 57–66.