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An efficient hydrophilic interaction liquid chromatography separation of 7 phospholipid classes based on a diol column

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ABSTRACT

A hydrophilic interaction liquid chromatography (HILIC) – ion trap mass spectrometry method was developed for separation of a wide range of phospholipids. A diol column which is often used with normal phase chromatography was adapted to separate different phospholipid classes in HILIC mode using a mobile phase system consisting of acetonitrile, water, ammonium formate and formic acid. An efficient between-class separation of seven phospholipid classes including phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinostol, phosphatidylcholine, phosphatidylserine, sphingomyelin and lysophosphatidylcholine was successfully achieved within 14 min using a gradient elution which starts with 90% of organic solvent and ends with 70% of organic solvent. 53 mM formic acid (in both organic phase and aqueous phase) and 60 mM ammonium formate (only in aqueous phase) were used as mobile phase modifier. The relatively high amount of ammonium formate was essential to obtain well-shaped peaks of each phospholipid class, especially phosphatidylserines; actually, no negative effect due to ammonium formate was observed for electrospray-mass spectrometry detection in real-life samples. Good chromatographic separation between different lipid classes was obtained (*Rs*, from 0.73 to 4.97) and well-shaped peaks (*tailing factor*, from 0.98 to 1.20) were obtained. The developed method was fully validated and the satisfactory performance characteristics such as linearity (R^2, 0.990–0.999), retention time stability (RSD < 1%), within day repeatability (RSD, 5–13%), between day variation (RSD, 7–14%) and recoveries (99.6–115.5%) indicated the gradient HILIC method was appropriate for profiling of plasma phospholipids. The method was successfully applied to separate phospholipids extracts from human plasma, mouse plasma and rat plasma.

1. Introduction

Phospholipids (PLs) are the primary structural constituents of biological membranes. Phospholipid (PL) molecules consist of a polar phosphoryl head group and one or two non-polar fatty acid tails with varying numbers of carbons and double bonds [1] (Fig. 1). PLs can be divided into two major families glycerophospholipids (GPs) and sphingomyelins (SMs). The GPs have in common a glycerol backbone and they differ in their polar head group, i.e., ethanolamine, choline, glycerol, inositol, and serine. Sphingomyelins (SMs) differ from GPs as they have a sphingosine base as the backbone instead of a glycerol.

In addition to their membrane function PLs are important in the emulsification of neutral fat and cholesterol deposits in blood vessels [2,3], intelligence improvement [4] and cell activation [5–7]. Next, PL metabolism is very closely associated with many different diseases. Furthermore, PLs have already been recognized to be important signaling molecules [8–16] and potential biomarkers for ovarian cancer [17–20], diabetes mellitus [21,22] and many other diseases [23–27]. Due to this apparent important role of PLs in biology, identification and quantification of PLs in biological samples are very important.

HPLC separation of PL classes is widely reported using either reversed phase liquid chromatography (RPLC) or normal phase liquid chromatography (NPLC) [28–31]. Mainly two basic solvent systems, viz. acetonitrile (ACN)-based solvent systems [30] and isopropanol (IPA)-based solvent systems [31], have been reported

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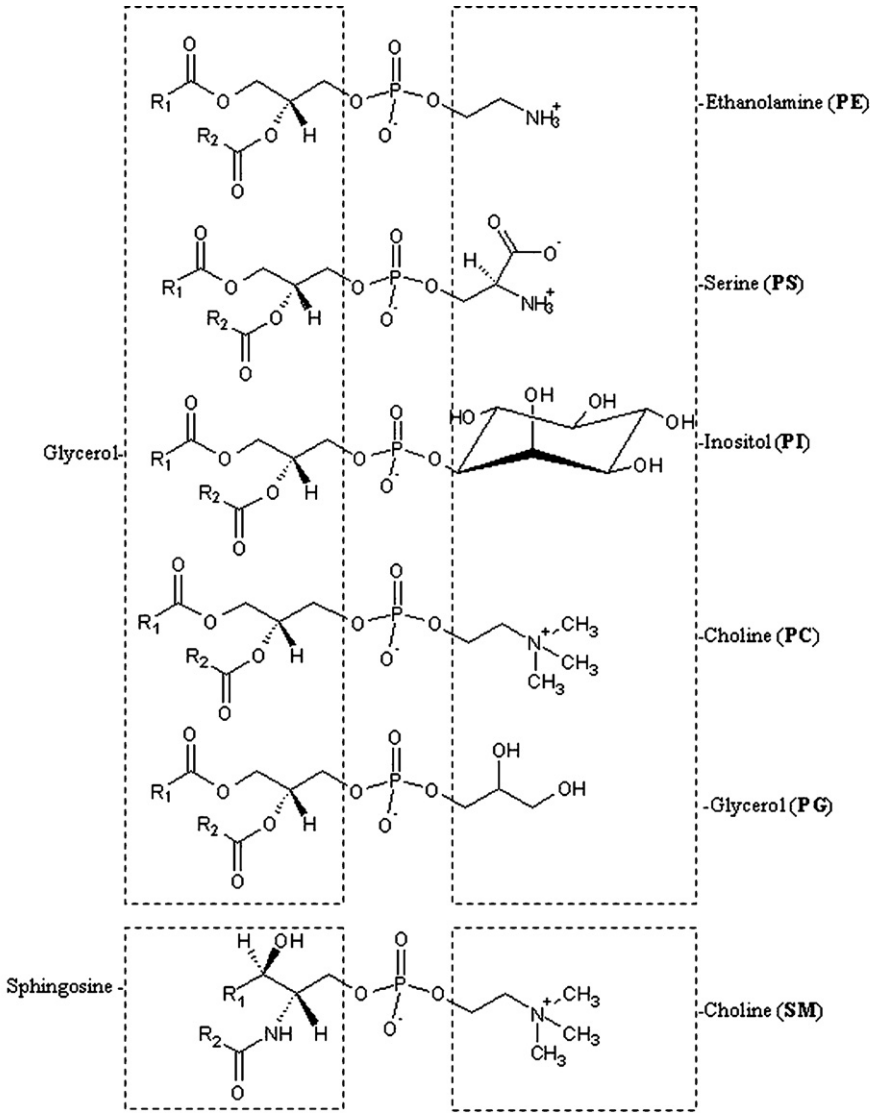

Fig. 1. Molecular structure of PLs. Each class has characteristic polar head and varying fatty acids chains R_1 and R_2. LPCs (not shown) have similar structure as PC with one fatty acids chain less. Under acidic condition, the choline-containing phospholipids such as sphingomyelins (SMs), phosphatidylcholines (PCs), phosphatidylethanolamines (PEs) and lyso-phosphatidylcholines (LPCs) are either positively charged or neutral depending on the actual pH. Phosphatidylglycerols (PGs), phosphatidylinositols (PIs) are neutral at low pH and become negatively charged at high pH and phosphatidylserines (PSs) are anionic lipids.

as mobile phase systems in the past 30 years. Some limited work though is reported on the use of chloroform ($CHCl_3$) as important mobile phase constituent [32], but due to the effects on (public) health, these systems have not been further evaluated in this study and the use of either ACN or IPA based solvents are the preferred choice. Some of the PLs are charged molecules and may require a counter ion in solution for efficient solution by RPLC system. Whereas sulphuric and phosphoric acid have been used for PLs as pH modifiers in liquid chromatography using ultraviolet (UV) detection, they are incompatible with evaporative light scattering detection and mass spectrometry (MS) detection [33–35]. Organic pH modifiers/buffers such as triethylamine, acetic acid, formic acid (FA), ammonia, ammonium formate (AmFm) and ammonium acetate (AmAc) are commonly applied [36–38]. Various modifications to silica like diol, nitrile, nitro, methyl, phenylcyano or phenylsulphonate substituent groups have been reported for PL separation. Each of these stationary phases offer specific selectivity.

Both RPLC and NPLC systems, with a variety of detectors, have been reported in the literature for separations both between PL classes and within PL class. Still, both have their limitations for separation of PLs. With NPLC, PL class separation was achieved but retention time shifting has been reported; this may be due to solvent mixing effects, slow equilibration of the stationary phase or may be due to phase separation since the mobile phase mixtures used often contain water and a water-insoluble solvent [36,39–40]. With RPLC, different compounds within the various PL classes were separated successfully but between-class separation was not obtained completely [41,42]. As a result, ion suppression of lipids of a certain class can occur due to presence of lipids of another class [43]. Therefore, the aim of the current study is to develop a separation method which can provide better class separation.

hydrophilic interaction liquid chromatography (HILIC) is highly capable of separating polar and hydrophilic compounds. In HILIC mode, the retention of solutes is increased if the percentage of organic solvent is increased. The high composition of organic solvent in the mobile phase which is required to retain polar compounds by HILIC has a positive effect on the ionization efficiency in ESI [44]. Therefore, HILIC coupled with ESI-MS has been widely used to measure polar compounds such as peptides, amino acids, sugars, proteins, nucleic acids and monosaccharides [45–51]. Up to

now, HILIC applications to separation of PLs were sparsely reported. The separation of a mixture of PCs, PEs and PIs on an amino column by gradient elution was reported [52]. Using a silica gel column and IPA–ACN mobile phase system, Zheng et al. [53] successfully separated lipid extracts from *Leishmania donovani*. Schwalbe-Herrmann et al. [54] separated at least four classes of PLs with isocratic elution in HILIC mode using a silica-based HILIC method. However, gradient elution usually gives a faster overall analysis, narrower peaks and better resolution without loss in linearity and repeatability compared with isocratic elution [55]. Therefore, a gradient elution HILIC method might be more suitable and efficient for PLs separation.

This paper describes the development, validation and application of a gradient HILIC method using a diol column for separating PL classes. In this paper, a diol column and an ACN–water mobile phase system containing ammonium formate (AmFm) and formic acid (FA) were explored to separate PL classes for, to our best knowledge, the first time. The method was validated to determine several performance characteristics (retention time stability, linearity, intermediate precision and recovery) and subsequently the applicability of the method for the analysis of PLs in various (blood) plasma samples.

2. Experimental

2.1. Chemicals and materials

ULC-MS grade IPA, $CHCl_3$, water and HPLC-S grade ACN, methanol (MeOH) were purchased from Biosolve (Valkenswaard, The Netherlands). FA (99+%) was purchased from ACROS OGANICS (New Jersey, NJ, USA). AmFm (≥99.995%) was obtained from Sigma Aldrich (St. Louis, MO, USA). PLs standards of 1,2-Dimyristoyl -*sn*-Glycero-3-[phospho-rac-(1-glycerol)](sodium salt) PG (C14:0/14:0), 1,2-Diheptadecanoyl -*sn*-Glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) PG (C17:0/17:0), 1,2-Dipentadecanoyl-*sn*-Glycero-3 phosphoethanolamine PE (C15:0/15:0), and 1,2-Diheptadecanoyl-*sn*-Glycero-3-phosphoethanolamine PE(C17:0/17:0), 1,2-Diheptadecanoyl-*sn*-Glycero-3-phosphocholine PC (C17:0/17:0), 1,2-Dinonadecanoyl-*sn*-Glycero-3-phosphocholine PC (C19:0/19:0), 1,2-Dimyristo-yl-*sn*-Glycero-3-[phospho-L-serine](sodium salt) PS (C14:0/14:0), 1,2-Dipalmitoyl-*sn*-Glycero-3-[phospho-L-serine](sodium salt) PS (C16:0/16:0), 1-Nonadecanoyl-2Hydroxy-*sn*-Glycero-3-phosphocholine LPC (C17:0), and 1-Heptadecanoyl-2Hydroxy-*sn*-Glycero-3-phosphocholine LPC (C19:0) were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. Instrumentation

An LC–ion trap-MS system (LCQ_{DECA}, Thermo Fisher Scientific, Somerset, NJ, USA) was used for this study. The LC part consisted of a Surveyor autosampler and quaternary MS pump. The ion trap mass spectrometer was equipped with an electrospray ionization (ESI) source for mass spectral detection.

2.2.1. HPLC

PL class separation was performed on a diol column (250 mm × 3 mm I.D., 5 μm particles, pore size 100 Å, NUCLEOSIL 100-5 OH, MACHEREY-NAGEL, Düren, Germany). The column was operated under gradient conditions with mobile phase A: 53 mM FA in ACN (pH ≈ 4.0–4.5) and mobile phase B: 60 mM AmFm and 53 mM FA in water (pH 3.64). The binary gradient program consisted of: 0–2 min: 90% A (and 10% B), 2–15 min: from 90% A to 70% A, 15–17 min: 70% A, 17–17.5 min: from 70% A to 90% A, 17.5–30 min: 90% A. The column temperature was set to 50 °C. The flow rate was 500 μL/min. ACN/H_2O (70:30 (v/v)) containing 53 mM FA was used as washing solvent for syringe and needle flushing using the flush volumes of 2000 μL and 3000 μL, respectively, at a flush flow rate of 200 μL/s. The temperature of the autosampler tray was set to 15 °C. The injection volume was 10 μL.

2.2.2. Ion trap mass spectrometry

The ion source parameters were optimized with a 1 μg/mL standard solution of PC (C19:0/19:0) and chosen as follows: electrospray voltage, −4 kV; capillary temperature, 280 °C; sheath gas flow, 70 psi; auxiliary gas flow, 30 psi; capillary voltage offset, −21 V; tube lens offset, 20 V. Maximum injection time was 150 ms and the number of μscans was 3. All data were collected in the negative ionization mode with a scan range of m/z 400–1000 in centroid mode.

2.3. Preparation of standards

PG (17:0/17:0), PE (17:0/17:0), PC (19:0/19:0), PS (16:0/16:0) and LPC (19:0) were chosen as internal standard compounds. PG (14:0/14:0), PE (15:0/15:0), PC (17:0/17:0), PS (14:0/14:0) and LPC (17:0) were chosen as calibration standard (CS) compounds. They were used for the normalization and calibration of the same PL class. According to the retention time and plasma concentration level, PE standards were used for PIs and PC standards were used for SMs.

The PL reference compounds were accurately weighed and put in a 20 mL glass bottle. The standards mixture was dissolved in IPA/$CHCl_3$ (2:1 (v/v)). The calibration stock solution was prepared at a high concentration level and then diluted subsequently with IPA to a working solution at different concentration levels from C7 to C1 (Table 1). The internal standard solution was prepared at concentration level C4.

2.4. Sample preparation

PLs were extracted from plasma by IPA. This IPA method is a modification of the MeOH method by Zhao and Xu [56]. The IPA method can be summarized as follows: 10 μL of plasma was added to a 1.5 mL Eppendorf safe lock tube. 10 μL of internal standard solution was spiked to the withdrawn plasma. 140 μL of IPA was added and then vortexed for 30 s. After equilibration for 10 min at room temperature, the mixture was centrifuged at 10,000 × g for 5 min at 10 °C. 120 μL of supernatant was taken and mixed with 10 μL of IPA. The extracted samples were stored at −20 °C until analysis.

2.4.1. Sample preparation for method validation

Pooled plasma was prepared from blood samples obtained from healthy individual volunteers and animals (rat plasma from healthy Wistar rats, and mouse plasma from healthy mice). All different biosamples for method validation were prepared in the following way.

The calibration standard solutions were spiked into pooled plasma before extraction. 10 μL of plasma was added to a 1.5 mL Eppendorf safe lock tube. 10 μL of internal standard solution and 10 μL of calibration standard working solution (C0–C7 respectively) were added to the withdrawn plasma. 130 μL of IPA was added and then vortexed for 30 s. After equilibration for 10 min at room temperature, the extract was centrifuged at 10,000 × g for 5 min at 10 °C. 120 μL of supernatant was taken and mixed with 10 μL of IPA. The extracted samples were stored at -20 °C until analysis. So called quality control (QC) samples were prepared at level C4 in the same way. Blank samples were prepared using 10 μL of water instead of 10 μL plasma.

Table 1
Concentration level setting of calibration solution for method validation.

Concentration level (μg/mL)	PC 17:0/17:0	PE 15:0/15:0	PS 14:0/14:0	PG 14:0/14:0	LPC 17:0
C0	0.0	0.0	0.0	0.0	0.0
C1	8.8	3.8	2.9	2.0	5.7
C2	17.5	7.5	5.7	4.0	11.3
C3	35.0	15.0	11.3	8.0	22.5
C4*	70.0	30.0	22.5	16.0	45.0
C5	140.0	60.0	45.0	32.0	90.0
C6	280.0	120.0	90.0	64.0	180.0
C7	560.0	240.0	180.0	128.0	360.0
Stock solution	5600.0	2400.0	1800.0	1280.0	3600.0

* IS solution was prepared at this concentration level.

The calibration standard solutions were spiked into plasma after extraction for validation purposes, viz., determination of recovery. 10 μL of plasma was added to a 1.5 mL Eppendorf safe lock tube. 10 μL of internal standard solution and 140 μL of IPA were added and then vortexed for 30 s. After equilibration for 10 min at room temperature, the extract was centrifuged at 10,000 × *g* for 5 min at 10 °C. 120 μL of supernatant was taken out and mixed with 10 μL of calibration standard working solution (C0–C7). The extracted samples were stored at −20 °C until analysis.

2.5. Method validation

The method was validated by preparing and analyzing samples at three different days (i.e. days 1–3) as described below. All samples were prepared in triplicate and injected in triplicate which creates 9 data points per concentration level. A sequence of QC sample, blank and QC sample were placed every twelve samples in the whole sample list. All signals of detected compounds are calculated as relative responses, i.e. as the response ratio of the peak area of a compound and the peak area of the corresponding internal standard. Per class of compounds 1 internal standard and 1 calibration standard are used to semi-quantify the individual compounds. As is known (data not presented) response of the individual compounds depends as well on the fatty acyl chain length and the number of double bonds present in the acyl chain. However, only a limited number of standards is (commercially) available thus choices needed to be made knowing there could be a deviation in actual levels. We choose for the compounds indicated and evaluating the data obtained from the validation provided acceptable results to continue this work.

2.5.1. Linearity

Samples for the calibration curve were prepared by adding calibration standard working solutions (C0–C7) to pooled plasma. The calibration procedure was carried out in day 1 of the validation. Calibration curves were prepared using all 7 calibration concentration levels and the blank sample (C0), with at each concentration level 9 data points.

2.5.2. Intermediate precision

Intermediate precision was determined for the samples at concentration level C2 (low), C4 (middle) and C6 (high) prepared in triplicates on the three consecutive days. Within-day and between-day precision was determined by relative standard deviation (RSD) which was calculated using analysis of variance (ANOVA). Retention time repeatability was evaluated by comparing the retention times of 10 standards compounds from 247 injections.

In addition, 18 QC samples were used to evaluate precision as well as within- and between-day variation. In each QC sample 18 endogenous PLs were arbitrarily selected to calculate their performance characteristics (retention time, response ratio) using ANOVA.

2.5.3. Recoveries

The recovery was calculated using the following equation:

$$\text{Recovery}\ \ (\%) = \frac{\text{Ratio}_{\text{SBE}} \times 4/3}{\text{Ratio}_{\text{SAE}}} \times 100$$

In which $\text{Ratio}_{\text{SBE}}$ is the response ratio of samples spiked before extraction (SBE). $\text{Ratio}_{\text{SAE}}$ is response ratio of samples spiked after extraction (SAE). 4/3 is a correction for differences in dilution between SBE and SAE. The before extraction calibrants are diluted more than the after extraction calibrants (see Section 2.4.1).

2.6. Data processing

LCquan v2.5 (Thermo Fisher Scientific, Somerset, NJ, USA) was used for the determination of the peak areas in the reconstructed ion chromatograms of the characteristic *m*/*z* of each PL. The parameters for the integration were peak detection algorithm, ICIS; smoothing points, 7; window, 30 s; view width, 2 min; baseline parameter, 100; area noise factor, 5; peak noise factor, 10. Prior to data analysis, all PL peaks were normalized. The response ratio of each PL was calculated by division of its peak area by the peak area of its corresponding internal standard. On the basis of similar retention and similar concentration level, the internal standards for each class are respectively: PG (C17:0/17:0) for the PGs, PE (C17:0/17:0) for PEs and PIs, PC (C19:0/19:0) for the PCs and SMs, PS (C16:0/16:0) for the PSs and LPC (C19:0) for the LPCs. Statistical data analysis and data visualization were carried out using Microsoft Office Excel 2007 (Microsoft, Redmond, WA), Matlab version 2010b (Mathworks, 2010) and R version 2.10.1 (The R Foundation for Statistical Computing 2009).

3. Results and discussion

3.1. Method optimization

Two methods for extraction of lipids from plasma were compared, extraction by MeOH and extraction by IPA. The IPA method was a modification of the MeOH method by Zhao and Xu [56] and was compared with that MeOH extraction as reference. Since IPA has a lower polarity than MeOH, theoretically, IPA should have higher extraction efficiency for more apolar PLs. The peak areas of two selected compounds in each class were compared to evaluate the relative difference on extraction efficiency between the IPA method and the MeOH method. Fig. 2 shows the relative difference of peak areas for the different PLs. Compared to the original MeOH extraction the IPA method has a higher extraction efficiency for low abundant apolar PLs such as PGs (25%+ higher) and PEs (20%+ higher). For LPCs the extraction efficiency for the extraction by IPA was about 10% lower compared to MeOH extraction. However, since LPC concentrations are relatively high in plasma this did not influence successful detection. Because of the better performance on low abundant PLs the IPA method was considered more

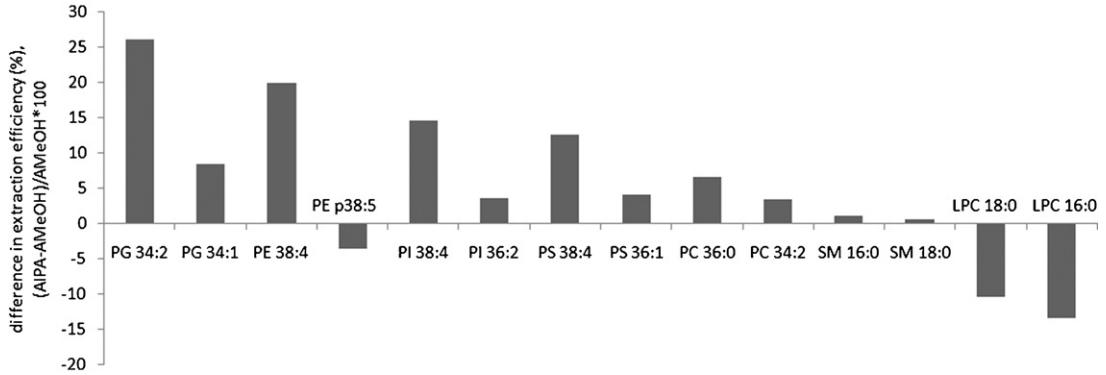

Fig. 2. The relative difference of peak areas of PLs obtained by LC–MS after extraction from human plasma by IPA compared to extraction by MeOH. Two endogenous compounds per PL class were evaluated.

suitable than the MeOH method for profiling of phospholipids in plasma.

Electrospray ionization (ESI) MS was used for detection. Negative ion electrospray was chosen because it gives more information-rich data with lower noise and background than positive ESI [36]. In negative ion mode, PGs, PEs, PIs, and PSs were detected as $[M-H]^-$ ions. PCs, SMs and LPCs were detected as formate adducts $[M+HCOO]^-$ ions due to the presence of AmFm in mobile phase. Actually, formate was added to the mobile phase as the uniform counter ions to yield the formation of $[M+HCOO]^-$ for electrical neutral lipids such as PCs, LPCs and SMs. Alternatively, AmAc can be used as buffer salt, but in that case the choline-containing PLs were to be quantified as the sum of the peak area of $[M-CH_3]^-$ and acetate adducts $[M+CH_3COO]^-$ ions, where the latter one, mostly is more intensive [53]. However, using the sum of two ions for one compound is expected to reduce the reliability of quantification. Therefore, AmFm was chosen as modifier for PLs separation and quantification using electrospray MS detection in negative ion mode.

The aim was to develop an efficient separation of phospholipid classes using gradient elution on a HILIC column. A diol column was chosen because of its water stability and long performance life [57]. The mobile phase system was chosen to be acetonitrile–water using AmFm and FA as modifiers. The higher organic solvent composition in the eluent resulted in more retention of polar compounds in HILIC. Actually, the first gradient explored started with 90% of ACN (containing 53 mM FA) and 10% of water (containing 53 mM FA and AmFm). During development of the method, we found that the AmFm concentration in aqueous phase plays a very important role in the separation of PLs. As discussed below, finally 60 mM AmFm was added to the aqueous phase because a better peak shape and sensitivity, especially for PSs, was obtained.

3.1.1. *Effect of AmFm amount on chromatographic behavior of PLs*

The result of the current study reveals that the concentration of AmFm highly influenced the peak shape and chromatographic behavior of PLs. We compared different AmFm concentrations (10 mM, 20 mM, 50 mM) in the aqueous phase only. It was found that all PLs benefited from a higher AmFm concentration as the peaks became sharper, i.e., the peak widths at 10% peak height decreased for the majority of PLs by 0.02 min (i.e. >6%). The effect was most prominent for PSs. The peak shape of PS (16:0/16:0) was dramatically improved as shown in Fig. 3. The retention times of negatively charged lipids (PG, PI and PS) were nonlinearly reduced by increase of AmFm amount. No retention changes for neutral lipids (i.e. PE, PC, SM and LPC) were observed.

Although several benefits are observed, it is difficult to interpret the exact interaction effects of AmFm on the separation of PLs. At higher pH the lipids, PLs apart from the PGs and PIs may become more charge neutral since the initial formic acid based mobile phase conditions will ensure that the amine groups are all fully charged; and at low pH and with high organic solvent content the phosphate and carboxyl groups might be only partially ionized. Raising the pH by increasing addition of AmFm will make the most of the lipids move towards a more charge neutral state. Peak broadening is generally produced where there is an equilibrium between ionized states. Somehow the higher concentration of ammonium creates stronger buffering thus less variation in the ionized states of the molecules.

The possible mechanism of the separation of PLs on a diol-bonded silica column in HILIC mode should be separately described for neutral lipids and negatively charged lipids. The elution behavior of neutral phospholipids depends on the molecular polarity. The elution of negatively charged phospholipids in HILIC-Diol mode is more complex. For PG and PI lipids, we just see that the retention times are reduced and the peak shape have the similar

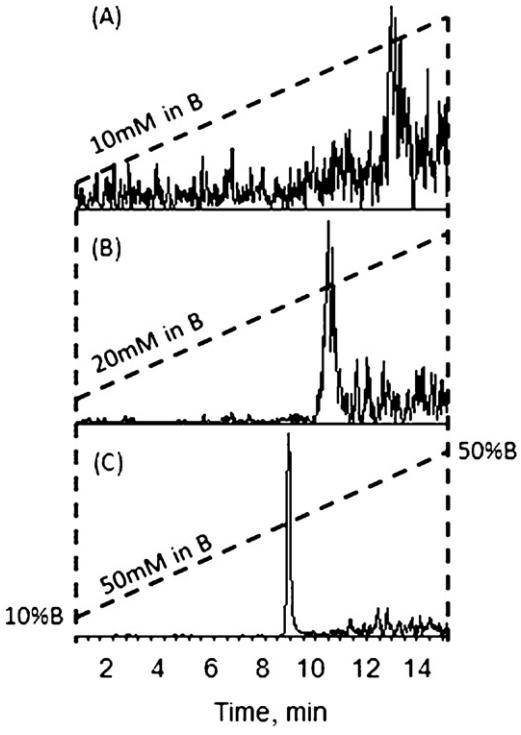

Fig. 3. The reconstructed ion HILIC–MS chromatogram of PS (16:0/16:0) obtained for increasing AmFm concentration. (A) 10 mM; (B) 20 mM; (C) 50 mM.

behavior with the neutral lipids. Elution of the PG and PI lipids, which will go from electrically neutral to negatively charged during elution, is attributed to hydrophilic interaction. Whereas for negatively charged PSs, the prominent improvement of peak shape and variation of retention reflect the elution behavior of PSs are more complex. An aqueous enriched layer is formed at the stationary phase, starting with OH groups on the surface of the stationary phase up to a certain distance from the surface, which can retain hydrophilic molecules. This water enriched stationary layer and organic solvent enriched mobile phase have different solvency for phospholipids. The changing polarity of the mobile phase, induced by the AmFm gradient caused elution of PLs. At the beginning of the gradient, just after injection, the hydrophilic PS species with negative charges prefer the surface water phase above the organic mobile phase. This effect originates from an interaction between PS and the weakly acidic Si–OH on the stationary phase. This interaction decreases during gradient development, due to the competing formate ions originating from the increasing AmFm in the mobile phase and subsequently in the stationary water enriched phase. Therefore the PS lipids were eluted more easily using the mobile phase containing relatively high AmFm. It is more likely that the amine groups will interact and this is why the PI and PG lipids elute earliest and the PCs and LPCs latest. Accordingly, the separation of negatively charged PLs likely depends on combined effect of hydrophilic interaction and electrostatic (ionic) interactions.

It is assumed that there is a characteristic AmFm concentration at which the PSs are almost entirely extracted. Under the gradient of 14 min using mobile phase B containing 50 mM AmFm, we calculated the concentration of AmFm in water phase as C_{A50} shown in Supplementary data, Table S1. In order to get more class resolution and individual resolution, we adapted the gradient to start with 10% B at 2 min and to end with 30% B at 15 min. For this gradient we calculated an optimum AmFm concentration of 60 mM (Supplementary data, Table S1).

The separation of neutral phospholipids with the diol-bonded silica column in HILIC mode was based on the polarity of compounds: the more polar the lipid, the higher was the retention. The elution of the negatively charged phospholipids likely depends on combined effect of hydrophilic interaction and electrostatic interactions.

3.1.2. Peak shape and separation efficiency

Well-shaped peaks are the precondition for a reliable quantification method. The tailing factor (*TF*) was monitored and minimized during the method development. The *TF* values should normally fall between 1.0 and 1.5 for a new column and an optimized method [58].

The *TF* of each reconstructed ion chromatogram peak was calculated with the following formula:

$$TF = \frac{\alpha}{2\beta}$$

In which α is the peak width at 5% of peak height. β is the difference between the retention time at the start of the peak (5% of peak height) and the retention time at the peak maximum.

TF of the reconstructed peaks shown in Fig. 4 ranged from 0.95 to 1.21 indicating that the developed method yields well-shaped peaks that can provide reliable integration data for quantification. Fig. 4 obviously indicates overlapping of subspecies of one family with subspecies of others, in particular if longer and unsaturated FAs are involved. It was noted that several peaks with different retention time were observed in one extracted chromatogram, e.g., PG, PE, PS. This is because that some of subspecies from different classes have the similar *m*/*z* masses in negative ionization mode.

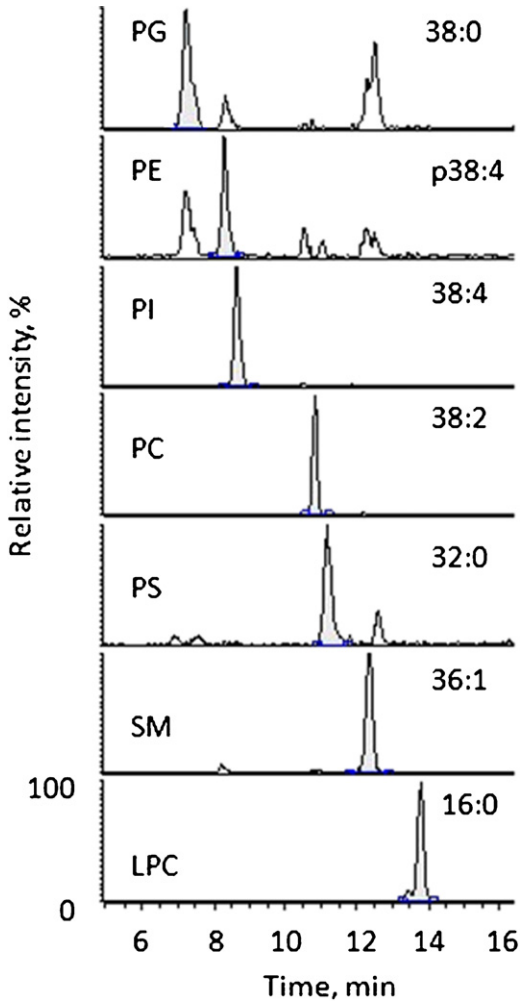

Fig. 4. Peak shape of seven PLs classes. The corresponding tailing factors are as follows: TF_{PG} = 1.03, TF_{PE} = 1.06, TF_{PI} = 1.08, TF_{PC} = 0.98, TF_{PS} = 1.20, TF_{SM} = 1.00, TF_{LPC} = 1.02.

To evaluate the separation efficiency, the chromatographic resolution was calculated using the half-height method [59]:

$$R_s = \frac{2\Delta T_R}{1.7(W_{1/2,A} + W_{1/2,B})}$$

In which ΔT_R is the relative retention time of two adjacent peaks. $W_{1/2,A}$ and $W_{1/2,B}$ are the peak widths of each individual peak at half height.

The observed resolution R_s between the peaks of two adjacent group peaks of the seven different PL classes ranged from 0.73 to 4.97 as obtained from Fig. 5(A). This efficient separation will provide good retention data for qualitative analysis of PL compounds.

3.1.3. Between-class separation of 7 PL classes

The current method allowed the class separation of 7 PL classes using a gradient elution. Fig. 6 depicts between-class separation of plasma PLs and the related mass/retention/intensity map. PGs were firstly eluted, followed by PEs, PIs, PCs, PSs, SMs and LPCs. In the current study, the retention time window of different lipid classes was firstly specified by appropriate lipid standards from different lipid classes. Then, the peaks were extracted according to their retention time and their *m*/*z* masses which are from our in-house lipids library established by our previous study. Based on all relevant information, the results indicate that SM and LPC display two peaks in HILIC lipid profile.

Table 2
Summary on performance characteristics of the developed method. Linearity, repeatability and recoveries.

Calibration standard compounds	Slope	Intercept	R^2	Linearity range (μg/mL)	RSD		Recoveries, %		
					Intra-day	Inter-day	High	Middle	Low
PG 14:0/14:0	0.48	−0.005	0.999	0.035–1.07	<13%	<14%	110.8	108.4	109.1
PE 15:0/15:0	0.40	0.008	0.994	0.24–14.77	<5%	<7%	102.3	101.3	103.5
PC 17:0/17:0	0.21	0.079	0.990	0.39–34.47	<6%	<8%	102.7	106.5	108.3
PS 14:0/14:0	0.71	0.031	0.987	0.22–11.08	<6%	<10%	100.7	105.6	115.5
LPC 17:0	0.29	0.045	0.997	0.12–22.15	<8%	<10%	99.6	102.5	101.4

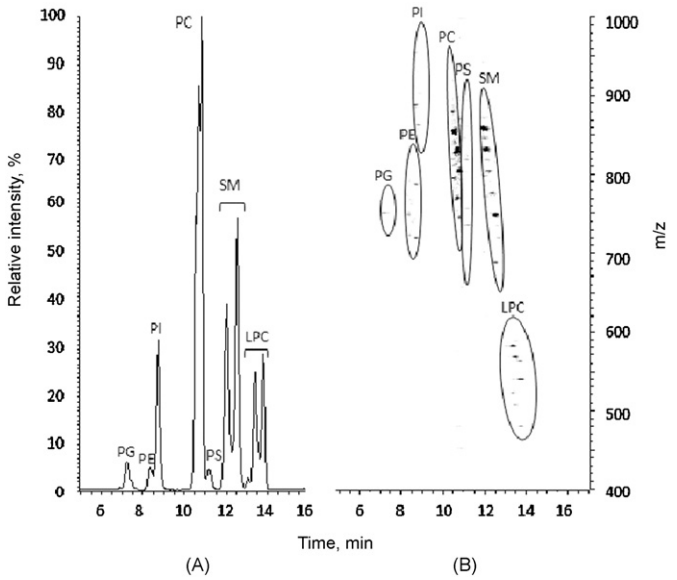

Fig. 5. Base peak chromatogram (A) and retention/mass/intensity map (B) of plasma extracts.

All of PLs were eluted within 14 min. The total run time for one injection was 30 min including column equilibration time. From the elution result, even 10% water in mobile phase still provided good retention of PLs. Although there was a little overlap between PEs and PIs (R_S, 1.06) as well as between PCs and PSs (R_S, 0.73), the class peaks were separated clearly. The retention information of each PL class contributed to the identification of PLs.

3.1.4. Within-class separation of individual PLs

Besides between-class separation, within-class separation of PLs was achieved to a certain extent in HILIC mode. Although the separation resolution is low which is mainly caused by the similar structure of the individual PLs in the same class, their retention differences can reflect the rule for HILIC separation of PLs which can be used as a basis for the molecular identification. In HILIC, the more polar compounds are retained longer. Within the same PL class, the polarity of different compounds depends on the chain length and degree of unsaturation of the fatty acids chains. Fig. 6 depicts the base peaks of three PI compounds (1) C38:4; (2) C36:2; (3) C34:2, three PC compounds (4) C38:4; (5) C36:2; (6) C34:2 and three LPC compounds (7) C18:0; (8) C18:1; (9) C18:2. From these compounds we can observe that (i) the retention time was shortened with the increasing chain length of the compounds and (ii) the retention time was prolonged with the increasing degree of unsaturation of the PL individuals. Although three PI compounds as well as three PC compounds have different degrees of unsaturation, the retention of these compounds increased with the chain length. The results of HILIC separation of PLs are consistent with Schwalbe-Herrmann's [54], indicating PLs have the regular behavior in HILIC mode. Accordingly, we can conclude that the length of fatty acid chain compared with degree of unsaturation is more effective on determining elution order of different PLs within the same class. So the retention of the individuals in the same class should be predicted by the chain length and by the degree of unsaturation when the fatty acid chains have the same length.

3.2. Method validation

Before measurements are performed in a new matrix, a method validation as discussed in Section 2.5 should be performed for that matrix. The next section (Section 3.3) discusses application of the method to rat-, mouse- and human plasma. In this section we limit ourselves to the validation of the method for application in human plasma. Results obtained for the other matrices are comparable. The samples were prepared as described in Section 2.4.1.

For all PLs investigated there were no significant shifts in retention time (RSD < 1%, a graph of retention times versus injection order is available as Supplementary material). Table 2 summarizes the validation results. All those results were based on response ratios calculated as described in Section 2.5.

The linearity results were obtained by weighted least squares with $1/s^2$ as weighing factor (s is the standard deviation on the 9 replicates at each level). For all classes except PG the method was linear over the complete calibration range (C0–C7) (Table 1). For PG the high levels (>C4) gave a lower response. This lower response is probably caused by detector saturation. The slope, intercept and

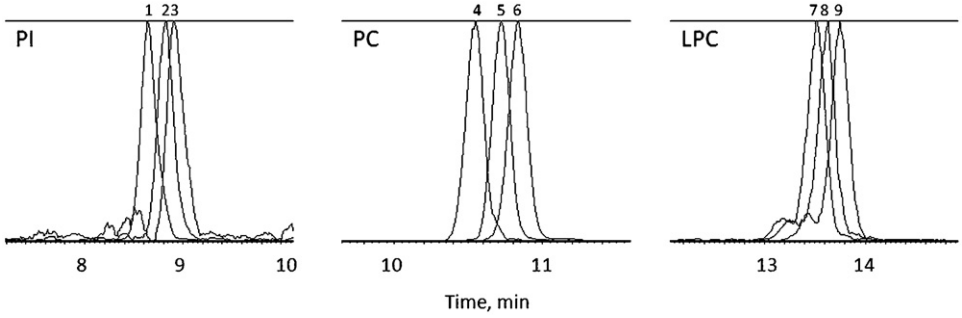

Fig. 6. Within-class separation of PLs. PIs: 1, C38:4; 2, C36:2; 3, C34:2. PCs: 4, C38:4; 5, C36:2; 6, C34:2. LPCs: 7, C18:0; 8, C18:1; 9, C18:2.

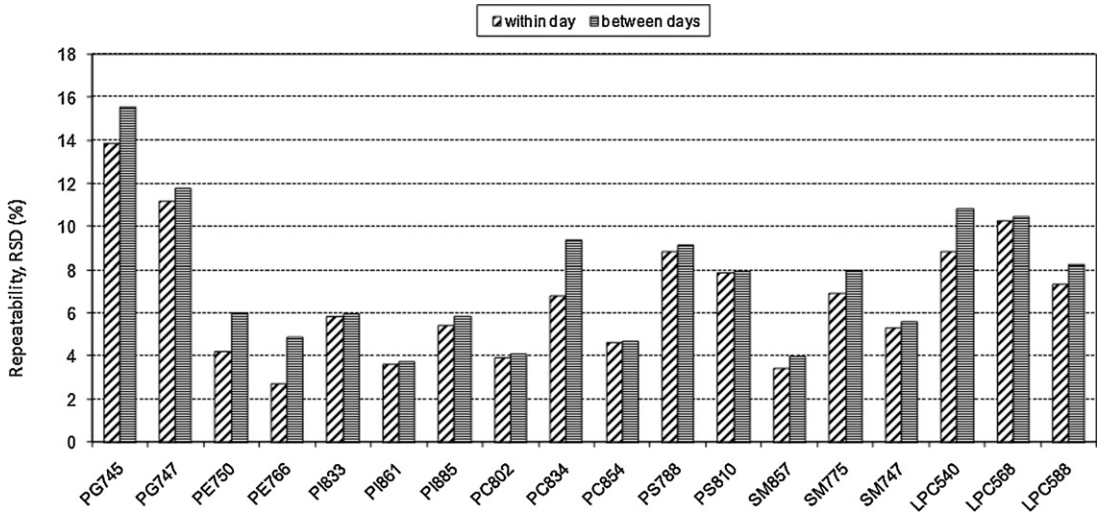

Fig. 7. Within-day and between-day RSDs of 18 selected endogenous compounds. The phospholipids were randomly selected, taking lipids of varying abundance (low, high, and middle) and various PL classes.

correlation coefficients (R^2) of the regression equations of calibration curves are presented in Table 2.

The within-day repeatability and between-day variation were calculated as the mean ratios of the peak area of calibration standards (non-endogenous) and the selected PL species (endogenous) from human plasma sample and the peak area of the corresponding internal standard spiked to the plasma sample prior extraction. The RSDs were calculated using ANOVA. The within-day RSD ranged from 5% to 13% for calibration standards (Table 2) and from 3% to 14% for the selected species (Fig. 7). The between-day RSD ranged from 7% to 14% for calibration standards and from 3% to 14% for the selected species. The relatively highest within- and between-day RSDs were found for PGs, but an RSD of 15% or less was considered acceptable for PLs profiling.

Recoveries were calculated as the ratio of the peak area of the PL compound in the sample spiked before extraction and in the samples spiked after sample preparation. The recoveries determined at three different levels ranged from 99.6% to 115.5%. Both recoveries and RSDs were considered acceptable for sample analysis from complex biological matrices indicating the combination of the IPA extraction method and the HILIC separation method is appropriate for quantification of PLs. Further work will focus on the application of this PLs profiling method to study diseases associated with PLs metabolism. The next section shows the application of this method for the separation of PLs in extracts from different bio-samples.

3.3. Application of the method

With the developed method, the PL extracts of human plasma, rat plasma and mouse plasma were separated successfully. In Fig. 8, the base peak chromatograms of all three samples detected in negative ionization mode are shown. The separations showed a very good resolution between the various PL classes. The peaks of PGs, PEs, PIs, PCs, PSs, SMs and LPCs were present in chromatograms of all extracts from different plasma. The intensity of PG is so strong in Fig. 8 that's because the samples were spiked with PLs standard including PG. Next as PC's are quite abundant in plasma and they all more or less co-elute they will show up in the total ion chromatogram as a relatively high peak. The presence of multiple peaks for PCs is caused by within-class separation of the individual PLs in PC class. PC is the most abundant PL class in plasma. Many of the individual PLs in PC have relatively high intensity. Therefore, we can see the multiple peaks for PC in Fig. 8. The peaks of each class show differences in different chromatograms, indicating PL components of different biological samples differ from each other.

For a further study, we will apply this method to investigate some specified disease associated with PLs metabolism.

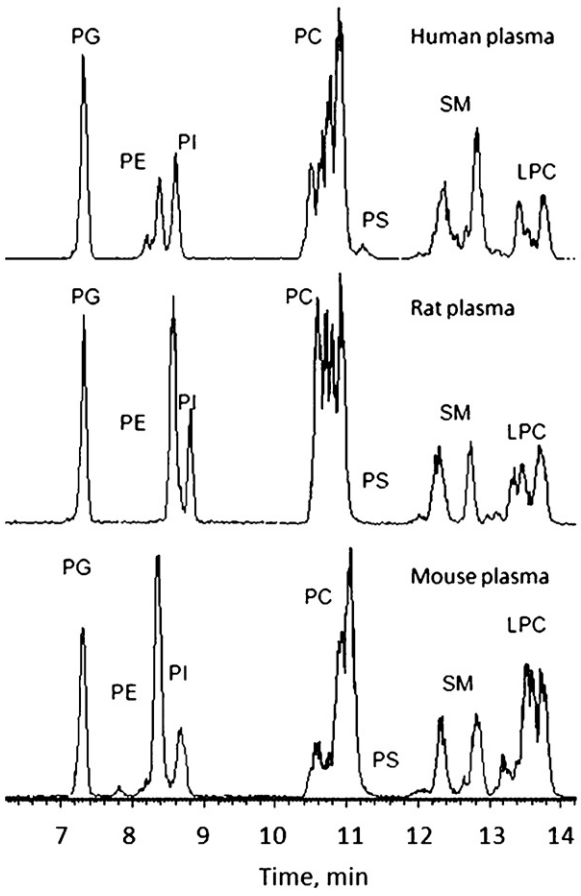

Fig. 8. Base peak chromatograms of PLs extracts of human plasma, rat plasma and mouse plasma by HILIC–ESI/MS.

4. Conclusions

HILIC is very promising for separation of PLs. A gradient HILIC method for plasma PL class separation was developed successfully. An efficient between-class separation of seven PL classes as well as within-class separation of PL individuals was achieved in HILIC mode by using a diol column and an ACN–water mobile phase system with AmFm as additive in the aqueous phase. The relatively high amount of AmFm in aqueous mobile phase is beneficial to well-shaped peaks of PLs, especially PSs, without adverse impact on ESI-MS detection. The developed method was fully validated. The satisfactory performance characteristics indicated the gradient HILIC method was a stable method for identification and quantification of PLs in a variety of plasma samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.11.034.

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