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PAPER

Optimized extraction of phospholipids and lysophospholipids for nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry†

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The efficiencies of four different methods for the extraction of phospholipids (PLs) and lysophospholipids (LPLs) from human plasma samples were examined by comparing extraction recovery values using nanoflow liquid chromatography-electrospray ionization-mass spectrometry (nLC-ESI-MS). For recovery measurements, six PL and six LPL standards of different head groups were spiked into a human plasma sample, and the peak areas of each individual species after extraction were measured from the chromatograms of the nLC-ESI-MS runs. Recovery was calculated by comparing the peak area of an extracted standard species with that of the same species' spike after extraction of the same plasma sample. For lipid extraction, four different extraction methods were examined: three based on the Folch method with different organic solvents such as $CHCl_3$, methyl-*tert*-butyl ether (MTBE), and MTBE/CH_3OH, and one relatively fast method involving CH_3OH only. Evaluations of recovery showed that the modified Folch method with MTBE/CH_3OH proposed in this study was effective for extracting most PL and LPL standards. Then, the four extraction methods were compared with the identified numbers of plasma PLs and LPLs, of which molecular structures can be confirmed by data-dependent, collision-induced dissociation experiments during nLC-ESI-MS-MS. These results demonstrated that the proposed method yielded the identification of 54 LPLs and 66 PLs from a plasma sample, which was the highest identification rate among the four methods.

1. Introduction

Over the past decade, lipidomics has advanced to a more prominent position in science as its applications to various types of human diseases, ranging from common adult diseases such as diabetes to more severe forms of cancer,[1,2] have become evident. Phospholipids (PLs) inside the human body are crucial players with the ability to control and serve multiple cellular functions and responses.[3,4] Numerous combinations of PLs, varying in the types and numbers of atoms included and in the lengths of their aliphatic chains, integrate to form a complex network of cellular lipids that are collectively referred to as a lipidome. With an infinite number of PLs, each having its own unique chemical and physical compositions, the further development in lipidomics is inevitable, especially when coupled with the advanced technology of mass spectrometry (MS). The fact that lipids are signaling molecules has stimulated a great deal of research to identify potential biomarkers of specific diseases. Among the different subclasses of lipids, lysophospholipids (LPLs) and PLs are often recognized as potential biomarkers of human diseases such as breast cancer,[5–7] ovarian cancer,[8,9] and prostate cancer.[10,11]

Intact PLs have been analyzed with simple thin layer chromatography (TLC) in conjunction with detection methods such as densitometry[12] or matrix-assisted laser desorption and ionization/time of flight mass spectrometry (MALDI-TOF/MS).[13] With rapid advances in the MS technique due to the adoption of electrospray ionization (ESI), the direct ESI-MS of intact PLs[14–17] or liquid chromatography (LC) coupled with ESI-MS[18–21] has expanded the scope of lipidomics toward efforts to discover potential biomarkers of adult diseases. Nanoflow LC-ESI-tandem MS (or nLC-ESI-MS-MS) is capable of separating PL and LPL species from tissue or urine samples at a detection limit of 2.2 fmol, as well as simultaneously characterizing molecular structures from fragment ion spectra.[22–24]

Depending on the objectives and types of targeted lipidomics, different protocols of lipid extraction may be followed, but one thing they have in common is that before any analytical approach is implemented, complete and successful extraction of targeted lipids from biological samples must be ensured in order to secure the validation of data. Therefore, the degree of recovery of the lipids is strongly associated with the optimization of results. Cellular lipids can be extracted from multiple sources including tissue, blood, and urine, in which an abundance of non-lipid groups such as proteins and sugars are present together. For the extraction of phospholipids, a number of methods have been

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utilized since the late 1800s.[25] Folch developed a lipid extraction method employing a mixture of chloroform and CH_3OH (2 : 1, v/v), which remains the most commonly and universally used technique.[26] Bligh and Dyer developed a simple and rapid method for the extraction of lipids that deployed a reduced solvent to sample ratio compared to the Folch method.[27] Most of the more recently developed protocols were derived from these classical methods; a few modifications were made to increase the recovery of lipids or to speed up tedious and time-consuming processes. Instead of chloroform, which has a relatively high toxicity for humans and the environment, methyl-*tert*-butyl ether (MTBE) is frequently used as a replacement due to its less severe health risks and ability to provide the same or better recovery of certain lipids.[28] Another method developed by Xu and Zhao involves only a single solvent, CH_3OH, instead of two as the classical methods.[29] The use of a single solvent eliminates phase separation, which facilitates a much quicker process while retaining high lipid recovery. In studies previously published by our laboratory using nLC-ESI-MS-MS, the classical Folch method using chloroform was used to extract lipids from tissue, cells, urine, and plasma;[22–24,30–32] however, the Folch method has often been criticized for its relatively low yield of lysophosphatidyl acid (LPA) and phosphatidyl acid (PA).

In the present study, we examined the efficiencies of known extraction methods, analyzing human blood samples spiked with typical PL and LPL standards using nLC-ESI-MS-MS, and identified an optimized extraction method for an improved simultaneous profiling of LPLs and PLs by combining and modifying two existing methods. Extraction efficiency was calculated by measuring the peak areas of the standard PL and LPL species run by nLC-ESI-MS, and recovery was estimated by comparing the peak areas of standards spiked into human plasma samples before and after lipid extraction. The results were analyzed further to compare the numbers of PL and LPL species identified from the plasma samples according to the different extraction methods.

2. Experimental

2.1. PL standards and plasma samples

Fourteen PL standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA): 18:0/18:0-phosphatidyl acid, 16:0/16:0-phosphatidylglycerol (PG), 15:0/15:0-PG, 16:0/16:0-phosphatidylserine (PS), 16:0/18:2-phosphatidylinositol (PI), 13:0/13:0-phosphatidylcholine (PC), 16:0/16:0-PC, 16:0/16:0-phosphatidylethanolamine (PE), 18:0-lysophosphatidyl acid, 18:0-lysophosphatidylglycerol (LPG), 18:0-lysophosphatidylserine (LPS), 18:1-lysophosphatidylinositol (LPI), 16:0-lysophosphatidylcholine, and 18:0-lysophosphatidylethanolamine. PLs and LPLs with fatty acyl chain lengths of 16 to 20 were selected specifically in order to assess whether extraction works regardless of biological matrix effects. The PL standards were dissolved in a solution of $CHCl_3$: CH_3OH (1 : 1, v/v), whereas the LPLs were dissolved in a slightly different solution of water : $CHCl_3$: CH_3OH (2 : 2 : 6, v/v/v). Both were diluted to desired concentrations in MeOH : CH_3CN (9 : 1, v/v). All standards except for 15:0/15:0-PG and 13:0/13:0-PC were mixed to a concentration of 25 pmol mL^{-1} and stored in a refrigerator at −20 °C.

Eight plasma samples were prepared to test the four extraction methods simultaneously. Four plasma samples were spiked with PL standard mixtures before extraction and the other four samples were spiked after extraction to serve as reference material. Blood plasma from a healthy individual was obtained with the help of Severance Hospital (Seoul, Korea) under informed consent. For each of the first four samples, 50 μL of plasma were placed in a 2 mL centrifuge tube, spiked with 15 μL of the PL standard mixture, and then diluted to a total volume of 1 mL with 0.1 M Tris buffered saline (TBS) solution, which was prepared with 4.0 g sodium chloride, 1.5 g Trizma base, and 0.1 g potassium chloride in a total 5.0 L of deionized water and adjusted to pH 7.4 by HCl. These four samples were tested using each of the extraction methods as explained in the next section. For reference material, another 50 μL of the same plasma was first diluted to a total volume of 1 mL with 0.1 M TBS solution and then extracted using each method. After extraction by each method, the plasma was spiked with 15 μL of the PL standard mixture. All eight samples were dried in a vacuum centrifuge over six hours.

2.2. Extraction protocols

2.2.1. Folch method with CH_3Cl. The extraction of standard PL and LPL species from each plasma sample by the classical Folch method with CH_3Cl was accomplished by adding 300 μL of CH_3OH to the dried powder from the previous sample preparation and briefly vortexing the mixture. After the addition of 600 μL of chloroform, the tube was vortexed for over an hour for incubation. Next, 180 μL of MS-grade water were added to the mixture for a more distinct phase separation and the mixture was vortexed at room temperature for 10 minutes for further incubation. The mixture was then centrifuged at 1000*g* for 10 minutes and the lower organic layer was transferred to a pre-weighed 2 mL centrifuge tube by a micropipette. After the organic phase was dried in a vacuum centrifuge, the extracted and dried lipids were dissolved in 80 μL of $CHCl_3$: CH_3OH (1 : 1, v/v), then diluted in 70 μL of H_2O : CH_3CN (9 : 1, v/v) and 225 μL of CH_3OH to a final concentration of 1 pmol μL^{-1}, and kept at −20 °C.

2.2.2. Folch method with methyl-*tert*-butyl ether. The Folch method with MTBE followed the previous procedure for the Folch method with CH_3Cl, except that the 600 μL of chloroform were replaced with 1000 μL of MTBE after the initial addition of 300 μL of CH_3OH to the dried powder.[28] The rest of the process was the same as the previous method.

2.2.3. Extraction with CH_3OH. Just after the initial addition of 300 μL of CH_3OH to the dried powder, the mixture was vortexed and then placed in an ice bath for 10 minutes, followed by centrifugation at 10 000*g* for five minutes. The supernatant was pipetted and transferred to a pre-weighed 2 mL centrifuge tube to continue drying in a vacuum centrifuge. The dissolution and dilution of the dried extract were the same as the last step in the previous methods.

2.2.4. Modified Folch method with MTBE/CH_3OH. After the initial addition of 300 μL of CH_3OH to the dried powder, it was vortexed briefly and then placed in an ice bath for 10 minutes.

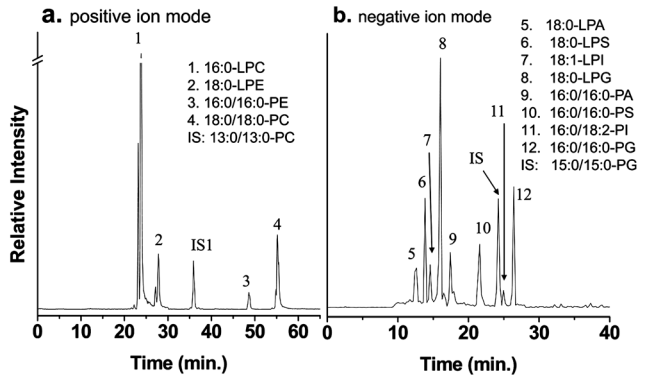

Fig. 1 Extracted chromatograms of six LPL and six PL standards spiked into a human plasma sample obtained at (a) positive and (b) negative ion modes of nLC-ESI-MS-MS.

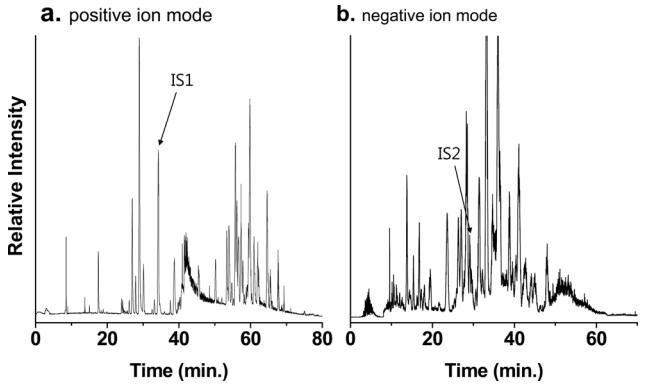

Fig. 2 Extracted chromatograms of extracted LPLs and PLs from 20 μg of human plasma resulting from the modified Folch method of MTBE/CH_3OH at (a) positive and (b) negative ion modes of nLC-ESI-MS-MS. Two internal standards (IS1 as 13:0/13:0-PC for positive ion mode and IS2 as 15:0/15:0-PG for negative ion mode) were added to the plasma extract.

Next, 1000 μL of MTBE were added to the mixture as it was described in Section 2.2.2 and it was vortexed for an hour. Then, 250 μL of MS-grade water were added and the resulting mixture was vortexed at room temperature for 10 minutes. Centrifuging at 1000*g* for 10 minutes formed a distinct phase separation, and the upper organic layer was transferred to a different centrifuge tube. Three hundred microlitres of CH_3OH were added to the lower aqueous layer and left in a shaker for 10 minutes. The lower phase was sonicated with a tip for 2 min and then centrifuged to collect the supernatant. The supernatant was combined with the previously collected upper organic layer and then dried in a vacuum centrifuge. The dissolution and dilution of the final dried extracts were the same as the procedures used in the previous methods.

2.3. Analysis by nLC-ESI-MS-MS

An LCQ Deca XP MAX ion trap mass spectrometer from Thermo Finnigan (San Jose, CA, USA), equipped with a 1200 capillary pump system including an autosampler from Agilent Technologies (Palo Alto, CA, USA), was used to analyze the recovery of lipids from each sample. For analyzing LPL and PL from plasma samples, an LTQ Velos ion trap mass spectrometer from Thermo Finnigan was used. A fused silica tubing (75 μm i.d., 360 μm o.d.) was melted with a torch and then pulled by hand in order to make a cone-shaped emitter end. After it was packed with reversed-phase C18 resin beads, 3 μm to 100 Å (Magic beads; Michrom Bioresources Inc., Auburn, CA, USA), it was cut into a length of 85 mm and used as an LC column. A capillary with 50 μm i.d. was directly connected to the packed column from the pump, using Pt wire for an electrical source, and a vent capillary tube (20 μm i.d., 360 μm o.d.) *via* a PEEK microcross, which was purchased from Upchurch Scientific (Oak Harbor, WA, USA). Two different composites of mobile phase solutions were utilized to load and analyze the samples. Solution A was composed of H_2O : CH_3CN (9 : 1, v/v) and B was isopropanol : acetonitrile (9 : 1, v/v). For both solutions, modifiers of 0.1% formic acid and 0.05% NH_4OH were added for positive and negative ion modes, respectively. An on–off valve was used at the end of the vent tube as a switch between the two flow modes, split and inject. During sample loading, a flow rate of 0.3 μL min^{-1} was applied from the pump to the packed column

Table 1 Percent recovery values ($n = 3$) of standard PLs and LPLs among different extraction methods. The internal standards were 13:0/13:0-PC and 15:0/15:0-PG in the positive and negative ion modes, respectively

PLs and LPLs	Retention time/ min	*m/z*	Folch with $CHCl_3$	Folch with MTBE	Only with CH_3OH	Modified Folch with MTBE and CH_3OH
(a) Positive ion mode						
16:0-LPC	23.8	496.0	78.7 ± 0.9	83.5 ± 0.5	74.5 ± 0.6	84.1 ± 0.6
18:0-LPE	27.8	481.9	79.9 ± 1.3	62.3 ± 2.1	93.5 ± 1.2	83.8 ± 0.4
16:0/16:0-PE	48.6	692.0	93.9 ± 0.7	66.8 ± 1.5	67.3 ± 1.3	72.1 ± 1.9
18:0/18:0-PC	55.2	790.3	99.6 ± 1.1	70.8 ± 1.7	95.0 ± 0.9	101.1 ± 0.9
(b) Negative ion mode						
18:0-LPA	12.7	437.1	58.5 ± 3.2	105.8 ± 2.0	81.6 ± 3.1	98.9 ± 4.0
18:0-LPS	13.9	524.0	60.4 ± 1.5	84.9 ± 2.6	95.9 ± 1.4	97.1 ± 5.9
18:1-LPI	14.6	597.1	77.9 ± 1.2	81.4 ± 1.0	88.4 ± 4.2	86.6 ± 3.1
18:0-LPG	16.0	511.2	74.6 ± 4.4	70.5 ± 0.4	97.8 ± 1.5	99.4 ± 1.9
16:0/16:0-PA	17.4	647.2	65.1 ± 5.6	100.2 ± 0.8	71.9 ± 2.0	98.4 ± 2.5
16:0/16:0-PS	21.6	734.3	86.0 ± 3.6	95.2 ± 2.2	92.9 ± 4.1	99.8 ± 2.9
16:0/18:2-PI	25.0	833.3	76.9 ± 1.9	91.6 ± 0.7	85.3 ± 1.6	84.4 ± 1.1
16:0/16:0-PG	26.4	721.4	103.9 ± 1.4	77.0 ± 3.2	102.8 ± 2.8	102.2 ± 1.9

for 14 minutes with solvent A. After 14 minutes, the valve was switched to the split mode, where a total flow of 6 μL min^{-1} was pumped out of the system while the column had a constant flow rate of 0.3 μL min^{-1} and the venting tube of 5.7 μL min^{-1}. A binary gradient elution was applied to both positive and negative ion modes to analyze the LPLs and PLs from the sample. In the negative ion mode, mobile phase B was increased to 55% over 1 min, 60% over the next 14 min, 100% over the following 10 min, and then was maintained at 100% for 35 minutes. In the positive ion mode, mobile phase B was ramped to 40% over 1 min, linearly increased to 100% over 69 min, and then maintained at 100% for 40 min. For ESI, 4.0 and 3.5 kV were applied for the analysis of PLs in negative and positive ion modes, respectively, at a normalized collision energy of 40%. Mass ranges were 350 to 950 amu for the negative and 400 to 900 amu for the positive ion mode. For a qualitative purpose, the data-dependent MS/MS analysis mode was used in all four extraction method runs. However, a full-scan mode was applied to quantify LPLs and PLs and the peak area of each species was calculated from the extracted chromatogram. Peak areas of each LPL and PL standard were calculated and divided by those of the corresponding internal standards from each run. Recoveries of each lipid species were calculated with the assumption that the peak area of each standard species spiked after extraction from diluted plasma was 100%.

2.4. Identification of plasma PLs and LPLs

To demonstrate whether the modified Folch method with MTBE/CH_3OH works better for the identification of real PLs and LPLs when the method is applied to a biological sample, the extraction was performed using human plasma to identify and compare the number of species extracted. Four 20 μL aliquots of a healthy donor's plasma (the same as that used in the above section) were dried in a vacuum centrifuge until they became powdery. PLs were extracted from the plasma samples according to the four methods explained previously. Identifications of plasma PLs and LPLs were based on the manual examination of characteristic fragment ions from collision-induced dissociation experiments.

3. Results and discussion

Among the 12 different classes of PLs and LPLs examined in this study, PC, LPC, PE, and LPE were analyzed in the positive ion mode of nLC-ESI-MS and the remaining species (PA, LPA, PS, LPS, PG, LPG, PI, and LPI) were detected in the negative ion mode. Fig. 1 shows the extracted chromatograms of the standard species spiked to human plasma sample from the (a) positive ion mode and (b) negative ion mode of nLC-ESI-MS runs. Depending on the hydrophobicity of the LPL and PL species, retention order appeared in such a way that the less hydrophobic LPLs with shorter acyl chains were eluted earlier than PLs with longer acyl chains. In the positive ion mode of nLC-ESI-MS runs, LPC (peak number 1 in Fig. 1a) appeared with split peaks with the latter peak more abundant, since the regioisomers (lyso/16:0-PC and 16:0/lyso-PC) can be separated in a capillary column with reversed phase due to the difference in geometrical structures, as reported previously.[32] While the positive ion mode of nLC-ESI-MS led to the separation of regioisomers, the negative ion mode of nLC-ESI-MS runs did not show a complete separation of regioisomers of species 5–8 (peaks 5–8 in Fig. 1b) since relatively fast separation conditions were applied. Two internal standards (13:0/13:0-PC for positive ion mode and 15:0/15:0-PG for negative ion mode) having odd-numbered fatty acyl chains, which are not normally found in the human body, were spiked into all of the plasma extract samples containing the 12 target standard species for the

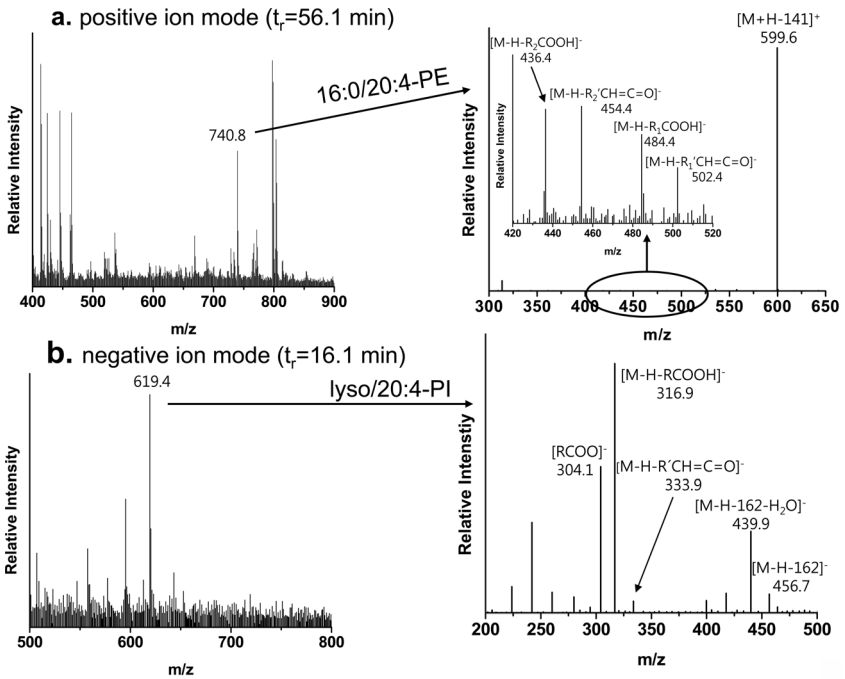

Fig. 3 nLC-ESI-MS spectra at (a) t_r = 54.57 min in the positive and (b) t_r = 16.06 min in the negative ion modes on the left along with CID spectra of each parent ion at *m/z* 740.8 and 619.4 on the right.

Table 2 Comparison of identified PL and LPL species from a healthy human plasma sample among four different extraction methods

			t_r/min			
Class	Molecular species	*m/z*	Folch with $CHCl_3$	Folch with MTBE	Only with CH_3OH	Modified Folch with MTBE and CH_3OH
(a) Positive ion mode						
LPC	lyso/14:0	468.5	22.5	22.7	22.7	23.1
	lyso/20:6	542.4	23.2	23.4	22.9	23.8
	14:0/lyso	468.5	23.4	23.5	23.1	23.9
	20:6/lyso	542.5	23.9	24.1	23.7	24.4
	lyso/22:6	568.4	25.5	25.7	25.2	26.1
	lyso/20:4	544.4	25.6	25.7	25.8	26.1
	22:6/lyso	568.5	26.2	26.3	26.3	26.7
	20:4/lyso	544.4	26.3	26.5	26.4	26.8
	lyso/16:0	496.5	27.1	27.3	27.0	27.7
	lyso/20:3	546.4	27.3	27.6	27.1	28.0
	lyso/18:2	520.3	28.5	28.0	27.8	28.6
	20:3/lyso	546.4	28.6	28.4	28.2	28.7
	16:0/lyso	496.5	28.7	28.9	28.5	28.8
	lyso/18:1	522.5	29.0	29.0	28.6	29.4
	18:2/lyso	520.4	29.0	29.1	28.9	29.5
	18:1/lyso	522.5	29.6	29.6	29.1	30.0
	lyso/18:0	524.5	32.4		32.2	32.9
	18:0/lyso	524.4	33.5		33.2	33.9
LPE	lyso/20:5	500.4			24.5	
	lyso/22:6	526.4	26.9	26.4	26.7	27.4
	20:5/lyso	500.4			26.9	
	lyso/20:4	502.5	27.0	26.6	26.9	27.6
	22:6/lyso	526.4	27.4	27.1	27.3	28.1
	20:4/lyso	502.5	27.5	27.2	27.6	28.3
	lyso/16:0	452.6			29.7	30.5
	16:0/lyso	452.6			30.4	31.1
	lyso/18:0	482.6	34.4	34.5	33.2	34.4
	18:0/lyso	482.6	35.4	35.4	34.1	35.3
PC	14:0/18:2	730.8	51.3	51.2	51.1	52.0
	16:1/18:2	756.9	52.4	52.7	52.3	52.8
	16:0/18:3	756.9	53.5	53.2	53.4	53.8
	16:0/20:5	780.9	53.6	53.5	53.5	53.9
	14:0/18:1	732.8	53.7	53.9		
	18:2/18:2	782.8	53.7	53.8	53.6	54.2
	16:0/16:1	732.8	54.3	54.5	54.2	55.0
	16:0/20:4	782.8	54.8	54.9	54.6	55.3
	16:1/18:1	758.7		55.1	54.9	55.6
	16:0/22:6	806.8	55.0	55.5	55.2	55.7
	16:0/18:2	758.8	55.5	55.6	55.4	56.1
	18:1/22:6	832.8	55.5	55.7		56.2
	18:1/18:2	784.8	56.3	56.1	56.2	56.7
	16:0/20:3	784.9	56.9	57.1	56.8	57.2
	20:2/18:2	810.7		57.0		57.5
	18:0/20:5	808.8	57.3	57.5	57.1	57.6
	16:0/18:1	760.9	58.0	58.1	57.9	58.5
	16:0/22:4	810.7		58.2	58.0	58.7
	18:0/22:6	834.8	58.6	58.8	58.6	59.2
	18:1/18:1	786.8	58.7	58.8	58.6	59.3
	16:0/20:2	786.8		58.8	58.6	59.3
	18:0/18:2	786.8	59.2	59.3	59.2	59.8
	18:0/20:4	810.8	60.5		59.5	60.0
	18:0/20:3	812.8	60.7	60.8	60.6	61.2
	18:0/16:0	762.8	60.7	60.9	60.6	61.4
	18:0/18:1	788.9	61.5	61.7	61.5	62.1
	18:0/22:4	838.7	61.5	61.8	61.4	62.2
	18:0/20:2	814.8		62.3	62.0	62.8
PE	16:0/20:4	740.8	56.6			56.1
	16:0/22:6	764.8		56.2	56.1	56.7
	18:1/22:6	790.7	56.8	56.9		57.5
	18:1/20:4	766.9	57.1	57.2	57.0	57.7
	18:1/18:2	742.9	57.1	57.3	57.0	57.8
	18:0/22:6	792.8	57.2	57.4	57.2	57.8

Table 2 (*Contd.*)

Class	Molecular species	*m/z*	t_r/min			
			Folch with $CHCl_3$	Folch with MTBE	Only with CH_3OH	Modified Folch with MTBE and CH_3OH
	22:0/16:0	776.9	58.8			59.4
	18:1/18:1	744.8	60.2	59.8	59.6	60.7
	20:0/18:2	772.9			61.1	61.8
	22:0/18:2	800.8	64.6		64.5	
(b) Negative ion mode						
LPA	lyso/14:0	381.4	11.8	11.9	12.0	11.9
	lyso/16:1	407.5	11.9			12.2
	14:0/lyso	381.4	12.3	12.5	12.6	12.5
	lyso/20:4	457.3	12.5	12.3	12.8	12.5
	lyso/18:2	433.3	12.7	12.7	12.9	12.5
	lyso/18:3	431.4	12.7		12.9	12.7
	16:1/lyso	407.5	12.8			13.0
	lyso/18:1	435.2		13.3	13.2	13.1
	lyso/16:0	409.3	12.8	13.4	13.6	13.2
	20:4/lyso	457.3	13.5	13.6	13.8	13.5
	18:3/lyso	431.4	13.6		13.9	13.6
	lyso/22:6	481.5	13.9			
	18:2/lyso	433.3	13.9	14.2	14.0	13.9
	18:1/lyso	435.2		14.2	14.5	14.0
	22:6/lyso	481.5	14.4			
	lyso/18:0	437.3	14.6	14.6	14.6	14.1
	16:0/lyso	409.3	14.7	14.9	14.7	14.9
	18:0/lyso	437.3	15.9	15.7	16.0	16.0
LPG	lyso/14:0	455.4	12.9		13.0	13.1
	lyso/16:0	483.5	13.9		13.9	14.0
	14:0/lyso	455.4	13.9		14.2	14.2
	lyso/18:2	595.3				15.0
	16:0/lyso	483.5	15.1		15.3	15.3
	18:2/lyso	595.3				15.7
	lyso/18:0	511.3		18.1	19.5	19.4
	18:0/lyso	511.3		19.5	21.3	20.7
LPI	lyso/20:4	619.4			17.1	16.1
	20:4/lyso	619.4			18.5	17.4
	lyso/18:0	599.3				18.8
	18:0/lyso	599.3				19.3
PA	22:6/18:0	746.6	40.5	39.4	40.5	39.3
	20:4/18:0	723.7	40.5	39.8		39.8
	20:4/20:1	748.7	40.6	40.0	41.0	40.0
	20:4/20:2	750.6	41.0		41.8	40.1
	18:2/22:6	742.6	41.8	40.8	42.0	40.4
	22:6/20:0	776.1			43.2	41.1
PG	18:1/18:2	771.7	30.0	31.5	31.9	30.3
	16:0/18:1	747.7	31.8	30.7		31.1
	18:1/18:1	773.4	33.1	31.4	31.7	32.5
	18:1/18:0	775.7		32.4		32.6
	16:0/18:0	749.6			35.0	36.1
	16:1/20:4	766.7	37.9		37.6	36.9
	16:1/18:1	744.7	45.0	43.3	44.7	43.7
	22:6/18:0	820.6	43.4			42.3
PI	16:0/20:4	857.6	29.7	29.0	30.1	29.4
	16:0/22:6	881.6	29.8	29.7		29.5
	16:0/18:1	835.6	30.8	29.7	30.3	29.6
	16:0/20:3	859.6	29.8	29.8	30.7	29.7
	16:0/18:2	833.6	30.0	30.1	30.8	30.0
	18:1/20:4	883.6		30.5	31.0	30.3
	16:0/18:0	837.6		30.7	31.0	30.5
	18:1/18:1	861.6	31.1	30.9	31.5	30.7
	18:0/22:6	909.6	31.6	31.2	32.1	31.1
	18:0/18:2	861.6	31.6	31.4	32.6	31.2
	18:0/20:4	885.6	31.8	31.2	32.5	31.6
	18:0/22:5	911.6	31.8	31.5	32.5	31.6
	18:0/20:2	889.7	32.2	31.9		31.9
	18:0/18:1	863.7	32.5	32.0	33.3	32.1
	18:0/22:4	913.7		32.3		32.3
	18:0/20:3	887.6	34.1	34.6	34.6	34.6

Table 3 Number of identified PL and LPL species from each extraction method

	Number of species found			
Class	Folch with $CHCl_3$	Folch with MTBE	Only with CH_3OH	Modified Folch with MTBE/CH_3OH
LPC	18	16	18	18
LPE	6	6	10	8
LPA	16	12	14	16
LPG	4	2	6	8
LPI	0	0	2	4
PC	23	27	25	27
PE	8	6	7	9
PA	5	4	5	6
PG	6	5	5	8
PI	13	16	13	16
Total	99	94	105	120

compensation of spectral fluctuation for each nLC-ESI-MS run. For enhanced accuracy and precision, a total of three runs were carried out at each ion mode for a sample from each extraction method. Table 1 lists the average percent recoveries obtained for each PL and LPL species extracted by the four different methods. The recovery values of each species were calculated by the ratios of the relative peak areas of each PL standard (compared to that of an internal standard) from an extracted chromatogram of a nLC-ESI-MS run of a sample spiked with standards before extraction to those of the same standard species spiked into a plasma extract sample after extraction. The peak area of each individual component was calculated from the extracted chromatogram corresponding to each specific *m/z* value from the entire chromatogram of a nLC-ESI-MS run. Among the data obtained with the first three methods (the classical Folch method with $CHCl_3$, the Folch method with MTBE, and extraction with CH_3OH only) in Table 1, the highest overall recovery values of LPLs were achieved by the extraction method with CH_3OH only except for the cases of 16:0-LPC and 18:0-LPA, while the Folch method with MTBE proved to be efficient in the extraction of the PL classes (except for poor recovery of 18:0/18:0-PC and 16:0/16:0-PG). Since CH_3OH is more hydrophilic than the other solvents used ($CHCl_3$ and MTBE), it is likely that this characteristic contributes to the high recovery rate of LPLs, which are more hydrophilic than PLs, as compared to the other methods.

When a modified Folch method (the last column in Table 1) was used with a sequential use of solvents, CH_3OH–MTBE–CH_3OH, it resulted in the highest recoveries of five species (LPC, PC, LPS, LPG, and PS) and nearly the maximum recoveries of LPA, LPI, PA, and PG within the statistical interval as well. Two notable recoveries were LPE and PE, for which the modified Folch method with MTBE/CH_3OH achieved the second highest recoveries among the four methods. Therefore, when studies of all major classes of LPLs and PLs are required, the modified Folch method with MTBE/CH_3OH is the most effective procedure among the four candidates. However, when LPE or PE is targeted specifically, then the method of extraction with CH_3OH only or the classical Folch method with chloroform, respectively, is the most efficient choice. In terms of reproducibility of the modified Folch method with MTBE/CH_3OH, not only small standard deviations from each peak shown in Table 1, but also small coefficients of variation (mostly less than 4.0%) support a good precision of the method.

The four extraction methods examined in this study were evaluated for their efficiency in the qualitative analysis of plasma PLs and LPLs. The structural identification of each molecular species can be achieved from the fragment ion spectra of each individual species obtained by data-dependent collision-induced dissociation experiments during nLC-ESI-MS-MS, as explained in earlier reports.[30–32] Fig. 2 shows the base peak chromatograms of a plasma sample (20 μg injection) extracted by the modified Folch method with MTBE/CH_3OH and analyzed by nLC-ESI-MS-MS in both positive and negative ion modes. Data obtained using other extraction methods are not shown in this article. Fig. 3 represents MS and MS-MS spectra of the two species found when extraction is made with the modified Folch method of MTBE/CH_3OH. Fig. 3a shows MS spectra of $t_r = 56.1$ min at positive ion mode of nLC-ESI-MS along with the data dependent CID spectra of the parent ion of *m/z* 740.8 showing distinct fragmentation patterns. In the upper right side of spectra, a prominent base peak is observed at *m/z* 599.6, $[M + H - 141]^+$, from the loss of phosphoethanolamine ($HPO_4(CH_2)_2NH_3$, 141 amu) along with characteristic fragment ions at *m/z* 484.4 and 436.4 representing the loss of acyl chains in the form of carboxylic acid ($[M + H - RCOOH]^+$) from sn-1 and sn-2 position, respectively. Fragment ions of *m/z* 502.4 and 454.4 represent the loss of acyl chain in the form of ketene ($[M + H - R'CH{=}C{=}O]^+$). From these, it is identified as 16:0/20:4-PE. At negative ion mode of nLC-ESI-MS-MS, it is demonstrated with an identification of lyso/20:4-PI in Fig. 3b when methanol is involved with the Folch method (see the third and the last column in Table 1). In Fig. 3b, parent ions of *m/z* 619.4 detected at $t_r = 16.1$ min of the negative ion mode of nLC-ESI-MS are assigned as lyso/20:4-PI which shows a regioisomer having a hydroxide group at the sn-1 position and an acyl chain (20:4) at sn-2. Fragment ions at *m/z* 456.7 and 439.9 in Fig. 3b represent the loss of the inositol head group ($C_6O_5H_{11}$, 162 Da) from parent ions before and after the additional loss of water molecules, respectively. Fragmentation in the acyl chain resulted in the formation of $[RCOO]^-$ at *m/z* 304.1, $[M - H - RCOOH]^-$ at *m/z* 316.9 and $[M - H - R'CH{=}C{=}O]^-$ at *m/z* 333.9. For the determination of sn-1 (lyso at sn-1) from sn-2 regioisomers, the fact that the intensity of $[M - H - RCOOH]^-$ is much larger than that of $[M - H - 162 - H_2O]^-$ shows evidence of lyso/20:4-PI. The sn-2 regioisomers (20:4/lyso-PI) exhibit a longer retention time ($t_r = 17.1$ min) than the sn-1 isomer with the ratio of the two characteristic ions, $[M - H - RCOOH]^-/[M - H - 162 - H_2O]^- < 1$, reversed (included in the ESI figure†) as observed in an earlier report.[32] The PLs and LPLs identified from each extraction method are compared in Table 2, which lists lipid species by head group and retention time, and the total numbers of identified species of each head group are compared in Table 3. As observed with the recovery evaluations in Table 1, LPLs were poorly detected by the Folch method with MTBE (36 LPLs in Table 3), while they were found in greater numbers (54 LPLs in total) when the modified Folch method with MTBE/CH_3OH was employed. As confirmed by the recovery study, the final extraction method provided a total of 120 species (54 LPLs and 66 PLs) from a healthy human plasma sample, while the conventional

Folch method (with chloroform) detected only 99 species (44 LPLs and 55 PLs).

4. Conclusions

We performed systematic examination of the efficiency of extraction of PLs and LPLs from a blood plasma sample by comparing the three existing extraction methods with a modified method designed for this study. A modified Folch method using MTBE/CH_3OH was found to be the most effective among the four methods tested for the extraction of most PLs and LPLs, except for the LPE and PE groups. The latter two groups showed higher recovery with the CH_3OH-only method and the Folch method with chloroform, respectively. In terms of the total number of identified PL and LPL species, the modified Folch method with MTBE/CH_3OH provided better efficiency in qualitative analysis than the conventional Folch method with $CHCl_3$. The modified Folch method of MTBE/CH_3OH can be applied in both chemical and clinical studies when PLs and LPLs from biological samples need to be extracted.

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