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# On-line two-dimensional capillary strong anion exchange/reversed phase liquid chromatography–tandem mass spectrometry for comprehensive lipid analysis

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ABSTRACT

An on-line two-dimensional liquid chromatography method was developed for comprehensive lipid profiling by coupling strong anion exchange (SAX) and nanoflow reversed-phase liquid chromatography (nRPLC) prior to electrospray ionization-tandem mass spectrometry (2D-SAX/nRPLC–ESI-MS/MS). Lipids can be classified into four different types according to the electrical propensities of the lipids: anionic, weak anionic, neutral polar, and special lipids. In 2D-SAX/nRPLC, various lipids can be fractionated in the first dimension (SAX: 5 μm to 100 Å, 5.0 cm × 75 μm i.d.) by step elution (methanol and salt solution), followed by the molecular separation of lipids in the second dimension (RP: 3 μm to 100 Å, 7.0 cm × 75 μm i.d.) with binary gradient LC. Since the elution of lipids from SAX can be achieved with a very small volume of eluent delivered from an autosampler, it can be simply implemented with an LC–ESI-MS instrument for full automation, and the salt step elution, including the two-step injection procedure, can be used for the selective analysis of the desired lipid fraction. For nRPLC–ESI-MS/MS run in either positive or negative ion mode, a common ionization modifier (0.05% ammonium hydroxide with 5 mM ammonium formate) was introduced into the binary mobile phase solutions so that 2D-LC–MS could be operated in both ion modes without changing the mobile phase solutions. The developed on-line 2D-SAX/nRPLC–ESI-MS/MS was evaluated with 22 different standard lipids for the optimization of the salt step elution and was applied to a healthy human plasma lipid extract, resulting in the identification of a total of 303 plasma lipids, including 14 different classes.



## 1. Introduction

Lipids are mostly water-insoluble biological molecules having a wide variety of polarities and are major components of biological membranes. Lipids play important roles in energy storage and signal transduction between cells, and are involved in human diseases such as diabetes, atherosclerosis, Alzheimer's disease, and cancer [1–3]. Lipids can be classified into eight categories: fatty acyls (FA), glycerophospholipids (GP or phospholipids), glycerolipids (GL), sphingolipids (SP), sterol lipids (ST), prenol lipids, saccharolipids, and polyketides [4,5]. Since each group of lipids contains a different combination of acyl chains, the simultaneous analysis of the entire lipidome is a complicated task. High-performance liquid chromatography (HPLC) in combination with electrospray ionization-mass spectrometry (ESI-MS) has been commonly used as a sensitive method for lipid analysis. While normal-phase liquid chromatography (NPLC) utilizing stationary phases such as silica [6–8], amine [9], and diol [10,11], including hydrophilic interaction chromatography [12,13], is an effective technique for resolving different lipid classes or subclasses (i.e., different head groups of phospholipids), it is incapable of separating lipids by individual molecular species or of estimating the relative abundances of individual components. Reversed-phase liquid chromatography (RPLC) is widely used for lipid analysis [14–17] and is effective for identifying low-concentration lipid species, since individual lipid species can be separated by the hydrophobic interactions between the alkyl chains (i.e., C18) on the stationary phase and the lipid fatty acyl chains. Recent reports show the profiling of more than 500 lipids from human plasma sample using several analytical methods including GC–MS and LC–MS/MS for each lipid category separately [18] and the identification of 444 lipids from rat plasma using ultrahigh performance LC–MS (UPLC–MS) with enhanced resolution and speed [19]. Nanoflow RPLC–ESI-MS/MS has been powerfully employed for characterizing phospholipids (PLs) from

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human plasma and urine samples with limit of detection (LOD) at low fmol/μL levels [20–23]. However, the comprehensive separation and characterization of lipids is still demanding due to the complexity of the lipids.

To overcome this limitation, multidimensional separation methods that are either off-line or on-line have been investigated. Strong cation exchange (SCX) chromatography [24,25] and NPLC [26,27] were used to fractionate different lipid classes in an off-line mode and the collected fractions were analyzed by RPLC in the second dimension. With anion exchange HPLC, phosphatidylinositide levels of cells were specifically analyzed with conductivity detection [28]. On-line two-dimensional liquid chromatography (2D-LC) approaches with silver ion HPLC coupled with RPLC (Ag-LC/RPLC) [29,30] or with a combination of NPLC and RPLC have been made [31,32]. Ag-LC/RPLC is useful to differentiate the different degrees of unsaturation in acyl chains by Ag ion interactions with double bonds, but is too specific for TG profiles and not sufficient to analyze the complicated lipid mixtures. On-line 2D-NPLC/RPLC was developed by introducing a solvent evaporation interface between NP and RP columns to remove the organic mobile phase used in the NPLC process [31]. While 2D-NPLC/RPLC–ESI-MS/MS with a solvent evaporation interface expanded the capability of phospholipid identification to a great extent, it has some limitations when applied to a wide variety of lipid classes, since earlier work focused on 12 subclasses of phospholipids. Besides the need for a vacuum pump, the detection limit of this method was relatively high (55–65 fmol) [32].

In this study, an on-line 2D capillary strong anion exchange/nanoflow RPLC (2D-SAX/nRPLC) separation with ESI-MS/MS has been developed to simultaneously analyze 22 lipid classes including four categories of lipids (GP, GL, SP, and ST). Since lipids can be classified into four different types according to their electrical propensities, namely anionic, weak anionic, neutral polar, and special lipids [33], anionic lipids can be differentiated from neutral lipids by SAX resin. However, SAX has not previously been fully integrated into the 2D-LC separation of lipid classes with the exception of an anion exchanger cartridge being used to purify gangliosides from tissues [34]. In case of using SCX for lipid separation, lipid affinity to SCX resin is so weak that selective separation of different lipid classes cannot be made. In this study, an on-line 2D-SAX/nRPLC is assembled by coupling a capillary SAX column (5 μm to 100 Å SAX resins in 5.0 cm × 75 μm i.d.) with a capillary RP analytical column (3 μm to 100 Å C18 resins in 7.0 cm × 75 μm i.d.) using two six-way valves for a full automation. Ionic solutions of different concentrations can be delivered for step elution (methanol or salt solution) from the autosampler to the SAX column, and lipid fractions of different electric propensities desorbed from the SAX column are loaded into a short C18 trap prior to being transferred to the analytical column. Then, typical binary gradient nRPLC separation of each lipid fraction followed by ESI-MS/MS analysis can be achieved as reported in several recent studies. The configuration of on-line capillary 2D-SAX/nRPLC is analogous to that of the dual-purpose sample trap for on-line SCX/nRPLC–MS/MS system for peptides [35]. While in the dual trap (SCX/RP) system, organic mobile phases for gradient RPLC elution after each salt step elution pass through SCX trap throughout 2D-separations, the current 2D-SAX-RPLC configuration utilizes a separate SAX column prior to a RP trap so that RPLC mobile phases can bypass the SAX column during gradient elution, preventing the unwanted release of bound lipids from SAX. To carry out a sequential nRPLC–MS run in both positive and negative ion modes depending on the type of lipids eluted from the SAX column by each salt step fractionation, a common ionization modifier for both ion modes was first introduced by investigating the ionization effect of modifiers so that nLC–ESI-MS/MS runs could be carried out without changing the mobile phase solution containing a different ionization modifier. Evaluations of 2D-SAX/nRPLC–ESI-MS/MS were made with 22 standard lipids of different classes to optimize the salt step concentrations of ammonium acetate solution for consecutive RPLC separations and to determine the limits of detection (LOD) and recovery rates. The developed method was applied to a human plasma lipid extract sample.

## 2. Materials and methods

### 2.1. Materials

All lipid standards used for the optimization of SAX/nRPLC–ESI-MS/MS were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Twenty-two standard lipids represented each lipid class, including four different categories (GP, GL, SP, and ST), and they are listed by their polar and ionic natures in Table 1 along with the LOD and recovery values obtained with SAX/nRPLC–ESI-MS/MS, which will be discussed later. All solvents [$CH_3CN$, isopropanol, $CH_3OH$, $H_2O$, $CHCl_3$, and methyl *tert*-butyl ether (MTBE)] were HPLC grade. Lipid standards were first dissolved in solvent A [6:3:1 (v/v) $CH_3OH/CHCl_3/H_2O$] and then diluted with $CH_3OH$ for nLC–ESI-MS/MS. Ammonium hydroxide (AH) and ammonium formate (AF) from Sigma Aldrich (St. Louis, MO, USA) were used as ionization modifiers to be added to mobile phase solutions. Capillary tubes with 20, 50, and 75 μm i.d. (360 μm o.d. for all) were purchased from Polymicro Technology, LLC (Phoenix, AZ, USA).

### 2.2. Lipid extraction from plasma sample

A human plasma sample was obtained from a healthy male volunteer (age 26). The extraction of lipids from the plasma sample followed the modified Folch method with MTBE/$CH_3OH$ reported in an earlier work [36]. Briefly, 0.3 mL of $CH_3OH$ was added to 0.1 mL of the plasma sample and after a short vortexing, the mixture was placed in an ice bath for 10 min. Then, 1.0 mL of MTBE was added to the mixture and it was vortexed for an hour. Next, 0.25 mL of MS-grade water was added to the mixture, which was then vortexed at room temperature for 10 min. After the mixture was centrifuged at 5000 rpm for 10 min, the upper organic layer was transferred to a different centrifuge tube; then, 0.3 mL of methanol was added to the remaining aqueous layer and agitated in a shaker for 10 min. The mixture was sonicated with a tip for 2 min and centrifuged to collect the portion in the supernatant. The previously removed organic fraction was added to the collected supernatant and then the mixture was dried in a vacuum centrifuge. The dried lipid extracts were dissolved in solvent A, diluted to a total volume of 500 μL, and stored. For SAX/nRPLC–ESI-MS/MS analysis, the final lipid solution in storage was diluted to a concentration of 5 μg/μL in $CH_3OH$.

### 2.3. Capillary SAX/RPLC–ESI-MS/MS

The capillary SAX column, a C18 trapping column, and capillary RPLC columns were prepared in our laboratory. The capillary SAX column was packed with a methanol slurry of 5-μm 100-Å Nucleosil SB from Macherey-Nagel Co. (Düren, Germany) to 5.0 cm in a capillary tube (75 μm i.d.) ended with a sol-gel frit (2 mm in length) under a constant pressure of He (1000 psi). The sol–gel frit was simply formed by baking at 100 °C for 2 h after immersing the capillary end very briefly in a solution of 1:4 (v/v) formamide/potassium silicate. The C18 trapping column was similarly packed with RP resins [5-μm 100-Å from Michrom Bioresources Inc. (Auburn, CA, USA)] for 1.0 cm in a frit-ended capillary (75 μm i.d.). The analytical column was packed with a methanol slurry of RP resins [3-μm 100-Å Watchers ODS-P from Isu Industry Co. (Seocho, South Korea)] in a 7.0 cm long pulled tip capillary, of which one end of the capillary

**Table 1**
Observed $m/z$ values, retention times, concentrations, calculated LODs, and recovery values of the 22 lipid standards shown in Fig. 3 by 2D-SAX-nRPLC–ESI-MS. RSD in retention time ($t_r$) is 0.20–2.17%.

| Group | Classes | Acyl chain | $m/z$ | $t_r$ (min) | Conc. (pmol/μL) | $R^2$ | LOD (fmol) | Recovery (area %) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Breakthrough | Methanol fraction | 10 mM AA | 250 mM AA | 1 M AA |
| Neutral polar lipids | LPC | 16:0 | 496.5[a] | 12.19[f], 12.68[g] | 0.45 | 0.997 | 0.93 ± 0.02 | 0.5 ± 0.2 | 99.5 ± 0.2 | N.D. | N.D. | N.D. |
| | PC | 12:0/12:0 | 622.6[a] | 18.33 | 0.93 | 0.992 | 0.64 ± 0.05 | 0.3 ± 0.2 | 99.7 ± 0.2 | N.D. | N.D. | N.D. |
| | SM | d18:1/12:0 | 647.6[a] | 20.48 | 0.86 | 0.997 | 1.08 ± 0.04 | 0.3 ± 0.2 | 99.7 ± 0.2 | N.D. | N.D. | N.D. |
| | DG | 18:1/18:1 | 638.5[b] | 28.55 | 1.99 | 0.998 | 1.37 ± 0.32 | 0.4 ± 0.2 | 99.6 ± 0.2 | N.D. | N.D. | N.D. |
| | TG | 16:0/18:1/18:2 | 874.8[b] | 33.66 | 1.27 | 0.991 | 1.12 ± 0.63 | 0.3 ± 0.3 | 99.7 ± 0.3 | N.D. | N.D. | N.D. |
| | CE | 18:2 | 666.7[b] | 34.64 | 1.15 | 0.996 | 1.18 ± 0.23 | 0.2 ± 0.1 | 99.8 ± 0.1 | N.D. | N.D. | N.D. |
| Weak anionic lipids | So | d18:1 | 300.4[a] | 13.78 | 1.56 | 0.996 | 1.32 ± 0.80 | N.D. | 99.4 ± 0.2 | 0.6 ± 0.2 | N.D. | N.D. |
| | Sa | d18:0 | 302.4[a] | 14.53 | 1.75 | 0.999 | 1.53 ± 0.50 | N.D. | 99.7 ± 0.2 | 0.3 ± 0.2 | N.D. | N.D. |
| | LacCer | d18:1/12:0 | 850.6[c] | 20.26 | 3.28 | 0.993 | 2.04 ± 0.81 | N.D. | 1.0 ± 0.2 | 99.0 ± 0.2 | N.D. | N.D. |
| | MHC | d18:1/12:0 | 688.5[c] | 20.87 | 4.11 | 0.992 | 1.82 ± 0.14 | N.D. | 1.1 ± 0.2 | 98.9 ± 0.2 | N.D. | N.D. |
| | Cer | d18:1/12:0 | 526.6[c] | 22.29 | 4.12 | 0.990 | 1.46 ± 0.19 | N.D. | 1.3 ± 0.3 | 98.7 ± 0.3 | N.D. | N.D. |
| | LPE | 14:0 | 424.5[d] | 8.07[f], 8.79[g] | 2.38 | 0.998 | 1.63 ± 0.06 | N.D. | 1.4 ± 0.2 | 98.6 ± 0.2 | N.D. | N.D. |
| | PE | 12:0/12:0 | 578.5[d] | 17.71 | 3.69 | 0.991 | 1.53 ± 0.20 | N.D. | 3.3 ± 0.2 | 96.7 ± 0.2 | N.D. | N.D. |
| Anionic lipids | LPG | 18:0 | 511.4[d] | 10.70[f], 11.24[g] | 0.67 | 0.990 | 0.72 ± 0.05 | N.D. | N.D. | 98.6 ± 0.2 | 1.4 ± 0.2 | N.D. |
| | PG | 12:0/12:0 | 609.6[d] | 14.10 | 0.91 | 0.997 | 0.95 ± 0.06 | N.D. | N.D. | 98.4 ± 0.2 | 1.6 ± 0.2 | N.D. |
| | LPI | 18:1 | 597.5[d] | 8.08[f], 8.52[g] | 0.64 | 0.986 | 1.35 ± 0.32 | N.D. | N.D. | 97.8 ± 0.4 | 2.2 ± 0.4 | N.D. |
| | PI | 16:0/18:2 | 833.6[d] | 20.93 | 1.68 | 0.990 | 8.16 ± 0.26 | N.D. | N.D. | 97.8 ± 0.3 | 2.2 ± 0.3 | N.D. |
| | LPS | 18:0 | 524.5[d] | 9.31[f], 9.83[g] | 2.17 | 0.989 | 1.06 ± 0.05 | N.D. | N.D. | 0.8 ± 0.1 | 99.2 ± 0.1 | N.D. |
| | PS | 14:0/14:0 | 678.7[d] | 17.68 | 1.75 | 0.991 | 6.59 ± 0.12 | N.D. | N.D. | 0.5 ± 0.1 | 99.5 ± 0.1 | N.D. |
| | LPA | 18:0 | 437.5[d] | 6.98[f], 7.41[g] | 3.69 | 0.982 | 6.25 ± 1.63 | N.D. | N.D. | 0.6 ± 0.3 | 99.4 ± 0.3 | N.D. |
| | PA | 12:0/12:0 | 535.5[d] | 11.69 | 4.76 | 0.986 | 9.00 ± 1.26 | N.D. | N.D. | 0.4 ± 0.2 | 99.6 ± 0.2 | N.D. |
| | CL | $(18:1)_4$ | 727.8[e] | 32.82 | 3.49 | 0.990 | 7.56 ± 0.93 | N.D. | N.D. | 0.3 ± 0.1 | 99.7 ± 0.1 | N.D. |

[a] $m/z$ of $[M+H]^+$.
[b] $m/z$ of $[M+NH_4]^+$.
[c] $m/z$ of $[M+HCOO]^-$.
[d] $m/z$ of $[M-H]^-$.
[e] $m/z$ of $[M-2H]^{2-}$.
[f] Lyso/acyl-LPL.
[g] Acyl/lyso-LPL.

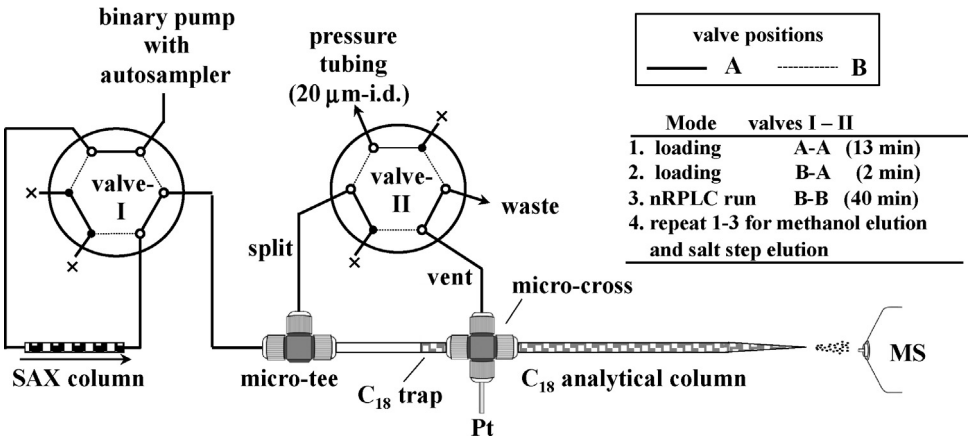


**Fig. 1.** Configuration of the on-line 2D SAX/nRPLC–ESI-MS/MS setup with a SAX column (5 μm to 100 Å Nucleosil SB, 75-μm i.d. ×5.0 cm), C18 trap (5 μm to 100 Å C18, 75-μm i.d. ×1.0 cm), and a pulled tip analytical column (3 μm to 100 Å C18, 75-μm i.d. ×7.0 cm). Valve positions (A for solid line and B for dotted line) of both six-way valves (I and II) vary depending upon the loading and nRPLC run modes.

(75 μm i.d.) was pulled to create a sharp needle (tip i.d. ~10 μm) for a direct ESI emitter.

The connection of the capillary SAX column with an analytical capillary RPLC column was achieved using a six-way valve of an autosampler equipped in a Model 1260 capillary pump system from Agilent Technologies (Waldbronn, Germany) and another six-way valve equipped in an LTQ Velos ion trap mass spectrometer from Thermo Fisher Scientific Inc. (San Jose, CA, USA). The valve configuration for the on-line SAX/nRPLC operation will be described later in Section 3. The salt step elution was accomplished by delivering different concentrations (10 mM to 1 M) of ammonium acetate solution from the autosampler to the SAX column. The nRPLC separation was carried out with a binary gradient elution. Mobile phase A was water with 0.05% ammonium hydroxide and mobile phase B was 1:3:6 (v/v) $CH_3OH/CH_3CN$/isopropanol with 0.05% AH and 5 mM AF added as modifiers. The binary gradient elution began with 100% mobile phase A, ramped to 30% B for 1 min, increased to 70% B over 15 min, and then rose to 100% B over 15 min. The mobile phase was maintained at 100% B for 10 min for completion. The control of HPLC pump was made by Agilent Chemstation from Agilent Technologies.

The ESI voltage for the MS analysis was 3.0 kV and the MS capillary temperature was set at 250 °C. The mass range for the precursor scan was 250–1100 amu. Collision-induced dissociation (CID) for MS/MS analysis was made for the three most intense ions from each precursor scan in data-dependent mode with a collision energy of 40%. MS data collection was made with Xcaliber software from Thermo Fisher Scientific. Identification of lipid molecular structures were made with the LiPilot [37] software developed in our laboratory, and the search results were confirmed manually.

## 3. Results and discussion

### 3.1. Selection of a common ionization modifier

Prior to the performance evaluation of 2D-SAX/nRPLC–ESI-MS/MS, it was necessary to develop a common ionization modifier to be added to the mobile phases that could be used for both positive and negative ion modes without changing mobile phases. This facilitates on-line 2D separation when sequential nRPLC–ESI-MS/MS runs of consecutive fractions from SAX need to be performed in both ionization modes of MS, depending on lipid classes. In an earlier evaluation of ionization modifiers for the analysis of phospholipids (PLs) [38], a modifier mixture of 0.05% AH with 1 mM AF was found to be useful for all PLs in negative ion mode. Phosphatidylcholines (PCs) and sphingomyelins (SMs) can be detected in negative ion mode as formate adducts. However, neutral polar lipids like TGs and diacylglycerols (DGs), in addition to cholesteryl esters (CE), are not detected in negative ion mode, while they can be ionized well in positive ion mode as ammonium adducts with the help of AF. The role of AF as a common modifier was tested by measuring the ionization efficiencies of ten lipid classes in positive ion mode. Fig. 2 shows a graph of the MS intensity of a number of neutral polar and weak anionic lipids (positive ion mode) with four different modifiers; the molecular structures of all the standard lipids are listed in Table 1. Since formic acid (0.1%) is not a good modifier in negative ion mode for most anionic lipids, it is not suitable as a common modifier for both ion modes, but was included for the comparison of the ionization efficiencies of PCs under other modifiers in positive ion mode. Apparently, 5 mM AF alone provided better ionization efficiencies among all 10 lipid classes. In the previous study [38], it was found that 1 mM AF with 0.05% AH showed slightly better efficiency in ionization of anionic lipids. Therefore, the mixture of 0.05% AH and 5 mM AF was selected as a common and highly efficient modifier that can be used for both ion modes.

### 3.2. Evaluation of SAX/nRPLC–MS/MS

On-line 2D-SAX/nRPLC was established by coupling a capillary SAX column with a C18 trap prior to a C18 analytical column in series using two six-way valves, as shown in Fig. 1. When a lipid mixture sample was loaded onto the SAX resin from an autosampler as in the solid-line configuration (position A of valve I) of Fig. 1, species that were not retained in the SAX column could be transferred to the C18 trap. Sample loading was done at 1 μL/min of 100% mobile phase A (water with 0.05% AH only) by the two-step loading method: positions A-A for both valves (I and II) for 13 min (Mode 1 in Fig. 1) and positions B-A for valves I and II for 2 min (Mode 2). During the first 13 min, it was expected that any non-retaining lipid species that passed through the SAX column could be trapped in the C18 trap and that salts or impurities could be vented via micro-cross through valve II. During the second period (2 min) of loading with valve I in position B (Mode 2), any remaining lipid species left in the capillary connecting tube between valve I and the micro-tee could be delivered to the C18 trap. This procedure is essential, since any species left in the capillary connecting tube could be wasted out through the micro-tee when gradient LC elution began with valve II in position B. After the two-step sample loading, the position of valve II was set to B (Mode 3), and then a binary gradient nRPLC separation for the breakthrough fraction

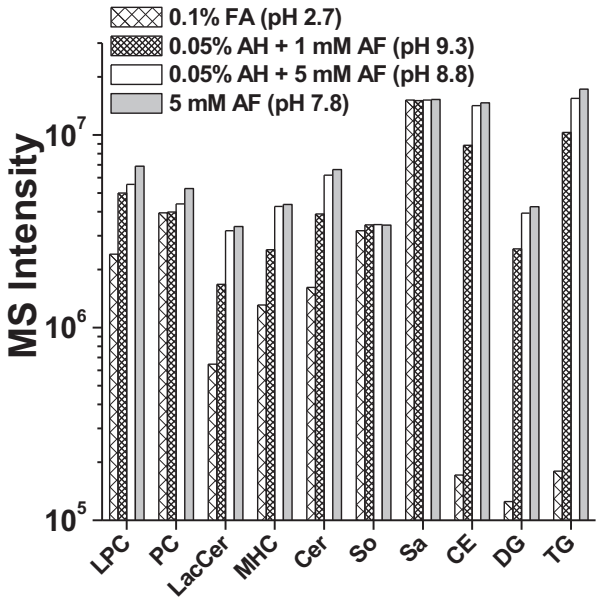


**Fig. 2.** Comparison of ESI-MS intensities of neutral polar and weak anionic lipids in positive ion mode among different ionization modifiers (typically used in negative ion mode) in comparison with FA, showing that a mixture of 0.05% AH + 5 mM AF is effective in positive ion mode. FA, formic acid; AH, ammonium hydroxide; AF, ammonium formate.

was carried out. With both valves in position B, the mobile phase solutions from the binary pump bypassed the SAX column so that the lipid species in the SAX column would not be desorbed, and the relatively fast pump flow, which was set at 16 μL/min to reduce dwell time, could be reduced to 300 nL/min by flow splitting at the micro-tee just before the C18 trap leading to the analytical column for nanoflow separation. Flow rate was controlled by varying the length of a narrow-bore (20 μm i.d.) pressure tube at valve II. After the breakthrough LC run, an initial methanol step elution prior to the salt step was applied, since neutral lipids including TG, DG, PC, and SM can be desorbed from the SAX column simply by delivering 5 μL of $CH_3OH$ from a microvial in the autosampler with mobile phase A following the two-step loading procedure. The volume of methanol (5 μL) was optimized to be a minimum plug so that lipids desorbed from the SAX column were readily trapped in the C18 trap without passing through. The nRPLC–ESI-MS/MS run followed the two-step loading of the methanol fraction to the C18 trap. The first salt step fractionation began by delivering 5 μL of 10 mM ammonium acetate (AA) solution from the autosampler into the SAX column and then a gradient nRPLC run was performed. Further salt step elution was carried out at two more concentration conditions: 250 mM and 1 mM AA solution. It was found that the last salt step (1 mM) was sufficient to desorb residual lipids from the SAX column.

The performance of the capillary SAX/nRPLC–ESI-MS/MS was evaluated with a mixture of 22 standard lipid species of different classes by selecting proper salt step concentration intervals for on-line nRPLC–ESI-MS/MS analysis. The initial sample loading was 1 μL of lipid standard mixtures (0.45–4.76 pmol/μL of each species; see detailed concentration information in Table 1). Fig. 3a shows the base peak chromatogram (BPC) of a breakthrough nRPLC–ESI-MS/MS run in which the chromatogram did not show a significant elution of lipids at the present MS intensity scale. The inset chromatogram is enlarged by 200 times, and shows some peaks from the neutral polar lipids listed in Table 1. However, the relative peak area of each species eluted at a breakthrough run compared with those from the following nRPLC run of the $CH_3OH$ fraction was less than 0.5% (this will be discussed later). Fig. 3b shows the BPC of the nRPLC–ESI-MS/MS run of the methanol fraction in positive ion mode, which represents the successful separation of PLs, including the phosphocholine head group (LPC, PC, and SM), DG, TG, and CE, along with two weakly anionic lipids, sphingosine (So) and sphinganine (Sa). To calculate the recovery percentage of each species during the entire separation, three internal standards were added to the standard lipid mixtures: 13:0/13:0-PC for IS-1, 15:0/15:0-phosphatidylglycerol (PG) for IS-2, and 12:0/12:0-phosphatidylserine (PS) for IS-3 (0.5 pmol/μL for each IS). Fig. 3c shows the BPC obtained from the LC run of the lipid fraction desorbed during the first salt step elution (10 mM AA), and Fig. 3d shows that for the second salt step fraction at 250 mM AA. Compared with Fig. 3b, a notable difference in chromatogram 3c is the elution of phosphatidylethanolamine (PE) and LysoPE (LPE). Since PEs and LPEs usually elute together with PCs in one-dimensional (1D) nLC–ESI-MS-MS run in positive ion mode, ionizations of PE and LPE species are usually suppressed by relatively abundant PC species. However, due to the stepwise fractionation of lipids in SAX, PCs and PEs can be analyzed in separate LC runs. Moreover, the PLs typically detected in negative ion mode in nLC–ESI-MS-MS runs, such as phosphatidyl acid (PA), PS, and cardiolipin (CL), did not elute in the 10 mM fraction, and were desorbed with the 250 mM AA solution, as seen in Fig. 3d. It is noteworthy that the regioisomers of 18:0-lysophosphatidylserine (LPS) shown in Fig. 3d were resolved in such a way that lyso/18:0-PS elutes (a small shoulder peak of LPS) earlier than 18:0/lyso-PS as it was confirmed with the difference in CID spectra of regioisomers separated by nLC in an earlier study [23].

In order to evaluate the recovery of each lipid species in five different elution steps, all five LC–ESI-MS/MS runs were run in positive ion mode and repeated in negative ion mode. Then average peak area of each species ($n = 3$) was calculated from extracted ion chromatograms and a total peak area of each species in different steps was set to be 100% either in positive or negative ion mode. Thus in Table 1, peak area of d18:1/12:0-LacCer (majority of it was eluted in 10 mM AA step) in both methanol and 10 mM AA fractions were based on chromatograms obtained in negative ion mode, while those of d18:1-So were based on chromatograms obtained in positive ion mode. By comparing the peak areas of each species among the different step fractions, as listed in Table 1, the current salt step interval was suitable to differentiate the lipid species exclusively in each run. For instance, it was found that the recovery values of the first eight species (LPC ~ Sa) in Table 1 were >99% in the methanol fraction, although less than 1% of each species eluted in the breakthrough elution. While five weak anionic lipids (lactosylceramide (LacCer), monohexosylceramide (MHC), Ceramide (Cer), LPE, and PE) eluted in the methanol fraction in small amounts, most of them eluted with 10 mM AA (96.7–99.0%). Moreover, most of the four anionic lipids (lysophosphatidylglycerol (LPG), PG, lysophosphatidylinositol (LPI), and phosphatidylinositol (PI)) eluted at 10 mM, while 1.4–2.2% of them were retained in the SAX column. After finishing the salt step elution with 250 mM AA, a final elution with 1 M AA solution was performed, but no single species was detected. This shows that 250 mM can be used for the final clean-up step for any remaining lipids in the SAX column. This recovery study shows that it is possible to quantify specific target lipids at a desired fraction using SAX/nRPLC–ESI-MS/MS.

### 3.3. Application to plasma lipids

The developed method was applied to a human plasma lipid extract sample, and the five BPCs obtained for the consecutive step fractions ((a) breakthrough, (b) methanol, (c) 10 mM AA, (d) 250 mM AA, and (e) 1 M AA) are shown in Fig. 4. Data-dependent MS/MS experiments were carried out for the eluting species during each nRPLC run. As observed with the BPCs of the standard

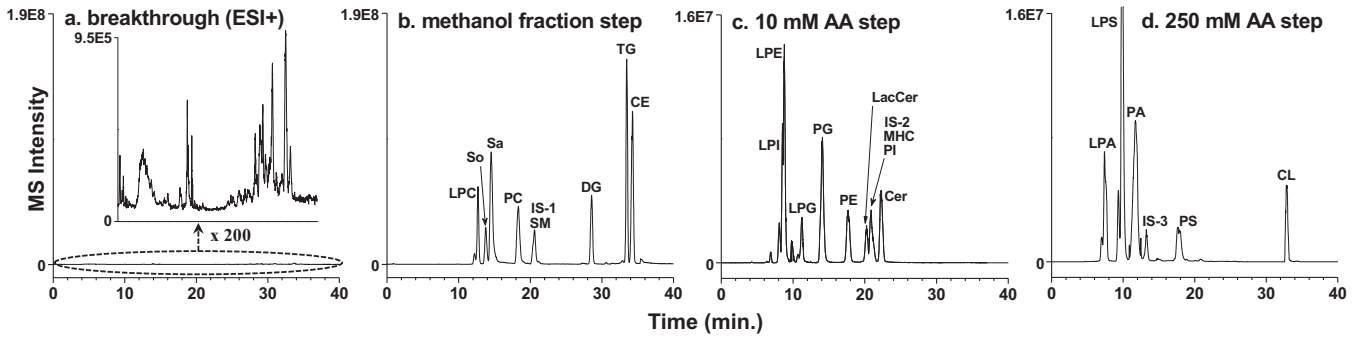


**Fig. 3.** Base peak chromatograms of the (a) breakthrough fraction, (b) methanol fraction, (c) 10 mM AA salt step fraction, and (d) 250 mM AA fraction of 22 standard lipid mixtures (listed in Table 1) by SAX/nRPLC–ESI-MS. Three internal standards (IS1: 13:0/13:0-PC, IS2: 15:0/15:0-PG, and IS3: 12:0/12:0-PS) were used.

lipid mixtures in Fig. 3, an nRPLC run of the breakthrough fraction of the plasma sample did not show significant elution of lipids. However, the peak intensity of the inset chromatogram in Fig. 4a was eight times higher than that of Fig. 3a (7.7E6 in Fig. 4a vs. 9.5E5 in Fig. 3a), showing that a considerable amount of non-polar components contained in the plasma sample that were not retained in the SAX column eluted during the breakthrough run. These were not identified from the CID spectra. In Fig. 4b and c, a significant fraction of lipids eluted during the methanol and first salt step elutions, while relatively few peaks appeared at 250 mM in Fig. 4d. The final run in Fig. 4e was made with the fraction at the elution of 1 M AA, resulting in broad peaks. Examination of the CID spectra obtained during the nRPLC–ESI-MS/MS run in Fig. 4e revealed that they were unknown species that did not belong to the lipid categories investigated in this study.

Identifications of lipid molecular structures were made from the MS/MS spectra obtained from the nRPLC–ESI-MS/MS experiments for each step fraction using LiPilot software against the database built in our lab, and the search results were confirmed manually. A total of 303 lipid species were identified from the human plasma sample by the 2D-SAX/nRPLC–ESI-MS/MS experiments, and they are listed with their molecular structures in Table S1 of the Supporting Information. Lipid molecules marked with a star (*) in Table S1 represent additional identifications from the 2D-LC experiments (68 species), while the others (235 species) were commonly found in both the 1D-LC and 2D-LC experiments. The numbers of lipid molecules identified with or without 2D separation are compared in Table 2. We found that on-line 2D-SAX/nRPLC separation increased the capability for detecting lipid species that could not be identified when 1D-nRPLC alone was used without SAX due to spectral congestion. For instance, Fig. 5a and b shows a comparison of a

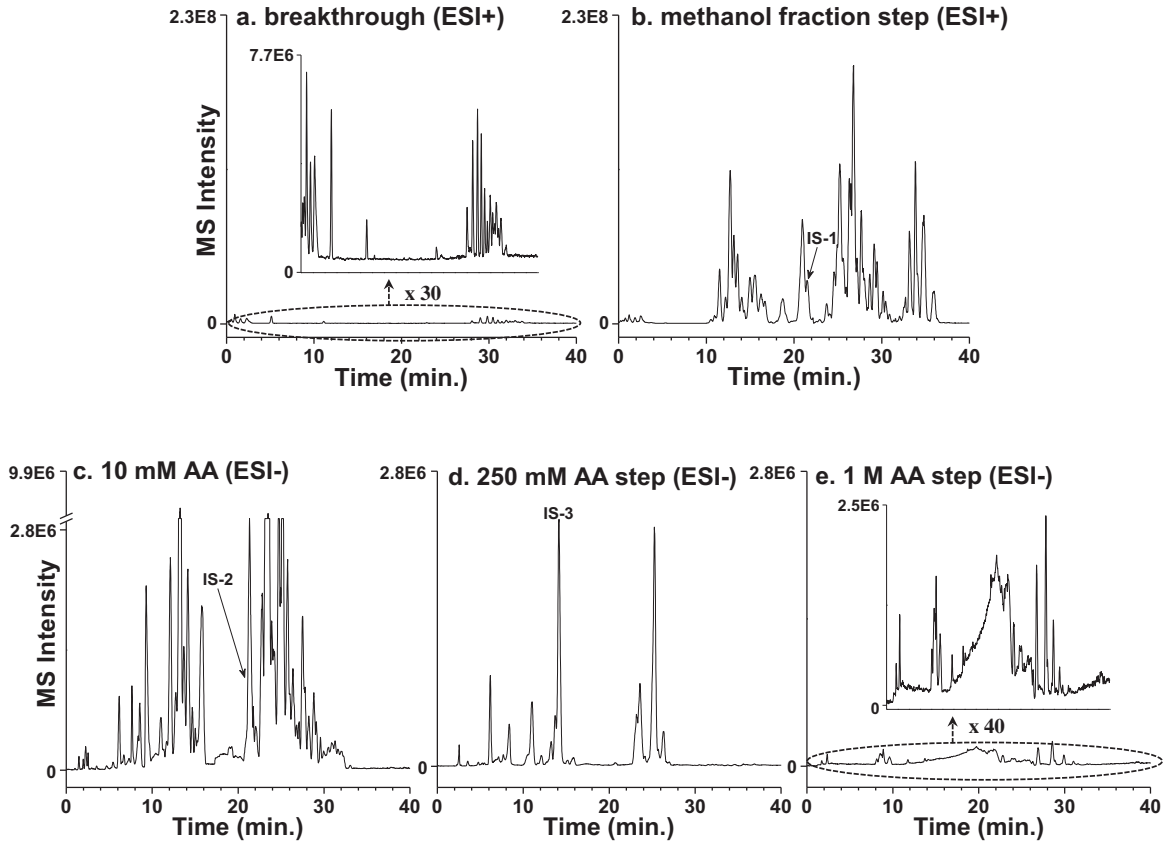


**Fig. 4.** Base peak chromatograms of human plasma lipid extract sample obtained at the five different step fractionations: (a) breakthrough, (b) methanol, (c) 10 mM, (d) 250 mM, and (e) 1 M AA by 2D-SAX/nRPLC–ESI-MS/MS.

**Table 2**
Number of identified lipid species from a healthy human plasma sample obtained by on-line 2D-SAX/nRPLC–ESI-MS/MS compared with results obtained from 1D-nRPLC–ESI-MS/MS alone.

| Classes | Number of species found | | Classes | Number of species found | | Classes | Number of species found | |
|---|---|---|---|---|---|---|---|---|
| | 1D-nLC | 2D-SAX/nLC | | 1D-nLC | 2D-SAX/nLC | | 1D-nLC | 2D-SAX/nLC |
| LPC | 16 | 23 | Sa | 1 | 1 | LPS | 4 | 4 |
| PC | 51 | 56 | LPE | 16 | 19 | PS | 0 | 1 |
| DG | 9 | 11 | PE | 9 | 17 | LPA | 6 | 7 |
| TG | 44 | 57 | LPG | 5 | 6 | PA | 6 | 10 |
| CE | 12 | 12 | PG | 6 | 9 | MHC | 3 | 4 |
| SM | 11 | 18 | LPI | 8 | 11 | Cer | 5 | 6 |
| So | 1 | 1 | PI | 22 | 30 | **Total** | **235** | **303** |

precursor MS scan of the plasma sample at $t_r$ = 13.60 min of 1D-nRPLC (ESI+) alone with that of the methanol fraction (ESI+) at $t_r$ = 13.60 min of the 2D-SAX/nRPLC run. The MS spectrum of the 1D-nRPLC run shows several lipids, including LPE and LPC, along with a few un-identified molecular species from the CID experiments. In contrast, the nRPLC run of the methanol fraction during 2D separation in Fig. 5b shows a simpler MS scan spectrum with the depletion of 18:1-LPE and 18:0-LPE species as well as ions (marked with *) at $m/z$ 536.2, 554.3, and 559.4 that were not identified. Moreover, 20:2-LPC ($m/z$ 548.5, $[M+H]^+$) was clearly identified from the characteristic fragment ion spectrum shown at the right side of Fig. 5b. The latter ion was detected in Fig. 5a, but its CID spectrum was not obtained due to spectral congestion. A more distinct difference between 1D and 2D separations is demonstrated in Fig. 5c and d, which were obtained at the same $t_r$ = 23.19 min for the plasma sample and the 10 mM AA fraction, respectively. Fig. 5c shows a number of lipid species (molecular structures from CID), including PCs, PA, and SM, whereas Fig. 5d shows only three intense PI molecules including 18:0/22:5-PI, which is shown with the CID spectrum at the right of Fig. 5d, and which was not detected due to ionic suppression from highly abundant species, leading to a failure in achieving MS/MS spectra in1D experiment. This is because the PC and SM molecules were already desorbed from the SAX column during the methanol step elution, and the PA molecules were not yet desorbed during the 10 mM salt step elution. In expressing acyl chain information of TG molecules in Table S1 in the Supporting Information, they were listed with the total number of carbons and double bonds. Though the information of individual acyl chain structure can be obtained from CID spectra of a precursor ion, a complete identification of three acyl chain locations is not possible since separation of TG positional isomers (isobaric species with different combinations of acyl chains) by nLC cannot be made completely. Therefore, the possible combinations of TG positional isomers were listed in Table S2 in the Supporting Information.

Among the 14 classes of lipids listed in Table 2, it is obvious that the numbers of LPC, SM, and PE molecules were significantly

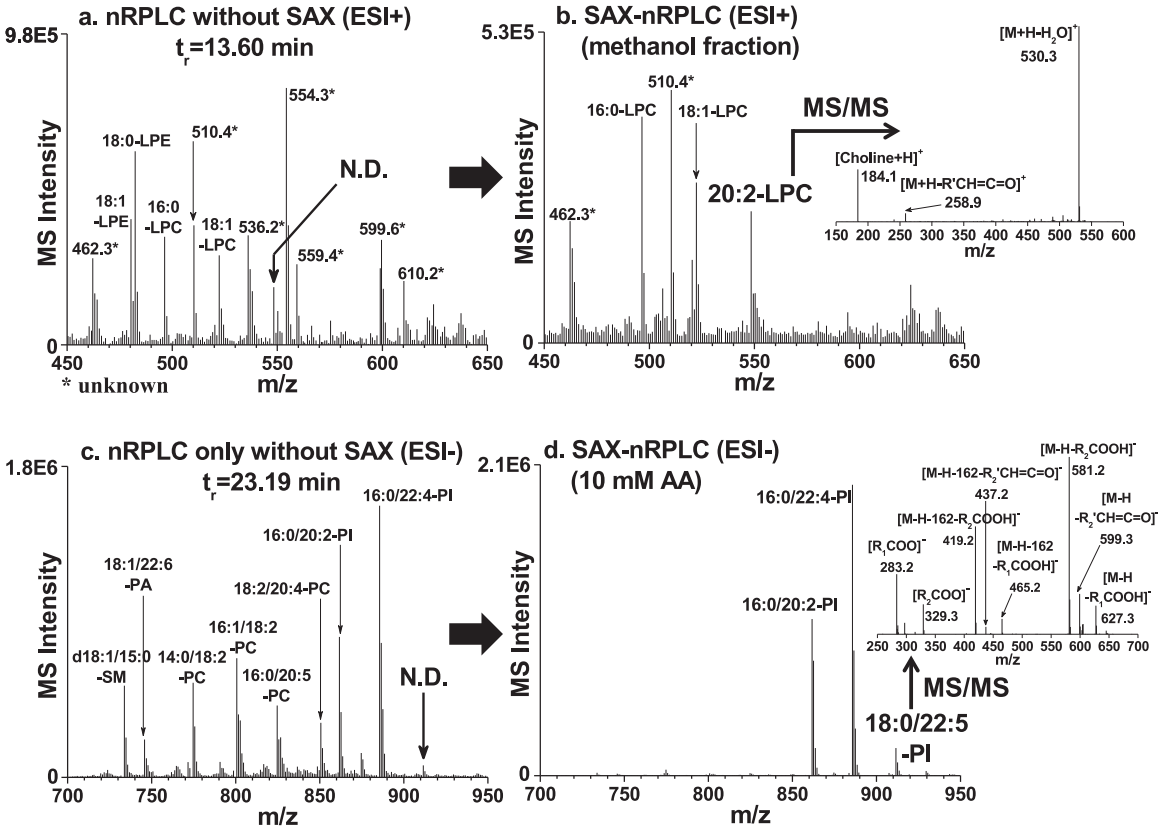


**Fig. 5.** Comparison of MS spectra of the plasma lipid extract sample obtained by only 1D-nRPLC–ESI-MS/MS with that obtained by 2D-SAX/nRPLC–ESI-MS/MS. MS spectra were obtained at $t_r$ = 13.60 min (positive ion mode) for (a) the lipid extract sample by 1D-LC without SAX and (b) the methanol fraction by 2D-SAX/nRPLC–ESI-MS/MS. Additional MS spectra were obtained at $t_r$ = 23.19 min in negative ion mode for (c) the lipid extract sample and (d) the 10 mM AA fraction by 2D-LC.

increased due to the use of 2D-LC separation, which supports that 2D-SAX/nRPLC is effective in the fractionation of lipid classes and molecular separation, especially for less abundant species. Fine control of the salt concentration interval may increase the fractionation capability, but there will be some overlap where the same species is eluted in several salt step fractions. The present 2D separation can be applied for the analysis of specific lipid classes or for the isolation of target molecules from SAX by depleting unwanted lipid fractions. The latter can be achieved simply by delivering, for instance, a methanol or AA solution to the SAX column from the autosampler by adjusting the six-way valve at position A during loading Mode 1 with an on-off valve connected at the end of the blocked port connected to the split tubing of the micro-tee. This can bypass the loading of unwanted lipid fractions into the C18 trap.

The current results were compared with those from LC–MS based methods recently reported. Quehenberger et al. [18] reported 588 lipids structurally identified from a NIST human plasma standard with quantitative information. However, it is rather difficult to compare the two results directly since 588 lipids were identified by using gas chromatography–MS and LC–MS in 7 different laboratories by targeting different classes of lipids of which extraction method was customized to each lipid class. Gao et al. [19] reported an identification of a total 444 rat plasma lipids with top-down/bottom-up approaches by using 1D-UPLC–MS. The report showed identification of 142 TG, 105 PC, and 30 free sterols species which were much higher than our results (57 TG, 56 PC, and no sterols which were non-targeted by LiPilot program) due to the higher peak capacity of UPLC than nLC. Besides these differences, our results showed a higher number of identification in most other lipid classes due the capability of detecting low abundant species. In addition, all identified phospholipids species in our study showed complete molecular structures with acyl chain location.

## 4. Conclusions

This study shows the development of an on-line 2D-SAX/nRPLC–ESI-MS/MS that can be utilized for the stepwise fractionation of various lipids (neutral polar, weak anionic, and anionic lipids) by capillary SAX, followed by the molecular-level separation of lipid fractions with nRPLC and the structural determination of lipid molecules from MS/MS spectra. By implementing SAX and C18 trapping columns prior to the nRPLC separation, the impurities or salts contained in a lipid sample can be removed prior to LC–MS/MS by an on-line method, which is helpful to lower the detection limit. First, the orthogonal separation of lipids in SAX in the first dimension reduces the complexity of the lipid mixtures, making it possible to detect less abundant lipid species with nRPLC in the second dimension. The 2D-SAX/nRPLC–ESI-MS/MS method was effective in expanding the identified number of lipid molecules of different categories. Since only a few microliters of salt solution from the autosampler are needed in the salt step fractionation to elute lipids from SAX column, it can be simply implemented with most LC–MS instruments for full automation without the need for an additional LC pump. Moreover, salt step fractionation using an autosampler, as well as the two-step sample loading procedures, can be used to bypass unwanted lipid fractions and to selectively analyze a desired lipid fraction, which can be useful for the quantitative analysis of target molecules. One of the important features of this study is the selection of a common ionization modifier (a mixture of 0.05% AH and 5 mM AF) for the successful operation of 2D LC–MS run in both positive and negative ion modes using the same set of binary mobile phase solutions. This facilitates the speedy analysis of a lipid sample without the need to wait for the solvent exchange for each ion mode. Our initial evaluation of the on-line 2D-SAX/nRPLC–ESI-MS/MS system has demonstrated its capability of analyzing a total of 303 plasma lipids from fourteen different classes, including SM, So, Sa, DG, TG, CE, MHC, Cer, PC, PE, PG, PI, PA, PS, and lysophospholipids. The present study reveals the precision in separation (less than 2.17% RSD in retention time) of individual lipid molecules as well as LOD levels (0.93–1.46 fmol/μL) similar to those reported in 1D-nLC–ESI-MS/MS. Moreover, the current method relies on the identification of lipid species of which molecular structures are fully determined by MS/MS. Further evaluations will be made to expand the capability of analyzing the other lipid classes.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.08.069.

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