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On-line high speed lipid extraction for nanoflow liquid chromatography-tandem mass spectrometry

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ABSTRACT

An on-line lipid extraction method is introduced by utilizing a short capillary extraction column using HILIC and C4 particles prior to nanoflow liquid chromatography-tandem mass spectrometry (nLC–MS/MS). The on-line extraction using a urine sample spiked with PL standards showed similar or slightly higher recovery values (86%–96%) of phospholipids (PLs) compared to those obtained by the conventional off-line extraction based on the Folch method with or without using the air-exposed drying process. In this study, we demonstrated that PL oxidation can occur during the air-exposed drying process of lipid extracts in standard liquid–liquid extraction procedures, which was confirmed by the oxidized PL (OxPL) molecules that were generated from an off-line extraction using a few PL standards. Quantitative comparison of these OxPL species between on- and off-line extraction followed by nLC–MS/MS with multiple reaction monitoring (MRM) analysis showed a significant decrease (2–10 fold) in unwanted OxPL species when on-line extraction was employed. While the number of identified PLs from a urine sample was somewhat lower than those by off-line extraction, the number of OxPLs was significantly reduced (from 70 to 22) with on-line extraction. The new method offers high speed (~5 min) automated extraction of PLs with nLC–MS/MS analysis and presents the possibility of handling a biological sample with a very limited amount of lipids.

1. Introduction

Lipidomics is the study of lipid networks in biological systems and generally needs molecular characterization and quantitation of lipids to elucidate their functions and interactions with proteins, cells, and other metabolites. Lipids are the main components of cellular membranes; moreover, they are involved in important functions such as energy storage, signal transduction between cells and proteins, cell proliferation, and death [1,2]. Recently, using lipidomic analysis for developing biomarkers has attracted considerable attention because lipids are known to be directly or indirectly related to the development of various adult diseases such as diabetes, cardiovascular diseases, and some cancers [3–7]. However, since lipids are very diverse in their molecular structures and polarities, extracting and analyzing lipid species of different categories simultaneously is difficult; therefore, lipidomic analysis required comprehensive and accurate analytical methods.

Mass spectrometry (MS) plays an important role in lipid analysis because of the simultaneous detection of a number of molecular ions and determination of their molecular structures. Liquid chromatography-electrospray ionization-tandem MS (LC-ESI–MS/MS) provides separation of complicated lipid mixtures in their intact states, followed by both qualitative and quantitative lipid profiling [8–10]. Nanoflow reversed-phase LC (nRPLC) with ESI–MS/MS has been utilized for analyzing phospholipids (PLs) from human plasma and urine samples with a limit of detection (LOD) of low fmol levels [11–13]; moreover, ultrahigh performance LC (UPLC) with ESI–MS/MS with enhanced resolution and speed has been utilized for identifying more than 400 lipids from rat plasma [14]. Recently, hydrophilic interaction chromatography (HILIC) method has shown its applicability to separate various lipid species from egg yolk [15]. Supercritical fluid chromatography (SFC) coupled with MS has been utilized for lipid profiling [16,17] and ultrahigh-performance SFC has demonstrated its high-throughput capability of analyzing 436 lipids in 6 min [18]. Although the abovementioned approaches can accelerate the performance and speed of lipid analysis, sample preparation, which includes lipid extraction from biological materials, requires attention because extracting all lipid classes simultaneously with a high recovery rate is difficult. Typical lipid extraction based on liquid–liquid extraction (LLE) has been widely utilized using the Folch method [19] or the Bligh and Dyer method [20]; however, these methods generally require large amounts of samples and removal of water when handling urine or blood samples requires

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a few hours. The solid phase extraction method offers a reduced matrix effect with higher selectivity compared to LLE; however, completing the extraction/purification steps, including the use of a series of solvents, requires time [21,22]. The quick, easy, cheap, effective, rugged, and safe (QuEChERS) method was applied for extracting lipids from human plasma and urine samples. Note that it shows high speed (<15 min) preparation of lipids with levels of lipid identification comparable to those of the Folch method [23]; however, adsorbents used to remove water in the QuEChERS method are not re-usable, which makes it less suitable for on-line extraction. Although the above methods provide an efficient extraction/clean-up of lipids, special care is required when extracting lipids from biological samples because, depending on the lipid classes to be analyzed, each method requires a slight modification for selecting organic solvents. Moreover, there is a possibility of inducing unwanted oxidation of lipid molecules from exposure to air during the drying step. Lipid oxidation generally occurs in biological systems via enzymes and/or reactive oxygen species (ROS); moreover, lipid oxidation typically results in hydroxylation of unsaturated acyl chains of PLs, cleavage of acyl chain into short-chain products or lysophospholipids (LPLs), and the dissociation of head groups of PLs [24,25]. Because oxidation of lipids alters biological membrane structures and leads to an increase in oxidized low-density lipoproteins, which is a key factor for the development and progression of age-related chronic diseases such as atherosclerosis [26,27], minimizing external lipid oxidation during extraction for accurate lipid analysis is important. In a recent report on the quantitative profiling of oxidized PLs in different lipoproteins from patients with coronary artery disease, special care was required to prevent unwanted lipid oxidation during extraction [28]. External lipid oxidation can be minimized by using N_2 during the drying steps or by using an on-line lipid extraction followed by direct analysis using MS or LC–MS. On-line lipid extraction may offer high speed extraction, which reduces the entire analysis time, however it has been hardly found except a report in which laser capture microdissection was utilized to collect fluorescently labeled brain tissue patches and carry out on-column lipid extraction prior to LC–MS^n [29]. The latter method demonstrated an identification of 58 lipids, but it was limited to mostly PC and PE with few Cer, and etc.

This study introduces an on-line lipid extraction device that can be utilized between an autosampler and an LC column during LC-ESI–MS/MS. This device is designed with a capillary based extraction column with a combination of packing materials, which can be applied for high speed lipid extraction by injecting urine samples after a simple treatment to remove proteins or macromolecules. The efficiency of the on-line lipid extraction column was evaluated using various particles (C4, C8, C18, and HILIC) packed in a short capillary tube by comparing the recoveries from a urine sample spiked with standard PLs between on-line and few off-line extraction methods. Also this study reveals that PL oxidation can occur with air exposure during the drying steps in typical extraction methods by confirming the molecular structures of the produced OxPLs when a few standard PLs underwent known extraction methods. Finally, the new method was applied for the comparison of urine samples' OxPL profiles between on- and off-line extraction methods.

2. Experimental

2.1. Materials & chemicals

Nineteen PL standards were purchased from Avanti Polar Lipid, Inc. (Alabaster, AL, USA): 14:0-LPE (lysophosphatidylethanolamine), 18:0-LPE, 12:0/12:0-PE (phosphatidylethanolamine), 14:0/14:0-PE, 14:0-LPG (lysophosphatidylglycerol), 18:0-LPG, 12:0/12:0-PG, 15:0/15:0-PG, 16:0-LPC (lysophosphatidylcholine), 16:0/16:0-PC (phosphatidylcholine), 20:0/20:0-PC, 14:0/14:0-PS (phosphatidyserine), 18:0/18:0-PS, 16:0/18:2-PI (phosphatidylinositol), d18:0/12:0-SM (sphingomyeline), d18:1/16:0-SM for the extraction efficiency test, 16:0/18:1-PE and 18:0/22:6-PG for the experiments to confirm oxidation products, and 16:0/16:0-PG as an internal standard (IS). Each PL standard was dissolved in 1:1 chloroform/MeOH at a concentration of 1 nmol/μL to make a stock solution. For the case of polar PLs like lysophospholipids, use of chloroform was minimized then small quantity of water (<15%) was added to enhance dissolvation. Then each stock solution was mixed to make a mixture at a concentration of 50 pmol/μL of each lipid in 8:2 MeOH/water for spiking to urine sample. A human urine sample was obtained from a healthy male volunteer (age 27) and stored under −20 °C before usage. Ammonium hydroxide and ammonium formate were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), HPLC grade solvents (acetonitrile, methanol, isopropanol, and water) were obtained from J. T. Baker (Phillipsburg, NJ, USA). $MgSO_4$ and CH_3COONa, which were used for QuEChERS, were obtained from Agilent Technologies (Palo Alto, CA, USA).

2.2. Off-line lipid extraction

Three extraction methods were utilized in this study. In the conventional Folch method, 200 μL of either urine or water both spiked with standard lipid mixtures was first mixed with 40 μL of methanol and 100 μL of chloroform. After 30 min, the mixture was centrifuged at 5000*g* for 10 min. The lower phase was then collected and dried in the vacuum centrifuge or under dried N_2 gas without using a vacuum centrifuge. For the Folch method with methyl-t-butylether/methanol (Folch with MTBE/MeOH), 200 μL of either urine or water both spiked with standard lipids was mixed with 40 μL of methanol and 100 μL of MTBE followed by vortexing for 30 min [30]. After centrifuging at 5000*g* for 10 min, the top layer containing lipids was collected and the bottom layer was transferred to another vial to extract remaining lipids by adding 100 μL of methanol followed by centrifugation for another 10 min. The supernatant was then collected and combined with the previously collected top layer. This mixture was dried under vacuum centrifuge or under dried N_2 gas to avoid exposure to air. Dried lipid extracts are dispersed in a solvent mixture composed of 10 μL of chloroform, 90 μL of 1:9 (v/v) acetonitrile:methanol, and 100 μL of LC mobile phase A (to be described later). The modified QuEChERS method for extraction of lipids follows the procedure described in the earlier report [23]. Briefly, the above described 200 μL of either urine or water both spiked with standard lipids is added to a prepacked extraction kit (250 mg of $MgSO_4$, 50 mg of CH_3COONa, and a glass ball) and mixed with 400 μL of 2:1 (v/v) chloroform:methanol for 1 min. The mixture is then centrifuged at 10,000*g* for 10 min, and the upper layer was transferred to 50 mg of C18 particles. The latter is centrifuged again at the same condition, and the upper layer is added to a separate vial and diluted to a final volume of 200 μL using the mobile phase A for nLC-ESI–MS/MS analysis.

2.3. On-line lipid extraction column

The on-line extraction column was prepared in the laboratory by packing various particles in a capillary (100 μm I.D. × 360 μm O.D. × 4 cm). Tested bead materials were Nucleodur® HILIC (5 μm), which was obtained from Macherey-Nagel (Duren, Germany); Magic® C4 and C8 resins (5 μm, 200 Å each), which was obtained from Bruker-Michrom (Auburn, CA, U.S.A.); and Watchers® ODS-P C18 particles (3 μm, 100 Å) from Isu Industry Corp. (Seoul, Korea). For packing the on-line extraction column, one end of the capil-

lary was connected to an in-line microfilter assembly from IDEX Health & Science LLC (Oak Harber, WA, USA) and the capillary was packed with each bead in a slurry state (water:methanol in 3:7 [v/v]) under helium at 1000 psi. Subsequently, the column was cut to a total length of 4 cm, whereas the other end of the capillary was connected to another in-line microfilter assembly. The column was coupled to an analytical column via two 6-port valves, which were equipped in the LC system and MS instrument. Note that all connections to and from the column were made using capillary tubes (50 μm I.D. and 360 μm O.D.).

2.4. nLC-ESI–MS/MS of PLs

For structural determination of PLs and OxPLs, a Model 1260 Infinity Capillary Pump system, equipped with an autosampler from Agilent Technologies (Palo Alto, CA, USA), was used with a LTQ Velos ion trap mass spectrometer from Thermo Finnigan (San Jose, CA, USA). For quantitative analysis, nanoACQUITY UPLC from Waters (Milford, MA, USA), coupled with a TSQ Vantage triple stage quadrupole MS from Thermo Finnigan, was used with the multiple reaction monitoring (MRM) method. Both LC-ESI–MS/MS systems utilized the same capillary LC analytical column which was prepared in our laboratory by pulling one-end of capillary tube (100 μm I.D. and 360 μm O.D.) into a sharp needle using a flame torch and packed with Watchers® ODS-P C18 particles (3 μm–100 Å) for 7 cm under He at 1000 psi.

Mobile phase solutions and the binary gradient elution condition used for both LC-ESI–MS/MS systems are described subsequently. Mobile phase A was H_2O:CH_3CN (90:10, v/v), and mobile phase B was composed of CH_3CN:CH_3OH:isopropanol (20:20:60, v/v). Ammonium formate (10 mM) and ammonium hydroxide (0.05%) which were developed for as effective ionization modifier in previous paper [31] were added to both mobile phase solutions to carry out mixed mode ionization. For gradient elution, mobile phase composition of B was ramped from 0% to 60% for 0.5 min, slowly increased to 80% for 10 min, to 97% for 4 min, and then maintained at 97% for 20 min. During qualitative analysis, using nLC with LTQ ion trap MS, PC, PE, and SM were detected in the positive ion mode with capillary temperature at 300 °C, 3.0 kV of ESI voltage, MS scan range of 400–950 amu, and 40% collision energy for CID. In the negative ion mode, PG, PI, and PA were detected with a capillary temperature at 300 °C, 2.5 kV of ESI voltage, MS scan range of 350–1000 amu, and 45% collision energy. During quantitative analysis using nUPLC and TSQ triple stage quadrupole MS, the same LC run conditions were used, but detection of ions was automatically switched between positive and negative ion modes with capillary temperature at 250 °C, 3.0 kV ESI voltage, and scan width 2.0. Precursor/product ions monitored via MRM method are listed in Table S1 of Supplementary data.

3. Results & discussion

3.1. Performance evaluation of on-line lipid extraction

The on-line lipid extraction method developed in this study is based on the use of a 4-cm long capillary column located in front of the analytical column via two 6-way valves as shown in Fig. 1. For the performance evaluation of the on-line extraction column, various chromatographic packing particles (C4, C8, C18, HILIC (4 cm each), and a combination of C4 + HILIC (2 + 2 cm)) were tested by comparing recovery values of sixteen PL standards spiked to a urine sample among different particles. For this test, 200 μL of a spiked urine sample was mixed with 100 μL methanol and 100 μL water. This mixture was then sonicated for 5 min in order to induce aggregation of proteins or enzymes and to dissolve lipids from the

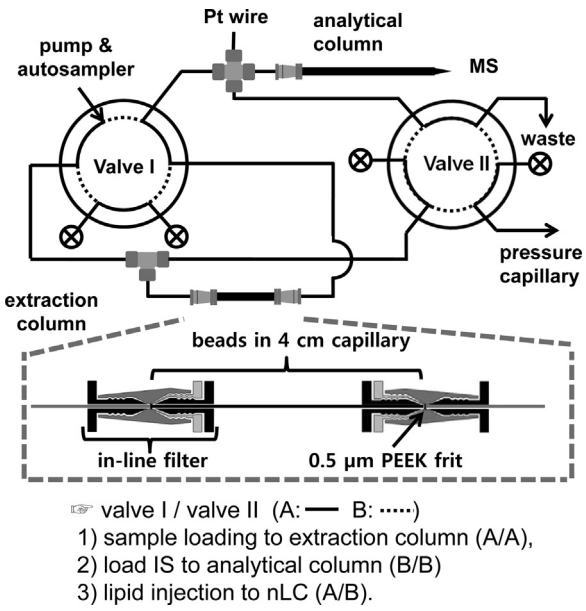

Fig. 1. System configuration of on-line lipid extraction for nLC-ESI–MS/MS. Note that the on-line lipid extraction column, the autosampler, and a capillary analytical column are assembled using two 6-way valves, which automatically are controlled. Valve positions I/II are A/A for sample loading to lipid extraction column, B/B for the direct loading of IS to analytical column, and A/B for elution of lipids from extraction column and nLC-ESI–MS/MS run (A for solid line and B for dotted line).

vesicular form of lipids in urine. The mixture was then centrifuged at 5000*g* for 5 min to remove aggregates before injection. On-line extraction and nLC-ESI–MS/MS of lipids can be performed in three stages with full automation. In the first step, 20 μL of sample was loaded from an autosampler onto the extraction column with the valve configurations I/II as A/A (A: solid line) at 2 μL/min of 100% mobile phase A (Fig. 1) for 5 min which was the time period for sample loading and solid phase extraction. With the valves at A/A, the mobile phase solution washed any salt and non-partitioning species contained in the sample, bypassed the analytical column (due to its back pressure), and exited through valve II. After 5 min of sample loading, the valve configuration was changed to B/B (B: dotted line) to load an IS (3 pmol of 16:0/16:0-PG) to the analytical column directly from the autosampler in order to compensate for signal fluctuations during repeated nLC-ESI–MS/MS. Since 16:0/16:0-PG was not identified from urine sample utilized in this study, it was selected as an IS. This step is necessary to mix an IS with a sample without passing it through the on-line lipid extractor; this assures consistent IS signals regardless of the performance of on-line extraction. In the last step, both valves were changed to A/B so that flow from the pump was delivered to the on-line extractor for desorbing lipid species and then to the analytical column for the beginning of gradient elution. At this stage, the pump flow rate was ramped to 20 μL/min, but it splits just before the extraction column so that only 600 nL/min of flow entered the on-line extraction column and then the analytical column by controlling the length of a pressure capillary tube connected to valve II.

For the validation of on-line lipid extraction, relative peak areas (vs. IS) of sixteen PL standards spiked to a urine sample were compared among different bead materials. Bars in Fig. 2a represent the peak area of each PL standard relative to that obtained with the C4 + HILIC extraction column which was set as 100. When HILIC particles (white bars) were utilized alone for on-line extraction, less hydrophobic PLs, including LPLs (No. 1, 2, and 5) and PGs with short acyl chains (No. 7 and 8), showed higher recoveries, and most PLs with long acyl chains yielded poor recoveries. However, C4 showed

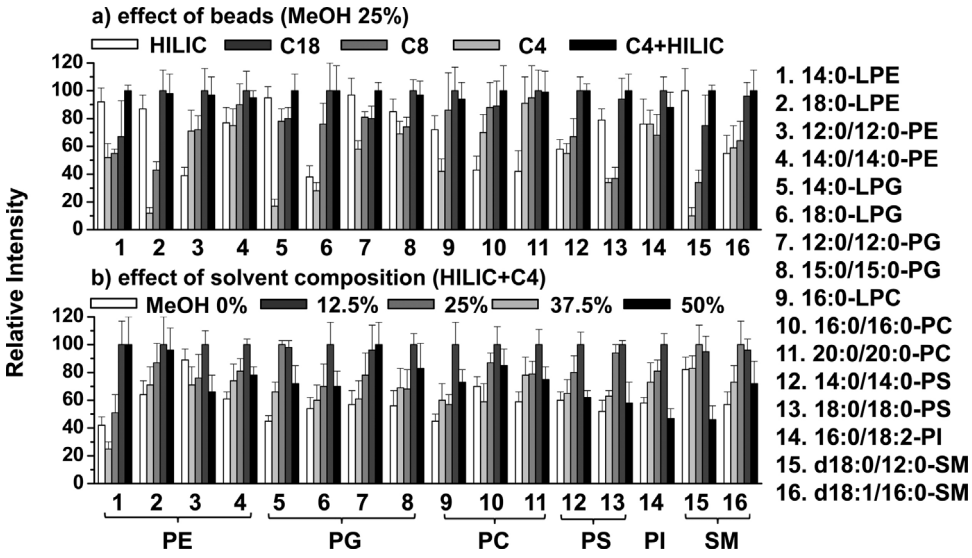

Fig. 2. a) Effect of various particles (C4, C8, C18, HILIC, and a mixed C4 + HILIC) packed in 4 cm long on-line extraction column on the extraction of 16 PL standards prior to nLC-ESI–MS/MS with MRM. Note that each peak area value was plotted relative to the peak of the highest recovery. Percentage of MeOH in the standard mixture was 33.3%. (n = 3) b) Effect of volume percentage of MeOH added to PL standards on the on-line lipid extraction in which each datum was relative to peak of highest recovery. (n = 3).

an opposite trend in which long chain PLs were significantly recovered. In order to take advantage of the partitioning properties of each bead, C4 and HILIC particles were packed in a series (2 cm each). C18 and C8 did not show a consistent recovery pattern among the PL standards on a wide polarity scale. Since methanol was added to urine sample at 25% (v/v) to remove proteins or other hydrophilic species, it may have interfered with the lipid partitioning of the C18 and C8 particles of the on-line extraction column during sample loading. In order to evaluate the influence of the amount of methanol added to the urine sample on the binding efficiency, the volume percentage of MeOH was varied (0%–50%) by varying volume of MeOH added to 200 μL of the PL-spiked urine sample followed by dilution to a total volume of 400 μL with distilled water. In this case, the mixed bead (C4 + HILIC in 2 cm each) was used. When MeOH was not added to the urine sample in Fig. 2b, recoveries of most PL standards were very poor, indicating that dissolution of lipids in the urine sample was not well achieved. As the MeOH amount increased, recoveries of most PL standards improved until 37.5% at which point they started to deteriorate. Based on results from this experiment, the use of the mixed bead extraction column together with 37.5% methanol (4:3:1 – urine:MeOH:H_2O) were selected for further experiments.

The effects of on-line extraction on the additional band broadening together with extended elution time due to the increased pathway was evaluated by comparing the separation of PL standards with and without on-line extraction. Fig. 3 shows the comparison of base peak chromatograms (BPCs) from ten PL standards without (a) and with on-line extraction (b) before nLC, indicating that additional peak broadening after on-line extraction was not observed at all except for the substantial increase in elution time at about 2 min. The delay period (~5 min) before the beginning of MS signals corresponds to the time period including sample loading and on-line extraction.

3.2. Efficiency between on-line & off-line extraction methods

The performance of the on-line lipid extraction method was evaluated by comparing its extraction efficiency of sixteen standard PLs spiked to a urine sample with the conventional Folch, the Folch with MTBE/MeOH, both with or without using N_2 for drying, and the QuEChERS methods (Table 1). In this case, a urine sample (125 μL) spiked with 16 PL standards was mixed with 75 μL of water (for off-line extraction) or 75 μL of MeOH (for on-line extraction) in order to adjust the final concentration of standards as 100 fmol/μL. In cases of off-line extractions, dried lipid powder was re-dissolved in a mixture of 10 μL of chloroform, 90 μL of 1:9 (v/v) acetonitrile:MeOH, 10 μL of IS (2 pmol/μL), and 90 μL of LC mobile phase A. Then 20 μL of the mixture was injected to nLC–MS/MS. For on-line extractions, 20 μL of the above urine mixture (with MeOH) was loaded onto the on-line extraction column and 1 μL of IS (1 pmol/μL) was loaded to an analytical column following the above IS injection step (B/B valve of Fig. 1). Recovery values from nLC–MS/MS analysis listed in Table 1 are the relative values with respect to the peak area obtained from the urine-free standard mixtures. While the conventional Folch method showed recovery values of 70%–90%, the Folch with MTBE/MeOH method showed >90% recovery values, as seen in a previous study [30]. Both methods exhibited increased recovery values when N_2 was used in the drying step. This can be from the oxidation of lipids by the

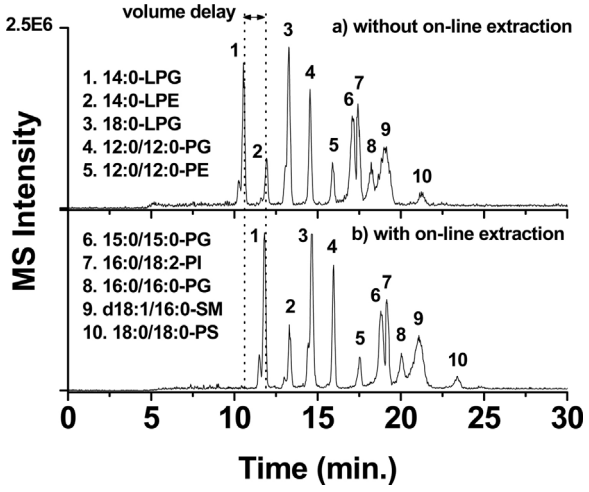

Fig. 3. Comparison of BPCs of 10 PL standards with or without using on-line extraction prior to nLC-ESI–MS/MS, showing no significant change in peak width and area except the volume delay due to the increased path between the extraction column and the valve-I in Fig. 1.

Table 1
Comparison of extraction efficiencies of 16 PL standards which were spiked to a urine sample between off-line and on-line extraction. Data represent the recovery value of each PL species relative to the nLC-ESI–MS/MS peak area of the PL standard without undergoing extraction. For on-line extractions, a mixed bead (C4 + HILIC) was utilized prior to nLC-ESI–MS/MS. (n = 3).

standard	*m/z*	Folch		Folch with MTBE/MeOH		QuEChERS	on-line extraction
		speedvac	under N_2	speedvac	under N_2		
14:0-LPE	424.3	72.9 ± 6.5	77.4 ± 7.2	94.6 ± 13.5	94.4 ± 14.2	78.7 ± 9.2	90.2 ± 7.9
18:0-LPE	480.4	76.5 ± 9.4	76.8 ± 11.8	89.3 ± 11.4	95.0 ± 12.3	90.3 ± 13.9	86.3 ± 12.1
12:0/12:0-PE	578.4	84.3 ± 9.8	82.8 ± 14.3	91.8 ± 15.0	97.3 ± 11.5	98.9 ± 14.4	91.8 ± 15.0
14:0/14:0-PE	634.5	88.9 ± 7.2	96.3 ± 9.7	97.5 ± 15.8	92.8 ± 7.6	94.5 ± 13.3	103.1 ± 10.8
14:0-LPG	455.3	72.4 ± 8.2	69.1 ± 8.7	100.3 ± 10.5	97.3 ± 9.8	92.5 ± 9.1	89.6 ± 15.8
18:0-LPG	511.3	77.7 ± 10.6	76.2 ± 8.0	101.6 ± 8.3	97.8 ± 9.0	98.8 ± 13.4	97.4 ± 10.2
12:0/12:0-PG	609.4	82.3 ± 5.1	85.4 ± 4.6	91.8 ± 4.5	95.7 ± 6.1	90.2 ± 10.9	90.1 ± 8.3
15:0/15:0-PG	693.5	89.2 ± 5.9	91.2 ± 6.4	91.2 ± 7.7	95.6 ± 7.6	91.7 ± 8.3	95.6 ± 9.7
16:0-LPC	496.4	70.9 ± 9.2	71.5 ± 8.5	91.5 ± 8.5	91.1 ± 7.2	85.5 ± 6.8	86.9 ± 7.5
16:0/16:0-PC	734.5	83.9 ± 8.6	80.2 ± 8.4	89.4 ± 9.0	88.6 ± 12.0	91.6 ± 11.5	89.3 ± 12.7
20:0/20:0-PC	846.6	92.7 ± 9.9	94.4 ± 6.0	97.9 ± 8.7	96.0 ± 7.9	89.1 ± 8.1	93.0 ± 9.2
14:0/14:0-PS	678.5	84.9 ± 10.5	81.0 ± 9.6	92.2 ± 11.9	94.5 ± 14.0	95.2 ± 13.9	94.5 ± 13.6
18:0/18:0-PS	790.6	84.2 ± 10.2	88.1 ± 11.4	90.8 ± 12.1	90.1 ± 13.1	90.2 ± 12.8	88.8 ± 11.2
16:0/18:2-PI	833.6	90.5 ± 2.5	90.2 ± 5.9	92.0 ± 6.5	90.7 ± 8.1	93.0 ± 7.8	92.8 ± 4.2
d18:0/12:0-SM	693.5	89.1 ± 6.5	87.8 ± 6.3	95.6 ± 10.3	94.6 ± 14.1	96.6 ± 7.6	89.9 ± 9.9
d18:1/16:0-SM	747.5	85.7 ± 5.6	88.6 ± 5.5	90.6 ± 8.3	93.4 ± 7.0	93.1 ± 8.8	89.8 ± 9.1

air-exposure during the drying process without N_2. Compared to the LLE methods, the QuEChERS method yielded better recoveries while all LPLs showed relatively poor recoveries. In the case of on-line extractions, this method yielded recovery values much higher than those of the Folch method and similar to those with Folch with MTBE/MeOH using N_2. This supports the idea that the on-line extraction method can be utilized for high speed lipid extraction with full automation.

3.3. On-line lipid extractor for prevention of oxidation

In order to investigate oxidized lipids that were produced during the lipid extraction process, PL extracts obtained by off-line extraction were analyzed by nLC-ESI–MS/MS with structural determination of Ox-PL species. A mixture of four PL standards was treated by varying extraction methods. Because oxidation of PLs typically occurs at unsaturated acyl chains, three standard PLs, which have only one unsaturated acyl chain (16:0/18:1-PE, 16:0/18:2-PI, and 18:0/22:6-PG) together with one saturated PG standard (12:0/12:0-PG), were selected to examine oxidation patterns and easily track their original PL molecules. The mixture was diluted to 200 μL using water at a concentration of 10 pmol/μL for each species and extracted by the three extraction methods: conventional Folch method followed by drying with N_2 or SpeedVac, and QuEChERS.

Fig. 4 shows the nLC-ESI–MS/MS results by injecting 3 μL of the standard lipid extract prepared with the Folch method along with SpeedVac drying. Fig. 4a shows the BPC of the four PL standards along with the elution of LPI and LPG, which are presumed to be oxidized products during extraction procedure or impurities of Std. samples. The inserted chromatogram (100 × magnification), which was obtained by direct injection of the same mixture without extraction, does not show a peak of 18:2-LPI but a very small peak of 12:0-LPG; therefore, LPI and most of LPG are believed to have been produced during extraction. Fig. 4b shows the collision-induced dissociation (CID) spectra of a precursor ion, *m/z* 747.5, which was observed at t_r = 29.0 min (not visible in Fig. 4a); moreover, it shows typical fragment ions of $[R_1COO]^-$ and $[R_2COO]^-$ at *m/z* 283.4 and 327.4, which originated from the acyl chain 18:00 and 22:6, respectively. This can be easily confirmed to have originated from 18:0/22:6-PG; however, four fragment ions at *m/z* 419.2, 437.3, 463.3, and 481.3 indicate that these were produced by the loss of each acyl chain in the form of carboxylic acid ($[M\text{-}H\text{-}RCOOH]^-$) or ketene ($[M\text{-}H\text{-}R'CH{=}C{=}O]^-$) but occurred after the glycerol head group ($-CH_2CHOHCH_2OH$, 75 Da) of the PG molecule was replaced with a H atom. Because there was no other PL molecule having the same acyl chain combination and no PA standard was added to the PL mixtures, the precursor ion (*m/z* 747.5) in Fig. 4b was determined to be 18:0/22:6-PA, which is an oxidized product of 16:0/22:6-PG obtained by the loss of its head group. Moreover, this loss resulted in an increase in the retention time to 29.0 min due to the increase of hydrophobicity. Because 16:0/22:6-PA was not identified when the same standard mixture was directly injected, it is presumed to be from extraction-induced oxidation. Fig. 4c shows the CID spectra of a precursor ion *m/z* 741.5 at t_r = 14.2 min, which was confirmed as a short chain product after the truncation of the unsaturated acyl chain (22:6) into a shorter chain, which was terminated with an aldehyde (18:0/15:4CHO-PG). Typical fragmentation patterns showing the loss of acyl chains from both precursor ions as $[M\text{-}H\text{-}RCOOH]^-$ and the precursor ion without the head group as $[M\text{-}H\text{-}74\text{-}RCOOH]^-$ represent the identification of PG molecule. The molecular structure of the precursor ion *m/z* 853.5 at t_r = 16.8 min in Fig. 4d was identified as a hydroperoxylated product of PG, i.e., 18:0/22:6 + OO-PG, which was obtained from the fragment ions that were produced after the loss of a water molecule from the precursor ($[M{+}H{-}H_2O]^-$ at *m/z* 835.5) and the loss of two oxygen atoms from sn-2 acyl chain ($[R_2COO\text{-}O_2]^-$ at *m/z* 327.4), in addition to the typical fragment ions. Examining other oxidized products showed systematic oxidations, which produced long- and short-chain products, including LPLs. A detailed study about oxidation patterns of Std. PLs was described in previous paper published from our Lab [32].

In Table 2, these products are listed and the degree of oxidation among different extraction methods is compared. It is observed from the results seen in Table 2 that more of OxPLs were produced from PLs with a large degree of unsaturation (18:0/22:6-PG). Most long- and short-chain products were not found in the original standard mixture without extraction, indicating that extraction of lipids appears to induce oxidation. Clearly, the dissociation of the head groups occurred with all four PL standards after extraction by the occurrence of PA molecules corresponding to the acyl chain structures of their origins e.g., 12:0/12:0-PA originated from 12:0/12:0-PG. Table 2 shows that SpeedVac drying in the Folch method induced various PLs oxidations, while Folch with drying under N_2 and the QuEChERS method yielded a smaller number of OxPLs. The reason for less oxidation with QuEChERS over the other methods is the lack of a drying step. However, LPLs (12:0-LPG, 16:0-LPE, 18:1-LPE, 16:0-LPI, 18:2-LPI, 18:0-LPA, 18:0-LPG,

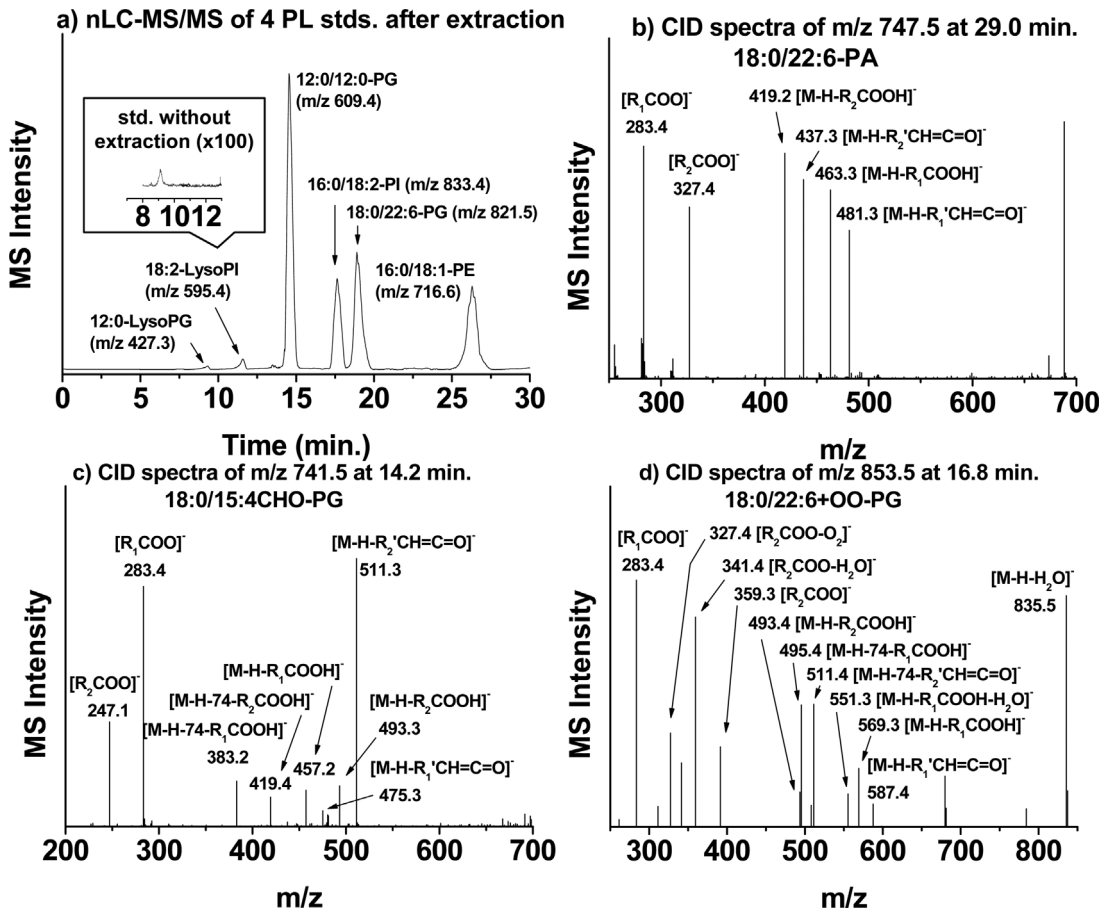

Fig. 4. a) Base peak chromatogram of the four standard PLs with oxidized products from nLC-ESI–MS/MS analysis of the PL extracts, which were treated using the Folch method. The inside panel is the enlarged (×100) chromatogram of the same standard mixture without undergoing extraction. b) CID spectra of ions of *m/z* 747.5 a 29.0 min showing an oxidized product identified as 18:0/22:6-PA originated from 18:0/22:6-PG, c) CID spectra of 18:0/15:4CHO-PG (*m/z* 741.5, 14.2) and d) 18:0/22:6 + OO-PG (*m/z* 853.5, 16.8).

Table 2
List of oxidized PL species detected by nLC-ESI–MS/MS run of the four PL standard mixtures which underwent extraction procedures; 0: without extraction, 1: Folch method with SpeedVac, 2: Folch with drying under N_2, 3: QuEChERS.

			methods							methods			
R1	R2	*m/z*	0	1	2	3	R1	R2	*m/z*	0	1	2	3
from 12:0/12:0-PG							from 18:0/22:6-PG						
12:0-LPG		427.3	√	√	√	√	18:0-LPG		511.3	√	√	√	√
from 16:0/18:1-PE							22:6-LPG		555.4	√	√	√	√
16:0-LPE		453.1	√	√	√	√	18:0	22:6 + O	837.5	√	√	√	
18:1-LPE		479.2		√			18:0	22:6 + OO	853.5		√		
from 16:0/18:2-PI							18:0	22:6 + 2O	853.5		√	√	
16:0-LPI		571.4	√	√	√	√	18:0	22:6 + 3O	869.3		√	√	
18:2-LPI		595.3	√	√	√	√	18:0	22:6 + O&OO	869.4		√		
16:0	18:2 + O	849.4		√	√		18:0	22:6 + 4O(OO)	884.7		√		
16:0	18:2 + OO	865.5		√			18:0	15:4CHO	741.6		√	√	
PAs from PE, PI, PG							18:0	15:4CHO + O	757.5		√		
18:0-LPA		437.5	√	√	√	√	18:0	21:5CHO	823.5		√		
12:0	12:0	535.4		√	√		18:0	12:2CHO	701.6		√		
16:0	18:1	673.5		√		√	18:0	14:3CHO	728.5		√		
16:0	18:2	671.5		√			18:0	14:3CHO + O	744.8		√		
18:0	22:6	747.6		√	√	√	18:0	21:5CHO + OO	855.5		√		
							number of oxidized products			8	27	14	9

and 22:6-LPG) originating from all standards were found regardless of the extraction method, including the results from analyzing the original lipid mixture (shown in the method 0 of Table 2). Because all LPLs were identified from the non-extracted standard mixture, the possibility that some of these LPL molecules were impurities present in the original standard or produced during ESI cannot be excluded.

The degree of oxidation was evaluated by quantifying OxPL species and their original PLs (4 PLs used in Table 2) between on- and off-line extraction methods (Table 3). In one set of experiments, the standard mixture (dispersed in 5:3 [v/v] H_2O:MeOH to avoid the

Table 3
Quantitative comparison of OxPL species produced by off-line extraction using SpeedVac drying with those with on-line extraction obtained by nLC-ESI–MS/MS with MRM. (n = 3).

Head	R1	R2	no extraction	Folch with MTBE/MeOH	with on-line extraction
PG	12:00	12:00	100.0 ± 10.6	91.7 ± 3.2	90.4 ± 5.6
	12:0-LPG		0.2 ± 0.2	4.3 ± 1.3	0.6 ± 0.3
PA	12:00	12:00		1.9 ± 0.9	0.2 ± 0.5
PE	16:00	18:01	100.0 ± 4.5	92.2 ± 2.5	92.3 ± 6.9
	16:0-LPE		0.4 ± 0.1	6.4 ± 3.4	0.4 ± 0.1
PA	16:00	18:01		0.7 ± 0.3	
PI	16:00	18:02	100.0 ± 9.8	90.8 ± 6.0	95.3 ± 8.6
	16:0-LPI		3.9 ± 2.3	8.8 ± 1.7	4.0 ± 1.8
	18:2-LPI			11.4 ± 2.8	
	16:00	18:2+O		4.9 ± 3.5	0.4 ± 0.8
PG	18:00	22:06	100.0 ± 8.8	86.4 ± 4.2	91.6 ± 8.9
	18:0-LPG		2.8 ± 1.3	6.4 ± 3.7	2.7 ± 1.2
	18:00	22:6+O	1.8 ± 1.4	4.8 ± 2.7	1.5 ± 0.6
	18:00	22:6+O&OO		0.1 ± 0.1	
	18:00	22:6+4O(OO)		0.8 ± 0.4	
	18:00	12:2CHO		2.5 ± 1.7	
	18:00	14:3CHO+O	0.1 ± 0.1	1.3 ± 0.9	0.2 ± 0.2
	18:00	21:5CHO+OO		5.4 ± 3.0	
PA	18:0-LPA		0.5 ± 0.3	0.9 ± 0.6	

matrix effect) was analyzed with or without undergoing on-line extraction prior to nLC-ESI–MS/MS using the MRM quantitation listed in Table 3. The nLC–MS/MS peak area of each standard without extraction was set as a reference (adjusted as 100.0) for each head group. For instance, the value 0.2 ± 0.2 of 12:0-LPG without extraction indicated that the relative peak area of the LPG species is 0.2 relative to that of 12:0/12:0-PG. By comparing the peak area values of original, un-oxidized PL molecules between the three different runs, recovery values after the Folch with MTBE/MeOH and after on-line extraction were 86.4–92.2 and 90.4–95.3, respectively, similar to those observed in Table 1. These results indicated that it was inevitable to have some loss due to extraction. However, decreases in peak area values of original PLs were not only a result of extraction but also of oxidation during extraction, mostly from the off-line extraction. It has been noted that more of OxPL species were detected with the off-line extracted sample, and their relative peak area in each head group significantly increased. LPL species, especially, were found to significantly increase in their relative levels after off-line extraction in Table 3: 4.3 from 0.2 for 12:0-LPG, 6.4 from 0.4 for 16:0-LPE, and 11.4 for 18:2-LPI. Moreover, some of these species were observed with the sample without having any extraction, but their amounts were considerably smaller than those after off-line extraction. Since retention time of LPL species was obviously different from that of the corresponding original PL molecule as observed in Fig. 4a, it can be presumed that LPL species were already contained at a very low level in each standard material. OxPL species found in PI and PG showed that hydroxylation occurred easily. However, these OxPL species were significantly reduced when on-line extraction was used for the standard mixture. Peak area values of LPL species with on-line extraction were decreased to the levels found with the non-extracted sample. Levels of hydroxylated species (16:0/18:2 + O-PI and 18:0/22:6 + O-PG) were significantly reduced to the levels of those without extraction, and other long chain or short chain products were not found with on-line oxidation. Moreover, dissociation of head groups was not found except in 12:0/12:0-PG but the amount of 12:0/12:0-PA was decreased by nearly 10 folds with on-line extraction. From Table 3, it appeared that oxidation of PL molecules during off-line extraction can be significantly minimized by using the on-line extraction method developed in this study.

Table 4
Total number of identified PLs from a human urine sample by Folch with MTBE/MeOH (left) and on-line extraction methods (right).

	PA	PG	PI	PS	PC	PE	SM	Total
PLs	15/11	12/10	17/14	10/7	18/16	17/16	4/4	93/78
OxPLs	15/5	16/6	18/5	4/0	9/2	8/4	–	70/22

3.4. Comparison of OxPL species from urine sample between on-line and off-line extraction

The on-line extraction method was applied to a healthy human urine sample. The effect of on-line extraction on inhibiting PL oxidation was evaluated by identifying OxPL species in comparison to those found from urinary PL extracts using Folch method with MTBE/MeOH. BPCs of urinary lipids between on-line and off-line extraction are compared in Fig. S1 of Supplementary data. Individual PL and OxPL species identified from off-line and on-line extraction methods are listed in Table S2. Numbers of PL species identified from each head group are compared in Table 4. Phospholipids with typical chain structures having polyunsaturated acyl chains such as 16:0/20:4, 18:0/20:4, and 18:0/22:6 were identified in most head groups from both extraction methods. Most of oxidized forms (16:0/20:4 + O, 18:0/20:4 + O, 18:0/20:4 + OO) derived from these chain structures were profiled from off-line extraction methods. Meanwhile, an oxidized form of 18:0/22:6 + O was found in PA, PG, and PI, and PE from both methods, supporting that these molecules existed in urine sample. It shows that the total number of PLs was somewhat reduced to 78 with on-line extraction from 93 with off-line extraction. A relatively smaller number of identified PLs with on-line extraction can originate from the difference in the volumes of urine utilized in on-line and off-line methods. While on-line extraction used 12.5 μL of urine (total 20 μL of mixture with methanol) injected to the extraction column, the off-line extraction began with 125 μL of urine (total 200 μL of mixture) and injection of extracted lipid to LC–MS/MS was made with 1/10 of this extract which is an equivalent amount of original urine. When 12.5 μL of urine was extracted by the same off-line method after diluting into 200 μL with methanol, identified number of PLs was 38, which was very low. For the case of OxPLs (without including LPLs), the identified number of OxPLs was found to be reduced to 22 with on-line extraction while 70 OxPL species were found from the

off-line method. Since OxPL species were also found from plasma samples of a healthy normal person [28], most of twenty-two OxPLs identified from on-line extraction can be thought of as natural OxPL species contained in the urine sample. Decrease in the number of OxPLs can be originated from the utilization of on-line procedure, however a possibility caused by relatively poor detection limit of on-line method cannot be excluded as the number of identified lipids was reduced. From this study, it was demonstrated that the on-line extraction method is conducive in preventing unwanted oxidation of PL molecules as well as the reduction of the entire analysis time by avoiding off-line extraction procedure, which normally takes more than half a day. Since on-line lipid extraction is achieved during the sample loading (5 min) to extraction column, it is a simple and rapid extraction method. By including the initial pre-treatment of urine sample (10 min for sonication and centrifugation), the entire process prior to the nLC–MS/MS analysis takes only about 15 min in total.

4. Conclusions

An on-line lipid extraction method to expedite lipidomic analysis has been introduced by implementing a short capillary extraction column serially packed with two different particles (HILIC and C4 resins in 4 cm), which provides a high speed automatic lipid extraction followed by LC–MS/MS analysis. This study, showed that recovery values of PLs can be maintained in the range of 86%–96%, which is similar to or slightly higher than the typical off-line extraction method. Furthermore, this study revealed that systematic oxidation of PLs can occur during the air-exposed drying process from the confirmation of molecular structures of OxPLs formed from standard PLs mixtures and these unwanted OxPLs can be significantly minimized with on-line extraction. While identified number of urinary PLs with on-line extraction was slightly less than that with off-line extraction, the number of OxPLs was significantly reduced from 70 to 22 in the off-line method due to blocking the chance of air-exposure for extracted lipids.

This on-line extraction method offers the possibility to handle a biological sample with very low amount of lipids without inducing an unwanted oxidation of PLs. In addition, it can be utilized for a high speed screening analysis of fresh urine samples when target PL lists are established. Continued studies are needed to minimize impurities, which may induce spectral noise in the MS analysis, and to expand the applicability for different types of biological samples such as blood plasma or tissue.

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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.2016.08.021.

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