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Rapid and simple extraction of lipids from blood plasma and urine for liquid chromatography-tandem mass spectrometry

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ABSTRACT

A simple and fast lipid extraction method from human blood plasma and urine is introduced in this study. The effective lipid extraction from biological systems with a minimization of the matrix effect is important for the successful qualitative and quantitative analysis of lipids in liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). The method described here is based on the modification of the quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction method, which was originally developed for pesticide residue analysis in food, for the purpose of isolating lipids from biological fluids. Applicability of QuEChERS method for lipids was evaluated by varying organic solvents for the extraction/partitioning of lipids in $MgSO_4$/CH_3COONa for the removal of water and by varying sorbents (primary secondary amines, graphitized carbon black, silica, strong anion exchange resins and C18 particles) for the dispersive solid-phase extraction (dSPE) step. This study shows that 2:1 (v/v) $CHCl_3$/CH_3OH is effective in the extraction/partitioning step and that 50 mg of C18 particles (for 0.1 mL plasma and 1 mL of urine) are more suitable for sample cleanup for the dSPE step of the QuEChERS method. Matrix effects were calculated by comparing the recovery values of lipid standards spiked to both plasma and urine samples after extraction with those of the same standards in a neat solution using nanoflow LC-ESI-MS/MS, resulting in improved MS signals due to the decrease of the ion suppression compared to the conventional Folch method. The modified QuEChERS method was applied to lipid extracts from both human urine and plasma samples, demonstrating that it can be powerfully utilized for high-speed (<15 min) preparation of lipids compared to the Folch method, with equivalent or slightly improved results in lipid identification using nLC-ESI-MS/MS.

1. Introduction

Lipids are major components of biological membranes and are involved in a number of metabolic processes, such as intercellular interactions, secretion, and energy storage [1]. Due to the important roles of lipids, a comprehensive lipid profile is necessary to study the mechanisms of lipid-related diseases, such as diabetes, Alzheimer's disease, atherosclerosis, and breast cancer [2,3]. Since lipids are so diverse their molecular structures, which are classified into eight categories based on their polar nature and common molecular backbone structures [4,5], it is difficult to simultaneously analyze all lipid classes.

Analytical methods that are frequently employed for lipid analysis are liquid chromatography-mass spectrometry (LC-MS) and direct analysis with MS via electrospray ionization (ESI). LC provides highly sensitive separation of lipid classes, depending on their polarity, in normal-phase LC (NPLC), or high-resolution separation of individual lipid molecules based on hydrophobicity of alkyl chains in lipids in reversed-phase LC (RPLC) [6–9]. With the coupling of LC with ESI-MS or ESI-MS/MS, lipid profiling can be conducted both qualitatively and quantitatively, along with the structural determination [10,11]. ESI-MS provides a fast analysis of lipids along with structural determination using high-resolution MS or from tandem MS experiments; however, ion suppression of highly abundant species can be of concern when complex lipid mixtures are to be analyzed [12–14].

Despite the high performances of these sophisticated analytical methods, the important pre-requisite for the successful analysis is the efficient extraction of highly complicated lipid mixtures of biological origin, such as cells, tissues, plasma, and urine, along with the removal of interfering substances, such as proteins, sugars, and other small molecules. Since lipids are so diverse in their hydrophobic and hydrophilic properties, it is often complicated to simultaneously extract all lipid classes with a high recovery

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rate. Traditionally, lipid extraction has been carried out with the Folch method [15] or the Bligh and Dyer method [16] based on liquid-liquid extraction. The liquid-liquid extraction (LLE) method requires a large sample, but generally yields low recovery as well as showing a matrix effect in LC-MS analysis [17,18]. Particularly for plasma or urine samples, the removal of water without losing recovery is an essential step. When the typical freeze-drying method is added, it often takes a long time (~12 h for urine) [19–21]. While a solid-phase extraction (SPE) method is effective in reducing the matrix effect and more selective than the LLE method, it requires a long period of time to carry out a series of purification/extraction processes, such as the preconditioning of sorbent materials, use of multiple solvents, and solvent waste fractionation steps [22–24]. Recently, a rapid extraction/cleanup approach, known as the QuEChERS (quick, easy, cheap, effective, rugged, and safe) method, was introduced by Anastassiades et al. in 2003 [25] for the multi-residue analysis of pesticides contained in food, and it was rapidly applied to various foods and environmental samples [26–28]. The method was further developed to extract polycyclic aromatic hydrocarbons in fish [29], veterinary drug residues in animal tissue [30], mycotoxins in cereals [31], and pharmaceuticals from whole blood [32,33]. It is known that the QuEChERS method reduces the preparation time (< 30 min) required to complete the extraction and clean-up processes. The extraction/partitioning step of QuEChERS involves extraction with CH_3CN partitioned from aqueous matrix using anhydrous magnesium sulfate to absorb water and sodium acetate to enhance phase separation. The second step of QuEChERS is the dispersive solid-phase extraction (dSPE) to cleanup impurities by using sorbents such as primary secondary amine (PSA), graphitized carbon black (GCB), and C18 particles.

This study focuses on the applicability of a QuEChERS-based approach toward the extraction and cleanup of various lipids from human blood plasma and urine samples for nanoflow LC-ESI-MS/MS analysis. The QuEChERS method, to our knowledge, has not been applied for lipid extraction. An initial evaluation was conducted by the recovery test of the extraction/partitioning step by varying organic solvents (CH_3CN, CH_3OH, $CHCl_3/CH_3OH$, and MTBE/CH_3OH) with nineteen lipid standards, followed by a recovery test of the dSPE step using different sorbents such as PSA, GCB, silica, strong anion exchange (SAX) resins, and C18 particles. To evaluate the influence of the matrix, recovery values of lipid standards spiked to both plasma and urine samples before and after extraction using the modified QuEChERS method selected from the initial evaluation were compared to calculate "matrix effects" using the equation proposed by Matuszewki et al. [34].

2. Experimental

2.1. Reagents

A total of 19 standard lipid molecules used for the evaluation of the proposed extraction method were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA): 12:0-lysophosphatidylcholine (LPC), 12:0/12:0-phosphatidylcholine (PC), 12:0- lysophosphatidylethanolamine (LPE), 12:0/12:0-phosphatidylethanolamine (PE), 14:0-lysophosphatidylglycerol (LPG), 12:0/12:0-phosphatidylglycerol (PG), 16:0/18:2- phosphatidylinositol (PI), 14:0/14:0-phosphatidylserine (PS), 18:0-lysophosphatidic acid (LPA), 12:0/12:0-phosphatidic acid (PA), $(14:0)_4$-cardiolipin (CL), d18:0/12:0-sphingomyeline (SM), d18:1/12:0-monohexosylceramide (MHC), d18:1/12:0-lactosylceramide (LacCer), d18:1/12:0-Ceramide (Cer), d18:0-sphinganine (Sa), 18:2- cholesterol ester (CE), 18:1/18:1-diacylglycerol (DG), and 16:0/18:1/18:2-triacylglycerol (TG). Ammonium formate, ammonium hydroxide, and chloroform were all of MS grade from Sigma (St. Louis, MO, USA). All solvents used for nLC-MS/MS and for extraction were all HPLC grade from Avantor Performance Materials (Phillipsburg, NJ, USA): water, CH_3OH, CH_3CN, and isopropanol. Lipid standards were dissolved in 1:1 (v/v) $CHCl_3$:CH_3OH solution except for the lysophospholipids (LPC, LPE, LPA, and LPG), which were dissolved in 1:1:3 (v/v/v) H_2O:$CHCl_3$:CH_3OH. Each standard was diluted to 9:1 (v/v) CH_3OH:CH_3CN for nLC-ESI-MS/MS analysis. For the preparation of the standard lipid mixture sample, the concentration of each standard was adjusted to 50 μmol/L. Internal standards (13:0/13:0-PC and 15:0/15:0-PG for positive and negative ion mode, respectively) were added to extracted sample at a concentration of 1 μmol/L after all sample preparation procedures.

For the removal of water and extraction/partitioning of lipids in the first step of the QuEChERS method, $MgSO_4$ and CH_3COONa from Agilent Technologies (Palo Alto, CA, USA) were utilized. To reduce the matrix effect through the second step, sorbent materials used were PSA (primary secondary amine), GCB (graphitized carbon black), and end-capped C18 particles (grafted silica) from Agilent and SAX (strong anion exchange) and silica from Macherey-Nagel Co. (Düren, Germany).

Human urine and plasma samples were obtained from a healthy male volunteer (age 27).

2.2. Lipid extraction

2.2.1. Folch method for plasma and urine samples

Extraction of lipids from standard mixtures, plasma, and urine samples by the Folch method followed the methods utilized in earlier studies [35,39]. For plasma samples, 100 μL of a plasma mixture containing standards was dried in a vacuum centrifuge, a model Bondiro MCFD 8508 freeze dryer with a concentrator from Ilshin Lab Co. (Yangju, Korea), for 6 h. To the dried powder, 300 μL of CH_3OH and 600 μL of chloroform were added in sequence. After vortexing for 1 h, 180 μL of MS-grade water was added for phase separation followed by centrifugation at 5000 rpm for 5 min. Then, the lower organic layer was transferred to another centrifuge tube and dried in the vacuum centrifuge. The resulting dried lipids were reconstituted in 200 μL of 1:1 (v/v) $CHCl_3$:CH_3OH and diluted in 200 μL of CH_3OH. The final solution was kept at 4 °C.

For the extraction of urine samples, 1 mL of urine in a 15-mL centrifuge tube was first frozen in liquid nitrogen and then dried in the vacuum centrifuge for 12 h. Before freeze-drying the urine sample, the centrifuge tube was covered with MilliWrap, a PTFE membrane filter with 0.45 μm pores, from Millipore (Billerica, MA, USA), without the screw cap to prevent lipids from evaporating, a measure applied to all drying steps. The resulting powder was dissolved in 0.90 mL of 2:1 (v/v) $CHCl_3$:CH_3OH and left for 1 h at room temperature. After adding 180 μL of MS-grade water, it was centrifuged at 5000 rpm for 5 min. The rest of the procedures are the same as those applied for plasma samples.

2.2.2. Modified QuEChERS method

In the first step of the QuEChERS method for the extraction/partitioning of lipids, 100 μL of plasma was placed in a 2-mL Eppendorf tube containing the 0.150-g pre-packed extraction preparation (125 mg of $MgSO_4$, 25 mg of CH_3COONa, and a glass ball). Then, 200 μL of an extraction solvent and 25 μL of water were added in sequence. To select an efficient extraction solvent, four different organic solvents (CH_3CN, CH_3OH, 2:1 (v/v) $CHCl_3$:CH_3OH, and 2:1 (v/v) methyl-*tert*-butyl ether (MTBE):CH_3OH) were utilized to test the recovery rate of each lipid. Since MTBE has been reported as an efficient organic solvent for the modification of the Folch method [38,39], it was utilized for comparison with the Folch method. For urine samples, 1 mL of urine was placed in a 15-mL Falcon tube containing 1.50 g of the same pre-packed extract

preparation with an increased volume. Then, 2 mL of an extraction solvent and 0.25 mL of water were added. Each mixture was vortexed for 1 min and centrifuged at 5000 rpm for 5 min.

In the second step (dSPE), a 150-μL portion of supernatant liquid was taken by pipette and transferred to a 2.0-mL Eppendorf tube containing solid-phase extraction (SPE) sorbents for sample clean-up. SPE sorbents tested in this study were PSA, GCB, Silica, SAX, and C18. The mixture was shaken for 30 s and centrifuged at 5000 rpm for 5 min. A 100-μL portion of the upper layer was transferred to a separate vial and diluted with 100 μL CH_3OH, at which point the final solution was ready for nLC-ESI-MS/MS analysis.

2.3. LC-MS/MS

Two different LC-MS/MS systems were employed in this study. For the evaluation of the recovery rate of lipids upon the use of different organic solvents during the extraction/partitioning step and dispersive solid phase extraction (dSPE) step of QuEChERS method, an ultrahigh performance liquid chromatography (UPLC) system coupled to a Xevo TQ mass spectrometer from Waters (Milford, MA, USA) was utilized. UPLC was utilized to increase the speed (less than 6 min for each run) in the optimization of QuEChERS method since a high resolution separation condition for standard lipids was not necessary. However when it was needed to evaluate the recovery and matrix effect after spiking standards to plasma and urine samples, nanoflow LC-ESI-MS/MS was adopted for the better separation of individual components from complicated lipid mixtures.

Standard lipid mixtures extracted from the first step of the QuEChERS method were quantified by using UPLC-ESI-MS/MS with a multiple reaction monitoring (MRM) method. For UPLC, an Atlantis Silica HILIC (3.0 μm, 2.1 × 100 mm) analytical column from Waters was utilized at 0.5 μL/min, with an injection of 1 μL of the standard lipid mixtures extracted from different solvents. Mobile phases for binary gradient elution were (A) 90/10 (v/v) CH_3CN/CH_3OH and (B) 50/40/10 (v/v/v) $H_2O/CH_3OH/CH_3CN$, both of which were added with 5 mM ammonium formate and 0.05% ammonium hydroxide. Gradient elution condition that was selected from optimization work began with 100% mobile phase A, ramped to 20% B for 2 min, increased to 90% B over 5 min, and maintained at 90% B for 3 min until completion. Analyses for each lipid extract (from different solvents) were repeated five times. For MS/MS, different cone voltages and collision voltages were applied for each lipid class with a fixed capillary voltage of 3.0 kV. The *m/z* values of the precursor ion and MRM quantifier ion of each lipid class are listed in Table 1. For quantitation, the peak area of each species was calculated relative to the area of an internal standard (13:0/13:0-PC for positive ion and 15:0/15:0-PG for negative ion mode) added to each extracted lipid standard mixture by using MassLynx from Waters.

Nanoflow LC-ESI-MS/MS was utilized to evaluate recovery values and matrix effect of lipid standards spiked to human plasma and urine samples, and to characterize plasma and urinary lipid mixtures. Capillary RPLC columns (75 μm-i.d. × 6 cm) were prepared by packing C18 particles, 3-μm 100-Å Watchers ODS-P from Isu Industry Co. (Seocho, South Korea), in a pulled-tip capillary of which one end was pulled by flame to create a sharp needle for direct ESI. An LTQ Velos ion trap mass spectrometer from Thermo Finnigan (San Jose, CA, USA) equipped with a model 1200 capillary pump system and an autosampler from Agilent Technologies (Palo Alto, CA, USA) were utilized. The connection of the analytical column between the LC and ESI-MS/MS was the same as described in previous literature [35]. Sample loading (fixed at 1 μL for all types of samples) from the autosampler was conducted at 0.6 μL/min directly to the analytical column without flow splitting. During separation, the pump flow rate was set at 14 μL/min, which was split into 300 nL/min at the microcross located just before the column. All valve operations were controlled automatically. Mobile phases for nLC-ESI-MS/MS were (A) 9/1 (v/v) H_2O/CH_3CN and (B) 2:3:5 (v/v) CH_3OH/CH_3CN/isopropanol. Both mobile phases contained 5 mM ammonium formate and 0.05% ammonium hydroxide. Mobile phase compositions used for nLC-ESI-MS/MS was different from those selected for UPLC since both systems utilized different stationary phases (HILIC and C18). Gradient elution began with 100% mobile phase A, ramped to 40% B for 1 min, increased to 80% B over 15 min, and then to 100% over 20 min. After separation, the gradient was maintained at 100% B over 10 min for column washing.

The capillary temperature of the MS was set at 200 °C, with 3.0 kV for ESI. For data-dependent MS^n analysis, the collision energy was fixed at 40%. The *m/z* range for the precursor scan was 250–1000. Structural determination of lipids from plasma and urine samples was conducted with LiPilot software [36], which was developed in our laboratory. Confirmation of identified lipids was manually performed.

Table 1
Standard lipid molecules examined in MRM mode during UPLC-ESI-MS/MS and their characteristic *m/z* values for both precursor ions and quantifier ions.

Classes	Cone/collision voltage (V)	Type of precursor/quantifier ions	Molecular species (*m/z* of precursor/quantifier ions)
LPC	30/20	$[M+H]^+/[M+H\text{-}RCOOH]^+$	lyso/12:0 (440/240) 12:0/lyso (440/240)
PC	30/20		12:0/12:0 (622/422)
SM	30/20		d18:1/12:0 (647/447)
LPE	25/15		lyso/12:0 (426/226) 12:0/lyso (426/226)
PE	25/15		12:0/12:0 (580/380)
MHC	20/40		d18:1/12:0 (644/444)
LacCer	20/40		d18:1/12:0 (806/606)
Cer	20/40		d18:1/12:0 (482/282)
Sa	30/15	$[M+H]^+/[M+H\text{-}H_2O]^+$	d18:0 (302/284)
DG	20/18	$[M+NH^4]^+/[M+H\text{-}RCOOH]^+$	18:1/18:1 (638/339)
CE	35/15		18:2 (666/369)
TG	40/25		16:0/18:1/18:2 (874/601)
LPG	45/30	$[M-H]^-/[RCOO]^-$	lyso/14:0 (455/227) 14:0/lyso (455/227)
PG	45/30		12:0/12:0 (609/199)
PI	70/40		16:0/18:2 (833/225)
PS	40/40		14:0/14:0 (678/227)
LPA	35/30		lyso/18:0 (437/283) 18:0/lyso (437/283)
PA	35/30		12:0/12:0 (535/199)
CL	70/70	$[M-2H]^{2-}/[RCOO]^-$	$(14:0)_4$ (619/227)

3. Results and discussion

3.1. Effect of organic solvent on lipid recovery in the extraction/partitioning step

The first step of the QuEChERS method involves the extraction/partitioning step, in which lipids are partitioned in organic solvent while the aqueous portion is adsorbed by a solid sorbent, $MgSO_4$. In order to select a good solvent or solvent mixture for lipids, nineteen lipid standard mixtures (10 pmol each) dissolved in 9:1 (v/v) CH_3OH:CH_3CN solution were spiked into the 0.150-g prepacked extraction preparation (125 mg $MgSO_4$/25 mg CH_3COONa) and 25 μL of water was added. Then lipids were extracted by varying organic solvents: CH_3CN:CH_3OH, 2:1 (v/v) $CHCl_3$:CH_3OH, and 2:1 (v/v) MTBE/CH_3OH. After extraction, internal standards (1 pmol of 13:0/13:0-PC (IS-1) for positive ion mode and 15:0/15:0-PG (IS-2) for negative ion mode) were added to the plasma sample to

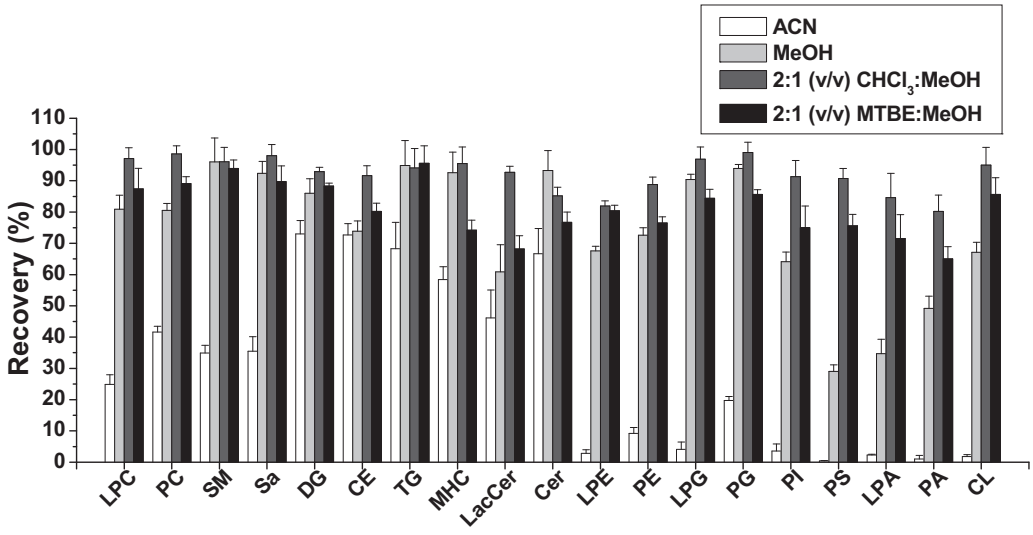

Fig. 1. Effects of organic solvents on the recovery values (%) of 19 standard lipids during the extraction/partitioning step of the QuEChERS method on recovery with 2:1 (v/v) $CHCl_3$:CH_3OH. The vortexing period was 1 min. Recovery values (%) were calculated by comparing the peak area of each lipid before and after extraction from MRM analysis of each quantifier ion by UPLC-ESI-MS/MS (n = 5).

compensate run to run variation in MS signals. Recoveries of individual lipid standards were calculated by comparing the peak area of each lipid with respect to that of IS before and after extraction from MRM analysis of each quantifier ion by UPLC-ESI-MS/MS, and the average recovery values (n = 5) are plotted in Fig. 1. Separation of lipid standards at each ion mode (positive and negative) was achieved within 6 min by using UPLC-ESI-MS/MS, as shown with the base peak chromatograms of the standard lipids in Figure S1 of the Supporting Information. For instance, the MRM transition of $[M+H]^+ \rightarrow [M+H\text{-}RCOOH]^+$ was monitored for the following standards: LPC, PC, SM, LPE, PE, MHC, LacCer, and Cer. Types of quantifier ions of all lipid standards, along with their m/z values, are listed in Table 1. While CH_3CN was utilized for a typical extraction solvent in the QuEChERS method, recovery values (based on peak area) of most lipid standards shown in Fig. 1 were very low (<50%). When tested with other organic solvents (CH_3OH, 2:1 (v/v) $CHCl_3$:CH_3OH, and 2:1 (v/v) MTBE:CH_3OH) typically utilized for lipid extraction, it was found that a mixture of $CHCl_3$:CH_3OH performed the best among them (recovery values are listed in the first column of Table 2), and thus it was selected as the extraction/partitioning solvent. During the extraction/partitioning step with the selected solvent, the vortex duration was varied from 1 min to 5, 10, 20 and 30 min, resulting in no critical difference among them, as shown in Figure S2. Therefore, a 1-min vortex duration was applied hereafter.

3.2. Effect of adsorbent materials for dispersive solid-phase extraction on lipid recovery

The dSPE step of the QuEChERS method for the clean-up process was evaluated using five different adsorbent materials for the same lipid standard mixtures. These experiments were made with lipid standard extracts obtained from the solvent extraction/partitioning step using the solvent mixture established in the previous test, 2:1 (v/v) $CHCl_3$:CH_3OH, and the efficiency of the dSPE process was

Table 2
Recovery values (%) of the extraction/partitioning step and the dispersive-solid phase extraction (dSPE) using different adsorbents in the QuEChERS method modified in this study by UPLC-ESI-MS/MS.

Lipid class	Recovery (%) of extraction/ partitioning Step ($CHCl_3$/MeOH,$MgSO_4$)	Recovery (%) of dSPE Step (%)								
		PSA	GCB	Silica	SAX	C18				
		50 mg				25 mg	50 mg	100 mg	200 mg	400 mg
LPC	95.1 ± 2.5	11.5 ± 1.0	94.9 ± 0.4	39.9 ± 3.1	83.7 ± 1.0	96.2 ± 2.8	95.1 ± 2.6	95.2 ± 3.1	92.8 ± 1.0	91.1 ± 4.6
PC	97.8 ± 2.0	86.4 ± 5.4	101.8 ± 1.1	35.9 ± 2.3	84.9 ± 0.8	85.8 ± 3.6	89.8 ± 3.1	88.7 ± 2.3	82.4 ± 0.8	80.7 ± 5.1
SM	97.0 ± 3.6	71.6 ± 0.9	96.4 ± 2.1	40.4 ± 1.8	86.0 ± 2.7	88.6 ± 2.1	90.4 ± 5.3	87.9 ± 1.8	85.8 ± 2.7	84.0 ± 3.9
Sa	97.5 ± 2.8	65.7 ± 1.2	61.7 ± 1.3	90.5 ± 0.6	84.5 ± 1.2	93.5 ± 0.9	93.7 ± 2.7	94.1 ± 0.6	91.3 ± 1.2	85.8 ± 3.0
DG	92.1 ± 1.1	92.0 ± 1.4	82.5 ± 1.6	97.7 ± 0.4	74.8 ± 1.8	88.7 ± 1.5	89.9 ± 3.9	83.4 ± 0.4	87.8 ± 1.8	80.1 ± 2.8
CE	90.3 ± 2.4	84.7 ± 0.3	0.3 ± 1.4	97.4 ± 1.9	86.0 ± 1.9	91.1 ± 2.5	91.3 ± 5.1	80.0 ± 1.9	75.9 ± 1.9	72.9 ± 4.3
TG	93.2 ± 4.8	88.3 ± 0.6	3.6 ± 1.5	92.6 ± 1.1	82.4 ± 0.6	85.2 ± 4.8	92.5 ± 1.8	74.8 ± 1.1	77.4 ± 3.6	54.6 ± 2.8
MHC	95.2 ± 3.4	98.8 ± 0.5	73.9 ± 1.6	93.4 ± 2.8	66.4 ± 0.5	89.5 ± 5.8	89.5 ± 2.0	85.2 ± 2.8	84.8 ± 4.5	77.1 ± 4.9
LacCer	93.3 ± 1.4	99.5 ± 1.2	89.1 ± 2.6	85.8 ± 2.4	59.1 ± 0.6	93.8 ± 6.7	94.1 ± 7.3	90.8 ± 2.4	84.7 ± 4.6	83.1 ± 8.5
Cer	85.2 ± 1.6	84.3 ± 0.3	76.2 ± 2.1	81.4 ± 2.6	60.4 ± 1.8	81.3 ± 7.4	80.6 ± 2.6	58.8 ± 2.6	54.6 ± 1.8	60.3 ± 4.7
LPE	80.5 ± 1.2	2.1 ± 0.2	57.9 ± 1.9	82.4 ± 2.3	61.3 ± 2.1	80.6 ± 2.8	80.4 ± 6.4	78.7 ± 2.3	78.0 ± 2.8	75.4 ± 4.1
PE	88.4 ± 1.8	17.4 ± 1.1	71.7 ± 0.8	80.0 ± 2.9	62.9 ± 0.9	88.6 ± 3.6	89.2 ± 4.7	77.7 ± 2.9	78.8 ± 2.9	77.1 ± 2.7
LPG	94.9 ± 3.0	22.1 ± 1.3	87.9 ± 1.6	95.0 ± 3.6	19.7 ± 2.6	90.7 ± 1.1	90.2 ± 5.3	61.7 ± 3.6	61.3 ± 2.6	60.0 ± 0.8
PG	97.5 ± 2.4	43.8 ± 2.1	72.8 ± 0.6	93.1 ± 4.2	23.4 ± 2.0	97.8 ± 0.9	96.1 ± 3.6	84.3 ± 4.2	70.9 ± 5.0	47.9 ± 1.7
PI	90.1 ± 4.1	3.3 ± 0.8	62.0 ± 1.8	89.4 ± 4.0	21.7 ± 0.9	89.2 ± 1.8	91.0 ± 1.3	73.5 ± 4.0	66.9 ± 2.9	55.7 ± 2.9
PS	88.6 ± 2.8	2.8 ± 0.3	72.8 ± 2.3	83.5 ± 3.5	12.8 ± 0.6	85.3 ± 5.4	83.6 ± 3.6	70.2 ± 6.4	68.5 ± 2.6	65.0 ± 6.4
LPA	85.6 ± 5.6	2.2 ± 0.2	73.3 ± 0.6	77.6 ± 2.5	12.8 ± 1.7	82.6 ± 3.8	82.4 ± 2.6	70.9 ± 6.5	69.4 ± 5.7	66.4 ± 3.5
PA	80.1 ± 4.0	3.7 ± 2.2	81.8 ± 4.8	78.4 ± 4.2	11.1 ± 2.1	77.9 ± 3.2	80.1 ± 5.6	68.4 ± 6.2	67.0 ± 2.6	63.9 ± 3.8
CL	94.8 ± 4.1	86.4 ± 2.3	38.7 ± 2.4	99.0 ± 3.6	1.7 ± 1.2	83.1 ± 4.7	83.6 ± 6.3	66.4 ± 3.6	59.9 ± 1.9	48.8 ± 2.5

Table 3
Recovery (R_Q) and the calculated matrix effect (ME (%)) of the modified QuEChERS method for lipid standards spiked into both urine and plasma. nLC-ESI-MS/MS was utilized.

Lipid class	R_Q (%)						ME (%)			
	Urine			Plasma			Urine		Plasma	
	10 pmol	1 pmol	0.1 pmol	10 pmol	1 pmol	0.1 pmol	Folch	QuEChERS	Folch	QuEChERS
LPC	98.1 ± 1.1	96.2 ± 3.3	96.5 ± 2.4	95.7 ± 0.5	95.1 ± 3.1	96.3 ± 1.7	2	5	3	8
PC	92.2 ± 2.2	92.8 ± 4.3	89.4 ± 4.2	97.4 ± 2.4	99.7 ± 6.5	95.8 ± 1.0	21	15	13	13
SM	91.3 ± 0.9	90.1 ± 3.4	91.9 ± 5.5	94.4 ± 1.4	90.8 ± 2.3	88.9 ± 1.4	4	6	6	9
Sa	88.6 ± 5.7	90.3 ± 5.5	92.1 ± 3.9	83.8 ± 0.9	84.7 ± 1.0	83.5 ± 1.1	2	6	1	8
DG	88.6 ± 2.6	89.3 ± 2.8	88.1 ± 0.6	86.1 ± 2.6	89.7 ± 0.5	88.3 ± 2.3	5	10	−14	13
CE	92.5 ± 4.3	92.7 ± 4.0	94.6 ± 3.2	92.1 ± 0.6	92.3 ± 1.3	91.8 ± 1.3	−16	5	−16	4
TG	92.7 ± 4.2	91.2 ± 3.9	91.8 ± 2.1	90.7 ± 2.6	93.4 ± 2.8	91.6 ± 0.4	−19	8	−17	10
MHC	89.3 ± 1.9	89.0 ± 2.8	87.9 ± 1.7	89.2 ± 0.5	89.4 ± 0.3	89.8 ± 2.2	3	11	1	13
LacCer	99.0 ± 7.5	97.4 ± 0.4	94.7 ± 3.1	93.5 ± 2.4	94.6 ± 0.7	93.8 ± 3.2	−31	8	−26	12
Cer	91.7 ± 6.1	89.7 ± 3.1	89.9 ± 1.1	89.8 ± 1.4	90.6 ± 3.0	91.6 ± 1.9	3	5	1	12
LPE	89.5 ± 2.5	87.3 ± 1.1	86.8 ± 1.6	91.8 ± 2.8	90.9 ± 0.4	90.6 ± 4.4	1	3	1	7
PE	91.9 ± 3.2	90.6 ± 1.1	90.8 ± 0.5	89.1 ± 3.7	89.7 ± 0.1	88.2 ± 0.5	−27	8	−14	5
LPG	94.4 ± 1.5	89.8 ± 1.2	91.5 ± 1.2	95.6 ± 0.6	90.4 ± 0.3	91.2 ± 2.0	4	7	10	9
PG	95.5 ± 3.3	94.6 ± 2.2	96.8 ± 4.1	97.4 ± 0.8	96.0 ± 1.3	96.8 ± 1.8	2	8	2	8
PI	93.4 ± 4.9	95.1 ± 1.5	91.9 ± 5.1	96.9 ± 3.5	93.7 ± 5.1	90.1 ± 1.6	23	12	5	16
PS	87.1 ± 0.6	84.8 ± 1.2	86.3 ± 3.8	91.2 ± 1.9	93.7 ± 0.4	90.8 ± 0.5	−10	3	−12	6
LPA	81.9 ± 0.9	82.7 ± 1.5	80.2 ± 2.2	80.8 ± 0.4	82.3 ± 2.2	80.7 ± 1.7	−18	17	−15	11
PA	80.3 ± 1.3	79.4 ± 3.4	81.7 ± 1.6	82.4 ± 1.0	84.1 ± 3.2	82.6 ± 3.0	−8	12	−10	9
CL	84.9 ± 4.8	84.4 ± 1.2	82.1 ± 3.6	85.8 ± 3.3	83.9 ± 1.7	83.4 ± 2.8	−10	14	−12	16

examined by comparing the peak area of each lipid with or without carrying out the dSPE step using UPLC-ESI-MS/MS. Table 2 lists the measured recovery values of lipid standards obtained from different adsorbents such as PSA, GCB, silica, SAX, and different amounts of C18 particles. It shows that PSA yielded poor recovery values for most lipid standards except neutral molecules, such as DG, TG, MHC, LacCer, and Cer. GCB yielded a poor recovery for non-polar lipids, such as CE and TG (<10% in recovery), and anionic CL (<40%), and silica adsorbents showed strong binding affinity with polar species, such as PC and SM (<40% of recovery). SAX yields with poor recovery for anionic lipids, such as PG, PI, PS, PA, and CL (<25%). While typical adsorbents utilized in the QuEChERS method showed large variations in recovery, with critical failures for specific type of lipids, C18 particles show relatively good recoveries (above 80%) for all species. When the amount of C18 adsorbents was varied, recovery values with 50 mg of C18 particles were the best, and the efficiency was lowered again when adsorbent amounts were increased to 100, 200, and 400 mg. Therefore, 50 mg of C18 particles was selected for the optimum amount of adsorbent for dSPE.

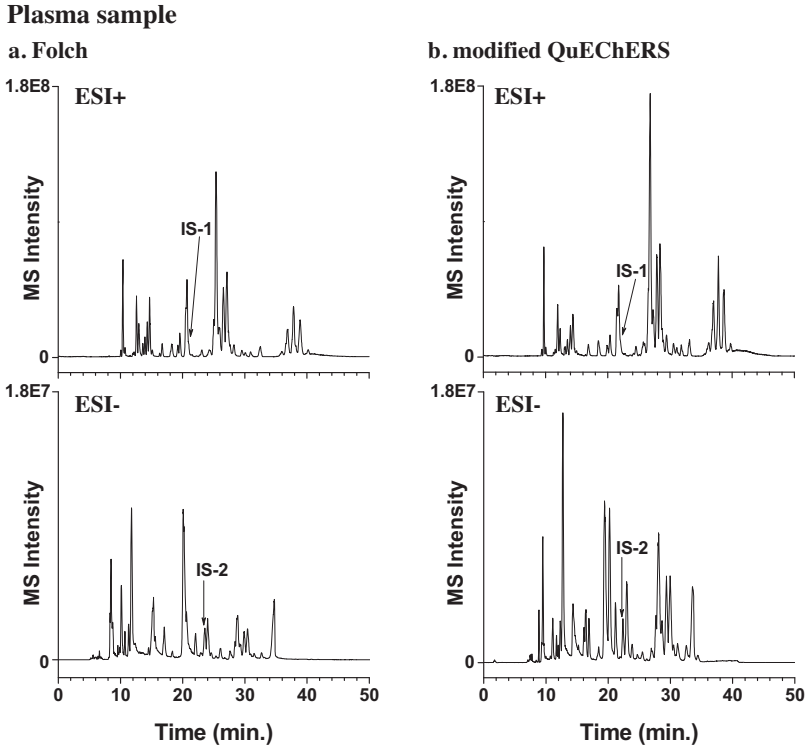

Fig. 2. Base peak chromatograms (BPCs) of lipid extracts from the plasma sample extracted with (a) the Folch and (b) the modified QuEChERS methods by nLC-ESI-MS/MS (internal standards IS-1 and IS-2 were added).

Table 4
List of 235 lipid molecules identified from a healthy human plasma sample obtained from the modified QuEChERS method by nLC-ESI-MS/MS. Lipids marked with an asterisk (*) represent the species that were not found from the Folch extraction method.

Classes	Acyl chain (m/z, t_r (min))			
PC[a] (69)	14:0/lyso (468.4, 9.74)	14:0/22:6 (778.6, 20.57)	14:0/18:1 (732.7, 22.83)	18:0/20:4 (810.7, 24.90)
	20:6/llyso (542.4, 9.85)	18:2/20:5 (804.6, 21.22)	18:1/20:5 (806.6, 22.88)	18:1/18:1 (786.8, 24.91)
	18:3/lyso (518.4, 9.87*)	18:3/18:2 (780.6, 21.28)	18:2/22:5 (832.6, 23.15)	16:0/20:2 (786.8, 25.01)
	16:1/lyso (494.5, 10.26)	14:0/18:2 (730.8, 21.36)	16:0/16:1 (732.7, 23.15)	18:0/22:5 (836.5, 25.11)
	lyso/18:2 (520.4, 10.64)	14:0/20:4 (754.6, 21.41)	16:0/20:4 (782.7, 23.17)	18:0/18:2 (786.8, 25.50)
	lyso/20:4 (544.4, 10.68)	16:1/16:1 (730.6, 21.48)	16:1/18:1 (758.7, 23.28)	16:0/22:4 (810.7, 25.52)
	22:6/lyso (568.4, 10.89)	16:1/22:6 (804.7, 21.52)	16:0/18:2 (758.7, 23.31)	18:2/22:4 (834.7, 25.58)
	20:4/lyso (544.4, 10.99)	20:4/20:4 (830.7, 21.67)	16:0/22:6 (806.6, 23.31)	18:0/20:3 (812.7, 25.60)
	18:2/lyso (520.5, 11.01)	16:0/20:5 (780.6, 21.80)	18:1/20:4 (808.8, 23.32)	18:0/22:6 (834.7, 25.77)
	lyso/16:0 (496.5, 11.44)	22:6/20:4 (854.6, 21.81)	18:0/20:5 (808.8, 23.41)	18:0/16:0 (762.8, 25.96)
	22:5/lyso (570.4, 11.49)	16:1/18:2 (756.6, 21.86)	18:1/18:2 (784.8, 23.60)	16:0/18:0 (762.8, 26.00)
	20:3/lyso (546.4, 11.83)	16:1/20:4 (780.6, 22.18)	18:0/18:3 (784.8, 23.81)	18:0/22:4 (838.7, 26.10)
	16:0/lyso (496.5, 11.97)	16:0/18:3 (756.6, 22.23)	16:0/22:5 (808.8, 23.97)	18:0/18:1 (788.9, 26.18)
	lyso/18:1 (522.4, 11.97)	18:2/20:4 (806.6, 22.23)	16:0/16:0 (734.7, 24.20)	16:0/20:1 (788.8, 26.24)
	18:1/lyso (522.5, 12.42)	18:3/18:2 (780.6, 22.26)	16:0/20:3 (784.9, 24.26)	18:1/20:1 (814.7, 26.51*)
	20:2/lyso (548.5, 13.00*)	18:2/18:2 (782.7, 22.32)	16:1/20:3 (782.7, 24.42)	
	lyso/18:0 (524.4, 13.73)	20:3/20:4 (832.6, 22.74)	16:0/18:1 (760.9, 24.49)	
	18:0/lyso (524.4, 14.15)	18:1/22:6 (832.6, 22.80)	18:1/22:5 (834.7, 24.61)	
SM[a] (13)	d18:1/14:0 (675.6, 20.31)	d18:1/18:1 (729.7, 22.66)	d18:1/23:1 (799.7, 27.34)	d18:1/24:0 (815.8, 29.28)
	d18:1/16:1 (701.7, 20.83)	d18:1/18:0 (731.8, 24.08)	d18:1/24:1 (813.8, 28.08)	
	d18:1/15:0 (689.7, 21.28)	d18:1/20:1 (757.8, 24.63*)	d18:1/23:0 (801.8, 28.53)	
	d18:1/16:0 (703.5, 22.16)	d18:1/21:0 (773.6, 26.89)	d18:1/25:1 (827.8, 29.06*)	
So[a] (1)	d18:1 (300.4, 13.93)			
Sa[a] (1)	d18:0 (302.4, 14.45)			
DG[b] (9)	16:0/16:1 (584.5, 26.36*)	18:1/18:2 (636.6, 26.83)	16:0/18:1 (612.6, 27.96)	
	16:1/18:1 (610.5, 26.67)	16:1/18:0 (612.6, 27.69)	18:1/18:1 (638.7, 27.98)	
	16:0/18:2 (610.5, 26.81)	16:0/16:0 (586.6, 27.84)	18:0/18:0 (642.7, 30.57)	
CE[b] (12)	20:5 (688.7, 34.43)	20:4 (690.7, 35.44)	20:3 (692.8, 36.68)	20:2 (694.8, 38.14)
	22:6 (714.7, 34.92)	16:1 (640.6, 36.07)	18:1 (668.6, 37.73)	18:0 (670.6, 39.52)
	18:3 (664.5, 35.10)	18:2 (666.6, 36.34)	16:0 (642.6, 37.97)	20:1 (696.7, 39.98)
TG[b] (39)	58:11 (942.8, 32.42)	46:2 (792.8, 33.45)	52:4 (872.9, 35.03)	50:1 (850.8, 37.12)
	48:4 (816.9, 32.66)	60:11 (970.9, 33.51)	56:7 (922.9, 35.09)	56:3 (930.9, 37.42)
	52:7 (866.9, 32.75)	48:3 (818.9, 33.56)	54:5 (898.7, 35.09)	54:2 (904.9, 37.67)
	56:9 (918.8, 32.81)	50:4 (844.9, 33.80)	56:6 (924.9, 35.56)	50:0 (852.9, 38.47)
	50:5 (842.9, 32.93)	58:8 (948.9, 34.32)	54:4 (900.9, 35.76)	52:1 (878.9, 38.52)
	54:4 (892.9, 32.96)	58:9 (946.8, 34.42)	50:2 (848.9, 35.81)	56:2 (932.9, 38.70)
	58:10 (944.8, 33.03)	46:1 (794.9, 34.47)	48:1 (822.9, 35.81)	54:1 (906.9, 38.97)
	52:6 (868.9, 33.09)	54:6 (896.9, 34.62)	56:5 (926.9, 36.41)	52:0 (880.9, 39.94)
	56:8 (920.8, 33.25)	48:2 (820.9, 34.62)	54:3 (902.8, 36.52)	54:0 (908.9, 40.47)
	54:7 (894.8, 33.27)	50:3 (846.9, 34.76)	52:2 (876.9, 37.12)	
PE[c] (25)	20:5/lyso (498.5, 10.00)	18:2/lyso (476.4, 12.02)	18:0/lyso (480.4, 14.11)	20:0/20:4 (794.6, 27.80)
	lyso/14:0 (424.5, 10.29)	20:4/lyso (500.5, 12.03)	16:0/20:4 (738.6, 25.53*)	18:0/20:2 (770.8, 27.95)
	lyso/20:5 (498.4, 10.91)	16:0/lyso (452.4, 12.83)	18:1/20:4 (764.6, 25.67)	20:0/18:2 (770.8, 28.08)
	lyso/18:2 (476.4, 11.55)	22:5/lyso (526.4, 12.95)	16:0/22:5 (764.6, 25.83)	20:2/18:0 (770.8, 28.19)
	lyso/20:4 (500.5, 11.57)	lyso/18:1 (478.6, 13.33)	18:0/22:6 (790.6, 26.35)	
	lyso/22:6 (524.4, 11.61)	18:1/lyso (478.5, 13.60)	18:0/20:4 (766.8, 27.36)	
	22:6/lyso (524.4, 11.93)	lyso/18:0 (480.4, 13.64)	18:0/22:4 (794.6, 27.36)	
PG[c] (11)	lyso/14:0 (455.5, 8.72)	18:1/lyso (509.5, 10.73*)	16:1/16:1 (717.6, 21.42)	18:1/18:2 (771.7, 23.99)
	14:0/lyso (455.5, 9.06)	lyso/18:0 (511.6, 11.42)	16:0/16:1 (719.6, 22.93)	16:0/18:1 (747.6, 24.91)
	22:6/lyso (555.5, 9.76)	18:0/lyso (511.6, 11.79)	16:1/18:1 (745.6, 23.38)	
PI[c] (30)	lyso/18:3 (593.5, 8.21)	18:2/18:3 (855.6, 19.02)	16:0/22:5 (883.5, 21.39)	18:0/18:2 (861.5, 22.65)
	20:4/lyso (619.4, 9.93)	16:0/18:3 (831.6, 19.90)	16:0/20:3 (859.5, 21.67)	18:1/20:3 (885.5, 22.72)
	18:2/lyso (595.5, 9.94)	16:0/20:4 (857.6, 19.95)	16:0/18:1 (835.6, 21.91)	18:1/18:1 (861.5, 22.72)
	lyso/16:0 (571.5, 10.18)	18:2/18:2 (857.6, 20.04)	18:0/20:5 (883.5, 21.97)	16:0/22:4 (885.5, 22.86)
	16:0/lyso (571.4, 10.44)	16:0/18:2 (833.5, 20.88)	18:0/18:3 (859.5, 22.04)	16:0/20:2 (861.5, 23.07)
	lyso/18:1 (597.4, 11.34)	18:1/18:3 (857.6, 20.92)	18:0/16:1 (835.6, 22.07)	18:0/20:3 (887.6, 23.33)
	18:1/lyso (597.4, 11.87)	18:1/20:4 (883.5, 21.07)	18:0/22:6 (909.5, 22.46)	
	18:0/lyso (599.4, 12.62)	18:1/18:2 (859.5, 21.19)	18:0/20:4 (885.5, 22.65)	
PS[c] (5)	lyso/14:0 (468.7, 7.96)	lyso/18:0 (524.5, 11.57)	18:0/20:4 (810.6, 25.87*)	
	14:0/lyso (468.5, 8.05)	18:0/lyso (524.4, 11.93)		
PA[c] (12)	lyso/20:4 (457.6, 7.96)	lyso/18:1 (435.6, 8.43)	14:0/14:0 (591.5, 16.47)	18:0/18:2 (699.6, 25.92)
	lyso/18:2 (433.4, 8.12)	lyso/18:0 (437.6, 9.25)	18:1/20:4 (721.6, 23.54)	18:1/18:1 (699.6, 26.44)
	lyso/16:0 (409.7, 8.25)	18:0/lyso (437.5, 9.83)	18:0/22:6 (747.7, 24.91)	18:0/18:1 (701.6, 27.30)
MHC[c] (3)	d18:1/22:0 (828.7, 26.67)	d18:1/24:1 (854.7, 27.81)	d18:1/23:0 (842.8, 27.91)	
Cer[c] (5)	d18:1/22:0 (666.7, 29.35)	d18:1/24:0 (694.8, 32.25)	d18:1/26:0 (722.8, 35.35)	
	d18:1/24:1 (692.8, 30.96)	d18:1/25:0 (708.8, 33.96)		

[a] m/z of $[M+H]^+$.
[b] m/z of $[M+NH_4]^+$.
[c] m/z of $[M-H]^-$.

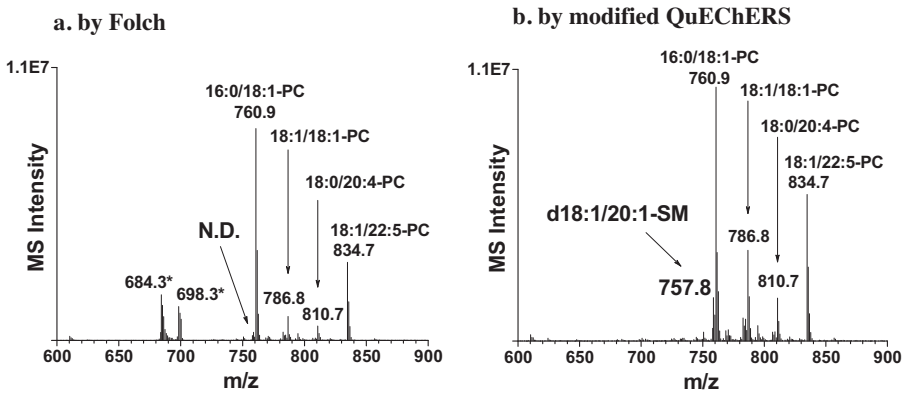

Fig. 3. Comparison of MS spectra (at t_r = 24.6 min.) between plasma lipid extracts prepared with (a) the conventional Folch method and (b) the modified QuEChERS method during nLC-ESI-MS/MS at positive ion mode.

3.3. Evaluation of matrix effect

To evaluate the matrix effect of the QuEChERS method modified in this study, the same lipid standards were spiked into both the plasma and urine samples from a healthy human and the resulting mixtures were processed with the QuEChERS method. In this case, recovery calculations were made by comparing the peak areas of standards spiked before and after the QuEChERS extraction. The percent recovery, R_Q (%), of the QuEChERS extraction can be expressed as

$$R_Q(\%) = \frac{A_{sb}}{A_{sa}} \times 100 \quad (1)$$

where A_{sb} and A_{sa} represent the peak area of a standard lipid spiked before extraction of plasma or urine sample and the corresponding peak area of a standard lipid spiked after extraction, respectively. The matrix effect as a percentage, ME (%), can be calculated as [37]

$$\mathrm{ME}(\%) = \left(\frac{A_{sa}}{A_n} - 1\right) \times 100 \quad (2)$$

where A_n represents the peak area of a same standard in a neat solution (no extraction), which is the same as the re-constitution solution used for a lipid sample spiked after extraction. Equation (2) was modified by subtracting 1 from the quotient of the original equation [34] of Matuszewki et al., so that a negative value of ME (%) indicates suppression of ionization, while a positive value indicates a signal enhancement of the analyte. Table 3 lists the calculated R_Q values of standard lipids spiked into both urine and plasma samples by varying the spiked amount of each standard between 10, 1, and 0.1 pmol, as well as the ME values of each standard, compared between the Folch and the modified QuEChERS method. All lipid standards spiked into both the urine and plasma samples showed relatively good R_Q values of around 90% at the three different spiked amounts. These results are similar to those obtained with the modified Folch method with MTBE/MeOH [39], demonstrating that the QuEChERS method customized in this study can be successfully employed for lipid extraction. The matrix effect of the QuEChERS method was compared with that of the conventional Folch method for 1 pmol of each spiked standard. For both plasma and urine samples, the calculated ME (%) values of the Folch method in Table 3 showed negative values for CE, TG, LacCer, PE, PS, LPA, PA, and CL, while the modified QuEChERS method exhibited positive values for all the species examined, demonstrating that ion suppression for the specified lipid species was substantially decreased to induce signal enhancement by the modified QuEChERS method.

3.4. Lipid identification from plasma and urine

The QuEChERS method modified in this study was applied to extract lipids from both plasma and urine samples, and the efficiency with a matrix effect was investigated further. Fig. 2 compares the base peak chromatograms of lipid extracts from the plasma sample using both the Folch and the modified QuEChERS method, showing that peak intensities from the latter method were increased for both positive and negative ion mode. For qualitative purposes, nLC-ESI-MS/MS was employed for the lipid analysis of both urine and plasma extracts. By examining the MS spectra shown in Fig. 3 which was obtained from both extraction methods at a same retention time, t_r = 24.6 min in Fig. 2, two presumed impurities detected at m/z 684.3 and 698.3 from the Folch method were not detected in the MS spectrum from the modified QuEChERS method. Moreover, the d18:1/20:1-SM species (m/z 757.8), which was not detected in the sample from the Folch method due to the ion suppression, was clearly identified from the QuEChERS method with CID spetra. From data-dependent MS/MS experiments, followed by lipid identification using the LiPilot software, a total of 235 lipid species from 14 different classes (69 PC, 13 SM, 1 So, 1 Sa, 9 DG, 12 CE, 39 TG, 25 PE, 11 PG, 30 PI, 5 PS, 12 PA, 3 MHC, and 5 Cer) were identified from the plasma lipid extracts using the QuEChERS method, while 217 species were identified from the Folch method. The number of identified lipids is less than 588 lipid species reported by Quehenberger et al. [40], however, this reported number was summation of the results from 7 different laboratories using GC-MS and LC-MS with different extraction methods which was specifically optimized for different lipid classes. The detailed molecular structures of identified lipid molecules are listed in Table 4. Similar experiments performed with lipid extracts from the urine sample resulted in an identification of a total 116 lipid species from 15 different classes (17 PC, 9 SM, 1 So, 1 Sa, 1 DG, 15 DG, 2 CE, 15 TG, 15 PE, 9 PG, 18 PI, 9 PS, 9 PA, 4 MHC, 4 Cer, and 4 CL) with the modified QuEChERS method, and the detailed lists are in Table S1 of the Supporting Information. Identified lipid species from the modified QuEChERS method were the same as those found from the Folch method.

4. Conclusions

This study shows that high-speed lipid extraction from both plasma and urine samples can be powerfully achieved with a reduction of the matrix effect by using the QuEChERS method. Compared to the conventional lipid extraction method, which includes freeze-drying that takes more than 12 h, the modified QuEChERS method suggested in this study significantly reduced

the sample preparation period (< 15 min). The extraction recovery values were around 90%, which is analogous to the recovery that can be obtained with the Folch method. By utilizing C18 particles in the dSPE step of the QuEChERS method, the matrix effect was substantially decreased to reduce ion suppression, resulting in the enhancement of MS detection of lipid species. The current study validated the modification of the QuEChERS method by evaluating the extraction recovery values and percent matrix effect (ME (%)) using standard lipids spiked into both the plasma and urine samples. Application of the modified QuEChERS method to the lipid extracts from both urine and plasma samples shows that equivalent numbers or better results can be obtained in the identification of lipid molecules in comparison to the results from the Folch method.

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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.2014.01.024.

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