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ORIGINAL ARTICLE

Increasing lipidomic coverage by selecting optimal mobile-phase modifiers in LC–MS of blood plasma

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Abstract Reversed-phase ultrahigh-performance liquid chromatography–mass spectrometry (UHPLC–MS) is the method of choice for lipidomic profiling of blood plasma. For comprehensive screening, lipids need to be screened in both positive and negative electrospray mode. We here show that optimal results for increased lipidome coverage are only obtained if two different mobile-phase modifier systems are used, as opposed to using a single common modifier for both ionization modes. Specifically, ammonium acetate and ammonium formate were tested with and without acidifiers on standards representing 16 lipid classes, as well as on 164 blood plasma lipids from 11 lipid classes. Optimal coverage was obtained using 10 mM ammonium formate in ESI(+) and 10 mM ammonium acetate in ESI(−), both without acidification. Importantly, detection of free fatty acids was suppressed at more than 50-fold lower signal intensity if ammonium formate or formic acid as acidifier was used in ESI(−), whereas cholesteryl esters were hampered by up to 10-fold lower peak heights when ammonium acetate was used under ESI(+) conditions. Using charged surface hybrid C18 columns had slight advantages for lipids detected in blood plasma extracts, except for phosphatidic acids for which bridged ethylene hybrid C18 columns showed clear advantages.

Keywords Liquid chromatography–mass spectrometry · Lipidomics · Blood plasma · Modifiers · Mobile phase · Optimization

Abbreviations

BEH	Ethylene bridged hybrid
CE	Cholesteryl ester
Cer	Ceramide
CSH	Charged surface hybrid
DG	Diacylglycerol
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
MG	Monoacylglycerol
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
RPLC	Reversed-phase liquid chromatography
SM	Sphingomyelin
TG	Triacylglycerol

1 Introduction

Many liquid chromatography (LC) configurations have been described for the analysis of complex lipid mixtures. The three most important ones include reversed-phase LC

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(RPLC), normal-phase LC (NPLC), and hydrophilic interaction LC (HILIC) (Cajka and Fiehn 2014; Sandra and Sandra 2013). Among these methods, RPLC has been the most widely used method, accounting for about 70 % of all reported liquid chromatography–mass spectrometry (LC–MS)-based analyses of complex lipids (Cajka and Fiehn 2014). Typical methods in RPLC-based lipidomics use a short (50–150 mm) microbore column (1–2.1 mm I.D.) with sub-2 μm or 2.6–2.8 μm (fused-core) particle size and C18 or C8-modified sorbent (Cajka and Fiehn 2014). As a weak mobile phase, water or mixtures with organic solvent such as methanol, acetonitrile, isopropanol or tetrahydrofuran are used, while a strong mobile phase consists primarily of isopropanol or tetrahydrofuran mixed with other solvents (acetonitrile, methanol, water). Use of mobile-phase modifiers is highly recommended to improve LC separation as well as ionization and detection of lipids.

Indeed, a variety of combinations of modifiers such as ammonium formate (Bojic et al. 2014; Castro-Perez et al. 2010; Chen et al. 2014, 2013; Whiley et al. 2012), ammonium formate with formic acid (Bird et al. 2013; Camont et al. 2013; Chitraju et al. 2012; Giera et al. 2012; Gregory et al. 2013; Gurdeniz et al. 2013; Isaac et al. 2011; Witting et al. 2014; Yamada et al. 2013a), ammonium acetate (Choi et al. 2014; Cui et al. 2013; Ding et al. 2008; Donovan et al. 2013; Fernandez et al. 2014; Gao et al. 2012; Gaudin et al. 2014; Pizarro et al. 2013; Sartain et al. 2011; Xia et al. 2011; Yamada et al. 2013b), ammonium acetate with acetic acid (Gallart-Ayala et al. 2013; Hummel et al. 2011; Ollero et al. 2011), and ammonium acetate with formic acid (Chitraju et al. 2013, 2012; Fauland et al. 2011; Hilvo et al. 2011; Knittelfelder et al. 2014) were reported. Buffer salts (ammonium formate and ammonium acetate) were added in the mobile phases typically at a concentration of 5–10 mM, while acids (formic acid and acetic acid) were used at 0.05–0.2 % (Cajka and Fiehn 2014).

Strangely enough, however, no systematic evaluation of these mobile-phase modifiers in RPLC–MS-based lipidomics has been conducted so far with clear rationale which modifiers provide the best results. Recently, the effect of ionization modifiers in basic pH has been evaluated for nanoflow LC–MS with capillary columns for comprehensive analysis of blood plasma and urine lipids (Bang et al. 2012; Bang and Moon 2013). A mixture of 0.05 % ammonium hydroxide with 1–5 mM ammonium formate (pH 9.3–8.8) was found to be effective in the analysis of complex lipid mixtures. However, using acid or neutral mobile phase during RPLC–MS analysis is preferred because current C18 stationary phases have typically lower stability in basic pH resulting also in lower temperature limits during separation.

In this study, we evaluated five combinations of LC–MS modifiers most frequently reported in RPLC–MS-based lipidomics papers. Our aim was to answer key questions such as: What is/are the most suitable modifier(s) in RPLC–MS-based lipidomics? How do the modifiers influence the formation of molecular species during electrospray ionization (ESI)? Which molecular species are the most suitable for subsequent MS/MS analysis forming intensive fragments? To answer these questions, mobile-phase modifiers were evaluated based on (1) the signal intensity of lipid standards and their peak widths, (2) the intensity of multiple lipid species for each lipid class in blood plasma, and (3) the intensity of fragment ions during MS/MS analysis with the attempt to increase the lipidome coverage. The results strongly emphasize that two different solvent modifiers must be used if researchers want to achieve truly comprehensive lipidomic profiles.

2 Materials and methods

2.1 Human plasma samples

For this study we used disodium EDTA plasma (HMPLEDTA) from Bioreclamation IVT (Westbury, NY, USA). Samples were stored at −80 °C prior to analysis.

2.2 Chemicals

LC–MS-grade solvents and modifiers were obtained from Fisher Scientific, Hampton, NH, USA (water, acetonitrile, methanol), Sigma–Aldrich/Fluka, St. Louis, MO, USA (isopropanol, formic acid, ammonium formate, methyl *tert*-butyl ether, toluene), Merck (ammonium acetate), and T.J. Baker (acetic acid).

Lipid standards lysophosphatidylethanolamine LPE(17:1), lysophosphatidylcholine LPC(17:0), phosphatidylcholine PC(12:0/13:0), phosphatidylethanolamine PE(17:0/17:0), phosphatidylglycerol PG(17:0/17:0), phosphatidylinositol PI(12:0/13:0), phosphatidylserine PS(17:0/14:1), phosphatidic acid PA(12:0/13:0), d_7-cholesterol, sphingomyelin SM(d18:1/17:0), ceramide Cer(d18:1/17:0), monoacylglycerol MG(17:0/0:0/0:0), diacylglycerol DG(12:0/12:0/0:0) and triacylglycerol d_5-TG(17:0/17:1/17:0) were obtained from Avanti Polar Lipids, Alabaster, AL, USA with the exceptions of 12-[[(cyclohexylamino)carbonyl]amino]-dodecanoic acid (CUDA) (Cayman Chemical, Ann Arbor, MI, USA), d_3-palmitic acid (CDN Isotopes, Pointe-Claire, Quebec, Canada), and cholesteryl ester CE(22:1) (Nu-Chek Prep, Elysian, MN, USA).

2.3 Sample preparation

Extraction of plasma lipids was carried out using a biphasic solvent system of cold methanol, methyl *tert*-butyl

ether (MTBE), and water with some modifications (Matyash et al. 2008). In more detail, 225 µL of cold methanol was added to a 20 µL blood plasma aliquot in a 1.5 mL Eppendorf tube, and then vortexed (10 s). Then, 750 µL of cold MTBE was added, followed by vortexing (10 s) and shaking (6 min) at 4 °C. Phase separation was induced by adding 188 µL of MS-grade water. Upon vortexing (20 s) the sample was centrifuged at 14,000 rpm for 2 min. The upper organic phase was collected in two 350 µL aliquots and evaporated. Dried extracts were resuspended using 110 µL of a methanol/toluene (9:1, v/v) mixture containing CUDA (internal standard for quality control of injection) with support of vortexing (10 s) and centrifuged at 14,000 rpm for 2 min prior to LC–MS analysis.

2.4 Liquid chromatography conditions

The RPLC–MS analyses were performed using an Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA) with a pump (G4220A), a column oven (G1316C), and an autosamples (G4226A). Lipids were separated on an Acquity UPLC CSH C18 column (100 × 2.1 mm; 1.7 µm) (Waters, Milford, MA, USA) coupled to an Acquity CSH C18 VanGuard pre-column (5 × 2.1 mm; 1.7 µm) (Waters). For comparison, we also used an Acquity UPLC BEH C18 column (100 × 2.1 mm; 1.7 µm) (Waters) coupled to an Acquity BEH C18 VanGuard pre-column (5 × 2.1 mm; 1.7 µm) (Waters). Both columns were maintained at 65 °C at a flow-rate of 0.6 mL/min. The mobile phases consisted of (A) 60:40 (v/v) acetonitrile:water with different modifiers (see Sect. 3) and (B) 90:10 (v/v) isopropanol:acetonitrile with different modifiers (see Sect. 3).

To eliminate errors during preparation of the mobile phases for particular experiments, three liters of each mobile phase (A) and (B) without modifiers were prepared first. After careful mixing, each mixture was split into 500 mL aliquots to which mobile-phase modifiers were added subsequently. To enhance solubilization of ammonium formate and ammonium acetate after their addition in the mobile phase, both salts were dissolved first in small volume of water before their addition in the mobile phases (0.631 g ammonium formate or 0.771 g ammonium acetate/1 mL water/1 L mobile phase). Each mobile phase with modifiers was mixed, sonicated for 15 min to achieve complete dissolving of modifiers (salts), mixed again, and then sonicated for another 15 min.

The separation was conducted under the following gradient: 0 min 15 % (B); 0–2 min 30 % (B); 2–2.5 min 48 % (B); 2.5–11 min 82 % (B); 11–11.5 min 99 % (B); 11.5–12 min 99 % (B); 12–12.1 min 15 % (B); 12.1–15 min 15 % (B). A sample volume of 1.7 and 5 µL was used for the injection in ESI(+) and ESI(−), respectively. Sample temperature was maintained at 4 °C.

2.5 Mass spectrometry

Mass spectrometric detection of lipids was performed on an Agilent 6530 QTOFMS system (Agilent). For each polarity, three functions were set-up. The first function collected the data without collision energy, while the second and the third function acquired the data with a collision energy of 25 and 40 eV, respectively ("All Ions MS/MS"). The acquisition rate was 2 Hz per function, thus, the actual acquisition rate set-up in the software was 6 Hz.

The other parameters were MS^1 mass range, *m/z* 50–1700; MS^2 mass range, *m/z* 50–1700; capillary voltage, +3 kV/−3 kV; nozzle voltage, +1 kV/−1 kV; gas temperature, 325 °C; drying gas (nitrogen), 8 L/min; nebulizer gas (nitrogen), 35 psi; sheath gas temperature, 350 °C; sheath gas flow (nitrogen), 11 L/min.

The instrument was tuned using an Agilent tune mix (mass resolving power ~10,000 FWHM). A reference solution (*m/z* 121.0509, *m/z* 922.0098 in ESI(+) and *m/z* 119.0360, *m/z* 966.0007 (formate adducts), *m/z* 980.0164 (acetate adducts) in ESI(−)) was used to correct small mass drifts during the acquisition.

2.6 Raw data processing

For the data processing MassHunter Qualitative (B.05.00) and Quantitative (B.05.01) Analysis (Agilent) software programs were used. Chromatographic peaks were annotated based on different levels (Sumner et al. 2007). Level 1: authentic reference standard available (lipid standards), level 2: putatively annotated plasma lipids based on their unique MS/MS fragmentation patterns using the open-access LipidBlast mass spectral library with NIST MS search software (Kind et al. 2013), level 3: putatively characterized compound classes (free fatty acids). Prior to further calculation combining data for different detected molecular species for each particular lipid (e.g., $[M + NH_4]^+$ and $[M + Na]^+$ for TG) was conducted.

3 Results and discussion

3.1 Selection of mobile-phase modifiers for evaluation

In general, the sensitivity of LC–MS-based lipidomic profiling methods can be increased by (1) increasing the concentration of the sample, (2) using ultrahigh performance liquid chromatography, (3) improving the ionization efficiency, and/or (4) utilizing a more sensitive detector.

Different modifiers such as ammonium formate or acetate and formic or acetic acid have been reported in RPLC–MS analysis of complex lipids (Cajka and Fiehn 2014). However, no systematic comparison of these modifiers has been conducted so far with respect to their effects on improving or hampering the electrospray ionization efficiency for specific lipid classes. To fill this knowledge gap we focused on following combinations of mobile-phase modifiers reported as the most frequent in LC–MS-based lipidomics papers: (1) ammonium formate (Bojic et al. 2014; Castro-Perez et al. 2010; Chen et al. 2014, 2013; Whiley et al. 2012), (2) ammonium formate with formic acid (Bird et al. 2013; Camont et al. 2013; Chitraju et al. 2012; Giera et al. 2012; Gregory et al. 2013; Gurdeniz et al. 2013; Isaac et al. 2011; Witting et al. 2014; Yamada et al. 2013a), (3) ammonium acetate (Choi et al. 2014; Cui et al. 2013; Ding et al. 2008; Donovan et al. 2013; Fernandez et al. 2014; Gao et al. 2012; Gaudin et al. 2014; Pizarro et al. 2013; Sartain et al. 2011; Xia et al. 2011; Yamada et al. 2013b), (4) ammonium acetate with acetic acid (Gallart-Ayala et al. 2013; Hummel et al. 2011; Ollero et al. 2011), and (5) ammonium acetate with formic acid (Chitraju et al. 2013, 2012; Fauland et al. 2011; Hilvo et al. 2011; Knittelfelder et al. 2014).

The experimental design of the study is shown in Fig. 1. For our evaluation, we used 10 mM concentration of salts (ammonium formate and ammonium acetate) and 0.1 % addition of acids (formic acid and acetic acid) as typical concentrations in these papers. The lipid standard mixtures and the lipid extracts were separated using a short microbore column (100 × 2.1 mm I.D.) with 1.7 µm particle size with C18 sorbent, which represents the currently preferred method in LC–MS-based lipidomics (Cajka and Fiehn 2014). Specifically, we used an Acquity UPLC CSH C18 column, which incorporates the latest generation of stationary phase sorbent. This column is based on ethylene bridged hybrid (BEH) particles incorporating a low level surface charge leading to improved peak symmetry and loading of the sample in low ionic-strength mobile phases. For comparison purposes, we also used an Acquity UPLC BEH C18 column because of its frequent use in both metabolomics and lipidomics studies (Cajka and Fiehn 2014). We used LC elution mobile phases that are frequently used in lipidomics, specifically (A) acetonitrile:water (60:40, v/v) and (B) isopropanol:acetonitrile (90:10, v/v). In combination with C18-modified particles for LC columns, these solvent combinations permitted effective elution and separation of complex lipid mixtures.

For the comparison of buffer modifier effects, we kept all other LC parameters constant, such as the mobile phases (A) and (B) mixtures and the LC gradient, as well as column flow-rate and temperature. Our focus was to observe the influence of particular modifiers on retention time and signal intensity of lipids during positive and negative electrospray ionization (ESI), peak widths, formation of molecular species, and intensity of fragments during MS/MS analysis of different lipid classes.

3.2 Effect of LC mobile-phase modifiers on signal intensity of lipid standards in pure solvents

We first evaluated the effect of different mobile-phase modifiers on ionization efficiency of a mixture of lipid standards and verified results using two different C18 columns that are frequently used for LC–MS-based lipidomics (Cajka and Fiehn 2014). Sixteen reference compounds were evaluated to represent 16 different lipid classes, from very polar lipids such as lysophosphatidylethanolamines to very nonpolar lipids such as cholesteryl esters (Table 1). Data were acquired over the

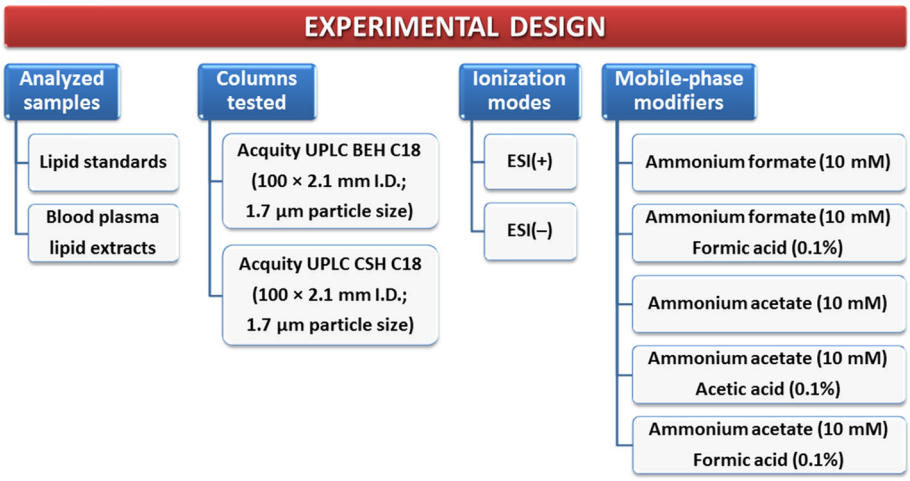

Fig. 1 Experimental design of the study focused on selecting optimal mobile-phase modifiers in LC–MS of blood plasma

Table 1 The effect of mobile-phase modifiers on the ionization efficiency of lipid standards during LC–MS analysis

		BEH-C18 Column					CSH-C18 Column				
Lipid Standard	**ESI Mode**	**AmF**	**AmF/FA**	**AmAc**	**AmAc/AA**	**AmAc/FA**	**AmF**	**AmF/FA**	**AmAc**	**AmAc/AA**	**AmAc/FA**
CE(22:1)	(+)	48%	60%	8%	12%	62%	94%	97%	16%	16%	73%
Cer(d18:1/17:0)	(+)	95%	92%	27%	35%	71%	92%	95%	30%	34%	57%
d_7-Cholesterol	(+)	98%	92%	0%	1%	78%	95%	92%	1%	0%	59%
DG(12:0/12:0/0:0)	(+)	79%	76%	20%	27%	54%	96%	89%	24%	28%	44%
LPC(17:0)	(+)	93%	97%	33%	33%	67%	98%	94%	36%	39%	52%
MG(17:0/0:0/0:0)	(+)	56%	97%	27%	30%	69%	70%	55%	35%	27%	40%
PC(12:0/13:0)	(+)	71%	98%	24%	26%	49%	70%	86%	28%	31%	37%
PE(17:0/17:0)	(+)	91%	88%	85%	82%	56%	95%	86%	95%	97%	76%
PG(17:0/17:0)	(+)	80%	61%	27%	31%	68%	97%	67%	35%	37%	55%
SM(d18:1/17:0)	(+)	84%	90%	34%	34%	61%	94%	95%	33%	45%	53%
d_5-TG(17:0/17:1/17:0)	(+)	90%	93%	34%	41%	97%	90%	79%	37%	37%	50%
Cer(d18:1/17:0)	(−)	71%	62%	95%	77%	96%	36%	43%	98%	62%	46%
LPC(17:0)	(−)	98%	74%	93%	80%	90%	73%	53%	94%	82%	54%
LPE(17:1)	(−)	87%	68%	72%	69%	79%	70%	59%	95%	83%	62%
d_3-Palmitic acid	(−)	3%	0%	96%	47%	1%	11%	5%	96%	67%	6%
PA(12:0/13:0)	(−)	48%	50%	74%	87%	97%	7%	6%	43%	34%	4%
PC(12:0/13:0)	(−)	77%	58%	79%	72%	71%	72%	56%	98%	82%	56%
PE(17:0/17:0)	(−)	77%	63%	92%	66%	70%	86%	81%	99%	86%	81%
PI(12:0/13:0)	(−)	82%	56%	98%	91%	75%	80%	79%	99%	90%	69%
PS(17:0/14:1)	(−)	73%	66%	95%	79%	90%	29%	35%	92%	94%	48%
SM(d18:1/17:0)	(−)	84%	61%	93%	68%	74%	83%	62%	96%	95%	60%

Each value represents an average of six technical replicate injections. Percentages are relative to the highest peak intensity for each lipid across all samples. Legend *AmF* ammonium formate, *AmF/FA* ammonium formate with formic acid, *AmAc* ammonium acetate, *AmAc/AA* ammonium acetate with acetic acid, *AmAc/FA* ammonium acetate with formic acid

two-day consecutive injections. Six technical replicates per study group were injected. We observed very low analytical errors of less than 2 % absolute standard deviation for all lipids that were abundantly detected (Table S1). Averages of the six replicates are given in Table 1 as percent of the maximal value detected in the set. Tune results acquired over this time confirmed that there was no significant drift in mass spectrometry sensitivity, excluding any relevant systematic bias.

Marking lipids that demonstrated sufficient (>70 %) to excellent peak heights (>90 %) (Table 1), it became clear that no single LC modifier gave satisfactory lipid detections under both positive and negative ionizations conditions. This is an important observation, because all currently published untargeted LC–MS-based lipidomics protocols have employed the same modifier(s) for both polarities (Bird et al. 2013; Bojic et al. 2014; Camont et al. 2013; Castro-Perez et al. 2010; Chen et al. 2014, 2013; Chitraju et al. 2013, 2012; Choi et al. 2014; Cui et al. 2013; Ding et al. 2008; Donovan et al. 2013; Fauland et al. 2011; Fernandez et al. 2014; Gallart-Ayala et al. 2013; Gao et al. 2012; Gaudin et al. 2014; Giera et al. 2012; Gregory et al. 2013; Gurdeniz et al. 2013; Hilvo et al. 2011; Hummel et al. 2011; Isaac et al. 2011; Knittelfelder et al. 2014; Ollero et al. 2011; Pizarro et al. 2013; Sartain et al. 2011; Whiley et al. 2012; Witting et al. 2014; Xia et al. 2011; Yamada et al. 2013a, b). For ESI(+), some lipids showed very large differences between modifiers. For example, cholesterol showed very large peak intensity alterations between 0 and 1 % average peak heights under ammonium acetate with or without acetic acid, and cholesteryl ester CE(22:1) with 8–12 % intensity in comparison to optimal buffer modifiers of 92–98 % (cholesterol) and 94–97 % (cholesteryl ester) when using ammonium formate, with or without formic acid as acidifier. These are very relevant differences of up to 10–20-fold magnitude, hence confirming our hypothesis that buffer modifiers carry significant impact in overall lipidomics performance in LC–MS studies. Only phosphatidylethanolamine PE(17:0/17:0) showed very little effects in ESI(+) of less than 2-fold peak height differences between the best and the worst buffer modifier.

Overall, findings confirmed previous reports that, ESI in positive ion is preferred for detection of neutral lipids such as lysophosphatidylcholines, phosphatidylcholines, diacylglycerols, triacylglycerols, cholesteryl esters and sphingomyelins. ESI(+) permits also ionization of weak anionic lipids such as lysophosphatidylethanolamine, phosphatidylethanolamine and ceramides (Bang et al. 2012; Bang and Moon 2013). Interestingly, one single

modifier stood out as being optimal for most of the lipids that were detected under positive electrospray conditions. Use of ammonium formate provided the highest peak intensities of a range of lipid standards in positive ion mode when using a CSH-C18 column, specifically for lysophosphatidylcholine, phosphatidylcholine, monoacylglycerol, diacylglycerol, triacylglycerol, cholesterol, sphingomyelin, ceramide, phosphatidylglycerol, phosphatidylethanolamine and cholesteryl ester. Ammonium formate with acidification showed somewhat less optimal results for the monoacylglycerol and phosphatidylglycerol standards.

Two columns were tested, the charged surface hybrid C18 column (CSH) and the bridged ethylene column (BEH). While there were overall few differences between these two columns, overall results were slightly better for the CSH column for which all positive ESI amenable lipids showed satisfactory to excellent peak heights using ammonium formate, specifically if no formic acid is used for additional acidification. Use of ammonium acetate is surely discouraged as given by the comparatively poor results for almost all lipids. It is interesting to note, though, that addition of formic acid somewhat improves the poor performance of ammonium acetate as a buffer modifier, notably cholesterol, ceramide and cholesteryl ester.

On the contrary, ammonium formate gave mostly disappointing results in negative electrospray mode, specifically for the free fatty acid palmitate and phosphatidic acid PA(12:0/13:0). Best results were achieved when using ammonium acetate without addition of further acidifiers and using the BEH column; indeed, good detectability of free fatty acids in ESI(−) has been previously reported (Bang et al. 2012; Bang and Moon 2013). Higher pH values of the mobile phase increase deprotonation of fatty acids, and, thus, improve ESI(−) ionization efficiency (Kanicky and Shah 2002). Ammonium acetate yields a buffer pH 7.0 for the mobile phase, close to the pK_a of high-carbon chain free fatty acids that range from pH 8.3–10.2 (Kanicky and Shah 2002). It is therefore understandable that other modifiers yield poorer results due to lower final pH values. However, the buffer pH for ammonium formate is only 0.4 pH units lower compared to ammonium acetate. The significant decline of fatty acid signal intensity suggests an additional suppressing effect of formate in the mobile phases. For lysophosphatidylethanolamine LPE(17:1) and phosphatidylcholine PC(12:0/13:0), results were slightly better for the CSH column than using the BEH column, even though phosphatidic acid PA(12:0/13:0) showed only half the peak heights when using a CSH column in comparison to BEH column. This compound showed poorer peak shape and overall chromatographic behavior when using the CSH column. Indeed, phosphatidic acids tend to elute as extensively broad peaks under the RPLC conditions generally used and thus NPLC/HILIC or direct infusion MS are used for their analysis (Ogiso et al. 2008). Overall, for samples for which PA lipids are suspected, the BEH column should be used. However, these lipids were not detectable in blood plasma, and hence, the CSH column was kept for performance testing in blood plasma lipidomics even for negative ESI.

With the exception of PA(12:0/13:0) both columns provided very narrow chromatographic peaks. Specifically, 5–8 s narrow peaks were obtained for early and middle eluting lipid standards [LPC(17:0), LPE(17:1), PI(12:0/13:0), d_3-palmitic acid, PC(12:0/13:0), d_7-cholesterol, PS(17:0/14:1), MG(17:0/0:0/0:0), DG(12:0/12:0/0:0)] and 7–9 s for late eluting lipids [PG(17:0/17:0), Cer(d18:1/17:0), SM(d18:1/17:0), PE(17:0/17:0), d_5-TG(17:0/17:1/17:0), CE(22:1)]. Peak widths of lipid standards were slightly wider when using a BEH-C18 column in comparison to peak widths obtained by CSH-C18 chromatography.

Expectedly, differences in final mobile phase pH values also impacted chromatographic retention times. When comparing mobile phase with ammonium formate and formic acid (pH 3.2) and the counterpart ammonium acetate that yields a pH 7.0, the highest retention time shift was observed for PG(17:0/17:0) (28 and 17 s for CSH and BEH columns, respectively), PA(12:0/13:0) (26 and 8 s for CSH and BEH chromatography, respectively), PS(17:0/14:1) (20 and 12 s for CSH and BEH, respectively), and d_3-palmitic acid (42 and 44 s for CSH and BEH, respectively). For all other lipid standards, the retention shift was less than 20 s for both columns.

3.3 Signal intensity of lipids in blood plasma extracts versus ionization mode

Next, we compared the signal intensity of lipids detected in blood plasma extracts when using different modifiers. Visual inspection of raw data indicated significant differences in lipid profiles for both positive and negative ion modes based on the mobile-phase modifiers used. We selected 164 representative lipids covering 11 lipid classes (Table S1) and compared their average peak heights for each combination of mobile-phase modifiers, relative to the most intense peaks across all samples. Using this targeted approach, it became more apparent which lipid classes ionized better under the conditions of the different mobile-phase modifiers used.

As Table 2 shows, ammonium formate gave sufficient (>70 %) to excellent peak heights (>90 %) for both tested columns using either ammonium formate alone, or in

conjunction with formic acid in ESI(+). Specifically, (lyso)phosphatidylcholines and diacylglycerols showed slightly higher peak abundances when using a BEH column in comparison to a CSH column under ammonium formate modifiers, which was found opposite for a subtle increase in peak intensities for triacylglycerides. In a similar manner to the tests conducted on lipid standards, all lipid classes showed 2- to 5-fold decreases in signal intensity when using ammonium acetate buffers instead of ammonium formate variants.

For negative ESI profiling of blood plasma lipids, again, ammonium acetate proved to be the most suitable buffer modifier in comparison to all other variants (Table 2). As there were no PA lipids detectable in blood plasma, the CSH column proved even more successful than the BEH column. All tested lipid classes (LPC, LPE, PC, PE, PI, SM, ceramides and fatty acids) showed 9–22 % more intensive peaks in comparison between CSH and BEH columns (Table 2).

Importantly, free fatty acids showed more than 50-fold increase in peak intensity between the worst and the best conditions tested. Use of ammonium acetate as a buffer modifier is crucial for this lipid class. Shorter retention times of free fatty acids was observed when using ammonium acetate modifier in the mobile phase (pH 7.0) as compared to other modifier combinations with lower mobile phase pH (Fig. 2). These results are consistent with previous observations published by Schiesel et al. who compared separation of fatty acids using RPLC–MS/MS. At pH 5.0, retention was remarkably increased for fatty acids in comparison to pH 9.5 (Kanicky and Shah 2002).

Our results suggest that many blood plasma lipids may be detected at either low signal intensity or remain undetected if the wrong buffer modifiers are chosen. These results demonstrate how much of an impact mobile-phase modifiers have on lipidomic coverage and that these should be optimized for each ESI polarity. To illustrate the lipidome coverage, Fig. 3 shows total ion chromatograms of separated blood plasma lipids on a CSH-C18 column acquired in ESI(+) and ESI(−) with ammonium formate and ammonium acetate mobile-phase modifiers, respectively. If instruments are used under positive/negative polarity switching, such as an orbital-ion trap analyzer, sub-optimal results will be achieved because a single mobile-phase modifier would need to be selected.

3.4 Intensity of fragment ions versus mobile-phase modifiers used

While specific lipids may be detected at lower abundance in negative electrospray ionization, adducts and MS/MS mass spectral fragmentation nevertheless yields further important information. We first focused on lysophosphatidylcholine, phosphatidylcholine, sphingomyelin, and ceramide lipid classes in ESI(−) due to the formation of different primary adducts (formate, $[M + HCOO]^-$ or acetate,

Table 2 The effect of mobile-phase modifiers on ionization efficiency (intensity of molecular species) of blood plasma lipids during LC–MS analysis

			BEH-C18 Column					CSH-C18 Column				
Lipid Class	No. of Lipids	ESI Mode	AmF	AmF/FA	AmAc	AmAc/AA	AmAc/FA	AmF	AmF/FA	AmAc	AmAc/AA	AmAc/FA
CE	8	(+)	83%	82%	19%	35%	82%	81%	88%	25%	23%	65%
Ceramides	4	(+)	85%	80%	28%	32%	53%	82%	80%	34%	32%	42%
DG	5	(+)	91%	84%	27%	37%	61%	76%	85%	38%	43%	47%
LPC	8	(+)	97%	85%	34%	34%	58%	86%	83%	38%	36%	44%
PC	18	(+)	93%	91%	21%	26%	54%	86%	92%	28%	28%	45%
PE	3	(+)	82%	72%	72%	71%	74%	91%	84%	86%	79%	70%
SM	9	(+)	86%	85%	30%	32%	55%	91%	87%	39%	36%	50%
TG	22	(+)	85%	80%	27%	35%	69%	90%	89%	36%	34%	59%
Ceramides	11	(−)	42%	38%	69%	71%	66%	43%	37%	80%	55%	61%
Fatty acids	18	(−)	7%	2%	74%	40%	2%	7%	2%	93%	54%	3%
LPC	4	(−)	74%	57%	76%	72%	76%	70%	63%	97%	76%	69%
LPE	4	(−)	86%	66%	71%	73%	87%	80%	79%	91%	82%	75%
PC	22	(−)	90%	71%	76%	57%	89%	84%	72%	88%	68%	77%
PE	6	(−)	67%	53%	77%	63%	71%	68%	58%	86%	71%	69%
PI	6	(−)	57%	36%	70%	60%	50%	64%	38%	92%	60%	42%
SM	16	(−)	85%	63%	79%	60%	78%	80%	66%	91%	65%	76%

Each value represents an average of six technical replicate injections. Percentages are relative to the highest peak intensity for each lipid across all samples

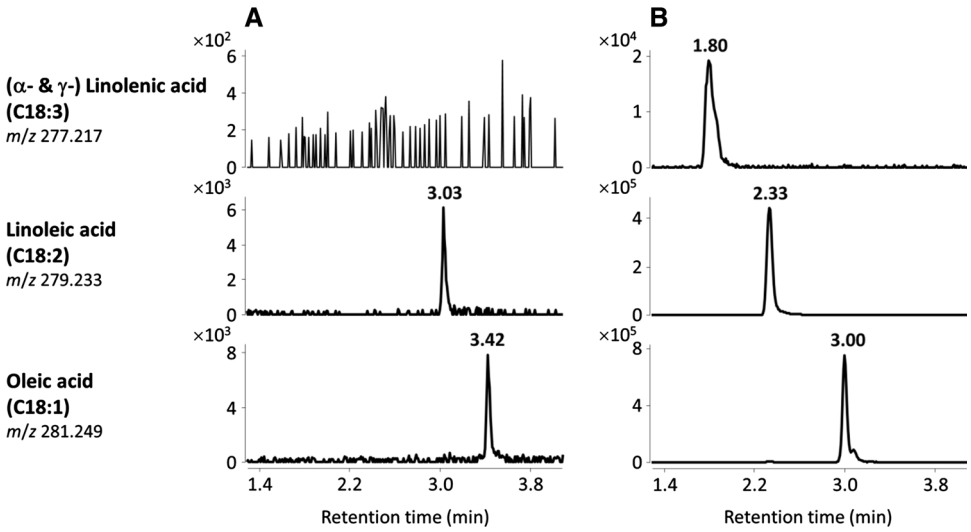

Fig. 2 Detection of selected free fatty acids in blood plasma lipid extracts. Lipids were separated using a charged surface hybrid C18 column with (**a**) 10 mM ammonium formate and 0.1 % formic acid mobile-phase modifiers, and (**b**) 10 mM ammonium acetate mobile-phase modifier

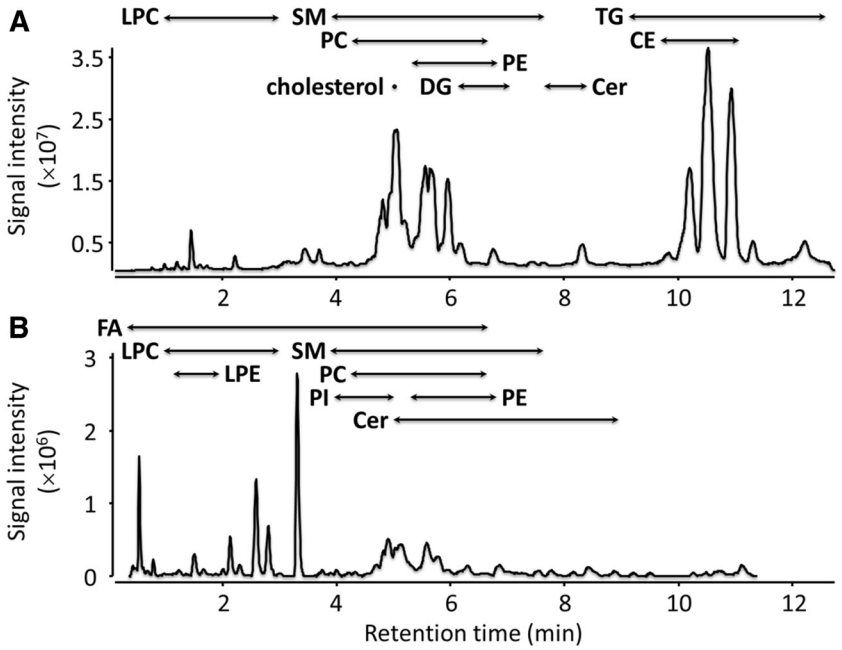

Fig. 3 Total ion chromatogram of blood plasma lipids separated on a CSH-C18 column using (**a**) 10 mM ammonium formate mobile-phase modifier, ESI(+), and (**b**) 10 mM ammonium acetate mobile-phase modifier, ESI(−). Elution of particular lipid classes for each ionization mode is highlighted by retention time ranges

$[M + CH_3COOH]^-$), depending on mobile-phase modifiers used. Using selected lipid standards we calculated a fragmentation ion ratio using peak heights from extracted ion chromatograms for fragment ion(s) and corresponding precursor ions. This procedure allowed us to assess if there was a significant difference in intensity of fragment ions originating from different adducts. To this end, we utilized All Ions MS/MS methodology as data independent acquisition, DIA. In general, DIA provides low-energy, intact, molecular spectra and corresponding high-energy fragment spectra simultaneously without preselecting any specific ions (Shah et al. 2013; Tsugawa et al. 2015). For the All Ions MS/MS experiments, a wide isolation window (*m/z* 50–1700) followed by fragmentation of precursor ions at 25 and 40 eV and detection of all fragment ions simultaneously for each collision energy setup was used.

Only slight differences in the fragmentation ion ratios (Table 3) were observed for LPC(17:0), PC(12:0/13:0) and SM(d18:1/17:0). For Cer(d18:1/17:0), the fragment ions formed from acetate adducts were 3–4-times more intensive than fragment ions originated from formate adducts making them better for identification of this lipid class. Fragmentation ratio values larger than 1 for All Ions MS/MS are due to adding up data from two MS/MS experiments because the All Ions MS/MS approach does not allow averaging MS/MS spectra.

Table 3 Fragmentation ratios for selected lipids using All Ions MS/MS

Lipid	Precursor ion	Fragment ion 1	Fragment ion 2	Fragment ion 3	Ratio 1	Ratio 2	Ratio 3
LPC(17:0)	$[M + HCOO]^-$	$[M–CH_3]^-$	FA *sn*-1		0.54 ± 0.03	0.44 ± 0.02	
	m/z 554.346	*m/z* 494.324	*m/z* 269.248				
	$[M + CH_3COO]^-$	$[M–CH_3]^-$	FA *sn*-1		0.62 ± 0.03	0.43 ± 0.02	
	m/z 568.361	*m/z* 494.324	*m/z* 269.248				
PC(12:0/13:0)	$[M + HCOO]^-$	$[M–CH_3]^-$	FA *sn*-1	FA *sn*-2	0.64 ± 0.11	1.56 ± 0.39	1.59 ± 0.32
	m/z 680.450	*m/z* 620.429	*m/z* 199.167	*m/z* 213.185			
	$[M + CH_3COO]^-$	$[M–CH_3]^-$	FA *sn*-1	FA *sn*-2	0.74 ± 0.11	1.67 ± 0.23	1.59 ± 0.12
	m/z 694.466	*m/z* 620.429	*m/z* 199.167	*m/z* 213.185			
Cer(d18:1/17:0)	$[M + HCOO]^-$	$[M–H]^-$	FA	Long-chain base fragment	0.20 ± 0.09	0.08 ± 0.03	0.02 ± 0.01
	m/z 596.525	*m/z* 550.520	*m/z* 294.2795	*m/z* 263.2249			
	$[M + CH_3COO]^-$	$[M–H]^-$	FA	Long-chain base fragment	0.81 ± 0.03	0.31 ± 0.02	0.07 ± 0.01
	m/z 610.542	*m/z* 550.520	*m/z* 294.2795	*m/z* 263.2249			
SM(d18:1/17:0)	$[M + HCOO]^-$	$[M–CH_3]^-$	$[C_4H_{11}NO_4P]^-$		1.73 ± 0.10	0.23 ± 0.03	
	m/z 761.581	*m/z* 701.560	*m/z* 168.043				
	$[M + CH_3COO]^-$	$[M–CH_3]^-$	$[C_4H_{11}NO_4P]^-$		1.95 ± 0.06	0.21 ± 0.02	
	m/z 775.597	*m/z* 701.560	*m/z* 168.043				

Fragmentation ratio calculated using the equation: Ratio = peak height of fragment ion (MS/MS)/peak height of precursor ion (MS^1). The ratio expressed as mean ± standard deviation; $n = 6$. For calculation a sum of peak heights of fragment ions from both All Ions MS/MS experiments (25 and 40 eV) used

4 Concluding remarks

Here, we present an evaluation of commonly used mobile-phase modifiers in the analysis of complex lipids in blood plasma using RPLC–MS. Our findings support the use of different combinations of modifiers for each ESI polarity to enhance the detection of lipids and therefore increase the lipidome coverage compared to the commonly used approach using the same modifiers for both ionization modes.

Use of mobile phases with 10 mM ammonium formate permits obtaining high signal intensity for lipids ionized/detected in ESI(+) with a CSH-C18 or BEH-C18 column. Overall, the use of ammonium acetate is not recommended in ESI(+) due to significant decrease of signal intensity for most of the lipids. For ESI(−) mode, 10 mM ammonium acetate should be used as it results in larger than 50-fold signal enhancement of free fatty acids due to neutral pH of the mobile phase. Formation of acetate adducts has also positive effect on increasing the signal intensity of fragment ions during MS/MS analysis for ceramide lipids as opposed to formate adducts.

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Compliance with ethical standard

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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