

The influence of citrate, EDTA, and heparin anticoagulants to human plasma LC–MS lipidomic profiling

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Abstract Lipid profiling of human plasma by liquid chromatography-electrospray ionization coupled to mass spectrometry (LC–ESI-MS) is being used to identify biomarkers of health, disease, and treatment efficacy. However, there is no consensus on the choice of anticoagulant to perform and compare lipidomic measurements. This study assessed the effect of the anticoagulants citrate, EDTA, and heparin, on eight synthetic and 80 plasma lipids, and compared lipidomic data among anticoagulants. Lipid extraction was affected distinctively by the anticoagulant of choice likely due to the different physicochemical properties among anticoagulants. Peak areas of seventy endogenous lipids showed significant differences between citrate–heparin and EDTA–heparin comparisons similar to those observed for synthetic lipids. Only ten endogenous lipid species showed comparable peak areas among the three anticoagulants. Correction by a structurally related internal standard only partly eliminated differences among anticoagulants (ANOVA, P value <0.001). However, comparisons among anticoagulants

were possible for most endogenous lipids after correction of peak areas by the sum of areas of its lipid class. Our observations indicate that the choice of anticoagulant distinctively impact the peak response of most lipid species by LC–ESI-MS. Lipidomic data from plasma obtained with different anticoagulants should address differences in matrix effects and extraction procedures since ion strength, plasma pH, and different physicochemical properties among anticoagulants influence lipid extraction and LC–ESI-MS analysis.

Keywords Anticoagulant · EDTA · Citrate · Heparin · Lipidomics · LC–MS

1 Introduction

The human plasma lipidome comprises thousands of lipids of chemically distinct classes. In addition to their role as structural components of membranes, several lipid species have been identified as markers of disease or signaling molecules (Gross and Han 2011). An increasing number of research studies indicate the important role of lipids in metabolic syndrome, Alzheimer's disease, cancer, and aging (Quehenberger and Dennis 2011; Gross and Han 2011). During the past decade, several strategies for the analysis of lipids have been developed to give rise to the field of lipidomics. High throughput lipidomic profiling by liquid chromatography coupled to mass spectrometry is one of the most widely used techniques due to its accuracy, robustness, and capacity to simultaneously assess several lipid classes. The most commonly used ionization method is electrospray followed by mass spectrometry (ESI-MS). Matrix effects (ME) with ESI are common and have been extensively reported (Jessome and Volmer 2006). The

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quantitative measure of a matrix factor is generally defined as the peak response of an analyte in the presence of matrix compared to the peak response of the analyte in the absence of matrix. The ratio of these two responses could be equal to one indicating no ME, lower than one indicating ion suppression, and if higher than one ion enhancement (Eq. 1). Ion suppression could occur when one or more analytes coelute thus, preventing an efficient ionization. Ion enhancement is observed when analyte loss is prevented during column separation or by increased ionization efficiency caused by supplemented salts or matrix analytes (Mastovska et al. 2005).

Despite plasma being the most widely available biological fluid for lipidomic analysis, there is no consensus on the choice of anticoagulant to assess lipid species by LC–ESI–MS. Maybe, because in most cases the nature of an anticoagulant is maintained constant throughout a study and the effect of different anticoagulants is seldom a problem as long as the same blood preparation procedure is used (Yu et al. 2011).

The choice of anticoagulant has been proven to affect measurements of small molecules (Christensen and Stalker 1991; Mei et al. 2003), metabolic profiling, (Barton et al. 2009; Mori 2012), and classic clinical parameters (Evans et al. 2001; Stein and Goodier 1986; Yi et al. 2011).

Heparin, EDTA, and citrate are commonly used anticoagulants. Citrate and EDTA chelate Ca^{2+} , a critical factor of coagulation, while heparin binds to several enzymes responsible for the coagulation cascade. Commercially available blood collection tubes have made plasma sampling a standardized procedure. The anticoagulants EDTA and heparin are present in solid form in blood collection tubes. Citrate however, is added as a premeasured liquid to attain a 0.38 % (%v/v) final blood citrate concentration. Citrate added as a liquid adds another factor of variation because if the tube is not properly filled with blood the expected 1:9 anticoagulant-to-blood ratio is not attained and dilution artifacts can be introduced. Altered citrate-to-blood ratios have shown to significantly affect several hemostatic measurements (Johnstone 1993).

Some of the future challenges of lipidomics include increasing the number of concomitantly identified species and comparison of lipid levels from different studies or different biological matrices. Hence, for quality control and for reliable lipidome comparisons, it is necessary to determine whether lipidomic data determined by LC–ESI–MS is influenced by the choice of anticoagulant.

Here, we investigated the effect of the anticoagulants, sodium citrate, potassium EDTA (K_2EDTA), and lithium heparin on the peak areas, matrix factors, and recovery values of synthetic lipids. Also we assessed and compared the effect of each anticoagulant on the peak areas of 80 highly abundant plasma lipids from seven different classes.

2 Materials and methods

2.1 Materials

Blood collection tubes (Vacutainer, Becton–Dickinson, Breda, The Netherlands), were used to obtain plasma with K_2EDTA , lithium heparin, or sodium citrate. Blood was collected in duplicate tubes for each anticoagulant, and processed at the Center for Human Drug Research (CHDR, Leiden, The Netherlands). Interindividual variability in the concentration of endogenous lipids is also a source of ME variations. Therefore, in an attempt to eliminate interindividual variation and to assess differences in ME due to anticoagulants alone, we collected blood from only one female volunteer under fasting conditions. Blood was withdrawn in one collection tube after another using the same catheter and sequentially replacing the blood collection tube, citrate first, followed by heparin, and finally by EDTA. Immediately after, plasma was separated by centrifugation at 1,300 rcf for 10 min at room temperature. Plasma was aliquoted into Eppendorf tubes and kept at $-80\text{ }^\circ\text{C}$ until lipid extraction and LC–MS analysis a week later. Plasma pH measurements were performed using a Hana pH-meter (Hana Instruments, IJsselstein, The Netherlands) with an IntelliProbe (Sentron, Roden, The Netherlands).

Synthetic phospholipids, lysophosphatidylcholine LPC (17:0), LPC (19:0), phosphoethanolamine PE (15:0/15:0), PE (17:0/17:0), phosphatidylcholine PC (17:0/17:0), and PC (19:0/19:0) were obtained from Avanti Polar Lipids Inc. (Alabaster AL, USA), triacylglycerols TG (51:0) and TG (45:0) were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). A solution of dichloromethane and methanol (2:1) was used to prepare matrix free solutions and to solubilize synthetic lipids. These lipids were spiked before and after extraction to plasma and to matrix-free solutions at two concentration levels, LPC (19:0), LPC (17:0), TG (45:0), and TG (51:0), low 32 $\mu\text{g/ml}$, high 160 $\mu\text{g/ml}$; PE (15:0/15:0) and PE (17:0/17:0) low 64 $\mu\text{g/ml}$, high 320 $\mu\text{g/ml}$; and PC (17:0/17:0), PC (19:0/19:0), low 96 $\mu\text{g/ml}$, high 480 $\mu\text{g/ml}$. Lipids were extracted from six independent replicates of each anticoagulant plasma and from three independent replicate matrix-free solutions. All samples were injected in duplicate.

2.2 Lipid profiling by LC–ESI–MS

Lipids from plasma aliquots (30 μl) were extracted according to the method of Bligh and Dyer with slight modifications (Bligh and Dyer 1959). Lipidomic analysis was performed using an optimized version of the method reported by Hu et al. (2008). Briefly, lipid extracts were separated by reverse phase-UPLC using an Acquity UPLC system equipped with binary pump and autosampler at $15\text{ }^\circ\text{C}$ (Waters, Milford

MA, USA). Lipids were separated in a T3 UPLC column 1.8 μm , 2.1 \times 100 mm (Acquity Waters) at 55 $^{\circ}\text{C}$ using a dynamic gradient from 68 to 3 % mobile phase A in 17 min, followed by equilibration for a total run of 20 min. Mobile phase A consisted of water and acetonitrile (40:60 %v/v), supplemented with 10 mM ammonium formate. Mobile phase B contained acetonitrile, isopropanol (10:90 %v/v), and 10 mM ammonium formate. All solvents were UPLC grade (Biosolve, Valkenswaard, The Netherlands). The UPLC was coupled to a QToF mass spectrometer, 6530 Accurate Mass QToF LC-MS (Agilent Technologies, Santa Clara, CA, USA) equipped with electrospray ionization ion source with jet stream nebulizer and Agilent Mass Hunter Acquisition software. Data were acquired using the following settings: gas temperature 325 $^{\circ}\text{C}$, nozzle voltage 1,000 V, gas flow 10 l/min, sheath gas flow 12 l/min, Vcap 3,500 V, mass range 450–1,600 m/z , in positive ion mode. This method is capable of assessing 180 different endogenous lipid species, 80 highly abundant lipids with an accuracy of RSD < 15 % were selected for this study. Precision and accuracy of the analytical method was determined by including quality control samples prepared for each anticoagulant and injected every 10 samples. Independent replicates and quality control samples were injected in duplicates and randomized within a group of samples of the same anticoagulant. After data acquisition, we used a targeted method and data mining to assess the peak areas of eight synthetic lipids and 80 endogenous lipids using MassHunter Quantitative Analysis Software (v.B.04.00, Agilent Technologies, 2008).

2.3 Lipid nomenclature

Lipids names and abbreviations were assigned according to Lipid Maps nomenclature (<http://www.lipidmaps.org>). The analytical method determines total lipid composition, as number of carbon atoms and double bonds without specifying the location of double bonds or the stereochemistry of the acyl chains. The following accepted abbreviations were used: ChoE, cholesteryl esters; DG, diacylglycerol; phosphocholines, PC and LPC for lyso species; sphingomyelins, SM; triglycerides, TG; phosphoethanolamines, PE and LPE for lyso species. The alkyl ether linkage is represented by the “O-” prefix e.g. PC (O-34:1), TG (O-50:2) the numbers within parenthesis refer to the total number of carbons of the fatty acyl chains followed by the number of double bonds of all the chains.

2.4 Calculations

ME, process efficiency (matrix and extraction effects), and recovery values were calculated using Eqs. 1–3, respectively. Equation 1 compares the signal of post-extraction additions of

the analyte extracted from a matrix to the signal obtained of the analyte extracted from a matrix-free solution (Matuszewski 2006; Peoples et al. 2008; Viswanathan et al. 2007). Equation 2 compares the signal of pre-extraction addition of the analyte in matrix to the signal of the analyte in solution added after extraction (Taylor 2005). Recovery was calculated (Eq. 3) to measure ME due to plasma components that are co-extracted with the analyte. Citrate blood collection tubes contained 0.30 ml of citrate buffer causing a dilution of the blood sample by a factor of 1.1. Inclusion of this dilution factor in our calculations did not modify results. Data is reported considering a dilution factor. Differences among anticoagulants were determined by ANOVA followed by a Fisher’s least significant differences (LSD) post hoc test and corrected for multiple testing by false discovery rate (FDR) using MetaboAnalyst 2.0 (Xia et al. 2009) and R statistical programming system (<http://www.r-project.org/>). Data was normalized by dividing the mean centered values by the standard deviation using the autoscaling option in MetaboAnalyst, normalized results were used to generate PCA plots. Differences between post and pre-extraction addition were determined by Student’s *t* test using Microsoft Excel. Anticoagulants ionic strength was calculated using the supplier information for sodium citrate (27.09 mg/ml), K_2EDTA (18 mg/tube), and lithium heparin (102 IU equivalent to 1.02 mg).

Relative ratios (RR) of each lipid species were obtained by dividing the area under the curve of each lipid by the area under the curve of its assigned internal standard. The RR of PC, PC-O, and SM species were quantified by the intensity ratios of their peaks to the internal standard PC (17:0/17:0); LPC species—to the internal standard LPC (19:0); PE and LPE species—to the internal standard PE (17:0/17:0); cholesteryl ester, diacylglycerol, and TG species—to the internal standard TG (51:0). Ratios of lipid class were calculated by dividing the lipid peak area by the sum of areas of lipids of the same class.

% Matrix Effect

$$\frac{\text{Lipid peak area after extraction from plasma}}{\text{Lipid peak area after extraction from matrix-free solution}} \times 100 \quad (1)$$

% Process Efficiency

$$\frac{\text{Lipid peak area before extraction from plasma}}{\text{Lipid peak area after extraction from matrix-free solution}} \times 100 \quad (2)$$

% Recovery

$$\frac{\text{Lipid peak area before extraction}}{\text{Lipid peak area after extraction}} \times 100 \quad (3)$$

3 Results

3.1 Comparison of synthetic lipid peak areas before versus after extraction

Peak areas of synthetic lipids were compared before and after extraction from plasma obtained with three different anticoagulants. Total and extracted ion chromatograms showed slight differences among anticoagulants (Fig. 1). ANOVA analyses indicated that when lipid standards undergo extraction, peak areas of all lipids, except PC (19:0/19:0) differ from each other, suggesting that lipid extraction is differently affected by each matrix. For example, citrate plasma peak areas are significantly different from those from EDTA and heparin (See pre-extraction addition, Table 1). Moreover, PE (17:0/17:0) and PC (17:0/17:0) peak areas in EDTA and heparin also differed from each other. We detected fewer differences among anticoagulants for post-extraction addition comparisons, mainly areas of synthetic lipid extracted from citrate plasma showed significant differences compared to EDTA or heparin (Table 1). Together these observations indicate that the lipid extraction process influences the peak response in an anticoagulant-dependent manner.

To further explore these differences, we compared lipid peak areas spiked before versus after extraction for one

anticoagulant at a time. Again comparisons show that the nature of the anticoagulant has an impact on the area of extracted lipid standards. Comparing post- versus pre-extraction peak areas differences are evident for citrate, for which all lipid standards showed the largest P-values (Student's *t* test, *P* value $<1.0 \times 10^5$). In contrast, peak areas before and after extraction of LPC (17:0), PE (17:0/17:0), and TG (51:0) were comparable when using plasma from EDTA or heparin, but peak areas were significantly different before and after extraction for the rest of the synthetic lipids. Notably, for citrate plasma lipid peak areas showed statistically significant differences before and after extraction. These observations confirm that liquid–liquid extraction affected peak area and that the influence of the extraction process is analyte and anticoagulant dependent with citrate being the anticoagulant that most significantly affects the extraction of these synthetic lipids.

3.2 Matrix effects and recovery calculations

We calculated ME using Equation 1 at two concentration levels, low (32–96 $\mu\text{g/ml}$) and high concentration range (160–480 $\mu\text{g/ml}$). We detected ion enhancement for LPC (19:0), PE (15:0/15:0), PC (17:0/17:0), and TG (45:0) and ion suppression for LPC (17:0) in all anticoagulants. PC (19:0/19:0) and TG (51:0) showed almost no ME. ANOVA

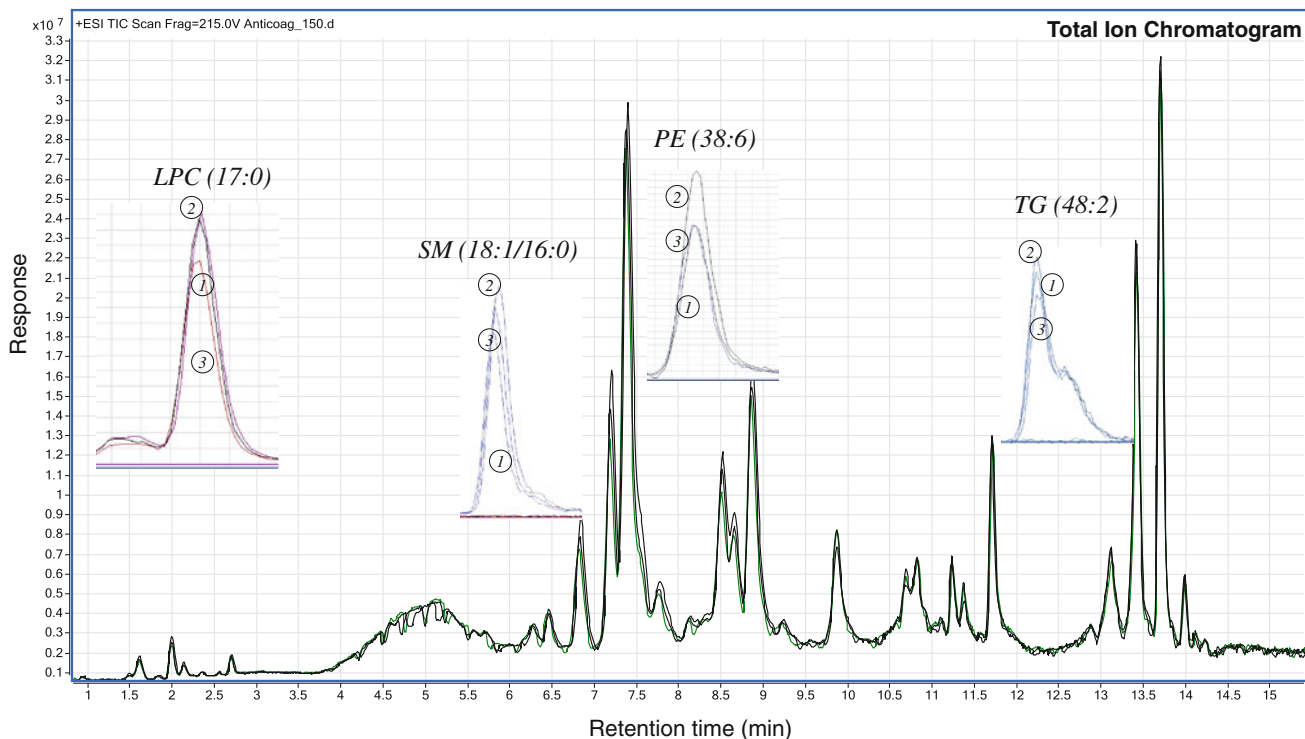


Fig. 1 Overlay of total ion chromatogram and extracted ion chromatogram of several lipid species in plasma obtained with citrate (1), EDTA (2), and heparin (3) anticoagulants

Table 1 Effect of different anticoagulants on peak areas (10^5) of lipid standards before and after lipid extraction

Lipid addition	No matrix	1. Citrate	2. EDTA	3. Heparin	Differences ^a	<i>P</i> value
LPC (19:0)						
Pre-extraction	5.2 ± 0.17	5.7 ± 0.18	6.0 ± 0.28	5.8 ± 0.14	1–2	<0.001
Post-extraction	10.0 ± 0.29	12.0 ± 0.25	11.0 ± 0.26	11.0 ± 0.41		
LPC (17:0)						
Pre-extraction	3.9 ± 0.26	5.1 ± 0.29	6.2 ± 0.16	6.0 ± 0.10	2–1; 1–3	<0.001
Post-extraction	9.0 ± 0.30	6.5 ± 0.14	6.3 ± 0.18	6.3 ± 0.18		
PE (15:0/15:0)						
Pre-extraction	2.8 ± 0.08	11.0 ± 0.72	13.0 ± 0.75	12.0 ± 0.04	2–1; 2–3	<0.001
Post-extraction	4.5 ± 0.12	21.0 ± 1.0	21.0 ± 1.5	21.0 ± 1.5		
PE (17:0/17:0)						
Pre-extraction	1.7 ± 0.07	1.8 ± 0.09	2.3 ± 0.08	2.2 ± 0.05	2–1; 2–3; 1–3	<0.001
Post-extraction	2.9 ± 0.09	2.4 ± 0.06	2.3 ± 0.03	2.2 ± 0.06	3–1	<0.001
PC (17:0/17:0)						
Pre-extraction	8.0 ± 0.49	12.6 ± 0.62	16.1 ± 0.64	14.2 ± 0.44	2–1; 2–3, 1–3	<0.001
Post-extraction	13.0 ± 0.49	15.0 ± 0.58	16.0 ± 0.22	15.0 ± 0.59	2–1; 2–3	<0.001
PC (19:0/19:0)						
Pre-extraction	11.7 ± 1.0	11.1 ± 0.55	11.6 ± 0.61	11.1 ± 0.36		
Post-extraction	18.6 ± 1.1	20.0 ± 0.47	19.0 ± 0.54	19.0 ± 1.0		
TG (45:0)						
Pre-extraction	5.1 ± 0.25	5.5 ± 0.41	5.9 ± 0.44	5.3 ± 0.36	3–2	0.015
Post-extraction	7.3 ± 0.38	8.7 ± 0.19	8.6 ± 0.66	8.7 ± 0.54		
TG (51:0)						
Pre-extraction	4.5 ± 1.3	5.3 ± 0.32	6.8 ± 0.33	6.1 ± 0.42	2–1	<0.001
Post-extraction	6.7 ± 0.25	6.6 ± 0.20	6.4 ± 0.42	6.0 ± 0.36	1–3	0.020

^a Comparisons among anticoagulants were obtained by ANOVA, Fisher's LSD post hoc test

P-values were corrected for multiple testing by FDR; blanks indicate no significant differences among anticoagulants. Comparisons between pre- and post-extraction additions were performed with Student's *t* test. Values depict mean ± standard deviation

analyses showed that citrate ME differed from those of heparin and EDTA for all lipid standards, while PC (17:0/17:0) seems to also differ between EDTA and heparin. When exploring ME at a higher concentration range, PE (15:0/15:0) and TG (45:0) ME were concentration dependent, ME at high concentrations were lower compared to those at low concentrations. For example, ME of PE (15:0/15:0) at low concentration, ME = 518 ± 33 %; at high concentration, ME = 230 ± 6.7 % (*P* < 0.001). For the rest of synthetic lipids, ME were similar for the two concentration levels (Table 2).

Recovery of synthetic lipids from plasma ranged from 50.8 to 104.5 % (Eq. 3) for all three anticoagulants. ANOVA analyses indicate that lipid recovery from plasma citrate differs from that of EDTA and heparin (Table 2). Recovery values in plasma citrate were higher for LPC (19:0), PC (19:0/19:0), and TG (45:0), and lower for LPC (17:0), PE (34:0), PC (17:0/17:0), and TG (51:0) compared to values obtained from EDTA or heparin. We performed principal component analysis (PCA) to identify whether recoveries of synthetic lipids are discriminated based on the choice of anticoagulant. Figure 2, shows that data from citrate stand separately from those of EDTA and heparin. This confirms that recovery of synthetic lipids from citrate plasma differ from those obtained with EDTA or heparin.

Calculations of process efficiency using Eq. 2 did not show differences among anticoagulants for synthetic lipids, LPC (17:0), PC (19:0/19:0), PE (15:0/15:0), and TG (51:0). Since Eq. 2 accounts for recovery (extraction efficiency) and ME in the same equation, it adds an additional variable in the determination of ME (Taylor 2005). The purpose of using Eq. 2 was to determine if simultaneous assessment of ME and extraction efficiency would generate comparable data among anticoagulants. However, calculation of process efficiency with Eq. 2 provided very different values compared to those of Equation 1 for most synthetic lipids. Equation 2 detects differences among anticoagulants for only half of the lipid standards, and does not detect most differences between citrate and EDTA previously observed with Eq. 1. Equation 2, masks differences between matrices by accounting for the extraction process so, it may not provide with informative values when extraction affects peak response and when attempting to identify the effect of a matrix alone.

3.3 Effect of different anticoagulants on endogenous lipid species

Next, we measured and compared peak areas of several endogenous lipid species (*n* = 80) in plasma obtained with sodium citrate, K₂EDTA, or lithium heparin. Most of the

Table 2 Matrix effect and recovery mean values (%) of synthetic lipids in different anticoagulants

	1. Citrate	2. EDTA	3. Heparin	Differences ^a	<i>P</i> value
Matrix effect					
LPC (19:0)	129 ± 5.4	115 ± 4.3	115 ± 6.2	1–2; 1–3	<0.001
LPC (17:0)	77.1 ± 3.2	68.1 ± 2.8	66.1 ± 3.8	1–2; 1–3	<0.001
PE (15:0/15:0)	512 ± 20.3	470 ± 16.6	468 ± 26	1–2	0.045
PE (17:0/17:0)	89.5 ± 4.1	78.8 ± 2.2	75.9 ± 3.3	1–2; 1–3	<0.001
PC (19:0/19:0)	118 ± 8.1	102 ± 7.9	106 ± 10.6	1–2; 1–3	0.030
PC (17:0/17:0)	126 ± 7.9	121 ± 4.4	112 ± 6.7	1–3; 2–3	0.013
TG (45:0)	132 ± 7.7	118 ± 12.3	120 ± 11.8		
TG (51:0)	109 ± 6.2	96.1 ± 6.9	91.1 ± 5.9	1–2; 1–3	0.001
Recovery					
LPC (19:0)	54.9 ± 1.9	52.5 ± 2.8	50.8 ± 2.1	1–2; 1–3	<0.001
LPC (17:0)	88.1 ± 4.7	99.4 ± 5.1	96.9 ± 3.1	2–1; 3–1	<0.001
PE (15:0/15:0)	62.2 ± 5.5	62.9 ± 3.7	56.6 ± 3.8	1–3; 2–3	0.002
PE (17:0/17:0)	86.3 ± 4.2	100 ± 3.7	99.9 ± 3.7	2–1; 3–1	<0.001
PC (19:0/19:0)	63.3 ± 3.5	61.9 ± 3.4	57.0 ± 3.3	1–3; 2–3	<0.001
PC (17:0/17:0)	91.6 ± 5.1	99.9 ± 4.6	95.7 ± 3.2	2–1; 3–1; 2–3	<0.001
TG (45:0)	71.3 ± 6.4	65.5 ± 6.5	63.0 ± 5.0	1–2; 1–3	0.033
TG (51:0)	90.9 ± 7.1	104 ± 8.8	105 ± 8.6	2–1; 3–1	<0.001
Process efficiency					
LPC (19:0)	122 ± 2.9	116 ± 6.2	113 ± 4.9	1–3	0.040
LPC (17:0)	145 ± 9.8	159 ± 11.7	154 ± 6.7		
PE (15:0/15:0)	443 ± 23.3	453 ± 34.6	420 ± 4.8		
PE (17:0/17:0)	117 ± 5.9	133. ± 7.9	126 ± 4.7	2–1	0.020
PC (19:0/19:0)	105 ± 7.9	99.9 ± 11.7	95.4 ± 9.2		
PC (17:0/17:0)	175 ± 8.4	204 ± 18.2	180 ± 6.9	2–1; 2–3	0.020
TG (45:0)	119 ± 9.3	116 ± 7.1	105 ± 5.3	1–3; 2–3	0.040
TG (51:0)	137 ± 8.9	161 ± 15.2	143 ± 9.5		

^a Comparisons among anticoagulants were obtained by ANOVA, Fisher's LSD post hoc test

P values were corrected for multiple testing by FDR; blanks indicate no significant differences among anticoagulants. Values depict mean ± standard deviation

lipid species ($n = 71$) showed significant differences among all or between two anticoagulants. Only a few lipids, cholesteryl esters ChoE (18:2), ChoE (20:4), diacyl glycerol DG (36:3), and five triglyceride species, showed comparable peak areas in the three anticoagulants (Table 3; Supplemental Fig. SF3).

Analysis of peak areas of endogenous lipids showed that 53 of these 71 lipids differed significantly between citrate versus heparin and EDTA versus heparin, but not between citrate versus heparin comparisons, indicating that peak areas of 53 lipid species are comparable only between citrate and heparin.

Graphical representation of ANOVA's results of Table 3 are presented in Fig. 3a, in which PCA analysis depicts separate groups of peak areas of endogenous lipids for each anticoagulant.

Analysis by lipid class revealed that peak areas of most LPC, PC, SM, and half of PE and TG species were different again, for citrate–EDTA and EDTA–heparin comparisons. The latter indicates the possibility of a reliable citrate-to-heparin comparison of peak areas of 70 % of endogenous lipids, but not between citrate and EDTA or heparin and EDTA. With some exceptions and with large variations

within the triglyceride class, peak area differences among anticoagulants tend to be similar for lipids of a same lipid class. This supports the notion that the ionization efficiency of most lipids during the electrospray process depends on the charge density of each lipid, while the ionization efficiencies of individual molecular species are independent of the aliphatic chain length within each lipid class (Gross and Han 2011). Note that eight lipids, LPC (16:0), PC (40:4), PE (O-38:5), PE (O-36:5), PE (36:3), SM (d18:1/16:0), TG (48:3), and TG (48:2) showed peak area differences among all three anticoagulants, suggesting that comparability of peak areas are dependent on the anticoagulant of choice and on the individual lipid molecule (Fig. 3b). These eight lipid species are good hydrogen bond acceptors, poor hydrogen bond donors, and belong to five different classes. However, their log-*P* values range from 5.0 to 14.2 indicating that their extraction to an organic phase is likely to differ significantly.

In most cases, the use of an internal standard compensates for ME, since the analyte of interest and its internal standard are similarly affected by the matrix. Therefore, we corrected the peak areas of endogenous lipids by a structurally related internal standard to calculate RR and explored differences

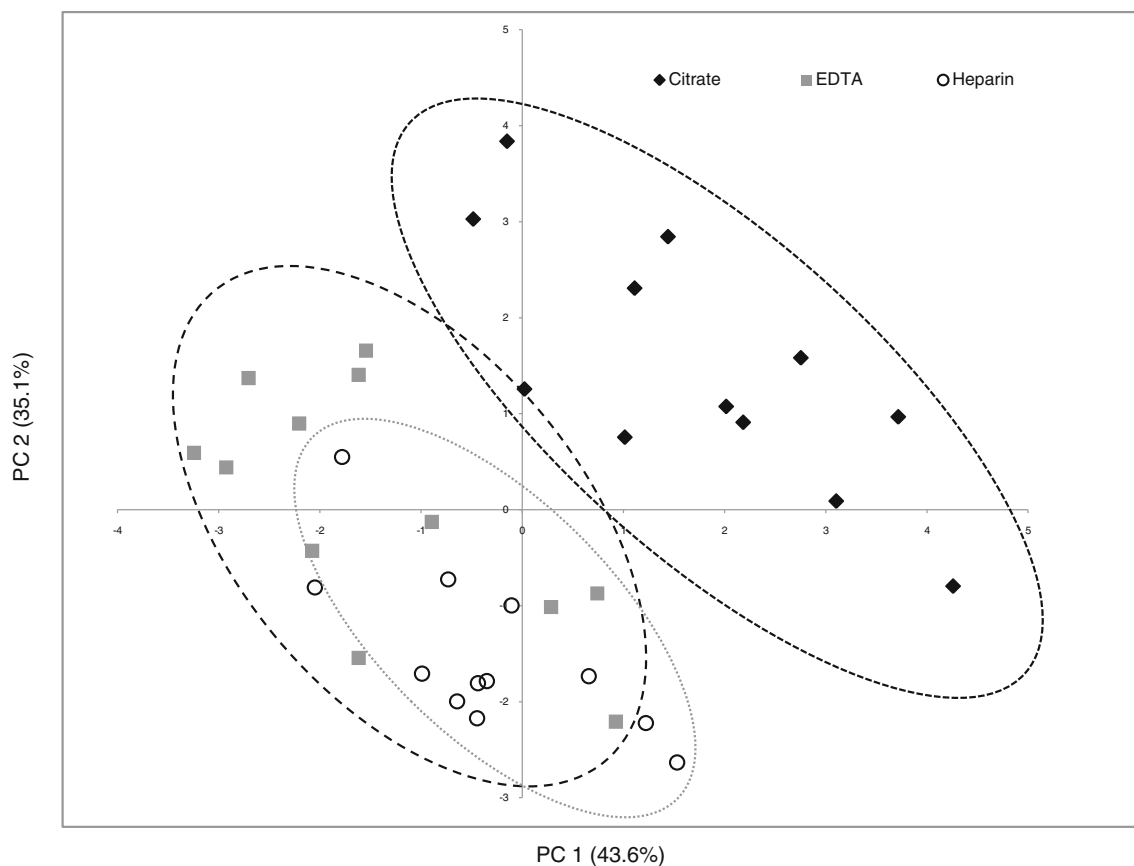


Fig. 2 PCA score plot of the effect of different anticoagulants on the recovery of synthetic lipids

among anticoagulants. ANOVA results showed that RR of most lipid species (88 %, $n = 70$) measured in citrate plasma were not comparable to RR determined in heparin or EDTA plasma. Sixty-six percent ($n = 53$) of all lipid RR values from citrate plasma differ from those of heparin and EDTA. Moreover, RR values of 32.5 % of lipid species from all classes differ among the three anticoagulants i.e. RR of these 26 lipids cannot be compared among any three anticoagulants. Many of the differences between citrate and heparin and EDTA were also observed for peak areas (uncorrected). These differences however were not always observed for the same lipid species or among the same anticoagulant, likely because the anticoagulant of choice influences the analysis of internal standards (used to calculate RR) differently to that of endogenous lipids. Besides lipidomic RR differences in citrate–heparin and citrate–EDTA a third of all lipid species also showed significant differences between EDTA and heparin. ANOVA analysis revealed that RR of only 10 lipid species seem to be comparable between anticoagulants (Supplemental Table ST1; Fig. SF1), and only five of these lipids are the same as those that were comparable for peak areas: ChoE (18:2), ChoE (20:4), DG (36:3), TG (51:1), and TG (60:2). In summary, almost all relative lipid ratios differed between citrate and

heparin, and half of these, also differed between citrate and EDTA. Thirty percent of these RR were also different when comparing EDTA versus heparin.

Finally, in an attempt to find a factor that corrected peak areas among anticoagulants and provided comparable data, we calculated the contribution of each individual lipid area to the total area of its lipid class, and performed comparisons of these ratios among anticoagulants. For example, the contribution of PC (32:0) was calculated by dividing the peak area of PC (32:0) by the sum of all PC lipid species. ANOVA analysis of these “class ratios” suggests that this could be a potential useful approach. Almost 80 % of lipid class ratios were comparable among anticoagulants. Statistical differences were still present for seventeen lipid species of the PC (1), PE (3), SM (2), and TG (11) classes. TG species showed the largest variations among the three anticoagulants (Supplemental Table S2). PCA analysis showed that lipid class ratios of all anticoagulants cluster closer together approaching the conformation of one large group (Supplemental Fig. 2).

We investigated whether the content of double bonds or fatty acid chain-length correlated with the differences observed among anticoagulants. Peak areas of lipids belonging to the same lipid class showed the same differences between anticoagulant comparisons. In addition, TG

Table 3 Effect of different anticoagulants on the peak area (10^5) of plasma lipids

	1. Citrate	2. EDTA	3. Heparin	Differences ^a	<i>P</i> value
ChoE (18:2)	2.3 ± 0.27	2.3 ± 0.16	2.3 ± 0.22		
ChoE (20:4)	0.87 ± 0.08	0.94 ± 0.11	0.87 ± 0.11		
ChoE (22:6)	0.55 ± 0.04	0.60 ± 0.07	0.54 ± 0.07		
DG (36:3)	0.26 ± 0.02	0.28 ± 0.04	0.26 ± 0.02		
LPC (14:0)	0.37 ± 0.01	0.48 ± 0.06	0.40 ± 0.02	2-1; 2-3	<0.001
LPC (O-16:1)	0.49 ± 0.02	0.57 ± 0.03	0.51 ± 0.04	2-1; 3-1; 2-3	<0.001
LPC (16:1)	1.2 ± 0.05	1.4 ± 0.07	1.2 ± 0.09	2-1; 2-3	<0.001
LPC (16:0)	50 ± 1.9	58 ± 2.6	50 ± 3.9	2-1; 2-3	<0.001
LPC (18:2)	32 ± 1.1	37 ± 1.8	32 ± 2.8	2-1; 2-3	<0.001
LPC (18:1)	20 ± 0.76	23 ± 1.1	20 ± 1.6	2-1; 2-3	<0.001
LPC (18:0)	22 ± 0.89	25 ± 1.39	22 ± 1.67	2-1; 2-3	<0.001
LPC (22:6)	0.96 ± 0.02	1.1 ± 0.05	0.97 ± 0.08	2-1; 2-3	<0.001
LPE (18:0)	0.28 ± 0.03	0.37 ± 0.05	0.30 ± 0.01	2-1; 2-3	<0.001
PC (32:2)	4.6 ± 0.18	5.3 ± 0.22	4.6 ± 0.30	2-1; 2-3	<0.001
PC (32:1)	17 ± 0.54	21 ± 0.94	18 ± 1.6	2-1; 2-3	<0.001
PC (32:0)	21 ± 0.87	25 ± 1.3	20 ± 1.5	2-1; 2-3	<0.001
PC (O-34:3)	17 ± 0.68	20 ± 0.93	17 ± 1.1	2-1; 2-3	<0.001
PC (O-34:2)	4.7 ± 0.21	5.6 ± 0.44	4.6 ± 0.28	2-1; 2-3	<0.001
PC (O-34:1)	8.0 ± 1.03	9.5 ± 0.45	7.6 ± 0.78	2-1; 2-3	<0.001
PC (34:4)	1.0 ± 0.13	1.3 ± 0.11	0.56 ± 0.07	2-1; 2-3	<0.001
PC (34:2)	831 ± 21	911 ± 30	821 ± 45	2-1; 2-3	<0.001
PC (34:1)	340 ± 13	389 ± 17	338 ± 21	2-1; 2-3	<0.001
PC (O-36:5)	17 ± 0.76	20 ± 1.1	17 ± 1.2	2-1; 2-3	<0.001
PC (36:5)	17 ± 0.84	20 ± 1.1	17 ± 1.4	2-1; 2-3	<0.001
PC (36:2)	476 ± 14	523 ± 20	465 ± 28	2-1; 2-3	<0.001
PC (36:1)	62 ± 2.7	74 ± 3.3	62 ± 4.4	2-1; 2-3	<0.001
PC (O-38:4)	7.6 ± 0.38	8.8 ± 0.49	7.4 ± 0.55	2-1; 2-3	<0.001
PC (38:6)	88 ± 3.3	102 ± 5.2	89 ± 6.6	2-1; 2-3	<0.001
PC (38:4)	124 ± 5.1	145 ± 7.1	124 ± 8.2	2-1; 2-3	<0.001
PC (40:6)	25 ± 0.95	29 ± 1.5	25 ± 1.8	2-1; 2-3	<0.001
PC (40:4)	12 ± 0.59	11.6 ± 0.51	10.9 ± 0.38	1-2; 1-3; 2-3	<0.001
PE (O-36:5)	1.5 ± 0.06	2.0 ± 0.11	1.4 ± 0.10	2-1; 1-3; 2-3	<0.001
PE (36:3)	0.31 ± 0.02	0.35 ± 0.01	0.33 ± 0.01	2-1; 3-1; 2-3	<0.001
PE (O-38:5)	2.8 ± 0.10	3.4 ± 0.17	2.6 ± 0.19	2-1; 1-3; 2-3	<0.001
PE (38:6)	2.8 ± 0.10	3.4 ± 0.18	2.9 ± 0.27	2-1; 2-3	<0.001
PE (38:2)	14 ± 0.68	16 ± 0.90	14 ± 0.99	2-1; 2-3	<0.001
SM (d18:1/14:0)	13 ± 0.53	15 ± 0.72	13 ± 0.98	2-1; 2-3	<0.001
SM (d18:1/15:0)	7.2 ± 0.32	8.0 ± 0.39	6.9 ± 0.55	2-1; 2-3	<0.001
SM (d18:1/16:1)	13 ± 0.52	16 ± 0.66	13 ± 1.02	2-1; 2-3	<0.001
SM (d18:1/16:0)	97 ± 3.7	125 ± 6.0	110 ± 7.3	1-3; 2-1; 2-3	<0.001
SM (d18:1/17:0)	2.0 ± 0.18	2.5 ± 0.29	2.2 ± 0.21	2-1; 2-3	0.005
SM (d18:1/18:0)	16 ± 0.61	21 ± 1.0	18 ± 1.3	2-1; 2-3	<0.001
SM (d18:1/20:1)	5.1 ± 0.24	5.8 ± 0.31	5.0 ± 0.36	2-1; 2-3	<0.001
SM (d18:1/20:0)	15 ± 0.57	18 ± 0.82	15 ± 0.95	2-1; 2-3	<0.001
SM (d18:1/21:0)	7.9 ± 0.27	9.2 ± 0.4	8.0 ± 0.57	2-1; 2-3	<0.001
SM (d18:1/22:1)	14 ± 0.54	16 ± 0.75	14 ± 1.1	2-1; 2-3	<0.001
SM (d18:1/22:0)	31 ± 1.0	37 ± 1.8	30 ± 2.0	2-1; 2-3	<0.001
SM (d18:1/23:0)	14 ± 0.68	16 ± 1.0	14 ± 1.1	2-1; 2-3	<0.001

Table 3 continued

	1. Citrate	2. EDTA	3. Heparin	Differences ^a	<i>P</i> value
SM (d18:0/24:0)	15 ± 0.61	18 ± 0.88	15 ± 0.9	2–1; 2–3	<0.001
TG (42:0)	0.88 ± 0.04	0.83 ± 0.06	0.82 ± 0.05	1–2; 1–3	0.015
TG (44:2)	0.32 ± 0.03	0.29 ± 0.03	0.28 ± 0.02	1–3	0.015
TG (44:1)	0.58 ± 0.08	0.64 ± 0.05	0.59 ± 0.05		
TG (44:0)	1.4 ± 0.09	1.3 ± 0.08	1.22 ± 0.11	1–2; 1–3	0.013
TG (46:2)	1.2 ± 0.07	1.2 ± 0.08	1.19 ± 0.05	1–3; 2–3	<0.001
TG (46:1)	2.2 ± 0.15	2.2 ± 0.18	2.0 ± 0.12	1–3; 2–3	0.003
TG (46:0)	2.7 ± 0.20	2.5 ± 0.19	2.4 ± 0.23	1–3	0.012
TG (48:3)	1.5 ± 0.08	1.6 ± 0.06	1.4 ± 0.07	2–1; 1–3; 2–3	<0.001
TG (48:2)	5.0 ± 0.26	5.4 ± 0.28	4.8 ± 0.26	2–1; 1–3; 2–3	<0.001
TG (48:1)	8.4 ± 0.86	9.5 ± 0.69	8.2 ± 0.61	2–1; 2–3	<0.001
TG (48:0)	4.6 ± 0.36	4.5 ± 0.41	4.2 ± 0.39	1–3; 2–3	0.045
TG (50:3)	11 ± 0.56	13 ± 0.7	11 ± 0.89	2–1; 2–3	<0.001
TG (50:2)	36 ± 1.5	38 ± 1.2	29 ± 1.8		
TG (50:1)	34 ± 1.7	38 ± 2.3	33 ± 1.9	2–1; 2–3	<0.001
TG (50:0)	3.6 ± 0.25	2.6 ± 0.17	3.3 ± 0.36	1–2	0.045
TG (51:3)	4.6 ± 0.28	5.3 ± 0.3	4.5 ± 0.26	2–1; 2–3	<0.001
TG (51:1)	2.3 ± 0.25	2.7 ± 0.34	2.4 ± 0.23		
TG (52:4)	30 ± 1.5	36 ± 1.7	30 ± 2.4	2–1; 2–3	<0.001
TG (52:3)	130 ± 5.1	154 ± 7.4	125 ± 11	2–1; 2–3	<0.001
TG (52:2)	214 ± 9.0	241 ± 10	210 ± 14	2–1; 2–3	<0.001
TG (54:3)	113 ± 4.9	129 ± 5.5	112 ± 7.4	1–2; 1–3	<0.001
TG (54:1)	1.8 ± 0.06	2.2 ± 0.22	1.9 ± 0.14	2–1	0.005
TG (54:0)	0.82 ± 0.07	1.5 ± 0.31	0.61 ± 0.06	2–1; 2–3	<0.001
TG (56:7)	5.0 ± 0.47	5.5 ± 0.26	4.7 ± 0.33	2–1; 2–3	0.043
TG (56:0)	0.48 ± 0.06	0.55 ± 0.02	0.37 ± 0.01	2–1; 2–3	<0.001
TG (57:2)	2.9 ± 0.15	2.8 ± 0.13	2.5 ± 0.30		
TG (58:3)	0.39 ± 0.05	0.38 ± 0.03	0.35 ± 0.04	2–3	0.020
TG (58:2)	0.45 ± 0.03	0.48 ± 0.04	0.41 ± 0.02	1–3	0.043
TG (58:1)	0.41 ± 0.02	0.44 ± 0.04	0.41 ± 0.03	1–3; 2–3	<0.001
TG (60:3)	0.22 ± 0.02	0.21 ± 0.02	0.21 ± 0.03		
TG (60:2)	0.28 ± 0.02	0.33 ± 0.03	0.29 ± 0.03		

^a Comparisons among anticoagulants were obtained by ANOVA, Fisher's LSD post hoc test

P values were corrected for multiple testing by FDR; Blanks indicate no significant differences among anticoagulants. Values depict mean ± standard deviation

species with few double bonds tend to have comparable peak responses among anticoagulants. Supplemental Table ST1 lists endogenous lipids, their double-bond content, and chain length, no additional differential patterns were observed when considering these variables.

4 Discussion

The application of lipidomics for the study of disease, health, and the efficacy of therapy has importantly increased during the last several years and has generated a large amount of data

on several hundreds of lipid species (Graessler et al. 2009; Bergheanu et al. 2008). Lipidomics thus, has reached a point in which comparison of the lipid profile of several studies will try to confirm and generate new hypotheses from different study cohorts. However, different studies are performed using different anticoagulants hence, we sought to determine whether lipidomic data determined by LC–ESI–MS is influenced by the choice of anticoagulant. Here, we observed that the use of different anticoagulants distinctively impact peak response areas of the here studied synthetic lipids in plasma. Consequently, recovery values and matrix factors of these eight synthetic lipids were mostly not comparable. Most

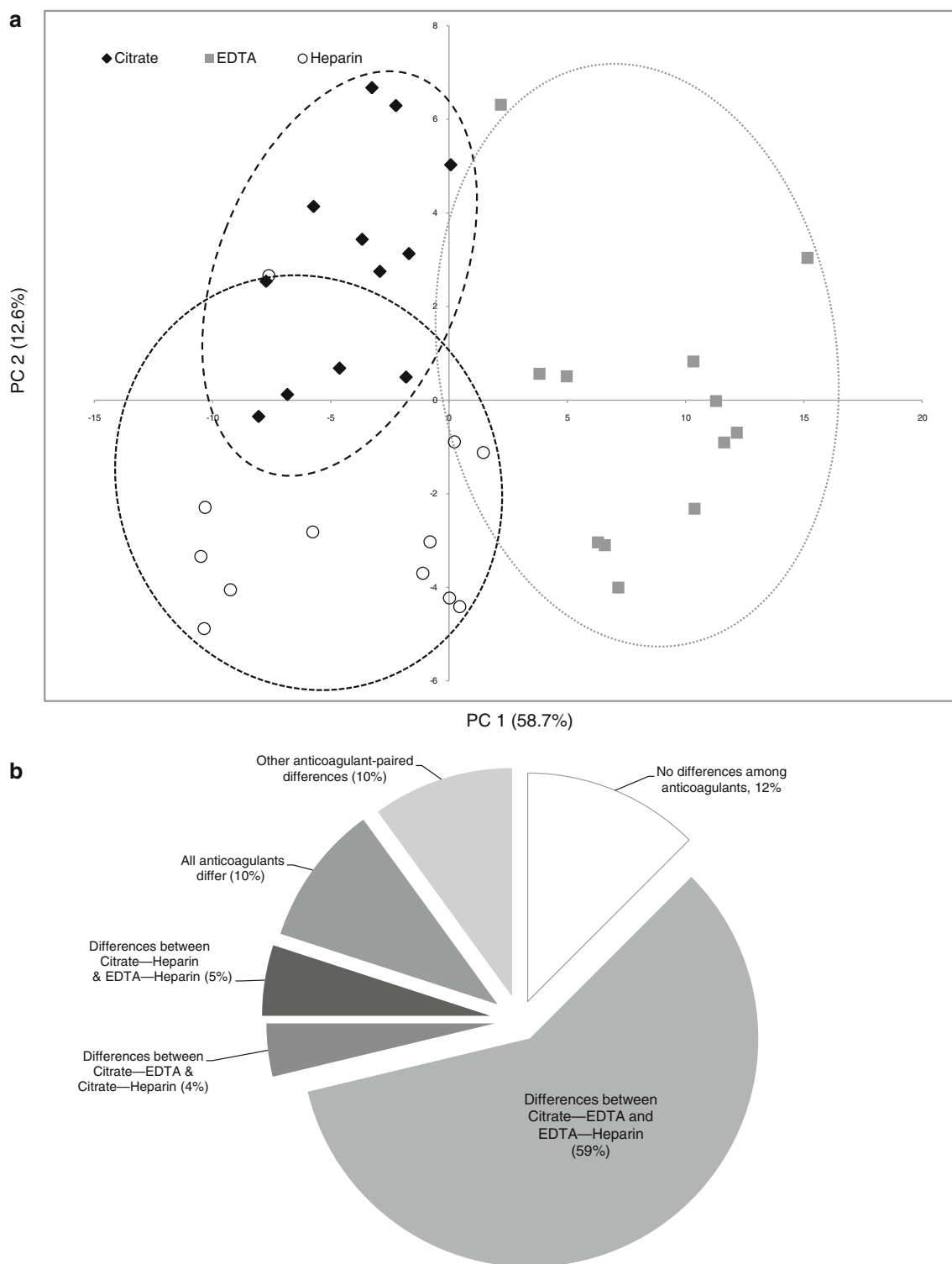


Fig. 3 **a** PCA score plot of the effect of different anticoagulants on the peak areas of endogenous plasma lipids. **b** Proportion of lipid species that differ between anticoagulant comparisons

synthetic lipids showed a ME above 100 % suggesting ion enhancement, which could be a consequence of plasma components, LC tubing interactions, or column blocked active sites preventing analyte loss during separation. These

variables could play a role in generating a higher signal in matrix-containing versus matrix-free solutions.

Endogenous plasma lipids also showed differences in peak areas among the three anticoagulants tested. Area

normalization by a structurally similar and arbitrarily selected internal standard did not eliminate peak area differences among anticoagulants. However, normalization by total lipid class rendered 80 % of lipid ratios comparable among all three anticoagulants.

Lipid extraction eliminates most of the anticoagulant salt before LC–MS analysis. However, it is unknown whether the chemistry of the anticoagulant, pH, and blood-dilution differences among anticoagulants affect lipid extraction or ESI-ionization. Several physico-chemical differences of the anticoagulants here tested may influence lipid extraction and could be responsible for the observed differences. For example, plasma pH was higher in sodium citrate (pH = 8.3) and potassium EDTA (pH = 8.2) compared to lithium heparin (pH = 7.6). Moreover, ionic strength was significantly higher in sodium citrate ($I = 0.209 \text{ mol/dm}^3$) and potassium EDTA ($I = 0.090 \text{ mol/dm}^3$) versus lithium heparin ($I = 0.00014 \text{ mol/dm}^3$). A higher ionic strength decreases protein solvation which would probably enhance protein denaturation and lipid release from lipoproteins and thus extraction into the organic phase. Another possibility is that variations in plasma pH distinctively influence lipid extraction. For example, the fraction of phospholipids negatively charged at physiological pH is almost 100 %, and since phospholipids bind with relative specificity to sodium or potassium but distinctively less to lithium, this could particularly influence lipid extraction (Kirschner 1958). Table 3 shows that for all phospholipid species peak areas are the highest in K₂EDTA (except PC 40:2), and peak areas are similar between citrate and heparin. Interestingly, for several TG species ($n = 8$) with none up-to three double bonds peak areas are higher in sodium citrate compared to K₂EDTA or heparin. This suggests that lipid extraction and LC–MS analysis of lipid classes is distinctively affected by ion strength or the chemical nature of the paired cation of the anticoagulant. Intriguingly, ME (Eq. 1) on synthetic lipids is higher in citrate but peak areas of endogenous lipids are higher in EDTA (Tables 2, 3). Sodium citrate and K₂EDTA generate similar plasma pH values, but citrate has a higher ionic strength, it is unclear how these properties would differentially influence lipid extraction in synthetic versus endogenous lipids. We can only speculate that the alkaline cations, potassium and sodium might influence lipid extraction by differentially altering the ion flux on the lipoprotein surface.

The proportion of lithium, in the organic phase after Bligh and Dyer extraction is likely negligible, and although we cannot explain the role of lithium in the differences observed, previous investigations have also reported differential LC–MS peak responses of small molecules with lithium heparin (Mei et al. 2003).

We performed a Bligh and Dyer extraction which together with an LC–ESI-MS method have been validated numerous times. These validations have been performed in plasma

obtained with sodium citrate, K₂EDTA, and sodium heparin, using the same or identical UPLC and mass spectrometer instruments. We observed that plasma citrate caused ion enhancement for several synthetic lipids. In addition, personal communications from other lipidomic studies indicated that sodium citrate confers ME, peak shape, peak response, and even validation parameters slightly different to those observed with sodium heparin or K₂EDTA. For example, linearity of lipid standards was $R^2 > 0.99$ in sodium heparin and K₂EDTA for all synthetic lipids, but $R^2 > 0.97$ for PE (15:0/15:0) and PC (19:0/19:0) in sodium citrate (Szymańska et al. 2011; Hu et al. 2008). The use of citrate as an anticoagulant requires accuracy to avoid dilution artifacts during blood sampling since its high ionic strength and pH seem to influence lipid extraction. Interestingly, Mori et al. recently suggested the use of EDTA instead of citrate or heparin for LC–MS analysis of specific lipids (Mori 2012). Together these observations would motivate us to avoid sodium citrate for future lipidomic analyses.

In an attempt to normalize lipid peak areas and generate comparable data among anticoagulants, we determined the individual contribution of a lipid to a lipid class and compared these ratios among anticoagulants. We observed that 80 % of lipid peak areas normalized by the total area of their lipid class were comparable among anticoagulants (Supplemental Table ST2 and Fig. SF2). In contrast, normalization by an internal standard rendered only 12 % of comparable data. This indicates that the selected internal standards and endogenous lipids are not equally affected by the anticoagulant. The use of several internal standards per lipid class or labeled internal standards to overcome differences between endogenous lipids and internal standards during lipidomic analysis is warranted.

5 Conclusions

Our results indicate that ME and recovery of synthetic lipids using the selected internal standards are influenced by the choice of anticoagulant. Except for 10 endogenous lipid species, the peak area of plasma lipids measured by LC–ESI-MS was differentially affected by each matrix. Differences in peak areas were not eliminated after normalization with an internal standard, but most of them were eliminated after correction by the total area of its lipid class. Our observations suggest that ME should be explored for new extraction schemes, choice of internal standards, and when changing the blood anticoagulant. The high ionic strength and pH of sodium citrate may not be ideal for classical lipid extraction or LC–ESI-MS analysis.

In conclusion, caution should be exercised when comparing lipidomic data of plasma obtained with different anticoagulants, especially for large metabolome data sets.

Investigation of statistical methods and correction factors such as calculation of class ratios may aid to generate comparable lipidomic data for different anticoagulants.

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References

- Barton, R. H., Waterman, D., Bonner, F. W., Holmes, E., Clarke, R., Nicholson, J. K., et al. (2009). The influence of EDTA and citrate anticoagulant addition to human plasma on information recovery from NMR-based metabolic profiling studies. *Molecular BioSystems*, 6(1), 215–224. doi:10.1039/b907021d.
- Bergheanu, S. C., Reijmers, T., Zwinderman, A. H., Bobeldijk, I., Ramaker, R., Liem, A.-H., et al. (2008). Lipidomic approach to evaluate rosuvastatin and atorvastatin at various dosages: Investigating differential effects among statins. *Current Medical Research and Opinion*, 24(9), 2477–2487. doi:1185/03007990802321709.
- Bligh, E., & Dyer, W. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37, 911–917.
- Christensen, J. M., & Stalker, D. (1991). Ibuprofen piconol hydrolysis invitro in plasma, whole-blood, and serum using different anticoagulants. *Journal of Pharmaceutical Sciences*, 80(1), 29–31. doi:10.1002/jps.2600800108.
- Evans, M. J., Livesey, J. H., Ellis, M. J., & Yandle, T. G. (2001). Effect of anticoagulants and storage temperatures on stability of plasma and serum hormones. *Clinical Biochemistry*, 34(2), 107–112.
- Graessler, J., Schwudke, D., Schwarz, P. E. H., Herzog, R., Shevchenko, A., & Bornstein, S. R. (2009). Top-down lipidomics reveals ether lipid deficiency in blood plasma of hypertensive patients. *PLoS One*, 4(7), e6261.
- Gross, Richard. W., & Han, X. (2011). Lipidomics at the interface of structure and function in systems biology. *Chemistry & Biology*, 18(3), 284–291.
- Hu, C., van Dommelen, J., van der Heijden, R., Spijksma, G., Reijmers, T. H., Wang, M., et al. (2008). RPLC-ion-trap-FTMS method for lipid profiling of plasma: Method validation and application to p53 mutant mouse model. *Journal of Proteome Research*, 7(11), 4982–4991. doi:10.1021/pr800373m.
- Jessome, L. L., & Volmer, D. A. (2006). Ion suppression: A major concern in mass spectrometry. *LCGC North America*, 24, 498–510.
- Johnstone, I. B. (1993). The importance of accurate citrate to blood ratios in the collection of canine blood for hemostatic testing. *Canadian Veterinary Journal (Revue Veterinaire Canadienne)*, 34(10), 627–629.
- Kirschner, L. B. (1958). The cation content of phospholipids from swine erythrocytes. *Journal of General Physiology*, 42(2), 231–241.
- Mastovska, K., Lehotay, S. J., & Anastassiades, M. (2005). Combination of analyte protectants to overcome matrix effects in routine GC analysis of pesticide residues in food matrixes. *Analytical Chemistry*, 77(24), 8129–8137. doi:10.1021/ac0515576.
- Matuszewski, B. K. (2006). Standard line slopes as a measure of a relative matrix effect in quantitative HPLC-MS bioanalysis. *Journal of Chromatography B—Analytical Technologies in the Biomedical and Life Sciences*, 830(2), 293–300. doi:10.1016/j.jchromb.2005.11.009.
- Mei, H., Hsieh, Y. S., Nardo, C., Xu, X. Y., Wang, S. Y., Ng, K., et al. (2003). Investigation of matrix effects in bioanalytical high-performance liquid chromatography/tandem mass spectrometric assays: Application to drug discovery. *Rapid Communications in Mass Spectrometry*, 17(1), 97–103. doi:10.1002/rcm.876.
- Mori, T. A., Mas, E., Barden, A. E., Zahra, P., & Croft, K. D. (2012). Measurement of resolvins and protectins using liquid chromatography-tandem mass spectrometry: Comparison of plasma and serum levels in humans. *ISSFAL Conference: Poster*, Vancouver, Canada.
- Peoples, M. C., Halquist, M. S., Ismaiel, O., El-Mammi, M. Y., Shalaby, A., & Karnes, H. T. (2008). Assessment of matrix effects and determination of niacin in human plasma using liquid-liquid extraction and liquid chromatography-tandem mass spectrometry. *Biomedical Chromatography*, 22(11), 1272–1278. doi:10.1002/bmc.1057.
- Quehenberger, O., & Dennis, E. A. (2011). The human plasma lipidome. *New England Journal of Medicine*, 365(19), 1812–1823. doi:10.1056/NEJMr1104901.
- Stein, P. E., & Goodier, D. W. (1986). Effect of in vitro sodium citrate anticoagulant on results of arterial blood gas analysis. *Journal of Clinical Pathology*, 39(9), 1046.
- Szymańska, E., van Dorsten, F., Troost, J., Paliukhovich, I., van Velzen, E., Hendriks, M., et al. (2011). A lipidomic analysis approach to evaluate the response to cholesterol-lowering food intake. *Metabolomics*, 1–13. doi:10.1007/s11306-011-0384-2.
- Taylor, P. J. (2005). Matrix effects: The Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. *Clinical Biochemistry*, 38(4), 328–334. doi:10.1016/j.clinbiochem.2004.11.007.
- Viswanathan, C. T., Bansal, S., Booth, B., DeStefano, A. J., Rose, M. J., Sailstad, J., et al. (2007). Quantitative bioanalytical methods validation and implementation: Best practices for chromatographic and ligand binding assays. *Pharmaceutical Research*, 24(10), 1962–1973. doi:10.1007/s11095-007-9291-7.
- Xia, J., Psychogios, N., Young, N., & Wishart, D. S. (2009). MetaboAnalyst: A web server for metabolomic data analysis and interpretation. *Nucleic Acids Research*, 37(suppl 2), W652–W660. doi:10.1093/nar/gkp356.
- Yi, J., Craft, D., & Gelfand, C. A. (2011). Minimizing preanalytical variation of plasma samples by proper blood collection and handling. *Methods in Molecular Biology*, 728(2), 137–149. doi:10.1007/978-1-61779-068-3_8.
- Yu, Z., Kastenmaller, G., He, Y., Belcredi, P., Maller, G., Prehn, C., et al. (2011). Differences between human plasma and serum metabolite profiles. *PLoS One*, 6(7), e21230.