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RESEARCH PAPER

Validation of lipidomic analysis of human plasma and serum by supercritical fluid chromatography–mass spectrometry and hydrophilic interaction liquid chromatography–mass spectrometry

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Abstract

Ultrahigh-performance supercritical fluid chromatography–mass spectrometry (UHPSFC/MS) has a great potential for the high-throughput lipidomic quantitation of biological samples; therefore, the full optimization and method validation of UHPSFC/MS is compared here with ultrahigh-performance liquid chromatography–mass spectrometry (UHPLC/MS) in hydrophilic interaction liquid chromatography (HILIC) mode as the second powerful technique for the lipid class separation. First, the performance of six common extraction protocols is investigated, where the Folch procedure yields the best results with regard to recovery rate, matrix effect, and precision. Then, the full optimization and analytical validation for eight lipid classes using UHPSFC/MS and HILIC-UHPLC/MS methods are performed for the same sample set and applied for the lipidomic characterization of pooled samples of human plasma, human serum, and NIST SRM 1950 human plasma. The choice of appropriate internal standards (IS) for individual lipid classes has a key importance for reliable quantitative workflows illustrated by the selectivity while validation and the calculation of the quantitation error using multiple internal standards per lipid class. Validation results confirm the applicability of both methods, but UHPSFC/MS provides some distinct advantages, such as the successful separation of both non-polar and polar lipid classes unlike to HILIC-UHPLC/MS, shorter total run times (8 vs. 10.5 min), and slightly higher robustness. Various types of correlations between methods (UHPSFC/MS and HILIC-UHPLC/MS), biological material (plasma and serum), IS (laboratory and commercially mixtures), and literature data on the standard reference material show the intra- and inter-laboratory comparison in the quantitation of lipid species from eight lipid classes, the concentration differences in serum and plasma as well as the applicability of non-commercially available internal standard mixtures for lipid quantitation.

Keywords Supercritical fluid chromatography · Hydrophilic interaction liquid chromatography · Mass spectrometry · Lipidomics · Validation · Quantitation · Plasma · Serum · Matrix effect

Introduction

Lipids are important biologically active substances, which are involved in signaling, membrane constituents, and energy storage mechanisms [1–3]. The alteration of lipid concentrations in body fluids may reflect pathological states, and the determination of characteristic lipid profiles could allow the prediction of healthy and disease states [4, 5]. However, lipid concentrations in humans are not only affected by the pathological state but also by other factors leading to a high biological variability, such as dietary intake, circadian rhythm, and hormonal cycle [6–8]. For diagnostic purposes, sensitive, robust, accurate, and precise methods capable to determine lipid

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concentrations are essential [9, 10], as variances in the analysis may lead to inconclusive results.

Mass spectrometry (MS)–based methods using either the direct infusion (also called shotgun) or the coupling to UHPLC are most commonly used for the quantitative lipidomic analysis [11]. The lipid class separation by HILIC-UHPLC [12–14] or UHPSFC [15, 16] is a powerful tool for the accurate quantitation, as one IS per lipid class allows the quantitation of all lipid species within the class, because they elute at the same or very similar retention times, which leads to the same matrix and ionization effects. The separation is based on the interaction of polar head groups of lipids with the polar stationary phase. In UHPSFC/MS, the mobile phase consists of supercritical carbon dioxide with organic modifiers, typically methanol or other alcohols, with or without additives, such as water and salts. The release of a new generation of UHPSFC instruments and commercially available interfaces to MS enables more stable and highly reproducible analysis with comparable performance to UHPLC and the advantage of higher speed of the analysis by employing higher flow rates due to the lower viscosity of the mobile phase and the flat plateau in van Deemter curve [15–22]. The potential of UHPSFC/MS for the high-throughput lipidomic analysis has been first demonstrated in 2015 [15] and later compared with UHPLC/MS and shotgun MS for the analysis of biological samples with the comparison of the analysis time, number of detected lipids, sample consumption, and solvent consumption [16].

The accurate quantitation of lipid species in biological samples based on MS is still challenging due to the lack of reproducibility in intra- and inter-laboratory studies. The full method validation, the use of multiple IS for each lipid class, and a quality control system may improve the overall comparability of lipidomic results obtained with different methods and in various laboratories [23].

The goal of this study is the optimization of all steps in the lipid analysis from the sample preparation through MS measurements including the full method validation for both UHPSFC/MS and UHPLC/MS. Validation results obtained with both methods are compared to each other with the special focus on precision and accuracy in order to assess the applicability of UHPSFC/MS in comparison to the well-established HILIC-UHPLC/MS technique for biological studies. The validation is performed for human plasma and serum, and differences between both types of body fluids are investigated. Finally, both methods are applied for the lipidomic quantitation of plasma, serum, and the NIST Standard Reference Material 1950 human plasma (further termed as NIST plasma) using deuterated and other exogenous IS. The quantitation performance is evaluated by the comparison of lipid concentrations obtained by both methods and with literature values.

Materials and methods

Chemicals and solvents

The following Chromasolv solvents and other chemicals (Honeywell, Riedel-de Haën, Germany) of LC-MS or HPLC grade were purchased from Sigma-Aldrich (St. Louis, MI, USA): acetonitrile (ACN), methanol (MeOH), 2-propanol (IPA), hexane, ethyl acetate (EtOAc), butanol (BuOH), sodium hydrogen bisphosphate, ammonium carbonate, formic acid, acetic acid (AcOH), ammonium formate (NH_4FA), and ammonium acetate (NH_4OAc). Lichrosolv solvents chloroform and methyl-*tert*-butyl ether (MTBE) were purchased from Merck (Darmstadt, Germany). Supercritical carbon dioxide ($scCO_2$) with 99.995% purity was purchased from Messer (Bad Soden, Germany), and water was obtained from an in-house Milli-Q water purification system (Millipore, Molsheim, France). Lipid standards including the SPLASH Lipidomix were obtained from Avanti Polar Lipids (Alabaster, AL, USA), Nu-Chek (Elysian, MN, USA), or Merck.

Standard mixtures

A mixture of lipid standards containing oleoyl (18:1) fatty acyls was selected as the system suitability standard (SSS), namely, monoolein, diolein, triolein, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine, 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (sodium salt), N-oleoyl-D-erythro-sphingosylphosphorylcholine, N-oleoyl-D-erythro-sphingosine, D-erythro-sphingosine, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-(1′-rac-glycerol) (sodium salt), D-lactosyl-ß-1,1' N-palmitoyl-D-erythro-sphingosine, D-galactosyl-ß-1,1' N-lauroyl-D-erythro-sphingosine, and D-glucosyl-ß-1,1′-N-palmitoyl-D-erythro-sphingosine. Stock solutions of 2.5 mg/mL in $CHCl_3$/MeOH (1:1, *v/v*) of each standard were prepared, afterwards mixed together and diluted with $CHCl_3$/MeOH (1:1, *v/v*) in order to obtain the final concentration of 25 ng/μL. This solution was further 1:5 (*v/v*) diluted before the analysis resulting in the concentration of 5 ng/μL. The SSS was used to control the instrument performance covering the whole chromatographic range without the presence of interfering matrix. The internal standard mixture (IS Mix) for the quantitation of lipid classes was prepared with final concentrations shown in Table 1 and the procedure for the preparation of IS Mix (Table S1 in Electronic Supplementary Material (ESM)).

Table 1 Concentrations of internal standards in our IS mix and the Splash Lipidomix calculated in nmol/mL of human plasma

Lipid class	Internal standard	Concentration in IS Mix (nmol/mL)	Concentration in SPLASH Lipidomix (nmol/mL)
Cholesteryl esters	CE 16:0 d7	342.0	400.3
Ceramides	Cer 30:1	2.7	
	Cer 35:1	2.3	
	Cer 36:1 d7	2.2	
Diacylglycerols	DG 24:2	11.3	
	DG 36:2 d5	5.8	
	DG 33:1 d7	5.7	12.0
Hexosyl ceramides	LacCer 30:1	2.4	
	GlcCer 30:1	2.5	
Cholesterol	Chol d7	231.6	200.1
Phosphatidyl inositol	PI 33:1 d7	5.7	8.2
Lysophosphatidyl choline	LPC 17:0	34.6	
	LPC 18:1 d7	34.8	36.0
Lysophosphatidyl ethanolamine	LPE 14:0	6.0	
	LPE 18:1 d7		8.1
Lysophosphatidyl glycerol	LPG 14:0	2.7	
Lysophosphatidyl serine	LPS 17:1	2.4	
Lysophosphatidic acid	LPA 14:0	2.1	
Monoacylglycerol	MG 19:1	12.1	
	MG 18:1 d7	11.4	4.0
Phosphatidic acid	PA 28:0	5.7	
	PA 33:1 d7		8.0
Phosphatidyl choline	PC 33:1 d7	106.2	160.1
	PC 28:0	113.4	
	PC 44:2	57.0	
	PC 44:0	95.8	
Phosphatidyl ethanolamine	PE 28:0	12.6	
	PE 33:1 d7	11.3	6.0
Phosphatidyl serine	PS 28:0	5.5	
	PS 33:1 d7		4.0
Phosphatidyl glycerol	PG 28:0	5.6	
	PG 33:1 d7		28.8
Sphingomyelin	SM 30:1	34.6	
	SM 36:2 d9	34.7	32.1
Sulfohexosylceramide	SHexCer 30:1	0.1	
Triacylglycerol	TG 48:1 d7	57.1	52.0
	TG 57:3	57.0	
Fatty acid	FA 18:1 d9	27.5	

Biological samples

Plasma (heparin-lithium 9 mL tubes) and serum (Z serum clot activator 9 mL tubes) of 38 male and 38 female healthy volunteers were collected. Pooled samples with equal ratios of 38 males and 38 females were prepared separately for plasma, serum, and also mixed pooled sample of plasma and serum used for the optimization of sample preparation.

UHPSFC/MS conditions

UHPSFC analyses were carried out on an Acquity UPC^2 instrument from Waters (Milford, MA, USA) using the Viridis BEH column (100 × 3 mm, 1.7 μm). The following linear gradient was employed using $scCO_2$ and MeOH with 30 mM ammonium acetate and 1% H_2O used as a modifier: 0 min, 1% modifier; 1.5 min, 16% modifier; 4 min, 51% modifier; 7 min, 51% modifier; 7.51 min,

1% modifier; and the equilibration with the total run time of 8 min. The automatic back-pressure regulator (ABPR) was set to 1800 psi, the column temperature to 60 °C, the flow to 1.9 mL/min, and the injection volume was 1 μL. The injection needle was washed after each injection with hexane/IPA/H_2O (2:2:1, *v/v/v*).

UHPSFC was coupled to the hybrid quadrupole–time of flight (QTOF) mass spectrometer Synapt G2-Si from Waters by using the commercial interface kit (Waters). The make-up solvent had the identical composition as the modifier (MeOH with 30 mM ammonium acetate and 1% H_2O) with a flow rate of 0.25 mL/min. The following parameters were set for QTOF measurements: sensitivity mode applying positive ion electrospray ionization (ESI) mode, the mass range of *m/z* 150–1200, the capillary voltage of 3 kV, the sampling cone of 20 V, the source offset of 90 V, the source temperature of 150 °C, the desolvation temperature of 500 °C, the cone gas flow of 50 L/h, the desolvation gas flow of 1000 L/h, and the nebulizer gas flow of 4 bar. The peptide leucine enkephalin was used as the lock mass. The analysis was done in the continuum mode with a scan time of 0.1 s and the lock mass scanning.

HILIC-UHPLC/MS conditions

HILIC-UHPLC was performed on an Agilent 1290 Infinity series UHPLC system (Agilent Technologies, Waldbronn, Germany) with the following conditions: the column Viridis BEH Waters (100 × 3 mm, 1.7 μm), the temperature of separation 40 °C, the flow rate 0.5 mL/min, and the injection volume 1 μL. The injection needle was washed with the mixture of MeOH/ACN/IPA/H2O (1:1:1:1, *v/v/v/v*) after each injection. The linear gradient was set as follows: 0 min, 100% mobile phase A; 5 min, 84% of A; 5.5 min, 84% of A; 5.51 min, 100% A; and 10.5 min, 100% A; where the mobile phase A was ACN/H_2O (96/4, *v/v*); and the mobile phase B was ACN/H_2O (2/98 *v/v*). Both phases contained 8 mM of ammonium acetate. The total run time including the equilibration is 10.5 min [24].

The UHPLC system was coupled to a Xevo G2-XS-QTOF mass spectrometer (Waters, Milford, MA, USA). The data were acquired in the sensitivity mode using ESI in the positive ion mode under the following conditions: the capillary voltage of 3 kV, the sampling cone of 20 V, the source offset of 90 V, the source temperature of 150 °C, the desolvation temperature of 500 °C, the cone gas of 50 L/h, and the desolvation gas flow of 1000 L/h. Mass spectra were measured in the *m/z* range of 150–1200 with the scan time of 0.5 s using the continuum mode and the lock mass scanning. The peptide leucine enkephalin was used as the lock mass.

Data analysis

The data in both UHPSFC/MS and HILIC-UHPLC/MS were acquired and pre-processed with MassLynx. First, the raw data file size was minimized for better data processing handling using the noise reduction tool named Waters Compression Tool. For better mass accuracy, lock mass correction was applied, and for further reduction of the data, file size spectra were converted from continuum to centroid mode using the Accurate Mass Measure tool in MassLynx. The scan range, which corresponds to the retention time window, for each lipid class was determined by comparing the first and last samples within a sequence. The MarkerLynx tool was used to apply this scan ranges for each lipid class to all samples within a sequence, resulting in the identification of all *m/z* with the corresponding intensity in all samples for the defined retention time window. The resulting tables containing *m/z* vs. intensities for each sample were exported as .txt files to the LipidQuant Excel script for automated data processing [16]. Then, the table of *m/z* vs. intensities was filtered by the comparison of individual *m/z* values with the embedded database for each lipid class with a predefined tolerance of mass window (5 mDa). The resulting filtered tables for each lipid class, where only natural lipids and IS are included corresponding to certain retention time window and with a maximum mass deviation of 5 mDa from the theoretical *m/z* were exported to another Excel file and used for the calculation of validation parameters. For the quantitation of lipid species, LipidQuant automatically calculated concentrations by the ratio of intensities of lipid species to intensities of lipid class IS with the known concentrations including the automatic isotopic correction for all lipid species.

Comparison of six extraction protocols

The generic protocol used 25 μL of pooled sample spiked with 17.5 μL IS Mix (before or after the extraction) and 3 mL of particular organic solvent placed in glass vials, which was homogenized for 15 min in the ultrasonic bath at 40 °C. When samples reached ambient temperature, 600 μL of H_2O was added in case of all protocols except for single-phase extraction protocol (MTBE/MeOH/$CHCl_3$), and then samples were vortexed for 1 min. The organic layer was removed with a glass pipette after 3 min of centrifugation (3000 rpm, 866 g). The organic layer was evaporated under a stream of nitrogen and stored at − 80 °C. Before the analysis, the residue was dissolved in 500 μL $CHCl_3$/MeOH (1/1, *v/v*) and vortexed for 1 min. Samples were filtered through 0.2 μm syringe filter, and transferred to injection vials. The following solvent mixtures were tested with some minor modifications in comparison to the initial protocols to keep the same generic setup for the mutual comparison of extraction protocols (Table S2 in ESM): (1) Folch [25], 2000 μL $CHCl_3$ and 1000 μL MeOH;

(2) MTBE [26], 2400 μL MTBE and 720 μL MeOH; (3) BuHe (unpublished method), 1200 μL BuOH and 1800 μL heptane; (4) MMC [27], single-phase extraction using 1500 μL MeOH, 750 μL MTBE, and 750 μL $CHCl_3$; (5) BuMe [28], addition of 1100 μL BuOH and 400 μL MeOH before homogenization, and afterwards 1100 μL heptane and 400 μL EtOAc, and after vortexing for 1 min 600 μL of 1% acetic acid were added; (6) 3Phase [29], 960 μL hexane, 960 μL EtOAc, 720 μL ACN, and 960 μL H_2O, where middle and upper layers were collected in this three-phase extraction protocol.

Sample preparation for validation

The sample of 25 μL of biological material (pooled plasma, pooled serum, plasma, or serum samples) was spiked with IS Mix either before or after the extraction. The modified Folch extraction protocol was used, where 2 mL $CHCl_3$ and 1 mL MeOH were added to 25 μL of biological material, and then the mixture was homogenized for 15 min at 40 °C in an ultrasonic bath. When samples reached ambient temperature, 600 μL of 250 mM ammonium carbonate buffer was added, and samples were extracted with the support of ultrasonication for 15 min. After the centrifugation at 3000 rpm (3 min), the organic layer was transferred to 8 mL vial using a glass pipette. Then, 2 mL $CHCl_3$ was added to the water layer, and the solution was again ultrasonicated. After the centrifugation, the organic layer was taken out, and both organic layers were combined and evaporated under the stream of nitrogen at 30 °C. The extract residue was stored at − 80 °C. The residue was dissolved in 500 μL $CHCl_3$/MeOH (1:1, *v/v*) and vortexed for 1 min after samples reached ambient temperature. For UHPSFC and UHPLC analysis, samples were further diluted 1:5 (*v/v*) with $CHCl_3$/MeOH (1:1, *v/v*) before the analysis.

Method validation

The bioanalytical method validation guidelines prepared by the Food and Drug Administration (FDA) [30], the European Medicines Agency (EMA) [31], and International council for harmonization of technical requirements for pharmaceuticals for human use [32] were followed as closely as possible together with recommendations in review articles focused on the method validation [33, 34]. The ESM contains the complete data obtained during the method optimization of sample preparation (ESM Table S3) and the validation of UHPSFC/MS and HILIC-UHPLC/MS methods (ESM Table S4). The validation was performed for the individual IS from the IS Mix as representatives for the corresponding lipid class species. It is assumed that the lipid class IS performs equally as naturally occurring lipid species from the same lipid class. All sample groups used for the validation were processed by two operators in triplicates to have a final sample group size of 6 samples for samples spiked before or after the extraction at L/M/H concentration levels.

Calibration curves

Three types of calibration curves were prepared always in duplicate both for serum and for plasma: (1) the calibration using the neat IS Mix without matrix, (2) the extracted pooled sample spiked after the extraction with different concentrations of IS Mix (ESM Table S1a), and (3) the pooled sample spiked with IS Mix at different concentration levels before the extraction (ESM Table S1a). Calibration curves were constructed in order to evaluate the linear range and the accuracy. Concentrations of individual calibration points were back-calculated by the regression equation in order to determine the error in concentration, which should be < 15%. The linearity was tested by applying the Mandel's fitting test. Therefore, theoretical intensities for samples with known concentrations were calculated by linear and quadratic regression equations obtained from calibration curves. The squares for intensity differences between experimentally determined and predicted intensities were calculated for each concentration point for linear and quadratic fits. The sum of linear fit differences was divided by N-2 and for the quadratic fit by N-3 (residual variances), whereby N is the total number of measurements. The residual variance for the linear fit multiplied with N-2 was subtracted by the product of N-3 and the residual variance of the quadratic fit and finally divided by the residual variance of the quadratic fit. The obtained F_{exp} value was compared to the tabular F value, and the linearity was accepted, when $F_{exp} < F$ [35, 36].

Lower limit of quantitation

The lower limit of quantitation (LLOQ) is the lowest concentration of an analyte in the sample, which can be reliably quantified with required precision expressed with the relative standard deviation (RSD) < 20% and accuracy error ± 20%. Calibration curves spiked with IS Mix at different concentration levels before the extraction were measured at the beginning and the end of the sequence resulting in 2 × 2 calibration curves (duplicates × measurements) for plasma and 2 × 2 calibration curves for serum. Concentrations of individual calibration points were back-calculated using individual regression equations. The error of individual calibration points was calculated by the subtraction of the back-calculated concentration minus the theoretical concentration and divided by the theoretical concentration. The RSD of back-calculated concentrations of calibration points measured at the beginning and the end of the sequence (2 × 2) was used for precision. The LLOQ was determined as the lowest calibration point, which fulfills the requirement of < 20% precision and ± 20% accuracy for all calibration curves.

Selectivity

The IS mixture has to be tested for its suitability to exclude any matrix or system-related interferences, which could lead to quantitation errors. The selectivity was calculated by the ratio of the response of blank matrix samples to the response of samples spiked with low level IS mixture after the extraction. The selectivity was determined for 3 replicates of pooled sample, 3 randomly selected samples for females, and 3 samples for males. The acceptance criterion for the analytical method to differentiate the IS from matrix compounds or system interferences is fulfilled, if responses of lipids in IS Mix are < 20% of the response of LLOQ.

Carry-over

Carry-over describes the appearance of analytes in an analytical run, even though the sample does not contain these analytes due to the contamination from previous runs. The carry-over effect was determined by evaluating solvent blanks injected after injections of the highest calibration points spiked before the extraction. The response in solvent blanks for the *m/z* corresponding to lipids in IS Mix should be < 20% of the response of LLOQ.

Dilution integrity

The dilution should not affect the precision and accuracy. Samples spiked before the extraction with twice the concentration of the high level were 5 times diluted in order to fit within the calibration range. Concentrations were calculated by regression equations of calibration curves spiked before the extraction. The precision was calculated by the RSD of calculated concentrations for the diluted samples. The error was determined by the subtraction of calculated values from theoretical concentrations and divided by the theoretical concentration. The precision should be < 20% at the LLOQ and < 15% above the LLOQ and the error ± 15%.

Accuracy and precision

The accuracy describes the closeness of mean experimental results to the true value. However, the determination of true values in biological samples is rather challenging. The determination of the accuracy using the neat standards or blank matrix samples spiked with IS Mix after the extraction does not take into account the sample preparation effects, such as the recovery rate. Consequently, calibration curves for samples spiked before the extraction were used for the determination of accuracy and precision. Samples were spiked with IS Mix at low, medium, and high (L/M/H) concentration levels before extraction. The regression equations of two extracted calibration curves measured at the beginning and the end of the sequence were used for the calculation of concentrations of individual samples. For the determination of the accuracy, calculated concentrations of samples were subtracted by the theoretical concentration and divided by the theoretical concentrations for each level. The mean of the accuracies was calculated for a single day, i.e., by using calibration curves measured at the beginning of the sequence for the determination of concentrations (within run accuracy) or for multiple days by using the calibration curves measured at the beginning and the end of the sequence for determination of concentrations (between run accuracy). This error should be ± 20% for samples with low level spike and ± 15% for samples spiked with medium and high level.

The repeatability was determined by % RSD of 6 consecutive measurements of the same sample for L/M/H levels of spike, which describes the variance of the injector and the analysis.

The within-run precision was % RSD of samples spiked before the extraction from only one operator, which describes the variance of sample preparation. The between-run precision was calculated from % RSD obtained for two extracted calibration curves measured at the beginning of the sequence and at the end after 3 days of measuring describing the time dependent instrumental variance. The inter-mediate precision was calculated as % RSD of samples spiked before the extraction at different concentration levels, which describes the variance of different operators.

The reproducibility was determined as % RSD for the calculated concentrations of 6 replicates for L/M/H levels of spiked samples before the extraction obtained by two different analytical methods describing the analytical method variance.

Extraction recovery

The extraction recovery was determined by the comparison of the signal intensity of samples spiked before and after the extraction at L/M/H concentration levels. Samples from both operators were used for the calculation of the extraction recovery (6 times for each L/M/H level).

Process efficiency

The process efficiency was calculated by the ratio of signal intensities for samples spiked before the sample preparation in comparison to neat standards. The influence of the whole sample preparation protocol and the matrix effect is reflected in the process efficiency. The process efficiency was determined for L/M/H concentration levels using 6 replicates.

Matrix effect

The matrix effect was determined by the ratio of responses of samples spiked after the extraction to responses of diluted neat

standards. The determination of matrix effects was performed for randomly selected samples (3 females and 3 males) and also for the pooled sample. The matrix may affect the ionization process using MS leading to the ion suppression or enhancement. The RSD of the matrix effect describes the variance in ionization events and should be ± 15%, termed here as the matrix factor. If the matrix effect is repeatable, then the ion enhancement improving the sensitivity is advantageous and not considered as the exclusion criterion. The matrix effect of individual samples and the pooled sample was determined for L/M/H concentration levels.

Extraction yield

The extraction yield was calculated as the ratio of responses of extracted standards at various concentration levels to the response of diluted standards without the extraction.

Results and discussion

Chromatographic separation

The main goal of this study is the full validation of two lipid class separation approaches and the comparison of the performance of UHPSFC/MS with the established HILIC-UHPLC/MS for the high-throughput lipidomic quantitation of human plasma and serum. All conditions for both methods are the same (identical samples, sequence table, chromatographic column, etc.) with the exception of mass spectrometers, where two QTOF instruments from the same vendor and the instrumental setting as close as possible have been used to guarantee the mutual comparability of results [37]. Differences in sensitivity and signal response, caused by the detector rather than the ion source, as the same model and settings were used, are compensated by the internal standard for the quantitation and by the fact that usually ratios are used for the calculation of validation parameters.

In HILIC-UHPLC, non-polar lipid classes elute in the void volume of the system due to the absence of polar functional groups (Fig. 1b, d), which hampers the possibility of quantitation of DG and MG due to common fragment ions with coeluting TG. Both non-polar and polar lipid classes are well retained in UHPSFC (Fig. 1a, c), therefore all non-polar acylglycerol classes (TG, DG, and MG) can be quantified due to their baseline separation. Chromatographic conditions of UHPSFC and UHPLC are optimized here in comparison to our previously published methods [15, 16] towards the higher throughput, because the total analysis time including washing and re-equilibration is reduced by about 40% for UHPSFC (8 min) and 30% for UHPLC (10.5 min) resulting in the throughput in real practice of over 150 samples/day for UHPSFC and 115 samples/day for UHPLC. Further improvements of MS conditions are implemented towards the automation of measurements and data processing, such as increased sensitivity (sensitivity mode instead of resolution mode setting in the vendor software), the significant reduction of file size (the continuum mode of data recording at higher scan rates with the subsequent noise reduction and the conversion from continuum to centroid data format), and improved mass accuracy (after run lock mass correction).

The correlation plot of decadic logarithms of retention factors obtained with UHPSFC and HILIC-UHPLC for SM, PC, and LPC illustrates that both methods provide the similar type of lipid class separation (Fig. 2a), but the close examination of retention factors inside individual classes shows only the partial correlation for SM (Fig. 2b), but no visible correlation for PC (Fig. 2c) and LPC (Fig. 2d). The adsorption as well as the partition mechanisms may contribute to the separation of lipids in UHPSFC depending on the mobile phase composition, where the increasing amount of polar modifier (up to 51%) switches conditions from supercritical to subcritical state [22], but this fact does not influence the reproducibility and robustness.

The comparison of chromatograms of human plasma extracts (Fig. 1a, b) and lipid standards in SSS (Fig. 1c, d) clearly illustrates the potential of current UHPSFC/MS and HILIC-UHPLC/MS approaches for the quantitation of additional polar lipid classes, but selective extraction protocols have to be developed due to the low concentration of these lipids in plasma and serum samples, which disables their quantitation using one generic method in parallel with the overwhelming majority of PC, TG, and CE classes. This problem cannot be solved by a simple increase of injected sample amount due to detector saturation and faster contamination of the mass spectrometer caused by these prevailing lipid classes, which is not coherent with high-throughput analysis of large cohorts. The use of reversed-phase (RP) UHPLC coupled to MS can bring about 2–3 times more separated species due to the isomeric separation in comparison to the lipid class separation approaches (HILIC or UHPSFC), and the better separation in RP also reduces the risk of MS detector saturation. On the other hand, the better separation in RP requires multiple IS per lipid class, because IS does not coelute with the lipid species from the same class, but the applicability of RP-UHPLC/MS for the lipidomic quantitation was proven in previous works as well [37, 38].

Comparison of extraction protocols

Six extraction protocols well known in the lipidomic and metabolomic field were compared (Table S3a and S3b in ESM) to find the best method for the MS quantitation coupled to the lipid class separation. The pooled sample of plasma and serum was always spiked before and after the extraction with IS mixture. For unbiased comparison, the overall organic solvent content was kept constant for all extraction protocols

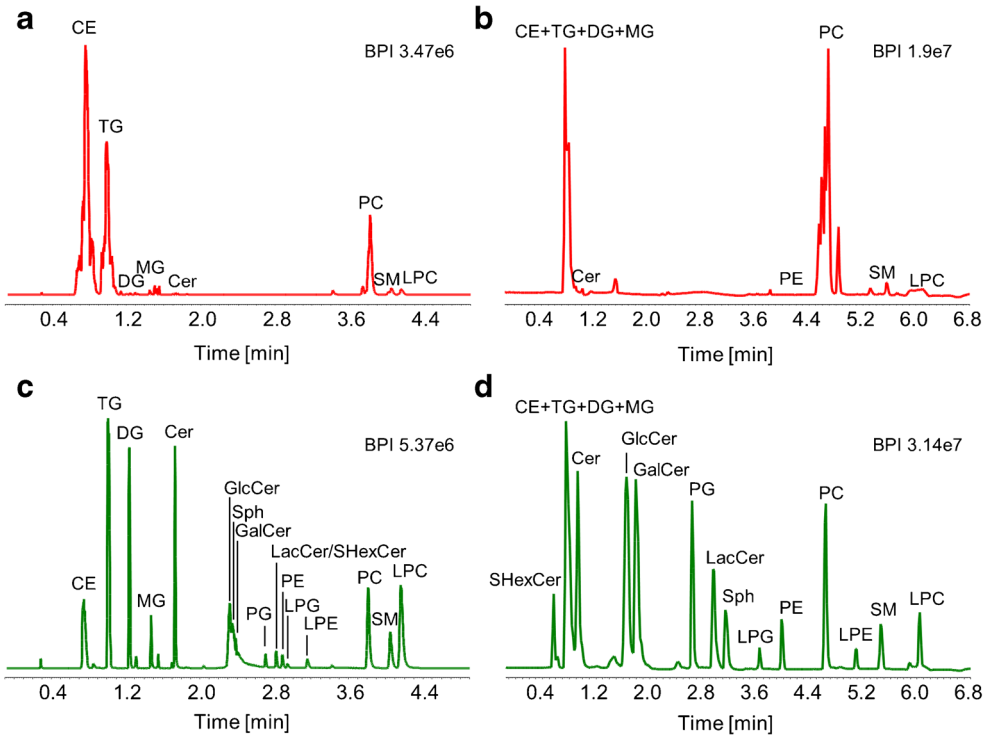

Fig. 1 Chromatograms of pooled human plasma spiked with the medium level concentration of IS **a** UHPSFC/MS and **b** HILIC-UHPLC/MS; and of the system suitability standard **c** UHPSFC/MS and **d** HILIC-UHPLC/MS

(details in "Material and methods"). The highest recovery rate for non-polar lipid classes was observed with the Folch extraction and for polar lipid classes with MMC (the example for SM in Fig. 3, the full data in Table S1 and Table S3a in ESM) [27, 39]. The RSD of signal intensities of samples spiked before the extraction was in general < 15% for all extraction protocols, but Folch and BuHe showed the best precision. The Folch protocol provided the best average recovery rate of 92% (Table S3a in ESM) with all values within the interval 87–106% except for the problematic behavior of PE, where BuHe protocol showed the best performance among all protocols. In case of HILIC, the best results are obtained with MMC with the average recovery rate of 95%. Matrix effects follow a comparable pattern for all extraction protocols (Table S3b in ESM) except for monophasic extraction protocol (MMC) with stronger enhancement effects, mainly for polar lipid classes.

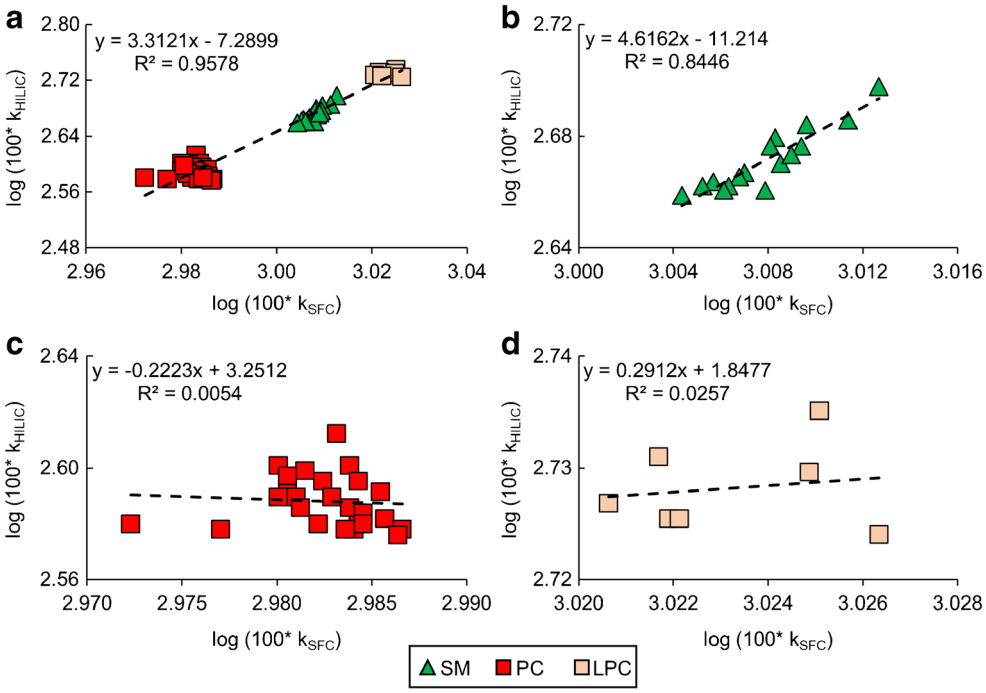

Fig. 2 Correlation of retention factors (decimal logarithm) obtained by UHPSFC/MS and HILIC-UHPLC/MS for lipid classes **a** PC (red squares), SM (green triangles), and LPC (orange squares); and for lipid species inside these classes **b** SM, **c** PC, and **d** LPC

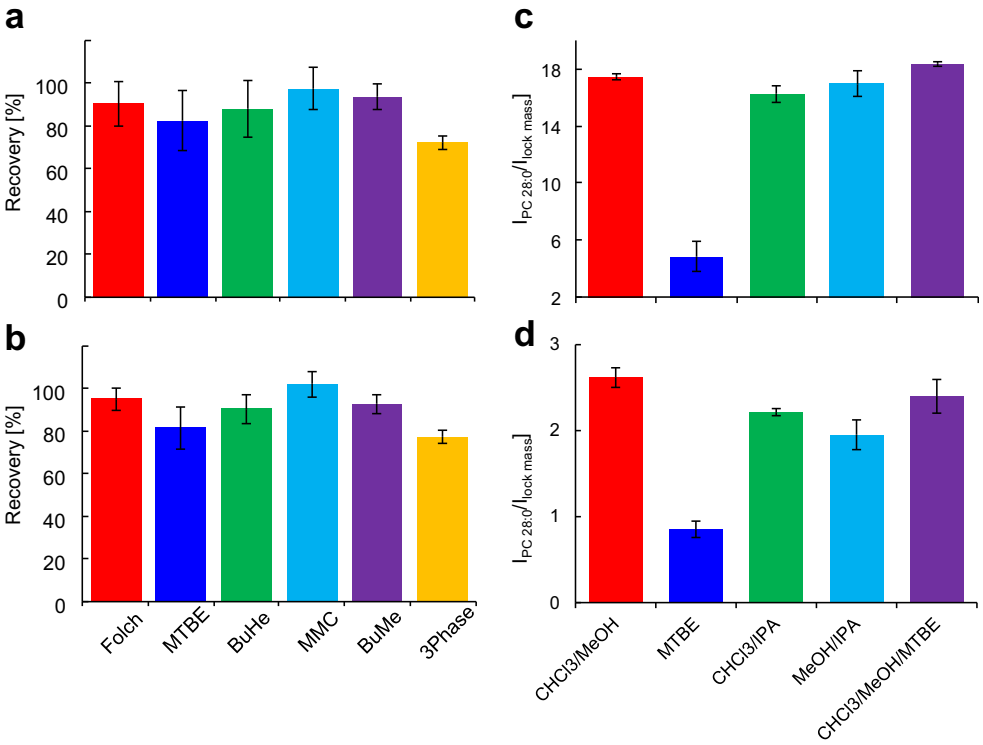

Fig. 3 Comparison of recovery rates obtained for SM 36:2 d9 standard using various extraction protocols (details in "Materials and methods") using **a** UHPSFC/MS and **b** HILIC-UHPLC/MS. Influence of the type of dissolution solvent for the extract on the signal response of PC 28:0 standard normalized to the lock mass using **c** UHPSFC/MS and **d** HILIC-UHPLC/MS. Error bars illustrate standard deviations

The key issue of enhancement effects is that signals are stable over the time, as discussed later in the method validation chapter. The Folch extraction was selected as the extraction protocol of choice for the validation with the addition of double extraction to increase the recovery of polar lipid classes. The influence of the addition of $CHCl_3$/MeOH (2:1, *v/v*) or only $CHCl_3$ in the second extraction step on the recovery rate was investigated (Fig. S1 in ESM) with the conclusion that the addition of $CHCl_3$ gave the highest recovery rates.

Optimization of extraction additives

The type of additive and pH value can influence the extraction equilibrium of ionic and ionizable lipids towards one phase in the liquid-liquid extraction system, which may enhance the extraction recovery. The following types of additives were tested in comparison to pure water without any additive: acetic acid, ammonium formate, ammonium acetate, ammonium carbonate, sodium hydrogen phosphate, and sodium chloride (Table S3c in ESM). Two operators performed all extractions in triplicate using the pooled sample of plasma and serum, i.e., 2×3 extracts for all additives. The signal increase was observed for all additives with only minor differences among them. The nature of the additive has almost no influence on the signal response, the response is higher using an additive in comparison to pure water for the extraction, and the precision of the sample preparation increases. The highest signals were measured for ammonium formate and ammonium carbonate considering both methods. Ammonium carbonate led to slightly higher responses for PE 33:1 d7 and Cer d30:1 using HILIC-UHPLC/MS and was further used for the validation in order to improve sensitivity for those lipids (the example for LPC 18:1 d7 in Fig. S2 and the full data in Table S3c in ESM).

Optimization of dissolution solvent used for the extract

Various polarities of polar head groups in lipids lead to differences in their solubility. Therefore, we tested the influence of various organic solvent mixtures used for the dissolution of extract residue after the nitrogen evaporation on the signal using UHPSFC/MS (Fig. 3c) and HILIC-UHPLC/MS (Fig. 3d). Each experiment was performed in triplicate. The following solvent mixtures (always the same volumetric ratios) were selected in line with common workflows in the lipidomic analysis: $CHCl_3$/MeOH, MTBE, $CHCl_3$/IPA, MeOH/IPA, and $CHCl_3$/MeOH/MTBE. Results were evaluated by relating the intensity of the IS to the total intensity of the lock mass regularly measured during the chromatographic run. The example of PC 28:0 standard is shown in Fig. 3 and the full data in Table S3d in ESM. In general, for all tested $CHCl_3$ containing mixtures and also MeOH/IPA, no significant difference in the response depending on the dissolution solvent was observed. The highest response was observed for $CHCl_3$/MeOH/MTBE followed by $CHCl_3$/MeOH and the lowest for pure MTBE considering all lipid class IS and both methods; therefore, MTBE is not recommended for the dissolution of the extract.

Optimization of dilution solvent

The dilution solvent in chromatography-MS analysis can significantly influence the peak shape and has to be optimized to the avoid peak distortion. In UHPSFC, aprotic solvents are recommended; however, it strongly depends on the properties of the analyte and starting chromatographic conditions. Several solvent mixtures were tested using UHPSFC/MS (Table S3e in ESM). The best response was obtained for hexane, IPA, and $CHCl_3$ (7:1.5:1.5, *v/v/v*) followed by $CHCl_3$/MeOH (1:1, *v/v*), ACN/MTBE (1:1, *v/v*), ACN/MeOH (1:1, *v/v*), and $CHCl_3$/MeOH/MTBE (1:1:1, *v/v/v*). In general, differences in the signal response depending on the diluting solvent were small. For better comparability of both methods and simplification with regard to the dissolving solvent (see above), $CHCl_3$/MeOH (1:1) was selected for further experiments.

Testing of spikes at various concentration levels

The validation experiments should be performed at different concentration levels. The natural abundance of lipid species in biological samples may differ significantly with the lipid class. The quantitation error of low abundant lipid species is more pronounced in comparison to high abundant lipid species. The special focus was put on low and medium abundant lipid species, as a consequence suitable spike levels close to the LLOQ (low level), slightly above (medium level), and above the LLOQ (high level) for the validation had to be determined. The suitable spike level for the validation was evaluated by spiking blank matrix with various volumes of IS Mix (7.5; 10; 12.5; 15; 20; 25; and 35 μL) and calculating the LLOQ from this testing calibration curve. For the validation, 15 μL for low level, 20 μL for medium level, and 35 μL for high level spike of IS Mix were used.

Full method validation for biological samples

The full method validation was performed in line with FDA and EMA guidelines for bioanalytical validations [30, 31] and applied for human plasma as well as human serum samples using UHPSFC/MS and HILIC-UHPLC/MS (Table S4 in ESM). The comparison of the IS Mix used in our group and the commercially available SPLASH Lipidomix is presented for these samples as well as for NIST SRM 1950 human plasma. The retention time stability, repeatability, intermediate precision, within-run and between-run precision, and LLOQ values are slightly better for HILIC-UHPLC/MS then for UHPSFC/MS. The higher retention shift in UHPSFC/MS in comparison to UHPLC/MS may be caused by the rather harsh conditions using $scCO_2$ and methanol as the mobile phase in UHPSFC/MS due to the intrinsic acid rather than the column chemistry as the same type of columns were used for both methods. The comparison of retention shifts obtained for plasma and serum using UHPSFC/MS shows that the retention stabilizes over time, as the retention shift is less pronounced in serum samples measured after plasma samples. Differences in precision are most probably caused by instrumental performance differences rather than the sample preparation, as the same samples were analyzed with both methods. Results suggest that the injection process in UHPSFC/MS is not as repeatable as for UHPLC/MS, which needs further improvement in the optimization of UHPSFC instruments by vendors similar as it was performed for UHPLC in the last decade. The selectivity, carry-over, the dilution integrity, and within-run and between-run accuracy are better for UHPSFC/MS. The lower accuracy of HILIC-UHPLC/MS may be caused by an increased contamination of the mass spectrometer during the sequence, because no splitting of the mobile phase is used in UHPLC unlike to UHPSFC; therefore, a higher absolute amount of lipidomic extracts is introduced to the mass spectrometer. This results in the faster signal drop reflected by the regularly measured SSS (Fig. S3 in ESM), which may cause some troubles for larger batches (over ca. 500 samples in total). The extraction recovery was between 85 and 115% for all lipid class standards except for PE, where the recovery is only about 30%. PE and Chol d7 was not determined with UHPSFC/MS due to too low sensitivity, and CE due to the extensive in-source fragmentation. The process efficiency reflects the influence of the whole sample preparation protocol, matrix effects, and measurement performance. As the recovery rate is around 100%, the process efficiency is mainly influenced by the matrix effect. HILIC-UHPLC/MS data shows that the process efficiency is around 50–60% for non-polar lipid species due to the ion suppression, because non-polar lipids coelute in or close to the void volume. For polar lipid classes, the process efficiency and matrix effects are within 85–115% independent of the concentration level. For UHPSFC/MS, no suppression effects are observed for non-polar lipid species, as the process efficiency and matrix effects are within 85–115%. On the other hand, significant ion enhancement is observed for polar lipid classes LPC, PC, and SM. The matrix effect (Fig. 4) was evaluated for the pooled sample as well as for individual randomly selected female and male samples obtained from healthy volunteers. The RSD of the pooled sample and individual samples here defined as matrix factor should be < 15%. In fact, the ion enhancement as matrix effect can be beneficial with regard to the sensitivity on condition that results are repeatable, which was confirmed by RSD < 15%. The extraction yield describes the effect of sample preparation for neat standards, and was 80–120% for all lipid classes using both methods. The RSD of back calculated concentrations of samples spiked before the extraction using calibration curves obtained with both methods gives the reproducibility and describes the differences in the quantitation by both methods. Even though the reproducibility was affected by the pronounced signal drop during the sequence

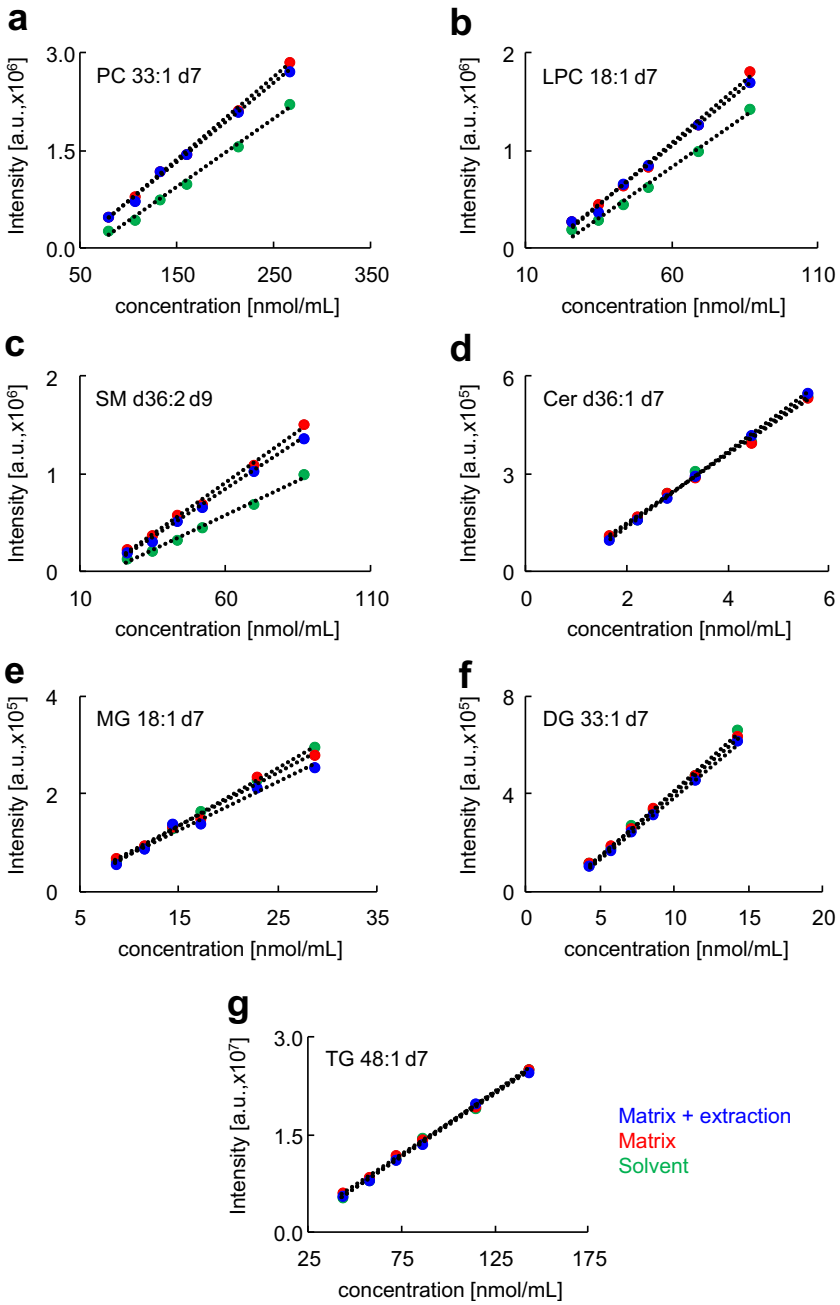

Fig. 4 Calibration curves of internal standard **a** PC 33:1 d7, **b** LPC 18:1 d7, **c** SM d36:2 d9, **d** Cer d36:1 d7, **e** MG 18:1 d7, **f** DG 33:1 d7, **g** TG 48:1 d7 measured by UHPSFC/MS for calibration solutions prepared in the neat solvent without matrix (green), spiked after (red), and before the extraction of human plasma (blue)

for HILIC-UHPLC/MS, results are mainly below 20% for plasma and 30% for serum. The normalization of samples with the average or median of quality control samples may improve the method comparison, as illustrated by Triebl et al. [40]. However, for unadorned performance comparison of both methods, no normalization was applied. The validation was partially repeated for the NIST plasma using our IS Mix and the NIST plasma, pooled plasma, and serum samples for the commercially available Splash Lipidomix at the medium concentration level. The selectivity, within-run accuracy, within-run precision, extraction recovery, matrix effect, and matrix factor were evaluated. Concentrations of the deuterated IS in the IS Mix and the SPLASH-Lipidomix are comparable (Table 1) with the exception of DG, which is about two times higher in the SPLASH-Lipidomix in comparison to the IS Mix. The precision is slightly better for HILIC-UHPLC/MS, the recovery rate is mostly between 90 and 100%, the ion suppression is observed for non-polar analytes using HILIC-UHPLC/MS, and the ion enhancement for polar lipid standards using UHPSFC/MS as matrix effect influencing the process efficiency. The comparison of all validation parameters shows the repeatability of the validation independent of the used matrix and IS mixture for both methods. Generally, results for plasma and serum are within the acceptance criteria

for all concentration levels according to the guidelines for all lipid class IS and both methods. Therefore, both methods and the different IS mixtures were tested for their quantitation performance of natural occurring lipid species in the NIST plasma, pooled serum, and pooled plasma.

Application of validated methods to human blood analysis

Samples spiked with IS Mix at the medium concentration level before the extraction were used for the quantitation. The quantitation performance for pooled plasma, pooled serum, and the NIST plasma was compared for the commercially available SPLASH Lipidomix and our internal IS Mix (Table S5 and Fig. S4 in ESM). Multiple IS per lipid class are present in our internal IS Mix, which enables the evaluation of possible differences in the quantitation of lipid species with different IS (Table S6 in ESM). Dependences of decadic logarithms of concentrations are used for the visualization of various types of correlations (Fig. 5) determined by UHPSFC/MS (Fig. 5a–c), HILIC-UHPLC/MS (Fig. 5d–f), and the method and literature data comparison (Fig. 5g–i). The first correlation (Fig. 5a) shows an excellent match between the quantitation of NIST plasma by our IS Mix and SPLASH Lipidomix, which confirms the applicability of both IS mixtures except for DG. The DG class is not frequently quantified in other published papers, and their quantitation requires further improvements, and probably the development of dedicated method would be the best solution. The correlation of human plasma from two different resources (our internal pooled sample and NIST SRM 1950) shows high similarity (Fig. 5b). Figure 5 c illustrates the comparison of pooled plasma and pooled serum from the same group of donors, collected at the same time, and processed the identical way.

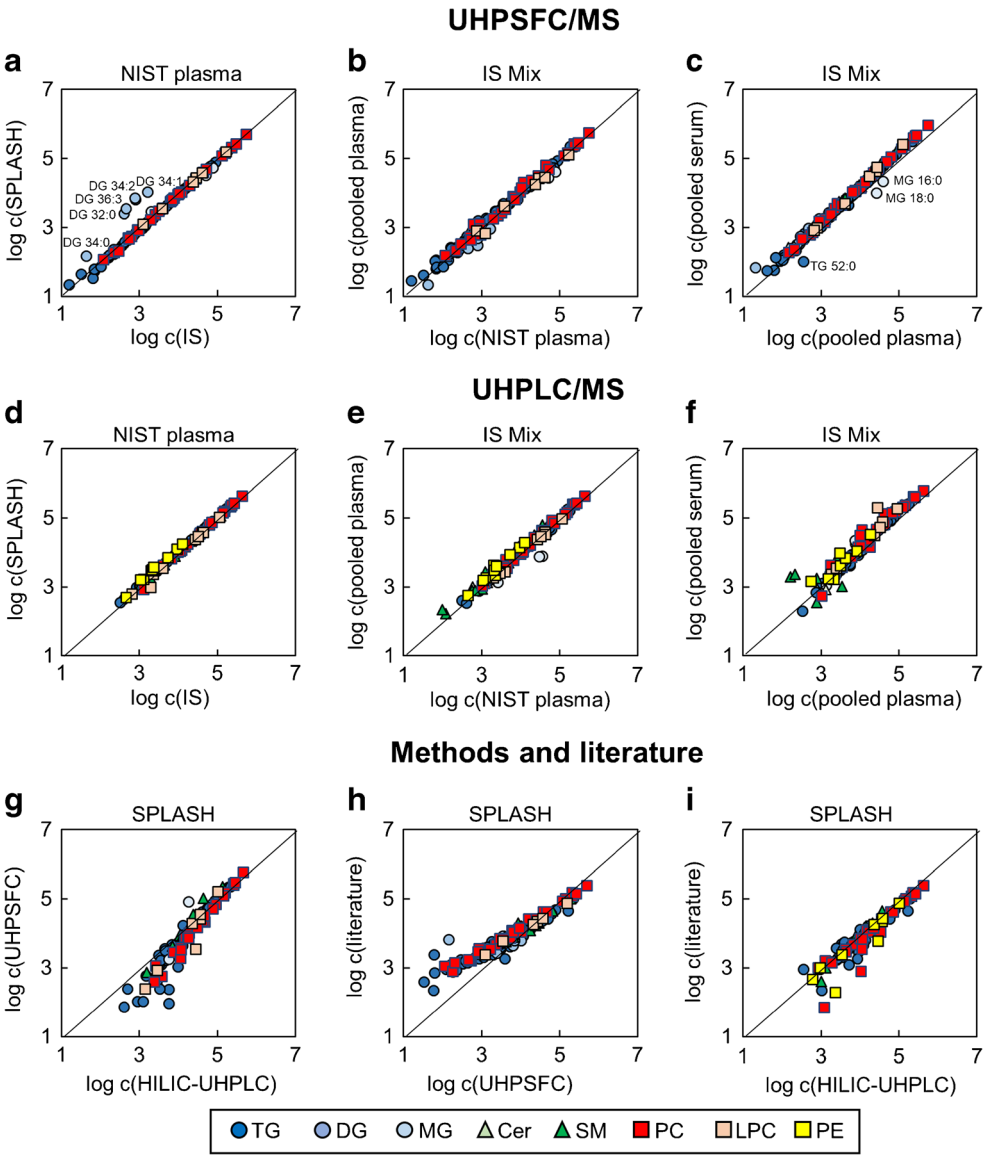

Fig. 5 Correlations of lipid concentrations (pmol/mL, decimal logarithm) using the IS Mix or the SPLASH Lipidomix for the quantitation of body fluids determined by UHPSFC/MS **a** IS Mix vs. SPLASH Lipidomix for NIST plasma, **b** pooled plasma vs. NIST plasma using the IS Mix, **c** pooled plasma vs. pooled serum using the IS Mix; determined by HILIC-UHPLC/MS **d** IS Mix vs. SPLASH Lipidomix for NIST plasma, **e** pooled plasma vs. NIST plasma using the IS Mix, **f** pooled plasma vs. pooled serum using the IS Mix; and the inter-method comparison **g** UHPSFC/MS vs. HILIC-UHPLC/MS using the SPLASH Lipidomix for the quantitation, and the correlation of quantitation results of UHPSFC/MS and HILIC-UHPLC/MS with the literature [9] **h** UHPSFC/MS vs. literature values, and **i** HILIC-UHPLC/MS vs. literature values

Concentrations in serum are about 20–30% higher (Fig. S4 in ESM) than in plasma, but no other visible differences are observed (except for few DG and MG probably caused technical problems with these classes). HILIC-UHPLC/MS data are shown in Fig. 5d–f for the same type of correlations as shown for UHPSFC/MS data in Fig. 5a–c. Slightly lower variations in these correlations are observed for UHPSFC/MS; therefore, we consider this method as superior in this comparison. The direct comparison of quantitative data on NIST plasma by both approaches demonstrates (Fig. 5g and ESM Table S5) that some variations exist mainly for non-polar glycerolipid classes eluting in the void volume in case of HILIC-UHPLC, which could cause worse accuracy of their determination. The correlation of published consensus values by Bowden et al. [9] and by Quehenberger et al. [41] with our UHPSFC/MS (Fig. 5h and ESM Figs. S4 and S5) and HILIC-UHPLC/MS (Fig. 5i) data shows RSD < 20% for about 1/3 of determined lipids, 20–40% for another 1/3, and > 40% for the last 1/3. The deeper analysis of these discrepancies is complicated, because the true information on all concentrations is unknown. These correlations indicate relatively good data quality for main glycerophospholipid and sphingolipid classes, but also some discrepancies in the quantitation of glycerolipid classes depending on the laboratory and used method, which highlights the importance of harmonization in the lipidomic quantitative workflows (Tables S5 and S6 in ESM) [23]. The use of multiple IS allowed the estimation of the quantitation error by using one IS for the quantitation of other IS from the same lipid class (Table S6 in ESM). The error was < 15% for TG, MG, SM, and LPC for UHPSFC/MS and for TG, SM, PC, and LPC for HILIC-UHPLC/MS (Table S6 in ESM).

Conclusions

The method optimization and analytical validation have been performed for two lipid class separation MS approaches (UHPSFC/MS and HILIC-UHPLC/MS). Validation results of UHPSFC/MS are comparable to those of more established HILIC-UHPLC/MS method. This work confirms the applicability of UHPSFC/MS for reliable, comprehensive, and high-throughput lipidomic quantitation of both non-polar and polar lipid classes. However, further improvements of the instrumental injection repeatability of UHPSFC/MS are still needed to reach the same level of injection repeatability as in UHPLC/MS. On the other hand, the use of a splitter in our UHPSFC/MS configuration resulted in slower contamination of the mass spectrometer in comparison to UHPLC/MS configuration. Quantitative results are correlated with HILIC-UHPLC/MS and also with the consensus paper on the lipidomic characterization of NIST plasma used as the standard reference material. The use of multiple IS per each lipid class improves the robustness of quantitation, because possible interferences for one IS can be solved by the use of alternative IS for the same class. The comparison of pooled plasma and serum obtained from the same subjects and measured under identical conditions shows no relevant differences for measured lipid classes, but serum concentrations are about 20–30% higher. However, an in-depth study for the determination of differences in the lipidome of serum and plasma using individual samples is needed. We highly encourage the use of the analytical validation of quantitative methods for the lipidomic analysis, which should improve the reproducibility and inter-laboratory comparability of published data. We believe that this study brings additional supporting arguments for the wider acceptance of UHPSFC/MS in high-throughput quantitative workflows in lipidomics. This study has proved that our IS Mix provides the same robustness and accuracy of lipidomic quantitation by lipid class separation–MS approaches as pre-mixed SPLASH Lipidomix.

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Compliance with ethical standards

The study was approved by the ethical committee at University Hospital Olomouc, Czech Republic, and all healthy volunteers signed informed consent.

Conflict of interest The authors declare that they have no conflict of interest.

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