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Lipidomic analysis of biological samples: Comparison of liquid chromatography, supercritical fluid chromatography and direct infusion mass spectrometry methods

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ABSTRACT

Lipidomic analysis of biological samples in a clinical research represents challenging task for analytical methods given by the large number of samples and their extreme complexity. In this work, we compare direct infusion (DI) and chromatography – mass spectrometry (MS) lipidomic approaches represented by three analytical methods in terms of comprehensiveness, sample throughput, and validation results for the lipidomic analysis of biological samples represented by tumor tissue, surrounding normal tissue, plasma, and erythrocytes of kidney cancer patients. Methods are compared in one laboratory using the identical analytical protocol to ensure comparable conditions. Ultrahigh-performance liquid chromatography/MS (UHPLC/MS) method in hydrophilic interaction liquid chromatography mode and DI-MS method are used for this comparison as the most widely used methods for the lipidomic analysis together with ultrahigh-performance supercritical fluid chromatography/MS (UHPSFC/MS) method showing promising results in metabolomics analyses. The nontargeted analysis of pooled samples is performed using all tested methods and 610 lipid species within 23 lipid classes are identified. DI method provides the most comprehensive results due to identification of some polar lipid classes, which are not identified by UHPLC and UHPSFC methods. On the other hand, UHPSFC method provides an excellent sensitivity for less polar lipid classes and the highest sample throughput within 10 min method time. The sample consumption of DI method is 125 times higher than for other methods, while only 40 μL of organic solvent is used for one sample analysis compared to 3.5 mL and 4.9 mL in case of UHPLC and UHPSFC methods, respectively. Methods are validated for the quantitative lipidomic analysis of plasma samples with one internal standard for each lipid class. Results show applicability of all tested methods for the lipidomic analysis of biological samples depending on the analysis requirements.

1. Introduction

Lipidomics [1] is a branch of metabolomics aimed at the analysis of lipid species and their biological functions in organism. Lipids play multiple roles in cellular functions, act as messengers in cell signaling, and they are potential biomarkers for some serious human diseases. According to their definition [2,3], lipids represent varied groups of compounds with large differences in their structures and physicochemical properties, which is demanding for analytical techniques used in the lipidomic analysis. Nowadays, mass spectrometry (MS) is a key technique in the lipidomic analysis of biological samples thanks to significant advances in last years. Mainly two approaches are used in the MS lipidomic analysis, such as direct infusion (DI)-MS lipidomics (often called shotgun lipidomics) and liquid chromatography/MS lipidomics, which are used almost equally in the literature [4,5].

DI-MS lipidomics represents analytical methods without any preseparation step. Lipid extracts are directly infused into the ion source of mass spectrometer, where all lipids are ionized together. Lipids are detected based on MS(/MS) spectra acquired by high resolving power/high mass accuracy analyzers [6–9] or more often using MS/MS scans (*i.e.*, precursor ion (PI), neutral loss (NL), and selected reaction monitoring (SRM) scans) based on the characteristic fragmentation behavior of individual lipid classes measured on

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triple quadrupole or quadrupole-linear ion trap mass spectrometers [10–13]. These methods are widely used for the analysis of large sample sets in a clinical research, because they are high-throughput and easy to automate with robotic systems providing accurate and reproducible quantitative data. On the other hand, determination of isomers, isobaric species or trace species is often difficult as they are analyzed in the mixture.

These problems are overcome by separation step in the case of chromatography/MS lipidomics, where different chromatographic modes can be selected for the separation of all kinds of isomerism. Furthermore, the separation of lipids avoids of ion suppression effect during the ionization process, which can be serious mainly for species with ionic head groups. Liquid chromatography (LC) is the most widely used chromatographic technique for the comprehensive lipidomic analysis. Lipids can be separated into individual species using reversed-phase (RP) separation [14–21] according to their fatty acyl composition, *i.e.*, fatty acyl length and number of double bonds (DB). Hydrophilic interaction liquid chromatography (HILIC) enables a class separation of lipids according to their polarity and charge [14,15,22–26], while lipids differing in the fatty acyl composition coelute together in one chromatographic peak corresponding to the lipid class. HILIC methods usually do not provide the separation of nonpolar lipids, which are not retained and elute in the column void volume. These lipids are separated using normal phase (NP) mode according to their polarity [22,25,27–29], but this mode cannot be used for more polar lipids due to their strong retention under such conditions. Serial coupling of HILIC and RP columns in mixed-mode liquid chromatography combines lipid class separation of lipids with better selectivity of lipid species within the lipid class [30]. HILIC and DI methods have been combined into one mass spectrometry-based lipidomics platform for the analysis of phospholipids and sphingolipids [31]. In last years, supercritical fluid chromatography (SFC) has become more popular chromatographic technique in metabolomic analyses including lipidomics. We have demonstrated that SFC using sub-two μm hybrid silica column has a great potential as the comprehensive and high-throughput screening method enabling the class separation of nonpolar and polar lipids in one 6 min analysis [32]. C18 columns are often used for SFC separations of individual lipid species according to the fatty acyl composition, such as the lipidomic analysis of intact [33] or methylated [34] species, the separation of TG [35], oxidized PC [36], carotenoids [37] or TG regioisomers [38].

The quantitative lipidomic analysis brings many challenges for analysts due to enormous complexity of lipidomic samples, because the utilization of corresponding standards for all species is impossible. Selected exogenous lipids with unusual composition of fatty acyls are used as internal standards (IS) for each lipid class (*e.g.*, short/long fatty acyls, combination of odd and even carbon or deuterated fatty acyls) and the concentration of individual species is calculated as the ratio of their signals related to the signal of IS [6,9,11–13,24,32]. This simplification using IS for all species within given lipid class brings reliable quantitative data for case/control studies.

The aim of this work is a comparison of chromatography/MS and DI-MS approaches for the lipidomic analysis of biological samples. For this purpose, HILIC ultrahigh-performance liquid chromatography (UHPLC)/MS and ultrahigh-performance supercritical fluid chromatography (UHPSFC)/MS methods are compared with DI-MS method in one laboratory using identical analytical protocol. Lipids in tumor tissue, surrounding normal tissue, plasma and erythrocytes pooled samples of kidney cancer patients are identified using each method to demonstrate their capabilities for the analysis of various lipid classes in complex biological matrices. Each method is validated for the quantitative lipidomic analysis of plasma samples using IS per each lipid class and the lipidomic composition of 6 plasma samples is determined.

2. Experimental

2.1. Materials

Acetonitrile, 2-propanol, methanol (all HPLC/MS grade), hexane, chloroform stabilized with 0.5–1% ethanol (both HPLC grade), ammonium acetate, and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was prepared with a Milli-Q ReferenceWater Purification System (Molsheim, France). Carbon dioxide 4.5 grade (99.995%) was purchased from Messer Group Gmbh (Bad Soden, Germany). Lipid class IS were used for the method validation and quantitative analysis, *i.e.*, DG 12:1/0:0/12:1, MG 19:1/0:0/0:0, and TG 19:1/19:1/19:1 purchased from Nu-ChekPrep (Elysian, MN, USA), D7-CE 16:0, Cer d18:1/12:0, HexCer d18:1/12:0 (Glucosyl(ß)Cer d18:1/12:0), D7-cholesterol, Hex2Cer d18:1/12:0 (Lactosyl(ß)Cer d18:1/12:0), LPC 17:0/0:0, LPE 14:0/0:0, LPG 14:0/0:0, LPS 17:1/0:0, PA 14:0/14:0, PC 14:0/14:0, PE 14:0/14:0, PG 14:0/14:0, PS 14:0/14:0, SM d18:1/12:0, and SulfoHexCer d18:1/12:0 (MonoSulfoGalactosyl(ß)Cer d18:1/12:0) purchased from Avanti Polar Lipids (Alabaster, AL, USA). Stock solutions of individual IS at the concentration of 2 mg/mL were prepared in a chloroform – 2-propanol (1:4, v/v) mixture. A stock solution of IS mixture for calibration samples was prepared by mixing of IS stock solutions at 1:1 ratio. Stock solutions of IS mixtures for the method validation and the quantitative analysis of plasma samples were prepared by mixing of IS stock solutions at ratios listed in Table S1.

2.2. Biological samples

Samples of tumor tissue, surrounding normal tissue, plasma, and erythrocytes were obtained from kidney cancer patients in cooperation with the Faculty Hospital Olomouc (Czech Republic) based on the approval of ethical committee at the Faculty Hospital Olomouc. The total lipid extracts were prepared from 25 μL of plasma/erythrocytes or 25 mg of tissue according to the modified Folch procedure [23], then evaporated by a gentle stream of nitrogen and redissolved in 0.5 mL of chloroform – 2-propanol (1:1, v/v) mixture. Plasma samples for the quantitative analysis were spiked before the extraction with appropriate IS mixture at middle concentration level (Table S1). Total lipid extract stock solutions were diluted 20 times by chloroform – 2-propanol (1:4, v/v) mixture for UHPLC/MS, 20 times by hexane – 2-propanol – chloroform (7:1.5:1.5, v/v/v) mixture for UHPSFC/MS and 12 times by chloroform – methanol – 2-propanol (1:2:4, v/v/v) mixture containing 7.5 mM of ammonium acetate and 1% of acetic acid for DI-MS analyses. The nontargeted identification and the method validation were carried out using pooled samples prepared from 20 random patient samples with males and females in equal proportions.

2.3. UHPLC/MS and UHPSFC/MS conditions

UHPLC experiments were performed on a liquid chromatograph Agilent 1290 Series (Agilent Technologies, Waldbronn, Germany) using the following conditions: Acquity UPLC BEH HILIC column (150 mm × 2.1 mm, 1.7 μm, Waters), the flow rate 0.4 mL/min, the injection volume 1 μL, the autosampler temperature 8 °C, the column temperature 40 °C, and the gradient: 0 min – 100% A, 10 min – 84% A + 16% B, where A was acetonitrile – water (96:4, v/v) mixture containing 7 mM of ammonium acetate, and B was water containing 7 mM of ammonium acetate.

UHPSFC experiments were performed on an Acquity UPC^2 instrument (Waters, Milford, MA, USA) using our previously devel-

Table 1
Observed ions and their average relative abundances in positive-ion ESI full-scan mass spectra of IS.

IS	Observed ion	Elemental formula	Theoretical *m/z*	Relative abundance [%]		
				UHPLC	UHPSFC	DI
D7-CE 16:0	$[M+K]^+$	$C_{43}H_{69}D_7KO_2$	670.5916		0.4	0.3
	$[M+Na]^+$	$C_{43}H_{69}D_7NaO_2$	654.6177		9.7	8.5
	$[M+NH_4]^+$	$C_{43}H_{73}D_7NO_2$	649.6623		0.4	100
	$[M+H]^+$	$C_{43}H_{70}D_7O_2$	632.6357		0.1	0
	$[M+H\text{-}FA]^+$	$C_{27}H_{38}D_7$	376.3955		100	3.3
Cer d18:1/12:0	$[M+K]^+$	$C_{30}H_{59}KNO_3$	520.4127		0.2	0.5
	$[M+Na]^+$	$C_{30}H_{59}NaNO_3$	504.4387		8.5	9.7
	$[M+H]^+$	$C_{30}H_{60}NO_3$	482.4568		2.7	100
	$[M+H\text{-}H_2O]^+$	$C_{30}H_{58}NO_2$	464.4462		100	17.1
	$[M+H\text{-}2H_2O]^+$	$C_{30}H_{56}NO$	446.4356		4.7	1.1
	$[M+H\text{-}FA\text{-}H_2O]^+$	$C_{18}H_{36}NO$	282.2791		0.7	0
	$[M+H\text{-}FA\text{-}2H_2O]^+$	$C_{18}H_{34}N$	264.2686		6.7	0.6
DG 12:1/0:0/12:1	$[M+K]^+$	$C_{27}H_{48}KO_5$	491.3133		0.1	1.0
	$[M+Na]^+$	$C_{27}H_{48}NaO_5$	475.3394		11.9	15.5
	$[M+NH_4]^+$	$C_{27}H_{52}NO_5$	470.3840		0	100
	$[M+H]^+$	$C_{27}H_{49}O_5$	453.3575		0.8	7.6
	$[M+H\text{-}H_2O]^+$	$C_{27}H_{47}O_4$	435.3469		100	21.9
	$[M+H\text{-}FA]^+$	$C_{15}H_{27}O_3$	255.1955		7.8	1.3
Hex2Cer d18:1/12:0	$[M+K]^+$	$C_{42}H_{79}KNO_{13}$	844.5183	2.6	0.1	0.7
	$[M+Na]^+$	$C_{42}H_{79}NNaO_{13}$	828.5444	27.1	43.4	22.3
	$[M+H]^+$	$C_{42}H_{80}NO_{13}$	806.5624	100	100	100
	$[M+H\text{-}H_2O]^+$	$C_{42}H_{78}NO_{12}$	788.5519	20.9	19.1	6.7
	$[M+H\text{-}Hex2]^+$	$C_{30}H_{58}NO_2$	464.4462	11.5	22.6	2.7
	$[M+H\text{-}Hex2\text{-}H_2O]^+$	$C_{30}H_{56}NO$	446.4356	0.9	1.9	0.8
	$[M+H\text{-}Hex2\text{-}FA\text{-}H_2O]^+$	$C_{18}H_{36}NO$	282.2791	0.3	0.4	0
	$[M+H\text{-}Hex2\text{-}FA\text{-}2H_2O]^+$	$C_{18}H_{34}N$	264.2686	2.7	5.2	0.4
HexCer d18:1/12:0	$[M+K]^+$	$C_{36}H_{69}KNO_8$	682.4655	3.6	0.3	0.8
	$[M+Na]^+$	$C_{36}H_{69}NNaO_8$	666.4915	34.7	27.8	5.8
	$[M+H]^+$	$C_{36}H_{70}NO_8$	644.5096	78.5	58.8	100
	$[M+H\text{-}H_2O]^+$	$C_{42}H_{78}NO_{12}$	626.4990	100	100	7.1
	$[M+H\text{-}Hex]^+$	$C_{30}H_{58}NO_2$	464.4462	26.8	28.1	2.4
	$[M+H\text{-}Hex2\text{-}FA\text{-}H_2O]^+$	$C_{18}H_{36}NO$	282.2791	0.6	0.4	0
	$[M+H\text{-}Hex2\text{-}FA\text{-}2H_2O]^+$	$C_{18}H_{34}N$	264.2686	4.8	4.4	0.3
D7-Cholesterol	$[M+H\text{-}H_2O]^+$	$C_{27}H_{38}D_7$	376.3955		100	100
LPC 17:0/0:0	$[M+K]^+$	$C_{25}H_{52}NO_7PK$	548.3113	1.1	0.1	3.4
	$[M+Na]^+$	$C_{25}H_{52}NO_7PNa$	532.3374	14.4	1.7	10.8
	$[M+H]^+$	$C_{25}H_{53}NO_7P$	510.3554	100	100	100
	$[M+H\text{-}H_2O]^+$	$C_{25}H_{51}NO_6P$	492.3449	0.8	0.5	0.3
	$[phosphocholine]^+$	$C_5H_{15}NO_4P$	184.0733	0.2	0.3	0.1
LPE 14:0/0:0	$[M+K]^+$	$C_{19}H_{40}KNO_7P$	464.2174	3.2	1.5	1.9
	$[M+Na]^+$	$C_{19}H_{40}NNaO_7P$	448.2435	50.0	14.4	5.7
	$[M+H]^+$	$C_{19}H_{41}NO_7P$	426.2615	100	100	100
	$[M+H\text{-}H_2O]^+$	$C_{19}H_{39}NO_6P$	408.2510	21.8	21.3	0.7
	$[M+H\text{-}phosphoethanolamine]^+$	$C_{17}H_{33}O_3$	285.2424	14.8	19.3	0.7
LPG 14:0/0:0	$[M+K]^+$	$C_{20}H_{41}KO_9P$	495.2120	24.4	5.6	2.3
	$[M+Na]^+$	$C_{20}H_{41}NaO_9P$	479.2380	48.8	23.8	31.5
	$[M+NH_4]^+$	$C_{20}H_{45}NO_9P$	474.2826	0	0	100
	$[M+H]^+$	$C_{20}H_{42}O_9P$	457.2561	3.5	6.3	92.8
	$[M+H\text{-}H_2O]^+$	$C_{20}H_{40}O_8P$	439.2455	22.1	18.5	11.0
	$[M+H\text{-}2H_2O]^+$	$C_{20}H_{38}O_7P$	421.2350	91.9	81.2	1.9
	$[M+H\text{-}phosphoglycerol]^+$	$C_{17}H_{33}O_3$	285.2424	100	100	6.4
LPS 17:1/0:0	$[M+K]^+$	$C_{23}H_{44}KO_9NP$	548.2385			2.8
	$[M+Na]^+$	$C_{23}H_{44}NaO_9NP$	532.2646			23.5
	$[M+H]^+$	$C_{23}H_{45}O_9NP$	510.2827			100
MG	$[M+Na]^+$	$C_{22}H_{42}NaO_4$	393.2975		52.9	17.7
19:1/0:0/0:0	$[M+NH_4]^+$	$C_{22}H_{46}NO_4$	388.3421		0	100
	$[M+H]^+$	$C_{22}H_{43}O_4$	371.3156		51.8	23.3
	$[M+H\text{-}H_2O]^+$	$C_{22}H_{41}O_3$	353.3050		100	4.0
PA 14:0/14:0	$[M+K]^+$	$C_{31}H_{61}KO_8P$	631.3736			0.8
	$[M+Na]^+$	$C_{31}H_{62}NO_8P$	615.3996			12.2
	$[M+NH_4]^+$	$C_{31}H_{65}NO_8P$	610.4442			100
	$[M+NH_4\text{-}H_3PO_4]^+$	$C_{31}H_{59}O_4$	495.4408			8.6
PC 14:0/14:0	$[M+K]^+$	$C_{36}H_{72}KNO_8P$	716.4627	2.5	0	6.2
	$[M+Na]^+$	$C_{36}H_{72}NNaO_8P$	700.4888	6.1	1.7	16.0
	$[M+H]^+$	$C_{36}H_{73}NO_8P$	678.5068	100	100	100
	$[phosphocholine]^+$	$C_5H_{15}NO_4P$	184.0733	0.3	0.2	0.5
PE 14:0/14:0	$[M+K]^+$	$C_{33}H_{66}KNO_8P$	674.4158	1.5	0.3	2.1
	$[M+Na]^+$	$C_{33}H_{66}NNaO_8P$	658.4418	8.8	7.9	10.9
	$[M+H]^+$	$C_{33}H_{67}NO_8P$	636.4599	100	100	100
	$[M+H\text{-}phosphoethanolamine]^+$	$C_{31}H_{59}O_4$	495.4408	22.1	20.1	5.0

Table 1 (*Continued*)

IS	Observed ion	Elemental formula	Theoretical *m/z*	Relative abundance [%]		
				UHPLC	UHPSFC	DI
PG 14:0/14:0	$[M+K]^+$	$C_{34}H_{67}KO_{10}P$	705.4103	7.1	0	1.6
	$[M+Na]^+$	$C_{34}H_{67}NaO_{10}P$	689.4364	14.5	7.7	11.0
	$[M+NH_4]^+$	$C_{34}H_{71}O_{10}P$	684.4810	5.6	3.7	100
	$[M+H]^+$	$C_{34}H_{68}O_{10}P$	667.4545	5.6	3.9	7.9
	[M+H-phosphoglycerol]$^+$	$C_{31}H_{58}O_4$	495.4408	100	100	18.4
PS14:0/14:0	$[M+K]^+$	$C_{34}H_{66}KO_{10}NP$	718.4056			1.4
	$[M+Na]^+$	$C_{34}H_{66}NaO_{10}NP$	702.4317			21.0
	$[M+H]^+$	$C_{34}H_{67}O_{10}NP$	680.4497			100
	[M+H-phosphoserine]$^+$	$C_{31}H_{59}O_4$	495.4408			6.7
SM d18:1/12:0	$[M+K]^+$	$C_{35}H_{71}KN_2O_6P$	685.4681	1.2	0.1	5.1
	$[M+Na]^+$	$C_{35}H_{71}N_2NaO_6P$	669.4942	22.4	2.1	7.6
	$[M+H]^+$	$C_{35}H_{72}N_2O_6P$	647.5123	100	100	100
	[phosphocholine]$^+$	$C_5H_{15}NO_4P$	184.0733	1.0	0.9	0.2
SulfoHexCer	$[M+Na]^+$	$C_{36}H_{69}NNaO_{11}S$	746.4484		0.5	14.9
d18:1/12:0	$[M+NH_4]^+$	$C_{36}H_{73}N_2O_{11}S$	741.4930		0	32.3
	$[M+H]^+$	$C_{36}H_{70}NO_{11}S$	724.4664		27.5	100
	$[M+H\text{-}SO_3]^+$	$C_{36}H_{70}NO_8$	644.5096		75.3	5.1
	$[M+H\text{-}H_2SO_4]^+$	$C_{36}H_{68}NO_7$	626.4990		100	19.0
	[M+H-Hex-FA-H_2O]$^+$	$C_{18}H_{36}NO$	282.2791		1.1	0
	[M+H-Hex-FA-2H_2O]$^+$	$C_{18}H_{34}N$	264.2686		9.3	0
TG 19:1/19:1/19:1	$[M+Na]^+$	$C_{60}H_{110}O_6Na$	949.8195		6.5	11.2
	$[M+NH_4]^+$	$C_{60}H_{114}NO_6$	944.8641		100	100
	$[M+H]^+$	$C_{60}H_{111}O_6$	927.8375		0.5	0
	[M+H-FA]$^+$	$C_{41}H_{75}O_4$	631.5660		39.8	1.7

oped UHPSFC/MS method for the lipidomic analysis [32] with the following conditions: Acquity BEH UPC^2 column (100 mm × 3 mm, 1.7 μm, Waters), the flow rate 1.9 mL/min, the injection volume 1 μL, the autosampler temperature 8 °C, the column temperature 60 °C, the active back pressure regulator (ABPR) pressure 1800 psi, and the gradient of methanol – water (99:1, v/v) mixture containing 30 mM of ammonium acetate as a modifier: 0 min – 1%, 5 min – 51%, 6 min – 51%.

Both UHPLC and UHPSFC experiments were performed using the identical hybrid quadrupole – travelling wave ion mobility – time of flight mass spectrometer Synapt G2Si (Waters) in the resolution mode with positive-ion ESI and the mass range *m/z* 50–1200 with the following setting of tuning parameters: the capillary voltage 3.0 kV, the sampling cone 20 V, the source offset 90 V, the source temperature 150 °C, the drying temperature 500 °C, the cone gas flow 0.8 L/min, the drying gas flow 17 L/min, and the nebuliser gas flow 4 bar. Leucine enkephaline was used as the lock mass for all experiments. UHPLC instrument was connected directly with the mass spectrometer by capillary from column to ESI probe. UHPSFC instrument was connected with the mass spectrometer *via* the commercial interface kit (Waters) composed of two T-pieces enabling the backpressure control and mixing of column effluent with a make-up liquid. The mixture of methanol – water (99:1, v/v) at the flow rate 0.25 mL/min delivered by HPLC 515 pump (Waters) was used as a make-up liquid.

2.4. DI-MS conditions

Experiments were performed on a quadrupole-linear ion trap mass spectrometer 6500 QTRAP (Sciex, Concord, ON, Canada) equipped by ESI probe with the following setting of tuning parameters: the ionspray voltage 5200 V, the curtain gas 20 psi, the source temperature 50 °C, the ion source gas(*1*) 15 psi, and the ion source gas(*2*) 10 psi. MS/MS scans were measured with the scan rate 1000 Da/s, the declustering potential 80 V, the entrance potential 10 V, and the collision energy 12 eV (cholesterol, CE), 20 eV (DG, MG), 25 eV (PA, SulfoHexCer), 30 eV (PE, LPE, PS, LPS, PG, LPG, TG), and 35 eV (PC, LPC, SM, Cer, HexCer, Hex2Cer). Samples were introduced by a flow injection using a liquid chromatograph Agilent 1290 Series (Agilent Technologies) consisted of Agilent 1290 binary pump and Agilent 1260 autosampler. 50 μL of sample was injected into the flow rate 3 μL/min of chloroform – methanol – 2-propanol (1:2:4, v/v/v) mixture containing 7.5 mM of ammonium acetate and 1% of acetic acid with the analysis time 12 min, and the autosampler temperature 20 °C. LC/MS system was washed after each analysis with methanol – 2-propanol – water (2:2:1, v/v/v) mixture containing 7.5 mM of ammonium acetate and 1% of acetic acid.

2.5. Method validation

The system suitability test was carried out before the validation procedure at two concentration levels. Calibration samples with dilution factors of 10 (high level) and 5000 (low level) were injected 6 times to evaluate the repeatability (relative standard deviation, RSD, %) of peak areas using UHPLC/MS and UHPSFC/MS methods and signal intensities using DI-MS method. Validation parameters such as selectivity, accuracy, precision, calibration curve, limits of detection and quantification, matrix effect, carry-over and stability were studied according to EMEA [39], extraction efficiency was studied according to FDA [40]. Individual parameters were determined for IS representing properties of the lipid class. The selectivity was determined using 6 extracts of randomly selected plasma samples spiked before the extraction with the IS mixture at middle concentration level (Table S1) and 6 extracts of appropriate non-spiked plasma samples. The accuracy and precision were studied using the pooled plasma sample spiked after the extraction at low and high concentration levels (Table S1). The intra-day accuracy and intra-day precision were studied in a single run using three samples per concentration level. The inter-day accuracy and inter-day precision were evaluated among three independent runs in two different days using three samples at the high concentration level. Two sets of 13 calibration samples of IS with and without matrix were prepared by dilution of the IS mixture stock solution (1:1) with dilution factors of 10, 25, 50, 75, 100, 500, 1000, 5000, 10,000, 25,000, 50,000, 75,000, and 100,000. Each calibration sample with matrix contained 5% of the pooled plasma extract. The lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ) corresponded to the first and the last points of linearity range, respectively. The limit of detection (LOD) was determined based on signal to noise ratio (S/N = 3) observed from reconstructed ion chro-

matogram (UHPLC/MS and UHPSFC/MS) or NL and PI mass spectra (DI-MS) of IS. Extracts of pooled plasma spiked with IS (Table S1) before the extraction and extracts of pooled plasma spiked after the extraction were prepared for each tested method separately and used to investigate the extraction efficiency. The matrix effect was evaluated according to Matuszewski [41] by comparison of the calibration slope of IS prepared in matrix (pooled plasma) with the slope of pure IS. The carry-over was evaluated for each IS by the injection of blank sample with the pure solvent after the calibration sample at high concentration level (dilution factor of 10). The reliability of results obtained within analysis of large sample sets was evaluated by on-instrument and freeze-and-thaw stability tests. The stability of spiked plasma extract at middle concentration level was measured in autosampler at certain time intervals: 0, 4, 8, 12, 16, and 24 h. Sample for freeze-and-thaw experiment was analyzed immediately after complete unassisted thawing in autosampler.

2.6. Data processing and quantitation

Ion intensities of positive-ion ESI full-scan mass spectra of lipid class peaks were used for the data evaluation of UHPLC/MS and UHPSFC/MS experiments, while intensities of MS/MS scans were used for DI-MS. Raw UHPLC/MS and UHPSFC/MS data were processed using MarkerLynx XS software, *i.e.*, individual ESI full-scans corresponding to lipid class peak were combined together (Fig. S1) with the peak separation 50 mDa and markers extracted with the intensity threshold 3000. Raw data from DI-MS experiments were extracted using LipidView software with the mass tolerance 0.3 Da, the minimum S/N = 5 and the minimum intensity 1%. All *.txt* data were further processed using our excel macro script for the detection and quantitation of lipids. Lipid species were detected according to accurate *m/z* values with the mass tolerance 10 mDa or MS/MS scans based on the database compiled from identified lipids in the pooled sample followed by the isotopic correction of ion intensities. Concentration of lipid specie was calculated from corrected ion intensity related to the intensity of lipid class IS. Ions and MS/MS scans of lipid classes used for the method validation and the quantitative analysis of lipids are listed in Table S2. Multivariate data analysis of quantitative results was performed using the SIMCA software, version 13.0 (Umetrics, Umeå, Sweden). Pareto scaling was used for data normalization defined as the mean-centered data divided by the square root of the standard deviation. Pareto scaling reduced the influence of intense peaks, while emphasizing weaker peaks. Logarithm function was used for data transformation to improve the normal distribution. The lipid nomenclature followed the LIPID MAPS system [2,3] and the shorthand notation for lipid structures [42].

3. Results and discussion

3.1. Lipidomic analysis

The goal of this work is the comparison of three different analytical methods for the lipidomic analysis of biological samples in a clinical research, such as chromatography/MS methods using UHPLC and UHPSFC together with DI-MS method. UHPLC/MS and DI-MS methods are used in this work as the most widespread methods for the lipidomic analysis, while UHPSFC/MS method is selected as a new method showing a great potential for the high-throughput and comprehensive lipidomic analyses [32]. These methods are compared in one laboratory using the standardized lipidomic analysis workflow as described in Fig. 1. First, the pooled sample is prepared from samples in the sample set to characterize averaged concentrations of individual lipids. Afterward, the total lipid extract of the pooled sample is prepared using chloroform – methanol – water extraction and used for the detailed nontargeted characterization of a lipid profile. The unambiguous identification of lipid species using UHPLC/MS and UHPSFC/MS methods is based on their retention times and positive/negative-ion full-scan and tandem mass spectra measured with high mass accuracy and high resolving power. MS/MS scans in positive and negative-ion modes based on the characteristic fragmentation behavior of lipid classes are used for identification of lipid species in case of DI-MS method. Total number of carbon atoms and double bonds (CN:DB) is identified for all lipid species, because species with identical CN:DB differing in fatty acyl composition are not resolved using tested methods. The detailed knowledge of the sample lipid profile is used for the selection of exogenous lipid species as IS, *i.e.*, lipids not identified in the sample or with concentrations bellow limit of quantitation. Then, each method is validated for the given type of sample and selected IS. Finally, validated methods are applied for the targeted quantitative analysis of individual samples with the addition of IS. Only species identified in the nontargeted characterization of pooled sample are quantified by the relation of their intensity in MS spectrum to the intensity of IS with known concentration. Obtained data are further processed and evaluated.

3.2. UHPLC and UHPSFC separation of lipids

The lipid class separation of total lipid extracts is used in this work, which brings several benefits over the lipid species separation. The main advantage is in the quantitative analysis, because IS coelute with species of the given lipid class in one peak under the identical composition of a gradient and matrix influencing the ionization efficiency of all species and IS at the same level. In case of the RP lipid species separation, IS differing in fatty acyl composition are separated from other species and elute under different conditions. The lipid class separation enables easier identification of lipid species, because all species from one lipid class differing in fatty acyl composition elute in one peak without the overlap with species from other lipid classes, which is typical for the RP separation of total lipid extracts [18,20]. Ion suppression effects among different lipid classes are also eliminated using the lipid class separation. Finally, lipid class separation methods can be generally faster due to the separation of only several lipid classes compared to the separation of hundreds species in the case of species separation mode.

HILIC-UHPLC method using 1.7 μm particle bridged ethylene hybrid silica column and a gradient of acetonitrile – water – ammonium acetate mobile phase is used based on our previous experiments. Our UHPSFC method for the lipidomic analysis [32] based on 1.7 μm particle bridged ethylene hybrid silica column with a gradient of methanol – water – ammonium acetate mixture as a modifier is used for UHPSFC experiments. Fig. 2A and B shows the UHPLC/MS and UHPSFC/MS chromatograms of the lipid class IS. The retention of lipids using both methods is governed mainly by their polarity, *i.e.*, retention times are higher for more polar lipid classes. Lipid species from one lipid class differing only in the fatty acyl composition have similar polarity, and they coelute in one peak corresponding to the given lipid class (Figs. 3 and 4). UHPLC method does not provide the resolution of some lipid classes eluting in the column void volume (Fig. 2A), which disables their proper identification and quantification. Mainly nonpolar lipids (CE and TG) show poor retention, but also species containing one hydroxyl group (Cer, DG, MG, and cholesterol), where the effect of their nonpolar part is more pronounced than the effect of the polar functional group. A similar behavior has been also observed for our previous HILIC methods with different silica columns [14,15] in agreement with general properties of HILIC mode showing lower selectivity for less polar species. On the other hand, UHPSFC method pro-

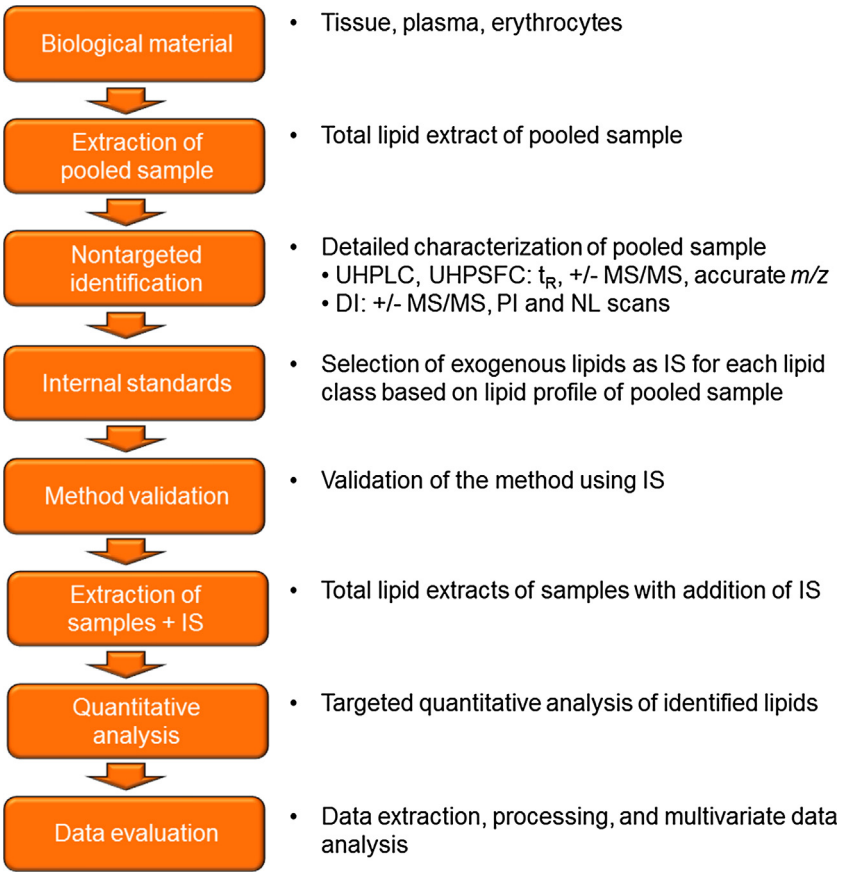

Fig. 1. Analysis workflow used in the lipidomic analysis of biological material.

vides the separation of all lipid classes in one analysis including nonpolar species (Fig. 2B). This selectivity is given by the nature of used mobile phase starting from normal-phase like conditions of nonpolar carbon dioxide with very low concentration of the polar modifier at the beginning of the gradient (1%) changing up to HILIC like conditions with high concentration of the modifier (51%) containing 1% of water. The retention behavior of lipids using UHPLC method is also influenced by a charge of lipid polar head group demonstrated by poor retention of SulfoHexCer containing very polar sulfate group, which is well retained using UHPSFC. The selectivity of both methods is similar except for LPG/PE and PC/LPE pairs showing an inverse retention order, *i.e.*, PE > LPG and LPE > PC using UHPLC compared to PE < LPG and LPE < PC using UHPSFC. 1,3-DG/1,2-DG and 1-MG/2-MG positional isomers are well resolved using UHPSFC, but positional isomers of more polar lyso- lipids are not resolved at all compared to the baseline resolution achieved by UHPLC (*i.e.*, 1-LPG/2-LPG, 1-LPE/2-LPE, and 1-LPC/2-LPC). UHPSFC method enables a partial resolution of lipid species within the lipid class peak according to the fatty acyl length and DB number, as described in our previous work [32]. This behavior is used as another tool for the unambiguous identification of lipids using UHPSFC. The partial separation of lipids according to the fatty acyl length is also achieved by UHPLC method, but no separation of species with 0–3 DB and only lower retention of species containing 4 and more DB are observed.

3.3. Nontargeted identification of lipids

ESI is used in this work for all tested methods and applied for the nontargeted identification of lipids in biological samples representing by tumor tissue, surrounding normal tissue, erythrocytes, and plasma pooled samples. The identical quadrupole – time of flight mass spectrometer with high resolving power/high mass accuracy is used for UHPLC and UHPSFC experiments to assure the same detection conditions for both methods differing only in used chromatographic technique. Individual lipids are identified from combined spectra of ESI full-scans corresponding to lipid class peaks (Fig. S1) based on accurate *m*/*z* with the mass error mostly bellow 5 ppm and based on fragment ions in tandem mass spectra. Different mass spectrometer is used for DI, because this approach requires mass analyzer for specific MS/MS scans (PI and NL scans). DI experiments are performed on a quadrupole-linear ion trap mass spectrometer with low resolving power and low mass accuracy. The identification of lipids using all methods is based on well-known fragmentation behavior of lipids, *i.e.*, characteristic fragment ions and neutral losses of individual lipid classes. The main observed ions and their relative abundances in positive-ion ESI full-scan mass spectra of IS are listed in Table 1. The comparison of UHPLC and UHPSFC shows clearly the effect of chromatographic method, because the same mass spectrometer is used for the detection. Relative abundances of fragment ions do not show significant differences between both methods for all lipids except for Hex2Cer d18:1/12:0, where fragment ions formed by the neutral losses of hexoses using UHPLC have half relative abundance compared to UHPSFC, *i.e.*, the relative abundance of $[M+H-Hex2]^+$ is 11.5% using UHPLC *vs.* 22.6% using UHPSFC, $[M+H-Hex2-H_2O]^+$ is 0.9% *vs.* 1.9% and $[M+H-Hex2-FA-2H_2O]^+$ is 2.7% *vs.* 5.2%. More significant differences are observed for $[M+Na]^+$ and $[M+K]^+$ adduct ions given by the combination of different nature of mobile phases and the concentration of water. The relative abundance of adduct ions for all species is significantly higher using UHPLC than UHPSFC except for $[M+Na]^+$ of Hex2Cer d18:1/12:0. The lower relative abundance of

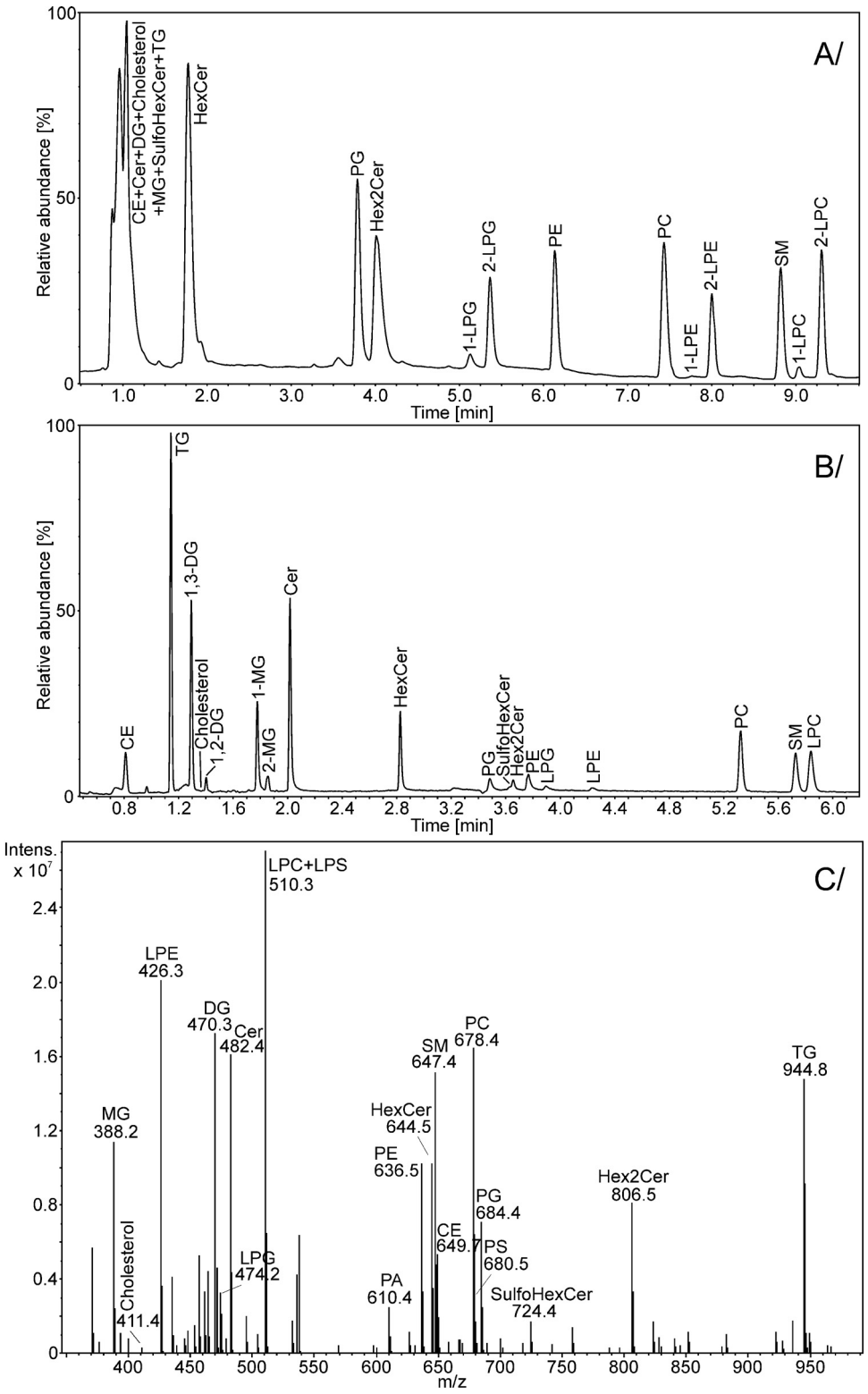

Fig. 2. Analyses of lipid internal standards using tested methods. ESI positive-ion total ion chromatograms using (A) UHPLC/MS and (B) UHPSFC/MS methods and (C) positive-ion full-scan ESI mass spectrum using DI-MS method. UHPLC conditions: Acquity UPLC BEH HILIC column (150 × 2.1 mm, 1.7 μm, Waters), the flow rate 0.4 mL/min, the column temperature 40 °C and the gradient: 0 min – 100% A and 10 min – 84% A + 16% B, where A is acetonitrile – water mixture (96:4, v/v) containing 7 mM of ammonium acetate, and B is water containing 7 mM of ammonium acetate. UHPSFC conditions: Acquity BEH UPC^2 column (100 × 3 mm, 1.7 μm, Waters), the flow rate 1.9 mL/min, the column temperature 60 °C, the ABPR pressure 1800 psi, and the gradient of methanol – water mixture (99:1, v/v) containing 30 mM of ammonium acetate as the modifier: 0 min – 1%, 5 min – 51%, and 6 min – 51%. Peak annotation: CE – cholesteryl ester, Cer – ceramide, DG – diacylglycerol, Hex2Cer – dihexosylceramide, HexCer – hexosylceramide, LPC – lysophosphatidylcholine, LPE – lysophosphatidylethanolamine, LPG – lysophosphatidylglycerol, LPI – lysophosphatidylinositol, LPS – lysophosphatidylserine, MG – monoacylglycerol, PA – phosphatidic acid, PC – phosphatidylcholine, PE – phosphatidylethanolamine, PI – phosphatidylinositol, PG – phosphatidylglycerol, PS – phosphatidylserine, SM – sphingomyelin, SulfoHexCer – sulfohexosylceramide, and TG – triacylglycerol.

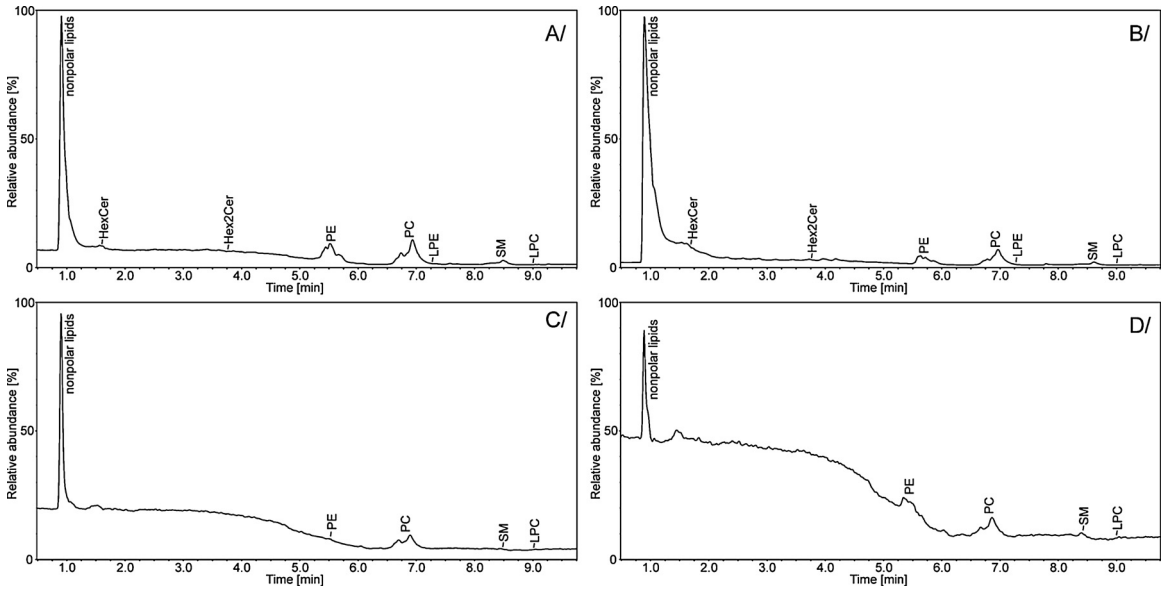

Fig. 3. Positive-ion UHPLC/ESI–MS chromatograms of (A) normal tissue, (B) tumor tissue, (C) plasma, and (D) erythrocytes pooled samples. UHPLC conditions and peak annotation are identical as for Fig. 2.

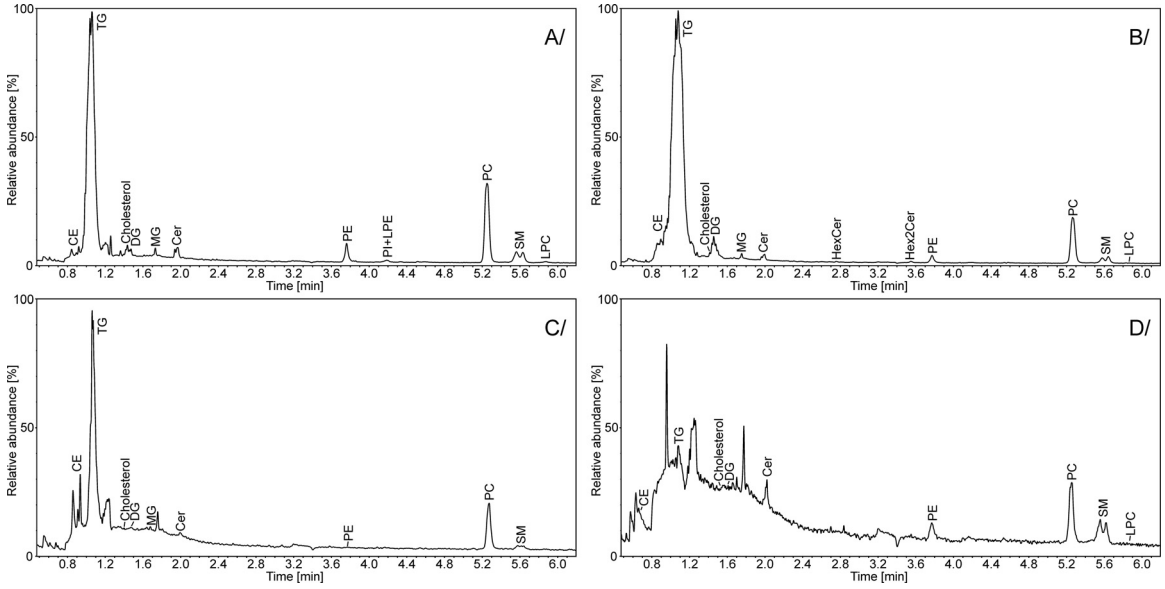

Fig. 4. Positive-ion UHPSFC/ESI–MS chromatograms of (A) normal tissue, (B) tumor tissue, (C) plasma, and (D) erythrocytes pooled samples. UHPSFC conditions and peak annotation are identical as for Fig. 2.

adduct ions using UHPSFC method is favorable for the analysis of complex samples, because adduct ions can overlap with protonated molecules of species with different composition of fatty acyls, and their resolution is more complicated requiring ultrahigh resolving power instrument or additional MS/MS experiments. Species with different CN:DB composition of fatty acyls are resolved by UHPSFC method due to their partial separation.

Relative abundances of $[M+Na]^+$ and $[M+K]^+$ using DI method are comparable to both chromatography/MS methods, but significantly higher abundances of $[M+NH_4]^+$ and $[M+H]^+$ is observed for many species, *i.e.*, higher relative abundance of $[M+NH_4]^+$ for D7-CE 16:0, MG 19:1/0:0/0:0, and PG 14:0/14:0, $[M+H]^+$ for Cer d18:1/12:0, and HexCer d18:1/12:0, and both ions for DG 12:1/0:0/12:1, LPG 14:0/0:0, and SulfoHexCer d18:1/12:0 species. On the other hand, significantly lower intensities of fragment ions in positive-ion ESI full-scan mass spectra of IS are observed in all cases, *e.g.*, the relative abundance of $[M+H\text{-}H_2O]^+$ of HexCer is 100% in both chromatography/MS methods compared to 7.1% in DI method, *etc.* This shows a softer ionization process using DI method given by the different composition of solvents with high concentration of the additives advancing the formation of molecular adducts, but also different construction of ion sources have to be considered.

Isobaric species with low differences in molecular weights (the same nominal mass) cannot be resolved using DI method due to a low resolving power and mass accuracy of quadrupole-linear ion trap mass spectrometry detection and the same characteristic fragment ions in positive-ion MS/MS spectra of both isobars. For example, protonated molecules of species differing by one additional methylene unit minus one DB and hydroxyl group (both $\Delta m/z = 16$) (*e.g.,* Cer d18:0/21:0 (611.6211) *vs.* Cer d18:0/20:1(OH) (611.5847)) or acyl and ether/plasmalogen species with one additional methylene unit (*e.g.*, PC 33:1 ($m/z = 746.5695$) *vs.* PC

Table 2
Number of identified lipid species in normal tissue, tumor tissue, plasma, and erythrocytes pooled samples.

Lipid class	Normal tissue			Tumor tissue			Plasma			Erythrocytes			Total
	UHPLC	UHPSFC	DI	UHPLC	UHPSFC	DI	UHPLC	UHPSFC	DI	UHPLC	UHPSFC	DI	
CE		13	10		26	13		13	11		6	6	28
Cer		22	14		22	15		13	13		21	13	35
Des			4			4			5			1	5
DG		42	18		43	18		19	13		10	9	47
Hex2Cer	3		5	4	4	5			4			1	6
HexCer	4		6	5	5	6			5			2	7
Cholesterol		1	1		1	1		1	1		1	1	1
LPA			1			1							1
LPC	5	8	11	5	8	11	4		9	2	2	4	13
LPE	7	7	7	4		10			8			4	13
LPG			2			2			6			5	7
LPI			4			4							4
LPS			3			3							3
MG		4	5		2	5		2	4			3	5
PA			9			8						10	10
PC	41	40	46	49	50	46	27	41	41	24	38	38	67
PE	35	29	42	35	30	48	9	5	39	27	23	41	50
PG			16			16			13			6	19
PI		6	25			27			12			12	30
PS			31			31			14			19	35
SM	24	30	26	23	24	26	12	22	20	11	30	24	33
SulfoHexCer			11			10			4				12
TG		135	34		153	34		111	28		42	8	179
Total	119	337	331	125	368	344	52	227	250	64	173	207	610

O-34:1/P-34:0 (746.6058)). Latter can be resolved in negative-ion mode MS^n spectra based on carboxylate fragment ions [43]. These isobars are resolved using UHPLC and UHPSFC methods thanks to higher resolving power/mass accuracy detection and different retention times.

In total, 610 lipid species have been identified in pooled samples of tumor tissue, surrounding normal tissue, plasma, and erythrocytes using three tested methods (Table S3). Table 2 shows numbers of identified lipid species within lipid classes using individual methods representing their different identification capability. The total number of identified species using UHPLC method is lower compared to other two methods, because less polar species are not identified/quantified due to their elution in the column void volume. These coeluted species can be resolved based on high resolving power ESI mass spectra, but their identification is very complicated mainly due to their overlap and response suppression by impurities eluted in the column void volume. The total numbers of identified species using UHPSFC and DI methods are comparable, but they differ significantly within individual lipid classes. In general, more species of nonpolar lipids are identified using UHPSFC compared to DI method, *i.e.*, CE, TG, Cer and DG. Mainly the number of TG species is significantly larger using UHPSFC showing higher sensitivity of this method for TG, *e.g.*, 135 *vs.* 34 TG in normal tissue, 153 *vs.* 34 in tumor tissue, *etc*. On the other hand, DI method shows better sensitivity for polar lipids, such as LPA, LPG, LPI, LPS, PA, PI, PG, PS, and SulfoHexCer. These species are usually not detected at all using both chromatography/MS methods due to low sensitivity given by the different composition of solvents and strong peak tailing using silica columns [44] in case of acidic lipids (*i.e.*, PA, LPA, PS, and LPS).

3.4. Method validation

Each method is validated for the quantitative analysis of lipids in plasma samples of kidney cancer patients. We apply a widely used strategy of lipidomic quantitation with one IS for each lipid class, while variations in ionization efficiency among individual species within given class differing in fatty acyl composition are neglected. The selection of appropriate IS for such kind of quantitative analysis is critical due to enormous complexity of lipidomic samples. Therefore, we select IS after the untargeted characterization of pooled samples based on the detailed knowledge of lipid profiles. It is difficult to find exogenous lipid species inside the lipidomic pattern with similar composition of fatty acyls, which are not present in samples or overlapped with isobaric species. Commercially available lipid quantitative standards provided by Avanti Polar Lipids use unusual combination of even and odd fatty acyls, but these species can be also overlapped with common endogenous species of identical nominal mass, especially when MS instruments with lower resolwing power are used. For example, PC 17:0/14:1 (PC 31:1, MW = 717.5309) can be overlapped with isobaric PC O-32:1/P-32:0 (MW = 717.5672), PC 17:0/20:4 (PC 37:4, MW = 795.5778) with PC O-38:4/P-38:3 (795.6142), PE 17:0/14:1 (PE 31:1, MW = 675.4839) with PE O-32:1/P-32:0 (675.5203), *etc*. We use mainly IS with shorter fatty acyls for the analysis of plasma samples, such as 14:0 or combinations of 18:1 and 12:0 fatty acyls in case of ceramide species (Table S1), because they are not detected in the plasma pooled sample. These IS are also widely used by other lipidomic groups [6,9,11,13].

IS are used for the method validation as representatives of all species within the lipid class. The system suitability test has been performed before the validation procedure showing acceptable values of RSD below 7.0% for all analyzed IS and tested methods. Pooled plasma spiked with the mixture of IS is used for the determination of linearity range, calibration slope, limits of detection, and quantitation (Table 3). Calibration curves are constructed for individual IS showing coefficient of determination (R^2) above 0.99. The linearity range of IS in the plasma matrix is in most cases two up to four orders of magnitude for all tested methods. LLOQ are usually lower in DI, while in UHPSFC vary among individual lipid classes due to wide differences in their responses. ULOQ is mostly lower using DI method and does not differ significantly among individual lipids, *i.e.*, ULOQ of most IS are between 2000 and 3000 pmol/mL in the calibration mixture. This is probably the critical concentration of lipids in the calibration mixture, where lipid-lipid interactions and/or aggregation of lipids start to have significant effect on the ionization efficiency of lipids limiting their linearity range [1].

Table 3
Results of the methods validation in plasma matrix.

IS	Linearity range [pmol/mL]			Calibration slope						Limit of detection [pmol/mL]		
	UHPLC	UHPSFC	DI	UHPLC		UHPSFC		DI		UHPLC	UHPSFC	DI
				with matrix	without matrix	with matrix	without matrix	with matrix	without matrix			
D7-CE 16:0		2–15832	2–2043			4.249	3.8135	0.0604	0.0726		0.4	1.3
Cer d18:1/12:0	5–4887	4–2077	1–2680	13.219	15.7370	9.7338	8.5860	0.1583	0.2334	2.1	0.2	0.3
DG 12:1/0:0/12:1		2–2211	3–2853			8.6865	8.2069	0.1024	0.1643		0.4	1.7
Hex2Cer d18:1/12:0	15–15335	130–2607	2–1602	6.1303	7.3858	0.0468	0.0458	0.0423	0.0457	6.2	75.3	0.8
HexCer d18:1/12:0	7–7313	6–6216	2–2005	7.6242	10.2260	1.339	1.1936	0.1583	0.1792	1.7	1.1	0.9
D7-Cholesterol		254–25400	8–1639			0.0156	0.0128	0.0009	0.0014		74.7	5.1
LPC 17:0/0:0	10–24252	8–4123	5–2658	3.823	4.6268	4.4757	4.2790	0.1707	0.2400	0.2	0.8	1.3
LPE 14:0/0:0	28–27665	47–9406	2–3034	0.9721	1.0059	0.1009	0.0806	0.0706	0.0893	0.3	1.8	1.2
LPG 14:0/0:0	10–10332	220–4391	3–2970	1.1144	1.5148	0.0435	0.0224	0.0289	0.0344	0.1	110.1	1.4
LPS 14:0/0:0			5–2660					0.0334	0.0519			2
MG 19:1/0:0/0:0		5–10802	2–1832			1.2084	1.3280	0.0399	0.0586		0.6	0.9
PA 14:0/14:0			1–2178					0.1235	0.1761			0.5
PC 14:0/14:0	7–17365	30–5904	4–1904	6.0489	7.7336	7.5383	4.6280	0.3334	0.5116	0.7	0.6	1.2
PE 14:0/14:0	7–7406	16–3147	1–2031	4.3202	5.6060	0.5294	0.4088	0.2201	0.2921	0.2	0.6	0.4
PG 14:0/14:0	7–6836	15–2905	1–1936	9.9834	12.5590	0.4008	0.3390	0.1646	0.2083	0.1	1.1	0.4
PS 14:0/14:0			1–1899					0.1414	0.1864			0.5
SM 18:1/12:0	7–18197	6–15468	10–1996	3.8316	4.7180	5.1779	4.8480	0.2653	0.3521	0.4	0.9	1.7
SulfoHexCer 18:1/12:0		68–900	2–892			0.1242	0.0852	0.0746	0.0828		42.1	0.9
TG 19:1/19:1/19:1		1–1439	4–3481			14.314	15.1920	0.0105	0.0151		0.3	1.4

Calibration curves of IS with and without pooled plasma as the matrix are measured to demonstrate the matrix effects. Responses of all IS are suppressed in the plasma matrix using UHPLC and DI methods, what can be demonstrated by the lower calibration slopes of IS with matrix than without matrix (Table 3). The average decrease of IS calibration slopes in the plasma matrix is 20% using UHPLC (from 16 to 26% for most lipids) and 27% using DI method (from 10% to 37%). The different effect is observed with UHPSFC method, where the response enhancement in the matrix is observed for the majority of IS with the average increase of calibration slopes of 21%. This behavior can be ascribed clearly to the properties of UHPSFC method, because the identical detection is used also for UHPLC while showing the response suppression of all IS. Quantitation limits obtained for studied methods show comparable results depending on the ionization efficiency of individual lipids. The lowest LOD are achieved for 6 IS using UHPLC, 6 using UHPSFC, and 4 using DI method, respectively. On-instrument and freeze-and-thaw stability tests show suitable results for all methods ranged from 80 to 120% and from 90 to 110%, respectively. All tested methods fulfill validation requirements for bioanalytical and clinical applications, showing variation in the extraction recovery within the range of 79–110% for most of IS, accuracy ranged from 85 to 114%, and precision up to 9.5% (Table S4).

3.5. Comparison of methods

The lipidomic analysis of biological samples in clinical research puts special requirements on analytical methods, such as throughput of samples, comprehensiveness, sample and solvent consumptions, *etc*. Selected parameters characterizing three tested lipidomic methods are compared in Table 4. The analysis time of UHPLC method is 10 min providing the separation of only polar lipid classes compared to 6 min analysis of both polar and nonpolar lipid classes using UHPSFC method. The method time including the time of analysis and the time of column equilibration is 20 min for UHPLC and 10 min for UHPSFC method. The equilibration time of UHPSFC is only 4 min without any compromise of retention time stability, while longer equilibration is required for UHPLC method (10 min) to achieve stable retention times due to generally slower equilibration of HILIC conditions and the use of lower flow rate compared to UHPSFC method. The analysis time of DI method is 12 min, which is double compared to UHPSFC, but provides comprehensive information about a wide range of lipid classes. The total method time is 23 min including time before analysis (2 min) to reach stable conditions after the sample injection and time for system washing after the analysis (9 min).

The highest number of lipid classes is identified using DI method including 23 nonpolar and polar lipids. Moreover, acidic lipids are determined using DI, which are not detected at all using UHPLC and UHPSFC methods. The number of identified lipid classes is lower using UHPLC method (7), because many nonpolar classes elute in the column void volume disabling their proper identification based on the MS and retention data. The highest total number of identified lipid species is achieved with UHPSFC method due to excellent ionization efficiency of nonpolar lipids, mainly TG.

The amount of injected sample is critical in some biological applications with limited quantity of sample. 1 μL of sample solution containing 0.05 μL of the total lipid extract is injected on column in UHPLC and UHPSFC analyses. Both methods provide comparable results of sensitivity depending on the ionization efficiency of individual lipid classes, which can differ between both methods, as demonstrated on slopes of calibration curves (Table 3). The similar sensitivity is also provided by DI method, but significantly higher amount of the sample is used. 50 μL of sample solution containing 6.25 μL of the total lipid extract is injected in DI method, which is 125 times more than using UHPLC and UHPSFC

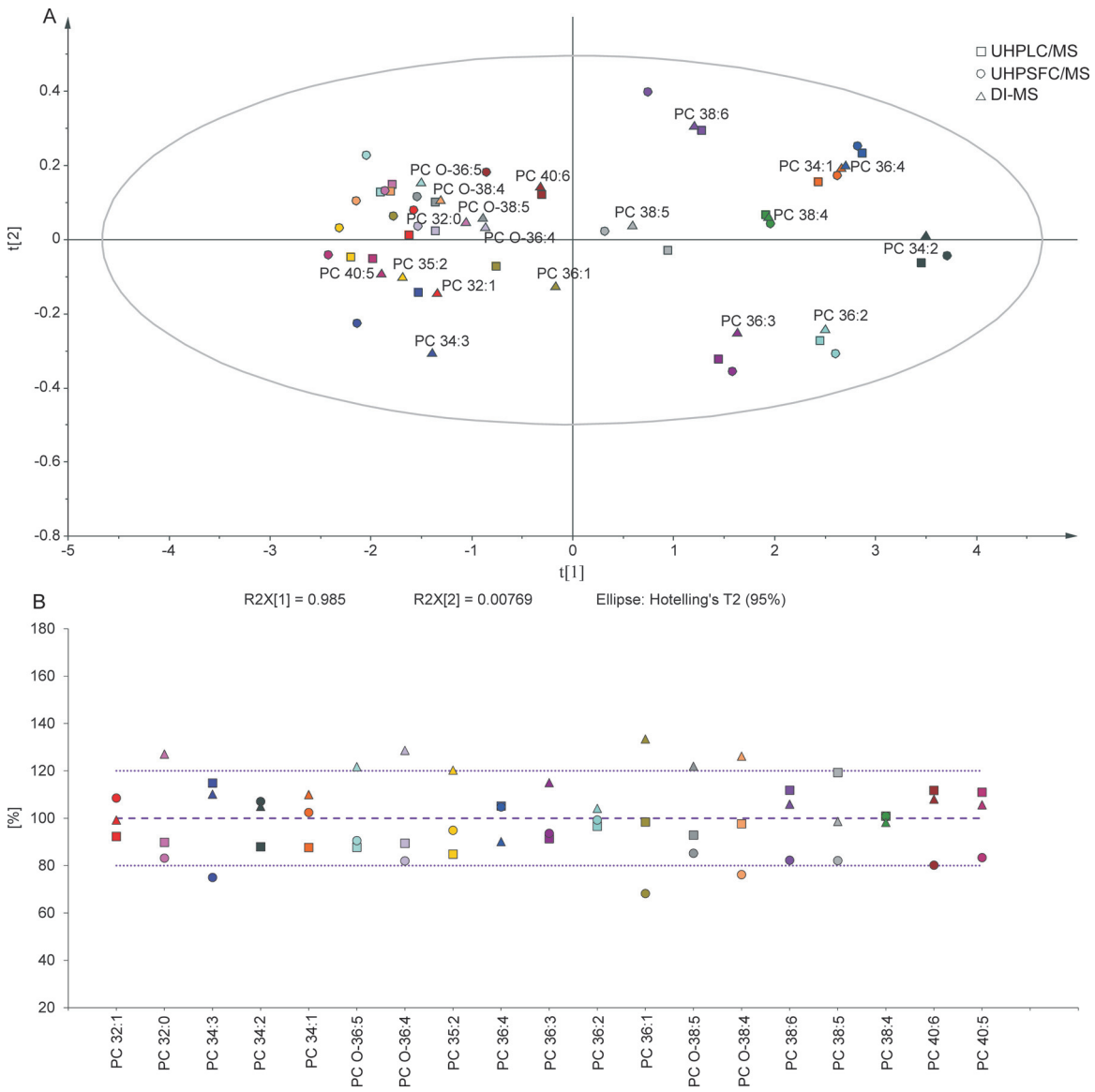

Fig. 5. Comparison of quantitation results of kidney cancer patient plasma samples using three tested methods. (A) The principal component analysis score plot of phosphatidylcholines of 6 plasma samples and (B) the difference plot of phosphatidylcholines of one sample using UHPLC/MS (square), UHPSFC/MS (circle), and DI-MS (triangle) methods.

Table 4
Comparison of selected parameters of tested lipidomic methods.

Method parameter	UHPLC	UHPSFC	DI
Analysis time [min]	10	6	12
Method time [min]	20	10	23
Identified lipid classes	7	14	23
Identified lipid species	132	450	390
Injection volume [μL]	1	1	50
Total lipid extract consumption [μL/analysis]	0.05	0.05	6.25
Flow rate [mL/min]	0.4	1.9	0.003
Organic solvent consumption [mL/analysis]	3.53	4.89[a]	0.04

[a] Consumption corresponds to both solvent of mobile phase (3.40 mL) and make-up liquid (1.49 mL) used for ESI.

methods. It is given by the fact that the sample is infused during the whole analysis, but individual lipids are measured only for short time corresponding to MS/MS scans. The sample consumption can be reduced by widely used nano-flow infusion system equipped by nanoESI with typical flow rates in hundreds of nL/min. On the other hand, the typical sample consumption for this experimental setup is 10 μL [9,12], which is only five times less compared to 50 μL injected in our DI method. The amount of organic solvent used for one analysis is very low for DI method, because only 3 μL/min flow rate of sample solution is used. The solvent consumptions for one analysis using UHPLC and UHPSFC methods are comparable (3.53 vs. 3.40 mL), but make-up liquid is used in case of UHPSFC to support ESI process with final consumption of organic solvent 4.89 mL.

Overall intensity of UHPLC and UHPSFC analyses obtained using the same mass spectrometer is comparable, but differences in response can be observed for individual lipid classes (Fig. 2, Table 3). UHPSFC method (Fig. 2B) shows excellent responses for nonpolar lipids (*e.g.*, Cer, DG, and TG), while responses of Hex2Cer, LPE, LPG, PE, and PG classes is lower compare to UHPLC (Fig. 2A). The lower response is given by different nature of mobile phases, because identical mass spectrometer is used for both UHPSFC and UHPLC methods. These classes elute in the time region, where UHPSFC mobile phase is changing from supercritical state to subcritical state due to higher amount of the modifier in the gradient [32]. ESI ionization efficiency is influenced causing lower response of lipid classes. UHPLC method shows more universal responses for separated polar lipids (Fig. 2A), but the response of nonpolar lipids cannot be compared due to their coelution. The DI method using the quadrupole – ion trap mass spectrometer provides lower distribution of lipid responses compared to UHPSFC method. Responses of lipids using DI can be also influenced by ion suppression effects according to the composition of sample, because all species are ionized together without any preseparation step. Especially charged species may compete for the charge during ESI process influencing their responses.

Validated methods are applied for the quantitative analysis of lipids in plasma samples of 6 kidney cancer patients. PC, SM and PE classes are identified using all tested methods, but PE species have concentration bellow the limit of quantification. Quantitative results of PC (Fig. 5A) and SM (Fig. S2A) species from 6 measured plasma samples using 3 tested methods are compared using the principal component analysis (PCA) score plot. Fig. 5A depicts the PCA score plot of 57 variables representing quantitative results of 19 PC species (labeled by different colors) measured using 3 methods (UHPLC/MS – square, UHPSFC/MS – circle, and DI-MS – triangle) in 6 samples. The first component in the PCA plot explains 98.5% and the second component explains 0.8% of the variation (Table S5). Each point in the PCA score plot represents concentrations of one PC in 6 measured plasma samples using given method. Individual PC species are represented by 3 points corresponding to 3 tested methods. Clustering of these 3 points shows a good correlation of measure concentrations in all samples among tested methods. The difference plot (Fig. 5B) is used to demonstrate relative differences of measured concentrations among tested methods for one plasma sample. Concentrations of individual PC species are related to the average species concentration from three tested methods. The variation of concentrations among tested methods is for most species within the range of ± 20%, which is good correlation of quantitative results among three different lipidomics methods based on the quantitation using only one IS for all lipid class species neglecting their various fatty acyls composition. Similar results are observed for SM species (Fig. S2) with slightly higher variation for species at low concentration in the plasma sample. These results confirm that this kind of quantitative analysis provides reliable quantitative results for clinical research especially for the case/control comparison.

4. Conclusions

The aim of this work is the comparison of three analytical methods (UHPLC/MS, UHPSFC/MS, and DI-MS) for the lipidomic analysis of biological samples in a clinical research. All experiments are performed in one laboratory using the same analytical protocol providing uniform conditions for the direct comparison of tested methods, which cannot be achieved using literature data measured under different conditions. Our goal is to demonstrate advantages and limitations of tested methods and not analytical techniques in general, because various conditions can be used by other authors providing different results. Obtained results show applicability of all tested methods for the lipidomic analysis of biological samples according to analysis requirements in respect to advantages and limitations of individual methods. UHPLC method can be applied mainly for the analysis of polar lipid classes, but is not convenient for the analysis of less polar lipids due to their elution in the column void volume disabling their proper identification and quantification. UHPSFC method can be used for comprehensive and high-throughput lipidomic analyses providing the separation and identification of lipids within the whole range of polarities in 6 min analysis. DI method provides the most comprehensive information about the composition of lipids covering 23 lipid classes in 12 min analysis. On the other hand, DI method does not enable the identification of isobaric species due to low resolving power/low mass accuracy MS detection. Calibration parameters of individual IS using all methods show generally similar sensitivity, but with response differences among individual lipid classes. UHPSFC method provides large differences among individual lipid classes with excellent response mainly for nonpolar lipids, but lower response for polar species. DI method provides more uniform response for most lipid classes bringing advantage for the identification of lipid classes with lower response in UHPLC and UHPSFC. The method time of UHPSFC method for the analysis of one sample is 10 min providing two times higher sample throughput than using UHPLC and DI methods. The main drawback of DI method is significantly higher sample consumption compared to other methods, but only 40 μL of organic solvent is used for one sample analysis compared to 3.5 and 4.9 mL using UHPLC and UHPSFC, respectively. Quantitative results using one IS for each lipid class show good correlation among tested methods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2017.10.022.

References

[1] X.L. Han, R.W. Gross, Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: a bridge to lipidomics, J. Lipid Res. 44 (2003) 1071–1079.
[2] E. Fahy, S. Subramaniam, H.A. Brown, C.K. Glass, A.H. Merrill, R.C. Murphy, C.R.H. Raetz, D.W. Russell, Y. Seyama, W. Shaw, T. Shimizu, F. Spener, G. van Meer, M.S. VanNieuwenhze, S.H. White, J.L. Witztum, E.A. Dennis, A comprehensive classification system for lipids, J. Lipid Res. 46 (2005) 839–861.
[3] E. Fahy, S. Subramaniam, R.C. Murphy, M. Nishijima, C.R.H. Raetz, T. Shimizu, F. Spener, G. van Meer, M.J.O. Wakelam, E.A. Dennis, Update of the LIPID MAPS comprehensive classification system for lipids, J. Lipid Res. 50 (2009) S9–S14.
[4] T. Čajka, O. Fiehn, Comprehensive analysis of lipids in biological systems by liquid chromatography-mass spectrometry, TrAC Trends Anal. Chem. 61 (2014) 192–206.
[5] M. Li, L. Yang, Y. Bai, H. Liu, Analytical methods in lipidomics and their applications, Anal. Chem. 86 (2014) 161–175.
[6] C.J. Fhaner, S.C. Liu, H. Ji, R.J. Simpson, G.E. Reid, Comprehensive lipidome profiling of isogenic primary and metastatic colon adenocarcinoma cell lines, Anal. Chem. 84 (2012) 8917–8926.
[7] H.S. Jonasdottir, S. Nicolardi, W. Jonker, R. Derks, M. Palmblad, A. Ioan-Facsinay, R. Toes, Y.E.M. van der Burgt, A.M. Deelder, O.A. Mayboroda, M. Giera, Detection and structural elucidation of esterified oxylipids in human synovial fluid by electrospray ionization-fourier transform ion-cyclotron mass spectrometry and liquid chromatography-ion trap-MS3: detection of esterified hydroxylated docosapentaenoic acid containing phospholipids, Anal. Chem. 85 (2013) 6003–6010.

[8] F. Li, X. Qin, H. Chen, L. Qiu, Y. Guo, H. Liu, G. Chen, G. Song, X. Wang, F. Li, S. Guo, B. Wang, Z. Li, Lipid profiling for early diagnosis and progression of colorectal cancer using direct-infusion electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry, Rapid Commun. Mass Spectrom. 27 (2013) 24–34.
[9] K. Schuhmann, R. Almeida, M. Baumert, R. Herzog, S.R. Bornstein, A. Shevchenko, Shotgun lipidomics on a LTQ Orbitrap mass spectrometer by successive switching between acquisition polarity modes, J. Mass Spectrom. 47 (2012) 96–104.
[10] O. Quehenberger, A.M. Armando, A.H. Brown, S.B. Milne, D.S. Myers, A.H. Merrill, S. Bandyopadhyay, K.N. Jones, S. Kelly, R.L. Shaner, C.M. Sullards, E. Wang, R.C. Murphy, R.M. Barkley, T.J. Leiker, C.R.H. Raetz, Z. Guan, G.M. Laird, D.A. Six, D.W. Russell, J.G. McDonald, S. Subramaniam, E. Fahy, E.A. Dennis, Lipidomics reveals a remarkable diversity of lipids in human plasma, J. Lipid Res. 51 (2010) 3299–3305.
[11] K. Yang, H. Cheng, R.W. Gross, X. Han, Automated lipid identification and quantification by multidimensional mass spectrometry-based shotgun lipidomics, Anal. Chem. 81 (2009) 4356–4368.
[12] L.A. Heiskanen, M. Suoniemi, H.X. Ta, K. Tarasov, K. Ekroos, Long-term performance and stability of molecular shotgun lipidomic analysis of human plasma samples, Anal. Chem. 85 (2013) 8757–8763.
[13] P. Wiesner, K. Leidl, A. Boettcher, G. Schmitz, G. Liebisch, Lipid profiling of FPLC-separated lipoprotein fractions by electrospray ionization tandem mass spectrometry, J. Lipid Res. 50 (2009) 574–585.
[14] E. Cífková, M. Holčapek, M. Lísa, Nontargeted lipidomic characterization of porcine organs using hydrophilic interaction liquid chromatography and off-line two-dimensional liquid chromatography-electrospray ionization mass spectrometry, Lipids 48 (2013) 915–928.
[15] M. Lísa, E. Cífková, M. Holčapek, Lipidomic profiling of biological tissues using off-line two-dimensional high-performance liquid chromatography mass spectrometry, J. Chromatogr. A 1218 (2011) 5146–5156.
[16] E.J. Ahn, H. Kim, B.C. Chung, G. Kong, M.H. Moon, Quantitative profiling of phosphatidylcholine and phosphatidylethanolamine in a steatosis/fibrosis model of rat liver by nanoflow liquid chromatography/tandem mass spectrometry, J. Chromatogr. A 1194 (2008) 96–102.
[17] O. Berdeaux, P. Juaneda, L. Martine, S. Cabaret, L. Bretillon, N. Acar, Identification and quantification of phosphatidylcholines containing very-long-chain polyunsaturated fatty acid in bovine and human retina using liquid chromatography/tandem mass spectrometry, J. Chromatogr. A 1217 (2010) 7738–7748.
[18] K. Sandra, A.d.S. Pereira, G. Vanhoenacker, F. David, P. Sandra, Comprehensive blood plasma lipidomics by liquid chromatography/quadrupole time-of-flight mass spectrometry, J. Chromatogr. A 1217 (2010) 4087–4099.
[19] Y. Sato, I. Suzuki, T. Nakamura, F. Bernier, K. Aoshima, Y. Oda, Identification of a new plasma biomarker of Alzheimer's disease using metabolomics technology, J. Lipid Res. 53 (2012) 567–576.
[20] M. Holčapek, M. Ovčačíková, M. Lísa, E. Cífková, T. Hájek, Continuous comprehensive two-dimensional liquid chromatography-electrospray ionization mass spectrometry of complex lipidomic samples, Anal. Bioanal. Chem. 407 (2015) 5033–5043.
[21] M. Narvaez-Rivas, Q.B. Zhang, Comprehensive untargeted lipidomic analysis using core-shell C30 particle column and high field orbitrap mass spectrometer, J. Chromatogr. A 1440 (2016) 123–134.
[22] U. Sommer, H. Herscovitz, F.K. Welty, C.E. Costello, LC-MS-based method for the qualitative and quantitative analysis of complex lipid mixtures, J. Lipid Res. 47 (2006) 804–814.
[23] E. Cífková, M. Holčapek, M. Lísa, M. Ovčačíková, A. Lyčka, F. Lynen, P. Sandra, Nontargeted quantitation of lipid classes using hydrophilic interaction liquid chromatography-electrospray ionization mass spectrometry with single internal standard and response factor approach, Anal. Chem. 84 (2012) 10064–10070.
[24] M. Scherer, G. Schmitz, G. Liebisch, High-throughput analysis of sphingosine 1-phosphate, sphinganine 1-phosphate, and lysophosphatidic acid in plasma samples by liquid chromatography-tandem mass spectrometry, Clin. Chem. 55 (2009) 1218–1222.
[25] P. Olsson, J. Holmback, B. Herslof, Separation of lipid classes by HPLC on a cyanopropyl column, Lipids 47 (2012) 93–99.
[26] I. Losito, R. Patruno, E. Conte, T.R.I. Cataldi, F.M. Megli, F. Palmisano, Phospholipidomics of human blood microparticles, Anal. Chem. 85 (2013) 6405–6413.
[27] M. Holčapek, E. Cífková, B. Červená, M. Lísa, J. Vostálová, J. Galuszka, Determination of nonpolar and polar lipid classes in human plasma, erythrocytes and plasma lipoprotein fractions using ultrahigh-performance liquid chromatography – mass spectrometry, J. Chromatogr. A 1377 (2015) 85–91.
[28] P.M. Hutchins, R.M. Barkley, R.C. Murphy, Separation of cellular nonpolar neutral lipids by normal-phase chromatography and analysis by electrospray ionization mass spectrometry, J. Lipid Res. 49 (2008) 804–813.
[29] D.G. McLaren, P.L. Miller, M.E. Lassman, J.M. Castro-Perez, B.K. Hubbard, T.P. Roddy, An ultraperformance liquid chromatography method for the normal-phase separation of lipids, Anal. Biochem. 414 (2011) 266–272.
[30] S. Granafei, P. Azzone, V.A. Spinelli, I. Losito, F. Palmisano, T.R.I. Cataldi, Hydrophilic interaction and reversed phase mixed-mode liquid chromatography coupled to high resolution tandem mass spectrometry for polar lipids analysis, J. Chromatogr. A 1477 (2016) 47–55.
[31] T.T. Zhang, S. Chen, X.L. Liang, H. Zhang, Development of a mass-spectrometry-based lipidomics platform for the profiling of phospholipids and sphingolipids in brain tissues, Anal. Bioanal. Chem. 407 (2015) 6543–6555.
[32] M. Lísa, M. Holčapek, High-throughput and comprehensive lipidomic analysis using ultrahigh-performance supercritical fluid chromatography-mass spectrometry, Anal. Chem. 87 (2015) 7187–7195.
[33] T. Yamada, T. Uchikata, S. Sakamoto, Y. Yokoi, S. Nishiumi, M. Yoshida, E. Fukusaki, T. Bamba, Supercritical fluid chromatography/Orbitrap mass spectrometry based lipidomics platform coupled with automated lipid identification software for accurate lipid profiling, J. Chromatogr. A 1301 (2013) 237–242.
[34] J.W. Lee, S. Nishiumi, M. Yoshida, E. Fukusaki, T. Bamba, Simultaneous profiling of polar lipids by supercritical fluid chromatography/tandem mass spectrometry with methylation, J. Chromatogr. A 1279 (2013) 98–107.
[35] E. Lesellier, A. Latos, A.L. de Oliveira, Ultra high efficiency/low pressure supercritical fluid chromatography with superficially porous particles for triglyceride separation, J. Chromatogr. A 1327 (2014) 141–148.
[36] T. Uchikata, A. Matsubara, S. Nishiumi, M. Yoshida, E. Fukusaki, T. Bamba, Development of oxidized phosphatidylcholine isomer profiling method using supercritical fluid chromatography/tandem mass spectrometry, J. Chromatogr. A 1250 (2012) 205–211.
[37] A. Matsubara, T. Bamba, H. Ishida, E. Fukusaki, K. Hirata, Highly sensitive and accurate profiling of carotenoids by supercritical fluid chromatography coupled with mass spectrometry, J. Sep. Sci. 32 (2009) 1459–1464.
[38] J.W. Lee, T. Nagai, N. Gotoh, E. Fukusaki, T. Bamba, Profiling of regioisomeric triacylglycerols in edible oils by supercritical fluid chromatography/tandem mass spectrometry, J. Chromatogr. B 966 (2014) 193–199.
[39] European Medicines Agency, Guideline on bioanalytical method validation, Committee for Medicinal Products for Human Use, 192217/2009, Rev. 1 Corr. 2, 21 July 2011.
[40] Food and Drug Administration, Guidance for Industry on Bioloanalytical Method Validation, Federal Register, 23 May 2001.
[41] B.K. Matuszewski, Standard line slopes as a measure of a relative matrix effect in quantitative HPLC-MS bioanalysis, J. Chromatogr. B 830 (2006) 293–300.
[42] G. Liebisch, J.A. Vizcaino, H. Kofeler, M. Trotzmuller, W.J. Griffiths, G. Schmitz, F. Spener, M.J.O. Wakelam, Shorthand notation for lipid structures derived from mass spectrometry, J. Lipid Res. 54 (2013) 1523–1530.
[43] F.F. Hsu, I.J. Lodhi, J. Turk, C.F. Semenkovich, Structural distinction of diacyl-, alkylacyl, and alk-1-enylacyl glycerophosphocholines as M-15 (−) ions by multiple-stage linear ion-trap mass spectrometry with electrospray ionization, J. Am. Soc. Mass Spectrom. 25 (2014) 1412–1420.
[44] E. Cífková, R. Hájek, M. Lísa, M. Holčapek, Hydrophilic interaction liquid chromatography-mass spectrometry of (lyso)phosphatidic acids, (lyso)phosphatidylserines and other lipid classes, J. Chromatogr. A 1439 (2016) 65–73.