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Reversed phase LC/MS/MS method for targeted quantification of glycerophospholipid molecular species in plasma

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ABSTRACT

The relationship between lipid status and metabolism, infant development and health has widely been studied, but the importance of individual glycerophospholipid species for biological functions in infants has hardly been considered. We developed a method for quantitative analyses of plasma glycerophospholipids from small sample volume. Proteins were precipitated with methanol, which eliminated further sample preparation. The supernatant was analysed by reversed-phase HPLC using a gradient of water, methanol and isopropanol as mobile phase. Electrospray ionisation in negative mode in combination with tandem mass spectrometry enabled detection of specific fatty acids as fragments of glycerophospholipid species. With this combination of chromatography and mass spectrometry, PC, lyso-PC, PE and lyso-PE species and their relevant isobaric compounds were quantified. Method validation showed a linear working range between 0.05 μmol/L and 10 μmol/L in diluted plasma samples. The intra-assay coefficients of variation ($n = 6$) ranged from 1.1% to 13.9%. Results were comparable with data of the human metabolome database and gas chromatographic fatty acid analyses. All quantitatively important PE and PC species are covered. The method can be applied for investigating dietary effects on plasma GP composition from small plasma volumes.

1. Introduction

Glycerophospholipids (GP) are major constituents of cell membranes and have important structural and functional roles in mammalian cells [1]. They also play important roles in signal transduction and as precursors for many other biologically relevant molecules [2]. Based on the polar head group at position sn-3 of the glycerol backbone, GP are divided into several classes. The major GP classes in plasma are glycerophosphocholines (PC) and glycerophosphoethanolamines (PE) [3]. Each of both GP classes consists of a multitude of molecular species, defined by the numerous combinations of fatty acids (FA), varying in chain length and double bonds, bound to positions sn-1 (FA1) and sn-2 (FA2).

The plasma GP molecular species composition is modified by dietary fat intake [4], body size [5], desaturase activity genetic variation in FADS polymorphisms [2,6,7] and metabolic disorders [8]. Methods for specific differentiation and sensitive quantification of GP molecular species are needed to understand GP composition, metabolism, regulation at the level of molecular species, and the relation to biological outcomes. In contrast to global FA composition the relation of individual GP molecular species to biological functions has received little attention. Quantification of GP as individual molecular species, including specification of the head group and FA moieties, appears important for the understanding of cellular and physiological processes [9].

Modern approaches for GP analysis are dominated by mass spectrometry, which is often combined with HPLC or gas chromatography, requiring transesterfication of GP FA to FA methylesters [10]. PC are commonly analysed in positive ion mode mass spectrometry due to the dominant choline fragment [11–13]. The fragmentation and ionisation is very sensitive but it is not possible to separate PC with the same molecular masses (isobars), such as PC 16:0/22:5, PC 18:0/20:5 and PC 18:1/20:4. However, this is of importance for studies with a focus on long chain polyunsaturated fatty acid (PUFA) status and its relationship to health outcomes and physiological parameters downstream of FA. Inclusion of PC and PE species as a potential mediating link into such studies requires the application of an analytical method, which specifically identifies

Abbreviations: GP, glycerophospholipid; PC, glycerophosphocholine; PE, glycerophosphoethanolamine; LPC, lyso-glycerophosphocholine; LPE, lyso-glycerophosphoethanolamine; FA, fatty acid; PUFA, polyunsaturated fatty acid; FA1, fatty acid at position sn-1; FA2, fatty acid at position sn-2; SD, standard deviation; SMRM, schedule multiple reaction monitoring; MRM, multiple reaction monitoring; CID, collision induced dissociation; QIR, qualifier ion ratio; LOQ, limit of quantification; CV, coefficient of variation; TAG, triacylglycerols; RT, retention time.

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the FA within the GP molecule. There is a clear need for investigating the effects of different diets (e.g., breast milk vs. infant formula in infants, or supplementation with fish oil in adults) on the formation of specific GP including the FA of interest and their incorporation into circulating lipoproteins and membranes. This can be achieved by negative ionisation mass spectrometry with fragmentation of GP into negatively charged FA and corresponding detection of these fragments [14].

A variety of methods for the quantification of GP species in biological samples have been described, including methods to profile the phospholipidome [15] or to quantify individual molecules [12]. We aimed to develop a LC/MS/MS method to separate and quantify plasma GPL molecular species of PC, PE, and the single FA species lyso-PC (LPC) and lyso-PE (LPE) in one single run from small sample volumes suitable for applications in infant studies with a simple and fast sample preparation [16].

2. Materials and methods

2.1. Biological material and reagents

Anonymous leftover plasma samples from patients at the Dr. von Hauner Children's Hospital were used for method development and validation. Individual or pooled plasma samples were aliquoted and stored at −80 °C until analysis. Results were presented as mean values ± standard deviation (SD). The ethical committee of the University of Munich Medical Faculty approved this procedure without informed consent.

GP standards were purchased from Avanti Polar Lipids Inc. (Alabaster, USA). Ammonium acetate (puriss, p.a.), acetic acid (puriss, p.a.), 2,6-di-tert-butyl-4-methylphenol (BHT; Purum), methanol (LC–MS, Chromasolv) and isopropanol (LC–MS, Chromasolv) were obtained from Fluka (Taufkirchen, Germany) and water (Chromasolv) from Sigma–Aldrich (Taufkirchen, Germany).

2.2. Sample preparation

Proteins were precipitated by the addition of 90 μL of methanol to 10 μL plasma in a 96-well filter plate (Millipore, Billerica, USA). The methanol contained 20 g/L BHT and 1 μmol/L of each internal standard LPC 13:0, LPE 13:0, PC 15:0/15:0 and PE 15:0/15:0. After shaking for 10 min at 200 U/min (IKA, Staufen, Germany) the mixture was cooled down for 10 min to −20 °C.

Precipitated proteins were removed by filtering the methanol/water phase into a 96-well plate (Agilent Technologies, Waldbronn, Germany) by centrifugation at 2330 × *g* with a Rotina 38R (Hettich, Tuttlingen, Germany) for 10 min at 4 °C. Further methanolic solution was added to achieve desired dilutions. The well plate was sealed with a solvent resisted pre-slit well cap for 96-well plates (Thermo Fisher Scientific, Rochester, USA).

2.3. High-performance liquid chromatography and mass spectrometry

A 1200-SL HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with degasser, pump, autosampler and column oven was used. The separation was performed on a 10 cm Kinetex reversed phase HPLC column with 2.1 mm inner diameter, 100 Å pore size and 2.6 μm particle size (Phenomenex, Torrance, USA). A modification of the mobile phase described by Ahn et al. and Hein et al. was used in a step gradient [17,18]. Mobile phase A consisted of 10 mmol/L ammonium acetate and 1 mmol/L acetic acid in water/methanol (60:40) and mobile phase B consisted of 10 mmol/L ammonium acetate and 1 mmol/L acetic acid in isopropanol/methanol (90:10). The pH-value of the mobile phases was adjusted to 5.7 with ammonium acetate and acetic acid to keep the analytes within a stable ionic state between the pK_a-values of 1.8 and 9.2 [17]. The gradient started by holding 40% mobile phase B for 4 min, followed by a linear ramp up to 65% B within 2 min. By holding 65% B for 8 min optimal separation of PC and PE species was achieved. Then mobile phase B was raised up to 95% within 4 min and held for 2 min at 95% B before going back to the starting conditions of 40% B within 0.5 min. With this final cleaning step triacylglycerols and cholesteryl esters were eluted from the column. Equilibration step was set to 3.5 min, flow rate to 350 μL/min at a column temperature of 55 °C and injection volume to 2 μL.

The HPLC system was directly coupled to an API 4000 QTRAP LC/MS/MS system (Applied Biosystems, Darmstadt, Germany) equipped with a turbo ion spray source. The operation mode was negative and ion spray voltage was set to −4500 V. Curtain gas was set to 30, collision gas was set to medium, ion source gas 1 was set to 60, and ion source gas 2 was set to 70. Nitrogen was used for all gas flows. The ion source was heated to 500 °C.

Quantitative analysis was performed in scheduled multiple reaction monitoring (SMRM) mode. In SMRM mode adapted multiple reaction monitoring (MRM) modes for specific transitions can be defined for different retention time (RT) windows. SMRM detection window was set to 120 s. A FA containing fragment of each analyte was utilized for quantification and another fragment of the molecule, depending on the kind of analyte, was used as qualifier ion. Collision induced dissociation (CID) parameters, collision cell exit potential, declustering potential and collision energy were specifically optimized for each group of analytes (Table 1). Entrance potential was set to −10 for all analytes. Data were post processed with Analyst 1.5.1 software (Applied Biosystems, Darmstadt, Germany).

2.4. Method development

The Kinetex column was compared to a Zorbax C18 (Agilent Technologies, Waldbronn, Germany) 50 mm × 4.6 mm, 1.8 μm particle size and a Gemini-NX C18 (Phenomenex, Torrance, USA) 100 mm × 2.1 mm, 3 μm particle size. A linear gradient starting at 40% mobile phase B rose to 100% mobile phase B within 10 min at a constant flow rate of 300 μL/min was used. A standard mixture of PE 14:0/14:0 and PE 16:0/18:1 was used at a concentration of 1 μmol/L.

A broad range of analytes, shown in Supplemental Table S1, is identified in pre-experiments by scanning plasma samples for specific head group parameters [11]. CID parameters were optimized during direct infusion of standard solutions.

As the detector signal intensity depends on the acyl chain length, the degree of unsaturation, and the position of the FA at the glycerol backbone different standards were measured at concentration of 1.0 μmol/L [19]. LPC 16:0, LPC 18:0, LPC 18:1, PC 16:0/14:0, PC 16:0/18:0, PC 16:0/18:1, PC 18:1/16:0, PC 16:0/18:2, PC 16:0/20:4 and PC 18:0/22:6 were analysed to study the considered influencing factors.

2.5. Quantification

LPC 13:0, LPE 13:0, PC 15:0/15:0 and PE 15:0/15:0 were used as internal standards for LPC, LPE, PC and PE, respectively. External quantification was done using 6 standards in 3 concentrations. LPC were quantified with LPC 16:0 and LPE with LPE 16:0. Quantification of PC with two different FA (PC *X*/*Y*) was done using PC 16:0/18:1 and PC containing two identical FA (PC *X*/*X*) using PC 16:0/16:0. PE 16:0/18:1 was chosen for PE containing different FA (PE *X*/*Y*) and PE 14:0/14:0 for PE with two identical FA (PE *X*/*X*). The areas of PC and PE species with FA 18:1 and 18:2 in sn-1 were corrected by multiplication of 1.24.

Table 1
Ions (Q1), fragments (Q3) and the optimized ion source parameters (DP – declustering potential, CE – collision energy, and CXP – collision exit potential) used for the detection of analysed glycerophospholipid groups (ID).

Q1	Q3	ID	DP	CE	CXP
$[M+Ac]^-$	$[M-CH_3]^-$	LPC_quant	−60	−24	−5
$[M+Ac]^-$	$[FA-H]^-$	LPC_qual	−60	−45	−5
$[M-H]^-$	$[PO_3]^-$	LPE_quant	−70	−85	−11
$[M-H]^-$	$[FA-H]^-$	LPE_qual	−70	−35	−15
$[M+Ac]^-$	$[FA-H]^-$	PC *X*/*X*_quant	−40	−55	−5
$[M+Ac]^-$	$[M-CH_3]^-$	PC *X*/*X*_qual	−40	−32	−11
$[M+Ac]^-$	$[FA1-H]^-$	PC *X*/*Y*_quant	−40	−55	−5
$[M+Ac]^-$	$[FA2-H]^-$	PC *X*/*Y*_qual	−40	−55	−5
$[M-H]^-$	$[FA-H]^-$	PE *X*/*X*_quant	−60	−50	−13
$[M-H]^-$	$[PO_3]^-$	PE *X*/*X*_qual	−60	−115	−1
$[M-H]^-$	$[FA1-H]^-$	PE *X*/*Y*_quant	−60	−50	−13
$[M-H]^-$	$[FA2-H]^-$	PE *X*/*Y*_qual	−60	−50	−13

2.6. Identification

For the unambiguous identification the RT and a qualifier ion ratio (QIR) were used since the ratio between fragments of an analyte is known to be a steady property of the molecule [20,21]. In Table 1 the transitions for each group of analytes are shown. The QIR was calculated by the ratio of the areas of two different fragments of the molecule (ID: *_quant, ID: *_qual).

2.7. Validation

Specificity was achieved by two individual SMRM transitions and the retention time of each analyte. For the establishment of the correct retention time for the scheduled mode a plasma sample was prepared and all analytes were measured in classical MRM mode. The linear range of the method was established using LPC 16:0, LPE 16:0, PC 16:0/16:0, PC 16:0/18:1, PE 14:0/14:0 and PE 16:0/18:1. Fifteen methanolic solutions with concentrations in the range of 0.05 μmol/L to 10.00 μmol/L were prepared and measured. This corresponded to GP molecular species plasma concentrations from 1 μmol/L to 200 μmol/L considering the dilution by a factor of 20 routinely applied in sample preparation. The lowest concentration of the linear range (0.05 μmol/L) was prepared 6 times and each solution was injected once for the verification of the limit of quantification (LOQ). The precision of the method was determined by six independent analyses of aliquots of one plasma sample. For the evaluation of accuracy virtual FA concentrations were calculated from measured GP molecular species and compared to FA concentrations measured by gas chromatography with flame ionisation detection according to Glaser et al. [16]. Furthermore, the applicability of different sample dilutions was tested by measuring 3 plasma samples with dilutions of 1:10, 1:20 and 1:50. For initial practice testing 11 plasma samples were analysed.

2.8. Statistics

The measured concentrations of GP were expressed as mean ± SD in μmol/L. The coefficient of variation (CV), expressed as percentage, was used as a measure of analytical precision. Linearity was estimated by weighted (1/X) linear regression. Statistical analyses were performed with PASW Statistics, version 18.0.0 (IBM, NY, USA).

3. Results

3.1. Method development

For the optimization of chromatographic separation three HPLC columns were initially tested by analysing a standard mixture of PE 14:0/14:0 and PE 16:0/18:1. With a resolution of 9.22 the Kinetex column turned out superior compared to the Zorbax-SB and the Gemini-NX columns, which showed resolutions of 5.95 and 6.41, respectively. The chosen mobile phase with a high isopropanol content in B was found to achieve a satisfactory separation of the GP species within 15 min (Fig. 1) and to wash triacylglycerols (TAG) and cholesteryl ester from the column towards the end of each run (data not shown).

Differentiation between isobaric compounds was achieved in negative ion mode. The ionisation of the positive choline head group was achieved by formation of adducts with acetate anions as described by Kerwin et al. [22]. As initial tests revealed only an about 5 times higher signal intensity for the analysis of a PC 16:0/18:1 standard in positive ion mode compared to negative ion mode, we considered the negative ion mode more suitable for our application.

The peak areas of CID produced FA fragments of analysed diacylphospholipid standards differed, depending on chain length, degree of unsaturation and position of binding at the glycerol backbone. In Table 2 detector signal areas of the transitions for FA1 and FA2 from different standard compounds are shown. There were differences in the areas for FA2 comparing different substances and comparing the areas for both FA for a given substance. However, the signal areas of FA 16:0 and 18:0 at sn-1 were nearly constant between the different substances. Therefore, quantification via the calibration curve set up with FA1 of PC 16:0/18:1 seemed valid for all PC with saturated FA1. For non naturally occurring PC 18:1/16:0,

Table 2
Areas of mass transitions (mean ± SD) and the qualifier ion ratio (QIR) of different phosphatidylcholine (A) and lyso-phosphatidylcholine (B) standards ($n = 8$). QIR of phosphatidylcholine species was calculated by dividing the area of fatty acids located at the glycerol at position sn-1 (FA1) by the area of the corresponding sn-2 fatty acid (FA2). QIR of lyso-phosphatidylcholine species was calculated by dividing the area of $[M-15]^-$ fragment by the area of the fatty acid (FA) fragment.

A

Analyte Name	FA1	FA2	FA1/FA2
	Area ± SD	Area ± SD	QIR ± SD
PC 16:0/14:0	1.70E+05 ± 4.84E+03	2.92E+05 ± 8.73E+03	0.58 ± 0.02
PC 16:0/18:0	1.80E+05 ± 4.69E+03	3.48E+05 ± 7.30E+03	0.52 ± 0.01
PC 16:0/18:1	1.93E+05 ± 8.09E+03	3.27E+05 ± 9.40E+03	0.59 ± 0.02
PC 16:0/18:2	1.91E+05 ± 5.37E+03	4.01E+05 ± 1.62E+04	0.48 ± 0.03
PC 16:0/20:4	1.80E+05 ± 4.69E+03	2.25E+05 ± 7.09E+03	0.80 ± 0.02
PC 18:0/22:6	1.80E+05 ± 6.32E+03	7.32E+04 ± 3.32E+03	2.46 ± 0.16

B

Analyte Name	[M−15]	FA	[M−15]/FA
	Area ± SD	Area ± SD	QIR ± SD
LPC 16:0	819500 ± 27501	859667 ± 39963	0.95 ± 0.05
LPC 18:0	794167 ± 28520	882333 ± 30362	0.90 ± 0.01
LPC 18:1	731333 ± 12675	807667 ± 31703	0.91 ± 0.04

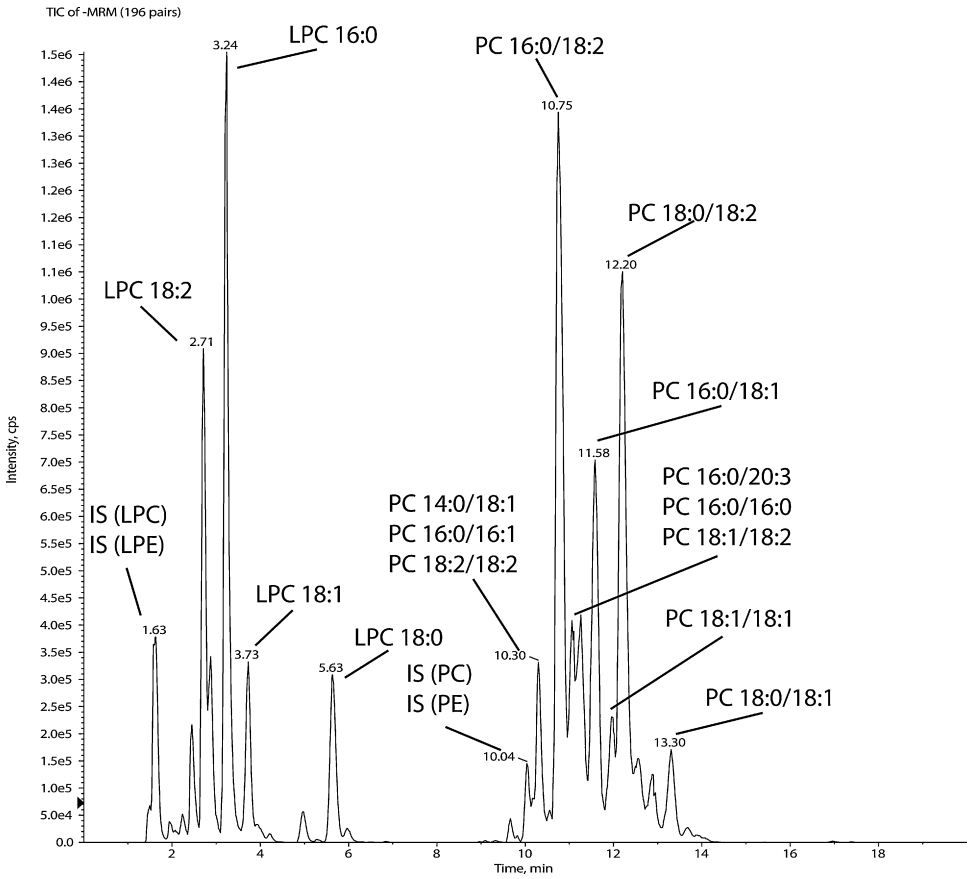

Fig. 1. Total ion chromatogram of a plasma sample analysed on a Kinetex column with the mobile phase described in Section 2. Only the largest peaks were labeled.

containing FA 18:1 at sn-1, a significant reduction of the signal of FA1 by 19.13 ± 6.04% was observed, compared to PC 16:0/18:1. The signal intensities of species, containing FA 18:1 at sn-1, were multiplied by 1.24, to compensate for this difference and enabled the application of the calibration curve for PC with saturated FA1. FA2 was used as qualifier ion by calculating the QIR of FA1 to FA2 (Table 1). As indicated in Table 2A the areas for FA2 were decreasing for the PUFA 20:4 and 22:6. This caused an increase of the QIR from 0.5 for PC 16:0/18:2 to 2.5 for PC 18:0/22:6. The QIR for PC 18:1/18:1 and PC 18:2/18:2 were similar, while clearly different from the QIR of PC 16:0/16:0 (Table 5). This justifies the factor of 1.24 also for the FA 18:2, but this has to be confirmed by a corresponding standard, which was not available to us.

Analysed LPC standards (LPC 16:0, LPC 18:0 and LPC 18:1) differed only marginally in signal areas of fragments. Thus, for LPC quantification, application of one calibration, obtained for LPC 16:0, for all LPC species was found sufficient (Table 2B).

3.2. Validation

Chromatography was checked for the separation of species with masses differing only by 2 Da, corresponding to one double bond. This separation was achieved for all analytes included. Fig. 2 shows this separation for PC 16:0/20:2 ($[M+Ac]^-$ = 844.6 Da) and PC 16:0/20:3 ($[M+Ac]^-$ = 842.6 Da) from a plasma sample as an example. The chromatogram of mass transition 844.6 Da → 255.2 Da representing PC 16:0/20:2 ($[M+Ac]^-$ = 844.6 Da) shows that the [M+2] isotopomer of PC 16:0/20:3 ($[M+Ac]^-$ = 842.6 Da + 2 Da) is well separated from PC 16:0/20:2.

Order of retention and correct RT for all analytes were determined by measuring a plasma sample in MRM mode to detect all selected GP molecular species in a chromatographic run. The RT extended with increasing number of carbon atoms, while double bonds decreased the RT. Thus, PC 16:0/18:2 and PC 16:0/20:4 eluted almost simultaneously. This effect receded with increasing number of double bonds. The RT differences between PC and corresponding PE were independent of carbon chain length of the FA and number of double bonds and were for all species about 0.3 min. RT for all analytes are listed in Supplemental Table S1.

Establishment of a satisfactory linear range is demanding for the quantification of GP, because of the large concentration differences between species, e.g., PC 16:0/18:2 can be expected several orders of magnitude higher than PC 16:0/22:6 concentrations. The linear working range was smaller than the concentration range of GP in plasma and thus, it was difficult to include a wide spectrum of species into a single analytical run. For linear range determination a standard mixture was diluted, measured and corresponding peak areas relative to internal standard areas were linearly related to the applied concentrations using the reciprocal of the concentrations as weighing factor. The estimated correlation coefficients were greater than or equal 0.99 over the linear range accepted for LPC 16:0, LPE 16:0, PC 16:0/16:0, PC 16:0/18:1, PE 14:0/14:0 and PE 16:0/18:1. In Fig. 3 the calibration curve of PC 16:0/18:1 is shown.

As limit for quantification 0.05 μmol/L, the lowest point of the linear range, was defined, this corresponds to 1.0 μmol/L in undiluted plasma if the sample was diluted 1:20. With this smallest concentration, the CV for all standard measurements were below 10%. The signal to noise ratio at this concentration ranged from 13 for LPE 16:0 to 129 for PE 14:0/14:0. Calibration curves were linear up to concentrations of 10 μmol/L, this corresponds to 200 μmol/L in plasma taking the dilution factor 20 into account.

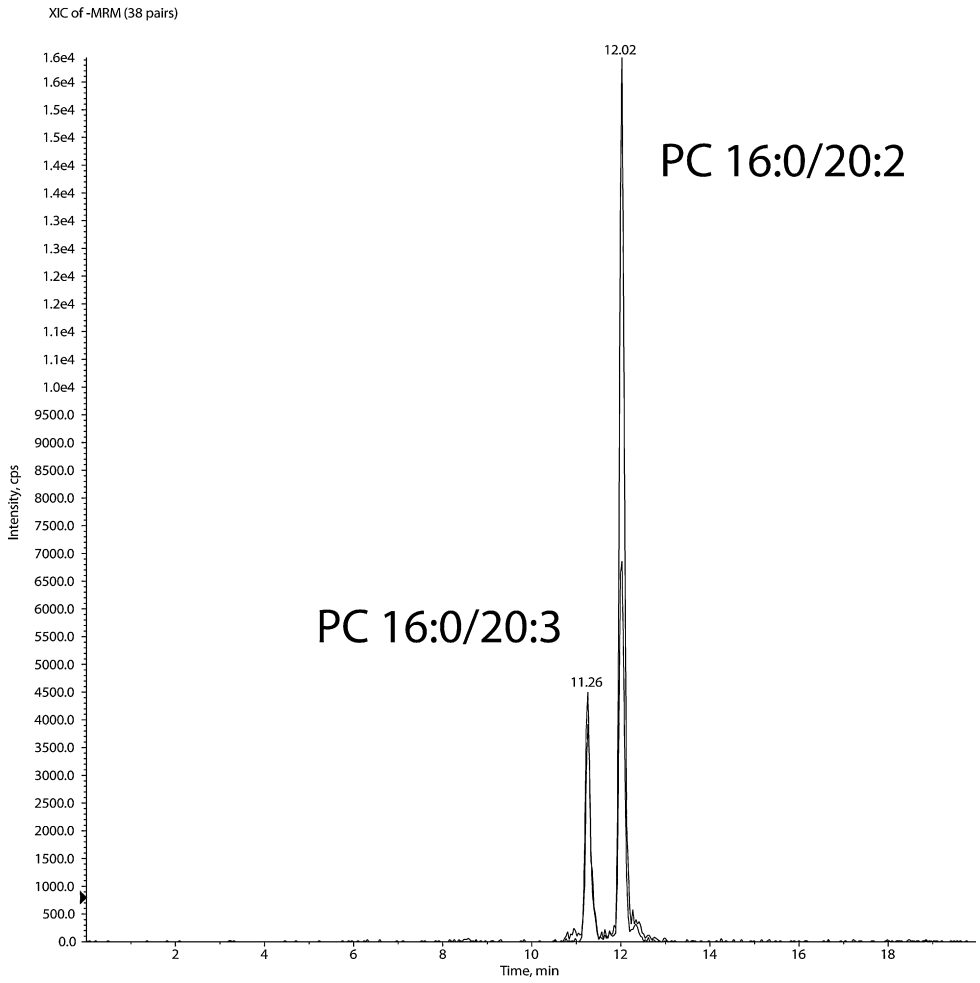

Fig. 2. Extracted ion chromatogram of mass transition 844.6 Da → 255.2 Da, demonstrating the chromatographic separation of PC 16:0/20:2 and PC 16:0/20:3 [M+2] in a plasma sample.

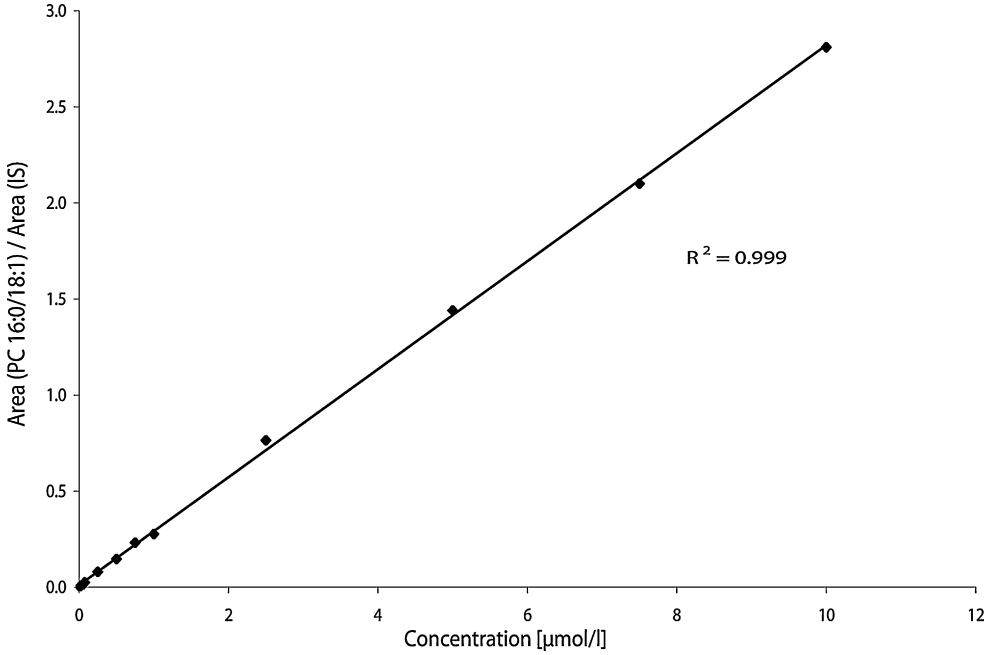

Fig. 3. Calibration curve of PC 16:0/18:1. The area ratio of the ion $[FA1-H]^-$ in relation to the internal standard area was used on the ordinate and the concentration was plotted on the abscise.

Table 3
Concentration (mean ± SD) and coefficient of variation (CV) of independent analyses of aliquots of a pooled plasma sample ($n = 6$).

Analyte name	Mean ± SD (μmol/L)	CV (%)	Analyte name	Mean ± SD (μmol/L)	CV (%)
LPC 14:0	2.58 ± 0.11	4.39	PC 16:0/20:5	11.04 ± 0.35	3.14
LPC 15:0	1.69 ± 0.10	5.64	PC 16:0/22:4	3.99 ± 0.40	10.01
LPC 16:0	151.17 ± 1.91	1.26	PC 16:0/22:5	10.24 ± 0.48	4.67
LPC 16:1	4.56 ± 0.28	6.14	PC 16:0/22:6	32.53 ± 1.49	4.59
LPC 18:0	49.83 ± 0.78	1.56	PC 18:0/16:0	6.96 ± 0.41	5.85
LPC 18:1	23.27 ± 0.38	1.64	PC 18:0/18:1	31.40 ± 0.78	2.49
LPC 18:2	30.20 ± 1.05	3.47	PC 18:0/18:2	121.12 ± 2.73	2.25
LPC 20:3	2.51 ± 0.08	3.22	PC 18:0/20:2	2.18 ± 0.23	10.76
LPC 20:4	6.41 ± 0.29	4.50	PC 18:0/20:3	23.65 ± 0.63	2.67
LPC 22:6	1.78 ± 0.15	8.21	PC 18:0/20:4	41.51 ± 0.72	1.74
LPE 16:0	3.31 ± 0.17	5.11	PC 18:0/20:5	4.23 ± 0.28	6.6
LPE 18:0	3.63 ± 0.08	2.18	PC 18:0/22:4	1.41 ± 0.06	4.32
LPE 18:1	2.41 ± 0.13	5.43	PC 18:0/22:5	2.62 ± 0.21	7.89
LPE 18:2	3.43 ± 0.17	5.07	PC 18:0/22:6	14.19 ± 0.41	2.91
LPE 20:4	2.28 ± 0.32	13.86	PC 18:1/18:1	26.24 ± 0.43	2.01
LPE 22:6	1.43 ± 0.11	7.87	PC 18:1/18:2	57.37 ± 1.17	2.52
PC 14:0/18:1	3.10 ± 0.26	8.41	PC 18:1/20:4	13.98 ± 1.51	13.42
PC 14:0/18:2	2.76 ± 0.20	7.11	PC 18:2/18:2	18.39 ± 0.56	3.79
PC 16:0/14:0	5.71 ± 0.21	3.64	PE 16:0/18:1	1.55 ± 0.16	10.59
PC 16:0/16:0	16.23 ± 0.18	1.09	PE 16:0/18:2	2.06 ± 0.21	10.36
PC 16:0/16:1	16.65 ± 0.67	4.00	PE 16:0/20:4	2.64 ± 0.08	2.88
PC 16:0/18:1	195.39 ± 3.92	2.00	PE 16:0/22:5	3.83 ± 0.13	3.42
PC 16:0/18:2[a]	266.63 ± 4.27	1.60	PE 16:0/22:6	3.35 ± 0.24	7.19
PC 16:0/18:3	5.86 ± 0.26	4.42	PE 18:0/18:2	3.40 ± 0.13	3.79
PC 16:0/20:1	1.87 ± 0.15	8.01	PE 18:0/20:4	6.91 ± 0.33	4.73
PC 16:0/20:2	5.99 ± 0.33	5.51	PE 18:0/22:6	2.18 ± 0.11	4.86
PC 16:0/20:3	60.34 ± 2.56	4.24	PE 18:1/18:2	1.65 ± 0.12	9.19
PC 16:0/20:4	100.91 ± 1.95	1.94			

[a] Concentration was outside the linear range.

As the matrix in biological samples is complex and inhomogeneous, method precision determined with plasma samples is a crucial parameter. Fifty five analytes shown in Table 3 exceeds the level of 1 μmol/L at the 1:20 dilution. Precision determination showed that 49 analytes were measured with a CV below 10% and only PC 16:0/22:4, PE 16:0/18:2, PE 16:0/18:1, PC 18:0/20:2, PC 18:1/20:4 and LPE 20:4 showed a CV between 10% and 14%.

As the linear range defined by the instrumental set up is too small to cover expected concentrations the applicability of different dilution factors was tested. Three different plasma samples were measured in 3 different dilutions (1:10, 1:20 and 1:50) and the concentrations of GP, which could be measured in at least two of the dilutions (concentrations between 0.05 μmol/L and 10.0 μmol/L injected into the LC–MS/MS), were compared by regression analysis. Plasma concentrations obtained with the dilution 1:20 (55 GP molecular species) were compared to results from dilutions 1:10 (59 GP molecular species) or 1:50 (37 GP molecular species). Slopes of 1.00 and 1.04 and coefficients of determination of 0.9742 and 0.9703 were calculated, respectively. Fig. 4 shows the linear correlation between the 1:10 and 1:20 dilution data.

Table 4 shows results obtained by gas chromatographic quantification of GP FA of a plasma sample after their transesterifiaction into FA methyl esters compared to FA concentrations calculated from GP molecular species measured by LC/MS/MS. The calculated FA concentrations were derived by summing up the GP molecular species containing the corresponding FA and taking into account the molecular composition (e.g., 1 μmol PC 16:0/16:0 yields 2 μmol FA 16:0). The proportion of FA covered by the LC/MS/MS analysis varied between 37% for FA 20:1 and almost 90% for eicosatrienoic-, plamitic- and eicosapentaenoic acids.

4. Discussion

We developed a sensitive, specific, precise, and fast reversed phase LC/MS/MS method with a simple and robust sample preparation for the quantification of GP molecular species from small plasma volumes.

A simple methanol protein precipitation was found adequate for sample preparation, as polar phospholipids were dissolved in high yield in the methanolic phase, whereas the non polar lipids precipitated with the proteins [16,23]. The advantage of this sample preparation was less time and solvent consumption in comparison to common extraction methods according to Folch [24] and easy handling of small plasma volumes was enabled. Furthermore, Folch extraction has been shown to extract lipids not quantitatively from plasma [25].

There is a multitude of GP molecular species in human plasma and quantification has to rely on a limited number of standard components. Therefore, it is necessary to identify groups of analytes

Table 4
Comparison of fatty acid (FA) concentrations calculated from measured glycerophospholipid molecular species by LC/MS/MS or directly measured by gas chromatography (GC).

FA	LC (μmol/L)	GC (μmol/L)	LC/GC (%)	LC (%)	GC (%)
14:0	14.15	21.96	64	0.55	0.70
15:0	1.69	–	–	0.07	–
16:0	921.04	1036.36	89	36.10	33.10
16:1	21.21	35	61	0.83	1.12
17:0	–	12.02	–	–	0.38
18:0	315.20	415.4	76	12.36	13.27
18:1	382.52	455.88	84	14.99	14.56
18:2	525.36	624.76	84	20.59	19.95
18:3	5.86	10.16	58	0.23	0.32
18:4	–	1.98	–	–	0.06
20:0	–	6.56	–	–	0.21
20:1	1.87	5.05	37	0.07	0.16
20:2	8.17	10.57	77	0.32	0.34
20:3	86.50	97.69	89	3.39	3.12
20:4	174.63	261.17	67	6.85	8.34
20:5	15.26	17.44	88	0.60	0.56
22:0	–	6.02	–	–	0.19
22:1	–	0.92	–	–	0.03
22:4	5.40	8.51	63	0.21	0.27
22:5	16.69	25.79	65	0.65	0.82
22:6	55.46	78.15	71	2.17	2.50
Total	2551.02	3131.39	81	100.00	100.00

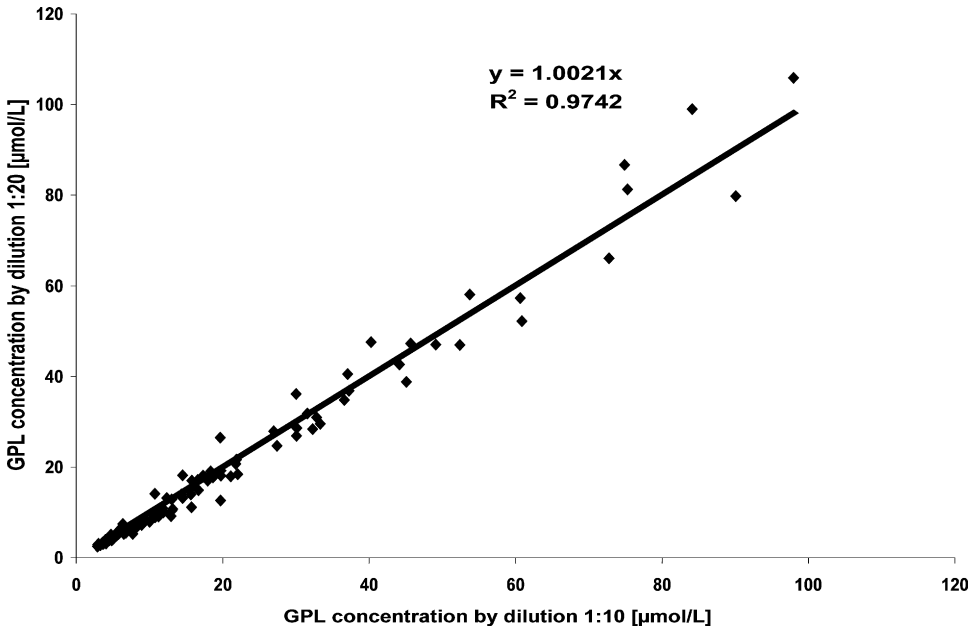

Fig. 4. Linear correlation between glycerophospholipid molecular species concentrations measured in 1:20 and 1:10 dilutions, showing almost identity of the results. A similar curve without significant intercept was obtained for the comparison between 1:20 and 1:50 (data not shown).

that behave similar and can be quantified using the same standard for calibration. A similar behavior was demonstrated in detail for diacyl-PC species containing saturated FA1, which was independent of FA2. For diacyl-PC species containing an unsaturated FA1 a lower signal response was obtained. Unfortunately, physiological relevant PC species, containing an unsaturated FA1, were not available to us and so the factor of 1.24 as correction for the signal areas of concerning species was implemented. Also, lyso-PC species were studied in more detail and the usage of one calibration for all measured lyso-PC species seemed valid.

Grouping and correction for unsaturated FA1, developed for PC species, were transferred to PE species, as the molecular structure is very similar to PC species. FA moieties had the same influence on chromatographic behavior for PE species as for PC species, with PE species eluting constantly 0.3 min after corresponding PC species. Further physical properties as molecular mass, melting point [26] and polarity justify the assumption of analogue mass spectrometric behavior. Correspondingly, the mass spectrometric behavior of LPC species was assumed for LPE species, concerning chromatographic retention and physical properties.

Finally, six quantification groups of GP molecular species were considered, depending on the polar head group (choline or ethanolamine), number of acyl residues, and in the diacyl-GP groups it was differentiated, whether FA1 and FA2 were different or identical. Species of each group were quantified by the corresponding external standard substance, considering the factor for diacyl-GP species, containing unsaturated FA1.

The dependence of the position of esterification to glycerol on the detector signal and the use of FA1 for quantification require that the distribution of the FA between positions does not vary significantly between samples. In general the shorter and less unsaturated FA are located at sn-1 position in GP from human samples [1]. This was tested by determining the ratio of response of FA1 and FA2 in the analysed GP molecular species of 11 plasma samples. The small observed variation between the samples indicates that variation of positional distribution does not introduce relevant additional error into the results. Furthermore, this justifies the use of the ratio between the two signal intensities for the calculation of a QIR (Table 5). For lyso-GP molecular species the QIR was calculated as the quotient of the $[M-CH_3]^-$ and $[PO_3]^-$ fragment for LPC and LPE, respectively and the single FA fragment. This ratio showed small variation between the samples as well. For PE 18:1/18:1 a QIR of 35.4 ± 2.5 was calculated (Table 5). This high ratio was caused by the low detecting fragment $[PO_3]^-$ used for species of PE with identical FA.

Emphasis in method development has been put on chromatographic separation of species, with small mass differences, as the natural abundance of M + 1 and M + 2 isotopomeres can be up to 55% and 16%, respectively, of the nominal mass. In normal phase HPLC and so called shot gun methods, without HPLC separation, analytical differentiation of these molecules is not possible [13,27]. Wrong quantification may result unless a sophisticated isotope correction is included into the data processing [28]. With the Kinetex HPLC-column the separation of all considered species, which differed by only 2 Da was achieved within 15 min. This was a remarkable reduction of retention time in contrast to some established RP methods. Hein et al. developed a comparable system in 2009. They separated PC and PE species within a run time of about 50 min [17]. Taguchi et al. separated phospholipids from cultured cells within 60 min [15] and the Ahn et al. method required about 80 min for the separation of PC and PE species [18].

The validation process proved that the developed method was specific, sensitive, precise and reliable in the range of 0.05 μmol/L to 10 μmol/L GP molecular species concentration in the injected solution. As the measured concentrations did not depend on the dilution factor the linear range of the method could easily be extended if required. The LOQ of 0.05 μmol/L was comparable to published methods using positive or negative ion mode [29,30]. Although a high number of PE and PC molecular species could be detected, the LC/MS/MS analysis did not include all species. For the estimation of the proportion of total GP bound FA covered by the HPLC analysis we compared the FA concentrations derived from measured GP molecular species to the FA concentrations measured directly by gas chromatography. The gas chromatographic analysis was specific for GP, but due to the transfer of FA into their methyl esters did not distinguish between species [16]. About 80% of total FA detected by gas chromatography were detected by LC/MS/MS. This difference was due to the exclusion of ether-linked GP (approx.

Table 5
Glycerophospholipid concentrations (mean ± SD) and the qualifier ion ratios (QIR, mean ± SD) of 11 plasma samples analysed with the developed LC/MS/MS method.

Analyte name	Mean conc. ± SD (μmol/L)	Mean QIR ± SD	Analyte name	Mean conc. ± SD (μmol/L)	Mean QIR ± SD
LPC 14:0	2.74 ± 0.82	1.17 ± 0.04	PC 16:0/20:5	17.55 ± 7.36	1.14 ± 0.03
LPC 15:0	1.86 ± 0.57	1.05 ± 0.04	PC 16:0/22:4	5.54 ± 1.97	0.63 ± 0.03
LPC 16:0	111.10 ± 50.14	0.91 ± 0.01	PC 16:0/22:5	20.12 ± 6.52	0.89 ± 0.04
LPC 16:1	3.95 ± 1.70	1.00 ± 0.05	PC 16:0/22:6	33.12 ± 14.11	2.17 ± 0.05
LPC 18:0	38.59 ± 20.88	0.98 ± 0.01	PC 18:0/16:0	5.63 ± 1.57	0.44 ± 0.05
LPC 18:1	24.44 ± 13.25	0.90 ± 0.03	PC 18:0/18:1	29.75 ± 4.71	0.32 ± 0.00
LPC 18:2	38.06 ± 22.97	0.89 ± 0.02	PC 18:0/18:2	117.44 ± 21.88	0.31 ± 0.00
LPC 18:3	1.39 ± 0.82	1.22 ± 0.14	PC 18:0/18:3	1.83 ± 0.66	0.35 ± 0.04
LPC 20:3	3.95 ± 2.35	0.98 ± 0.02	PC 18:0/20:2	2.23 ± 0.43	0.35 ± 0.01
LPC 20:4	8.61 ± 5.98	1.84 ± 0.05	PC 18:0/20:3	29.55 ± 6.91	0.35 ± 0.01
LPC 22:5	1.04 ± 0.56	2.18 ± 0.22	PC 18:0/20:4	48.61 ± 11.14	0.49 ± 0.01
LPC 22:6	2.55 ± 0.87	7.83 ± 0.65	PC 18:0/20:5	5.92 ± 2.90	0.84 ± 0.04
LPE 16:0	3.13 ± 1.21	0.10 ± 0.01	PC 18:0/22:4	1.84 ± 0.45	0.49 ± 0.03
LPE 18:0	3.51 ± 1.52	0.11 ± 0.00	PC 18:0/22:5	4.65 ± 1.69	0.64 ± 0.02
LPE 18:1	3.67 ± 2.60	0.11 ± 0.01	PC 18:0/22:6	14.43 ± 6.08	2.30 ± 0.11
LPE 18:2	5.39 ± 3.06	0.11 ± 0.01	PC 18:1/18:1	24.40 ± 6.65	1.27 ± 0.21
LPE 18:3	0.64 ± –	0.16 ± –	PC 18:1/18:2	54.89 ± 8.73	0.39 ± 0.01
LPE 20:3	0.66 ± 0.18	0.14 ± 0.01	PC 18:1/18:3	2.72 ± 1.53	0.78 ± 0.14
LPE 20:4	3.22 ± 1.72	0.23 ± 0.02	PC 18:1/20:4	16.97 ± 5.79	0.58 ± 0.02
LPE 22:5	0.76 ± 0.25	0.30 ± 0.12	PC 18:2/18:2	25.11 ± 9.61	1.35 ± 0.10
LPE 22:6	1.39 ± –	0.72 ± –	PE 16:0/18:1	2.06 ± 1.11	0.41 ± 0.03
PC 14:0/16:1	0.99 ± 0.44	0.59 ± 0.03	PE 16:0/18:2	3.29 ± 1.71	0.38 ± 0.03
PC 14:0/18:1	4.00 ± 1.26	0.38 ± 0.02	PE 16:0/20:3	0.72 ± 0.28	0.44 ± 0.03
PC 14:0/18:2	4.55 ± 1.55	0.34 ± 0.01	PE 16:0/20:4	4.43 ± 3.35	0.78 ± 0.04
PC 16:0/14:0	6.43 ± 2.00	0.98 ± 0.15	PE 16:0/22:5	5.59 ± 1.55	0.82 ± 0.17
PC 16:0/16:0	16.28 ± 2.88	1.53 ± 0.04	PE 16:0/22:6	6.85 ± 6.05	2.93 ± 0.26
PC 16:0/16:1	18.52 ± 6.11	0.46 ± 0.03	PE 18:0/18:1	1.00 ± 0.37	0.32 ± 0.01
PC 16:0/18:1	195.54 ± 33.30	0.40 ± 0.01	PE 18:0/18:2	4.17 ± 1.59	0.33 ± 0.01
PC 16:0/18:2	283.10 ± 49.84	0.38 ± 0.00	PE 18:0/20:3	0.95 ± 0.44	0.34 ± 0.01
PC 16:0/18:3	9.60 ± 3.58	0.44 ± 0.01	PE 18:0/20:4	9.45 ± 5.92	0.58 ± 0.02
PC 16:0/20:1	2.15 ± 0.89	0.76 ± 0.21	PE 18:0/22:6	2.89 ± 2.32	2.66 ± 0.30
PC 16:0/20:2	6.26 ± 1.78	0.43 ± 0.02	PE 18:1/18:1	1.36 ± 0.52	35.40 ± 2.48
PC 16:0/20:3	74.55 ± 20.29	0.44 ± 0.01	PE 18:1/18:2	2.12 ± 0.58	0.42 ± 0.04
PC 16:0/20:4	119.71 ± 38.75	0.64 ± 0.00	PE 18:1/20:4	1.92 ± 1.50	0.62 ± 0.06

5% of PC by mass; approx. 43% of PE by mass [31]) and all non choline or ethanolamine containing species from the LC/MS/MS analysis, while they contribute to the FA concentrations measured by gas chromatography. Furthermore, GP molecular species with concentrations below 1 μmol/L were not included into the LC/MS/MS results, but in the FA methyl ester analysis they contributed to the corresponding concentrations. The limit of detection for the LC/MS/MS method might explain why some of the FA, which contribute only a small percentages to total GP FA are largely underrepresented in the LC/MS/MS results (e.g., FA 20:1, Table 4) or are not quantified at all (e.g., FA 17:0, Table 4). The somewhat lower detected proportions of most long chain PUFA reflect the exclusion of plasmalogens from the LC/MS/MS analysis, as plasmalogens are a reservoir of arachidonic acid and docosahexaenoic acid [32]. Nevertheless, the LC/MS/MS analysis provides a good overview of the FA composition, as the FA composition calculated from the LC/MS/MS data is very similar to the composition measured by gas chromatography (Table 4).

In plasma samples 12 LPC, 9 LPE, 33 PC and 14 PE species could be identified and quantified. Although accuracy could not be strictly quantified by analysing certified reference material, independent gas chromatography analysis confirmed the plausibility of the results. Furthermore, the concentrations found in the analysed 11 plasma samples agreed very well with data given in the human metabolome database [33].

The outstanding advantage of our method was the clear separation of isobaric compounds, enabling unambiguous identification of GP molecular species due to the combination of the short reversed phase chromatography with the negative ion mode and two specific mass transitions per analyte. Using our method for PC 16:0/22:5 a concentration of 20.1 ± 6.5 μmol/L, for PC 18:0/20:5 a concentration of 5.9 ± 2.9 μmol/L, and for PC 18:1/20:4 a concentration of 21.1 ± 5.8 μmol/L could be determined, while in positive mode using the phophocholine fragment for detection only the aggregated concentration of these species would have been obtained as 47.1 μmol/L PC 38:5. This example illustrates the importance of the specific quantification of molecular species as PC 18:1/20:4 is a major source for pro-inflammatory eicosanoids derived from arachidonic acid [2] and the description PC 38:5 does not enable any allocation to the *n*−3 or *n*−6 series.

A limitation of our method is that the separation of FA isomers in respect to the position of the double bonds is not possible. Thus, a distinction between species with an *n*−3 or *n*−6 FA with identical numbers of carbon atoms and double bonds could not be made. This seemed less critical in cases, where one FA largely dominates. Concentrations of species containing FA 20:3*n*−3 or FA 20:4*n*−3 could not be determined, but the assumption that the detector signal fully corresponds to species containing FA 20:3*n*−6 or FA 20:4*n*−6 is well justified as the concentrations of these *n*−3 FA are small compared to the *n*−6 FA [16]. Nevertheless, for FA 18:3 and FA 22:5 the contribution of *n*−3 and *n*−6 isomers could be similar [16] and thus positions of the double bonds have to be considered undefined in the results. While this limitation could only be overcome by the availability of improved stationary phases or drastically prolonged chromatography times the method provides the potential to include more GP molecular species (e.g., phosphatidylserine, plasmalogens) and the sensitivity might be improved by using larger sample volumes and preconcentration of the injection solution.

To the best of our knowledge this is the first method for the quantification of GP molecular species in negative ion mode mass spectrometry with the specificity of two individual mass transitions and reversed phase HPLC. Isobaric compounds were identified and quantified separately and isotope correction was avoided by chromatographic separation. Time for samples preparation was reduced to a minimum and the major GP classes (LPC, LPE, PC and PE) were analysed within a short single chromatographic run. Thus,

the method is suitable for large interventional and observational studies. Due to the small sample volume of 10 μL the method can be applied in studies in children and infants.

Conflict of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.09.043.

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