analytical chemistry

Article

pubs.acs.org/ac

Nontargeted Quantitation of Lipid Classes Using Hydrophilic Interaction Liquid Chromatography–Electrospray Ionization Mass Spectrometry with Single Internal Standard and Response Factor Approach

Eva Cífková,[†] Michal Holčapek,*[,†] Miroslav Lísa,[†] Magdaléna Ovčačíková,[†] Antonín Lyčka,[‡,§] Frédéric Lynen,[⊥] and Pat Sandra[#]

[†]Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Studentská 573, 532 10 Pardubice, Czech Republic

[‡]Research Institute for Organic Syntheses, Rybitví 296, 533 54 Pardubice-Rybitví, Czech Republic

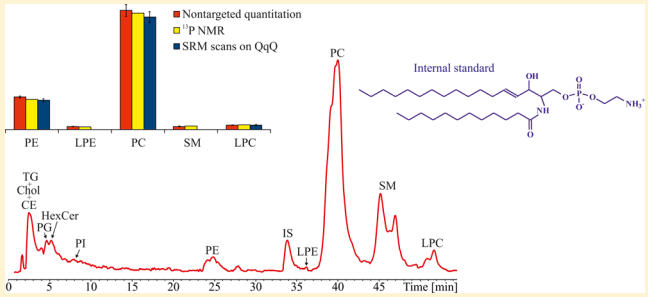
[§]Faculty of Science, University of Hradec Králové, Rokitanského 62, 500 03 Hradec Králové 3, Czech Republic

[⊥]Department of Organic Chemistry, Ghent University, Krijgslaan 281 S4-bis, B-9000 Ghent, Belgium

[#]Research Institute for Chromatography, President Kennedypark 26, B-8500 Kortrijk, Belgium

Supporting Information

ABSTRACT: The identification and quantitation of a wide range of lipids in complex biological samples is an essential requirement for the lipidomic studies. High-performance liquid chromatography–mass spectrometry (HPLC/MS) has the highest potential to obtain detailed information on the whole lipidome, but the reliable quantitation of multiple lipid classes is still a challenging task. In this work, we describe a new method for the nontargeted quantitation of polar lipid classes separated by hydrophilic interaction liquid chromatography (HILIC) followed by positive-ion electrospray ionization mass spectrometry (ESI-MS) using a single internal lipid standard to which all class specific response factors (RFs) are related to. The developed method enables the nontargeted quantitation of lipid classes and molecules inside these classes in contrast to the conventional targeted quantitation, which is based on predefined selected reaction monitoring (SRM) transitions for selected lipids only. In the nontargeted quantitation method described here, concentrations of lipid classes are obtained by the peak integration in HILIC chromatograms multiplied by their RFs related to the single internal standard (i.e., sphingosyl PE, d17:1/12:0) used as common reference for all polar lipid classes. The accuracy, reproducibility and robustness of the method have been checked by various means: (1) the comparison with conventional lipidomic quantitation using SRM scans on a triple quadrupole (QqQ) mass analyzer, (2) ^{31}P nuclear magnetic resonance (NMR) quantitation of the total lipid extract, (3) method robustness test using subsequent measurements by three different persons, (4) method transfer to different HPLC/MS systems using different chromatographic conditions, and (5) comparison with previously published results for identical samples, especially human reference plasma from the National Institute of Standards and Technology (NIST human plasma). Results on human plasma, egg yolk and porcine liver extracts are presented and discussed.



Lipids are hydrophobic or amphipathic small molecules that originate entirely or in part by carbanion-based condensations of thioesters (fatty acids, glycerolipids, glycerophospholipids, sphingolipids) or carbocation-based condensations of isoprene units (prenols and sterols).[1] Lipids are divided according to the structural and biosynthetic complexity into fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides categories.[1] Another division is based on the number of hydrolysis products into simple (nonpolar) and complex (polar) lipids.[2] The identification and quantitation of all lipids in complex biological samples can lead to the understanding how lipids function in a biological system and to the elucidation of mechanisms of lipid-related diseases including obesity, atherosclerosis, cancer, cardiovascular diseases, etc. The disruption of lipid metabolism may be associated with these diseases as well as the occurrence of modified lipids generated by free radicals oxidation.[2]

HPLC/MS is the most powerful analytical tool for nontargeted characterization of the lipidome in biological

Received: September 3, 2012
Accepted: October 17, 2012
Published: October 17, 2012

dx.doi.org/10.1021/ac3024476 | *Anal. Chem.* 2012, 84, 10064–10070

samples. HILIC and reversed-phase (RP) systems are typically used in the separation of lipids. Individual lipid classes can be separated according to their polarity using silica[3−6] or diol columns[7−10] in the HILIC mode or according to their hydrophobicity (the length of fatty acyl chains and the number of double bonds) using C_{18} columns[11−13] in the RP mode. Nonaqueous reversed-phase (NARP)[14−16] HPLC can be used in the separation of nonpolar lipids (triacylglycerols, diacylglycerols, monoacylglycerols, and fatty acids). Gas chromatography (GC) with flame ionization detection is typically applied for fatty acid profiling after transesterification into fatty acid methyl esters,[17] but it does not provide any information on intact polar lipids unlike HPLC/MS.

The most popular MS ionization techniques for the lipid analysis are electrospray ionization (ESI)[3,4,7,9,11−13,18,19] and matrix-assisted laser desorption/ionization (MALDI)[20−26] for the identification of polar lipids, while atmospheric pressure chemical ionization (APCI)[27,28] or atmospheric pressure photoionization (APPI)[27,29] are preferred for nonpolar lipids. MALDI has been coupled to the thin-layer chromatography (TLC)[23] for the analysis of phospholipids from egg yolk and for biological samples as well.[30]

The conventional method for quantitative analysis of lipids by MS is based on SRM scans on QqQ mass spectrometers.[3,13,19,31−35] This targeted quantitation can be used in the determination of lipids with known fragmentation behavior, because the previous knowledge of precursor ions and their specific product ions are required for SRM transitions. Advantages of the SRM approach are high sensitivity and selectivity, especially when combined with the analyte specific retention times in HPLC. On the other hand, the limitation of SRM quantitation is that this determination is, in principle, targeted and therefore limited to lipid molecules with predefined transitions, which may lead to the loss of information on unexpected lipids. The promising untargeted lipidomic quantitation has been introduced recently using the combination of ultrahigh resolving power (>100 000) and subppm mass accuracy of Fourier transform (orbitrap and ion-cyclotron resonance) instruments, which enables the identification and quantification of lipid species directly using nontargeted MS/MS data.[36−39] This strategy requires the most expensive Fourier transform mass spectrometers, which are not available to all lipid researchers. ^{31}P NMR is an alternative method for the absolute quantitation of the lipid classes containing phosphorus, because individual lipid species inside lipid classes have almost identical chemical shifts and NMR does not require any RFs for the quantitation.[23,40,41] Drawbacks of this approach are the lack of structural information unlike MS and very low sensitivity (high concentrations and several hours for the signal accumulation are required).

The goal of our work has been the development of a novel nontargeted broad spectrum lipidomic quantitation technique using HILIC-HPLC/ESI-MS applied to the determination of all separated lipid classes. Such a method should provide comprehensive information on the lipidome of biological samples without the loss of lipids and the need of defined SRM transitions and without the need of expensive internal standards (ISs) for each lipid class. Nontargeted quantitation is based on the peak integration of individual lipid classes in the HILIC mode multiplied by their RFs and correlated by only single IS (sphingosyl PE, d17:1/12:0) for all lipid classes. A similar quantitation approach based on the use of the RFs was successfully developed for the NARP-HPLC/APCI-MS analysis of triacylglycerols (TGs) and applied in several lipidomic studies focused on TGs.[15,17,42,43] In this work, this strategy is extended to the quantitation of several lipid classes with different polarities in one HPLC/MS run.

EXPERIMENTAL SECTION

Chemicals and Standards. Acetonitrile, 2-propanol, methanol (all HPLC gradient grade), chloroform (HPLC grade, stabilized by 0.5−1% ethanol), methyl-*tert*-butyl ether (MTBE), and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). 1,2-Dioleoyl-*sn*-glycero-3-phosphoglycerol (18:1/18:1-PG), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoglycerol (18:1-LPG), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18:1/18:1-PE), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (18:1-LPE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18:1/18:1-PC), 1-oleoyl-*sn*-sphing-4-enine-1-phosphocholine (18:1-SM), and 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (18:1-LPC) for the determination of RFs, 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine (17:0/17:0-PC), 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine (17:0/17:0-PE), and 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (17:0-LPC) as ISs for the conventional quantitation using SRM transitions, and N-dodecanoyl-heptadecasphing-4-enine-1-phosphoethanolamine (sphingosyl PE, d17:1/12:0) as the IS for nontargeted quantitation with RFs were purchased from Avanti Polar Lipids (Alabaster, AL). The human plasma standard reference material (NIST plasma) from the National Institute of Standards and Technology (Gaithersburg, MD) was prepared from plasma samples of 100 individuals between 40 and 50 years of age including an equal number of men and women and with a racial distribution that reflects the United States population. Egg and porcine liver samples were purchased at local stores.

Sample Preparation. Total lipid extracts from egg yolk, NIST human plasma, and porcine liver were prepared according to a modified Folch procedure[44] using a chloroform/methanol/water system. Briefly, approximately 0.5 g of lipid tissue and 50 μL of 3.3 mg/mL sphingosyl PE were homogenized with 10 mL of a mixture of chloroform/methanol (2:1, v/v), and the homogenate was filtered using a coarse filter paper. Then, 2 mL of 1 mol/L NaCl was added, and the mixture was centrifuged at 3000 rpm for 5 min at room temperature. The chloroform (bottom) layer containing the lipids was evaporated by a gentle stream of nitrogen and redissolved in a chloroform−2-propanol mixture (1:1, v/v) for the HILIC analysis. The modified Bligh and Dyer method[45] was performed in the same way as the Folch method, except that the mixture of chloroform−methanol was in the ratio 1:2 (v/v). For MTBE extraction,[46] approximately 0.5 g of lipid tissue and 50 μL of 3.3 mg/mL sphingosyl PE were homogenized with 15 mL mixture of MTBE/methanol (4:1, v/v). Then, 3 mL of water was added, and the organic (upper) layer containing the lipids was collected. The aqueous (bottom) layer was extracted again using the mixture MTBE/methanol/water (10:3:2.5, v/v/v). The organic (upper) layer was collected and combined with the organic extract from the previous step, evaporated by a gentle stream of nitrogen, and redissolved in chloroform−2-propanol mixture (1:1, v/v) for the HILIC analysis. Bligh and Dyer and MTBE extraction methods were used only for the comparison of extraction recoveries, but in all other measurements only the Folch extraction was used.

dx.doi.org/10.1021/ac3024476 | Anal. Chem. 2012, 84, 10064−10070

HILIC-HPLC/ESI-MS Conditions. Nontargeted quantitation with RFs was performed on a liquid chromatograph Agilent 1200 series (Agilent Technologies, Waldbronn, Germany) coupled to ESI-MS detection on the Esquire 3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany). RFs of individual lipid classes and peak areas were determined using the total ion current chromatograms in the positive-ion ESI-MS mode in the mass range m/z 50−1000 with the following setting of tuning parameters: pressure of the nebulizing gas 60 psi, drying gas flow rate 10 L/min, and temperature of the drying gas 365 °C. Conditions used for measurements in the negative-ion mode were identical except for the polarity. The data were acquired and evaluated using the Data Analysis software (Bruker Daltonics). Total lipid extracts were fractionated into lipid classes using a Spherisorb Si column (250 × 4.6 mm, 5 μm, Waters, Milford, MA), a flow rate of 1 mL/min, an injection volume of 10 μL, column temperature of 40 °C, and a mobile phase gradient: 0 min, 94% A + 6% B; 60 min, 77% A + 23% B, where A was acetonitrile and B is 5 mM aqueous ammonium acetate. The injector needle was washed with the mobile phase before each injection.

Conventional quantitation using SRM transitions was performed on a liquid chromatograph Alliance 2690 (Waters) coupled to the Micromass Quattro Micro QqQ mass spectrometer (Waters). The following parameters of the ESI-MS source were set up for the positive-ion mode: mass range m/z 50−1000, capillary voltage 3.3 kV, extractor voltage 3 V, RF lens voltage 0.1 V, temperature of ESI source 100 °C, temperature of drying gas 300 °C, nebulization gas flow rate 500 L/h, collision energy 25 V for PE and 30 V for PC and LPC, the cone voltage 30 V for PE and 40 V for PC and LPC. SRM scans for lipid classes were performed with a dwell time of 0.2 s. The data were acquired and processed using MassLynx software (Waters).

^{31}P NMR Conditions. ^{31}P NMR spectra were measured on a Bruker Avance II 400 spectrometer at 202.46 MHz. The samples were dissolved in chloroform−2-propanol mixture (1:1, v/v). Deuterium oxide (placed in a 4 mm coaxial insert having a capillary in the measurement area) was used as a lock compound. ^{31}P chemical shifts were referred to the signal of PE ($\delta(^{31}P)$ = 0.01).

RESULTS AND DISCUSSION

Development of New Nontargeted Quantitation of Lipid Classes Using HILIC-HPLC/ESI-MS. The prerequisite for the development of our nontargeted lipidomic quantitation is a good chromatographic separation of individual lipid classes. For this purpose, we developed a HILIC-HPLC/ESI-MS method, which enables the separation of up to 19 lipid classes in a wide range of polarities in one analytical run.[5] The method has been applied to the analysis of NIST human plasma mixed with a known amount of IS and extracted using the modified Folch procedure[5] (Figure 1A). The HILIC chromatogram of human plasma shows 12 lipid classes eluting in order of polarity, i.e., nonpolar lipids (TG, Chol, CE), PG, HexCer, PI, PE, IS, LPE, PC, SM, and LPC. Unfortunately, the HILIC-HPLC method does not enable the separation of nonpolar lipids, because they coelute in one chromatographic peak close to the void volume of the system. The negative-ion ESI mode (Figure 1B) is more sensitive for anionic lipid classes, such as HexCer, PI, and PE.

The basic idea of the novel nontargeted lipidomic quantitation is based on the peak integration of lipid classes

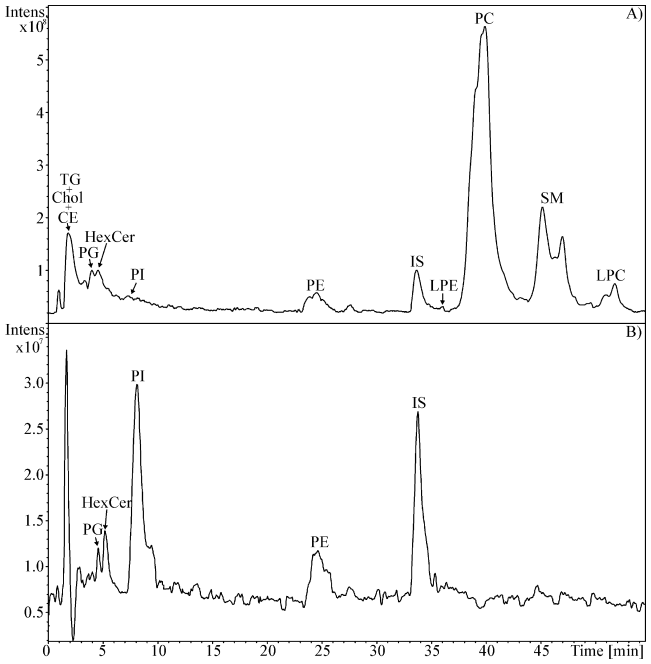


Figure 1. HILIC-HPLC/MS separation of total lipid extract from the NIST human plasma sample with the addition of internal standard (sphingosyl PE, d17:1/12:0) using Spherisorb Si column (250 × 4.6 mm, 5 μm) in: (A) positive-ion ESI-MS, and (B) negative-ion ESI-MS modes. HPLC conditions: flow rate 1 mL/min, separation temperature 40 °C, gradient 0 min−94% A + 6% B, 60 min−77% A + 23% B, where A is acetonitrile and B is 5 mM aqueous ammonium acetate. Peak annotation: TG, triacylglycerols; Chol, cholesterol; CE, cholesteryl esters; PG, phosphatidylglycerols; HexCer, hexosylceramides; PI, phosphatidylinositols; PE, phosphatidylethanolamines; IS, internal standard; LPE, lysophosphatidylethanolamines; PC, phosphatidylcholines; SM, sphingomyelins; LPC, lysophosphatidylcholines.

in the HILIC chromatogram followed by the multiplication of obtained peak areas by their RFs related to the single IS common for all lipid classes. The rather difficult task is the selection of a suitable IS with an appropriate retention behavior (i.e., no coelution with peaks of other lipid classes in HILIC chromatograms), which does not commonly occur in nature and has similar extraction behavior as for determined lipid classes. Polydeuterated phosphatidylcholine (18:0/18:0−PC, D79) was the first compound tested as IS with the assumption that polydeuterated and nondeuterated species could be separated. Unfortunately, the HILIC method did not provide any visible separation of polydeuterated and nondeuterated lipid species. The second compound tested as IS was N-dodecanoyl-heptadecasphing-4-enine-1-phosphoethanolamine (sphingosyl PE, d17:1/12:0) (Figure 2), which complies with all requirements for the IS in the nontargeted lipidomic quantitation. Sphingosyl PE elutes between the PE and LPE peaks, well separated from all lipid classes occurring in

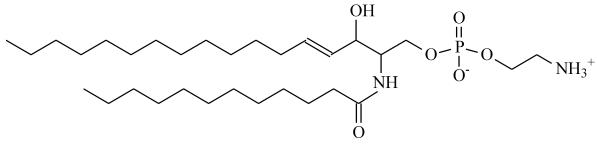


Figure 2. Structure of N-dodecanoyl-heptadecasphing-4-enine-1-phosphoethanolamine (sphingosyl PE, d17:1/12:0) used as an internal standard.

dx.doi.org/10.1021/ac3024476 | Anal. Chem. 2012, 84, 10064−10070

biological samples studied in our laboratory so far (lipid extracts from plants, animal and human body fluids and tissues such as plasma, serum, sebum, sperm, various organs with and without tumors, etc.). Moreover, sphingosyl PE provides an acceptable intensity in both positive-ion and negative-ion ESI-MS modes.

First, calibration curves were measured for the IS and these lipid classes (PG, LPG, PE, LPE, PC, SM, LPC) represented by standards containing oleoyl (Δ9*cis*-C18:1) acyls. The oleic acid has been selected as one of the most common fatty acids occurring in biological samples consistently with our previous method developed for TGs.[15] Calibration curves are linear at least in the concentration range from 5 to 1000 μg/mL for PC, SM, and LPC, and from 25 to 800 μg/mL for PG, LPG, PE, and LPE. Each lipid class is described by parameters of the linear dependency, $y = ax + b$, where y is the peak area, x is the concentration, and r^2 is the regression coefficients (Table 1).

Table 1. Parameters of Calibration Curves for Individual Lipid Classes Represented by Standards Containing Oleoyl (Δ9*cis*-C18:1) Acyl (retention times (t_R), Slopes (a), Intercepts (b), Regression Coefficients (r^2), and Response Factors (RF))

lipid class	t_R [min]	a	b	r^2	RF
PG	4.7	183.1	14.6	0.9996	0.318
LPG	8.4	271.8	23.1	0.9993	0.214
PE	24.8	196.6	2.2	0.9991	0.296
IS	**33.9**	**58.1**	**−0.6**	**0.9998**	**1.000**
LPE	36.1	112.7	−1.1	0.9984	0.516
PC	39.8	550.7	9.5	0.9997	0.106
SM	45.3	857.6	3.8	1.0000	0.068
LPC	51.2	488.6	2.8	0.9998	0.119

RFs are calculated as the ratio of the slope of the calibration dependency obtained for the IS to slopes of calibration dependencies of individual lipid classes. Obtained values of RFs are constant over months of measurements on the same instrument at identical conditions (deviation below 4%). This approach can be transferred to other instruments, but calibration dependencies must be measured first to obtain RFs valid for this particular instrument under given chromatographic conditions.

Peak areas of individual lipid classes and IS are integrated in the HILIC-HPLC/ESI-MS chromatogram of the total lipid extract with added IS. The concentration of IS is calculated from the calibration dependency, $c_{IS} = (A_{IS} + 0.6)/58.1$. Concentrations of individual lipid classes (Figure 3A) are calculated from the ratio of the peak area of lipid class to the peak area of IS multiplied by the RF_{class} and c_{IS}. For example, the concentration of PCs is calculated from the ratio of the peak area of PC to the peak area of IS multiplied by the RF_{PC} and c_{IS}. This new approach was compared with the conventional way of quantitation based on SRM transitions measured on a QqQ mass analyzer (Figure 3B), which is typically used in the shotgun setup without a chromatographic separation. However, identical HILIC conditions were applied here to ensure the direct comparison of quantitative data obtained by both approaches. Main disadvantages of quantitation using SRM scans are the necessity of ISs for each lipid class and the ability to quantify only lipids with previously determined SRM transitions. On the other hand, the requirement of our method

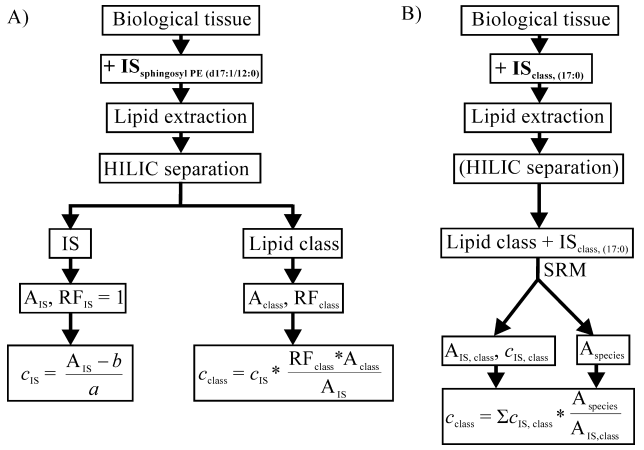


Figure 3. General schemes of both quantitation approaches: (A) novel nontargeted quantitation of lipid classes using single internal standard and response factor approach in HILIC-HPLC/ESI-MS, (B) conventional targeted quantitation using SRM scans on QqQ mass spectrometer. Abbreviations: IS, internal standard; A, peak area; RF, response factor; *c*, concentration; *a*, slope; *b*, intercept; SRM, selected reaction monitoring.

is the necessity of chromatographic resolution of lipid classes to be determined.

Extraction Recovery and Robustness of the Whole Method. Three basic extraction approaches are used in the lipidomic analysis and compared in this paper: (A) chloroform−methanol (2:1, v/v) extraction according to Folch,[44] (B) chloroform−methanol (1:2, v/v) extraction according to Bligh and Dyer,[45] and (C) MTBE extraction according to Shevchenko et al.[46] Extraction recoveries of these three extraction approaches (details in Experimental Section) are compared for Cer, PE, LPE, PC, SM, and LPC standards containing oleoyl acyl (Figure 4). White columns show chromatographic peak areas obtained from the standard solution without any extraction (100%), while other columns show extraction recoveries of individual approaches calculated as the mean peak area from six measurements together with standard deviations. All methods mostly provide results with good mutual agreement with few exceptions, e.g., lower recovery of LPE for Folch extraction, but all other values are in the interval 62−99%. The use of IS is essential for the reliable quantitation in accordance with established practice in HPLC/MS. When extraction recoveries are measured in human plasma and related to the IS (Figure S-1), then variability among individual extraction methods is rather low and in principle any of these methods can be used in the quantitation, as illustrated on the example of PE, PC, SM, and LPC. The additional test on the robustness was performed by the analysis of the porcine liver sample by three different persons, who extracted the same sample using the Folch extraction and each extract was injected two times into HPLC/MS, i.e., in total 6 chromatograms for one sample (Figure S-2). The mutual agreement is again satisfactory.

Verification of General Applicability of Our Method. Principal questions about the general applicability of our method based only on one IS and the use of RFs are the following: (1) stability of RFs over longer period of time, (2) applicability of the method on different HPLC/MS systems with different chromatographic conditions, (3) the verification that the approach based only on the single IS and RF approach can provide accurate results for various biological samples in

dx.doi.org/10.1021/ac3024476 | *Anal. Chem.* 2012, 84, 10064−10070

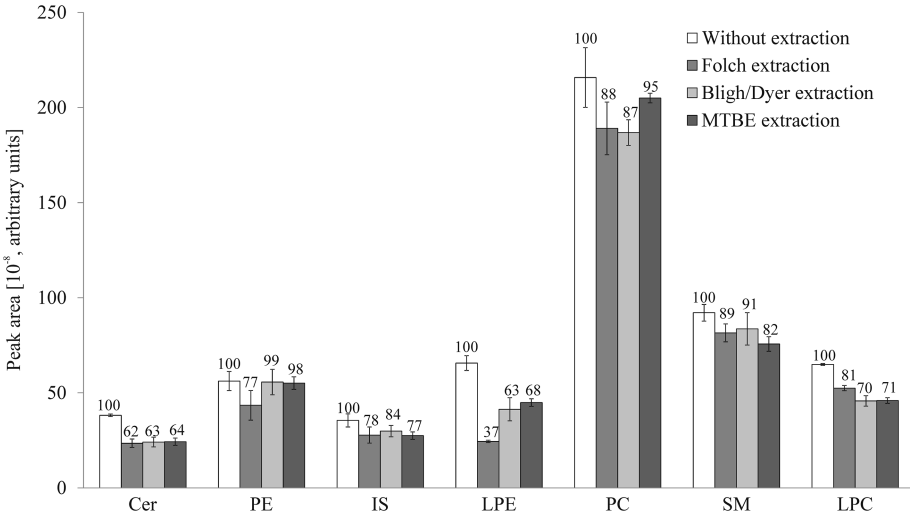


Figure 4. Comparison of extraction efficiency of Folch, Bligh and Dyer, and methyl-*tert*-butyl ether (MTBE) methods for ceramides (Cer), PE, IS, LPE, PC, SM, and LPC standards containing oleoyl (Δ9*cis*-C18:1) acyl. Reported relative recoveries (in percentages) are the standard mean of six measurements (three times extraction, each extract measured two times) together with standard deviations.

agreement with established techniques used in lipidomic quantitation. First, calibration curves were remeasured after a few months, and relative differences of RFs were lower than 4%. Then calibration curves were also measured on a different type of instrument (Q-TOF) using faster analysis under ultrahigh-performance liquid chromatography conditions optimized for main phospholipid and sphingolipid classes occurring in human plasma (clinical study in progress), and again good correlation was obtained in the measurement of identical human plasma samples.

The next step of the method validation was the comparison with reference methods used in lipidomic quantitation (SRM approach on QqQ and ^{31}P NMR) supported by the comparison with previously reported data on identical samples. Lipid species containing fatty acids with an odd carbon number are used as ISs in the SRM-based quantitation, PE (17:0/17:0), PC (17:0/17:0), and LPC (17:0). Individual SRM transitions (Table S-1) were assigned in previous off-line two-dimensional HILIC × RP-HPLC/MS measurements.[5] The first comparison is shown for the total lipid extract from egg yolk (Figure 5 and Table S-2), which was divided into three identical parts and used for measurements by three techniques. In general, the agreement between three completely different approaches is acceptable. In the case of PE and PC, values obtained by nontargeted quantitation with RFs are slightly higher compared to values obtained by targeted SRM approach, which could be explained by the fact that some minor lipid species are not defined in the SRM approach. Relative differences between nontargeted and targeted approaches are 0.5% for LPC, 5.6% for PC, and in the worst case 9.8% for PE (calculated from Table S-2). Values for LPE and SM with SRM are missing due to the lack of IS for these classes. Figure 6 shows an example of

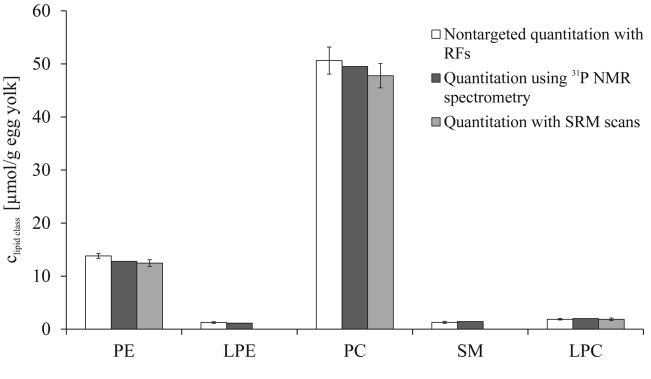


Figure 5. Comparison of concentrations (μmol/g) of PE, LPE, PC, SM, and LPC in the egg yolk using nontargeted quantitation of lipid classes using single internal standard and response factor approach in HILIC-HPLC/ESI-MS (white columns), the quantitation using ^{31}P NMR spectroscopy (dark columns), and the quantitation using SRM scans on QqQ mass spectrometer (gray columns).

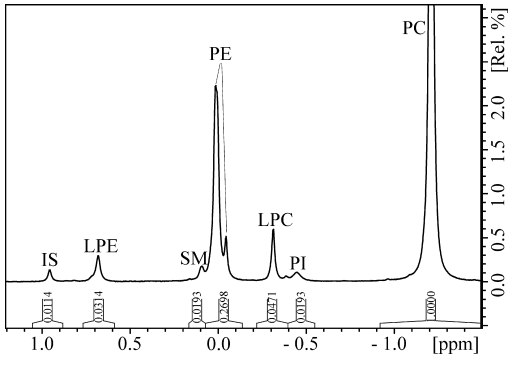


Figure 6. ^{31}P NMR spectrum of egg yolk extract measured in the chloroform−2-propanol mixture (1:1, v/v) at 202.46 MHz.

the ^{31}P NMR spectrum of egg yolk extract measured in chloroform−2-propanol mixture (1:1, v/v) at 202.46 MHz. The main advantage of NMR spectroscopy is no need of any RFs, because the signal is directly proportional to the number of measured nuclei, i.e., ^{31}P in our case.[23,40,41] The effect of fatty acyl chain length as well as the number and positions of double bonds on chemical shifts of individual lipids inside classes is rather small; therefore, the ^{31}P NMR spectrum of total lipid extract (Figure 6) shows peaks of lipid classes in a fashion very similar to that for HILIC-HPLC (Figure 1), which brings an ideal situation for the mutual comparison of obtained results. The last comparison is provided for the analysis of NIST human plasma (Figure 7 and Table S-3) by nontargeted

dx.doi.org/10.1021/ac3024476 | *Anal. Chem.* 2012, 84, 10064−10070

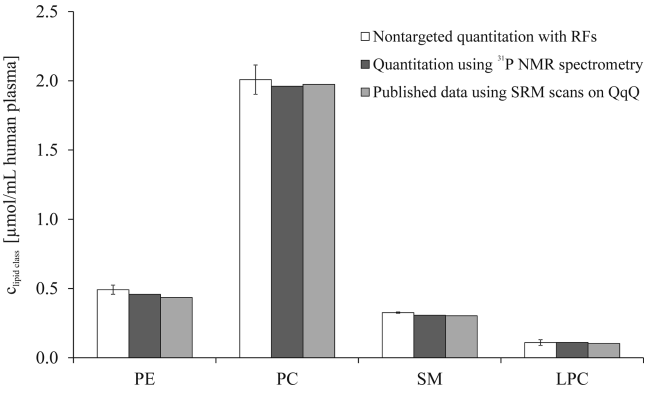


Figure 7. Comparison of concentrations (μmol/mL) of PE, PC, SM, and LPC in the NIST human plasma using nontargeted quantitation of lipid classes using single internal standard and response factor approach in HILIC-HPLC/ESI-MS (white columns), the quantitation using ^{31}P NMR spectroscopy (dark columns), and previously published data[34] (gray columns).

lipidomic quantitation with RFs in HILIC-HPLC/ESI-MS, ^{31}P NMR, and previously published data[34] obtained in eight different laboratories mainly by SRM on QqQ, except for classes of fatty acids and sterols quantified by GC/MS. The mutual correlation among three different analytical methods is rather good, especially considering the complexity of lipidomic plasma extracts and the number of individual lipid molecules inside particular lipid classes. Our method provides slightly higher concentrations for most lipid classes, which can be explained the same way as for the egg yolk.

Quantitation of Individual Lipid Species. The HILIC-HPLC/ESI-MS method can also be used in quantitation of lipid molecular species within individual lipid classes using relative intensities of characteristic ions in ESI mass spectra obtained by the peak integration in the HILIC chromatogram. Relative abundances of SM species have been determined based on $[M + H]^+$ ions (Table 2) and compared with the literature data.[47] The comparison of results shows a good match except for d24:1/18:1-SM. Moreover, absolute concentrations of lipid species can be calculated for the known total SM concentration in the egg yolk (in μmol/g) determined from the SM peak area in the HILIC chromatogram multiplied by the relative peak area of individual SM species. This approach is applicable for other lipid classes as well, but in certain cases the exact quantitation of individual species inside the class can be complicated by isobaric interferences of $[M + Na]^+$ ions with $[M + H]^+$ ions for lipids with the fatty acyl chain longer by two carbon atoms and additional three double bonds, e.g., protonated 36:4-PC has the same nominal mass m/z 782 as sodiated 34:1-PC, which requires either the ultrahigh-resolution typical for Fourier transform ion cyclotron resonance mass spectrometer or negative-ion mode, as illustrated on the example of PI determined in NIST human plasma and compared with previously published data on the same sample (Figure S-3). Another possible solution is the preferential formation of desired adduct ions achieved by the addition of a selected ion into the mobile phase.[48] Isobaric interferences do not occur for the SM class due to the absence of fatty acyl chains with three or more double bonds, so the accurate quantitation (Table 2) can be performed.

Table 2. Comparison of Relative Abundances (%) of Individual SM in the Egg Yolk Sample between Our Data Obtained by HILIC-HPLC/ESI-MS and Previously Published High-Performance Thin-Layer Chromatography Data[47] and the Absolute Concentrations (μmol/g) of Individual SM Species

sphingomyelin	HILIC-HPLC/ESI-MS (%)	HPTLC[47] (%)	HILIC-HPLC/ESI-MS (concentration, μmol/g)
d16:0/18:1	61	66	0.78
d18:0/18:1	9	10	0.12
d18:1/18:1	2	1	0.03
d20:0/18:1	2	4	0.03
d22:0/18:1	5	6	0.06
d22:1/18:1	1	1	0.01
d23:0/18:1	2	2	0.03
d24:0/18:1	4	5	0.05
d24:1/18:1	14	3	0.18
others	0	2	0.00
total	100	100	1.28

CONCLUSIONS

The novel nontargeted lipidomic quantitation method for the comprehensive lipidomic analysis enables the quantification of all lipid classes separated by HILIC-HPLC. Concentrations of individual lipid classes are obtained by the peak integration in the HILIC mode multiplied by their RFs related to a single IS. Concentrations of individual lipid species inside lipid classes can be also determined as relative abundances of particular peaks in mass spectra multiplied by the total concentration of the lipid class. The correlation with earlier published data on the lipidomic characterization of NIST human plasma,[34] egg yolk,[47] our comparison with established SRM approach on QqQ mass spectrometer, and also the ^{31}P NMR quantitation confirms the accuracy and precision of our results and applicability for various types of biological samples. In general, our nontargeted method is better suited for the quantitation of lipid classes, while the conventional SRM targeted approach has better sensitivity for selected lipid molecules and is faster compared to rather long HPLC/MS runs in the HILIC mode. Nontargeted HILIC-HPLC/ESI-MS quantitation can be used in the comprehensive lipidomic characterization of multiple lipid classes, such as clinical studies searching for lipidomic differences between healthy volunteers and disease patients. Two comprehensive lipidomic studies are in progress in our group using the described quantitative method, namely the lipidomic characterization of porcine organs and tissues and the clinical study of lipoprotein plasma fractions and erythrocytes of cardiovascular disease patients (over 1000 samples per year). The preliminary results confirm the robustness and reliability of this quantitative assay.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +420-466037087. Fax: +420-466037068. E-mail: Michal.Holcapek@upce.cz.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the grant project No. 206/11/0022 sponsored by the Czech Science Foundation (E.C., M.H., M.L., and M.O.). We acknowledge the help of Pawel Pasikowski and Blanka Červená with the validation of the extraction procedure and Vitaliy Chagovets for the critical reading of the manuscript.

■ REFERENCES

(1) Fahy, E.; Subramaniam, S.; Brown, H. A.; Glass, C. K.; Merrill, A. H.; Murphy, R. C.; Raetz, C. R. H.; Russell, D. W.; Seyama, Y.; Shaw, W.; Shimizu, T.; Spener, F.; van Meer, G.; VanNieuwenhze, M. S.; White, S. H.; Witztum, J. L.; Dennis, E. A. *J. Lipid Res.* **2005**, *46*, 839.
(2) Christie, W. W. http://lipidlibrary.aocs.org/ downloaded April 2012.
(3) Axelsen, P. H.; Murphy, R. C. *J. Lipid Res.* **2010**, *51*, 660.
(4) Hutchins, P. M.; Barkley, R. M.; Murphy, R. C. *J. Lipid Res.* **2008**, *49*, 804.
(5) Lísa, M.; Cífková, E.; Holčapek, M. *J. Chromatogr. A* **2011**, *1218*, 5146.
(6) McLaren, D. G.; Miller, P. L.; Lassman, M. E.; Castro-Perez, J. M.; Hubbard, B. K.; Roddy, T. P. *Anal. Biochem.* **2011**, *414*, 266.
(7) Uran, S.; Larsen, A.; Jacobsen, P. B.; Skotland, T. *J. Chromatogr. B* **2001**, *758*, 265.
(8) Harrabi, S.; Herchi, W.; Kallel, H.; Mayer, P. M.; Boukhchina, S. *Food Chem.* **2009**, *114*, 712.
(9) Pang, L. Q.; Liang, Q. L.; Wang, Y. M.; Ping, L.; Luo, G. A. *J. Chromatogr. B* **2008**, *869*, 118.
(10) Wang, C.; Xie, S. G.; Yang, J.; Yang, Q.; Xu, G. W. *Anal. Chim. Acta* **2004**, *525*, 1.
(11) Retra, K.; Bleijerveld, O. B.; van Gesteil, R. A.; Tielens, A. G. M.; van Hellemond, J. J.; Brouwers, J. F. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 1853.
(12) Sandra, K.; Pereira, A. D.; Vanhoenacker, G.; David, F.; Sandra, P. *J. Chromatogr. A* **2010**, *1217*, 4087.
(13) Shaner, R. L.; Allegood, J. C.; Park, H.; Wang, E.; Kelly, S.; Haynes, C. A.; Sullards, M. C.; Merrill, A. H. *J. Lipid Res.* **2009**, *50*, 1692.
(14) Cvačka, J.; Hovorka, O.; Jiroš, P.; Kindl, J.; Stránský, K.; Valterová, I. *J. Chromatogr. A* **2006**, *1101*, 226.
(15) Holčapek, M.; Lísa, M.; Jandera, P.; Kabátová, N. *J. Sep. Sci.* **2005**, *28*, 1315.
(16) Lísa, M.; Velínská, H.; Holčapek, M. *Anal. Chem.* **2009**, *81*, 3903.
(17) Lísa, M.; Netušilová, K.; Franěk, L.; Dvořáková, H.; Vrkoslav, V.; Holčapek, M. *J. Chromatogr. A* **2011**, *1218*, 7499.
(18) Lytle, C. A.; Gan, Y. D.; White, D. C. *J. Microbiol. Methods* **2000**, *41*, 227.
(19) Sommer, U.; Herscovitz, H.; Welty, F. K.; Costello, C. E. *J. Lipid Res.* **2006**, *47*, 804.
(20) Cvačka, J.; Svatoš, A. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2203.
(21) Al-Saad, K. A.; Siems, W. F.; Hill, H. H.; Zabrouskov, V.; Knowles, N. R. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 373.
(22) Fuchs, B.; Schiller, J. *European J. Lipid Sci. Technol.* **2009**, *111*, 83.
(23) Fuchs, B.; Schiller, J.; Suss, R.; Schurenberg, M.; Suckau, D. *Anal. Bioanal. Chem.* **2007**, *389*, 827.
(24) Rohlfing, A.; Muthing, J.; Pohlentz, G.; Distler, U.; Peter-Katalinic, J.; Berkenkamp, S.; Dreisewerd, K. *Anal. Chem.* **2007**, *79*, 5793.
(25) Stubiger, G.; Belgacem, O. *Anal. Chem.* **2007**, *79*, 3206.
(26) Stubiger, G.; Belgacem, O.; Rehulka, P.; Bicker, W.; Binder, B. R.; Bochkov, V. *Anal. Chem.* **2010**, *82*, 5502.
(27) Holčapek, M.; Jandera, P.; Zderadička, P.; Hrubá, L. *J. Chromatogr. A* **2003**, *1010*, 195.
(28) Lísa, M.; Holčapek, M. *Chem. Listy* **2005**, *99*, 195.
(29) Cai, S. S.; Short, L. C.; Syage, J. A.; Potvin, M.; Curtis, J. M. *J. Chromatogr. A* **2007**, *1173*, 88.
(30) Stubiger, G.; Pittenauer, E.; Belgacem, O.; Rehulka, P.; Widhalm, K.; Allmaier, G. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 2711.
(31) Ahn, E. J.; Kim, H.; Chung, B. C.; Kong, G.; Moon, M. H. *J. Chromatogr. A* **2008**, *1194*, 96.
(32) Berdeaux, O.; Juaneda, P.; Martine, L.; Cabaret, S.; Bretillon, L.; Acar, N. *J. Chromatogr. A* **2010**, *1217*, 7738.
(33) Hsu, F. F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 1036.
(34) Quehenberger, O.; Armando, A. M.; Brown, A. H.; Milne, S. B.; Myers, D. S.; Merrill, A. H.; Bandyopadhyay, S.; Jones, K. N.; Kelly, S.; Shaner, R. L.; Sullards, C. M.; Wang, E.; Murphy, R. C.; Barkley, R. M.; Leiker, T. J.; Raetz, C. R. H.; Guan, Z. Q.; Laird, G. M.; Six, D. A.; Russell, D. W.; McDonald, J. G.; Subramaniam, S.; Fahy, E.; Dennis, E. A. *J. Lipid Res.* **2010**, *51*, 3299.
(35) Takatera, A.; Takeuchi, A.; Saiki, K.; Morisawa, T.; Yokoyama, N.; Matsuo, M. *J. Chromatogr. B* **2006**, *838*, 31.
(36) Fauland, A.; Kofeler, H.; Trotzmuller, M.; Knopf, A.; Hartler, J.; Eberl, A.; Chitraju, C.; Lankmayr, E.; Spener, F. *J. Lipid Res.* **2011**, *52*, 2314.
(37) Sato, Y.; Nakamura, T.; Aoshima, K.; Oda, Y. *Anal. Chem.* **2010**, *82*, 9858.
(38) Schuhmann, K.; Almeida, R.; Baumert, M.; Herzog, R.; Bornstein, S. R.; Shevchenko, A. *J. Mass Spectrom.* **2012**, *47*, 96.
(39) Taguchi, R.; Ishikawa, M. *J. Chromatogr. A* **2010**, *1217*, 4229.
(40) Schiller, J.; Arnold, K. *Med. Sci. Monit.* **2002**, *8*, MT205.
(41) Spyros, A.; Dais, P. *Prog. Nucl. Magn. Reson. Spectrosc.* **2009**, *54*, 195.
(42) Lísa, M.; Holčapek, M. *J. Chromatogr. A* **2008**, *1198*, 115.
(43) Lísa, M.; Holčapek, M.; Boháč, M. *J. Agric. Food Chem.* **2009**, *57*, 6888.
(44) Folch, J.; Lees, M.; Stanley, G. H. S. *J. Biol. Chem.* **1957**, *226*, 497.
(45) Bligh, E. G.; Dyer, W. J. *Can. J. Biochem. Physiol.* **1959**, *37*, 911.
(46) Matyash, V.; Liebisch, G.; Kurzchalia, T. V.; Shevchenko, A.; Schwudke, D. *J. Lipid Res.* **2008**, *49*, 1137.
(47) Ramstedt, B.; Leppimaki, P.; Axberg, M.; Slotte, J. P. *Eur. J. Biochem.* **1999**, *266*, 997.
(48) Hsu, F. F.; Bohrer, A.; Turk, J. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 516.