Journal of Chromatography A, 1450 (2016) 76–85

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Retention behavior of lipids in reversed-phase ultrahigh-performance liquid chromatography–electrospray ionization mass spectrometry

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ARTICLE INFO

Article history:
Received 4 March 2016
Received in revised form 10 April 2016
Accepted 29 April 2016
Available online 3 May 2016

Keywords:
Lipids
Lipidomics
Ultrahigh-performance liquid chromatography
Mass spectrometry
Human plasma
Retention behavior

ABSTRACT

Reversed-phase ultrahigh-performance liquid chromatography (RP-UHPLC) method using two 15 cm sub–2 μm particles octadecylsilica gel columns is developed with the goal to separate and unambiguously identify a large number of lipid species in biological samples. The identification is performed by the coupling with high-resolution tandem mass spectrometry (MS/MS) using quadrupole – time-of-flight (QTOF) instrument. Electrospray ionization (ESI) full scan and tandem mass spectra are measured in both polarity modes with the mass accuracy better than 5 ppm, which provides a high confidence of lipid identification. Over 400 lipid species covering 14 polar and nonpolar lipid classes from 5 lipid categories are identified in total lipid extracts of human plasma, human urine and porcine brain. The general dependences of relative retention times on relative carbon number or relative double bond number are constructed and fit with the second degree polynomial regression. The regular retention patterns in homologous lipid series provide additional identification point for UHPLC/MS lipidomic analysis, which increases the confidence of lipid identification. The reprocessing of previously published data by our and other groups measured in the RP mode and ultrahigh-performance supercritical fluid chromatography on the silica column shows more generic applicability of the polynomial regression for the description of retention behavior and the prediction of retention times. The novelty of this work is the characterization of general trends in the retention behavior of lipids within logical series with constant fatty acyl length or double bond number, which may be used as an additional criterion to increase the confidence of lipid identification.

1. Introduction

Lipids fulfill multiple essential roles within all eukaryotic cells in living organisms [1]. Living cells contain thousands of different lipid molecules that fall into eight lipid categories according to LIPID MAPS classification, namely fatty acyls, glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids, saccharolipids and polyketides [1–5] containing many classes and subclasses. The dysregulation of the lipid metabolism contributes to numerous serious human diseases, such as obesity, diabetes, cardiovascular diseases and cancer. Therefore, they are investigated as possible biomarkers of these diseases [6–9].

Lipidomic analysis starts with the liquid – liquid lipid extraction from biological materials using organic solvents. The most frequently used extraction procedures are based on chloroform – methanol – water systems according to Folch et al. [10] or Bligh and Dyer [11], or the extraction using methyl *tert*-butyl ether solvent instead of chloroform [12]. Gas chromatography–mass spectrometry is an established approach for fatty acyl profiling [13]. Various analytical strategies are used in the lipidomic analysis using nontargeted and targeted lipidomic approaches [14–18]. Another possible division of lipidomic approaches is according to used analytical methodology. Shotgun lipidomics using triple quadrupole instruments and characteristic precursor ion and neutral loss scans [19–21] is well established approach for the fast quantitation of lipid molecular species from extracts of biological samples without a chromatographic separation. The second approach is the use of liquid chromatography–mass spectrometry (LC/MS) coupling, where various chromatographic modes can be selected depending on the required type of separation, such as reversed-phase (RP) LC [22–25], normal-phase (NP) LC [26,27], hydrophilic interaction liquid chromatography (HILIC) [14,15], silver-ion LC [13,28,29] and chiral LC [30,31]. The RP separation mode coupled with MS is widely used in a comprehensive lipidomic analysis to identify individual molecular species in different biological samples [22,32–34], where lipids are separated according to the length of fatty acyl chains and

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http://dx.doi.org/10.1016/j.chroma.2016.04.082
0021-9673/

the number and position of double bonds (DB) [22]. In the RP mode, mobile phases are typically composed of mixture of water containing volatile buffers and polar organic solvents, such as methanol, acetonitrile and 2-propanol. RP mode provides intra- and interclass separation of lipid species, especially in ultrahigh-performance liquid chromatography (UHPLC) configuration [22], but on the other hand the quantitation is more demanding, because the lipid class internal standards do not coelute with analytes unlike the lipid class separation in HILIC or NP modes. NP-LC is particularly suitable for the separation of nonpolar lipid classes, where individual nonpolar lipid classes are separated based on their polarity [27]. HILIC separation allows the lipid class separation, where individual lipid classes are separated according to their polarity and electrostatic interactions [14,15]. HILIC and RP modes have relatively good complementarity of retention mechanisms, therefore various modes of their 2D-LC coupling have been already applied for the lipidomic analysis [35–37]. The HILIC-like separation can be also achieved in ultrahigh-performance supercritical fluid chromatography (UHPSFC) on silica columns, but with shorter analysis time and more efficient separation [38]. The silver-ion LC is a special chromatographic mode based on the formation of weak reversible complexes of silver ions with π electrons of DB, which enables the resolution of triacylglycerols (TG) and diacylglycerols (DG) isomers differing in the number, positions and geometry of DB [13,28,29]. The most demanding separation task is a chiral resolution, which has been applied to TG enantiomers [30,31].

The main goal of our work is the study of the retention behavior of individual lipids in RP-UHPLC to describe general dependences of retention times on the carbon number (CN) and the DB number. For this purpose, RP-UHPLC method with two C18 columns in series is optimized and coupled to high-resolution MS/MS to unambiguously identify the large number of lipids. The retention data are collected for lipid extracts of human plasma, human urine and porcine brain samples. Individual lipid species are identified based on accurate *m/z* values of their molecular adducts and characteristic fragment ions in their MS/MS spectra measured in positive- and negative-ion modes. Relative dependences of retention times on the CN or the DB number are fitted with the second degree polynomial regressions.

2. Material and methods

2.1. Chemicals and standards

Acetonitrile, 2-propanol, methanol, (all LC/MS gradient grade), hexane (HPLC grade), chloroform (HPLC grade, stabilized by 0.5–1% ethanol), ammonium acetate, sodium chloride, sodium methoxide, standards of cholest-5-en-3ß-yl octadecanoate [cholesteryl ester (CE) 18:1] and 3ß-hydroxy-5-cholestene [cholesterol (Chol)] were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was prepared with a Milli-Q Reference Water Purification System (Molsheim, France). Standards of polar lipid classes containing C18:1(9Z) fatty acyl(s), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), ceramide (Cer), and sphingomyelin (SM d18:1/12:0), ceramide (Cer d18:1/12:0), cholesteryl (d7) ester (Chol d7 16:0) and oleic acid-d9 (FA d9 18:1) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Nonpolar lipid standards of TG 18:1/18:1/18:1, TG 19:1/19:1/19:1 and DG 18:1/18:1 were purchased from Nu-Chek Prep (Elysian, MN, USA). The lipid nomenclature follows the shorthand notation for lipid structures published by Liebisch et al. [39] and the LIPID MAPS [2] classification system. Samples of human plasma and urine were obtained from healthy volunteers from the research team. Porcine brain was obtained from the local store.

2.2. Sample preparation

Blood was collected to heparin-lithium tubes and ultracentrifuged to obtain plasma. The total lipid extracts of human plasma, human urine and porcine brain tissue were prepared according to Folch procedure [10] using the chloroform – methanol – water solvent system with minor modifications [14,15]. Human plasma (50 μL) was homogenized with 3 mL of the chloroform – methanol (2:1, v/v) mixture, while porcine brain tissue (50 mg) and human urine (2 mL) were homogenized with 6 mL of the chloroform-methanol mixture (2:1, v/v) in the ultrasonic bath at 40 °C for 10 min. Then, deionized water (600 μL for human plasma and 1200 μL for porcine brain) was added (no additional water for human urine), and the mixture was centrifuged at 3000 rpm for 3 min under ambient conditions. The chloroform (bottom) layer containing lipids was collected, evaporated by a gentle stream of nitrogen and redissolved in 1 mL of the chloroform – 2-propanol (1:1, v/v) mixture for the RP-UHPLC/ESI-MS analysis.

2.3. RP-UHPLC conditions

Experiments were performed with an Agilent 1290 Infinity series (Agilent Technologies, Santa Clara, CA, USA). Two identical Acquity UPLC BEH C_{18} columns (150 mm × 2.1 mm, 1.7 μm, Waters, Milford, MA, USA) were coupled in series and used for the separation of total lipid extracts under the following conditions. Flow rate 180 μL/min, injection volume 2 μL, column temperature 40 °C, mobile phase gradient 0 min – 21.5% of solvent A and 78.5% of solvent B, 160 min – 100% of solvent B, where solvent A was 5 mmol/L aqueous ammonium acetate and solvent B was the mixture of 99.5% of acetonitrile – 2-propanol (1:2, v/v) and 0.5% water, the concentration of ammonium acetate in solvent B was also 5 mmol/L. The system backpressure reached 1000 bar during the gradient analysis.

2.4. ESI-MS conditions

The hybrid QTOF mass spectrometer (micrOTOF-Q, Bruker Daltonics, Bremen, Germany) with an ESI source was used as the detector under the following conditions: capillary voltage 4.5 kV, nebulizing gas pressure 1.0 bar, drying gas flow rate 8 L/min and drying gas temperature 200 °C. ESI mass spectra were measured in the range of *m/z* 50–1500 in positive- and negative-ion modes. Argon as the collision gas at the collision energy of 20–25 eV was used for MS/MS experiments. MS/MS spectra are recorded in both polarity modes using the data independent mode for all ions exceeding the instrumental intensity threshold of 10^4. The external calibration of the mass scale was performed with sodium formate clusters before individual measurements together with the internal recalibration using the most abundant known lipids. The data were acquired using the DataAnalysis software (Bruker Daltonics).

3. Results and discussion

3.1. RP-UHPLC separation of lipids

The goal of our RP-UHPLC analysis is the identification of the large number of lipid species, which is then used for the study of the retention behavior of individual lipids in logical series with the constant number of carbon atoms or DB. For this purpose, we have selected the coupling of two 15 cm C18 columns with sub–2 μm particles (150 mm × 2.1 mm, 1.7 μm) and aqueous ammonium acetate – acetonitrile – 2-propanol gradient, which

Table 1
Numbers of identified lipids in studied biological samples.

Lipid class	Human plasma	Human urine	Porcine brain	Total
FA	31	20	39	39
LPC	10	0	8	11
LPE	5	0	6	6
SM	33	1	21	33
PI	13	0	6	13
PG	1	0	5	6
PE	30	18	28	33
PC	50	2	40	57
Sulfatides	0	0	7	7
Cer	0	0	13	13
HexCer	0	0	12	12
DG	12	0	11	15
Chol + SE	22	0	9	22
TG	139	6	106	149
Total	346	47	311	416

provided the best performance in our previous work on 2D-LC/MS using single C18 column in the first dimension [35]. Optimized conditions with two C18 columns (details in Section 2) result in higher peak capacity ($P_C = 377$ calculated as $Pc = 1 + (t_g/1.7 \times w_{1/2})$ [40]) in comparison to one C18 column ($P_C = 140$) [40]. The number of real identifications in case of optimized RP separation (416) is 10% higher than the theoretical peak capacity due to the use of reconstructed ion currents. The analysis time of 160 min is acceptable for the study of retention behavior but not for the routine lipidomic analysis, where faster gradients on shorter columns are preferred.

Individual lipid species are separated in the RP mode according to the CN and the DB number, which is defined as the equivalent carbon number (ECN) and calculated as the total CN of all fatty acyls minus two times the DB number (ECN = CN-2DB). The ECN model has been initially developed for TG and other nonpolar lipids [41–43], but it is also applicable for polar lipid classes [35,44]. Few exceptions from this rule can be observed for phospholipids containing the combination of highly polyunsaturated and saturated fatty acyls, which are retained more strongly and elute in higher ECN groups, *e.g.*, PC 18:0/20:5 and PC 18:0/22:6 (both with ECN 28) elute in the group with ECN 30. The similar behavior is observed for all polar lipid classes in RP-UHPLC. Polar lipid classes (FA, LPC, LPE, SM, PI, PG, PE, PC, sulfatides, Cer, and HexCer) and DG are eluted over a broad range of retention times. Nonpolar lipids (CE and TG) have the highest retention in the RP mode, while the most polar lysophospholipids are retained the least (Fig. 1 and Table S1).

3.2. Identification of individual lipids using RP-UHPLC/ESI-MS

The identification of individual lipids is based on our previous experiences with retention, ionization and fragmentation behavior of various classes of lipids [6,14,15,35,38,44,45]. Table S1 lists only unambiguously identified lipids based on retention behavior characteristics, accurate m/z values (better than 5 ppm in most cases) and characteristic fragmentation behavior in both polarity modes. In total, 416 lipid species from 14 lipid classes including FA, LPC, LPE, SM, PI, PG, PE, PC, sulfatides, Cer, HexCer, DG, Chol, CE and TG have been positively identified in total lipid extracts of human plasma, human urine and porcine brain (Tables 1 and S1). The Venn's diagram shows differences among studied samples, how many lipids are shared among individual matrices and what identifications are unique for some samples (Fig. S2). The lipid species level (*e.g.*, PE 36:4) is the first annotation style based just on retention times and accurate m/z values in full-scan mass spectra. The fatty acyl/alkyl level (*e.g.*, PE 16:0_20:4) is used when the information of attached fatty acyls is known from MS or MS/MS spectra, but without *sn*-differentiation. The slash separator (*e.g.*, PE 16:0/20:4) indicates the known preference of *sn*-position according to the shorthand lipid notation recommended by Liebisch et al. [39]. Reported lipids are also correlated with our previous papers on the lipidomic characterization of biological tissues [6,44,45] and body fluids [38] to confirm the identification and avoid any error in the list of identified lipids.

The identification of FA, PI, PE, sulfatides and PG classes is based on deprotonated molecules $[M-H]^-$ in negative-ion ESI mass spectra. The characteristic fragment ion for lipid classes containing choline moiety (LPC, SM and PC) is m/z 184 in the positive-ion mode. Further observed ions for lipid classes containing choline moiety are protonated $[M+H]^+$ and sodiated $[M+Na]^+$ molecules in the positive-ion mode. The neutral loss of $\Delta m/z$ 141 is typical for positive-ion ESI mass spectra of PE, while negative-ion ESI-MS/MS provides information on the position of attached fatty acyls based on the ratio of $[R_1COO]^-/[R_2COO]^-$ ions, where $[R_2COO]^-$ ion is more abundant. This ratio is changed for the combination of saturated and highly polyunsaturated (C20:5 or C22:6) fatty acyls in PE due to the formation of $[R_i]^-$ ions from $[R_iCOO]^-$ ions caused by the neutral loss of carbon dioxide [46]. This approach is used for other phospholipid classes as well, as discussed in more details in our previous works [14,15]. The regioisomeric determination of lysophospholipids (LPL) is based on the knowledge of retention order of 1-LPL and 2-LPL standards. Observed ions for DG are protonated $[M+H]^+$ and sodiated $[M+Na]^+$ molecules and loss of water $[M+H-H_2O]^+$ in the positive-ion mode. The characteristic fragment ion for lipid species containing cholesterol (Chol and CE) is m/z 369. ESI mass spectra of Cer and HexCer exhibit characteristic ions related to the type of base, *e.g.*, m/z 264 for 18:1 and m/z 266 for 18:0, which enables the accurate identification of fatty acyl position. The interpretation of positive-ion ESI mass spectra of TG is based on $[M+NH_4]^+$ and $[M+H-R_iCOOH]^+$ ions. In some cases, more lipids with identical formula are reported with different retention times, such as FA 18:1 (9.8 and 10.4 min), FA 17:0 (10.6 and 11.3 min), FA 21:0 (21.2 and 22.8 min), PC 18:1/22:6, (40.0 and 40.6 min), PC 16:0/20:4 (41.0 and 44.0 min), *etc*. The most likely explanation for observed isomers is different positions of DB for unsaturated fatty acyls and branching for saturated fatty acyls, but we cannot report this information in tables due to missing verification with standards.

Fig. S1 shows TIC chromatograms of RP-UHPLC/ESI-MS analysis of the lipid standard mixture in positive-ion (Fig. S1A) and negative-ion (Fig. S1B) modes. The lipid standard mixture contains CE, Cer, Chol, DG, FA, GlcCer, LacCer, LPC, LPE, PA, PC, PE, PG, PS, SM and TG lipid class representatives with 18:1 fatty acyls and it is used throughout this work for the method development. Table S2 reports the major ions for individual lipid classes observed in both polarity modes. Fig. 1 depicts TIC chromatograms of measured lipid extracts: **A/**human plasma containing 11 lipid classes (FA, LPC, LPE, SM, PI, PG, PE, PC, DG, CE and TG), **B/**human urine containing 5 lipid classes (FA, SM, PE, PC and TG), and **C/**porcine brain containing 14 lipid classes (FA, LPC, LPE, SM, PI, PG, PE, PC, sulfatides, Cer, HexCer, DG, CE and TG). The porcine brain extract is selected due to known lipidomic complexity, which yields the detection of additional lipid classes not occurring in other studied samples, *i.e.,* sulfatides, Cer and HexCer. The type of glycosylation cannot be determined, therefore HexCer annotation is used instead of GluCer for the standard. These RP conditions with very long gradient time are purposely selected for the description of retention behavior of various lipid classes, but our conclusions are also applicable for shorter gradients used in the routine lipid analysis. This identification list will be used as the supporting information in our future quantitative lipidomic studies using either faster UHPLC/MS or shotgun MS approaches.

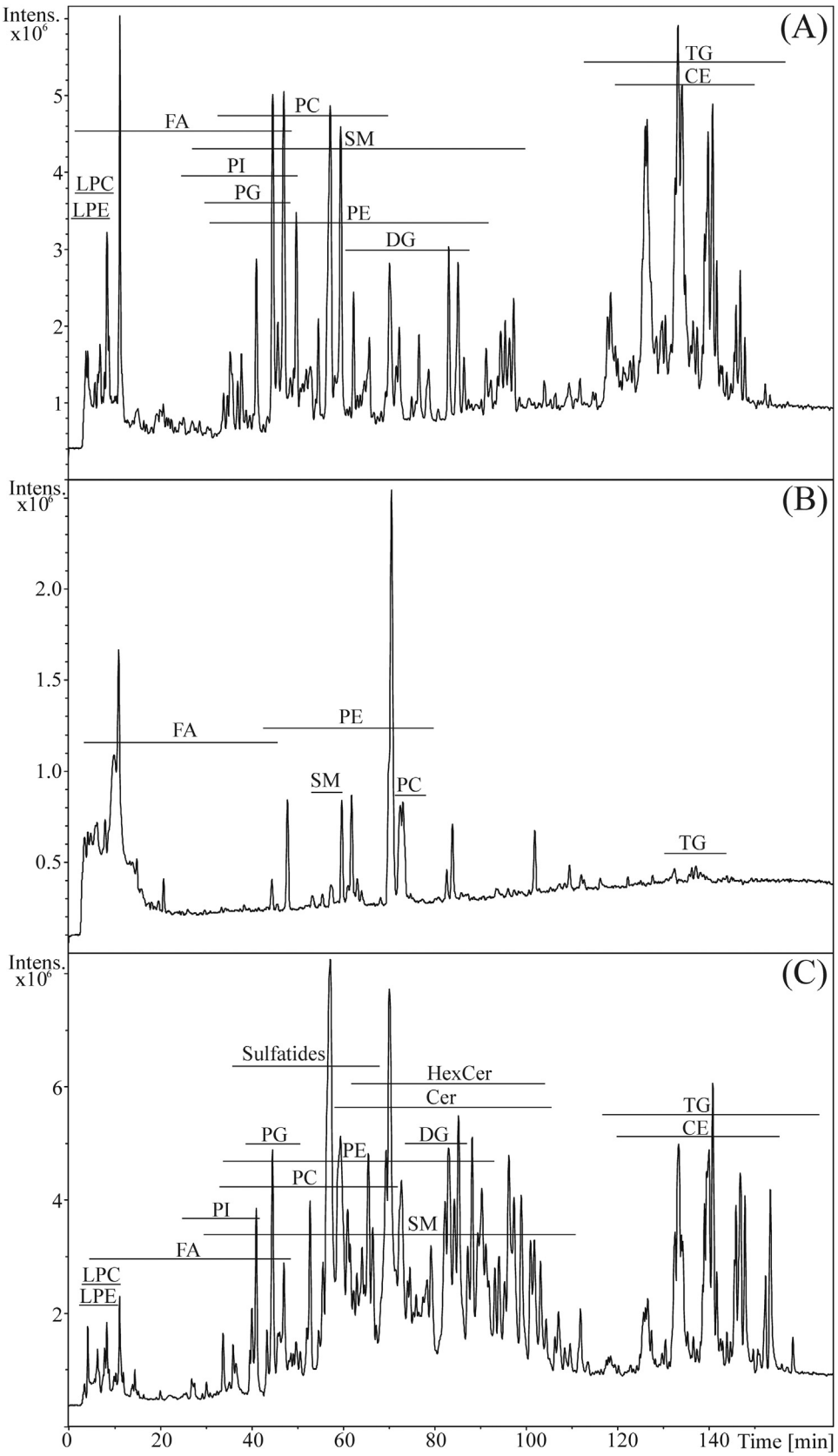

Fig. 1. Positive-ion RP-UHPLC/ESI-MS total ion current chromatograms of total lipid extracts of: (**A**) human plasma, (**B**) human urine, and (**C**) porcine brain. Conditions: two Acquity UPLC BEH C_{18} columns (150 mm × 2.1 mm, 1.7 μm) coupled in series, flow rate 180 μL/min, injection volume 2 μL, column temperature 40 °C, mobile phase gradient of acetonitrile, 2-propanol and 5 mmol/L aqueous ammonium acetate (other details in Material and methods). FA are detected only in the negative-ion mode. *Abbreviations:* CE – cholesteryl ester, Cer – ceramide, DG – diacylglycerol, FA – fatty acid, HexCer – hexosyl ceramide, LPC – lysophosphatidylcholine, LPE – lysophosphatidylethanolamine, PC – phosphatidylcholine, PE – phosphatidylethanolamine, PI – phosphatidylinositol, PG – phosphatidylglycerol, SM – sphingomyelin, and TG – triacylglycerol.

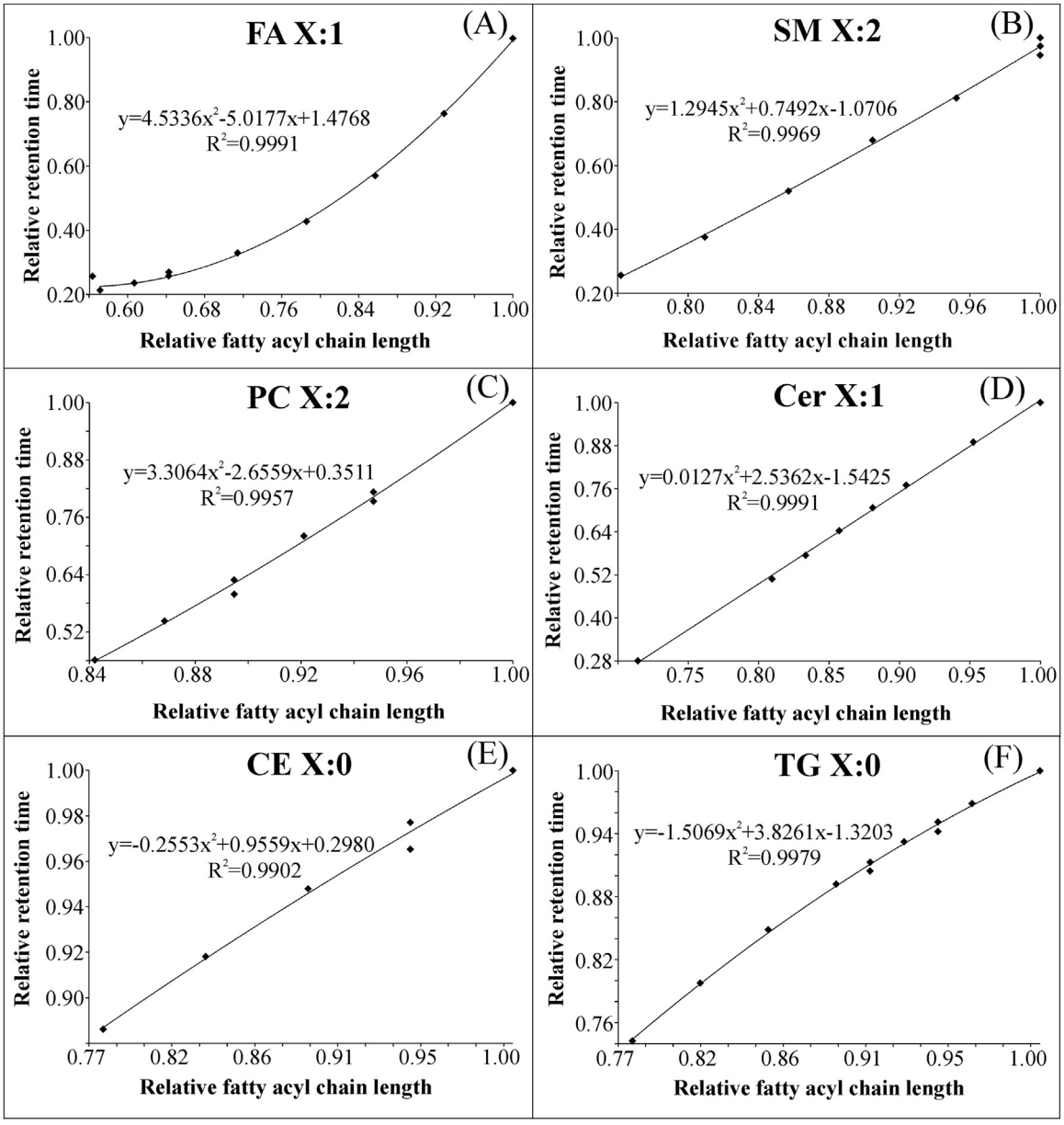

Fig. 2. Plots of polynomial dependences of relative retention times of lipids on the relative CN: (**A**) FA X:1 (X is from 16 to 28), (**B**) SM X:2 (X is from 32 to 42), (**C**) PC X:2 (X is from 32 to 38), (**D**) Cer X:1 (X is from 34 to 42), (**E**) CE X:0 (X is from 14 to 18), and (**F**) TG X:0 (X is from 42 to 54).

3.3. *Study of retention behavior of various lipid classes*

For all lipid classes studied, dependences of retention times on the CN (X) or the DB number (Y) can be fitted with the second degree polynomial regression $y = ax^2 + bx + c$. These curves can be plotted either as retention times *vs.* CN (or DB number) or as relative retention times *vs.* relative CN (or relative DB number). The use of relative units is more universal, because coefficients of polynomial regression do not depend on the column length or diameter. The highest value in a particular plot is equal to 1.00 and other values are recalculated. Typical dependences are shown in Fig. 2 for dependences on the CN for **A**/FA X:1, **B**/SM X:2, **C**/PC X:2, **D**/Cer X:1, **E**/CE X:0, and **F**/TG X:0. Table 2 shows that correlation coefficients R^2 are mostly better than 0.99, especially for curves containing more data points. Fig. 3 and Table 3 summarize results of dependences on DB number for: A/FA 18:Y, B/PI 36:Y, C/PE 40:Y, D/PC 34:Y, E/CE 18:Y, and F/TG 56:Y. Correlation coefficients are slightly worse for DB dependences, which is caused by a lower number of data points per curve compared to fatty acyl chain dependences and also multiple points caused by lipids with identical CN:DB composition but different fatty acyl composition. Polynomial equations still enable satisfactory prediction of retention times, because relative errors of retention time calculations using polynomial equations are better than 5%, which confirms the applicability of polynomial regressions as the supplementary identification criterion in addition to MS data.

Polynomial equations can be also applied for the prediction of retention times of missing lipids in their logical series. Fig. 4 shows an illustration how this approach can be used for the identification of unknown lipid inside the measured series. These lipid standards are not occurring in measured biological samples, therefore these standards are added and then experimental retention times are correlated with predicted retention times with relative errors lower than 5%, as illustrated on the example of TG 19:1/19:1/19:1 (Fig. 4**A**). Mass accuracies of molecular adducts (Fig. 4**B**) and product ions (Fig. 4**C**) are better than 5 ppm, which results in very high confidence of such identification. Other examples are shown in

Table 2
Parameters of polynomial regressions $y = ax^2 + bx + c$ of relative retention times of individual lipid series on the relative CN.

Lipid series	a	b	c	R^2	X
FA X:0	3.7991	−3.8555	1.0368	0.9949	12, 14, 15, 16, 17, 18, 20, 21,22, 23, 24, 25, 26
FA X:1	4.5336	−5.0177	1.4768	0.9991	16, 17, 18, 20, 22, 24, 26, 28
FA X:3	6.9790	−9.0233	3.0443	0.9910	18, 20, 22
SM X:0	−1.2578	5.3851	−3.1273	1.0000	34, 36, 40
SM X:1	0.3365	2.1286	−1.4651	0.9960	30, 32, 33, 34, 36, 38, 39, 40, 41, 42, 43
SM X:2	1.2945	0.7492	−1.0706	0.9969	32, 34, 36, 38, 40, 42
PI X:1	21.0477	−35.6225	15.5747	1.0000	32, 34, 36
PE X:1	−6.9475	15.1790	−7.2317	1.0000	34, 36, 40
PE X:2	−2.2415	7.0608	−3.8193	1.0000	34, 36, 40
PE X:4	−11.2849	24.7328	−12.4479	1.0000	36, 38, 40
PE X:5	3.0113	−1.6411	−0.3695	0.9999	36, 38, 40, 42
PE X:6	44.5140	−82.0360	38.5210	1.0000	38,40,42
PC X:1	6.2750	−8.6052	3.3241	0.9952	32, 34, 36
PC X:2	3.3064	−2.6559	0.3511	0.9957	32, 33, 34, 35, 36, 38
PC X:3	6.3464	−8.0278	2.6421	0.9626	34, 36, 38
PC X:4	−8.3879	19.8431	−10.4552	0.9458	36, 38, 40
Sulfatides X:1	1.6537	0.4987	−1.1487	1.0000	36, 38, 41, 42
Sulfatides X:2	−1.5463	6.9934	−4.4471	1.0000	42, 43, 44
Cer X:1	0.0127	2.5362	−1.5425	0.9991	34, 35, 36, 37, 38, 40, 42
Cer X:2	−2.7252	7.9161	−4.1909	1.0000	36, 38, 42
HexCer X:0	1.4186	−1.3316	0.9035	0.9620	36, 40, 42, 44
CE X:0	−0.2553	0.9559	0.2980	0.9902	14, 15, 16, 17, 18
CE X:1	−0.2321	0.9920	0.2383	0.9994	14, 16, 17, 18
TG X:0	−1.5069	3.8261	−1.3203	0.9979	42, 44, 46, 48, 49, 50, 51, 52, 54
TG X:1	−1.7123	4.2233	−1.5138	0.9921	44, 46, 48, 49, 50, 52, 53, 54, 56
TG X:2	−1.5937	4.0467	−1.4598	0.9930	46, 48, 49, 50, 51, 52, 53, 54, 56, 58
TG X:3	−0.9837	3.0144	−1.0457	0.9787	48, 49, 50, 51, 52, 53, 54, 56, 58
TG X:4	−0.1748	1.8097	−0.6370	0.9219	50, 52, 54, 56
TG X:5	1.6848	−1.6437	0.9517	0.9339	52, 54, 56

Table 3
Parameters of polynomial regressions $y = ax^2 + bx + c$ of relative retention times of individual lipid series on the relative DB number.

Lipid series	a	b	c	R^2	Y
FA 18:Y	0.5002	−1.2249	1.0007	0.9934	0, 1, 2, 3
FA 20:Y	0.8193	−1.6202	0.9707	0.9882	0, 1, 2, 3, 4, 5
FA 22:Y	0.7217	−1.5616	1.0041	0.9998	0, 1, 3, 4, 5, 6
FA 24:Y	1.3396	−2.2112	1.0000	1.0000	0, 1, 6
LPC 18:Y	0.4519	−1.0203	0.9452	0.9799	0, 1, 2
LPE 18:Y	−0.2105	−0.0351	1.0000	1.0000	0, 1, 2
SM 34:Y	−0.1743	−0.1677	1.0000	0.9830	0, 1, 2
SM 40:Y	−0.0247	−0.1926	1.0000	0.9870	0, 1, 2
SM 42:Y	0.1313	−0.5940	1.1834	0.9783	1, 2, 3
PI 36:Y	0.6095	−1.3926	1.3181	0.9863	1, 2, 3, 4
PI 38:Y	0.4390	−1.5755	1.6932	0.9622	3, 4, 5, 6
PE 34:Y	0.0870	−0.4319	1.0000	1.0000	0, 1, 2
PE 36:Y	0.0886	−0.7954	1.1483	0.9725	1, 2, 3, 4, 5
PE 38:Y	0.6683	−1.7895	1.7471	0.9208	3, 4, 5, 6
PE 40:Y	−0.3108	−0.4074	1.0724	0.9977	1, 2, 4, 5, 6, 7
PC 32:Y	0.0229	−0.4313	1.0000	0.9895	0, 1, 2
PC 34:Y	0.1973	−0.6858	0.9998	0.9878	0, 1, 2, 3
PC 36:Y	0.1975	−0.9098	1.1679	0.9830	1, 4, 5
PC 38:Y	0.0965	−0.8828	1.2945	0.9658	2, 3, 4, 5, 6
DG 34:Y	0.0535	−0.3395	1.0000	0.9980	0, 1, 2
DG 36:Y	0.1018	−0.5536	1.1673	0.9978	1, 2, 3
CE 18:Y	0.0210	−0.1889	1.0000	0.9995	0, 1, 2, 3
CE 20:Y	−0.2639	0.1622	0.9977	0.9945	3, 4, 5
TG 46:Y	−0.0151	−0.1126	1.0000	0.9828	0, 1, 2
TG 48:Y	0.0069	−0.1685	1.0002	0.9731	0, 1, 2, 3
TG 50:Y	−0.0221	−0.1716	0.9977	0.9744	0, 1, 2, 3, 4
TG 52:Y	0.0014	−0.2243	0.9999	0.9854	0, 1, 2, 3, 4, 5
TG 54:Y	−0.0160	−0.2182	0.9965	0.9720	0, 2, 3, 4, 5, 6
TG 56:Y	−0.0263	−0.2148	1.0268	0.9856	1, 2, 3, 4, 5, 6, 7

Table S3 for FA d9 18:1 (relative error 2.3%), SM d18:1/12:0 (4.9%), Cer d18:1/12:0 (1.9%) and CE d7 16:0 (1.8%).

The prediction of retention times with defined accuracy better than 5% yields additional identification point together with accurate *m*/*z* values of (de)protonated molecules, molecular adducts and diagnostic fragment ions, which increases the confidence of identification. The regularity in retention times of homologous series is known for long time, but we report here for the first time the well-defined identification criteria for lipids with possible applicability as additional identification point similarly as for LC/MS determination of compounds in forensic toxicology and doping analysis [47].

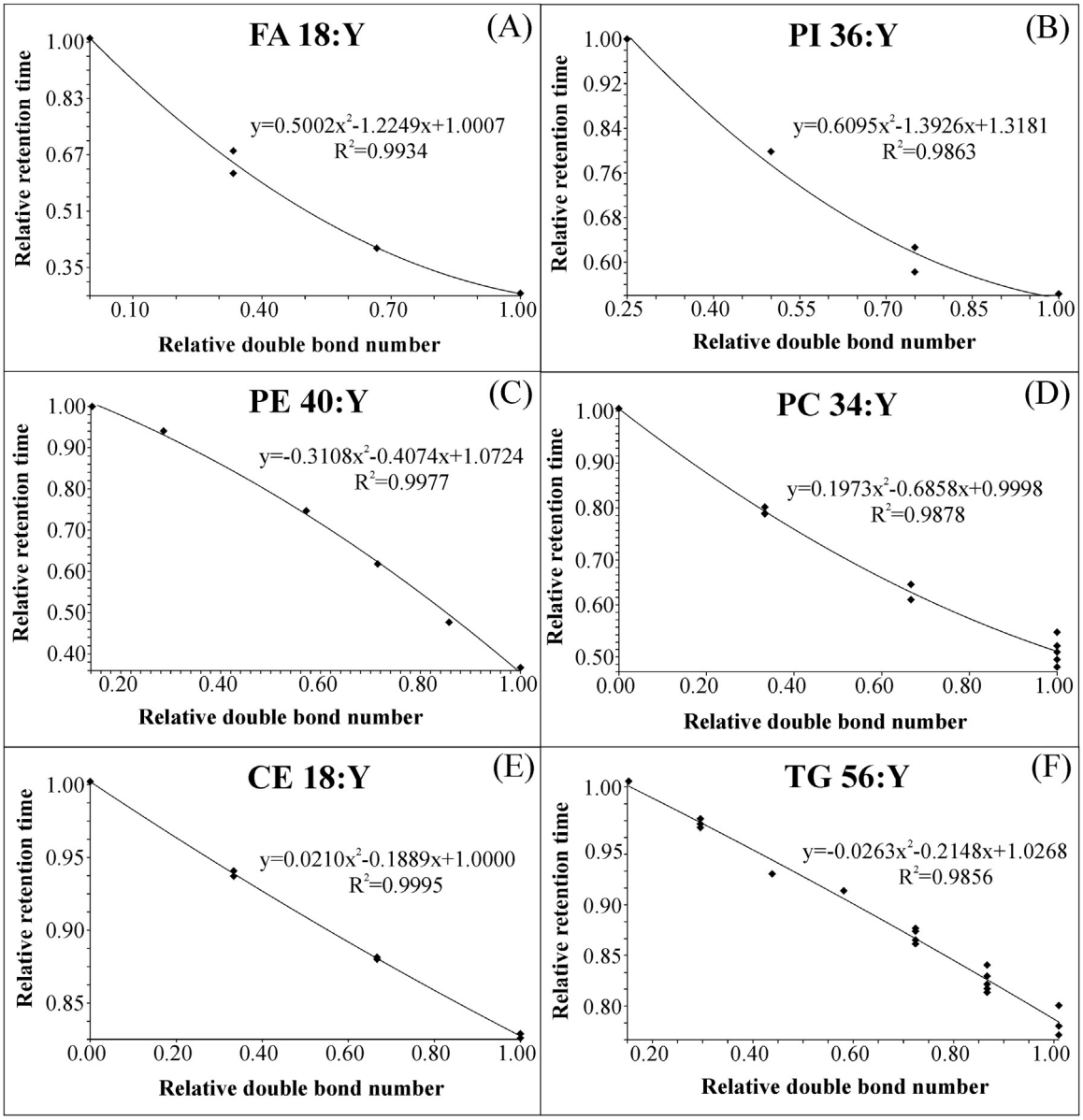

Fig. 3. Plots of polynomial dependences of relative retention times of lipids on the relative DB number: (**A**) FA 18:Y (Y is from 0 to 3), (**B**) PI 36:Y (Y is from 1 to 4), (**C**) PE 40:Y (Y is from 1 to 7), (**D**) PC 34:Y (Y is from 0 to 3), (**E**) CE 18:Y (Y is from 0 to 3), and (**F**) TG 56:Y (Y is from 1 to 7).

3.4. Comparison of retention behavior with other studies

The general applicability of our approach for the construction of retention times dependences has been tested on RP-LC/MS data previously published by a different group [48] and our UHPSFC/MS data [38] obtained by the chromatographic mode with the different retention mechanism. Results are illustrated on examples of **A/**PC X:2, **B/**TG X:1, **C/**PC 38:Y, and **D/**TG 58:Y in Fig. 5, Tables S4 and S5 for RP-LC and of **A/**PC X:1, **B/**TG X:1, **C/**PC 36:Y, and **D/**TG 54:Y in Fig. 6, Tables S6 and S7. Polynomial regressions have a general applicability for the correlation of relative retention times *vs.* the relative CN or relative DB number. In few cases, *a* quadratic coefficients are so small that these curves can be considered as the linear regression. Relative errors in case of UHPSFC data [38] are affected by very small retention times (*ca.* 1 min) and resulting negligible differences among individual TG (in the range of tens of milliseconds), but relative errors of retention time calculation are always better than 5% without any exception. We suggest this approach as other identification point to be generally used in UHPLC/MS and UHPSFC/MS to strengthen the advantage of chromatographic separation for the confident lipidomic identification based on the regularity in retention times of lipid homologous series.

4. Conclusions

The RP-UHPLC/ESI-MS method enables the separation and identification of large number of individual lipid species in human plasma, human urine and porcine brain samples. This method is applied for the study of retention behavior of various polar and nonpolar lipid classes, where polynomial dependences of relative retention times both on relative CN and relative DB number are observed. The regularity in the retention behavior of lipid homologous series is systematically studied, which results in the suggestion of the second degree polynomial regressions for the description of retention patterns of lipid logical series. Polynomial regressions of retention times can be applied as an additional criterion for the identification of unknown lipids in addition to MS and MS/MS data, which increases the confidence of LC/MS identi-

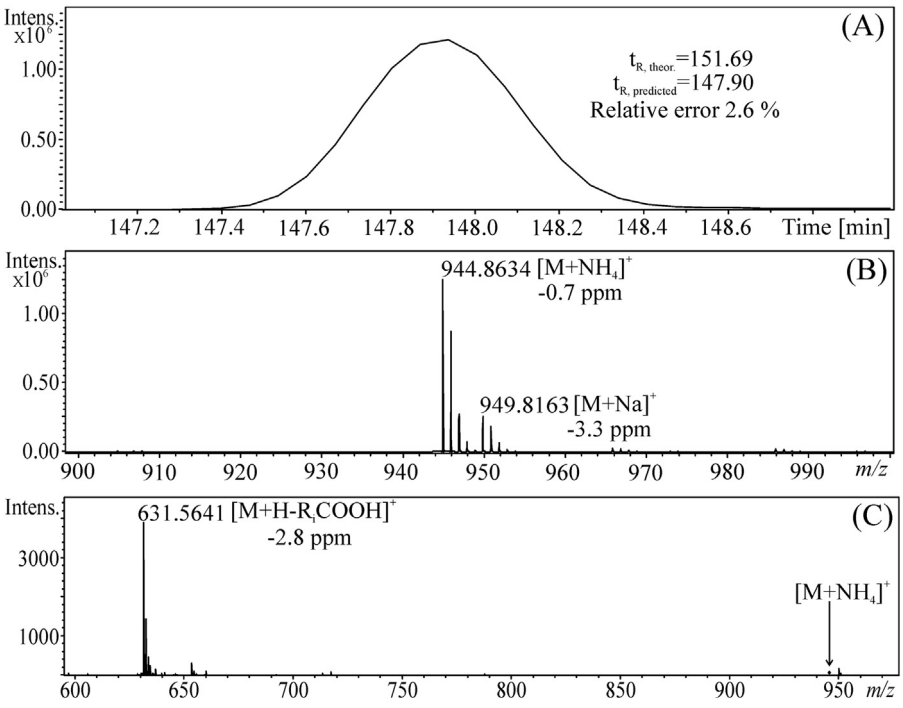

Fig. 4. RP-UHPLC/ESI-MS records of TG 19:1/19:1/19:1 standard: (**A**) reconstructed positive-ion chromatogram, (**B**) ESI-MS spectrum, and (**C**) MS/MS spectrum of $[M+NH_4]^+$ at *m*/*z* 944.8634.

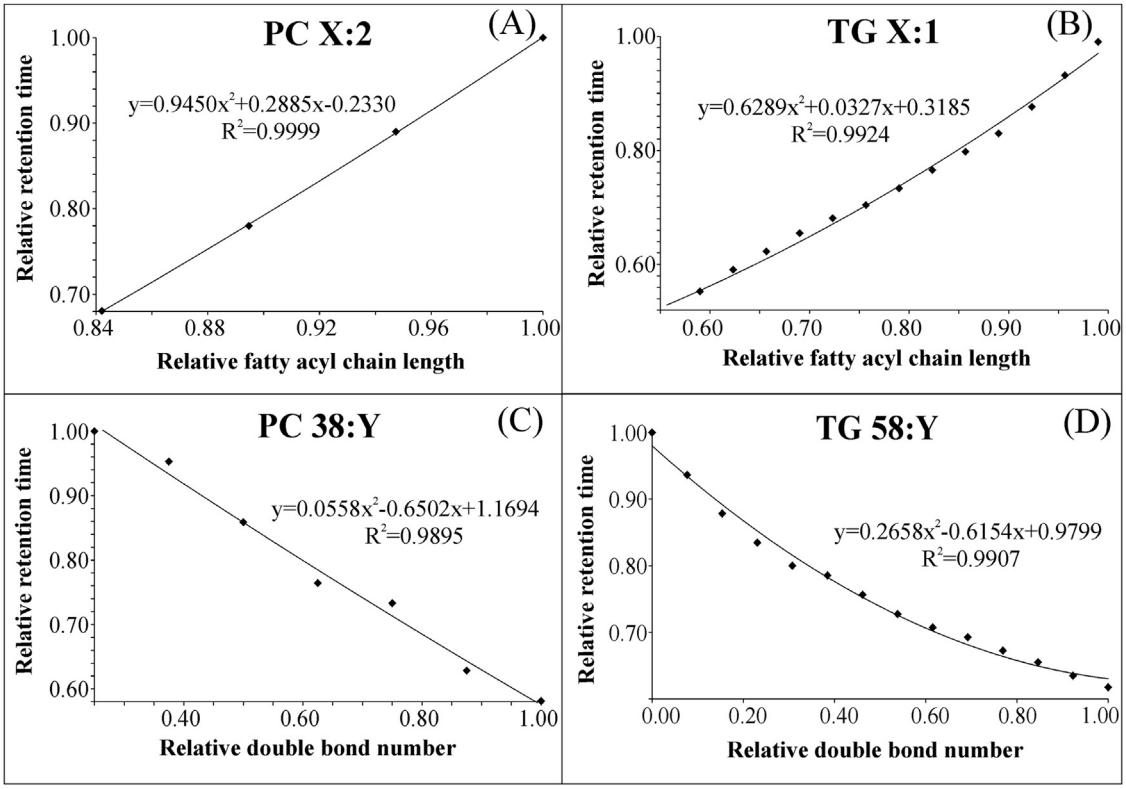

Fig. 5. Plots of polynomial dependences of relative retention times of PC and TG on the relative CN (X) and the relative DB number (Y) from RP-HPLC data [48]: (**A**) PC X:2 (X is from 32 to 38), (**B**) TG X:1 (X is from 34 to 60), (**C**) PC 38:Y (Y is from 2 to 8), and (**D**) TG 58:Y (Y is from 0 to 13).

fication. Another possible application is the prediction of retention times of missing lipids in their logical series, which may bring additional identifications of lipids at expected time windows, which is worthy mainly for trace species or lipids suffering from low ionization efficiencies, because a good quality MS and MS/MS data may not be available in such cases. The logical series with different fatty acyl lengths are based on the higher number of data points and they provide slightly better correlation coefficients, therefore they are recommended as the first choice for the identification.

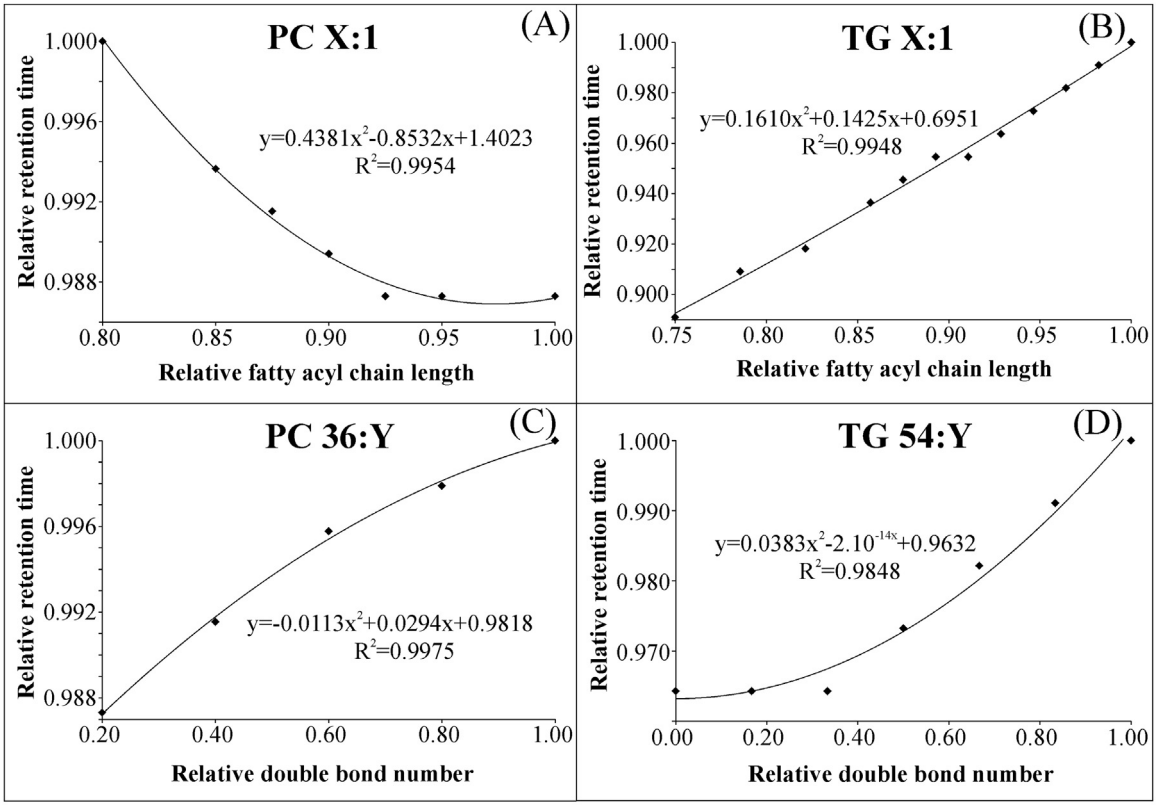

Fig. 6. Plots of polynomial dependences of relative retention times of PC and TG on the relative CN (X) and the relative DB number (Y) from UHPSFC data [38]: (**A**) PC X:1 (X is from 32 to 40), (**B**) TG X:1 (X is from 42 to 56), (**C**) PC 36:Y (Y is from 1 to 5), and (**D**) TG 54:Y (Y is from 0 to 6).

Acknowledgments

This work was supported by the ERC CZ grant project LL1302 sponsored by the Ministry of Education, Youth and Sports of the Czech Republic.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.04.082.

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