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Characterization of fatty acid and triacylglycerol composition in animal fats using silver-ion and non-aqueous reversed-phase high-performance liquid chromatography/mass spectrometry and gas chromatography/flame ionization detection

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ABSTRACT

Fatty acid (FA) and triacylglycerol (TG) composition of natural oils and fats intake in the diet has a strong influence on the human health and chronic diseases. In this work, non-aqueous reversed-phase (NARP) and silver-ion high-performance liquid chromatography with atmospheric pressure chemical ionization mass spectrometry detection and gas chromatography with flame-ionization detection (GC/FID) and mass spectrometry detection are used for the characterization of FA and TG composition in complex samples of animal fats from fallow deer, red deer, sheep, moufflon, wild boar, cock, duck and rabbit. The FA composition of samples is determined based on the GC/FID analysis of FA methyl esters. In total, 81 FAs of different acyl chain length, double bond (DB) number, branched/linear, *cis*-/*trans*- and DB positional isomers are identified. TGs in animal fats contain mainly monounsaturated and saturated FAs. High amounts of branched and *trans*-FAs are observed in the samples of ruminants. In NARP mode, individual TG species are separated including the separation of *trans*- and branched TGs. Silver-ion mode provides the separation of TG regioisomers, which enables the determination of their ratios. Great differences in the preference of unsaturated and saturated FAs in the *sn*-2 position on the glycerol skeleton are observed among individual animal fats. Unsaturated FAs are preferentially occupied in the *sn*-2 position in all animal samples except for wild boar with the strong preference of saturated FAs in the *sn*-2 position.

1. Introduction

The composition of dietary triacylglycerols (TGs) and their fatty acids (FAs) has significant effects on the prevention or genesis of serious chronic diseases, such as cardiovascular diseases, diabetes, and cancer [1–3]. The correct ratio of saturated and polyunsaturated fatty acids in plant or animal tissues in our diet is especially important, as reflected by recommended daily income of these FAs by some national health organizations, *e.g.*, National Institute of Health in USA. The presence of unusual FAs with special biochemical properties should be also taken into account [4,5]. Wide differences in the FA composition can be found among various types of animal adipose tissues, *e.g.*, ruminants, poultry or fish. Animal fats are characterized by a high content of saturated FAs that have higher temperature and oxidation stability in comparison to unsaturated FAs, but on the other hand their higher content in the diet increases the risk of coronary heart diseases. On the other hand, fish oils are a predominant source of ω-3 polyunsaturated FAs, mainly eicosapentaenoic (Δ*5,8,11,14,17*-C20:5, EPA) and docosahexaenoic (Δ*4,7,10,13,16,19*-C22:6, DHA) acids, precursors of eicosanoids that reduce the inflammation in the body and they also play a crucial role in the prevention of atherosclerosis or heart attack [6]. In the natural samples, *cis*-configuration of double bonds (DBs) is predominant, but small amounts of FAs (<5%) with *trans*-configuration (*trans*-FAs) are present in ruminant meats and milk as products of rumen bacteria. Other products of rumen bacteria are odd- and branched-chain FAs having the important role to maintain an optimal fluidity of the microbial cell membrane or FAs with conjugated DBs as an intermediate in the biohydrogenation of unsaturated acids [4,7].

Nutrition properties of TG mixtures are given by their FA composition differing in acyl chain lengths and number, position and

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configuration (*cis*-/*trans*-) of DBs. The most widespread method for the analysis of FA profiles is gas chromatography with flame ionization detection (GC/FID) of fatty acid methyl esters (FAMEs) after the catalyzed transesterification of TGs. This method enables fast, simple and reliable characterization of very complex samples based on FA profiles. High-performance liquid chromatography (HPLC) with mass spectrometry (MS) detection is a powerful tool for the characterization of TGs in complex natural mixtures. Two chromatographic techniques are most widespread in the analysis of TGs in natural samples, *i.e.*, non-aqueous reversed-phase (NARP) HPLC and silver-ion chromatography. In NARP-HPLC [8–22], retention times of TGs increase with the increasing equivalent carbon number (ECN) defined as the carbon number (CN) in all acyl chains minus twice the number of DBs (ECN = CN − 2DB). The separations of TGs differing in the position [15,17,22] and configuration [20–23] of DBs or the partial resolution of regioisomers [24] and TGs containing linear and branched FAs [25] have been reported as well. The retention times of TGs can be significantly reduced without the loss of resolution by the use of ultra-high performance liquid chromatography with sub-two micron particles column [26,27].

Silver-ion chromatography [28–34] is based on the formation of weak reversible complexes of silver ions impregnated on the silica or mostly bounded to the ion-exchange stationary phase with π electrons of DBs of unsaturated TGs. The mixture of hexane/acetonitrile is a typical mobile phase used in silver-ion chromatography but with a poor reproducibility due to the low miscibility of these solvents. The addition of 2-propanol into the mobile phase improves the miscibility of these solvents which provides a better reproducibility of retention times among analyses within one or more days [33]. Chlorinated mobile phases are also frequently used in silver-ion HPLC using dichloromethane or dichloroethane with the addition of other polar modifiers at low concentration, typically acetonitrile, acetone, and methanol [31,32,35]. In silver-ion chromatography, separation of TGs is governed mainly by the number of DBs. Double bond positional isomers [32,33], *cis*-/*trans*-isomers [23,28,30] or regioisomers ($R_1R_1R_2$ vs. $R_1R_2R_1$) [23,28,33] can be also separated. The orthogonality of silver-ion and NARP modes can be demonstrated in two dimensional separation for very complex samples in on-line [36–38] or off-line [23,39,40] setup.

The main goal of this work is the characterization of FA and TG composition of selected animal samples important in the nutrition and foodomics. NARP-HPLC and silver-ion HPLC with APCI-MS detection are used for the analysis of TGs according to acyl chain lengths and the number of DBs and FA composition of samples is determined using GC/FID analysis of FAMEs after the transesterification of TGs. The composition of TGs and unusual FAs in analyzed animal fats is discussed.

2. Materials and methods

2.1. Materials

Acetonitrile (HPLC gradient grade), 2-propanol, hexane (both HPLC grade) and sodium methoxide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Model mixtures of FAME standards GLC#617 (C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, Δ*9*-C14:1, C15:0, Δ*10*-C15:1, C16:0, Δ*9*-C16:1, C17:0, Δ*10*-C17:1, C18:0, Δ*9*-C18:1, Δ*9t*-C18:1, Δ*9,12*-C18:2, Δ*9t,12t*-C18:2, Δ*6,9,12*-C18:3, Δ*9,12,15*-C18:3, C20:0, Δ*11*-C20:1, Δ*11,14*-C20:2, Δ*8,11,14*-C20:3, Δ*11,14,17*-C20:3, Δ*5,8,11,14*-C20:4, Δ*5,8,11,14,17*-C20:5, C21:0, C22:0, Δ*13*-C22:1, Δ*13,16*-C22:2, Δ*7,10,13,16*-C22:4, Δ*4,7,10,13,16*-C22:5, Δ*7,10,13,16,19*-C22:5, Δ*4,7,10,13,16,19*-C22:6, C23:0, C24:0, Δ*15*-C24:1) and GLC#566 (C8:0, C10:0, C11:0, Δ*10*-C11:1, C12:0, C13:0, Δ*12*-C13:1, C14:0, Δ*9*-C14:1, C15:0, C16:0, Δ*9*-C16:1, C17:0, Δ*10*-C17:1, C18:0, Δ*9*-C18:1, Δ*11t*-C18:1, Δ*9,12*-C18:2, Δ*6,9,12*-C18:3, Δ*9,12,15*-C18:3, C19:0, C20:0, Δ*11*-C20:1, Δ*11,14*-C20:2, Δ*8,11,14*-C20:3, Δ*11,14,17*-C20:3, Δ*5,8,11,14*-C20:4, Δ*5,8,11,14,17*-C20:5, C21:0, C22:0, Δ*13*-C22:1, Δ*13,16*-C22:2, Δ*7,10,13,16*-C22:4, Δ*4,7,10,13,16*-C22:5, Δ*7,10,13,16,19*-C22:5, Δ*4,7,10,13,16,19*-C22:6, C23:0, C24:0, Δ*15*-C24:1) were purchased from Nu-Chek-Prep (Elysian, MN, USA). Standards of methyl 15-methylheptadecanoate (*ai*C18:0) and methyl 16-methylheptadecanoate (*i*C18:0) were purchased from Larodan Fine Chemicals (Mälmo, Sweden). Samples of adipose tissues from fallow deer (*Dama dama*), red deer (*Cervus elaphus*), sheep (*Ovis aries*), moufflon (*Ovis musimon*), wild boar (*Sus scrofa*), cock (*Gallus gallus*), duck (*Anas platyrhynchos domesticus*) and rabbit (*Oryctolagus cuniculus*) were obtained from the local veterinarian. The amount of 20 g of the sample was crushed in a homogenizer with 20 mL of hexane for 10 min. The mixture was filtered and hexane was evaporated under a mild stream of nitrogen yielding the pure animal fat.

2.2. NARP-HPLC/APCI-MS

NARP-HPLC experiments were performed on a chromatographic apparatus consisting of a Model 616 pump with a quaternary gradient system, a Model 996 diode-array UV detector, a Model 717+ autosampler, a thermostated column compartment and a Millennium chromatography manager (all from Waters, Milford, MA, USA). Samples were analyzed using the following HPLC conditions: two chromatographic columns Nova-Pak C_{18} (300 mm × 3.9 mm and 150 mm × 3.9 mm, 4 μm, Waters) connected in series, a flow rate of 1 mL/min, an injection volume of 10 μL, column temperature of 25 °C and the mobile phase gradient according to Ref. [23]: 0 min – 80% A + 20% B, 121 min – 40% A + 60% B, 122 min – 80% A + 20% B, where A is acetonitrile and B is a mixture of hexane–2-propanol (1:1, v/v). The injector needle was washed with the mobile phase before each injection. The column hold-up volume, t_M, was 3.2 min for the system with 300 + 150 mm Nova-Pak C_{18} columns.

The Esquire 3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany) with positive-ion APCI was used in the mass range *m/z* 50–1200 with the following setting of tuning parameters: pressure of the nebulizing gas 70 psi, drying gas flow rate 3 L/min, temperatures of the drying gas and APCI heater were 350 °C and 400 °C, respectively. Individual reconstructed ion current chromatograms were used to support the identification of coeluting peaks.

2.3. Silver-ion HPLC/APCI-MS

Silver-ion HPLC experiments were performed on a liquid chromatograph Agilent 1200 Series (Agilent Technology, Waldbronn, Germany). Samples were analyzed using the following HPLC conditions according to Ref. [33]: three silver-ion chromatographic columns ChromSpher Lipids (250 mm × 4.6 mm, 5 μm, Varian, Palo Alto, CA, USA) connected in series, the flow rate of 1 mL/min, the injection volume of 1 μL, column temperature of 25 °C, and the mobile phase gradient: 0 min – 100% A, 140 min – 61% A + 39% B, where A is the mixture of hexane–2-propanol–acetonitrile (99.8:0.1:0.1, v/v/v) and B is the mixture of hexane–2-propanol–acetonitrile (96:2:2, v/v/v). The mobile phase was prepared freshly every day. Silver-ion columns were conditioned at 50 μL/min using the initial mobile phase composition overnight and at 1 mL/min for 1 h before the first analysis. The injector needle was washed with the mobile phase after each injection. The chromatographic system was equilibrated between injections for 45 min.

The hybrid quadrupole time-of-flight (QqTOF) analyzer micrOTOF-Q (Bruker Daltonics, Bremen, Germany) with positive-

ion APCI was used in the mass range *m/z* 50–1200 with the following tuning parameters: flow of the nebulizing and drying gas 5 and 3 L/min, respectively, temperatures of the drying gas and APCI heater 300 °C and 400 °C, respectively.

2.4. GC/FID and GC/MS analyses of FAMEs

FAMEs were prepared using a standard procedure with sodium methoxide according to Ref. [41]. Briefly, the amount of 5 mg of the sample and 1 mL of 0.25 M sodium methoxide in methanol was heated on a water bath for 10 min at 65 °C. After the reaction, water saturated with sodium chloride was added and then FAMEs were extracted from the mixture using hexane.

GC/FID experiments were performed on the gas chromatograph with flame ionization detection Agilent 7890 (Agilent Technologies, Waldbronn, Germany) using TR-FAME column (70% cyanopropyl polysilphenylene-siloxane), 30 m length, 0.25 mm ID, 0.25 μm film thickness (Thermo Scientific, Waltham, USA). GC conditions were as follows: the injection volume was 1 μL, the split ratio was 1:15, the flow rate of nitrogen as a carrier gas was 1.3 mL/min, the temperature program: the initial temperature 140 °C, ramp to 235 °C at 4 °C/min. Injector and detector temperatures were 250 °C and 280 °C, respectively. Table 1 shows the arithmetic mean values of relative weight concentrations calculated from three replicate measurements.

GC/MS experiments were performed on a gas chromatograph Agilent 6890N coupled to Agilent 5975B mass spectrometer (both Agilent Technologies) using the identical TR-FAME column as for GC/FID experiments. GC conditions were as follows: the injection volume was 1 μL, the split ratio was 1:15, the flow rate of helium as the carrier gas was 1.2 mL/min, the temperature program: the initial temperature 100 °C, ramp to 235 °C at 3 °C/min, the injector temperature was 250 °C. The MS detection conditions: temperatures of ion source and quadrupole were 230 °C and 150 °C, respectively, electron ionization with electron energy 70 eV was used in the mass range *m/z* 25–600.

2.5. Definition of abbreviations

Identified TG species are annotated using initials of FA trivial names (or by CN:DB) arranged according to their stereochemical positions (*sn*-1, *sn*-2 and *sn*-3), *e.g.*, 1-octadecenoyl-2-octadecadienoyl-3-hexadecanoyl-*sn*-glycerol is annotated as OLP. *Sn*-1 and *sn*-3 positions cannot be resolved in this work and FAs in these positions are arranged according to their decreasing molecular masses. The positions and number of branching in TGs cannot be exactly determined by HPLC/MS, therefore we annotate such TGs with a prefix *b*, for example *b*SSS means that this TG contains three C18:0 acyl chains and one or more of them are branched C18:0 acid(s) with unknown position(s) of branching. The regioisomeric positions of branched FAs are not determined. Abbreviations of FAs: M – myristic (C14:0); P – palmitic (C16:0); Po – palmitoleic (Δ*9*-C16:1); Ma – margaric (C17:0); Mo – margaroleic (Δ*9*-C17:1); S – stearic (C18:0); O – oleic (Δ*9*-C18:1); Va – vaccenic (Δ*11t*-C18:1); L – linoleic (Δ*9,12*-C18:2); Ln – linolenic (Δ*9,12,15*-C18:3); A – arachidic (C20:0); G – gadoleic (Δ*9*-C20:1). FAs in GC/FID chromatograms are annotated using CN:DB with position(s) and *cis*-/*trans*- (*t*) configuration of DBs and branching (*i* – iso, *ai* – anteiso, *b* – branched FA with unknown position(s) of branching). Unsaturated and branched FAs identified by GC/MS without identical standards are annotated without position(s) of DBs and branching, respectively.

3. Results and discussion

3.1. NARP-HPLC/APCI-MS

NARP-HPLC method with APCI-MS detection using C_{18} column in the total length of 45 cm, separation temperature 25 °C and the gradient of acetonitrile/2-propanol/hexane [23] is used for the analysis of TGs from animal fats (Figs. 1A and 2A, Table S1). Retention times of TGs in NARP-HPLC are given by the total number of carbon atoms and DBs in TGs usually expressed by ECN value, *i.e.*, ECN = CN − 2DB. The retention of TGs increases with the increasing ECN, while the length and unsaturation of individual fatty acyls in TGs affect their retention inside individual ECN groups. TGs with the higher number of DBs have lower retention times than saturated TGs with the same ECN, *e.g.*, OOO (t_R = 60.5 min) has slightly lower retention in comparison to OOP (62.0 min), POP (63.8 min), or even PPP (65.5 min), all with ECN = 48 (Table S1). TG isomers with *cis*- and *trans*-configuration of DBs are also separated using NARP-HPLC method (Figs. 1A and 2A). *Trans*-FAs have the straight arrangement of acyl chains with similar physicochemical properties as for saturated FAs, therefore TGs containing *trans*-FAs are more retained in NARP-HPLC than *cis*-TGs, *e.g.*, SOO with t_R = 69.6 min and its *trans*-isomer SOVa with t_R = 70.8 min (Table S1).

Peaks of isomers of saturated TGs with the same CN of fatty acyls and shifted retention times are identified in ruminant samples. These isomers correspond to TGs containing branched (*b*) FAs as confirmed by GC/FID data, where numerous *b*FAs in ruminant samples are found. TG isomers containing linear and *b*FAs provide identical APCI mass spectra without any diagnostic fragment ions or measurable differences in relative abundances of ions, therefore they cannot be differentiated based on mass spectra only. On the other hand, NARP-HPLC/APCI-MS data are used for the resolution of both isomers based on shifts in retention times determined using reconstructed ion current chromatograms (RICs) of $[M+H–RCOOH]^+$ ions as shown in Fig. 3. TGs with *b*FAs have slightly lower retention times in comparison to their linear analogs (Fig. 3B), *e.g.*, *b*SSP (t_R = 78.7 min) containing *b*FA has lower retention time than SSP (79.8 min) containing linear acyl chains with the same CN:DB. Only one peak of *b*TGs with the same CN is identified in NARP-HPLC chromatograms from ruminant fats in contrast to expected combinations of *b*FAs with different positions and number of branching identified in GC/FID experiments, therefore all isomers of *b*TGs coelute in one peak. This is in agreement with the literature [25], where TGs with *iso*- and *anteiso*-FAs are not separated under the gradient elution, only TGs containing two or three *b*FAs are partially separated under the isocratic elution with extremely long retention times in the range of hundreds of minutes.

APCI full-scan positive-ion mass spectra of analyzed TGs provide $[M+H]^+$ and $[M+NH_4]^+$ ions and also abundant fragment ions $[M+H–RCOOH]^+$ formed by cleavage of FAs from glycerol. Ratios of $[M+H–RCOOH]^+$ fragment ions are used for the determination of prevailing FA in the *sn*-2 position according to well known rule that the neutral loss of FA from this position provides the fragment ion with a lower relative abundance in comparison to *sn*-1 and *sn*-3 positions [12,13,42].

3.2. Silver-ion HPLC/APCI-MS

Samples of animal fats are analyzed using three ion-exchange based silver-ion HPLC columns connected in series in the total length of 75 cm and hexane/2-propanol/acetonitrile gradient [33]. TGs are separated into groups according to their number of DBs. The higher number of DBs means stronger interactions with silver ions in the stationary phase resulting in higher retention times (Figs. 1B and 2B, Table S1). TGs inside individual DB groups are partially separated according to acyl chain lengths, as demonstrated

Table 1
Relative weight concentrations [%] of FAs[a] identified in analyzed samples from GC/FID of FAMEs, their retention times (t_R) and response factors (RF).

t_R (min)	Fatty acid[b]	RF	Fallow deer	Red deer	Sheep	Moufflon	Wild boar	Cock	Duck	Rabbit
4.3	C10:0	1.33	0.1	0.1	0.1	0.1	0.1			0.1
4.9	C11:0	1.24				0.1				
5.1	*i*C12:0	1.16[c]				0.2				
5.4	*b*C13:0	1.11[c]				0.3				
5.6	C12:0	1.16	0.1	0.4	0.1	0.1	0.1	0.1	<0.1	0.2
5.9	*b*C13:0	1.11[c]				0.4				
6.0	*i*C13:0	1.11[c]		0.1						
6.1	*b*C14:0	1.07[c]				0.4				
6.4	C13:0	1.11	0.1	0.1		0.1				<0.1
6.5	*b*C14:0	1.07[c]				0.1				
6.8	*b*C14:0	1.07[c]				0.3				
6.9	*i*C14:0	1.07[c]	1.1	0.3	0.1	0.1				0.1
7.0	*b*C15:0	1.03[c]				0.3				
7.2	*b*C15:0	1.03[c]				0.2				
7.4	C14:0	1.07	4.8	5.7	2.1	2.8	1.3	0.5	0.7	3.4
7.6	*b*C14:0	1.07[c]	0.1		<0.1	0.8				
7.7	*b*C14:0	1.07[c]				0.4				
7.8	*b*C14:0	1.07[c]	0.1			0.9				
7.9	*b*C14:0	1.07[c]	0.1			0.4				
8.0	*i*C15:0	1.03[c]	0.4	0.8	0.2					0.1
8.1	Δ*9*-C14:1	1.11	0.2	<0.1				0.1	0.1	0.1
8.3	*ai*C15:0	1.03[c]	0.6	1.0	0.2					0.2
8.5	*b*C16:0	1.00[c]				0.1				
8.6	C15:0	1.03	2.0	1.6	0.6	1.6	<0.1	0.1	0.1	1.1
8.8	C15:1	1.00	0.1	0.1	0.1					0.3
8.9	*b*C16:0	1.00[c]	0.1			0.9				
9.0	*b*C16:0	1.00[c]	<0.1			0.7				
9.3	*i*C16:0	1.00[c]	9.5	0.4	0.2	0.6				0.5
9.6	*b*C17:0	0.99[c]	0.3			0.4				
9.9	C16:0	1.00	25.0	24.8	19.8	16.0	25.7	19.8	21.6	33.5
10.1	C16:1	1.00[c]		0.1	0.1			0.1	0.1	0.1
10.2	*b*C17:0	0.99[c]	0.1			1.0				
10.3	*b*C17:0	0.99[c]	0.2	0.2	0.1	0.4			<0.1	
10.4	*b*C17:0	0.99[c]	0.1		0.1	0.9				
10.5	C16:1	1.02[c]	0.6	0.8	0.6	1.5	0.3	0.5	0.5	0.4
10.6	Δ*9*-C16:1	1.02	1.1	0.7	0.6	2.2	2.5	2.5	4.3	3.0
10.7	*i*C17:0	0.99[c]	0.4	0.5	0.4					
10.9	*ai*C17:0	0.99[c]	1.5	0.7	0.8	1.4	0.1		0.1	0.3
11.1	C17:1	0.99[c]	0.1	0.3						
11.2	*b*C18:0	0.97[c]				0.1				
11.3	C17:0	0.99	3.1	1.5	2.2	2.6	0.2	0.3	0.2	1.4
11.6	C16:2	0.99[c]	<0.1	<0.1	0.1					<0.1
11.9	Δ*10*-C17:1	0.99	0.4	0.1	0.5	2.7	0.2	0.1	0.1	0.7
12.0	*i*C18:0	0.96	2.0	0.1	0.2	0.7				0.1
12.2	*ai*C18:0	0.97				0.1				
12.7	C18:0	0.97	22.2	36.7	31.6	7.7	11.7	10.5	5.1	8.5
13.0	Δ*9t*-C18:1	0.98	0.3	0.4	0.8	0.5	0.1	0.3	0.3	0.1
13.1	Δ*11t*-C18:1	0.98[c]	1.0	2.7	5.6	2.3				0.2
13.2	Δ*9*-C18:1	0.98	15.8	12.3	25.8	34.5	38.2	49.5	48.8	23.2
13.3	Δ*11*-C18:1	1.00	0.3	0.7	0.5	0.7	2.8	2.2	1.7	1.1
13.4	C18:1	0.98[c]	0.1	0.2	0.1	0.1				0.1
13.5	C18:1	0.98[c]	0.5			0.2	0.1	<0.1	0.1	0.1
13.6	Δ*9t,12t*-C18:2	0.99		0.5	0.8	0.3				0.1
13.7	C18:1	0.98[c]	0.3	0.2	0.4	0.7				
13.9	C18:2	0.98[c]	0.1	<0.1	0.1	0.4		0.1	0.1	<0.1
14.1	C19:0	1.00	0.4	0.6	0.6	0.5		0.1	0.1	0.2
14.2	Δ*9,12*-C18:2	0.98	1.4	1.8	1.3	2.0	12.3	10.2	12.7	13.3
14.5	C18:2	0.98[c]		0.1						
14.6	C19:1	1.00[c]	0.1	0.2	0.2	0.3	<0.1	0.1	0.1	0.2
14.8	Δ*6,9,12*-C18:3	0.97						0.1	<0.1	<0.1
15.3	Δ*9,12,15*-C18:3	0.97	0.9	1.2	1.0	1.4	1.3	0.7	1.1	5.5
15.5	C20:0	0.97	0.5	0.5	0.2	0.1	0.2	0.2	0.1	0.2
15.7	C18:2	0.98[c]	0.1	0.1	0.7	1.5	0.1		0.1	0.1
15.9	C18:4	1.00[c]	0.2	0.2	0.2	0.2		0.1	0.2	
16.0	Δ*11*-C20:1	0.98	0.1	0.1	0.1	0.1	1.0	1.0	0.4	0.3
16.2	C20:1	0.98[c]			0.1	0.1	<0.1			
16.6	C18:3	0.97[c]			0.1	0.1				
16.7	C20:2	0.97[c]						0.1	0.1	
16.9	C21:0	0.96	0.1	0.1						
17.0	Δ*11,14*-C20:2	0.97		<0.1		<0.1	0.6	0.1	0.1	0.1
17.6	Δ*8,11,14*-C20:3	0.96				<0.1	0.1	0.1	0.1	<0.1
18.0	Δ*5,8,11,14*-C20:4	0.99	0.1	<0.1	<0.1	<0.1	0.1	0.1	0.3	0.1
18.1	Δ*11,14,17*-C20:3	0.96		<0.1		<0.1	0.3			0.1
18.2	C22:0	0.93	0.1	0.1	<0.1					<0.1
18.7	C22:1	0.95[c]							<0.1	

Table 1 (*Continued*)

t_R (min)	Fatty acid[b]	RF	Fallow deer	Red deer	Sheep	Moufflon	Wild boar	Cock	Duck	Rabbit
18.8	Δ*13*-C22:1	0.95					<0.1	<0.1		
19.2	Δ*5,8,11,14,17*-C20:5	0.97			<0.1					
19.6	C23:0	0.96	0.1	0.1						
20.8	C24:0	0.96	0.1	0.1						
20.9	Δ*7,10,13,16*-C22:4	0.96					0.1	0.1	0.1	0.1
22.0	Δ*7,10,13,16,19*-C22:5	0.97	0.2		0.1	0.1	0.1	0.1	0.1	0.2
	Others	1.00[c]	0.7	0.7	0.5	4.3	0.4	0.2	0.5	0.6

[a] Standard deviations of all relative weight concentrations in this table are <0.03 as calculated from three replicates.
[b] FAs are annotated using CN:DB with position(s) and *cis*-/*trans*- (*t*) configuration of DBs and branching (i – iso, ai – anteiso, b – branched FA with unknown position(s) of branching). Unsaturated and branched FAs identified by GC/MS without identical standards are annotated without position(s) of DBs and branching, respectively.
[c] The response factor of FAs without an identical standard is set the same as for the standard with the identical CN:DB, *e.g.*, iC13:0 and bC13:0 is the same as for C13:0.

on retention times of the pair of SOS (37.3 min) and SOP (37.7 min), both with DB = 1 differing by two methylene units, *i.e.*, the difference in retention is approximately 0.4 min per two methylene units (Table S1). RICs of protonated molecules, ammonium adducts and fragment ions from HPLC/APCI-MS analysis are used for the identification of TGs in highly complex animal fats (*e.g.*, Figs. 3 and 4) even for peaks with the high number of coeluted species, for example in the group of TGs with DB = 0 with very small differences in retention times of individual saturated TGs coeluting in one peak. In silver-ion HPLC, *trans*-FAs have lower retention times in comparison to *cis*-isomers with the same CN:DB, but they elute before the DB minus one group, for example SSS (t_R = 17.6 min, without DB) < SVaS (t_R = 26.8 min, 1 *trans*-DB) < SOS (t_R = 37.3 min, 1 *cis*-DB). Differences of retention times between *cis*- and *trans*-TGs decrease for the higher number of DBs, but they still elute in separated groups between two DB groups with all *cis*-FAs and the identification of *trans*-TGs in the sample can be done even without the need of MS detection.

Silver-ion HPLC method enables the baseline separation of TG regioisomers up to three DBs and at least the partial resolution of regioisomers with four to seven DBs. Regioisomers with the higher number of DBs in fatty acyls in outer positions (*sn*-1 and *sn*-3) are retained more strongly in comparison to TGs with the same unsaturation in the *sn*-2 position (Fig. 4C and D), *e.g.*, SLS (t_R = 51.4 min) and SSL (52.5 min). All TG regioisomers are at least partially separated except for $R_1R_2R_3$ and $R_2R_1R_3$ type regioisomers, where R_1 and R_2 are different saturated FAs that elute in one peak, *e.g.*, SPO and OSP regioisomers are not resolved. The determination of exact composition of individual regioisomers by a simple integration of their peak areas is impossible in highly complex animal samples due to the coelution with other species. For this reason, RICs of protonated molecules and $[M+H–RCOOH]^+$ fragment ions are used to support the proper determination of regioisomeric ratios (Fig. 4). The precision of this procedure is limited by the huge number of isobaric species in the samples and the increment of A+2 isotopic peaks to the intensity of reconstructed ion currents, but it still provides acceptable results for most biochemical applications. Fig. 4 shows a different procedure for the rough determination of regioisomeric composition inside DB groups with the different unsaturation level of individual FAs, *i.e.*, saturated (Sa), monounsaturated (Mo) and diunsaturated (D) FAs. The ratio of regioisomers is determined based on the ratio of peak areas of both groups of regioisomers. It is confirmed that the ratio for all pairs within particular unsaturation groups of regioisomers is similar, as illustrated on the example shown in Fig. 4A and B. The validity of this presumption has been confirmed by the peak areas of individual regioisomers from RICs of fragment ions, as illustrated on selected examples in Fig. 4C, 4D. Regioisomers with unsaturated FAs in the *sn*-2 position, *i.e.*, SaMoSa (Fig. 4A), SaDSa and MoMoSa (Fig. 4D), have higher relative concentrations in comparison to peaks of isomers with unsaturated FAs in *sn*-1/3 positions, *i.e.*, SaSaMo, SaSaD and MoSaMo. This procedure for the rough determination of regioisomeric ratios together with known retention rules in silver-ion HPLC can be easily applied with any HPLC detector, if an HPLC/MS system is not available in the laboratory.

3.3. Analysis of FA composition using GC/FID

The FA composition of animal fats is characterized by the GC/FID analysis of FAMEs after the transesterification of TGs with sodium methoxide (Figs. 1C and 2C, Table 1). At first, the carrier gas flow rate, initial temperature and temperature program have been carefully optimized using complex FAME standard mixtures to achieve the separation of maximum number of species including the separation of all types of isomerism, *i.e.*, *cis*-/*trans*-, linear/branched and DB positional isomers. Individual FAs are identified based on retention times of identical standards or electron ionization (EI) mass spectra from GC/MS analysis. Positions of DBs and branching of some isomers without commercially available standards cannot be clearly identified due to the lack of diagnostic fragment ions in EI mass spectra. These FAs are annotated by their CN:DB but without positions of DBs and branching, *e.g.*, *b*C17:0 in Fig. 1C.

In optimized GC/FID method, all FAMEs including different isomers are baseline or at least partially separated. Retention times of FAMEs increase with the increasing acyl chain length and the number of DBs. Positions of DBs in the acyl chain (DB positional isomers) also influence retention times of FAMEs. DB positional isomers with a greater distance between the first DB and the ester group are retained more strongly, for example C18:1 positional isomers Δ*9*-C18:1 with t_R = 13.2 min and Δ*11*-C18:1 with t_R = 13.4 min (Table 1). FAMEs containing *trans*-configuration of DBs have lower retention times in comparison to their *cis*-isomers, as demonstrated on elaidic (Δ9*t*-C18:1) and oleic (Δ*9*-C18:1) FAs with retention times 13.0 and 13.2 min, respectively. Branched FAs have the lower retention in comparison to their linear analogs. For example, FAs containing 18 carbon atoms that are methyl branched on the first (*iso, i*) or the second (*anteiso*, *ai*) carbon atom have lower retention times in comparison to their C18:0 linear analog, *i.e.*, *i*C18:0 (t_R = 12.0 min), *ai*C18:0 (t_R = 12.2 min) and C18:0 (t_R = 12.7 min). Some *b*FAs are identified based on retention shifts and EI mass spectra, but the determination of branching position or the number of branching in multibranched FAs is not possible without identical standards, which are not commercially available.

Peak areas of FAMEs in GC/FID chromatograms are used for the determination of FA profiles in analyzed animal fats. These areas are multiplied by response factors (RFs) of identical standards related to the palmitic acid. Response factors of individual FAMEs are in the range from 0.93 to 1.03 for FAs with 15 to 24 carbon atoms and 0 to 5 DBs including all isomers, *i.e.*, positional and *cis*-/*trans*-DB isomers and linear/branched isomers of acyl chains (Table 1). Higher differences of RF values can be observed only for FAs with short acyl chains (Δ*9*-C14:1 – 1.11, C14:0 – 1.07, C13:0 – 1.11, C12:0 – 1.16, C11:0 – 1.24 and C10:0 – 1.33), but it is still acceptable for the reliable quantitation due to the low concentration of these FAs.

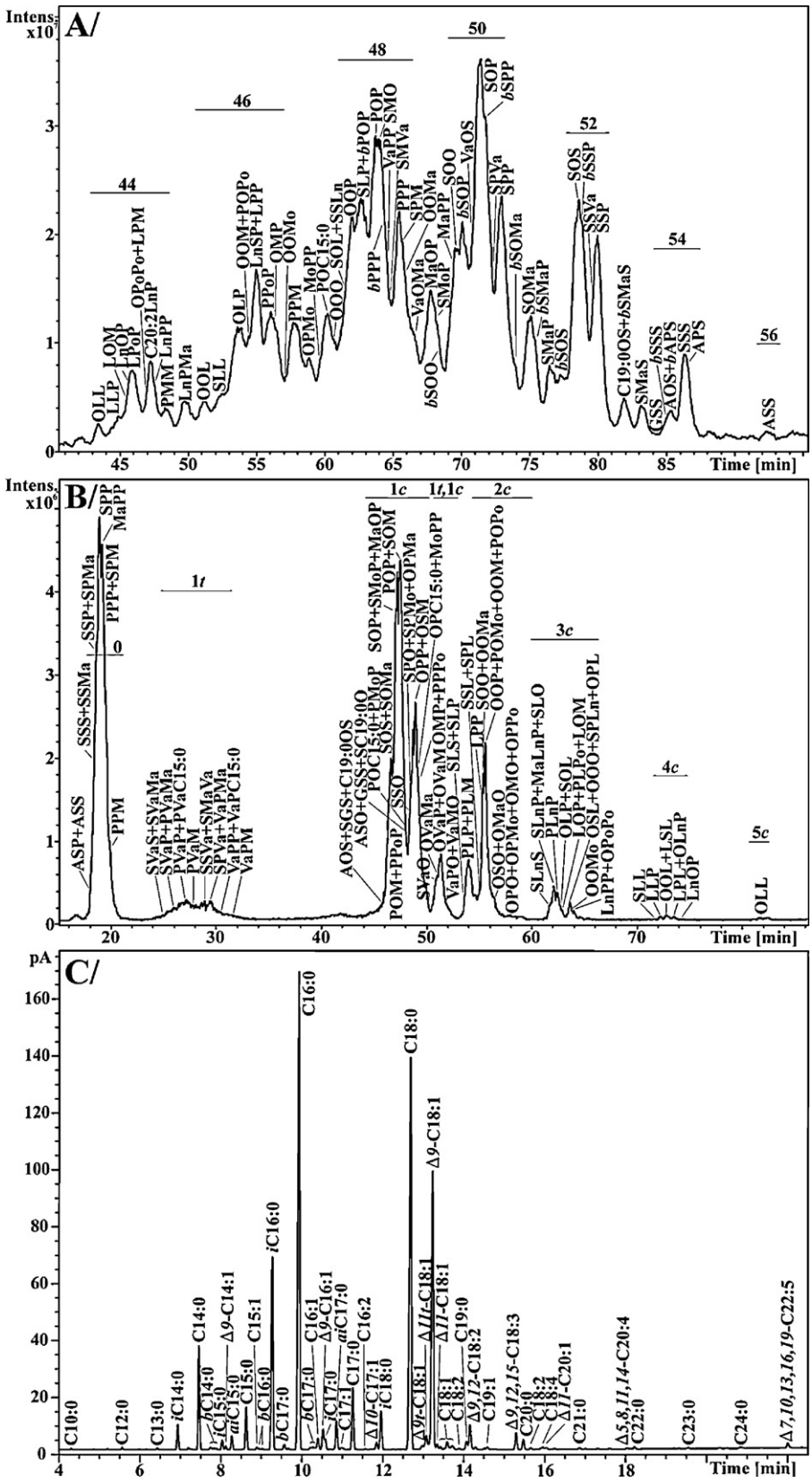

Fig. 1. Analysis of fallow deer (*Dama dama*): (A) NARP-HPLC/APCI-MS analysis of TGs, (B) silver-ion HPLC/APCI-MS analysis of TGs, and (C) GC/FID analysis of FAMEs. NARP-HPLC conditions: two Nova-Pak C18 columns (300 and 150 mm × 3.9 mm, 4 μm) connected in series, flow rate 1 mL/min, column temperature 25 °C, gradient 0 min – 80% A + 20% B, 121 min – 40% A + 60% B, 122 min – 80% A + 20% B, where A is acetonitrile and B is a mixture of hexane–2-propanol (1:1, v/v). Silver-ion HPLC conditions: three ChromSpher Lipid columns (250 mm × 4.6 mm, 5 μm) connected in series, flow rate of 1 mL/min, column temperature 25 °C, gradient 0 min – 100% A, 140 min – 61% A + 39% B, where A is the mixture of hexane–2-propanol–acetonitrile (99.8:0.1:0.1, v/v/v) and B is the mixture of hexane–2-propanol–acetonitrile (96:2:2, v/v/v). GC/FID conditions: TR-FAME column (30 m length, 0.25 mm ID, 0.25 μm film thickness), the flow rate of nitrogen as a carrier gas 1.3 mL/min, temperature program: the initial temperature 140 °C, ramp to 235 °C at 4 °C/min.

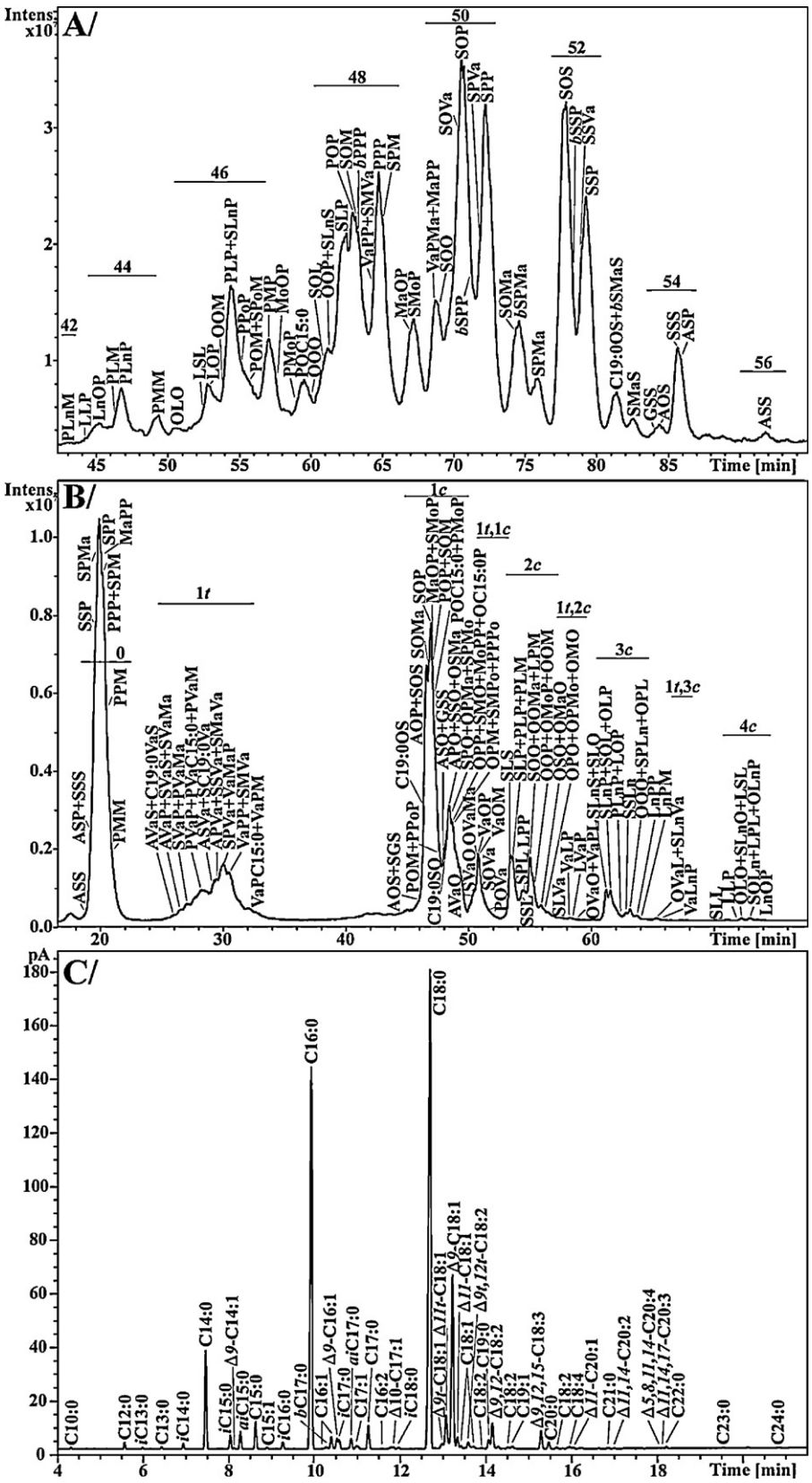

Fig. 2. Analysis of red deer (*Cervus elaphus*): (A) NARP-HPLC/APCI-MS analysis of TGs, (B) silver-ion HPLC/APCI-MS analysis of TGs, and (C) GC/FID analysis of FAMEs. HPLC and GC conditions are identical as for Fig. 1.

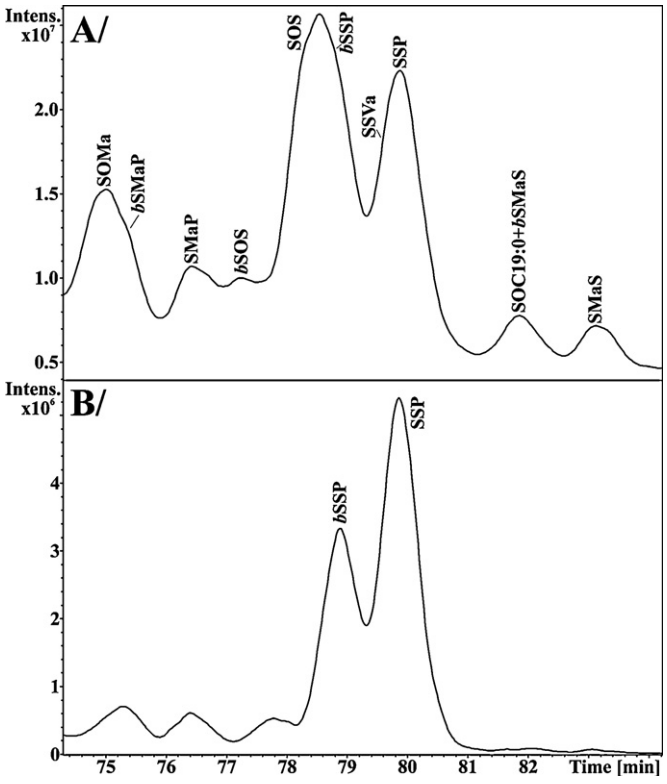

Fig. 3. NARP-HPLC/APCI-MS identification of TGs containing linear (SSP) and branched (*b*SSP) FAs using the total ion current chromatogram (A) and the reconstructed ion current chromatogram ($m/z = 579$) corresponding to the ion $[M+H-RCOOH]^+$ for the neutral loss of C18:0 (B) in fallow deer (*Dama dama*) sample. HPLC conditions are identical as for Fig. 1.

Response factors of FAs without identical standards are set identical as the closest FAME standard with the same CN:DB values.

3.4. Analysis of animal fats

NARP-HPLC, silver-ion HPLC and GC-FID methods have been applied for the analysis of animal fats from fallow deer (Fig. 1), red deer (Fig. 2), wild boar (Fig. 5), duck (Fig. 6), sheep (Fig. S1), moufflon (Fig. S2), cock (Fig. S3) and rabbit (Fig. S4) samples to achieve the maximum information for the characterization of FA and TG composition of these samples. Tables 1 and 2 show the FA composition of analyzed fats using GC/FID method. Properly optimized GC/FID method provides the separation and identification of a wide range of FAs even in complex animal fats. In total, 81 FAs have been detected in 8 animal fats ranging from 29 in wild boar to 61 in moufflon (Table 2). The number of identified FAs in individual animal samples is significantly higher than in most common fatty samples, for example from 7 to 18 FAs have been identified in a wide range of plant oils [18,43]. The enormous complexity of animal samples is given by the presence of many *cis*-/*trans*-, positional and branched FA isomers. Ruminant fats (fallow deer, red deer, sheep and moufflon) are rather complex due to the action of bacteria in their rumen that produce a number of unusual FAs, mainly branched and natural *trans*-FAs. 29 *b*FAs have been detected in all ruminants, from which 25 *b*FAs are found in the most complex moufflon sample. The most abundant *b*FAs are *i*C16:0 with the concentration up to 9.5%, *ai*C17:0 up to 1.5% and *i*C18:0 up to 2.0% in fallow deer. The concentration of *b*FAs in ruminant fats ranges between 2.2% in sheep and 16.6% in fallow deer in comparison to the trace amount in samples of omnivores (wild boar, cock and duck) with the concentration < 0.1% (Table 2). The highest concentration of *b*FAs is observed in samples of wild animals, *i.e.*, fallow deer (16.6%) and moufflon (12.0%), given

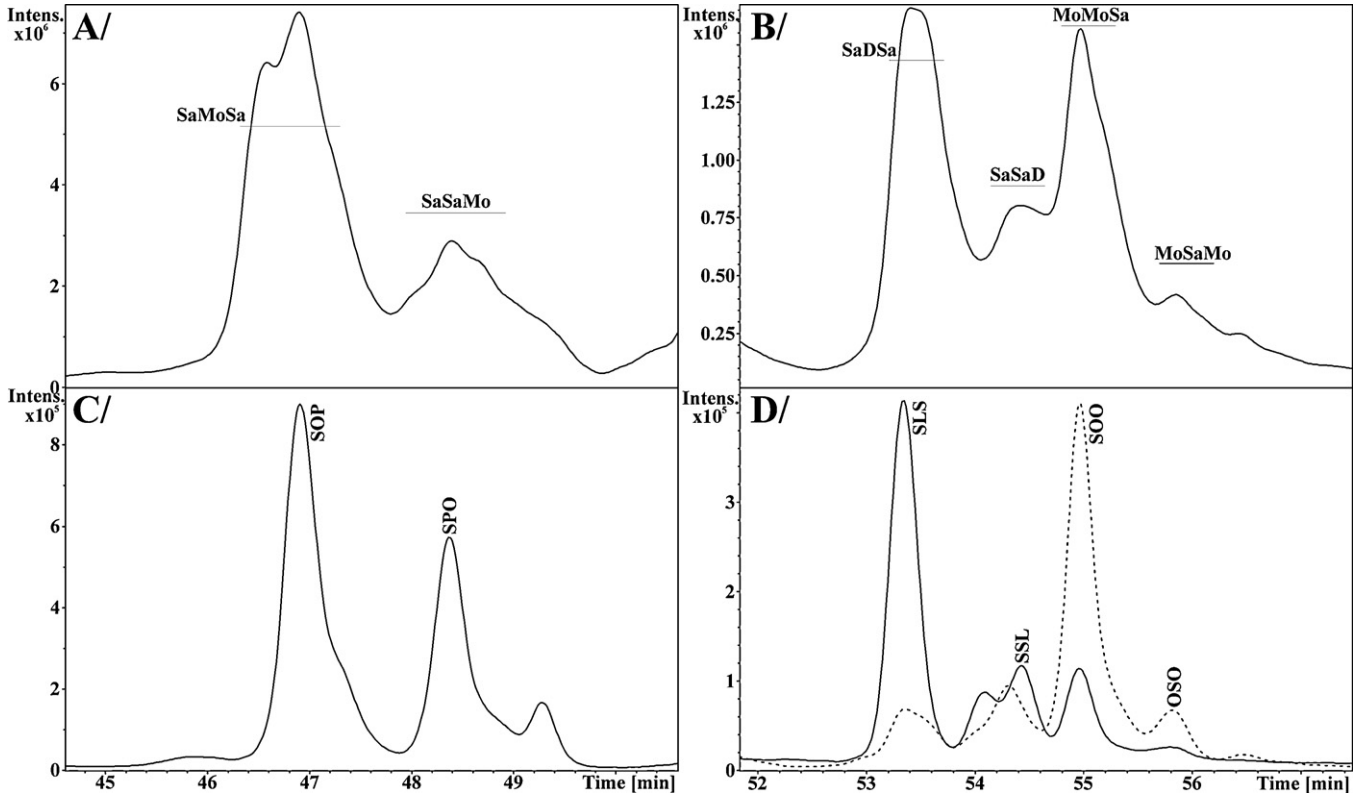

Fig. 4. Determination of TG regioisomers ratios in red deer (*Cervus elaphus*) using silver-ion HPLC/APCI-MS. Total ion chromatograms of regioisomers with 1 (A) and 2 (B) DBs and reconstructed ion current chromatograms of regioisomers SOP/SPO ($m/z = 579$) (C) and SLS/SSL ($m/z = 605$) and SOO/OSO ($m/z = 607$, dash line) (D). Sa – saturated, Mo – monounsaturated and D – diunsaturated FAs. HPLC conditions are identical as for Fig. 1.

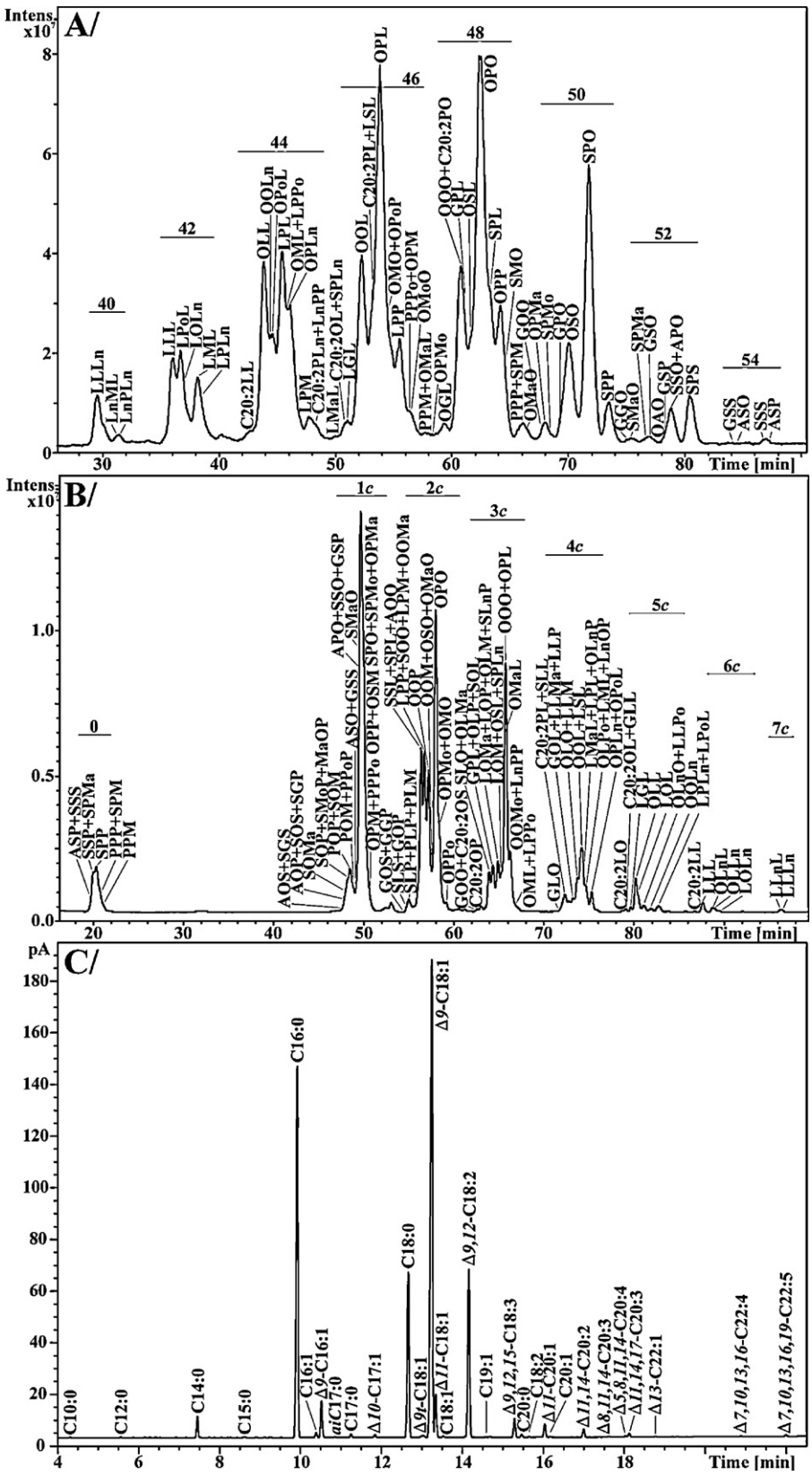

Fig. 5. Analysis of wild boar (*Sus scrofa*): (A) NARP-HPLC/APCI-MS analysis of TGs, (B) silver-ion HPLC/APCI-MS analysis of TGs, and (C) GC/FID analysis of FAMEs. HPLC and GC conditions are identical as for Fig. 1.

probably by their different diet. Three naturally occurring *trans*-FAs are identified in analyzed animal fats (*i.e.*, Δ*9t*-C18:1, Δ*11t*-C18:1 and Δ*9t,12t*-C18:2). Ruminant samples contain *trans*-FAs from 1% up to 7%, where vaccenic acid (Δ*11t*-C18:1) with the concentration up to 5.6% in sheep sample is the most abundant *trans*-FA. On the other hand, their concentrations in herbivore (rabbit) and omnivore samples are only trace (at most 0.4% of *trans*-FAs in rabbit).

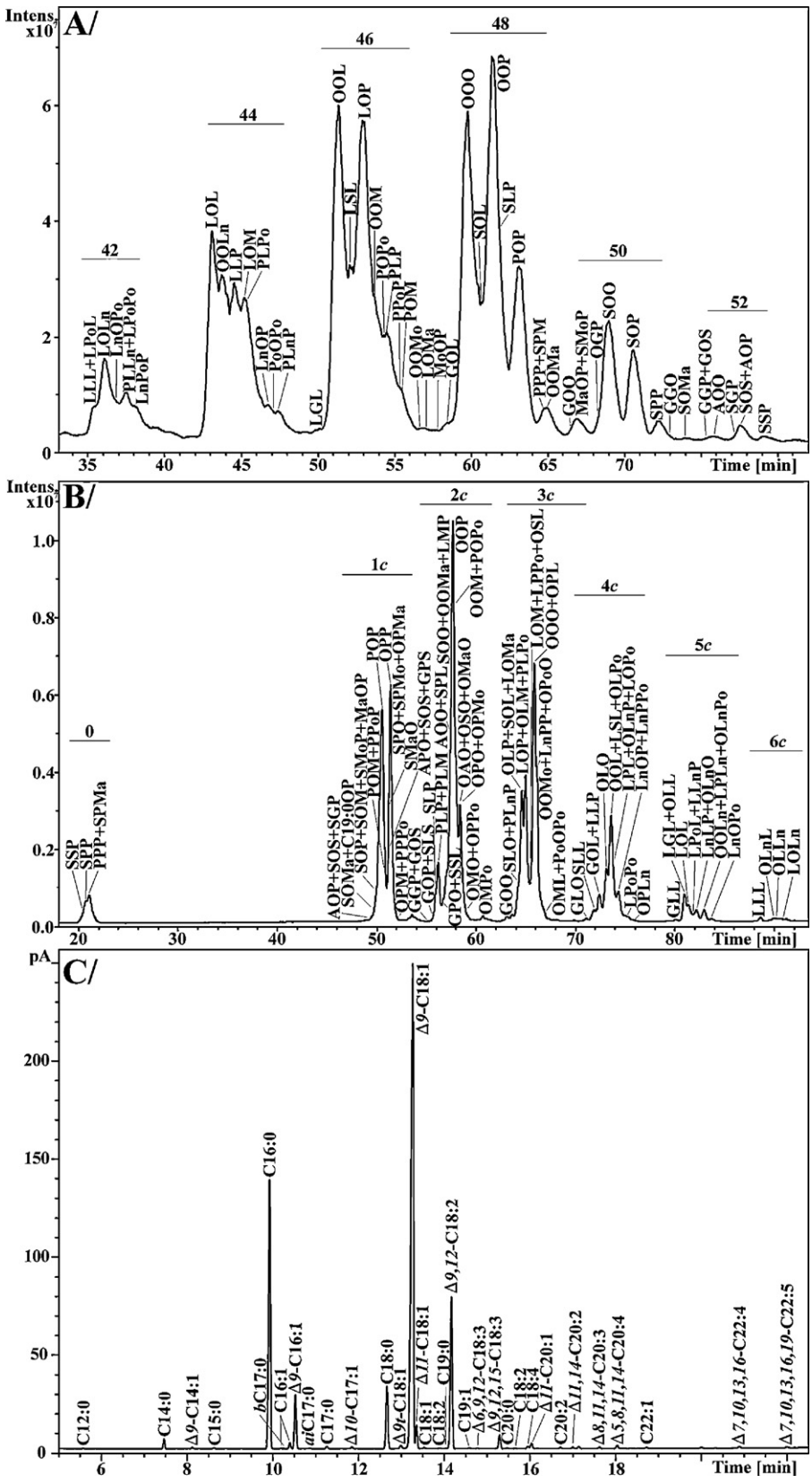

Fig. 6. Analysis of duck (*Anas platyrhynchos domesticus*): (A) NARP-HPLC/APCI-MS analysis of TGs, (B) silver-ion HPLC/APCI-MS analysis of TGs, and (C) GC/FID analysis of FAMEs. HPLC and GC conditions are identical as for Fig. 1.

Table 2 shows the sums of FAs identified in analyzed samples. All samples of animal fats are composed mainly from saturated (28–76%) and monounsaturated (19–57%) FAs, while the content of polyunsaturated FAs is rather low (3–20%) in contrast to common plant oils composed mainly from mono- (15–65%) and polyunsaturated (10–70%) FAs with low concentration of saturated FAs

Table 2
The number of identified FAs and the relative weight concentrations [%] of saturated, monounsaturated, polyunsaturated, essential (linoleic and linolenic acids), branched and *trans*-FAs in analyzed samples using GC/FID analysis of FAMEs.

	Number of FAs	Saturated FAs (%)	Monounsaturated FAs (%)	Polyunsaturated FAs (%)	Essential FAs (%)	Branched FAs (%)	*Trans*-FAs (%)
Fallow deer	53	75.4	21.1	2.9	2.3	16.6	1.3
Red deer	49	76.4	18.9	4.0	3.0	4.0	3.5
Sheep	45	59.7	35.3	4.5	2.3	2.2	7.1
Moufflon	61	43.6	45.9	6.1	3.5	12.0	3.2
Wild boar	29	39.4	45.4	14.8	13.6	0.1	0.1
Cock	31	31.7	56.3	11.7	10.9	0	0.3
Duck	34	28.0	56.5	15.0	13.8	0.1	0.3
Rabbit	44	50.0	29.8	19.7	18.8	1.3	0.4

Table 3
Relative peak areas [%] of DB groups (*c* – *cis*-, *t* – *trans*-) in analyzed samples from silver-ion HPLC/APCI-MS.

	DB group											
	0	1*t*	1*c*	1*t*,1*c*	2*c*	1*t*,2*c*	3*c*	1*t*,3*c*	4*c*	5*c*	6*c*	7*c*	8*c*
Fallow deer	31.8	5.1	44.0	4.1	11.9		2.6		0.4	0.1			
Red deer	36.3	13.7	34.5	4.1	8.2	0.1	2.7	0.1	0.3				
Sheep	17.7	13.5	33.3	11.8	17.8	0.8	3.8	0.5	0.7	0.1			
Moufflon	9.3	2.5	39.1	6.8	28.9	3.3	8.1	0.5	1.4	0.1			
Wild boar	4.4		31.1		32.3		20.3		8.2	2.8	0.8	0.1	
Cock	2.6		27.0		33.8		24.3		9.8	2.2	0.3		
Duck	2.7		22.4		32.5		27.0		11.6	3.2	0.6		
Rabbit	11.1		29.1		26.0		18.1		10.0	3.9	1.3	0.4	0.1

Table 4
Regioisomeric composition of selected TGs in animal fats compared to sunflower oil as a representative plant oil.

Regioisomers	Sunflower oil[a]	Pig[a]	Cattle[b]	Fallow deer	Red deer	Sheep	Moufflon	Wild boar	Cock	Duck	Rabbit
POP/OPP	100/0	8/92	63/37	66/34	74/26	61/39	76/24	10/90	78/22	52/48	51/49
OOP/OPO	98/2	12/88	94/6	87/13	77/23	94/6	95/5	18/82	87/13	53/47	67/33
PLP/LPP	100/0	1/99	61/39	57/43	64/36	65/35	0/0	8/92	53/47	56/44	52/48
LLP/LPL	97/3	9/91	62/38	61/39	57/43	54/46	59/41	9/91	52/48	58/42	54/46
OLP/LOP/OPL	63/36/1	3/12/85	49/36/15	50/38/12	57/28/15	56/34/10	46/30/24	6/9/85	54/37/9	41/36/23	47/35/18

[a] Data from Ref. [33].
[b] Data from Ref. [23].

(10–25%) [18,43]. Animal fats also contain low concentrations of essential FAs (linolenic and linoleic acids) from 2.3% up to 18.8%, which is significantly lower than in plant oils. High concentrations of saturated FAs and low content of essential and other polyunsaturated FAs in animal fats is not favorable in the human diet due to the potential for the cardiovascular diseases. On the other hand, they contain FAs with special functions in the human organism, such as *b*FAs and vaccenic acid (precursor of conjugated linolenic acids), therefore the reasonable proportion of both animal and plant fats in the human diet is recommended.

FA profiles from GC/FID experiments are used for the simple characterization of animal fats, but the information about the composition of TGs is completely lost. NARP and silver-ion HPLC together with APCI-MS enables the separation and identification of intact TGs in analyzed samples providing information about their FA composition. TGs with 38 up to 56 ECN values are identified in animal fats using NARP-HPLC/APCI-MS method including TGs with branched and *trans*-FAs (Table S1) that are identified based on their different retention behavior. TGs with 0 up to 8 *cis*-DBs are separated using silver-ion HPLC. TGs containing one *trans*-DB and combinations with up to four *cis*-DBs are identified in rumen samples thanks to their significantly lower retention times in comparison to *cis*-TGs. Table 3 shows the relative peak areas of particular DB groups from silver-ion HPLC that are in agreement with the FA composition from GC/FID analysis (Tables 1 and 2).

Silver-ion HPLC/APCI-MS data provide information about the composition of TG regioisomers, which is important information for the nutrition due to the stereospecificity of human lipases and therefore different bioavailability of FAs on the glycerol skeleton. The preference of FAs with different unsaturation degree in the *sn*-2 position strongly depends on the origin of the sample. Table 4 shows the comparison of regioisomeric composition of TGs with common saturated, mono- and diunsaturated FAs in sunflower oil and animal fats. Plant oils have a very strong preference of unsaturated FAs in the *sn*-2 position with negligible concentration of regioisomers with saturated FAs in the *sn*-2 position [33]. In contrast to plant oils, pig and wild boar samples have an inverse regioisomeric composition with the strong preference of saturated FAs in the *sn*-2 position, for example regioisomers of PLP/LPP with the ratio 100/0 in sunflower vs. 1/99 in pig and 8/92 in wild boar samples, *etc*. Other animal samples have the preference of unsaturated FAs (mono and diunsaturated FAs) in the *sn*-2 position, but the high amount of regioisomers with saturated FAs in the *sn*-2 position is also present in the samples, *e.g.*, ratio of POP/OPP isomers in the samples range between 78/22 in cock and 51/49 in rabbit. Saturated FAs in the *sn*-2 position in red deer (Fig. 2B) and sheep (Fig. S1B) samples are preferred only in TGs containing one DB with *trans*-configuration, *i.e.*, TGs containing vaccenic acid, which preferentially occupies *sn*-1/3 positions.

4. Conclusions

The detailed characterization of TG composition of 8 animal fats using NARP-HPLC/APCI-MS, silver-ion HPLC/APCI-MS and GC/FID methods is described in this work. 81 FAs including a high number of branched/linear and *cis*-/*trans*-DB isomers have been detected using GC/FID method. NARP mode enables the identification of

intact TGs containing branched and *trans*-FAs due to the different retention behavior in comparison to their linear and *cis*-FAs analogs. The ratios of TG regioisomers are determined based on silver-ion HPLC data. Unsaturated FAs are preferred over saturated FAs in the *sn*-2 position in ruminant and herbivore samples, while saturated FAs are strongly preferred in the *sn*-2 position in wild boar sample. The combination of three analytical techniques is time consuming, but it brings the maximum information content about highly complex animal samples including the identification of different types of isomerism. We have identified 282 TGs including regioisomers, 21 of them containing *b*FAs and 55 *trans*-FAs.

Acknowledgments

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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.chroma.2011.07.032.

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