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Research Article

Orthogonality of silver-ion and non-aqueous reversed-phase HPLC/MS in the analysis of complex natural mixtures of triacylglycerols

The goal of this work is the study of possibilities of two basic separation modes used in the analysis of complex triacylglycerol (TG) samples of plant oils and animal fats, *i.e.* non-aqueous reversed-phase (NARP) and silver-ion HPLC coupled with atmospheric pressure chemical ionization mass spectrometry (APCI-MS). The orthogonality of both separation modes is tested for complex TG mixtures containing fatty acids (FAs) with different acyl chain lengths, different number, positions and geometry of double bonds (DBs) and different regioisomeric positions of FAs on the glycerol skeleton. The retention in NARP mode is governed by the equivalent carbon number, while the retention in silver-ion chromatography increases with the increasing number of DBs with a clear differentiation between *cis*- and *trans*-FAs. Moreover, silver-ion mode enables at least the partial resolution of regioisomeric TG mixtures including *cis*-/*trans*-regioisomers, as illustrated on two examples of randomization mixtures. Off-line 2D coupling of both complementary modes (NARP in the first dimension and silver-ion in the second dimension) yields the superior chromatographic selectivity resulting in the highest number of identified TGs ever reported for studied samples. Off-line 2D chromatograms are processed with the home-made software providing various ways of data visualization.

Keywords: 2D liquid chromatography / Regioisomers / Silver-ion chromatography / *Trans* fatty acids / Triacylglycerols
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1 Introduction

Triacylglycerols (TGs) from plant oils and animal fats are an important part of human diet due to their high nutritional value. They are the source of energy, essential FAs (linoleic and linolenic acids), fat-soluble vitamins (A, D, E and K), *etc.* [1–4]. Natural samples of TGs are complex mixtures of tens up to hundreds of TG species with different physico–chemical properties given by the type of esterified FAs. TGs are characterized by the total carbon number (CN) and the number, position and configuration (*cis*-/*trans*-) of double bonds (DBs) in FA acyl chains. TGs can also differ in the stereospecific positions of FAs on the glycerol skeleton (*sn*-1, 2 or 3) yielding TG regioisomers. If TG has different FAs in *sn*-1/3 positions, then the carbon atom in the *sn*-2 position becomes a chiral center. The combination of different FAs and above mentioned types of isomerism in TGs lead to an enormous number of TG species in natural samples. The characterization of natural mixtures based on the determination of esterified FAs is not sufficient to describe all physico–chemical and nutritional properties of samples. The determination of stereospecific positions of FAs in TGs is important due to the stereospecific environment in the human organism. Another issue is the identification of *trans*-FAs (TFAs) in TGs, which are assumed to have harmful health effects. The characterization of TGs in complex natural mixtures including the determination of different types of isomerism is a challenging task requiring the combination of various separation modes, careful optimization of separation conditions and the use of appropriate detection technique.

Non-aqueous reversed-phase (NARP) HPLC coupled to MS enables the identification of the highest number of TG species in natural samples [5–19]. The retention of TGs depends on the equivalent carbon number (ECN) defined as the total CN in all acyl chains minus two times the number of DBs. The retention of TGs increases depending on the

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Abbreviations: A, arachidic; **APCI,** atmospheric pressure chemical ionization; **CN,** carbon number; **DB,** double bond; **2D,** two-dimensional; **E,** elaidic; **ECN,** equivalent carbon number; **FA,** fatty acid; **G,** gadoleic; **L,** linoleic; **Ln,** alpha-linolenic; **γ-Ln,** gamma-linolenic; **M,** myristic; **Ma,** margaric; **Mo,** margaroleic; **NARP,** non-aqueous reversed-phase; **O,** oleic; **P,** palmitic; **Po,** palmitoleic; **S,** stearic; **St,** stearidonic; **TFA,** *trans*-fatty acid; **TG,** triacylglycerol

ECN. Under optimized NARP-HPLC conditions (*i.e.* column packing, column length, separation temperature, mobile phase gradient, *etc.*) [11, 13], TGs within one ECN group can be separated according to the length and unsaturation of FAs, the position [12, 16, 19] and configuration of DBs [17, 20]. NARP system has a lower selectivity for the separation of TG regioisomers and their partial separation is feasible only with the multiple column coupling and very long retention times in the range of 100–200 min [21, 22], which is not practical for the routine use.

TGs differing in the DB number are well separated using silver-ion normal-phase HPLC [23–29]. This method is based on the formation of weak reversible complexes of silver ions immobilized on the stationary phase (impregnated silica or ion-exchange column) with π electrons of DBs during the sample elution throughout the chromatographic column. The retention of TGs increases with increasing unsaturation of TGs and it is also affected by the position [26, 28] and geometry [23, 29] of DBs and partially by the length of acyl chain [26]. The steric availability of DBs for the interaction with silver ions in regioisomers enables their separation under carefully optimized conditions [23, 26], *i.e.* column packing and column length, mobile phase composition or gradient steepness. The identification of TG regioisomers is also possible using MS detection based on different relative abundances of fragment ions formed by the neutral losses of FAs from *sn*-1/3 and *sn*-2 positions [10, 18, 30–33]. This approach is often applied for the assignment of prevailing FA in the *sn*-2 position, however the calibration curves for mixtures of both regioisomers have to be measured for the quantitative determination of *sn*-2 occupation [10, 30–32] using the same instrument and ionization technique. Standards of regioisomers are commercially available only at very limited range and their synthesis by the randomization procedure from mono-acid TG standards enables to measure relative abundances of fragment ions for pure compounds [26]. Atmospheric pressure chemical ionization (APCI) is the most frequently used ionization technique for TGs analysis due to their non-polar character, but the electrospray ionization can be applied as well due to the formation of ammonium adducts [34].

The combination of NARP and silver-ion separations in 2D chromatography using either on-line [35–37] or off-line [38–40] mode with APCI-MS detection promises the identification of the highest number of TGs in complex mixtures from plant oils and animal fats. On-line separation with silver-ion separation in the first dimension and NARP in the second dimension enables the fast separation of TGs in two chromatographic modes without the intervention of operator. In contrast, off-line setup requires the fraction collection in the first dimension and their analysis in the second dimension is more laborious, but the retention window in the second dimension is not limited by the fraction collection time in the first dimension.

The main goal of our work is the development of off-line 2D HPLC/MS method applicable for the separation of the highest possible number of TGs including the resolution of TG regioisomers and TGs containing TFA. TGs are separated in the first NARP dimension according to the ECN and collected 1 min fractions are then analyzed with silver-ion HPLC in the second dimension followed by APCI-MS identification. The possibility of off-line 2D HPLC for a wide range of TGs is illustrated with complex TG mixtures prepared by the randomization procedure. Selected examples of complex plant oil (blackcurrant oil) and animal fat (beef tallow) are discussed.

2 Materials and methods

2.1 Materials

Acetonitrile, 2-propanol and hexane (HPLC gradient grade solvents) and sodium methoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standards of tripalmitin (PPP, C16:0), tristearin (SSS, C18:0), triolein (OOO, 9-C18:1), trielaidin (EEE, 9*t*-C18:1), trilinolein (LLL, 9,12-C18:2), trilinolenin (LnLnLn, 9,12,15-C18:3) and triarachidin (AAA, C20:0) were purchased from Nu–ChekPrep (Elysian, MN, USA). Blackcurrant oil was prepared in the laboratory using the extraction with hexane according to ref. [10]. Beef tallow was prepared from the fat tissue using the same procedure after the homogenization of tissue with hexane in a homogenizer.

2.2 Randomization

Standards of mixed-acid TGs ($R_1R_1R_2$, $R_1R_2R_2$, *etc.*) were prepared from mono-acid triacylglycerols ($R_1R_1R_1$) using the randomization procedure according to Ref. [26]. Briefly, the mixture of 25 mg of OOO, 25 mg of EEE and 100 mg of sodium methoxide were weighed into a dry boiling flask with the addition of 2 mL of hexane dried with molecular sieves. The mixture was heated for 30 min in a water bath under the reflux condenser at constant temperature 75°C. Then, the reaction mixture was extracted with water and three times with 1 mL of methanol to remove sodium methoxide. The hexane phase containing the randomized analyte was used for the HPLC analysis. The same procedure was applied for the mixture of 15 mg of PPP, 15 mg of SSS, 15 mg of OOO, 15 mg of LLL, 15 mg of LnLnLn, 15 mg of AAA and 180 mg of sodium methoxide.

2.3 NARP-HPLC

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 Millenium chromatography manager (all from Waters, Milford, MA, USA). Samples

were analyzed using the following HPLC conditions: two chromatographic columns Nova-Pak C_{18} (300 × 3.9 and 150 × 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 UV detection at 205 nm. The mobile phase gradient with the slope of 0.65%/min was used for the analysis of TGs from plant oils (method 1) according to ref. [11]: 0 min–100% acetonitrile, 106 min–31% acetonitrile + 69% 2-propanol, 109 min–100% acetonitrile. The maximum backpressure at the end of gradient was about 270 bars. The mobile phase gradient with the slope of 0.33%/min was used for the analysis of TGs from animal fats (method 2): 0 min–80% A + 20% B, 121 min–40% A + 60% B, 122 min–80% A + 20% B, where A was acetonitrile and B was 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 3.20 min for the system with 300+150 mm Nova-Pak C_{18} columns. The automated fraction collector Gilson FC203B (Middleton, WI, USA) was used for collecting 1 min fractions in the NARP mode using 2 mL vials. Solvents from collected fractions were evaporated using a mild stream of nitrogen and then fractions were redissolved in 150 μL of the initial mobile phase used for the silver-ion HPLC. These fractions were subsequently analyzed by silver-ion HPLC/MS.

2.4 Silver-ion HPLC

Silver-ion HPLC experiments were performed on the liquid chromatograph Agilent 1200 Series (Agilent Technology, Waldbronn, Germany). The final silver-ion HPLC method for the analysis of plant oils and animal fats used the following conditions [26]: three silver-ion columns Chrom-Spher Lipids (250 × 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). Mobile phases were prepared fresh every day. Silver-ion columns were conditioned at 50 μL/min of the initial mobile phase composition overnight and then at 1 mL/min for one hour before the first analysis. The injector needle was washed with the mobile phase before each injection. The chromatographic system was equilibrated between injections for 30 min. The hybrid quadrupole time-of-flight analyzer micrOTOF-Q (Bruker Daltonics, Bremen, Germany) with positive-ion APCI was used in the mass range 50–1200 *m/z* with the following tuning parameters: the flow of the nebulizing and drying gas 5 and 3 L/min, respectively, temperatures of the drying gas and APCI heater 300 and 400°C, respectively. Reconstructed ion current chromatograms were used to support the identification of coeluting peaks.

2.5 Evaluation of 2D data

2D data were processed using the software developed in the laboratory [41] based on the Python programming language with Matplotlib 2D plotting library. The data from mass spectrometer were exported as netCDF files. Reconstructed ion chromatograms were converted into the ASCII format and the matrix with rows corresponding to the fraction collection period was created. 2D chromatograms were constructed as contour plots with the first dimension retention times from NARP-HPLC on the *x*-axis and the second dimension retention times from silver-ion HPLC on the *y*-axis. Retention data of all TGs detected in this work are summarized in Table 1.

3 Results and discussion

3.1 Comparison of retention mechanisms in NARP and silver-ion HPLC

First, the optimum TG test mixture has been selected for the demonstration of possibilities and limits of both NARP and silver-ion separation modes. Two types of isomerism are critical for the HPLC separation, *i.e.* regioisomerism ($R_1R_1R_2$ *versus* $R_1R_2R_1$) and geometrical isomerism of DBs (*cis- versus trans-*). Therefore, mono-acid TGs containing *cis*-FA (oleic acid, 9-C18:1, O) and *trans*-FA (elaidic acid, 9*t*-C18:1, E) have been randomized according to the procedure described in our previous work [26] yielding a randomization mixture of TGs at identical concentration ratios: OOO, OOE, OEO, EOO, OEE, EOE, EEO and EEE. Doublets OOE/EOO and OEE/EEO are enantiomers and hence they cannot be resolved in non-chiral environment, so their coelution must be expected in both studied systems. NARP systems provide the partial separation of species differing in the total number of TFA, but regioisomers are coeluting under these conditions (Fig. 1A). On the other hand, silver-ion mode yields more than sufficient separation of TGs differing in the total number of TFA but also partial separation of regioisomers EOE/OEE and OOE/OEO (Fig. 1B). It is interesting to note that the retention of TFAs is slightly higher in NARP mode, but significantly lower in silver-ion compared with *cis*-FAs. It is in agreement with well-known fact that the physico-chemical properties of TFA are closer to saturated FAs due to the linear arrangement of alkyl chains containing *trans*-DB similarly as for saturated chains. This simple rule is generally valid for the retention of all TGs in both separation modes, which helps in the planning of optimal conditions for 2D separation of target analytes and also the data interpretation.

The second example (Fig. 2) is the randomization mixture of the same amounts of six mono-acid TGs (PPP, SSS, OOO, LLL, LnLnLn and AAA) with different number of DBs and lengths of FA acyl chains providing theoretically $n^3 = 216$ (for $n = 6$) of different TGs considering all types of isomerism [26]. When enantiomers are neglected, there is

Table 1. Retention times of all TGs detected in silver-ion mode and NARP mode for studied samples in this work

TG[a]	DB	t_R in silver-ion mode (min)[b]	ECN	t_R in NARP mode (min)	
				Method 1[c]	Method 2[d]
AAA	0	16.9	60	117.5	–
AAS[e]		17.1	58	113.4	–
AAP[e]		17.5	56	109.0	–
ASS[e]		17.5	56	109.1	92.2
SSS		17.6	54	104.6	86.2
ASP[e]		17.6	54	104.7	86.5
SSMa[e]		17.8	53	–	83.2
APP[e]		17.9	52	99.6	–
SSP[e]		17.9	52	99.7	79.8
SPMa[e]		18.1	51	–	76.5
SPP[e]		18.3	50	94.4	72.9
SMaM[e]		18.5	49	–	67.8
PPP		18.7	48	88.7	65.5
SPM[e]		18.7	48	–	65.6
AOA	1	36.1	56	107.4	–
AOS		36.9	54	102.6	84.8
SGS		36.9	54	–	84.4
SC19:00[e]		37.3	53	–	81.8
AOP		37.3	52	97.5	78.2
SOS		37.3	52	97.6	78.3
SGP		37.3	52	–	77.8
SOMa		37.5	51	–	74.8
SOP		37.7	50	92.3	71.3
POMa		37.9	49	–	67.7
SMoP		37.9	49	–	67.8
POP		38.1	48	87.0	63.8
SOM		38.1	48	–	64.0
POC15:0		38.3	47	–	60.2
PPoP		38.4	46	–	56.1
POM		38.4	46	81.3	56.2
AAO		38.6	56	107.4	–
GSS		38.6	54	–	84.4
ASO[e]		38.6	54	102.6	84.8
GSP[e]		38.9	52	–	77.8
APO[e]		38.9	52	97.5	78.2
SSO		38.9	52	97.6	78.3
SMaO[e]		39.1	51	–	74.8
SPO[e]		39.3	50	92.3	71.3
OPMa[e]		39.7	49	–	67.7
SPMo[e]		39.6	49	–	67.8
OPP		39.7	48	87.0	63.8
OSM[e]		39.7	48	–	64.0
PC15:00[e]		39.9	47	–	60.2
PPPo		40.0	46	–	56.1
OPM[e]		40.0	46	81.3	56.2
SOE	2	48.6	50	–	70.8
EOP		48.9	54	–	63.2
ALA		50.9	54	102.2	–
ALS		51.1	52	96.9	–
ALP		51.4	50	91.8	–
SLS		51.4	50	91.9	–
SLP		51.7	48	86.6	62.9
PLP		52.0	46	80.9	55.2
AAL		52.1	54	102.2	–
ASL[e]		52.2	52	96.9	–
APL[e]		52.5	50	91.8	–
SSL		52.5	50	91.9	–
SPL[e]		52.8	48	86.6	62.9
GSO		52.8	52	–	76.3
AOO		52.8	52	96.0	76.7
LPP		53.0	46	80.9	55.2
GPO		53.1	50	–	69.1
SOO		53.1	50	90.8	69.6
OOMa		53.2	49	–	66.0
OOP		53.4	48	85.4	62.0
OAO		53.8	52	96.0	–
OSO		54.0	50	90.8	69.6
OMaO		54.2	49	–	66.0
OPO		54.3	48	85.4	62.0
OPMo		54.5	47	–	58.3
OMO		54.6	46	–	54.4
OPPo		54.6	46	–	54.8
ALnA	3	58.3	52	97.3	–
ALO		58.6	50	90.4	–
ALnS		58.6	50	92.2	–
SLnS		58.7	48	86.9	–
ALnP		58.9	48	86.8	–
SLnP		59.0	46	81.4	55.2
SLO		59.2	48	85.1	61.4
AOL		59.2	50	90.4	–
OLMa		59.4	47	–	57.6
OPoPo		59.8	44	–	47.5
PLnP		59.8	44	75.4	–
OLP		59.8	46	79.3	53.6
SOL		59.8	48	85.1	61.4
AALn		60.1	52	97.3	–
LOP		60.5	46	79.3	53.6
OAL		60.7	50	90.4	–
ASLn[e]		60.7	50	92.2	–
SSLn		61.0	48	86.9	–
APLn[e]		61.1	48	86.8	–
OSL		61.2	48	85.1	61.4
OOO		61.6	48	84.0	60.5
SPLn[e]		61.7	46	81.4	55.2
OPL		61.8	46	79.3	53.6
LnPP		61.9	44	75.4	–
ALL	4	68.6	48	84.8	–
ALnO		68.6	48	85.4	–
SLL		69.0	46	79.0	–
LLP		69.4	44	73.1	45.6
AOLn		69.7	48	85.4	–
OLO		70.0	46	77.9	52.0
LAL		70.0	48	84.8	–
OOL		70.4	46	77.9	52.0
LSL		70.4	46	79.0	53.2
SLnO		70.4	46	80.0	–
SOLn		70.7	46	80.0	–
LPL		70.8	44	73.1	45.6
OLnP		70.9	44	74.0	–
OALn		71.2	48	85.4	–
γLnOP		71.4	44	74.0	–

Table 1. Continued

TG[a)]	DB	t_R in silver-ion mode (min)[b)]	ECN	t_R in NARP mode (min)	
				Method 1[c)]	Method 2[d)]
OSLn		71.8	46	80.0	–
OPLn		72.2	44	74.0	–
LnOP		72.2	44	74.7	–
GLL	5	75.3	46	77.2	–
ALnL		76.6	46	79.6	–
ALLn		76.7	46	79.6	–
OLL		77.0	44	71.8	–
SLnL		77.1	44	73.8	–
SLLn		77.3	44	73.8	–
LOL		77.4	44	71.8	–
LALn		78.2	46	79.6	–
LSLn		78.8	44	73.8	–
LLnP		79.3	42	67.8	–
LnLP		79.5	42	67.8	–
OLnO		79.7	44	72.6	–
OOLn		80.4	44	72.6	–
LPLn		80.9	42	67.8	–
γLnLP		81.2	42	68.5	–
OOγLn		81.3	44	73.3	–
GLLn	6	85.3	44	72.1	–
LLL		85.4	42	65.3	–
GLγLn		86.6	44	72.8	–
OLnL		86.7	42	66.4	–
OLLn		86.8	42	66.4	–
ALnLn		87.0	44	74.3	–
LOLn		87.2	42	66.4	–
SLnLn		87.5	42	68.5	–
LnLnP		88.2	40	62.1	–
LOγLn		88.6	42	67.1	–
LnALn		88.7	44	74.3	–
LnSLn		89.2	42	68.5	–
LnPLn		89.7	40	62.1	–
γLnPLn		91.1	40	62.8	–
γLnγLnP		91.5	40	63.5	–
SLSt		91.5	42	69.6	–
LLnL	7	92.1	40	59.6	–
LLLn		92.3	40	59.6	–
LLγLn		93.7	40	60.4	–
OLnLn		94.1	40	60.8	–
LnOLn		94.5	40	60.8	–
γLnOLn		95.2	40	61.6	–
γLnOγLn		95.8	40	62.3	–
OLSt		96.6	40	61.9	–
LOSt		97.0	40	61.9	–
LnPSt		98.1	38	57.2	–
LLnLn	8	101.6	38	54.0	–
LnLLn		101.8	38	54.0	–
γLnLLn		102.6	38	54.7	–
LLSt		104.3	38	55.1	–
γLnLγLn		104.4	38	55.4	–
LnOSt		105.4	38	56.1	–
γLnOSt		106.0	38	56.8	–
StPSt		108.5	36	52.6	–
LnLnLn	9	110.1	36	48.3	–
LnLSt		111.0	36	49.4	–
γLnLnLn		111.4	36	49.0	–
γLnLSt		112.5	36	50.1	–
γLnγLnLn		112.9	36	49.7	–
γLnγLnγLn		114.3	36	50.6	–
LnLnSt	10	120.2	34	43.5	–
γLnLnSt		121.9	34	44.2	–
γLnγLnSt		123.1	34	44.9	–

a) Abbreviations used for fatty acids: **A**–arachidic (C20:0); **E**–elaidic (Δ9*trans*-C18:1), **G**–gadoleic (Δ9-C20:1), **L**–linoleic (Δ9,12-C18:2), **Ln**–alpha-linolenic (Δ9,12,15-C18:3), γ-**Ln**–gamma-linolenic (Δ6,9,12-C18:3), **M**–myristic (C14:0), **Ma**–margaric (C17:0), **Mo**–margaroleic (Δ9-C17:1), **O**–oleic (Δ9-C18:1), **P**–palmitic (C16:0), **Po**–palmitoleic (Δ9-C16:1), **S**–stearic (C18:0), **St**–stearidonic (Δ6,9,12,15-C18:4).

b) Silver-ion HPLC – three ChromSpher Lipids columns in the total length 75 cm, flow rate 1 mL/min, injection volume 1 μL, column temperature 25°C, 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).

c) NARP method 1 – two chromatographic columns Nova-Pak C_{18} in the total length 45 cm, flow rate 1 mL/min, injection volume 10 μL, column temperature 25°C, mobile phase gradient: 0 min–100% acetonitrile, 106 min–31% acetonitrile + 69% 2-propanol, 109 min–100% acetonitrile.

d) NARP method 2 – mobile phase 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 the mixture of hexane–2-propanol (1:1, v/v), other conditions are identical as for method 1.

e) These regioisomers cannot be resolved.

still $(n^3+n^2)/2 = 126$ different TGs. Figure 2A shows the NARP chromatogram, where some groups are strongly coeluting and they can be resolved only by reconstructed ion current chromatogram of appropriate m/z values due to APCI-MS detection. Regioisomers are not resolved in this mode; therefore, we use peak annotations with the asterisk for non-resolved regioisomers (Fig. 2). The retention is driven by the ECN with the partial or sometimes full separation inside ECN groups. Silver-ion chromatogram (Fig. 2B) shows quite different picture, where the retention is governed by the DB number, while the carbon chain length has only a minor effect on the retention. The unique feature of silver-ion chromatography is the ability to separate regioisomers under optimized conditions. The illustrative example of retention mechanism in this separation mode is the TG group containing one DB (Fig. 2B), which forms a mirror-like picture of two quintuplet peaks AOA/AOS/AOP+SOS/SOP/POP and AAO/ASO/APO+SSO/SPO/OPP. The regioisomeric position has a stronger effect on the retention than the carbon chain length. Unsaturations in the side *sn*-1/3 positions enable a stronger interaction with silver ions and hence higher retention than

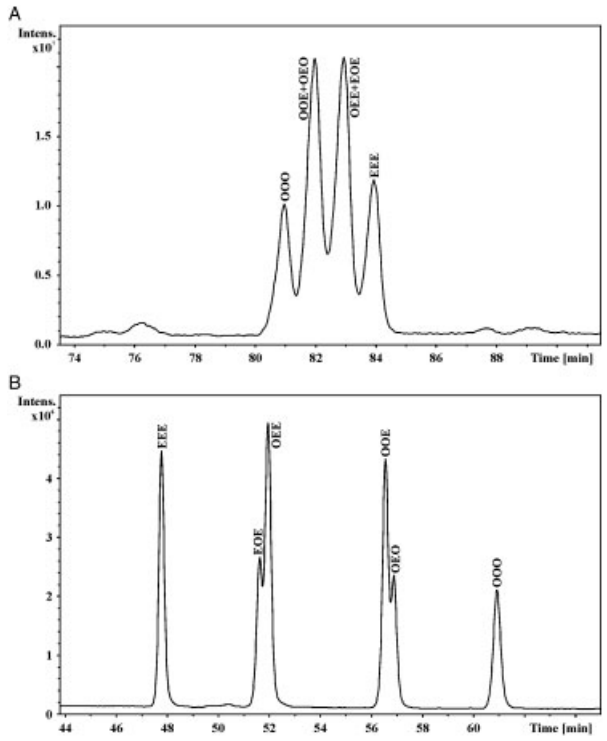

Figure 1. HPLC chromatograms of the randomization mixture of triolein (OOO, 9-C18:1) and trielaidin (EEE, 9*t*-C18:1) with APCI-MS detection: (A) NARP-HPLC using the method 1 – two Nova-Pak C_{18} columns in the total length 45 cm, flow rate 1 mL/min, column temperature 25°C, gradient of acetonitrile–2-propanol, (B) silver-ion HPLC – three ChromSpher Lipids columns in the total length 75 cm, flow rate 1 mL/min, column temperature 25°C, gradient of hexane–2-propanol–acetonitrile, see Section 2 for more details.

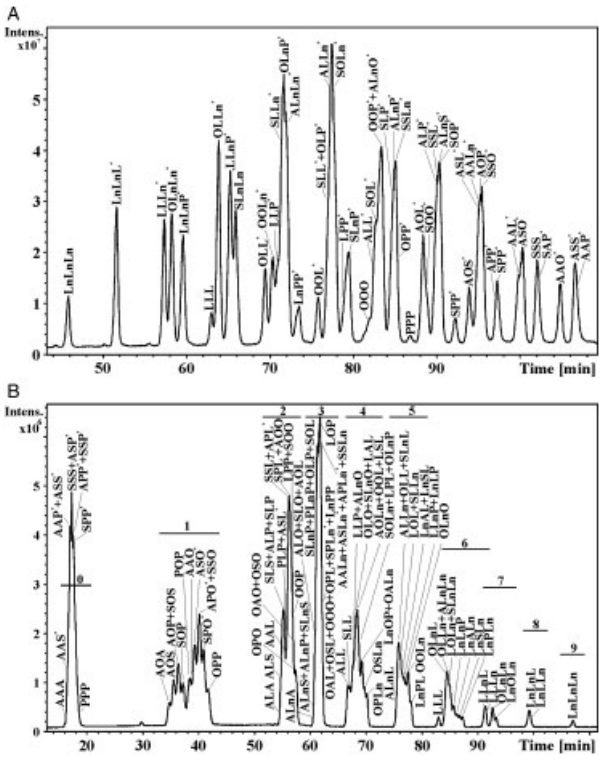

Figure 2. HPLC chromatograms of the randomization mixture of PPP, SSS, OOO, LLL, LnLnLn and AAA: (A) NARP chromatogram using the method 1, (B) silver-ion chromatogram with labeled DB groups, conditions identical as for Fig. 1. Non-resolved regioisomers are labeled with the asterisk.

regioisomers with the same unsaturation in the middle *sn*-2 position, for example POP < OPP. The longer saturated alkyl chain means the lower retention in silver-ion mode (A < S < P). Figure 3 is a compilation of 2D record obtained from NARP and silver-ion data showing relatively good coverage of 2D separation space. Colored labeling of DB and ECN groups improves the visualization of this plot containing labeled sections for 0–7 DBs and 40–54 ECNs, which is in total 64 sections out of which 27 sections are occupied by peaks of TGs, while the remaining 37 are empty, which is affected by the initial choice of mono-acid TGs used for the randomization. Figure 3 shows only the zoomed region of whole 2D chromatogram to enable a reasonable visibility of annotation. The full data are summarized in Table 1.

On the other hand, silver-ion chromatograms provide rather complex records of many strongly overlapping peaks within individual DB groups complicating the interpretation. If the silver-ion separation is coupled in off-line 2D separation with NARP, then the superior chromatographic performance is obtained providing the separation, identification and quantitation of the highest number of TG isomers. On-line 2D limits the analysis time in the second dimension typically to 1 min according to time used for the modulation, but this time is not sufficient either for the silver-ion separation of regioisomers or good resolution in NARP mode. Therefore, off-line 2D coupling enables to explore the full separation power of both dimensions resulting in the highest possible number of identified TGs at cost of higher total analysis time of 3 days running for 24 h per day automatically in case of blackcurrant oil including all steps, *i.e.* automated fraction collection in the first dimension and half an hour equilibration between individual injections in the second dimension.

3.2 Analysis of TGs in plant oils

Blackcurrant oil is one of the most complex plant oils analyzed in our laboratory [10, 11, 13–16, 26] containing a high proportion of essential FAs and both α- and γ-linolenic acids; therefore, this sample is selected for 2D analysis as a chromatographic challenge. Figure 4A depicts a zoomed region of 2D separation of blackcurrant oil, where the first dimension is NARP method 1 using the coupling of two NovaPak C_{18} columns in the total length of 45 cm. Collected 1 min fractions by automated fraction collector are then analyzed in the second dimension by silver-ion chromatography using the coupling of three ChromSpher Lipid columns in the total length of 75 cm (see Section 2 for more

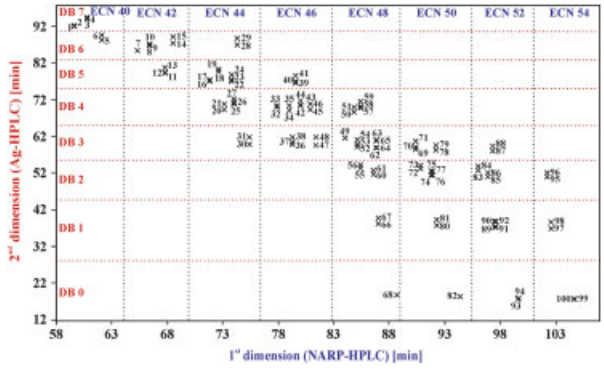

Figure 3. Reconstructed 2D plot compiled from NARP and silver-ion data shown in Fig. 2. Peak annotation:1-LLnL; 2-LLLn; 3-OLnLn; 4-LnOLn; 5-LnLnP; 6-LnPLn; 7-LLL; 8-OLnL; 9-OLLn; 10-LOLn; 11-LLnP; 12-LnLP; 13-LPLn; 14-SLnLn; 15-LnSLn; 16-OLL; 17-LOL; 18-OLnO; 19-OOLn; 20-LLP; 21-LPL; 22-SLnL; 23-SLLn; 24-LSLn; 25-OLnP; 26-LnOP; 27-OPLn; 28-ALnLn; 29-LnALn; 30-PLnP; 31-LnPP; 32-OLO; 33-OOL; 34-SLL; 35-LSL; 36-OLP; 37-LOP; 38-OPL; 39-ALnL; 40-ALLn; 41-LALn; 42-SLnO; 43-SOLn; 44-OSLn; 45-PLP; 46-LPP; 47-SLnP; 48-SPLn+PSLn; 49-OOO; 50-ALL; 51-LAL; 52-SLO; 53-SOL; 54-OSL; 55-OOP; 56-OPO; 57-ALnO; 58-AOLn; 59-OALn; 60-SLP; 61-SPL+PSL; 62-ALnP; 63-APLn+PALn; 64-SLnS; 65-SSLn; 66-POP; 67-OPP; 68-PPP; 69-ALO; 70-AOL; 71-OAL; 72-SOO; 73-OSO; 74-ALP; 75-APL+PAL; 76-SLS; 77-SSL; 78-ALnS; 79-ASLn+SALn; 80-SOP; 81-SPO+PSO; 82-SPP+PSP; 83-AOO; 84-OAO; 85-ALS; 86-ASL+SAL; 87-ALnA; 88-AALn; 89-AOP; 90-APO+PAO; 91-SOS; 92-SSO; 93-APP+PAP; 94-SSP+SPS; 95-AAL; 96-ALA; 97-AOS; 98-ASO+SAO; 99-SSS; 100-APS+ASP+PAS.

details). Fractions without detected peaks in the first dimension are not analyzed in the second dimension to save instrumental time. 2D plot shows colored labeling of 3–7 DBs and 40–46 ECNs (in total 20 sections), where 50% of them contain chromatographic peaks. Peaks in other boxes cannot be expected, because such combinations are not common in the nature.

The home-made software [41] has been previously used for the processing of comprehensive 2D chromatograms, but this work extends its applicability also for the successful visualization of off-line 2D data (Fig. 4B). The *z*-axis (out of plane) is in the logarithmic scale because of better visualization of small peaks. Another way of the presentation of 2D data is three-dimensional chromatogram shown in Fig. 6. Each presentation style of 2D data shown here has some advantages and drawbacks. Dot plots (Figs. 4A and 6A) are well suited for the precise observation of retention times in both dimensions, but the information on peak intensities and chromatographic resolution is lost. Three-dimensional presentation (Fig. 5) shows quickly an overall appearance of 2D record. Contour plots (Figs. 4B and 6B) are something in between, because retention times can be determined relatively easily from these records, the chromatographic resolution can be seen, but some problems may be with small and/or coeluting peaks. For these reasons, contour plots are considered the most suitable for the visualization of 2D chromatograms [35–37, 41].

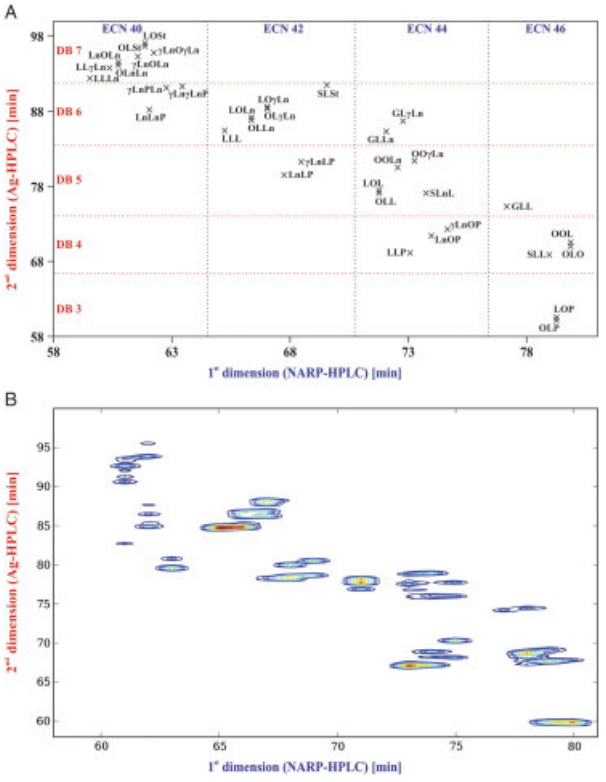

Figure 4. Off-line 2D chromatograms of blackcurrant oil using NARP in the first dimension and silver-ion mode in the second dimension after the fraction collection each minute: (A) dot plot with the peak annotation and DB and ECN labeling, (B) contour plot showing peak intensities, NARP and silver-ion conditions identical as for Fig. 1.

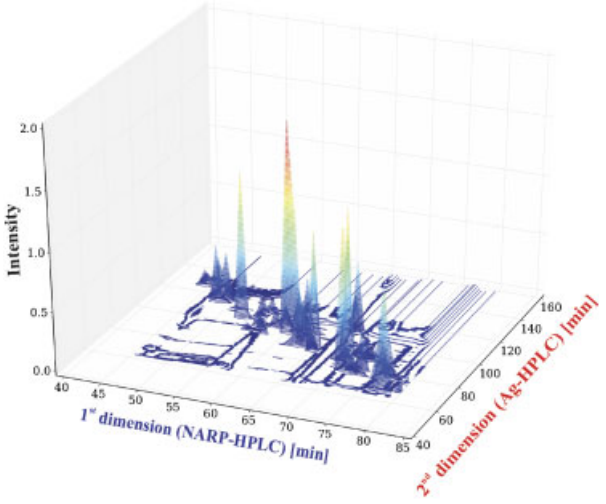

Figure 5. Three-dimensional presentation of 2D chromatogram of blackcurrant oil, conditions identical as for Fig. 1.

3.3 Analysis of TGs in animal fats

Some animal fats are known for their content of TFAs, which are monitored in our diet due to possible negative

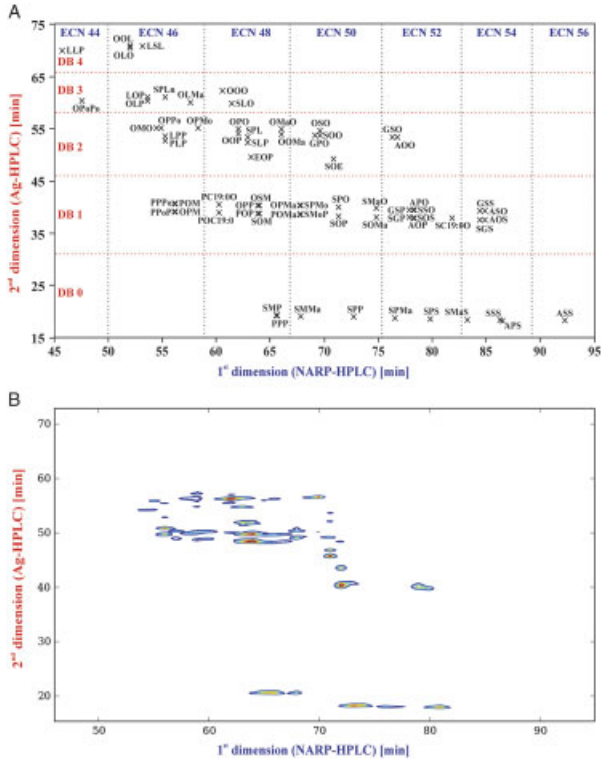

Figure 6. 2D chromatograms of beef tallow using NARP method 2 for the first dimension and silver-ion mode for the second dimension after the fraction collection each minute: (A) dot plot with the peak annotation and DB and ECN labeling, (B) contour plot showing peak intensities.

health effects if consumed in higher amounts. Beef tallow (Fig. 6) contains mainly saturated and monounsaturated FAs with a significant portion of TFA, which can be easily identified in the silver-ion mode due to significantly lower retention in comparison with *cis*-FAs. In principle, NARP separation mode is also applicable for the separation of *cis*-/*trans*-isomers, but the resolution is much worse in comparison with the silver-ion mode. There is also a notable amount of FAs containing the odd number of carbon atoms (*i.e.* C17:0, C17:1, C19:0) unlike to plant oils, where odd number FAs are present only at trace levels. For a better clarity, odd number ECN groups are not labeled in Fig. 6A, *e.g.* SMaO and SOMa with ECN = 51 and DB = 1 can be found in the section corresponding to ECN = 50.

4 Concluding remarks

This work demonstrates the potential of off-line 2D combination of NARP-HPLC in the first dimension followed by the silver-ion HPLC analysis of collected fractions in the second dimension. This experimental setup provides the highest separation capacity of TGs in complex natural samples of plant oils and animal fats, as demonstrated on selected examples of blackcurrant oil as a highly complex plant oil containing both α- and γ-Ln and beef tallow as a representative of animal fats containing also TFAs. The randomization synthesis of regioisomeric TG standards with the selected composition has played an important role in the method development. NARP mode enables the separation of highly complex TG mixtures according to their ECN and also inside individual ECN groups, while the retention in silver-ion mode is governed mainly by the number and geometry of DBs with possible resolution of regioisomers. Both systems show quite good complementary as a prerequisite for 2D analysis. The disadvantage of presented approach is long total analysis time, therefore this method is recommended only for highly detailed characterization of complex samples, but not intended for the routine quality control of TG mixtures of lower complexity.

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