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Comparison of various types of stationary phases in non-aqueous reversed-phase high-performance liquid chromatography–mass spectrometry of glycerolipids in blackcurrant oil and its enzymatic hydrolysis mixture

Miroslav Lísa[a], Michal Holčapek[a,*], Helena Sovová[b]

[a] University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry, Studentská 573, 532 10 Pardubice, Czech Republic
[b] Institute of Chemical Process Fundamentals of the ASCR, v. v. i., Rozvojová 135, 165 02 Prague, Czech Republic

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ABSTRACT

The selection of column packing during the development of high-performance liquid chromatography method is a crucial step to achieve sufficient chromatographic resolution of analyzed species in complex mixtures. Various stationary phases are tested in this paper for the analysis of complex mixture of triacylglycerols (TGs) in blackcurrant oil using non-aqueous reversed-phase (NARP) system with acetonitrile–2-propanol mobile phase. Conventional C_{18} column in the total length of 45 cm is used for the separation of TGs according to their equivalent carbon number, the number and positions of double bonds and acyl chain lengths. The separation of TGs and their more polar hydrolysis products after the partial enzymatic hydrolysis of blackcurrant oil in one chromatographic run is achieved using conventional C_{18} column. Retention times of TGs are reduced almost 10 times without the loss of the chromatographic resolution using ultra high-performance liquid chromatography with 1.7 μm C_{18} particles. The separation in NARP system on C_{30} column shows an unusual phenomenon, because the retention order of TGs changes depending on the column temperature, which is reported for the first time. The commercial monolithic column modified with C_{18} is used for the fast analysis of TGs to increase the sample throughput but at cost of low resolution.

1. Introduction

The selection of appropriate column during the development of chromatographic method for the separation of complex mixtures of triacylglycerols (TGs) in plant oils and animal fats containing tens to hundreds of species with small differences in physicochemical properties is a crucial step to achieve the highest separation selectivity. Properties of TGs are given by the number, position(s) and configuration (*cis*-/*trans*-) of double bonds (DBs), acyl chain lengths, and stereochemical position (*sn*-1, 2 and 3) of fatty acids (FAs) on the glycerol skeleton (regioisomers and optical isomers) [1]. Individual chromatographic techniques do not allow the separation of all types of TG isomers and usually some compromise has to be made. Two main chromatographic techniques are used in the analysis of TG mixtures, i.e., silver-ion chromatography and non-aqueous reversed-phase high-performance liquid chromatography (NARP-HPLC). The silver-ion chromatography [2–9] is based on the formation of weak reversible complexes of silver ions with π electrons of DBs of unsaturated TGs. Ion-exchange or silica columns impregnated with silver ions are usually used with hexane-based mobile phases. In silver-ion chromatography, retention times of TGs are influenced by the number, position [5,8] and configuration [2,7,9] of DBs in acyl chains and partially by the acyl chain lengths [5]. The resolution of TG regioisomers ($R_1R_1R_2$ vs. $R_1R_2R_1$) using silver-ion chromatography is possible under carefully optimized chromatographic conditions [2,5,7].

The highest number of identified TGs has been reached in NARP-HPLC with atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) using the connection of C_{18} columns in the total length 45 cm and acetonitrile–2-propanol gradient [10–14]. In the NARP-HPLC system with the conventional C_{18} column, TGs are separated according to the equivalent carbon number (ECN) defined as the total number of carbon atoms (CN) minus two times the number of DBs, i.e., ECN = CN − 2DB. Retention times of TGs increase with the increasing ECN and TGs inside one ECN group are separated according to the composition of attached FAs, mainly the lengths of acyl chains and the number of DBs. The separation of TGs differing in the position [14–16] and configuration [17,18] of DBs or partial resolution of regioisomers [19,20] has been reported as well. Long hydrophobic alkyl chains of C_{30} stationary phase provide stronger interactions of non-polar compounds containing the long

* Corresponding author. Tel.: +420 46 6037087; fax: +420 46 6037068.
E-mail address: Michal.Holcapek@upce.cz (M. Holčapek).

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alkyl chain resulting in at least comparable or even better resolution in comparison to conventional C_{18} stationary phase. C_{30} columns are widely used for the separation of non-polar carotenoids or vitamins [21–23], but no separation of TGs using these columns has been reported so far.

NARP-HPLC separations of TGs using conventional C_{18} columns with 3–5 μm particle size provide good results but at the cost of long retention times in the range of tens of minutes. Nowadays, an effort to reduce the analysis time is of great interest due to the requirement on higher sample throughput. Decreasing the particle size enables to reduce the retention time in comparison to conventional columns. Columns with sub-two micron particles are used for ultra high-performance liquid chromatography (UHPLC) analysis with significantly shorter retention times at the same chromatographic resolution [24]. On the other hand, the use of sub-two micron particles results in higher system backpressure, therefore the special chromatographic system designed for higher pressures have to be used for UHPLC analysis. Monolithic stationary phases are characterized by large-sized through-pores resulting in the low flow resistance and the lower system backpressure. Its higher permeability enables the utilization of higher flow rates and increase of the sample throughput. Silica-based monoliths modified by C_{18} alkyl phase have been used for the analysis of TGs from plant oils [25].

The main goal of our work is the comparison of different NARP systems for the separation of TGs in complex natural samples. Conventional C_{18}, sub-two micron C_{18} particles, C_{30} column and C_{18} monolithic column in NARP systems are tested using blackcurrant oil containing the complex mixture of TGs including DB positional isomers. The analysis time, retention behavior, chromatographic resolution and the number of identified TGs using individual columns are discussed. NARP-HPLC system is also applied for the analysis of mixture of more polar hydrolysis products after the partial enzymatic hydrolysis of TGs in blackcurrant oil.

2. Experimental

2.1. Materials

Acetonitrile, 2-propanol (both HPLC gradient grade) and hexane (HPLC grade) were purchased from Sigma–Aldrich (St. Louis, USA). The solvents of mobile phase were degassed by continuous stripping with helium during the analysis. The hydrolysis of blackcurrant oil was performed with immobilized *sn*-1 and *sn*-3 selective enzyme in supercritical carbon dioxide [26]. The oil was extracted from seeds donated by Zemcheba (Chelčice, Czech Republic) and its main fraction containing TGs [27] was used. The reaction was carried out in a continuous-flow packed-bed reactor at temperature 40 °C and pressure 15 MPa with oil dissolved in supercritical carbon dioxide saturated with water. The enzyme Lipozyme was supplied by Fluka Chemie AG (Buchs, Switzerland). Blackcurrant oil and its hydrolysis mixture were dissolved in an acetonitrile–2-propanol–hexane mixture (1:1:1, v/v/v) at the concentration of 1% (w/v) and 10 μl was injected for the analysis in triplicate.

2.2. Chromatographic and detection conditions

2.2.1. HPLC analysis

All HPLC experiments were performed using the 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). NARP-HPLC conditions for the analysis of TGs with conventional C_{18} column were used according to Ref. [10], i.e., two chromatographic columns Nova-Pak C_{18} (150 mm × 3.9 mm and 300 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 a mobile phase gradient with a slope of 0.65%/min: 0 min-100% acetonitrile, 106 min-31% acetonitrile + 69% 2-propanol, 109 min-100% acetonitrile. ProntoSil C_{30} column (250 mm × 4.6 mm, 3 μm, Bischoff, Leonberg, Germany) was used for the analysis of TGs with the same HPLC conditions as for conventional C_{18} column. Experiments with monolithic stationary phase were performed using Chromolith Performance RP-18e column (100 mm × 4.6 mm, Merck, Darmstadt, Germany), the flow rate of 5 mL/min, the injection volume of 5 μL, column temperature of 25 °C and the isocratic elution with the mobile phase composition 85% acetonitrile + 15% 2-propanol. T-piece with the split ratio 1:4 was used for splitting of the flow rate before APCI-MS. The column hold-up volume, t_M, was measured with uracil – 3.20 min for the system with 300 + 150 mm Nova-Pak C_{18} columns and 3.00 min for the system with 250 mm C_{30} ProntoSil column. The injector needle was washed with the mobile phase before each injection. The UV detection at 205 nm and APCI-MS were coupled in series. The Esquire 3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany) in the mass range m/z 50–1200 was used with the following setting of tuning parameters: the pressure of the nebulizing gas of 70 psi, the drying gas flow rate of 3 L/min, temperatures of the drying gas and APCI heater were 350 and 400 °C, respectively.

2.2.2. UHPLC analysis

NARP-UHPLC experiments were performed on an Acquity UPLC system consisting of binary pump, diode-array UV detector, autosampler, thermostated column compartment and Empower chromatography data software (Waters, Milford, MA, USA). Acquity BEH C_{18} column (150 mm × 2.1 mm, 1.7 μm, Waters) was used with the flow rate of 0.4 mL/min, the injection volume of 1 μL, column temperature of 30 °C and the mobile phase gradient with the slope of 2%/min: 0 min-100% acetonitrile, 23 min-54% acetonitrile + 46% 2-propanol, 24 min-100% acetonitrile. Mobile phase gradient with the slope of 2%/min and the following composition: 0 min-80% acetonitrile + 20% 2-propanol, 14 min-52% acetonitrile + 48%

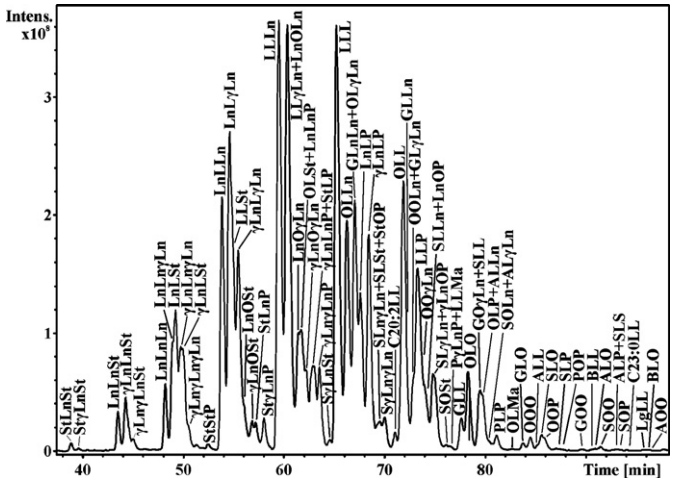

Fig. 1. NARP-HPLC/APCI-MS analysis of triacylglycerols in blackcurrant oil using conventional C_{18} column. *Conditions*: two Nova-Pak C_{18} columns (150 mm × 3.9 mm and 300 mm × 3.9 mm, 4 μm) connected in series, APCI-MS detection in positive-ion mode, flow rate 1 mL/min, column temperature 25 °C, gradient 0 min-100% acetonitrile, 106 min-31% acetonitrile + 69% 2-propanol (see Section 2 for more details and the explanation of used notation of identified TGs).

Table 1
Triacylglycerols (TG) identified in the blackcurrant oil with their equivalent carbon numbers (ECN), molecular weights (MW), retention times (t_R) and relative retention (r) using conventional C_{18} column and C_{30} column with column temperatures 25 and 40 °C.

TG	ECN	MW	C_{18} (25 °C)		C_{30} (25 °C)		C_{30} (40 °C)	
			t_R (min)[a]	r[b]	t_R (min)[a]	r[b]	t_R (min)[a]	r[b]
StLnSt	32	868	38.8	0.631	33.5[c]	0.598	25.2[c]	0.543
StγLnSt		868	39.5	0.644	n.d.	–	n.d.	–
LnLnSt	34	870	43.5	0.715	38.5[c]	0.696	29.4[c]	0.645
γLnLnSt		870	44.2	0.727	n.d.	–	n.d.	–
γLnγLnSt		870	44.9	0.739	n.d.	–	n.d.	–
LnLnLn	36	872	48.3	0.800	43.5[c]	0.794	33.9[c]	0.756
LnLnγLn		872	49.0	0.812	n.d.	–	n.d.	–
LnLSt		872	49.4	0.819	43.7[c]	0.798	34.2[c]	0.763
γLnLnγLn		872	49.7	0.824	n.d.	–	n.d.	–
γLnLSt		872	50.1	0.832	n.d.	–	n.d.	–
γLnγLnγLn		872	50.6	0.840	n.d.	–	n.d.	–
StStP		846	52.6	0.876	n.d.	–	n.d.	–
LnLLn	38	874	54.0	0.901	48.8[c]	0.898	38.9[c]	0.878
LnLγLn		874	54.7	0.913	n.d.	–	n.d.	–
LLSt		874	55.1	0.924	51.1	0.943	40.9[c]	0.927
γLnLγLn		874	55.4	0.926	n.d.	–	n.d.	–
LnOSt		874	56.1	0.938	51.1[c]	0.943	40.9[c]	0.927
γLnOSt		874	56.8	0.950	n.d.	–	n.d.	–
StLnP		848	57.2	0.957	58.2	1.082	45.2[c]	1.032
StγLnP		848	58.0	0.972	58.8	1.094	n.d.	–
LLLn	40	876	59.6	1.000	54.0[c]	1.000	43.9[c]	1.000
LLγLn		876	60.4	1.014	n.d.	–	n.d.	–
LnOLn		876	60.8	1.022	56.2[c]	1.043	45.8[c]	1.046
LnOγLn		876	61.6	1.035	n.d.	–	n.d.	–
OLSt		876	61.9	1.041	56.4	1.047	46.1	1.054
LnLnP		850	62.1	1.044	63.2	1.180	50.2[c]	1.154
γLnOγLn		876	62.3	1.048	n.d.	–	n.d.	–
γLnLnP		850	62.8	1.057	63.8	1.192	n.d.	–
StLP		850	63.1	1.062	64.3	1.202	50.6	1.164
γLnγLnP		850	63.5	1.069	64.5	1.206	n.d.	–
SγLnSt		876	64.5	1.087	n.d.	–	n.d.	–
LLL	42	878	65.3	1.000	59.2	1.000	49.1	1.000
OLLn		878	66.4	1.018	61.4[c]	1.039	51.0[c]	1.041
GLnLn		904	66.8	1.024	63.4	1.075	53.0	1.085
OLγLn		878	67.1	1.029	n.d.	–	n.d.	–
LnLP		852	67.8	1.040	69.0	1.174	55.6[c]	1.141
γLnLP		852	68.5	1.052	69.7	1.187	n.d.	–
SLnγLn		878	69.2	1.063	75.1	1.283	60.2[c]	1.241
SLSt		878	69.6	1.069	n.d.	–	n.d.	–
StOP		852	69.8	1.072	n.d.	–	n.d.	–
SγLnγLn		878	69.9	1.074	75.7	1.294	n.d.	–
C20:2LL	44	906	70.8	0.985	n.d.	–	n.d.	–
OLL		880	71.8	1.000	66.5	1.000	56.0	1.000
GLLn		906	72.1	1.004	68.4[c]	1.030	58.0[c]	1.038
OOLn		880	72.6	1.012	68.7[c]	1.035	58.1[c]	1.040
GLγLn		906	72.8	1.015	n.d.	–	n.d.	–
LLP		854	73.1	1.019	74.7	1.129	61.0	1.094
OOγLn		880	73.3	1.022	n.d.	–	n.d.	–
SLLn		880	73.8	1.029	81.0	1.228	65.6[c]	1.181
LnOP		854	74.0	1.032	77.1	1.167	63.0[c]	1.132
SLγLn		880	74.5	1.039	81.8	1.241	n.d.	–
γLnOP		854	74.7	1.042	77.8	1.178	n.d.	–
SOSt		880	75.9	1.060	n.d.	–	n.d.	–
PγLnP		828	76.1	1.063	n.d.	–	n.d.	–
LLMa	45	868	76.3	1.066	n.d.	–	n.d.	–
GLL	46	908	77.2	0.991	73.5	0.997	62.9	0.998
OLO		882	77.9	1.000	73.7	1.000	63.0	1.000
GOγLn		908	78.8	1.012	n.d.	–	n.d.	–
SLL		882	79.0	1.015	86.8	1.185	70.8	1.130
OLP		856	79.3	1.019	82.6	1.126	68.3	1.088
ALLn		908	79.6	1.023	n.d.	–	n.d.	–
SOLn		882	80.0	1.028	n.d.	–	n.d.	–
ALγLn		908	80.3	1.032	n.d.	–	n.d.	–
PLP		830	80.9	1.040	n.d.	–	n.d.	–
OLMa	47	870	82.3	1.059	n.d.	–	n.d.	–

Table 1 (*Continued*)

TG	ECN	MW	C_{18} (25 °C)		C_{30} (25 °C)		C_{30} (40 °C)	
			t_R (min)[a]	r[b]	t_R (min)[a]	r[b]	t_R (min)[a]	r[b]
GLO		910	83.1	0.989	80.6	0.997	69.5	0.994
OOO		884	84.0	1.000	80.8	1.000	69.9	1.000
ALL		910	84.8	1.010	n.d.	–	n.d.	–
SLO	48	884	85.1	1.014	n.d.	–	n.d.	–
OOP		858	85.4	1.017	90.6	1.126	75.2	1.079
SLP		858	86.6	1.032	n.d.	–	n.d.	–
POP		832	87.0	1.037	n.d.	–	n.d.	–
GOO		912	89.0	0.979	n.d.	–	n.d.	–
BLL		938	90.0	0.991	n.d.	–	n.d.	–
ALO		912	90.4	0.995	n.d.	–	n.d.	–
SOO	50	886	90.8	1.000	n.d.	–	n.d.	–
ALP		886	91.8	1.011	n.d.	–	n.d.	–
SLS		886	91.9	1.013	n.d.	–	n.d.	–
SOP		860	92.3	1.017	n.d.	–	n.d.	–
C23:0LL	51	952	92.4	1.018	n.d.	–	n.d.	–
LgLL		966	94.9	0.988	n.d.	–	n.d.	–
BLO	52	940	95.5	0.995	n.d.	–	n.d.	–
AOO		914	96.0	1.000	n.d.	–	n.d.	–

[a] n.d. means not detected.
[b] Relative retention is defined as $r = (t_R - t_M)/(t_S - t_M)$, where t_M is 3.2 min for the system with C_{18} column and 3.0 min for the system with C_{30} column and t_S are retention times of standards for particular ECN groups, i.e., LLLn (ECN = 32–40), LLL (42), OLL (44, 45), OLO (46, 47), OOO (48), SOO (50, 51) and AOO (52).
[c] Coelution of triacylglycerols containing Ln and γLn acids.

2-propanol, 15 min-80% acetonitrile + 20% 2-propanol was used for fast UHPLC analysis. The baseline drift in UV chromatograms was suppressed using the chromatographic software.

2.3. Notation of identified species

TGs, diacylglycerols (DGs) and monoacylglycerols (MGs) were annotated using initials of trivial names of FAs arranged according to their *sn*-1, *sn*-2 and *sn*-3 positions, i.e., **P**, palmitic (C16:0); **Ma**, margaric (C17:0); **S**, stearic (C18:0); **O**, oleic (Δ9-C18:1); **L**, linoleic (Δ9,12-C18:2); **Ln**, linolenic (Δ9,12,15-C18:3); **γ-Ln**, gamma-linolenic (Δ6,9,12-C18:3); **St**, stearidonic (Δ6,9,12,15-C18:4); **A**, arachidic (C20:0); **G**, gadoleic (Δ9-C20:1); **C20:2**, eicosadienoic (Δ9,11-C20:2); **B**, behenic (C22:0); **C23:0**, tricosanoic (C23:0); **Lg**, lignoceric (C24:0).

3. Results and discussion

3.1. NARP-HPLC analysis of TGs and enzymatic hydrolysis products using conventional C_{18} column

Blackcurrant oil is one of the most complex plant oils typical by high content of linolenic (Δ9,12,15-C18:3, Ln) and gamma-linolenic (Δ6,9,12-C18:3, γLn) acids, both with 3 DBs but in different positions in the acyl chain (i.e., DB positional isomers). Blackcurrant oil is selected as a testing TG mixture based on its complexity which is challenging for NARP-HPLC technique commonly used in the analysis of natural TG mixtures. TG composition is determined using NARP-HPLC method with two conventional C_{18} columns (4 μm particles) in total length 45 cm, acetonitrile–2-propanol gradient and column temperature 25 °C according to our previous optimization [10]. The conventional C_{18} columns provide the separation of TGs according to the ECN and TGs within one ECN group are separated based on the saturation degree and the length of acyl chains of attached FAs (Fig. 1). DB positional isomers are also well resolved in this NARP-HPLC system, where TGs with γLn acid have higher retention in comparison to TGs containing Ln acid (Table 1), e.g., LnLnLn (t_R = 48.3 min) and LnLnγLn (t_R = 49.0 min), etc. The average difference between TGs containing one γLn instead of Ln is about 0.7 min. TG species are identified based on the retention behavior and masses of protonated molecules $[M+H]^+$, low abundant ammonium adducts $[M+NH_4]^+$ and $[M+H-R_iCOOH]^+$ fragment ions observed in their positive-ion full scan APCI mass spectra. Prevailing FA in *sn*-2 position is determined based on lower relative abundances of $[M+H-R_iCOOH]^+$ fragment ions arising by the cleavage of FA from this position in comparison to *sn*-1 and *sn*-3 positions [28,29]. *Sn*-1 and *sn*-3 positions cannot be resolved in NARP-HPLC system and are considered as equivalent. Totally, 83 TGs including DB positional isomers have been identified in this work which is the highest number of identified TGs reported so far in comparison to 56 TGs reported in Ref. [8] or 21 in Ref. [28].

The NARP system is also applied for the analysis of more polar glycerolipids (DGs and MGs) and FAs (Fig. 2) prepared by partial enzymatic catalyzed hydrolysis of blackcurrant oil in supercritical carbon dioxide during the preparation of FAs used as food supplements. The hydrolysis mixture containing unreacted TGs and reaction products DGs, MGs and FAs are analyzed in one analytical run without any fractionation. Fig. 2(A) illustrates HPLC separation of hydrolysis mixture using the conventional C_{18} column, where groups of TGs and DGs are clearly resolved with the partial coelution of polar MGs and FAs. The coelution of these polar compounds is given by the same ECN, because the retention in NARP systems is mainly governed by hydrophobic interactions with only limited effect of polarity. For better separation of FAs and MGs species, acetonitrile/water isocratic step at the beginning of gradient can improve the separation [29] or normal-phase chromatography on silica yields a class separation of individual lipid classes based on their polarity [30]. On the other hand, the excellent resolution of TGs would be partially lost by the addition of water in the initial part of gradient (data not shown). The partial resolution of FA and MG groups is still sufficient for the identification of individual species based on their APCI mass spectra. Deprotonated molecules $[M-H]^-$ in negative-ion and $[M+H-H_2O]^+$ ions in positive-ion full scan APCI mass spectra are used for the identification of FAs and MGs, respectively. In total, 6 FAs, 4 MGs and 27 DGs have been identified in the hydrolysis mixture. A zoomed region of this separation (Fig. 2(B)) illustrates that individual DG species are well resolved according to their ECN including the separation of DGs within individual ECN groups and *sn*-1,2- and *sn*-1,3-DG regioisomers. 1,2- and 2,3-DGs are optical isomers, which cannot be resolved in non-chiral sys-

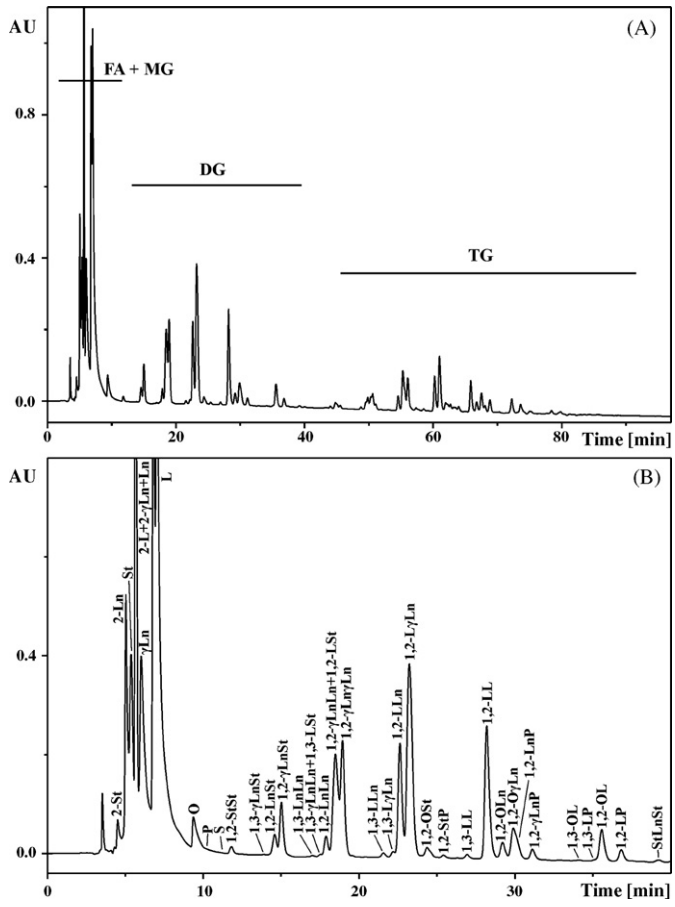

Fig. 2. NARP-HPLC analysis of triacylglycerols (TG) hydrolysis products after the partial enzymatic hydrolysis of blackcurrant oil by *sn*-1/3 selective enzyme Lipozyme using conventional C_{18} column (A) and detail of separation of fatty acids (FA), monoacylglycerols (MG) and diacylglycerols (DG) (B). Conditions identical as for Fig. 1.

tem and therefore they are considered equivalent in this work, i.e., 1,2-DG corresponds to the mixture of 1,2- and 2,3-DGs. Masses of protonated molecules $[M+H]^+$ and $[M+H-H_2O]^+$ fragment ions in positive-ion full scan APCI mass spectra are used for the identification of individual DG species. Non-specific chemical hydrolysis of TGs provides three DG regioisomers (1,3-, 1,2- and 2,3-DG) in ratio 1:1:1. 1,2- and 2,3-optical isomers cannot be separated in NARP-HPLC, hence the final chromatogram contains two peaks of DG regioisomers at the ratio 1,3-DG:1,2-DG = 1:2 (not shown). In this study, *sn*-1 and *sn*-3 specific enzyme Lipozyme is used for the hydrolysis of TGs providing almost exclusively one peak of 1,2-DG regioisomer (Fig. 2(B)). Peaks of 1,3-DGs have very low relative peak areas (1–5%) in comparison to 1,2-DGs (95–98%), which confirms expected specificity of this enzyme (Table 2).

3.2. NARP-UHPLC analysis of TGs in blackcurrant oil

The NARP-HPLC using conventional C_{18} column in total length 45 cm with 4 μm particles provides an excellent resolution of TGs, but retention times up to 96 min are rather long (Table 1). The goal of method transfer to UHPLC has been to maintain the resolution, while the analysis time should be reduced as much as possible. UHPLC method has been developed with the help of the software supplied with UHPLC system from Waters for the method transfer from the conventional HPLC to UHPLC system. Consequentially, we have tested effects of individual chromatographic parameters on the separation, i.e., flow rate, column temperature,

Table 2
Relative peak areas (UV at 205 nm) of 1,3- and 1,2-diacylglycerol regioisomers identified in the hydrolysis mixture of triacylglycerols after the partial enzymatic hydrolysis of blackcurrant oil by *sn*-1/3 selective enzyme Lipozyme.

Diacylglycerol	1,3-Diacylglycerol		1,2-Diacylglycerol	
	t_R (min)	Peak area (%)	t_R (min)	Peak area (%)
γLnSt	13.8	1.2	14.9	98.8
LnLn	16.9	5.2	17.8	94.8
γLnLn + LSt	17.4	1.3	18.4	98.7
LLn	21.5	3.6	22.5	96.4
LγLn	22.1	1.8	23.1	98.2
LL	26.9	2.6	28.1	97.4
OL	34.0	1.6	35.5	98.4
LP	35.1	5.0	36.7	95.0

gradient steepness, initial composition and sample injection volume. The flow rate in the range of 0.2–0.4 mL/min has no significant effect on the chromatographic resolution, because it corresponds to the flat region in van Deemter curve of sub-two micron particle columns [24]. Increased column temperature had tremendous negative effect on the chromatographic resolution of TGs in NARP-HPLC mode [10,11], but this effect is not so significant in UHPLC. Therefore, higher column temperature can be used to reduce the system backpressure in UHPLC, which enables higher flow rates and decreased analysis time. The gradient steepness and initial gradient composition have relatively low effect on the resolution, but significant effect on the analysis time. Therefore, a compromise between the resolution and analysis time is made. Maximum

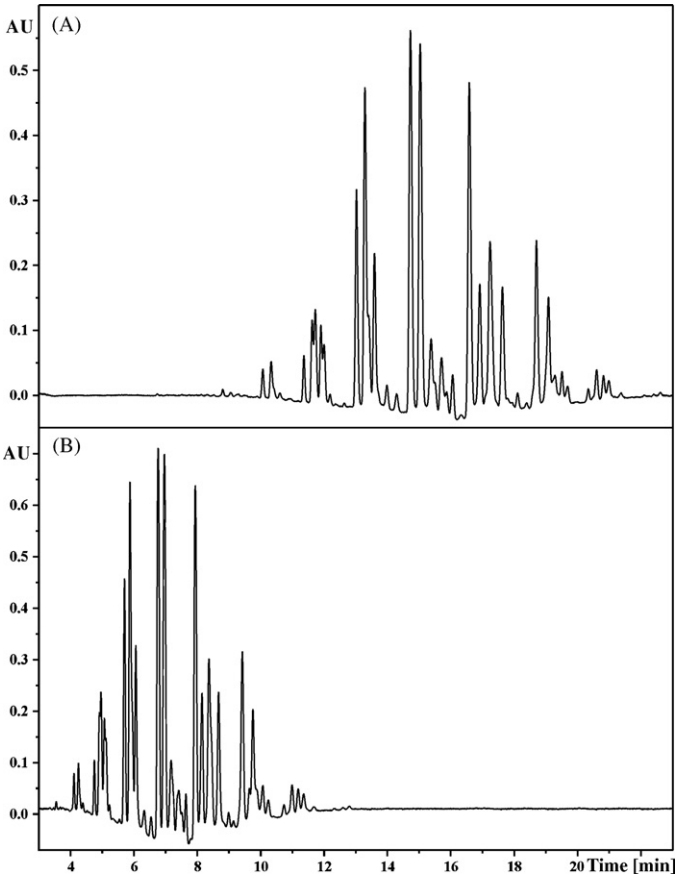

Fig. 3. NARP-UHPLC analysis of triacylglycerols in blackcurrant oil using two gradients: (A) 0 min-100% acetonitrile, 23 min-54% acetonitrile + 46% 2-propanol and (B) 0 min-80% acetonitrile + 20% 2-propanol, 14 min-52% acetonitrile + 48% 2-propanol. *Conditions*: Acquity UPLC BEH C_{18} column (150 mm × 2.1 mm, 1.7 μm), UV detection at 205 nm, flow rate 0.4 mL/min, column temperature 30 °C.

sample injection volume without any effect on the resolution is 2 μL.

Final NARP-UHPLC separation of TGs in blackcurrant oil using the flow rate 0.4 mL/min, column temperature 30 °C, the slope of gradient of 2% 2-propanol/min, 100% acetonitrile as initial mobile phase and 1 μL injection volume provides a similar picture as with conventional HPLC but within 22 min (Fig. 3(A)). The analysis time is significantly reduced by the change of initial gradient composition to 80% acetonitrile + 20% 2-propanol (Fig. 3(B)). TGs are eluted within 12 min with only small loss of resolution. DB positional isomers are separated with average difference between TGs containing one γLn instead of Ln about 0.16 min. The magnification of critical TG groups for the separation (Fig. 4) shows that UHPLC provides better resolution, as demonstrated on the group with ECN = 36 at the beginning of gradient (compare HPLC chromatogram in Fig. 4(A) with UHPLC in Fig. 4(C)) and ECN = 44 at the end of run (compare Fig. 4(B) and (D)). Moreover, retention times of TGs in UHPLC are reduced almost 10 times in comparison to the conventional HPLC providing significantly higher sample throughput.

3.3. NARP-HPLC analysis of TGs using C_{30} column

C_{30} column is tested for the analysis of TGs using the same chromatographic conditions as for the conventional C_{18} column (Fig. 5(A)). The total analysis time using 25 cm C_{30} column with 3 μm particles is comparable to conventional NARP-HPLC analysis but with significantly lower resolution and the number of identified TG species decreased to 38 from initial 83 with C_{18} column (Table 1). TGs containing only unsaturated FAs are separated according to ECN. In contrast to C_{18} column, DB positional isomers (Ln vs. γLn) for TGs with three unsaturated chains are not resolved using C_{30} column and they elute in one peak. The ECN retention mechanism is changed for TGs containing already one saturated FA on the glycerol skeleton. The retention of these TGs is higher in comparison to TGs containing only unsaturated FAs and they elute in higher ECN groups, e.g., StLnP with ECN = 38 elutes between ECN groups 40 and 42, etc. This phenomenon is probably due to the stronger interaction of straight saturated chains of FAs with long alkyl chains of C_{30} stationary phase. Stronger interactions of saturated alkyl chains also enable the partial separation of DB positional isomers unlike to TGs containing only unsaturated FAs.

The change of column temperature in conventional NARP-HPLC system with C_{18} columns affects retention times of TGs at the same level without changing the retention order [10]. By the way we have never seen changes in the retention order of TGs among our extensive NARP C_{18} studies and data published by other groups. The situation is different with C_{30} column. Fig. 5 shows the comparison of TG analysis using C_{30} column at 25 °C (A) and 40 °C (B) column temperature. The retention of TGs containing at least one saturated FA chain significantly decreases in comparison to

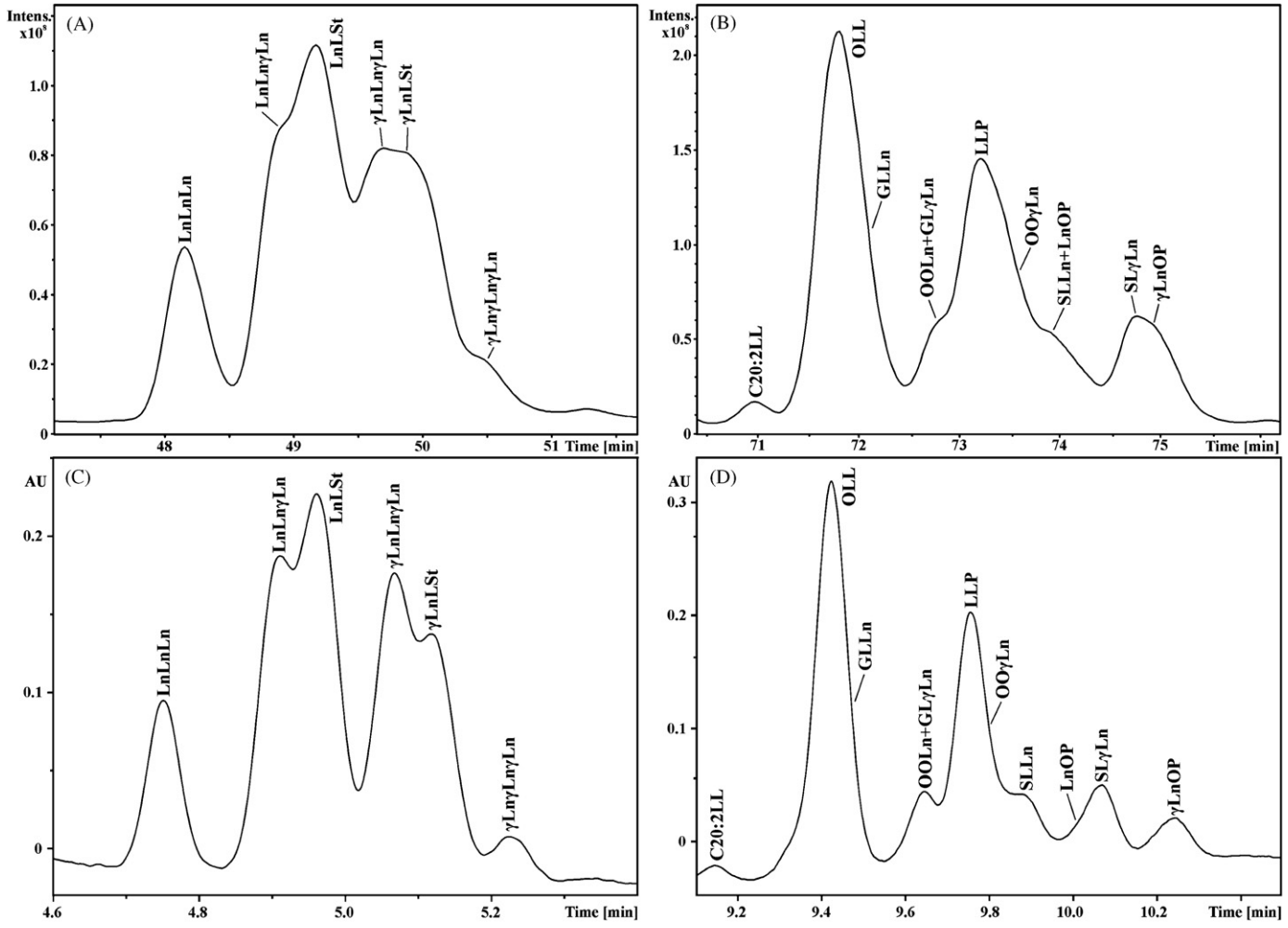

Fig. 4. Details from NARP-HPLC (A and B) and NARP-UHPLC (C and D) separations of critical groups of TGs in blackcurrant oil for comparison of the separation at the beginning of the chromatographic run (A and C) and at the end of gradient (B and D). Conditions identical as for Figs. 1 and 3.

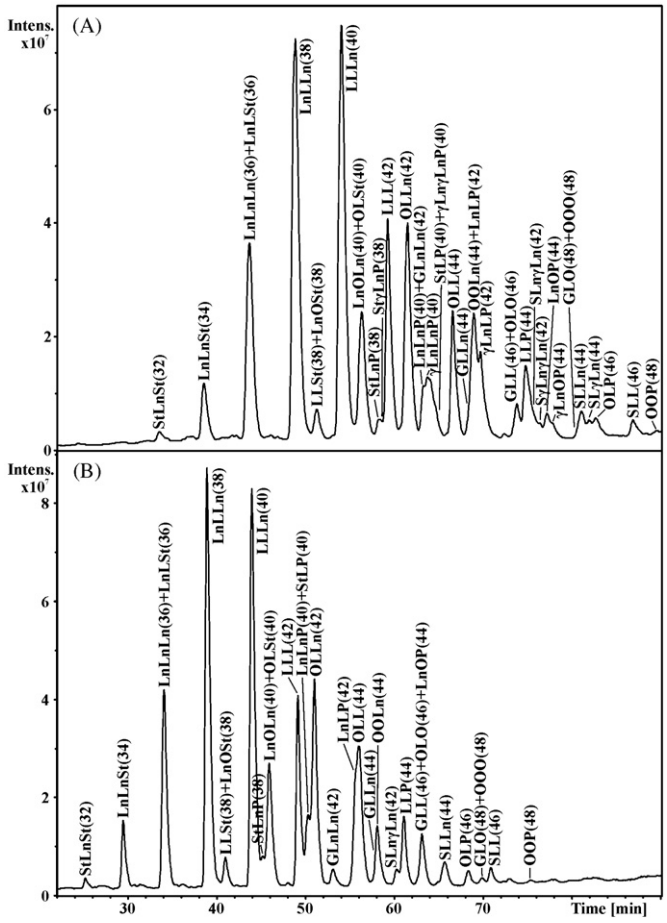

Fig. 5. NARP-HPLC/APCI-MS analysis of triacylglycerols in blackcurrant oil using C_{30} column and column temperatures: (A) 25 °C and (B) 40 °C. *Conditions*: ProntoSil C_{30} column (250 mm × 4.6 mm, 3 μm), APCI-MS detection in positive-ion mode, flow rate 1 mL/min, gradient 0 min-100% acetonitrile, 106 min-31% acetonitrile + 69% 2-propanol.

TGs containing only unsaturated FAs at higher column temperature resulting in changed retention order, e.g., StLnP with ECN = 38 elute in group ECN = 40. Moreover, the resolution of DB positional isomers for TGs containing saturated FAs is completely lost and they elute in one peak. Shifts of retention times of TGs with different column temperature can be visualized by relative retention (Table 1) with higher difference for TGs containing one saturated FA, e.g., $\Delta r = 0.050$ for StLnP in comparison to $\Delta r = 0.014$ for LnOSt, etc. C_{30} stationary phase is strongly temperature dependent and the conformation of C_{30} alkyl chains partially changes at higher temperature from straight *trans n*-alkyl conformation to gauche confirmation [21] which probably causes lower interactions with saturated acyl chains. Changes in the gradient steepness or in the gradient composition only shift retention times, but without notable effect on the chromatographic resolution of TGs unlike to the temperature dependence.

3.4. NARP-HPLC analysis of TGs using the monolithic column

Silica-based monolithic columns modified by C_{18} alkyl phase are used for the analysis of TGs in the blackcurrant oil using acetonitrile–2-propanol isocratic elution (Fig. 6). Low flow resistance of monolithic columns enables the utilization of high flow rates (5 mL/min) to increase the sample throughput. The retention of TGs increases with increasing ECN, but individual groups are not clearly separated. Only 21 TG species with higher concentration

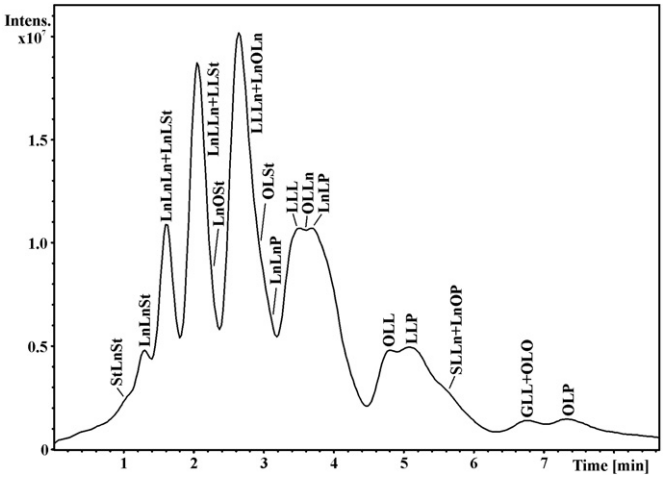

Fig. 6. NARP-HPLC/APCI-MS analysis of triacylglycerols in blackcurrant oil using C_{18} monolithic column. *Conditions*: Chromolith Performance RP-18e column (100 mm × 4.6 mm), flow rate 5 mL/min, column temperature 25 °C, isocratic elution with 85% acetonitrile and 15% 2-propanol, T-piece splitting 1:4 before positive-ion APCI-MS detection.

are identified in the blackcurrant oil in comparison to 83 TGs identified with the conventional C_{18} column. On the other hand, the total analysis time of TGs using C_{18} modified monolithic column is only 8 min, which is 12 times lower in comparison to conventional NARP-HPLC with C_{18} columns. Therefore, this simple and fast separation can be used only for rough determination of TG profiles.

4. Conclusions

This work demonstrates the utilization of different stationary phases in the analysis of TGs using NARP system with acetonitrile–2-propanol gradient. Blackcurrant oil containing high number of TG species including DB positional isomers is used for the characterization of chromatographic behavior of TGs on different columns. Conventional C_{18} column in the total length of 45 cm enables the separation and identification of 83 TGs species including DB positional isomers. This system is successfully applied for the separation of TGs with their more polar hydrolysis products in one analytical run. DG regioisomers are resolved in this system and their relative peak areas confirm the stereoselectivity of enzyme Lipozyme. The separation of TGs using NARP-UHPLC analysis enables almost 10 times reduction of the total analysis time with slightly improved chromatographic resolution in comparison to the conventional NARP-HPLC. The analysis of TGs using C_{30} column provides only 38 identified TG species and no resolution of DB positional isomers of unsaturated TGs. Higher column temperature using C_{30} column causes a change in the retention order of TGs containing saturated FAs which is a phenomenon reported for the first time. Monolithic stationary phase modified by C_{18} alkyl chains enables fast separation of TGs into ECN groups which is applicable only for the determination of rough TG profile.

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