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# Characterization of triacylglycerol and diacylglycerol composition of plant oils using high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

Michal Holčapek, Pavel Jandera*, Petr Zderadička, Lucie Hrubá

*Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Nám Čs Legií 565, 53210 Pardubice, Czech Republic*

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**Abstract**

Triacylglycerols (TGs) and diacylglycerols (DGs) in 16 plant oil samples (hazelnut, pistachio, poppy-seed, almond, palm, Brazil-nut, rapeseed, macadamia, soyabean, sunflower, linseed, *Dracocephalum moldavica*, evening primrose, corn, amaranth, *Silybum arianum*) were analyzed by HPLC–MS with atmospheric pressure chemical ionization (APCI) and UV detection at 205 nm on two Nova-Pak $C_{18}$ chromatographic columns connected in series. A single chromatographic column and non-aqueous ethanol–acetonitrile gradient system was used as a compromise between the analysis time and the resolution for the characterization of TG composition of five plant oils. APCI mass spectra were applied for the identification of all TGs and other acylglycerols. The isobaric positional isomers can be distinguished on the basis of different relative abundances of the fragment ions formed by preferred losses of the fatty acid from *sn*-1(3) positions compared to the *sn*-2 position. Excellent chromatographic resolution and broad retention window together with APCI mass spectra enabled positive identification of TGs containing fatty acids with odd numbers of carbon atoms such as margaric (C17:0) and heptadecanoic (C17:1) acids. The general fragmentation patterns of TGs in both APCI and electrospray ionization mass spectra were proposed on the basis of $MS^n$ spectra measured with an ion trap analyzer. The relative concentrations of particular TGs in the analyzed plant oils were estimated on the basis of relative peak areas measured with UV detection at 205 nm.


*Keywords:* Positional isomers; Vegetable oils; Triacylglycerols; Diacylglycerols; Acylglycerols; Glycerols

## 1. Introduction

The plant oils are complex mixtures containing triacylglycerols (TGs) of various fatty acids, such as oleic (O), linoleic (L), linolenic (Ln), stearic (S), palmitic (P), etc., differing in the acyl chain lengths and their positions *sn*-1, 2 or 3 on the glycerol skeleton, and in the number and positions of the double bonds in the acyl chains. The standard notation of TGs employs the initial of fatty acid names, arranged in the order of their position on the glycerol skeleton (see Tables 1 and 2).

Non-aqueous reversed-phase high-performance liquid chromatography (NARP-HPLC) has been

*Corresponding author. Tel.: +420-46-603-7023; fax: +420-46-603-7068.
*E-mail address:* pavel.jandera@upce.cz (P. Jandera).

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Table 1
Abbreviations of acyls and characteristic fragment ions of triacylglycerols (for structures, see Fig. 1)

| Trivial Name | Symbol | CN:DB | ECN | Ion B | Ion B-$H_2O$ | Ion C | Ion C-$H_2O$ |
|---|---|---|---|---|---|---|---|
| Myristic | M | C14:0 | 14 | 285 | 267 | 211 | 193 |
| Palmitoleic | Po | C16:1 | 14 | 311 | 293 | 237 | 219 |
| Palmitic | P | C16:0 | 16 | 313 | 295 | 239 | 221 |
| Heptadecanoic | Mo | C17:1 | 15 | 325 | 307 | 251 | 233 |
| Margaric | Ma | C17:0 | 17 | 327 | 309 | 253 | 235 |
| Linolenic | Ln | C18:3 | 12 | 335 | 317 | 261 | 243 |
| Linoleic | L | C18:2 | 14 | 337 | 319 | 263 | 245 |
| Oleic | O | C18:1 | 16 | 339 | 321 | 265 | 247 |
| Stearic | S | C18:0 | 18 | 341 | 323 | 267 | 249 |
| Gadoleic | G | C20:1 | 18 | 367 | 349 | 293 | 275 |
| Arachidic | A | C20:0 | 20 | 369 | 351 | 295 | 277 |
| Behenic | B | C22:0 | 22 | 397 | 379 | 323 | 305 |
| Lignoceric | Li | C24:0 | 24 | 425 | 407 | 351 | 333 |

widely used for the separation of complex samples of natural TGs. The retention in NARP-HPLC increases with increasing equivalent carbon number (ECN) defined as the total carbon number (CN) in all acyl chains minus two times the number of double bonds (DB), i.e. ECN=CN−2DB. Under optimized separation conditions, the separation of some TGs with the same ECNs is also possible, for example the pair LLL and OLLn, or the group of OOO, OOP, OPP and PPP can be resolved [1–3].

The silver ion HPLC in normal-phase systems is a widespread technique for the separation of lipids, such as TGs, based on selective interactions of silver ions with species differing in the number and positions of double bonds [4–8]. The selectivity is usually poor for TGs differing only in the lengths of alkyl chains, therefore NARP-HPLC usually provides a better resolution of complex TG mixtures such as plant oils. Adlof et al. [8] used a series of four silica gel columns modified with silver ions to separate the stereochemical isomers of conjugated linoleic acid enriched TGs.

Since acylglycerols do not absorb the UV radiation above 220 nm, the UV detection is possible only at very low wavelengths and necessitates the highest purity HPLC solvents. The UV detection at 205 or 210 nm can provide linear calibration curves and very good sensitivity even with gradient elution technique [9]. Various less common detectors were used in past in the HPLC of TGs, such as a moving wire detector [10], a density detector [11] and a flame ionization detector [12]. Nowadays, evaporative light scattering detection (ELSD) is widely used for HPLC of lipids that do not absorb in the UV region [1–3]. In contrast to these detectors, a mass spectrometric detector provides valuable information about the analyte structure. The HPLC–MS enables a positive identification of individual acylglycerols without the need for authentic reference standards. The atmospheric pressure chemical ionization [12–20] is most frequently used ionization technique for HPLC–MS analysis of TGs, because it enables—in addition to the molecular mass ($M_r$) determination—the identification of the individual acyls. The relative abundances of protonated molecules $[M+H]^+$ can be very low in the APCI mass spectra of saturated TGs, such as PPP or SSS, but even in this case, the $M_r$ values of TGs can be easily determined from the $[M+H\text{-}RCOOH]^+$ ions of the individual acyls and the retention data. The relative abundances of $[M+H\text{-}RCOOH]^+$ ions also enable the identification of the acyl in the middle *sn*-2 position, because of a lower relative abundance than statistically expected in comparison with the $[M+H\text{-}RCOOH]^+$ ions of the acyls in the *sn*-1(3) positions [20–22]. Atmospheric pressure chemical ionization (APCI) ionization technique was also coupled with supercritical fluid chromatography and applied for the analysis of soyabean and sunflower oils [6].

Electrospray ionization (ESI) yields cationised TGs molecules ($[M+Na]^+$, $[M+K]^+$ or $[M+NH_4]^+$-depending on the mobile phase composition) without any protonated molecule [23,24, our unpublished results]. In the ESI mass spectra of TGs,

Table 2
The masses of protonated molecules $[M+H]^+$, the equivalent carbon numbers (ECN), the retention times $t_R$ in minutes (HPLC method 1, see Section 2) and separation factors of triacylglycerols (TG) identified in the plant oils

| TG | ECN | $[M+H]^+$ | Retention time ($t_R$) | Separation factor ($r$) |
|---|---|---|---|---|
| LnLnLn | 36 | 873 | 81.5 | 0.864 |
| LLnLn | 38 | 875 | 87.9 | 0.934 |
| LLLn | 40 | 877 | 94.0 | 1.000 |
| PoLnPo | | 825 | 94.6 | 1.007 |
| OLnLn | | 877 | 94.9 | 1.010 |
| LnLnP | | 851 | 96.7 | 1.029 |
| LLnMo | 41 | 865 | 96.9 | 1.032 |
| LnLnMa | | 865 | 97.8 | 1.041 |
| LLL | 42 | 879 | 99.7 | 1.000 |
| LLPo | | 853 | 99.9 | 1.002 |
| PoLPo | | 827 | 100.2 | 1.005 |
| PoPoPo | | 801 | 100.6 | 1.009 |
| OLLn | | 879 | 100.8 | 1.011 |
| PoLM | | 801 | 101.6 | 1.019 |
| LLM | | 827 | 101.7 | 1.020 |
| PoPoM | | 775 | 102.1 | 1.025 |
| LnLP | | 853 | 102.6 | 1.030 |
| SLnLn | | 879 | 103.4 | 1.038 |
| LLMo | 43 | 867 | 103.5 | 1.039 |
| MoPoPo | | 815 | 103.8 | 1.042 |
| LLnMa | | 867 | 104.4 | 1.048 |
| GLLn | 44 | 907 | 105.0 | 0.992 |
| OLL | | 881 | 105.8 | 1.000 |
| OLPo | | 855 | 106.3 | 1.005 |
| PoOPo | | 829 | 106.8 | 1.010 |
| OLnO | | 881 | 106.9 | 1.011 |
| LLP | | 855 | 107.5 | 1.016 |
| OPoM | | 803 | 108.2 | 1.023 |
| OLnP | | 855 | 108.4 | 1.025 |
| OLM | | 829 | 108.5 | 1.026 |
| PPoPo | | 803 | 108.7 | 1.028 |
| PLM | | 803 | 109.4 | 1.035 |
| PPoM | | 777 | 110.1 | 1.041 |
| PLnP | | 829 | 110.1 | 1.041 |
| OPoMo | 45 | 843 | 109.6 | 1.037 |
| OLMo | | 869 | 109.6 | 1.037 |
| LLMa | | 869 | 110.9 | 1.049 |
| PMoPo | | 843 | 111.6 | 1.056 |
| GLL | 46 | 909 | 111.7 | 0.992 |
| OLO | | 883 | 112.6 | 1.000 |
| OOPo | | 857 | 112.9 | 1.003 |
| SLL | | 883 | 114.0 | 1.013 |
| LOP | | 857 | 114.2 | 1.014 |
| OOM | | 831 | 114.3 | 1.015 |
| POPo | | 831 | 114.8 | 1.020 |
| GPoPo | | 857 | 115.2 | 1.024 |
| PLP | | 831 | 116.2 | 1.033 |
| SLnP | | 857 | 116.5 | 1.035 |
| POM | | 805 | 116.6 | 1.036 |
| PPoP | | 805 | 116.8 | 1.038 |
| OOMo | 47 | 871 | 116.3 | 1.033 |
| OLMa | | 871 | 117.5 | 1.044 |
| OMoP | | 845 | 118.0 | 1.049 |
| MaPM | | 793 | 119.8 | 1.065 |
| GLO | 48 | 911 | 118.4 | 0.995 |
| OOO | | 885 | 119.0 | 1.000 |
| SPM | | 807 | 119.2 | 1.002 |
| ALL | | 911 | 120.1 | 1.009 |
| GOM | | 859 | 120.1 | 1.009 |
| GLP | | 885 | 120.1 | 1.009 |
| SLO | | 885 | 120.6 | 1.014 |
| OOP | | 859 | 120.9 | 1.016 |
| SLP | | 859 | 122.2 | 1.027 |
| POP | | 833 | 122.6 | 1.031 |
| PPP | | 807 | 125.1 | 1.052 |
| OOMa | 49 | 873 | 124.1 | 1.044 |
| MaOP | | 847 | 125.6 | 1.056 |
| GLS | 50 | 913 | 123.9 | 0.978 |
| GOO | | 913 | 124.6 | 0.983 |
| BLL | | 939 | 125.7 | 0.992 |
| ALO | | 913 | 126.1 | 0.995 |
| GOP | | 887 | 126.3 | 0.997 |
| SOO | | 887 | 126.7 | 1.000 |
| AOPo | | 887 | 126.8 | 1.001 |
| ALP | | 887 | 127.9 | 1.010 |
| SLS | | 887 | 128.0 | 1.010 |
| SOP | | 861 | 128.7 | 1.016 |
| SPP | | 835 | 131.4 | 1.038 |
| SOMa | 51 | 875 | 131.1 | 1.035 |
| GOG | 52 | 941 | 129.7 | 0.969 |
| LiLL | | 967 | 130.8 | 0.977 |
| BOL | | 941 | 131.4 | 0.982 |
| GSO | | 915 | 131.9 | 0.986 |
| AOO | | 915 | 132.0 | 0.986 |
| BLP | | 915 | 133.0 | 0.994 |
| SOS | | 889 | 133.8 | 1.000 |
| AOP | | 889 | 134.0 | 1.002 |
| LiOL | 54 | 969 | 136.0 | 1.017 |
| BOO | | 943 | 136.9 | 1.024 |
| LiLP | | 943 | 137.8 | 1.030 |
| BLS | | 943 | 138.0 | 1.032 |
| BOP | | 917 | 138.7 | 1.037 |
| GGS | | 943 | 139.0 | 1.039 |
| SSS | | 891 | 139.5 | 1.043 |
| AOS | | 917 | 139.6 | 1.044 |
| LiOO | 56 | 971 | 141.5 | 1.058 |
| LiOP | | 945 | 143.0 | 1.070 |

The separation factor $r = t'_R/t'_S$ is the net retention time $t'_R$ related to the retention time $t'_S$ of a standard compound in each group of ECNs. The common TGs in plant oils are selected as standard compounds, i.e. LLLn for ECN = 36–41, LLL for ECN = 42 and 43, OLL for ECN = 44 and 45, OLO for ECN = 46 and 47, OOO for ECN = 48 and 49, SOO for ECN = 50 and 51, SOS for ECN = 52–56.

the relative abundances of the $[M+H\text{-}RCOOH]^+$ ions are lower but still sufficient for the identification of the individual acyls. The $[M+Na]^+$ and $[M+K]^+$ ions are more stable and hence the fragmentation in MS–MS and multiple MS ($MS^n$) experiments is more difficult in comparison to the APCI technique. The interpretation of ESI–$MS^n$ spectra can be complicated by the presence of both protonated and sodiated fragment ions. ESI was applied for HPLC–MS analysis of corn and sunflower oils [23] and to the capillary electrochromatography–MS analysis of evening primrose, arachide, corn, walnut and salad oils [25]. The dual parallel ESI and APCI mass spectrometer was applied for the identification of complex TGs oxidation products [24].

Matrix-assisted laser desorption/ionization (MALDI) is an alternative ionization technique for TGs, but the coupling of MALDI with HPLC is less common. Direct MALDI–MS analysis of plant oils allows only the identification of main TGs [26–29], but minor or trace components may not be detected. In the MALDI mass spectra of TGs, the sodiated molecules occur, but protonated molecules are usually absent and the neutral losses of fatty acids are similar as in the APCI and ESI mass spectra.

In this work, we have developed a high-resolution HPLC–APCI-MS method enabling the separation and identification of TGs with identical ECNs. We have applied this method for the characterization of 16 plant and vegetable oils. Furthermore, we have compared benefits of the APCI and ESI mass spectra in MS and $MS^n$ modes for the identification of TGs. The special attention has been paid to the determination of acyls in the *sn*-2 position, studied in detail on the complete set of the positional isomers of TGs containing palmitic and oleic acids.

## 2. Experimental

### 2.1. Materials

Acetonitrile and hexane were purchased from Merck (Darmstadt, Germany), 2-propanol from Research Institute for Organic Synthesis (Pardubice-Rybitví, Czech Republic). De-ionized water was doubly distilled in glass with addition of potassium permanganate. The solvents were filtered through a 0.45-μm Millipore filter and degassed by continuous stripping with helium. The standards of OOO, OOP,

Table 3
Masses of the characteristic fragment ions A of triacylglycerols

| *m/z* of fragment ion A | Possible structures | *m/z* of fragment ion A | Possible structures |
|---|---|---|---|
| 495 | MM | 591 | OMa, SMo |
| 521 | PoM | 593 | SMa |
| 523 | PM | 595 | LnLn |
| 535 | MoM | 597 | LLn |
| 537 | MaM | 599 | LL, OLn |
| 545 | LnM | 601 | OL, SLn |
| 547 | LM, PoPo | 603 | OO, SL, GPo |
| 549 | PPo, OM | 605 | SO, GP, APo |
| 551 | PP, SM | 607 | SS, AP |
| 561 | MoPo | 627 | GLn |
| 563 | MoP, MaPo | 629 | GL, ALn |
| 565 | MaP | 631 | GO, AL |
| 571 | LnPo | 633 | AO, GS |
| 573 | LnP, LPo | 635 | AS, BP |
| 575 | OPo, LP | 659 | GG, BL |
| 577 | OP, SPo, GM | 661 | AG, BO |
| 579 | SP, AM | 663 | AA, BS, LiP |
| 585 | LnMo | 687 | LiL |
| 587 | LMo, LnMa | 689 | LiO |
| 589 | OMo, LMa | | |

OPO, OPP, POP, PPP, LLL and LnLnLn were purchased from Sigma–Aldrich (St. Louis, MO, USA).

### 2.2. High-performance liquid chromatography

The chromatographic apparatus consisted 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). 10 μl sample volumes, the flow-rate 1 ml/min, the column temperature 40 °C and the UV detection at 205 nm were used in all experiments with two gradient-elution HPLC methods. The void volume $t_M$ is 2.05 min. The injector needle was washed with the mobile phase before each injection.

HPLC method 1 was used for all oils except of five samples stated bellow: 0 min—70% aqueous acetonitrile, 20 min—100% acetonitrile, 36 min—100% acetonitrile, 132 min—60% 2-propanol+40% acetonitrile, 135 min—70% aqueous acetonitrile, two chromatographic columns Nova-Pak $C_{18}$ (150×3.9 mm, 7 μm) connected in series (Waters).

HPLC method 2 was used for *Dracocephalum moldavica*, evening primrose, corn, amaranth and *Silybum arianum* oils: 0 min—100% acetonitrile, 56 min—30% acetonitrile+70% ethanol with a single chromatographic column Nova-Pak $C_{18}$.

### 2.3. Mass spectrometry

The outlet of the UV detector of the liquid chromatograph was connected to a VG Platform quadrupole mass spectrometer (Micromass, Manchester, UK) using APCI in the positive-ion mode. The data was acquired in the mass range $m/z$ 35–1000. The mass spectra were taken at different points across the chromatographic peaks to find out possible

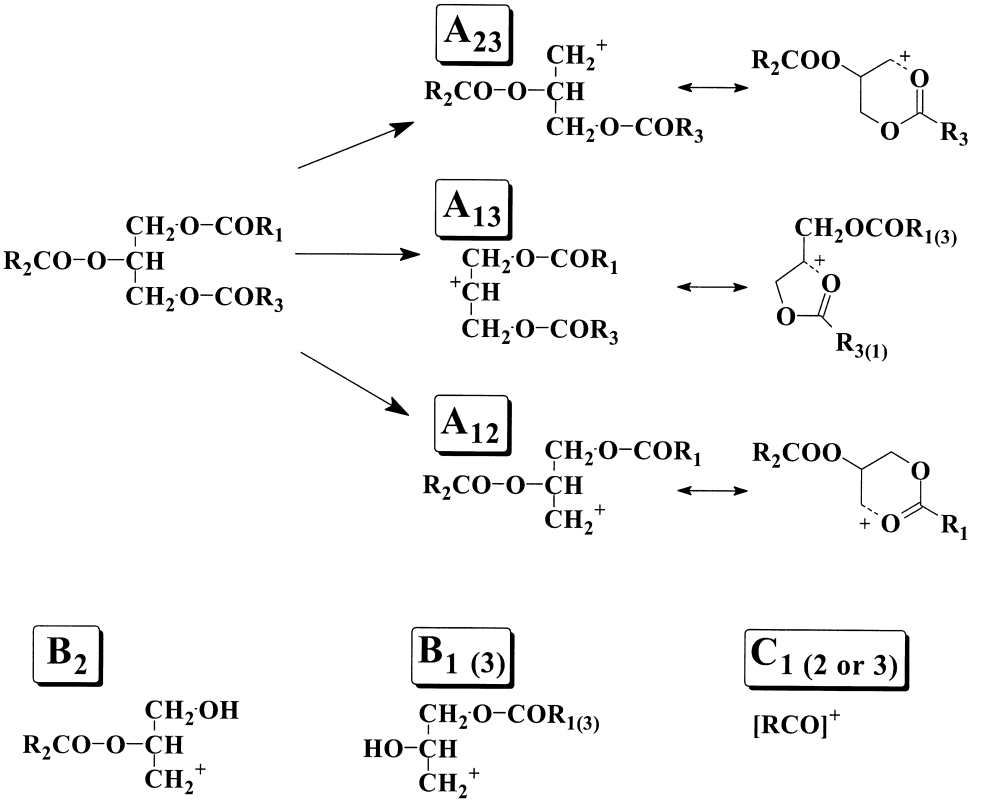


Fig. 1. Structures and notation of the fragment ions of triacylglycerols and other acylglycerols used in this work.

coelutions. The temperature was held at 500 °C in the APCI probe and at 120 °C in the ion source, the cone voltage was 20 V. The ion trap analyzer Esquire3000 (Bruker Daltonics, Bremen, Germany) was used for $MS^n$ measurements in the mass range $m/z$ 50–1000. For APCI measurements, the pressure of the nebulising gas was 70 p.s.i., the flow-rate of the drying gas was 5 l/min, the temperatures of the drying gas and APCI heater were 350 and 400 °C, respectively. For ESI measurements, the direct infusion technique at the flow-rate of 5 μl/min was used with the ion source temperature 300 °C, the flow-rate and the pressure of nitrogen were 4 l/min and 10 p.s.i., respectively.

### 2.4. Sample preparation

Common oils (rapeseed, sunflower, soyabean and linseed oils) were bought on the market. Palm and macadamia oils were obtained from Milo Olomouc, Czech Republic. The samples of *Dracocephalum moldavica*, evening primrose, corn, amaranth and *Silybum arianum* oils were obtained from Galena (Opava, Czech Republic). Other oils (almond, poppy seed, hazel nut, Brazil-nut and pistachio oils) were prepared in the laboratory using the following procedure. First, salts were removed from the food products by washing with water. Then 10–15 g of nuts or seed were carefully crashed in a mortar to fine particles, which were mixed with 15 ml of hexane and stirred occasionally for 15 min. The solid particles was filtered out using a rough filter paper and then the extract was filtered again through a fine filter with 0.2-μm pores. From the filtered extract, hexane was evaporated at room temperature overnight yielding the pure plant oil.

For HPLC analysis, the samples of plant oils and of a reaction mixture of rapeseed oil transesterification were dissolved in acetonitrile–2-propanol (1:1, v/v) solvent mixture yielding 3% (w/v) solutions except for the palm oil sample, which had to be dissolved in acetonitrile–2-propanol–hexane (1:1:1, v/v) due to a high content of less polar saturated TGs.

For direct infusion mass spectrometric experiments, the stock solutions containing 100 mg/l of LnLnLn, LLL, OOO, OOO, OOP, OPO, OPP, POP or PPP standards in hexane–2-propanol (1:1) were diluted 100 times with the same solvent mixture to prepare working solutions used for the measurement of mass spectra of pure compounds. For measurements of the calibration curves, the working solutions of OOP/OPO and OPP/POP were mixed yielding samples with 0:100, 20:80, 40:60, 60:40, 80:20 and 100:0 molar ratios.

The TG samples for ESI measurements were diluted with hexane–2-propanol (1:1) to the final concentration 3.3 and 1 m*M* of ammonium acetate was added.

## 3. Results and discussion

### 3.1. Identification of triacylglycerols using the APCI mass spectra and notation of fragment ions

Table 1 gives the symbols, the carbon numbers (CN) and the double bond (DB) numbers of acyls

Table 4
The relatives abundances of the molecular adducts and the fragment ions A for triacylglycerols PPP, SSS, PoPoPo, OOO, LLL and LnLnLn measured with APCI and ESI ionization techniques

| TG | Double bonds | APCI | | ESI | | | |
|---|---|---|---|---|---|---|---|
| | | Ion A | $[M+H]^+$ | Ion A | $[M+NH_4]^+$ | $[M+Na]^+$ | $[M+K]^+$ |
| PPP | 0 | 100 | 0.03 | 82.6 | 100 | 71.7 | 8.1 |
| SSS[a] | 0 | 100 | 0 | – | – | – | – |
| PoPoPo[a] | 3 | 100 | 6.3 | – | – | – | – |
| OOO | 3 | 100 | 7.1 | 41.7 | 26.9 | 100 | 13 |
| LLL | 6 | 27.1 | 100 | 22.7 | 18.1 | 100 | 3.6 |
| LnLnLn | 9 | 8.3 | 100 | 16.4 | 13.1 | 100 | 3.1 |

1 m*M* of ammonium acetate was added to the solution for ESI measurements (see Section 2).
[a] Data obtained from HPLC–APCI-MS analysis of plant oils.

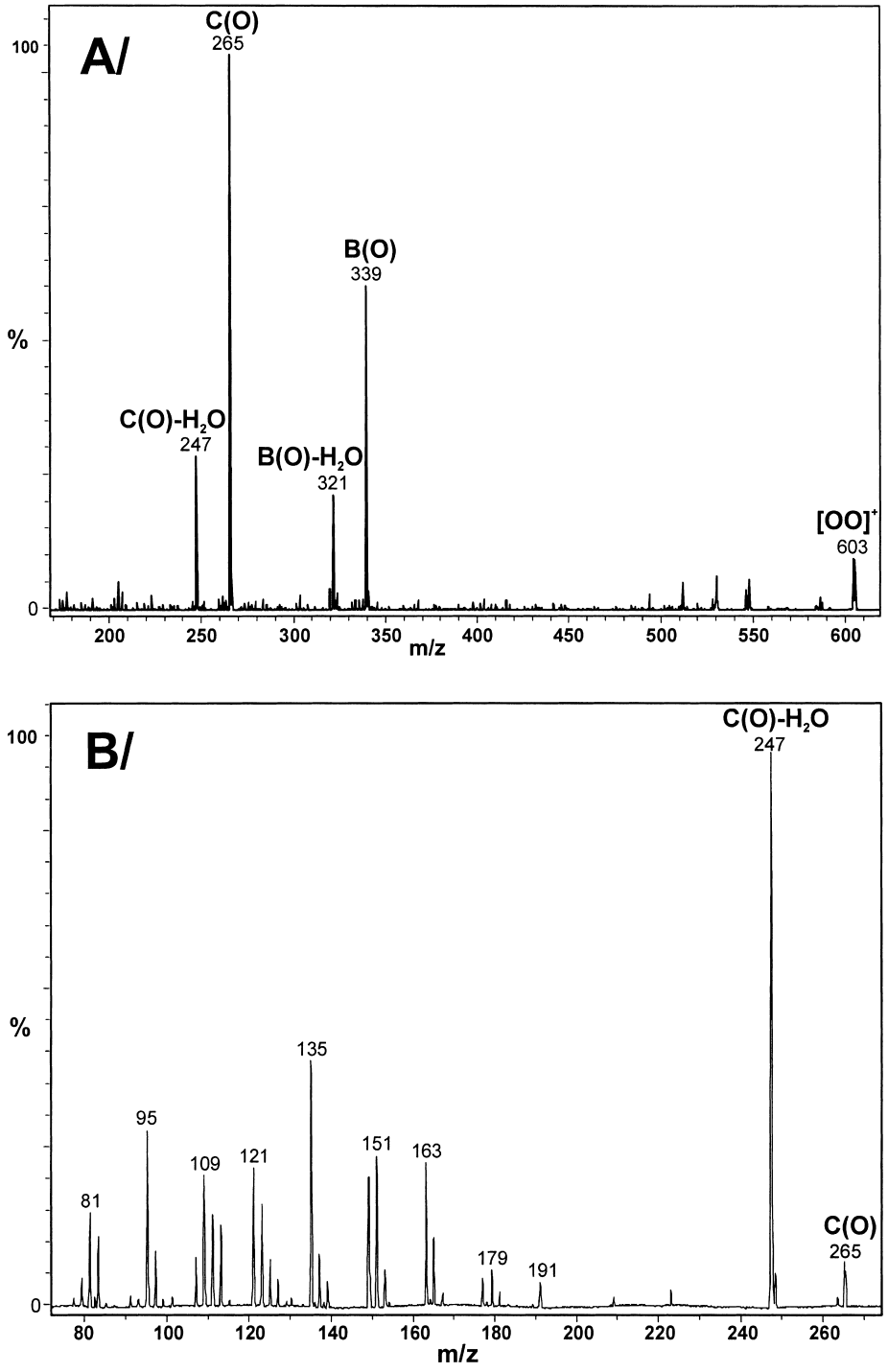


Fig. 2. Positive-ion APCI mass spectra of OOP: A/MS–MS spectrum of $m/z$ 603.7 (ion A), B/MS–MS–MS spectrum of $m/z$ 603.7 (ion A)—265.0 (ion C).

found in the plant oil samples. Table 2 lists the equivalent carbon numbers (ECNs), the masses of protonated molecules, the retention times and the separation factors measured using the HPLC method 1 with the UV detection at 205 nm for all TGs identified in 16 plant and vegetable oils, i.e. for 97 TGs in total. The $m/z$ values of the structurally most important fragment ions A and of the fragment ions B, B-$H_2O$, C and C-$H_2O$ observed in MS–MS spectra are listed in Tables 1 and 3. The $M_r$ values can be calculated from the masses of the individual acyl ions C as follows: $M_r = C_1 + C_2 + C_3 + 89$, the $m/z$ of the ions $A = C_1 + C_2 + 73$, and the $m/z$ of the ions $B = C_1 + 74$. The structures of the fragment ions observed both in the APCI and ESI mass spectra are shown in Fig. 1. The notation of the fragment ions A, B and C is used consistently, in agreement with our previous work [10]. For a better clarity, only the integer parts of the $m/z$ values are listed in the tables. The exact values are by approximately 0.8 higher for $[M+H]^+$ ions of TGs in Table 2 (e.g. 879.8 for LLL), by 0.5 higher for ions A in Table 3 (e.g. 551.5 for $[PP]^+$) and by 0.2 or 0.3 higher for all fragment ions listed in Table 1.

Positive-ion APCI and ESI mass spectra of LnLnLn, LLL, OOO and PPP were investigated in the MS and $MS^n$ modes. The relative abundances of important ions are listed in Table 4. The APCI mass spectra of TGs show primarily two structurally important ions, $[M+H]^+$ and the ions A (see Fig. 1). In the ESI mass spectra, adduct ions are observed instead of protonated molecules. The addition of the alkali metal [31] or ammonium [32] ions enhances the sensitivity and improves the signal stability in the ESI mode. The ratios of the $[M+H]^+$ ions to the ions A in the APCI mass spectra strongly depend on the number of double bonds in the TGs molecules. Even though the relative abundances of the $[M+H]^+$ ions are less than 0.1% for saturated TGs, the ions A and the retention times provide enough information for their unambiguous identification. In the ESI mass spectra, the molecular adduct ions of saturated TGs are much more abundant, which is advantageous for the $M_r$ determination. Fig. 1 presents typical ions observed in the MS and $MS^n$ spectra of TGs. Fig. 2 shows $MS^2$ and $MS^3$ spectra of OOP. Unfortunately, no characteristic fragment ions were found to enable the assignment of the positions of double bonds. The general fragmentation path starts from the protonated molecule via ion(s) A to fragment ions B and C with subsequent losses of water (Fig. 2A) and finally leads to low-mass fragment ions of the aliphatic series (Fig. 2B). The base peaks in the MS–MS spectra of the ions A of most TGs are the fragment ions C, except for polyunsaturated TGs, such as LnLnLn, where the ion C-$H_2O$ is more abundant. The fragmentation behaviour is similar for APCI and ESI, but the dissociation of the sodiated molecules

Table 5
The relative abundances of diacylglycerol fragment ions $[OO]^+$, $[OP]^+$ and $[PP]^+$ for all positional isomers of triacylglycerols composed of oleic and palmitic acids and the parameters of the calibration curves for the determination of the positional isomers

| TG | $[OO]^+$ | | | | $[OP]^+$ | $[PP]^+$ | | | |
|---|---|---|---|---|---|---|---|---|---|
| | APCI | APCI Ref. [24] | ESI | ESI Ref. [24] | APCI, ESI and Ref. [24] | APCI | APCI Ref. [24] | ESI | ESI Ref. [24] |
| OPO | 20.6 | 16.7 | 15.7 | 24.4 | 100 | – | – | – | – |
| OOP | 45.5 | 50.7 | 67.4 | 67.7 | 100 | – | – | – | – |
| POP | – | – | – | – | 100 | 28.1 | 29.3 | 33 | 23.3 |
| OPP | – | – | – | – | 100 | 92.2 | 87.4 | 98.8 | 67.7 |

| $TG_1/TG_2$ | $a$ | $b$ | $R^2$ |
|---|---|---|---|
| OOP/OPO | 5.12±2.41 | 0.37±0.04 | 0.974 |
| OPP/POP | 17.54±6.26 | 1.18±0.10 | 0.970 |

$Y = a + b \times TG_1/TG_2$, ±standard deviations, where $TG_1/TG_2$ is the molar ratio of TG positional *sn*-1(3) and *sn*-2 isomers, $Y$ is the response ratio of A ions ($[OO]^+/[OP]^+$ for the OOP/OPO isomers and $[PP]^+/[OP]^+$ for OPP/POP isomers); $R^2$ is the correlation coefficient.

produced by ESI requires a higher energy and the ESI $MS^n$ spectra contain both sodiated and non-sodiated fragment ions.

Plant oils usually contain a mixture of positional isomers differing by the acyl position, *sn*-2 vs. *sn*-1(3), such as the pairs of OOP/OPO and POP/OPP [5]. Three identical acyls on the glycerol backbone ($R_1R_1R_1$) provide only a single ion A, while the $R_1R_1R_2$ TGs produce two different types of ions A with the statistical abundance ratios 2:1, and the $R_1R_2R_3$ TGs three different types of ions A with the statistical abundance ratios 1:1:1. The loss of RCOOH from the equivalent side positions *sn*-1 and *sn*-3 is preferred comparing to the cleavage from the middle position *sn*-2 [16–18]. The relative abundances of the different types of the $[M+H-RCOOH]^+$ ions enable the identification of the acyl predominant in the *sn*-2 position, which has been explained by the energetic preference of this cleavage in comparison to *sn*-1(3), however without a more detailed reasoning [20–22]. Fig. 1 shows possible resonance structures of the fragment ions A involving the cyclic systems with more stable six-member rings for $A_{23}$ and $A_{12}$ ions, increasing their relative abundances with respect to $A_{13}$ ions forming a less stable five-member ring. Similar explanation was used earlier for the relative abundances of the positional isomers of 1,2- and 1,3-propylene glycol benzoyl derivatives [30].

The acyls in the *sn*-2 position are determined on the basis of different relative abundances of the $A_{13}$ ions with respect to $A_{12}$ and $A_{23}$ ions and are attributed to the middle letter in the abbreviation used in the TGs notation (for example OPO means

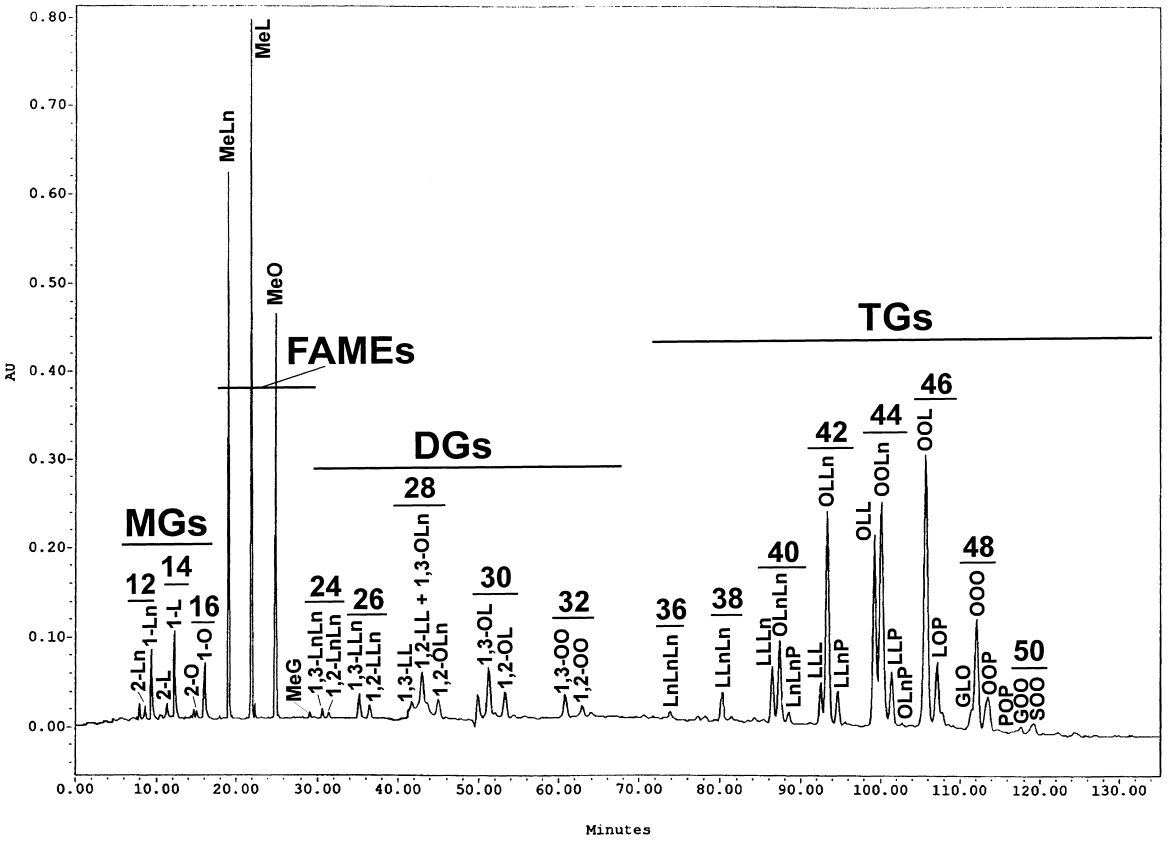


Fig. 3. Chromatogram of mono- (MGs), di- (DGs), triacylglycerols (TGs) and fatty acid methyl esters (FAMEs) in the transesterification reaction mixture of rapeseed oil with methanol. The HPLC method 1, UV detection at 205 nm, peak numbers correspond to the ECN.

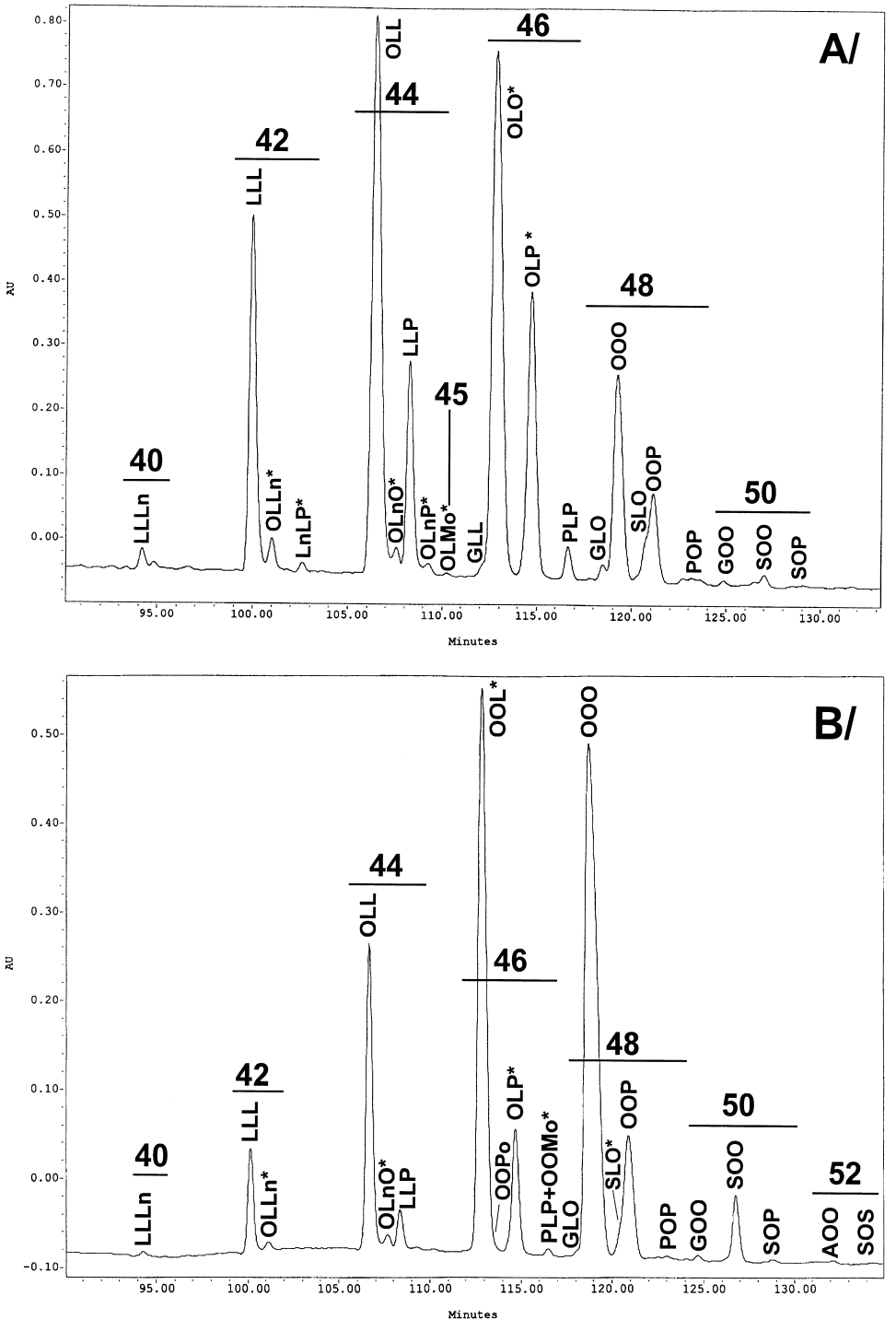


Fig. 4. Chromatograms of triacylglycerols in: (A) pistachio oil, (B) hazelnut oil. The HPLC method 1, UV detection at 205 nm, peak numbers correspond to the ECN.

Table 6
The relative peak areas of the individual TGs in the plant oil samples. The data were measured with the HPLC method 1 (see the Section 2), UV detection at 205 nm

| TG | Hazelnut | Pistachio | Poppy-seed | Almond | Palm | Brazil-nut |
|---|---|---|---|---|---|---|
| LnLnLn | – | – | – | – | – | – |
| LLnLn | – | – | – | – | – | – |
| LLLn | 0.1 | 0.6 | 2.8 | – | – | 0.1 |
| PoLnPo | – | – | – | – | – | – |
| OLnLn | – | – | – | – | – | – |
| LnLnP | – | – | – | – | – | – |
| LLnMo | – | – | 0.2* | – | – | – |
| LnLnMa | – | – | – | – | – | – |
| LLL | 3.7 | 11.7 | 38.6 | 8.7 | 0.4 | 14.8 |
| LLPo | – | – | – | – | – | – |
| PoLPo | – | – | – | – | – | – |
| PoPoPo | – | – | – | – | – | – |
| OLLn | 0.5* | 1.0* | 1.1* | 0.1 | – | 0.2 |
| PoLM | – | – | – | – | – | – |
| LLM | – | – | – | – | – | 0.06 |
| PoPoM | – | – | – | – | – | – |
| LnLP | – | 0.3* | 0.7* | – | – | 0.09 |
| SLnLn | – | – | – | – | – | – |
| LLMo | – | – | 0.1 | 0.1 | – | – |
| MoPoPo | – | – | – | – | – | – |
| LLnMa | – | – | – | – | – | – |
| GLLn | – | – | – | – | – | – |
| OLL | 12.3 | 24.8 | 19.9 | 27.6 | 2.2 | 16.7 |
| OLPo | – | – | – | – | – | – |
| PoOPo | – | – | – | – | – | – |
| OLnO | 0.7* | 0.9* | – | – | – | – |
| LLP | 1.6 | 7.1 | 18.1 | 4.8 | 8.3 | 13.0 |
| OPoM | – | – | – | – | $<0.05$ | – |
| OLnP | – | 0.5* | – | – | 1.9 | – |
| OLM | – | – | – | – | – | 0.07 |
| PPoPo | – | – | – | – | – | – |
| PLM | – | – | – | – | 1.1* | $<0.05$ |
| PPoM | – | – | – | – | – | – |
| PLnP | – | – | – | – | – | – |
| OPoMo | – | – | – | – | – | – |
| OLMo | – | $<0.05$ | – | 0.1* | – | – |
| LLMa | – | – | 0.2* | – | – | 0.05 |
| PMoPo | – | – | – | – | – | – |
| GLL | – | 0.5 | 0.2 | – | – | – |
| OLO | 28.2* | 25.2* | 6.1* | 28.0 | 5.3 | 13.1* |
| OOPo | $<0.05$ | – | – | – | – | – |
| SLL | – | – | +LOP* $\Sigma=9.5$ | – | – | – |
| LOP | 5.2* | 11.8* | +SLL $\Sigma=9.5$* | 11.3* | +OOM* $\Sigma=23.9$ | 16.7* |
| OOM | – | – | – | – | +LOP $\Sigma=23.9$* | – |
| POPo | – | – | – | – | – | – |
| GPoPo | – | – | – | – | – | – |
| PLP | +OOMo* $\Sigma=0.2$ | 1.1 | 0.5 | +OOMo $\Sigma=0.5$ | +POM $\Sigma=17.7$ | 2.6 |
| SLnP | – | – | – | – | – | – |

Table 6. Continued

| TG | Hazelnut | Pistachio | Poppy-seed | Almond | Palm | Brazil-nut |
|---|---|---|---|---|---|---|
| POM | – | – | – | – | +PLP Σ=17.7 | – |
| PPoP | – | – | – | – | – | – |
| OOMo | +PLP Σ=0.2* | – | – | +PLP Σ=0.5 | – | – |
| OLMa | – | – | – | – | – | <0.05 |
| OMoP | – | – | – | – | <0.05 | – |
| MaPM | – | – | – | – | <0.05 | – |
| GLO | 0.1 | 0.5 | – | 0.1* | – | <0.05 |
| OOO | 36.5 | 8.9 | 0.4 | 13.3 | +SPM Σ=3.8 | 4.6 |
| SPM | – | – | – | – | +OOO Σ=3.8 | – |
| ALL | – | – | – | – | – | +SLO+OOP Σ=10.0 |
| GOM | – | – | – | – | – | – |
| GLP | – | – | – | – | – | – |
| SLO | 1.4* | 1.3 | <0.05 | 1.8 | +OOP Σ=17.2 | +ALL+OOP Σ=10.0 |
| OOP | 6.1 | 3.4 | 1.5 | 2.7 | +SLO Σ=17.2 | +ALL+SLO Σ=10.0 |
| SLP | – | – | 0.1 | – | 2.5 | 2.6 |
| POP | 0.06 | 0.1 | <0.05 | 0.1 | 11.2 | 0.6 |
| PPP | – | – | – | – | 0.06 | – |
| OOMa | – | – | – | <0.05 | <0.05 | – |
| MaOP | – | – | – | – | <0.05 | – |
| GLS | – | – | – | – | – | – |
| GOO | 0.2 | 0.1 | – | <0.05 | – | – |
| BLL | – | – | – | – | – | – |
| ALO | – | – | – | – | – | 0.2 |
| GOP | – | – | – | – | – | – |
| SOO | 2.8 | 0.4 | <0.05 | 0.6 | 1.7 | 2.3 |
| AOPo | – | – | – | – | – | – |
| ALP | – | – | – | – | 0.3 | – |
| SLS | – | – | – | – | – | 1.2 |
| SOP | 0.09 | 0.06 | – | – | 1.9 | 0.7 |
| SPP | – | – | – | – | 0.07* | – |
| SOMa | – | – | – | – | <0.05 | – |
| GOG | – | – | – | – | – | – |
| LiLL | – | – | – | – | – | – |
| BOL | – | – | – | – | – | – |
| GSO | – | – | – | – | – | – |
| AOO | <0.05 | – | – | – | 0.09 | <0.05 |
| BLP | – | – | – | – | – | – |
| SOS | 0.1 | – | – | – | 0.2 | 0.3 |
| AOP | – | – | – | – | – | – |
| LiOL | – | – | – | – | – | – |
| BOO | – | – | – | – | 0.05 | – |
| LiLP | – | – | – | – | – | – |
| BLS | – | – | – | – | – | – |
| BOP | – | – | – | – | <0.05 | – |
| GGS | – | – | – | – | – | – |
| SSS | – | – | – | – | – | – |

Table 6. Continued

| TG | Hazelnut | Pistachio | Poppy-seed | Almond | Palm | Brazil-nut |
|---|---|---|---|---|---|---|
| AOS | – | – | – | – | – | 0.06 |
| LiOO | – | – | – | – | <0.05 | – |
| LiOP | – | – | – | – | <0.05 | – |
| Total number of TGs | 21 | 21 | 20 | 18 | 33 | 28 |

| TG | Rapeseed | Macadamia | Soyabean | Sunflower | Linseed |
|---|---|---|---|---|---|
| LnLnLn | <0.05 | – | 0.09 | – | 30.4 |
| LLnLn | <0.05 | – | 1.9* | – | 18.7 |
| LLLn | 2.7 | – | 10.3 | – | 5.3 |
| PoLnPo | – | 0.2 | – | – | – |
| OLnLn | 3.7 | – | 2.4* | – | 13.5 |
| LnLnP | 2.3 | – | 0.4 | – | 6.9 |
| LLnMo | – | – | 0.2* | – | – |
| LnLnMa | – | – | – | – | – |
| LLL | 1.1 | – | 18.9 | 33.3 | 0.9 |
| LLPo | – | 0.3 | – | 0.1 | – |
| PoLPo | – | 1.3 | – | – | – |
| PoPoPo | – | 2.6 | – | – | – |
| OLLn | 5.7 | – | 6.2 | 2.6 | 5.9 |
| PoLM | – | 0.5 | – | – | – |
| LLM | – | – | 0.9 | 0.5 | – |
| PoPoM | – | 1.2* | – | – | – |
| LnLP | 0.7* | – | 4.0 | – | 3.0 |
| SLnLn | – | – | 0.7 | – | 4.1 |
| LLMo | – | – | 0.2 | 0.06 | – |
| MoPoPo | – | 1.0 | – | – | – |
| LLnMa | – | – | – | – | – |
| GLLn | – | – | – | 0.06 | – |
| OLL | 8.0 | 0.9 | 14.9 | 25.1 | 1.0 |
| OLPo | – | 3.9* | – | – | – |
| PoOPo | – | 8.2 | – | – | – |
| OLnO | 11.1* | – | 1.3* | 0.8* | 4.2* |
| LLP | 1.1 | – | 12.0 | 12.6 | – |
| OPoM | – | 1.66 | – | – | – |
| OLnP | 2.3 | – | 2.4 | – | 3.1* |
| OLM | – | – | – | – | – |
| PPoPo | – | 2.6* | – | – | – |
| PLM | – | – | +OLMo Σ=0.4 | – | – |
| PPoM | – | 1.0 | – | – | – |
| PLnP | 0.06 | – | 0.2 | – | 0.5* |
| OPoMo | – | 0.7 | – | – | – |
| OLMo | – | – | +PLM Σ=0.41 | <0.05 | – |
| LLMa | – | – | 0.2 | 0.1 | – |
| PMoPo | – | 0.4 | – | – | – |
| GLL | +OLO Σ=23.5 | – | 0.2 | 0.3* | – |
| OLO | +GLL Σ=23.50 | 6.4 | 6.0* | 6.1* | 0.8* |
| OOPo | – | 16.1 | – | – | – |

Table 6. Continued

| TG | Rapeseed | Macadamia | Soyabean | Sunflower | Linseed |
|---|---|---|---|---|---|
| SLL | – | – | +LOP<br>Σ=8.7 | +LOP*<br>Σ=11.4 | 1.1 |
| LOP | 5.4* | 2.7* | +SLL<br>Σ=8.7 | +SLL<br>Σ=11.4* | 0.6 |
| OOM | – | – | – | – | – |
| POPo | – | +GPoPo<br>Σ=6.1 | – | – | – |
| GPoPo | – | +POPo<br>Σ=6.1 | – | – | – |
| PLP | 0.7 | – | 1.4 | 0.6 | – |
| SLnP | – | – | 0.2 | – | – |
| POM | – | +PPoP<br>Σ=1.0 | – | – | – |
| PPoP | – | +POM<br>Σ=1.0 | – | – | – |
| OOMo | 0.2* | 0.9 | – | – | – |
| OLMa | – | – | 0.09 | – | – |
| OMoP | – | +GLO<br>Σ=1.1* | – | – | – |
| MaPM | – | – | – | – | – |
| GLO | 1.2 | +OMoP*<br>Σ=1.1 | 0.1 | 0.07 | – |
| OOO | 16.8 | +GOM*<br>Σ=19.4 | 0.8 | 0.5 | – |
| SPM | – | – | – | – | – |
| ALL | – | – | +GLP*<br>Σ=0.3 | 0.5 | – |
| GOM | – | +OOO<br>Σ=19.4* | – | – | – |
| GLP | – | – | +ALL<br>Σ=0.3 | – | – |
| SLO | 2.1 | – | +OOP<br>Σ=2.3 | +OOP<br>Σ=2.1 | – |
| OOP | 4.3 | 9.9 | +SLO<br>Σ=2.32 | +SLO<br>Σ=2.1 | – |
| SLP | – | +POP<br>Σ=1.2 | 0.7 | 0.7 | – |
| POP | 0.8 | +SLP<br>Σ=1.2 | 0.2 | 0.2 | – |
| PPP | – | – | – | – | – |
| OOMa | – | – | – | – | – |
| MaOP | – | – | – | – | – |
| GLS | 0.5 | – | – | – | – |
| GOO | 1.3 | 3.1 | – | – | – |
| BLL | – | – | 0.4 | 1.1 | – |
| ALO | 0.3* | – | 0.2 | – | – |
| GOP | 0.8 | 1.2 | – | – | – |
| SOO | 1.2 | – | 0.2 | <0.05 | – |
| AOPo | – | 3.5 | – | – | – |
| ALP | – | – | +SLS+SOP<br>Σ=0.3* | +SLS<br>Σ=0.2 | – |

Table 6. Continued

| TG | Rapeseed | Macadamia | Soyabean | Sunflower | Linseed |
|---|---|---|---|---|---|
| SLS | – | – | +ALP+SOP Σ=0.3* | +ALP Σ=0.2 | – |
| SOP | 0.5 | 0.4 | +ALP+SLSΣ=0.3* | 0.06 | – |
| SPP | – | – | – | – | – |
| SOMa | – | – | – | – | – |
| GOG | 0.3 | 0.3 | – | – | – |
| LiLL | 0.3 | – | 0.1 | 0.4 | – |
| BOL | 0.5 | – | 0.2 | 0.3* | – |
| GSO | – | – | – | – | – |
| AOO | 0.4 | 0.3 | <0.05 | – | – |
| BLP | – | – | 0.1 | 0.09* | – |
| SOS | 0.07 | – | <0.05 | <0.05 | – |
| AOP | – | <0.05 | <0.05 | – | – |
| LiOL | 0.06 | – | 0.06 | <0.05* | – |
| BOO | 0.1 | – | <0.05 | <0.05 | – |
| LiLP | – | – | – | 0.08 | – |
| BLS | – | – | 0.05 | – | – |
| BOP | – | – | <0.05 | – | – |
| GGS | <0.05 | – | – | – | – |
| SSS | – | – | – | – | – |
| AOS | – | – | – | – | – |
| LiOO | <0.05 | – | <0.05 | <0.05 | – |
| LiOP | – | – | – | – | – |
| Total number of TGs | 38 | 37 | 52 | 36 | 16 |

The data marked with an asterisk correspond to the TGs, in which the predominating isomer in the *sn*−2 position could not be identified unambiguously because of a small peak intensity, because of coelution with another TGs yielding the fragment ions A with the same masses, or because of low concentration differences between the positional *sn*−2 and *sn*−1(3) isomers. The same notation is used in Table 7 and Figs. 4 and 6.

palmitoyl in the *sn*-2 position). The positions *sn*-1 and *sn*-3 are considered as identical like in most previous works, in spite of different symmetry and consequently different biological properties. The *sn*-1 and *sn*-3 positional isomers can be principally separated by silver ion HPLC [7], but cannot be distinguished by NARP-HPLC. In our notation, the acyl with a higher mass is consistently attributed to the *sn*-1 position, e.g. SLO. The unsaturated fatty acids tend to occupy the middle *sn*-2 position, especially the linoleoyl has the strongest preference for the *sn*-2 position. If the unambiguous determination of the *sn*-2 acyl is complicated by a very low concentration, coelutions with TGs containing the fragment ions A with identical masses or low concentration differences between the *sn*-2 and *sn*-1(3) positional isomers, then such TG is marked with asterisk in the tables and figures, e.g. OLO*****. For more precise attribution of the *sn*-2 predominant acyl or for the determination of the isomeric ratios, the mass spectra of the authentic standards should be measured. Both the results of our present work and of Ref. [24] show that the attribution of the *sn*-2 acyl to the less intense fragment ion A of oleoyl and palmitoyl containing positional isomers may be oversimplified and should be revised. When applying strictly this approach to the identification of OOP in Table 5, the *sn*-2 acyl could be misidentified as the OPO or as a mixture of OPO/OOP.

The relative ratios of $[OO]^+$, $[PO]^+$ and $[PP]^+$ fragment ions A were measured for TGs standards containing oleic and palmitic acyls, i.e. OOO, OOP, OPO, OPP and POP (Table 5) using both APCI and ESI ionization techniques and the results were compared with the recently published data [24]. The positional isomers OOP/OPO and OPP/POP yield the mass spectra showing significant differences in the relative ratios of $[OO]^+/[OP]^+$ and $[PP]^+/[OP]^+$ ions. The relative abundances of the isomeric ions A depend to some extent on the ionization

technique (APCI vs. ESI) and on the instrument type (Table 5), which suggests that the relative abundances of the $[OO]^+$, $[PO]^+$ and $[PP]^+$ ions should be measured with authentic standards on a particular instrument and cannot rely on the published values of the relative ratios. To determine the ratios of *sn*-2 and *sn*-1(3) isomers, we measured the calibration mixtures of OOP and OPO (see Section 2 for details) and plotted the mean values of the ratios of relative abundances of $[OO]^+/[OP]^+$ ions from five consecutive injections vs. the relative percentage of the OOP isomer in the OOP/OPO mixture to obtain the calibration curves, whose parameters *a* and *b* are listed in Table 5. The correlation coefficients $R^2$ indicate that this method can be applied for a rough estimate of the ratios of the *sn*-2 and *sn*-1(3) isomers of TGs.

### *3.2. Characterization of triacylglycerols and diacylglycerols in plant oils by HPLC–APCI-MS*

Several chromatographic columns and mobile phase gradients were tested to provide optimum separation of TGs. The best results were obtained with the Nova-Pak $C_{18}$ columns and acetonitrile–2-propanol gradients. A first aqueous–organic gradient step is introduced to improve the separation of more polar MGs and DGs present in the samples of the reaction mixtures of biodiesel production. The mobile phases without chlorine-containing solvents, which are often used in NARP-HPLC of TGs, improve the stability of the mass spectrometric signal and require a less frequent ion source cleaning. Two Nova-Pak $C_{18}$ columns connected in series in series and combined aqueous acetonitrile and 2-propanol–acetonitrile gradient steps improved the resolution not only for TGs with the same ECN, which is significantly better than in earlier reported HPLC analyses, especially for TGs with lower ECNs, but also for DGs and MGs poorly separated from some lower ECN TGs in the earlier works. The separation of MGs, DGs and TGs in a single run is illustrated in Fig. 3. Almost all TGs with equal ECNs are clearly separated in this work, whereas only partial resolution—if any—of the individual TGs was apparent from the chromatograms in Refs. [13,17,20,22,26], e.g. LLL/OLLn/LLnP are well resolved using the present HPLC method on the contrary to previous works. Furthermore, the groups of TGs with different ECNs are very well separated from each other, so that there is enough space for complete separation and unambiguous identification of TGs with odd ECNs, such as OLMo in Fig. 4A and OOMo in Fig. 4B.

The results obtained with the HPLC method 1 for 11 plant oil samples are summarized in Table 6, where the relative peak areas of all identified TGs are calculated from the UV chromatograms at 205 nm. The separation is illustrated by the examples of pistachio (Fig. 4A) and hazelnut (Fig. 4B) oils. As reported in all earlier publications [1–3,14–26], the TGs are eluted in the order of increasing ECNs and

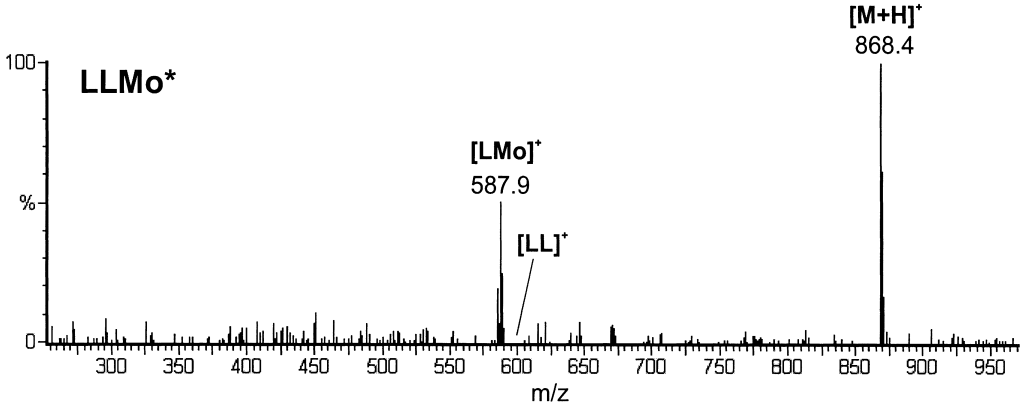


Fig. 5. Positive–ion APCI mass spectrum of a triacylglycerol containing acyl with an odd number of carbon atoms: LLMo identified in soyabean oil.

most TGs with identical ECNs are more or less separated, except for positional isomers $R_1R_1R_2$ and $R_1R_2R_1$. Two methylene groups contribute to the retention slightly higher than the decrement of one double bond. This is illustrated by the retention order of the TGs with ECN=48: OOO<OOP<POP<PPP. This is the second time when the presence of unusual fatty acid TGs with odd numbers of carbon atoms was identified in some TGs at low concentration levels (see Table 6). Fig. 5 presents an example of mass spectrum, on the basis of which LLMo was unambiguously identified in soyabean oil. However, in the earlier work reporting such TGs [20], their peaks were not clearly resolved from other TGs and their identification is not illustrated by the spectra or peak annotation in chromatograms. We found TGs containing fatty acids with odd numbers of carbon atoms in most plant oils. These TGs consist of one acyl with odd number of carbon atoms (C17:0 or C17:1) and two acyls of most abundant fatty acids for a particular plant oil, for example LLMo, OLMo, LLMa and SOMo in sunflower oil, LLMa and OLMa in Brazil-nut oil, OOMo in hazelnut oil, MoPoPo, OPoMo, PMoPo, OOMo,

Table 7
The relative peak areas and retention times in minutes of individual TGs in the plant oil samples

| TG | $t_R$ | *Dracocephalum moldavica* | Evening primrose | Corn | Amaranth[a] | *Silybum arianum* |
|---|---|---|---|---|---|---|
| LnLnLn | 18.6 | 38.2 | – | – | – | – |
| LLnLn | 21.8 | 21.4 | – | – | – | 0.2 |
| LLLn | 25.1 | 6.7 | – | 2.2 | 1.0 | 0.8 |
| OLnLn | 25.6 | 8.6 | – | 0.06 | 0.3 | 0.3 |
| LnLnP | 26.1 | 6.8 | – | – | – | – |
| LnLnMa | 27.3 | 0.4 | – | – | – | – |
| LLL | 28.4 | 1.2 | 27.5 | 29.1 | 10.3 | 24.5 |
| OLLn | 28.8 | 4.1 | 4.4 | 1.7 | 1.3 | 1.7 |
| LnLP | 29.6 | 3.0* | 1.0 | 0.7* | 0.9 | 0.4* |
| SLnLn | 29.8 | 3.2 | – | – | – | – |
| LLnMa | 30.4 | 0.1 | – | – | – | – |
| OLL | 32.0 | 0.8 | 24.6 | 24.9 | 19.2 | 23.6 |
| LLP | 32.4 | 1.9 | 15.0 | 16.6 | 20.7 | 12.3 |
| OLnP | 33.0 | 1.9 | 1.2 | 0.08 | 0.5 | – |
| PLnP | 33.6 | 0.2 | – | – | – | – |
| OLO | 35.2 | 0.5* | +SLL Σ=9.7* | +SLL Σ=8.9* | 11.3* | +SLL Σ=8.7* |
| SLL | 35.5 | – | +OLO* Σ=9.7 | +OLO* Σ=8.9 | – | +OLO* Σ=8.7 |
| OLP | 36.0 | 0.4 | 9.3 | 9.3 | 16.7 | 11.1 |
| PLP | 36.4 | 0.4 | 1.4 | 1.7 | 7.1* | 0.9 |
| OOO | 38.2 | – | 1.5 | 1.3 | 2.7 | 0.5 |
| SLO | 38.4 | – | +OOP Σ=2.6* | +OOP Σ=2.0* | 4.8* | +OOP Σ=7.8* |
| OOP | 38.7 | – | +SLO* Σ=2.6 | +SLO* Σ=2.0 | – | +SLO* Σ=7.8 |
| POP | 39.3 | – | 0.5 | 1.0 | 2.0 | 0.9 |
| SOO | 41.5 | – | 0.5 | 0.4 | 0.9 | 3.7 |
| SOP | 42.3 | – | 0.1 | 0.2 | 0.5 | 0.5 |
| SOS | 44.3 | – | 0.2 | – | – | 1.5 |
| SSP | 45.0 | – | – | – | – | 0.3 |
| SSS | 46.9 | – | – | – | – | 0.3 |
| Total number of TGs | – | 18 | 17 | 18 | 16 | 21 |

The data were measured with the HPLC method 2 (see Section 2); UV detection at 205 nm
[a] In addition to TGs, amaranth oil contains high amount of squalene ($t_R$=9.9 min).

Table 8
Diacylglycerols identified in the plant oils

| DG | ECN | 1,3-DG | | 1,2-DG | | Hazelnut | Pistachio | Poppy seed | Almond | Palm | Brazil-nut | Rapeseed | Macadamia | Soyabean | Sunflower | Linseed |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $t_r$ | $r$ | $t_r$ | $r$ | | | | | | | | | | | |
| LnLn | 24 | 33.3 | 0.520 | 35.2 | 0.551 | – | – | – | – | – | – | – | – | – | – | Yes |
| LLn | 26 | 41.7 | 0.659 | 44.1 | 0.699 | – | – | Yes | – | Yes | – | Yes | – | Yes | – | – |
| PoPo | 28 | 49.8 | 0.794 | 53.0 | 0.847 | – | – | – | – | – | – | – | Yes | – | – | – |
| LL | 28 | 51.3 | 0.819 | 53.8 | 0.860 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | – | Yes | Yes | – |
| OPo | 30 | 61.5 | 0.988 | 64.1 | 1.032 | – | – | – | – | – | – | – | Yes | – | – | – |
| OL | 30 | 62.2 | **1.000** | 64.5 | 1.038 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | – |
| PPo | 30 | 63.4 | 1.020 | 66.3 | 1.068 | – | – | – | – | – | – | – | Yes | – | – | – |
| LP | 30 | 63.5 | 1.022 | 66.6 | 1.073 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | – | Yes | Yes | – |
| OO | 32 | 71.8 | 1.160 | 75.4 | 1.220 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | – |
| OP | 32 | 74.3 | 1.201 | 77.4 | 1.253 | Yes | Yes | Yes | Yes | | | | | | | |
| PP | 32 | 76.3 | 1.234 | 80.4 | 1.303 | – | – | – | – | | | | | | | |
| SO | 34 | 88.0 | 1.429 | 90.7 | 1.474 | – | – | – | – | | | | | | | |

The separation factor $r = t'_R/t'_S$ is the net retention time $t'_R$ related to the retention time $t'_S$ of diacylglycerol 1,3-OL, $t_R$ in min.

OMoPo in macadamia oil and LnLnMa and LLnMa in *Dracocephalum moldavica* oil, etc. (see Tables 6 and 7). DGs containing the main fatty acids found in TGs were also detected in all plant oils (Table 8).

We compared the results of the present HPLC–MS method for the analysis of TGs in plant oils with the previous publications [1–3,5,6,14,20,25] and we found the identical elution order for all TGs, which is probably generally valid for NARP-HPLC (Table 2). The best resolution of the critical pairs of TGs was achieved in our work which resulted in the highest number of TGs reported so far: 52 TGs in the soyabean oil (one of the most complex plant oils) in our work (38 TGs in Ref. [20]; LnLnLn, LnOLn, LnLMo, LLM, SLnLn, LLMo, PLM, OLMo, LLMa, SLnP, OLMa, GLO, ALL, GLP, BLS, BOP and LiOO are missing; they additionally found LStL, OLSt and PoGG; 22 TGs in Ref. [6]; 19 TGs in Ref. [3] and 12 TGs in Ref. [17]); 38 TGs in the rapeseed oil (26 TGs in Ref. [20]; OOLn, PLnP, GLL, OMoO, SLO, SLG, GOP, GOG, LiLL, SOS, GGS and LiOO are missing there); 21 TGs in hazelnut oil (17 TGs in Ref. [20], OLnO, PLP, OOMo, GLO and SLO are missing, they additionally found AOP). These examples clearly demonstrate that improved chromatographic resolution with the present HPLC method 1 simplifies the identification of trace and isobaric TGs. As far as we know, the TG composition of some less widespread plant oils is published here for the first time.

The HPLC method 2 without the first acetonitrile–water gradient step is a compromise between the excellent resolution of the HPLC method 1 and the analysis time, which is reduced from 140 to 50 min. Figs. 4 and 6 illustrates impaired resolution, for example OLL/OLnO/LLP and OLP/PLP TGs using the method 2. Furthermore, the HPLC method 2 does not provide satisfactory separation of less retained and more polar MGs and DGs. However, it provides comparable resolution of TGs as the best earlier published separations [3,20,22] and can be applied for routine analysis of main TGs, such as in the present work to five plant oil samples (Table 7): *Dracocephalum moldavica* (Fig. 6A), evening primrose, corn, amaranth and *Silybum arianum* (Fig. 6B) oils.

In addition to identified TGs and DGs, some plant oils also contain small amounts (in the range of

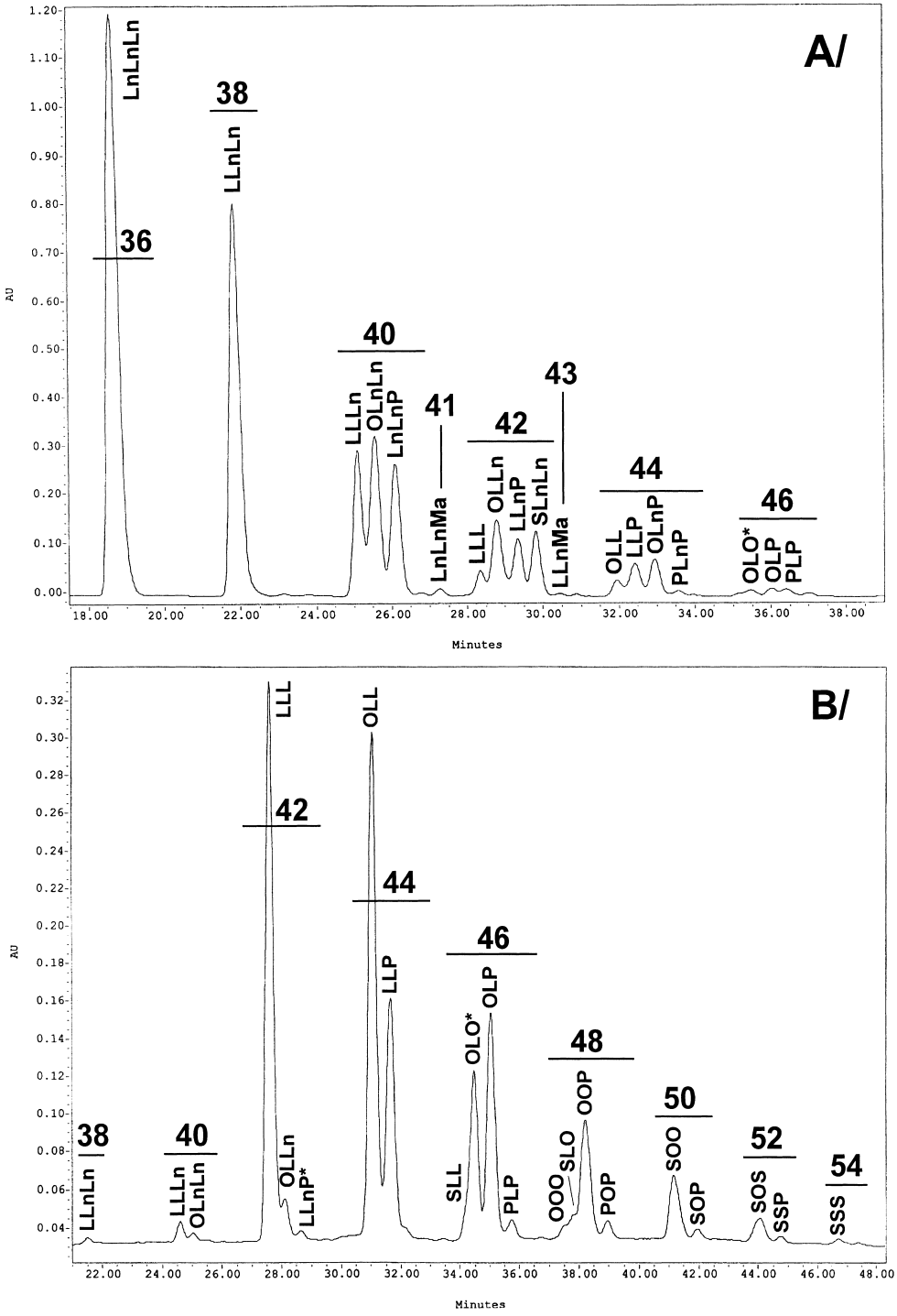


Fig. 6. Chromatograms of triacylglycerols in: (A) *Dracocephalum moldavica* oil, (B) *Silybum arianum* oil. The HPLC method 2, UV detection at 205 nm, peak numbers correspond to the ECN.

tenths of per cent) of TG oxidation products in accordance with previous works of Byrdwell and Neff [18,24], especially plant oils containing higher contents of polyunsaturated fatty acids (rapeseed and linseed oils) and commercial products purchased in the local supermarket (e.g. sunflower frying oil). The oxidation products are eluted earlier than corresponding TGs, but their APCI mass spectra are very complex [18]. In the present work, the oxidation products did not complicate the analysis of the individual TGs in the plant oil mixtures.

## 4. Conclusions

The combined aqueous–organic/non-aqueous gradient HPLC method 1 with two chromatographic columns Nova-Pak $C_{18}$ in series yields improved chromatographic resolution with respect to the earlier reported methods when applied for the analysis of complex TG mixtures. This method allowed not only the complete characterization of the TG composition of 11 plant oils including the trace and coeluting TGs, but it also enabled the separation and identification of mono- and diacylglycerols in a single run, together with TGs. The oils obtained from various plants differ in the content of saturated and unsaturated fatty acids with different acyl chain lengths and in their complexity starting from the most simple linseed oil (16 TGs and one DG) to the most complex soyabean oil (52 TGs and 6 DGs). In several plant oils, the unusual TGs containing fatty acids with odd numbers of carbon atoms (C17:0 and C17:1) were unambiguously identified. The HPLC method 2 offers a compromise between the time of analysis and the separation selectivity. It was applied for the identification of main TGs in five plant oils. In total, 97 TGs and 12 DGs were identified in 16 plant oil samples which—to our best knowledge—is the highest number of TGs identified in the individual plant oils ever reported. Several TGs are detected in this work for the first time. The mass spectra of the authentic standards of TGs containing oleoyl and palmitoyl acyls measured with the direct infusion APCI and ESI showed that the relative abundances of the fragment ions A depend not only on the stereochemical positions of the acyls, but also on the type of the two acyls in the ion A. Hence, the correct attribution of the acyl in the *sn*-2 position is not straightforward, especially for the TGs containing both saturated and unsaturated acyls. The unsaturated acyls prefer *sn*-2 position, especially the linoleoyl. The contents of TGs in the plant oil samples were estimated on the basis of their peak areas measured with the UV detection at 205 nm. In spite of neglecting the response factors, the data are in agreement with previous works.

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## References

[1] A. Stolyhwo, H. Colin, G. Guiochon, Anal. Chem. 57 (1985) 1342.
[2] A.J. Palmer, F.J. Palmer, J. Chromatogr. 465 (1989) 369.
[3] S. Héron, PhD thesis, Université Paris 6, Paris, 1992.
[4] W.W. Christie, J. Chromatogr. 454 (1988) 273.
[5] M.-B. Macer, A. Holmqvist, J. Sep. Sci. 24 (2001) 179.
[6] P. Sandra, A. Medvedovici, Y. Zhao, F. David, J. Chromatogr. A 974 (2002) 231.
[7] P. Février, A. Binet, L. Dufossé, R. Grée, F. Yvergnaux, J. Chromatogr. A 923 (2001) 53.
[8] R.O. Adolf, A. Menzel, V. Dorovska-Taran, J. Chromatogr. A 953 (2002) 293.
[9] K. Geeraert, D. DeSchepper, J. High Resolut. Chromatogr. 6 (1983) 123.
[10] M. Holčapek, P. Jandera, J. Fischer, B. Prokeš, J. Chromatogr. A 858 (1999) 13.
[11] K.V.V. Nurmela, L.T. Satama, J. Chromatogr. 435 (1988) 139.
[12] R.V. Flor, L.T. Hecking, B.D. Martin, J. Am. Oil Chem. Soc. 70 (1993) 199.
[13] W.C. Byrdwell, Lipids 36 (2001) 327.
[14] W.C. Byrdwell, E.A. Emken, W.E. Neff, R.O. Adlof, Lipids 31 (1996) 919.
[15] W.E. Neff, W.C. Byrdwell, K.R. Steidley, G.R. List, G. Snowder, J. Liq. Chromatogr. Relat. Technol. 25 (2002) 985.
[16] W.E. Neff, W.C. Byrdwell, G.R. List, J. Liq. Chrom. Relat. Technol. 24 (2001) 837.
[17] A. Jakab, K. Héberger, E. Forgács, J. Chromatogr. A 976 (2002) 255.

[18] W.E. Neff, W.C. Byrdwell, J. Chromatogr. A 818 (1998) 169.
[19] M. Holčapek, P. Jandera, J. Fischer, Crit. Rev. Anal. Chem. 31 (2001) 53.
[20] H.R. Mottram, S.E. Woodbury, R.P. Evershed, Rapid Commun. Mass Spectrom. 11 (1997) 1240.
[21] H.R. Mottram, R.P. Evershed, Tetrahedron Lett. 37 (1996) 8593.
[22] H.R. Mottram, Z.M. Crossman, R.P. Evershed, Analyst 126 (2001) 1018.
[23] O. Sjövall, A. Kuksis, H. Kallio, Lipids 37 (2002) 81.
[24] W.C. Byrdwell, W.E. Neff, Rapid Commun. Mass. Spectrom. 16 (2002) 300.
[25] P. Sandra, A. Dermaux, V. Ferraz, M.M. Dittmann, G. Rozing, J. Microcol. Sep. 9 (1997) 409.
[26] A. Jakab, K. Nagy, K. Héberger, E. Forgács, Rapid Commun. Mass. Spectrom. 16 (2002) 2291.
[27] G.R. Asbury, K. Al-Saad, W.F. Siems, R.M. Hannam, H.H. Hill, J. Am. Soc. Mass Spectrom. 10 (1999) 983.
[28] F.O. Ayorinde, E. Elhilo, C. Hlongwane, Rapid Commun. Mass. Spectrom. 13 (1999) 737.
[29] F.O. Ayorinde, B.E. Eribo, K.V. Balan, J.H. Johnson, L.W. Wan, Rapid Commun. Mass. Spectrom. 13 (1999) 937.
[30] M. Holčapek, H. Virelizier, J. Chamot-Rooke, P. Jandera, C. Moulin, Anal. Chem. 71 (1999) 2288.
[31] F.F. Hsu, J. Turk, J. Am. Soc. Mass Spectrom. 10 (1999) 587.
[32] J.T. Lin, C.L. Woodruff, T.A. McKeon, J. Chromatogr. A 782 (1997) 41.