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Quantitation of triacylglycerols in plant oils using HPLC with APCI-MS, evaporative light-scattering, and UV detection

The main constituents of plant oils are complex mixtures of TGs differing in acyl chain lengths, number and positions of double bonds, and regioisomerism. A non-aqueous reversed-phase HPLC method with acetonitrile–2-propanol gradient and 30+15 cm NovaPak C_{18} columns makes possible an unambiguous identification of the highest number of TGs ever reported for these oils, based on positive-ion APCI mass spectra. A new approach to TG quantitation is based on the use of response factors with three typical detection techniques for that purpose (APCI-MS, evaporative light-scattering detection, and UV at 205 nm). Response factors of 23 single-acid TGs (saturated TGs from C7 to C22, 7 unsaturated TGs), 4 mixed-acid TGs, diolein and monoolein are calculated from their calibration curves and related to OOO. Due to differences between saturated and unsaturated acyl chains, the use of response factors significantly improves the quantitation of TGs. 133 TGs containing 22 fatty acids with 8–25 carbon atoms and 0–3 double bonds are identified and quantified in 9 plant oils (walnut, hazelnut, cashew nut, almond, poppy seed, yellow melon, mango, fig, date) using HPLC/APCI-MS with a response factor approach. Average parameters and relative fatty acid concentrations are calculated with both HPLC/APCI-MS and GC/FID.

Key Words: Plant oil; Vegetable oil; Triacylglycerol; Quantitation; Response factor

Received: February 21, 2005; revised: June 3, 2005; accepted: June 16, 2005

DOI 10.1002/jssc.200500088

1 Introduction

Plant oils are complex mixtures of various compound classes, where the main constituents are triacylglycerols (TGs) consisting of saturated and unsaturated fatty acids (FAs), such as oleic (O), linoleic (L), linolenic (Ln), stearic (S), palmitic (P), *etc.*, differing in their acyl chain lengths and their stereochemical positions *sn*-1, 2, or 3 on the glycerol skeleton, and in the number and positions of the double bonds in the acyl chains. They may also differ in *cis*/*trans* configuration of double bonds and *R/S* optical isomerism of TGs with three different acyl chains. The standard notation of TGs employs the initials of the fatty acid trivial names arranged in the order of their positions on the glycerol skeleton. Mostly, *sn*-1 and *sn*-3 positions are not discriminated. Information about the distribution and type of FAs on the glycerol backbone is quite important for lipid digestion and metabolism, because FAs at the *sn*-1 and *sn*-3 positions are digested first by lipases yielding *sn*-2 monoacylglycerols and free FAs [1, 2].

Non-aqueous reversed-phase high-performance liquid chromatography (NARP HPLC) has been widely used for the separation of complex natural lipid samples [3–25]. 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 most TGs within the same ECNs group is also possible, for example the critical pair LLL/OLLn or the group of OOO, OOP, OPP, and PPP can be resolved [3–5]. The separation of TGs differing in the position(s) of double bond(s) is also feasible [6]. On the other hand, NARP HPLC is not suitable for the separation of three types of isomerism, *i.e.*, regioisomers, *R/S* isomers, and *cis*/*trans*

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Abbreviations: NARP HPLC, non-aqueous reversed-phase high-performance liquid chromatography; MS, mass spectrometry; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; ELSD, evaporative light-scattering detection; GC, gas chromatography; FID, flame ionization detection; TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol; FA, fatty acid; ECN, equivalent carbon number; CN, carbon number; DB, double bond; sn, stereochemical numbering; RF, response factor; Cy, caprylic acid; C, capric acid; La, lauric acid; M, myristic acid; Po, palmitoleic acid; P, palmitic acid; Mo, margaroleic acid; Ma, margaric acid; Ln, linolenic acid; L, linoleic acid; O, oleic acid; S, stearic acid; G, gadoleic acid; A, arachidic acid; B, behenic acid; Lg, lignoceric acid.

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isomers. Various mobile phase systems, mostly in gradient elution mode, are described in the literature, such as 2-propanol/acetonitrile [3, 7, 8], 2-propanol/acetonitrile/hexane [9, 25], acetone/acetonitrile [5, 10–13], acetonitrile/chloroform [14], 100% propionitrile [4], acetonitrile/dichlormethane [15–21], *etc.* The common feature of the mentioned separation systems is a low polarity of mobile phase components, because TGs are not soluble in water or common aqueous–organic mobile phases used in reversed-phase HPLC. An aqueous–organic step at the beginning of gradient may improve the chromatographic resolution of more polar acylglycerols (di- and monoacylglycerols) without sacrificing the resolution of TGs [9].

The alternative separation technique frequently employed in the lipid analysis is silver ion HPLC in normal-phase systems. The separation principle of this technique is based on the strong interactions between silver ions and π-electrons from the double bonds [26]. Ag^+ HPLC is very successful in the separation of lipids differing in the number [26, 27] and positions of double bonds [28] and *cis*/*trans* isomerism, too [29]. The retention increases in order of increasing number of double bonds, but the method has a low separation selectivity for lipids differing only in the saturated part of molecules, which are usually not separated [26–29] and the reproducibility is low. Numerous studies can be found in the literature on the separation of various lipid classes using silver ion HPLC [26–29 and citations therein]. The separation mechanism of silver ion chromatography is complementary to NARP HPLC, so that off-line or on-line coupling of these two separation modes should considerably improve the number of resolved compounds. Recent results on 2D separation of TGs in plant oils [12] are encouraging, but the number of identified TGs for a given plant oil is still lower than with a properly optimized NARP system. Further improvements in this area are likely in the near future.

In addition to NARP and silver ion HPLC, capillary electrochromatography [25], supercritical fluid chromatography [30–31], and subcritical fluid chromatography [32] have also been successfully applied for the separation of complex TG mixtures with similar chromatographic resolution to NARP or in the case of capillary electrochromatography even slightly better resolution for TGs with higher ECNs [25].

Among the detection techniques not providing structural information, evaporative light-scattering detection (ELSD) is the most widespread in TG analysis [33, 34], but the non-linear response of this detector is a clear disadvantage for the quantification. The other possibility is UV detection at very low wavelengths (*e.g.*, 205 nm) [3, 8, 9], which requires the use of HPLC gradient-grade solvents, but provides a linear response unlike ELSD. The blank gradient should be subtracted to avoid baseline drift during the gradient. Refractive-index detection is often used in routine analyses [35–37], but it cannot be applied for gradient analyses typically used for complex TG mixtures and nowadays is often replaced by ESLD or MS detection.

The coupling of HPLC and mass spectrometry (HPLC/MS) is a powerful tool in lipid analysis, because it provides both structural information and usually also the highest sensitivity among all available chromatographic detectors [9]. Atmospheric pressure chemical ionization (APCI) is the most frequently used ionization technique for TG analysis because of easy coupling to non-aqueous mobile phase systems used in NARP HPLC and high ionization efficiency for non-polar TG molecules. The presence of both protonated molecules $[M+H]^+$ and fragment ions $[M+H-R_iCOOH]^+$ is important for structure elucidation [3, 4, 7–14, 17–23]. Electrospray ionization (ESI) mass spectra exhibit $[M+Na]^+$ and $[M+K]^+$ ions instead of protonated molecules and also fragment ions, such as $[M+Na-R_iCOOH]^+$ and $[M+Na-R_iCOONa]^+$, but in a lower relative intensity [3, 21, 27]. The abundance of molecular adducts with alkali metal ions significantly depends on the salt content in the solution. Coupling with Ag^+ HPLC may require the use of post-column make-up flow of other polar solvent both for ESI and APCI, because typical mobile phases contain more than 98% of hexane [12, 29], which is not favorable for the ionization process. APCI mass spectra provide information on the predominant fatty acid in the *sn*-2 position. The precise ratio of regioisomers can also be obtained by the measurement of calibration curves with both positional isomers [11, 14]. In principle, the same approach is applicable with ESI.

The chromatographic quantitation of TGs is usually based only on the relative areas of chromatographic peaks neglecting potential differences in the relative responses of TGs differing in the number of double bonds and acyl chain lengths. The obvious advantage of such an approach is its simplicity, but it may lead to significant systematic errors in the determination of TG concentration. Due to the enormous number of TGs occurring in natural samples, the calibration curves can be constructed only for a small part of identified single-acid TGs. To our best knowledge, only few authors [17, 37] have attempted to quantify complex natural TG mixtures using a more sophisticated approach than normalized chromatographic peak areas or a limited number of TG standards. The first approach [17] is based on the measurement of calibration sets of SSS, OOO, LLL, LnLnLn, PPP, and PoPoPo with APCI-MS and the use of response factors calculated in different ways and related to deuterated d_{12}-PPP standard. The other approach [37] used for isocratic HPLC with refractive index detection relies on the use of RFs determined with authentic single-acid TG standards and related to OOO.

The main goal of our work to develop a method suitable for reliable quantitation of TGs in complex natural samples based on the comparison of three frequently used detection techniques for gradient elution HPLC of TGs (APCI-MS, ELSD and UV at 205 nm). First, the HPLC separation has to be carefully optimized to achieve the highest possible chromatographic resolution and to reduce the number of possible coelutions. Then, the RFs of authentic standards of single-acid TGs are determined by comparing their calibration curves. The model for the calculation of RFs for mixed-acid TGs with APCI-MS is proposed and applied for the determination of TG composition in 9 edible plant oils prepared in the laboratory – walnut, hazelnut, cashew nut, almond, poppy seed, yellow melon, mango, fig, and date plant oils. The quantitative results for TGs in some plant oils are compared with validated gas chromatography–flame ionization detection (GC/FID) determination of fatty acid methyl esters (FAMEs) obtained by transesterification of TGs.

2 Experimental

2.1 Materials

Acetonitrile, 2-propanol and hexane were purchased from Merck (Darmstadt, Germany). De-ionized water was prepared with a Demiwa 5-roi purification system (Watek, Ledeč nad Sázavou, Czech Republic). The solvents were filtered through a 0.45-μm Millipore filter and degassed by continuous stripping with helium. The standards of trimyristin (MMM, C14:0), tripalmitin (PPP, C16:0), tripalmitolein (PoPoPo, C16:1), trimargarin (MaMaMa, C17:0), triolein (OOO, C18:1), trilinolein (LLL, C18:2), α-trilinolenin (α-LnLnLn, C18:3) and the mixture of tri-, di-, and monoolein were purchased from Sigma-Aldrich (St. Louis, USA); tristearin (SSS, C18:0), γ-trilinolenin (γ-LnLnLn, C18:3), model mixtures of TG standards GLC#435 (all saturated single-acid TGs from C7 to C22) and GLC#406 (C16:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1 and C22:1), model mixture of FAMEs standards GLC#85 (C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C14:1, C15:0, C15:1, C16:0, C16:1, C17:0. C17:1, C18:0, C18:1, C18:1T, C18:2, C18:3, C18:3γ, C20:0, C20:1, C20:2, C22:0, C22:1, C20:3, C20:4, C22:2, C22:6 and C24:1), GLC#1A (C16:0, C18:0, C18:1, C18:2 and C18:3), and GLC#06A (C16:0, C18:0, C20:0, C22:0 and C24:0) were purchased from Nu-Chek-Prep (Elysian, USA).

2.2 Chromatographic and detection conditions

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). The final HPLC method for the analyses of all plant oils and the calculation of RFs used the following conditions: two chromatographic columns Nova-Pak C_{18} (300 × 3.9 and 150 × 3.9 mm, 4 μm, Waters) connected in series, flow rate 1 mL/min, injection volume 10 μL, column temperature 25°C, and mobile phase gradient with the steepness 0.65%/min: 0 min–100% acetonitrile, 106 min–31% acetonitrile–69% 2-propanol, 109 min–100% acetonitrile. 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 30 + 15 cm Nova-Pak columns. The UV detection at 205 nm and positive-ion APCI-MS connected in series were used in most experiments. All UV chromatograms were baseline subtracted using the analysis with a blank injection. The Esquire 3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany) was used in the mass range *m/z* 50–1200 with the following setting of tuning parameters: pressure of the nebulizing gas 70 psi, drying gas flow rate 3 L/min, the temperatures of the drying gas and APCI heater were 350°C and 400°C, respectively. For quantitative evaluation of all standards and samples, reconstructed ion current chromatograms in the region *m/z* 300–1200 were used. Individual reconstructed ion current chromatograms were used to support the identification of coeluting peaks. A Sedex 75 (Alfortville, France) was employed for determination of response factors with evaporative light-scattering detection (ELSD) (connected in series with UV detector) using a nebulizing temperature of 60°C; the flow rate and pressure of nitrogen were 10 L/min and 2.4 bar, respectively.

2.3 Sample preparation

10–15 g of each sample (walnut, hazelnut, cashew nut, almond, poppy seed, yellow melon seed, mango stone, fig stone, and date seed) was weighed and then carefully crushed in a mortar to fine particles, which were mixed with 15 mL of hexane, and the mixture was stirred occasionally for 15 min. The solid particles were filtered out using a coarse filter paper and then the extract was filtered again using a fine filter with 0.45-μm pores. From the filtered extract, hexane was evaporated overnight at room temperature yielding a pure plant oil. The oil samples were dissolved in an acetonitrile–2-propanol–hexane mixture (2:2:1, *v/v/v*) to prepare a 3% solution (*w/v*); 10 μL of this solution was injected for HPLC analysis.

2.4 Calibration curves and limits of detection

The stock solutions of unsaturated TGs (PoPoPo, OOO, LLL, and LnLnLn) at the concentration 3 g/L and of saturated TGs (MMM, PPP, MaMaMa, and SSS) at 0.15 g/L were dissolved in acetonitrile–2-propanol–hexane mixture (2:2:1, *v/v/v*). These solutions were diluted with the same solvent mixture yielding the working solutions at 5,

10, 50, 100, and 150 mg/L for saturated and 5, 10, 100, 300, and 500 mg/L for unsaturated TGs. For the standard mixture of tri-, di- and monoolein, the stock solution at 3.33 g/L in acetonitrile–2-propanol–hexane mixture (2:2:1, *v/v/v*) was diluted with the same solvent mixture for the calibration set of 50, 100, 300, and 500 mg/L. All calibration curves were measured using a 10 μL injection volume of working solutions in three repeated analyses with three detection techniques (APCI, ELSD, and UV at 205 nm), and the average peak areas were used for the construction of calibration curves. For reliable quantitation, concentrations of individual TGs in analyzed samples should not be higher than a verified linear range, because negative deviations from the linear calibration dependences in APCI-MS were observed for high concentrations in this and previous [17] work. Samples with TGs concentrations outside the linear calibration range must be diluted. The limits of detection (LOD) at $S/N = 3$ were determined with the injection volume 10 μL and averaged for particular saturation groups: APCI-MS – 2 mg/L for saturated, 3 mg/L for monounsaturated, 2 mg/L for diunsaturated, and 1 mg/L for triunsaturated; ELSD – 4 mg/L for saturated, 10 mg/L for monounsaturated, 14 mg/L for diunsaturated, and 15 mg/L for triunsaturated TGs; UV detection – 100 mg/L for saturated, 13 mg/L for monounsaturated, 4 mg/L for diunsaturated, and 2 mg/L for triunsaturated.

2.5 Preparation of fatty acid methyl esters and their gas chromatographic analysis with flame ionization detection

Fatty acid methyl esters (FAMEs) were prepared from TGs in plant oils using a standard procedure with sodium methoxide [38]. FAMEs mixtures were analysed by gas chromatography–flame ionization detection (GC/FID) on a Varian CP 3800 with a CP-8410 autosampler and a CP-1177 injector (Varian Analytical Instruments, Walnut Creek, CA, USA) using a BTR-Carbowax-30W-0.5F silica capillary column, 30 m length, 0.32 mm ID, 0.5 μm film thickness (Quadrex, Woodbridge, CT, USA). GC conditions were as follows: injection volume 1 μL, split ratio 1:40, flow rate of nitrogen as a carrier gas 0.7 mL/min, temperature program: initial temperature 160°C hold for 6 min, then ramp to 200°C at 20 K/min, hold for 10 min, ramp to 240°C at 5 K/min and hold for 20 min with a total analysis time of 46 min. Injector and detector temperatures were 250 and 270°C, respectively.

3 Results and discussion

3.1 Nomenclature and general conventions about TGs

Table 1 summarizes all fatty acids (FAs) identified in individual TGs together with their trivial names, abbreviations, carbon numbers (CN), double bond (DB) numbers, and equivalent carbon numbers (ECN). **Table 2** lists ECNs, molecular weights (MWs), RFs measured with APCI-MS, retention times t_R, and the relative retention r measured using the HPLC method with 45 cm total column length for 133 TGs identified in 9 plant oils consisting of 22 fatty acids. The masses and structures of fragment ions have been described in our previous work [3]. Plant oils usually contain a mixture of regioisomers. Three identical acyl chains on the glycerol backbone (single-acid $R_1R_1R_1$ type) provide only a single ion $[M+H-R_1COOH]^+$, while mixed-acid $R_1R_1R_2$ type produces two different $[M+H-R_1COOH]^+$ and $[M+H-R_2COOH]^+$ ions with a statistical abundance ratio of 1:2, and the $R_1R_2R_3$ type has three different $[M+H-R_1COOH]^+$, $[M+H-R_2COOH]^+$, and $[M+H-R_3COOH]^+$ ions with a statistical abundance ratio of 1:1:1. Neutral loss of R_iCOOH from the equivalent side positions *sn*-1 and *sn*-3 is preferred over cleavage from the middle position *sn*-2, which can be applied for determination of the acid predominant in the *sn*-2 position [3, 4, 8–11, 14]. The type (mainly unsaturation degree) may also influence the relative intensity of $[M+H-R_iCOOH]^+$ ions, so the published data [3, 11, 14, 21] on the ratios of authentic standards of selected regioisomers are taken into consideration for the empirical determination of *sn*-2 acyl chain. For precise determination of regioisomeric

Table 1. Systematic and trivial names of fatty acids found in TGs of studied plant oils listed with their abbreviations, carbon numbers (CN), double bond (DB) numbers, and equivalent carbon numbers (ECN).

Systematic name	Trivial name	Abbreviation	CN:DB	ECN
Octanoic	Caprylic	Cy	C8:0	8
Decanoic	Capric	C	C10:0	10
Dodecanoic	Lauric	La	C12:0	12
Tetradecanoic	Myristic	M	C14:0	14
Pentadecanoic	–	–	C15:0	15
Hexadecanoic	Palmitic	P	C16:0	16
cis-9-Hexadecenoic	Palmitoleic	Po	C16:1	14
Heptadecanoic	Margaric	Ma	C17:0	17
cis-10-Heptadecenoic	Margaroleic	Mo	C17:1	15
Octadecanoic	Stearic	S	C18:0	18
cis-9-Octadecenoic	Oleic	O	C18:1	16
cis-9,12-Octadecadienoic	Linoleic	L	C18:2	14
cis-9,12,15-Octadecatrienoic	Linolenic	Ln	C18:3	12
Nonadecanoic	–	–	C19:0	19
Eicosanoic	Arachidic	A	C20:0	20
cis-11-Eicosenoic	Gadoleic	G	C20:1	18
cis-11,14-Eicosadienoic	–	–	C20:2	16
Heneicosanoic	–	–	C21:0	21
Docosanoic	Behenic	B	C22:0	22
Tricosanoic	–	–	C23:0	23
Tetracosanoic	Lignoceric	Lg	C24:0	24
Pentacosanoic	–	–	C25:0	25

Table 2. Triacylglycerols (TG) identified in studied plant oils listed with their equivalent carbon numbers (ECN), molecular weights (MW), retention times t_R, relative retention *r*, and response factors (RF) determined with APCI-MS.

TG	ECN	MW[a)]	t_R	*r*[b)]	*RF*
LnLnLn	36	872	48.3	0.800	0.40
LnLLn	38	874	54.0	0.901	0.46
LaLLa		718	54.8	0.915	4.22
LaOC		692	55.3	0.924	8.22
MOCy		692	55.9	0.934	26.07
MLaLa		666	56.6	0.947	4.95
LnLnMo	39	862	57.7	0.966	0.54
LnLnC15:0		836	58.7	0.984	0.85
LLLn	**40**	**876**	**59.6**	**1.000**	**0.51**
LLLa		798	60.3	1.012	2.39
LnOLn		876	60.6	1.018	0.60
OOCy		746	61.4	1.032	25.48
MLLa		746	61.5	1.034	3.13
LaOLa		720	61.8	1.039	4.36
LnLnP		850	62.1	1.044	0.71
POCy		720	63.0	1.060	25.59
PLaLa		694	63.8	1.074	4.47
MMLa		694	63.8	1.074	3.86
LnLMo	41	864	63.3	1.066	0.59
LLL	**42**	**878**	**65.3**	**1.000**	**0.57**
LLPo		852	65.7	1.006	0.82
OLLn		878	66.4	1.018	0.66
LLM		826	66.7	1.023	1.30
OLLa		800	67.0	1.027	2.54
OOC		774	67.6	1.037	6.54
LnLP		852	67.8	1.040	0.76
MLM		774	68.2	1.047	2.04
PLLa		774	68.3	1.048	2.64
SLnLn		878	68.5	1.052	0.47
MOLa		748	68.5	1.052	3.27
SOCy		748	69.9	1.074	25.35
PMLa		722	70.7	1.087	3.38
LLMo	43	866	69.0	1.060	0.65
LLC15:0		840	70.3	1.081	0.96
LnLMa		866	70.7	1.087	0.59
C20:2LL	44	906	70.8	0.985	0.50
OLL		**880**	**71.8**	**1.000**	**0.71**
OLPo		854	72.2	1.006	0.97
OLnO		880	72.6	1.012	0.80
LLP		854	73.1	1.019	0.82
OLM		828	73.7	1.028	1.45
SLLn		880	73.8	1.029	0.53
LnOP		854	74.0	1.032	0.91
OOLa		802	74.1	1.034	2.68
ALnLn		906	74.3	1.036	0.40
PLM		802	75.1	1.048	1.55
SLLa		802	75.2	1.050	2.41
MOM		776	75.5	1.054	2.18
POLa		776	75.6	1.055	2.79
PLnP		828	75.7	1.057	1.01
OLMo	45	868	75.6	1.055	0.79
LLMa		868	76.3	1.066	0.65
MoLP		842	76.4	1.067	0.90
OLnMa		868	77.0	1.076	0.74
GLL	46	908	77.2	0.991	0.50
OLO		**882**	**77.9**	**1.000**	**0.86**
OOPo		856	78.3	1.005	1.11
SLL		882	79.0	1.015	0.58
OLP		856	79.3	1.019	0.96
GOLa		830	79.4	1.020	2.47
ALLn		908	79.6	1.023	0.46
OOM		830	79.7	1.024	1.59
POPo		830	79.8	1.025	1.22
SOLn		882	80.0	1.028	0.67
BLnLn		934	80.1	1.029	0.42
PLP		830	80.9	1.040	1.07
SLM		830	80.9	1.040	1.32
PPoP		804	81.3	1.046	1.32
POM		804	81.3	1.046	1.70
SOLa		804	81.3	1.046	2.55
SLnP		856	81.4	1.047	0.78
OOMo	47	870	81.5	1.048	0.94
OLMa		870	82.3	1.059	0.79
C21:0LLn		920	82.3	1.059	0.45
MoOP		844	82.7	1.064	1.04
C23:0LnLn		948	82.9	1.067	0.40
GLO	48	910	83.1	0.989	0.64
OOO		**884**	**84.0**	**1.000**	**1.00**
ALL		910	84.8	1.010	0.51
GOM		858	85.0	1.012	1.38
BLLn		936	85.1	1.014	0.48
SLO		884	85.1	1.014	0.73
OOP		858	85.4	1.017	1.11
SLP		858	86.6	1.032	0.83
BLLa		858	86.6	1.032	2.36
SLnS		884	86.9	1.036	0.54
AOLa		832	87.0	1.037	2.48
POP		832	87.0	1.037	1.21
SOM		832	87.0	1.037	1.46
PPP		806	88.7	1.058	1.32
C23:0LLn	49	950	87.8	1.047	0.46
OOMa		872	88.4	1.054	0.94
MaOP		846	89.7	1.071	1.04
GOO	50	912	89.0	0.979	0.79
GLS		912	89.9	0.990	0.51
BLL		938	90.0	0.991	0.53
LgLLn		964	90.2	0.993	0.46
ALO		912	90.4	0.995	0.66
GOP		886	90.4	0.995	0.89

Table 2. Continued ...

SOO		**886**	**90.8**	**1.000**	**0.87**
ALP		886	91.8	1.011	0.76
SLS		886	91.9	1.013	0.60
SOP		860	92.3	1.017	0.98
AOM		860	92.3	1.017	1.39
BOLa		860	92.3	1.017	2.50
SPP		834	94.4	1.041	1.08
C23:0OLa	51	874	94.7	1.045	2.48
SOMa		874	95.0	1.048	0.81
LgLL	52	966	94.9	0.988	0.51
BLO		940	95.5	0.995	0.68
GOS		914	95.7	0.997	0.66
AOO		**914**	**96.0**	**1.000**	**0.80**
LgLM		914	96.7	1.008	1.25
BLP		914	96.8	1.009	0.78
ALS		914	96.9	1.010	0.53
LgOLa		888	97.1	1.012	2.48
AOP		888	97.5	1.016	0.91
SOS		888	97.6	1.017	0.74
SSP		862	99.7	1.040	0.85
C19:0OS	53	902	100.2	1.045	0.70
LgLO	54	968	100.5	1.048	0.66
BOO		942	101.0	1.054	0.82
LgLP		942	101.9	1.064	0.76
BLS		942	102.0	1.065	0.55
AOS		916	102.6	1.071	0.67
SSS		890	104.6	1.093	0.61
C23:0OO	55	956	103.3	1.079	0.80
LgOO	56	970	105.5	1.102	0.80
LgLS		970	106.5	1.113	0.53
LgOP		944	106.9	1.117	0.91
BOS		944	107.0	1.119	0.69
C25:0OO	57	984	107.7	1.126	0.80
C23:0OS		958	109.2	1.142	0.67

[a] For better clarity, the decimal places are neglected in this table.

[b] Relative retention $r = (t_R - t_M)/(t_S - t_M)$, where t_M is 3.20 min and t_S are retention times of standards for particular ECN groups (printed in bold), *i.e.*, LLLn for ECN = 41 and lower, 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, AOO for 52 and higher.

ratios, the accurate determination of calibration curves for both regioisomeric standards is essential [11, 14]. The *sn*-2 acids in TGs are denoted according to the prevailing acids identified in the studied plant oils, *e.g.*, OLP corresponds to linoleoyl prevailing in the *sn*-2 position. TGs marked with asterisk (*e.g.*, OLP*) signify that the determination of middle *sn*-2 acid is not unambiguous due to: 1) the coelution with other TG with the same masses of $[M+H-R_iCOOH]^+$ ions, 2) the concentrations of regioisomers being similar, 3) the concentration of TG being so low that *sn*-2 acid cannot be determined clearly. The positions *sn*-1 and *sn*-3 are considered as equivalent, because these regioisomers cannot be distinguished by NARP HPLC or mass spectrometry, so acids in *sn*-1 and *sn*-3 positions are ordered by decreasing mass, *i.e.*, SLO (not OLS). Identified TGs are sorted into three groups: major TGs (>5 weight% in a particular oil), minor TGs (>0.5% and <5%), and trace TGs (<0.5%), which is found useful for easier comparison and discussion of results. These conventions are used through the whole paper including figures and tables.

3.2 Quantitation using the response factor approach

Natural TG (or lipid in general) samples contain very complex mixtures, but commercial standards are available only for a limited range of TGs, mostly single-acid type $R_1R_1R_1$. Moreover, less common TGs are expensive and polyunsaturated TGs are prone to oxidation, hence quantitation based on the calibration curves for all TGs is practically impossible even if only major TGs are taken into account. For this reason, a suitable approach for the quantitation of complex TG mixtures with a limited range of authentic TG standards (mainly single-acid type) is sought in this work. TGs with different number and positions of double bonds, and lengths of acyl chains, differ in the relative responses with common HPLC detection techniques used for TGs (APCI-MS, ELSD, and UV at 205 nm). This leads to systematic errors in the quantitation based on the relative peak areas. The main goal of our work is to improve the accuracy and precision of TG quantitation using appropriate RFs. For this purpose, the calibration curves of 23 single-acid TGs were measured **(Table 3**) and the RFs of mixed-acid TGs calculated. The RF of OOO, as one of the most widespread natural TGs, is set to RF = 1.00 for all detection techniques and other RFs are expressed relative to this standard value. The use of calibration curves with 5 calibration points should provide better precision than the RFs based on a single point only. For detection techniques providing linear concentration responses (*e.g.*, APCI-MS and UV), the ratio of calibration slopes a(OOO)/a(TG) is used for the calculation of RFs of individual TGs. For detection techniques with non-linear detector response (*e.g.*, ELSD), the ratios of y values y(OOO)/y(TG) at different concentration levels have to be used instead of calibration slopes. The ratios of y values decrease slightly with increasing concentration (**Table 4**), but RFs are relatively stable within a limited concentration range (50–500 mg/L) with the relative standard deviations always lower than 3.2%. RFs should not be used for concentrations outside this range because of non-linear dependence. This is, of course, a serious draw-

Table 3. Response factors (RF) of 23 single-acid TG standards, 4 mixed-acid TG standards and representatives of diacylglycerols (diolein, OO) and monoacylglycerols (monoolein, O) determined with APCI-MS, ELSD and UV detection at 205 nm[a].

TG (CN : DB)	APCI				ELSD					UV			
	a	*b*	r^2	RF	*a*	*b*	*c*	r^2	RF	*a*	*b*	r^2	RF
C7 : 0	0.229	−0.001	0.995	97.20	1.692	0.038	0.006	1.000	0.32	0.223	−0.001	1.000	7.57
C8 : 0	0.299	0.004	0.992	74.44	1.235	0.281	0.002	1.000	0.44	0.217	0.001	1.000	7.77
C9 : 0	0.572	0.004	0.998	38.91	1.118	0.776	−0.010	0.998	0.48	0.211	0.004	0.997	8.00
C10 : 0	1.263	−0.006	0.991	17.62	0.980	0.951	−0.005	0.999	0.55	0.230	0.009	0.991	7.33
C11 : 0	2.052	0.001	0.995	10.85	1.493	1.289	−0.021	1.000	0.36	0.215	0.006	0.991	7.85
C12 : 0	3.684	−0.007	0.999	6.04	1.990	1.936	−0.039	1.000	0.27	0.243	−0.002	0.994	6.94
C13 : 0	5.166	−0.010	0.998	4.31	3.569	2.032	0.040	1.000	0.15	0.208	0.003	1.000	8.11
C14 : 0	8.033	0.010	0.999	2.77	7.835	1.141	−0.001	0.999	0.07	0.237	0.001	1.000	7.12
C15 : 0	12.700	0.024	0.999	1.75	8.091	1.705	0.013	1.000	0.07	0.227	0.002	0.999	7.43
C16 : 0	16.904	0.303	0.994	1.32	12.778	1.948	−0.009	1.000	0.04	0.241	0.001	0.997	7.00
C16 : 1	16.749	0.715	0.991	1.33	0.447	2.004	−0.119	0.993	1.16	2.132	−0.002	0.999	0.79
C17 : 0	27.590	−0.082	0.995	0.81	14.665	2.697	−0.028	1.000	0.04	0.241	−0.003	0.997	7.00
C18 : 0	36.451	−0.539	0.998	0.61	20.143	2.342	−0.053	1.000	0.03	0.261	−0.004	0.991	6.46
C18 : 1	**22.258**	**1.540**	**0.991**	**1.00**	**0.526**	**1.600**	**0.023**	**1.000**	**1.00**	**1.687**	**0.001**	**0.999**	**1.00**
C18 : 2	39.268	1.563	0.992	0.57	1.084	1.119	−0.075	0.992	0.49	10.512	0.058	0.999	0.16
αC18 : 3	55.618	2.733	0.993	0.40	1.853	0.800	0.008	0.997	0.29	22.788	0.482	0.995	0.07
γC18 : 3	76.808	2.212	0.993	0.29	1.670	1.134	−0.052	0.994	0.32	35.709	0.416	0.996	0.05
C19 : 0	45.635	−0.883	0.970	0.49	23.896	2.602	−0.085	0.999	0.02	0.287	−0.005	1.000	5.88
C20 : 0	56.277	−1.512	0.974	0.40	37.033	1.600	−0.086	0.999	0.01	0.303	−0.002	1.000	5.57
C20 : 1	62.552	0.012	1.000	0.36	4.637	2.038	0.004	1.000	0.12	1.960	−0.008	0.998	0.86
C21 : 0	56.605	−1.986	0.958	0.39	25.331	3.418	−0.149	0.997	0.02	0.322	−0.003	1.000	5.24
C22 : 0	48.118	−1.695	0.958	0.46	7.698	1.871	−0.079	0.995	0.07	0.278	−0.002	1.000	6.07
C22 : 1	52.990	0.059	0.999	0.42	7.018	1.284	0.006	1.000	0.08	1.394	0.003	0.996	1.21
OPP	19.697	0.232	0.997	1.13	–	–	–	–	–	0.912	−0.002	0.997	1.85
POP	20.230	0.263	0.994	1.10	–	–	–	–	–	1.163	−0.010	1.000	1.45
OPO	21.198	0.173	0.996	1.05	–	–	–	–	–	1.268	−0.009	0.995	1.33
OOP	21.822	0.274	0.996	1.02	–	–	–	–	–	1.638	−0.006	1.000	1.03
OO	9.676	−0.035	1.000	2.30	0.483	0.972	-0.039	0.999	1.10	2.270	0.006	1.000	0.74
O	1.655	−0.354	1.000	13.45	0.225	0.180	-0.023	0.996	2.39	3.061	0.035	1.000	0.55

[a] RFs are expressed relative to OOO, which is set to 1.00 for all detection techniques. For APCI-MS and UV detection, *a* and *b* values are coefficients of the linear calibration dependence $y = a \cdot x + b$ and RFs are calculated as $RF(TG) = a_{OOO}/a_{TG}$, because *b* values can be neglected. For ELSD, *a*, *b*, and *c* values are coefficients of the quadratic calibration dependences $y = a \cdot x^2 + b \cdot x + c$ and RFs are calculated as the arithmetic mean of y_{OOO}/y_{TG} ratios calculated at 50, 100, 200, and 500 mg/L for OOO and individual TGs. r^2 is the value of coefficient of determination, *y* corresponds to the peak areas and *x* is the concentration in g/L.

back for reliable quantitation of lipid samples. Moreover, the differences among RFs are as high as two orders of magnitude. The non-linear response of ELSD and large differences among individual TGs do not allow us to find a suitable model for the calculation of RFs of mixed-acid TGs. It has been found during our measurements that various parameters (nebulizing gas flow rate and temperature, detector type, mobile phase composition) may have a notable effect on RFs, which causes low method robustness.

The responses of saturated TGs with UV detection at low wavelengths are very low. Unfortunately, LODs of the order of 10^2 mg/L are not sufficient in practice, which basically disqualifies the applicability of the UV detector for the quantitation of natural samples containing saturated TGs. Moreover, considerable differences are observed among RFs of saturated (5.24–8.11), monounsaturated (0.79–1.21), and polyunsaturated (0.05–0.16) TGs. When only one double bond is introduced into a saturated TG molecule, the response is notably increased, for example RF(PPP) = 7.00, RF(OPP) = 1.85, and RF(POP) = 1.45. If the RFs of mixed-acid TGs are calculated as an arithmetic mean of individual FA contributions, then the calculated value is completely misleading, for example RF(OPP or POP) = (2 × RF(PPP) + RF(OOO))/3 = 5.00.

Table 4. Calculation and statistical evaluation of response factors for triacylglycerols and diacylglycerol diolein (OO) and monoacylglycerol monoolein (O) using ELSD.

TG (CN:DB)	Response factors						
	Concentration level [mg/L]				Arithmetic mean	Standard deviation	Relative standard deviation [%]
	50	100	200	500			
C7:0	0.330	0.320	0.316	0.313	0.32	0.006	2.0
C8:0	0.450	0.438	0.432	0.428	0.44	0.008	1.9
C9:0	0.492	0.481	0.476	0.473	0.48	0.007	1.5
C10:0	0.559	0.548	0.542	0.539	0.55	0.008	1.4
C11:0	0.367	0.360	0.356	0.354	0.36	0.005	1.4
C12:0	0.275	0.270	0.267	0.265	0.27	0.004	1.4
C13:0	0.155	0.151	0.149	0.148	0.15	0.003	1.8
C14:0	0.071	0.069	0.068	0.068	0.07	0.001	1.7
C15:0	0.069	0.067	0.066	0.065	0.07	0.001	2.1
C16:0	0.044	0.042	0.042	0.041	0.04	0.001	2.7
C16:1	1.146	1.161	1.168	1.173	1.16	0.010	0.9
C17:0	0.038	0.037	0.036	0.036	0.04	0.0008	2.1
C18:0	0.028	0.027	0.026	0.026	0.03	0.0008	2.8
C18:1	**1.000**	**1.000**	**1.000**	**1.000**	**1.00**	**–**	**–**
C18:2	0.504	0.495	0.490	0.487	0.49	0.006	1.3
α-C18:3	0.299	0.291	0.288	0.285	0.29	0.005	1.8
γ-C18:3	0.330	0.322	0.319	0.316	0.32	0.005	1.6
C19:0	0.023	0.023	0.022	0.022	0.02	0.0005	2.5
C20:0	0.015	0.015	0.014	0.014	0.015	0.0005	3.2
C21:0	0.022	0.021	0.021	0.021	0.02	0.0004	2.2
C20:1	0.119	0.116	0.115	0.114	0.12	0.002	1.8
C22:0	0.072	0.070	0.069	0.069	0.07	0.001	1.7
C22:1	0.079	0.077	0.076	0.075	0.08	0.001	1.8
OO	1.111	1.100	1.095	1.091	1.10	0.007	0.7
O	2.441	2.390	2.364	2.348	2.39	0.035	1.5

Therefore, simple averaging is not suitable for that purpose and some weighting factors have to be introduced to balance the different contributions from saturated and unsaturated acyl chains. Such a model would be very laborious and due to the insufficient sensitivity for saturated TGs, this approach was abandoned as meaningless and our attention was focused on APCI-MS.

The responses with APCI-MS are linear, the sensitivity is sufficient for all TGs regardless of the degree of unsaturation and the differences among individual TGs usually found in natural plant oils are much lower compared to the other two detection techniques (Table 3). When only common acyl chain lengths (C14–C22) are considered, then all RFs are within the approximate range of 0.4–2.8. For shorter FAs, the increase of RFs is observed, but these FAs are not found in common plant oils (or at maximum at trace levels) except in date seed oil. **Figure 1** illustrates a dependence of RFs of saturated TGs on carbon number with the inset showing details of unsaturated TGs. In the case of APCI-MS, the arithmetic mean is applicable for the calculation of RFs of mixed-acid TGs (for FAs C14

Table 5. Concentrations [mg/g] of 133 triacylglycerols (TG) identified in 9 plant oil samples using the APCI-MS detection and response factor approach.

TG	Walnut	Hazelnut	Cashew	Almond	Poppy seed	Yellow melon	Mango stone	Fig seed	Date seed
LnLnLn	6							57	
LnLLn	35							77	
LaLLa									36
LaOC									17
MOCy									48
MLaLa									9
LnLnMo								<0.1	
LnLnC15:0								<0.1	
LLLn	97				17		<0.1	60	
LLLa									15
LnOLn	13							82	
OOCy									39
MLLa									38
LaOLa									121
LnLnP	11							62	
POCy									28
PLaLa									5
MMLa									4
LnLMo					1			0.1	
LLL	135	15	17	24	203	115	1	29	1
LLPo		0.3	1						
OLLn	68	2			9	0.3	0.4	101	
LLM									6
OLLa									37
OOC									16
LnLP	53				8	2	0.3	72	
MLM									7
PLLa									25
SLnLn	2							21	
MOLa									92
SOCy									14
PMLa									5
LLMo	1			0.3	1				
LLC15:0						1			
LnLMa	<0.1							<0.1	
C20:2LL	<0.1				0.2			<0.1	

Table 5. Continued ...

OLL	111	58	55	103	137	133	7	55	3
OLPo		0.4	0.4						
OLnO	18	2	1				1	43	
LLP	85	15	31	30	153	116	2	38	0.3
OLM									12
SLLn	12							20	
LnOP	14		1				2	35	
OOLa									110
ALnLn								2	
PLM									4
SLLa									15
MOM									39
POLa									47
PLnP	1				< 0.1		< 0.1	1	
OLMo		<0.1	<0.1	1	<0.1				
LLMa	0.4		<0.1	<0.1	1	2		<0.1	
MoLP				<0.1					
OLnMa								<0.1	
GLL	2		<0.1		1	1		0.1	
OLO	52	133	93	149	59	64	17	32	7
OOPo		2	1						
SLL	16		26		26	39	3	8	
OLP	33	53	39	88	58	56	8	16	2
GOLa									18
ALLn								4	
OOM									35
POPo		0.2	0.1						
SOLn	3						2	5	
BLnLn								3	
PLP	4	2	13	4	7	8	1	0.3	0.2
SLM									3
PPoP			<0.1						
POM									25
SOLa									22
SLnP	<0.1						1	0.1	
OOMo		2		1					
OLMa	<0.1	<0.1	0.3	0.4	<0.1	<0.1		<0.1	
C21:0LLn								<0.1	

Table 5. Continued ...

MoOP		0.3	<0.1	1					
C23:0LnLn								<0.1	
GLO	0.1	<0.1	0.1		<0.1	0.3	<0.1	<0.1	<0.1
OOO	18	243	113	213	13	19	29	12	13
ALL	4				2	4		0.1	
GOM									2
BLLn								1	
SLO	11	9	46	10	11	22	16	3	4
OOP	9	141	85	86	10	13	28	9	17
SLP	2	3	12	<0.1	2	6	9	<0.1	<0.1
BLLa									2
SLnS							2	<0.1	
AOLa									6
POP	1	13	31	3	2	1	13	1	6
SOM									10
PPP						<0.1	<0.1		
C23:0LLn								<0.1	
OOMa		2	1	0.3		<0.1	0.4		<0.1
MaOP			<0.1						
GOO	<0.1	3	2	0.3	<0.1	<0.1	0.4	<0.1	1
GLS	<0.1								
BLL			0.2		<0.1	<0.1		<0.1	
LgLLn								<0.1	
ALO	<0.1	1		<0.1	<0.1	1	0.1	<0.1	0.3
GOP							1		1
SOO	3	41	81	19	2	6	81	2	7
ALP	<0.1				<0.1	<0.1		<0.1	<0.1
SLS	<0.1		5		<0.1	2	12	<0.1	<0.1
SOP	<0.1	9	38	1	1	<0.1	74	<0.1	2
AOM									3
BOLa									8
SPP			<0.1			<0.1	<0.1		
C23:0OLa									<0.1
SOMa							1		<0.1
LgLL					<0.1	<0.1			
BLO			1			<0.1	<0.1		0.2
GOS							1		
AOO	<0.1	2	8	<0.1		<0.1	8	<0.1	2

Table 5. Continued ...

LgLM									<0.1
BLP						<0.1			<0.1
ALS			0.3			<0.1	0.1		<0.1
LgOLa									1
AOP		<0.1	1			<0.1	1		1
SOS		1	19			<0.1	143		0.2
SSP							1		
C19:0OS							<0.1		
LgLO							0.1		
BOO							2		
LgLP							<0.1		
BLS							<0.1		
AOS							18		
SSS							2		
C23:0OO							<0.1		
LgOO							2		
LgLS							<0.1		
LgOP							2		
BOS							4		
C25:0OO							<0.1		
C23:0OS							<0.1		

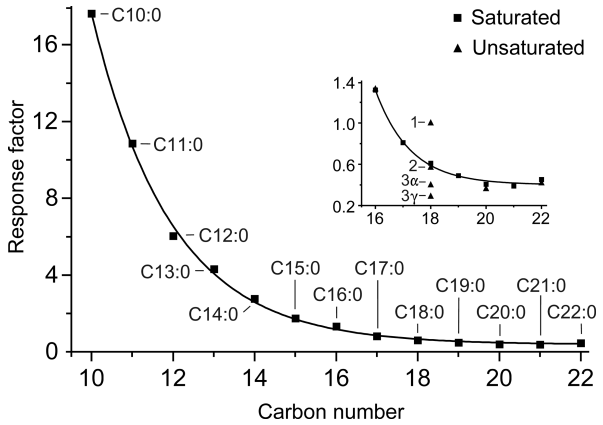

Figure 1. Dependence of response factors of saturated single-acid TGs measured by APCI-MS on the carbon number in the acyl chain (fitted with the equation $y = 2925 \times \exp(-0.5134x) + 0.3824$, $R^2 = 0.999$). Unsaturated single-acid TGs are shown in inset detail.

and higher), for example the calculated value for RF(OOP or OPO) = 1.11 *vs.* experimental values RF(OOP) = 1.02 and RF(OPO) = 1.05. The approach is validated by comparison with the standard GC/FID method (discussed in Section 3.3). RFs of 133 TGs occurring in studied plant oils are calculated this way (listed in Table 2) and the individual peak areas are multiplied by the corresponding RF yielding the real concentration of TG in a given plant oil. Each oil is analysed in triplicate, then the concentrations are averaged, and final values for 9 studied oils are shown in **Table 5**.

The calculation of RFs of mixed-acid TGs neglects the differences – if any – between the regioisomers. Based on our measurements of OPO/OOP and OPP/POP regioisomers, these differences are low with UV detection and nearly negligible with APCI-MS (Table 3). Anyway, distinguishing RFs for both regioisomers would not be beneficial in practice, because the natural lipid sources typically contain both regioisomers in a certain ratio (not identical for all plant oils and animal fats) and regioisomers coelute in all NARP HPLC systems.

3.3 Comparison of HPLC/APCI-MS and GC/FID results of plant oils

APCI-MS is used for the determination of TG relative concentrations in 9 plant oils (Table 5): walnut oil (**Fig. 2.a**),

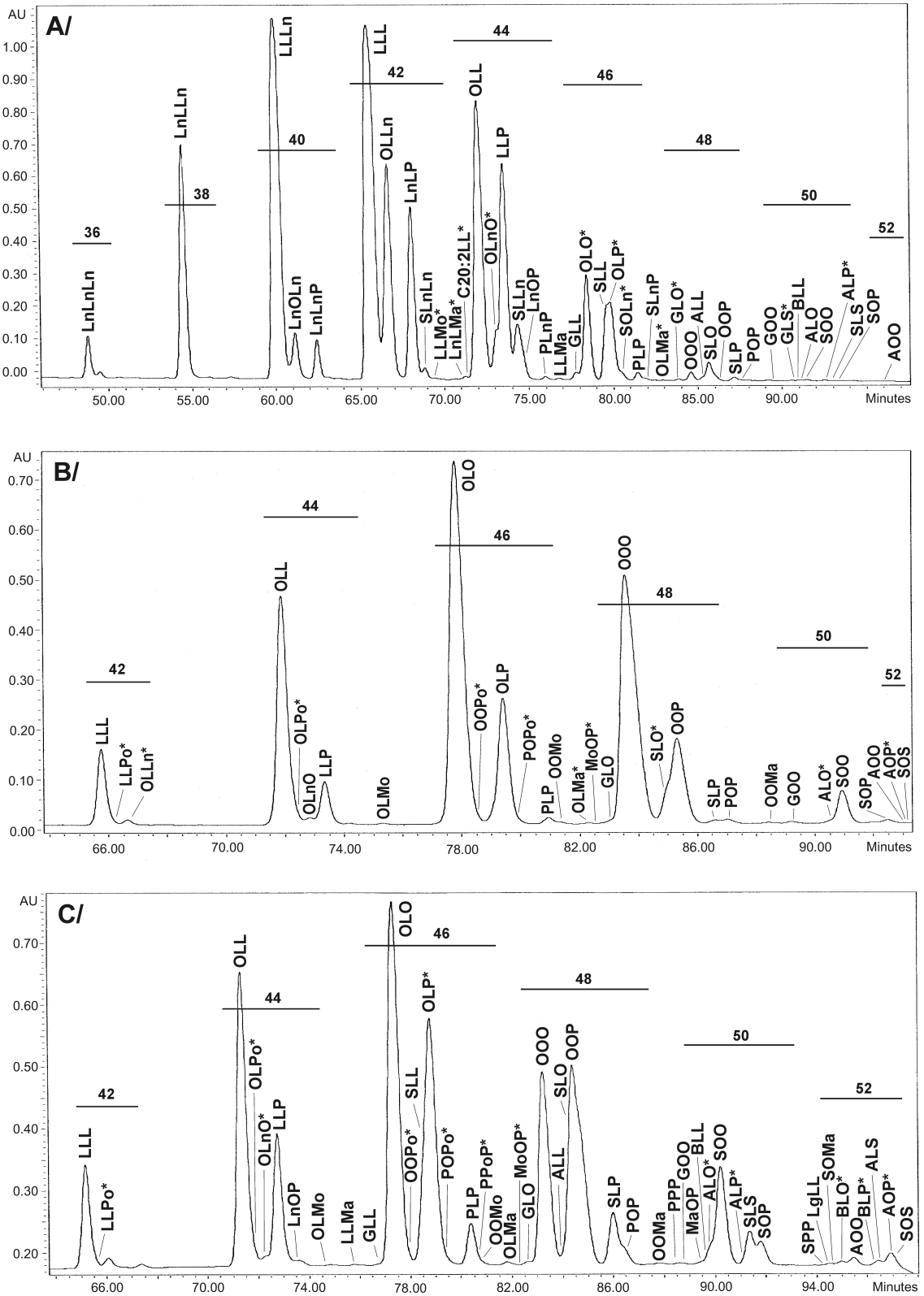

Figure 2. Chromatographic separation of plant oils: A) walnut; B) hazelnut; C) cashew nut. Experimental conditions: 30 + 15 cm Nova-Pak columns connected in series, UV detection at 205 nm, flow rate 1 mL/min, column temperature 25°C, injection volume 10 μL, mobile phase gradient 0 min–100% acetonitrile, 106 min–31% acetonitrile–69% 2-propanol, 109 min–100% acetonitrile. Numbers correspond to ECNs.

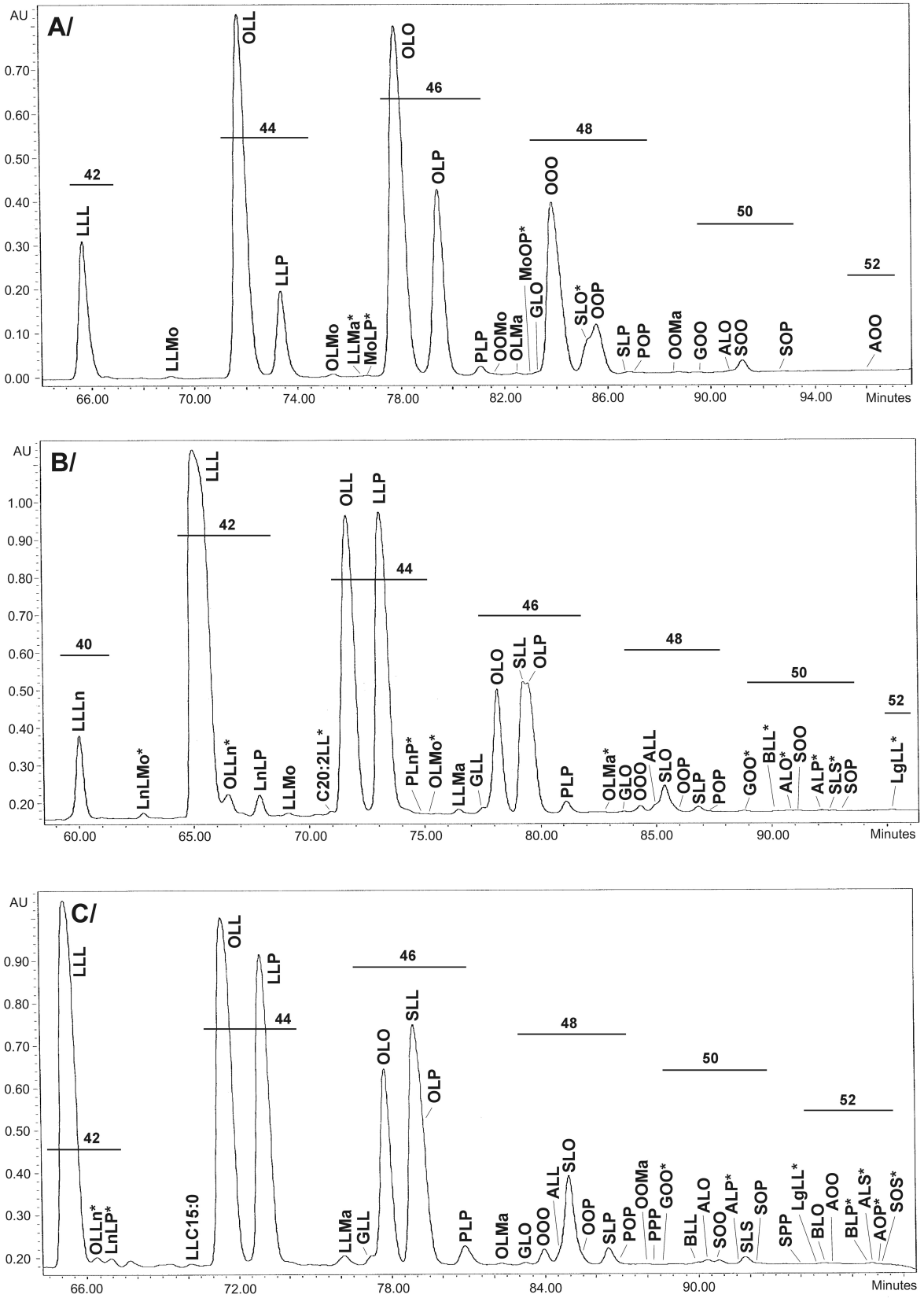

Figure 3. Chromatographic separation of plant oils: A) almond; B) poppy seed; C) yellow melon. All conditions are identical to those for **Figure 2**.

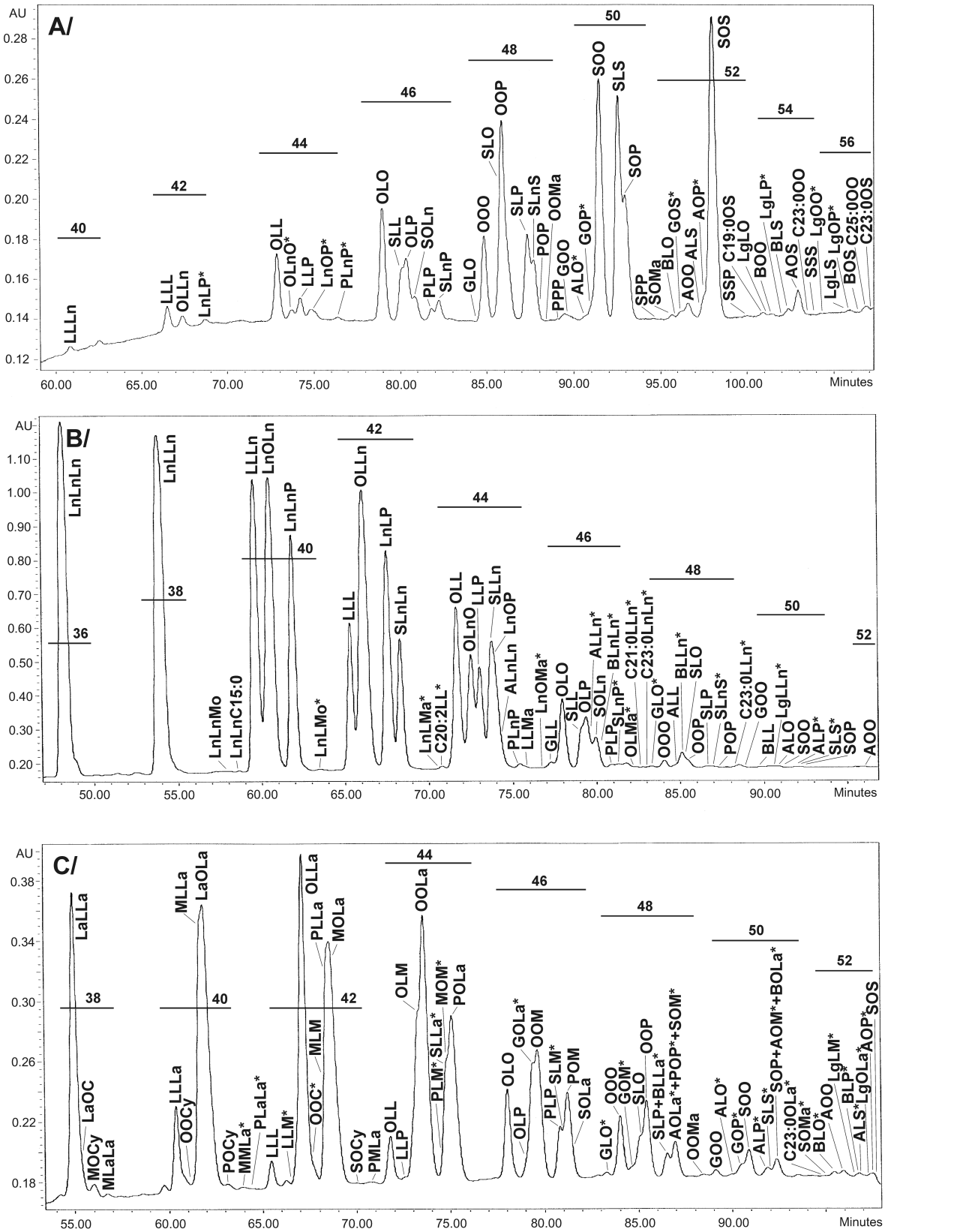

Figure 4. Chromatographic separation of plant oils: A) mango stone; B) fig seed; C) date seed. All conditions are identical to those for **Figure 2**.

Table 6. Comparison of relative concentrations [weight%] of individual FAMEs calculated from GC/FID[a] and from HPLC/APCI-MS of TGs[b].

Fatty acid	Walnut		Hazelnut		Cashew		Almond		Poppy seed		Yellow melon		Fig seed	
	GC	LC	GC	LC	GC	LC	GC	LC	GC	LC	GC	LC	GC	LC
C14:0	< 0.05	–	< 0.05	–	< 0.05	–	0.05	–	< 0.05	–	0.06	–	< 0.05	–
C15:0	< 0.05	–	< 0.05	–	–	–	< 0.05	–	< 0.05	–	< 0.05	0.05	< 0.05	–
C16:0	6.81	8.42	7.05	10.50	11.97	12.94	6.70	9.44	8.62	10.88	8.76	10.88	6.89	8.75
C16:1	0.08	–	0.26	0.08	0.36	0.09	0.56	–	0.12	–	0.08	–	0.07	–
C17:0	< 0.05	< 0.05	0.05	0.09	0.11	0.05	0.05	< 0.05	0.06	0.05	0.08	0.05	0.05	< 0.05
C17:1	< 0.05	< 0.05	< 0.05	0.09	< 0.05	< 0.05	< 0.05	0.13	< 0.05	0.09	–	–	< 0.05	< 0.05
C18:0	1.74	2.01	2.45	2.87	11.69	11.73	1.23	1.37	1.86	1.95	5.22	1.95	2.61	2.34
C18:1	17.28	19.33	77.40	63.39	57.02	51.92	67.35	62.09	14.86	18.53	22.88	18.53	19.23	20.00
C18:2	60.58	53.40	12.17	17.53	17.06	22.58	23.56	26.97	73.25	66.73	61.55	66.73	29.70	28.97
C18:3[c]	12.65	16.53	0.07	0.18	0.14	0.09	< 0.05	–	0.66	1.61	0.20	1.61	40.68	39.52
C20:0	0.07	0.17	0.11	0.14	0.87	0.44	0.06	< 0.05	0.10	0.10	0.22	0.10	0.18	0.25
C20:1	0.18	0.09	0.12	0.14	0.16	0.10	0.07	< 0.05	0.07	0.05	0.13	0.05	0.28	–
C20:2	< 0.05	< 0.05	< 0.05	–	–	–	–	–	< 0.05	< 0.05	< 0.05	–	< 0.05	–
C22:0	< 0.05	< 0.05	< 0.05	–	0.18	0.05	< 0.05	–	< 0.05	< 0.05	0.05	< 0.05	0.07	0.18
C24:0	< 0.05	–	< 0.05	–	0.18	< 0.05	< 0.05	–	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Others	0.42	–	0.21	–	0.25	–	0.30	–	0.28	–	0.69	–	0.14	–
Av. rel. error [%][d]	–	16.3	–	32.0	–	12.4	–	18.6	–	16.1	–	29.5	–	7.8

a) Calculated according to Ref. [39].
b) Calculated as the sum of relative contributions of individual FAs in identified TGs.
c) C18:3 is the sum of α- and γ-linolenic acid, when the relative concentration of γ-linolenic acid is always lower than 0.1%.
d) Calculated as average relative difference between LC and GC determinations for fatty acids $\geqslant$ 1%.

hazelnut oil **(Fig. 2.b)**, cashew nut oil (**Fig. 2.c**), almond oil (**Fig. 3.a**), poppy seed oil (**Fig. 3.b**), yellow melon seed oil (**Fig. 3.c**), mango stone oil (**Fig. 4.a**), fig seed oil (**Fig. 4.b**), and date seed oil (**Fig. 4.c**). TGs are identified on the basis of their molecular weights determined from the presence of protonated molecules $[M+H]^+$ in APCI mass spectra, characteristic fragment ions $[M+H-R_iCOOH]^+$ providing an easy identification of individual acids and the position of the most abundant acid in the middle *sn*-2 position, too. Finally, the correctness of the identification is verified by the retention order and relative retention (see Table 2). For the unambiguous identification of trace peaks, the reconstructed ion current records of selected *m/z* values are used to clearly confirm the presence or absence of selected ions in particular peaks, which makes it possible to solve coelutions and even to identify peaks at trace levels.

The *sn*-2 position corresponds to the prevailing acid, but mostly both regioisomers are present. The regioisomeric purity could be determined on the basis of calibration curves of both authentic standards. Concerning the discrimination of OLL vs. LOL (or other TGs differing only by two mass units), the contribution of two ^{13}C atoms to the A+2 ions has to be subtracted first, for example the formula of $[OO]^+$ is $C_{39}H_{71}O_4$ and the theoretically calculated abundance of isotopic peak A+2 is 10.5%. When in previously published data [20] on OLL ($[OL]^+ : [LL]^+ = 100 : 70$) and LOL ($[OL]^+ : [LL]^+ = 100 : 23$) regioisomeric pairs are also taken into account, then the distribution of oleoyl and linoleoyl in the *sn*-2 position is close to 1 : 1 (*e.g.*, almond oil – $[OL]^+$: $[OO]^+ = 100 : 56$, cashew oil – 100 : 55, and hazelnut oil – 100 : 53).

To verify the precision and accuracy of the HPLC/APCI-MS method, 7 plant oils were transesterified using a standard procedure with sodium methoxide and analyzed by GC/FID [39]. First, the RFs of a wide range of FAMEs were measured by GC/FID (see Experimental part), then the relative concentrations of individual FAs were calculated. For all identified TGs in plant oils, the relative weight contributions of individual FAs are summarized and compared with GC/FID results (**Table 6**). This comparison is somewhat affected by the fact that trace FAs are not identified in HPLC/APCI-MS, because one FA may be distributed among many different combination in TGs, which results in TG concentrations below the detection limit. Further, the coelution of trace TGs with more abundant

Table 7. Comparison of average carbon numbers (aCN), equivalent carbon numbers (aECN) and double bond (aDB) numbers, total TG weight%, and the number of identified TGs in 9 plant oils calculated from GC/FID analyses (GC) and HPLC/APCI-MS analyses (LC).

Plant oil source	Latin name	Total TGs [%, *w/w*]	No. of TGs	Average parameter					
				aCN		aECN		aDB	
				GC	LC	GC	LC	GC	LC
Walnut	*Juglans regia L.*	82	43	17.77	17.82	14.26	14.32	1.77	1.75
Hazelnut	*Corylus avellana L.*	75	30	17.82	17.78	15.78	15.71	1.02	1.04
Cashew	*Anacardium occidentale*	73	46	17.76	17.74	15.92	15.80	0.92	0.97
Almond	*Prunus dulcis*	72	25	17.81	17.80	15.50	15.49	1.15	1.16
Poppy seed	*Papaver somniferum L.*	73	33	17.78	17.77	14.51	14.66	1.64	1.56
Yellow melon seed	*Cucumis melo L.*	61	37	17.70	17.77	14.77	14.89	1.47	1.44
Mango stone	*Mangifera indica L.*	49	53	–	17.86	–	16.69	–	0.58
Fig seed	*Ficus carica L.*	85	54	17.85	17.82	13.82	13.91	2.01	1.96
Date seed	*Phoenix dactylifera*	98	66	–	15.26	–	14.18	–	0.53
Average relative error [%] [a]				–	0.2	–	0.6	–	2.6

[a] Calculated as average relative difference between LC and GC determinations.

TGs with the same or similar retention times may complicate the identification of trace FAs. GC determination is free of such problems, because FAMEs elute well separated in a single peak unlike the distribution of FAs among many TGs with different retention times in HPLC. If we keep in mind all sources of potential errors, then the correlation is acceptable. The average relative errors are similar to those in the previous publication on TG quantitation [17]. To our best knowledge, Ref. [17] is the only work published in the literature on the systematic use of RFs for TG determination in complex natural mixtures.

Table 7 summarizes so-called average parameters calculated for plant oils on the basis of GC/FID and HPLC/APCI-MS data, *i.e.*, average carbon number (aCN), average double bond (aDB) number, and average equivalent carbon number (aECN). There is excellent agreement (average relative errors are 0.2, 0.6, and 2.6%) between both methods. Except for date seed oil (15.26), the aCN is very close to the typical 18 carbon atoms in the FA moiety and nearly identical for all samples (from 17.70 to 17.85), which is caused by the prevailing presence of oleic, linoleic, and linolenic fatty acids. Higher differences can be found in the aDB number (from 0.53 to 2.01) and aECN (from 13.82 to 16.69). These average values are useful for the characterization of the type of natural oil, as indicated by our preliminary results on a wider range of various plant oils (this work is in progress). The total TG content in date seed oil (98%) seems to be slightly overestimated, which may be due to the presence of FAs with shorter acyl chains with higher RFs probably associated with increased systematic error in the calculation of RFs of corresponding mixed-acid TGs.

3.4 Analysis of complex mixtures containing tri-, di-, and monoacylglycerols

In this work, 23 single-acid TGs were measured and their RFs determined. RFs of mixed-acid TGs were calculated for APCI-MS. Finally, the RFs of diolein as a representative of diacylglycerols and monoolein as a representative of monoacylglycerols were determined using pure standards (Table 3). The RF ratios of OOO/OO and OOO/O are used for the calculation of RFs of DGs or MGs and then applied for the quantitation of these acylglycerol classes. In this approximation, the differences between positional isomers 1,2-DGs *vs.* 1,3-DGs and 1-MGs *vs.* 2-MGs are neglected. If a more reliable quantitation is needed, then the whole procedure as for TGs should be repeated, including the discrimination between regioisomers.

The chromatographic system optimized for the analysis of TGs used in this work can also be applied for the analysis of more polar acylglycerols, such as DGs and MGs. **Fig. 5** shows the separation of the reaction mixture of biodiesel production from rapeseed oil by transesterification with methanol at half-reaction time. The groups of TGs and DGs are fully separated including the separation of 1,3-DG and 1,2-DG positional isomers, where 1,3-DG is eluted first. There is some peak overlap in the region of MG and methyl esters of FAs. If the analysis of these more polar acylglycerols is the main goal, then initial gradient delay or an initial step with aqueous acetonitrile could improve the separation, similarly to our previous work with aqueous-organic gradient mobile phases [9]. **Table 8** lists concentrations of TGs, DGs, and MGs deter-

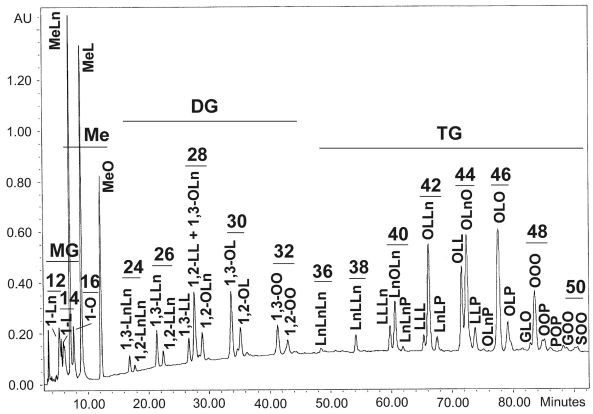

Figure 5. Chromatographic separation of the reaction mixture of rapeseed oil transesterification with methanol containing the groups of tri- (TG), di- (DG), and monoacylglycerols (MG) and methyl esters (Me) of fatty acids. All conditions are identical as for Fig. 2.

mined with APCI-MS in the transesterification mixture of rapeseed oil.

4 Conclusion

A NARP HPLC separation method with acetonitrile–2-propanol mobile phase gradient has been developed and used for unambiguous identification of 133 TGs in 9 plant oils. A knowledge of plant oil composition, including the identification of *sn*-2 acids and reliable quantitation, is very important from a nutritional point of view. Based on the comparison of three detection techniques (APCI-MS, ELSD, and UV at 205 nm), APCI-MS detection is recommended for the analysis and quantitation of TGs for the following reasons: a) unambiguous identification even for strongly coeluting and trace peaks; b) determination of acid prevailing in *sn*-2 position or possible quantitation of regioisomers based on the calibration curves; and c) linear calibration curves (unlike ELSD) with relatively low differences among common C14–C22 fatty acids (unlike UV and ELSD detection). UV detection cannot be recommended because of large differences between saturated and unsaturated TGs and insufficient sensitivity for saturated TGs. Concerning the differences between saturated and polyunsaturated TGs, polyunsaturated TGs have significantly higher relative responses than saturated ones with UV detection (absorption of UV light by unsaturated chains), slightly higher with APCI-MS (easier ionization of π-electrons), but lower with ELSD (probably because less light is scattered by folded conformations of unsaturated chains). The total amount of TGs in the studied plant oils lies in the range of 49% (mango stone oil)–98% (date seed oil). The remaining part (*i.e.*, 2–51%) corresponds to more polar lipids and other compound classes present in these oils. The suggested HPLC/APCI-MS approach with RFs is applicable to the characterization of plant oils, as confirmed by acceptable correlation with validated GC/FID method for FAMEs. GC/FID and HPLC/APCI-MS results are used for the calculation of averaged parameters (aCN, aECN, and aDB), which can characterize the type of plant oil. GC/FID of transesterified FAMEs is an established method for the determination of FA composition; however, unlike the HPLC/APCI-MS method presented in this work, it does not provide any information on TG composition.

Table 8. Concentrations [mg/g] of individual triacylglycerols (TGs), diacylglycerols (DGs) and monoacylglycerols (MGs) determined with APCI-MS detection in the reaction mixture of transesterification of rapeseed oil with methanol.

Compound	*c* [mg/g]	Compound	*c* [mg/g]
TGs		**DGs**	
LnLnLn	0.3	1,3-LnLn	2
LnLLn	3	1,2-LnLn	0.1
LLLn	8	1,3-LLn	5
LnOLn	19	1,2-LLn	2
LnLnP	4	1,3-LL	4
LLL	6	1,2-LL	5
OLLn	33	1,3-OLn	12
LnLP	6	1,2-OLn	5
OLL	26	1,3-OL	9
OLnO	52	1,2-OL	2
LLP	8	1,3-OO	38
OLnP	1	1,2-OO	14
OLO	61	**MGs**	
OLP	17	1-Ln	4
GLO	2	1-L	5
OOO	85	1-O	31
OOP	22		
GOO	5	POP	1
SOO	8		

Acknowledgments

This work was supported by the grant project No. MSM0021627502 sponsored by the Ministry of Education, Youth and Sports of the Czech Republic and projects Nos. 203/04/0917 and 203/05/2106 sponsored by the Grant Agency of the Czech Republic.

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