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Analytical monitoring of the production of biodiesel by high-performance liquid chromatography with various detection methods

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Abstract

Gradient elution reversed-phase high-performance liquid chromatography (RP-HPLC) was used for the determination of compounds occurring during the production of biodiesel from rapeseed oil. Individual triacylglycerols (TGs), diacylglycerols, monoacylglycerols and methyl esters of oleic, linoleic and linolenic acids and free fatty acids were separated in 25 min using a combined linear gradient with aqueous–organic and non-aqueous mobile phase steps: 70% acetonitrile+30% water in 0 min, 100% acetonitrile in 10 min, 50% acetonitrile+50% 2-propanol–hexane (5:4, v/v) in 20 min and 5 min final hold-up. Another method with a non-aqueous linear mobile phase gradient [from 100% methanol to 50% methanol+50% 2-propanol–hexane (5:4, v/v) in 15 min] was used for fast monitoring of conversion of rapeseed oil triacylglycerols to fatty acid methyl esters and for quantitation of residual TGs in the final biodiesel product. Sensitivity and linearity of various detection modes (UV detection at 205 nm, evaporative light scattering detection and mass spectrometric detection) were compared. The individual sample compounds were identified using coupled HPLC–atmospheric pressure chemical ionization mass spectrometry in the positive-ion mode.

Keywords: Biodiesel; Detection, LC; Acylglycerols; Glycerols; Fatty acid methyl esters

1. Introduction

In the last decade, biodiesel has been introduced as an environmentally more friendly substitute for fossil diesel fuels. Biodiesel is produced from renewable sources by transesterification of triacylglycerols (TGs) of fatty acids in vegetable oils (e.g., rapeseed, sunflower or soybean oil) to methyl esters (MEs) of fatty acids [1]. The presence of even small amounts of the original unconverted oil compounds in biodiesel can cause engine problems and results in increased production of hazardous emissions [2]. Hence, a sensitive and reliable analytical method is needed to monitor the contents of TGs, diacylglycerols (DGs) and monoacylglycerols (MGs) in biodiesel. Maximum allowed concentration limits are specified in the national standards, e.g., in the Czech Republic (0.24%) [3], Austria [4], Italy [5], Germany [6] and France [7] and will be included in the European standard specifications based on the EN 590 for fossil diesel fuels [8–10].

Gas chromatography (GC) can be used for the determination of MEs, but it is less convenient for the analysis of non-volatile acylglycerols, which need to be derivatized before the analysis by tri-

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methylsilylation [11–13] or acetylation [14] of the free hydroxyl groups in MGs and DGs. GC determination of underivatized TGs is feasible at high column temperatures (approximately 350°C) and requires a short capillary column with a good temperature stability [14,15]. On-line coupling of normal-phase high-performance liquid chromatography (HPLC) with GC was employed for the determination of acetylated MGs and DGs and of TGs in biodiesel [14]. GC was also used for the determination of residual glycerol in biodiesel after derivatization [16]. Screening analysis of unknown seed oils with attention to GC and GC–mass spectrometry (MS) of fatty acid derivatives and methods for the determination of the configuration of double bonds in the fatty acids were reviewed by Spitzer [17]. GC methods for the analysis of TGs usually utilize volatile derivatives of fatty acids [17,18], but a large number of derivatives suitable for HPLC with UV or fluorescence detection was also proposed [18].

HPLC makes feasible direct analysis of all biodiesel components without derivatization. However, acylglycerols and methyl esters do not absorb in the UV region at wavelengths higher than 220 nm, which causes detection problems. Various detection techniques have been employed for the determination of TGs to substitute conventional UV detection [19,20], including the use of moving wire detection [21], density detection [22], flame ionization detection (FID) [23], refractive index detection [24–27], evaporative light scattering detection (ELSD) [28–34] and mass spectrometric detection [35–37]. Non-aqueous reversed-phase (NARP) HPLC has become the established technique for the separation of TGs, both in the isocratic [31–33,35] and in the gradient elution mode [20,23,33,34,36–40]. NARP-HPLC with acetonitrile–dichloromethane (68:32) allows distinguishing between the TGs with the same molecular masses and different position of the double bonds, e.g., LLL vs. OLLn [31,32] (see Table 1 for notations). With 100% propionitrile as the mobile phase [35] it is possible to separate individual TGs and DGs differing in the equivalent carbon number (ECN), which is defined as ECN=CN−2DB, where CN is the number of carbon atoms in the acyl chains of acylglycerol and DB is the number of double bonds. In NARP-HPLC with a linear gradient from 100% methanol to 100% 2-propanol, the retention times of TGs can be predicted [41] on the basis of the experimental retention times of various synthetic standards [20].

Silver-phase HPLC has been often used for separation of lipids differing in the number and position of double bonds [42–44]. This separation technique was successfully coupled with atmospheric pressure chemical ionization (APCI) MS [45] or with electrospray ionization (ESI) MS [46]. Silver ions form weaker complexes with γ-linoleic acyls than with α-linoleic acyls in TGs. Further, separation of isomeric TGs SOO from OSO and SSO from SOS (where S=saturated fatty acid and O=monounsaturated fatty acid) was possible with silver-phase HPLC [46]. In addition to silver-phase HPLC, Christie reviewed also the applications of chiral HPLC to the structural analysis of TGs. Chromatography on a strong cation-exchange column in the silver ion form was used to separate and determine eight geometrical isomers of the phenacyl esters of linolenic acid [47].

In addition to RP-HPLC, normal-phase chromatography on a cyanopropyl silica column can separate partially transesterified lipids into fractions containing lower alkyl esters, free fatty acids, TGs, 1,3-DGs, 1,2-DGs and MGs [30]. Similar group separation, but without distinguishing between 1,3- and 1,2-diacylglycerols, was reported using a cyanopropyl silica column coupled with two gel-permeation chromatographic columns [22]. Normal-phase HPLC was used for the separation of TGs according to the number of double bonds with partial separation according to the acid chain length, the esters with longer chains being eluted first [48]. However, normal-phase chromatography is not suitable for the separation of the individual compounds in the same ester class.

Sandra et al. [49] compared separations of TGs using microcolumn HPLC and non-aqueous capillary electrochromatography (CEC) with 10 m*M* ammonium acetate in acetonitrile–2-propanol–hexane (57:38:5) as the mobile phase. CEC provided better resolution of TGs differing in the position of double bonds (e.g., LLL from OLLn) than HPLC.

MS with various ionization techniques has been successfully used for structure determination of lipids. Barber et al. [50] suggested the fragmentation pattern of TGs using electron ionization (EI). Later,

Table 1
Retention times (t_R) and molecular masses of main components identified during the transesterification of rapeseed oil with methanol[a]

t_R (min)	r	Name	Notation	ECN[b]	Molecular mass
Fatty acids					
1.53	0.293	Linolenic acid	Ln acid	–	278.2
2.02	0.554	Linoleic acid	L acid	–	280.2
3.05	1.000	Oleic acid	O acid	–	282.3
Monoacylglycerols (MGs)					
3.23	0.458	2-Monolinolenin	2-Ln	12	352.3
3.63	0.536	1-Monolinolenin	1-Ln	12	352.3
4.09	0.627	2-Monolinolein	2-L	14	354.3
4.43	0.694	1-Monolinolein	1-L	14	354.3
5.49	0.902	2-Monoolein	2-O	16	356.3
5.99	1.000	1-Monoolein	1-O	16	356.3
Methyl esters of fatty acids (MEs)					
9.06	0.658	Methyl ester of linolenic acid	MeLn	–	292.2
11.23	0.833	Methyl ester of linoleic acid	MeL	–	294.3
13.30	1.000	Methyl ester of oleic acid	MeO	–	296.3
15.23	1.156	Methyl ester of gadoleic acid	MeG	–	324.3
Diacylglycerols (DGs)					
15.36	0.785	1,3-Dilinolenin	1,3-LnLn	24	612.5
15.60	0.798	1,2-Dilinolenin	1,2-LnLn	24	612.5
16.47	0.845	1,3-Linoleoyl-linolenoyl-glycerol	1,3-LLn	26	614.5
16.76	0.861	1,2-Linoleoyl-linolenoyl-glycerol[c]	1,2-LLn	26	614.5
17.43	0.897	1,3-Dilinolein	1,3-LL	28	616.5
17.60	0.906	1,3-Oleoyl-linolenoyl-glycerol	1,3-OLn	28	616.5
17.73	0.913	1,2-Dilinolein	1,2-LL	28	616.5
17.87	0.921	1,2-Oleoyl-linolenoyl-glycerol[c]	1,2-OLn	28	616.5
18.43	0.951	1,3-Oleoyl-linoleoyl-glycerol	1,3-OL	30	618.5
18.70	0.966	1,2-Oleoyl-linoleoyl-glycerol[c]	1,2-OL	30	618.5
19.33	1.000	1,3-Diolein	1,3-OO	32	620.5
19.60	1.015	1,2-Diolein	1,2-OO	32	620.5
Triacylglycerols (TGs)					
20.63	0.839	Trilinolenin	LnLnLn	36	872.7
21.23	0.864	Dilinolenoyl-linoleoyl-glycerol[c]	LLnLn	38	874.7
21.87	0.891	Dilinoleoyl-linolenoyl-glycerol[c]	LLLn	40	876.7
21.97	0.895	Dilinolenoyl-oleoyl-glycerol[c]	OLnLn	40	876.7
23.50	0.918	Trilinolein	LLL	42	878.7
22.60	0.922	Oleoyl-linoleoyl-linolenoyl-glycerol[c]	OLLn	42	878.7
23.17	0.946	Dilinoleoyl-oleoyl-glycerol[c]	OLL	44	880.8
23.27	0.951	Dioleoyl-linoleoyl-glycerol[c]	OOLn	44	880.8
23.80	0.973	Dioleoyl-linoleoyl-glycerol[c]	OOL	46	882.8
24.43	1.000	Triolein	OOO	48	884.8
25.00	1.024	Dioleoyl-gadoleoyl-glycerol[c]	OOG	50	912.8

[a] Relative retention times $r=t'_R/t'_S$ are the net retention times t'_R related to the net retention time t'_S of a standard compound in each group (standards: O acid, 1-O, MeO, 1,3-OO, OOO). HPLC conditions of method 2 as described in Section 2.4.

[b] ECN is the equivalent carbon number, ECN=CN−2DB, where CN is the number of carbon atoms in all acyls and DB is the number of double bonds.

[c] Positional isomers (e.g., 1,2-OO-3-L vs. 1,3-OO-2-L or 1-O-2-L vs. 1-L-2-O) are not distinguished.

the positive-ion mode chemical ionization (CI) with ammonia was applied for the structure analysis of diacyl phosphatidylcholine [51] and CI in the negative-ion mode for the analysis of TGs [52,53]. CI is a softer ionization technique than EI and the CI mass spectra yield protonated molecular ions of diacyl phosphatidylcholine in contrast to EI. More recent ionization techniques, like thermospray ionization (TSI), ESI and APCI are superior to EI and CI in the field of the lipid analysis for the following reasons: (1) the molecular mass determination is easier; (2) the structural information can be also obtained, as the fragmentation process can be enhanced by collisionally induced dissociation (CID) in-source or in the MS–MS arrangement; (3) the possibility of direct coupling of soft ionization MS with HPLC makes feasible the analysis of complex mixtures of natural lipids. ESI is the softest ionization technique as it yields only the protonated molecular ions of TGs with no fragmentation, so that an MS–MS instrument is necessary to obtain structural information on the acyl chains [54]. ESI-MS–MS was used to localize the positions of double bonds of polyunsaturated fatty acids [55]. However, the performance of ESI is not optimum with non-aqueous mobile phases. Both molecular mass and structural information on phospholipids and related compounds was obtained with TSI [40]. A very efficient method for the structure elucidation of TGs using ESI- and fast atom bombardment (FAB) MS–MS has been reported recently [56].

Various HPLC–MS methods for the lipid analysis have been recently reviewed [57,58]. The most successful ionization technique in this field is probably APCI [35–37,59], which provides a sensitive HPLC detection, both the structural and the molecular mass information and full compatibility with common HPLC conditions for the separation of TGs. APCI-MS was also applied for the analysis of TGs containing hydroxy [60], hydroperoxy [61] and epoxy [37] groups.

Supercritical fluid chromatography (SFC) coupled with EI-MS was applied for the characterization of the butter fat TGs [62] and more recently SFC–APCI-MS has elucidated information on both the molecular masses and on the acyl chain composition in TGs [63,64]. The relative abundance of the $[M+H\text{-}R_iCOOH]^+$ ion makes possible distinguishing between α- and γ-linoleic residues in the same regiospecific position on the glycerol backbone [64].

In the present work, UV detection, ELSD and APCI-MS are compared with respect to their sensitivity, compatibility with gradient elution and possibility of peak identification of TGs without authentic standards. To our knowledge, these three techniques for HPLC detection of TGs have not been compared so far. The objective of the present work is the development of an HPLC method suitable for the separation of TGs, DGs, MGs and MEs occurring during the production of biodiesel from the rapeseed oil. According to the literature [8], TGs in rapeseed oil contain mainly oleic (60%), linoleic (20%) and linolenic (8%) acids with lower concentrations of some other fatty acids, such as palmitic (4%), gadoleic (2–3%) and stearic (1–2%) acids. We have focussed our attention on the esters of the three most abundant acids with different ECNs. The separation of TGs within the class of the same ECN is presently being investigated. Finally, the fragmentation in APCI-MS is discussed and compared with earlier published data.

2. Experimental

2.1. Materials

Methanol and hexane were obtained from Baker (Deventer, The Netherlands), acetonitrile from LabScan (Dublin, Ireland) and 2-propanol from VÚOS Rybitví (Pardubice, Czech Republic). All solvents were of HPLC-grade and were used as obtained, without further purification. Deionized water was doubly distilled in glass with addition of potassium permanganate. A glass cartridge column, Separon SGX C_{18} (particle size 7 μm, 150×3 mm I.D.), was obtained from ECOM (Prague, Czech Republic). Triolein, trilinolein and trilinolenin were purchased from Sigma–Aldrich (Prague, Czech Republic). The samples of transesterified rapeseed oil were obtained from the Department of Physical Chemistry, University of Pardubice.

2.2. The chromatographic instrument

The chromatographic apparatus consisted of a

Model 616 pump with a quaternary gradient system, a Model 996 diode-array detector, a Model 717+ autosampler, a thermostatted column compartment and a Millennium chromatography manager (all from Waters, Milford, MA, USA). Some experiments were performed on a HP 1090M liquid chromatograph equipped with a diode-array detector, an autosampler, a three-solvent delivery system and a thermostatted column compartment (all from Hewlett-Packard, Palo Alto, CA, USA), connected to an ELSD system PL-EMD 950 (Polymer Labs., Shropshire, UK). The temperature of the ELSD system was kept at 60°C and the flow-rate of the nebulising air at 10 l/min.

2.3. The mass spectrometric instrument

For HPLC–MS experiments, the Waters liquid chromatograph was connected to a VG Platform quadrupole mass spectrometer (Micromass, Manchester, UK) via an APCI probe. The mass spectrometric data were acquired in the range m/z 35–1000 at a scan duration of 1.9 s in the positive-ion APCI mode. A 3.20 kV potential was applied on the discharge needle. The temperature was held at 500°C in the APCI probe and at 120°C in the ion source. A 20 V voltage was applied on the cone. Nitrogen was used as the drying, sheath and nebulising gas. For quantitative analysis of synthetic standards of triolein, trilinolein and trilinolenin, the chromatograms were reconstructed using (1) the ion current of selected characteristic ions with m/z 335, 337, 339, 595.5, 599.5, 603.5, 873.5, 879.5 and 885.5 (see Table 2) and (2) the total ion current in the range m/z 200–1000.

2.4. Procedures

The solvents were filtered through a 0.45-μm Millipore filter prior use and degassed by continuous stripping with helium. The injection volumes of 10 μl and the flow-rate of 1 ml/min were used in all experiments. The column temperature was held constant at 40°C. All samples were dissolved in 2-propanol–hexane (5:4, v/v).

HPLC method 1: reservoir A contained methanol, reservoir B contained a mixture of 2-propanol–hexane (5:4, v/v). A linear gradient from 100% A to 50% A+50% B in 15 min was employed.

HPLC method 2: reservoir A contained water, reservoir B contained acetonitrile and reservoir C contained 2-propanol–hexane (5:4, v/v). A 25-min ternary gradient with two linear gradient steps was employed: 30% A+70% B in 0 min, 100% B in 10 min, 50% B+50% C in 20 min, followed by isocratic elution with 50% B+50% C for the last 5 min. The retention times of all the compounds identified by APCI-MS are given in Table 1.

2.5. Quantitative analysis

The calibration curves were measured in two series of experiments. Each data point was obtained from at least two repeated measurements. For the first set of experiments, a sample of rapeseed oil was dissolved in 2-propanol–hexane (5:4) to yield the 1% (v/v) stock solution, which was diluted with the same solvents to prepare 0.16, 0.32, 0.48, 0.64 and 0.80% calibration solutions for the UV detection and ELSD. For APCI-MS, these solutions were further diluted ten times. The parameters of the dependences

Table 2
The masses of characteristic ions A–D (see fragmentation scheme in Fig. 5) of TGs containing gadoleic (G), oleic (O), linoleic (L), linolenic (Ln) and palmitic (P) acyls

R_1	R_2	Ion A	R_1	R_2	Ion A	R	Ion B	Ion C	Ion D
G	G	659.6	O	Ln	599.5	G	367.3	293.3	275.2
G	O	631.6	L	L	599.5	O	339.3	265.3	247.2
G	L	629.6	L	Ln	597.5	L	337.3	263.2	245.2
G	Ln	627.5	Ln	Ln	595.5	Ln	335.3	261.2	243.1
G	P	605.6	O	P	577.5	P	313.3	239.2	221.2
O	O	603.6	L	P	575.5				
O	L	601.5	Ln	P	573.5				

of the peak areas on the concentrations of the most abundant TGs in rapeseed oil (OOO, OOL, OLL and OOLn, LLL and OLLn, LLLn and OLnLn) are listed in Table 3.

The standard calibration solutions containing 50–300 mg/l of each triolein (OOO), trilinolein (LLL) and trilinolenin (LnLnLn) in 2-propanol–hexane (5:4) were used in the second series of experiments for direct comparison of the three detection methods. The calibration parameters of OOO, LLL and LnLnLn are listed in Table 4.

3. Results and discussion

3.1. HPLC separation

The non-aqueous RP-HPLC method 1 with a 15 min gradient was used for rapid determination of the yield of transesterification of rapeseed oil. Fig. 1 illustrates the separation of a mixture of rapeseed oil and of biodiesel under these conditions. The individual MEs and TGs of linolenic, linoleic and oleic acids were separated from each other (except for the

Table 3
The constants of the calibration curves (peak areas, *A*, versus the mass, *m*, of the rapeseed oil in 10 μl sample) determined by linear (UV detection at 205 nm, and positive-ion APCI-MS) and non-linear (ELSD) regression[a]

ECN[c]	Main TGs	Slope (IU/ng)[d]	Intercept (IU)[d]	Correlation coefficient	c[e] (IU)[d]	b[e] (IU/ng)[d]	a[e] (IU/ng^2)[d]	Coefficient of determination
UV 205 nm[b]								
40	OLnLn+LLLn	22 950	−33 200	0.993				
42	OLLn+LLL	49 870	−55 300	0.997				
44	OLL+OOLn	85 750	−67 500	0.998				
46	OOL	67 050	−47 200	0.999				
48	OOO	27 920	−19 700	0.993				
ELSD[b]								
40	OLnLn+LLLn				1.966	−0.135	0.022	0.995
42	OLLn+LLL				10.968	−1.187	0.127	0.999
44	OLL+OOLn				38.986	−4.293	0.557	1.000
46	OOL				42.881	−2.2560	0.814	1.000
48	OOO				32.513	1.614	0.933	0.999
APCI-MS[f] (data evaluated only for the fragment ions A)								
40	OLnLn+LLLn	3170	−60	0.990				
42	OLLn+LLL	16 140	700	0.996				
44	OLL+OOLn	58 050	2510	0.996				
46	OOL	126 800	11 190	0.997				
48	OOO	208 200	25 250	0.999				
APCI-MS[f] (data evaluated for the mass range m/z 200–1000)								
40	OLnLn+LLLn	15 090	3210	0.991				
42	OLLn+LLL	51 970	2930	0.993				
44	OLL+OOLn	137 000	4380	0.996				
46	OOL	213 020	19 820	0.998				
48	OOO	289 400	20 100	0.997				

[a] HPLC conditions of method 1 as described in Section 2.4.
[b] Calibration range 15–100 μg of the rapeseed oil.
[c] ECN=Equivalent carbon number
[d] IU=Integration units, arbitrary.
[e] *a*, *b*, *c* are the constants of the second-degree polynomial dependence $A=am^2+bm+c$.
[f] Calibration range 1.5–10 μg of the rapeseed oil.

Table 4
The constants of the calibration curves [peak areas, *A*, versus the masses, *m*, of the pure standards of triolein (OOO), trilinolein (LLL) and trilinolenin (LnLnLn) in 10 μl sample injected] determined by linear (UV detection at 205 nm and positive-ion APCI–MS) and by non-linear (ELSD) regression[a]

TGs	Slope (IU/ng)[b]	Intercept (IU)[b]	Correlation coefficient	Limit of detection (ng)	c[c] (IU)[b]	b[c] (IU/ng)[b]	a[c] (IU/ng^2)[b]	Coefficient of determination	Limit of detection (ng)
UV 205 nm									
LnLnLn	42 800	−9800	0.998	60					
LLL	83 860	−45 600	0.992	30					
OOO	30 940	−7200	0.998	80					
ELSD									
LnLnLn					−10.64	0.460	$1.46\cdot10^{-4}$	0.997	1500
LLL					−74.51	1.143	$2.409\cdot10^{-3}$	0.998	400
OOO					−188.87	5.288	$1.9653\cdot10^{-2}$	0.999	70
APCI-MS (data evaluated only for the fragment ions A)									
LnLnLn	11 570	13 800	0.987	70					
LLL	41 210	29 600	0.988	20					
OOO	144 020	89 200	0.991	6					
APCI-MS (data evaluated for the mass range m/z 200–1000)									
LnLnLn	33 610	93 800	0.989	500					
LLL	72 810	89 600	0.980	200					
OOO	219 670	123 600	0.991	100					

[a] HPLC conditions of method 1 as described in Section 2.4. The concentration range 50–300 mg/l in the calibration solutions was used in all detection modes.
[b] IU=Integration units, arbitrary.
[c] a, b, c are the constants of the second-degree polynomial dependence $A=am^2+bm+c$.

combinations LLLn/OLnLn, LLL/OLLn and OLL/OOLn, with the same ECN – for the notation of the sample compounds see Table 1). The peaks of DGs are buried in the baseline noise between the peaks of MEs and of TGs. HPLC method 1 was intended for rapid quantitative determination of the yield of the transesterification reaction of rapeseed oil with methanol by comparing the sums of the areas of the peaks of MEs and TGs using UV detection at 205 nm.

HPLC method 2 combining aqueous–organic and NARP gradient elution HPLC was used to obtain more detailed information on the individual sample components. It provides, in addition to similar resolution of the individual TGs present in rapeseed oil as in HPLC method 1, improved resolution of MEs and nearly complete resolution of all C_{18} free acids with different numbers of double bonds and of their mono- and diacylglycerols (Fig. 2). The retention increases in the order: free fatty acids <MGs<MEs<DGs<TGs. As expected, the MGs, DGs and TGs are eluted in order of increasing ECN. All MGs, MEs and DGs of the three most abundant acids (oleic, linoleic and linolenic) are resolved, except for the LL and OLn with the same ECN. Further, the positional isomers of DGs are partially resolved. 1,3-DGs are eluted before 1,2-DGs, possibly because of the steric shielding of the central hydroxy group in the 1,3-isomer. The separation of the components in the reaction mixture after the transesterification of rapeseed oil is accomplished within 25 min. An example of the chromatogram of a real sample of biodiesel prepared by the transesterification of rapeseed oil is shown in Fig. 3.

APCI-MS can be used to reconstruct the selected ion chromatograms from the total ion chromatograms using characteristic ions of the individual DGs or TGs for their identification and for the deconvolution

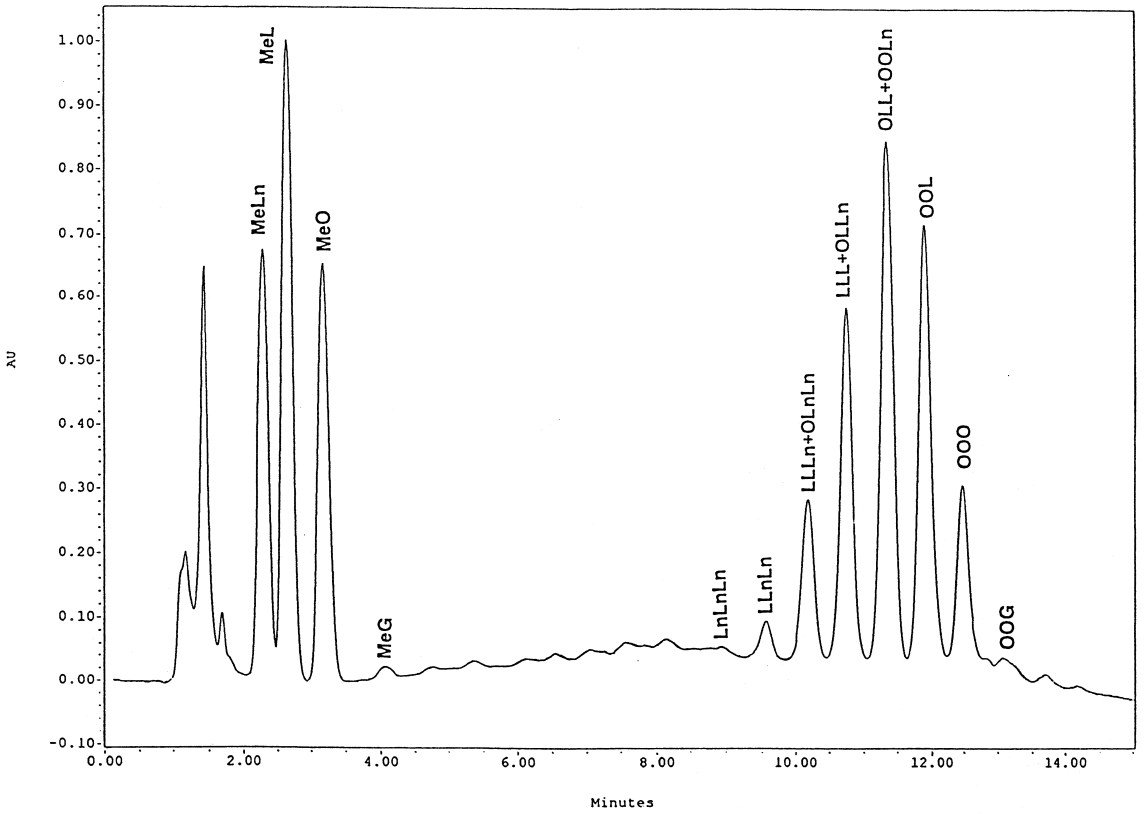

Fig. 1. HPLC separation of a mixture of rapeseed oil and biodiesel. HPLC method 1 (see Section 2.4), UV detection at 205 nm, flow-rate 1 ml/min, injection volume 10 μl. Notation of compounds as in Table 1.

of overlapping peaks (Fig. 4). This is possible because of characteristic differences in the masses of the fragment ions produced by subsequent losses of fatty acids with different numbers of double bonds and different masses. For example, the mass spectrum of OLL contains fragment ions with m/z 601 (loss of linoleic acid, m/z 280) and m/z 599 (loss of oleic acid, m/z 282) from the $[M+H]^+$ ion (m/z 881). The $[M+H]^+$ ion of OOLn with the same mass may lose linolenic acid (fragment ion m/z 603) or oleic acid (fragment ion m/z 599). The selected ion chromatogram reconstructed at m/z 601 contains only the peak of OOL and the chromatogram reconstructed at m/z 603 only the peak of OOLn. The chromatograms reconstructed in this way may be used to deconvolve overlapping peaks and to determine the exact retention times of the coeluted TGs and DGs. Fig. 4 shows an example of such deconvolution of the overlapping peaks of OLL and OOLn in the reconstructed selected ion chromatograms at m/z 337 and 335, respectively. The masses of the fragment ions suitable for this purpose are given in Table 1, which lists also the retention times of the individual compounds. The values of t_R in Table 1 may not exactly match the chromatograms recorded on another instrument because of possible differences in gradient dwell volumes and in column hold-up volumes, hence the table lists also the relative retention times, related to an easily identifiable compound in each group: O acid, 1-O, MeO, 1,3-OO and OOO. The retention times of the peaks with the same EON but with different distribution of double bonds differ from each other by ca. 0.1 min, which is too small a difference to allow their separation by HPLC. Work is in progress to improve the separation by optimizing the type of the chromatographic column and the separation conditions. Nevertheless, it is possible to identify and to de-

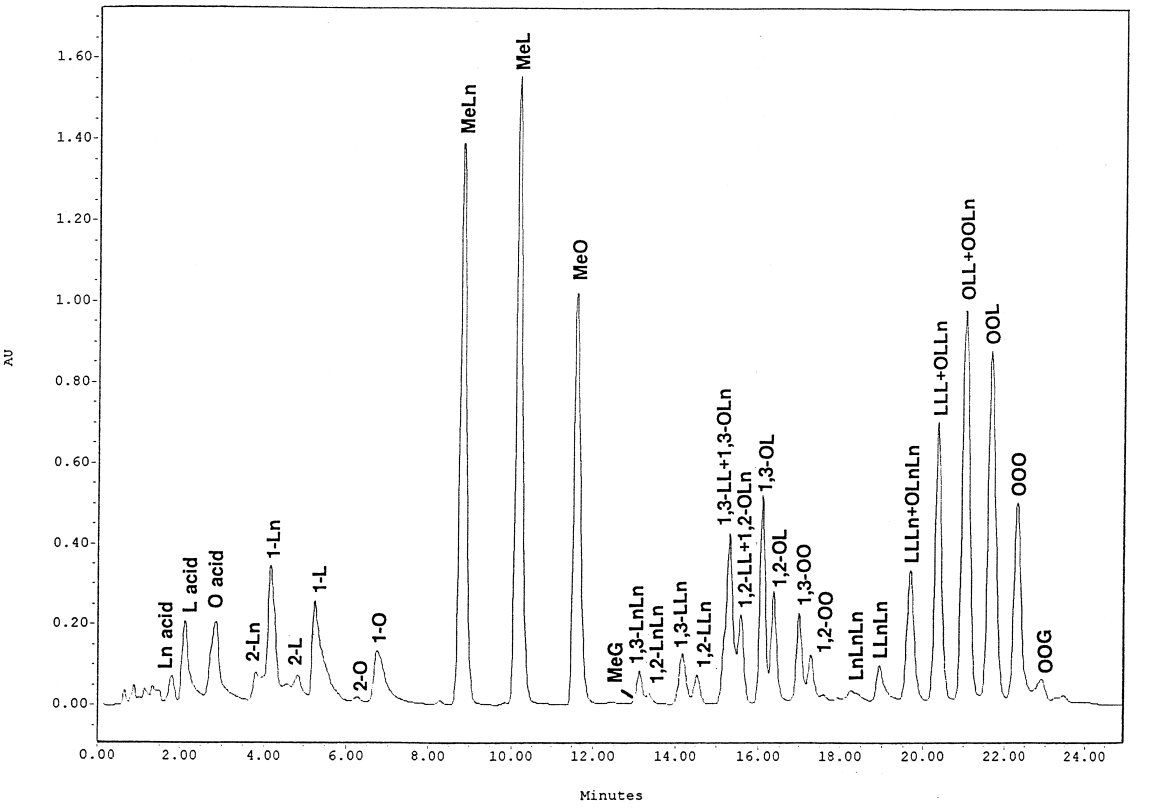

Fig. 2. HPLC separation of a reaction mixture of rapeseed oil methanolysis. HPLC conditions of method 2 (see Section 2.4), UV detection at 205 nm, flow-rate 1 ml/min, injection volume 10 μl. Notation of compounds as in Table 1.

termine the coeluted compounds using HPLC–MS with reconstructed selected ion chromatograms.

3.2. Peak identification in the positive-ion APCI mass spectra of acylglycerols and fatty acid methyl esters

The molecular masses of the individual compounds can be readily determined from the peaks of $[M+H]^+$ ions and of the characteristic molecular adducts with alkali metal ions – $[M+Na]^+$ (22 m/z units higher) and $[M+K]^+$ (38 m/z units higher). The APCI-MS fragmentation patterns of the fatty acid esters studied in the present work show some common features (Fig. 5). Characteristic fragment ions are formed by the loss of one acid molecule from the $[M+H]^+$ ions of TGs (ion A in Fig. 5) and DGs (ion B in Fig. 5) or of a molecule of water from the $[M+H]^+$ ions of DGs (ion A in Fig. 5) and of MGs (ion B in Fig. 5, base peak). Another abundant fragment ion in the mass spectra of TGs is formed by subsequent losses of an acid molecule and an acyl R_iCO (ion B in Fig. 5). Further, acyl ions (C in Fig. 5) and dehydrated acyl ions (D in Fig. 5) are observed in the mass spectra of MGs and MEs. The masses of all important ions of TGs, DGs, MGs and MEs containing oleic, linoleic, linolenic, gadoleic and palmitic acids are listed in Table 2. Representative mass spectra of several compounds of each class are shown in Figs. 6–9. Note the peak of the ion A with m/z 577 in the spectrum of OOO in Fig. 6 corresponding to TGs with one or two palmitic acyls, coeluted with OOO.

The base peak in the mass spectra of TGs with five and more double bonds corresponds to the protonated molecule (Fig. 6, LLL, OLLn). The mass spectra of TGs with three or two different acyls exhibit three or two $[M+H-R_iCOOH]^+$ ions differ-

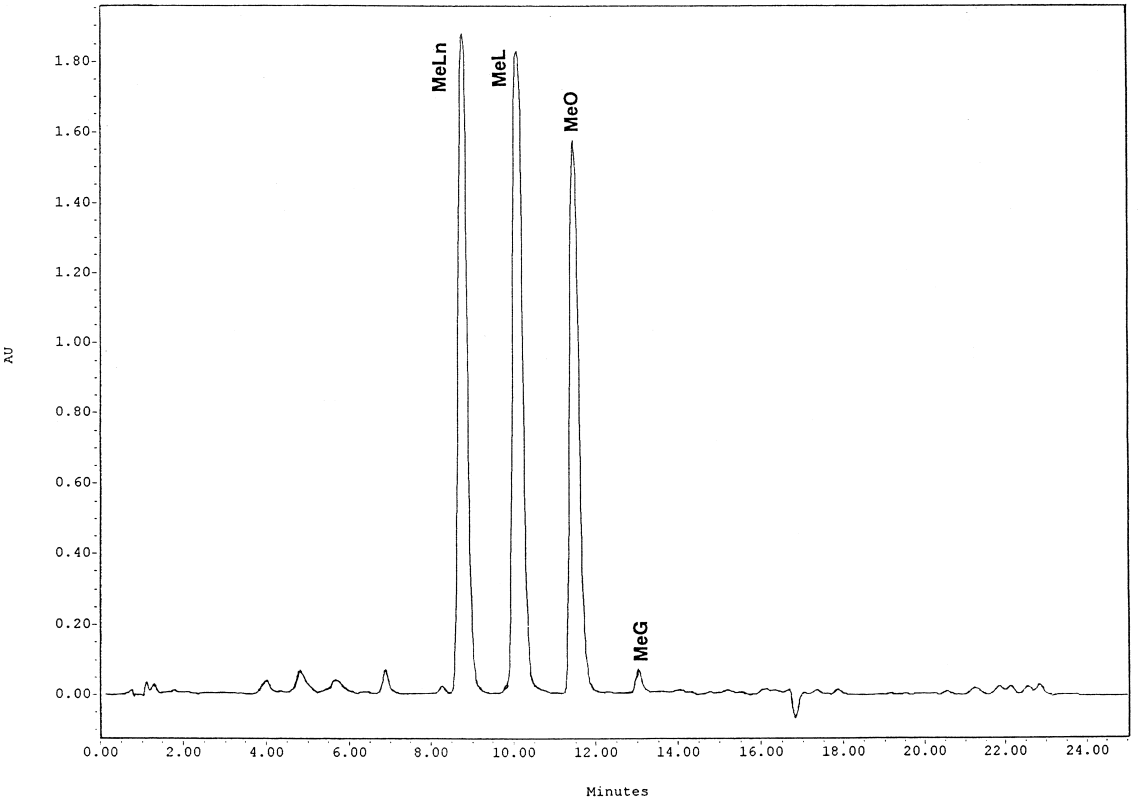

Fig. 3. HPLC separation of components of final biodiesel, conditions as in Fig. 2.

ing by two units, in the spectra of TGs with three identical alkyls only one $[M+H\text{-}R_iCOOH]^+$ ion appears. The fragmentation described in the present work is similar as in the mass spectra obtained with EI [37], TSI [40] and ESI [56], where the ions formed by the losses of an acyl, water or alcohol molecule from the molecular ions of acylglycerols were observed. The ions A correspond to the base peaks in the EI [62], ESI [54,56], TSI [40] and APCI [35,36,40,45,61,62,64] mass spectra of TGs. The relative abundance of this ion depends on the position of the acyl in the TGs molecule [45,62], on the type of acyl and on the position of double bond in the acyl (such as α-linolenic versus γ-linolenic acyls) group [64]. The ions B and C were observed in ESI [54,56] and TSI [40] mass spectra of TGs. Crawford and Plattner observed the ions B and C in some compounds during the analysis of phospholipids [51]. The mass spectrum of triolein (Fig. 6) is very similar to the earlier published APCI mass spectrum [35], where the acyl ion C was observed instead of the ion B in the present work. This difference can probably be attributed to a lower in-source collisionally induced dissociation energy in the present work. The APCI mass spectra of TGs show similar fragmentation patterns as the EI mass spectra [50].

In the mass spectra of DGs with three and more double bonds, the base peaks correspond to the protonated molecules. Isomeric 1,2-DGs can be distinguished from 1,3-DGs on the basis of the ratio of the protonated molecules or of the ions A to the ions B (Fig. 7). This ratio is always higher for 1,3-DG than for 1,2-DG isomers. This rule can be applied for the identification of unknown 1,2-DG and 1,3-DG isomers, when the standards are not available.

The base peak in the mass spectra of MGs (Fig. 8) corresponds to the loss of water, the protonated molecule is also apparent. Other important ions are

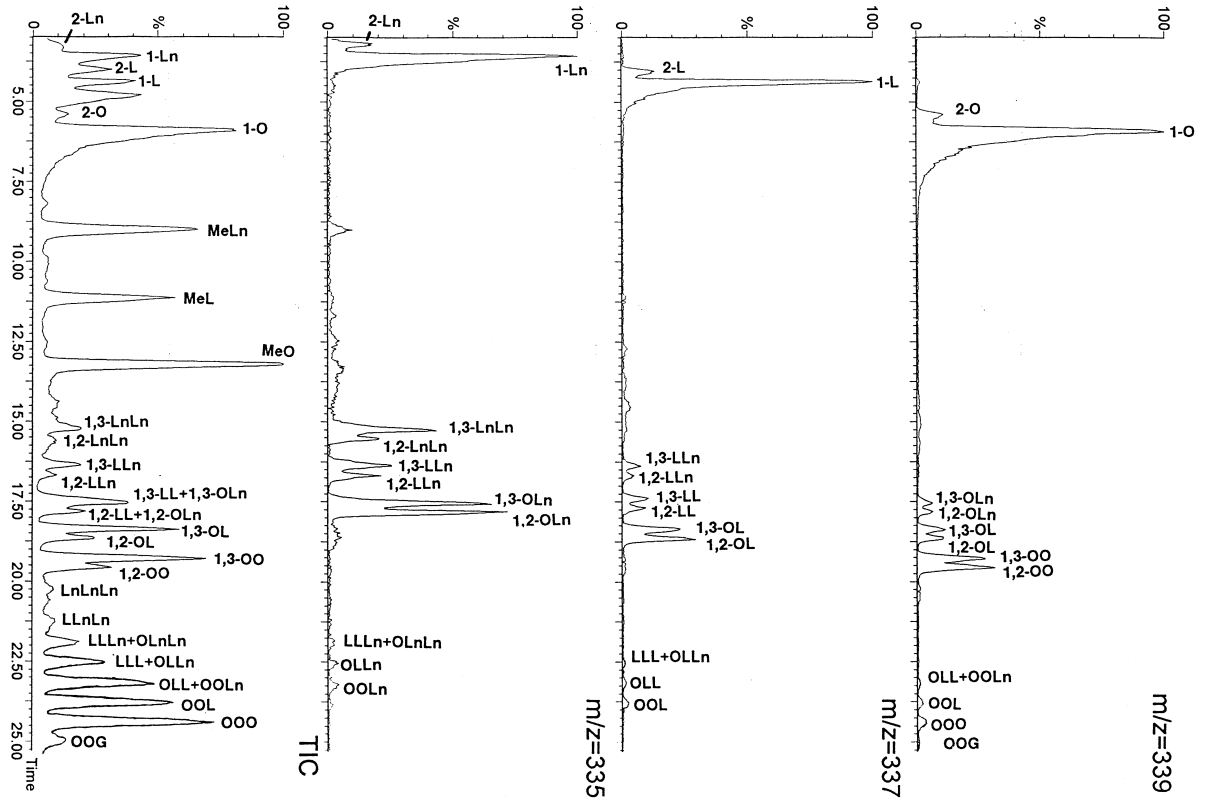

Fig. 4. HPLC separation of a reaction mixture of rapeseed oil methanolysis, APCI-MS, HPLC conditions as in Fig. 2. Total ion current (TIC) chromatogram and selected ion current chromatograms of ions m/z 339, 337 and 335 are shown. Time scale in min.

$[R_iCO]^+$ and $[R_iCO\text{-}H_2O]^+$. In the mass region below m/z 200, the ions attributed to the aliphatic series formed by the fragmentation of alkyl chains are observed like in the EI mass spectra of MGs.

We have not found an earlier work reporting the mass spectra of free DGs and MGs, probably because of their low volatilities. GC–EI-MS was used for the determination of the positions of double bonds and methyl branching in the nicotinoyl derivatives of DGs and MGs [65,66] and mono- and digalactosyl diacylglycerols were analysed using FAB-MS–MS [67].

The APCI mass spectra of MEs (Fig. 9) are very close to their EI mass spectra with characteristic aliphatic ion series, but the relative abundances of the high-mass ions (ions $[M+H]^+$, C and D) are higher. The base peak in the APCI mass spectra corresponds either to the ion C (e.g., MeO) or to the $[M+H]^+$ ion (e.g., MeLn).

In contrast to all other studied compounds, free fatty acids give no response in positive-ion APCI-MS, but good signals are obtained in negative-ion APCI-MS, where $[M-H]^-$ ions are observed. The abundances of the fragment ions in these spectra are very low.

In addition to the determination of the position of the acyls in the acylglycerols, the determination of the total number and the positions of the double bonds in both acylglycerols and free acids is important for the identification of the individual components in fat samples. For this purpose, the ESI-MS–MS technique is useful [55].

3.3. Quantitative analysis

From among various detection techniques applied in the HPLC of acylglycerols, UV detection at 200–

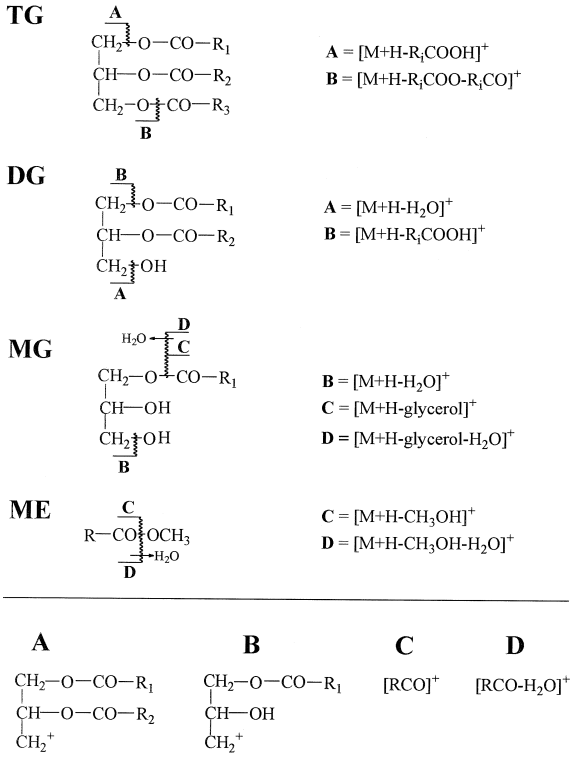

Fig. 5. Fragmentation scheme of triacylglycerols (TGs), diacylglycerols (DGs), monoacylglycerols (MGs) and fatty acid methyl esters (MEs) using positive-ion APCI-MS. A, B, C and D are the most important fragment ions.

205 nm, ELSD and MS are most suitable for the purpose of the present work, because of their compatibility with gradient elution necessary for satisfactory resolution of complex sample mixtures containing MEs, MGs, DGs and TGs. The calibration curves generally are linear for UV detection and MS over a broad concentration range, which is not the case with ELSD. The response factors for the individual sample compounds differ from one another with each of the three detection methods (Tables 3 and 4). Natural samples may contain 40 to 100 or more individual TGs, so that the construction of the calibration curves using pure standards for each species would not be practical. Further, many compounds are coeluted even in well-designed chromatographic systems, so that the quantitation of coeluted compounds in overlapped peaks on the basis of the authentic standards would necessarily be subject to errors [68,69].

With MS, compounds with different molecular masses can be distinguished and chromatograms can be reconstructed using selected ions to make the quantification possible. However, this approach can be applied to the coeluted free fatty acids, MEs, MGs and DGs, but is not fully successful with TGs, as some species with different acyl combinations (see, e.g., Fig. 4) or positional isomers cannot be distinguished by their mass spectra. Further, this method does not eliminate the need for a large number of pure calibration standards. Hence, simplified quantitation methods are required.

For the quantitative analysis of residual acylglycerols in biodiesel, the sums of the normalized peak areas corresponding to the main compound classes, MEs, MGs, DGs and TGs can be used, but for accurate analysis it is very important that the fatty acid composition of all the components in a methanolysis mixture should be the same for all compound classes [22]. However, transesterification can produce a randomization of the fatty acid chains among the species, which would diminish natural preferences for the distribution of the fatty acids in the original plant oil.

A quantification method was suggested taking into account non-equal fatty acid distribution in TGs, which employs the response factors determined for each TG as the ratio of peak areas between the original oil sample and the same sample after chemical randomization (interesterification), for which the distribution of the fatty acids was determined by GC–FID, where response factors are not necessary [57]. Another method of quantification relies on multiplication of the response factors for the fatty acids determined by GC–FID to produce the final response factor for each TG [57].

For the characterization of quality of biodiesel prepared by transesterification of plant oils, the information on the concentration of the residual acylglycerols and of free glycerol is of primary importance. To a first approximation, the contents of the acylglycerols containing only three main individual acids (O, L and Ln) can be used for the information on the acid distribution in MGs, DGs and TGs with equal ECN, because the contents of other acids such as stearic, palmitic, gadoleic, etc., are 1–4% or less. The concentrations of MEs, MGs, DGs and TGs with equal ECN containing the three

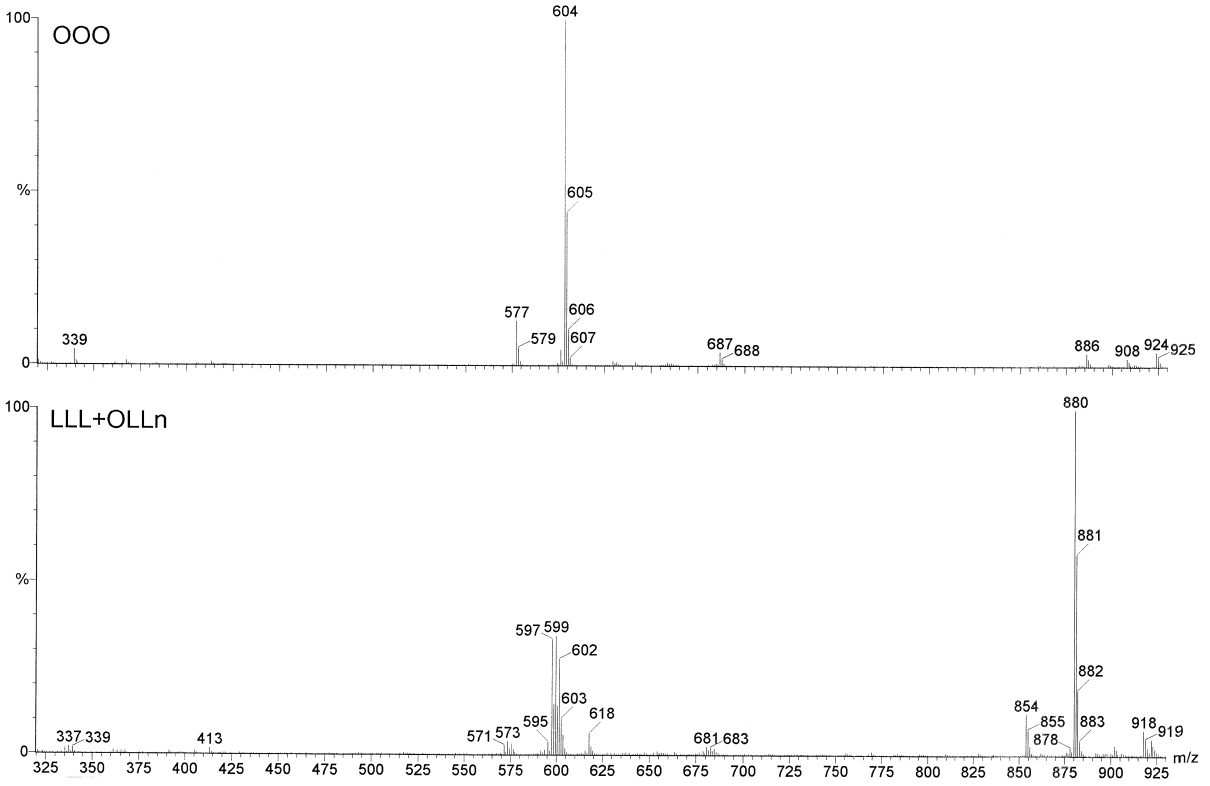

Fig. 6. Positive-ion APCI mass spectrum of triolein (OOO) and mixed spectrum of trilinolein and oleoyl-linoleoyl-linolenoyl-glycerol (OLLn), masses of important ions are given in Table 1.

acids can be determined from the chromatograms obtained by the present HPLC method 2. For this purpose, we compared low-wavelength UV detection (at 205 nm), ELSD and two variants of APCI-MS: (1) based on the selected ion chromatograms solely of the ions A and (2) evaluated from the chromatograms in the m/z range 200–1000.

Table 3 shows the constants of the dependences of the peak areas on the concentrations of TGs with equal ECN in diluted rapeseed oil using UV detection at 205 nm, ELSD and APCI-MS based on the fragment ions A and on the ion current evaluated in the range m/z 200–1000. Table 4 shows the constants of the calibration curves of pure OOO, LLL and LnLnLn standards for the same detection modes as in Table 3. The constants of the calibration curves for UV detection and APCI-MS were determined by linear regression, whereas second-degree polynomial non-linear regression had to be used to fit the non-linear calibration data for ELSD.

3.3.1. UV detection

Weak absorbance of acylglycerols at wavelengths higher than 220 nm was considered as a major disadvantage of UV detection. We measured the calibration curves at 205 nm as a compromise between the sensitivity and the baseline noise. The correlation coefficients 0.992–0.999 indicate acceptable linearity. The limits of detection (LODs) defined as the signal-to-noise ratio=2 increase in the order: LLL<LnLnLn<OOO (Table 4) and are comparable with the LOD for ELSD of OOO, but are at least an order of magnitude better for LLL and LnLnLn. The differences between the response factors of the three TGs (the slopes of the calibration curves) are lesser than with the other detection techniques used in the present work.

3.3.2. Evaporative light scattering detection

ELSD is widely used for the determination of acylglycerols and other lipids. The calibration curves

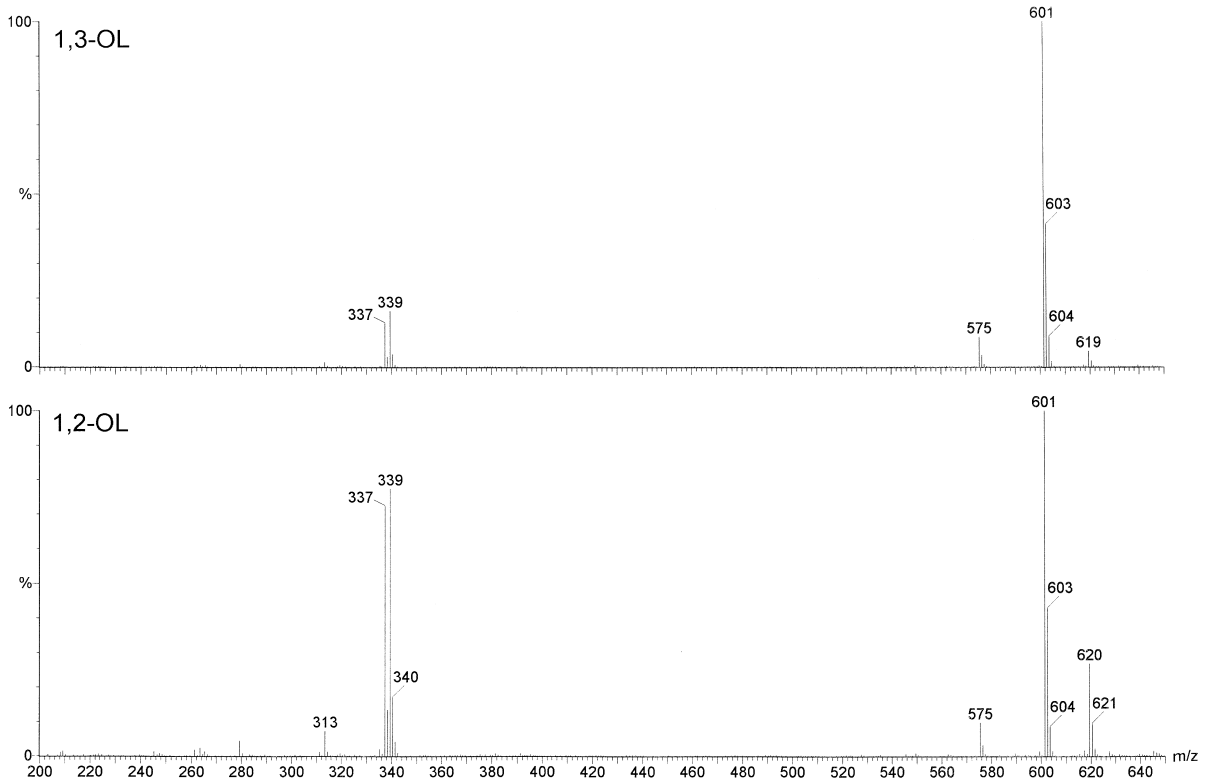

Fig. 7. Positive-ion APCI mass spectra of 1,3-oleoyl-linoleoyl-glycerol (1,3-OL) and 1,2-oleoyl-linoleoyl-glycerol (1,2-OL).

with ELSD generally are non-linear and are usually fitted by polynomial dependences [30,70,71]. In our work, the concentration dependences of the experimental peak areas of TGs was adequately described by quadratic calibration curves (Fig. 10A). The coefficients of determination for the quadratic relationship are between 0.994 and 0.996. ELSD shows a strong decrease of the sensitivity (increasing LOD) with increasing number of double bonds in acylglycerol molecules (Table 4). The LODs for LLL and LnLnLn are approximately an order of magnitude higher than for UV detection.

3.3.3. Positive-ion APCI-MS

APCI-MS can be routinely applied for HPLC analyses using the conventional analytical columns and is compatible with gradient elution. Moreover, it offers additional structural information. APCI-MS of the base peak A is at least an order of magnitude more sensitive than MS using the total ion current (TIC) in the range m/z 200–1000 and than ELSD (Table 4). With APCI-MS, deviations from the linearity of the calibration curves occur for the samples containing TGs in concentrations higher than 0.1–0.3% (v/v). Hence, appropriate dilution is necessary to achieve good linearity of the calibration curves with APCI-MS (Fig. 10B). The LODs increase and the sensitivity decreases with increasing number of double bonds in TGs. However, this effect is lower than with ELSD, especially in the TIC detection mode. The LODs for the method using extracted signals of the base peaks are comparable with or better than for the UV detection at 205 nm.

3.3.4. Comparison of the detection modes using normalized peak areas

All the detection modes tested meet the sensitivity of determination, with the exception of ELSD for TGs containing three or more double bonds. The neglection of the presence of palmitic, stearic and

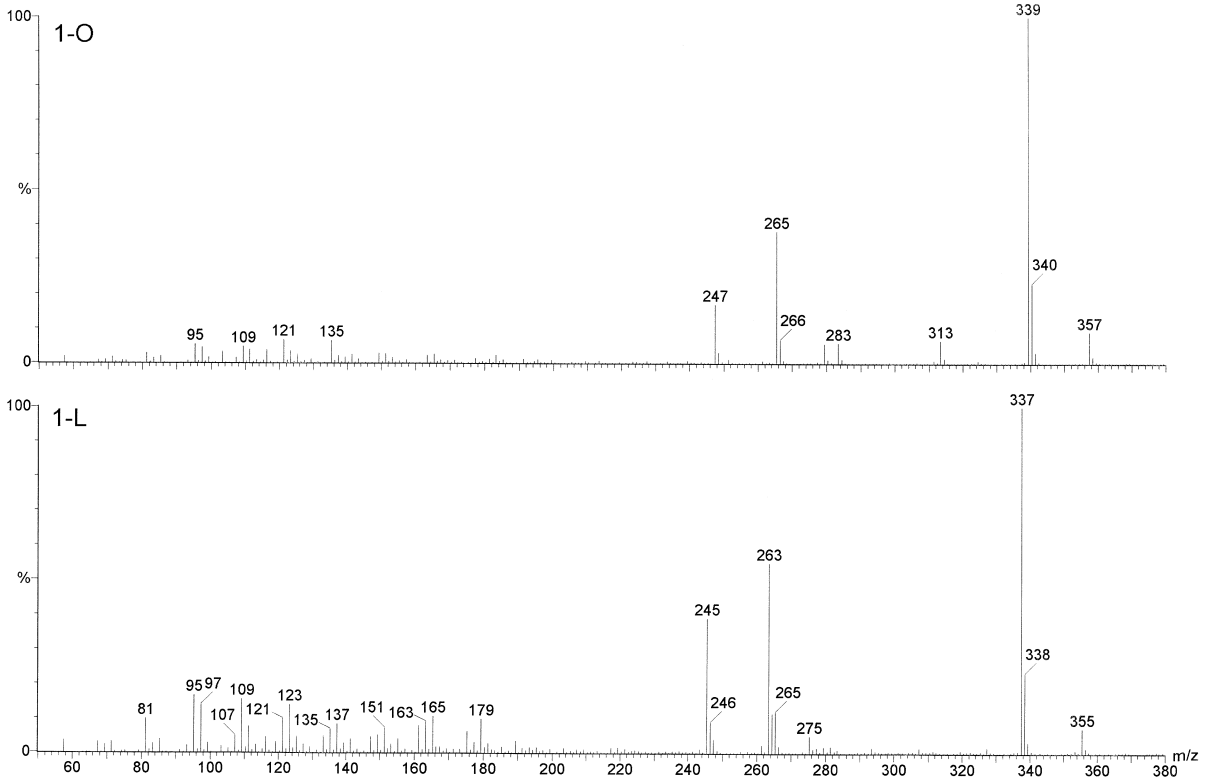

Fig. 8. Positive-ion APCI mass spectra of 1-monoolein (1-O) and 1-monolinolein (1-L).

other minor acids in the present method leads to errors in quantification. Because of the low contents of the TGs in the final biodiesel products, these errors are not very significant in this specific analytical case, where only a quick estimation of the relative amounts of different classes of compounds is desired.

Different slopes of the calibration curves for the individual TGs obtained with all detection methods tested (Table 4) cause differences between the contents of the individual TGs evaluated using the normalized peak areas method with each detection mode. This is illustrated by the data in Table 5, which compares the concentrations of TGs in a rapeseed oil sample evaluated by HPLC method 1 from normalized peak areas for various detection modes. When no correction factors are used, ELSD yields higher concentrations of OOL and lower concentrations of OOO and OLnLn+LLLn than UV detection. The results obtained with the two variants of APCI-MS and with ELSD are more close to each other, but significant differences are still apparent. The APCI-MS data evaluated solely for the fragment ions A result in lower contents of TGs with higher number of double bonds than the data evaluated for all ions in the mass range m/z 200–1000, in agreement with increasing abundance ratio of the A: $[M+H]^+$ ions for the TGs with lower numbers of double bonds. Hence, the evaluation of the APCI mass spectra in the m/z range 200–1000 can be expected intuitively to yield more realistic concentration ratios of the individual TGs than the evaluation based on the A ions only. This is supported by lower differences between the slopes of the calibration curves for the wide-mass-range evaluation variant in comparison to the quantitation based solely on the A ions (Tables 3 and 4). On the other hand, the data evaluated for the fragment ions A result in significantly decreased detection limits.

To refine the results of quantitation, we used peak

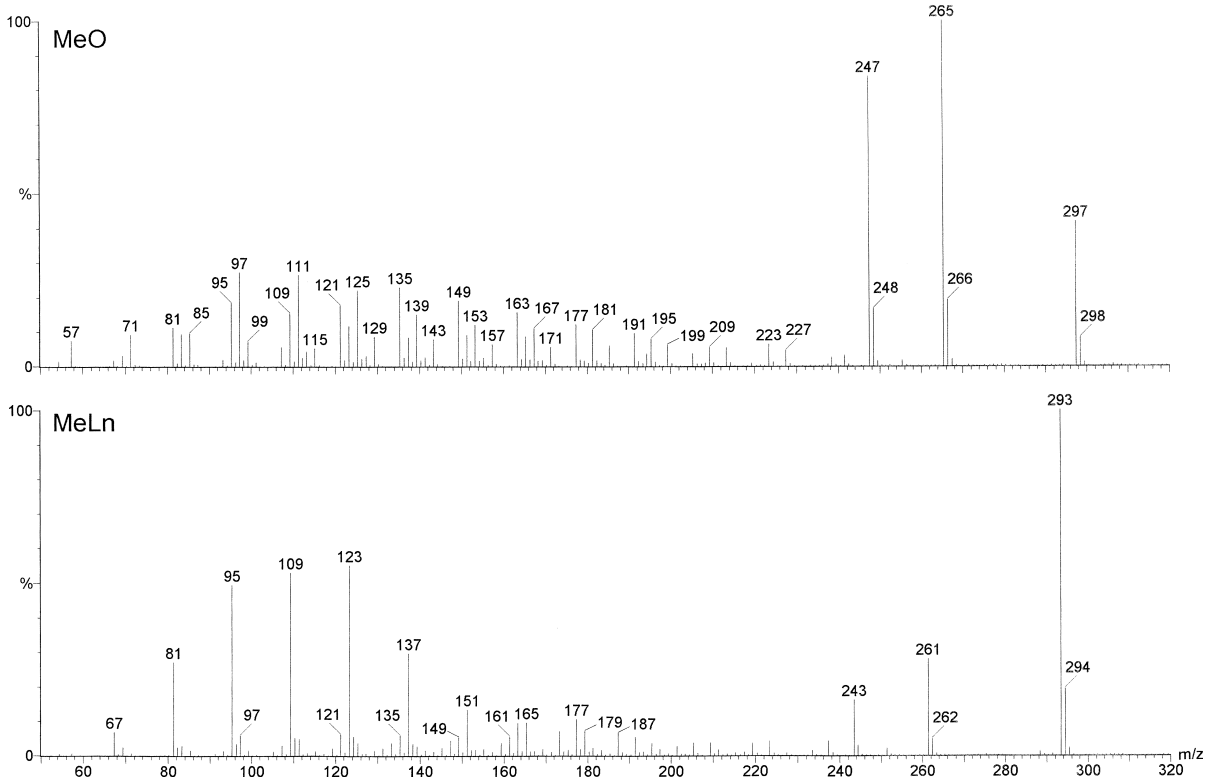

Fig. 9. Positive-ion APCI mass spectra of methyl esters of oleic (MeO) and linoleic (MeLn) acids.

areas multiplied by the correction factors obtained by averaging the slopes of the calibration plots from Table 4 according to the number of the three main acyls, O, L and Ln, in the TGs with equal ECN and the average contents of the acids in the rapeseed oil (60% O, 20% L and 8% Ln). This approach includes significant simplifications in neglecting minor acids present in the rapeseed oil (palmitic, stearic and gadoleic). Nevertheless, the differences between the contents of the individual TGs determined in the individual detection modes diminished with respect to the uncorrected normalized peak areas results, especially for the data evaluated using UV detection. The correction factors had little effect on the results obtained with APCI-MS, especially for the data evaluated in the m/z range 200–1000.

From the results of the present work, APCI-MS compares favorably with UV detection and ELSD as the reproducibility and the LODs are concerned, but further experiments will be necessary for more generally valid conclusions. The sensitivity of APCI-MS based on the data evaluated in the m/z range 200–1000 is appropriate for the determination of the TGs with different ECN in biodiesel products, as the linearity range of this detection method matches with the maximum allowed concentration of residual acylglycerols given by the regulations. Further, APCI-MS offers valuable structural information and possibility of deconvolution of overlapped chromatographic peaks using extracted ion chromatograms.

4. Conclusions

A gradient-elution NARP-HPLC method was developed, which allows the separation and the determination of methyl esters and triacylglycerols in

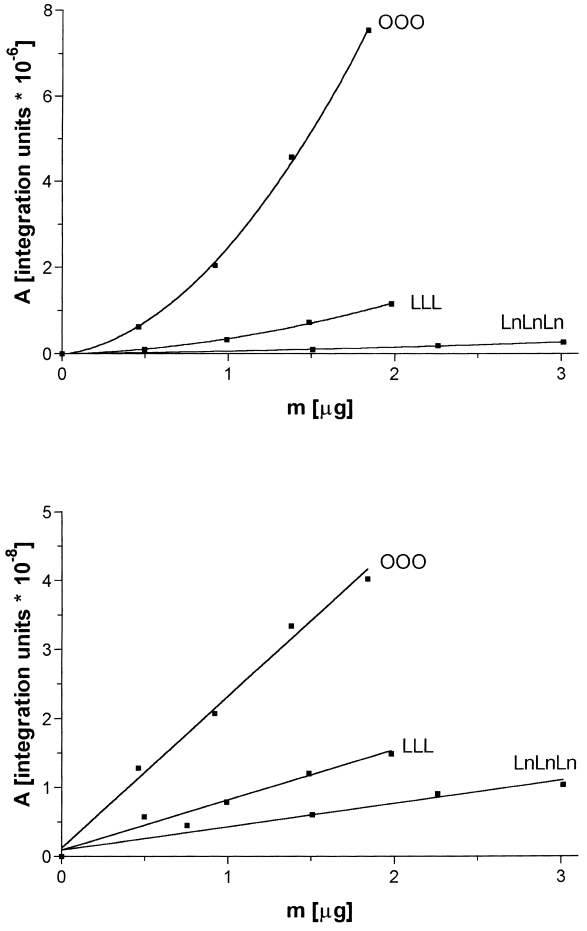

Fig. 10. Calibration curves of pure standards of triolein (OOO), trilinolein (LLL) and trilinolenin (LnLnLn) using HPLC method 1 with ELSD (A) and APCI-MS (B) in the range m/z 200–1000; m=sample amount; A=peak areas.

biodiesel produced from the rapeseed oil by transesterification with methanol. Using a combined aqueous–organic and non-aqueous gradient elution, the resolution is improved, so that the separation of all free acids, methyl esters, mono-, di- and triacylglycerols differing in their ECNs is possible in a single run in approximately 25 min. All individual compounds in sample mixtures can be identified using positive-ion APCI-MS, as the mass spectra yield both protonated molecules and characteristic fragment ions, which facilitates the determination of the structure of individual species. The general fragmentation patterns for the individual classes of compounds in the positive-ion APCI mode are similar as in the electron ionization mass spectra, but the relative abundances of the molecular ions are higher.

The determination of TGs with different ECNs is possible using UV detection at 205 nm, ELSD and APCI-MS, but the contents of the individual acids using normalized peak areas are subject to errors and the results differ for the individual detection methods tested. ELSD is less convenient than the other detection methods with a linear response, as its response should be fitted by quadratic calibration plots. APCI-MS and especially ELSD show a decrease of the sensitivity with increasing number of double bonds in the individual TGs. However, the sensitivity of UV detection is also different for the individual TGs. APCI-MS is the best suited detection mode for the analysis of rapeseed oil and of biodiesel. It elucidates additional structural information on the acylglycerols present in natural oils and in the synthetic products of their transesterification. As the retention increases with increasing equivalent carbon number, the conditions of HPLC method 2 combining aqueous–organic and non-aqueous gradient elution can be used also for the separation of other types of lipid samples. The contribution of two methylene groups to the increase in the retention in the reversed-phase systems employed in this work is slightly higher than the contribution of one double bond to the decrease in the retention. A few coeluted di- and triacylglycerols with the same molecular masses can be resolved by reconstructed ion current chromatograms using adequately selected fragment ions.

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Table 5
Relative amounts of triacylglycerols (in %, v/v, arithmetic means from 15 experiments±standard deviation) in a rapeseed oil sample determined with various detection methods using the normalized peak areas method (the meaning of the correction factors is explained in the text)

	ECN[a]				
	40	42	44	46	48
	Main TGs				
	OLnLn+LLLn	OLLn+LLL	OLL+OOLn	OOL	OOO
Data without correction factors					
UV 205 nm	9.1±0.3	19.3±0.3	33.7±0.5	26.4±0.3	11.5±1.0
ELSD	0.9±0.1	4.6±0.2	20.4±0.5	32.8±0.2	41.4±0.7
APCI-MS[b]	0.8±0.1	3.9±0.2	14.1±0.3	30.7±0.4	50.5±0.8
APCI-MS[c]	2.1±0.3	7.4±0.2	19.4±0.4	30.2±0.3	41.0±0.6
Data with correction factors					
UV 205 nm	4.1±0.3	8.4±0.3	30.4±0.5	30.4±0.3	26.7±1.0
ELSD	1.4±0.1	7.2±0.2	12.0±0.5	36.7±0.2	42.7±0.7
APCI-MS[b]	0.9±0.1	4.3±0.2	14.4±0.3	30.8±0.4	49.6±0.8
APCI-MS[c]	2.1±0.3	7.4±0.2	19.3±0.4	30.2±0.3	41.0±0.6

[a] ECN=Equivalent carbon number.
[b] Data evaluated only for the fragment ions A.
[c] Data evaluated for the mass range m/z 200–1000.

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