



DOCTORAL THESIS

CHARACTERIZATION OF PLANT OILS BASED ON THEIR TRIACYLGLYCEROL CONTENT BY HPLC/APCI-MS AND MALDI-TOFMS

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Paper I
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1. PREFACE

Nutrition has regained prominence in recent years due to the increasing number of cancer and cardiovascular diseases. From the various food products the edible oils play an important role in human nutrition due to their everyday consumption and biologically important compounds present in them. The composition of the different types of plant oils is considerably diverse, but in general they contain significant amount of triacylglycerols (*ca.* 97%) and biologically also important minor compounds. The consumption of the various types of edible oils is rather one-sided and country dependent. In eastern European countries mainly sunflower and corn oils (in addition of lard) are used in everyday nutrition. These oils contain relatively low amount of nutritionally valuable compounds. Some countries have better position in the consumption of important lipids. The Mediterranean diet includes significant amount of olive oil containing relatively high amount of naturally occurring antioxidants such as phenolic compounds. The adulteration of biologically important oils (such as olive) with other cheap oils also makes more difficult the spreading of the good quality oils.

Despite the large number of publications describing the importance of oils in nutrition, biochemistry and also many other areas of science, only few articles are focused on their analysis. This doctoral thesis attempts to perform a comprehensive examination of various types of oils focusing on their triacylglycerol compositions. The influence of the upcoming results on human nutrition is not clear yet, however it is hoped, that more attentions will be paid on the examination of different types of oils in relation to their nutritional values.

2. DIFFERENTIATION OF PLANT OILS BASED ON THEIR TRIACYLGLYCEROL ANALYSIS

2.1. INTRODUCTION

2.1.1. Composition of vegetable oils

The importance of various edible oils in nutrition has regained prominence in recent years. This is mainly due to the biologically important major and minor compounds present in the oils. Major compounds are triacylglycerols (TAGs) present in 95-98%, and minor compounds are different varieties of compounds present in 5-2%, such as wax esters, hydrocarbons, tocopherols, tocotrienols, phenolic compounds, phospholipids, carotenoids, free fatty acids [1-4]. Monoacylglycerols and diacylglycerols are also present in small amounts originated from incomplete triacylglycerol biosynthesis or hydrolysis of triacylglycerols. The quantity and the type of the major and minor compounds depend strongly on the variety of the oil. Thus the analysis of the oils is of great importance, since it characterizes the quality of the oils. The characterization of various types of oils can be performed by analyzing the major [5-9], minor [10,4] or both major and minor compounds [11-12] of the oils.

2.1.2. Structure of triacylglycerols

Triacylglycerols -the major compounds of oils- are esters of glycerol in which each of the three hydroxyl groups is esterified with a fatty acid. Triacylglycerols (and also glycerols) possess reflective symmetry (it can be superimposed on its mirror image) and do not possess rotational symmetry, since a triacylglycerol cannot be superimposed on itself by rotation. The two primary hydroxyl groups are thus distinguishable from each other, and it is feasible to use a stereochemical numbering system (*sn*-1, *sn*-2, *sn*-3) [13]. According to that, if an analytical

technique is not capable to distinguish between the *sn*-1 and *sn*-3 positions, indication has to be used in TAGs names (referring to the asymmetry), e.g. 1(3)-stearoyl-2-linoleoyl-3(1)-palmitoyl glycerol.

Four main type of TAG can be distinguished based on the structure of the molecule (without distinguish the *sn*-1 and *sn*-3 positions):

- (i) AAA-type, homogenous (monoacidic) TAG as trilinoleoyl glycerol (LLL);
- (ii) ABA-type, mixed symmetric TAG containing two different fatty acids as 1,3-dipalmitoyl-2-linoleoyl glycerol (PLP);
- (iii) AAB-type, mixed asymmetric TAG containing two different fatty acids as 1(3), 2-distearoyl-3(1)-oleoyl glycerol (SSO);
- (iv) ABC-type, mixed TAG containing three different fatty acids as 1(3)-linolenoyl-2-linoleoyl-3(1)-palmitoyl glycerol (LnLP).

2.1.3. Composition of plant oil triacylglycerols

Five different fatty acids are predominantly esterified in plant oil triacylglycerols. They are palmitic (16:0), stearic (18:0), oleic (18:1, *cis*-9), linoleic (18:2, *cis*-*cis*-9,12) and linolenic (18:3, *cis*-*cis*-*cis*-9,12,15) acids. Beside of these fatty acids there are many hundreds of others, some of which may contribute to a major proportion in individual seed oils. Examples are erucic acid (22:1, *cis*-13) in rapeseed oil and ricinoleic acid (18:1, *cis*-9, 12-OH) in castor oil [13].

The different fatty acids have stereospecific distribution on the glycerol backbone, rather than a completely random or “restricted random” distribution. Seed oils frequently have (poly)unsaturated fatty acids, such as oleic, linoleic and linolenic acids at the *sn*-2 position. Saturated fatty acids, as palmitic and stearic acids are predominantly found at *sn*-1 and *sn*-3

positions. In TAGs where (poly)unsaturated fatty acids make up more than 1/3 of the total fatty acids there is an “overflow” to *sn*-1 and *sn*-3 positions, with a slight preference for *sn*-1 position [13].

2.1.4. Analysis of plant oil triacylglycerols by conventional techniques

The TAG composition of plant oils is traditionally determined by conventional analytical techniques, including high performance liquid chromatography (HPLC) with refractive index detection (RID) [14] or evaporative light-scattering detector (ELSD) [15,16]; silver ion-HPLC (Ag-HPLC) [17,18] and gas chromatography/flame ionization detector (GC/FID) [19,20]. However these techniques were and some of them are still successfully used for the analysis of plant oil triacylglycerols each technique has its own disadvantage. The main disadvantage of RID is that, it is only compatible with isocratic elution. ELSD is a universal detector compatible with all eluting solvents also in gradient elution, however careful calibration is required for the analysis. The applicability of UV detection is also rather limited, since TAGs have low wavelengths absorbance maximum and considering relatively high background of the commonly used solvents. In silver ion-HPLC the silver ions interact with double bonds. Thus unsaturated TAGs are eluted according to the degree of unsaturation. Beside of this advantage there are some critical TAG pairs, which cannot be separated from each other, such as tripalmitoyl glycerol (PPP) and 1(3),2-dipalmitoyl-3(1)-stearoyl glycerol (PPS) as well as 1(3),2-dilinoleoyl-3(1)-palmitoyl glycerol (PLL) and 1(3),2-dilinoleoyl-3(1)-stearoyl glycerol (SLL). GC with thermally stable columns (approximately. up to 380°C) can also be used for the analysis of oils. The main limitation factor of using this technique is the degradation of high molecular weight unsaturated TAGs, such as trilinolenoyl glycerol (LnLnLn).

2.1.5. Analysis of positional isomer triacylglycerols by mass spectrometry

Analysis of positional isomer TAGs by mass spectrometry started by using electron impact ionization in positive mode (EI) [21]. During the EI fragmentation of TAG molecules loss of acyloxymethylene moiety occurs from the *sn*-1 and *sn*-3 but not from the *sn*-2 position of glycerol, beside of many other cleavages and rearrangements [21]. This makes possible to distinguish the acyl group attached to *sn*-2 position from those attached to *sn*-1 and *sn*-3 positions. Chemical ionization (CI) in negative mode was found to be more sensitive ionization method for analysis of TAGs than EI. In addition it offers the possibility of identification the positional isomers [22-25]. During the fragmentation of TAGs in negative mode CI, the most intense ions are $[M-H]^-$, $[M-H-RCOOH]^-$, $[M-H-RCOOH-74]^-$ and $[M-H-RCOOH-100]^-$. (The structure of the $[M-H-RCOOH-74]^-$ and $[M-H-RCOOH-100]^-$ ions are unknown.) The $[M-H-RCOOH]^-$, $[M-H-RCOOH-74]^-$, $[M-H-RCOOH-100]^-$ fragment ions occurs primarily by the loss of fatty acids from the *sn*-1 and *sn*-3 positions, make it also possible to distinguish the acyl group attached to the *sn*-2 position from those attached to the *sn*-1 and *sn*-3 positions. Despite of this advantage, neither EI nor CI is suitable to couple with separation techniques capable separating complex mixtures of TAGs without any degradation.

The atmospheric pressure chemical ionization (APCI) technique is one of the most often used ionization methods for analysis of TAGs by mass spectrometry and high performance liquid chromatography/mass spectrometry (HPLC/MS). The main reason for this is that APCI yields simple mass spectra from TAGs and also provides the distinction the acyl group attached to the *sn*-2 position from those to the *sn*-1 and *sn*-3 positions [26-33]. APCI spectra of TAGs typically contain protonated molecular ion $[M+H]^+$ and “diacylglycerol” fragment ion(s) $[M+H-RCOOH]^+$. The diacylglycerol fragment ion(s) (F_1 , F_2 and F_3) originates from the protonated molecular ion by the loss of a fatty acid (Figure 1). The probability of the fatty

acid loss from the *sn*-1, *sn*-3 positions are predominate and equally preferable during the fragmentation [28]. The identification of TAGs from the APCI mass spectra is based on the masses of the protonated molecule- and the diacylglycerol fragment ions [34,35]. The positional isomers are identified from the relative abundances of the diacylglycerol fragment ions [28]. For example, in the mass spectrum of ABC-type TAG the least abundant diacylglycerol fragment ion corresponds to the loss of a fatty acid from the *sn*-2 position (F_3 in Figure 1). AAB and ABA types of TAGs can also be distinguished based on the diacylglycerol fragment ions ratio ($[AA]^+/[AB]^+$). This fragment ions ratio is close to 1 (*ca.* 0.9) in the mass spectra of AAB, and much lower than 1 (*ca.* 0.3) in the mass spectra of ABA [28]. This is explained on the same basis as the ratio of the diacylglycerol fragment ions from ABC type TAG.

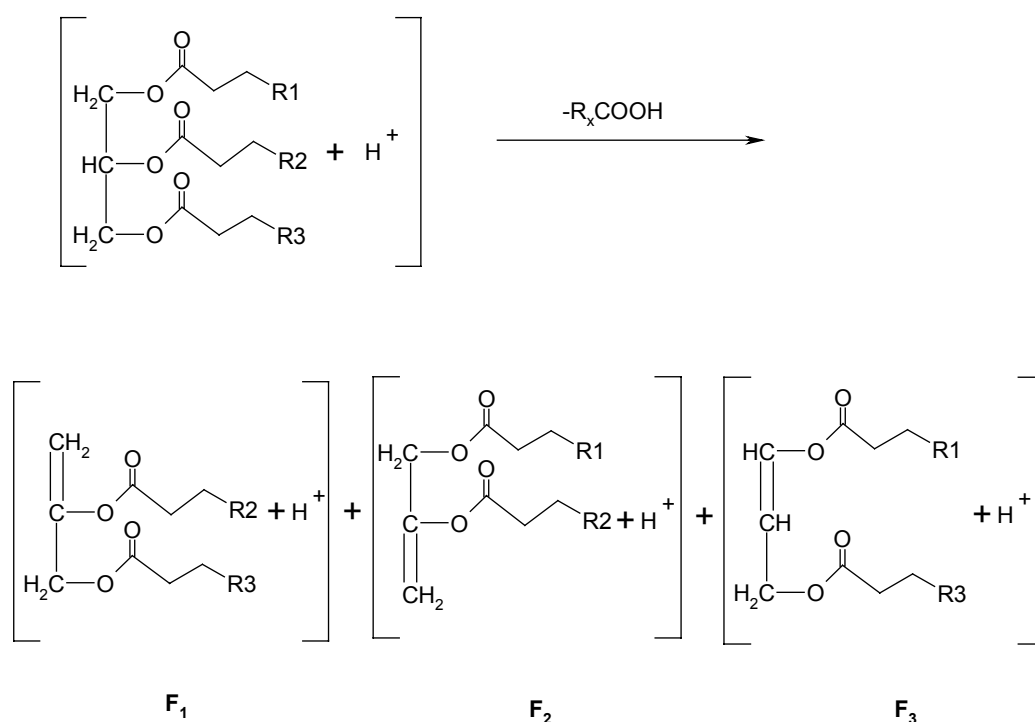


Figure 1. The most dominant APCI fragmentation pathway of triacylglycerols. (The sketched objects depict possible ion structures, but not necessary represent the real ones.)

2.1.6. Analysis of plant oil triacylglycerols by mass spectrometry

HPLC/APCI-MS (high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry) is the most useful technique from the various analytical techniques for analysis of plant oil triacylglycerols. This technique combines the advantages of both the HPLC and the APCI/MS techniques. These advantages are (i) high capability of separating complex mixture of TAGs, (ii) identification of non- or partially resolved HPLC peaks and (iii) identification of various positional isomers [11, 26, 27, 30-32, 36, 37].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) has also been successfully used for analysis of plant oil triacylglycerols [38-41]. The most commonly used MALDI matrices such as 2,5-dihydroxybenzoic acid (DHB), α -cyano-4-hydroxycinnamic acid (CCA) and *trans*-3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid, SA) were applied during the analysis of TAGs. Rarely used matrix such as $K_4Fe(CN)_6$ has also been successfully used for analysis of TAGs [42]. However MALDI-TOFMS is capable for characterization of complex mixture of TAGs, the identification of the positional isomers with this technique has not been reported.

Despite of the huge success and wide application of electrospray ionization (ESI or ES), its application for TAG analysis is still limited [33,43]. This tendency might be linked to the phenomena, that the operation of ESI is based on charge attachment to the target molecules via their transfer from the liquid phase into the gas phase. The considerably apolar TAG molecules undergo such process harder than polar compounds.

Tandem mass spectrometry is an efficient technique for analyzing complex mixtures, thus its application with APCI ionization is a promising tool in the field of oil characterization [33].

2.1.7. Statistical calculations

Mass spectrometric and chromatographic analyses, in most cases, are not adequate to characterize food products according to their chemical composition. Using appropriate multivariate mathematical statistical analysis on the data obtained from the mass spectrometric and/or chromatographic analyses can help to solve the problem. Linear discriminant analysis (LDA), a common multivariate analysis, is one of the most frequently used supervised pattern recognition methods [44]. LDA is designed to find explicit boundaries between given classes, in order to discriminate among them. The combined variable (latent variable) calculated in this way is the linear combination of the original variables. These functions are called roots (or canonical varieties). For better visualization of the results these roots can be plotted against one another in the score plot.

LDA and also PCA (principal component analysis) have been successfully used to classify honey [45], cocoa butter and wine samples [46] based on pyrolysis/mass spectrometry (Py/MS) data. Multivariate statistical analysis on chromatographic data also has been applied successfully to classify various oils such as classification of Cretan olive oils based on triacylglycerol profile measured by HPLC/RID [5] or determination of adulteration in sesame seed oil based also on triacylglycerol profile as measured by HPLC/RID [6]. Evaluation of GC/FID data by statistical calculations for characterization of various oils based on fatty acid and TAG compositions and for detection of adulteration in olive oil was also performed [8,10]. Evaluation of HPLC/APCI-MS data with statistical calculations was also published [11]. This method was used to detect adulteration of olive oil by hazelnut oil.

2.2. EXPERIMENTAL

2.2.1. Materials

Set of cold-pressed oil samples consisting of 6 almond, 4 avocado, 5 corn germ, 11 grape seed, 4 linseed, 3 mustard seed, 11 olive, 4 peanut, 3 pumpkin seed, 3 sesame seed, 5 soybean, 7 sunflower, 2 walnut and 5 wheat germ (73 pieces total) were purchased from local grocery stores and factories (Table 1). The oils, which were purchased from the same factories or stores, were originated from different batches.

HPLC grade acetonitrile and trifluoroacetic acid (TFA) were both obtained from Riedel-de Haën (Seelze, Germany). HPLC grade acetone and chloroform were purchased from Koch-Light (Haverhill, England) and Carlo Erba (Milano, Italy), respectively. 2,5-dihydroxybenzoic acid (DHB) was purchased from Aldrich (Steinheim, Germany). 1(3),2-dioleoyl-3(1)-palmitoyl glycerol (POO), 1,3-dipalmitoyl-2-oleoyl glycerol (POP) and 1(3),2-dioleoyl-3(1)-stearoyl glycerol (SOO) standards were purchased from SIGMA. The purity of the standards were approx. 99%.

Table 1. Type and the source of the various oil samples.

Oil varieties	Source of the samples									Number of samples
	Factory 1	Factory 2	Factory 3	Factory 4	Factory 5	Factory 6	Factory 7	Factory 8	Stores	
Almond	4	1	1							6
Avocado	2		2							4
Corn germ				4					1	5
Grape seed	2	2							7	11
Linseed		2		1	1					4
Mustard seed		1			2					3
Olive									11	11
Peanut		1	2						1	4
Pumpkin seed		1		1	1					3
Sesame seed		1			1				1	3
Soybean		2				3				5
Sunflower				2			2	2	1	7
Walnut		2								2
Wheat germ	3	1		1						5
									Total	73

2.2.2. Instrumentation

2.2.2.1. HPLC/APCI-MS

HPLC/APCI-MS analyses were carried out using a Shimadzu HPLC equipment (Kyoto, Japan) consisting of high-pressure gradient system (LC10-AD, FCV-10AL), autoinjector (SIL-10AD), on-line membrane degasser (DGU-14A) and column oven (CTO-10AS), coupled to a Shimadzu QP2010 mass spectrometer fitted with an APCI source. The APCI capillary, source and block temperatures were 300, 200 and 200°C, respectively, and corona probe high voltage was 4.5 kV. High purity nitrogen was used as nebuliser gas, at a flow rate of 2 L·min⁻¹. CDL (curve desolvation line), Q array and Q array RF potentials were set to -35, 60 and 150 V, respectively. The detector gain was 1.5 kV. Positive ion spectra were recorded over the range of m/z 200-1000, with a scan speed of 1000 amu·sec⁻¹.

The TAGs in oils were separated on an ODS column (Purospher, RP-18e, 125x4 mm, 5 µm, Merck, Darmstadt, Germany) with acetone-acetonitrile eluent system, at a flow rate of 0.6 mL·min⁻¹. Two-stepped linear gradient was applied during the analysis: acetone concentration from 20% to 66% in 3 min, hold at 66% for 13.5 min, then from 66% to 80% in 1 min and finally hold at 80% until 30 min. Autosampler and column oven were set to 20 and 25°C, respectively. The injection volume was 5 µL. Two or three parallel experiments were performed on each analyte.

2.2.2.2. HPLC/APCI-MS/MS and APCI-MS/MS

HPLC/APCI-MS/MS and APCI-MS/MS analyses were carried out using a PE Sciex API 2000 triple-quadrupole mass spectrometer coupled to a Perkin-Elmer HPLC (Perkin-Elmer Sciex, Toronto, Canada). The APCI source temperature was set to 300°C. Needle current was

set to 4 μA . High purity nitrogen was used as nebuliser, auxiliary and curtain gas and was set to 60, 40 and 40 p.s.i., respectively (1 p.s.i.= 6894.76 Pa). The collision gas was also high purity nitrogen and was set to a value of 4 (arbitrary unit). Orifice plate lens (OR) and focusing ring lens (RNG) potentials were set to 96 and 290 V, respectively. Collision energy (defined as the potential differences between Q0 and RO2 lenses) was scanned between -10 and -70 V; and -55 V was the best compromise for intense diacylglycerol ions. The detector gain was 2.0 kV. Positive ion product ion mass spectra of the $[\text{M}+\text{H}]^+$ ions of twelve TAGs (LLL_n: 877.7, LLL: 879.7, LnLP: 853.7, LLO: 881.7, PLL: 855.7, OOL: 883.7, PLO: 857.7, PLP: 831.7, OOO: 855.7, POO: 859.7, POP: 833.7 and SOO: 887.7 Da) were recorded over the range of m/z 20-900, with a scan speed of $200 \text{ amu}\cdot\text{sec}^{-1}$. The product ion spectra of the first nine TAGs were recorded measuring soybean oil by HPLC/APCI-MS/MS using the same chromatographic column and conditions as described above (Chapter 2.2.2.1.). The injection volume was 20 μL . The product ion spectra of POO, POP and SOO were recorded measuring standard solutions ($0.2 \text{ mg}\cdot\text{mL}^{-1}$ in aceton:ACN=2:1) by APCI-MS/MS with continuous flow sample infusion at a flow rate of $20 \mu\text{L}\cdot\text{min}^{-1}$.

2.2.2.3. MALDI-TOFMS

MALDI-TOFMS analysis was carried out on a Bruker BIFLEX mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) in reflectron mode, using a nitrogen laser at 337 nm, accelerating and reflectron voltages of 19.5 and 20.0 kV, respectively.

Positive ion mass spectra were accumulated from 50-80 acquisitions. Minimum of five parallel experiments were performed on each analyte. Spectra were recorded over the range of m/z 0-1300.

2.2.3. Preparation of sample solutions for HPLC/APCI-MS and -MS/MS analyses

Each oil was diluted in acetone/acetonitrile (2:1, v/v) to a concentration of 1% (v/v).

2.2.4. Preparation of sample and matrix solutions for MALDI-TOFMS analysis

Each oil was diluted in chloroform to a concentration of 0.4% (v/v). 10 mg DHB was dissolved in 1 mL acetone containing 0.25% TFA and stored in refrigerator (+5°C). This matrix solution was spotted on the multiprobe target and allowed to dry for 10 s. Afterwards the analyte solution in chloroform was spotted on the top of the matrix [41].

One multiprobe target was used strictly for one analyte solution in order to avoid the accidental mixing of different samples. Between the measurements the targets were thoroughly cleaned. The sample-matrix mixtures were wiped off with strips of cotton soaked with chloroform, acetone and acetonitrile, respectively. After that, the targets were sonicated for 10 minutes in chloroform, acetone and acetonitrile, respectively. Finally the targets were risen with the solvents mentioned above.

2.2.5. Calculations

Statistica 5.5 software package (StatSoft Inc., Tulsa, OK, USA) was applied to complete linear discriminant analysis. LDA calculations were performed on the relative TAGs peaks areas and the relative TAG content of the various plant oils calculated from the ion chromatograms of the HPLC/APCI-MS measurements, and from the spectra recorded from the MALDI-MS measurements, respectively.

2.3. RESULTS AND DISCUSSION

2.3.1. HPLC/APCI-MS analysis of plant oils

Seventy-three edible oil samples (14 different varieties) were analyzed by HPLC/APCI-MS. The possible number of TAGs built from five different fatty acids is 75 (including the positional isomers). Many different TAGs were detected, but the most abundant in the measured oils were twelve different ones with the following possible structures: LLLn, LLL, LnLP, LLO, PLL, OOL, PLO, PLP, OOO, POO, POP and SOO (Table 2, Figure 2-5). One-one total ion chromatograms (TICs) from each type of oil are shown in Figure 2-5.

Table 2. Ions observed in APCI mass spectra of the most abundant TAGs in oils.

TAG	Ions in the APCI mass spectra of TAGs ^a							SIC ^b	
	$[M+H]^+$	$[M+H-R_{1(3)}COOH]^+$	$[M+H-R_{3(1)}COOH]^+$	$[M+H-R_2COOH]^+$	m_1	m_2			
	<i>m/z</i>	F ₁	<i>m/z</i>	F ₂	<i>m/z</i>	F ₃	<i>m/z</i>	<i>m/z</i>	
LLLn	877.7	LL	599.5	LLn	597.5	LLn	597.5	877.1	877.9
LLL	879.7	LL	599.5	LL	599.5	LL	599.5	879.1	879.9
LnLP	853.7	LnL	597.5	LP	575.5	LnP	573.5	853.1	853.9
LLO ^c	881.8	LL	599.5	LO	601.5	LO	601.5	881.2	881.9
PLL	855.7	PL	575.5	LL	599.5	PL	575.5	855.1	855.9
OOL	883.8	OO	603.5	OL	601.5	OL	601.5	601.0	601.9
PLO	857.8	PL	575.5	OL	601.5	PO	577.5	577.0	577.9
PLP	831.7	PL	575.5	PL	575.5	PP	551.5	551.0	551.9
OOO	885.8	OO	603.5	OO	603.5	OO	603.5	603.0	603.9
POO ^d	859.8	PO	577.5	OO	603.5	PO	577.5	577.0	577.9
POP ^d	833.8	PO	577.5	PO	577.5	PP	551.5	577.0	577.9
SOO ^d	887.8	SO	605.5	OO	603.5	SO	603.5	605.0	605.9

^a: $[M+H]^+$ indicates the pseudo-molecular ion, $[M+H-R_{1(3)}COOH]^+$ and $[M+H-R_{3(1)}COOH]^+$ indicate the diacylglycerol fragment ions containing fatty acids in the 2, 3(1) and in the 2, 1(3) positions, respectively; $[M+H-R_2COOH]^+$ indicates the diacylglycerol fragment ion containing fatty acids in the 1, 3 positions. ^b: m_1 and m_2 specify the mass range used for SIC (single ion chromatograms). ^c: The positions of L and O fatty acids were elucidated in Section 4. ^d: The structure was confirmed by comparison with the mass spectra of the respective positional isomer standard.

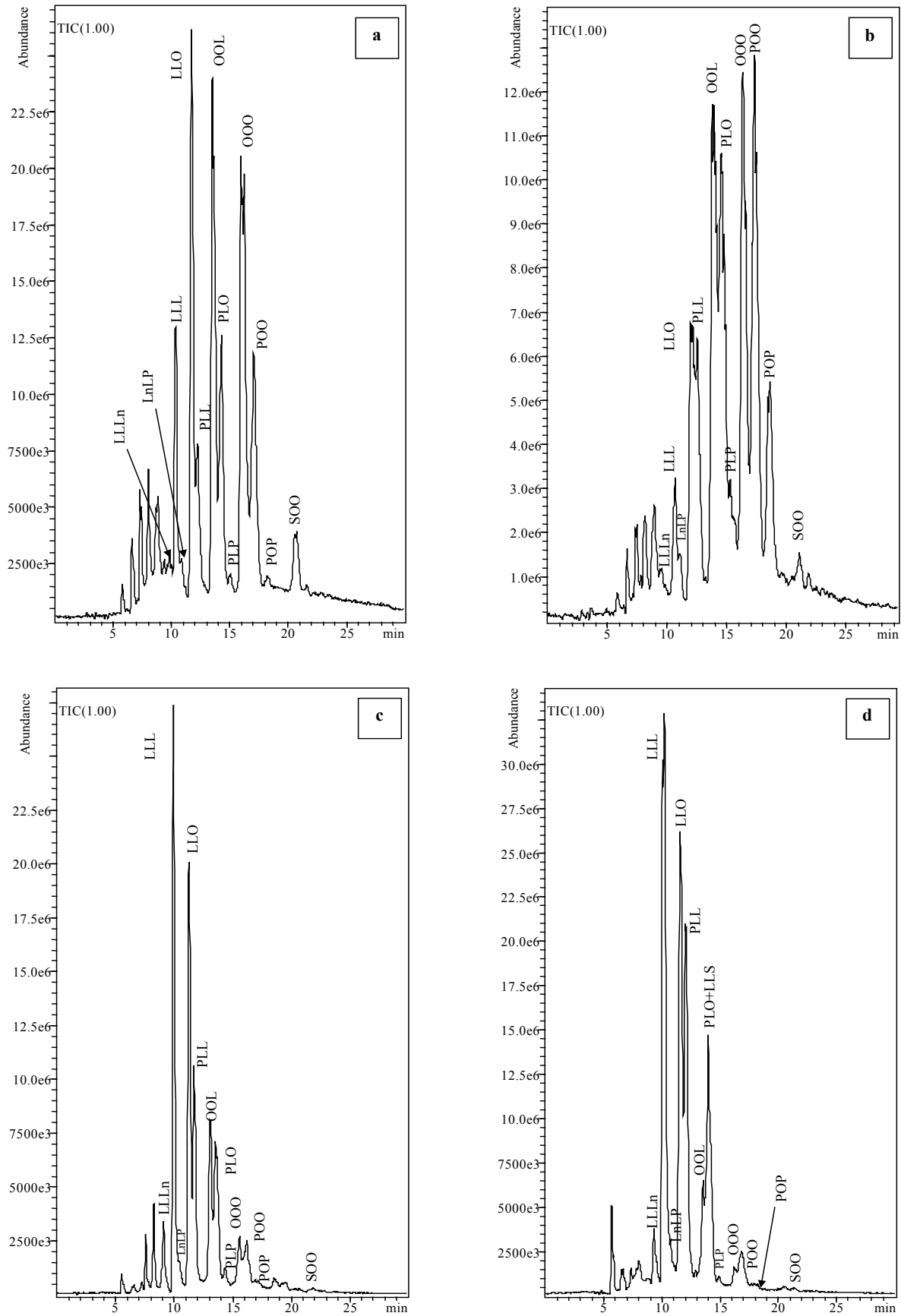


Figure 2. HPLC/APCI-MS profiles of almond oil (a), avocado oil (b), corn germ oil (c) and grape seed oil (d).

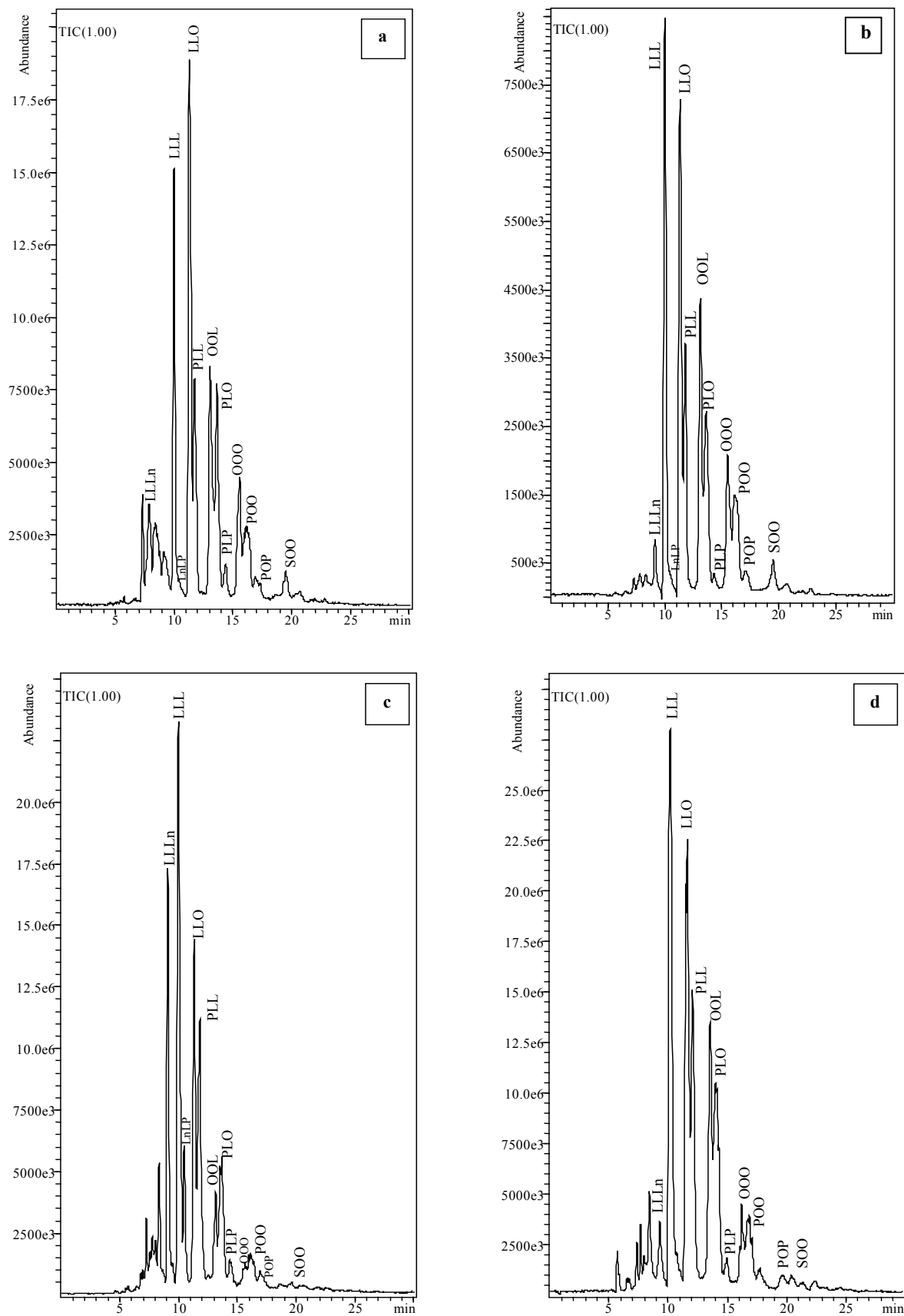


Figure 4. HPLC/APCI-MS profile of pumpkin seed oil (a), sesame seed oil (b) soybean oil (c) and sunflower oil (d).

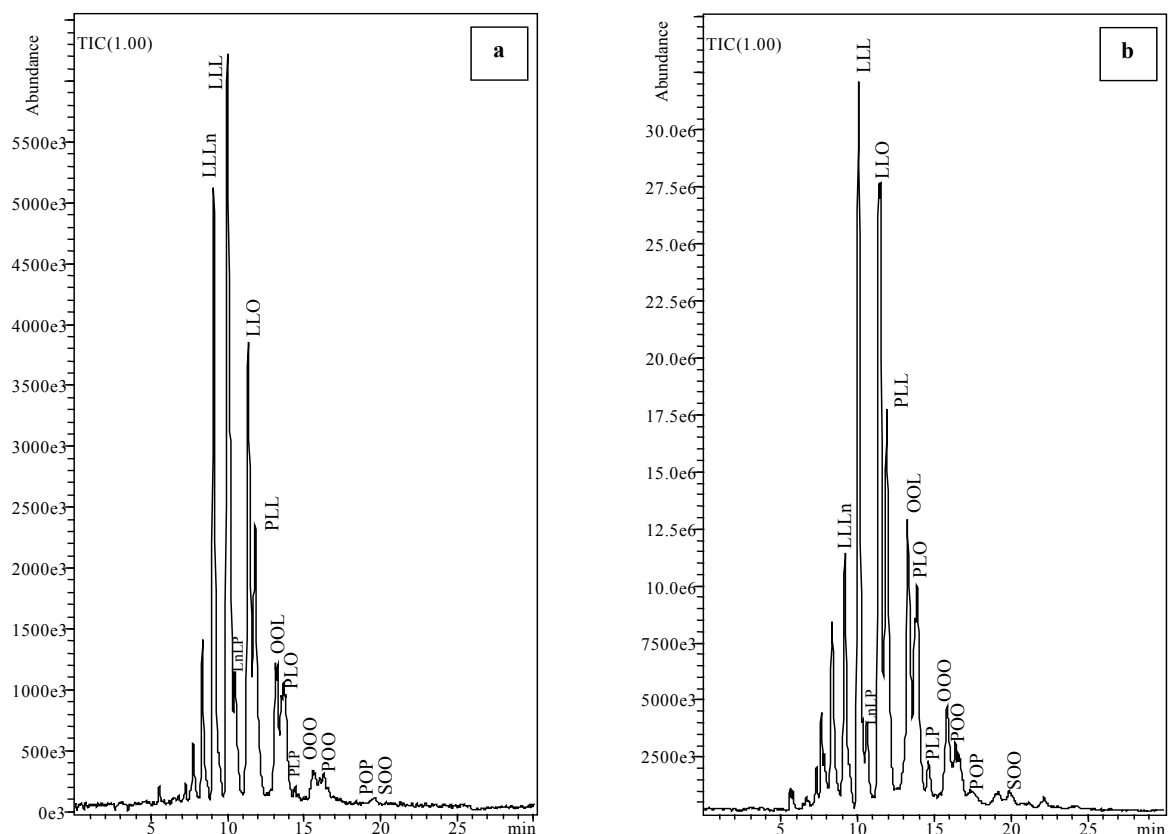


Figure 5. HPLC/APCI-MS profile of walnut oil (a) and wheat germ oil (b).

The possible structure of TAGs were elucidated according to the m/z values of the diacylglycerol ($[M+H-RCOOH]^+$) and the protonated molecular ions $[M+H]^+$ (Table 2), and to the relative abundances of the diacylglycerol ions measured by HPLC/APCI-MS (Section 2.1.5). Product ion mass spectra of the twelve TAGs were also recorded in order to verify the origin of the respective diacylglycerol fragments from the $[M+H]^+$ ions. Figure 6/a and 6/b show single and product ion APCI mass spectra of POP, respectively. The relative abundances of the diacylglycerol fragment ions ($[PP]^+$ and $[PO]^+$) show slight difference (Figure 6/a-b), which might be a consequence of different instrument designs. The verification of the LLO, POO, POP and SOO positional isomers was carried out by analyzing of respective standards under identical conditions by HPLC/APCI-MS (Table 2). Figure 6/c show APCI mass spectrum of POP standard. Figure 6/a and 6/c shown similar spectra. In all cases sodiated, ammoniated *etc.* adducts were not significant.

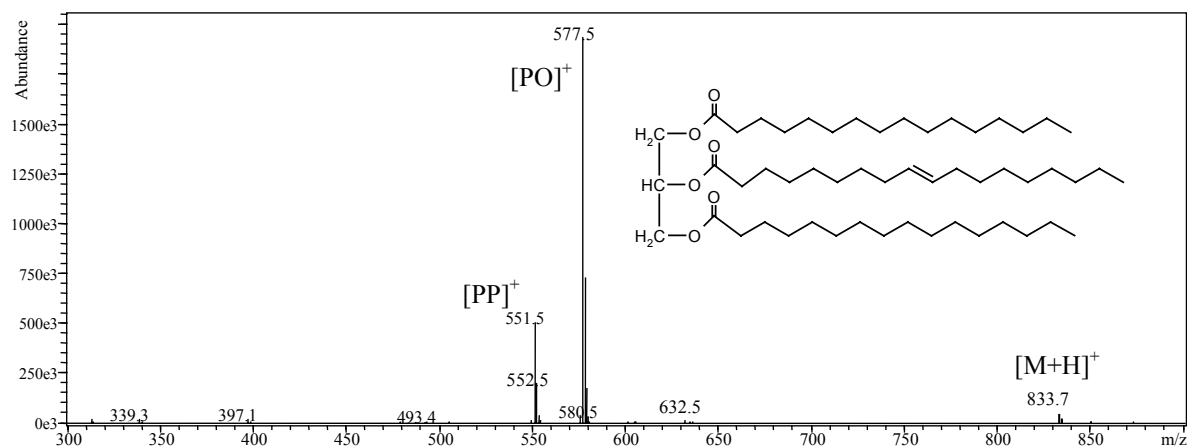


Figure 6/a. APCI mass spectrum of POP from avocado oil, measured by HPLC/MS.

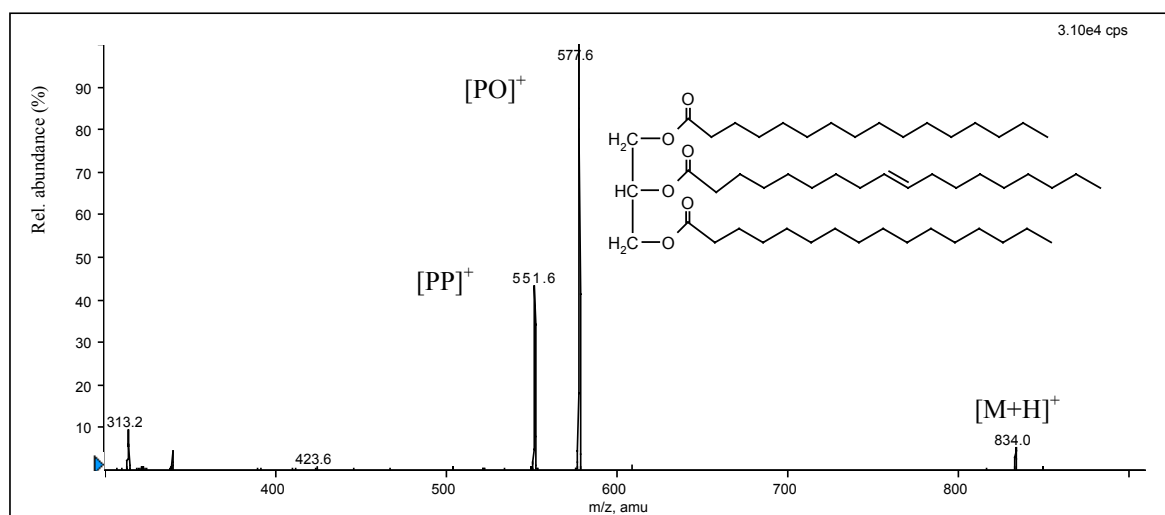


Figure 6/b. Product ion (m/z 833.7, [M+H]⁺) APCI mass spectrum of POP standard measured by direct MS/MS.

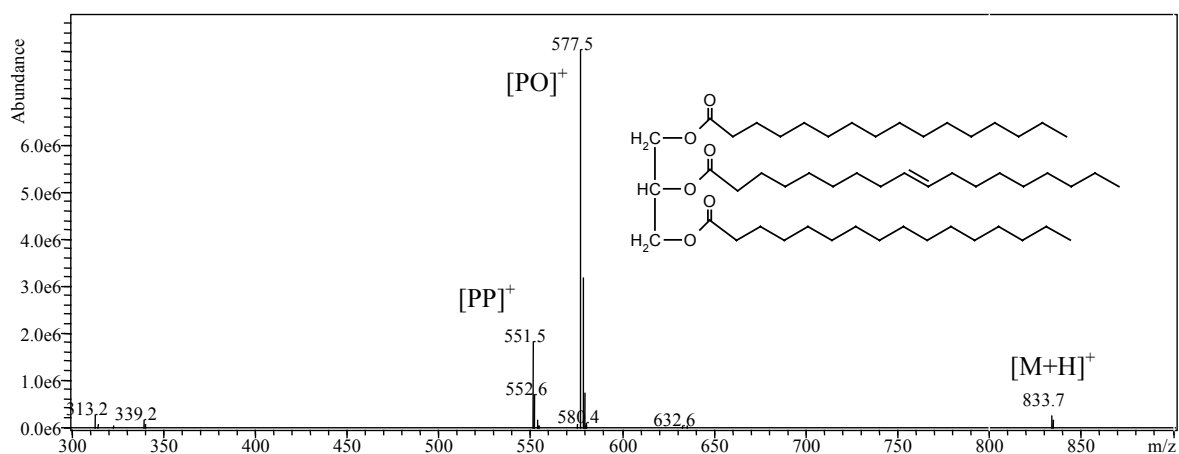


Figure 6/c. APCI mass spectrum of POP standard, measured by HPLC/MS.

The positional isomers were the same in the oils with few exceptions. The distinction between the positional isomers was not obvious in two cases; between LLO and LOL; and between OOL and OLO. The ion abundance of $[\text{OL}]^+ / [\text{LL}]^+$ was slightly larger than 1 in the samples of almond, avocado, grape seed, mustard seed, pumpkin seed, sunflower soybean, walnut and wheat germ oils. It is quite possible that small amount of LOL as well as LLO are present in these oils. The ion abundance ratio of $[\text{OL}]^+ / [\text{OO}]^+$ was also slightly higher than 1 in the samples of almond, pumpkin seed and soybean oils. It is also quite possible that small amount of OLO in addition to OOL are present in these oils. This assumption was examined in Chapter 4. The structures of the other TAGs in the various oils were in good agreement with the published data [5-7,20,27,30,37,47-49].

Identification of positional isomers is generally based on the masses of the protonated molecular and diacylglycerol fragment ions and the ion abundances of the diacylglycerol fragments [11,26-28,30-37]. In our work the above described complementary measurements (MS/MS and measurements of standards) were also performed in order to get more reliable information about the possible structure of the compounds. It should be noted, that despite of these complementary measurements, the aim of this study was not the exact structure elucidation of TAGs. This would require other measurements such as exact mass determination *etc.*

All mass spectra measured by HPLC/APCI-MS showed significant $[\text{M}+\text{H}]^+$ ions. The abundance of protonated molecular ions was strongly dependent on the number of double bonds presented in TAGs, as described previously [33,49]. Highly saturated TAGs showed low intense $[\text{M}+\text{H}]^+$ ions while highly unsaturated TAGs possessed abundant $[\text{M}+\text{H}]^+$ ions. Figure 6/a-b, 7 and 8 show mass spectra of a highly saturated (POP; 53:1, POO; 55:2) and highly unsaturated (LnLP, 55:5) TAGs with high and low $[\text{M}+\text{H}]^+$ ion abundance,

respectively. The applied potential on the Q-array lens (corresponds to in-source collision-induced dissociation region of the instrument) influenced also the relative intensity of the $[M+H]^+$ and the diacylglycerol fragment ions. In HPLC/APCI-MS experiments 60 V was the optimum value of the Q-array lens potential. This value of the Q-array lens potential was the best compromise for repeatable and intense both $[M+H]^+$ and fragment ion formation.

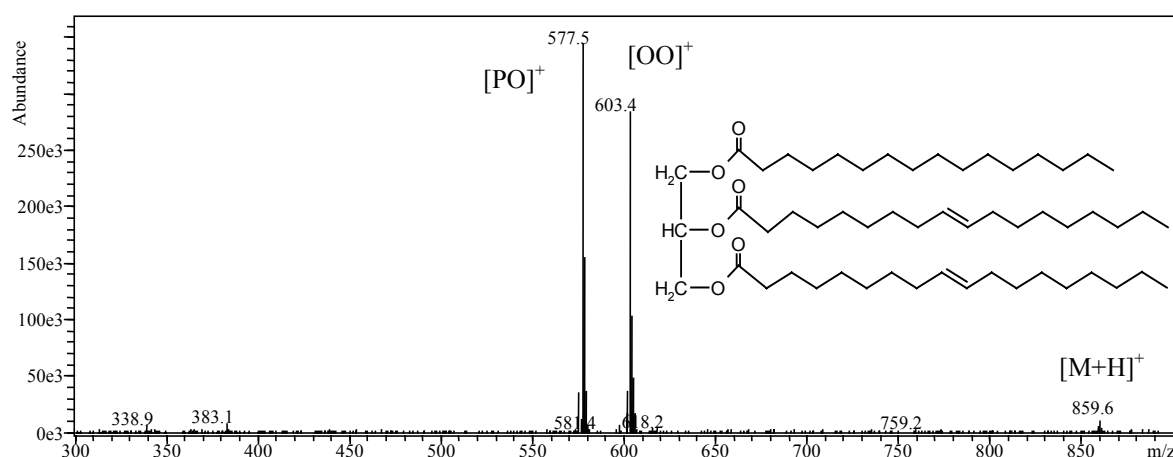


Figure 7. APCI mass spectrum of 1(3)-palmitoyl-2,3(1)-dioleoyl glycerol (POO) from vegetable oil.

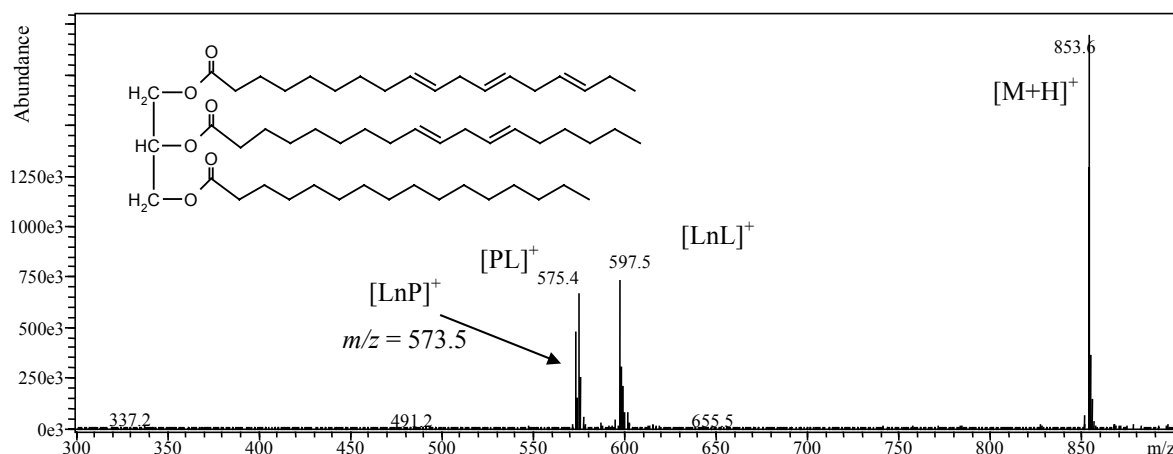


Figure 8. APCI mass spectrum of 1(3)-linolenoyl-2-linoleoyl-3(1)-palmitoyl glycerol (LnLP) from vegetable oil.

The TAGs were eluted within 21 min (Figure 2-5, Table 3) and total run time was set to 30 min. The SD values of the retention times were low indicating the good repeatability of the measurements (Table 3). Considerable amount of TAGs containing unsaturated fatty acid (oleic acid) as OOO, OOL was found in almond, avocado, olive and grape seed oils (Figure 2/a,b,d, 3/c). Considerable amount of TAGs containing polyunsaturated fatty acids (linoleic and linolenic acids) as LLLn, LLL and LLO were found in corn germ, grape seed, mustard seed, pumpkin seed, sesame seed, soybean, sunflower, walnut and wheat germ oils (Figure 2/c,d 3/b, 4/a-c, 5/a,b).

Table 3. The most abundant TAGs found in oils by HPLC/APCI-MS (Purospher RP-18 column 125x4mm, 5 μ m, in acetone/acetonitrile gradient @ 0.6 mL \cdot min $^{-1}$).

TAG	Molecular mass (Da)	CN:DB ^a	t _r mean \pm SD ^b (min)
LLLn	876.7	57:7	9.23 \pm 0.22
LLL	878.7	57:6	10.09 \pm 0.21
LnLP	852.7	55:5	10.56 \pm 0.18
LLO ^c	880.8	57:5	11.47 \pm 0.26
PLL	854.7	55:4	11.92 \pm 0.30
OOL	882.8	57:4	13.31 \pm 0.32
PLO	856.8	55:3	13.95 \pm 0.38
PLP	830.7	53:2	14.70 \pm 0.45
OOO	884.8	57:3	15.77 \pm 0.45
POO ^c	858.8	55:2	16.64 \pm 0.53
POP ^c	832.8	53:1	17.67 \pm 0.64
SOO ^c	886.8	57:2	19.95 \pm 0.70

^a: carbon number:double-bond number, ^b: the mean value and standard deviation of the retention time, ^c: The retention time was confirmed by comparing to a respective positional isomer standard.

Single ion chromatograms (SICs) were created by plotting the ion chromatograms of one of the most intense peak present in the mass spectra of the twelve TAGs (LLLn, LLL, LnLP, LLO, PLL, OOL, PLO, PLP, OOO, POO, POP and SOO). The mass ranges used for SICs are shown in the last two column of Table 2.

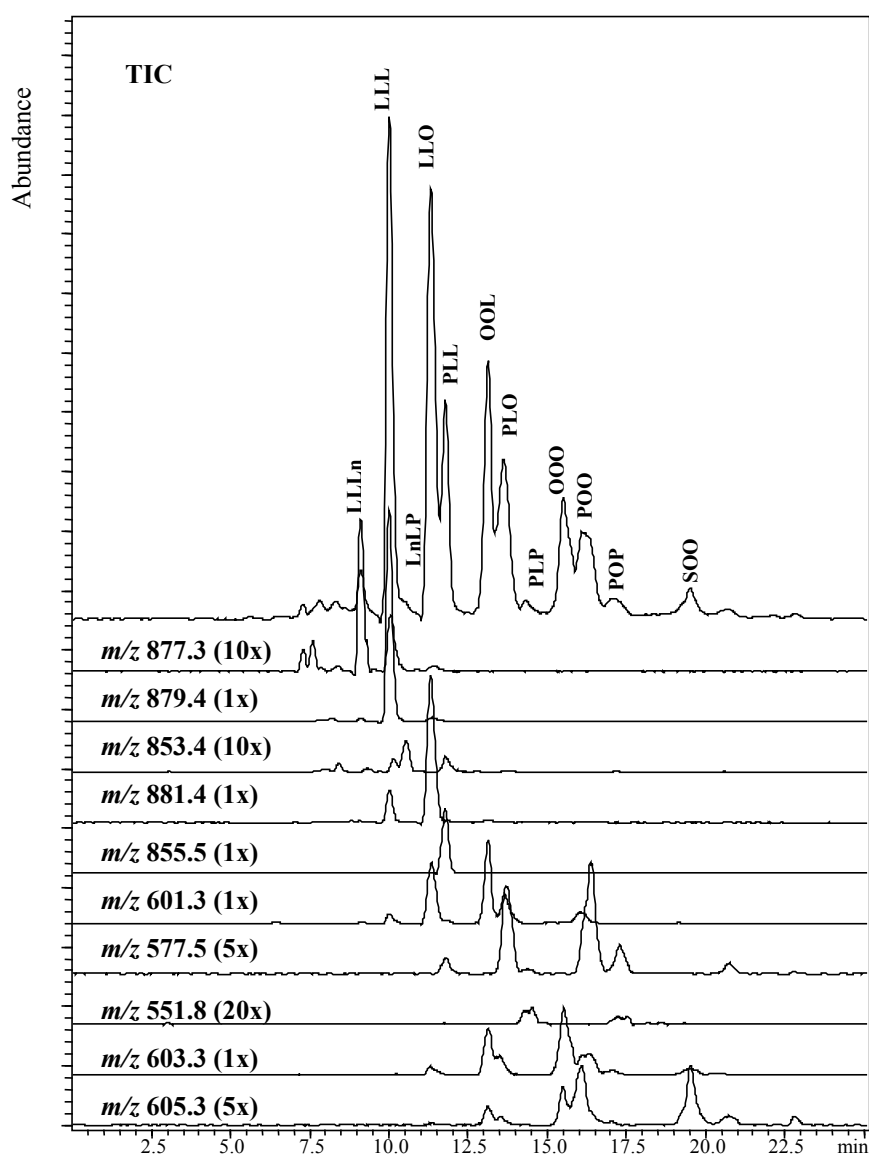


Figure 9. Total ion chromatogram (TIC) and single ion chromatograms (SIC) of a sesame seed oil measured by HPLC/APCI-MS.

SICs in the case of LLLn, LLL, LnLP, LLO and PLL were created by plotting ion chromatograms of adequate protonated molecular ion peaks due to their relatively high abundances (Table 2, Figure 7). SICs in the case of OOL, PLO, PLP, OOO, POO, POP and SOO were created by plotting ion chromatograms of one of the diacylglycerol fragment ion peaks (Table 2). The latter mentioned triacylglycerols were highly saturated and due to that the intensity of the pseudo-molecular ion peak was not sufficiently high in each case (Figure 8). Total ion and single ion chromatograms of one of the measured sesame seed oil are shown in Figure 9.

The HPLC/APCI-MS measurements of each oil were performed in order to calculate relative peak areas of TAGs for the statistical calculation. These relative peak areas were calculated by the individual TAG peak areas normalized to the sum of the selected twelve TAG peak areas. The values of the calculated relative TAGs peaks areas in the various oils are shown in Table 4. The RSD values of small and large peaks were around 15 and 4%, respectively (data are not presented here).

Table 4. *The mean values of the relative TAGs peaks areas (%) in various plant oils, calculated from the HPLC/APCI-MS measurements.*

Sample	TAG											
	LLLn	LLL	LnLP	LLO	PLL	OOL	PLO	PLP	OOO	POO	POP	SOO
Almond 1	0,58	6,05	0,20	10,41	2,04	21,08	3,87	0,83	40,22	10,26	0,95	3,50
Almond 2	0,49	7,86	0,19	9,35	2,21	18,01	2,95	0,57	43,70	9,96	1,21	3,50
Almond 3	0,53	10,57	<0.10	7,66	2,42	13,57	1,66	0,39	48,59	9,58	0,85	4,18
Almond 4	0,45	11,09	<0.10	7,67	2,57	13,14	1,66	0,36	47,84	10,44	0,93	3,86
Almond 5	<0.10	3,92	<0.10	11,08	2,35	22,43	3,45	0,44	44,40	8,79	0,46	2,69
Almond 6	0,27	7,10	<0.10	4,73	1,66	15,22	1,83	0,11	53,57	9,37	0,83	5,23
Avocado 1	<0.10	0,83	1,05	2,46	2,35	10,27	11,14	4,54	32,00	23,39	10,78	1,18
Avocado 2	<0.10	0,84	1,32	2,36	2,60	9,68	12,26	3,94	31,88	24,80	9,28	1,03
Avocado 3	<0.10	0,80	1,29	2,88	2,63	10,71	12,31	3,06	32,80	23,62	9,20	0,72
Avocado 4	<0.10	0,38	0,26	1,62	1,56	9,46	14,19	1,92	33,16	25,87	10,52	0,96
Corn germ 1	1,05	30,25	0,38	24,91	12,03	12,60	3,83	1,52	8,11	3,33	0,86	1,13
Corn germ 2	1,49	31,74	0,36	20,44	8,95	16,06	4,64	1,89	8,47	3,49	1,16	1,31
Corn germ 3	1,45	32,84	0,52	24,65	11,33	11,73	4,17	1,68	7,38	2,95	0,76	0,73
Corn germ 4	1,60	41,38	0,46	30,54	12,51	6,45	1,92	0,09	3,43	0,95	0,19	0,48
Corn germ 5	3,26	39,82	0,97	33,23	15,98	2,90	1,01	0,08	1,82	0,67	0,13	0,21

Sample	TAG											Table 4 cont.
	LLLn	LLL	LnLP	LLO	PLL	OOL	PLO	PLP	OOO	POO	POP	SOO
Grape seed 1	5,26	39,95	1,22	19,76	14,85	7,23	4,13	0,66	3,68	1,84	0,60	0,82
Grape seed 2	4,81	41,19	1,34	20,41	14,35	7,09	4,03	0,76	3,05	1,91	0,45	0,60
Grape seed 3	4,53	38,41	0,99	22,20	16,93	6,52	4,31	0,77	2,92	1,47	0,44	0,50
Grape seed 4	2,11	30,32	0,37	18,94	13,56	11,37	6,09	0,21	6,83	6,78	1,74	1,67
Grape seed 5	2,32	36,63	0,35	19,20	13,21	11,89	5,80	0,22	4,84	3,44	0,95	1,18
Grape seed 6	3,24	32,15	0,50	19,14	12,65	12,06	5,99	0,30	8,15	3,33	0,72	1,77
Grape seed 7	3,56	33,72	0,74	20,45	13,16	12,35	5,81	0,34	5,42	2,64	0,79	1,02
Grape seed 8	2,94	39,18	0,52	18,21	15,19	11,77	5,75	0,30	2,92	1,92	0,57	0,75
Grape seed 9	3,32	38,05	0,64	19,78	15,08	10,75	5,53	0,24	3,01	2,18	0,56	0,86
Grape seed 10	3,30	33,37	0,53	21,44	14,18	11,16	5,97	0,20	5,90	2,40	0,71	0,85
Grape seed 11	1,53	34,86	0,34	19,66	14,22	11,32	6,28	0,23	6,53	3,23	0,75	1,06
Linseed 1	23,69	26,37	4,20	18,27	7,66	8,03	2,15	1,03	5,03	1,87	0,38	1,32
Linseed 2	28,67	25,94	4,85	17,07	6,12	6,57	1,67	0,61	4,86	1,74	0,36	1,55
Linseed 3	36,65	26,99	5,04	16,04	5,69	3,81	0,98	0,36	3,08	0,76	0,18	0,42
Linseed 4	41,09	21,10	6,94	17,22	5,28	2,58	0,79	0,37	2,52	1,10	0,24	0,78
Mustard seed 1	1,08	40,12	0,40	31,82	9,94	7,49	1,79	0,15	4,72	1,27	0,32	0,91
Mustard seed 2	1,78	50,50	0,45	24,81	9,42	5,75	2,01	0,24	3,63	0,81	0,21	0,60
Mustard seed 3	10,85	33,84	3,88	19,62	12,93	5,50	3,46	0,26	6,03	2,62	0,19	1,01
Olive 1	n.d. ^a	0,52	<0.10	2,80	0,76	10,87	2,47	0,45	52,61	18,87	4,61	6,30
Olive 2	0,22	2,15	0,22	6,92	1,95	11,34	2,18	0,58	46,43	18,14	3,85	6,07
Olive 3	0,09	1,49	<0.10	4,82	2,04	11,02	4,17	1,90	42,51	20,49	6,38	5,52
Olive 4	0,06	0,42	0,19	3,75	1,21	10,49	3,11	0,69	49,56	21,10	4,85	4,79
Olive 5	n.d. ^a	0,31	<0.10	1,94	0,45	11,89	2,29	0,09	53,47	18,55	3,81	7,20
Olive 6	n.d. ^a	1,97	0,38	8,40	6,33	14,55	7,74	0,73	30,43	18,64	7,21	3,63
Olive 7	n.d. ^a	1,05	0,22	5,54	1,83	11,79	3,88	0,13	47,97	18,77	4,35	4,48
Olive 8	n.d. ^a	1,01	0,06	5,34	1,57	10,52	2,55	0,20	52,08	16,78	4,36	5,53
Olive 9	n.d. ^a	0,54	<0.10	4,42	1,25	8,45	1,87	<0.10	59,36	15,73	2,48	5,84
Olive 10	n.d. ^a	1,91	<0.10	9,75	1,48	6,83	0,30	<0.10	63,24	13,05	1,53	1,91
Olive 11	n.d. ^a	0,20	<0.10	1,29	0,44	7,64	2,68	0,14	54,64	20,81	4,77	7,36
Peanut 1	4,33	8,86	0,74	6,65	2,22	13,14	1,82	0,60	46,10	10,13	1,32	4,08
Peanut 2	n.d. ^a	2,69	0,29	8,54	2,99	17,45	11,87	2,38	34,26	11,84	2,82	4,87
Peanut 3	n.d. ^a	10,17	0,30	17,78	6,47	13,33	1,48	0,18	37,27	7,53	0,96	4,53
Peanut 4	4,26	17,83	3,34	33,68	12,13	7,71	1,37	<0.10	14,08	3,47	0,53	1,59
Pumpkin seed 1	<0.10	19,42	<0.10	21,11	9,53	17,20	4,48	3,03	15,14	5,17	1,80	3,14
Pumpkin seed 2	<0.10	20,47	<0.10	17,81	10,29	18,57	4,40	2,64	15,37	5,70	1,35	3,40
Pumpkin seed 3	<0.10	20,56	<0.10	20,95	10,95	17,37	4,08	2,73	14,28	5,22	1,27	2,61
Sesame seed 1	1,08	21,38	0,15	14,80	11,68	15,82	5,02	0,53	19,28	5,91	1,33	3,02
Sesame seed 2	1,46	26,66	0,41	23,40	9,26	11,84	3,35	0,17	14,76	5,37	0,82	2,49
Sesame seed 3	0,34	12,56	0,11	13,94	5,47	22,59	6,69	0,75	20,70	8,30	2,57	5,97
Soybean 1	15,74	27,35	7,05	13,83	11,23	8,47	3,76	3,58	4,02	2,48	1,16	1,33
Soybean 2	16,96	26,35	6,36	14,63	12,13	8,44	3,77	3,77	3,55	2,20	0,92	0,93
Soybean 3	17,86	32,03	5,92	15,40	12,28	5,51	2,93	2,28	2,58	1,85	0,80	0,56
Soybean 4	18,54	33,03	4,62	17,39	9,41	7,18	3,01	1,89	3,70	2,01	0,53	0,64
Soybean 5	16,97	40,93	4,78	20,63	10,32	2,98	0,86	1,00	1,38	0,55	0,18	0,44
Sunflower 1	<0.10	29,34	n.d. ^a	21,96	10,90	15,69	3,74	1,68	10,73	2,84	0,63	2,48
Sunflower 2	0,87	31,70	n.d. ^a	21,48	10,40	16,39	4,50	1,88	8,24	2,57	0,59	1,38
Sunflower 3	<0.10	33,59	n.d. ^a	28,96	12,49	11,76	3,65	1,54	4,80	1,68	0,48	1,04
Sunflower 4	<0.10	35,34	n.d. ^a	27,42	11,57	12,55	3,47	1,40	5,68	1,61	0,29	0,68
Sunflower 5	<0.10	30,34	n.d. ^a	25,01	10,66	16,96	4,24	1,61	6,33	2,56	0,60	1,70
Sunflower 6	1,01	34,41	0,03	28,16	11,52	12,47	3,25	0,22	5,78	1,93	0,27	0,94
Sunflower 7	0,27	28,42	n.d. ^a	18,76	10,26	20,39	5,67	0,63	8,77	3,61	0,91	2,31
Walnut 1	20,92	30,62	6,28	13,75	9,46	8,19	2,80	1,06	4,53	1,42	0,35	0,62
Walnut 2	26,44	38,11	3,36	11,23	6,71	9,22	2,97	0,80	5,16	1,43	0,19	0,05
Wheat germ 1	5,01	32,99	2,39	23,16	9,41	11,28	3,08	2,04	7,60	1,85	0,38	0,81
Wheat germ 2	4,43	26,40	1,94	24,26	11,43	12,68	3,68	2,38	8,80	2,25	0,61	1,15
Wheat germ 3	14,08	25,86	7,79	12,69	15,41	5,03	3,67	5,63	5,27	2,75	1,45	0,36
Wheat germ 4	12,57	23,30	6,11	12,74	12,82	11,68	6,19	1,49	6,49	3,54	1,59	1,48
Wheat germ 5	5,01	29,18	2,08	17,76	10,31	18,19	5,20	0,86	6,33	2,79	0,75	1,53

^a not detected; data were calculated from two or three parallel experiments at each oil.

The relative TAGs peaks areas were quite similar values in the same variety of oils with few exceptions *e.g.* peanut oils and wheat germ oil samples (Table 4). Almond oils have high relative LLL, LLO, OOL, OOO and POO contents. Avocado oils contained relatively high saturated fatty content esterified on the glycerol backbone such as PLO, POO and POP. Corn germ, grape seed, mustard seed, soybean, sunflower, walnut and wheat germ oils were similar to each other due to their relatively high LLL, LLO, PLL and OOL contents. Corn germ, grape seed, mustard seed, sesame seed, soybean, sunflower, walnut and wheat germ oils are also called trilinolein-rich (high-trilinolein) oils. Almond, avocado, linseed, olive and peanut oils contained low level of trilinolein and due to that they are called low-trilinolein oils. Linseed oils have the highest polyunsaturated TAG content (LLL_n). The other oil varieties namely olive, peanut, pumpkin seed and sesame seed oils together with almond and avocado oils have relatively high OOO content.

2.3.2. MALDI-TOFMS analysis of plant oils

The same 73 edible oil samples (14 different varieties) were analyzed by MALDI-TOFMS. MALDI produced predominantly sodiated pseudo-molecular ions from the oil samples. The masses of these ions and the APCI-MS results served as base for the identification of the TAG compounds. Comparing the TAG intensities of the measured oils the most abundant TAGs were PLL, PLO, POO, LLL_n, LLL, LLO, OOL and OOO. The other TAGs -chosen at the data evaluation at APCI-MS (LnLP, PLP, POP and SOO)- had low intensities because of their relatively low concentrations. Good shot-to-shot repeatability was achieved by using DHB as matrix. MALDI-TOF spectra of one-one samples from the examined oil varieties are shown in Figure 10-13, in the range of m/z 860-940.

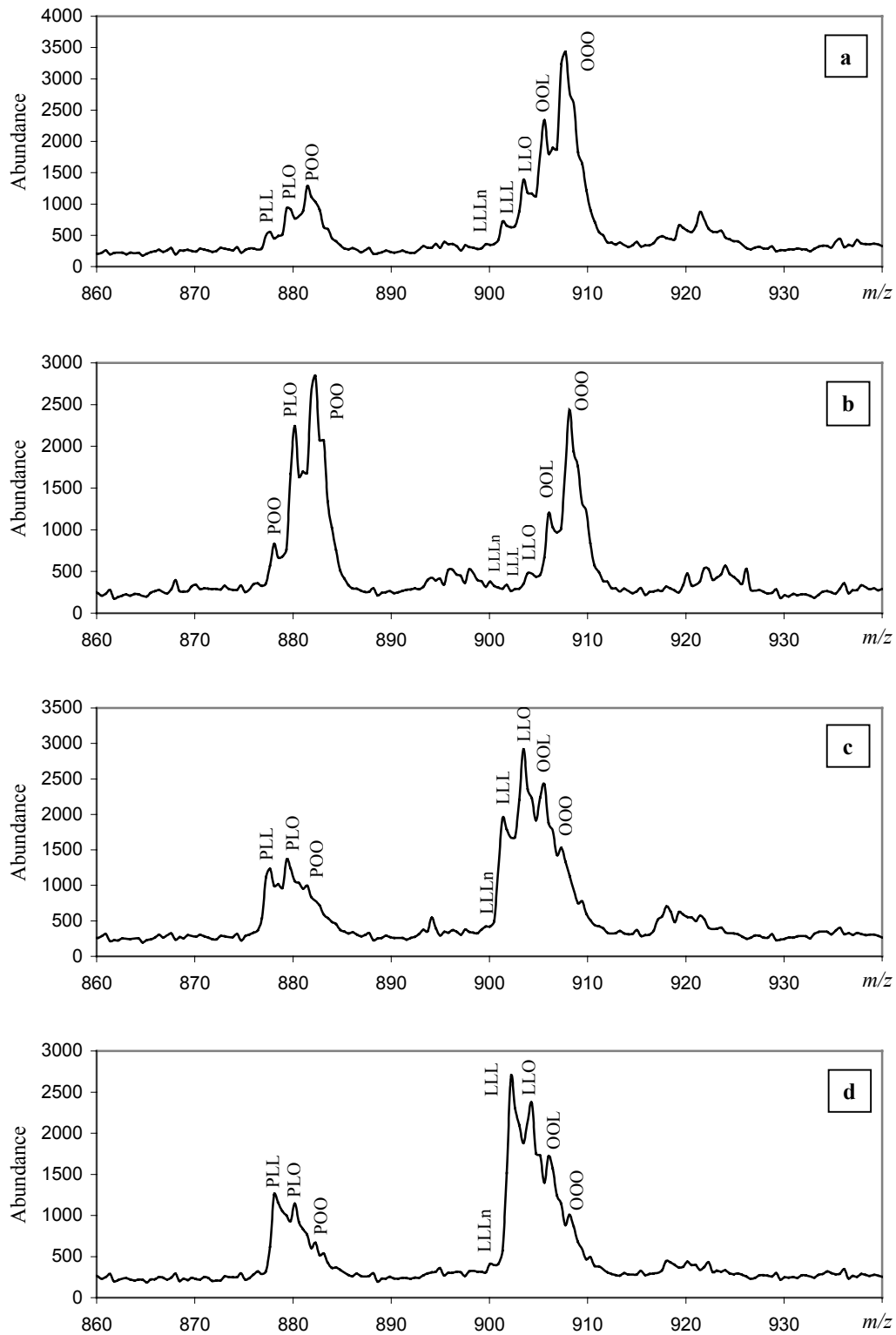


Figure 10. MALDI-TOF mass spectra of almond oil (a), avocado oil (b), corn germ oil (c) and grape seed oil (d); showing sodiated TAG peaks.

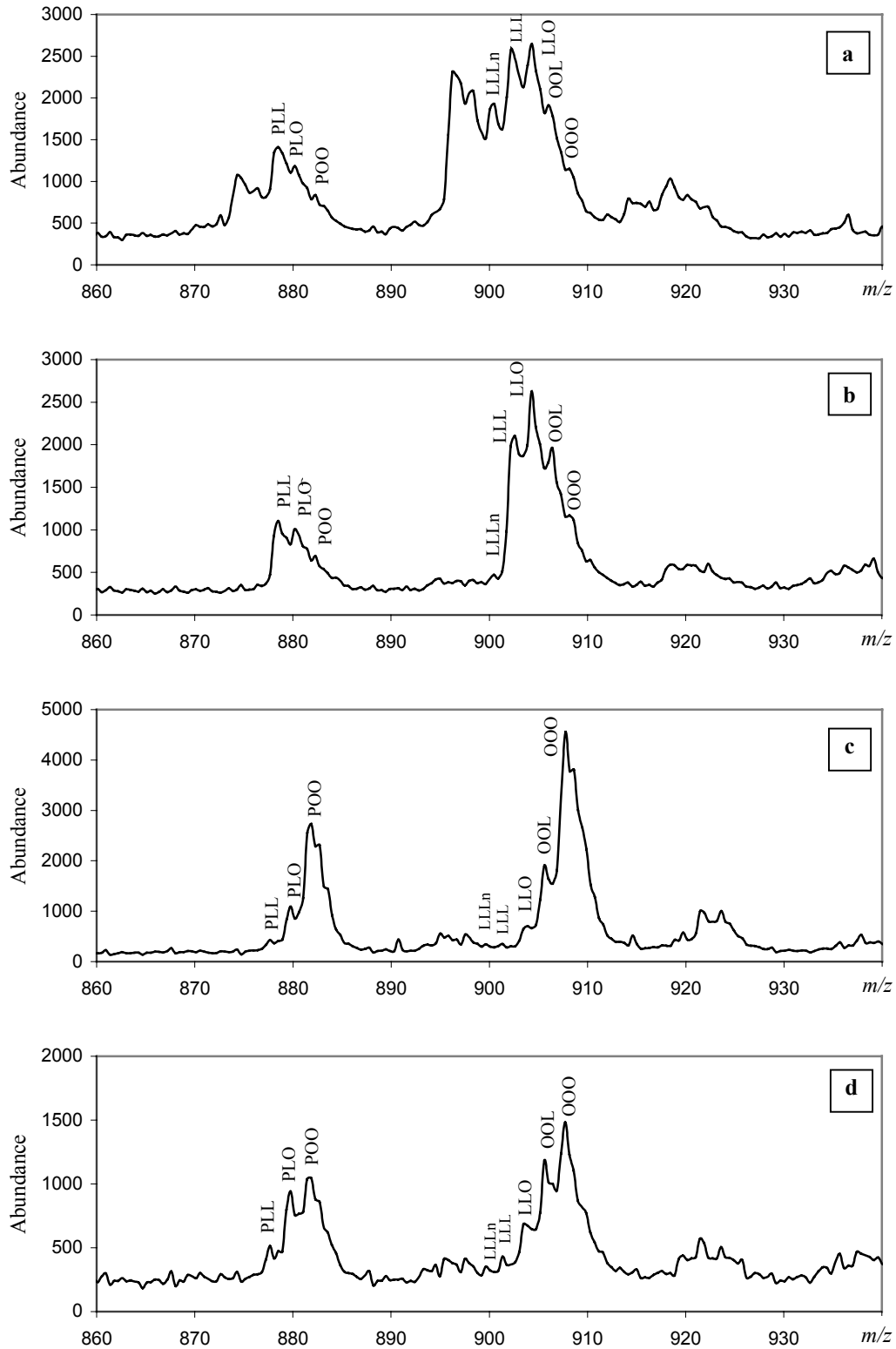


Figure 11. MALDI-TOF mass spectra of linseed oil (a), mustard seed (b), olive oil (c) and peanut oil (d); showing sodiated TAG peaks.

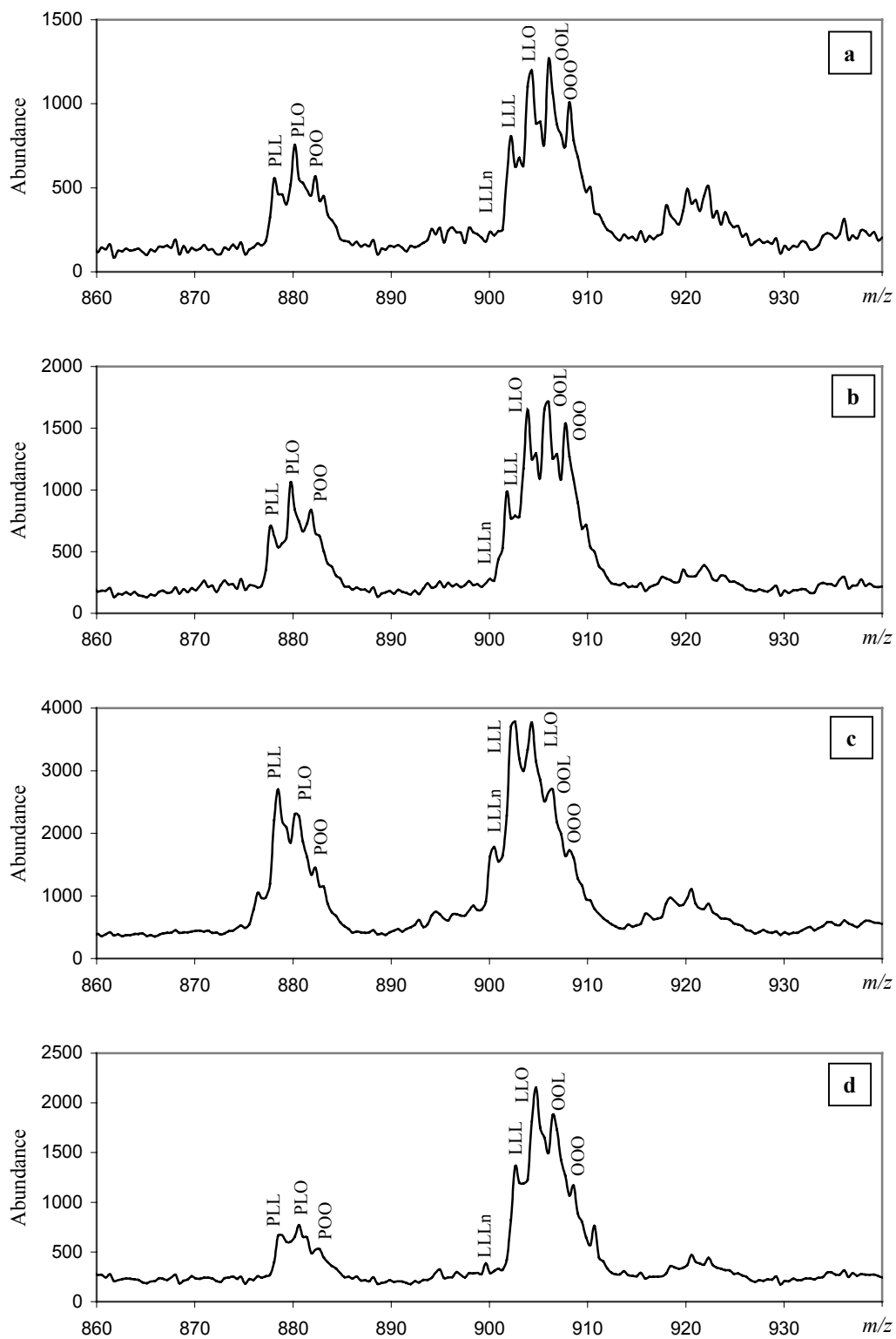


Figure 12. MALDI-TOF mass spectra pumpkin seed oil (a), sesame seed oil (b), soybean oil (c) and sunflower oil (d); showing saponified TAG peaks.

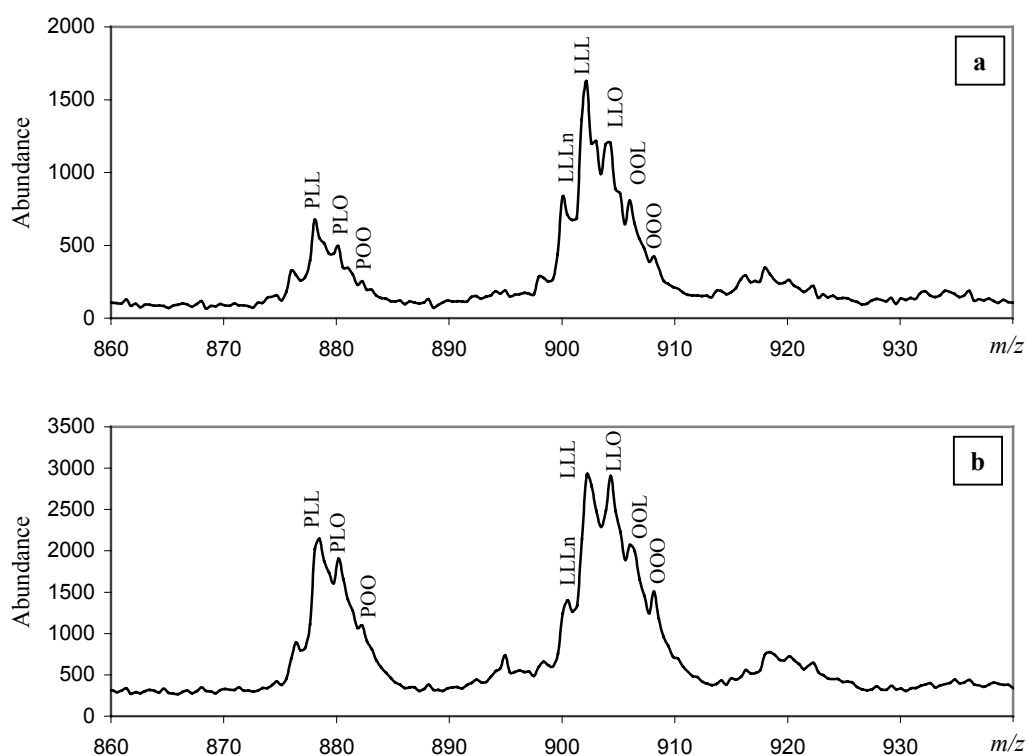


Figure 13. MALDI-TOF mass spectra of walnut oil (a) and wheat germ oil (b); showing sodiated TAG peaks.

The resolution of the MALDI-TOFMS instrument was approximately 300 (at half peak height, Figure 10-13). This resolution allowed only to „semi-resolve” the TAG peaks, however this resolution proved to be sufficient for characterization of the oils (Chapter 2.3).

Sodiated molecular ion abundances of the most abundant TAGs namely PLL, PLO, POO, LLLn, LLL, LLO, OOL and OOO (eight different) were readout from the MALDI-TOF mass spectra. These individual ion abundances were also normalized to the sum of the all (8) sodiated molecular ion abundances resulting in relative TAG contents. The values of the calculated relative TAG contents in the various oils are shown in Table 5. The RSD values of the relative TAG contents were around 7% (data are not presented here). These RSD values were 3% lower than relative TAGs peaks areas calculated from the HPLC/APCI-MS measurements (10%).

Table 5. *The mean values of the relative TAG contents (%) in various plant oils, calculated from the MALDI-TOF mass spectra.*

Sample	TAG							
	PLL	PLO	POO	LLLn	LLL	LLO	OOL	OOO
Almond 1	4,58	10,55	11,57	2,92	4,64	12,00	21,92	31,80
Almond 2	5,01	9,34	12,62	3,20	5,68	10,68	20,21	33,26
Almond 3	4,68	6,98	12,84	3,54	6,42	10,51	16,12	38,92
Almond 4	5,19	6,99	11,40	3,58	8,47	11,39	16,96	36,02
Almond 5	4,80	10,36	11,61	3,78	5,84	13,80	21,93	27,87
Almond 6	5,19	7,30	11,86	3,70	7,95	11,92	17,50	34,59
Avocado 1	7,73	20,02	27,07	4,05	3,82	4,76	10,86	21,69
Avocado 2	8,40	19,58	26,05	5,37	5,57	5,55	10,39	19,09
Avocado 3	7,71	19,91	27,67	4,73	4,42	4,92	10,15	20,49
Avocado 4	7,75	20,38	27,34	5,30	4,98	5,63	9,62	18,99
Corn germ 1	9,98	11,05	7,61	3,59	15,78	21,96	18,27	11,76
Corn germ 2	10,23	11,25	7,31	3,21	16,41	22,35	18,09	11,14
Corn germ 3	11,19	12,06	8,42	3,84	16,66	21,39	16,58	9,86
Corn germ 4	10,13	10,91	6,86	3,15	16,97	23,12	18,56	10,30
Corn germ 5	12,13	13,08	9,43	4,47	15,04	19,57	15,70	10,58
Grape seed 1	11,90	9,14	4,54	4,24	25,56	22,00	14,51	8,10
Grape seed 2	11,84	9,29	5,12	4,20	24,46	22,00	14,89	8,21
Grape seed 3	12,29	9,52	5,74	4,40	24,86	21,09	14,41	7,69
Grape seed 4	11,09	9,26	5,89	4,17	25,81	22,22	13,74	7,82
Grape seed 5	10,70	9,27	4,98	3,78	25,82	22,26	15,17	8,01
Grape seed 6	11,25	9,97	6,04	4,05	23,12	21,56	15,04	8,98
Grape seed 7	11,60	8,70	4,35	3,38	26,56	23,23	14,68	7,51
Grape seed 8	11,98	9,16	4,67	3,77	26,71	21,99	14,42	7,30
Grape seed 9	12,41	9,09	4,09	3,58	26,58	22,43	14,82	7,00
Grape seed 10	11,45	8,80	4,08	3,77	26,76	22,42	15,34	7,37
Grape seed 11	12,35	9,33	4,91	3,95	25,82	21,58	14,20	7,86
Linseed 1	9,88	8,34	5,90	14,10	19,19	19,45	14,31	8,82
Linseed 2	10,30	8,51	6,40	17,10	17,83	17,40	13,04	9,41
Linseed 3	10,16	8,12	6,52	18,45	18,22	17,22	12,37	8,95
Linseed 4	10,10	8,09	6,34	21,48	18,36	15,90	11,17	8,56
Mustard seed 1	10,04	10,88	9,95	5,45	14,27	19,13	17,33	12,96
Mustard seed 2	9,71	8,96	6,28	4,02	19,57	23,74	17,50	10,21
Mustard seed 3	11,95	11,29	8,69	8,13	16,45	17,64	14,60	11,25
Olive 1	3,73	8,42	21,97	3,51	3,87	5,38	13,70	39,41
Olive 2	4,63	8,95	21,94	3,17	3,27	6,17	14,27	37,59
Olive 3	5,33	11,86	22,53	4,95	5,27	6,85	13,70	29,52
Olive 4	5,09	9,43	21,72	5,02	5,53	6,02	12,43	34,76
Olive 5	2,99	7,06	21,35	3,64	3,41	4,77	13,28	43,51
Olive 6	7,36	18,61	23,62	3,06	2,82	7,82	15,41	21,30
Olive 7	4,58	10,54	22,28	4,01	4,71	6,72	14,33	32,83
Olive 8	4,56	8,98	20,59	5,88	5,62	6,39	12,66	35,33
Olive 9	3,64	9,03	24,41	3,05	2,95	5,39	13,57	37,96
Olive 10	4,01	8,35	23,38	3,56	3,74	6,09	13,63	37,23
Olive 11	3,88	7,88	23,46	3,72	3,73	5,82	11,92	39,58
Peanut 1	5,06	9,97	15,55	5,03	4,89	9,62	18,72	31,16
Peanut 2	5,29	11,78	16,43	3,92	4,38	11,55	19,57	27,07
Peanut 3	5,20	11,49	16,01	4,29	4,50	10,31	20,37	27,84
Peanut 4	7,01	13,87	16,33	5,82	5,83	10,64		22,87
Pumpkin seed 1	8,65	11,88	9,14	3,43	12,47	18,65	19,82	15,98
Pumpkin seed 2	9,43	13,22	11,35	3,91	8,52	15,24	20,22	18,11
Pumpkin seed 3	9,99	13,29	10,87	4,24	9,16	16,20	19,55	16,71
Sesame seed 1	7,91	10,76	8,60	3,18	12,54	20,18	21,08	15,75
Sesame seed 2	7,86	12,10	10,09	3,33	10,74	18,42	20,28	17,18
Sesame seed 3	7,76	12,21	11,31	4,56	8,97	16,82	20,29	18,08
Soybean 1	13,15	11,52	6,22	9,48	19,56	19,42	12,86	7,79
Soybean 2	13,58	11,81	7,22	10,32	18,24	18,17	12,76	7,91
Soybean 3	13,42	11,76	7,63	10,05	18,63	17,87	12,53	8,10
Soybean 4	12,61	11,46	7,23	9,16	18,82	18,61	13,47	8,64
Soybean 5	12,76	11,33	7,25	8,75	18,54	18,72	13,70	8,96

Samples	TAG							
	PLL	PLO	POO	LLLn	LLL	LLO	OOL	OOO
Sunflower 1	8,06	8,64	5,93	3,64	15,78	24,67	20,99	12,30
Sunflower 2	9,58	9,11	5,13	3,62	18,03	24,72	19,38	10,43
Sunflower 3	9,61	9,66	5,68	4,27	17,75	24,38	18,53	10,11
Sunflower 4	8,78	9,19	5,36	3,36	19,13	25,77	18,48	9,93
Sunflower 5	9,36	9,80	6,20	4,16	16,35	22,99	19,84	11,30
Sunflower 6	8,55	8,54	5,16	3,74	17,47	24,97	20,80	10,77
Sunflower 7	9,56	9,95	6,18	4,22	17,13	23,10	19,11	10,75
Walnut 1	10,47	7,83	4,46	12,95	24,09	19,83	13,06	7,31
Walnut 2	9,67	8,13	4,40	12,41	22,87	20,52	14,01	8,00
Wheat germ 1	9,91	9,37	5,30	4,30	16,37	23,29	20,02	11,44
Wheat germ 2	10,90	10,89	7,71	4,82	15,90	21,54	17,22	11,02
Wheat germ 3	18,61	15,25	8,30	6,68	15,64	15,50	11,22	8,80
Wheat germ 4	13,29	11,98	7,38	8,79	18,71	18,15	12,87	8,81
Wheat germ 5	11,04	9,80	6,01	5,21	18,92	23,00	16,94	9,08

Data were calculated from minimum of five parallel measurements at each oil.

The relative TAG contents in the same variety of oils were also found to have quite similar values (Table 5). Corn germ, grape seed, mustard seed, soybean, sunflower, walnut and wheat germ oils contained a relatively high amount of unsaturated TAGs such as PLL, LLL, LLO and OOL as found also at the HPLC/APCI-MS measurements (Chapter 2.3.1, TIC). Almond, avocado, olive, peanut, pumpkin seed and sesame seed oils contained relatively high OOO content as found also at the HPLC/APCI-MS measurements (Chapter 2.3.1, TIC).

2.3.3. Linear Discriminant Analysis (LDA)

LDA was applied both to the APCI-MS and MALDI-MS data matrices (Table 4 and 5). The relative TAGs peaks areas and TAG contents of the oils were considered as variables (12 and 8, for the APCI and MALDI analyses, respectively), and each variety of the oil was considered as a class (14 different ones). The relative TAGs peaks areas (HPLC/APCI-MS) and TAG contents (MALDI-TOFMS) are not identical with their concentration ratio in the oils. However, it has been assumed that the oils can be classified even by using them without the exact knowledge of the their concentration.

The possibility of overfitting in LDA calculation was checked by examination of whether the dimensionality (number of variables) exceeds the $(n-g)/3$ value (where n is the number specimens, and g is the number of groups) [50]. The value of the fraction was 19.7 $((73-14)/3)$, so the dimensionality did not exceed that value in either case.

LDA gave good results for the analysis of both APCI and MALDI data matrices. 68 samples (93.15%) were correctly classified from 73 various samples in both cases according to the summarized classification matrices (Table 6 and 7). Only one-one samples from the corn germ, peanut, sunflower, walnut and wheat germ oils were not classified correctly based on the HPLC/APCI-MS data (Table 6). Misclassification of samples can be explained by slightly various TAG contents (compared to same variety oils), originating from different provenance, blending, measuring or calculating errors. These outlier samples are natural phenomena, and can be deleted if there are a great number of samples per groups.

Table 6. Summary of the LDA classification matrix calculated from HPLC/APCI-MS data.

Oil classes	Total by oil	False identification	Correct (%)
Almond	6	0	100
Avocado	4	0	100
Corn germ	5	1	80
Grape seed	11	0	100
Linseed	4	0	100
Mustard seed	3	0	100
Olive	11	0	100
Peanut	4	1	75
Pumpkin seed	3	0	100
Sesame seed	3	0	100
Soybean	5	0	100
Sunflower	7	1	85.7
Walnut	2	1	50
Wheat germ	5	1	80
Total	73	5	93.15

The classification matrix calculated from the MALDI-TOFMS data shows a somewhat better result (Table 7). In this case the distinction among the different groups is better. Only samples from pumpkin seed (2) and wheat germ (3) oils were not classified correctly. A classification matrix was also calculated by LDA from the HPLC/APCI-MS results based on the same eight variables used in the MALDI-TOFMS LDA calculation. The result of this calculation was that 90.41% of the samples were correctly classified (not presented here).

The different canonical roots were plotted against one another in order to visualize the statistical results. Figure 14 and 15 show the three-dimensional score plots of roots with the highest discriminant power (all samples were included).

Table 7. Summary of the LDA classification matrix calculated from MALDI-TOFMS data.

Oil classes	Total by oil	False identification	Correct (%)
Almond	6	0	100
Avocado	4	0	100
Corn germ	5	0	100
Grape seed	11	0	100
Linseed	4	0	100
Mustard seed	3	0	100
Olive	11	0	100
Peanut	4	0	100
Pumpkin seed	3	2	33.3
Sesame seed	3	0	100
Soybean	5	0	100
Sunflower	7	0	100
Walnut	2	0	100
Wheat germ	5	3	40
Total	73	5	93.15

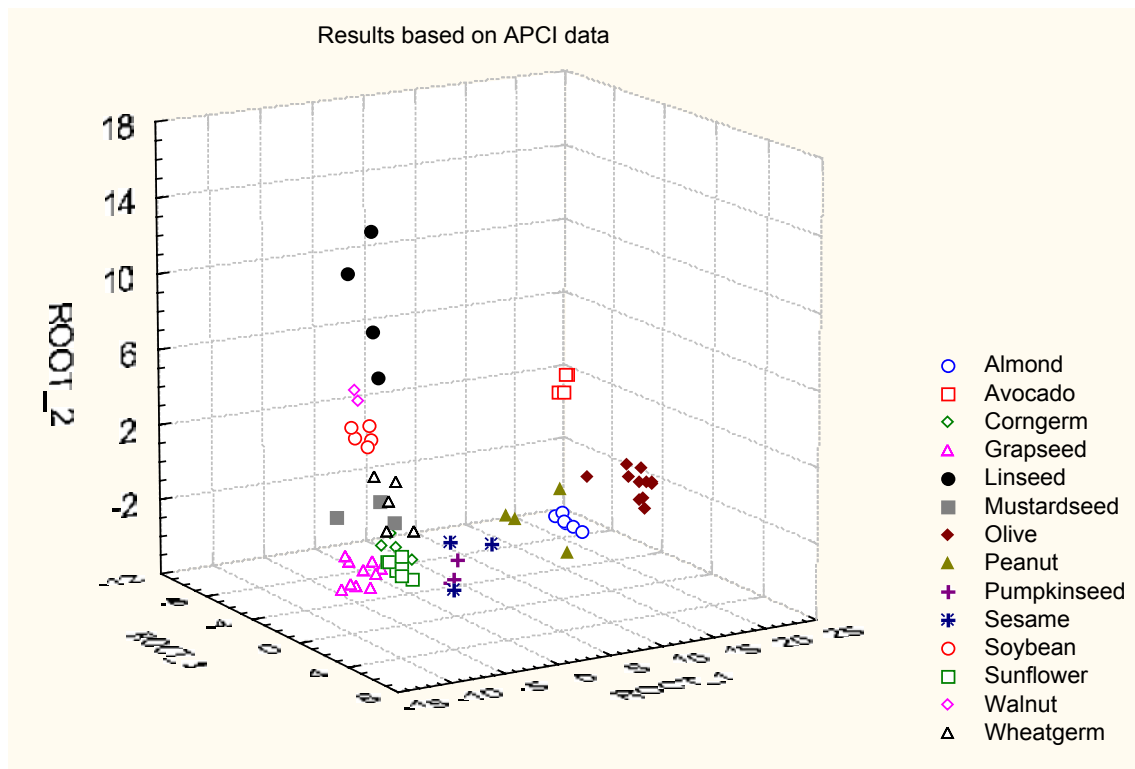


Figure 14. Three-dimensional score plot from LDA based on the HPLC/APCI-MS data.

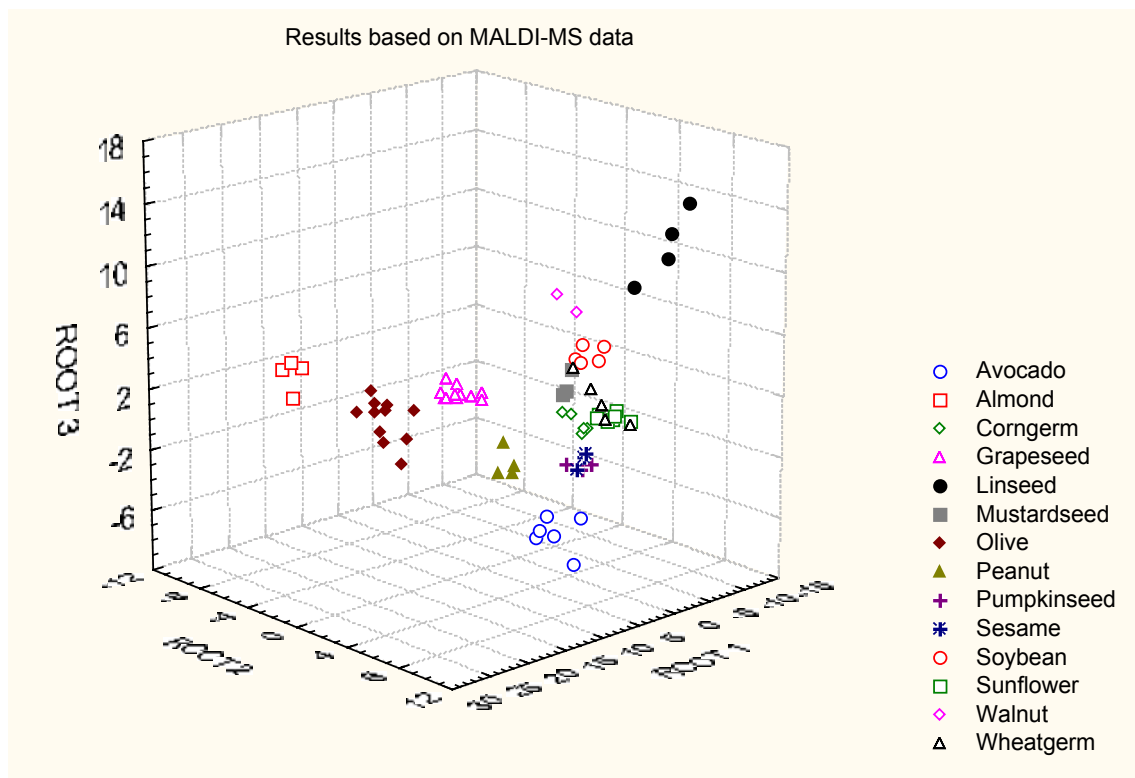


Figure 15. Three-dimensional score plot from LDA based on the MALDI-TOFMS data.

The first three roots mostly discriminate among the almond, avocado, grape seed, linseed, olive, soybean and walnut oils based on the HPLC/APCI-MS data (Figure 14). These oils are clearly separated in seven different clusters. Moreover, cluster of peanut oil samples was also clearly separated based on the MALDI-MS data (Figure 15). The first three roots can hardly discriminate among the corn germ, mustard seed, pumpkin seed, sesame seed and sunflower oil samples in both cases. Comparing the two score plots, the various oil clusters were somewhat better separated in Figure 15. This better result (calculated from the MALDI-TOFMS data) is probably due to the better accuracy of the MALDI-TOFMS experiments and data processing, but the difference was slight.

2.4. CONCLUSION

The main TAG compositions of various plant oils (almond, avocado, corn germ, grape seed, linseed, mustard seed, olive, peanut, pumpkin seed, sesame seed, soybean, sunflower, walnut and wheat germ, 2-11 different pieces from each) were analyzed using two different mass spectrometric techniques: HPLC/APCI-MS and MALDI-TOFMS. HPLC/APCI-MS measurements were performed using a relatively short 30-min gradient elution program with acetone-acetonitrile eluent systems on a microparticulate ODS column (Purospher, RP-18e, 125x4 mm, 5 μ m). The possible structure of the most abundant TAGs were LLL_n, LLL, L_nLP, LLO, PLL, OOL, PLO, PLP, OOO, POO, POP and SOO. The fatty acid distribution of the TAGs were in good agreement with the published data excepting the LLO and OOL isomers. MALDI-TOFMS measurements were performed in reflectron mode. During these experiments the most abundant TAGs were LLL_n, LLL, LLO, PLL, OOL, PLO, OOO and POO.

Relative TAG peak areas from HPLC/APCI-MS measurements and TAG contents from the MALDI-TOFMS measurements were calculated at each plant oil for the linear discriminant analysis (LDA). These relative TAG peak areas and contents measured by the mass spectrometric techniques in combination with LDA have been successfully used to distinguish between the oil varieties. In both cases successful classification of 93.15% of the samples were obtained. This great value of correct classification is unique in the LDA calculations indicating the efficiency of the applied methods. Almond, avocado, grape seed, linseed, mustard seed, olive, sesame seed and soybean oil varieties (eight different) were 100% correctly classified based on both the HPLC/APCI-MS and the MALDI-TOFMS data. Classifications of one-one samples from corn germ, peanut, sunflower, walnut and wheat germ oil varieties were not correct based on the HPLC/APCI-MS data. LDA calculation based on the MALDI-TOFMS data gave somewhat better result. In this case only samples from peanut and wheat germ oil varieties were not classified correctly. The correctly classified oil varieties formed clusters and were clearly separated from each other on the score plots.

Comparing the two mass spectrometric methods combined with the LDA calculation, MALDI-TOFMS provided somewhat better results in addition to much shorter analysis and data processing time than HPLC/APCI-MS.

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3. SEPARATION OF PLANT OIL TRIACYLGLYCEROLS ON A MONOLITHIC REVERSED-PHASE SILICA COLUMN

3.1. INTRODUCTION

3.1.1. Monolithic columns

Monolithic-type columns used in liquid chromatography were first described in the HPLC literature in 1989 [1]. These columns are synthesized by different materials to prepare organic (polymer-based) and inorganic porous materials [2] such as polyacrylamide [2-3], polyacrylate [4,5], poly(styrene-*co*-divinylbenzene) [6,7], and silica [2,8,9] monoliths. Monolithic columns built by continuous porous materials have higher total porosity than conventional columns packed with particles. This results in a lower pressure drop and enables the maintenance of higher mobile phase flow rates [10], while conventional columns are more pressure-limited. It is well known that reducing the particle size in a good quality conventional column (with a narrow particle-size distribution) reduces Eddy diffusion, and results in greater column efficiency (*HETP*-height equivalent of a theoretical plate). The contribution of Eddy diffusion to *HETP* [11]:

$$HETP_{Eddy} = 2 \cdot \lambda \cdot d_p$$

where λ is the packing factor and d_p is the average diameter of the particles (if the distribution of the particle-size is narrow). On the other hand this effect increases the pressure drop. The pressure drop (Δp) is inversely proportional to the square value of particle diameter, according to the substituted Darcy's law by the Carmen-Kozeny relationship [11,12]:

$$\Delta p = \frac{\Theta \cdot \eta \cdot L \cdot u}{d_p^2}$$

where Θ is a constant describes the column porosity, η is the viscosity of the solvent, L is the column length and u is the flow rate of the mobile phase.

Rod type columns with silica skeletons having relatively large through-pores (0.5-8 μm) are much more efficient at high flow rates, due to the flat van Deemeter curve compared to that of conventional columns packed with particles [13]. Unfortunately polymer-based monolithic columns often contain micropores, which reduce column efficiency, deform peak symmetry and have weak resistance to organic solvents [14,15].

3.1.2. Silica monolithic columns

Monolithic silica material was developed by Tanaka *et al.* [10], introduced by Cabrera *et al.* at HPLC '98 [16] and commercialized by Merck (Darmstadt, Germany) as SilicaRod columns, recently named Chromolith. The silica rods are obtained by hydrolysis and polycondensation of alkoxy silanes. The inner surface of the rod can be chemically modified in the same way with the conventional microparticulate porous silica, resulting *e.g.* amino, RP-18 support. This “fourth generation” HPLC material has excellent properties due to the biporous structure [16]. Columns typically consist of 0.5-8 μm through-pores (Figure 1/a) and 2-30 nm mesopores in the 0.3-5 μm silica skeletons [9,10].

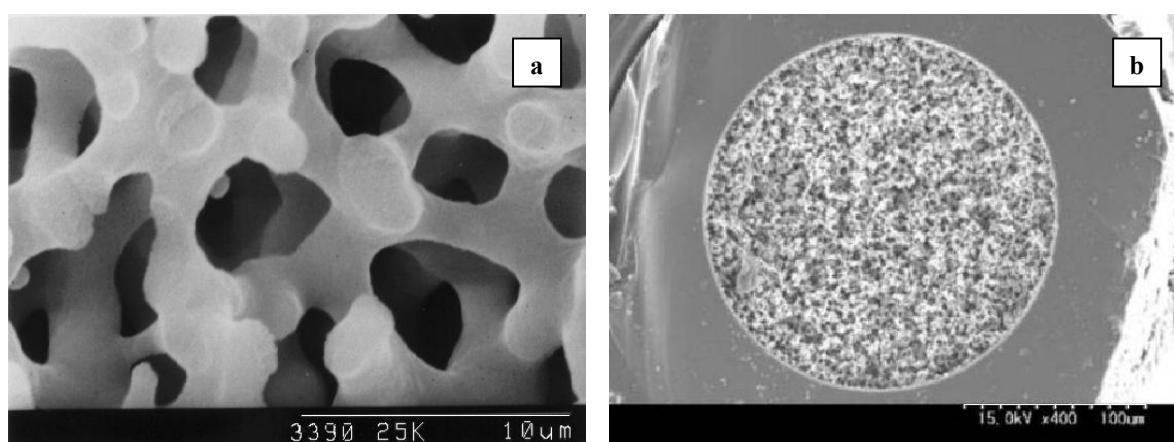


Figure 1. Electron micrograph of through-pores and silica skeletons in monolithic silica (a) and monolithic silica in a 200 μm I.D. capillary (b).

3.1.2.1 Properties of silica monolithic columns

Chromolith silica columns have separation efficiency of more than 80 000 theoretical plates per meter, which is equivalent to the performance of well-packed conventional HPLC columns with 5 μm particles [13]. Table 1 shows some main features of a conventional particulate and Chromolith (SilicaRod) columns.

Table 1. Main features of silica microparticulate and SilicaRod columns [13,17].

Main features	Column type	
	Purospher Si	Chromolith
Particle size	5 μm	not applicable
Through-pores	-	1.5-2 μm
Interstitial void	1.25-2 μm^{a}	-
Mesopores	120 Å	120 Å
Pore volume	1.1 $\text{mL}\cdot\text{g}^{-1}$	1 $\text{mL}\cdot\text{g}^{-1}$
Surface area	330-350 $\text{m}^2\cdot\text{g}^{-1}$	300-350 $\text{m}^2\cdot\text{g}^{-1}$
Total porosity ^b	ca. 66%	ca. 81%

^a The interstitial void of a microparticulate column is about 25-40% of the particle size;

^b Data measured after RP-18 derivatization

The main advantage of the chromolith column is that, it has total porosity of ca. 81% compared with the ca. 66% of the microparticulate porous silica (Table 1). This enables the use of higher mobile phase flow rates with relatively small pressure drop providing short analysis times.

3.1.3. Application

In recent years monolithic columns have been extensively studied [17-23] and several new types e.g. new polymeric-based and capillary silica monolithic columns (Figure 1/b) have been introduced for application in HPLC and CEC (capillary electrochromatography) [24-26].

The usage of silica monolithic columns in HPLC/MS has also been investigated [27-31], however contrary to all expectations the use of monolithic columns is still limited mainly due to the high solvent consumption. The main advantage of monolithic type silica column is that it provides excellent separation efficiency within short analysis times at low pressure drop. This may also be realized in HPLC/MS applications especially in high-throughput work.

3.2. EXPERIMENTAL

3.2.1. Materials

Five different cold pressed oil samples (peanut, pumpkin seed, sesame seed, soybean, wheat germ; 1-1 from each) were purchased at local grocery stores and factories; and dissolved in HPLC grade acetone-acetonitrile (2:1) to a concentration of 1%. Acetone and acetonitrile were obtained from Koch-Light (Haverhill, England) and Riedel-de Haën (Seelze, Germany), respectively.

3.2.2. Instrumentation

HPLC/MS analyses were carried out using the same Shimadzu (Kyoto, Japan) HPLC system coupled to the QP2010 mass spectrometer fitted with APCI source as described in Chapter 2.2. The APCI source parameters were optimized to triacylglycerol analysis and were also the same as described in Chapter 2.2. Spectra were obtained over the range of m/z 500-1000, with a scan speed of $500 \text{ amu}\cdot\text{sec}^{-1}$. The eluent was split after the HPLC column with a tee-union, and approximately $400 \mu\text{L}\cdot\text{min}^{-1}$ went to the MS interface.

The TAGs in oils were separated on a reversed-phase end-capped monolithic silica column (SilicaROD, RP-18e, $50\times 4.6 \text{ mm}$, Merck, Darmstadt, Germany), with acetone-acetonitrile eluent system, at a flow rate of $5 \text{ mL}\cdot\text{min}^{-1}$. Linear gradient was applied during the

analysis, acetone concentration from 10% to 66% in 10 min. Autosampler and column oven were set to 20 and 25°C, respectively. The injection volume was 5 μL . Each oil was measured five times.

3.3. RESULTS AND DISCUSSION

The flow rate coming from the monolithic column was shrunk by a tee-union flow splitter in order to obtain an optimal flow rate for the APCI ion source conditions. This means, it was slightly adjusted to make *ca.* 400 $\mu\text{L}\cdot\text{min}^{-1}$ flow rate in the peak tube directed to the MS.

The majority of TAG compounds in the oils were identified as described in Chapter 2.1.5. In all cases the protonated molecular $[\text{M}+\text{H}]^+$ and the diacylglycerol fragment $[\text{M}+\text{H}-\text{RCOOH}]^+$ ions did not form significantly adduct ions (sodiated, ammoniated etc.). APCI mass spectrum of PLO is shown in Figure 2.

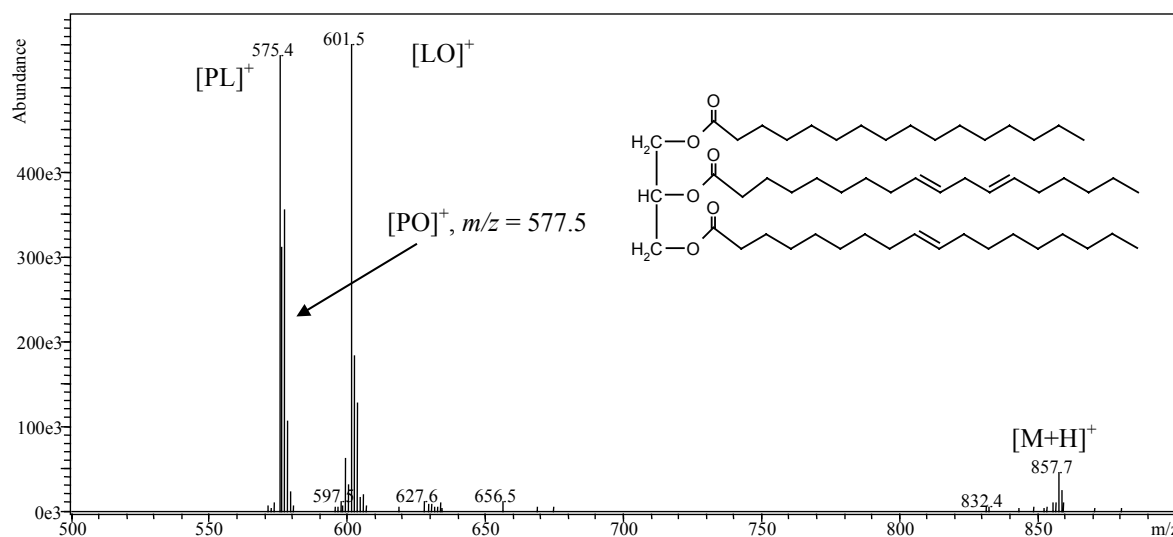


Figure 2. APCI mass spectrum of 1(3)-palmitoyl-2-linoleoyl-3(1)-oleoyl glycerol (PLO) from wheat germ oil, measured by HPLC/MS.

The TAGs were eluted within a short analysis time (8 min) for all samples due to the high flow rate ($5 \text{ mL}\cdot\text{min}^{-1}$) and the gradient elution applied on the monolithic silica column. This considerably short analysis time is unique compared with approximately 50-min HPLC runs described in the literature [32-34]. The pressure drop on the column was around 700 p.s.i. during the gradient measurements, with ± 20 p.s.i. fluctuation (the pressure drop is directly proportional to the viscosity of the eluent).

Total ion chromatograms (TIC) of peanut, pumpkin seed, and sesame seed, soybean and wheat germ oil are shown in Figure 3-4. Although in some cases the TAGs are not baseline separated (Figure 3-4), mass spectrometric detection enables baseline separation of different compounds plotting single ion chromatograms.

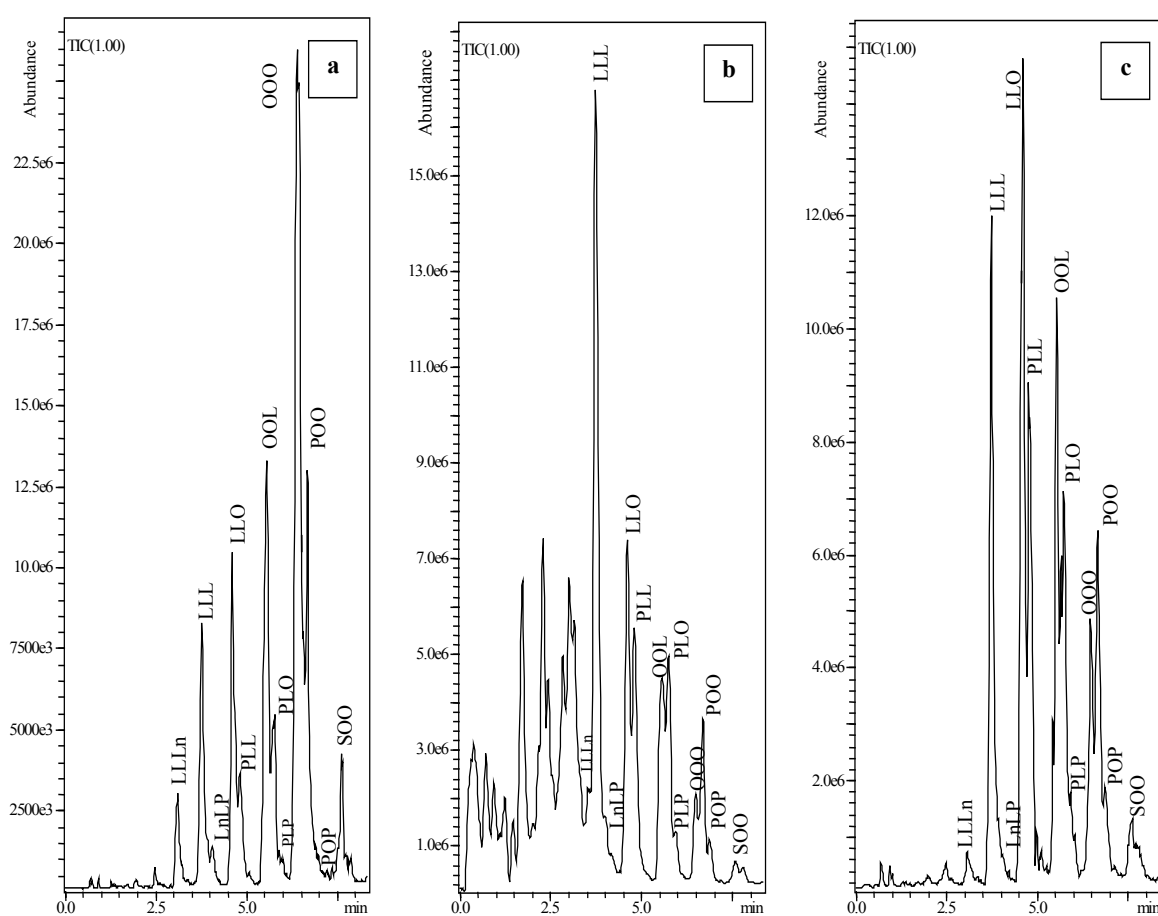


Figure 3. HPLC/APCI-MS profile of peanut oil (a), pumpkin seed oil (b) and sesame seed oil (c); compounds were separated on a RP-18e monolithic silica column @ $5 \text{ mL}\cdot\text{min}^{-1}$.

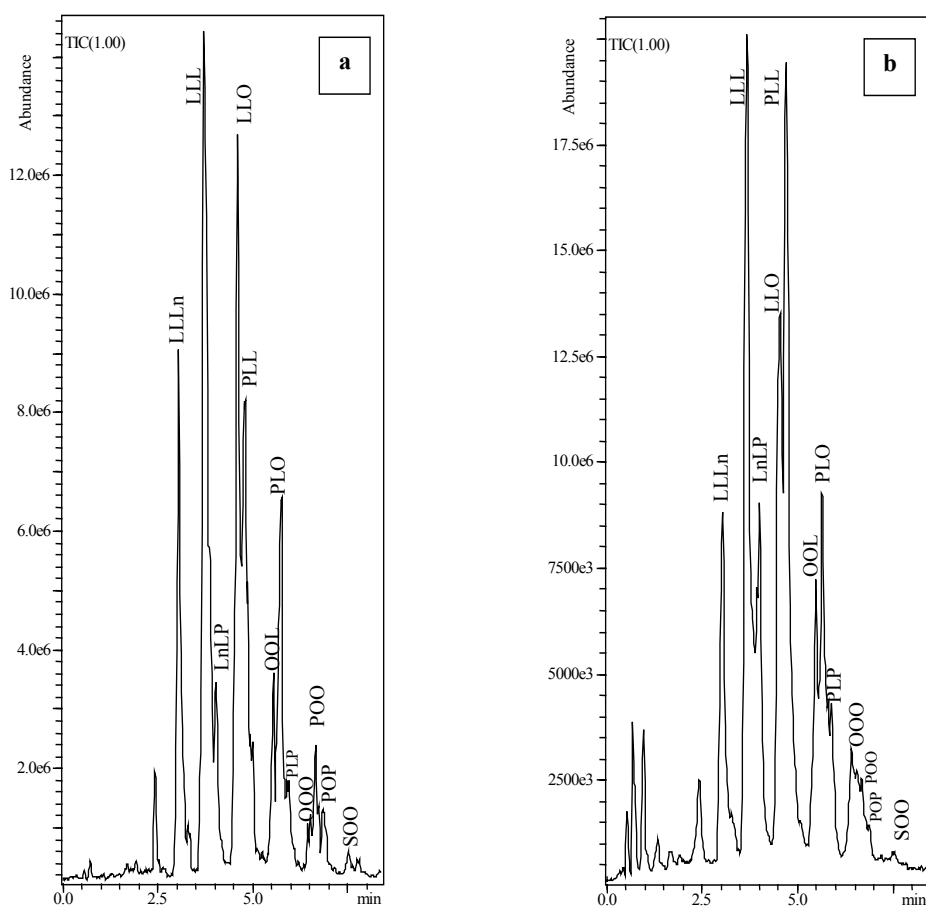


Figure 4. HPLC/APCI-MS profile of soybean oil (a) and wheat germ oil (b); compounds were separated on a RP-18e monolithic silica column @ $5 \text{ mL}\cdot\text{min}^{-1}$.

The mean value and the relative standard deviations (RSD) of the retention times of the most abundant TAGs are shown in Table 2. The same twelve TAGs (LLLn, LLL, LnLP, LLO, PLL, OOL, PLO, PLP, OOO, POO, POP and SOO) were the most abundant as observed in our previous measurements performed on the microparticulate RP silica column (Chapter 2.3.1). The RSD values of the retention times are low indicating the good repeatability even at high flow rate. The maximum RSD value of the retention time is 1.02% (RSD of the retention time of PLL, Table 2).

Table 2. *The mean values of the retention times of the most abundant TAGs found in oils on a SilicaROD RP-18e column 50x4.6mm, in acetone/acetonitrile gradient @ 5 mL·min⁻¹.*

TAG	t_{rmean}^a (min)	RSD ^b (%)
LLLn	3.07	0.75
LLL	3.74	0.91
LnLP	4.04	1.00
LLO	4.59	0.87
PLL	4.77	1.02
OOL	5.53	0.59
PLO	5.73	0.76
PLP	5.94	0.72
OOO	6.48	0.88
POO	6.69	0.85
POP	6.90	0.47
SOO	7.59	0.39

^{a,b} Data were calculated from twenty-five measurements (five parallel measurements per oils); ^b standard deviations of the retention times (%).

The relative peak areas of TAGs were also calculated from the single ion chromatograms (SIC) in order to examine the repeatability of the measurements. The mass ranges used for SICs were the same as in Chapter 2 (last two columns of Table 2 in Chapter 2.3). The peak areas of various TAGs in oils were integrated from the SIC chromatograms. The individual peak areas of different TAGs were normalized to the sum of the all TAG peak (eleven) areas at each oil, resulting relative peak areas (Table 3). The peak area of POP was not integrated in all cases, due to its low amount. Peak area of LLLn and LLL at pumpkin seed oil; and peak

area of SOO at soybean and wheat germ oil were also not integrated due to the same reason. The RSDs of relative peak areas of small peaks are around 12%, and of large peaks are around 7%. These RSD values are somewhat lower compared to the values calculated from the ODS results (Chapter 2.3.1).

Table 3. *The mean values of the relative TAG peak areas (%) in various plant oils, calculated from SICs of HPLC/APCI-MS measurements.*

TAG		Samples				
		Peanut oil	Pumpkin seed oil	Sesame seed oil	Soybean oil	Wheat germ oil
LLLn	A_{mean}^a	2.65	n.i. ^c	0.80	15.68	12.69
	RSD ^b	9.50	-	12.80	4.10	7.50
LLL	A_{mean}^a	7.23	23.15	23.03	34.54	29.02
	RSD ^b	3.10	7.70	10.40	7.00	8.70
LnLP	A_{mean}^a	0.57	n.i. ^c	0.21	4.54	7.75
	RSD ^b	12.60	-	6.60	6.40	7.20
LLO	A_{mean}^a	6.15	21.92	21.18	16.14	12.06
	RSD ^b	10.70	3.40	6.70	10.00	11.10
PLL	A_{mean}^a	1.78	11.17	7.76	13.19	16.46
	RSD ^b	11.00	10.90	6.40	9.60	11.60
OOL	A_{mean}^a	12.74	19.06	18.23	6.33	6.35
	RSD ^b	5.40	6.50	7.50	11.50	10.70
PLO	A_{mean}^a	1.53	4.38	4.95	3.73	5.39
	RSD ^b	11.20	12.30	10.40	12.10	4.10
PLP	A_{mean}^a	0.10	0.79	0.60	0.78	1.80
	RSD ^b	11.60	10.90	5.80	7.60	7.20
OOO	A_{mean}^a	51.72	10.77	13.99	3.09	5.94
	RSD ^b	1.90	11.80	8.10	10.80	8.30
POO	A_{mean}^a	10.55	6.07	5.53	1.98	2.55
	RSD ^b	6.90	12.70	8.20	10.30	12.70
SOO	A_{mean}^a	4.98	2.69	3.71	n.i. ^c	n.i. ^c
	RSD ^b	8.90	12.40	8.70	-	-

^{a,b} Data were calculated from five parallel measurements per oils; ^a mean value of the calculated relative peak area (%); ^b standard deviations of the retention times (%); ^c not integrated due to low amount.

3.4. CONCLUSION

It can be concluded from our data that adequate resolution of TAGs was achieved in various oils (peanut, pumpkin seed, sesame seed, soybean and wheat germ oil). The separation was performed on a monolithic RP silica column within a very short analysis time (10 min total run time) using gradient elution with acetone-acetonitrile eluent system. The short analysis time of separating extremely apolar compounds such as triacylglycerols ($\log P$ greater than 7) is unique compared to the long-run separations described in the literature [32-34], or to our previously described 30-min separation (Chapter 2.3.1). The repeatability of both the retention times and peak areas were also good. The maximum RSD value of the retention time was 1.02%. The RSD values of the peak areas were between 3.4 and 12.8%.

Although the separation was fast it should be also considered that the solvent consumption was high even during the short analysis time. 50 mL solvent was used on the monolithic column (10 min total run time) compared to the 18 mL on the microparticulate column (30 min total run time, Chapter 2.3.1) per HPLC runs. This corresponded to 277.78% higher solvent consumption.

3.5. REFERENCES

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4. QUANTIFICATION OF THE RATIO OF 1(3), 2-DILINOLEOYL-3(1)-OLEOYL AND 1,3-DILINOLEOYL-2-OLEOYL GLYCEROL POSITIONAL ISOMERS IN PLANT OILS

4.1. INTRODUCTION

4.1.1. *The importance of the fatty acid distribution of triacylglycerols in relation of their absorption and metabolism in humans*

The distribution and the type of the fatty acids on the glycerol backbone play a key role in lipid digestion, absorption and metabolism [1,2]. During digestion, fatty acids at the *sn*-1 and *sn*-3 positions are digested by lipases (TAG hydrolyzing enzymes) resulting *sn*-2 monoacylglycerols and free fatty acids. The absorption of long chain saturated fatty acids (chain length of C16 or longer) is much less efficient than their unsaturated counterparts, particularly when they form esters with glycerol its *sn*-1 or *sn*-3 positions [2,3]. Free saturated fatty acids with chain length of C16 or longer (*e.g.* palmitic acid: 16:0; or stearic acid: 18:0) can form insoluble salt in the presence of calcium or magnesium in intestine resulting in poor absorption. Since the *sn*-2 monoacylglycerols are not able to form salts they can be readily absorbed [1,3]. They can be re-esterified during biochemical steps and finally released into blood as TAGs. Fatty acids on the *sn*-2 position are conserved in this way [4]. Short and medium chain fatty acids can be absorbed as free fatty acids from the *sn*-1 and *sn*-3 positions, due to their better solubility in aqueous media. They provide fast energy delivery because they can be rapidly oxidized by the liver after secreted directly to the portal vein.

The first evidence on the importance of the positional isomer TAGs was published in 1968, when Tomarelli *et al.* described the fatty acid distribution of human milk fat and its effect on absorption [5]. In human milk fat over the 60% of palmitic acid is esterified at the *sn*-2 positions of the glycerol, whereas in formulas or cow's milk it is mainly at the *sn*-1 and

sn-3 positions of the glycerol [5-7]. The absorption of palmitic acid from human milk was found to be efficient [5,8] since the solubility of *sn*-2 monopalmitoyl-glycerol is better compared to the free palmitic acid released by lipases from the *sn*-1 or *sn*-3 positions. These facts have a great influence on infant nutrition since most of the energy is supplied from TAGs [9].

The lipid digestion and absorption of the different regioisomer TAGs is well described, however their metabolism is not fully understood. Not only the absorption and the energy delivery, but also the metabolic pathway of TAGs depends significantly on their fatty acid distribution although their contribution is probably moderate [4,10-13]. Most of the studies are focused on the health protection effect of various fatty acids, but without considering their positions on the glycerol into account [14,15]. It has been shown many years ago that mono- and polyunsaturated fatty acids have serum total cholesterol lowering and anticarcinogenic effects, whereas saturated fatty acids increase serum lipid and total cholesterol concentration accelerating atherosclerosis (constriction of the arteries) [16-18]. The effect of the mono- and polyunsaturated fatty acids distribution of TAGs on their metabolism has neither been intensively researched. According to Combe the position of linoleic acid in triacylglycerols might influence its metabolism by desaturation and elongation into essential fatty acids [19].

4.1.2. Human pancreatic lipase

Lipases can hydrolyze TAGs in human body. Human pancreatic lipase (HPL) is the major enzyme involved in TAG digestion. HPL is a 50 kDa glycoprotein containing 449 amino acids, which is directly secreted as an active enzyme. The structure of the enzyme was first described by Winkler *et al.* [20]. The enzyme contains two structural domains: a large N-terminal domain (1-336) and a smaller C-terminal domain (337-449). The N-terminal domain contains the active site with a catalytic triad formed by serine (152), asparagine (176) and histamine (263). In general, the serine hydroxyl group can be acylated by free fatty acids

resulting a fatty acyl-enzyme complex. HPL has stereoselectivity towards the *sn*-1 and *sn*-3 positions of TAGs. Beside of HPL, human gastric lipase (HGL) has also digestive activity [21], although the contribution to TAG digestion is much less [22]. HGL exhibits stereoselectivity towards the *sn*-3 position of TAGs. None of the two digestive lipases are able to hydrolyze directly the ester bond at the *sn*-2 position of TAGs; consequently, 1,3-diacylglycerols never detected [23].

4.1.3. “Structured” triacylglycerols

There was a major turning point to understand the importance of the distribution and the type of the fatty acids on the glycerol backbone. From that point not only the analytical and biochemical studies but also the production of “structured” TAGs became more intense [2,24,25]. The terms of “structured”, “designed” or “tailored” TAGs were introduced to TAGs obtained by structural modification of natural fats and oils through chemical and/or enzymatic procedures [24-26]. These modifications allow the improvement of their physical and nutritional properties. In relation to physical properties, modification can improve structuring, melting and storage behaviors, which are strongly correlated.

The improvement of the nutritional properties of the TAGs is of great importance in the food researches [25-29]. Specific “structured” TAG can be produced in order to reduce saturated fatty acid content of the TAG providing better clinical nutritional products for patients who need rapid energy delivery and also essential fatty acid uptake [29]. TAGs with medium-chain fatty acids (mainly C8:0 and C10:0) at the *sn*-1,3 position and long-chain polyunsaturated fatty acids (mainly C18:3, C20:5 or C22:5) at the *sn*-2 position can provide special nutrition source for the latter patients [2,29]. The medium-chain fatty acids provide rapid energy delivery. This is due to they can easily absorbed by the intestine cells, followed by they secretion directly to the portal vein and finally they are rapidly oxidized by the liver.

It is important that the medium-chain fatty acids are esterified on the *sn*-1 and *sn*-3 position and HPL can only digest in these positions. Long-chain polyunsaturated fatty acids at the *sn*-2 position of the glycerol backbone provide the delivery of the essential fatty acids to the required target through biochemical steps [30].

Moreover, a commercial product “Betapol” was marketed containing a “structured” lipid as a main component [31]. “Betapol” is developed for infant nutrition based on vegetable oils and having similar fatty acid distribution than human milk fat. This product eliminates the problems mentioned in Chapter 4.1.1, since the *sn*-2 position is substituted by a palmitic acid residue and other unsaturated fatty acids are placed at the *sn*-1 and *sn*-3 positions on the glycerol backbone.

Enzymatic procedures of the synthesis of “structured” TAG are mainly involved different lipases [32-34]. The enzymatic procedure goes under mild conditions and does not need costly instruments compared to the chemical modification, which usually involves special conditions such as vacuum or pressure and/or heat. The lipases can be classified into three major groups based on their regiospecificity (i) *sn*-1, *sn*-3, (ii) non regiospecific and (iii) specific lipases exhibiting selectivity toward particular fatty acids [35,36]. The intensively studied HPL and porcine pancreatic lipases belong to the first group.

4.1.4. Measuring the ratio of the positional isomer triacylglycerols by APCI-MS

The positions of fatty acids in a TAG can be measured by APCI-MS as described in Chapter 2.1.5. The positional isomers are identified from the masses and the relative abundance of the diacylglycerol fragment ions $[M+H-RCOOH]^+$ (or $[M-RCO_2]^+$) and the mass of the protonated molecular ion $[M+H]^+$ [37]. Accordingly, in the mixtures of ABA and AAB type TAGs the ratios of the diacylglycerol fragment ions ($[AA]^+/[AB]^+$) are proportional with the relative amounts of ABA or AAB [38].

4.2. EXPERIMENTAL

4.2.1. Materials

1(3),2-dilinoleoyl-3(1)-oleoyl glycerol (LLO) was purchased from Sigma (St. Louis, MO, USA) and dissolved in HPLC grade acetone-acetonitrile (2:1, v/v) to a concentration of 0.152 mg·mL⁻¹. 1,3-dilinoleoyl-glycerol and oleic acid were purchased from Nu-Chek Prep. (Elysian, MN, USA). The purity of the standards was higher than 99%. N,N'-dicyclohexylcarbodiimide (DCC) and 4-dimethyl-aminopyridine (DMAP) were purchased from Aldrich (Milwaukee, WI, USA). Kieselgel 60 was purchased from Merck (Darmstadt, Germany). The most commonly used edible oils namely grape seed, olive, pumpkin seed, soybean, sunflower and wheat germ were chosen for the measurements and purchased from local grocery stores and factories. Five different samples of each cold-pressed oil were used throughout the experiments totaling 30 samples (Table 1). The oils, which were purchased from the same factories or stores, were originated from different batches. Out of 5 grape seed oil samples, one was prepared from red grape seeds, the other from white grape seeds and the rests from mixed grape seeds. The samples were dissolved in HPLC grade acetone-acetonitrile (2:1, v/v) to give a final concentration of 0.1%. Acetone and acetonitrile were obtained from Koch-Light (Haverhill, England) and Riedel-de Haën (Seelze, Germany), respectively. Chloroform was purchased from Carlo Erba (Milano, Italy).

Oil varieties	Source of the samples								Number of samples	
	Factory 1	Factory 2	Factory 3	Factory 4	Factory 5	Factory 6	Factory 7	Factory 8		Stores
Grape seed	3	1							1	5
Olive		2							3	5
Pumpkin seed			2	1	1				1	5
Soybean					2	3				5
Sunflower				1			1		3	5
Wheat germ				1	1				3	5
									Total	30

Table 1. Type and the source of the various oil samples.

4.2.2. Preparation of 1,3-dilinoleoyl-2-oleoyl glycerol (LOL)

The 1,3-dilinoleoyl glycerol was esterified with oleic acid to obtain 1,3-dilinoleoyl-2-oleoyl glycerol (LOL). In order to run the reaction 1,3-dilinoleoyl-glycerol (300 mg, 0.49 mmol), oleic acid (137.5 mg, 0.49 mmol), DCC coupling reagent (100.5 mg, 0.49 mmol) and DMAP catalyst (10 mg) were dissolved in chloroform (10 mL). The reaction mixture was magnetically stirred under nitrogen atmosphere at 25°C for 24 hours in a 25-mL round-bottom flask. The proceeding of the reaction was monitored by HPLC/APCI-MS.

After the reaction was completed, the solution was evaporated to dryness in rotary evaporator. Chloroform (1 mL) was added to the light yellow residue and the product was purified by normal phase preparative column chromatography (silica gel) using chloroform as eluent under N₂ atmosphere. The column was prepared by Kieselgel 60 (53 g). The overall yield of the LOL was 72% (310 mg). The purified LOL was then analyzed by NMR and HPLC/APCI-MS.

4.2.3. Preparation of standard mixtures of LOL and LLO

Each standard was diluted in acetone/acetonitrile (2:1, v/v) to a concentration of 0.152 mg·mL⁻¹. Mixtures of LOL and LLO solutions were prepared from the stock solutions at 9 different ratios: 9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8, 1:9.

4.2.4. Instrumentation

4.2.4.1. NMR (¹³C, ¹H, 400 MHz, CHCl₃)

¹³C- and ¹H-NMR spectra of LLO and LOL were recorded on a Varian Unity-Inova 400 NMR spectrometer (Varian, USA).

4.2.4.2. HPLC/APCI-MS

HPLC/MS analyses were carried out using the same Shimadzu (Kyoto, Japan) HPLC system coupled to the QP2010 mass spectrometer fitted with APCI source, with using the optimized source parameters to TAG analyses as described in Chapter 2.2.2.1. Full scan mode analyses of the LLO and LOL standards were performed by the MS parameters described also in Chapter 2.2.2.1. Selected ion monitoring (SIM) was performed in positive ion mode for the analyses of the TAG standards (LLO, LOL) and the oils on the following ions: m/z 599.4 ($[LL]^+$ fragment), m/z 601.4 ($[LO]^+$ fragment) and m/z 881.6 ($[LLO+H]^+$ and/or $[LOL+H]^+$) with 0.2 amu mass window (micro scan) and 1 sec of dwell time (interval) parameters.

The same microparticulate RP silica column (Purospher, RP-18e, 125x4 mm, 5 μ m, Merck, Darmstadt, Germany) was used as described in Chapter 2, with acetone-acetonitrile eluent system, at a flow rate of 0.6 mL \cdot min⁻¹. The same two-stepped gradient program was applied for the HPLC/MS analyses of the LLO and LOL standards in full scan mode as described in Chapter 2.2.2.1. Isocratic eluent program was applied during the HPLC/MS analyses of the LLO and LOL standards and their mixtures in SIM mode with acetone concentration of 57% (acetonitrile of 43%) for 12.5 min. Linear gradient elution was used during the HPLC/MS analyses of the oil samples in SIM mode starting with acetone concentration of 57% hold for 11 min, followed by the linear gradient from 57% to 90% in 0.5 min, hold at 90% during 5 min, than from 90% to 57% in 0.5 min and finally equilibrate for 3 min at 57% (20 min total run time). Autosampler and column oven were set to 20 and 25°C, respectively. The injection volume was 5 μ L. The triacylglycerol standards (LLO, LOL) and oil samples were measured five and three times, respectively.

4.3. RESULTS AND DISCUSSION

4.3.1. NMR and MS analyses of LOL and LLO standards

The ^{13}C -NMR spectra of the two dilinoleoyl-oleoyl glycerol positional isomers showed significant differences in the region of olefinic carbons signals confirming the different structures (Figure 1). In the spectrum of LLO the olefinic carbon signals appeared at 130.27, 130.06, 130.02, 129.75, 128.15, 128.12, 127.96 and 127.94 ppm (Figure 1/a). The olefinic signals of LOL were detected at 130.27, 130.07, 130.05, 129.72, 128.12, and 127.95 ppm (Figure 1/b). In the case of LLO the two higher-field signals of LOL (128.12 and 127.95 ppm) splitted into four (128.15, 128.12, 127.96 and 127.94 ppm) indicating the disrupt of the symmetry.

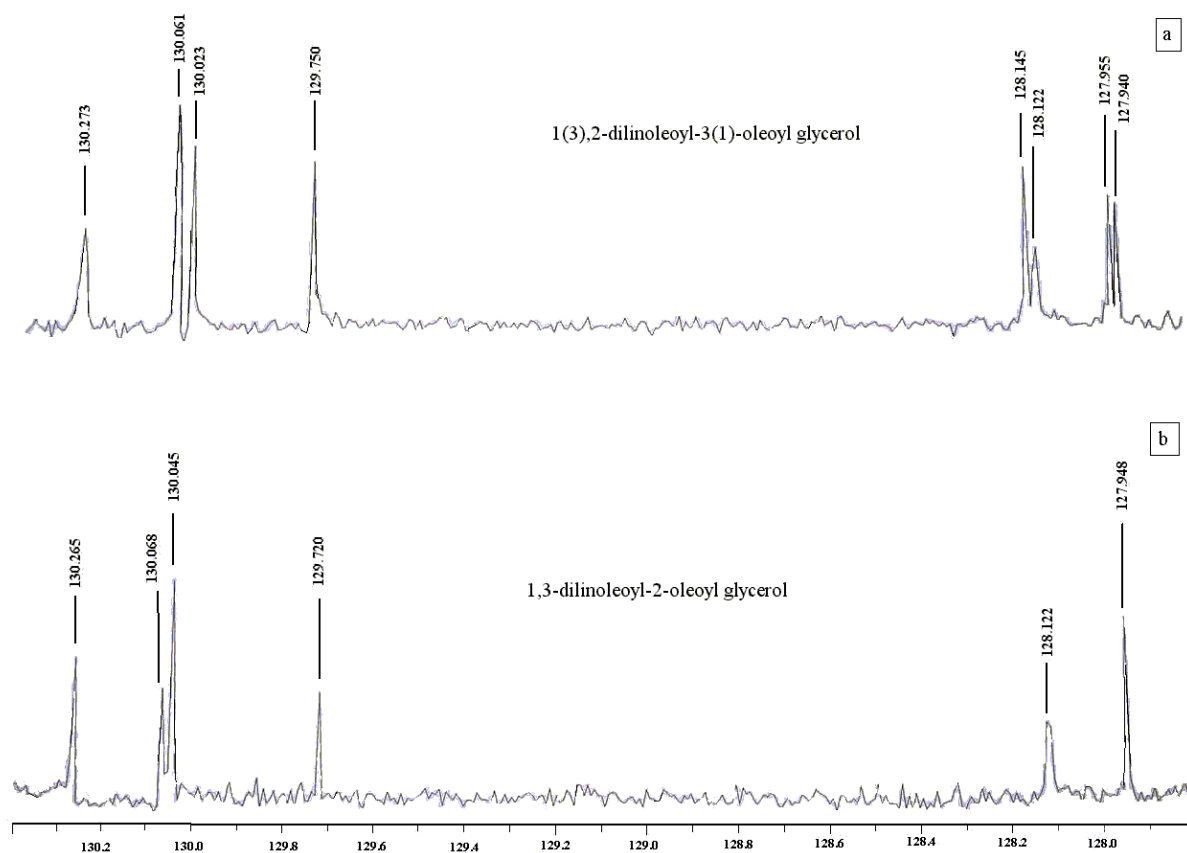


Figure 1. ^{13}C NMR spectra of 1(3),2-dilinoleoyl-3(1) oleoyl glycerol (a) and 1,3-dilinoleoyl-2-oleoyl glycerol (b).

The $^1\text{H-NMR}$ spectra showed no observable differences between the two positional isomers (LLO and LOL, $\text{C}_{50}\text{H}_{100}\text{O}_6$), δH [CDCl_3]: 0.89 (9 H, m, CH_3), 1.20-1.40 (48 H, m, CH_2), 1.63 (6 H, m, CH_2), 1.65-2.10 (12 H, m, $\text{CH}=\text{CH}-\text{CH}_2$), 2.31 (4 H, t, $J=7.4$ Hz, $2\times \text{CO}-\text{CH}_2$), 2.32 (2 H, t, $J=7.4$ Hz, $\text{CO}-\text{CH}_2$), 2.77 (4 H, m, $=\text{CH}-\text{CH}_2-\text{CH}=\text{, } J=6.5$ Hz), 4.15, 4.29 (2×2 H, $2\times\text{dd}$, $J_{\text{gem}}=11.9$ Hz, $J_{\text{vic}}=6.0$ and 4.3 Hz, $2\times \text{O}-\text{CH}_2$), 5.26 (1 H, m, $\text{O}-\text{CH}$), 5.28-5.42 (10 H, m, $\text{CH}=\text{CH}$) ppm).

HPLC/APCI-MS analysis of the synthesized LOL standard shows negligible amount of trilinoleoyl glycerol impurity (LLL, $[\text{M}+\text{H}]^+$: 879.8 Da, Figure 2). This impurity was originated from the dilinoleoyl glycerol standard, which was contained slight amount of trilinoleoyl glycerol. HPLC/APCI-MS analysis of the LLO SIGMA standard found no significant amount of impurities (not presented here).

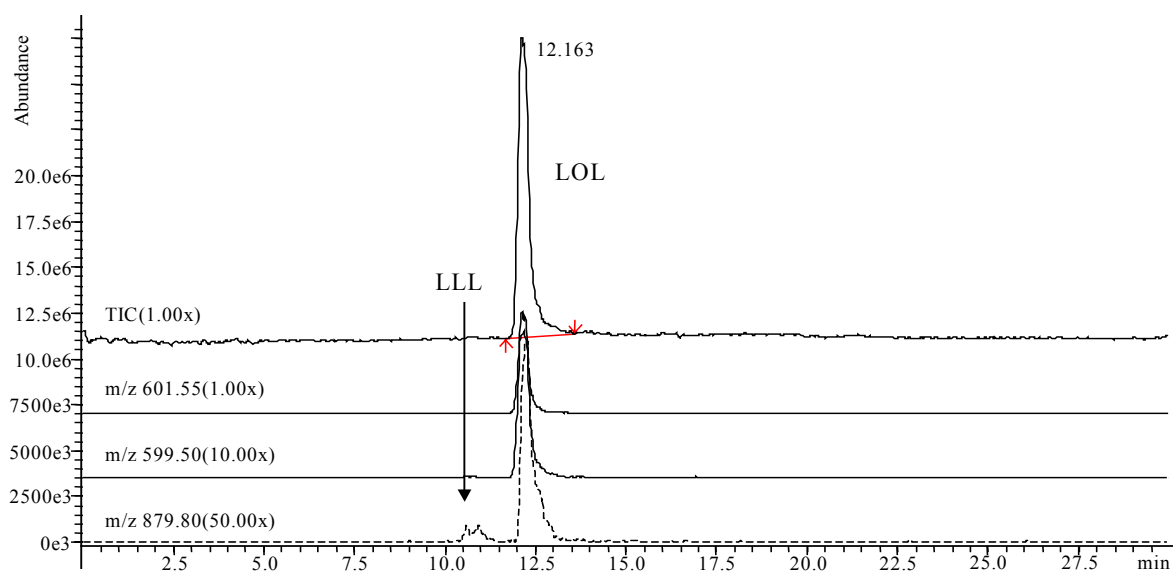


Figure 2. Total ion and single ion chromatograms of 1,3-dilinoleoyl-2-oleoyl glycerol (LOL) measured by HPLC/APCI-MS.

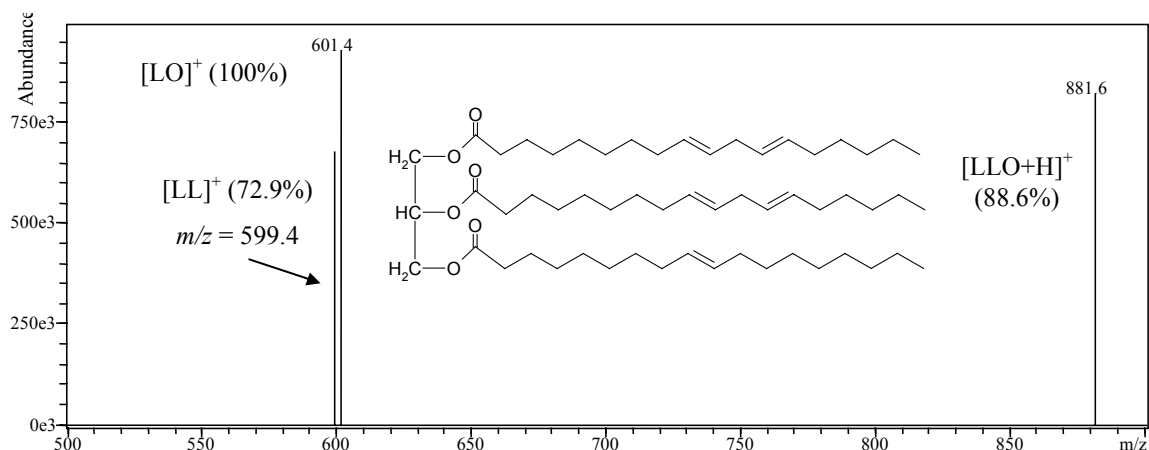


Figure 3. APCI mass spectrum of 1(3),2-dilinoleoyl-3(1)-oleoyl glycerol recorded in SIM mode (selected ions: 599.4: $[LL]^+$; 601.4: $[LO]^+$ and 881.6: $[M+H]^+$).

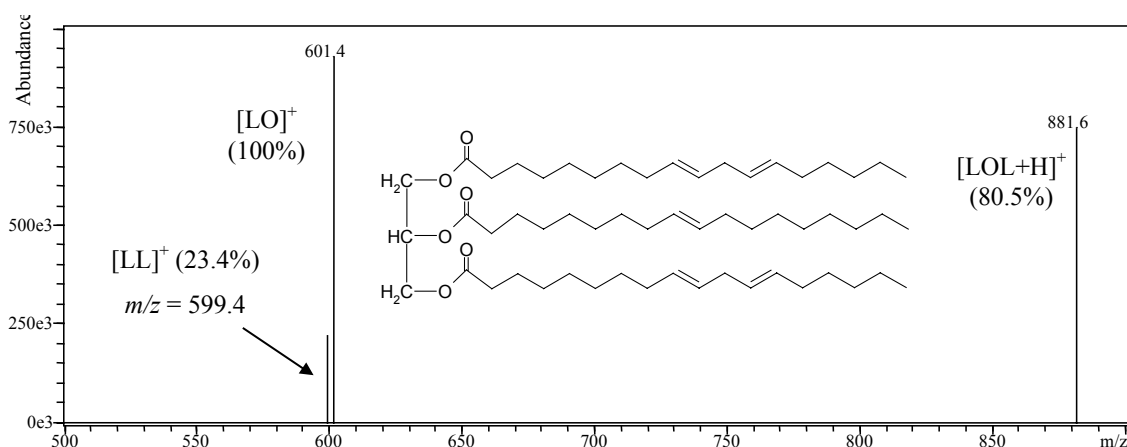


Figure 4. APCI mass spectrum of 1,3-dilinoleoyl-2-oleoyl glycerol recorded in SIM mode (selected ions: 599.4: $[LL]^+$; 601.4: $[LO]^+$ and 881.6: $[M+H]^+$).

Mass spectra of the two positional isomer triacylglycerols (LLO and LOL) exhibited different diacylglycerol fragment ions intensity ratios (Figure 3-4) as described in Chapter 2.1.5. The ratios of the $[LL]^+$ and $[LO]^+$ ions were around 7.0 and 2.3 in LLO and LOL, respectively (Table 2).

4.3.2. SIM measurements of LOL and LLO standard mixtures

The ratios of $[LL]^+$ and $[LO]^+$ ions in terms of percentage ($[LL]^+/[LO]^+$, %) were determined from the mass spectra of the dilinoleoyl-oleoyl glycerol (averaging *ca.* 50 scans followed by background subtraction) at each standard mixture. The measured ratios (%) are shown in Table 2. The relative standard deviations (RSD) of the ratios were low indicating a good repeatability of the measurements. The calibration curve of the ratios of $[LL]^+$ and $[LO]^+$ ions (%) is plotted in Figure 5. A clear linear relationship with high *r* coefficient (0.9942) was found indicating a good linear fit. The critical *r* value for 95% confidence interval and *n*=11 (degree of freedom) is 0.6020.

Table 2. The ratio of the $[LL]^+$ and $[LO]^+$ fragment ions (%) in mixtures of LOL and LLO at various percentage measured by HPLC/APCI-MS in SIM mode.

Concentration of LOL (% v/v) ^a	$[LL]^+/[LO]^+$ (%) ^b	RSD (%) ^c
100	22.89	0.84
90	25.65	1.12
80	29.91	0.81
70	34.58	0.42
60	39.38	1.50
50	44.91	1.58
40	49.75	2.03
30	54.99	1.60
20	59.90	1.87
10	66.04	1.41
0 (100% LLO)	70.28	2.89

^a: $100 \cdot \text{LOL} / (\text{LOL} + \text{LLO})$; ^{b, c}: Data were calculated from five parallel measurements;

^c: Relative standard deviation of the ratio of $[LL]^+$ and $[LO]^+$ fragments.

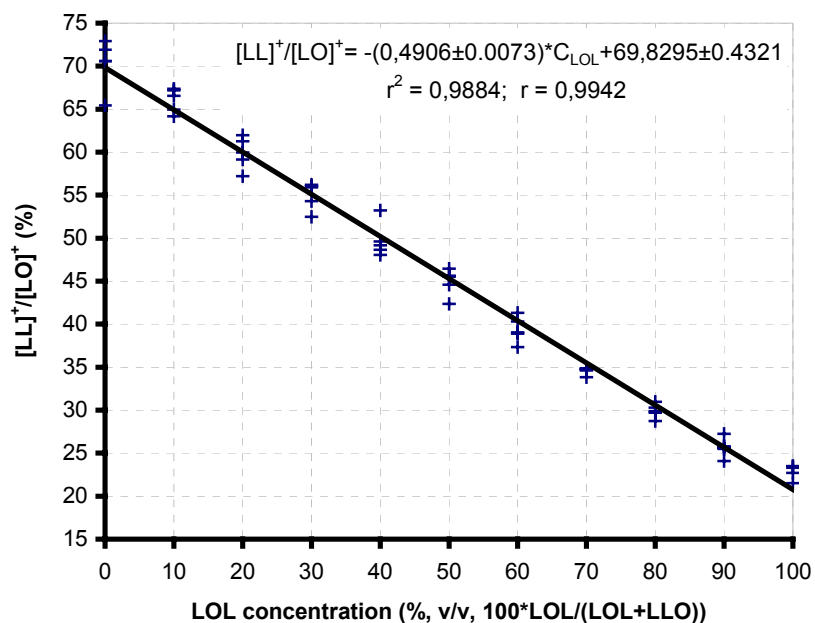


Figure 5. Ratio of the $[LL]^+$ and $[LO]^+$ fragment ions (%) in mixtures of LOL and LLO at various percentages (calibration curve).

4.3.3. Quantitation of LOL and LLO in vegetable oils

From the previously studied edible oils the most commonly used five different varieties such as grape seed, pumpkin seed, soybean, sunflower and wheat germ oils were chosen for the measurements in which the distinction between the LLO and LOL positional isomers was not obvious (Chapter 2.3.1). Olive oils were also measured in order to check the results published if it contains purely LLO isomer. Five-five samples were measured from each oil variety (30 pieces total).

HPLC/APCI-MS analyses in SIM mode were performed on the two diacylglycerol fragments ($[LL]^+$: 599.4 Da and $[LO]^+$: 601.4 Da) and the protonated molecular ion (881.6 Da) of dilinoleoyl-oleoyl glycerol in case of each oil. Total run time was 20 min and dilinoleoyl-oleoyl glycerol was eluted at 9.75 min (Figure 6-7).

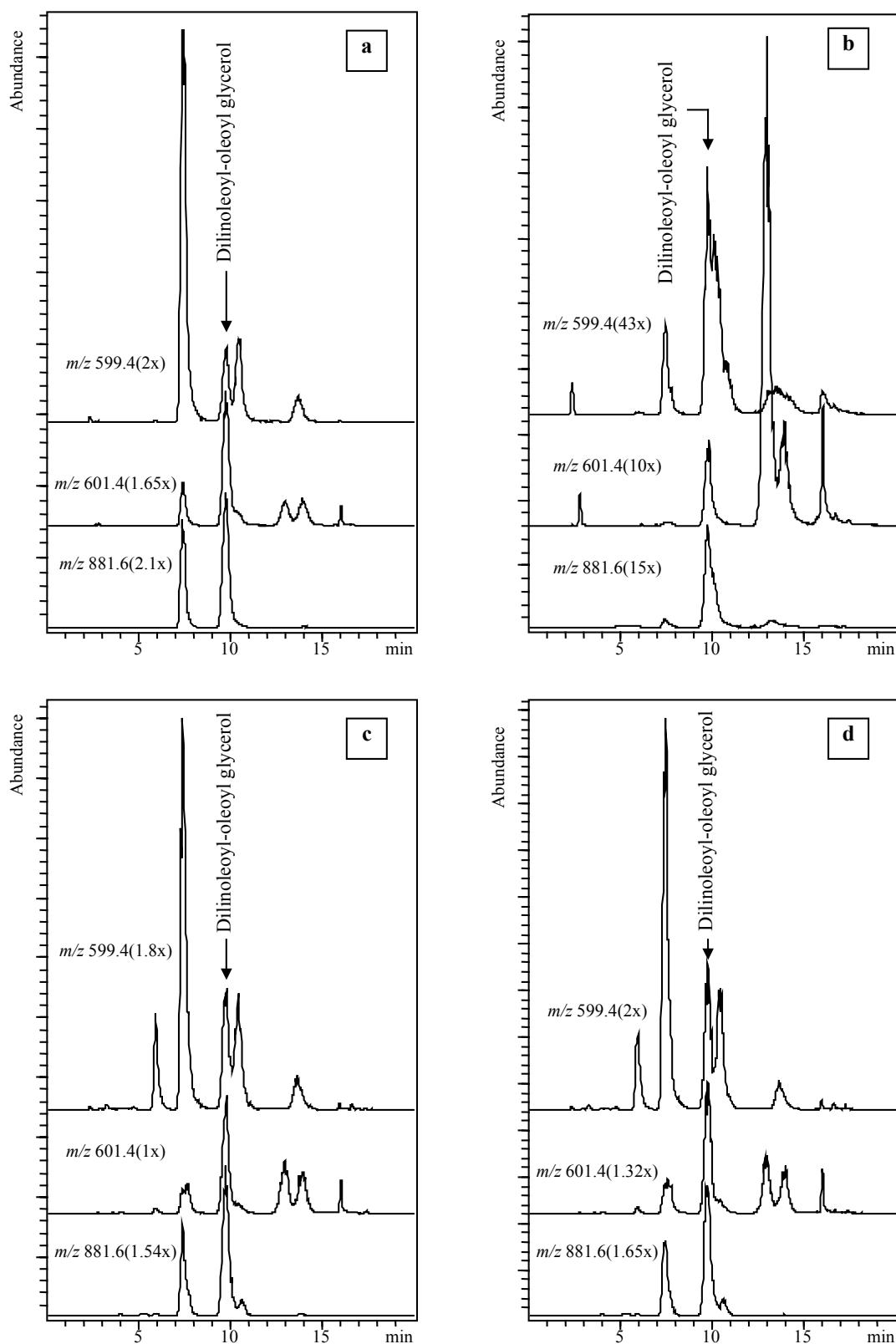


Figure 6. Selected ion chromatograms of 599.4 ($[LL]^+$), 601.4 ($[LO]^+$) and 881.6 ($[LOL+H]^+$ and/or $[LLO+H]^+$) ions from grape seed oil (a), olive oil (b), sunflower oil (c) and soybean oil (d) measured by HPLC/APCI-MS in SIM mode.

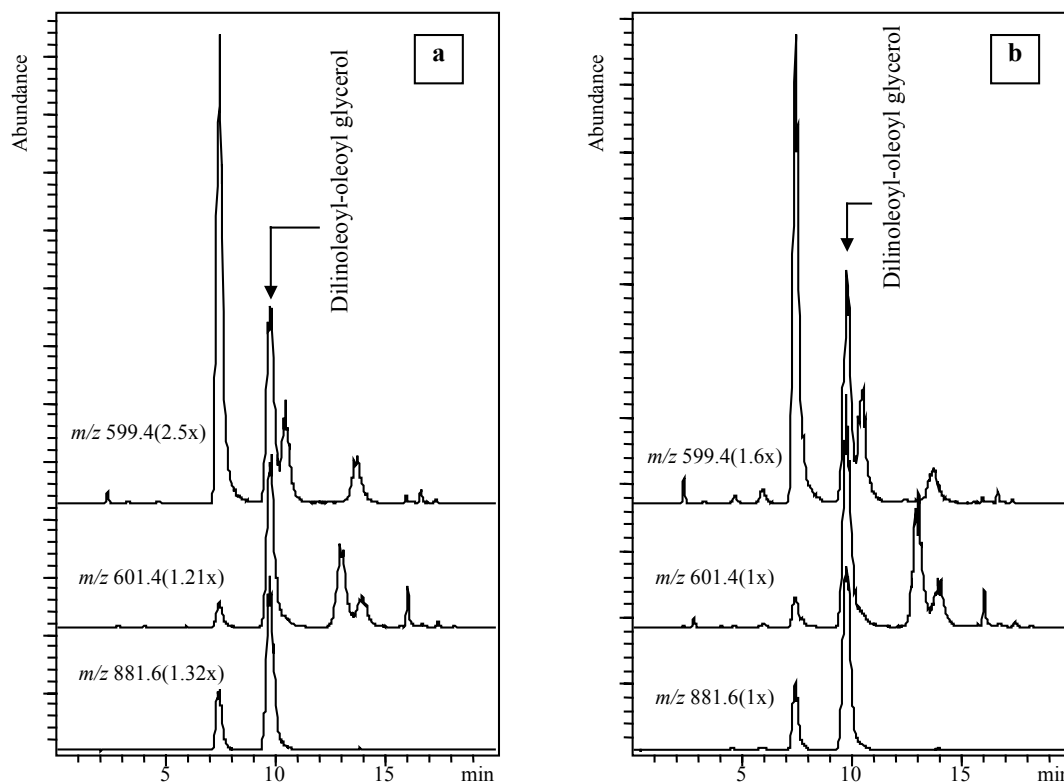


Figure 7. Selected ion chromatograms of 599.4 ($[LL]^+$), 601.4 ($[LO]^+$) and 881.6 ($[LOL+H]^+$ and/or $[LLO+H]^+$) ions from pumpkin seed oil (a) and wheat germ oil (b) measured by HPLC/APCI-MS in SIM mode.

The averaged mass spectra of dilinoleoyl-oleoyl glycerols were prepared from *ca.* 50 scans followed by background subtraction at each oil, similarly as described previously at the mixtures of the TAG standards. The $[LL]^+$ signal was not separated measuring olive oil as compared to that of other oils, due to of its relatively high PLL content (co-eluted peak, Figure 6/b).

The ratio of the $[LL]^+$ and $[LO]^+$ fragment ions (generated from dilinoleoyl-oleoyl glycerol) were obtained from the spectra in case of each oil. These ratios in various oils in term of percentage are shown in Table 3. The relative standard deviations (RSD) of the ratios were low indicating a good repeatability of the SIM measurements (Table 3). In most cases the RSD values of were around 2%, and the highest value was 4.81% (Soybean 4).

Table 3. $[LL]^+/[LO]^+$ (%) values and the calculated relative LOL content (%) of various edible oils measured by HPLC/APCI-MS in SIM mode.

Oil samples	$[LL]^+/[LO]^+$ (%) ^a	RSD (%) ^b	LOL content (%) ^c	LOL content for oil variety (%) ^d
Grape seed 1	48.52	2.94	43.4±2.9	44.2±2.6
Grape seed 2	48.36	1.02	43.8±1.0	
Grape seed 3	48.63	2.41	43.2±2.4	
Grape seed 4	48.10	4.45	44.3±4.4	
Grape seed 5	47.20	2.46	46.1±2.4	
Olive 1	68.65	0.77	2.4±1.1 ≈ 0	0
Olive 2	70.47	1.99	-1.3±2.9 ≈ 0	
Olive 3	69.79	2.50	0.1±3.6 ≈ 0	
Olive 4	71.26	1.81	-2.9±2.6 ≈ 0	
Olive 5	68.91	2.80	1.9±3.9 ≈ 0	
Pumpkin seed 1	64.74	1.40	10.4±1.8	13.9±4.3
Pumpkin seed 2	61.95	1.75	16.1±2.2	
Pumpkin seed 3	61.19	2.97	17.6±3.7	
Pumpkin seed 4	64.93	3.09	10.0±4.1	
Pumpkin seed 5	62.30	3.17	15.3±4.0	
Soybean 1	62.27	4.57	15.4±5.8	16.7±4.6
Soybean 2	61.04	3.87	17.9±4.8	
Soybean 3	60.14	2.46	19.8±3.0	
Soybean 4	62.67	4.81	14.6±6.1	
Soybean 5	62.00	3.35	16.0±4.2	
Sunflower 1	56.03	0.65	28.1±0.7	26.8±3.2
Sunflower 2	57.23	4.48	25.7±5.2	
Sunflower 3	55.38	2.88	29.5±3.3	
Sunflower 4	57.98	0.46	24.2±0.5	
Sunflower 5	56.78	2.33	26.6±2.7	
Wheat germ 1	60.91	1.90	18.2±2.4	15.9±2.9
Wheat germ 2	61.86	2.16	16.3±2.7	
Wheat germ 3	63.39	1.82	13.1±2.4	
Wheat germ 4	61.52	1.59	16.9±2.0	
Wheat germ 5	62.58	2.88	14.8±3.7	

^{a,b,c}: Data were calculated from three parallel measurements; ^b: Relative standard deviation of the ratio of $[LL]^+$ and $[LO]^+$ fragments; ^{c,d}: refer to all dilinoleoyl-oleoyl glycerol content ($100 \cdot \text{LOL}/(\text{LOL}+\text{LLO})$) (%) and were calculated from the calibration curve; ^d: calculated from 15 measurements (3 per oils).

The ratio of the $[LL]^+$ and $[LO]^+$ fragment ions show that not only LLO, but also significant amount of LOL isomer were present in grape seed, pumpkin seed, soybean, sunflower and wheat germ oils. The ratio of the $[LL]^+$ and $[LO]^+$ ions in olive oils were around 70%, confirming that the olive oil contains practically no LOL isomer, but purely LLO isomer.

Relative LOL contents refer to all dilinoleoyl-oleoyl glycerol content (the fourth column of Table 3, Figure 8) were calculated from the ratios based on the calibration curve (Figure 5). Grape seed oils contained the highest relative LOL content of the measured oils. The ratios of the positional isomers were around 1:1 since they contained around 44% LOL isomer (56% LLO). Sunflower oils also have a relatively high LOL isomer content. The ratios were around 27:73 of LOL:LLO. The other measured oils except olive oil also contained significant portion of LOL in addition to the LLO isomer. The ratios were around 15:85 of LOL:LLO in pumpkin seed, soybean and wheat germ oils.

From our data an important finding can also be concluded. The ratio of the LOL and LLO isomers seems to be slightly constant value per oil varieties (Table 3, Figure 8). The averaged LOL contents are shown in the last column of Table 3. Grape seed oil has the highest ratio, accounted for $44.2 \pm 2.6\%$ of LOL isomer (related to all dilinoleoyl-oleoyl glycerol isomers). It is also worth noting that grape seed oil samples were made from different types of grapes but the ratios of the LOL and LLO isomers were similar in all cases (Table 3, Figure 8). Although the exact species of the seeds are unknown, it is known the three of the samples were made from mixed seeds (red and white, Grape seed 1-3) and one-one samples were made from purely white (Grape seed 4) and red (Grape seed 5) grape seeds, respectively. Sunflower oil also contained a high relative LOL content ($26.8 \pm 3.2\%$) regardless of the origin of the samples.

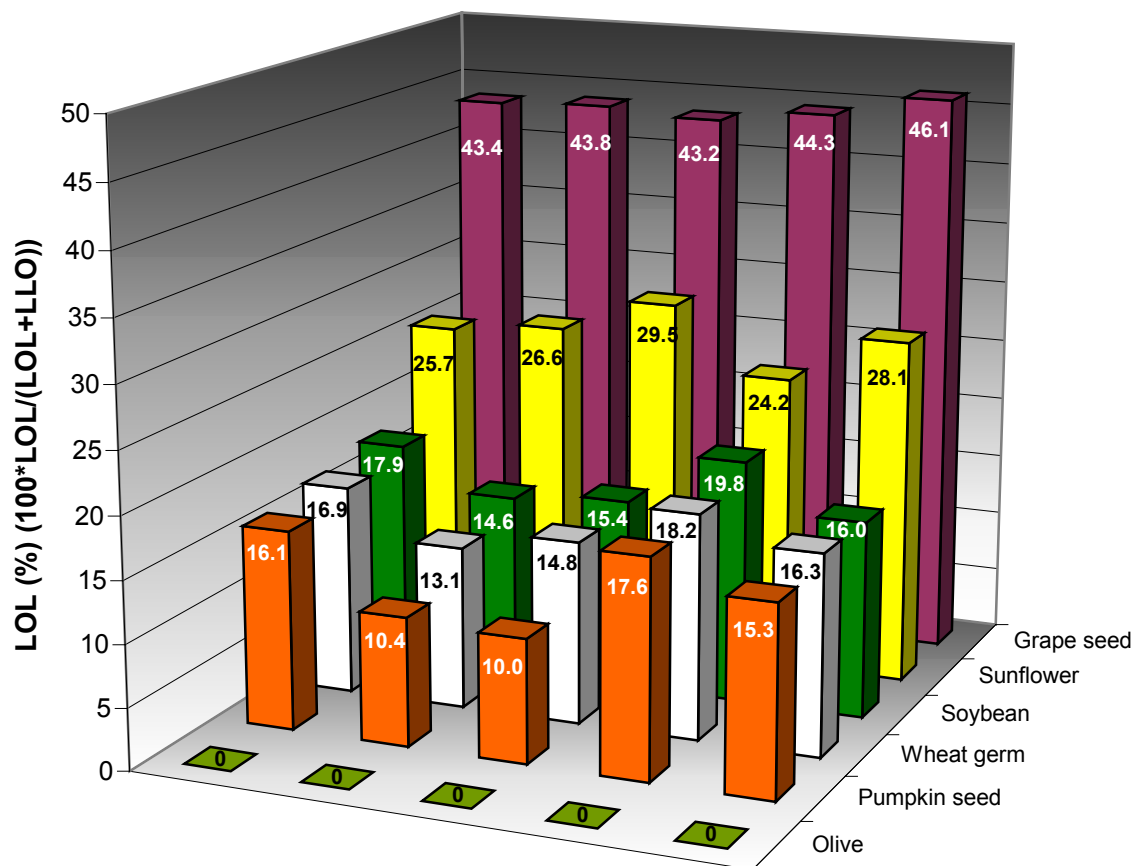


Figure 8. Relative content of LOL positional isomer refers to all dilinoleoyl-oleoyl glycerol isomers (LOL+LLO) in various vegetable oils measured by HPLC/APCI-MS in SIM mode.

There were no significant differences among the LOL:LLO isomer ratios in the soybean wheat germ and pumpkin seed oils. The LOL:LLO ratio was 15:83. The relative LOL contents in soybean wheat germ and pumpkin seed oils were $16.7 \pm 4.6\%$, $15.9 \pm 2.9\%$ and $13.9 \pm 4.3\%$, respectively. The grape seed, olive and sunflower oils can be clearly distinguished from other oils (pumpkin seed, soybean and wheat germ oils) based on the relative LOL content (Figure 8).

4.4. CONCLUSION

The ratio of LLO and LOL isomers in grape seed, olive, pumpkin seed, soybean, sunflower and wheat germ oils measured by HPLC/APCI-MS in SIM mode has not been studied so far (in most of the published oil analyses the structure of dilinoleoyl-oleoyl glycerol was declared as pure LLO isomer [39-43]).

HPLC/APCI-MS was found to be suitable method for quantitation of the LOL and LLO positional isomers based on their diacylglycerol fragments ratios. The commercially not available LOL isomer standard was successfully synthesized. The analysis of synthesized LOL and the supplied LLO standards were performed by ^{13}C -, ^1H -NMR and HPLC/APCI-MS analyses. The calibration curve of the diacylglycerol fragment ratio ($[\text{LL}]^+ / [\text{LO}]^+$, %) in various LOL (or LLO) concentrations was measured in SIM mode. This was found to be linear with a high r coefficient value ($r=0.9942$, $r_{\text{krit}}=0.6020$) making possible the determination of the exact ratio of the LOL and LLO isomers in various oils. The equation of the calibration curve was

$$\frac{[\text{LL}]^+}{[\text{LO}]^+} (\%) = -0.49 * \text{Conc}_{\text{LOL}} (\%) + 69.83$$

The results of the analyses confirmed our previous suspicion, that some of the oil varieties (grape seed, pumpkin seed, soybean, sunflower and wheat germ oils) contain both dilinoleoyl-oleoyl glycerol isomers (LOL and LLO), and the amount of the LOL isomer is significant. The measured dilinoleoyl-oleoyl glycerols ratios (relative LOL or LLO contents) in various oils were found to be a constant value per oil varieties. The RSDs of the relative LOL contents were between 0.5 and 6.1%, indicating the good repeatability of the SIM measurements. Grape seed oils contained the highest relative LOL content accounted for

44.2±2.6%. Sunflower oils contained also relatively high LOL content accounted for 26.8±3.2%. Pumpkin seed, soybean and wheat germ oils contained quite similar amount of LOL ratios accounted for 16.7±4.6%, 15.9±2.9% and 13.9±4.3%, respectively. Olive oils contained practically 100% of LLO isomer confirming the published data [40-42]. These results indicate that the unsaturated fatty acids such as linoleic and oleic acids have “non-random” distribution pattern in various oils.

The distributions of the various fatty acids on the glycerol backbone influence also the metabolism of TAGs. Thus our results have probably nutritional and biological importance as well.

4.5. REFERENCES

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5. SUMMARY

Two different methods were worked out for the characterization of plant oils based on their triacylglycerol profile. One was based on high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (HPLC/APCI-MS) analysis and the other on matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOFMS) analysis, both in combination with linear discriminant analysis (LDA). Successful classification of 14 different types of plant oils (almond, avocado, corn germ, grape seed, linseed, mustard seed, olive, peanut, pumpkin seed, sesame seed, soybean, sunflower, walnut and wheat germ) was obtained by LDA based on their HPLC/APCI-MS triacylglycerol profiles. Out of the 73 samples 68 were correctly classified (93.2%) indicating the correct classification of 9 different types of oils (almond, avocado, grape seed, linseed, mustard seed, olive, pumpkin seed, sesame seed and soybean oil). Successful classification of the 14 different types of plant oils was also obtained by LDA based on their MALDI-TOFMS triacylglycerol profiles. Out of the 73 samples 68 were correctly classified (93.2%) in this case also, indicating the correct classification of 12 different types of oils (almond, avocado, corn germ, grape seed, linseed, mustard seed, olive, peanut, sesame seed, soybean, sunflower and walnut oil). Comparing the two mass spectrometric methods combined with the LDA calculation, MALDI-TOFMS provided better results in addition of much shorter analysis and data processing time.

The HPLC analysis time in the HPLC/APCI-MS method was found to be a little bit long (30-min) for analysis of a great number of oil samples. In order to reduce the analysis time an adequate and repeatable fast separation (10-min) of plant oil triacylglycerols was worked out on a monolithic RP silica column using gradient elution program at 5 mL·min⁻¹ flow rate. This

short-run HPLC method was successfully coupled to APCI mass spectrometer by eluent flow splitting (ca. $400 \mu\text{L}\cdot\text{min}^{-1}$ went to the MS interface).

In the mass spectrum of LLO the ratio of the diacylglycerol fragment ions was found to be altered in some cases. This finding indicated that LOL isomer was present in addition to the LLO isomer in some types of oils. This assumption was confirmed in grape seed, olive, pumpkin seed, soybean, sunflower and wheat germ oils by quantitating the exact ratio of the LOL and LLO isomers using HPLC/APCI-MS in selected ion monitoring (SIM) mode. The measured calibration curve showed linear behavior making possible the determination of the exact ratio of the LOL and LLO isomers (relative LOL content) in various oils. This relative LOL content was found to be constant value per oil varieties. The relative standard deviations of the LOL contents were around 2.5%, indicating the good repeatability of the SIM measurements. The relative LOL contents in increasing order were accounted for $13.9\pm 4.3\%$, $15.9\pm 2.9\%$, $16.7\pm 4.6\%$, $26.8\pm 3.2\%$ and $44.2\pm 2.6\%$ in wheat germ, soybean, pumpkin seed, sunflower and grape seed oils, respectively. Olive oils contained practically 100% of LLO isomer confirming the published data. These results indicate that the unsaturated fatty acids such as linoleic and oleic acids have “non-random” distribution pattern in various oils creating the future possibility for the identification of different oils based on the LOL and LLO isomer ratio.

6. ABBREVIATIONS

APCI	Atmospheric pressure chemical ionization
APCI-MS	Atmospheric pressure chemical ionization-mass spectrometry
CDL	Curve desolvation line
CEC	Capillary electrochromatography
CI	Chemical ionization
CN	Carbon number
DB	Double bond
DCC	N,N'-dicyclohexyl-carbodiimide
DHB	2,5-dihydrobenzoic acid
DMPA	4-dimethyl-aminopyridine
EI	Electron impact
ELSD	Evaporative light-scattering detector
ESI (ES)	Electrospray ionization
ESI-MS	Electrospray ionization-mass spectrometry
GC/FID	Gas chromatography/flame ionization detection
GC/IRMS	Gas chromatography/isotope ratio mass spectrometry
GC/MS	Gas chromatography/mass spectrometry
HETP	Height equivalent of a theoretical plate
HGL	Human gastric lipase
HPL	Human pancreatic lipase
HPLC	High-performance liquid chromatography
HPLC/APCI-MS	High-performance liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry
L	Linoleic acid (18:2, C ₁₈ H ₃₂ O ₂)
LDA	Linear discriminant analysis
LLL	Trilinoleoyl glycerol
LLLn	1(3),2-dilinoleoyl-3(1)-linolenoyl glycerol
LLO	1(3),2-dilinoleoyl-3(1)-oleoyl glycerol
LLS	1(3),2-dilinoleoyl-3(1)-stearoyl glycerol
Ln	Linolenic acid (18:3, C ₁₈ H ₃₀ O ₂)

LnLP	1(3)-linolenoyl-2-linoleoyl-3(1)-palmitoyl glycerol
LOL	1,3-dilinoleoyl-2-oleoyl glycerol
MALDI-TOFMS	Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry
MS	Mass spectrometry
NP	Normal phase
NMR	Nuclear magnetic resonance
O	Oleic acid (18:1, C ₁₈ H ₃₄ O ₂)
ODS	Octadecyl silica (C ₁₈ silica)
OLO	1,3-dioleoyl-2-linoleoyl glycerol
OOL	1(3),2-dioleoyl-3(1)-linoleoyl glycerol
OOO	Trioleoyl glycerol
P	Palmitic acid (16:0, C ₁₆ H ₃₂ O ₂)
PCA	Principal component analysis
PLL	1(3),2-dilinoleoyl-3(1)-palmitoyl glycerol
PLO	1(3)-palmitoyl-2-linoleoyl-3(1)-oleoyl glycerol
PLP	1,3-dipalmitoyl-2-linoleoyl glycerol
POO	1(3),2-dioleoyl-3(1)-palmitoyl glycerol
POP	1,3-dipalmitoyl-2-oleoyl glycerol
Py/MS	Pyrolysis/mass spectrometry
RID	Refractive index detector
RP	Reversed phase
RSD	Relative standard deviation (%)
S	Stearic acid (18:0, C ₁₈ H ₃₆ O ₂)
SD	Standard deviation
SIC	Single ion chromatogram
SIM	Selected ion monitoring
SOO	1(3),2-dioleoyl-3(1)-stearoyl glycerol
SSO	1(3),2-distearoyl-3(1)-oleoyl glycerol
TAG	Triacylglycerol
TFA	Trifluoroacetic acid
t _r	Retention time
TIC	Total ion chromatogram

7. ACKNOWLEDGEMENTS

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Annamária Jakab

8. APPENDIX

LISTS OF THE PAPERS AND POSTER PRESENTATIONS

PAPER I

Differentiation of Vegetable Oils by Mass Spectrometry Combined with Statistical Analysis (A. Jakab, K. Nagy, K. Héberger, K. Vékey, E. Forgács)

Rapid Commun. Mass Spectrom. 2002; **16/24**: 2291-2297.

PAPER II

Comparative Analysis of Different Plant Oils by High-Performance Liquid Chromatography-Atmospheric Pressure Chemical Ionization Mass Spectrometry (A. Jakab, K. Héberger, E. Forgács)

J. Chromatogr. A, 2002; **976**: 255-263.

PAPER III

Characterization of Plant Oils on a Monolithic Silica Column by High-Performance Liquid Chromatography-Atmospheric Pressure Chemical Ionization Mass Spectrometry

(A. Jakab, E. Forgács)

Chromatographia, 2002; **56**: S69-S73.

PAPER IV (not presented here)

Quantitation of positional isomer dilinoleoyl-oleoyl-glycerols in Plant oils by Mass Spectrometry

(A. Jakab, I. Jablonkai, E. Forgács)

Submitted to *Rapid Commun. Mass Spectrom.*

POSTER PRESENTATION (not presented here)

- Quantification of positional isomer triacylglycerols in plant oils (A. Jakab, I. Jablonkai, E. Forgács) *21th Informal Meeting on Mass Spectrometry* (IMMS 21th), Antwerp, Belgium, 11-15. May 2003.
- Növényi olajok természetes antioxidáns tartalmának vizsgálata HPLC/MS-es (Jakab A., Forgács E.) *Elvlasztástudományi Vándorgyűlés*, Lillafüred, Hungary, 16-18. Oct. 2002.
- Növényi olajok csoportosítása lineáris diszkriminancia analízissel tömegspektrometriás adatok alapján (Jakab A., Nagy K., Héberger K., Vékey K., Forgács E.) *Chemometrics 2002*, Tata, Hungary, 29. Sept-1. Oct. 2002.
- Analysis of Plant Oil Triacylglycerols by HPLC/APCI-MS and MALDI-TOFMS (A. Jakab, K. Nagy, K. Vékey, E. Forgács) *20th Informal Meeting on Mass Spectrometry* (IMMS 20th), Primiero, Italy, 12-16. May 2002.
- Comparative Analysis of Different Plant Oils by HPLC/APCI-MS (A. Jakab, E. Forgács) *Seventh International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analyzers* (HTC-7) Bruges, Belgium, 6-8. Feb. 2002.
- Characterization of Plant Oils on a Monolithic Silica Column by HPLC/APCI-MS (A. Jakab, E. Forgács) *Balaton Symposium '01*, Siófok, Hungary, 2-4. Sept. 2001.

PAPERS DIRECTLY NOT RELATED TO THE THESIS (not presented here)

- Influence of Physico-Chemical Parameters of some Barbituric Acid Derivatives on their Retention on an Amide Embedded RP Silica Column
(A. Jakab, M. Prodán, E. Forgács) *J. Pharma. Biomed. Anal.*, 2002; **27**: 913-921.
- PCA Followed by Two-Dimensional Nonlinear Mapping and Cluster Analysis Versus Multilinear Regression in QSRR
(A. Jakab, G. Schubert, M. Prodán, E. Forgács) *J. Liq. Chromatogr. Rel. Technol.*, 2002; **25(1)**: 1-16.
- Determination of the Retention behavior of Barbituric Acid Derivatives in Reversed-Phase HPLC by using Quantitative Structure-Retention Relationships
(A. Jakab, G. Schubert, M. Prodán, E. Forgács) *J. Chromatogr. B.* 2002; **770**: 227-236.
- Study of the Retention Parameters of Barbituric Acid Derivatives in Reversed Phase HPLC by using Quantitative Structure-Retention Relationships
(A. Jakab, G. Schubert, M. Prodán, E. Forgács) *Chromatographia*, 2002; **56**: S55-S60.
- Three-dimensional Principal Component Analysis Used for the Study on Enzyme Kinetics. An Empirical Approximation for the Determination of the Dimensions of Component Matrices
(H. Morais, C. Ramos, E. Forgács, A. Jakab, T. Cserhádi, J. Oliviera, T. Illés, Z. Illés) *Quant. Struct. Act. Relat.*, 2001; **20**: 241-247.
- Separation of Ethoxylated Lauryl alcohol Oligomers and Ethoxylated Sorbitane Monoleate Oligomers on Porous Graphitized Carbon Column
(V. Németh-Kiss, A. Jakab, E. Forgács) *Chem. Anal.*, 2001; **46**: 613-619.

NYILATKOZAT

Alulírott Jakab Annamária kijelentem, hogy ezt a doktori értekezést magam készítettem és abban csak a megadott forrásokat használtam fel. Minden olyan részt, amelyet szó szerint, vagy azonos tartalomban, de átfogalmazva más forrásból átvettem, egyértelműen, a forrás megadásával megjelöltem.

Budapest, 2002-06-10.

aláírás