The Use of Atmospheric Pressure Chemical Ionization Mass Spectrometry with High Performance Liquid Chromatography and Other Separation Techniques for Identification of Triacylglycerols

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Abstract: Triacylglycerols (TAGs) are widely distributed, if not universal, in edible plants and edible plant triacylglycerols are nutrients forming an important part of the human diet. TAGs of higher plants consist mainly of esters of glycerol with fatty acids, many of which are essential as human nutrients and are therefore in the center of increasing attention. Despite their importance, the molecular species of each TAG class have not yet been fully characterized. The use of state-of-the-art techniques, especially in a combination such as that discussed in this review, permits the identification of not only TAG classes, but also their positional isomers and often also individual molecular species.

Atmospheric pressure chemical ionization mass spectrometry (APCI-MS) in combination with high-performance liquid chromatography (HPLC) has proven to be a very valuable technique for analysis of different lipids from a variety of sources. This paper describes direct analyses of TAGs from natural oils, frequently very unusual, using LC-MS/APCI.

Keywords: Triacylglycerols, Atmospheric pressure chemical ionization, Mass spectrometry, High-performance liquid chromatography, Gas chromatography, Supercritical fluid chromatography, LC-MS/APCI.

1. INTRODUCTION

Mass spectrometry (MS) with on-line atmospheric pressure chemical ionization (MS/APCI) in combination with high-performance liquid chromatography (HPLC), usually reversed-phase HPLC (RP-HPLC) has proven to be a very valuable technique for analysis of different lipids from a variety of sources. This paper describes analyses of TAGs from natural oils, frequently very unusual, using HPLC coupled with on-line MS/APCI (LC-MS/APCI). Some applications of MS/APCI to TAG analysis have been reviewed [1-6] and are also recorded on web pages (http://www.lipidlibrary.co.uk/lit_surv/ms_ms/apci_ms.htm).

TAG analysis has always presented a difficult analytical challenge. HTGC has been used for many years, but there are drawbacks to this approach. TAGs with numerous sites of unsaturation are subject to degradation at the temperatures used for HTGC. TAGs with long fatty chains and/or oxygen-containing functional groups require higher temperatures that often produce substantial column bleed. Numerous methods for HPLC of TAGs are available, but these, too, have limitations. The complexity of most TAG mixtures in naturally occurring fats and oils precludes complete structural analysis using mass spectrometry. Simple synthetic mixtures, such as model systems and TAG standards, can be analyzed by LC-MS without separation (by infusion) to allow facile qualitative characterization of all components. For more complex samples, chromatographic separation of components coupled to the information-rich APCI detectors clearly makes qualitative and quantitative analysis easier than without separation, as will be seen.

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This review does not deal with oxidation products of TAGs unless the plant oils themselves are composed of fatty acids having OH groups, such as castor oil. With some exceptions, the quantitation of TAGs has also not been treated here and we rather refer the reader to existing reviews and books [2,3].

2. RESULTS

2.1. Plant Oils

One of the most frequent applications of LC-MS/APCI is the analysis of plant oils. The last decade, during which this method has become more and more common, has witnessed the analysis of most of commonly used plant oils. A survey of the TAG sources, analytical and identification methods and conditions including references is given in Table **1**.

Special attention was paid to oils used for food preparation, such as canola oil (rapeseed oil with lowered content of erucic acid) and soybean oil. In 1996, LC-MS/APCI was used for analyzing canola oil, the spectral identification of the TAGs being based on the DAG (diacylglycerol) fragments and on the protonated molecular ion $[M+H]^+$, except trisaturates which gave no $[M+H]^+$. TAGs were identified and quantitated in normal, high-stearic acid and high-lauric acid canola varieties. The LC-MS/APCI identification of canola oil TAGs allowed their quantitation by RP-HPLC coupled with a commercial flame ionization detector (FID). There was agreement between fatty acid composition obtained by LC-MS and LC-FID. However, the TAG resolution obtained by LC-MS/APCI was superior to LC-FID in the qualitative identification of TAGs present in amounts below one percent. The oils of the modified canola varieties, compared to typical canola oil, contained increased content

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Table 1. Sources of TAGs, HPLC Techniques and Analytical Conditions Used for their Separation and Identification

Pub No	Column	Phase	Mobile Phase	Flow Rate
4	15x3x7 or 15x3.9x7	C-18	MeOH-iPrOH-Hex or ACN-iPrOH-water; grad	1 mL/min
5	15x3.9x7 or 30x3.9x7	C-18	ACN-iPrOH; grad	1 mL/min
7	25x4.6x5+25x4.6x10	C-18	PrCN-ACN-DCM; grad	1 mL/min
8	15x3x7	C-18	MeOH-iPrOH-Hex or ACN-iPrOH-water-Hex; grad	1 mL/min
9	25x4.6x5+25x4.6x10	C-18	PrCN+Hex; grad	1 mL/min
10	30x3.9x4 and 15x3.9x4	C-18	ACN-iPrOH; grad	1 mL/min
11	5x4.6	C-18	Acetone-ACN; grad	5 mL/min split 400 μL
12	12.5x4x5	C-18	Acetone-ACN; grad	0.6 mL/min
14	15x3.9x7 or two 15x3.9x7	C-18	ACN-EtOH or ACN-iPrOH-water; grad	1 mL/min
15	25x3x5	C-18	ACN-EtOH; grad	0.7 mL/min
16	25x4.6x5	C-18	ACN-DCM; grad	0.7 mL/min
17	5.5x2x3	C-18	MeOH-water-Hex+0.2% AcOH; grad	0.2 mL/min→0.7 mL/min
19	25x4.6x5+25x4.6x10	C-18	PrCN+ACN-DCM; grad	1 mL/min
20	two 25x4.6x5	C-18	ACN-DCM; grad	1 mL/min
21	two 15x4.6x3	C-18	ACN-MTBE-$CHCl_3$	1 mL/min
22	25x4.6x5	C-18	Acetone-ACN	1 mL/min
23	25x4.6x5	C-18	ACN-iPrOH-Hex+50mM $AcONH_4$	
24	25x10x5	C-18	PrCN	0.8 mL/min
25	two 25x4.6x5	C-18	ACN-DCM; grad	0.7 mL/min
26	two 25x4.6x5	C-18	ACN-DCM; grad	0.85 mL/min
27	two 25x4.6x5	C-18	ACN-DCM; grad	0.85 mL/min
28	25x10x5	C-18	PrCN	0.8 mL/min
29	25x4.6x5	C-18	PrCN	0.5 mL/min
32	25x4.6	C-18	EtOH-iPrOH; grad	0.8 mL/min
33	25x4.6	C-18	EtOH-iPrOH; grad	0.8 mL/min
35	25x2.1x5	C-18	ACN-$CHCl_3$; grad	0.25 mL/min
36	no HPLC-directly to MS		PrCN	1 mL/min
37	12.5x4x5	C-18	Acetone-ACN; grad	0.6 mL/min
38	25x4.6x5	C-18	ACN-iPrOH-Hex; grad	
39	25x4.6x5	C-18	PrCN	0.8 mL/min
42	two 25x4.6x5	C-18	ACN-DCM-$C_2H_4Cl_2$; grad	0.8 mL/min
43	25x2.1x5	C-18	ACN-$CHCl_3$; grad	0.25 mL/min
49	25x4.6x5	C-18	Acetone-ACN; 70:30	1 mL/min
49	25x4.6x5	Ag	Hex-ACN; 995:5	1 mL/min
50	25x4.6x5	C-18	ACN-iPrOH; grad	1 mL/min
50	25x4.6x5	Ag	Hex-ACN; 995:5	1 mL/min

(Table 1) contd.....

Pub No	Column	Phase	Mobile Phase	Flow Rate
51	15x1x5	Ag-Si	Hex-ACN; grad	0.05 mL/min
51	10x4.6	C-18	ACN-iPrOH; grad	4 mL/min
52	10x4.6	C-18	ACN-iPrOH; grad	4 mL/min
52	15x1x5	Ag-Si	Hex-ACN; 993:7	
53	two 25x4.6x5	C-18	ACN-iPrOH; grad	1 mL/min
53	25x4.6x5	C-18	Acetone-ACN; 70:30	1 mL/min
53	25x4.6x5	Ag-Si	Hex-ACN; 994:6	1 mL/min
54	15x1x5	Ag-Si	Hex-ACN; grad	0.011 mL/min
54	25x4.6x5	C-18	Acetone-ACN; 70:30	1 mL/min
64	two 25x4.6x5	C-18	ACN-DCM; grad	0.7 mL/min
67	25x4.6x5+25x4.6x10	C-18	PrCN+Hex; grad	1 mL/min
68	25x4.6x5+25x4.6x10	C-18	PrCN-ACN-DCM; grad	1 mL/min
69	25x4.6x5	Si	Hex-MTBE-AcOH; 85:15:0.4	1 mL/min
69	25x4.6x3	CN	Hex-MTBE-0.4% AcOH; grad	1 mL/min
70	25x4.6x3	Si	Hex-MTBE-AcOH; 82.5:17.5:0.4	0.5 mL/min
71	25x4.6x5	C-18	ACN-iPrOH-Hex; grad	

Ag - Silver-modified cation-exchange ligand-covered spherical silica

Table 1. Continuation A

Pub No	Capillary Voltage	Corona Voltage (current)	Vaporizer Temp	Nebulizer Gas	Drying Gas	Sheath Gas
4		20 V	500			
5			350	483 kPa	N 3 L/min	
7		6.0 μA	400		N 2 L/min	55 psi
8	3200 V	20 V	500	N	N	N
9		5.0 μA	225-240	N		55 psi
10			400	70 psi	N 3 L/min	
11	4500 V		300	N 2 mL/min		
12	4500 V		300	N 2 mL/min		
14		20 V	500	70 psi	N 5 L/min	
15	no data →					
16		6.0 μA	400			35 psi
17		4.0 μA	500	60 psi		
19		6.0 μA	400			60 psi
20		6.0 μA	350			35 psi
21		8.0 μA	300			N 6.75 bar
22		5.0 μA	400	2.5 L/min		

(Table 1 Continuation A) contd.....

Pub No	Capillary Voltage	Corona Voltage (current)	Vaporizer Temp	Nebulizer Gas	Drying Gas	Sheath Gas
23	3000 V		325	N 60 psi	N 4 L/min	
24		6.0 μA	400			60 psi
25						35 psi
26		6.0 μA	400		N 2 L/min	35 psi
27	no data →					
28		5.0 μA	450			60 psi
29	3000 V		450	150 L/h	400 L/h	
32						
33		5.0 μA	500			
35		5.0 μA	500			
36		5.0 μA	500	90 L/h		
37		5.0 μA	450			60 psi
38	4500 V		200	N 2 mL/min		
39	2000 V					
42		5.0 μA	450			60 psi
43		5.0 μA	400			N 50 psi
49		5.0 μA	400			
49	3000 V		400			
50						
50	3000 V		400	N 2 mL/min		
51						
51	3000 V		450	N 2 mL/min		
52						
52			400		N 2.5 l/min	
53						
53	3000 V		400	N 2 mL/min		
53						
54						
54	3000 V		400		N 2 L/min	
64						
67		6.0 μA	400			35 psi
68		5.0 μA	240			55 psi
69		6.0 μA	400		N 2 L/min	35 psi
69	no data →					
70	no data →					
71	2906			N	N	

Table 1. Continuation B

Pub No	Auxiliary Gas	Mass Range	Scan Time	Analyzed Samples
4		35-1000		rapeseed oil - biodiesel
5		50-1200		hazelnut, walnut, cashew nut, almond, poppy seed, sugar melon seed, rosehip, fig, date, black currant oils
7	5 mL/min	400-1100	1.75-2/s	canola oils: normal and genetically modified high-stearic and high-lauric varieties
8		35-1000	1.9 s	rapeseed oil - biodiesel
9	5 mL/min	400-1100	0.65 s	soybean oils: normal and genetically modified high-stearic and high-palmitic varieties
10		50-1200		walnut, hazelnut, cashew nut, almond, poppy seed, yellow melon seed, mango stone, fig stone, date seed
11		500-1000		peanut, pumpkin seed, sesame seed, soybean, and wheat germ
12		500-1000		almond, avocado, corn germ, grape seed, linseed, mustard seed, olive, peanut, pumpkin seed, sesame seed, soybean, sunflower, walnut, and wheat germ
14		35-1000		hazelnut, pistachio, poppy seed, almond, palm, brazil-nut, rapeseed, macadamia, soybean, sunflower, linseed, *Dracocephalum moldavica*, evening primrose, corn, amaranth, *Silybum arianum*
15				Madeira laurel oil
16	5 mL/min	400-1100	1.75-2/s	highly saturated edible fats: coconut, cocoa butter, palm, randomized palm, palm olein, and randomized palm olein oils
17	60 psi	100-1000		olive oil
19	25 mL/min			*Crepis alpina* and *Vernonia galamensis* seed oils
20	5 mL/min	400-1100	2.67 s	hydroxy-acid containing seed oils: castor bean oil, *Lesquerella fendleri* and *L. gordonii*
21		450-1050		castor oil
22		200-1000	2 s	hazelnut and olive oil and hazelnut-olive blends
23		65-950	0.1 m/z	interesterified rapeseed oil and tricaproin
24	20 mL/min	200-1000	2 s	beef, chicken, lamb, and pork
25	5 mL/min	400-1100	1.75-2/s	animal tallow and potential food formulation fats
26	5 mL/min	400-1100	1.75-2/s	potential margarine base stocks, including blends and interesterified mixtures
27				
28	20 mL/min	200-1000	2 s	bovine milk fat
29		100-1500	2 s	human milk
32				lipids from Saxon-Medieval cooking pots
33		422-1000	0.2 s	lipids from late Roman cooking pots
35		200-1200	0.2 s	lipids from Roman oil lamps
36		150-1200	1 s	palm oil, cocoa butter, beef, pork and chicken fats
37	20 mL/min	200-1000	2 s	TAG standards
38		200-1000		grape seed, olive, pumpkin seed, soybean, sunflower, and wheat germ oils
39				Perilla, corn, olive
42	20 mL/min	200-1000	2 s	black currant, blue poppy seed, evening primrose, extra virgin olive, hazelnut, maize, rapeseed, and soybean
43	N 5 mL/min	400-1100	0.8 s	α- and γ-linolenic acid-containing oils: cloudberry seed oil, evening primrose oil, borage oil, and black currant seed oil

(Table 1 Contiuation B) contd....

Pub No	Auxiliary Gas	Mass Range	Scan Time	Analyzed Samples
49		250-1100	1 s	TAG standards AAP, APA, PPA, PAP, DPD, DDP, DDLa, LaDLa, LaPD
49		450-1100		rice oil
50				rice oil
50		215-1100	0.2 s	donkey milk
51				donkey milk
51		300-900	0.2 s	donkey milk
52				donkey milk
52		500-1000	0.1 s	standarts
53				
53		450-1100		lard and tallow fats
53				
54				
54		400-900	0.2 s	soybean and linseed
64				
67	5 mL/min	150-1200	2 s	canola oil
68	5 mL/min	400-1100	2.5 s	SSS, OOO, LLL, LnLnLn
69	5 mL/min	400-1100	1.75-2/s	normal and interesterified soybean oils and lard
69				interesterified hydrogenated soybean oil and tributyrin, tricaproin, tricaprylin
70				interesterified hydrogenated soybean oil and triacetin
71		100-1000		lymph from rats

Commentary to Table **1**

The following main trends that can be inferred from Table **1**:

TAG separation was in most cases done on reversed-phase C18 columns, other types being used only exceptionally or in special cases (e.g. two-dimensional HPLC).

Column length considerably affects the separation; in many cases the authors used columns in series. This configuration was used even with 25 cm columns. Unfortunately, none of the studies mentions the number of theoretical plates, not even for the commercially available reference compound SSS.

The range of the mobile phases used is broad and includes mixtures of up to 4 components. Gradient elution is mostly employed, which is understandable in view of the large differences in polarity, chain lengths and unsaturation of the TAGs analyzed. With few exceptions, the mobile phase usually contains acetonitrile (propionitrile) and either alcohols or hydrocarbons.

The mobile phase flow rate with columns 4 – 4.6 mm in diameter is standard – about 1 mL/min.

Pressure or temperature change programming has not yet been used.

Descriptions of HPLC separations include complete data; this holds by no means for MS conditions, which are often disparate in terms of nomenclature used, and lacking in data description.

In general, the vaporization temperature was around 400 ^{o}C but in some cases it was 200 or 500 ^{o}C.

The nebulizer, drying, sheath and auxiliary gas used was in all cases nitrogen because of its inert nature, but its flow rate and pressure employed in individual studies vary considerably.

Corona voltage, as well as corona current are given in relatively narrow limits, i.e. about 3000 V for corona voltage and 5 μA for corona current.

The mass range used was of course in the range of TAG masses. It was in the interval of 200-1000 Da, the upper and lower limits being in some cases shifted to lower or higher values.

Scan time was in the range of 0.2 – 2 s in dependence on the instrument and data processing mode.

The types of analyzed samples are described in the table. The relevant table column clearly indicates that the analyzed materials were mostly common commercially available oils and fats, scant attention being paid to less conventional TAG sources and applications. One wonders why, despite the state-of-the-art instrumentation, no fish oil or microbial lipids (single cell oils) were analyzed. It is exactly this field, i.e. analysis of unconventional materials and samples, where the future of the method can be seen (see also our last paper [72]).

of TAGs known to be more oxidatively stable like SOL, SSL, SOO and SSO glycerols in high-stearic acid canola oil and LaLaL, LaLaO and LaOO glycerols in high-lauric acid canola oil, see Table **2** for shortcuts of FAs. These oils contained fewer linolenate-containing TAGs known to decrease oxidative stability. The LC-MS/APCI technique gave better resolution and quantitation of TAGs in the canola oils than the LC/FID. However, the LC/FID system gave satisfactory analyses suitable for many research programs, like the development of genetically modified canola varieties with oils of improved oxidative stability [7].

The content and structure of TAGs in wild high-erucic acid rapeseed oil (Fig. **1**), which is used as an environmentally friendly substitution of oil used for fuel production, was analyzed by gradient elution. RP-HPLC was used for the determination of compounds occurring during the production

Table 2. Systematic and Trivial Names of FAs Found in TAGs with their Abbreviations, Carbon Numbers (CN), Double Bond (DB) Numbers and Equivalent Carbon Numbers (ECN)

Systematic Name	Trivial Name	Abbreviation	CN:DB	ECN
Dodecanoic	Lauric	La	12:0	12
Hexadecanoic	Palmitic	P	16:0	16
Cis-9-Hexadecenoic	Palmitoleic	Po	16:1	14
Heptadecanoic	Margaric	Ma	17:0	17
Octadecanoic	Stearic	S	18:0	18
Cis-9-Octadecenoic	Oleic	O	18:1	16
Cis-9,12-Octadecadienoic	Linoleic	L	18:2	14
Cis-9,12,15-Octadecatrienoic	Linolenic	αLn	18:3	12
Cis-6,9,12-Octadecatrienoic	Pinolenic	γLn	18:3	12
Eicosanoic	Arachidic	A	20:0	20

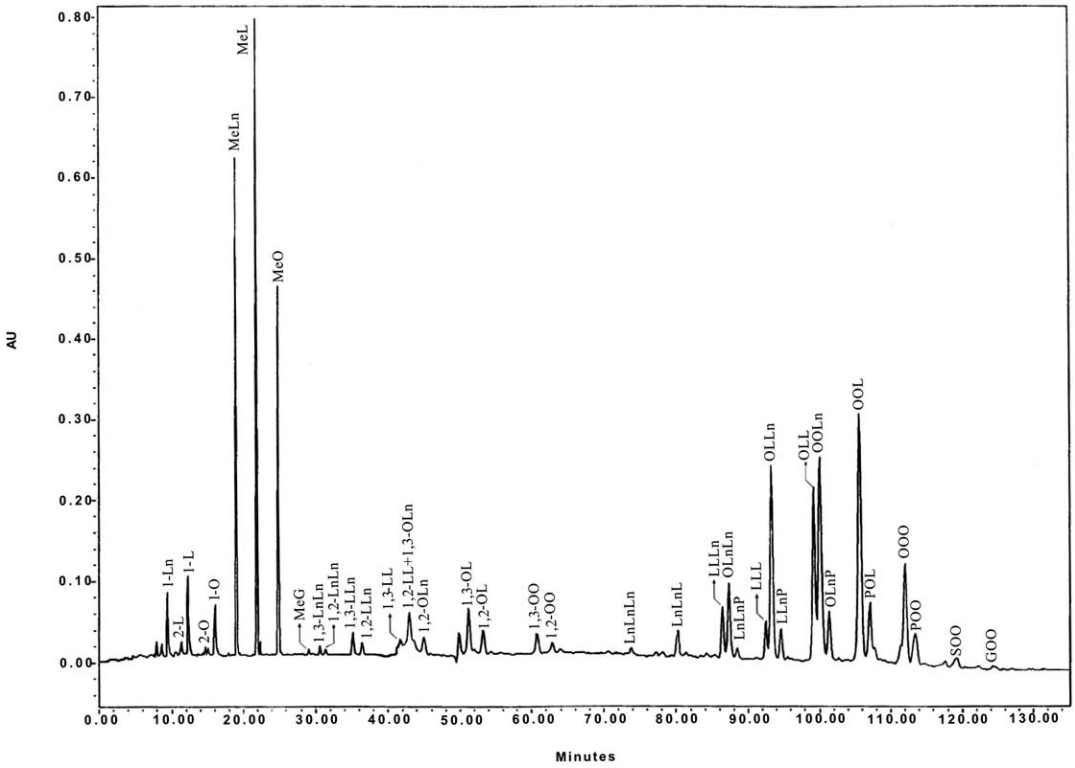

Fig. (1). The chromatogram of rapeseed oil partially transesterified with methanol. For sources and analytical methods and conditions see ref. [4].

of biodiesel from rapeseed oil. Individual TAGs, DAGs, MAGs (monoacylglycerols) and FAMEs (fatty acid methyl esters) of oleic, linoleic and linolenic acids and free fatty acids were separated in 25 min using a combined linear gradient with aqueous-organic and non-aqueous mobile phase steps: 70% acetonitrile+30% water in 0 min, 100% acetonitrile in 10 min, 50% acetonitrile+50% 2-propanol-hexane (5:4, v/v) in 20 min and 5 min final hold-up. Another method with a non-aqueous linear mobile phase gradient [from 100% methanol to 50% methanol+50% 2-propanol-hexane (5:4, v/v) in 15 min] was used for fast monitoring of conversion of rapeseed oil TAGs to fatty acid FAMEs and for quantitation of residual TAGs in the final biodiesel product. Sensitivity and linearity of various detection modes (UV detection at 205 nm, evaporative light scattering detection and mass spectrometric detection) were compared. The individual sample compounds were identified using LC-MS/APCI in the positive-ion mode [8].

Soybean oil TAGs from genetically modified soybean lines were conclusively identified by RP-LC-MS/APCI.

Spectral identification of the TAGs was based on the molecular $[M+1]^+$ ion and the 1(2)-, 2(3)- and 1(3)-DAG fragments. TAGs identified in high-stearic and high-palmitic soybean varieties were quantitated by RP-HPLC with FID. There was excellent agreement between the fatty acid composition calculated from the TAG composition and the fatty acid composition obtained by gas chromatography of the transmethylated oils. The oils of the modified soybean varieties, compared to typical soybean oil, contained increased content of TAGs known to be more oxidatively stable, such as LOS, LPS, and LPP glycerols, and less TAGs like LLL, known to decrease oxidative stability [9].

133 TAGs containing 22 fatty acids with 8-25 carbon atoms and 0-3 double bonds were identified and quantified in 9 plant oils (walnut, hazelnut - see Fig. **2**, cashew nut, almond, poppy seed, yellow melon, mango) using LC-MS/APCI with a response factor approach. A non-aqueous RP-HPLC method with acetonitrile-2-propanol gradient and 30+15 cm NovaPak C-18 columns makes possible an unambiguous identification of the highest number of TAGs ever reported for these oils, based on positive-ion APCI mass spectra. A new approach to TAG quantitation was based on the use of response factors with three typical detection techniques for the purpose (APCI, evaporative light-scattering detection, and UV at 205 nm). Response factors of 23 single-acid TAGs (saturated TAGs from C7 to C22, 7 unsaturated TAGs), 4 mixed-acid TAGs, diolein and olein were calculated from their calibration curves and related to OOO. Due to differences between saturated and unsaturated acyl chains, the use of response factors significantly improved the quantitation of TAGs. Average parameters and relative fatty acid concentrations were calculated with both LC-MS/APCI and GC/FID [10].

Five plant oils (peanut, pumpkin seed, sesame seed, soybean, and wheat germ) have been analyzed by LC-MS/APCI. Gradient elution was performed with acetone-acetonitrile mobile phases on a short monolithic silica column (50 mm x 4.6 mm). Identification of plant oil TAGs was based on the pseudomolecular ion $[M+H]^+$ and the DAG $[M-RCO_2]^+$ fragments. Positional isomers of TAGs were identified from the relative intensities of the $[M-RCOO]^+$ fragments. Principal-component analysis, used to find similarities and differences between the different oils, indicated that the different plant oils could be clearly differentiated according to their TAG composition [11].

The main TAG composition of different plant oils (almond, avocado, corn germ, grape seed, linseed, mustard seed, olive, peanut, pumpkin seed, sesame seed, soybean, sunflower, walnut and wheat germ) was analyzed using two different mass spectrometric techniques: LC-MS/APCI and MALDI-TOFMS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry). Linear discriminant analysis (LDA) as a multivariate mathematical statistical method was successfully used to distinguish different plant oils based on their relative TAG composition. With LDA analysis of either MS/APCI or MALDI/MS data, the classification among the almond, avocado, grape seed, linseed, mustard seed, olive, sesame seed and soybean oil samples was 100% correct. In both cases only 6 different oil samples from a total of 73 were not classified correctly [12].

TAGs and DAGs in 16 plant oil samples (hazelnut, linseed (Fig. **3**) [13], pistachio, poppy seed, almond, palm, Brazil-nut, rapeseed, macadamia, soyabean, sunflower, *Dracocephalum moldavica*, evening primrose, corn, amaranth, *Silybum arianum*) were analyzed by LC-MS/APCI and UV detection at 205 nm on two Nova-Pak C-18 chromatographic columns connected in series. A single chromatographic column and non-aqueous ethanol-acetonitrile gradient system was used as a compromise between the analysis time and the resolution for the characterization of TAG composition of five plant oils. APCI mass spectra were used for the identification of all TAGs and other acylglycerols. The isobaric positional isomers can be distinguished on the basis of different relative abundances of the fragment ions formed by preferred losses of the fatty acid from sn-1(3) positions compared to the sn-2 position. Excellent chromatographic resolu-

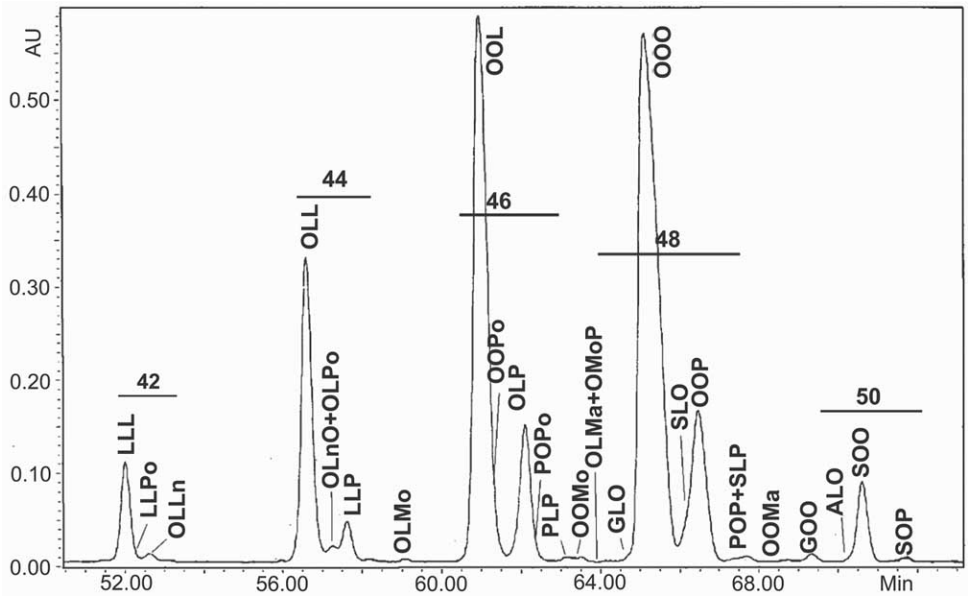

Fig. (2). HPLC of hazelnut oil. For sources and analytical methods and conditions see ref. [5].

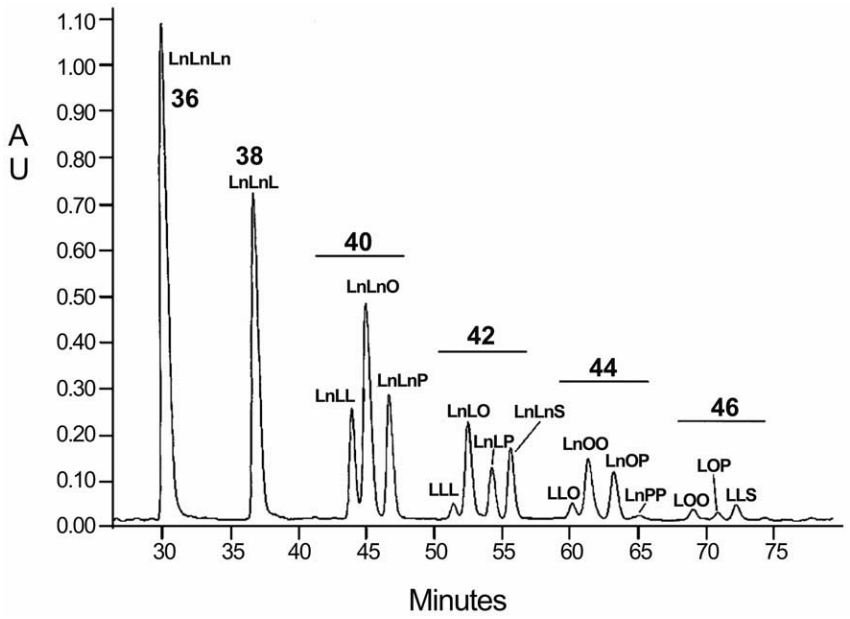

Fig. (3). HPLC chromatogram of fresh linseed oil. For sources and analytical methods and conditions see ref. [13].

tion and broad retention window together with APCI mass spectra enabled positive identification of TAGs containing fatty acids with odd numbers of carbon atoms such as margaric (17:0) and heptadecenoic (17:1) acids. The general fragmentation patterns of TAGs in both APCI and ESI mass spectra were proposed on the basis of spectra measured with an ion trap analyzer. The relative concentrations of particular TAGs in the analyzed plant oils were estimated on the basis of relative peak areas measured with UV detection at 205 nm [14].

Madeira laurel oil was fractionated by liquid extraction combined with TLC, and TAGs were analyzed by LC-MS/APCI. Eluted molecular species compositions of the eluted TAG in the complex natural mixture were determined by GC identification of FAME and by LC-MS/APCI analysis of the lipid. The APCI spectra of most TAG exhibited $[M+H]^+$ and $[M-RCOO]^+$ ions, which defined the molecular weight and the molecular association of fatty acyl residues, respectively. Despite the relatively high degree of saturation, with a saturated/unsaturated ratio of 0.70, neither totally saturated TAG nor mixed asymmetric TAG with two saturated FA (SSM or SSU, where S is saturated, M is unsaturated, and U is unsaturated) were found. This type of molecular structure provides a possible explanation for the relatively low m.p. (12-15^{o}C) and also the high oxidative resistance observed [15].

TAG compositions by area percent were obtained by RP-LC-MS/APCI of highly saturated fatty acid fats, such as coconut, cocoa butter, palm, randomized palm, palm olein, and randomized palm olein oils. Accurate identification and quantitation of these TAG compositions were obtained and proved by comparison of the fatty acid composition calculated from the TAG composition obtained by MS/APCI with the fatty acid composition obtained by gas chromatography of the FAMEs of the transmethylated oils. Also, MS/APCI accuracy was proved by comparison of the experimental TAG composition with the predicted TAG composition for randomized oils. Average absolute errors, with respect to TAG quantitation and identification, were less than 1%. This study identified and quantitated these TAGs present in proportions greater than 0.1% (oil, number of TAGs): coconut, 99; palm, 27; randomized palm, 28; palm olein, 28; randomized palm olein, 29, and cocoa butter, 19. Concentrations of UUU, UUS, USS, and SSS TAGs, which can be determined accurately from RP-LC-MS/APCI of the actual TAG species, affected the physical properties of food formulation fats [16].

RP-LC-MS/APCI method was used to compare olive oils from different geographic areas. An effective discrimination of extra virgin olive oils was described and combined with chemometric evaluation. The presented method is simple since the diluted oil sample is directly injected into the system, without any preliminary chemical derivatization or purification step. With this method, separation of DAGs, TAGs and sterols occurred within 20 min and was achieved using a C18 column. Detection was performed by positive APCI mass spectrometry which provided sensitivity to detect over 50 compounds in the sample. After extraction of data, stepwise discriminant function analysis was used to select the variables with the highest discriminative power. These variables were used to perform linear discriminant analysis and classify/predict the samples. One-hundred per cent classification and 99% prediction rate was achieved for olive oils obtained from Nocellara, Biancolilla and Cerausola cultivars. Reliability of prediction was tested by cross validation [17].

Some plant oils contain fatty acids that carry in their long chain an oxygen-containing functionality. One of the most common fatty acids of this type is ricinoleic acid (12-hydroxy-9-octadecenoic acid), found commonly in, e.g., castor oil. The following several studies deal with oils containing oxygen functionalities, although this review in general does not deal with oxidation products of TAGs and re-

fers rather to several outstanding reviews and book chapters devoted to this topic [2,3,18].

Unusual seed oils having significance for chemical synthesis (*Crepis alpina*), or with fatty acids, which contain functional groups important in the preparation of plastics (*Vernonia galamensis*) were analyzed by a RP-LC-MS/APCI. The method identified 16 TAGs in the *Crepis alpina* oil and 18 TAGs in *Vernonia galamensis* oil and showed greater sensitivity for detection of and improved identification of TAGs compared to previous analyses using the techniques of RP and Ag^+-LC with a FID. The most abundant *Crepis alpina* TAGs were: LCrCr (33.0%), CrCrCr (32.3%), PCrCr (11.5%), LLCr (6.7%) (Cr - crepenic acid). The remaining TAGs occurred at five or less mole percent abundance. The most abundant *Vernonia galamensis* TAGs were: VVV (43.3%), LVV (21.3%), OVV (7.9%), PVV (8.2%) and SVV (6.4%) (V - vernolic acid). The remaining TAGs occurred at four or less mole percent abundance. The studies provided new knowledge concerning the TAG composition of these oils and showed that the APCI technique is suitable for mass spectral identification of neutral molecules which do not contain a chargeable functional group [19].

RP-LC-MS/APCI was also used for direct analysis of intact TAGs from hydroxy-containing plant oils. Castor bean oil, *Lesquerella fendleri* and *L. gordonii* oils were separated into tri-hydroxy, di-hydroxy, mono-hydroxy and non-hydroxy TAGs using the RP-HPLC method. The APCI ionization source produced fragments representing loss of zero (protonated molecular ion), one, two, and three hydroxy groups. The primary fragments (base peaks) in the mass spectra resulted from loss of all hydroxy groups from the TAGs. Using the acetonitrile/methylene chloride solvent system, diagnostically important acetonitrile adducts were formed which allowed identification of the molecular weights of the hydroxy TAGs as well as confirmation of the number of hydroxy groups contained therein. A series of four adducts was formed: $[M+23]^+$, $[M+39]^+$, $[M+54]^+$, and $[M+59]^+$ [20].

A non-aqueous RP-HPLC method, based on two columns in series, has been used to separate the major TAGs from commercial castor oil and to perform either on-line negative ion APCI, or off-line positive ion matrix-assisted laser desorption ionization (MALDI)/MS. The resulting mass spectra showed chloride-attached TAG molecules $[M+Cl]^-$ in the case of negative-ion APCI, and sodium-attached TAG molecules $[M+Na]^+$ in the case of positive-ion MALDI. For MALDI time-of-flight (TOF)/MS, a liquid binary matrix system consisting of sodium ferrocyanide and glycerol was applied, resulting in excellent TAG sensitivity, which was necessary for the determination of trace amounts of TAGs in castor oil. Both techniques allowed unambiguous molecular mass determination of intact TAG molecules with no thermal degradation. Furthermore, seamless post source decay (PSD) fragment ion analysis by means of a curved field reflector TOF mass spectrometer allowed the determination of the fatty acid composition of each individual TAG. Castor oil contained eight different TAGs which were successfully determined by both APCI and MALDI techniques. In each TAG, at least two units of ricinoleic acid were present. The following fatty acids were determined by seamless PSD fragment ion analysis and APCI/MALDI molecular mass determination as TAG substructures: ricinoleic, palmitic, stearic, oleic, linoleic, linolenic, dihydroxy stearic acid and eicosenoic acid. Triricinolein was the dominant TAG [21].

The analysis of the TAG, tocopherol and sterol composition of hazelnut oil, olive oil and their mixtures (90% olive oil with 10% hazelnut oil, 70% olive oil with 30% hazelnut oil and 50% olive oil with 50% hazelnut oil) was published. The main TAGs were OOO, POO, LOO and SOO. The discriminant analysis showed that hazelnut oil, olive oil and oil mixtures were clearly separated according to their TAG composition [22].

LC-MS/APCI was used for identifying TAG molecular species in lymph samples from rats given either a structured lipid or safflower oil. The structured lipid was MLM-type (M, medium-chain fatty acid; L, long-chain fatty acid) and manufactured from caprylic acid (8:0) and the oil (safflower oil or high-oleic sunflower oil). The TAG composition of the lymph varied significantly between structured TAGs and safflower oil. DG fragment ions were found for all TAGs and the ammonium adduct molecular ion $[M+NH_4]^+$ could also be observed for all the TAGs at the selected conditions. Protonated molecular ions were formed from TAGs containing unsaturated FA, and FA fragment ions were also observed in the case of strong fragmentation. The lymph TAGs were identified from their ammonium adduct molecular ions and DG fragment ions. In addition to the intact MLM-type structured TAGs, the MLL- and LLL-type TAGs were also identified. The absorption pathway of MLM-type structured TAGs is most likely the same as that of conventional long-chain TAGs, i.e. they were hydrolyzed into 2-acylglycerol and medium-chain FAs, which were then used for resynthesis of TAGs. The study demonstrated the possibility to study the absorption pathway of TAG via identification of TAG species in biological samples [23].

2.2. Animal Fats

The second most widely analyzed group of natural mixtures containing TAGs are animal fats. The samples for analysis are obtained predominantly from domestic animals, mostly mammals, or from their milk.

LC-MS/APCI was applied to the characterization of TAGs in animal fats. The major TAGs in four fats (beef, chicken, lamb and pork) were identified and positional isomers assigned according to their APCI mass spectra. Beef and lamb fat TAGs were confirmed as containing higher proportions of saturated fatty acids compared with those of chicken and pork. LC-MS/APCI was also shown to be of value in providing regiospecific information for the fatty acids in individual TAG species. For example, beef and lamb fat were shown to contain both *cis*- and *trans*-isomers of the 18:1 fatty acid, whilst chicken and pork contained only the *cis*-isomer. When the position of fatty acid substitution was determined from the APCI spectra, the *cis*-18:1 was predominantly found in the 2-position of the TAG, while the *trans*-18:1 showed a preference for the 1/3-position. Similarly, it was confirmed that although the 2-position of beef, chicken and lamb fat TAGs was dominated by unsaturated fatty acids, in pork fat, a characteristically high proportion of palmitic acid was seen in this position. The TAGs identified

compared well with those reported previously. The distributions of 2-position fatty acids seen in lamb and pork fat compared favorably with those obtained by the more traditional method of lipase degradation. Although the distributions for chicken and beef showed some discrepancies, these can be attributed to weaknesses in the quantitation procedure or the specificity of the lipase. Overall, the technique of LC-MS/APCI has been shown to be very powerful for the regiospecific analysis of animal fats [24].

TAG compositions, in mole percent, were obtained by RP-LC-MS/APCI of lard and mutton tallow fractions under consideration for food formulation products. Accurate identification and quantitation of these TAG compositions were obtained and proved by comparison of the fatty acid composition calculated from the TAG composition which was obtained by MS/APCI with the fatty acid composition, and converted to mole percent as obtained by GC of the FAME of the transmethylated oils. Average absolute errors with respect to TAG quantitation and identification were less than 1%. This study identified and quantitated TAG concentrations equal to and greater than 0.1%. Concentration of TAG with various fatty unsaturated (U) and saturated (S) fatty acids with TAG, designated as UUU, UUS, USS, and SSS species, can be determined accurately from RP-HPLC/APCI-MS of the actual TAG species. These TAG species UUU, UUS, USS, and SSS affect the physical properties of food formulation fats [25].

Considerable attention was paid to *trans*-fatty acids arising as undesirable side products during the hydrogenation of plant oils and fats. Several margarine base stock candidates have previously been prepared for the purpose of finding better, more oxidatively stable food components: high-saturated vegetable oils, randomized vegetable oils, vegetable oil-hard stock blends, and interesterified vegetable oil-hard stock blends. TAG compositions of these products were determined using RP-LC-MS/APCI. TAG percent composition results for samples of known composition (randomized and interesterified samples) exhibited less average error when obtained by HPLC coupled with MS/APCI, after application of response factors, than the results obtained by HPLC coupled with a flame ionization detector. The fatty acid compositions calculated from the mass spectrometric data also exhibited less average error than the fatty acid compositions resulting from the flame ionization detector data. The average error of the fatty acid compositions determined by the mass spectrometer was the lowest for interesterified blend samples, followed by randomized samples, high-saturated fatty acid oils, normal oils, and blends. Analysis of the vegetable oil-hard stock blends by mass spectrometer required special treatment for the calculation of response factors [26].

The composition and structures of TAG in a commercially prepared hydrogenated soybean oil margarine base stock (39.7% *trans* FAs) were determined by LC-MS/APCI. The base stock was separated by preparative HPLC into four fractions. Fractions 1 and 4, constituting about 8% of the total, were shown to consist of LOO, PLO, and LLS and OSS and PSS, respectively. APCI will not distinguish between O (*cis*-C18:1), and E, (elaidic - *trans*-18:1). Thus, O and E may be used interchangeably in discussion of TAG isomer structures. Fraction 2 consisted of OOO and POO. Fraction 3 consisted of OOO, POO, OOS, and POS. About 80% of the total triglycerides consisted of OOO, POO, and OOS. The *trans* fatty acid content of the fractions was determined, and the results showed that 92% of the total *trans* content was found in fractions 2 and 3 [27].

Other studies focused on milk fats, either the milk of breast-feeding women or the milk of farm animals. Bovine milk TAGs have been characterized using LC-MS/APCI and HTGC-MS. The complex nature of the fat meant that pre-fractionation was necessary to provide simpler fractions for more detailed molecular analyses. Silica TLC gave rise to two fractions, one of which contained predominantly butyric acid containing TAGs. Gel permeation chromatography (GPC) gave rise to 16 fractions, which were subsequently analyzed using LC-MS/APCI. Twelve of the GPC fractions were also analyzed by HTGC-MS using a capillary column coated with a polarizable stationary phase. TAGs present in the fractions were correlated with those in the chromatogram of the whole milk fat through retention time comparison and the use of mass chromatograms. In total, 120 TAGs were identified [28].

Detailed analyses were undertaken to determine which TAG species in mature human milk are less affected by external factors and may thus be considered as TAG markers, as well as to determine which species are most influenced by these external conditions. Furthermore, the correlation between the TAG markers and their FAs was examined. In order to obtain the maximum variability of sampling conditions, 40 mature human milk samples (from six healthy women from Spain) were collected, on different days, at different times of the day, and from different breasts during and after both the baby's and mother's meal. The TAG composition was determined by HPLC with an evaporative light-scattering detector, and also with a LC-MS/APCI. FA compositions were determined by GC. Some TAG species were found in relatively constant levels in mature human milk, and could thus be considered as markers of the mature milk TAG profile. TAG species classified in this group were: LaMO, CaPO, LaCaO, LaPCa, LaOL, MPLn, LLO, LaOO, MPL, and MOL. On the other hand, concentrations of other TAG species (PaLS, MPO, PaOO, PPP, MPS, SPP, LOO, PPO, MOS, SSP, POL, and SOS) were found to vary considerably between samples and may thus be understood to be especially affected by the external factors. The TAG species present in mature human milk are affected in different ways by external factors such as dietary intake, nutritional status, length of lactation, time of the day, etc. Some TAGs may be considered as markers of mature human milk as they are relatively constant under a wide range of sampling conditions and do not depend on the factors mentioned [29].

LC-MS/APCI has also been used to analyze art and archaeological samples. The former studies concerned two conservation related problems: analysis of a white exudate on a painting by Mark Rothko (National Gallery of Art, Washington DC) and the analysis of components present in acetone (propanone) extracts of oil paint films [30].

The use of LC-MS/APCI with archaeological specimens is still rare. Animal fats are preserved at archaeological sites in association with unglazed pottery, human and animal re-

mains, and other deposits or hoards. HTGC and HTGC/MS has confirmed the presence of animal fats in lipid extracts of artifacts. Degradation products and pathways have been discerned through the analyses of archaeological finds and the products of laboratory and field-based decay experiments. Regiospecific analysis of intact TAGs by LC-MS/APCI provides a further criterion for establishing the origin of fats. Preparative GC has been employed to isolate individual fatty acids from archaeological pottery in sufficient amounts for C-14 dating [31].

Lipid extracts of shreds of archaeological late Roman cooking pots were analyzed using HTGC/MS and LC-MS/APCI. The detection of high amounts of saturated TAGs indicated that animal fat was processed in these pots. Part of the animal fat was characterized as originating from ruminants due to the presence of *trans*-fatty acids. The distribution of saturated TAGs and the higher concentration of stearic acid compared to palmitic acid in the transesterified lipid extract indicated that this was sheep fat. The results illustrate how complex mixtures can be unraveled and original contents of ancient ceramic vessels can be determined using specialized analytical equipment [32].

The lipid fraction of residues in ancient oil lamps found at the archaeological site of Sagalassos (south-west Turkey) was analyzed with LC-MS/APCI. The identification of TAGs detected with LC-MS/APCI showed that olive oil was predominantly used as a fuel for the antique oil lamps. The presence of large quantities of multiply unsaturated TAG and traces of saturated TAG indicated that also other oils and animal fat were added. Summarizing, the analysis of TAGs with LC-MS/APCI in lipid extracts of ancient ceramics proved to be a valuable method to reconstitute the original contents [33].

2.3. Positional Isomers of TAGs

TAG having the same equivalent carbon number (ECN) i.e., TAG having identical masses, but different distributions of unsaturation in the fatty acyl chains, such as SOO and SSL, which both have an ECN of 50 and a molecular weight of 886, composed critical pairs. When the chromatographic separation is less than optimal, these could not be differentiated based on the protonated molecule peaks, so the DAG fragment ions were crucial to differentiating these molecules. Based purely on statistical considerations, any ABA or AAB TAG should yield twice as much of the $[AB]^+$ ion as the $[AA]^+$ ion, since there are two A FA available to combine with the B FA to give an $[AB]^+$ ion. The ratio of $[AA]^+$ to $[AB]^+$ should be 1:2.

One key aspect of TAG analysis has always been determination of the positional placement of the fatty acyl chains on the glycerol backbone. Organisms (plants, animals and microorganisms) synthesize lipids with structural specificity. The knowledge of the configuration of TAG is an important aspect of biosynthetic, dietary, nutritional, metabolic and related studies. TAGs occur in nature as a mixture of positional isomers differing by the acyl position, sn-2 vs. sn-1(3), such as the pairs of OOP/OPO. The acyls in the sn-2 position are determined on the basis of different relative abundances of the $[M+H\text{-}RCOOH]^+$ ions with respect to $[OO]^+$, $[OP]^+$ and $[PO]^+$ ions and are attributed to the middle letter in the abbreviation used in the TAGs notation (for example OPO means palmitoyl in the sn-2 position). The sn-1 and sn-3 positional isomers can be principally separated by silver ion HPLC [7], but cannot be distinguished by RP-HPLC. The unambiguous determination of the sn-2 acyl is usually complicated by a very low concentration, coelutions with TAGs containing the fragment ions $[PO]^+$ and $[OP]^+$ with identical masses or low concentration differences between the sn-2 and sn-1(3) positional isomers. The results show that the attribution of the sn-2 acyl to the less intense fragment ion $[M+H\text{-}RCOOH]^+$ of oleoyl and palmitoyl containing positional isomers may be oversimplified. To provide a clue to the splitting of TAG, Figs. (**4, 5,** and **6**) give the mass spectra of TAG, DAG and monoacylglycerol. Fig. (**7**) shows the spectrum of an unusual TAG (LLMo, where Mo is heptadecenoic acid) [14].

The main problems encountered when analyzing TAGs by LC-MS/APCI are the regioisomeric composition of TAG and degree of unsaturation in the TAG. Three basic types, i.e. AAA-type TAG, an ABA/AAB/BAA, and type 3 (it has three different FA) have been analyzed and the percentage composition of their regioisomers was quantified [34].

Positional distribution of fatty acyl chains of TAGs in vegetable oils and fats (palm oil, cocoa butter) and animal fats (beef, pork and chicken fats) was examined by RP-LC-MS/APCI using a quadrupole mass spectrometer. Quantification of regioisomers was achieved for TAGs containing two different fatty acyl chains, i.e. P, S, O, and/or L. For seven pairs of AAB/ABA-type TAGs, namely PPS/PSP, PPO/POP, SSO/SOS, POO/OPO, SOO/OSO, PPL/PLP and LLS/LSL, calibration curves were established on the basis of the difference in relative abundances of the fragment ions produced by preferred losses of the fatty acid from the 1/3-position compared to the 2-position. In practice the positional isomers AAB and ABA yield mass spectra showing a significant difference in relative abundance ratios of the ions $[AA]^+$ to $[AB]^+$. The regression models show linear behavior that allows the determination of the proportion of each regioisomer in an AAB/ABA pair, within a working range from 10 to 1000 μg/ml and a 95% confidence interval of +/-3% for three replicates [35].

Fragmentation of TAGs by APCI allows identification of positional isomers. The relative intensities of the $[M\text{-}RCOO]^+$ ions provide information on the position of fatty acids within an ABC type triacylglycerol and enable ABA and AAB type molecules to be distinguished [36].

The ratios of TAG positional isomers containing two LL and one O moieties - namely LLO and LOL - were quantified in grape seed, olive, pumpkin seed, soybean, sunflower and wheat germ oils by LC-MS/APCI in selected ion monitoring (SIM) mode. Relative LOL contents (LOL/(LLO+LOL)) of the oils were calculated from the mass abundances of the $[LL]^+$ and $[LO]^+$ diacylglycerol fragment ions $[M+H\text{-}RCOOH]^+$ using a calibration curve. The calibration curve of the relative DG mass abundances was measured in SIM mode. The relative LOL contents were found to be relatively consistent for each oil variety. The relative LOL content in grape seed, sunflower, pumpkin seed, soybean and wheat germ oils accounted for 44.2, 26.8, 16.7, 15.9 and 13.9%, respectively. Only olive oils contained practically 100% of the LLO isomer. These results indicate

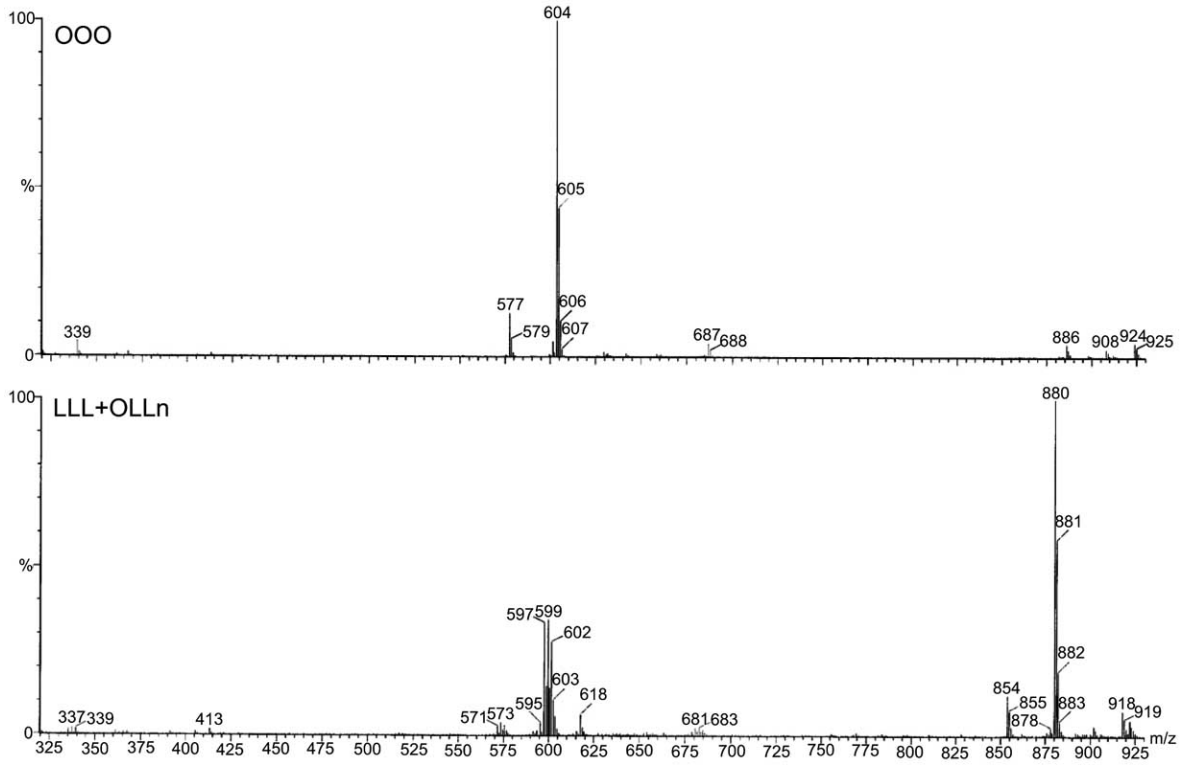

Fig. (4). Positive-ion APCI mass spectrum of OOO and mixed spectrum of LLL and OLLn. For sources and analytical methods and conditions see ref. [8].

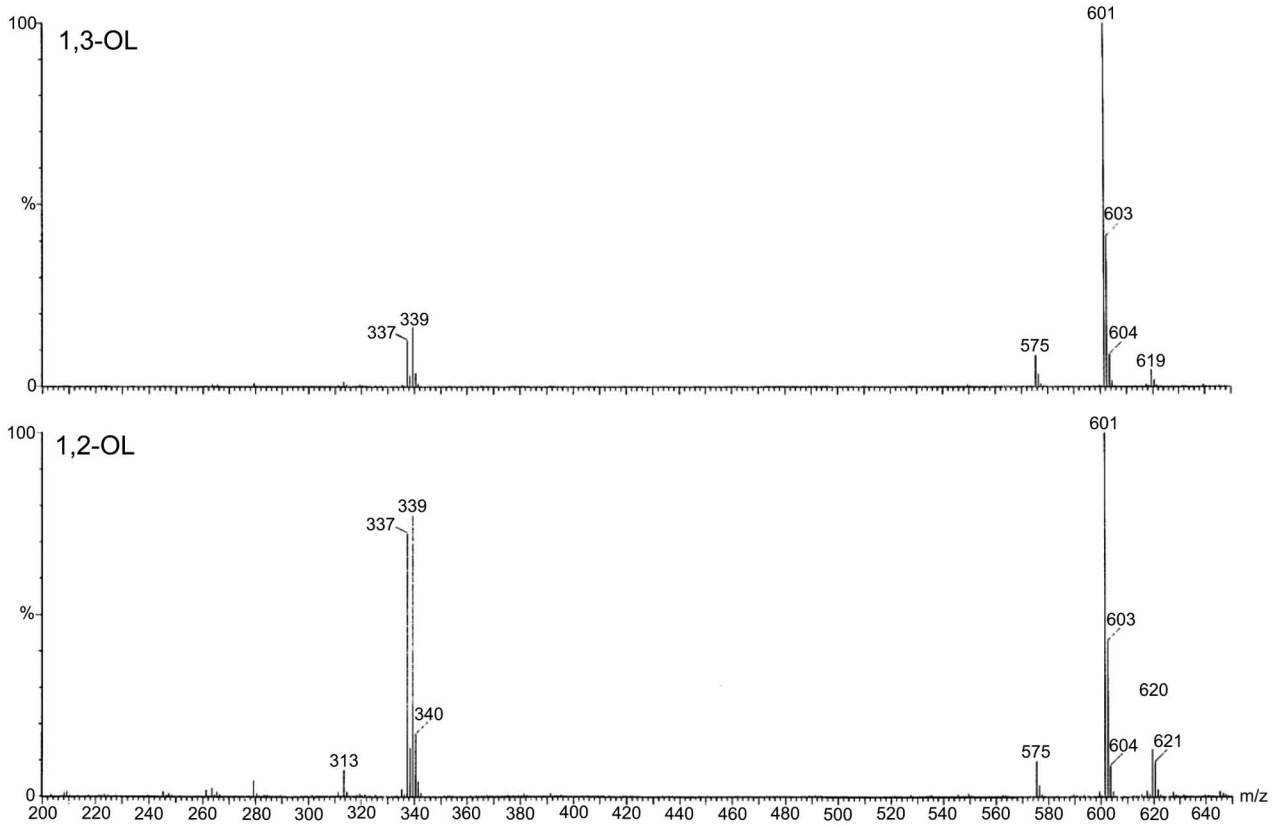

Fig. (5). Positive-ion APCI mass spectra of 1,3-OL and 1,2-OL. For sources and analytical methods and conditions see ref. [8].

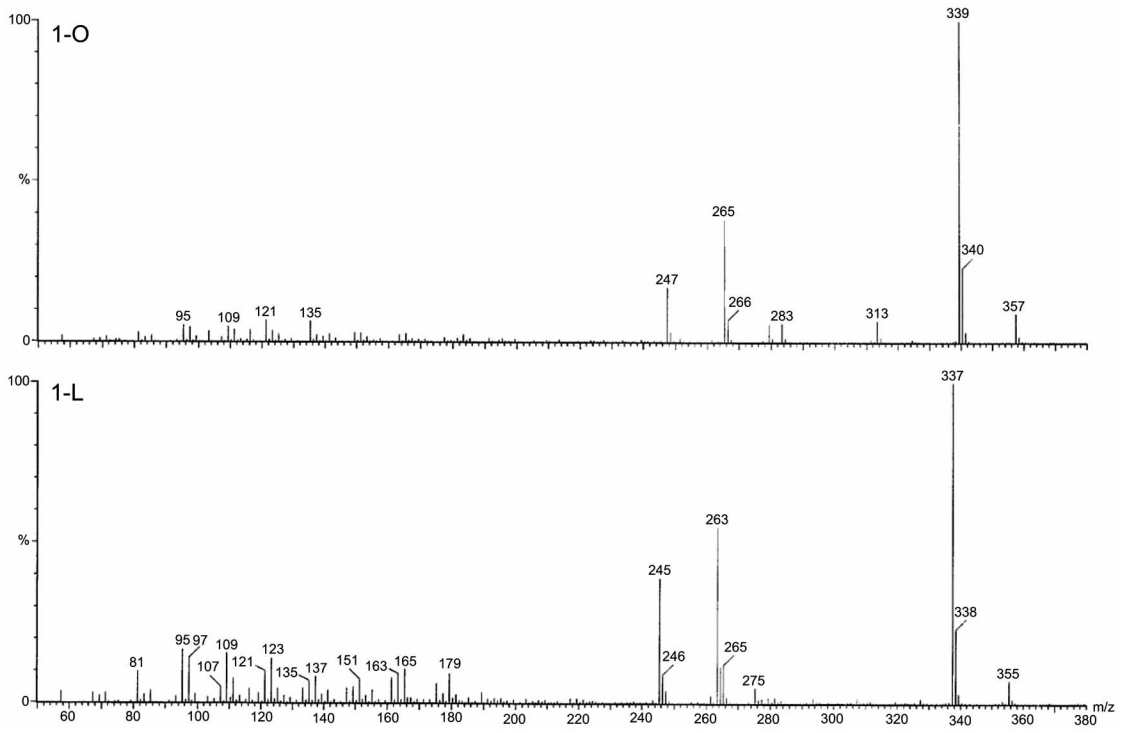

Fig. (6). Positive-ion APCI mass spectra of 1-O and 1-L. For sources and analytical methods and conditions see ref. [8].

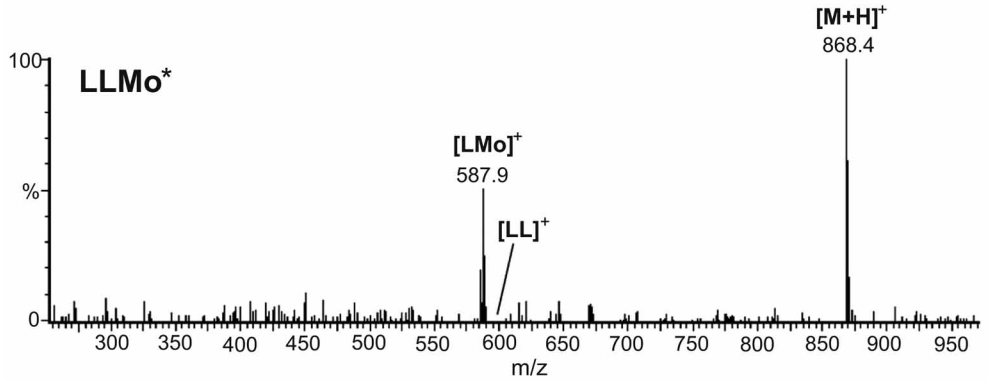

Fig. (7). Positive–ion MS/APCI of a TAGs with an odd number of carbon atoms: LLMo identified in soybean oil. For sources and analytical methods and conditions see ref. [14].

that the unsaturated fatty acids such as linoleic and oleic acids have "non-random" distribution patterns in various oils [37].

LC-MS/APCI of some plant TAGs gave mass spectra with the peaks of $[M+H]^+$ and $[M+H\text{-}RCOOH]^+$ ions, in which $[M+H]^+$ and RCOOH represent, respectively, the protonated molecular ion and FA at the sn-1-(or -3-) position of the TAG. It was possible to discriminate fatty acids with sn-1-(or -3-) and -2-positions [38].

LC-MS/APCI enables the positional isomers of individual triacylglycerols in mixtures to be identified. Application of LC-MS/APCI to the analysis of soybean oil allowed thirty nine TAGs and seven DGs to be positively identified. The compounds present were quantified and the distribution of fatty acids in the β-position calculated. This distribution compared favorably with that obtained using a more conventional regiospecific lipase degradation of the oil. The method was used with the following oils: blackcurrant, blue poppy seed, evening primrose, extra virgin olive, hazelnut, maize and rapeseed [39].

Another very frequent application of LC-MS/APCI is the separation of TAGs containing positional isomers of fatty acids, i.e. acids differing in the position and possibly geometry of the double bonds. The most widely analyzed were TAGs containing α- and/or γ-linolenic acids, which have outstanding dietary and nutritional properties.

Under collisional activation and with a fixed charge, ions containing long-chain structures undergo charge-remote fragmentations, generating products that provide information about the structure of these long chains. The applications, energetics, and mechanisms of charge-remote fragmentations of natural products including triacylglycerols were reviewed [40].

TAGs of the seed oils rich in α- and/or γ-linolenic acid moieties were separated by Ag^+-LC followed by MS/APCI detection. Mass spectra of most TAGs exhibited abundant $[M+H]^+$ and $[M-RCOO]^+$ ions, which defined the molecular weight and the molecular association of fatty acyl residues of a TAG, respectively. Silver ions formed weaker complexes with triacylglycerols containing γ-linolenic acid than with those containing α-linolenic acid, i.e., the elution order of molecules was ABLn-γ > ABLn-α, ALn-γLn-γ > ALn-γLn-α > ALn-αLn-α, and Ln-γLn-γLn-γ > Ln-γLn-αLn-α > Ln-αLn-αLn-α, where Ln-α = α-linolenic acid; Ln-γ = γ-linolenic acid, and A, B = fatty acids different from Ln. Furthermore, Ag^+-LC resulted in partial separation within equally unsaturated TAGs according to differences in the combined number of acyl carbons. Regioisomeric forms of TAGs were not determined from the seed oil samples, although differences were measured with reference compounds in the relative abundances of $[M-RCOO]^+$ ions formed by a loss of a fatty acyl residue from the sn-2 position and the sn-1/3 positions. Ag^+-LC-MS/APCI provided valuable information for structure elucidation of seed oil TAGs: 43 molecular species were identified from cloudberry seed oil, 39 from evening primrose oil, 79 from borage oil, 44 from alpine currant, and 56 from black currant seed oils. The quantitation requires to be studied further, especially in those cases where several molecular weight species of TAGs eluted in a single chromatographic peak [41].

TAGs of oils rich in α-and/or γ-linolenic acids were analyzed by RP-LC-MS/APCI. The APCI spectra of most TAGs exhibited $[M+H]^+$ and $[M-RCOO]^+$ ions, which defined the molecular weight and the molecular association of fatty acyl residues, respectively. RP-LC resulted in, at least, partial separation of TAGs containing α- and/or γ-linolenic acid moieties. Molecules containing α-linolenic acid residues exhibited a relatively weaker retention by the stationary phase than the corresponding molecules containing γ-linolenic acid residues. Good separation of triacylglycerols of cloudberry seed oil, evening primrose oil, borage oil, and black currant seed oil was achieved. Good quality mass spectra could be extracted even from minor chromatographic peaks representing 0.2% or less of the total TAGs [42].

The chemical synthesis of pure TAG regioisomers that contain long chain polyunsaturated FAs, such as arachidonic acid or docosahexaenoic acid, and saturated fatty acids, such as lauric acid or palmitic acid, at defined positions, was described. A single step methodology using (benzotriazol-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate (PyBOP), an activator of carboxyl group commonly used in peptide synthesis and occasionally used in carboxylic acid esterification, has been developed for structured TAG synthesis. Identification of the fatty acyl chains for each TAG species was confirmed by MS/APCI and NMR spectra. The generic described procedures can be applied to a large variety of substrates and were used for the production of specific TAGs of defined molecular structures, with high regioisomeric purity. Combination of MS and NMR was shown to be an efficient tool for structural analysis of TAG. In particular, some NMR signals were demonstrated to be regioisomer specific, allowing rapid positional analysis of LC-PUFA containing TAG [43].

2.4. Ag+-LC

A specific separation method involves the use of silver ions for impregnating the stationary phase. This area has been the subject of a number of reviews and book chapters devoted to both the theory and practice of TAG separation [44-47].

However, the use of silver-ion-impregnated column alone cannot provide a complete separation of TAGs, as seen in Figs. (**8**, **9**) published in the excellent paper by Dobson *et al.* [45].

The analytical benefits of automated comprehensive two-dimensional chromatographic methods have been exploited and emphasized by part of the chromatographic community, particularly in the past decade. The most important novelty of comprehensive multidimensional (MD) techniques, with respect to classical MD chromatography, is that the entire injected sample is subjected to two independent separation processes, thus greatly enhancing peak capacity. The unsuspectedly complex nature of many real-world samples has been elucidated through the enhanced resolving power of these techniques. The present review reports on the employment of comprehensive chromatographic methods [based on gas chromatography (GC), liquid chromatography (LC), LC-GC and packed supercritical fluid chromatography (pSFC)] in the field of lipid analysis. We also give brief information on the history and the state-of-the-art of the most widely-used techniques [48].

The determination of the TAG profile in real world matrices is rather difficult as these compounds present a complex composition and are characterized by similar physicochemical properties. This investigation is based on the HPLC multidimensional determination of the TAG profile in terms of TAG species and positional isomers in a rice oil sample. The off-line bi-dimensional system was attained through the coupling of non-aqueous reversed-phase HPLC and silver ion Ag^+-HPLC. The primary column eluate was fractionated and the fractions of interest were then injected onto the secondary column, allowing the separation of several TAG positional isomers, unresolved in the first dimension. Peak assignment was carried out by combining retention data with APCI spectra information. The fatty acid distribution along the glycerol backbone, determined by Ag^+-HPLC, was confirmed through diglyceride ion ratios derived from APCI analysis. Method validation, where both precision and accuracy were measured, was carried out in preliminary applications on standard compounds. The analytical results obtained showed that rice oil TAGs follow a distribution which can be considered typical for vegetable oils [49].

The separation and determination of TAGs, which are the main components of naturally occurring fats and oils, in milk fat is a challenging task due to the very complex nature of this matrix. The TAG fraction of donkey milk lipids has been characterized by using LC-MS/APCI. RP-HPLC has been used for TAG separation, silver ion Ag^+-HPLC being used as a second dimension to clarify and confirm the identification. The RP-HPLC eluate was fractionated and the fractions of interest were injected onto the Ag^+-HPLC column. In both cases peak assignment was carried out by combining retention data with APCI spectra information. In total, 55 TAGs

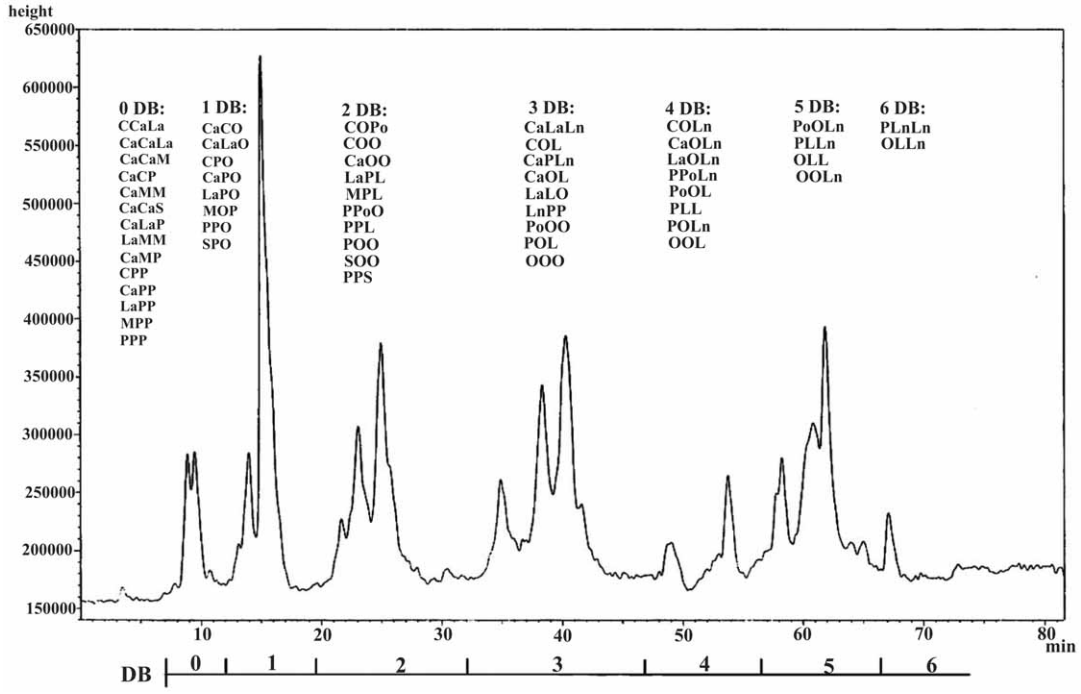

Fig. (8). Separation of TAGs from rat parametrial adipose tissue by HPLC on a Nucleosil 5SA column in the silver ion form. For sources and analytical methods and conditions see ref. [48].

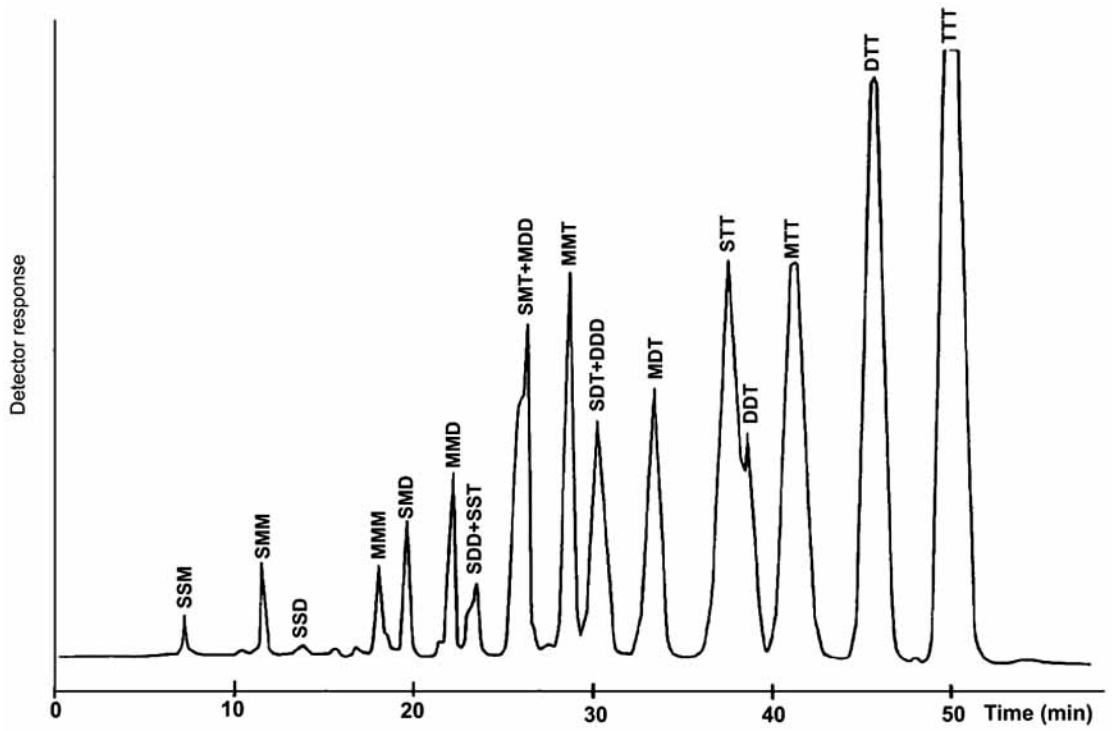

Fig. (9). Silver ion HPLC of TAGs from linseed oil. For sources and analytical methods and conditions see ref. [43].

in donkey milk fat were identified (without considering the positional isomers) and quantified on the basis of percentage peak areas in the RP-HPLC chromatogram (without the use of correction factors). Amongst the identified TAGs, POLn, POO, PPO, CaPO, POL, and PPoO proved to be the main components of the TAG fraction of donkey milk [50].

A sequel paper to the 2005 report concerning again donkey milk brought the method of 3D analysis (Ag-LC×RP-LC×MS/APCI) to near perfection. Efficient separation aided in an identification of nearly 60 TAGs [51].

A rice oil sample was separated by Ag^{+}-HPLC and RP-HPLC with APCI identification. The method was successful

in the separation of a high number of solutes, otherwise unachievable through one-dimensional LC. Furthermore, the use of APCI as detection system provided a third analytical dimension boosting the identification power of the comprehensive chromatographic method [52].

The presence of tallow in lard is not easy to determine, due to the similarity of the composition of these two animal fats, which differ mainly in the distribution of FA in the three positions of the glycerol molecule. The determination of the composition of the TAG fraction of lard, tallow, and their mixtures was investigated by HPLC in combination with APCI. The presence of tallow in lard was determined through the study of the sn-POP/sn-PPO ratio by multidimensional HPLC. The off-line bidimensional system was attained through the coupling of non-aqueous reversed phase RP-HPLC and silver ion Ag^+-HPLC. The primary column eluate was fractionated and the fraction containing POP/PPO isomers was injected onto the secondary column, allowing the separation of positional isomers, unresolved in the first dimension. Peak assignment was carried out by combining retention data with APCI spectral information. The fatty acid distribution along the glycerol backbone, determined by Ag^+-LC, was confirmed through DAG ion ratios derived from APCI analysis. Method validation was carried out in preliminary applications on standard TAGs. The analytical results obtained showed that even a 5% addition of tallow to lard modifies the distribution of positional isomers [53].

TAGs from soybean and linseed oils were analyzed by Ag-LC×RP-LC–APCI-MS in a study that is currently the last of the series of papers by Dugo *et al.* making use of the Ag-LC×RP-LC×MS/APCI method. The analyses were performed using a completely automated system, assembled using commercially available equipment. The system is easy to use and, when comprehensive analyses are not performed, the two HPLC systems can be used independently. Under the conditions presented in this work, the characterization of many other TAG profiles of a plant, vegetable or animal origin can be performed; this, however, has not yet been attempted [54].

2.5. Supercritical Fluid Chromatography

Supercritical fluid chromatography (SFC) is another option for separating TAGs. The theory of the method including its use with lipid compounds has been repeatedly described [55,56]. Examples of the use of SFC with APCI are given in Table **3**.

The first paper of this type [57] described a laboratory built SFC interface attached to an MS/APCI instrument. This interface design was used to demonstrate the first SFC-MS/APCI of cholesterol, cholesteryl palmitate, trilaurin, and the methyl ester of stearic acid, as well as others. These authors showed that, under some conditions, APCI produced abundant $[M+18]^+$ adduct ions with water. This occurred because the make-up gas used in their experiment was moistened by sparging it through water. The first SFC-MS/APCI mass spectrum of LaLaLa showed several differences compared to the spectra that are now more commonly observed from such fully saturated TAGs. Their APCI mass spectrum exhibited a protonated molecule as a base peak, with a large $[M+H_2O]^+$ adduct formed due to sparging of the make-up gas through water. The primary fragment formed (at about 18% abundance) was the DAG fragment ion $[M\text{-}RCOO]^+$.

A SFC, combined with a triple-quadrupole MS via a LC-APCI interface, was utilized in the analysis of berry oil TAGs. The separation of TAGs according to acyl carbon number and degree of unsaturation was accomplished on a 20 m x 50 μm i.d. SB-cyanopropyl-25 column. The resolution of TAGs in the reconstructed ion chromatogram and the sensitivity of the SFC-MS/APCI system were comparable to or slightly better than those obtained with a FID. TAGs formed diagnostic $[M+H]^+$ and $[M\text{-}RCOO]^+$ ions with all tested reactant ion solvents except with ammonium hydroxide in methanol, which formed abundant $[M+18]^+$ ions instead of $[M+H]^+$ ions. The abundance of the $[M+H]^+$ ion increased with increasing degree of unsaturation of a TAG, whereas the abundance of the $[M\text{-}RCOO]^+$ ion depended on the regiospecific distribution of the FA moiety between the sn-1/3 positions and the sn-2 position and on the number of double bonds [58].

This phenomenon was further addressed in another paper in black currant (*Ribes nigrum*) and alpine currant (*R. alpinum*) seed oils. The effect of the γ-linolenic acid (18:3n-6) residue on the elution of TAGs on a 25% cyanopropyl-25% phenyl-50% methylpolysiloxane stationary phase was confirmed by using capillary SFC-MS/APCI. The general elution rule on this stationary phase was that triacylglycerols having the same ACN + 2n value coeluted (ACN = acyl carbon number and n = combined number of double bonds in the acyl chains). The different effect of γ- and α-linolenic acid residues on the retention of TAGs and the use of SFC-MS/APCI allowed the study of a number of different linolenic acid residue isomer combinations in TAGs with an identical ACN and degree of unsaturation. Stearidonic acid (18:4n-3) residue was found to have a similar effect on the retention behavior of TAGs as that of γ-linolenic acid residue. The abundance of the $[M\text{-}RCOO]^+$ ion, formed by the loss of one FA moiety of a TAG, was found to be clearly higher in the case of γ-isomer than that of α-isomer in the identical regiospecific position. This indicates that the distance of the double bonds from the glycerol backbone in the acyl chain affects the stability of a TAG molecule in the MS/APCI system [59].

A completely different set of TAGs with a much wider spectrum of fatty acid chain lengths was identified in milk fat TAGs by means of SFC-MS/APCI with supercritical carbon dioxide. Ionization was achieved by introducing vapor of ammonia in methanol into the ionization chamber, which resulted in the formation of abundant $[M+18]^+$ and $[M\text{-}RCOO]^+$ ions of TAGs. These ions defined both the molecular weight and the FA constituents of a TAG, respectively. SFC on a nonpolar stationary phase provided an efficient separation of TAGs according to the combined number of carbon atoms in the acyl chains of a molecule. In addition to the identification of the major chromatographic peaks representing molecules with 26-54 acyl carbons, minor peaks representing TAGs with an odd number of acyl carbons were separated and identified. Furthermore, compositional information on partially separated isobaric TAGs, which differed substantially in the chain length of the fatty acyl residues, was achieved within some of the peaks. A new finding of

Table 3. Sources of TAGs, SFC Techniques and Analytical Conditions Used for their Separation and Identification

Pub No	Column	Phase	Temp	Mobil Phase	Flow Rate	Corona Voltage (current)
57	10x50x0.25	30% biphenyl-methylpolysiloxane	80	CO_2	no data →	
58	10x50; two 10x50	SB-octyl-50 SB-cyanopropyl-25	135	CO_2	0.37 mL/min; 0.17 mL/min	5.0 μA
59	two 10x50	SB-cyanopropyl-25	135	CO_2	0.18 mL/min	5.0 μA
60	10x50	SB-octyl-50	140	CO_2	0.37 mL/min	
61	25x4.6x5	Ag-Si	65	ACN-iPrOH 60:40+CO_2	1 mL/min	10 μA
62	0.25x250x5	NH_2	70	CO_2	no data →	

Table 3. Continuation A

Pub No	Nebulizer Gas	Drying Gas	Vaporizer Temp	Sheath Gas	Auxiliary Gas	Mass Range	Scan Time	Analyzed Samples
57	no data →							soybean and sunflower oils
58				N	N	450-950	0.7 s	cloudberry, sea buck-thorn
59			425	N 1 bar	N 5 mL/min	450-1000	0.7 s	black currant, alpine currant
60			425	N 15 psi	N 5 mL/min	200-950	0.8 s	cow milk
61	60 psi	4 L/min	400			500-1000		plant oils
62								*Commiphora guillaumini*

this study was the formation of abundant $[M+18]^+$ ions of saturated TAGs in addition to diagnostic fragment ions, being of primary importance in structure elucidation [60].

Characterization of TAGs in soybean and sunflower oils was achieved by Ag^+ packed-column SFC with MS detection. APCI and coordination ion spray with silver ions were used as ionization modes. Compared to UV detection, the sensitivity was increased by a factor of 100. Both ionization modes are generating similar structural information. Molecular ions $[M-H]^+$ or $[M-Ag]^+$ are observed in the mass spectra with the exception of the saturated TAGs for which only coordination ion spray gives intense molecular ions. The position at which the fatty acids are esterified to the glycerol backbone can be elucidated by APCI although it remains speculative whether this is valid for highly unsaturated TAGs because reference compounds are not available to prove this phenomenon [61].

SFC-MS/APCI was used to analyze a complex mixture of TAGs and DAGs extracted from the tree *Commiphora guillaumini*. The single components, including the ant attractant OO, were identified in both the positive and the negative mode [62].

2.6. Perspectives

One of the possible future trends in the area of lipid analysis is the complex analysis of all lipids in the sample, i.e. the analysis of not only TAGs but also other species. The current possibilities of a complex sample analysis are documented in several studies based mainly on two-dimensional technique, i.e. the use of two columns with different polarities with subsequent detection by APCI or ESI. They should at least give some hint of the enormous potential of LC-MS/APCI.

Analysis of phospholipids was performed using LC separation with two mass spectrometers in parallel providing ESI and APCI data simultaneously from a triple quadrupole instrument and a single quadrupole instrument, respectively. The outputs from UV-Vis and evaporative light scattering detectors were also acquired by the two mass spectrometers, respectively, for four detectors overall. This arrangement was used to identify and calculate percent areas for molecular species of dihydrosphingomyelin and sphingomyelin in commercially available bovine brain, in human plasma extract and in porcine lens extract. Molecular species of phosphatidylethanolamine and its plasmalogen, and phosphatidylcholine and its plasmalogen were identified and semi-quantitative analysis performed. Other findings concerned phospholipids, which were found to undergo dimerization in the electrospray source, giving masses representing combinations of the species present. TAGs gave usable mass spectra under APCI conditions, and mass spectra for phospholipids which underwent both protonation and sodium adduct formation in different chromatographic runs were obtained [63].

Another study performed by the same group on the same instrumentation concerned the analysis of TAGs in canola oil, analysis of triolein oxidation products, and analysis of TAG positional isomers separated using RP-HPLC. A triple quadrupole mass spectrometer was interfaced via an APCI interface to two RP-LC columns in series. An ion trap mass spectrometer was coupled to the same two columns using an ESI interface, with ammonium formate added as electrolyte. MS/ESI under these conditions produced abundant ammonium adduct ions from TAGs, which were then fragmented to produce MS/MS spectra and then fragmented further to produce MS/MS/MS spectra. MS-MS/ESI of the ammoniated adduct ions gave product ion mass spectra which were similar to mass spectra obtained by MS/APCI. MS-MS/ESI produced DAG fragment ions, and additional fragmentation (MS/MS/MS) produced $[RCO]^+$ ions, $[RCOO+58]^+$ ions, and other related ions which allowed the assignment of individual acyl chain identities. Analysis of oxidation products was also performed. MS/ESI and MS/APCI were found to be complementary techniques [64].

A commercially available bovine brain total lipid extract containing polar and non-polar lipid fractions has been separated in one analytical run using two HPLC systems in parallel, coupled to dual mass spectrometers in parallel. The whole extract of bovine brain was separated on amino phase (LC1) and reversed-phase (LC2) columns simultaneously, with detection using an ion trap mass spectrometer (MS1) and a tandem mass spectrometer (MS2) coupled to the two HPLC systems, respectively. A single injection was loaded onto an amine column to perform separation of phospholipid components. Non-polar components, such as TAGs, were unretained on this polar column. Using the diverter valve on the front of the ion trap mass spectrometer (MS1), the bolus of neutral lipids was redirected to a RP-LC system (LC2) on which the neutral lipids were separated using a second chromatographic system at the same time that the polar lipids were separated on LC1. MS/ESI on an ion trap mass spectrometer (MS1), with ammonium formate added as a sheath liquid, was used for analysis of the phospholipids. MS/ESI with ammonium formate incorporated into the solvent system was used on a tandem mass spectrometer (MS2) for identification of TAGs and other neutral lipids. This dual liquid chromatography/dual mass spectrometry (LC2/MS2) system represents a unified approach for a total lipid analysis in one experiment using a single sample injection [65].

Another possibility is the use of a column with CN phase and a silica column, as described below. A novel on-line system combining supercritical fluid extraction (SFE) and two-dimensional high performance liquid chromatography (2D-HPLC) was developed. A trap column and two three-port valves were employed to couple SFE and 2D-HPLC system, which was composed of a CN column and a monolithic silica column, connected by a 10-port dual-position valve. The analytes extracted by supercritical CO_2 were completely transferred to the 2D-HPLC system. After separation in two orthogonal modes, the eluents were delivered to APCI-tandem-MS for identification of the samples. In this way, sample preparation, separation, detection, and identification were integrated into an on-line system permitting analysis of the fruiting bodies of *Ganoderma lucidum*, and at least 73 components were resolved in the extract [66].

CONCLUSIONS

The future of TAG analysis can be seen in two areas. The first is the use of new ionization techniques such as, e.g., atmospheric pressure photoionization mass spectrometry, MS/MS combination, and also the use of ultra performance liquid chromatography. The other area offers the possibility of analyzing a much larger spectrum of samples. To our knowledge, LC-MS/APCI has not yet been used for analyzing materials such as fish oil, milk of most mammals including cows, various plant oils containing unusual positional FA isomers, TAGs contained in microorganisms including algae, yeasts, etc. We foresee explosive development in this area in the next decade.

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