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Quantitation of triacylglycerols from plant oils using charged aerosol detection with gradient compensation

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Abstract

Quantitative analysis of triacylglycerols (TGs) in plant oils and animal fats by normalization of peak areas can lead to erroneous results due to the large response differences with common HPLC detectors between the various TGs. The charged aerosol detector (CAD), that generates an almost universal response for non-volatile compounds, was combined with non-aqueous reversed-phase HPLC (NARP-HPLC) to develop a simple quantitative method, without need for RFs, for the analysis of complex natural TG mixtures from plant oils. Two 25 cm Hypersil ODS columns, connected in series, and a mobile phase gradient composed of acetonitrile, 2-propanol and hexane were used. Mobile phase compensation was applied, by mixing of the column effluent with the inversed gradient delivered by a second HPLC pump, for the suppression of the response dependency of the analytes on the mobile phase composition. Calibration curves of 16 saturated (from C7:0 to C22:0) and 3 unsaturated (C18:1, C18:2, C18:3) single-acid TG standards were measured and their RFs were compared with a previously described method using atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). The variation in response of the most common TGs (containing fatty acid chains from 12 to 19 carbons) could be reduced to less than 5% making the combination of NARP-HPLC with CAD and mobile phase compensation an adequate tool for fast quantitative analysis of TGs in common plant oils.

Keywords: HPLC; Charged aerosol detection; Triacylglycerols; Triglycerides; Plant oils

1. Introduction

Natural plant oils are complex mixtures of various non-polar compounds of which the triacylglycerols (TGs) make up to 90% of the content. TGs are triesters of fatty acids (FAs) and glycerol differing in length of the FA chains, in the number, position and *cis*/*trans* configuration of the double bonds (DBs), in the position of the FAs on the glycerol skeleton (regioisomers) and in optical isomerism for TGs containing three different FAs. TGs from plant oils represent an important part of the human diet, because they serve as a source of energy and of essential FAs that are used for the synthesis of lipid membranes. Knowledge of the qualitative and quantitative composition of TGs in plant oils is important for dietetical and nutritional reasons because of the influence of the different properties of each FA to the human organism.

The identification of TGs in plant oils which can contain several hundred types is challenging and requires an efficient separation before mass spectrometric characterization. High-performance liquid chromatography (HPLC) has been widely used for this purpose. Normal-phase HPLC with silver ion embedded columns (Ag-HPLC) allows for the separation of TGs differing in the number and position of the DBs [1–4]. The retention of the TGs thereby increases with increasing number of DBs. With non-aqueous reversed-phase HPLC (NARP-HPLC) [5–27] TGs differing in acyl chain lengths and in number and position of the DBs can be separated. The retention thereby increases with increasing equivalent carbon number (ECN) which is defined as the total carbon number in all acyl chains minus two times the amount of DBs, i.e. ECN = CN − 2DB. Under optimized conditions, the separation of the TGs in a single ECN group can also be achieved [10,16,19,23,24]. Relatively

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apolar solvents are usually used for the analysis of the TGs because of their insolubility in water and in common reversed-phase HPLC solvents. Typically combinations of acetonitrile (ACN)/2-propanol [14,16,22–24,26], ACN/2-propanol/hexane [11,12], ACN/acetone [8,13,15,17,19,20], propionitrile [10], ACN/chloroform [21] and ACN/dichloromethane [7,9,25] are used as mobile phases in gradient or isocratic NARP-HPLC mode.

The quantitation of TGs from natural mixtures is complicated by the large structural variation of the species leading to much differing detector response factors with common HPLC detectors, such as mass spectrometry, UV or evaporative light scattering detection (ELSD). Simple quantitation based on the relative peak areas while neglecting the differences in response factors is widely used in lipid analysis. However, the differences in chain lengths, the number and position of the DBs and other structural differences lead to highly different responses with common detectors used for TG analysis like UV absorbance at low wavelengths, ELSD and APCI-MS. It has been demonstrated that quantitation without taking the response factors (RFs) into consideration leads to large errors in the results depending on which detector is being used [23]. On the other hand, precise quantitation using the RFs obtained from calibration curves of each pure TG can be time consuming, expensive or not possible because of the lack of TG standards.

Nevertheless, several quantitation approaches based on the (partial) knowledge of the RFs have been developed for quantitation of TGs in plant oils. A method using APCI-MS and RFs has been described for the analysis of TGs in a wide range of plant oils and fats [23,27]. The RFs of fatty acids in the TGs were thereby calculated by the calibration curves of 23 single-acid TG standards and subsequently related to triolein. The RFs of TGs with a mixed fatty acid composition were then calculated as the arithmetic mean of the RFs of fatty acids in each TG. In another approach [9], the RFs of TGs with certain fatty acids were calculated by comparison of the fatty acid composition calculated from the TG composition using APCI-MS to the fatty acid composition obtained by a calibrated GC/FID method of transmethylated TGs. The RFs of TGs with a mixed fatty acid composition were then calculated by multiplication of the RFs of the fatty acids present in the TG. RFs have also been calculated by comparison of the RFs of randomized samples to the RFs of their statistically expected composition. All of these approaches yield satisfactory precision for quantitation of TGs in natural mixtures, but they are complicated and time consuming.

The quantitation of TGs using detectors of which the response is almost independent of the analyte structure allows simpler and faster quantitation without the need of RFs. HPLC combined with flame ionization detection (FID) [5–7,9] and with refractive index (RI) detection have been used for quantitation of TGs in plant oils [8,18]. Both detectors, however, do not allow gradient analysis and moreover they exhibit low sensitivity and robustness as they are highly dependent on the mobile phase composition, temperature or pressure variations during analysis.

Aerosol-based detectors like ELSD [25] and charged aerosol detection (CAD) [28–31] are gaining interest as they are almost universal detectors which can be used in combination with gradient analysis. Drawbacks of ELSD are that it requires non-linear calibration and sometimes lacks sensitivity [12]. The quantitation of a number of TGs by HPLC–ELSD was demonstrated whereby a non-linear equation was used for quantification of unknowns as opposed to the internal normalization method often wrongly applied in HPLC–ELSD as such [25]. Isocratic analysis was thereby performed on an oil containing nine TGs.

Just like for the ELSD, the response of the CAD is almost independent on the structure of non-volatile compounds [29], but it strongly depends on the amount of organic solvent in the mobile phase as it influences the transport efficiency of the nebulizer and the generated signal [32]. The responses of individual lipid classes may significantly differ [31], if the gradient compensation is not employed. The increasing response of more retained compounds in gradient elution is hindering quantitative analysis without knowledge of the RFs [33–35]. This response dependency can, however, be corrected by mixing of the column effluent with the inversed gradient delivered by a second HPLC pump, before introduction into the CAD. In this way, a constant mobile phase composition is reaching the detector [33,35]. The dilution of the analytes by mixing with the inversed mobile phase gradient thereby does not cause a loss of sensitivity because of the mass sensitivity of CAD.

The goal of this work was the development of a simple approach for the quantitation of TGs in complex natural mixtures from plant oils using NARP-HPLC in the gradient mode combined with universal CAD and mobile phase compensation. For this purpose, calibration curves of various single-acid saturated (from C7:0 to C22:0) and unsaturated (C18:1, C18:2, C18:3) TG standards were measured with and without gradient compensation and the response dependency on the length of fatty acid chains and on the number of DBs using CAD was evaluated. The developed method is applied to the analysis of real samples of complex TG mixtures from plant oils.

2. Experimental

2.1. Materials and samples

Acetonitrile and 2-propanol (LC–MS grade) were purchased from Biosolve (HA Valkenswaard, The Netherlands). Hexane was LC grade and was obtained from Sigma–Aldrich (St. Louis, USA). The TG standard mixtures GLC#435 (saturated single-acid TGs from C7:0 to C22:0) and GLC#437 (single-acid TGs C16:0, C18:0, C18:1, C18:2, C18:3 and C20:0) were purchased from Nu-Chek-Prep (Elysian, USA). Nitrogen 4.0 which was used as nebulizing gas for the CAD was purchased from Messner (Mechelen, Belgium). Samples of sunflower (*Helianthus annuus*), soybean (*Glycine soja*), grape seed (*Vitis vinifera*), sesame (*Sesamum indicum*) and linseed (*Linum usitatissimum*) oils were prepared in the laboratory by the following procedure. Each sample was carefully crushed in a mortar to obtain fine particles, which were then mixed with hexane. The mixture was stirred occasionally for 15 min. The solid particles were filtered out using filter paper and then the extract was filtered again using a syringe filter with 0.45 μm pores. Hexane was evaporated from the filtered extract yielding the pure plant oil. Samples of olive

(*Olea europea*) and palm oils (*Elaeis guineensis* Jacq.) were purchased from Augustus Oils Ltd. (Hampshire, England). The oil samples were dissolved in hexane to obtain a final concentration of 600 mg/L. Four microliters of this solution was injected for HPLC analysis.

2.2. Instrumentation

Experiments were performed on an HP1050 system from Agilent Technologies (Waldbronn, Germany) equipped with a quaternary gradient pump, autosampler and controlled by Chemstation software. A second HP1050 quaternary gradient pump programmed with the control panel and started by an external signal from the first HP1050 Series system was used for gradient compensation according to ref. [33]. Effluents from both pumps were connected using a T-piece before the Corona CAD detector (ESA Analytical, Aylsbury, Buckinghamshire, England). Data from the CAD detector were processed by a Peak Simple Chromatography Data System model 202 and Peak Simple software (both from SRI Instruments, Torrance, CA). The following parameters were used for the CAD. The acquisition range was set at 100 pA, the low filter was kept constant and the N_2 pressure was 35 psi. The data acquisition was triggered automatically using the external start signal from the LC autosampler and finished after a predetermined time. TGs in plant oils were identified using HPLC coupled to mass spectrometry. The chromatographic apparatus consisted of a Model 616 pump with a quaternary gradient system, a model 996 diode-array UV detector, a model 717+ autosampler, a thermostated column compartment and a Millennium chromatography manager (all from Waters, Milford, MA, USA) combined with an Esquire 3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany) equipped with a positive-ion atmospheric pressure chemical ionization (APCI) source. The following tuning parameters were used. The pressure of the nebulizing gas was 70 psi, the drying gas flow rate was set at 3 L/min, the temperatures of the drying gas and APCI heater were 350 °C and 400 °C, respectively.

2.3. HPLC conditions

Separations were carried out on two Hypersil ODS columns (250 mm × 4.6 mm I.D., 5 μm, Thermo Electron, San Jose, CA, USA) connected in series and equipped with a precolumn containing the same sorbent. The flow rate of mobile phase was set to 1 mL/min with the following gradient composition used for each analysis: 0 min – 20% A + 80% B, 80 min – 75% A + 25% B, where A is 2-propanol:hexane (1:1, v/v) and B is ACN. The column temperature was 30 °C and the injection volume was 4 μL. Each analysis was repeated three times. Two identical Hypersil ODS columns including the precolumn were connected to the second HPLC pump. The flow rate was thereby also set at 1 mL/min and an inverse gradient composition was applied for compensation i.e. 0 min – 80% A + 20% B, 80 min – 25% A + 75% B. With compensation a flow rate of 2 mL/min mobile phase is introduced into the CAD. Nebulization of the mobile phase at that high flow rate is problem free and within the CAD manufacturers' recommendations.

2.4. Calibration curves, response factors and limits of detection

The stock solutions were prepared by dissolving the GLC#435 and GLC#437 samples in hexane to obtain final concentrations of 63 mg/L (higher concentrations could not be prepared because of low solubility of saturated TGs from C18 to C22) and 333 mg/L of each TG, respectively. The calibration curves were constructed at six concentration levels: 2, 5, 10, 20, 40 and 63 mg/L for GLC#435 and 2, 10, 50, 100, 200 and 333 mg/L for GLC#437. The RFs of the TGs using CAD with mobile phase compensation were calculated as the ratio of the slope of the calibration curve to the slope of the curve obtained for triolein. The limit of detection (LOD) was determined according to the U.S. Environmental Protection Agency (EPA) recommended procedure [36]. Four microliters of a standard solution containing 2 mg/L of each analyte (which leads to an approximate S/N value of 10) was injected seven times and standard deviations of peak areas were calculated. LODs are obtained by multiplying the measured standard deviations by three.

3. Results and discussion

3.1. TG response factors with charged aerosol detection (CAD)

Aerosol-based detectors, such as ELSD and CAD, are nearly universal detectors providing non-volatile compounds are analyzed. TGs have extremely low vapour pressures so they can be detected with a good sensitivity. CAD provides some further benefits in terms of increased dynamic range and sensitivity compared to ELSD, hence this detector was used to develop a simple approach for quantitative TG analysis in plant oils. Two standard TG mixtures containing 16 saturated and 3 unsaturated single-acid TGs were therefore measured. The chromatograms (Fig. 1A and C) show an increasing baseline signal and TG response during gradient elution which is in agreement with previously published data [33,34]. The increased response is caused by an improved transport efficiency of the nebulizer [32] leading to a higher number of ions reaching the detector because of the increasing content of apolar solvent in the gradient.

This non-uniform response of TGs during gradient elution prohibits reliable quantitative analysis. For this reason, the response dependency was corrected by using the gradient mobile phase compensation method [33]. Briefly, the effluent from the HPLC column is mixed by a T-piece installed before the detector with the inverse gradient delivered by the second HPLC pump. The mobile phase reaching the CAD has a constant composition and therefore constant conditions are obtained for nebulizing the mobile phase with target analytes during the gradient elution. Fig. 1B and D show the separation of the standard TG mixtures using CAD with mobile phase compensation. RFs of TG standards analyzed using CAD with and without the gradient mobile phase compensation were calculated based on the peak areas obtained from the analysis of TGs at one concentration level. The response factors of the TGs are calculated as

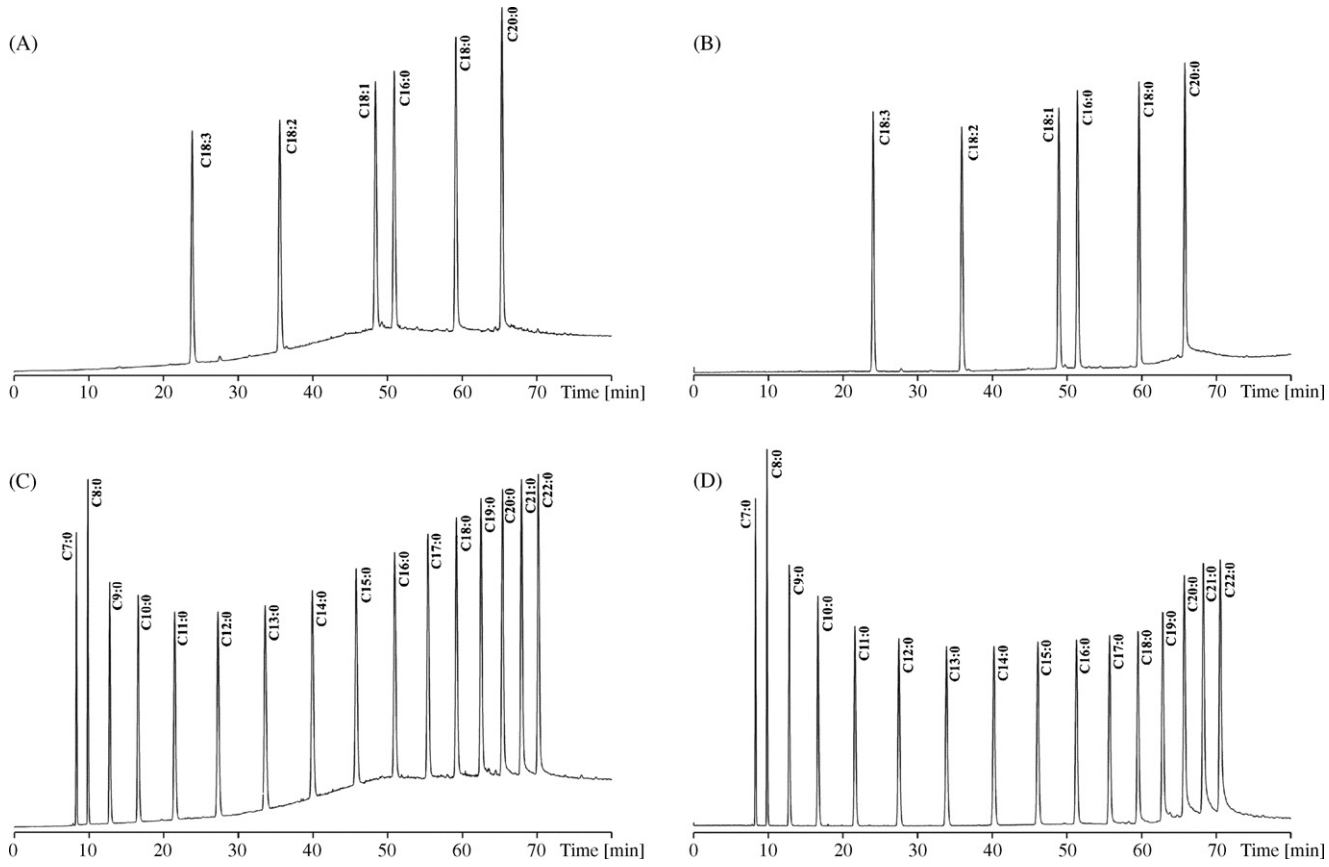

Fig. 1. HPLC separation of equal amounts of each single-acid triacylglycerol (TG) standard using charged aerosol detection: (A) analysis of the TG mixture #437 without and (B) with mobile phase compensation, (C) analysis of the TG mixture #435 without and (D) with mobile phase compensation. Conditions: two Hypersil ODS columns (250 mm × 4.6 mm, 5 μm) connected in series. Flow rate: 1 mL/min. Column temperature: 30 °C. Further details in Section 2.

the ratio of the TG peak areas to the peak area of triolein. The latter was selected because it is one of the most common TGs in nature. The mobile phase compensation significantly improved the response uniformity. For example, the response factor of TG C9:0 changed from 0.69 for the analysis without compensation to 0.83 for the analysis with compensation, for C10:0 from 0.75 to 0.85, for C18:0 from 1.15 to 1.02, for C18:3 from 0.84 to 0.92, etc.

Calibration curves were constructed for 19 single-acid TG standards using CAD with mobile phase compensation to determine the dependency of the response factors of the TGs on the chain length and on the number of DBs in the fatty acid chains. Table 1 shows the results obtained for the series of single-acid TG standards. Contrary to a precedent report [33,34] where the calibration curves required plotting on logarithmic co-ordinates to obtain linearity, in this work the equation of linear dependency, $y = ax + b$, fitted very well on linear co-ordinates, with correlation coefficients of 0.997 and better for all analyzed TG standards. For this reason, the calibration curves were considered linear in the studied concentration range (from LOD to 330 mg/L for the unsaturated TGs and from LOD to 67 mg/L for the saturated TGs). The response factors of the TGs slightly increased with increasing carbon number from 0.54 for the TG containing heptanoic acid (C7:0) to 1.38 for the TG containing behenic acid (C22:0). The lower response obtained for the TGs containing short acyl chains is probably not caused by an increased volatility as they still remain large molecules with very low volatilities [33]. This is also supported by observing the standard deviation of the peak areas, which would be expected to be worse for the volatile compounds using CAD. However, the latter is stable for all analyzed TGs over the whole concentration range (data not shown).

It can be observed from these data that the RFs using the CAD show a slight dependency on the number of carbon atoms in the TG molecules (Fig. 2). This is mainly the case for the TGs with acyl chains ranging from C7:0 to C11:0 with RFs ranging from 0.54 to 0.89 and for TGs containing chains from C20:0 to C22:0 with RFs ranging from 1.11 to 1.38. The dependency of the RFs is lower for TGs containing FAs with 12–19 carbon atoms in the acyl chain with values ranging from 0.94 to 1.05, which is an excellent result for compounds differing by 21 methylene units on the whole TG. The RFs obtained for the single-acid TGs containing FAs with 9–20 carbon atoms show a 20% variation which is still a good result for compounds differing by 33 methylene units and cover an almost doubling of the molecular mass (from 512 to 974 g/mol). The RFs of TGs containing 0, 1, 2 or 3 double bounds were also compared. For the TGs containing three stearic acid chains (C18:0) a response of 1.02 was obtained. For the di- (C18:2) and tri-unsaturated (C18:3) equivalents, a response of 0.98 and 0.92 were obtained, respectively. Hence, the presence

Table 1
Calibration parameters of 19 single-acid triacylglycerol (TG) standards using charged aerosol detection with mobile phase compensation with their corresponding retention times t_R and response factors (RFs)

TGs	t_R (min)	a	b	r^2	RFs-CAD	RFs-APCI[a]
C7:0	8.3	15.3	−31.0	0.999	0.54	97.20
C8:0	10.1	20.9	−12.1	0.999	0.74	74.44
C9:0	12.9	23.6	3.1	0.999	0.83	38.91
C10:0	16.7	24.3	15.4	0.997	0.86	17.62
C11:0	21.6	25.3	16.8	0.997	0.89	10.85
C12:0	27.4	26.7	23.4	0.997	0.94	6.04
C13:0	33.8	27.5	26.5	0.997	0.97	4.31
C14:0	40.1	27.1	31.6	0.997	0.95	2.77
C15:0	46.0	27.9	25.9	0.997	0.98	1.75
C16:0	51.1	28.8	21.5	0.997	1.01	1.32
C17:0	55.6	28.7	32.4	0.997	1.01	0.81
C18:3	24.1	26.2	78.7	0.999	0.92	0.40
C18:2	35.8	27.9	97.2	0.999	0.98	0.57
C18:1	**48.6**	**28.4**	**123.2**	**0.998**	**1.00**	**1.00**
C18:0	59.3	29.1	28.4	0.998	1.02	0.61
C19:0	62.8	29.7	5.7	0.998	1.05	0.49
C20:0	65.5	31.6	−2.5	0.998	1.11	0.40
C21:0	68.2	36.2	−71.6	0.999	1.27	0.39
C22:0	70.5	39.2	−89.6	0.998	1.38	0.46

a and b were obtained from the linear regression according to $y = ax + b$, where y represents the peak areas, x is the concentration in mg/L and r^2 is the correlation coefficient of the curve. The RFs of the TGs using charged aerosol detection (RFs-CAD) and atmospheric pressure chemical ionization (RFs-APCI) are related to triolein (C18:1) which is set to 1.00.

[a] Data from ref. [23].

and the amount of double bounds have only a minor influence on the response. The RFs of the saturated and unsaturated single-acid TG standards with carbon chains containing 12–19 atoms show a variation of only 5% except for C12:0 (6%) and C18:3 (8%) which is an acceptable result for most quantitative applications. Hence, simple quantitative analysis based on the relative peak areas without the knowledge of the RFs becomes possible for plant oil analysis.

In Table 1, the RFs obtained by CAD are also compared with the RFs measured with atmospheric pressure chemical ionization coupled to ion trap mass spectrometry [23]. The RFs of the TGs measured with APCI-MS show large differences. This is mainly the case for the TGs with shorter fatty acids due to the strong discrimination of low m/z values in the ion trap analyzer. In comparison to CAD, the RFs using APCI-MS can therefore

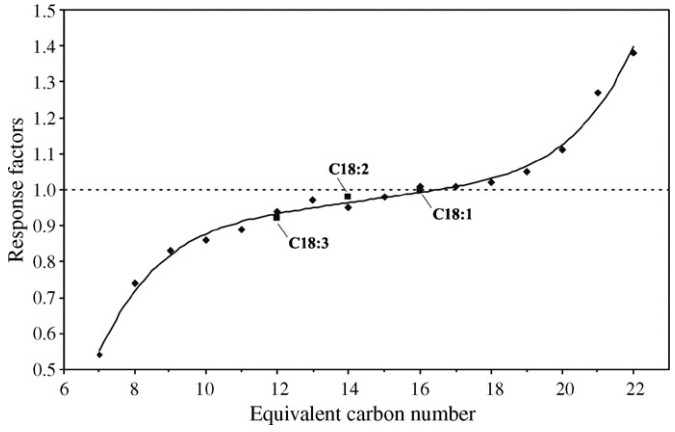

Fig. 2. Dependence of response factors obtained with corona charged aerosol detection on the equivalent carbon number of TGs. All TG standards are saturated except for oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) fatty acids.

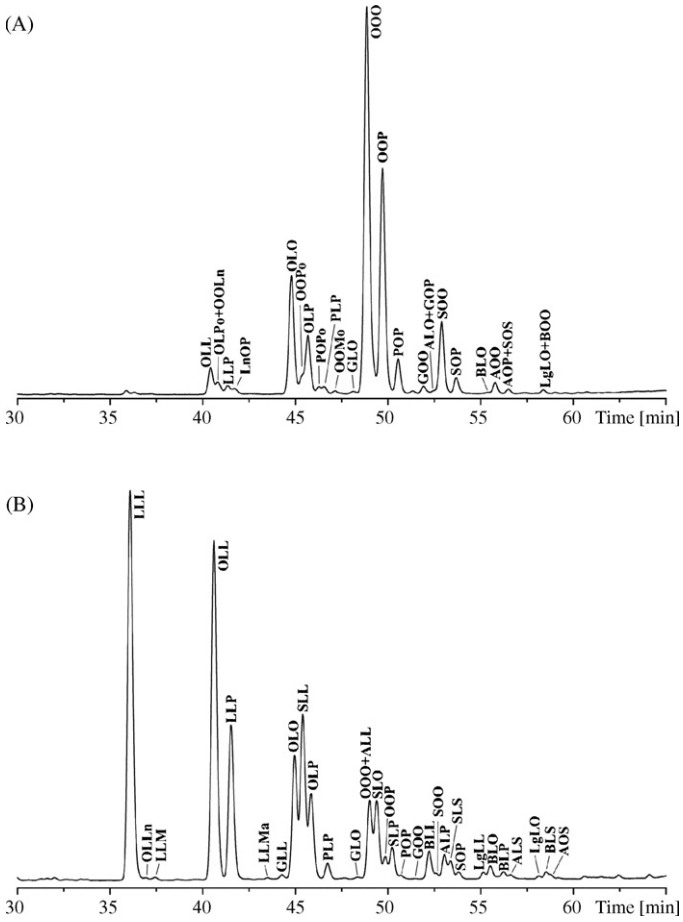

Fig. 3. Representative separations of studied plant oils using charged aerosol detection with mobile phase compensation: (A) olive oil (*Olea europea*) and (B) sunflower oil (*Helianthus annuus*). Conditions: two Hypersil ODS columns (250 mm × 4.6 mm I.D., 5 μm) connected in series. Flow rate: 1 mL/min. Column temperature: 30 °C. Further details in Section 2.

Table 2
Systematic and trivial names of fatty acids found in triacylglycerols of the studied plant oils listed with their abbreviations, carbon numbers (CN), number of double bond (DB) and equivalent carbon numbers (ECN)

Systematic name	Trivial name	Abbreviation	CN:DB	ECN
Tetradecanoic	Myristic	M	C14:0	14
Hexadecanoic	Palmitic	P	C16:0	16
cis-9-Hexadecenoic	Palmitoleic	Po	C16:1	14
Heptadecanoic	Margaric	Ma	C17:0	17
cis-10-Heptadecenoic	Margaroleic	Mo	C17:1	15
Octadecanoic	Stearic	S	C18:0	18
cis-9-Octadecenoic	Oleic	O	C18:1	16
cis-9, 12-Octadecadienoic	Linoleic	L	C18:2	14
cis-9, 12, 15-Octadecatrienoic	Linolenic	Ln	C18:3	12
Eicosanoic	Arachidic	A	C20:0	20
cis-11-Eicosenoic	Gadoleic	G	C20:1	18
cis-11, 14-Eicosadienoic	–	–	C20:2	16
Docosanoic	Behenic	B	C22:0	22
Tetracosanoic	Lignoceric	Lg	C24:0	24

Table 3
Triacylglycerols (TGs) identified in the studied plant oils listed according to their equivalent carbon number (ECN), retention times (t_R) and relative peak areas

TGs	ECN	t_R (min)	Relative peak areas (%)						
			Olive	Sunflower	Palm	Soybean	Grape	Sesame	Linseed
LnLnLn	36	24.1							19.9
LnLLn	38	27.8				1.5			13.4
LLLn	40	31.8				8.5	0.3	0.1	4.1
LnOLn		32.3				0.6			14.5
LnLnP		33.1				0.4			5.8
LLL	42	35.8		26.2		16.9	31.3	10.0	0.6
OLLn		36.3		0.1		5.3		0.3	7.7
LLM		37.2		0.1					
LnLP		37.3				4.0	0.1	0.1	7.5 + SLnLn
LLMo	43	38.3				0.1			
C20:2LL	44	39.6				0.1	0.1		
OLL		40.3	2.2	23.1	0.4	14.6	23.0	18.2	1.2
OOLn		40.6	1.1 + OLPo			1.1		0.3	7.4
LLP		41.2	0.7	9.6	1.8	12.8	12.9	6.6	
SLLn		41.4				1.1			2.6
LnOP		41.8	0.5		0.4				3.2
PLM		42.6			0.3				
PLnP		42.6				0.3			0.3
LLMa	45	43.2		<0.1		0.2	0.2		
GLL	46	44.0		0.2		0.2	0.4	0.1	0.2
OLO		44.6	11.0	7.1	1.4	5.6	7.1	17.2	2.2
OOPo		45.1	0.9						
SLL		45.2		9.9		4.0	8.5	3.4	0.3
OLP		45.6	5.5	4.9	8.6	7.8	6.7	9.9	3.5 + SOLn + BLnLn
OOM		46.0			0.4				
POPo		46.1	0.5						
PLP		46.3	0.5	0.9	8.7	2.5	0.2	1.5	
POM		46.4			1.6				
SLnP		46.4							0.5
PPM		46.9			0.7				
OOMo	47	46.9	0.2						
OLMa		47.4				0.2		0.1	
GLO	48	47.9	0.2	< 0.1		0.2	0.1	0.2	
OOO		48.6	41.8	4.3 + ALL	3.6	1.9 + ALL	1.4 + ALL	10.1	2.0
SLO		49.1		4.3	1.0	3.0	4.1	5.5	0.6
OOP		49.5	22.2	1.1	19.7	1.6	1.6	6.3	0.9
SLP		49.9		1.6	1.9	1.7	0.4	1.5	0.4
SLnS		50.4							0.2
POP		50.5	2.9	0.3	28.8	0.7	0.3	1.2	
PPP		51.3			8.4				
GOO	50	51.6	0.6	< 0.1					
BLL		51.9		1.5		0.4		0.3	
ALO		52.2	0.1 + GOP		0.2 + GOP		0.1	0.4	
SOO		52.5	6.1	0.3	2.7	0.2	0.8	3.4	0.7
ALP		52.8		1.2		0.6	0.2	0.7	0.1
SLS		53.1		0.8		0.5	0.2		0.2
SOP		53.5	1.3	0.4	6.2	0.5		1.4	
SPP		54.3			1.9				
LgLL	52	54.8		0.3		0.1			
BLO		55.2	0.1	0.7		0.2			
AOO		55.5	0.9		0.2			0.3	
BLP		56.0		0.3		0.2			
ALS		56.3		0.2		0.2			
AOP		56.3	0.4 + SOS		0.8 + SOS			0.7 + SOS	
APP		56.9			0.3 + SSP				
LgLO	54	58.1	0.3 + BOO	0.2				0.2 + BOO	
BLS		58.3		0.3		0.1			
AOS		58.6		0.1		0.1			

Table 4
Comparison of the relative amounts (wt%) of fatty acids (FAs) widely present in plant oils based on the measured triacylglycerol composition in the analyzed samples using HPLC/CAD and HPLC/APCI-MS

FAs	Olive		Sunflower		Palm		Soybean		Grape		Sesame		Linseed	
	CAD	APCI[a]	CAD	APCI[a]	CAD	APCI[a]	CAD	APCI[a]	CAD	APCI[a]	CAD	APCI[a]	CAD	APCI[a]
C16:0	12.0	11.8	6.8	7.7	47.6	40.6	11.6	11.7	7.2	9.4	10.3	10.9	7.1	6.9
C18:0	2.5	2.6	6.3	5.2	4.7	4.6	4.0	3.4	4.8	3.7	5.1	5.0	2.0	3.7
cis-9-C18:1	75.7	73.9	21.4	23.0	37.1	41.4	18.8	19.2	19.4	22.2	41.5	40.9	19.9	20.8
cis-9,12-C18:2	7.9	8.5	63.6	61.5	9.1	10.3	56.1	51.8	68.1	63.2	41.8	41.5	17.3	15.9
cis-9, 12,15-C18:3	0.5	0.8	<0.1	0.1	0.1	0.2	8.5	12.5	0.1	0.6	0.3	0.6	53.7	52.3
Others	1.4	2.4	1.9	2.5	1.4	2.9	1.0	1.4	0.4	0.9	1.0	1.1	<0.1	0.4

[a] Data from ref. [27].

not be neglected and have to be used for quantitative analysis of TGs in plant oils.

3.2. Quantitation of TGs in plant oils by NARP-HPLC/CAD

TGs from plant oils are an important part of the human diet and knowledge of the oil composition is therefore relevant from a nutritional point of view. NARP-HPLC combined with CAD and mobile phase compensation was used for the quantitation of TGs in plant oils (Fig. 3). TGs are generally noted by the initial of the FA trivial names (Table 2) arranged according to their *sn*-1, 2 and 3 positions. Identified TGs are listed in Table 3. *Sn*-1 and *sn*-3 positional isomers cannot be resolved in NARP-HPLC and are therefore considered equivalent. FAs in these positions are therefore arranged in order of decreasing molecular weights (for example, a TG containing two oleic and one linoleic acid group is noted as OOL). FAs in *sn*-2 position can be differentiated by characteristic differences in the ratio of the fragment ions by APCI-MS [16,23,24].

The retention times of TGs in NARP-HPLC depend on the acyl chain lengths and on the number and position of the DBs according to the equivalent carbon number (ECN = CN − 2DB). In this work, TGs with the same ECN were also partially separated with only a few overlapping peaks (Fig. 3 and Table 3) in this way providing sufficient separation for quantitation using CAD detection. As discussed above, the RFs of the TGs containing FAs with 12–19 carbon atoms and 0–3 double bonds range from 0.92 to 1.05 drastically simplifying quantitation. The main constituents of TGs in plant oils [23,24,26,27] are saturated fatty acids with 16 (palmitic) and 18 carbon atoms (stearic) and unsaturated fatty acids with 18 carbon atoms containing 1 (oleic acid), 2 (linoleic acid) or 3 double bonds (linolenic acid). Their total content in common plant oils make up 93–100% of the total FA content with a mean value of 98%. FAs with shorter or longer acyl chains or containing more DBs are unusual and typically they comprise only one fatty acid in the TGs. They are usually present at trace concentration levels in natural plant oils (up to 0.5%) with a few known exceptions, i.e. coconut oil contains 77% of the C6:0–C14:0 fatty acids and date seed oil contains 39% of the C8:0–C14:0 fatty acids. This means that the simple quantitation with CAD is applicable for a wide range of natural plant oils.

Table 3 lists concentrations of identified TGs in seven plant oils based on the relative peak areas using CAD and the gradient mobile phase compensation while neglecting the TG response factors. The FA content calculated from the TG composition using CAD and the gradient mobile phase compensation method is given in Table 4. These results are in a good agreement with the results obtained using a quantitative APCI-MS method with knowledge of the RFs [27]. The latter were previously confirmed by a validated GC/FID method [23].

3.3. Reproducibility and limit of detection

Universal detectors typically provide a lower reproducibility and sensitivity in comparison to common more selective HPLC detectors. The reproducibility of the results obtained with NARP-HPLC/CAD and mobile phase compensation was calculated from five consecutive analyses of single-acid TG standards and expressed as the relative standard deviation (RSD) of the peak areas. The mean values of the calculated RSDs were 5.8% at a concentration of 2 mg/L, 1.3% at 10 mg/L and 0.4% at 333 mg/L. The limit of detection with mobile phase compensation was calculated according to the U.S. EPA recommended procedure [35]. The LODs ranged from 0.1 to 0.5 mg/L with an average value 0.3 mg/L for the 19 TGs. The injection volume of 4 μL used for each analysis corresponds to 1.2 ng of analyte injected on column, which is an excellent result for a nearly universal detector. No explicit differences in the reproducibilities and LODs were observed among the TGs containing saturated, unsaturated, long or short fatty acid chains.

4. Conclusions

The recently introduced CAD was used for the quantitative analysis of TGs in complex natural mixtures. Because the response of the CAD depends on the composition of the mobile phase, gradient elution cannot be used for reliable quantitative analysis without knowledge of response factors. The mobile phase compensation method was used to improve the response uniformity of the analyzed TG standards under gradient elution conditions. The response factors of TGs containing saturated and unsaturated fatty acids from 12 to 19 carbon atoms showed

only 5% variation, which is sufficient for simple quantitative analysis without the need of response factors. The developed measuring method was applied for the quantitation of TGs in seven common edible plant oils containing mainly saturated and unsaturated fatty acids with 16 and 18 carbon atoms. Good reproducibility and excellent limits of detection with a nearly universal response was achieved. Compared to previously published quantitative methods based on the knowledge of RFs, the current method is cheaper (no TG standards are needed), faster (no calibration curves are required for the determination of relative concentrations) and the obtained precision is acceptable for most analytical purposes.

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