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Characterization of triglycerides in vegetable oils by silver-ion packed-column supercritical fluid chromatography coupled to mass spectroscopy with atmospheric pressure chemical ionization and coordination ion spray

P. Sandra[a,b,*], A. Medvedovici[c], Y. Zhao[b], F. David[d]

[a]*Department of Chemistry, University of Stellenbosch, CENSSUS, Private Bag X1, Matieland 7602, South Africa*
[b]*Department of Organic Chemistry, Ghent University, Krijgslaan 281-S4, B-9000 Ghent, Belgium*
[c]*Department of Chemistry, University of Bucharest, Sos. Panduri 90-92, Bucharest-5, Romania*
[d]*Research Institute for Chromatography, Kennedypark 20, B-8500 Kortrijk, Belgium*

Abstract

Characterization of triglycerides in vegetable oils was achieved by silver-ion packed-column supercritical fluid chromatography (SI-pSFC) with mass spectrometric detection. Hyphenation was made using commercially available liquid chromatography–mass spectrometry (LC–MS) interfaces without any modification. A make-up fluid was delivered through a T-piece placed before or after the SFC restrictor by means of a high pressure pump. Atmospheric pressure chemical ionization (APCI) and coordination ion spray (CIS) 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 exception of the saturated triglycerides for which only CIS gives intense molecular ions. The position at which the fatty acids are esterified to the glycerol backbone can be elucidated by pSFC–APCI although it remains speculative whether this is valid for highly unsaturated triglycerides because reference compounds are not available to proof this.

Keywords: Vegetable oils; Silver-ion packed-column supercritical fluid chromatography; Supercritical fluid chromatography; Triglycerides

1. Introduction

Non-aqueous reversed-phase liquid chromatography (RPLC) [1] and silver-ion chromatography (SIC) [2] are the most powerful chromatographic methods to separate complex mixtures of triglycerides (TGs) or triacylglycerols (TAGs) in natural oils. In RPLC on octadecyl silica the separation is based on the chain length of the fatty acids and the total number of double bonds. On silver ion-exchange columns TGs are separated according to the degree and the distribution of unsaturation. RPLC and SIC are complementary in nature for structure elucidation of TGs in natural oils. For SIC, silver ions can be loaded on classical silica packings or bound onto ion-exchange material. The latter method developed by Powell [3] and optimised by Christie [4,5] gives columns stable for a long period of time. In silver-ion liquid chromatography (LC) also posi-

*Corresponding author.
E-mail address: pat.sandra@richrom.com (P. Sandra).

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tional isomers can be separated but this only under very specific conditions [6,7]. The silver ion technique has been reviewed in Ref. [8].

Packed-column supercritical fluid chromatography (pSFC) on silver loaded stationary phases (SI-pSFC) has been applied to separate triglycerides and derivatized fatty acids (methyl or phenacyl esters) from natural or synthetic oil samples [9–14]. One of the main problems in TG analysis with fluid based separation techniques (LC and pSFC) is detection. TGs have absorbance maxima at low wavelengths, and considering the relatively high background of commonly used solvents, the applicability of UV in triglyceride analysis is rather limited. Since gradient elution is required to elute all TGs also refractive index (RI) detection can not be used. Most often applied today is the transport flame ionization detector (FID) and since this detector is no longer commercially available, the evaporative light scattering detector (ELSD). Both detectors, however, do not provide information on the TG structures. Interest in mass spectroscopic (MS) detection for TG analysis has grown considerably in the last decade [15] and since the introduction of indirect spraying and atmospheric pressure ionization techniques, the coupling of fluid based separation techniques like LC and pSFC to MS is a quasi-routine technique accessible to most laboratories. The features of atmospheric pressure chemical ionization (APCI) and atmospheric pressure electrospray ionization (ESI) for TG analysis by LC have been described in the literature [16–19]. Since TGs are relatively apolar, APCI is by far providing the best ionization. Also positional isomers could be assigned from APCI-MS data [20]. The group of Evershed thoroughly investigated RPLC–APCI for the characterization of TGs in vegetable oils [21] and animal fats [22].

The hyphenation of SFC to MS has been reviewed by Combs et al. [23]. Coupling pSFC to APCI-MS using commercial LC-interfaces with hardly any modification has been described [24,25].

For solutes difficult or impossible to ionize with ESI, Bayer et al., recently introduced the concept of coordination ion spray (CIS) [26,27]. CIS consists of introducing prior to nebulization a metal ion able to generate adducts with the non ionizable analyte molecule due to interactions mediated by electron density transfer. Applications in LC-CIS–ESI-MS were focused on unsaturated fatty acids methyl esters, vitamins, carotenes and estrogenic compounds. Similar results, although not explicitly mentioned as being CIS, were obtained for cyclodextrins, oligosaccharides, bafilomycins and crown ethers [28–30]. Transition metal ions such as Ag^{+}, Cu^{+}, Ni^{2+}, Pd^{2+} or alkaline ions (Li^{+}, Na^{+}, K^{+}, Rb^{+}, Cs^{+}, Ca^{2+} and Mg^{2+}) have been successfully used. CIS-ESI should also be a perfect technique for the MS analysis of triglycerides.

The goal of the present work was to construct a pSFC-MS coupling using a single quadrupole instrument for 4.6 mm I.D. columns without modifying commercially available LC-interfaces and allowing a simple transfer process of the column effluent to the ionization module, and to apply APCI and CIS to the analysis of triglycerides separated by silver ion SFC.

2. Experimental

2.1. Materials

Acetonitrile, isopropanol, methanol and chloroform were high-performance liquid chromatography (HPLC)-grade from Labscan (Analytical Science, Dublin, Ireland). Ammonium acetate and silver nitrate p.a. grade were purchased from Aldrich (Bornem, Belgium). Carbon dioxide, purity 4.5 and nitrogen were from Air Products, Sombreffe, Belgium. Soybean and sunflower oils for alimentary use were purchased from a local supermarket. The samples for pSFC were prepared in chloroform.

2.2. Abbreviations

Triglycerides are noted by means of three symbols corresponding to the fatty acids linked to the glycerol backbone. The symbols used for the fatty acids are P for palmitic acid (C_{16}:0), S for stearic acid (C_{18}:0), O for oleic acid (C_{18}:1), L for linoleic acid (C_{18}:2) and Ln for linolenic acid (C_{18}:3). Positional isomerization on the glycerone backbone is also indicated in the three letter symbols, e.g. PPO (P in sn 2; secondary alcohol) and POP (O in sn 2; secondary alcohol).

2.3. Silver-ion packed-column supercritical fluid chromatography (SI-pSFC)

A sulphonic silica based strong cation-exchanger column (Nucleosil 100-5 SA, 25 cm×4.6 mm I.D.× 5 μm, Cat. no. 720097.46) from Macherey Nagel (Düren, Germany) was loaded with silver ions according to the procedure described by Christie [5]. Briefly, the column was flushed with a 1% ammonium acetate solution at 0.5 ml/min for 1 h, followed by water for another 60 min. A silver nitrate solution (20% w/v) was injected 20 times with a 1 min interval in 50 μl aliquots. The column was finally washed for 20 min with water and for 1 h with methanol. The column was operated at 65 °C, a flow-rate of 1 ml/min and a mixture of acetonitrile/isopropanol (6/4) as modifier. The pressure was programmed from 150 bar (2 min) to 300 bar at 1.5 bar/min. The modifier profile was also programmed from 1.2% (2 min) to 7.2% (28 min) at 0.3%/min, next to 12.2% at 0.54%/min. Before starting actual analysis, the Ag-loaded column was conditioned by performing 20 blank runs with the described mobile phase. After this treatment, retention times were very stable. Between runs at least 15 min equilibration with neat CO_2 is required to eliminate saturation of the stationary phase with modifier. The column was installed in an Hewlett-Packard G1205A SFC instrument (Agilent Technologies, Little Falls, DE, USA) configured in the down stream mode and equipped with an HP 1050 Diode Array Detector which was operated at 210 nm. The injection volume was 5 μl.

2.4. Mass spectroscopy

An Agilent 1100 series MSD (Agilent Technologies, Waldbronn, Germany) equipped with an ESI and APCI interface was used.

The following working conditions were applied for APCI: spectral range from 500 to 1000 a.m.u., positive ionization mode, CID voltage 100 V, drying gas flow 4 l/min, nebulizing pressure 60 p.s.i., drying gas temperature 325 °C, vaporizer temperature 400 °C; capillary voltage 6 kV and corona current 10 μA. The make-up solvent, delivered by an Agilent 1100 LC pump, was methanol at a flow-rate of 1.5 ml/min at ambient temperature.

The operation conditions for CIS applying the ESI interface were: spectral range from 200 to 1200 a.m.u., positive ionization mode, CID voltage 250 V, drying gas flow 12 l/min, nebulising pressure 50 p.s.i., drying gas temperature 340 °C, capillary voltage 4 kV. The make-up solvent consisted of a silver nitrate methanolic solution with a concentration of 100 μg/ml Ag^+ and was delivered at a flow-rate of 1.0 ml/min at ambient temperature by an Agilent 1100 LC pump.

3. Results and discussion

In the coupling pSFC-MS, a make-up fluid must be added for two reasons. First of all, after the restriction the fluid density as well as its solvating properties are drastically reduced which results in precipitation, association of analytes (especially non-volatile ionic ones) and destruction of the chromatographic separation. Consequently, peak tailing and distortion may be observed in the chromatograms. A practical solution to the ''cold trapping effect'' of the solutes is heating of the transfer line. However, high temperatures are needed to volatilize the solutes and decomposition may occur. Moreover, the heating will generate a dry gaseous jet at the nebulizer tip and no ionization will take place [31]. Secondly, pure carbon dioxide is not ionized in the APCI source nor does it play any direct role in ion formation due to the lack of any abundant CO_2 derived primary ions [31]. It is therefore essential to add a polar modifier like methanol to the column effluent in order to obtain ionization. The first modification made on the original instrumental set-up (Fig. 1A), was the introduction of a zero dead volume Valco T-piece (Alltech, Lokeren, Belgium) (Fig. 1B) before the nozzle.

Through the T-piece, the make-up solvent is delivered by means of an Agilent 1100 isocratic high pressure pump. Despite its robustness and simplicity (this configuration was initially used) the life-time of the expensive nozzle was 1 month for APCI with pure methanol as make-up fluid and only 2 days for silver nitrate solutions as make-up fluid. The configuration shown in Fig. 1C, where the make-up fluid is added after expansion, was tested for TG samples and no problems were encountered with precipitation. However, the resolution decreased with

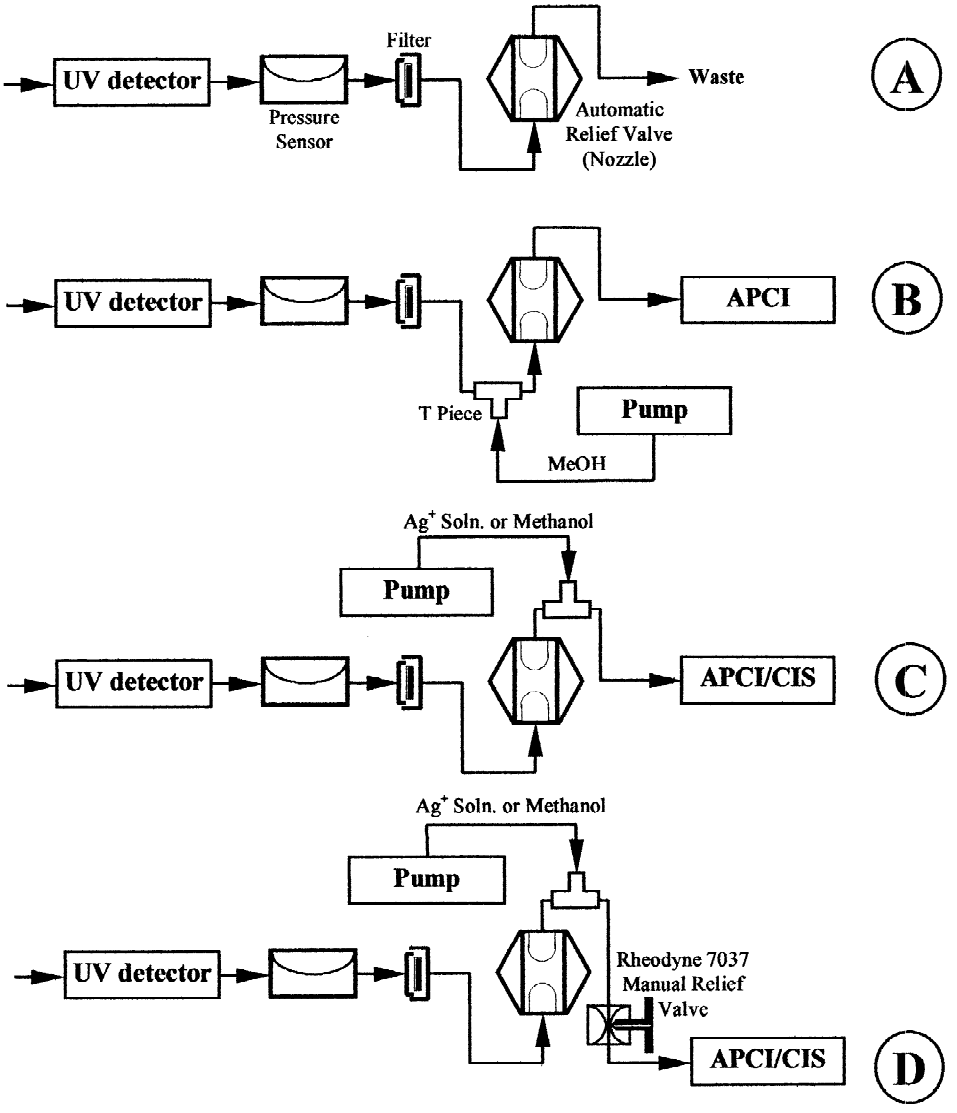

Fig. 1. Instrumental set-up for pSFC-MS using APCI and CIS-ESI ionization. (A) Original configuration of the SFC. (B) Configuration of the SFC with T-piece before the nozzle. (C) Configuration of the SFC with T-piece after the nozzle. (D) Configuration of the SFC with T-piece and relief valve after the nozzle.

roughly 15%. The final modification (Fig. 1D) consisted in inserting a manual Rheodyne 7037 diaphragm relief valve with an internal volume of 3 μl (Gilson, Villiers le Bel, France) and set at 100 bar between the electronic variable restrictor (nozzle) of the G1205A SFC instrument and the entrance of the MS interface. The diaphragm valve was introduced to add make-up solvent introduction before CO_2 expansion and simultaneously to preserve the electronic variable restrictor from a direct liquid flow input.

The SI-pSFC analysis of the triglycerides from soybean oil is shown in Fig. 2A for UV detection at 210 nm and in Fig. 2B for the APCI–MS detection.

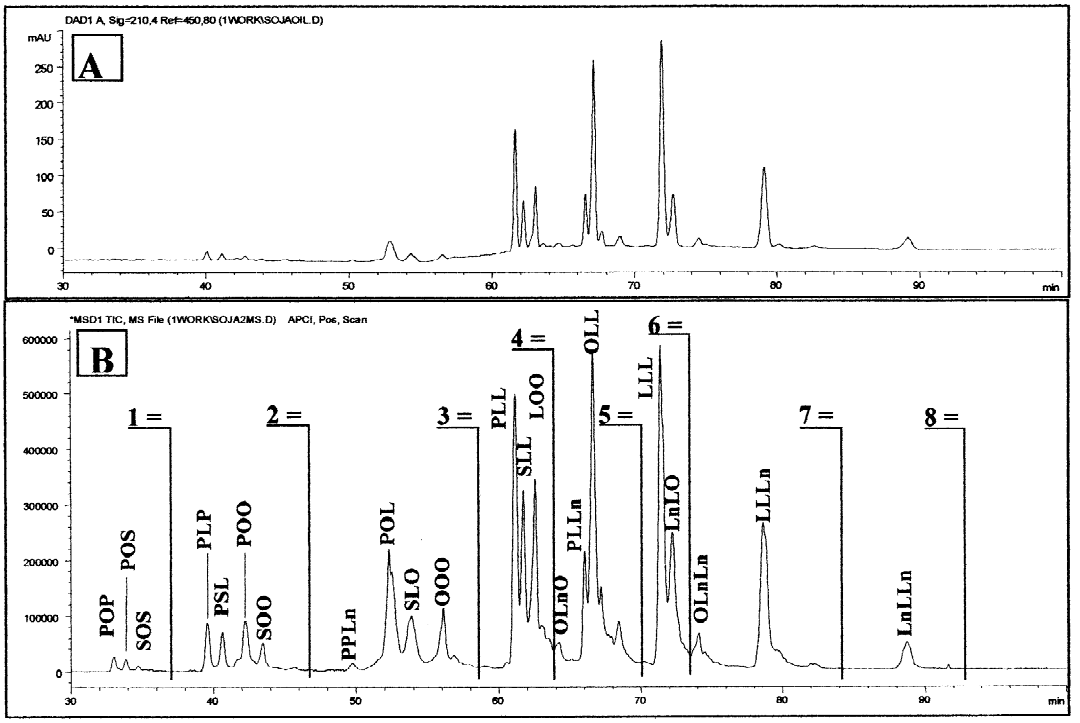

Fig. 2. SI-pSFC separation of soybean oil with UV at 210 nm (A) and APCI-MS (B). Conditions are given in the Experimental section. For TG identification see Table 1.

For the MS analysis, the sample was 100 times less concentrated, i.e. a 10% solution for UV and a 0.1% solution for MS, both in $CHCl_3$.

TGs are in first instance separated according to the number of double bonds (NDB) (Fig. 2B). Within each group, an additional separation is observed according to the carbon number (CN), i.e. the number of carbon atoms of the three fatty acids bonded to the glycerol backbone. This behaviour on the silver bonded phase, may be explained by the hydrophobic influence of the phenyl–propyl moieties present as spacers in the ion-exchange material between the sulphonic groups and the silica surface. Solutes characterized by the same CN elute according to the number of unsaturated fatty acids, e.g. SLL elutes before OOL. The elution order is supported by the MS data (see further). Some tailing can be observed in Fig. 2B for the large peaks. This is not due to the interface because small peaks are perfectly gaussian as is obvious from comparison of the peak profiles eluting around 40 to 46 min in both traces (PLP, PSL, etc.). The tailing effect is entirely due to the well known APCI effect. High solute quantities in the APCI source often give rise to peak tailing. Nevertheless, this is not affecting seriously the chromatographic resolution and peak identification. Mass spectra were taken by substracting the spectra at the beginning and at the end of each peak from the apex spectra.

Fig. 3 shows some spectra taken from the analysis of soybean oil (Fig. 2B) for TGs present at low (POP at 33.0 min, SOO at 43.5 min), medium (SLO at 53.9 min) and high (OLL at 66.6 min) concentration. The masses lower than 500 a.m.u. (acyl ions $[R_xCO]^+$ and monoglyceride ions $[(M+H)-R_xCOO-R_xCO]^+$) are not given because they are low in intensity and only provide information on the constituting fatty acids in each TG. R_x can be R_1, R_2 or R_3.

All spectra show a clear $[M+H]^+$ ion. Spectra of

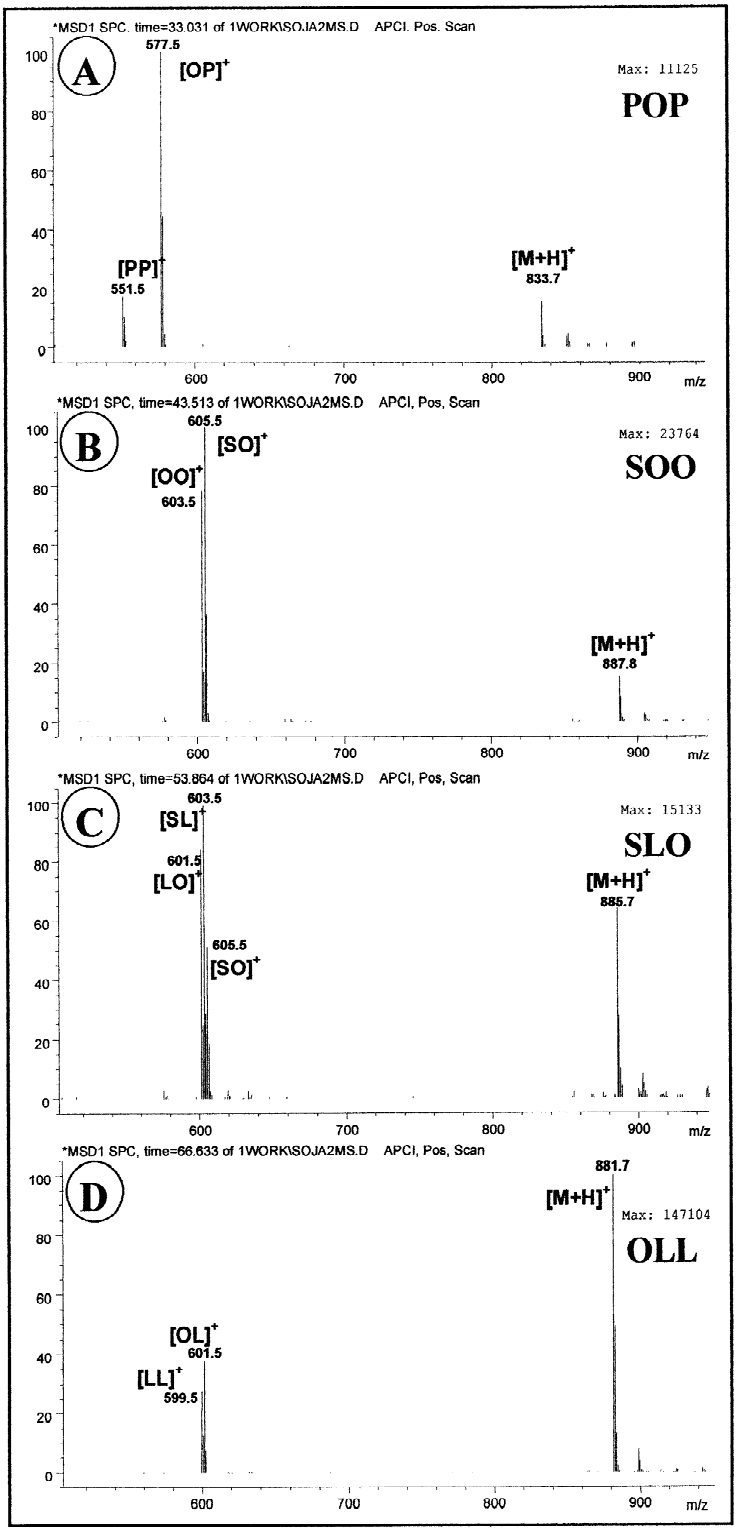

Fig. 3. Some representative APCI-MS spectra of the soybean oil analysis.

highly unsaturated TGs, e.g. OLL, possess for 100% the $[M+H]^+$ ion and fragment ions are not intense, while highly saturated TGs show $[M+H]^+$ ions of low intensity, e.g. POP, but very intense fragment ions. This is in agreement with LC–APCI-MS data using non-aqueous mobile phases [21,22]. The fragments ions correspond to the diglyceride ions $[(M+H)-R_xCOOH]^+$ in which R_x can be R_1, R_2 or R_3. Several collision induced voltages (CID) were applied, i.e. from 20 to 200 V and 100 V was the best compromise for both $[M+H]^+$ and fragment ion formation.

As already described in the literature for the RPLC–APCI-MS combination [20–22], cleavage of the fatty acid located in the middle position (sn2–secondary–R_2) of the glycerol backbone is energetically less favourable for steric reasons than cleavage at the end positions (sn1 and 3–primary–R_1/R_3). This results in intensity differences for the diglyceride ions and positional isomers can be elucidated based on the diglyceride ion ratios. Verification was carried out by analyzing several triglyceride standard positional isomers under identical conditions. RPLC–APCI-MS and SI-pSFC–APCI-MS provide the same information in this respect. This is clear from the spectra in Fig. 3. In our opinion, however, it is still speculative whether the intensity differences of diglyceride ions are generally applicable to elucidate positional isomers. On the one hand, verification for highly unsaturated TGs is impossible because of lack of standards, and on the other hand, the intensity of diglyceride ions for highly unsaturated TGs decreases in function of degree of unsaturation and intensity differences become smaller.

The identified TGs in soybean oil, taking the above considerations on positional isomers into consideration, are presented in Table 1.

One of the disadvantages of APCI-MS for TG analysis is that the more saturated are the TGs, the less intense is the $[M+H]^+$ ion. For fully saturated TGs no $[M+H]^+$ ion is detected. This is also valid for RPLC–APCI-MS [20–22] and illustrated in recent application notes of Waters [32] and Agilent

Table 1
MS data of the triglycerides separated in the chromatogram of soybean oil (Fig. 2)

Peak No.	(NDB)	Attribution	$[M+H]^+$ signal	Relative abundance (%)	Fragments in order of intensity		
					1 Attribution	2 Attribution	3 Attribution
1	1	POP[a]	833.5	16.0	PP	OP	–
2	1	POS	861.8	9.5	PO	SO	PS
3	1	SOS[a]	889.7	10.5	SO	SS	–
4	2	PLP	831.7	10.0	PP	PL	–
5	2	PSL	859.7	12.5	PL	SL	PS
6	2	POO[a]	859.7	27.0	PO	OO	–
7	2	SOO[a]	887.7	16.0	OO	SO	–
8	3	PPLn	829.7	100.0	PP	PLn	–
9	3	POL	857.7	46.0	PL	LO	PO
10	3	SLO	885.8	64.0	LO	SL	SO
11	3	OOO	885.7	12.0	OO	–	–
12	4	PLL	855.7	100.0	PL	LL	–
13	4	SLL	883.7	100.0	LL	SL	–
14	4	LOO	883.7	55.0	LO	OO	–
15	5	OLnO	881.7	100.0	OLn	OO	–
16	5	PLLn	853.7	100.0	PL	LLn	PL
17	5	OLL	881.7	100.0	LL	OL	–
18	6	LLL	879.7	100.0	LL	–	–
19	6	LnLO	879.7	100.0	LLn	LO	OLn
20	7	OLnLn	877.7	100.0	OLn	–	–
21	7	LLLn	877.7	100.0	LnL	LL	–
22	8	LnLLn	875.7	100.0	LnL	LnLn	–

[a] The position of the fatty acid was confirmed by comparison with the mass spectra of the respective positional standard isomer.

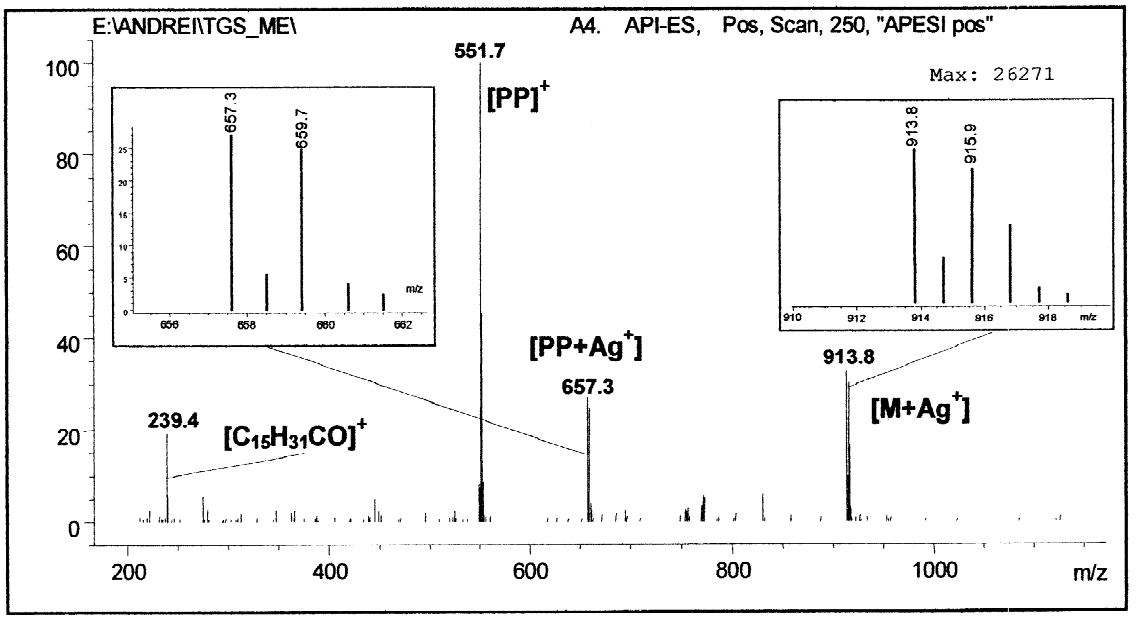

Fig. 4. Ag^+–CIS–ESI-MS spectrum of tripalmitin.

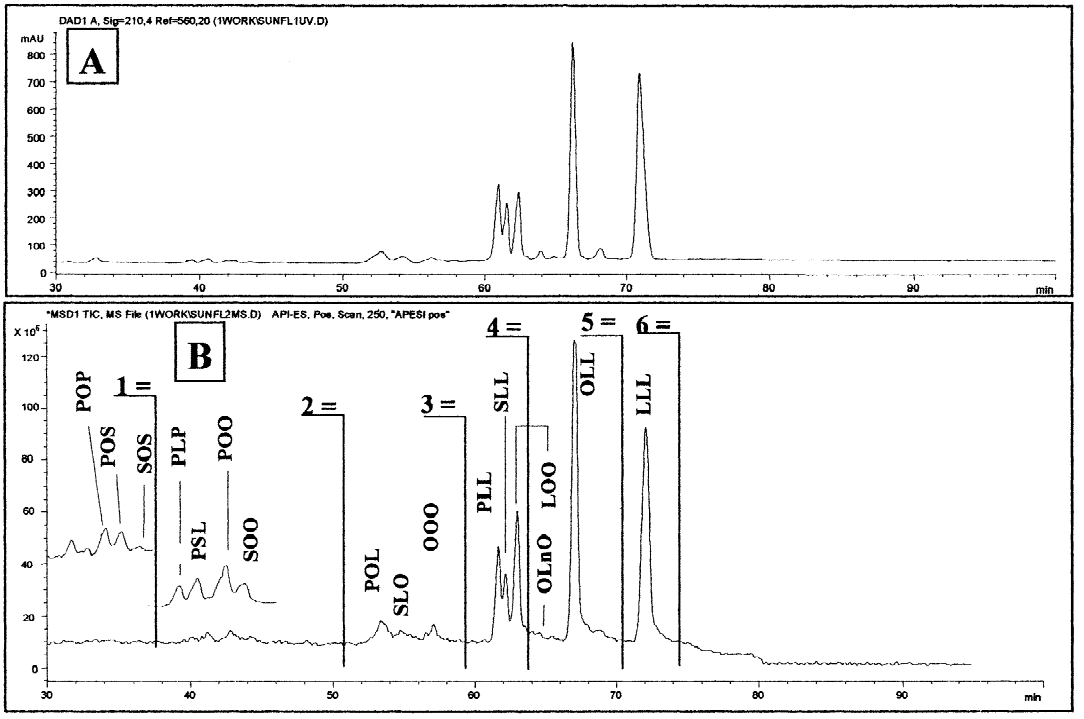

Fig. 5. SI-pSFC separation of sunflower oil with UV at 210 nm (A) and Ag^+–CIS–ESI-MS (B). Conditions are given in the Experimental section. For TG identification see Table 2.

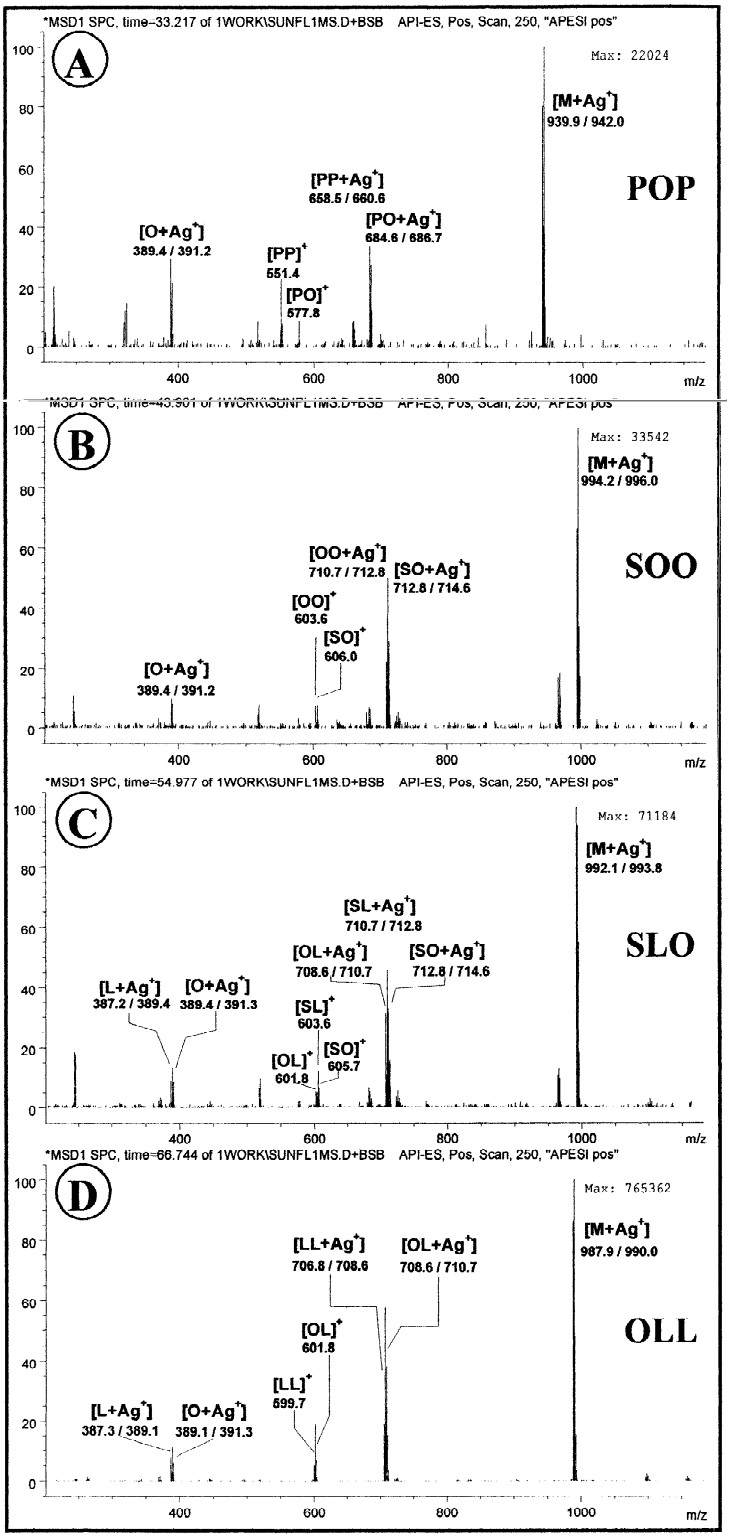

Fig. 6. Some representative Ag^+–CIS–ESI-MS spectra of the sunflower oil analysis.

Technologies [33]. In this context, CIS ionization was evaluated for pSFC-MS using the coupling shown in Fig. 1D. The following ionization parameters were optimized: influence of the counter ion of Ag^+, concentration of the Ag^+ ions in the make-up solvent, make-up solvent flow-rate, and CID or fragmentor voltage. Silver nitrate and acetate were evaluated but the latter gives ca. 50% less ionization and tailing of the peaks increased. The silver ion concentration in the make-up solvent was varied from 25 to 400 μg/ml doubling each time the concentration. Then, 100 μl/min was the optimal concentration. At lower concentrations, ionization decreased and peak asymmetry increased while at higher concentration frequent cleaning of the ESI interface was imposed to keep the sensitivity intact. Make-up solvent flows of 0.5, 1, 1.5 and 2 ml/min were used and best results in terms of ionization yield were at 1 ml/min. The CID voltage had the most important influence on the quality of the generated mass spectra. Below 200 V, the adduct $[M+Ag]^+$ is mainly formed together with a cluster representing $[M+Ag+AgNO_3]^+$. At voltages higher than 300 V, the intensity of $[M+Ag]^+$ drastically decreased, the cluster $[M+Ag+AgNO_3]^+$ was no longer detected while the intensity of fragments without silver coordination strongly increased. A good compromise was found at 250 V. Fig. 4 shows the Ag^+–CIS-ESI spectrum of the fully saturated TG tripalmitin (PPP) obtained by flow injection analysis under the optimized CIS conditions. Contrary to ACPI-MS the molecular ion is clearly represented by the $[M+Ag]^+$ ion. Because of the two isotopes of silver (^{107}Ag–51.35% and ^{109}Ag–48.65%), silver adducts are doublets (A+2 element). The coordination between silver and TGs in first instance is mediated by the π electrons of the double bonds in the fatty acids. However, as illustrated for PPP, silver also coordinates with the electronic pairs of the oxygen atoms from a –O–CO– moiety, while a saturated acyl group, i.e. $[C_{15}H_{31}CO]^+$ in Fig. 4 or fatty acid, can not form a complex. At least one double bond in the fatty acid chain is required for Ag-complexation (see further). Concerning the diglyceride fragment, both the non- and complexated ions are detected. However, when saturated, e.g. $[PP]^+$ vs. $[PP+Ag]^+$ in Fig. 4, the intensity of the complexated ion is lower than that of the free ion, while this is the reversed when the fatty acids are unsaturated.

The pSFC-UV–Ag–CIS–ESI-MS chromatograms of sunflower oil are shown in Fig. 5 and some representative spectra namely for POP at 33.2 min, SOO at 43.9 min, SLO at 55.0 min and OLL at 66.7

Table 2
MS data of the triglycerides separated in the chromatogram of sunflower oil (Fig. 5)

Peak number	NDB	Attribution	$[M+Ag]^+$	Rel. Abund. (%)	Fragments							
					$[R_xR_x+Ag]^+$			$[R_xR_x]^+$			$[RCOOH+Ag]^+$	
					Attribution	Attribution	Attribution	Attribution			Attribution	
1	1	POP	939.9/942.0	100	PP	PO	–	PP	OP	–	O	–
2	1	POS	968.1/969.9	100	PO	PS	SO	PO	–	–	O	–
3	1	SOS	995.7/997.7	100	SO	SS	–	–	–	–	O	–
4	2	PLP	937.8/939.9	100	PP	PL	–	PP	–	–	L	–
5	2	PSL	966.0/968.1	100	PS	PL	SL	PS	–	–	L	–
6	2	POO	966.0/968.1	100	PO	OO	–	PO	–	–	O	–
7	2	SOO	994.2/996.0	100	SO	OO	–	SO	OO	–	O	–
8	3	POL	963.9/966.0	100	PL	PO	OL	PL	PO	–	O	L
9	3	SLO	992.1/993.9	100	SO	SL	OL	SO	SL	LO	O	L
10	3	OOO	992.1/993.9	100	OO	–	–	OO	–	–	O	–
11	4	PLL	962.1/963.9	100	PL	LL	–	PL	–	–	L	–
12	4	SLL	990.0/992.1	100	SL	LL	–	SL	–	–	L	–
13	4	OOL	990.0/992.1	100	OL	OO	–	OO	OL		O	L
14	5	OLnO	987.9/990.0	100	OLn	OO	–	OO	–	–	O	–
15	5	OLL	987.9/990.0	100	OL	LL	–	OL	LL	–	O	L
16	6	LLL	986.1/987.9	100	LL	–	–	LL	–	–	L	–

min, are shown in Fig. 6. For comparison, the same TGs were selected in Fig. 6 (CIS) and in Fig. 3 (APCI). Main difference with APCI-MS is that, whatever the degree of unsaturation, the $[M+Ag]^+$ ion is always the most intense ion. Note that only unsaturated fatty acids ions are detected as Ag-complexes.

The triglycerides identified in sunflower oil are listed in Table 2. Elucidation of positional isomers was impossible with CIS–ESI-MS.

4. Conclusion

Coupling of pSFC to MS can easily be performed without modification of APCI and ESI interfaces used for LC–MS. A robust and versatile pSFC-MS set-up is obtained by a simple T-piece and a relief valve. Both APCI and CIS–ESI gives useful information for structure elucidation of TGs in vegetable oils. SIC-pSFC–APCI-MS provides the same information as RPLC–APCI-MS. Both techniques are complementary to each other because of the different separation mechanisms. In CIS–ESI, the $[M+Ag^+]$ ion shows the highest intensity irrespective of the degree of unsaturation. Even fully saturated TGs can be characterized by CIS–ESI. Both ionization techniques are rugged and are well tolerating the modifier and pressure SFC gradients.

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