ELSEVIER

Journal of Chromatography A, 810 (1998) 53–61

JOURNAL OF CHROMATOGRAPHY A

Silver-phase high-performance liquid chromatography–electrospray mass spectrometry of triacylglycerols

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Received 22 December 1997; received in revised form 23 March 1998; accepted 31 March 1998

Abstract

Silver-phase chromatography hyphenated with on-line electrospray mass spectrometry can be applied to characterize triacylglycerols present in vegetable oils with respect to their degree of unsaturation, the position of the most unsaturated fatty acid and the carbon number (CN). The CN information obtained with silver-phase HPLC–ESP–mass spectrometry is complementary to the unsaturation information obtained by silver-phase HPLC-flame ionization detection. Both information is essential to monitor or study modified vegetable oils on the presence of non-natural triacylglycerols. The quantitative results obtained with the method are in agreement with the results obtained in the silver-phase HPLC-flame ionization detection and with theoretical values calculated from the fatty acid distribution of the oil. Silver-phase HPLC–ESP-mass spectrometry gives direct information on fatty acid position and triacylglycerol CN, for each of the triacylglycerols in the sample. This in contrast with non-aqueous reversed-phase HPLC hyphenated with on-line atmospheric pressure chemical ionization-mass spectrometry, which requires a more extensive data processing. The results obtained with silver-phase HPLC–ESP-mass spectrometry can be presented in a three-dimensional overview (relative amount, CN, fatty acid position) serving as a fingerprint for the oil.

Keywords: Argentation chromatography; Palm Oil; Regio-specific isomers; Fatty acids; Triacylglycerols

1. Introduction

Natural oils and fats are complex mixtures consisting primarily of triacylglycerols (TAGs), with the number of TAGs being as high as 50 or more. Each individual TAG is described by four basic attributes: (a) the total carbon number (CN), which is the sum of the alkyl chain lengths of each of the three fatty acids (FAs); (b) the position of the three fatty acids on the glycerol backbone; (c) the degree of unsaturation; and (d) the position and configuration of the double bounds (DBs) in each FA.

Each of these parameters can be analyzed with well-described and widely used analytical techniques. The CN distribution and some (limited) information on the position of the fatty acids is obtained by high-temperature gas chromatography (HT-GC) or supercritical chromatography (SFC) [1,2]. The degree of unsaturation and CN distribution are obtained by non-aqueous reversed-phase high-pressure liquid chromatography (NARP-HPLC) [3]. Finally argentation chromatography provides information on the degree and the distribution of the

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0021-9673/98/$19.00
PII: S0021-9673(98)00277-5

unsaturation, and symmetric/asymmetric information [4]. No single analytical method is available which provides the combined CN, FA type and FA position information. Therefore, several authors proposed the combination of the above-mentioned techniques, e.g. the collection of peaks from Ag-HPLC which are analyzed by HT-GC.

Argentation chromatography can be performed in two different modes. In the first mode, silver ions are bound onto the column stationary phase by using ion-exchange resins as packing. A second method is to load silver ions onto a classical silica packing. The on-line coupling of silver ion-exchange HPLC with mass spectrometry has been described recently [5,6]. Although both silver HPLC systems separate TAGs in order of unsaturation, there is a marked difference between the methods. Silica stationary-phase systems coated with silver nitrate (Ag-HPLC) offer also a separation of regioisomeric TAGs, e.g. the separation of S_2O in the two regioisomers SOS and SSO (S, saturated FA; O, mono-unsaturated FA). Silver ion-exchange systems are only capable of separating regioisomers under specific conditions [7–9]. A review of the silver ion-exchange methodology is presented in Ref. [10]. The regioisomeric information is related to the physical properties of the oil or fat (for most physical and nutritional purposes only the differentiation between the sn2 and sn1=sn3 position is required, where sn≠ indicates the stereospecific numbering of the fatty acids on the glycerol backbone).

Ag-HPLC connected to conventional HPLC detectors like the flame ionization detector (FID) or the evaporative light scattering detector (ELSD), does not provide information on CN of the TAG or the carbon chain length of the different fatty acids. As non-natural types of TAGs can be formed in, e.g. modified or enzymatically transesterified oils, another distribution of TAGs can be expected than would typically be found in natural oils. We have directed our efforts on a new promising combination of argentation chromatography with electrospray mass spectrometry. The on-line combination provides full TAG characterization with respect to CN, position and type of FAs of TAG mixtures, e.g. samples of natural or modified edible oils. This technique is less likely to be influenced by thermal degradation of unsaturated TAGs as is the case with HT-GC [2] and HT-GC–MS–MS [11].

To analyze compounds by mass spectrometry the compounds have to be ionized, the preferred techniques are atmospheric pressure chemical ionization and electrospray ionization. Both are well described in the literature for the analysis of TAGs [12–14]. A comprehensive overview is given in Ref. [15].

2. Experimental

2.1. Materials

All pure TAGs were obtained from Larodan Fine Chemicals (Malmo, Sweden) or synthesized in-house from pure fatty acids. Solvents were HPLC grade (Lichrosolv, Merck, Darmstadt, Germany). Ammonium acetate, sodium acetate, acetic acid and silver nitrate were analytical grade (Merck).

A sample of a chemically fully interesterified palm oil (inPO) with a known triglyceride composition was used as a test sample. Pure TAGs were dissolved in toluene and standard solutions were prepared in a mixture of an apolar and polar solvent at concentrations of 1–500 pmol/μl. Direct injection of 10 μl of the standard solutions of TAGs was used to perform the tests on various parameters.

2.2. Silver-phase chromatographic conditions

A ternary-gradient HPLC system consisting of Gilson 305/306/306 pumps, an 811B dynamic mixer and an 805S manometer were used (Gilson, Villier-le-Bel, France). A 100×4.6 mm AgSi HPLC column was used throughout all experiments. These columns were prepared in-house with a balanced slurry packing technique. The packing material, Nucleosil, particle size 3 μm, pore size 100 Å, is loaded with a 10% (w/w) $AgNO_3$ solution in acetonitrile. Columns are preconditioned with the gradient and tested before actual use for sufficient resolution in the separation of a critical pair of regioisomeric TAGs (e.g. SOS/SSO).

The solvents used in the gradient were: toluene–hexane (50:50, v/v) (A); toluene–ethylacetate (75:25, v/v) (B), and toluene with 80 μl/l formic acid added (C). Gradients used: 0.0 min, 97% A and 3% B, 2.5 min; 96% A and 4% B, 8.0 min; 85% A and 15% B, 9.0 min; 75% A and 25% B, 13.0 min; 10% A and 90% B, 13.1 min; 100% C, 17.1 min;

97% A and 3% B, at 20.0 min end of program. The flow was 1.5 ml/min and injections (20 μl) were made with a Rheodyne loop-type HPLC injector (Rheodyne, Cotati, CA, USA).

2.3. solid-phase extraction supercritical fluid chromatography

The CN of the saturated fraction of the inPO sample was determined by SPE-SFC as an independent check on the MS results. A solution of the sample was extracted on a silver ion-loaded SPE cartridge (Varian/Bond Elut SCX, 1210–2040) to obtain the saturated TAG fraction. This sample was analyzed for the CN distribution on a SFC Mega 3000 series with FID detection (Carlo Erba, Milan, Italy), SFC column DB-17 (J&W, Folsom CA, USA) and supercritical CO_2 as carrier. The response of the system was checked with pure standards of saturated TAGs over the relevant CN range.

2.4. Optimization of the MS system

The optimization of the MS-system was performed on a Trio-2000 mass spectrometer (Micromass, Manchester, UK) by constant infusion with a Phoenix 20CU syringe pump (Fisons, Manchester, UK) into the electrospray ion source with a normal probe. An electrolyte (ammonium acetate or sodium acetate) was added to the solvent. All solvent was pumped to the ion source, without splitting. Conditions of the electrospray ion source and the flows applied are described with the optimization results.

2.5. On-line Ag-HPLC–MS measurements

The positive electrospray experiments were performed on two instruments: a Micromass Trio-2000 mass spectrometer and a Micromass Platform II mass spectrometer (Micromass, Manchester, UK).

The final conditions after optimization were: capillary voltage, 4.0 kV; cone voltage, 95 V; source temperature, 100°C; scan range, 100–950 Da; continuum scanning mode scan rate, 2 s/scan; nebulizer gas, nitrogen (10 l/h); bath gas, nitrogen (300 l/h); flow to the probe, 30 μl/min; electrolyte, 0.1 mmol/l sodium acetate in methanol.

Fig. 1 shows the configuration of the HPLC–MS system. After the splitting, 0.0025″ I.D. PEEK tubing was used to minimize peak broadening.

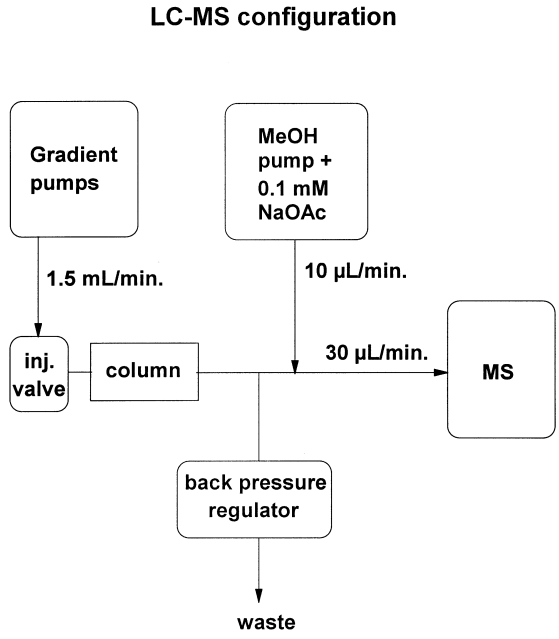

Fig. 1. Configuration of the HPLC system.

The electrospray source was cleaned and subsequently tuned with 20-μl injections (bypassing the column) of 1 pmol/μl tri-C18:0 TAG at a split flow-rate of 30 μl/min, prior to analysis.

A 10 mg/ml solution of the sample was prepared in toluene. Dilutions were prepared at 0.1, 0.2, 0.5, and 1.0 mg/ml in toluene–hexane (50:50) to be analyzed on the Trio-2000. On the Platform II only the 0.2 mg/ml solution was analyzed.

3. Results

3.1. Optimization of experimental ES-MS conditions

The optimization procedure was done by 10-μl loop-type injections of a solution of 13 pmol/μl tri-C18:0 TAG in a mixture of dichloromethane–methanol (70:30) with 30 mmol/l ammonium acetate in the methanol. The cone voltage was 55 V. In this way, pseudo-molecular $[M+NH_4]^+$ species (m/z 908) are formed, which are detected with a

signal-to-noise ratio equal to 60 at a flow-rate of 4 μl/min.

After replacing the dichloromethane by a mixture of toluene–hexane (50:50), which resembles better the actual Ag-HPLC conditions, the signal-to-noise ratio dropped to 30. By increasing the flow to the source to 30 μl/min, the signal-to-noise ratio dropped even further and the $[M+NH_4]^+$ ion was hardly visible. At a cone voltage of 100 V $[M+Na]^+$ and $[M-acyloxy]^+$ ions became visible in the spectrum, even after adding ammonium acetate. This indicates that $[M+NH_4]^+$ ions are completely fragmented to $[M\text{-}acyloxy]^+$ ions. Therefore, it is more appropriate to add sodium acetate and use the $[M+Na]^+$ ions instead of the $[M+NH_4]^+$ ions to quantify the TAGs. In the case of Na cationization the sensitivity is fairly independent for the flow between 10 and 100 μl/min. Above 100 μl/min the sensitivity strongly decreased. At a sodium acetate concentration of 0.1 mmol/l in methanol maximum sensitivity was obtained. At lower concentrations the sensitivity dropped, while at higher concentrations sodium acetate cluster ions became visible in the spectrum. The stability of the $[M+Na]^+$ ions prevents the further analysis by MS–MS.

3.2. Dynamic and linear range for saturated triglycerides at 100 μl/min

The dynamic range for saturated TAGs with different alkyl chain lengths (tri-C8:0, tri-C12:0 and tri-C18:0) was determined using Na cationization, a fixed-cone voltage of 95 V, and a fixed flow of 100 μl/min to the source, using the configuration shown in Fig. 1 with an adapted split ratio. All measurements were performed in triplicate. The concentrations varied from 20 to 1000 pmol/μl for tri-C8:0, 10 to 500 pmol/μl for tri-C12:0, and 20 to 200 pmol/μl for tri-C18:0 TAG.

Peak areas were measured by integrating the reconstructed ion chromatograms at *m/z* 493, 661 and 913, respectively. Tri-C18:0 TAG had the lowest dynamic range, with an upper concentration limit of about 100 pmol/μl. The TAGs with the longest alkyl chains are therefore the limiting factor in the dynamic range. At concentrations lower than 100 pmol/μl the response is almost linear (Fig. 2).

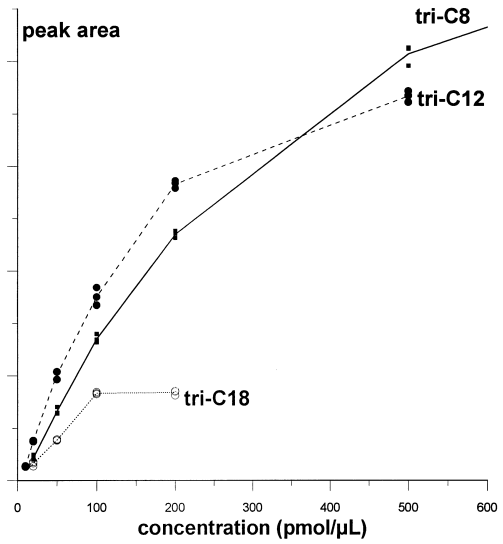

Fig. 2. Dynamic range and linearity of different saturated TAGs, via on-line measurements in triplicate. Flow-rate, 100 μl/min; cone voltage, 95 V.

3.3. Dynamic range for tri-C18:0 TAG at flow-rates of 10, 30 and 100 μl/min

Experimental conditions were the same as above.

The saturation level increases when the flow to the source is decreased (Fig. 3). If the experiment with

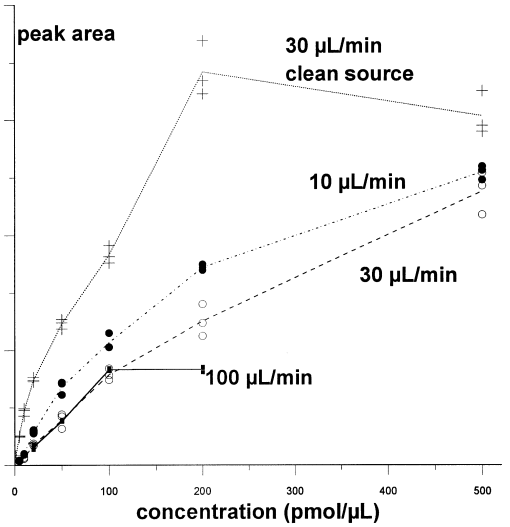

Fig. 3. Effect of the flow to the source on dynamic range and sensitivity for tri-C18:0 TAG. On-line measurements in triplicate. Cone voltage, 95 V.

30 μl/min was repeated after the source had been cleaned after 20 injections a significant improvement in sensitivity was obtained. The upper concentration limit is about 100–200 pmol/μl for tri-C18:0 TAG using a clean source. The lower-limit of the flow to the source is about 30 μl/min, at lower flows peak broadening is unacceptable. Adjustment and reproducibility of the splitter is also very critical below this lower limit.

The experiment with a flow of 30 μl/min was repeated on the Platform II mass spectrometer. It turned out that the signal-to-noise ratio on the Platform II is about 10 times better than on the Trio-2000. The upper limit of the dynamic range and the linearity of the two instruments are about the same.

3.4. Effects of overlapping peaks of saturated triglycerides.

Two, partly co-eluting, TAGs (tri-C18:0 and C18:0–C16:0–C18:0) were injected independently and as a mixture. Injections were made at three concentration levels (20, 50 and 100 pmol/μl) of each TAG. The cone voltage was fixed at 95 V and the flow to the source was 30 μl/min. At low concentrations (20 pmol/μl) the response of different co-eluting TAGs is comparable to the response obtained when they are injected separately. The presence of different TAGs does not influence the ionization of each other.

At higher concentrations this is no longer the case. Apparently the upper limit of the dynamic range, posed by the capacity of the electrospray ion source, is the sum of the concentrations of each individual TAG. Thus when peaks are co-eluting the dynamic range for each TAG is smaller.

3.5. Effect of the cone voltage on the response of saturated triglycerides

A mixture of tri-C8:0, tri-C10:0, tri-C12:0, tri-C14:0, tri-C16:0 and tri-C18:0 TAGs was injected onto the column. The concentration of each TAG was 20 pmol/μl. At this level the sum of the concentrations of the three co-eluting peaks of tri-C18:0, tri-C16:0 and tri-C14:0 TAGs is well below the upper limit of the dynamic range of the source. The cone voltage was varied between 55 and 105 V. The flow to the source was fixed at 30 μl/min. Peak areas of the $[M+Na]^+$ ions in the reconstructed ion chromatograms were plotted against the cone voltage (Fig. 4). At high cone voltages the responses of TAGs with long alkyl chains are the highest, whereas at low cone voltage the opposite is true. At about 95 V, responses for the long-chain TAGs are almost equal. This value for the cone voltage was used in further measurements.

3.6. Qualitative results obtained by on-line Ag-HPLC–ESP-MS

3.6.1. Chromatogram and spectra

A typical chromatogram obtained with the Platform II instrument is shown in Fig. 5. When analyzing an HPLC peak, the individual TAG species with different CN can be observed during the elution of the peak. TAGs with long alkyl chains elute at the front of the peak, while TAGs with short alkyl chains elute at the end of the peak. This is illustrated by the picture of the total ion current (TIC) and the reconstructed ion chromatograms of the $[M+Na]^+$ ions for the different CNs in a series of saturated TAGs (Fig. 6). One should keep in mind that the absolute intensity of these ion chromatograms is

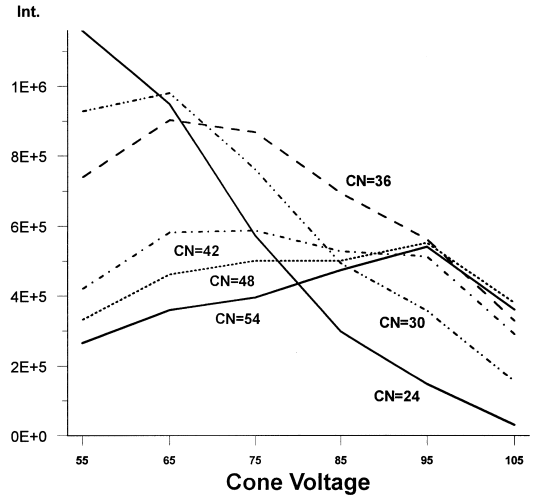

Fig. 4. Effect of the cone voltage (CV) on the peak areas of the $[M+Na]^+$ ions of saturated TAGs, triC8:0, triC10:0, triC12:0, triC14:0, triC16:0 and triC18:0; flow-rate, 30 μl/min. The best cone voltage for the higher CN species is 95 V.

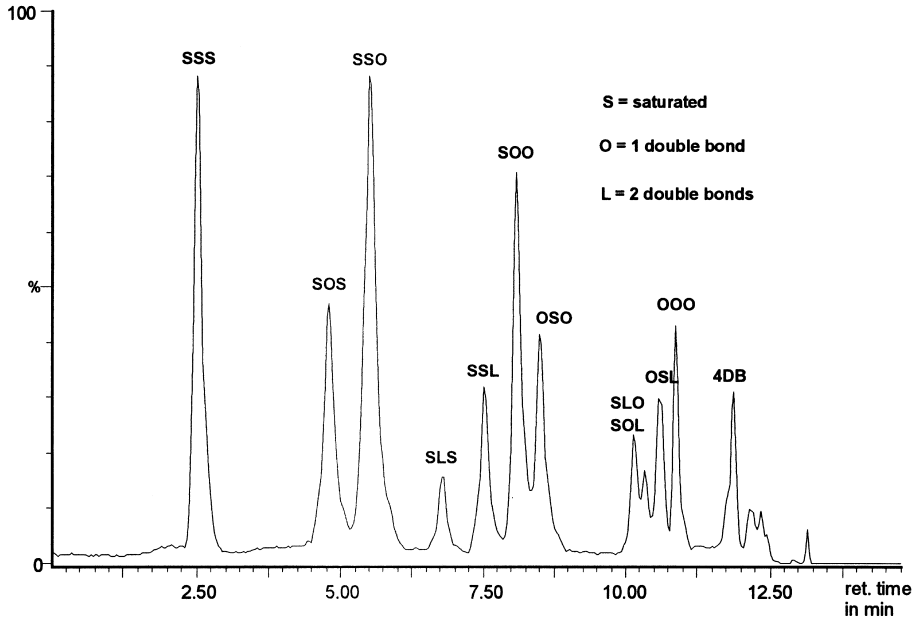

Fig. 5. Total ion current chromatogram of interesterified palm oil measured on the Platform II. Cone voltage, 95 V; flow-rate, 30 μl/min; 20-μl injection. For HPLC conditions see text.

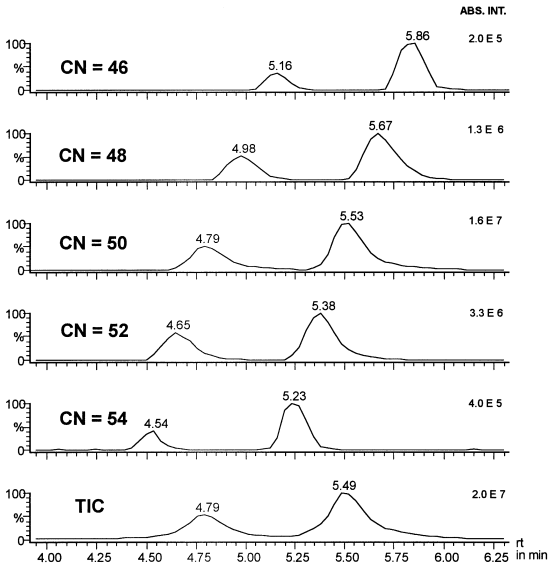

Fig. 6. Mass chromatograms of the $[M+Na]^+$ ions in the SOS and SSO peak. At the left is the SOS, at the right the SSO component. The total ion current is given on the lowest picture, the various CN by the reconstructed ion chromatograms in the other pictures. Absolute intensities are stated on the right.

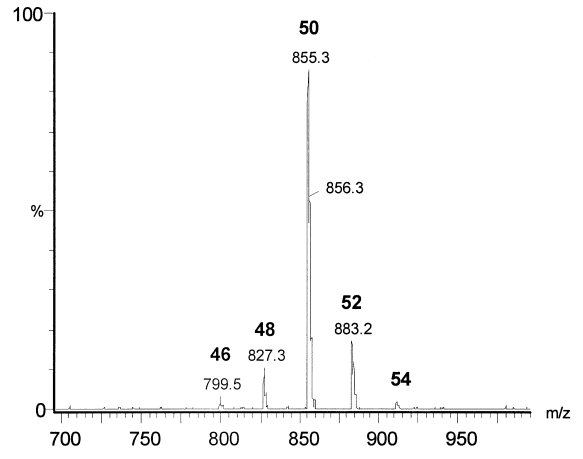

Fig. 7. Combined (between 5.1 and 6.0 min) electrospray mass spectrum for SSO peak. CNs are indicated in bold above the m/z for each individual TAG. Resolution is decreased in favour of sensitivity.

different. The individual spectra within one HPLC peak can be combined to give a single spectrum, e.g. Fig. 7 shows the combined spectrum of the SSO peak.

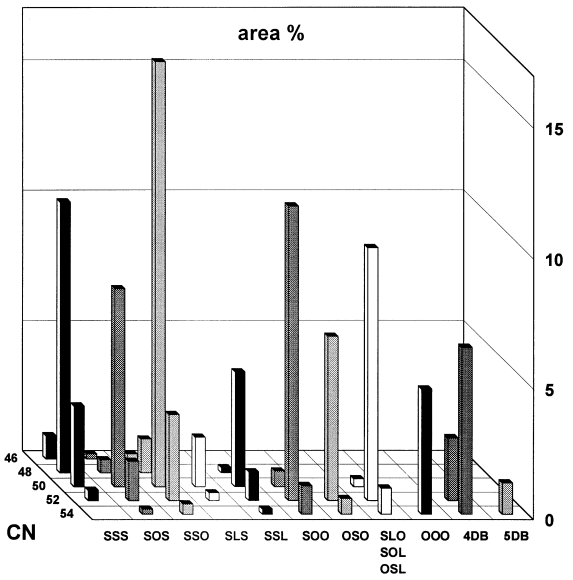

Fig. 8. Three-dimensional presentation of the composition of interesterified palm oil: on the *x*-axis from left to right the Ag-HPLC separation into TAG groups; on the *y*-axis the relative response in area % of the area of all peaks in the TIC picture; on the *z*-axis the CN distribution.

3.6.2. Three-dimensional presentation

If the responses of the specific ions formed by the triglycerides with different carbon numbers are plotted against retention time, a three-dimensional plot is obtained. The retention time axis shows the Ag-HPLC separation into TAG groups with increasing level of unsaturation (*x*-axis). The *z*-axis shows the CN distribution within each TAG class. The *y*-axis gives the relative area percentages and thus a good impression of the composition of the edible oil (Fig. 8). From this picture it becomes clear that the more unsaturated TAGs have a higher CN, and thus longer fatty acid chains.

3.7. Quantitative results obtained by on-line Ag-HPLC–ESP-MS

3.7.1. Quantitative comparison of peak areas of the compound groups

Peak areas were measured using the reconstructed ion chromatograms of the particular ions, including the ^{13}C isotopes. Resolution was decreased to get better sensitivity. The peak areas of all ions within a co-eluting peak (SSS, etc.) were combined to give the relative amount of that compound group. These results were in good agreement with the values obtained by Ag-HPLC–FID analyses and the calculated theoretical values based on complete fatty acid randomization (Table 1). Agreement for other oils with respect to their relative composition measured by Ag-HPLC–FID and Ag-HPLC–ESP-MS was always better than 5%. This is sufficient for our purpose of characterizing the edible oils.

The detection limit for each individual TAG was found to be about 400 ng/ml for the Trio-2000 and

Table 1
Area percentages obtained by LC-FID and LC-MS

Sample inPO		Ag-HPLC–FID (15 mg/ml) (%)	Ag-HPLC–ESP-MS (0.2 mg/ml)	
Compound group	Theoretical (%)		Trio-2000 (%)	Platform II (%)
SSS	14.7	14.9	14.8	13.8
SOS	9.9	10.9	9.9	9.6
SSO	21.0	20.9	21.5	19.6
SLS	2.5	2.5	2.1	2.6
SSL	5.7	5.5	5.9	5.9
SOO	14.2	13.3	13.0	13.8
OSO	7.5	8.1	7.0	8.7
SLO/SOL	7.5	7.1	11.0	8.7
OSL	4.2	3.8		5.5
OOO	5.1	5.2	4.8	5.8
4 DB	6.7	7.8	8.8	6.0
5 DB			1.2	n.d

Notation used for TAGs: S, saturated FA; O, mono-unsaturated FA; L, double unsaturated FA; DB, double bonds.

40 ng/ml for the Platform II. The latter instrument was expected to be much more sensitive due to the modern ion optics, where a hexapole is used instead of lenses for transfer of ions into the quadrupole. At concentration levels above 0.2 mg/ml the upper limit of the dynamic range is exceeded. In that case, the relative concentration of the major component (in this sample SSO) is underestimated and therefore the relative concentrations of the other compounds are overestimated.

Sample concentrations have to be balanced between concentration at the low end, where the linearity of the electrospray source is generally better, and high concentrations allowing more precise peak area determinations. The maximum concentration is about 0.25 mg/ml with a injection volume of 20 μl. In general, the sum of the concentrations of the TAGs in the highest HPLC peak has to be well below the upper limit of the dynamic range, which is around 100 pmol/μl. The minimum relative percentage of a component in a mixture is around 0.2% for analysis on the Trio-2000 and 0.02% for analysis on the Platform II.

In silver-phase HPLC the silver is stripped from the stationary phase. This is confirmed by the formation of $[M+Ag]^+$ ions for the highly unsaturated TAGs (DBs>3). These ions were identified by the characteristic isotope pattern of silver and the mass difference between the $[M+Na]^+$ ions and the $[M+Ag]^+$ ions. The formation of the $[M+Ag]^+$ ions depended on the condition of the ion source and the values of the voltages in that source. The peak areas of these ions have to be included in the calculations otherwise the contribution of these compound groups to the composition of the sample is too low. Because the responses of the $[M+Ag]^+$ ions are unknown, the formation of these ions should be prevented. However, the mechanism of the formation of these ions is unclear. The retention time is stable over months, but the condition of the column is checked always before starting a series of analyses.

3.7.2. Comparison of the quantitative results with off-line SPE-SFC

The quantitative results obtained for the saturated TAGs (SSS) were compared with off-line SPE-SFC. The HPLC–ESP-MS results are based on the 0.2 mg/ml injection of the oil sample. The peak area of the $[M+Na]^+$ ions is used for the calculation of the area percentage of the individual TAGs in the SSS group (Table 2). These results are in agreement with the theoretical composition and the results obtained by SPE-SFC–FID.

4. Conclusions

Electrospray is a suitable ionization method to perform on-line Ag-HPLC–MS analysis of TAGs, providing the CN distribution of the TAGs eluting for each Ag-LC peak. The quantitative results of the representative test sample are in agreement with results obtained with the Ag-HPLC–FID method and also with the theoretical composition of the sample. The total concentration of the TAGs of the highest HPLC peak has to be well below the upper limit of the dynamic range in order to achieve reliable results.

Table 2
Composition of the saturated fraction (SSS) obtained by off-line SPE-SFC-FID and LC–MS

SSS carbon number	Theoretical (%)	Peak area (%)		
		Ag-SPE-SFC–FID	HPLC–MS	
			Trio-2000	Platform II
44	0.3	0.3		0.3
46	0.9	0.8	0.9	1.1
48	10.0	8.2	10.4	11.2
50	2.8	2.3	3.1	3.1
52	0.5	0.5	0.4	0.4
54	0.1	0.1	[a]	0.02

[a]Below detection limit.

The detection limit of the Platform II mass spectrometer is about 10 times lower than on the Trio-2000, and the dynamic range is a factor of 10 higher.

Acknowledgements

We acknowledge our gratitude to the University of Utrecht, The Netherlands, for access to the Platform II mass spectrometer; and Bernard Vandeginste for his helpful discussions.

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