J Am Oil Chem Soc (2009) 86:19–25
DOI 10.1007/s11746-008-1316-9

ORIGINAL PAPER

Triacylglycerol Analysis of Fats and Oils by Evaporative Light Scattering Detection

Roeland Rombaut · Nathalie De Clercq · Imogen Foubert · Koen Dewettinck

Received: 14 April 2008 / Revised: 30 September 2008 / Accepted: 27 October 2008 / Published online: 20 November 2008

Abstract A high performance liquid chromatography method with evaporative light scattering detection was developed for the analysis of oils and fats, which enabled excellent separation of major and minor triacylglycerol (TAG) species in 33 min, including regeneration of the column. The influence of the mobile phase and temperature on separation and analysis time were evaluated with a cocoa butter standard. The influence of the drift tube temperature and flow of the nebulising gas on the evaporative light scattering detector output signal was investigated by means of a response surface experimental design. Especially the flow of the nebulising gas had a profound effect on the detector signal. An optimal separation was obtained when using a 150 × 3.0 mm C18 column with 3 μm particle diameter at 20 °C and an acetonitrile/dichloromethane gradient at 0.72 mL/min. The maximum response was attained when the ELSD detector was set at the minimum temperature (45 °C) and a gas flow of 1.2 L/min. Finally, the linearity of the detector was investigated. It was found that at very low concentrations, the signal tends to flatten towards zero, giving an underestimation for minor TAG species, especially for oils or fats with a mixed fatty acid composition.

R. Rombaut · N. De Clercq (✉) · I. Foubert · K. Dewettinck
Laboratory of Food Technology and Engineering,
Department of Food Safety and Food Quality,
Faculty of Bioscience Engineering, Ghent University,
Coupure Links 653, 9000 Ghent, Belgium
e-mail: N.declercq@ugent.be
URL: http://www.fte.UGent.be

Keywords Triacylglycerol · Oils and fats · Analysis · HPLC–ELSD

Abbreviations

CB	Cocoa butter
ELSD	Evaporative light scattering detector
HPLC	High performance liquid chromatography
L	Linoleic acid
Ln	Linolenic acid
O	Oleic acid
P	Palmitic acid
Po	Palmitoleic acid
S	Stearic acid
TAG	Triacylglycerol

Introduction

Analysis of triacylglycerol (TAG) species in oils and fats has gained increasing attention in the last decades. In the oil industry, it can be a tool for monitoring and optimising processes such as pressing, refining, fractionation, hydrogenation and interesterification of oils. In food research, it is used for studying crystallisation phenomena, for detecting adulteration of specialty fats and oils like cocoa butter (CB) [1], milk fat [2] and olive oil [3], and for recognition of virgin olive oils originating from a single cultivar [4], or from a protected designation of origin [5].

Reversed phase high performance liquid chromatography (RP-HPLC) has become the most popular separation and analysis method for TAG species in oils and fats. Numerous methods are published, mostly using silica based octadecylsilane packing as stationary phase and propionitrile, or a mixture of acetone, acetonitrile and

dichloromethane, as mobile phase, with or without gradient elution. These chromatographic techniques are extensively reviewed by Andrikopoulos [6], Buchgraber et al. [7], and Perona and Ruiz-Gutierrez [8].

For the chromatographic analysis of oils and fats, the use of an evaporative light scattering detector (ELSD) is generally preferred nowadays. In this detector, the solvent originating from the column is nebulised in a heated tube by means of a pressurised gas (compressed air, helium or nitrogen) and evaporates. The analyte, which has to be less volatile than the eluting solvent, passes as an aerosol and reflects and refracts a beam of conventional or laser light at the end of the tube. The scattered light is detected by a photodiode and is directly related with the quantity of the analyte and the droplet size. In the last decade, detectors equipped with a laser as a light source has become commercially available. These laser light scattering detectors outperform other models in sensitivity, stability and reproducibility over longer periods of analysis [9, 10]. The ELSD is a mass-sensitive detector that responds to any analyte less volatile than the mobile phase. It has a low background signal, a non-specific response (unlike a flame ionisation detector), is compatible with gradient elution (unlike a RI-detector), is compatible with a broad range of solvents, and has a signal independent of the degree of saturation and chain length (unlike an UV-detector). However, detector settings like flow of the nebulising gas, temperature of the nebuliser and heating tube and the flow rate and composition of the mobile phase highly affect the droplet size of the aerosol and thus indirectly the signal. For each type of analysis, detector settings should be optimised to ensure the highest sensitivity, and should be maintained rigorously. Otherwise, when working quantitatively, a recalibration and validation of the method is indispensable.

From a theoretical point of view, the response of the ELSD is sigmoidal upon increasing analyte concentrations [10]. For most HPLC–ELSD applications, the construction of standard calibration curves for each analyte allows accurate concentration estimation of unknown samples. Depending on the injection range, a linear or an exponential curve can be fitted perfectly on the data. This latter methodology however requires the existence of reference material and requires that the concentration of the unknown analyte is between the minimal and maximal value of the standard curve. For TAG analysis, both prerequisites are only partially fulfilled. High purity standards of TAGs with a mixed fatty acid composition are limited. However, even if reference material were commercially available, the diversity of TAG species in each oil would make it virtually impossible to construct a calibration curve for each TAG species. Moreover, in most oils and fats, a few TAG species (e.g., OOO in olive oil) are dominant, whereas the remaining fraction contains numerous other TAGs, albeit in very small quantities. As such, as there is no other option, in TAG analysis, relative peak areas are readily converted into relative TAG concentration, assuming linearity and uniformity of the detector signal, regardless of the TAG species and absolute concentration.

This study was performed to develop a HPLC–ELSD method, enabling a quick separation of the TAG species in oils and fats. For this purpose a simple mobile phase gradient was selected and the optimum column temperature determined. Secondly, the influence of the ELSD drift tube temperature and the gas flow of the nebuliser were investigated by means of experimental design. In the last part, linearity and uniformity of the ELSD output was investigated by means of a certified CB standard.

Materials and Methods

Materials

Oils and fats were obtained from a local grocery store. The oil or fat was dissolved in a concentration of 0.05–5 mg/mL in dichloromethane/acetonitrile 30/70 (v/v) prior to injection. Acetone, acetonitrile and dichloromethane were used as mobile phases and were HPLC grade (Acros Organics, Geel, Belgium). TAG standards of tricaprin, trilaurin, trimyristin, tripalmitin, tristearin, tritridecanoin and triheptadecanoin were obtained from Nu-Chek Prep, Inc. (Minnesota, USA). A certified CB standard (IRMM-801) was obtained from the European institute for reference materials and measurements (IRMM, Geel, Belgium).

Chromatography

Separation of TAG species was performed on a Thermo Finnigan Surveyor HPLC system with four solvent lines, degasser, autosampler and Atlas 2003 software (Thermo Electron Corporation, Brussels, Belgium) which was coupled to an Alltech ELSD 2000 evaporative laser light scattering detector (Grace Alltech, Lokeren, Belgium). N_2 was used as the nebulising gas at a flow of 1.2 mL/min, and a nebulising temperature of 45 °C. The gain was set at 1 and the impactor, a split option for the detection of semi-volatiles in combination with aqueous mobile phases, was disabled. The suction head of the dichloromethane solvent line was replaced by a Hastalloy C suction head, to prevent ghost peaks during gradient elution.

The column was a 150 × 3.0 mm Alltima HP C18 HL with 3 μm particle diameter (Grace Alltech, Lokeren, Belgium). A precolumn with a silica packing was used. The elution program is given in Table 1. The flow was maintained at 0.72 mL/min, which resulted in a back

Table 1 Mobile phase gradient. The solvent flow is maintained at 0.72 mL/min

Time (min)	Dichloromethane (% v/v)	Acetonitrile (% v/v)
0	30	70
25	51	49
26	70	30
27	70	30
28	30	70
33	30	70

pressure of 120–150 bar. The injection volume was 25 µL. The column and samples were thermostatted at 20 and 40 °C respectively. Standards of tritridecanoin and triheptadecanoin were added to correct for small retention time shifts, allowing correct peak identification.

Statistics

Experimental design was performed with Design Expert 5 (Stat-Ease Corporation, Minneapolis, USA), statistics with SPSS 12.0 and Sigmaplot 10.0 (SPSS, Inc., Chicago, USA).

Results

Mobile Phase Selection

Three different mobile phases were evaluated for the separation of TAG species: A two-stepped linear gradient of dichloromethane/acetonitrile, as described by Letter [11], a two-stepped linear gradient of acetone/acetonitrile, as described by Jakab et al. [12] and a linear gradient of dichloromethane/acetonitrile/acetone as described by Pons et al. [13]. Propionitrile, sometimes used as a mobile phase for isocratic separation, was not evaluated due to its high toxicity. The column and chromatographic system were as described in the materials and methods section. The column temperature was set at 20 °C. The CB standard was used for method evaluation. Chromatograms with the three solvent systems are given in Fig. 1. The acetone/acetonitrile gradient resulted in broad peaks and poor resolution of the PLS–POP and SLS–POS pairs in comparison with the two other mobile phases. The dichloromethane/acetonitrile system was preferred over the dichloromethane/acetonitrile/acetone system, as the gradient is less complex and the separation slightly better with regard to the same pairs as mentioned above. A column with a 3 mm internal diameter was preferred because the solvent use was decreased by 57.5% compared with a standard 4.6 mm internal diameter column. This results in a better evaporation process and in a more stable baseline.

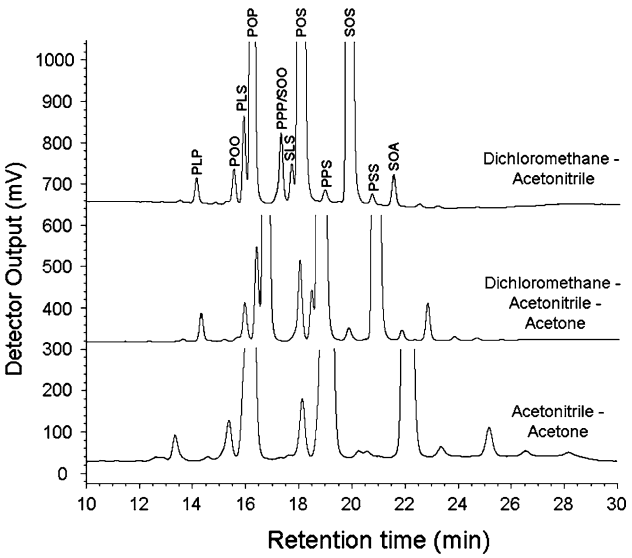

Fig. 1 Influence of mobile phase composition on the separation of cocoa butter TAG. The column temperature was set at 25 °C, the flow of mobile phase was maintained at 0.72 mL/min

Column Temperature

The influence of column temperature on retention time and peak separation was investigated by analysing CB at column temperatures of 15, 20, 25 and 30 °C. As can be seen from Fig. 2, with increasing temperature the retention time highly decreased, and the peaks of POO and PPP/SOO tended to join with the peaks of PLS and SLS, respectively. Inversely, at decreasing temperatures, PLS and SLS tended to join with the peaks of POP and POS, respectively. At 20 °C, PPS and PSS were completely resolved. Therefore, 20 °C was chosen as the optimum column temperature since the best separation is combined with a fairly acceptable retention time (<30 min).

Optimisation of Detector Settings

The influence of the flow of the nebulising gas and temperature settings of the ELS-detector were determined by means of an experimental design (response surface central composite orthogonal design). Design maxima (T_{max} = 105 °C and $Flow_{max}$ = 3.5 L/min) were the setting limits of the detector. Minima (T_{min} = 45 °C and $Flow_{min}$ = 1.2 L/min) were those values above which no change in baseline noise could be observed. For each combination (13 in total, 5 at the center point), a full chromatogram of a standard solution of tricaprin, trilaurin, trimyristin, tripalmitin and tristearin with a concentration of 0.2 mg/mL each was recorded. The total peak area was chosen as the response value. A quadratic model was fit to the data. Non-significant ($P > 0.1$) model coefficients were eliminated by a stepwise addition algorithm, resulting in a reduced

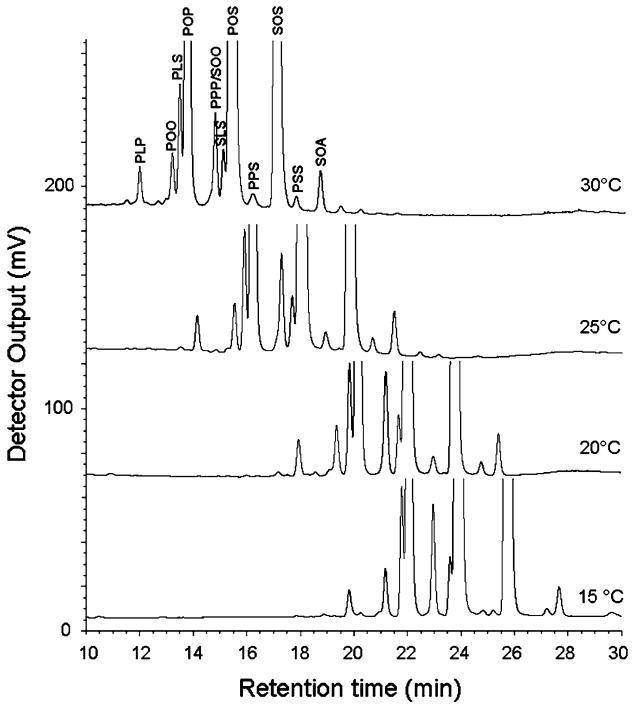

Fig. 2 Influence of column temperature on retention and separation of cocoa butter TAG using the dichloromethane/acetonitrile system. The flow of mobile phase was maintained at 0.72 mL/min

quadratic model. Analysis of variance (ANOVA) revealed that this reduced quadratic model was highly significant (Table 2). Determination coefficients were all close to 1, including the predicted determination coefficient, which gives information about how well the model fits each point in the design, and whether the model is prone to small variations of one of the data points. The lack of fit test was insignificant and no outliers were observed.

Table 2 Statistical parameters of the reduced quadratic model fitted to the experimental total peak area as a function of the nebuliser gas flow and drift tube temperature of the ELSD

ANOVA term	Total peak area
Sum of squares	
Model	7.72E + 14
Residual	7.96E + 11
Lack of fit	4.34E + 11
Pure error	3.62E + 11
Corrected total	7.73E + 14
Predicted residual	3.15E + 12
Significance	
Model	<0.0001
Lack of fit	0.433
Determination coefficients	
R^2	0.999
Predicted R^2	0.995

Table 3 Significance of the reduced quadratic model coefficients and final equation term for the prediction of the experimental total peak area as a function of gas flow and temperature of the ELSD

Model coefficient	*P*-value	Final equation term
Intercept		5.811E + 7
Temperature	<0.0001	−7.571E + 4
Temperature^2	1	2.03
Flow	<0.0001	−2.818E + 7
Flow^2	<0.0001	3.787E + 6
Temperature × flow	0.0117	1.486E + 4

Factors temperature, flow, and flow^2 were found highly significant, flow × temperature significant, and temperature^2 insignificant on the ELSD output (Table 3). In Fig. 3, the total peak area is given as a function of the drift tube temperature and flow of the nebulising gas. The response of the ELSD was highly dependent on the flow of the nebuliser gas, decreasing sharply at increasing gas flow. The effect of temperature was less pronounced. The ELSD output decreased slightly with increasing temperature, however less pronounced at higher gas flow rates. This latter phenomenon is expressed in the model by the significant interaction term flow × temperature. A maximum response was obtained at a minimum temperature (45 °C) and a gas flow of 1.2 L/min. At these conditions, the model predicts an ELSD output signal which is about 2.8 times higher than at intermediate settings ($T = 75$ °C

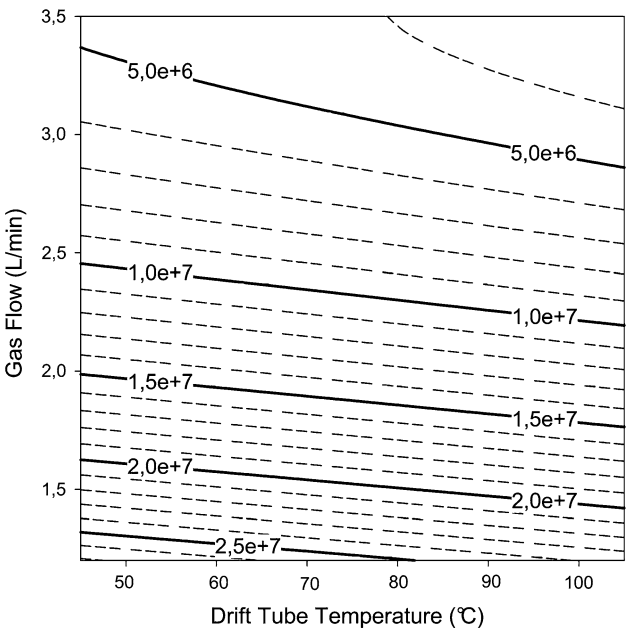

Fig. 3 Total peak area of a standard mixture of triacylglycerols (tricaprin, trilaurin, trimyristin, tripalmitin and tristearin at 0.2 mg/mL each) as a function of the temperature of the nebuliser/drift tube, and flow of the nebulising gas (nitrogen)

Table 4 Concentration range (25 μL injected on column), slope (±stdev), intercept (±stdev) and determination coefficient of linear calibration curves of individual cocoa butter standard triacylglycerols

Triacylglycerol	Concentration range (mg/mL)	Slope (10^6)	Intercept (10^6)	R^2
POP	0.018–0.888	29.2 ± 0.3	−1.08 ± 0.11	0.997
POS + SLS	0.046–2.283	26.7 ± 0.3	−0.88 ± 0.34	0.995
POO	0.002–0.111	23.4 ± 0.8	−0.21 ± 0.04	0.965
SOS	0.031–1.549	28.6 ± 0.2	−1.11 ± 0.16	0.998
PPP + SOO	0.003–0.171	26.2 ± 0.7	−0.34 ± 0.06	0.975

L linoleic acid, *O* oleic acid, *P* palmitic acid, *S* stearic acid

and flow = 2.35 L/min) and about 8 times higher than at maximum settings ($T = 105$ °C and 3.5 L/min). These values clearly point out the importance of thorough ELS detector calibration and of maintaining the experimental conditions (=detector settings) upon comparison of results.

Linearity of the ELSD

To investigate the linearity of the ELSD, a CB standard was used of which the normalised concentrations of POP (18.14%), POS (44.68%), POO (2.26%), SOS (31.63%) and SOO (3.29%) are certified. As the proposed HPLC method does not fully separate POS/SLS and PPP/SOO pairs, the reference values of POS and SOO were corrected with SLS (1.9%) and PPP (0.2%), respectively. This standard was injected in concentrations from 0.05 to 0.5 mg fat/mL with an 0.05 mg/mL interval, and from 0.5 to 5 mg CB/mL with an 0.5 mg/mL interval. In Table 4, the injection range for each individual CB TAG species is given, as well as the slope, intercept and determination coefficient of the linear calibration curve fitted to the experimental data. In Fig. 4, the absolute concentration of each CB TAG is plotted as a function of the peak area. From this figure, it can be clearly noticed that in the range 0.25–2 mg TAG/mL the ELSD response is linear and fairly uniform, irrespective of the TAG species. As such, if all TAG species would be situated in this concentration region, relative peak areas can be readily converted into relative concentrations. However, at smaller concentrations (Fig. 4, inset), it is clear that the ELSD response is far from linear, and that the curve tends to flatten when approaching zero. This can also be derived from the data in Table 4. For all linear calibration curves, the intercept is substantially negative, pointing out the non-linear part at low concentrations. For the main TAG species, POP, POS/SLS and SOS, this non-linear part has only limited influence on the parameters of the linear curve: the slope is fairly equal, and the determination coefficient is close to one. For the minor

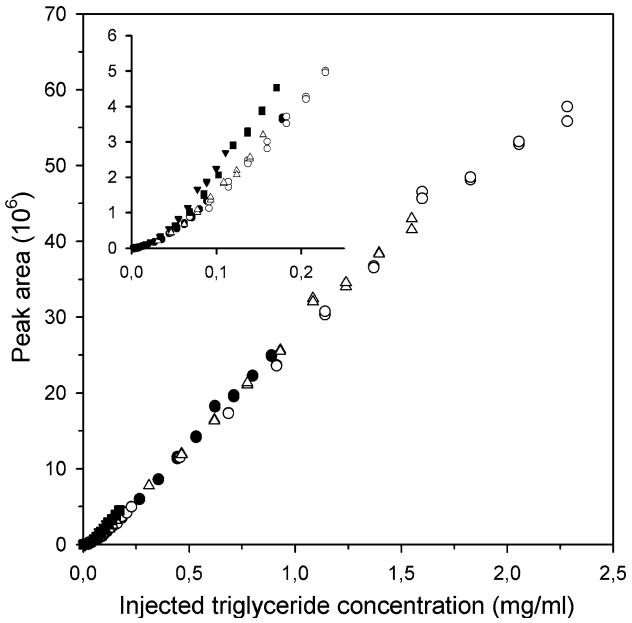

Fig. 4 Peak area as a function of injected concentration of POP (*filled circles*), POS/SLS (*open circles*), POO (*filled inverted triangles*), SOS (*open triangles*) and PPP/SOO (*filled squares*). Injection volume was 25μL. *L* linoleic acid, *O* oleic acid, *P* palmitic acid, *S* stearic acid

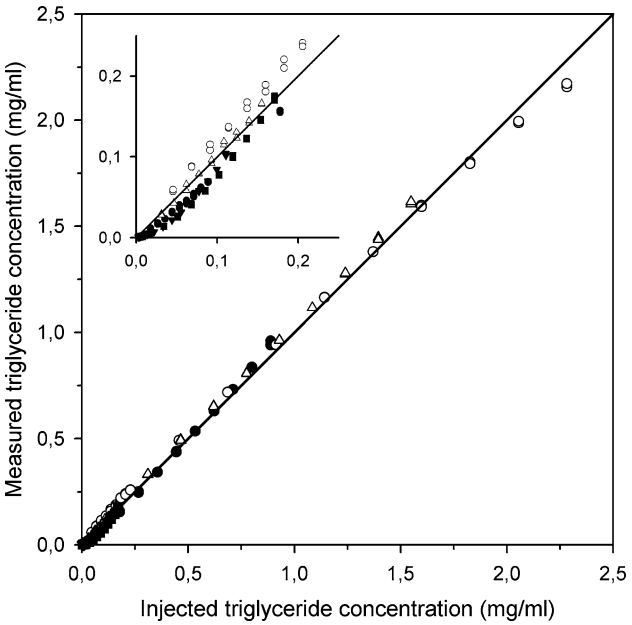

Fig. 5 Measured concentrations as a function of injected concentration of POP (*filled circles*), POS/SLS (*open circles*), POO (*filled inverted triangles*), SOS (*open triangles*) and PPP/SOO (*filled squares*). *L* linoleic acid, *O* oleic acid, *P* palmitic acid, *S* stearic acid

TAG species (POO and PPP/SOO) however, the effect of the non-linear part on the parameters of the linear calibration curves is substantial. Here, the intercept is smaller, and the slope and determination coefficient is lower than

Table 5 Relative peak area of each TAG species as a function of the injected cocoa butter concentration

Injected standard concentration (mg cocoa butter/mL)	Relative peak area of each triacylglycerol (% of total peak area)				
	POP	POS + SLS	POO	SOS	PPP + SOO
0.5	13.8	51.6	0.5	33.1	1.0
1.0	15.6	49.1	0.7	33.1	1.4
1.5	16.6	47.9	0.9	32.9	1.7
2.0	17.1	47.1	1.1	32.7	2.0
2.5	17.5	46.6	1.3	32.3	2.3
3.0	17.9	46.0	1.4	32.1	2.6
3.5	18.0	45.6	1.6	31.9	2.9
4.0	18.3	45.0	1.7	31.9	3.1
4.5	18.6	44.2	1.9	32.1	3.2
5.0	19.0	43.3	2.1	32.2	3.5
Certified reference (g TAG/100 g total TAG)	17.77 ± 0.25	45.66 ± 0.29	2.21 ± 0.16	30.98 ± 0.28	3.42 ± 0.17

L linoleic acid, *O* oleic acid, *P* palmitic acid, *S* stearic acid

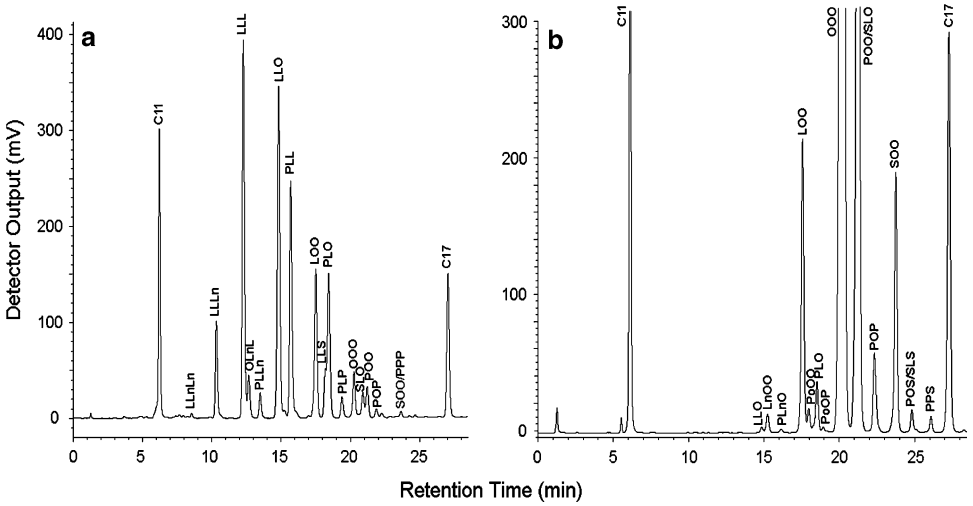

Fig. 6 HPLC–ELSD chromatogram of an oil with a symmetrical triacylglycerol distribution (**a**, soy oil) and an asymmetrical triacylglycerol distribution (**b**, olive oil). Chromatographic conditions as described in the method section. Standards of tritridecanoin and triheptadecanoin were added to correct for small retention time shifts, allowing correct peak identification. *L* linoleic acid, *Ln* linolenic acid, *O* oleic acid, *P* palmitic acid, *Po* palmitoleic acid, *S* stearic acid

for the major TAG species. At high concentrations (>2 mg/mL), it seems that the curve tends to flatten slightly, pointing out the sigmoidal response of the ELSD. The influence of this non-linearity at low concentrations (<0.5 mg/mL) is illustrated in Fig. 5, where the calculated (measured) concentration is plotted against the actual injected concentration, and given in Table 5, where the relative peak area of each TAG species is given as a function of the injected CB concentration. Ideally, in Fig. 5, the experimental data should be situated on the diagonal axis. However, at low concentrations, the minor species POO and PPP/SOO are underestimated and the main component POS/SLS is overestimated. At higher concentrations (>2 mg/mL), the main component POS seems to be underestimated. From Table 5, it can be seen that at injected CB concentrations of 3–4 mg CB/mL, the calculated relative peak area seems to correspond the best with the true value, as here, most of the TAG species are in the linear response of the detector. The latter is only possible with oils or fats with a symmetrical profile like soy oil (Fig. 6a). Here, there are some predominant TAG species, but the difference in peak area with minor species is rather small. As such, at oil concentrations of 3–5 mg soy oil/mL, all TAG species will be situated in the linear detector output, and relative peak areas will reflect the accurate relative TAG concentrations. However, for fats or oils with a distinctive asymmetrical TAG profile like olive oil (Fig. 6b), where OOO accounts for more than 50% of total TAG, it will be virtually impossible to define a concentration region where the ELSD response will be linear for all TAG species. As such, minor TAG species will be underestimated.

Conclusion

In spite of a non-linear response at low and high concentrations, the ELSD remains highly suitable as a detector for HPLC TAG analysis of fats and oils, as it is a highly sensitive detector with a uniform response and a broad linear output range, independent of the TAG species molecular structure. Compared with a refractive index detector, it is about 10–50 times more sensitive, and compatible with gradient elution, resulting in a better separation in a shorter analysis time. Compared to a charged aerosol detector or a flame ionisation detector, it has a more uniform response. For process optimisation (e.g., interesterification or fractionation) or TAG fingerprinting, where similar samples are compared with a reference, the proposed method is suitable, when working under similar analysis conditions (detector settings, injected oil concentration, etc.). However, when using the proposed method for quantitative accurate TAG analysis, one should take into account that for oils or fats with an asymmetrical TAG profile, a serious underestimation of minor TAG species can occur. As such, results should be interpreted with care.

References

1. Buchgraber M, Ulberth F, Anklam E (2004) Method validation for detection and quantification of cocoa butter equivalents in cocoa butter and plain chocolate. J AOAC Int 87:1164–1172
2. Lipp M (1995) Review of methods for the analysis of triglycerides in milk-fat—application for studies of milk quality and adulteration. Food Chem 54:213–221
3. Ollivier D (2003) Determination of virgin vegetable adulteration: application to the quality of virgin oils, particularly virgin olive oil. OCL 10:315–320
4. Aranda F, Gomez-Alonso S, del Alamo RMR, Salvador MD, Fregapane G (2004) Triglyceride, total and 2-position fatty acid composition of Cornicabra virgin olive oil: comparison with other Spanish cultivars. Food Chem 86:485–492
5. Ollivier D, Artaud J, Pinatel C, Durbec JP, Guerere M (2003) Triacylglycerol and fatty acid compositions of French virgin olive oils. Characterization by chemometrics. J Agric Food Chem 51:5723–5731
6. Andrikopoulos NK (2002) Chromatographic and spectroscopic methods in the analysis of triacylglycerol species and regiospecific isomers of oils and fats. Crit Rev Food Sci Nutr 42:473–505
7. Buchgraber M, Ullberth F, Emons H, Anklam E (2004) Triacylglycerol profiling by using chromatographic techniques. Eur J Lipid Sci Technol 106:621–648
8. Perona JS, Ruiz-Gutierrez V (2004) Analysis of neutral lipids: triacylglycerols. In: Nollet LML (ed) Handbook of food analysis. Marcel Dekker, New York, pp 275–312
9. Onken J, Berger RG (1998) Evaporative light scattering detector (ELSD) for the analysis in food chemistry. Dtsch Lebensm-Rundsch 94:287–292
10. Koropchak JA, Magnusson LE, Heybroek M, Sadain S, Yang XH, Anisimov MP (2000) Fundamental aspects of aerosol-based light-scattering detectors for separations. Adv Chromatogr 40:275–314
11. Letter WS (1993) A qualitative method for triglyceride analysis by HPLC using an evaporative light-scattering detector. J Liq Chromatogr 16:225–239
12. Jakab A, Heberger K, Forgacs E (2002) Comparative analysis of different plant oils by high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. J Chromatogr A 976:255–263
13. Pons MS, Bargallo AIC, Sabater MCL (1998) Evaluation by high-performance liquid chromatography of the hydrolysis of human milk triacylglycerides during storage at low temperatures. J Chromatogr A 823:467–474