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Journal of Chromatography A, 1078 (2005) 28–34

JOURNAL OF CHROMATOGRAPHY A

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# Study of the analysis of alkoxyglycerols and other non-polar lipids by liquid chromatography coupled with evaporative light scattering detector

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Received 9 December 2004; received in revised form 31 March 2005; accepted 6 April 2005

**Abstract**

An HPLC method with evaporative light scattering detection (ELSD) for the simultaneous analysis of various lipid classes, particularly alkoxyglycerols and acylglycerols with very similar structure and polarity, has been developed. These lipid classes are frequently found in numerous fats and oils such as shark liver oils and can serve as substrates for lipase-catalyzed reactions. This method utilizes a silica column and a gradient elution of isooctane, methyl *tert*-butyl ether and 2-propanol in different proportions. Separation between squalene, sterol esters, and fatty acid ethyl esters has been achieved in a time of analysis slightly higher than 8 min. In addition, a good resolution between 1,3-diacylglycerols and free sterols was also attained in the same run, with a broad range of concentrations. Excellent precision regarding the retention times was obtained. The limit of detection for the different lipid classes studied was below 1 μg. Intra-day and inter-day variation of retention times and areas was also evaluated. The relative standard deviation of intra-day variation for retention times and areas never exceeded of 0.1 and 10, respectively. The HPLC-ELSD method was also optimized to separate and quantify the hydrolysis products of alkoxyglycerols and acylglycerols (mono-esterified and non-esterified alkoxyglycerols and mono-esterified and di-esterified acylglycerols) at the same time, rendering a useful method for the study of lipase-catalyzed reactions and a wide variety of fats and oils. The present methodology not only separates 18 different lipid classes with a good reproducibility, but it is also able to estimate the relative proportion in which they are found in a broad range of concentrations.
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*Keywords:* Alkoxyglycerols; ELSD; HPLC; Normal-phase; Non-polar lipids

## 1. Introduction

Several HPLC methods for the analysis of lipid classes have been described. Some of these methods utilize cyanopropyl columns for the separation of lipid classes [1]. Nine lipid classes were also separated on a dihydroxypropyl column [2]. Up to 12 different lipid classes have been simultaneously analyzed using normal stationary phase enriched in hydroxyl groups, namely diol columns [3]. All these methods utilize evaporative light scattering detectors (ELSD) which have brought a major advance in the detection of lipid classes by HPLC. This detector is not limited by the nature of the solvent, flow rate, or ambient temperature. In addition, with ELSD no lipid derivatization is required and provides a quantitative response [4,5].

One of the difficulties of these normal-phased HPLC methods is the simultaneous separation of hydrocarbons, such as squalene, sterol esters, and fatty acid methyl or ethyl esters combined with a good resolution of more polar lipids in the same run. Some methods have been described for the separation of non-polar lipid classes using alumina as stationary phase [6,7]. Schaefer et al. have successfully separated wax esters, sterol esters, fatty acid methyl esters on a diol column although peak splitting of lipid classes was observed mostly due to the different degrees of saturation of the fatty acid residues coupled to the lipid.

In the present study we have developed an HPLC method for the analysis of non-polar lipids. In particular, we intended to study the separation of the lipid classes present in shark

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doi:10.1016/j.chroma.2005.04.015

liver oils and their hydrolysis products in combination with other lipids that are easily found in numerous fats and oils. Squalene, diesterified alkoxyglycerols (DEAG) and triacylglycerols (TAG) are the main lipids present in shark liver oils. Simultaneous HPLC analysis of these lipids has not been previously described. One of the main difficulties for the analysis of these compounds arises because DEAG and TAG have very similar structure and polarity. Because of that, these two lipids can co-elute in HPLC analysis. Hence, the present study describes a methodology for the simultaneous analysis of different alkoxyglycerols, squalene and other non-polar lipid classes. In addition, products of hydrolysis of DEAG such as, monoesterified alkoxyglycerols (MEAG) and non-esterified alkoxyglycerols (NEAG) can be also analyzed with this methodology. The resolution and quantification of the different compounds under study have been also evaluated.

## 2. Experimental

### 2.1. Materials

Diesterified alkoxyglycerols, non-esterified alkoxyglycerols, non-esterified dialkoxyglycerol (NEDAG), triolein, 1,3-diolein (1,3-DAG), 1,2-diolein (1,2-DAG), 1-monolein (MAG), cholesterol (sterols), squalene (SQ), cholesteryl oleate (sterol esters), palmitoyl palmitate (waxes), and linoleic acid (FFA) were purchased from Sigma (Madrid, Spain). α-, β-, δ-, and γ-tocopherol were obtained from Calbiochem (Darmstadt, Germany). Conjugated linoleic acid (FFA) and conjugated linoleic ethyl ester (FAEE) were kindly donated by Natural lipids (Hovdebygda, Norway). Both standards and real samples were accurately weighted and dissolved in chloroform/methanol 2:1 (v/v) prior analysis.

### 2.2. Apparatus

The analyses were effected on a Kromasil silica 60 column (250 mm × 4.6 mm, Análisis Vinicos, Tomelloso, Spain) coupled to a CTO 10A VP 2 oven, a LC-10AD VP pump, a gradient module FCV-10AL VP, a DGU-14A degasser, and a evaporative light scattering detector ELSD-LT from Shimadzu (IZASA, Spain).

## 3. Results and discussion

### 3.1. Development of the HPLC method

It is well established that separation in normal phase chromatography is based on the polar groups of the molecule regardless the non-polar side chain. This fact permits one to separate lipid classes regardless the number of carbon atoms and degree of saturation of the compound. However, the whole structure of the molecule contributes on the separation. Although the effect of alkyl groups to the retention is limited, it should be also taken into account. In addition, steric effects have also influence on the retention making possible the separation of *cis* and *trans* isomers. Hence, this type of chromatography is preferred when commercial mixtures of oils have to be analyzed because of the presence of complex mixture of chemical species.

It should be noted that some authors have reported lack of reproducibility with unmodified normal phases [8]. In order to improve the stability and the reproducibility of the silica column used, it has been described that the column can be flushed with mixtures of hexane/isopropanol [9]. Based on the results obtained by Schaefer et al. [3] several gradient of solvents consisting of different ramps and proportions of isoctane, MTBE, and isopropanol were assayed. Isoctane is the most apolar solvent that provides adequate initial conditions for the separation of the most apolar lipid classes under study (e.g. squalene, sterol esters, waxes, and fatty acid methyl esters). However, a small percentage of a more polar solvent should be added to improve the solubility of some compounds in the mobile phase. In addition, pure isooctane utilized as the initial conditions produced important delays in the retention times of the most polar lipids under study (e.g. monoacylglycerols). Hence, isoctane with 0.5% (v/v) of methyl *tert*-butyl ether (MTBE) was chosen as the initial conditions in our HPLC method. In order to elute the most polar lipids isopropanol should be utilized. It should be indicated that higher percentages of isopropanol were also studied in order to reduce the retention times of the most polar lipids under study. However if one needs to separate non-esterified alkoxyglycerols and monoacylglycerols, small percentage of isopropanol should be used. Consequently, percentages of isopropanol higher than 4% could not be utilized.

In our method, a non-modified silica column was used to avoid the peak splitting of some lipid classes that occurs on diol columns [3]. The ELSD conditions were 2.2 bar, 35 °C, and gain 3. The flow rate was 2 mL/min. A splitter valve to reduce the flow through the detector was used after the column and only 50% of the mobile phase was directed through the detector. According to the vendor specifications of the ELSD flow rates higher than 1 mL/min should be avoided for this type of detector. The gradient of solvents utilized is shown in Table 1. The column temperature was maintained at 35 °C. Although peak splitting could be considered an improvement in the chromatographic resolution, it is also inconvenient in order to quantify the different lipid classes that occur in commercial lipids. For example, the peak splitting of fatty acids present in commercial oils subdivides these chemical species in several subclasses based on the number of double bonds. This fact complicates the separation of different lipid classes and increases the risk of overlapping with other lipid classes and it makes difficult to find the adequate response factors for quantification purposes. To confirm that no peak splitting occurred in our method, two commercial mixtures of conjugated linoleic acid (FFA) and conjugated linoleic acid ethyl ester (FAEE) were injected. This product contains several fatty acids such as palmitic, oleic, and conjugated linoleic

Table 1
Gradient A mobile phase composition (%)

| Time (min) | Isooctane | MTBE[a] | Isopropanol |
|---|---|---|---|
| 0 | 99.5 | 0.5 | 0 |
| 3 | 99.5 | 0.5 | 0 |
| 3.01 | 98.5 | 1.5 | 0 |
| 20 | 89 | 11 | 0 |
| 30 | 75 | 25 | 0 |
| 30.01 | 71 | 27 | 2 |
| 50 | 71 | 27 | 2 |
| 50.01 | 75 | 25 | 0 |
| 60 | 75 | 25 | 0 |
| 61 | 99.5 | 0.5 | 0 |
| 70 | 99.5 | 0.5 | 0 |

[a] MTBE contained 0.01% (v/v) of formic acid.

acids among other fatty acid or fatty acid ethyl esters species, respectively. The chromatogram showed in Fig. 1 indicated that no peak splitting for FFA and FAEE were observed.

### 3.2. Mobile phases studied

Two different gradient elution systems were assayed. Gradient A is shown in Table 1. Gradient B was similar to Gradient A except that isooctane was replaced by hexane. The retention times and resolution of the different compounds studied is shown in Table 2. Both isooctane and hexane with 0.5% of MTBE enables the separation of SQ, SE, and FAEE in a time of analysis slightly higher than 8 min.

Resolution of each peak ($R_s$) was calculated according to the following equation:

$$R_s = \frac{R_{t_2} - R_{t_1}}{1/2(w_1 + w_2)}$$

Table 2
Retention times and resolution of the different compounds studied

| Compound | Gradient A | | Gradient B | |
|---|---|---|---|---|
| | $R_t$ | Resolution | $R_t$ | Resolution |
| Squalene | 1.7 | 1.1 | 1.6 | 1.1 |
| Sterol esters | 4.0 | 8.9 | 3.5 | 7.4 |
| Waxes | 4.4 | 0.8 | 3.8 | 0.9 |
| | 8.2 | 7.5 | 8.0 | 9.3 |
| FAEE | 8.7 | 2.2 | 8.4 | 1.2 |
| | | | 8.8 | 1.2 |
| DEAG | 12.0 | 11.4 | 11.9 | 8.2 |
| α-Tocopherol | 13.2 | 3.0 | 13.0 | 2.9 |
| TAG | 14.2 | 2.3 | 14.2 | 2.8 |
| β-Tocopherol | 15.7 | 3.7 | 15.5 | 3.2 |
| γ-Tocopherol | 17.0 | 2.9 | 16.9 | 3.1 |
| FFA | 18.2 | 2.5 | 18.2 | 2.8 |
| δ-Tocopherol | 19.7 | 3.1 | 19.5 | 2.8 |
| NEDAG | 24.8 | 9.9 | 24.5 | 9.6 |
| MEAG | 25.5 | 1.2 | 25.4 | 1.5 |
| Sterols | 26.7 | 2.6 | 26.7 | 2.7 |
| 1,3-DAG | 28.9 | 5.0 | 29.2 | 5.6 |
| 1,2-DAG | 33.3 | 7.0 | 33.5 | 6.9 |
| NEAG | 44.3 | 17.2 | 43.9 | 16.1 |
| MAG | 45.8 | 2.0 | 45.7 | 2.5 |

FAEE, fatty acid ethyl esters; DEAG, diesterified alkoxyglycerols; TAG, triacylglycerols; FFA, free fatty acids; NEDAG, non-esterified dialkoxyglycerols; MEAG, monoesterified alkoxyglycerols; 1,3-DAG, 1,3-diacylglycerols; 1,2-DAG, 1,2-diacylglycerols; NEAG, non-esterified alkoxyglycerols; MAG, monoacylglycerols.

where $R_t$ and $w$ are the retention time and the width of two adjacent peaks, respectively. The width utilized for the determination of the resolution corresponds to that at the base of the peak.

Similar retention times and resolutions were obtained with both gradients. However, peak splitting and poorer resolution

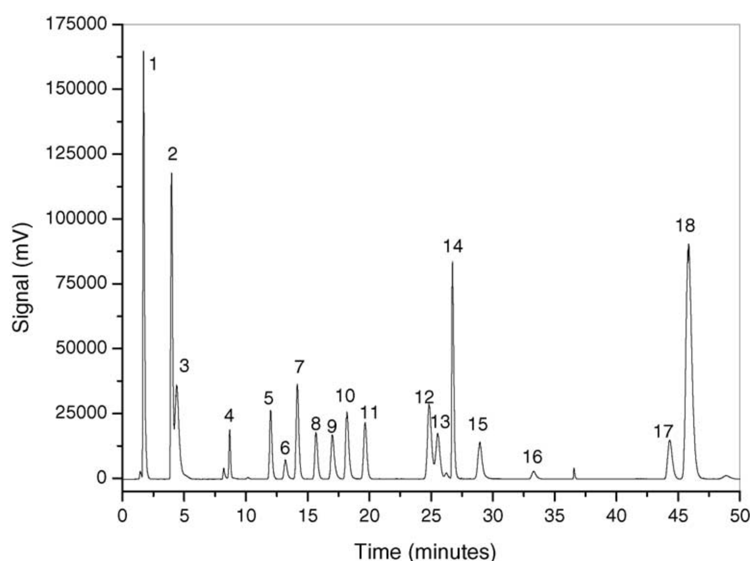


Fig. 1. Separation of the different lipid classes. (1) Squalene; (2) sterol esters; (3) waxes; (4) FAEE; (5) DEAG; (6) α-tocopherol; (7) TAG; (8) β-tocopherol; (9) γ-tocopherol; (10) FFA; (11) δ-tocopherol; (12) NEDAG; (13) MEAG; (14) sterols; (15) 1,3-DAG; (16) 1,2-DAG; (17) NEAG; (18) MAG. Micrograms injected 10–20.

for FAEE was obtained with Gradient B than that obtained with Gradient A. In addition, Gradient B produced higher noise in the baseline between 25 and 35 min compared to that of Gradient A. Hence, Gradient A was selected for further experiments.

Inspection of Table 2 indicates that waxes and sterol esters were less adequately resolved. It should be stated that improvements in the resolution of these two compounds increase the retention times of compounds such as monoacylglycerols non-esterified alkoxyglycerols which difficult the simultaneous analysis of all these lipid classes. In addition, taking into account the difficulties previously reported for the separation of these two compounds in normal-phased chromatography, the results obtained with both gradients are acceptable.

The results in Table 2 also point out that the resolution between NEDAG and MEAG is smaller than 1.7. However the resolution obtained (1.2 for Gradient A and 1.5 for Gradient B) is good enough for separation and quantification purposes.

### 3.3. Reproducibility

It has been previously described that the relative retention times are unsteady in normal-phased chromatography. For this reason, it is recommended that relative retention times should only be used as a guideline in peak identification. The use of known reference standards or alternative methods of chemical identification, such as mass spectroscopy, should be employed for peak identification.

Due to the complexity of the chromatography and detector variability, intra-day and inter-day variability of both retention times and response was evaluated for the different lipid classes under study. The relative standard deviation of intra-day variation for retention times and areas never exceeded of 0.1 and 10, respectively. To determine inter-day variation, three injections of the mixture ($n = 3$) of lipids under study were performed in three separate days during a total period of time of 1 week. The relative standard deviation (RSD) was taken as a measure of precision. Inspection of Table 3 indicates a very good reproducibility regarding the retention times and an acceptable precision in the response. It should be noted that the same sample was utilized for the three injections, over a period of time of 1 week. This fact could have produced partial evaporation and/or insolubilization of some compounds present in the mixture, which could have increased the inter-day variability of the response. It should also be indicated that the response of the different tocopherols analyzed could not be included because, as the time evolved, disappearance of the different tocopherols was observed.

In order to improve the reproducibility of a gradient chromatography the re-equilibration of the system plays a crucial role. Initially, several attempts to recondition the system in a single step were effected. However, lack of reproducibility in both the retention times and the responses were observed for the different lipids under study. Hence, the recondition of the

Table 3
Relative standard deviation (RSD) of the retention times and areas for the different lipid classes analyzed

| | Retention time (RSD) | Area (RSD) |
|---|---|---|
| Squalene | 0.9 | 10.6 |
| Sterol esters | 0.9 | 8.8 |
| Waxes | 1.2 | 13.1 |
| FAEE | 0.3 | 13.8 |
| DEAG | 0.4 | 10.8 |
| TAG | 0.4 | 15.9 |
| FFA | 0.3 | 18.1 |
| MEDAG | 0.5 | 7.6 |
| MEAG | 0.4 | 11.7 |
| Sterols | 0.9 | 8.7 |
| 1,3-DAG | 0.4 | 6.2 |
| 1,2-DAG | 0.6 | 6.9 |
| NEAG | 1.0 | 17.4 |
| MAG | 1.1 | 15.3 |

Table 4
Coefficients of the quadratic regression for SQ, FFA, MEAG, sterols, TAG, SE, 1,3-DAG, α-tocopherol, FAEE, NEAG, and MAG

| | SQ | FFA | MEAG | Sterols |
|---|---|---|---|---|
| $M_0$ | 11.437 | 11.368 | 11.021 | 10.994 |
| $M_1$ | 1.807 | 1.970 | 1.901 | 2.036 |
| $M_2$ | −0.132 | −0.134 | −0.086 | −0.150 |
| $R$ | 0.999 | 1.000 | 1.000 | 0.999 |

| | TAG | SE | 1,3-DAG |
|---|---|---|---|
| $M_0$ | 10.689 | 10.554 | 10.528 |
| $M_1$ | 1.969 | 1.833 | 2.172 |
| $M_2$ | −0.108 | −0.078 | −0.136 |
| $R$ | 1.000 | 0.999 | 0.999 |

| | α-Tocopherol | FAEE | NEAG | MAG |
|---|---|---|---|---|
| $M_0$ | 8.088 | 8.499 | 8.901 | 9.691 |
| $M_1$ | 3.411 | 3.212 | 2.666 | 2.030 |
| $M_2$ | −0.300 | −0.300 | −0.159 | −0.074 |
| $R$ | 1.000 | 0.999 | 1.000 | 1.000 |

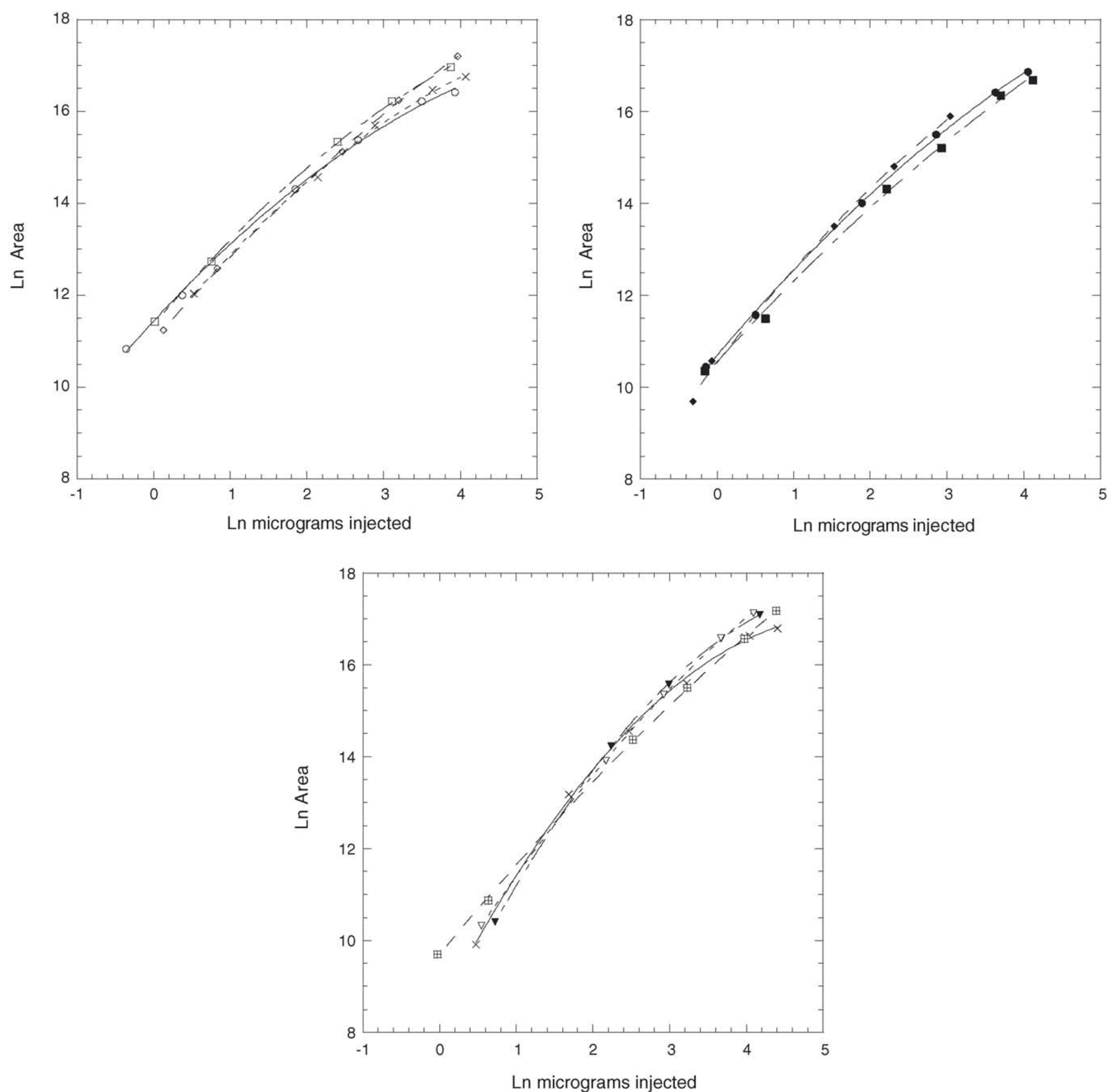


Fig. 2. Calibration curves of the different lipid classes studied: (◯) squalene; (□) FFA; (◊) MEAG; (×) sterols; (●) TAG; (■) SE; (⧫) 1,3-DAG; (▲) FAEE; (▼) α-tocopherol; (⊞) MAG; (▽) NEAG.

system utilized in the present study was effected in two consecutives steps (Table 1). The first step consisted on removing only the isopropanol utilized in the last part of the gradient elution. The second step permits one to recover the initial conditions for a subsequent analysis. This protocol improves the reproducibility of both relative retention times and responses of the different lipid classes studied. According to our results, it could be speculated that by progressively reducing the polarity of the mobile phases, a more efficient procedure of recovering the initial chromatographic conditions can be attained.

### 3.4. Response factors

Linear response curves have been reported for the detection of lipid classes by ELSD, although only for a narrow range of amount injected. Genge et al. prepared standard curves for non-polar lipids ranging from 1 to 12 μg and observed that the relationship between light scattering and solute concentration was generally linear, but second order polynomial regression analysis gave the best fit [10]. When dealing with a wide variety of standard amounts or concentrations, responses are better fitted to

polynomial curves [11]. Fig. 2 shows the calibration curves of the different lipid classes studied. The amount of sample injected was in the range between 1 and 50 μg injected. This broad range of concentrations studied permits one to simultaneously quantify minor constituents in fats and oil in concentrations as low as 1% (w/w) of the total and simultaneously with the rest of neutral lipid existing in the sample. Hence, this methodology not only separates 18 different lipid classes but also is able to estimate the relative proportion in which they are found in a broad range of concentrations.

The response of the ELSD was linearized in a double log plot. A quadratic regression equation of the form

$$\ln \text{area} = M_0 + M_1 \times \ln \text{micrograms injected} + M_2 \times (\ln \text{micrograms injected})^2$$

was utilized to obtain the calibration curves. The coefficients of the quadratic regression analysis and the correlation factors obtained are shown in Table 4. Inspection of Table 4 indicates very good correlation factors for all lipid classes studied.

Calibration curves of the different lipid classes studied were carried out. The results are shown in Fig. 2. It should be also noted that the response factors are mainly affected by the relative volatility of the compounds analyzed. Hence the two main response factors affected by the proportions of the different compounds included in each peak correspond to those of FAEE and FFA. Hence, for quantification purposes, the composition of both FFA and FAEE of each sample should be previously determined by means of different techniques such as gas chromatography. Then, individualized calibration curves for these two lipid classes and for each

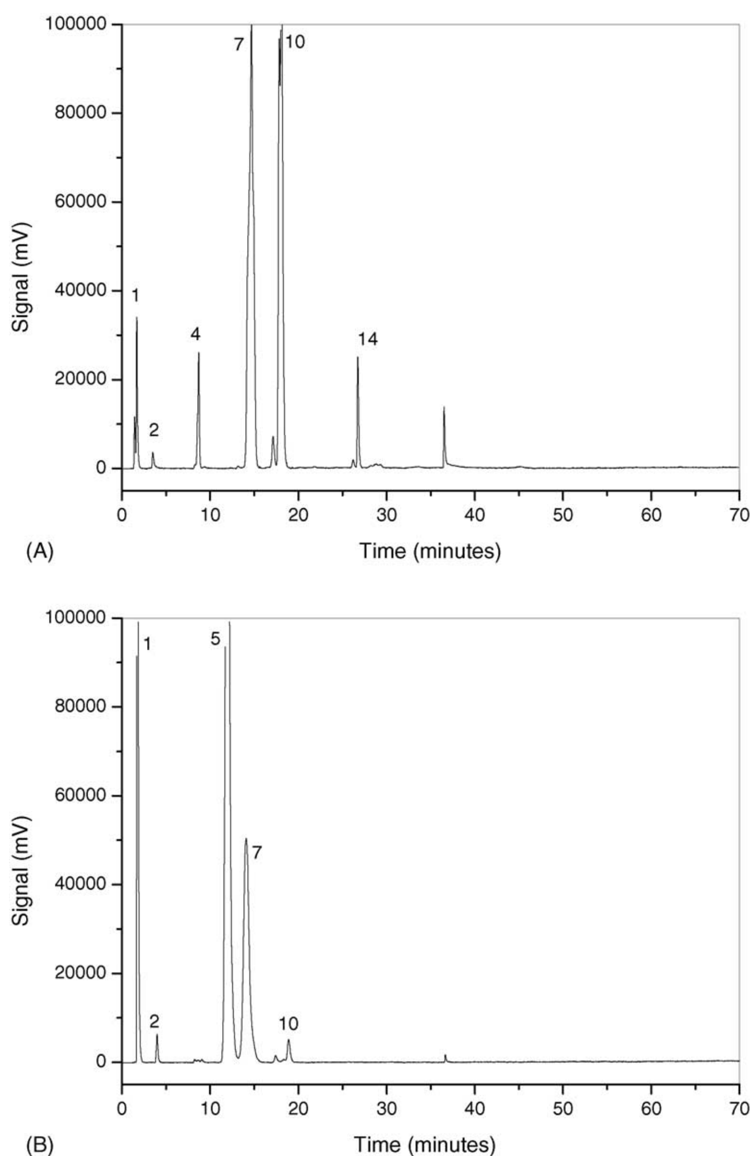


Fig. 3. HPLC analysis of a deodorizer distillate from sunflower oil and a raffinate from shark liver oil. Nomenclature is the same as in Fig. 1.

product to be analyzed could be effected. On the contrary for other neutral lipids, similar response factors are observed regardless the composition of the compounds included in each peak.

The calibration curves of the different lipid classes were grouped according to their response. Inspection of Table 4 permits one to determine how the different lipid classes studied respond. Hence, the quadratic term responsible of the curvature of the response is negative in all cases. The highest quadratic term was obtained for FAEE and for α-tocopherol with a value of −0.300 which indicates an important curvature of these two responses in the range of concentrations studied. The limit of detection for all lipids studied was below 1 μg.

### *3.5. Quantification of commercial samples*

In order to evaluate the present methodology analyses of a deodorizer distillate from sunflower oil and a raffinate from shark liver oil were effected (Fig. 3). Inspection of Fig. 3A indicates that both the major (FFA and TAG) and the minor constituents (squalene, sterols and γ-tocopherol) of the deodorizer distillate from sunflower oil can be simultaneously analyzed. Inspection of Fig. 3A and B indicates that no peak splitting is observed for DEAG, TAG, and sterols coming from a deodorizer distillate from sunflower oil and a raffinate from shark liver oil. These compounds in both samples are composed by numerous chemical species and the absence of peak splitting demonstrate that our methodology permits one to separate different lipid classes regardless the number of chemical species included in each of them. It should be also noted a very slight peak splitting for the peak corresponding to free fatty acids ($R_t = 18.2$). If one consider that this peak is formed by at least four different fatty acids (namely palmitic, stearic, oleic, and linoleic) this peak splitting can be considered almost negligible. Fig. 3B shows the good separation between DEAG and TAG and the presence of cholesterol esters and squalene in shark liver oils. Quantification of the different lipid classes present in the deodorizer distillate from sunflower oil indicates that the weight percentage of squalene, sterol esters, FAEE, TAGs, FFA, and sterols was ca. 2.4, 2.5, 14.7, 34.4, 20.1, and 3.3, respectively. Quantification of a raffinate from shark liver oil showed a percentage of squalene, sterol esters, DEAG, and TAG of ca. 3.1, 0.5, 59.4, and 30.9, respectively. The balance of these two products could consist in traces of other compounds not detected by the present method.

### *3.6. Concluding remarks*

The methodology herein described can be utilized for the analysis of acylglycerol mixtures and esterified and non-esterified alkoxyglycerols. Excellent reproducibility regarding the retention times and acceptable reproducibility regarding the responses can be obtained for the different lipid classes studied. A broad range of concentration can be utilized in order to simultaneously quantify major and minor constituents of numerous fats and oils. In addition monitoring the kinetics of lipase-catalyzed reactions involving sterols, alkoxyglycerols and acylglycerols is also possible.

## Acknowledgements

This work corresponds to preliminary studies of the project AGL2004-07227-C02-01 by Ministerio de Ciencia y Tecnologia (Spain). A postdoctoral contract (programa Ramon y Cajal) for Carlos Torres was provided by the Ministerio de Ciencia y Tecnologia (Spain). A predoctoral fellowship for Luis Vazquez, provided by Comunidad Autonoma de Madrid, is also acknowledged.

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