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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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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.

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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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