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Fast non-aqueous reversed-phase liquid chromatography separation of triacylglycerol regioisomers with isocratic mobile phase. Application to different oils and fats

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ABSTRACT

The distribution of fatty acid species at the *sn*-1/3 position or the *sn*-2 position of triacylglycerols (TAGs) in natural fats and oils affects their physical and nutritional properties. In fats and oils, determining the presence of one or two regioisomers and the identification of structure, where they do have one, as well as their separation, became a problem of fundamental importance to solve. A variety of instrumental technics has been proposed, such as MS, chromatography–MS or pure chromatography. A number of studies deal with the optimization of the separation, but very often, they are expensive in time. In the present study, in order to decrease the analysis time while maintaining good chromatographic separation, we tested different monomeric and polymeric stationary phases and different chromatographic conditions (mobile phase composition and analysis temperature) using Non-Aqueous Reversed Phase Liquid Chromatography (NARP–LC). It was demonstrated that mixed polymeric stationary bonded silica with accessible terminal hydroxyl groups leads to very good separation for the pairs of TAGs regioisomers constituted by two saturated and one unsaturated fatty acid (with double bond number: from 1 to 6). A Nucleodur C18 ISIS percolated by isocratic mobile phase (acetonitrile/2-propanol) at 18 °C leads to their separations in less than 15 min. The difference of retention times between two regioisomers XYX and XXY are large enough to confirm, as application, the presence of POP, SOP, SOS and PLP and no PPO, SPO, SSO and PPL in *Theobroma* cacao butter. In the same way, this study respectively shows the presence of SOS, SOP and no SSO, PSO in *Butyrospermum parkii* butter, POP, SOP, SOS and no PPO, PSO and SSO in *Carapa* oil and finally POP and no PPO in *Pistacia Lentiscus* oil.

1. Introduction

Triacylglycerols (TAGs) are the main constituents of plants oils and animal fats. They are esters of glycerol with three fatty acids (FA). They are characterized by their total carbon number (CN), their number of double bonds (DB), the type and stereospecific position of fatty acids on glyceryl moiety, but also the number, position, and configuration of DB in acyl chains. The digestion, absorption and physiology are deeply related to the TAGs structure, namely TAG molecular species and TAG positional isomer [1]. So, the stereospecific analysis of TAGs which is a challenging problem in the lipid analysis is of high importance. The determination of FA in *sn*-2 or *sn*-1/3 positions (TAGs regioisomers) was considered using different methods [2]. The oldest method was enzymatic hydrolysis of the TAGs [3,4] but difficult to carry and time-consuming. A method that uses chromatography but does not require chromatographic separation of the regioisomers relies on Mass Spectrometry. It provides information on the individual isomers based on the selective abundance of certain fragments or product ions. The loss of FA in the *sn*-2 position is assumed to be energetically less favorable than the loss of FA from the *sn*-1 or *sn*-3 position. Atmospheric pressure chemical ionization (APCI-MS) of TAGs produces $[M+H]^+$ and diacylglycerol fragment ions $[M+H\text{-}RCOOH]^+$. The abundance ratios of the $[M+H\text{-}RCOOH]^+$ ions are dependent on the positions of the FA on the glycerol backbone [5–9]. Electrospray ionization (ESI–MS) has also been used for regioisomer analysis of TAGs with formation of adducts containing sodium, lithium, or for example ammonium [8,10–14]. Negative ion chemical ionization tandem MS (NICI) has also been described for this analysis [8,15,16]. The relative abundance of $[M\text{-}H\text{-}RCOOH\text{-}100]^-$ ions obtained from $[M\text{-}H]^-$, was lower for FA originated from the secondary position than those originated from the primary positions of TAG molecule. Differen-

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Table 1
Nomenclature. Triacylglycerols are named according to the following fatty acid abbreviations.

Abbreviation	Name	Carbon number number of double bonds
P	Palmitic acid	16:0
S	Stearic acid	18:0
O	Oleic acid	18:1 (Z9)
L	Linoleic acid	18:2 (Z9, Z12)
Ln	Linolenic acid	18:3 (Z9, Z12, Z15)
E	Eicosapentaenoic acid	20:5 (Z5, Z8, Z11, Z14, Z17)
D	Docosahexaenoic acid	22:6 (Z4, Z7, Z10, Z13, Z16, Z19)

tial mobility spectrometry coupled to ESI–MS has been applied for the separation of lipids of different classes [17] and more recently for separation of simple unsaturated TAGs regioisomers [18]. However, most of these methods are based on the intensity difference or the intensity ratio of daughter ions of pairs of TAG regioisomers not or partially separated. In conjunction to these spectrometric techniques, allowing overcome this problem, pure chromatographic approaches were used. Even if it is well known that silver-ion chromatography suffers from a lower reproducibility of retention times in comparison to reversed-phase systems, it has been described for the TAGs regioisomers separation [8,19–21]. Hexane/acetonitrile mobiles phases have been described as allowing the separation of TAGs regioisomers. The addition of a small amount of 2-propanol into the mobile phase improves the retention times reproducibility. The temperature effect has been studied but is still not fully understood. The baseline separation of regioisomer TAGs pairs up to three double bonds and the partial separation of TAGs regioisomers with four to seven double bonds were achieved. The off-line 2D system coupling of non-aqueous reversed phase and silver-ion HPLC with APCI–MS have been shown to have high resolving power for TAGs regioisomers [22,23]. Reversed-Phase Liquid Chromatography is currently used to separate numerous TAGs. Momchilova et al. were the first to propose in 2004 regioisomeric TAGs separation in RPLC [24]. Good resolutions for five pairs of regioisomeric TAGs consisting of two palmitic acids (P) and one unsaturated FA (O, L, Ln, D, E) (Table 1) were reported using acetonitrile/alcohols or acetone mobile phases and a non–endcapped ODS stationary phase at 18 °C. They concluded that the selection of the stationary phase and the mobile phase strongly affect the separation of these regioisomeric TAGs. In 2006, they have tested stationary bonded silicas with different percentages of free silanol groups and different mobile phase compositions. The more polar solvents were better suited for stationary phases with high percentage of free silanol groups [25]. In all cases, the TAG with the unsaturated acyl residue in either *sn*-1 or *sn*-3 position was retained more strongly than in the corresponding *sn*-2 position. This may be due to a residual or polarity of the reversed- stationary phase, which would interact more strongly with the polarisable double bonds of the more unsaturated FA when it is located in the more accessible primary positions of the TAG molecule. Kuroda et al. [26] took account of polymeric and monomeric nature of ODS bonded silicas [27] for TAGs separation.They achieved separations of OPO/OOP and POP/PPO on a polymeric column using a recycle HPLC separation system with acetonitrile/2-propanol/hexane mobile phase and a low temperature. Other studies have shown that two Hichrom™ columns percolated by acetonitrile/2-propanol led to the separation of PoPoX/PoXPo (Po = C16:1, X = C15:0) and MaOO/OMaO (Ma = C17:0) [28]. All studies reported so far give nice separations of regioisomeric TAGs but with extremely long analysis times (100, 200 or 500 min). Shorter times (35–60 min) were obtained with the use of non-endcapped polymeric ODS bonded silica percolated by acetonitrile/2-propanol at 18 °C or acetonitrile/2-propanol/hexane at 22 °C [29] for TAGs with two palmitic acids and one highly unsaturated fatty acid (HUFA) residues. They could show that the HUFA binding position is predominant at the *sn*- 2 position in fish and at the *sn*-1 3 position in marine mammals. Finally, a C28 column with acetone/acetonitrile at 15 °C led in 20 min to the separation of regioisomers consisting of two long chain saturated FA and one short chain saturated FA, which are characteristically contained in milk fat [30].

In this study, we focus on RPLC for the regioisomers separation by testing different stationary phases. Our final goal was to propose separation with simple conditions and short time analysis.

2. Materials and methods

2.1. Reagents and samples

All solvents were HPLC grade. Acetonitrile (MeCN) was purchased from Baker (Deventer, The Netherlands), 2-propanol (iPrOH) and acetone (AcMe) from Merck (Darmstadt, Germany).

Except Figs. 5 a, 6 a, 7 a and 8 a, positional isomers of triacylglycerols (TAGs) are denoted throughout the text as AAB and ABA, where A and B denote different fatty acids residues. For example, ABA contains fatty acid A at positions *sn*-1 and *sn*-3, and fatty acid B at position *sn*-2 on the glycerol backbone. TAGs such as AAB and BAA exist as pairs of enantiomers and cannot be distinguished by the method described herein.

Triacylglycerols POP, PPO, SPO, PSO, SOP, SOS, SSO, PDP, PPD, PEP, PPE, PLnP, PPLn were from Cluzeau (Sainte Foy la Grande, France) or Larodan (Malmö, Sweden). Abbreviations are described in Table 1. Different oils and fats have been analysed: *Butyrospermum parkii* butter from Burkina Faso and *Theobroma cacao* butter from Dominican Republic bought to Aroma-Zone (Paris, France); *Pistacia Lenticus* from Tunisia and *Carapa* oil from Congo. All samples were dissolved in acetonitrile/acetone (50/50 v/v) and have been injected at least 3 times.

2.2. UHPLC instrument and working conditions

The UHPLC system consisted of a binary pump, an automated injector and a column oven, (Agilent 1200 Series, Massy, France) coupled to a model Sedex 85 evaporative light-scattering detector (Sedere, Alfortville, France). The nebulizing gas was air at 3.0 bars, the nebulization temperature was 40 °C and the gain PM was 11. Chromatograms were recorded with Chemstation acquisition software (Agilent Technologies, Massy, France).

Different columns have been tested: Uptisphere Strategy C18 NEC (100 × 4.6 mm, 2.2 μm), Uptisphere Strategy C18-2 (100 × 4.6 mm, 2.2 μm) from Interchim (Montluçon, France), Nucleodur C18 ISIS (250 × 4.6 mm, 5 μm and 50 × 2 mm, 1.8 μm) from Macherey-Nagel (Düren, Germany), Spheri-5 ODS Brownlee (250 × 4.6 mm, 5 μm) from Perkin Elmer (Waltham, MA, USA), Kromasil 100-C18 (250 × 4.6 mm, 5 μm) from Eka nobel (Bohus, Sweden) and Supersphere 100 RP 18e (250 × 4 mm, 4 μm) from Merck (Darmstadt, Germany).

3. Results and discussion

Six different C18 bonded stationary phases have been tested with the mobile phase conditions and temperature described by Momchilova [24] (acetonitrile/2-propanol 70/30, 18 °C, 0.8 mL/min). To choose these columns, we made the synthesis of reported results of Momchilova et al. and our own previous studies. It have previously been shown that the best separation of critical pairs of TAGs with same partition number or TAGs with double bond positional isomers of fatty acid was gained on polymeric C18 bonded silicas and to a lesser extent on very densely monomeric