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Enantioselective chromatography in analysis of triacylglycerols common in edible fats and oils

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ABSTRACT

Enantiomers of racemic triacylglycerol (TAG) mixtures were separated using two chiral HPLC columns with a sample recycling system and a UV detector. A closed system without sample derivatisation enabled separation and identification by using enantiopure reference compounds of eleven racemic TAGs with C12–C22 fatty acids with 0–2 double bonds. The prolonged separation time was compensated for by fewer pretreatment steps. Presence of one saturated and one unsaturated fatty acid in the asymmetric TAG favoured the separation. Enantiomeric resolution, at the same time with stronger retention of TAGs, increased with increasing fatty acid chain length in the *sn*-1(3) position. Triunsaturated TAGs containing oleic, linoleic or palmitoleic acids did not separate. The elution order of enantiomers was determined by chemoenzymatically synthesised enantiopure TAGs with a co-injection method. The method is applicable to many natural fats and oils of low unsaturation level assisting advanced investigation of lipid synthesis and metabolism.

1. Introduction

When two different fatty acids are esterified in the *sn*-1 and *sn*-3 positions of triacylglycerols (TAGs), the chiral molecule appears in either *S* or *R* configuration due to the centre of asymmetry in the *sn*-2 position. The enantiomers typically show different biochemical behaviour and technological properties, even though the chemical characteristics are close to each other (Stalcup, 2010). Analysis of the extremely wide selection of stereospecific and species-specific TAG structures in edible fats and oils is a challenge (Brockerhoff, 1971; Innis, 2011; Takagi & Ando, 1991).

Today, the detailed molecular structures of TAGs are still largely unknown (Itabashi, 2012). To understand the impact of the TAG regioisomers and enantiomers on metabolism, bioavailability, digestion, absorption, transport, and common health (Bracco, 1994; Kubow, 1996; Linderborg & Kallio, 2005; Small, 1991) as well as on physicochemical properties, (Foubert, Dewettinck, Van de Walle, Dijkstra, & Quinn, 2007; Itabashi, 2005) a fast and reliable molecular level enantiospecific analysis is of primary importance.

The TAG enantiomers have nearly identical chromatographic and spectroscopic behaviour, as well as physical properties, regardless of the different absolute configurations. In theory, they have the ability to rotate plane-polarised light in opposite directions. To measure the optical activity, a mixture must have an excess of one enantiomer, but in many cases the optical activity alone does not reveal the TAG configurations (Anderson, Sutton, & Pallansch, 1970; Brockerhoff, 1971; Schlenk, 1965). The enantiopure TAGs are known to possess cryptochirality (Mislow & Bickart, 1977) or being cryptoactive (Schlenk, 1965), referring to their extremely low specific optical rotation as being very close to zero and hardly measureable.

Several regiospecific mass spectrometric methods discriminate between the fatty acids in the secondary position (*sn*-2) from those in the primary positions (*sn*-1 and *sn*-3) (Currie & Kallio, 1993; Holčapek, Lísa, Jandera, & Kabátová, 2005; Kallio & Currie, 1993; Leskinen, Suomela, & Kallio, 2007; Lísa, Velínská, & Holčapek, 2009; Lísa et al., 2011). The loss of *sn*-2 fatty acids during tandem mass spectrometry is energetically less favourable than the loss of the fatty acids from the primary positions, whereas the positions *sn*-1 and *sn*-3 are regarded as equivalent.

The most frequently used methods of stereospecific analysis are enzymatic or chemical hydrolysis in combination with chromatography (Blasi et al., 2008; Boukhchina, Gresti, Kallel, & Bézard, 2003; Brockerhoff, 1971), *via* conversion to phospholipid derivatives followed by hydrolysis with stereospecific phospholipases. Instead of using phospholipid derivatives a chiral derivatising agent can be applied (Christie, Nikolova-Damyanova, Laakso, & Herslof, 1991;

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Laakso & Christie, 1990). The resolution of the diacylglycerol urethanes is achieved by adsorption chromatography. Also chiral-phase high-performance liquid chromatography (HPLC) is widely applied as an alternative method for resolution of the isolated diacylglycerol (DAG) and monoacylglycerol (MAG) derivatives in the stereospecific analysis of TAGs (Itabashi, 2005). One significant drawback in the laborious multistep methods is the acyl migration of fatty acids between the positions of glycerol (Compton, Vermillion, & Laszlo, 2007), and it is difficult to totally eliminate. In the end, the stereochemistry of individual TAG molecules remains unknown in these methods.

Iwasaki and coauthors were the first to present the enantiomeric separation of intact TAGs containing octanoic acid and eicosapentaenoic acid or docosahexaenoic acid, in combinations unlikely to be found in nature (Iwasaki et al., 2001). A recent study by Nagai et al. (2011) introduced a direct enantiomer resolution of naturally occurring TAGs without derivatisation, using HPLC in which the sample was recycled through the column several times. Other methods based on circulation of the analytes through one column, have also been developed (Charton, Bailly, & Guiochon, 1994; Grill, 1998). In four applications the enantiomeric separation of TAGs has been achieved by using polysaccharide-based chiral stationary phases (Iwasaki et al., 2001; Lísa & Holčapek, 2013; Nagai et al., 2011; Řezanka, Lukavský, Nedbalová, Kolouchová, & Sigler, 2012). Lísa and Holčapek (2013) separated TAG enantiomers and regioisomers with 1–8 double bonds and different chain lengths using two columns in the normal-phase mode. However, specific co-elution problems existed in analysis of TAGs with saturated and polyunsaturated fatty acids in primary positions. The retention behaviour of TAG enantiomers in chiral HPLC is highly complex and depends on the specific molecular structures. Thus, the use of pure enantiomeric reference compounds is essential.

The aim of the present study was to apply a sample recycling HPLC system based on two identical chiral columns (Trone, Vaughn, & Cole, 2006) to separate intact TAG enantiomers naturally found in many food fats and oils. The other objective was to determine the enantiomeric elution order to investigate the retention mechanisms.

2. Materials and methods

2.1. Chemicals and reference TAGs

Methanol was from J.T. Baker (Deventer, Netherlands). *n*-Hexane was purchased from Sigma–Aldrich Corporation (St. Louis, MO). Acetonitrile and 2-propanol were from VWR International (Radnor, PA). All solvents were either pro analysis or HPLC grade and used without further purification.

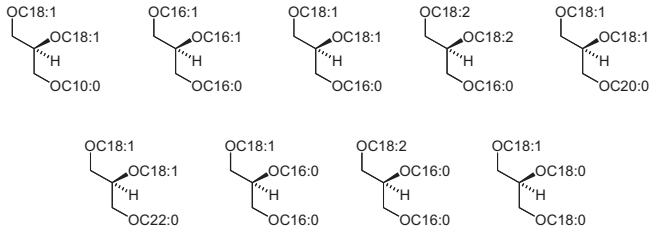

Fig. 1. The synthesised triacylglycerol enantiomers.

Fifteen racemic (Table 1) and nine enantiopure (Fig. 1) reference compounds were used for the HPLC analyses. All racemic TAGs were purchased from Larodan Fine Chemicals (Malmö, Sweden). Enantiopure structured (*S*)-TAGs with saturated, mono- or diunsaturated fatty acids at predetermined positions of the glycerol backbone were prepared as described by Kristinsson, Linderborg, Kallio, and Haraldsson (2014). In short, altogether 18 compounds, including MAG and DAG intermediates, and AAB-type enantiopure structured (*S*)-TAGs were synthesised. (*S*)-TAGs may be divided into two categories. Six of them possess one saturated fatty acyl group located in the *sn*-3 position and two identical unsaturated fatty acyl groups in the remaining *sn*-1 and *sn*-2 positions of the glycerol backbone. The saturated fatty acids belonging to the first category include decanoic (C10:0), palmitic (C16:0), arachidic (C20:0) and behenic (C22:0) acids, and the unsaturated fatty acids present include the monounsaturated palmitoleic (C16:1) and oleic (C18:1) acids, and the diunsaturated linoleic (C18:2) acid. The remaining three TAGs of second category possess two identical saturated acyl groups in the *sn*-2 and *sn*-3 positions and one unsaturated acyl group in the *sn*-1 position. The saturated fatty acids of the second category are limited to palmitic and stearic (C18:0) acids, and the unsaturated fatty acids are C18:1 and C18:2. The synthesis of the first category TAGs is based on a five-step chemoenzymatic process involving a highly regioselective *Candida antarctica* lipase, and the second category TAG products were synthesised by a fully chemical five-step synthetic route where no enzyme was needed. All intermediates and final TAG products were obtained in high chemical and regiopurity. No acyl migration was observed to take place during these reactions. Synthesised products were fully characterised by traditional synthetic organic chemistry methods including ^{1}H and ^{13}C NMR and IR spectroscopy, as well as high-resolution mass spectrometric analyses. Specific rotation was determined for all chiral compounds involved, and the melting point was determined for all compounds that were crystalline.

Table 1
Retention times (t_R, min) of all triacylglycerol racemates after the first column and separation factors (α).

TAG	Abbreviation	t_R	α
1,2-Dioleoyl-3-caproyl-*rac*-glycerol	*rac*-18:1-18:1-10:0	17.9	n.d.*
1,2-Dioleoyl-3-lauroyl-*rac*-glycerol	*rac*-18:1-18:1-12:0	19.8	1.006
1,2-Dioleoyl-3-myristoyl-*rac*-glycerol	*rac*-18:1-18:1-14:0	25.0	1.020
1,2-Dioleoyl-3-palmitoyl-*rac*-glycerol	*rac*-18:1-18:1-16:0	29.4	1.021
1,2-Dioleoyl-3-stearoyl-*rac*-glycerol	*rac*-18:1-18:1-18:0	34.9	1.022
1,2-Dioleoyl-3-arachidoyl-*rac*-glycerol	*rac*-18:1-18:1-20:0	40.0	1.045
1,2-Dioleoyl-3-behenoyl-*rac*-glycerol	*rac*-18:1-18:1-22:0	48.6	1.074
1,2-Dipalmitoleoyl-3-palmitoyl-*rac*-glycerol	*rac*-16:1-16:1-16:0	20.4	1.014
1,2-Dipalmitoleoyl-3-oleoyl-*rac*-glycerol	*rac*-16:1-16:1-18:1	21.6	n.d.
1,2-Dilinoleoyl-3-palmitoyl-*rac*-glycerol	*rac*-18:2-18:2-16:0	24.6	1.016
1,2-Dioleoyl-3-linoleoyl-*rac*-glycerol	*rac*-18:1-18:1-18:2	26.0	n.d.
1,2-Dipalmitoyl-3-linoleoyl-*rac*-glycerol	*rac*-16:0-16:0-18:2	27.3	1.030
1,2-Dipalmitoyl-3-elaidoyl-*rac*-glycerol	*rac*-16:0-16:0-tr18:1	30.9	n.d.
1,2-Dipalmitoyl-3-oleoyl-*rac*-glycerol	*rac*-16:0-16:0-18:1	31.0	1.027
1,2-Distearoyl-3-oleoyl-*rac*-glycerol	*rac*-18:0-18:0-18:1	40.0	1.035

* n.d.: not defined.