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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.

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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.

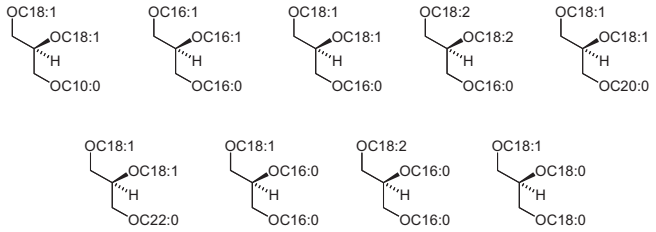

Fig. 1. The synthesised triacylglycerol enantiomers.

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.

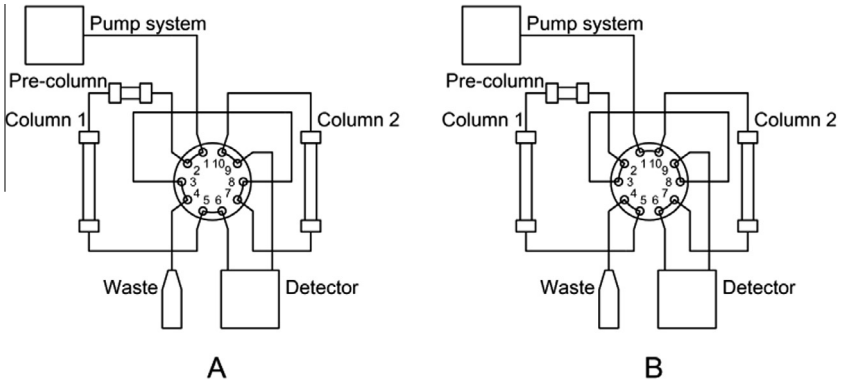

Fig. 2. Principle of the sample recycle HPLC equipment. The two different figures correspond to the both positions of the valve. (A) col. 1 → UV → col. 2, (B) col. 2 → UV → col. 1.

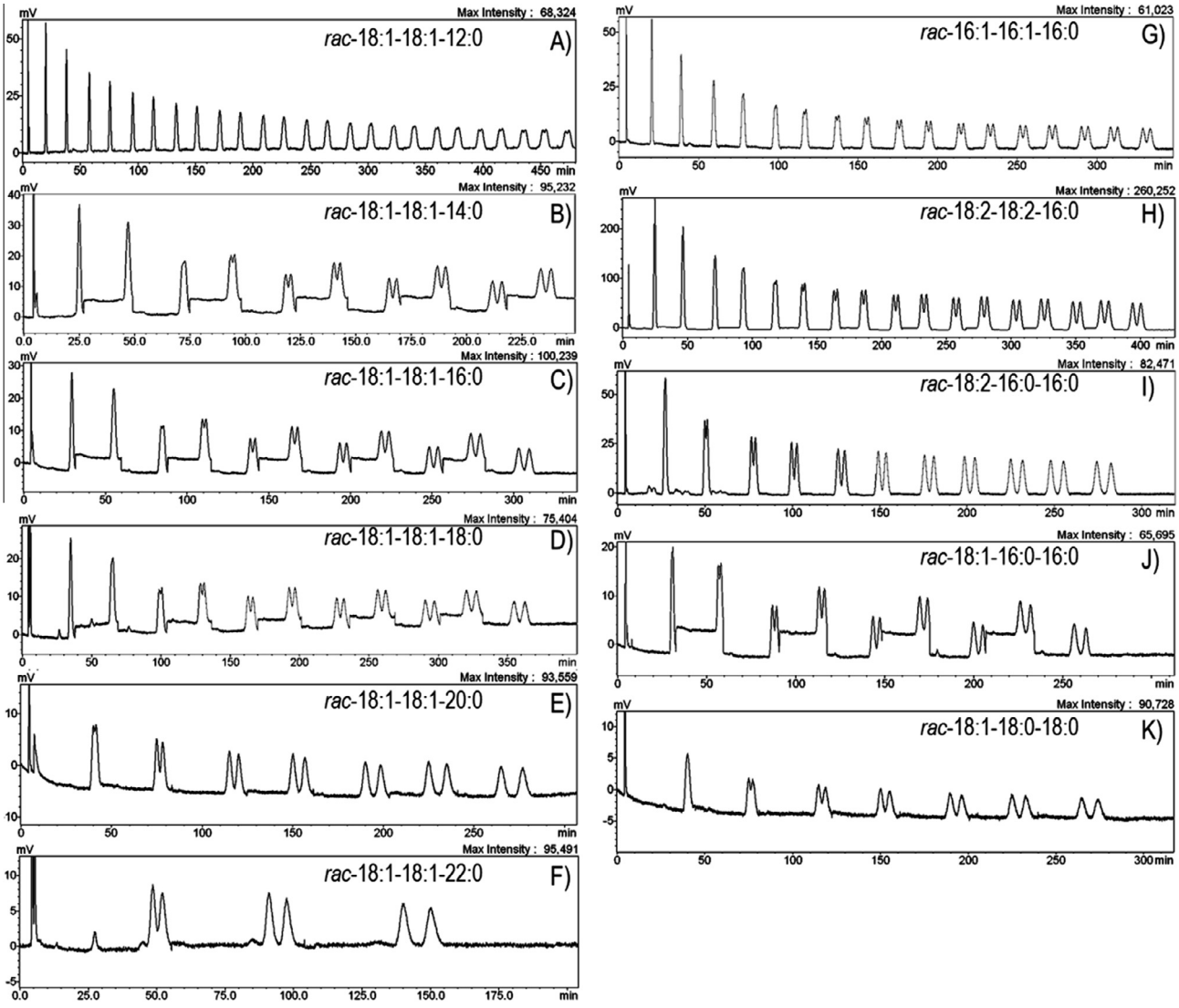

Fig. 3. UV chromatograms of eleven racemic triacylglycerols, (A) *rac*-18:1-18:1-12:0, (B) *rac*-18:1-18:1-14:0, (C) *rac*-18:1-18:1-16:0, (D) *rac*-18:1-18:1-18:0, (E) *rac*-18:1-18:1-20:0, (F) *rac*-18:1-18:1-22:0, (G) *rac*-16:1-16:1-16:0, (H) *rac*-18:2-18:2-16:0, (I) *rac*-18:2-16:0-16:0, (J) *rac*-18:1-16:0-16:0, (K) *rac*-18:1-18:0-18:0. Columns: CHIRALCEL OD-RH (150 × 4.6 mm, 5 μm), Mobile phase: methanol, Detector: UV SPD-20A at 205 nm.

2.2. Recycle HPLC instrumentation and chromatographic conditions

The CHIRALCEL OD-RH (150 × 4.6 mm, 5 μm) columns with cellulose tris(3,5-dimethylphenylcarbamate) stationary phases coated on 5 μm silica-gel and precolumn (10 × 4.0 mm, 5 μm) were obtained from Chiral Technologies Europe (Illkirch, France). The Shimadzu Prominence system (Kyoto, Japan) consisted of a SIL-20A autosampler, an LC-20AB pump, a CTO-10AC column oven,

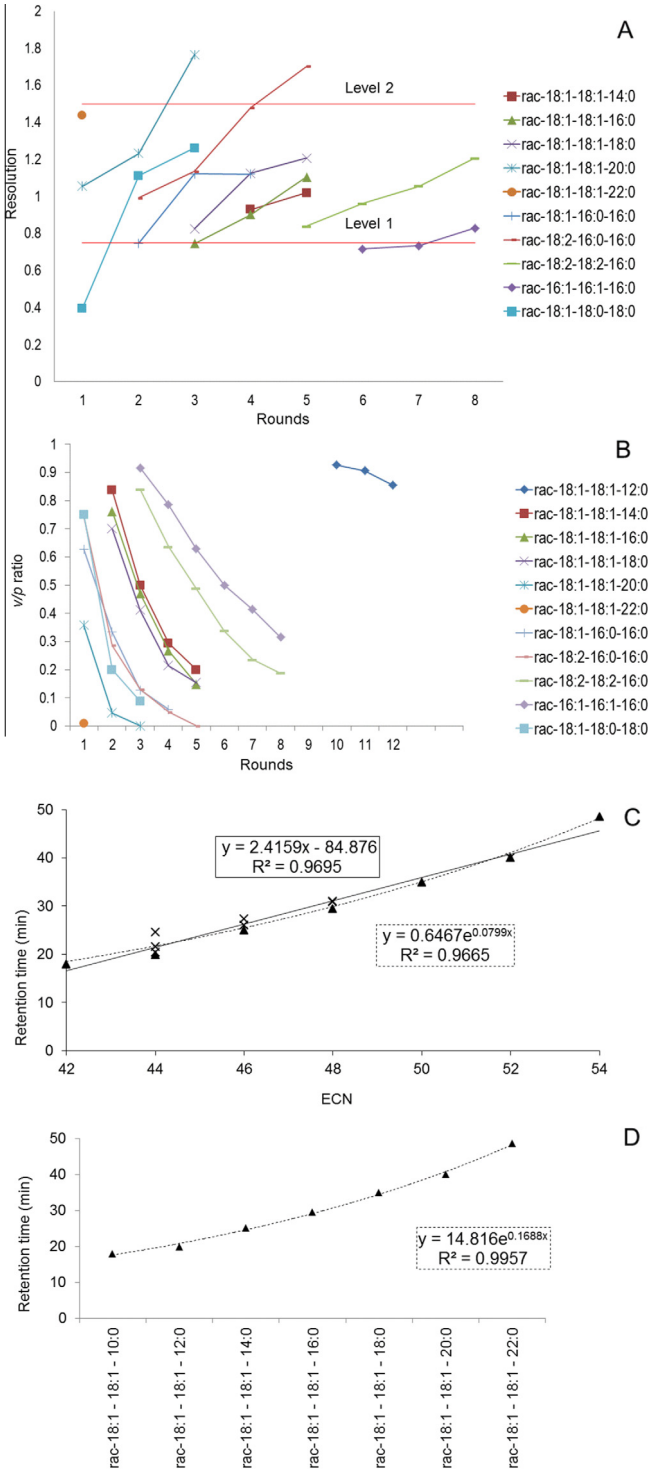

Fig. 4. Measure of separation. (A) Resolutions of ten triacylglycerol racemates after each round. Lower horizontal line corresponding to 50% of perfect separation (Level 1 was $R = 0.75$), upper line corresponding to perfect separation (Level 2 was $R = 1.5$). (B) The ratio of the valley height to the peak height of eleven triacylglycerol racemates after each round. (Round = 2 columns). (C) Elution order of fifteen racemates according to the equivalent carbon numbers. Triacylglycerols having C18:1 at the *sn*-1(3) and *sn*-2 positions and fatty acid C10:0 to C22:0 at the *sn*-3(1) position are marked as triangles, and others are marked as crosses. Exponential curve is marked as dashed lines. (D) Exponential curve fitted for the data points of triacylglycerols having C18:1 at the *sn*-1(3) and *sn*-2 positions and fatty acid from C10:0 to C22:0 at the *sn*-3(1) position.

and a DGU-20A5 degasser. The UV detector was an SPD-20A system, and fractions were collected automatically using an FRC-10A or manually. With the CHIRALCEL OD-RH columns, isocratic methanol at a flow rate of 0.5 mL/min at 25 °C was used. Detection was carried out at 205 nm with an SPD-20A UV detector. The CS3080 Sample Peak Recycler™ (Chiralizer Services, Newtown, PA) was installed in this system. The TAG enantiomers were resolved by rerunning the partially separated compounds after the first column *via* the UV detector to the second column. After the first round (consisting of two columns), recycling was continued through the identical columns until the desired resolution was attained. Either manual or automated (by using the LCsolution program, Shimadzu, Kyoto, Japan) switching between the columns was used.

The recycling system consisted of a controlling device and a high-pressure 10-port valve. The valve was set to pump the solvent through the first column *via* the injection valve and the pre-column (Fig. 2A). Once the last peak in the peak pair was passed through the UV detector, the enantiomer pair was presumed to be eluted completely onto the second column, and the position of the valve was changed (Fig. 2B). If the peak resolution was not satisfactory after passing through the second column, the LC Sample Recycler™ was triggered again (col. 1 → UV → col. 2) by timed switching. The analyte was circulating continuously through the first column, the detector, back to the valve and then back to the second column until a desired resolution was attained. The UV detector was applied to monitor in real time the separation after each of the columns without disturbing the run or destroying the sample.

2.3. Chiral resolution

Altogether, 15 pure racemic TAGs, which are commonly found in nature and in food oils, were diluted separately in *n*-hexane/2-propanol (3:2, v/v) to 1 g/L, and the injection volume was 15 μL. The enantiopurity of the synthesised TAGs was confirmed by injecting 10 μL of a sample containing 7–15 μg of enantiomer diluted in *n*-hexane/2-propanol. The elution order of enantiomers was determined by co-injection of enantiopure and racemic TAG. A 15 μg aliquot of the racemic TAG together with 5 μg of enantiopure TAG were diluted in *n*-hexane/2-propanol, and injected in 20 μL.

Separation of the enantiomeric TAGs was followed by determining the progress of total retention time (t_R), adjusted retention time (t'_R), separation factor (α), peak resolution (R_s), and valley-to-peak ratio (v/p) of the analytes. The separation factor, which shows the ability of the column to differentiate the analytes, was defined by the following equation:

$$\alpha = k_2/k_1, \text{ where } k = t'_R/t_M \quad (1)$$

In Eq. (1), t_M is the hold-up time, t'_R is the adjusted retention time of the analyte, and k is defined as the retention factor.

Peak resolution, in this context, means the separation of a racemate into the component enantiomers, and is calculated according to Eq. (2):

$$R_s = \frac{2(t_{R2} - t_{R1})}{w_{b_1} + w_{b_2}}, \text{ where } w_{b2} = \frac{4}{2.355} \cdot w_h \quad (2)$$

In Eq. (2), w_{b2} is the base width of the latter peak, and w_h is the width at half height. In peak pairs, where the bottom of the valley was higher than at the half height of the peak, peak resolution was

not determined. The ratio of the valley height to the peak height (Christophe, 1971) may be employed as a measure of separation even when the separation is very weak. It is calculated from Eq. (3):

$$v/p \text{ ratio} = h_v/h_p \quad (3)$$

In this equation, h_v is the valley height, and h_p is the peak height of the first peak of two.

3. Results and discussion

3.1. Separation of racemic TAGs

The chiral recycling HPLC method in polar-organic phase mode enabled successful enantioseparation of eleven out of fifteen racemic TAGs examined. Chiral separation with the column and conditions examined was effective when the asymmetric TAG molecule contained both saturated and unsaturated fatty acids.

The UV chromatograms of the eleven racemic TAGs separated into enantiomers using the recycle HPLC method are presented in Fig. 3. The progress of separation between the enantiomers is illustrated. Molecular weights of the isolated enantiomers were confirmed as protonated TAGs and ammonium adducts by atmospheric pressure chemical ionisation mass spectrometry. Retention of the compounds as well as the separation power increased with the corresponding length of the fatty acid in the *sn*-1(3) position, as illustrated in the chromatograms A–F of Fig. 3.

Four racemic reference mixtures analysed did not exhibit any enantioresolution in an applicable time (*rac*-18:1-18:1-10:0, *rac*-16:1-16:1-18:1, *rac*-18:1-18:1-18:2 and *rac*-16:0-16:0-*tr*18:1). Both automated and manual column switching was applied with *rac*-18:1-18:1-10:0, and after 32 elution cycles (running time 9.5 h) no enantiomeric separation was attained. After 10 columns, no resolution was achieved with the triunsaturated TAGs consisting of fatty acids with one or two double bonds. Theoretically, the sample can be recycled as many times as needed for the desired resolution to be obtained, but in practice peak broadening limits the number of cycles.

Trisaturated TAGs were not examined because their absorption of UV light is very low. Routinely used evaporative light scattering detection or mass spectrometry (Holčapek et al., 2005; Leskinen et al., 2007) are inoperable as the sole detectors in a recycling system, in which the detection method must be non-destructive. However, after isolation of an enantiomer it can be further analysed with LC–MS, as the mobile phase is LC–MS compatible.

3.2. Measure of separation

The chain length of fatty acids in TAGs had a significant impact on elution rate; the shorter the carbon chain, the faster the elution. A higher degree of unsaturation results in a shorter retention time as shown when comparing the TAGs *rac*-18:1-18:1-18:0 (t_R 34.9) and *rac*-18:1-18:0-18:0 (t_R 40.0). Table 1 lists all TAGs examined, along with their retention times (t_R) after the first column, and the separation factors (α). The list starts with seven TAGs, which possess a saturated fatty acid C10:0–C22:0 in the *sn*-1(3) position and two C18:1 fatty acids in the remaining positions. This is followed by TAGs with two or three unsaturated fatty acids, and finally TAGs with unsaturated fatty acid in a primary position along with two saturated fatty acids. The separation factor defines the distance of the peak maxima of the two enantiomers but does not take the peak widths into account. Together with the retention time, the separation factor is a useful value when evaluating the separation ability of the circulating system.

Peak resolution determines the progress of separation of the racemate into the enantiomers over time (Fig. 4A). The definition of one round, in this context, means the full cycle including columns 1 and 2 (Fig. 2). The improvement of the apparent resolution was not in linear correlation to the number of full cycles due to the non-ideal peak broadening. Resolution at level one (Fig. 4A) may be regarded as a sufficient measure for estimation of the proportions of the TAG enantiomers. During the rounds previous to those shown in Fig. 4A, either resolution was not observed or the widths of the peaks at the half height could not be defined. For partially overlapping peaks, v/p ratios were determined (Fig. 4B). The v/p ratio was determined for *rac*-18:1-18:1-12:0 although the resolution could not be calculated. The v/p values of TAGs examined decreased similarly, except *rac*-18:1-18:1-12:0 for which the enantiomeric separation was very slow.

3.3. Retention mechanism

The compounds were eluted in relation to the equivalent carbon number (ECN) defined as the number of carbon atoms in the fatty acid residues minus twice the number of double bonds (Fig. 4C). The r^2 values indicate sufficient fits of both the linear line ($r^2 = 0.970$) and exponential curve ($r^2 = 0.967$). When TAGs with C18:1 in the *sn*-1(3) and *sn*-2 positions and fatty acid from C10:0 to C22:0 in the *sn*-3(1) position are taken into account, the data fit to the exponential model perfectly ($r^2 = 0.996$), as illustrated

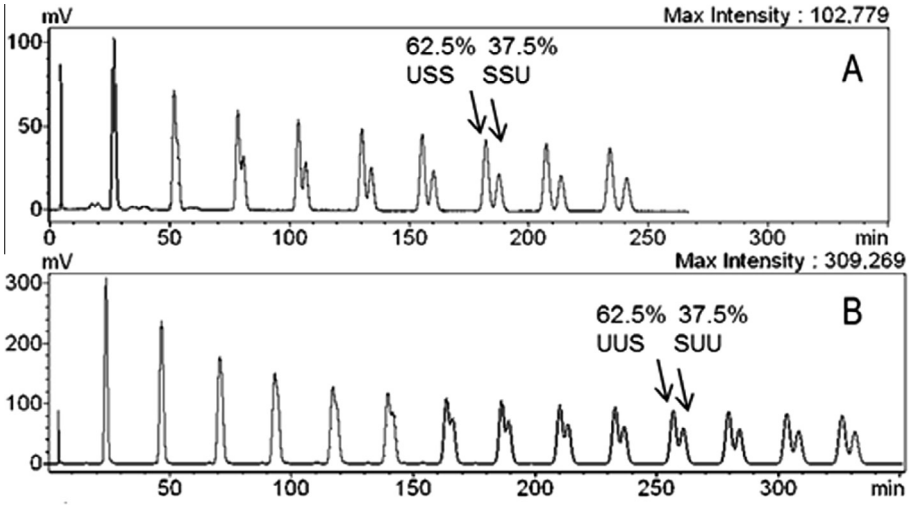

Fig. 5. UV chromatograms of (A) *rac*-18:2-16:0-16:0 co-injected with 25% of *sn*-18:2-16:0-16:0 and (B) *rac*-18:2-18:2-16:0 co-injected with 25% of *sn*-18:2-18:2-16:0. HPLC conditions identical to Fig. 3. U – unsaturated, S – saturated.

in Fig. 4D. The curves can be used to forecast retention behaviour of chiral TAG racemates under the chromatographic conditions examined. However, the curves can never fully replace enantiopure reference compounds.

3.4. Identification of enantiomeric TAGs

The UV chromatograms of nine synthesised enantiopure TAGs analysed by the chiral recycle HPLC technique showed an absence of impurities, and that no isomerisation occurred during synthesis. The elution order of stereoisomeric TAGs was determined by the co-injection technique. Fig. 5 shows chromatograms of the TAG mixtures of 75% *rac*-18:2-16:0-16:0 co-injected with 25% of *sn*-18:2-16:0-16:0 (Fig. 5A) and 75% *rac*-18:2-18:2-16:0 co-injected with 25% of *sn*-18:2-18:2-16:0 (Fig. 5B). After the first two column passes minimal visible resolution was noticed but from thereon a sequenced elution of *sn*-18:2-16:0-16:0 and *sn*-16:0-16:0-18:2 enantiomers, respectively, was recognisable. The analogous enantiomeric separation of *sn*-18:2-18:2-16:0 from *sn*-16:0-18:2-18:2 was visible only after the fifth column, and improved cycle by cycle. In these two examples, as in the analyses of all the other critical pairs of TAG enantiomers, the enantiomer with unsaturated fatty acid in the *sn*-1 position eluted faster than the enantiomer with the unsaturated fatty acid in the *sn*-3 position.

4. Conclusion

A straightforward sample recycling method, without enzymatic hydrolysis or derivatisation, offers reliable information on the enantiomeric composition of TAGs using fewer chemicals than traditional methods. The results can be used to characterise the chromatographic behaviour of TAG enantiomers. During one run the sample can pass up to thirty times through the column. Amount of stationary phase contact with the sample is multiplied contrary to traditional column chromatography, where two or three columns in series are the maximum due to the high back-pressure. This method is applicable for chiral racemates commonly found in nature that are only partially resolved, if the length of separation time and the peak broadening cause no restrictions.

Only a certain number of enantiomers may be separated from each other at a time, and the number of these compounds varies according to TAG composition of the sample. Thus, the individual natural oil may need to be pre-fractionated into more simple TAG groups or even single TAGs for the enantiomeric analysis.

With the columns and conditions examined separation of triunsaturated TAGs was practically impossible. However, the most common natural lipids comprise mixed fatty acid composition, and thus the limitation to TAGs with both saturated and unsaturated fatty acids often does not limit the analysis substantially.

In addition to analysis of stereospecific TAG molecular compositions and structural changes from natural matrices or processed food, the technique provide information about the enantiomeric purity of synthesised products. Knowledge of enantiopurity and stereospecific composition of TAGs enables more specific investigation of TAG fatty acid composition and its effects on health and lipid biosynthesis. The presented method contributes to the area of lipid research, which has been limited by the absence of reliable methods applicable for complex natural and biological samples.

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