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Enantiomeric separation of asymmetric triacylglycerol by recycle high-performance liquid chromatography with chiral column

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ABSTRACT

In our previous studies, we employed recycle HPLC for the separation of triacylglycerol (TAG)-positional isomers (PIs). In this study, a recycle HPLC system equipped with a polysaccharide-based chiral column was applied to the enantiomeric separation of some asymmetric TAGs having straight-chain C16–C18 acyl residues. As a result, 1,2-dipalmitoyl-3-oleoyl-*rac*-glycerol (*rac*-PPO), 1,2-dioleoyl-3-palmitoyl-*rac*-glycerol (*rac*-OOP), and 1,2-dipalmitoyl-3-linoleoyl-*rac*-glycerol (*rac*-PPL) were resolved into their respective enantiomers. However, neither 1,2-dioleoyl-3-linoleoyl-*rac*-glycerol (*rac*-OOL), consisting of only unsaturated fatty acids, nor 1,2-dipalmitoyl-3-stearoyl-*rac*-glycerol (*rac*-PPS), consisting of only saturated fatty acids, was resolved. These results suggest that the asymmetric TAGs, used in this study, having both a palmitic acid moiety and an oleic acid (or a linoleic acid) moiety at the *sn*-1 or *sn*-3 positions are resolved by the chiral column. This new chiral separation method can be used in combination with atmospheric pressure chemical ionization mass spectrometry to determine the *sn*-OOP/*sn*-POO ratio in palm oil. This method is applicable for the chiral separation of asymmetric TAGs in palm oil.

1. Introduction

Triacylglycerols (TAGs), which are composed of three fatty acids and a glycerol, are major components of natural edible oils and fats. The combinations of fatty acids on the glycerol backbone enable numerous TAG molecular species to be formed in nature, but these combinations are not formed at random. Many researchers have studied the positional distributions of fatty acids in TAGs from plants, animals, and fish [1–3]. For example, cocoa butter, which is used in chocolate manufacturing, contains symmetric TAGs having two saturated fatty acids (palmitic acid and stearic acid) at the *sn*-1 or *sn*-3 position and one unsaturated fatty acid (oleic acid) at the *sn*-2 position [4]. These TAG molecular species contribute to the physical properties of chocolate. Milk fat plays a crucial role in nutrition in infancy. The TAG molecular species in human milk fat is apt to bind a palmitic acid moiety at the *sn*-2 position, thus allowing infants to take in energy easily through the β-oxidation of saturated fatty acids [5–7]. Asymmetric distributions of fatty acids in TAGs of chicken plasma and eggs have been reported [8]. Palmitic acid is bound predominantly to the *sn*-1 position (approximately 70%) and oleic acid to the *sn*-2 and *sn*-3 positions in their TAGs. In the case of fish and sea mammal TAGs, researchers have focused on the binding positions of highly unsaturated fatty acids (HUFAs) on the glycerol backbone. TAGs from fish tend to bind HUFAs at the *sn*-2 position, whereas TAGs from sea mammals bind HUFAs at the *sn*-1 or *sn*-3 position [9–11]. Thus, each of the oils and fats in nature has a characteristic fatty acid distribution on the glycerol backbone because of its metabolic pathway or nutritional requirements [12,13]. Although stereospecific analyses of the TAGs mentioned above have been performed using various GC and HPLC methods, most of them could not separate intact TAG molecular species that included positional isomers (PIs) and enantiomers. Some oils and fats mentioned above, with symmetric or asymmetric fatty acid distributions, presumably consist of characteristic TAG molecular species, which may contribute to the properties of the oils and fats such as symmetric TAG molecular species in cocoa butter [4]. It is therefore important to analyze not only the fatty acid distributions but also the TAG molecular species to gain a better understanding of the physical, chemical, and nutritional properties of natural oils and fats.

The separation of TAG molecular species by HPLC has been studied so far [14]. In general, non-aqueous reversed-phase HPLC on ODS columns is used for TAG composition analyses, in which the elution order is dependent on the acyl chain length and the num-

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ber of double bonds in the TAG molecules [15,16]. Until now, a combination of reversed-phase HPLC and mass spectrometry (MS) has been used to identify many kinds of TAG molecular species, including TAG-PIs in natural edible oils [17–19]. Silver-ion HPLC is powerful method for the separation of TAG-PIs because silver ion can recognize the number, geometry, and position of the double bonds in the molecule [20,21]. Although widely used ODS columns having a monomeric ODS stationary phase are not effective for the separation of TAG-PIs, some ODS columns are capable of separating TAG-PIs. Momchilova et al. reported the separation of six kinds of TAG-PI pairs consisting of two palmitic acids and one unsaturated fatty acid on a non-endcapped ODS stationary phase [22,23]. For further high-resolution separation of TAG-PIs, we employed recycle HPLC, in which the target analytes pass through the same column repeatedly. We thus separated TAG-PIs, namely, 1,3-dioleoyl-2-palmitoyl-*sn*-glycerol (*sn*-OPO)/*rac*-OOP and *sn*-POP/*rac*-PPO, and showed that non-endcapped polymeric ODS stationary phases are effective in recognizing the structural differences between the TAG-PIs [24]. In addition, we found that the non-endcapped polymeric ODS stationary phase was effective for resolving TAG-PIs with one or two saturated fatty acid moieties, the chain length of which was at least 12-carbon [25]. Although reversed-phase and silver-ion HPLC have been used to separate TAG-PIs, these methods cannot be used to separate TAG-enantiomers. Meanwhile, Itabashi and Takagi reported the chiral separation of MAG and DAG [26,27]. Their method has been used for the stereospecific analysis of TAGs from natural oils and fats [28,29]. However, their methods required partial hydrolysis of the acyl groups of TAGs and precolumn derivatization of DAGs to 3,5-dinitrophenylurethanes (DNPUs) before the chiral HPLC analysis. On the other hand, Iwasaki et al. showed that some TAG-enantiomers can be directly resolved by chiral HPLC on a polysaccharide-based chiral stationary phase (CSP) without prior derivatization, but only if the TAG-enantiomers consist of very different acyl groups, such as 1-docosahexaenoyl-2,3-dicapryroyl-*sn*-glycerol (*sn*-DCC)/*sn*-CCD and 1-eicosapentaenoyl-2,3-dicapryroyl-*sn*-glycerol (*sn*-ECC)/*sn*-CCE [30]. Of the many applications of chiral-phase HPLC to glycerolipids reviewed by Itabashi [31], only Iwasaki's report showed that the separation of TAG-enantiomers with their intact structures kept. Nevertheless, their method could not be used to separate the major TAG molecular species having acyl residues with chain lengths of C16–C18 contained in edible oils and fats. No TAG-enantiomer separation without derivatization has been reported till date. Therefore, a method that enables the enantiomeric separation of intact asymmetric TAG molecular species in natural oils and fats is required for the identification and characterization of these molecules, as well as TAG-PI separation.

In this study, we screened polysaccharide-based chiral columns for the resolution of *rac*-PPO into *sn*-PPO and *sn*-OPP and attempted to resolve some other racemic asymmetric TAGs (*rac*-OOP, 1,2-dipalmitoyl-3-linoleoyl-*rac*-glycerol (*rac*-PPL), 1,2-dioleoyl-3-linoleoyl-*rac*-glycerol (*rac*-OOL), and 1,2-dipalmitoyl-3-stearoyl-*rac*-glycerol (*rac*-PPS)) into individual pairs of enantiomers, in combination with recycle HPLC system for high-resolution separation. To evaluate whether our method is applicable to the analysis of asymmetric TAGs in natural oils and fats, we determined the ratio of *sn*-OOP to *sn*-POO in palm oil through the use of chiral HPLC with the recycle system and atmospheric pressure chemical ionization (APCI)-MS. Because palm oil is rich in *sn*-OOP (or *sn*-POO) as well as *sn*-POP and its physical property may be affected by *sn*-OOP (or *sn*-POO), it will be worthwhile to know the enantiomeric ratio of *sn*-OOP to *sn*-POO. Finally, we discussed the TAG-enantiomer structures that had to be resolved by CSP with regard to the difference between the acyl groups and their binding positions on the glycerol backbone.

2. Experimental

2.1. Chemicals and materials

1,2-Dipalmitoyl-*sn*-glycerol (*sn*-1,2-PP), 1,2-dipalmitoyl-*rac*-glycerol (*rac*-1,2-PP), 1,2-dioleoyl-*sn*-glycerol (*sn*-1,2-OO), 1,2-dioleoyl-*rac*-glycerol (*rac*-1,2-OO), *sn*-POP, *sn*-OPO, *rac*-OOP, *rac*-OOL, linoleic acid, oleic acid, palmitic acid, and stearic acid were purchased from Sigma–Aldrich Corporation (St. Louis, MO). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 4-dimethylaminopyridine (DMAP) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Palm oil used was commercial product. All of the other reagents used were analytical grade and purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The chiral columns—CHIRALPAK IA, IB, and IC (4.6 mm i.d. × 250 mm, 5 μm, Daicel Chemical Industries, Ltd., Tokyo, Japan); CHIRALPAK AD-H, AS-H, AY-H, CHIRALCEL OD-H, OJ-H, and OZ-H (4.6 mm i.d. × 250 mm, 5 μm, Daicel Chemical Industries, Ltd.); and CHIRALPAK AD-RH, AS-RH, OD-RH, and OJ-RH (4.6 mm i.d. × 150 mm, 5 μm, Daicel Chemical Industries, Ltd.)—were screened for the chiral separation of *rac*-PPO.

2.2. Synthesis of asymmetric TAGs (sn-PPO, rac-PPO, sn-OOP, rac-PPS, and rac-PPL)

sn-1,2-PP, oleic acid, EDC, DMAP, and *N*-ethyldiisopropylamine (0.2 mmol of each) were reacted in dichloromethane at 0 °C for 12 h under argon [32]. The reaction product (*sn*-PPO) was purified by flash silica gel chromatography using a hexane/ethyl acetate mixture (90/10, v/v) as an eluent. *sn*-PPO was isolated in approximately 83% yields. Their structures were confirmed by APCI mass spectra, with *m/z* 834, 578, and 552 corresponding to a protonated molecule ($[PPO+H]^+$), loss of palmitate to give $[PO]^+$, and loss of oleate to give $[PP]^+$, respectively. The parameters of the mass spectrometer are mentioned below (Section 2.4.1). *rac*-PPO, *sn*-OOP, *rac*-PPS, and *rac*-PPL were synthesized and purified in the same way as *sn*-PPO. *rac*-PPO was synthesized from *rac*-1,2-PP and oleic acid, *sn*-OOP from *sn*-1,2-OO and palmitic acid, *rac*-PPS from *rac*-1,2-PP and stearic acid, and *rac*-PPL from *rac*-1,2-PP and linoleic acid. All the structures of asymmetric TAGs we synthesized were confirmed by APCI mass spectra and shown in Supplementary material 1–4.

2.3. Chiral column screening

2.3.1. Chiral column screening in normal-phase HPLC

The HPLC system used for screening in the normal-phase mode was composed of an on-line degasser (DGU-20A5, Shimadzu Corporation, Kyoto, Japan), a pump (LC-20AB, Shimadzu Corporation), autosampler (SIL-20A, Shimadzu Corporation), column oven (CTO-20AC, Shimadzu Corporation), and UV–visible detector (SPD-20A, Shimadzu Corporation). *rac*-PPO (1.7 mg/mL in hexane) was chromatographed on a chiral column, i.e., CHIRALPAK IA, IB, IC, AD-H, AS-H, AY-H, CHIRALCEL OD-H, OJ-H, and OZ-H. Hexane/ethanol (100/0.5, v/v) or hexane/2-propanol (100/0.5, v/v) were used as the mobile phase. The flow rate, wavelength of the UV–visible detector, column temperature, and injection volume were 1.0 mL/min, 210 nm, 25 °C, and 10 μL, respectively.

2.3.2. Chiral column screening in reversed-phase HPLC

The HPLC system used for screening in the reversed-phase mode was the same as that used in the normal-phase mode. *rac*-PPO (1.2 mg/mL in methanol) was chromatographed on a chiral column, i.e., CHIRALPAK IA, IB, IC, AD-RH, AS-RH, CHIRALCEL OD-RH, and OJ-RH. Methanol (100%) or acetonitrile (100%) was used as the mobile phase. The flow rate, wavelength of the UV–visible detector, col-

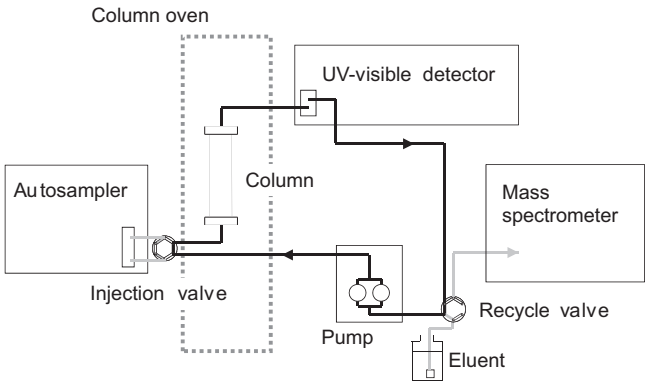

Fig. 1. Diagram of recycle HPLC with mass spectrometer.

umn temperature, and injection volume were 0.5 mL/min, 210 nm, 25 °C, and 10 μL, respectively.

2.4. Recycle HPLC conditions

2.4.1. Recycle HPLC conditions for TAG-enantiomer separation

The recycle HPLC system (Fig. 1) was composed of a recycle pump (PU712R, GL Sciences Inc., Tokyo, Japan), an autosampler (GL-7420, GL Sciences Inc.), a column oven (CO705C, GL Sciences Inc.), UV–visible detector (UV702, GL Sciences Inc.), and two automatic valves (VALVE UNIT 401, FLOM Co., Ltd., Tokyo, Japan), making the analytes pass through the same column repeatedly and cutting the dead volume of the autosampler during a recycle run. Operational software (EZChrom *Elite*, Agilent Technologies, Inc., Santa Clara, CA) was used to control the recycle HPLC system. An APCI-MS (Quattro micro API, Waters Corporation, Milford, MA) and its operational software (MassLynx Ver. 4.1, Waters Corporation) were used as a detector, in combination with the recycle HPLC system. A chiral column (CHIRALCEL OD-RH, 4.6 mm i.d. × 150 mm, 5 μm) and a guard cartridge (CHIRALCEL OD-RH, 4.0 mm i.d. × 10 mm, 5 μm, Daicel Chemical Industries, Ltd.) having cellulose tris-(3,5-dimethylphenylcarbamate) coated on silica gel as a stationary phase were used for the resolution of the TAG-enantiomers, where the column temperature was 25 °C. Methanol (100%) was used as the mobile phase at a flow rate of 0.5 mL/min. The injection volume was 20 μL. The wavelength of UV–visible detector was 205 nm.

Asymmetric TAGs (Fig. 2), *rac*-PPO, *sn*-POO, *rac*-OOP, and *sn*-OOP were dissolved in 2-propanol, adjusted to 1000 μg/mL, and used as standard solutions. *rac*-PPO/*sn*-PPO (or *rac*-OOP/*sn*-OOP) mixed solution was prepared by mixing of equal amounts of *rac*-PPO and *sn*-PPO (or *rac*-OOP and *sn*-OOP) standard solutions. *rac*-PPL, *rac*-OOL, and *rac*-PPS were dissolved in 2-propanol, adjusted to 500, 1000, 250 μg/mL, respectively, and used as standard solutions. A 20 μL aliquot of the standard solution was injected from the autosampler, and the peaks of the target TAG-enantiomers, having passed through the columns and UV–visible detector, were returned to the entrance of the recycle pump by a recycle valve during the recycle mode. After the target TAG-enantiomers were sufficiently resolved by the recycle run, they were forced into the mass spectrometer through the recycle valve, which changed the outlet flow pass of the UV–visible detector from the entrance of the recycle pump to the mass spectrometer. The APCI-MS parameters were as follows: polarity, APCI positive; corona current, 3.0 μA; source temperature, 120 °C; desolvation temperature, 450 °C; cone gas flow, 50 L/h; desolvation gas flow, 200 L/h; data acquisition mode, full scan over an m/z range of 500–1000; scan time, 0.5 s; and cone voltage, 20 V.

2.4.2. Recycle HPLC conditions for TAG-PI separation

The above-mentioned recycle HPLC system was also used to separate the TAG-PIs, *sn*-POP/*rac*- (or *sn*-) PPO, and *sn*-OPO/*rac*- (or *sn*-) OOP. TAGs, *sn*-POP/*rac*-PPO mixture, *rac*-PPO, *sn*-PPO, *sn*-OPO/*rac*-OOP mixture, *rac*-OOP, and *sn*-OOP were dissolved in 2-propanol, each of them was adjusted to 1000 μg/mL, and used as standard solutions. Tandem-jointed non-endcapped polymeric ODS columns

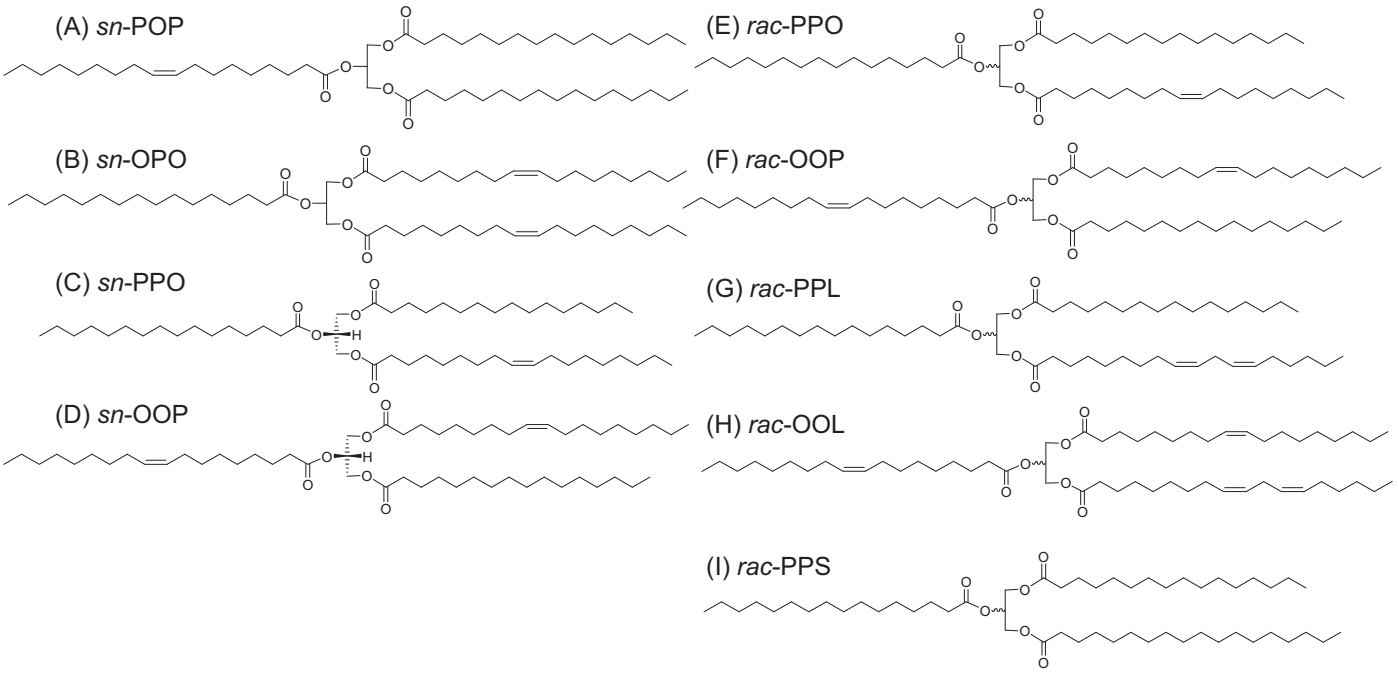

Fig. 2. The structures of symmetric and asymmetric TAGs used in this study. (A) 1,3-dipalmitoyl-2-oleoyl-*sn*-glycerol (*sn*-POP), (B) 1,3-dioleoyl-2-palmitoyl-*sn*-glycerol (*sn*-OPO), (C) 1,2-dipalmitoyl-3-oleoyl-*sn*-glycerol (*sn*-PPO), (D) 1,2-dioleoyl-3-palmitoyl-*sn*-glycerol (*sn*-OOP), (E) 1,2-dipalmitoyl-3-oleoyl-*rac*-glycerol (*rac*-PPO), (F) 1,2-dioleoyl-3-palmitoyl-*rac*-glycerol (*rac*-OOP), (G) 1,2-dipalmitoyl-3-linoleoyl-*rac*-glycerol (*rac*-PPL), (H) 1,2-dioleoyl-3-linoleoyl-*rac*-glycerol (*rac*-OOL), and (I) 1,2-dipalmitoyl-3-stearoyl-*rac*-glycerol (*rac*-PPS).

(Inertsil ODS-P, 4.6 mm i.d. × 250 mm, 5 μm, GL Sciences Inc.) were employed, and the column temperatures for the resolution of *sn*-POP/*rac*-PPO and *sn*-OPO/*rac*-OOP were 25 °C and 10 °C, respectively [24]. Acetonitrile/2-propanol/hexane (3/2/1, v/v/v) was used as the mobile phase at a flow rate of 1.0 mL/min. The injection volume was 10 μL. The wavelength of UV–visible detector was 205 nm. All of the APCI-MS parameters were the same as for the TAG-enantiomers, except for the cone voltage. To enhance the production of DAG ions by in-source collision-induced dissociation (CID), the cone voltage was adjusted to 40 V.

2.5. Analysis of the ratio of sn-OOP and sn-POO in palm oil

2.5.1. Preparative isolation of sn-OOP and sn-POO fraction by reversed-phase HPLC

A 20 μL aliquot of 0.5% acetone solution of palm oil was chromatographed on the polymeric ODS columns at 10 °C using recycle system. The HPLC conditions were shown in Section 2.4.2. After *sn*-OOP and *sn*-POO were separated from *sn*-OPO by three cycles of recycle run, a peak containing *sn*-OOP and *sn*-POO was collected and the solvent was evaporated.

2.5.2. Chiral separation of sn-OOP/sn-POO mixed fraction

The dried residue obtained in Section 2.5.1 was dissolved in 200 μL of 2-propanol and a 20 μL aliquot of the solution was analyzed by chiral HPLC with recycle system and APCI-MS. The HPLC condition used was shown in Section 2.4.1. For more selective detection of *sn*-OOP and *sn*-POO by APCI-MS, the multiple reaction monitoring (MRM) mode was used and the protonated molecule $[M+H]^+$ at *m/z* 860 and the $[M-RCO_2]^+$ ion at *m/z* 578 were chosen as the parent and the daughter ions. The cone voltage and collision energy were 20 and 26 V, respectively.

3. Results and discussion

3.1. Chiral column screening

We initially screened the chiral columns to resolve the *rac*-PPO into individual enantiomers. Of all the screened columns which have polysaccharide-based CSPs, only CHIRALCEL OD-RH in combination with methanol as the mobile phase slightly separated the *rac*-PPO peaks into those of individual enantiomers, with no other column resolving the *rac*-PPO (data not shown). We confirmed that none of the chiral columns we tested could separate *rac*-PPO into its individual enantiomers when acetonitrile was used as the mobile phase. The addition of distilled water to methanol (or acetonitrile) made it impossible to elute rac-PPO from the columns. Methanol should therefore be used as the mobile phase. Although Iwasaki et al. separated *sn*-ECC/*sn*-CCE and *sn*-DCC/*sn*-CCD using normal-phase HPLC on CHIRALCEL OD, which has the same CSP as CHIRALCEL OD-H and OD-RH [30], CHIRALCEL OD-H used with a normal-phase mode in our screening was not capable of separating *rac*-PPO. CHIRALPAK IA, IB, and AD-RH have 3,5-dimethylphenylcarbamate in their CSP as well as CHIRALCEL OD-RH, but they could not separate *rac*-PPO. In particular, CHIRALPAK IB employs the same chiral selector as CHIRALCEL OD-RH, except that it is immobilized on silica gel in CHIRALPAK IB. The immobilization of the chiral selector on silica gel could affect its conformation [33], which would make it impossible to resolve *rac*-PPO. CHIRALPAK AD-RH uses chiral coated stationary phase, just like CHIRALCEL OD-RH, but employs amylose-based chiral selector. CHIRALCEL OJ-RH has different chiral selector than CHIRALCEL OD-RH though it also employs cellulose, and its chiral selector is coated on silica gel just like CHIRALCEL OD-RH. These results indicate that the combination of the phenylcarbamate derivatives and polysaccharides in the chiral selector, and method for modifying the surface of silica gel using the chiral selector. Namely, the CSP of CHIRALCEL OD-RH, cellulose tris-(3,5-dimethylphenylcarbamate) coated on silica gel, is indispensable for the separation of *rac*-PPO. Use of methanol as the mobile phase is also necessary to separate them. The CSP of CHIRALCEL OD-RH in methanol presumably forms the higher-order structure required for the enantiomeric recognition of *rac*-PPO. Therefore, we employed CHIRALCEL OD-RH with methanol as the mobile phase for subsequent TAG enantiomer analyses, in combination with the recycle HPLC system for the high-resolution separation.

3.2. Elution order of each enantiomer of rac-PPO and rac-OOP

Fig. 3A shows the separation of *rac*-PPO into its enantiomers on CHIRALCEL OD-RH, on which the enantiomers were gradually resolved during a recycle run with the peaks monitored by UV light (205 nm). The baseline level gradually increased during the recycle run because the signal level of UV absorbance of *rac*-PPO was not sufficient for neglecting the increase of baseline level. After the separation, these peaks were detected by APCI-MS using the full scan mode. Fig. 4A–C shows extracted ion chromatograms (XICs) of *m/z* 834, corresponding to the protonated molecules of PPO, with Fig. 4A showing the separation of *rac*-PPO after the recycle run shown in Fig. 3A. To ascertain the elution order of a racemic mixture of *sn*-PPO and *sn*-OPP, *sn*-PPO was simultaneously chromatographed with *rac*-PPO. Fig. 4B and C shows XICs of *sn*-PPO and a mixture of *sn*-PPO/*rac*-PPO, respectively. These results reveal that *sn*-PPO is eluted after *sn*-OPP. In addition, all of the mass spectra of these enantiomers were nearly identical to each other (data not shown). Furthermore, to verify the chiral separation of *rac*-PPO, we examined whether *sn*-POP was contained in *rac*-PPO and *sn*-PPO because *sn*-POP could be generated in them as a by-product if *sn*-1,3-PP is generated from *sn*-1,2-PP (or *rac*-1,2-PP) by acyl migration during synthesis of *sn*-PPO (or *rac*-PPO) [34]. Fig. 5A shows the separation of *sn*-POP and *rac*-PPO obtained by the recycle HPLC system equipped with the non-endcapped polymeric ODS column described in our previous report [24]. None of

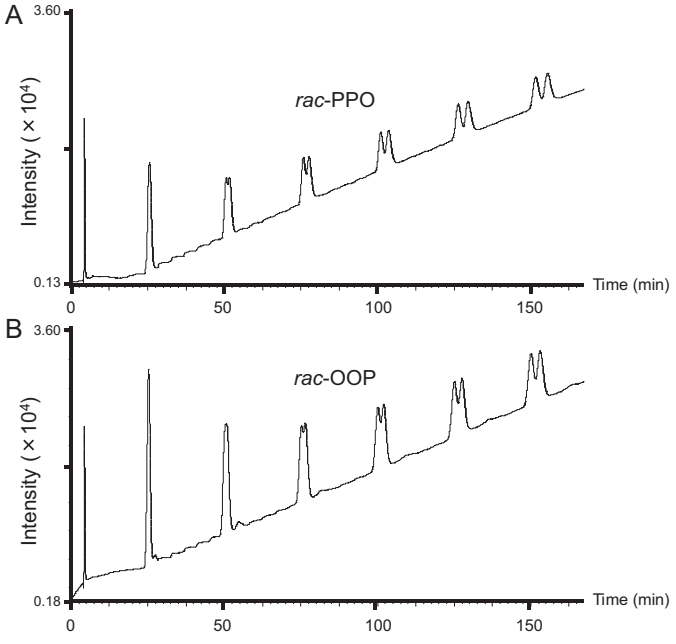

Fig. 3. Enantiomeric separation of (A) *rac*-PPO and (B) *rac*-OOP on a CHIRALCEL OD-RH column during a recycle run. Column, CHIRALCEL OD-RH (4.6 mm i.d. × 150 mm, 5 μm); column temperature, 25 °C; mobile phase, methanol; flow rate, 0.5 mL/min; detection, 205 nm UV.

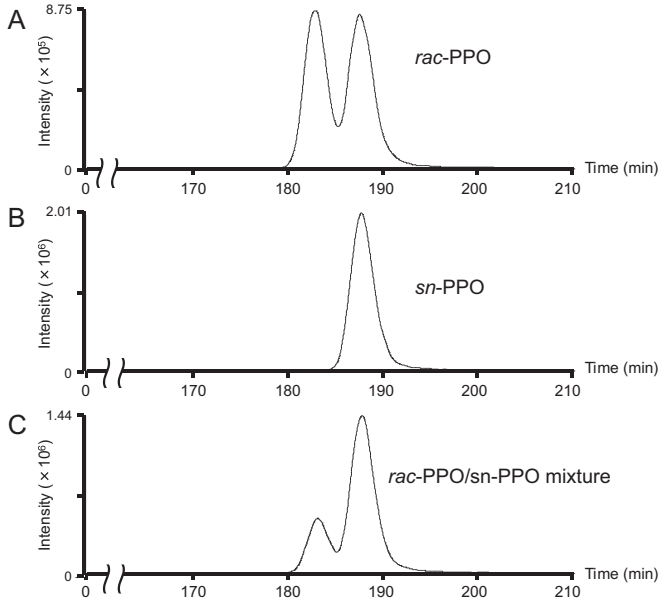

Fig. 4. Enantiomeric separation of (A) *rac*-PPO, (B) *sn*-PPO, and (C) *rac*-PPO/*sn*-PPO mixture after the end of recycle run shown in Fig. 3A. Column, CHIRALCEL OD-RH (4.6 mm i.d. × 150 mm, 5 μm); column temperature, 25 °C; mobile phase, methanol; flow rate, 0.5 mL/min; detection, APCI-MS (XIC of *m/z* 834).

the chromatograms of *rac*-PPO and *sn*-PPO showed contamination by *sn*-POP (Fig. 5B and C). It is likely that the carbon content of the polymeric ODS column and the column temperatures used in this separation might cause peak tailing. The carbon content of this column is higher than that of other ODS columns. The column temperatures for the separation of *sn*-POP/*rac*-PPO and *sn*-OPO/*rac*-OOP are 25 °C and 10 °C, respectively, which are close to the melting points of these TAGs. The APCI mass spectra shown in Fig. 6 also confirm the positional isomeric purities of *rac*-PPO and *sn*-PPO (Fig. 6C and D), because *rac*-PPO and *sn*-PPO formed DAG ions at

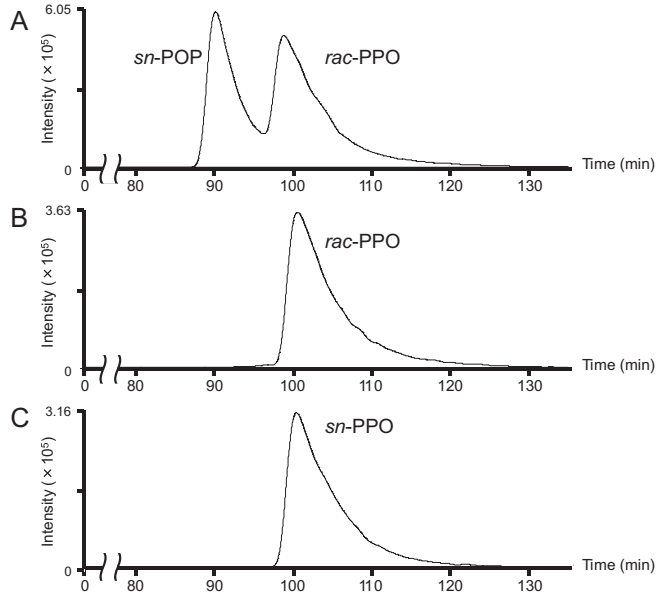

Fig. 5. Separation of TAG-PI on non-endcapped polymeric ODS columns using recycle HPLC. (A) *sn*-POP/*rac*-PPO mixture, (B) *rac*-PPO, and (C) *sn*-PPO. Column, tandem-jointed Inertsil ODS-P (4.6 mm i.d. × 250 mm, 5 μm); column temperature, 25 °C; mobile phase, acetonitrile/2-propanol/hexane (3/2/1, v/v/v); flow rate, 1.0 mL/min; detection, APCI-MS (XIC of *m/z* 851).

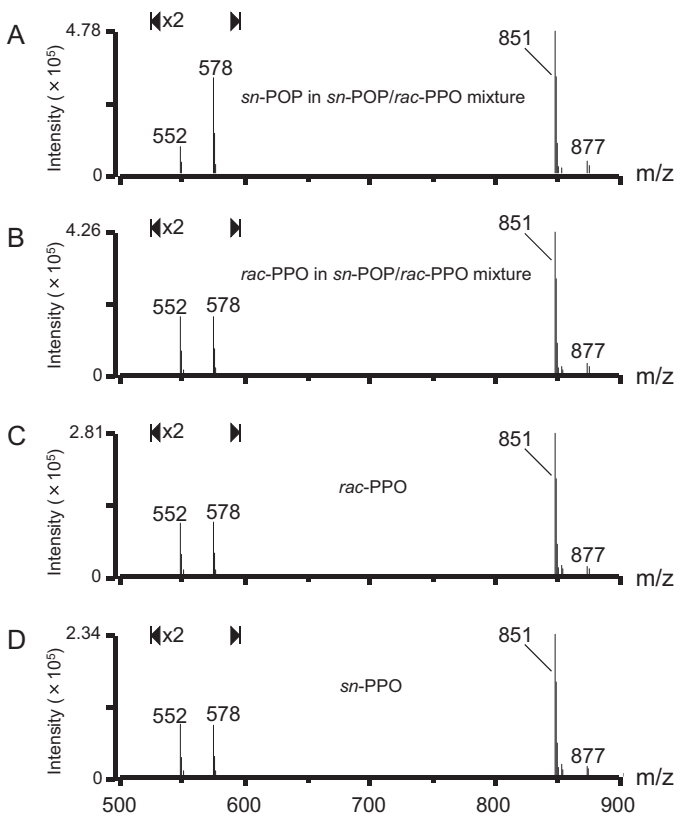

Fig. 6. Mass spectra of (A) *sn*-POP separated from *sn*-POP/*rac*-PPO mixture, (B) *rac*-PPO separated from *sn*-POP/*rac*-PPO mixture, (C) *rac*-PPO, and (D) *sn*-PPO. *m/z* 851, 578, and 552 are corresponding to $[PPO + NH_4]^+$, $[PO]^+$, and $[PP]^+$, respectively.

the same ratio, *m/z* 552 and 578, corresponding to $[PP]^+$ and $[PO]^+$, respectively.

Similarly, Fig. 3B shows the separation of *rac*-OOP into its enantiomers on the same chiral column as *rac*-PPO. Fig. 7A–C shows

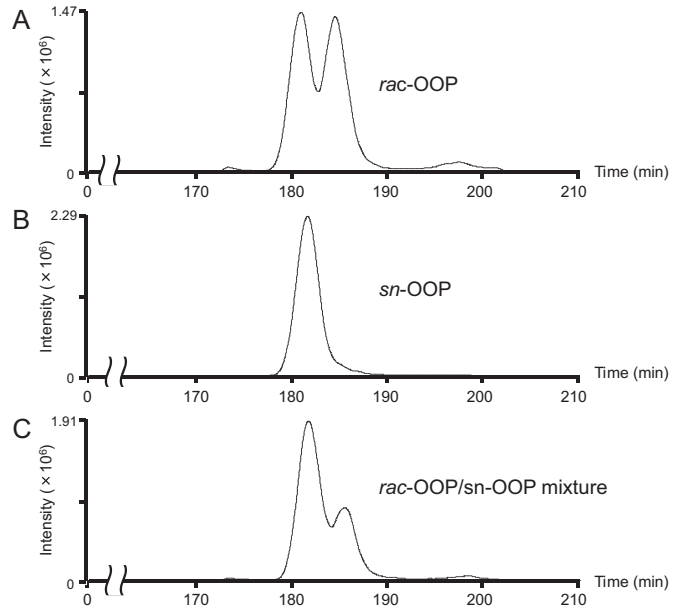

Fig. 7. Enantiomeric separation of (A) *rac*-OOP, (B) *sn*-OOP, and (C) *rac*-OOP/*sn*-OOP mixture after the end of recycle run shown in Fig. 3B. Column, CHIRALCEL OD-RH (4.6 mm i.d. × 150 mm, 5 μm); column temperature, 25 °C; mobile phase, methanol; flow rate, 0.5 mL/min; detection, APCI-MS (XIC of *m/z* 860).

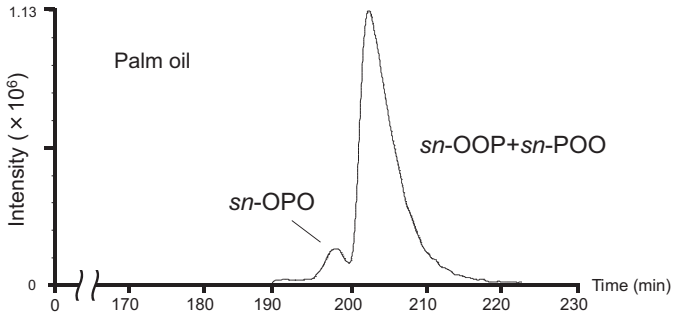

Fig. 8. Preparative isolation of *sn*-OOP and *sn*-POO in palm oil by non-endcapped polymeric ODS columns using recycle HPLC. Column, tandem-jointed Inertsil ODS-P (4.6 mm i.d. × 250 mm, 5 μm); column temperature, 10 °C; mobile phase, acetonitrile/2-propanol/hexane (3/2/1, v/v/v); flow rate, 1.0 mL/min; detection, APCI-MS (XIC of *m/z* 877).

XICs of *m/z* 860, corresponding to the protonated molecule of OOP, and Fig. 7A shows the separation of *rac*-OOP after the recycle run shown in Fig. 3B. Fig. 7B and C shows XICs of *sn*-OOP and a mixture of *sn*-OOP/*rac*-OOP, respectively. These chromatograms show that *sn*-OOP is eluted before *sn*-POO. All of the mass spectra of these enantiomers were nearly identical to each other (data not shown). No contaminations in *rac*-OOP and *sn*-OOP by *sn*-OPO were confirmed by the same HPLC condition except for the column temperature (10 °C), as along with *sn*-POP in *rac*-PPO and *sn*-PPO. The chromatograms and APCI mass spectra were shown in Supplementary material 5 and 6. These data indicate that *rac*-PPO and *rac*-OOP can be separated by CHIRALCEL OD-RH; *sn*-OPP and *sn*-OOP, which have an oleic acid moiety at the *sn*-1 position, are eluted before *sn*-PPO and *sn*-POO, respectively.

3.3. Analysis of the ratio of sn-OOP and sn-POO in palm oil

We carried out enantiomeric resolution of *sn*-OOP/*sn*-POO in palm oil using our method. Before this chiral separation, it was necessary to remove *sn*-OPO from the sample solution of palm oil by reversed-phase HPLC for TAG-positional isomer separation (Fig. 8) because *sn*-OPO was overlapping with *sn*-OOP and *sn*-POO and these TAGs were detected by the same MRM channel. This preparative isolation and chiral separation of *sn*-OOP/*sn*-POO in palm oil revealed that the *sn*-OOP/*sn*-POO ratio in palm oil was approximately 3/2 (Fig. 9). The peak area ratio was regarded as the amount ratio because the same responses were obtained from respective enantiomers of *rac*-OOP in XIC (Fig. 7A) and MRM (data not shown) chromatograms of APCI-MS.

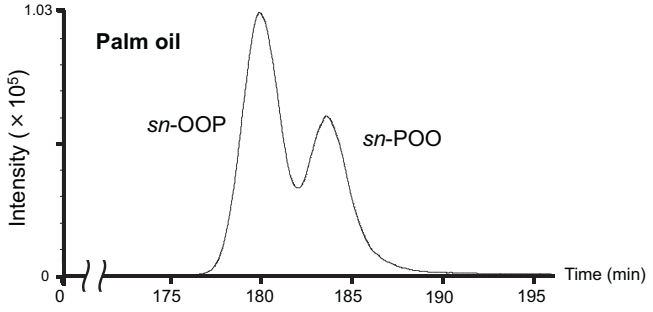

Fig. 9. Enantiomeric separation of *sn*-OOP/*sn*-POO fraction of palm oil. Column, CHIRALCEL OD-RH (4.6 mm i.d. × 150 mm, 5 μm); column temperature, 25 °C; mobile phase, methanol; flow rate, 0.5 mL/min; detection, APCI-MS (MRM mode, $[M+H]^+$ at *m/z* 860 and the $[M-RCO_2]^+$ ion at *m/z* 578 were chosen as the parent and the daughter ions).

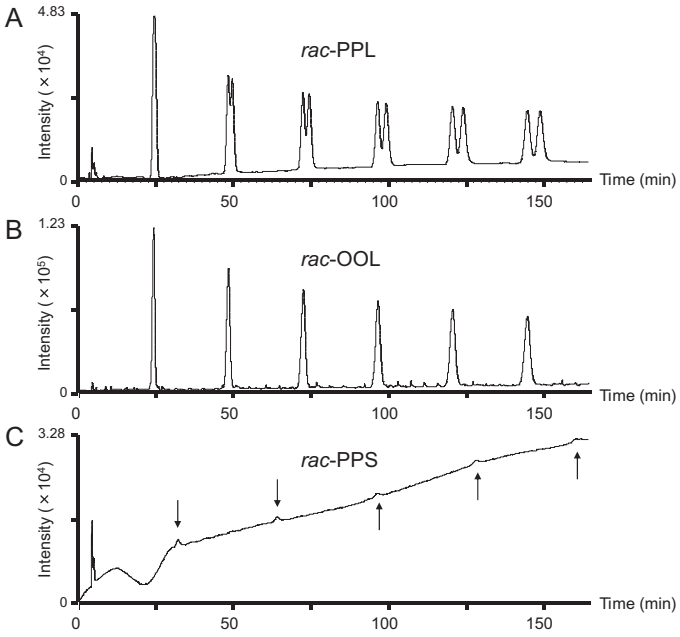

Fig. 10. UV chromatograms of (A) *rac*-PPL, (B) *rac*-OOL, and (C) *rac*-PPS under the condition of enantiomeric separation for *rac*-PPO and *rac*-OOP using a recycle run. Column, CHIRALCEL OD-RH (4.6 mm i.d. × 150 mm, 5 μm); column temperature, 25 °C; mobile phase, methanol; flow rate, 0.5 mL/min; detection, 205 nm UV.

3.4. The structures of asymmetric TAG for enantiomeric separation

To investigate the structural characteristics of asymmetric TAG, which need to be resolved on CHIRALCEL OD-RH, we examined whether *rac*-PPL, *rac*-OOL, and *rac*-PPS could be resolved by the same column. As a result, *rac*-PPL was separated (Fig. 10A), but *rac*-OOL and *rac*-PPS were not (Fig. 10B and C). The results for *rac*-PPO, *rac*-PPL, and *rac*-PPS suggest that an unsaturated fatty acid such as oleic acid or linoleic acid bound to the *sn*-1 or *sn*-3 position enables the resolution of asymmetric TAGs having two palmitic acid moieties on a CHIRALCEL OD-RH column. In the case of asymmetric TAGs having two oleic acid moieties, the data show that *rac*-OOL, which has a linoleic acid moiety at the *sn*-1 or *sn*-3 position instead of a palmitic acid in contrast to *rac*-OOP, could not be resolved. These chiral recognition mechanisms could be explained by the "three-point attachment model", which is generally cited for explaining the principle of chiral recognition [35,36]. In this model, at least three configuration-dependent attractive contact points between the chiral receptor and chiral substrate are necessary. In the case of asymmetric TAGs such as *sn*-PPO, the two kinds of palmitic acids bound at the *sn*-1 and *sn*-2 positions might show two different hydrophobic interactions, and one oleic acid at the *sn*-3 position shows π–π interactions with the chiral stationary phase. These three kinds of interactions may cause chiral separation of *rac*-PPO. This theory would also explain the chiral separation of *rac*-OOP and *rac*-PPL. Although we assumed that three-point attachment models consisting of only palmitic acid and oleic acid (or linoleic acid) enable chiral separation of *rac*-PPO, *rac*-OOP, and *rac*-PPL, we have to examine whether differences in the acyl chain length or the number and position of the double bonds in the asymmetric TAG molecule would cause three different interactions with the chiral stationary phase. Further investigation of the chiral separation of asymmetric TAGs having saturated fatty acids (short-, medium-, or long-chain fatty acids), monounsaturated fatty acids, and HUFAs and their isomers is necessary in future studies.

4. Conclusions

This study revealed that CHIRALCEL OD-RH having cellulose tris-(3,5-dimethylphenylcarbamate) coated on silica gel could resolve racemic mixtures of asymmetric TAGs, namely, *rac*-PPO, *rac*-OOP, and *rac*-PPL into the individual enantiomers, in combination with recycle HPLC. Our new chiral separation method in combination with APCI-MS was capable of determining the ratio of *sn*-OOP to *sn*-POO in palm oil. This is the first report of the enantiomeric separation of asymmetric TAGs found in nature. Further studies are necessary to clarify the ability of the chiral column to recognize other acyl moieties such as other HUFAs, trans fatty acids, and saturated fatty acids having different acyl chains lengths. The elucidation of the TAG structures that need to be resolved by the CSP will provide us with a detailed recognition mechanism. This method can be applied to analyses of asymmetric TAGs in nature, which will lead to new findings in studies on lipid metabolism and the physical properties of edible oils and fats.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.02.067.

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