Abundances of Triacylglycerol Positional Isomers and Enantiomers Comprised of a Dipalmitoylglycerol Backbone and Short- or Medium-chain Fatty Acids in Bovine Milk Fat

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Abstract: Bovine milk fat (BMF) is composed of triacylglycerols (TAG) rich in palmitic acid (P), oleic acid (O), and short-chain or medium-chain fatty acids (SCFAs or MCFAs). The composition and binding positions of the fatty acids on the glycerol backbone determine their physical and nutritional properties. SCFAs and MCFAs are known to characteristically bind to the sn-3 position of the TAGs in BMF; however, there are very few non-destructive analyses of TAG enantiomers binding the fatty acids at this position. We previously reported a method to resolve the enantiomers of TAGs, binding both long-chain saturated fatty acid and unsaturated fatty acid at the sn-1 and 3 positions, in palm oil, fish oil, and marine mammal oil using chiral HPLC. Here, we further developed a method to resolve several TAG enantiomers containing a dipalmitoyl (PP) glycerol backbone and one SCFA (or MCFA) in BMF. We revealed that the predominant TAG structure in BMF was homochiral, such as 1,2-dipalmitoyl-3-butyroyl-sn-glycerol. This is the first quantitative determination of many TAG enantiomers, which bind to a SCFA or MCFA, in BMF was evaluated simultaneously. Furthermore, the results indicated that the amount ratios of the positional isomers and enantiomers of TAGs consisting of a dipalmitoyl (PP) glycerol backbone and SCFA (or MCFA), resembled the whole TAG structures containing the other diacylglycerol backbones consisting of P, O, myristic acid, and/or stearic acid in BMF.

Key words: chiral separation, enantiomers, milk fat, triacylglycerol

1 INTRODUCTION

Triacylglycerols (TAGs), which are esters composed of three fatty acids and one glycerol molecule, are an important energy source¹. The structure of TAGs and the binding positions of the fatty acids on the glycerol backbone are shown in Fig. 1 using a Fischer projection². To better understand the structure of edible oils and fats, it is important to examine both the fatty acid composition and their binding positions on the glycerol backbone. Brockerhoff developed a stereospecific analytical method using phospholipase A₂ that enabled the analysis of the fatty acid composition at the sn-1, 2, and 3 positions³⁻⁵. Itabashi et al. developed another stereospecific method using chiral HPLC⁶⁻⁷. These analytical techniques have been used in many studies on the positional distributions of fatty acids in TAGs from various organisms in nature⁸⁻¹⁰.

Bovine milk contains about 3.5% to 5% total lipid, and 98% or more of the lipid is TAG¹¹. Bovine milk fat (BMF) is rich in palmitic acid (P) and oleic acid (O). Long-chain saturated fatty acids, such as myristic acid (M), P, and stearic
acid (S), are mainly distributed at the sn-1 and 2 positions\textsuperscript{12}. In addition, the milk fat of ruminants such as BMF distinctively contains short- and medium-chain fatty acids (SCFAs and MCFAs), including butyric acid (C\textsubscript{4}), caproic acid (C\textsubscript{6}), and caprylic acid (C\textsubscript{8}); these fatty acids are esterified predominantly at the sn-3 position\textsuperscript{10,12}. Similar trends are observed in rat milk fat (RMF) and human milk fat (HMF)\textsuperscript{13,14}. Although RMF and HMF do not contain C\textsubscript{4}–C\textsubscript{6} and C\textsubscript{4}–C\textsubscript{8}, respectively, the C\textsubscript{8} in RMF, and the capric acid (C\textsubscript{10}) and lauric acid (C\textsubscript{12}) in HMF, which are shorter fatty acids in their respective milk fats, are mainly bound to the sn-3 positions. These SCFAs and MCFAs at the sn-3 position are selectively hydrolyzed by lingual lipase or gastric lipase in the stomach, and then absorbed into gastric mucosa\textsuperscript{15,16}. The energy obtained by the action of these stereoselective lipases is essential for newborns, who require an efficient energy supply from milk fat.

The positional distribution of fatty acids in BMF\textsuperscript{12} indicates that the TAGs of BMF are likely to be homochiral. However, studies using the positional distribution of fatty acids do not show concrete TAG molecular species with intact structures, where TAG molecular species only refers to the combination of three fatty acids on the glycerol backbone without consideration of their binding positions. The physical and nutritional properties of oils and fats depend upon the TAG structure\textsuperscript{17}, which consists of both the constituent fatty acids and their binding positions. To investigate the structure of TAGs, their positional isomers and enantiomers should be analyzed. Herein, a TAG positional isomer refers to TAGs where the composition of the fatty acids is identical but only the fatty acid at the β-position (sn-2 position) is fixed, e.g., β-POP and β-PPO are positional isomers. The three letters refer to the combination of fatty acids on the glycerol backbone and β is a prefix that signifies that the fatty acid at the β-position is fixed\textsuperscript{2}. In β-PPO, whether O is bound at the sn-1 or 3 position is unclear. However, an asymmetric TAG could be comprised of enantiomers because the carbon atom at the sn-2 position forms a stereogenic center. When enantiomers are considered, β-PPO is recognized as a mixture of two enantiomers, sn-PPO and sn-OPP. A TAG enantiomer designated as ‘sn-PPO’ is a TAG binding two Ps and one.

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**Fig. 1** Structures of the triacylglycerol (TAGs) standards. A) Symmetric TAGs, B) asymmetric TAGs (single enantiomer), C) asymmetric TAG (racemate) comprised of two palmitic acids (Ps) and one short-chain or medium-chain fatty acid (SCFA or MCFA).
O at the sn-1, 2, and 3 positions, in that order. A mixture of an equal amount of sn-PPO and sn-OPP is designated as rac-PPO or rac-OPP, because it is racemic.

Recently, we developed two separation methods for TAG positional isomers and enantiomers. We showed that pairs of TAG positional isomers, which consisted of two long-chain saturated fatty acids and one other fatty acid (long-chain unsaturated fatty acid, SCFA, or MCFA), such as β-POP/β-PPO, β-PC₃P/β-PPC₄, or β-PC₁₀P/β-PPC₁₀, were resolved using reversed-phase HPLC on an octacosyl(C28) column. We also showed that racemic mixtures of asymmetric TAGs binding both long-chain saturated fatty acid and unsaturated fatty acid at the sn-1 and 3 positions, such as rac-PPO, rac-PPL, and rac-POO, were resolved using chiral HPLC on a polysaccharide-based chiral column. In the separation of TAG positional isomers using a C28 column, we showed that the difference between saturated and unsaturated fatty acids, and the difference in the length of the acyl chains, contributed to the resolution of the TAG positional isomers. However, there is no data on the chiral separation of asymmetric TAGs consisting of only saturated fatty acids, where the acyl chain lengths are different in the sn-1 and 3 positions, such as rac-PPC₄. Here, we examined whether our chiral HPLC method could separate racemic mixtures of asymmetric TAGs that consisted of two palmitic acids and one SCFA (or MCFA) in BMF. We then applied our two separation methods to the quantification of BMF TAG positional isomers and enantiomers consisting of two palmitic acids and one of C₆, C₈, C₁₀, or C₁₂, because P is a major fatty acid in BMF, and the structure of whole TAGs with SCFA or MCFA in BMF is presumably represented by the profile of TAG isomers comprised of dipalmitoyl glycerol backbone and SCFA (or MCFA).

2 EXPERIMENTAL PROCEDURES

2.1 Chemicals and Materials

The TAG isomers shown in Fig. 1 were in-house products (Tsukishima Foods Industry Co., Ltd., Tokyo, Japan). All of the other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The butter was purchased at a supermarket in Tokyo, Japan.

2.2 Lipid Extraction from Butter

Butter (1 g) was diluted in 9 mL of 1% saline solution. The diluted butter solution was then added to 20 mL of a chloroform and methanol (2/1, v/v) mixture in a test tube equipped with a screw cap, which was vigorously using a vortex mixer, and centrifuged at 1,500 × g for 10 min. The aqueous phase was extracted two more times using the same method. The combined bottom layers were collected and were concentrated using a rotary evaporator under vacuum. The BMF thus obtained was stored in an argon-purged screw-capped vial at −30°C until analysis.

2.3 Analysis of extracted ion chromatograms corresponding to diacylglycerol ions in BMF

BMF (8 mg) was dissolved in 1 mL of acetone. A 20 μL aliquot of the sample solution was then injected into an HPLC (Alliance e2695, Waters Corporation, Milford, MA) with a C28 reversed-phase column (Sunrise C28, 4.6 mm i.d. × 250 mm, 5 μm, ChromaNik Technologies Inc., Osaka, Japan) with a mass spectrometer (MS) detector using an atmospheric pressure chemical ionization (APCI) probe (Quattro micro API, Waters Corporation). The column temperature, and flow rate were 35°C and 1.0 mL/min, respectively. The composition of the mobile phase was varied during analysis using a linear gradient elution mode. The initial composition of the mobile phase was acetonitrile/acetone/acetonitrile (50/50, v/v); the ratio of acetonitrile was then linearly increased to acetonitrile/acetone (100/0, v/v) over a period of 30 min, and then held constant until all components were eluted. The corona current, cone voltage, source block temperature, desolvation temperature, desolvation gas flow rate, and cone gas flow rate of the APCI ion source were 3.0 μA, +20 V, 120°C, 450°C, 200 L/h, and 50 L/h, respectively. A total ion current chromatogram (TICC) was obtained using a full scan mode over the range of m/z 200–1000. The extracted ion chromatograms (EICs) of [TAG–RCOO]⁺, which corresponded to diacylglycerol (DAG) ions produced from the in-source collision-induced dissociation, were obtained by data processing of the TICC.

2.4 Quantification of TAG positional isomers

Five pairs of TAG positional isomer standards, sn-PC₃P/rac-PPC₄ (where n = 4, 6, 8, 10, and 12) were mixed together and dissolved in 2-propanol. The concentration of each TAG isomer was adjusted to either 250, 200, 150, 100, 50, or 5 μg/mL and 100 μg/mL of triundecanoin was added as an internal standard (IS) to obtain calibration curves. A sample solution of 50 mg of the BMF obtained in Section 2.2 and 1.0 mg of triundecanoin as the IS were weighed into a 10 mL volumetric flask, which was filled with 2-propanol. For a recovery test, 0.50 mg (50 μg/mL) of each of the TAG isomer standards, i.e., sn-PC₃P and rac-PPC₈ (where n = 4, 6, 8, 10, and 12), were added to the sample solution. The ammonium adduct ion of each TAG and the dipalmitoyl glycerol ion were selected as the precursor and product ions, respectively, for the selected reaction monitoring (SRM) channels. The SRM transitions for PPC₄, PPC₆, PPC₈, PPC₁₀, and PPC₁₂ were m/z 656 → 551, 684 → 551, 712 → 551, 740 → 551, and 768 → 551, respectively (Table 1). The cone voltage and collision energy were 35 V and 20 eV, respectively, for both PPC and PPC₈, 40 V and 20 eV, respectively, for PPC₆; and 40 V and 25 eV, respectively, for PPC₄.
Here, the standards of the TAG positional isomers were expressed as β-PCnP/rac-PPCn (where n = 4, 6, 8, 10, and 12) instead of sn-PCnP/rac-PPCn (where n = 4, 6, 8, 10, and 12) to identify the TAG positional isomers in the standards and BMF in the same way.

Recall that both PPC10 and PPC12. The SRM transition for the triundecanoyl IS was m/z 614→411. The cone voltage and collision energy for triundecanoyl were 30 V and 25 eV, respectively. The setup of LC-MS/MS and the C28 column were identical to that in Section 2.3. The column temperature, flow rate, and the mobile phase were 80/20, v/v; the ratio of acetone was then linearly increased up to acetone/acetonitrile (100/0, v/v) over 40 min, and then held constant until all components were eluted. The operating conditions of the APCI ion source were identical to those listed in Section 2.3.

The calibration curves were obtained using the peak areas of the SRM chromatograms, and the sample solutions were analyzed in the same way. The abundance ratios and recoveries of each of the TAG positional isomers in the standards and BMF were analyzed in the same way. The abundance ratios and recoveries of each of the TAG positional isomers in the standards and BMF were analyzed in the same way. The abundance ratios and recoveries of each of the TAG positional isomers in the standards and BMF were analyzed in the same way.

### Table 1

<table>
<thead>
<tr>
<th>TAG</th>
<th>SRM transition</th>
<th>Equation</th>
<th>r</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-PCP₄</td>
<td>656→551</td>
<td>y = 1.09 × 10⁻³ x</td>
<td>0.994</td>
<td>124</td>
</tr>
<tr>
<td>β-PPC₄</td>
<td>656→551</td>
<td>y = 1.71 × 10⁻³ x</td>
<td>0.994</td>
<td>105</td>
</tr>
<tr>
<td>β-PCP₆</td>
<td>684→551</td>
<td>y = 1.19 × 10⁻³ x</td>
<td>0.996</td>
<td>119</td>
</tr>
<tr>
<td>β-PPC₆</td>
<td>684→551</td>
<td>y = 1.98 × 10⁻³ x</td>
<td>0.994</td>
<td>106</td>
</tr>
<tr>
<td>β-PC₈</td>
<td>712→551</td>
<td>y = 1.08 × 10⁻³ x</td>
<td>0.990</td>
<td>119</td>
</tr>
<tr>
<td>β-PPC₈</td>
<td>712→551</td>
<td>y = 1.91 × 10⁻³ x</td>
<td>0.985</td>
<td>116</td>
</tr>
<tr>
<td>β-PC₁₀P</td>
<td>740→551</td>
<td>y = 1.14 × 10⁻³ x</td>
<td>0.986</td>
<td>110</td>
</tr>
<tr>
<td>β-PPC₁₀P</td>
<td>740→551</td>
<td>y = 1.77 × 10⁻³ x</td>
<td>0.987</td>
<td>113</td>
</tr>
<tr>
<td>β-PC₁₂P</td>
<td>768→551</td>
<td>y = 0.907 × 10⁻³ x</td>
<td>0.974</td>
<td>97</td>
</tr>
<tr>
<td>β-PPC₁₄P</td>
<td>768→551</td>
<td>y = 1.48 × 10⁻³ x</td>
<td>0.989</td>
<td>120</td>
</tr>
</tbody>
</table>

y: area ratio (analyte/IS), x: concentration (µg/mL)

**Limited of detection:** 1.7 µg/mL (S/N>3), **Limited of quantification:** 5.6 µg/mL (S/N>10)

#### 2.5 Fractionation of β-PPCₙ(where n=4, 6, 8, 10, and 12) from BMF

Collection of the asymmetric TAG positional isomers from the BMF was performed using the method described in Section 2.4. The concentration of the BMF was adjusted to be approximately 8 mg/mL using acetone, and 20 µL of the sample was injected into the C28 column. Each β-PPCₙ peak (where n = 4, 6, 8, 10, and 12) was detected by the SRM chromatograms using their retention times, and eight fractions of each were collected. The identical fractions were combined and concentrated under vacuum.

#### 2.6 Chiral separation of standards of TAG enantiomers using a recycle HPLC system with a chiral column

The asymmetric TAG standards, rac-PPC₄, rac-PPC₆, rac-PPC₈, rac-PPC₁₀, and rac-PPC₁₂, were dissolved in 2-propanol and their concentrations were adjusted to 1000 µg/mL. 20 µL of the sample solution was injected into a recycle HPLC system, which consisted of a recycle pump (PU712R, GL Sciences Inc., Tokyo, Japan), an autosampler (GL-7420, GL Sciences Inc.), a column oven (CO705C, GL Sciences Inc.), a UV-VIS detector (UV702, GL Sciences Inc.), and two automatic valves (VALVE UNIT 401, FLM Co., Ltd., Tokyo, Japan), which allowed the analytes to pass through the same column repeatedly and reduced the extra-column volume of the autosampler during additional passes, and a chiral column with a guard cartridge (CHIRALCEL OD-3R, 4.6 mm i.d. × 150 mm, 3 µm, and 4.0 mm i.d. × 10 mm, 3 µm, respectively, Daicel Corporation, Tokyo, Japan). The analytes were detected by MS using an APCI probe (Quattro micro API, Waters Corporation). The column temperature, flow rate, and the mobile phase were 25°C, 0.5 mL/min, and methanol, respectively. The recycle
HPLC system and MS system were controlled using their operational software, (EZChrom Elite, Agilent Technologies, Inc., Santa Clara, CA) and MassLynx Ver. 4.1, Waters Corporation, respectively. The operating conditions of the APCI source were identical to those listed in Section 2.3. A protonated molecule of each TAG and the dipalmityl glycerol ion were selected as the precursor and product ions, respectively, for the selected reaction monitoring (SRM) channels. The SRM transitions for PPC4, PPC6, PPC8, PPC10, and PPC12 were \( m/z \) 639→551, 667→551, 695→551, 723→551, and 751→551, respectively. The cone voltage and collision energy were 15 V and 10 eV, respectively. After each racemate was resolved into its individual enantiomers by passing through the column five times, their peaks were detected using MS. The elution order of the enantiomers was confirmed by comparing the retention times of a racemate with one of its enantiomers (e.g., \( \beta \)-PPC4 and \( sn \)-PPC4 (data not shown)).

### 2.7 Enantiomeric ratios of asymmetric TAGs in BMF

The asymmetric TAG fraction obtained in Section 2.5, which contained \( \beta \)-PPC4, \( \beta \)-PPC6, \( \beta \)-PPC8, \( \beta \)-PPC10, and \( \beta \)-PPC12, was dissolved in 2-propanol and its concentration was adjusted to approximately 1 mg/mL. The sample solution was analyzed using the setup and analytical conditions of the LC-MS/MS system that were described Section 2.6. The enantiomeric ratios were determined using the peak area ratios of the SRM chromatograms. The abundance ratios of each TAG enantiomer of \( \beta \)-PPC4, \( \beta \)-PPC6, \( \beta \)-PPC8, \( \beta \)-PPC10, and \( \beta \)-PPC12 in the BMF were calculated using equations (2) and (4).

\[
\text{sn-PPC}_n(\%) = \frac{\beta \text{-PPC}_n(\%) \times \text{Area}_{\text{sn-PPC}_n}}{\text{Area}_{\text{sn-PPC}_n} + \text{Area}_{\text{sn-C2PP}}} \tag{4}
\]

### 3 RESULTS AND DISCUSSION

#### 3.1 Analysis of extracted ion chromatograms corresponding to diacylglycerol ions in BMF

We examined the TAG molecular species in BMF using a C28 column at 40°C. Under these conditions, TAGs are eluted in ascending order of Partition number (PN) \(^{22}\), where PN = carbon number of three acyl chains \( 2 \times \) number of double bonds, and the TAG positional isomers were not separated. The mass spectrum of each peak in the total ion chromatogram (TICC) was difficult to analyze because several TAG peaks overlapped (Fig. 2F); however, the use of extracted ion chromatograms (EICs) facilitated the estimation of the fatty acid composition of the TAGs. Figures 2A–E show the EICs of \( m/z \) 495, 523, 577, 551, and 579, which were DAG ions (i.e., [TAG–RCOO]+), generated by the in-source collision-induced dissociation (CID). Because BMF contains greater than 10% of M, P, and O \(^{12}\), the \( m/z \) 495, 523, 577, 551, and 579 peaks most likely corresponded to [MM]+, [MP]+, [PO]+, [PP]+, and [PS]+, respectively. It was reported that BMF TAGs consist of two long-chain fatty acids (LCFAs) at the sn-1 and sn-2 positions, and one SCFA or MCFA at the sn-3 position \(^{10, 12, 23}\). The patterns of these chromatograms were similar to each other, which meant that the SCFA and MCFA might be bound to the glycerol backbones with two LCFAs in this way, though the binding positions of the fatty acids on the glycerol backbone could not be distinguished using these HPLC conditions. For example, in case of an EIC corresponding to \([PP]^+\) (Fig. 2D), the first five peaks corresponded to PPC4, PPC6, PPC8, PPC10, and PPC12. The fatty acid composition of each TAG was identified using the APCI/MS spectra (data not shown). The next peaks presumably corresponded to PPO (\( Po \): palmitoleic acid), PPM, PPO, PPP, and PPS according to PN though it was difficult to estimate fatty acid composing TAG molecular species because three or more DAG fragment ions were detected from coeluting peaks. The other EICs probably show that TAGs comprised of two LCFAs and one SCFA or MCFA as is the case with EIC of \([PP]^+\). Accordingly, we assumed that the actual amount ratio of the TAG positional isomers and enantiomers, which consisted of a dipalmityl glycerol backbone, and one SCFA or MCFA, represented the whole TAG structure, which contained other DAG backbones present in BMF. We analyzed the amount ratios of these TAG isomers because we could separate both the TAG positional isomers and enantiomers that contained a dipalmityl glycerol backbone.

#### 3.2 Quantification of TAG positional isomers

The objective of the separation and quantification of TAG positional isomers in this section is not only to examine the ratio of TAG positional isomers but also to fractionate asymmetric TAGs for chiral separation in a later section (Section 3.4). We previously reported that the separation of \( \beta \)-PC10P/\( \beta \)-PPC10 and \( \beta \)-PC12P/\( \beta \)-PPC12 was achieved using a C28 column at 15°C under isocratic conditions with either acetone or mixture of acetone and acetonitrile \(^{18-20}\). Although we have already reported that the ratio of \( \beta \)-PC10P/\( \beta \)-PPC10 in the milk and cheese of ruminants including cow \(^{20}\), the data indicated only the ratio of \( \beta \)-PC10P/\( \beta \)-PPC10 but not their amounts in milk fat. Meanwhile, we have already quantified \( \beta \)-PC12P/\( \beta \)-PPC12 in BMF \(^{19}\). In both cases, each enantiomer was not separated and quantified.

To separate the TAG positional isomers, \( \beta \)-PC10P/\( \beta \)-PPC10 (where \( n = 4, 6, 8, 10 \), and 12), we used a C28 reversed-phase column at 15°C and a solvent gradient of acetone and acetonitrile for the simultaneous separation of \( \beta \)-PC10P/\( \beta \)-PPC10 (where \( n = 4, 6, 8, 10 \), and 12). To selectively detect these TAGs, we used the SRM mode of the LC/MS/MS, and selected \([PPC_n+NH_4]^+\) and \([PP]^+\) as the precursor and product ions, respectively. Ammonium adduct ions are pre-
sumably caused by ammonium contained in acetonitrile as trace impurity. As a result, all the standards of TAG positional isomer pairs were resolved (Fig. 3A–E). Retention times of each pair of symmetric and asymmetric TAGs increased with carbon number of three acyl chains, and symmetric TAGs were eluted earlier. The linear calibration curves for each of the standard solutions of the TAG positional isomers were obtained over a range of 5–250 µg/mL, and all of the correlation coefficients were greater than 0.97 (Table 1). The values of slopes of β-PPC₄ were greater than those of β-PC₄P because formation of [PP]⁺ from β-PPC₄ in collision cell is energetically more favorable than that from β-PC₄P (where n = 4, 6, 8, 10, and 12) [24]. In BMF (Fig. 3F–J), no symmetric TAG positional isomers containing C₄, C₆, C₈ or C₁₀ were detected, but a small amount of that containing C₁₂ was detected (Table 2). The result that only β-PPC₄ was detected in BMF was consistent with our previous paper [19]. The detected ratio of β-PC₁₀P/β-PPC₁₀ conflicts with our previous report [20]. The reason for this is unclear, but this inconsistency might be caused by differences in the origin and season in which the milk was produced [25, 26]. Some of the positional distributions of C₁₀ and C₁₂, which were reported by several researchers, are also inconsistent. We suspect that the amount ratios of these TAG positional isomers might be affected by seasonal and regional variations in the positional distributions of fatty acids [12, 27]. However, at least we found that most TAG positional isomers that consisted of two palmitic acids and either C₄, C₆, or C₈ existed in the asymmetric form.
Chiral separation of standards of TAG enantiomers using a recycle HPLC system with a chiral column. First, we confirmed whether the standard solutions of racemic mixtures of the asymmetric TAGs \( \text{rac}_{\text{PPC}4}, \text{rac}_{\text{PPC}6}, \text{rac}_{\text{PPC}8}, \text{rac}_{\text{PPC}10}, \text{and} \text{rac}_{\text{PPC}12} \) were separated into their enantiomers on the CHIRALCEL OD-3R chiral stationary phase \( \text{CSP} \), which was the same as the CHIRALCEL OD-RH we used previously\(^2\). As a result, \( \text{rac}_{\text{PPC}} \) (where \( n = 4, 6, 8, 10, \) and 12) was resolved into their enantiomers using the CHIRALCEL OD-3R column and the recycle HPLC system (which makes the analyte pass through the column five times (Fig. 4A–E)). This is the first chiral separation of TAG enantiomers comprised of two Ps and one SCFA (or MCFA). Retention time of each pair of asymmetric TAGs increased with carbon number of three acyl chains, and

Table 2 Abundance ratios of triacylglycerol positional isomers and enantiomers comprised of dipalmitoylglycerol backbone with short- or medium-chain fatty acid in bovine milk fat (wt%).

<table>
<thead>
<tr>
<th>sn-PPC(_4)</th>
<th>sn-PC(_4)P</th>
<th>sn-C(_4)PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5 ± 0.2</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>sn-PPC(_6)</td>
<td>sn-PC(_6)P</td>
<td>sn-C(_6)PP</td>
</tr>
<tr>
<td>2.3 ± 0.2</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>sn-PPC(_8)</td>
<td>sn-PC(_8)P</td>
<td>sn-C(_8)PP</td>
</tr>
<tr>
<td>0.8 ± 0.1</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>sn-PPC(_10)</td>
<td>sn-PC(_10)P</td>
<td>sn-C(_10)PP</td>
</tr>
<tr>
<td>1.3 ± 0.1</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>sn-PPC(_12)</td>
<td>sn-PC(_12)P</td>
<td>sn-C(_12)PP</td>
</tr>
<tr>
<td>0.8 ± 0.1</td>
<td>0.12 ± 0.01</td>
<td>nd</td>
</tr>
</tbody>
</table>

Mean ± SD (n=3) \( \text{nd: not detected (less than} 0.1\text{wt%)} \)
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3.4 Enantiomeric ratios of asymmetric TAGs in BMF

To analyze the enantiomeric ratios of these asymmetric TAGs in BMF, the fractionation of asymmetric TAGs using reversed-phase HPLC was required before the chiral HPLC analysis because the asymmetric TAGs were indistinguishable from the symmetric TAGs when they overlapped on the SRM chromatogram. The chiral analysis of the asymmetric TAG fractions, β-PPC₄, β-PPC₆, β-PPC₈, β-PPC₁₀, and β-PPC₁₂, showed that each of the asymmetric TAGs was composed of mostly one enantiomer with SCFA or MCFA at the sn-3 position, namely, sn-PPC₄, sn-PPC₆, sn-PPC₈, sn-PPC₁₀, and sn-PPC₁₂ (Fig. 4F–J). If the positional distribution of C₁₀ and C₁₂ was considered, more sn-C₁₀PP and sn-C₁₂PP should be detected in the asymmetric TAG fractions. TAGs other than those with dipalmitoyl glycerol back-

Fig. 4  TAG enantiomer separation of bovine milk fat (BMF) asymmetric TAGs comprised of two palmitic acids and one SCFA (or MCFA). A) rac-PPC₄, B) rac-PPC₆, C) rac-PPC₈, D) rac-PPC₁₀, and E) rac-PPC₁₂ are selected reaction monitoring (SRM) chromatograms of the standard solutions, and F) β-PPC₄, G) β-PPC₆, H) β-PPC₈, I) β-PPC₁₀, and J) β-PPC₁₂ are SRM chromatograms of BMF.

Column: CHIRALCEL OD-3R (4.6 mm i.d. × 150 mm, 3 µm) with a guard cartridge (CHIRALCEL OD-3R, 4.0 mm i.d. × 10 mm, 3 µm), column temperature: 25°C, mobile phase: methanol, flow rate: 0.5 mL/min, the number of passages through the column by the recycle HPLC system: five passes for each TAG, ion source of MS: APCI positive, SRM transitions: m/z 639→551 for PPC₄; m/z 667→551 for PPC₆; m/z 695→551 for PPC₈; m/z 723→551 for PPC₁₀, and m/z 751→551 for PPC₁₂.
bones would bind C_{10} and C_{12} at their sn-1 and 2 positions. The result that most of the TAG isomers with a dipalmitoylglycerol backbone containing C_{6}, C_{8}, C_{10}, and C_{12} existed as sn-PPC_{4}, sn-PPC_{8}, sn-PPC_{10}, and sn-PPC_{12} in BMF were 4.5, 2.3, 0.8, 1.3, and 0.8 wt%, respectively, with a total of 9.7 wt% (Table 2). Furthermore, we found that the composition of the sn-PPC_{4}, sn-PPC_{8}, sn-PPC_{10}, and sn-PPC_{12} was consistent with the positional distribution of C_{4}, C_{8}, C_{10}, and P. The contents of sn-PPC_{4}, sn-PPC_{8}, sn-PPC_{10}, and sn-PPC_{12} in BMF were 4.5, 2.3, 0.8, 1.3, and 0.8 wt%, respectively, which were converted into weight percent using the positional distribution of fatty acids in BMF^{32}. Therefore, the composition of the TAG positional isomers and enantiomers, which were comprised of dipalmitoylglycerol backbone and SCFA (or MCFA), sufficiently represented the structure of all of the TAGs with SCFA (or MCFA) in BMF. These data indicate TAGs in BMF are homochiral. SCFA (or MCFA) at the sn-3 position of BMF plays an important role in nutrition in calf. Pregastric lipase of calf shows a specificity for C_{4}, C_{6}, C_{8}, and C_{10} and preferentially hydrolyzes ester bonds at the α positions (sn-1,3 positions)^{15,29}. These SCFA and MCFA would be efficient energy source for calf because they are selectively hydrolyzed by pregastrial lipase in the stomach and then absorbed into gastric mucosa as mentioned above. TAG in BMF is synthesized by acyltransferases in glycerol phosphate pathway^{19}. Bovine DAG acyltransferase probably binds SCFA (or MCFA) stereoselectively to the sn-3 position of 1,2-DAG^{19}. In this way, homochiral TAGs in BMF are likely synthesized by acyltransferase in mammary gland with consideration for calf nutrition. In addition to nutritional properties, BMF might have specific physical properties due to its homochiral structure. We recently reported a difference in the physical properties of rac-PPO and sn-PPO (or sn-OPP)^{29}, and the fact that the physical properties of a racemic TAG and one side of its enantiomers is different is of interest to researchers and workers in lipid science and the oil and fat industry. Future studies should focus on the nutritional and physical properties of the individual TAGs in BMF.

4 CONCLUSION

TAG positional isomers and enantiomers comprised of dipalmitoylglycerol backbone and SCFA (or MCFA) in BMF were resolved and quantified using C28 reversed-phase column and CHIRALCEL OD-3R chiral column. This is the first chiral separation of TAG enantiomers binding only saturated fatty acids at sn-1 and sn-3 positions, and the result indicates that the structure of BMF TAG is homochiral, which means SCFAs (or MCFAs) are bound predominantly to the sn-3 position of dipalmitoylglycerol backbone. Our result is consistent with previous researches on the positional distribution of fatty acids in BMF.

References

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