Actual Ratio of Triacylglycerol Positional Isomers in Milk and Cheese

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Abstract: Actual ratios of triacylglycerol (TAG) positional isomers in human, rat, and cow milk fat and cow, buffalo, goat, and sheep cheese fat were analyzed using HPLC–UV–atmospheric pressure chemical ionization–MS/MS system equipped with an octacosyl silylation column or polymeric ODS column. We substituted cheese fats for milk fats in parts of our study because milks from ruminants, with the exception of cows, are difficult to get in Japan. The actual ratio of β–PPC (the TAG consisting of two palmitic acids (P) and one capric acid (C), with the palmitic acid located at the β position) and β–PCP in human milk was different from those in ruminants, with more than half of the medium-chain fatty acids located at the β position even though other fats possessed it mainly at the α position. Palmitic acid was mainly located at the β position for human milk and rat milk; however, the location in ruminant cheese fat was mainly at the α position. The location of fatty acids is thought to be very important for infant nutrition. Particularly, the location of palmitic acid in case of human milk and of medium-chain fatty acids in case of ruminant milk was very characteristic and is considered to be very important to the fatty acids in milk fat.

Key words: actual ratio, infant nutrition, milk fat, triacylglycerol positional isomer

1 INTRODUCTION

Triacylglycerol (TAG) is a main component of lipids and consists of glycerol and three fatty acids1. The binding positions of the fatty acids on glycerol are simply categorized into two types with the primary and secondary alcohol groups on the glycerol defined as the α(sn-1, 3) position and the β(sn-2) position. In the case that an asymmetric carbon atom exists in TAG, the binding positions are categorized into three positions, the sn-1(α) position, the sn-2 (β) position, and the sn-3(α) position, according to the Fisher projection2.

Bovine milk contains about 3.5-5 g fat in 100 mL of milk and the dominant component is TAG3. It has been found that the milk TAG characteristically contains short- or middle-chain fatty acids and the fatty acids are mainly located at the sn-3(α) position in TAG. The lingual lipase, which exists in newborn infants, orally hydrolyzes fatty acids bound at the sn-3(α) position in TAG at a rate that is twice that at the sn-1(α) position to obtain free short- or middle-chain fatty acids4,5. The short- or middle-chain fatty acid is readily absorbed into the oral mucosa and changed to acyl-CoA, an energy source, in the cell because the fatty acid does not need to change to acylcarinitine when it passes the inner mitochondrial membrane6-7. For these reasons, milk fat and newborn infants possess an elaborate system to easily take in energy. The existence of these fatty acids in milk and dairy products gives them their characteristic flavor and the flavor is caused by the hydrolysis of fatty acids such as the short- and middle-chain fatty acids in milk fat8,9. Furthermore, it is known that the TAG in human milk mainly has palmitic acid located at the β(sn-2)

Abbreviations: APCI, atmospheric pressure chemical ionization; C28, octacosyl silylation; DGAT, diacylglycerol acyltransferase; GPAT, sn-glycerol-3–phosphate acyltransferase; MRM, multiple reaction monitoring; TAG, triacylglycerol; TLC, thin layer chromatography

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position\textsuperscript{10}, and the location of palmitic acid at the $\beta(sn-2)$ position improves the absorption of fat in the small intestine\textsuperscript{11}. This effect is also explained by the formation of fatty acid soap. Milk contains a large amount of not only milk fat, but also calcium ion. The hydrolyzed fatty acids that were originally located at the $\alpha$ position of TAG easily react with calcium ions to form fatty acid soap, and the formed soap is not absorbed in the small intestine\textsuperscript{11}. Thus, TAG in milk fat plays an important role in infant nutrition. Moreover, the combination of the three fatty acids in TAG, namely the TAG molecular species, is important for the melting point of milk fat. The melting point affects the stable dispersion of the milk fat in milk. Brown \textit{et al.} reported that 27°C was the mean melting point of milk fat\textsuperscript{12}. Winter \textit{et al.} reported that the three dominant TAG molecular species in human milk were dipalmitoyloleoylglycerol (PPO), combination of the abbreviations of three fatty acids indicates just the combination of three fatty acids in TAG) 11.8%, linoleoyloleypalmitoylglycerol (LOP) 10.0%, and dioleoylpalmitoylglycerol (OOP) 4.4\%.\textsuperscript{14} The combination ratio of the respective TAG molecular species in milk fat strongly affects the melting point\textsuperscript{15}.

The combination of the three fatty acids in milk fat and the binding position of the fatty acids are very important for constituting milk fat as mentioned above; however, there is no study that takes into account the binding position of the fatty acids in the TAG molecular species, namely the TAG positional isomer. For example, the location of palmitic acid in TAG is important in milk fat, but the ratio of 1,2 (or 2,3)–dipalmitoyl-3 (or 1)–oleoyl–sn–glycerol ($\beta$-PPO) to 1,3-dipalmitoyl-2-oleoyl-sn-glycerol ($\beta$-POP) in PPO, the most dominant TAG molecular species in human milk, has not been reported. Recently, we reported that the polymeric ODS column can simply resolve AAB-type TAG positional isomers in the case of saturated fatty acids with a carbon length of 10 or more located in TAG\textsuperscript{16–18}. Moreover, we also reported that an octacosyl silylation (C28) column can resolve AAB-type TAG positional isomer binding two saturated fatty acids for very short periods using acetone as a mobile phase\textsuperscript{19}. In this study, the abundance of respective TAG positional isomers binding palmitic acid in several kinds of milk and cheese were examined using a recycle HPLC-UV-atmospheric pressure chemical ionization (APCI)–MS/MS system equipped with a polymeric ODS or C28 column.

### 2 EXPERIMENTAL PROCEDURES

#### 2.1 Chemicals and materials

The TAG positional isomers shown in Table 1 were obtained from Tsukishima Foods Industry Co., Ltd (Tokyo, Japan). Oxytocin was purchased from Sigma-Aldrich Co. (St. Louis, MO), and other reagents were purchased.

#### Table 1 Structures of TAG positional isomers used in this study.

<table>
<thead>
<tr>
<th>Structures</th>
<th>$\beta$-PPC</th>
<th>$\beta$-PPO</th>
<th>$\beta$-POO</th>
<th>$\beta$-PPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-PCP</td>
<td>$\beta$-POP</td>
<td>$\beta$-OPO</td>
<td>$\beta$-PDP</td>
<td></td>
</tr>
</tbody>
</table>

Ex.)

![Diagram of TAG positional isomers](image)

**β-PPC (1,2 (or 2,3)–dipalmitoyl–3 (or 1)–capryl–sn–glycerol)**

This TAG has a fixed P at the $\beta$ position, and other fatty acids are located at the $\alpha$ position. In this case, the TAG is expressed as $\beta$-PPC.

**Notation of abbreviation in Table 1**

- Capric acid (C)
- Palmitic acid (P)
- Oleic acid (O)
- Docosahexaenoic acid (DHA:D)
from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cow milk, Italian mozzarella cheese made from buffalo milk, Dutch chevrette cheese made from goat milk, French Ossau-Iraty cheese made from sheep milk, and Japanese processed cheese made from cow milk were bought at a supermarket in Tokyo, Japan.

Human milk was obtained from a breast-feeding Japanese woman who agreed to participate in this study. Rat milk was collected from 12-week-old primiparous F344/N rats purchased from Japan SLC Inc. (Shizuoka, Japan). Rat experiments were performed in accordance with the Tokyo University of Marine Science and Technology guidelines for animal use and care provided by the ethics committee. The rats were fed an AIN-93G diet obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan) and allowed free access to food and water. The rats were housed individually in plastic cages lined with sawdust in a temperature- and light-controlled environment (23 ± 2°C and 12 h cycle, respectively). All the rats gave birth on the 21st day of the pregnancy. The neonatal rats were separated from the mother rats at 2 days after childbirth. The mother rats were anesthetized by inhalation administration of sevoflurane (Mylan Inc., Canonsburg, PA). Fifteen minutes after oxytocin injection, rat milk was obtained after disinfecting the mammary region with ethanol. The milk obtained from the rats at 2 days after childbirth.

2.2 Separation of TAG from lipid samples

Lipid samples from cow and human milk were extracted using the Folch procedure. The milk was diluted with a nine-fold volume of distilled water. The diluted milk was added to a two-fold volume of chloroform and methanol (2:1, v/v) mixture in a test tube equipped with a screw cap, mixed vigorously with a vortex mixer, and centrifuged at 1,500 × g for 10 min. The bottom layer was collected and dried under a stream of nitrogen. The residue was dissolved in hexane and spotted on a silica gel plate to separate the TAG fraction by thin layer chromatography (TLC). The spotted TLC plate was developed with a petroleum ether/diisopropyl ether/acetic acid (80/20/1, v/v/v) mixture. The TAG was recovered by scraping off the appropriate thick band and extracted using a chloroform/methanol (20/1, v/v) mixture. Lipid samples in rat milk were collected by centrifugation at 1,500 × g for 10 min at 4°C. The floating milk fat was gathered and used as rat milk lipid. The lipid was dissolved in hexane and the TAG fraction was separated by TLC. The lipid in the cheese was extracted using the Bligh and Dyer procedure. The TAG in the extracted lipid was separated using the same method as above. The extracted TAG was dried by rotary evaporation under vacuum, weighed, and then dissolved in 2-propanol. The diluted samples were adjusted to 2 mg/mL. The samples were stored in an argon-purged screw-capped vial at −40°C until they were analyzed.

2.3 Analyses of TAG positional isomers

The extracted TAG sample diluted with 2-propanol was injected into a recycle HPLC-UV-APCI-MS/MS system 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 (VALVEUNIT401, FLOM Co., Ltd., Tokyo, Japan) that made the analytes pass through the same column repeatedly and cut the dead volume of the auto sampler 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 (Mass Lynx Ver.4.1, Waters Corporation) were used as the detector, in combination with the recycle HPLC system. Tandem jointed non-encapped polymeric ODS columns (Inertsil ODS-P, 4.6 mm i.d. × 250 mm: 5 µm, GL Sciences Inc.) were used for the resolution of 1,2 (or 2,3) -dilinoleoyl-3 (or 1) -palmitoyl-sn-glycerol (β-OOP) and 1,3 -dilinoleoyl-2-palmitoyl-sn-glycerol (β-POO). An acetonitrile/2-propanol/hexane (3/2/1, v/v/v) mixture was employed as the mobile phase at a flow rate of 1.0 mL/min and a column temperature of 10°C. Multiple reaction monitoring (MRM) mode was used, and the ammonium ion adducted molecule [OOP + NH₄]⁺ at m/z 877 and the [OOP – ROO]⁺ ion at m/z 578 [PO⁺] were chosen as the parent and the daughter ions, respectively. The cone voltage and collision energy were 20 and 26 eV, respectively. An HPLC system ( Alliance e2695, Waters Corporation) and C28 column (octacosyl silylation column; Sunrise C28, 4.6 mm i.d. × 250 mm: 5 µm, ChromaNik Technologies Inc., Osaka, Japan) was used for the separation of the other types of TAG positional isomer pairs such as β-POO and β-POP, 1,2 (or 2,3) -dipalmitoyl-3 (or 1) -capryl-sn-glycerol (β-PPC) and 1,3 -dipalmitoyl-2-capryl-sn-glycerol (β-PCP), and 1,2 (or 2,3) -dipalmitoyl-2-capryl-sn-glycerol (β-POO) and 1,3 -dipalmitoyl-2-docosahexaenoyl-sn-glycerol (β-PDP). Acetone was employed as the mobile phase at a flow rate of 1.0 mL/min and a column temperature of 15°C. MRM mode was used, and the protonated molecule [M + H]⁺ at m/z 724 for PCP, 834 for POP, and 880 for PDP and the [M – RO2]⁺ ion at m/z 468 [PC⁺] for PCP, 578 [PO⁺] for POP, and 552 [PP⁺] for PDP were selected as the parent and the daughter ions, respectively.

2.4 Preparation of calibration curves for respective TAG positional isomers

Equal weights of a TAG positional isomer pair (e.g., β-OOP and β-POO, shown in Table 1) were mixed and dissolved in 2-propanol. The standard solutions were adjusted
to 50, 100, 200, and 400 μg/mL, and 20 μL of standard solution was introduced into the HPLC-UV-APCI-MS/MS system. Calibration curves for each TAG positional isomer were prepared by plotting the concentration on the x-axis and the chromatogram peak area on the y-axis. All the calibration curves represented a first-order equation passing through the origin. The actual ratios of the respective TAG positional isomer pairs in milk or cheese TAGs were calculated from the ratio of peak area on the chromatogram and adjusted by the ratio of the calibration curve equation slopes. The detection limit and quantification limit of this method were calculated using the signal/noise ratio (s/n). The detection limit and quantification limit were defined as s/n = 3 and 10, respectively.

3 RESULTS AND DISCUSSION

In this study, the AAB type TAG positional isomers binding characteristic fatty acids such as middle chain fatty acid in ruminant milk and DHA in human milk were selected as target TAGs. Furthermore, the dominant AAB type TAG molecular species consisting of P and O were also selected as target TAGs. The resolution of the respective TAG positional isomer pairs (standard compounds) and the representative MRM chromatograms for the TAG positional isomers in milk samples are indicated in Fig. 1-4. All the TAG positional isomer pairs were separated; however, the resolution of β-OOP and β-OPO required performing the recycle run four times. Other TAG positional isomer pairs were separated without using a recycle run. These results were consistent with our previous results. The detection limit and quantification limit were calculated from s/n = 3 and 10, respectively. From the results, the detection limit and quantification limit were 4 ng and 12 ng, respectively.

Relative ratios of the calibration curve slopes between β-AAB and β-ABA-type TAG positional isomers are summarized in Table 2. The actual ratios of β-AAB and β-ABA-type TAG positional isomers in milk or cheese were calculated using the ratio of calibration curve slopes and peak area on the MRM chromatogram (Table 3). In this study, cheese fat was used instead of milk fat from buffalo, goat, and sheep milk because it was very difficult to obtain these kinds of milk in Japan. Although it is generally recognized that milk fat in cheese is hydrolyzed by lipase during processing[5, 23, 24], comparison of the TAG positional isomer pair between cow milk and cow cheese indicated that the actual ratios of the respective TAG positional pairs were almost equal (Table 3). This result was interpreted to mean that the hydrolysis by lipase did not affect the actual ratio of the respective TAG positional pairs significantly. Consequently, in this study, the results obtained from milk and fat were directly compared without considering the type of food.

It has been reported that short- and medium-chain fatty acids are mainly located at the sn-3 position of ruminant milk fat and that lingual lipase, characteristically found in a baby’s mouth, hydrolyzes these fatty acids located at the sn-3 position of milk fat[6, 7]. These facts demonstrate that short- and medium-chain fatty acids are

![Fig. 1 HPLC-APCI–MS/MS chromatograms of β-PDP and β-PPD. (A) Standard compound, (B) Cheese (cow milk), (C) Rat milk.](image)

![Fig. 2 MRM chromatograms of β-POP and β-PPO. (A) Standard compound, (B) Cheese (cow milk), (C) Rat milk.](image)
important energy sources for a baby. For this study, capric acid, a medium-chain saturated fatty acid consisting of 10 carbon atoms, was selected for discussion because human milk does not contain fatty acids consisting of 8 or less carbon atoms. The relative ratio of β-PPC and β-PCP in human milk was different from that of ruminants, and more than half of the medium-chain fatty acids were located at the β position, namely 67.4% was β-PCP (Table 3). In fact, it had been previously reported that the medium-chain fatty acid was distributed mainly at the sn-3 position in human milk; however, this was not consistent with our results.

It is known that milk TAG is synthesized in mammary tissue by the glycerol-3-phosphate pathway. Three types of acyltransferase partake in this pathway. The sn-glycerol-3-phosphate formed from glycerol is esterified with fatty acids (acyl-CoA) at the sn-1 position by sn-glycerol-3-phosphate acyltransferase (GPAT). After the reaction, the 1-acyl-sn-glycerol-3-phosphate is further esterified with fatty acids at the sn-2 position by diacylglycerol acyltransferase (DGAT) to form TAG. Distribution of fatty acids at respective binding positions on TAG would be ascribed to

![Fig. 3](image_url) MRM chromatograms of β-PCP and β-PPC. (A) standard TAG positional isomer pairs, (B) cheese manufactured from cow milk, and (C) rat milk.

![Fig. 4](image_url) MRM chromatograms of β-OPO and β-OOP. (A) standard TAG positional isomer pairs, (B) cheese manufactured from cow milk, and (C) rat milk.

<table>
<thead>
<tr>
<th>TAG</th>
<th>MRM transition</th>
<th>Equation (<em>R</em>)</th>
<th>Ratio of equation slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-PCP</td>
<td>724 &gt; 468</td>
<td>y = 130.5 × (0.89)</td>
<td>β-PCP/β-PPC = 1.28</td>
</tr>
<tr>
<td>β-PPC</td>
<td>724 &gt; 468</td>
<td>y = 101.6 × (0.95)</td>
<td></td>
</tr>
<tr>
<td>β-POP</td>
<td>834 &gt; 578</td>
<td>y = 364.9 × (0.94)</td>
<td>β-POP/β-PPO = 1.49</td>
</tr>
<tr>
<td>β-PPO</td>
<td>834 &gt; 578</td>
<td>y = 244.5 × (0.96)</td>
<td></td>
</tr>
<tr>
<td>β-PDP</td>
<td>880 &gt; 552</td>
<td>y = 223.4 × (0.98)</td>
<td>β-PDP/β-PDD = 0.82</td>
</tr>
<tr>
<td>β-PPD</td>
<td>880 &gt; 552</td>
<td>y = 183.1 × (0.99)</td>
<td></td>
</tr>
<tr>
<td>β-OPO</td>
<td>877 &gt; 578</td>
<td>y = 3069 × (0.99)</td>
<td>β-OPO/β-POO = 1.78</td>
</tr>
<tr>
<td>β-POO</td>
<td>877 &gt; 578</td>
<td>y = 1723 × (0.99)</td>
<td></td>
</tr>
</tbody>
</table>

*R*: multiple correlation coefficient
the characteristic of these acyltransferases. Each acyltransferase probably has a favorable reaction substrate to synthesize TAG, and the variation of the TAG molecular species would be strongly affected by this factor. The differences of the distribution of the medium-chain fatty acid at the sn-1, 2, and 3 positions of PPC between human milk and ruminant milk may be due to these reasons; however, the exact details are unknown. In the case of TAG consisting of two oleic acids and one palmitic acid, palmitic acid mainly locates at the β position of human milk (β-POO: 75%) and rat milk (β-POO: 73%). This result is consistent with the report by Breckenridge et al. and also interpreted to mean that the location of oleic acid at the α position was important because the β-PPO was dominant in TAGs consisting of two palmitic acids and one oleic acid. In fact, the importance of the palmitic acid location at the β position in TAG has already been advocated by many researchers of human infant nutrition. Consequently, our result that β-OPO was dominant supports this hypothesis and the dominant location of oleic acid at the α position might be side result. The TAG was hydrolyzed by pancreatic lipase in the lumen to form two free fatty acids and one 2-monoacylglycerol. The free fatty acids and 2-monoacylglycerol form a micelle, which is absorbed in the small intestine. It is known that the fatty acid existing in 2-monoacylglycerol, when bound at the β position, is absorbed into the small intestine more efficiently than free fatty acids. Consequently, palmitic acid, as an important energy source, was located at the β position of human milk TAG so that it could be absorbed efficiently. Humans can synthesize palmitic acid, but cannot synthesize many medium-chain fatty acids, such as capric acid, like ruminants. Taking into account these facts, the key fatty acid as an efficient energy source in human and ruminant milk might be selected from the fatty acids that can be readily synthesized in each body.

Actual ratios of β-OPO and β-POO in ruminant milk TAG such as buffalo (β-POO: 65%), goat (β-POO: 80%), and sheep (β-POO: 63%) were opposite from that of non-ruminant milk. Based upon the distribution of β-OPO and β-POO in ruminant milk, it might be interpreted to mean that the location of oleic acid at the β position was important for ruminants; however, the β-PPO that was dominant in both ruminant and non-ruminant TAGs consisting of two palmitic acids and one oleic acid. According to these results, the location of oleic acid at the β position in ruminant TAG might not be as strong a messaging signal as palmitic acid at the β position in human TAG or the medium-chain fatty acid at the α position in ruminant TAG. Further study would be needed to evaluate this possibility. The location of DHA was also examined and the actual ratio of β-PPD and β-PDP was almost the same in human and rat milk.

Table 3  Actual ratios of TAG positional isomers in milk and cheese fats (%).

<table>
<thead>
<tr>
<th></th>
<th>Cow</th>
<th>Human</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-PCP/β-PPC</td>
<td>28.9 ± 2.1/71.1 ± 2.1</td>
<td>67.4 ± 0.7/32.6 ± 0.7</td>
<td>16.2 ± 1.2/83.8 ± 1.2</td>
</tr>
<tr>
<td>β-POO/β-POO</td>
<td>56.8 ± 1.1/43.2 ± 1.1</td>
<td>74.9 ± 0.5/25.1 ± 0.5</td>
<td>73.4 ± 1.2/26.6 ± 1.2</td>
</tr>
<tr>
<td>β-POP/β-PPO</td>
<td>12.4 ± 2.6/87.6 ± 2.6</td>
<td>2.7 ± 0.6/97.3 ± 0.6</td>
<td>2.9 ± 0.4/97.1 ± 0.4</td>
</tr>
<tr>
<td>β-PDP/β-PDD</td>
<td>N.D.</td>
<td>55.6 ± 3.6/44.4 ± 3.6</td>
<td>43.5 ± 1.3/56.5 ± 1.3</td>
</tr>
</tbody>
</table>

**Cheese**

<table>
<thead>
<tr>
<th></th>
<th>Cow</th>
<th>Buffalo</th>
<th>Goat</th>
<th>Sheep</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-PCP/β-PPC</td>
<td>29.2 ± 0.6/70.8 ± 0.6</td>
<td>30.7 ± 1.6/69.3 ± 1.6</td>
<td>16.4 ± 1.0/83.6 ± 1.0</td>
<td>29.0 ± 1.1/71.0 ± 1.1</td>
</tr>
<tr>
<td>β-POO/β-POO</td>
<td>58.4 ± 1.5/41.6 ± 1.5</td>
<td>34.8 ± 0.5/65.2 ± 0.5</td>
<td>20.0 ± 0.5/80.0 ± 0.5</td>
<td>37.0 ± 0.4/63.0 ± 0.4</td>
</tr>
<tr>
<td>β-POP/β-PPO</td>
<td>9.0 ± 0.6/91.0 ± 0.6</td>
<td>15.6 ± 0.5/84.4 ± 0.5</td>
<td>8.6 ± 0.3/91.4 ± 0.3</td>
<td>23.2 ± 0.3/76.8 ± 0.3</td>
</tr>
<tr>
<td>β-PDP/β-PDD</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

(Average Value ± S.D., n = 3)  
Not Detected: N.D.
Our next target is the analysis of the TAG enantiomer in milk TAG.

References
