Homochiral Asymmetric Triacylglycerol Isomers in Egg Yolk

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Abstract: The composition of triacylglycerol (TAG) positional isomer (-PI) and enantiomer (-E) in immature chicken egg yolk, mature chicken yolk, and chicken meat was examined. POO (consisting of one palmitic acid (P) and two oleic acids (Os)), PPO (consisting of two Ps and one O), and PPL (consisting of two Ps and one linoleic acid (L)) were treated as representative TAG molecular species in all the analytical samples because P, O, and L were the major fatty acids comprising egg and chicken meat. sn-POO (binding P at sn-1 position) was predominant in egg yolks, while sn-OOP and sn-OPO were present in chicken meat. This difference was ascribed to the different roles of these isomers as nutrients, because TAG in egg yolk is important for new born organisms and TAG in chicken meat is used for fat accumulation. The compositions of the TAG isomers in PPO and PPL in egg yolk were similar, and O and L did not bind at the sn-1 position. In contrast, all the isomers of PPO and PPL were found in chicken meat. These results imply that the TAG structure could be modified so that the nutrient requirement is fulfilled in egg yolk and chicken meat.

Key words: egg yolk, enantiomer, positional isomer, quantification, triacylglycerol

1 INTRODUCTION

Eggs are composed of important nutrients such as proteins, lipids, inorganic components, and a variety of vitamins¹. All the components of eggs are necessary for the growing embryo and serve as important nutrients for humans. For this reason, eggs are termed "complete nutrient food." The lipid in egg yolk is an indispensable component for the development of the brain, nervous system, and cells in the embryo because it contains docosahexaenoic acid (DHA), linoleic acid, palmitic acid (P), etc. Lipids comprise 65% triacylglycerol (TAG) and 30% phospholipid (PL), in addition to cholesterol, pigments, and carotenoids². TAG, the main component of egg yolk lipids, is composed of three fatty acid (FA) molecules esterified with one glycerol. Therefore, infinite combination patterns of the three FAs in TAG are possible. Such a combination in TAG is called "TAG molecular species," which are identified by three FA abbreviations; for example, ABC consists of three kinds of FAs, A, B, and C. Furthermore, the binding position of FAs on the glycerol moiety in TAG is categorized into two types. The primary and secondary alcohol groups on glycerol are defined as the α and β positions, respectively³. These two positions are distinguished by pancreatic lipase in the digestion process, and the lipase hydrolyzes only the FAs located at the α position of TAG⁴. In the case of a TAG consisting of two kinds of FAs, "A" and "B," a TAG with B at the α position (β-AAB) and a TAG

Abbreviations: APCI-MS, atmospheric pressure chemical ionization-mass spectrometer; ATGL, adipose triglyceride lipase; C₃₀C₁₆C₁₆, triundecanooate; DHA, docosahexaenoic acid; E, enantiomer; FA, fatty acid; HSL, hormone-sensitive lipase; IS, internal standard; L, linoleic acid; LOD, limit of detection; LOQ, limit of quantification; M, myristic acid; MGL, monoacylglycerol lipase; MRM, multiple reaction monitoring; O, oleic acid; P, palmitic acid; PI, positional isomer; PL, phospholipid; PUFA, polyunsaturated fatty acid; rac-POO, 1-palmitoyl-2,3-dioleoyl-rac-glycerol; rac-PPL, 1,2-dipalmitoyl-3-linoleoyl-rac-glycerol; rac-PPPO, 1,2-dipalmitoyl-3-oleoyl-rac-glycerol; S, stearic acid; SFA, saturated fatty acid; sn-OOP, 1,2-dioleoyl-3-palmitoyl-sn-glycerol; sn-OPO, 1,3-dioleoyl-2-palmitoyl-sn-glycerol; sn-PPO, 1,3-dipalmitoyl-2-oleoyl-sn-glycerol; sn-PPL, 1,2-dipalmitoyl-3-oleoyl-sn-glycerol; sn-PLP, 1,3-dipalmitoyl-2-linoleoyl-sn-glycerol; sn-PPL, 1,2-dipalmitoyl-3-linoleoyl-sn-glycerol; s/n, signal-to-noise ratio; TAG, triacylglycerol; UFA, unsaturated fatty acid; WAT, white adipose tissue

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with B at the β position (β-ABA or sn-ABA) exist. Although these TAGs consist of the same kinds and numbers of the respective FAs, they are differentiated as "TAG positional isomers (TAG-P)". In addition, the carbon atom connecting the secondary alcohol becomes a stereogenic center in the β-ABA type TAG. If the FA esterified with the secondary alcohol group is positioned on the left side and the hydrogen bound to the asymmetric carbon atom is located on the right side in the Fischer projection, the esterified position on the upper carbon atom of the stereogenic center is called “sn-1 position.” The esterified position on the secondary alcohol group at the stereogenic center is called “sn-2 position.” The residual position is called “sn-3 position”[2]. Accordingly, the TAG-PI β-ABA contains two kinds of TAG enantiomers (TAG-E): sn-ABA (1,2-diA-3-B-sn-glycerol) and sn-BAA (1-B,2,3-diA-sn-glycerol). Although there is no difference in the physical properties between a pair of TAG-Es, some organisms or enzymes can recognize only one enantiomer. For example, lingual lipase selectively hydrolyzes short- and medium-chain FAs located at the sn-3 position in rodent milk fat[6].

There are numerous reports on the lipid analysis of eggs. Christie et al. reported that avian egg preferentially binds saturated FA at the sn-1 position and unsaturated FA at the sn-2 and 3 positions[3]. Holub et al. found that phosphatidylcholine in egg yolk binds saturated FA at the sn-1 position and unsaturated FA at the sn-2 position[4]. Furthermore, incorporation of functional FAs into egg lipids from feeds has been investigated. Koppenol et al. reported that DHA and EPA in the feeds can be incorporated into the egg yolk[5]. It has also been reported that CLA in the feeds can be incorporated into the egg yolk. In addition, an increase in the amount of saturated FAs such as myristic acid (M), palmitic acid (P), and stearic acid (S) and a decrease in the amount of unsaturated fatty acid (UFA) such as oleic acid (O), arachidonic acid, and DHA in egg yolk were detected[6].

Many other studies concerning FA distributions in egg yolk lipids have also been conducted[7–10]; however, there are very few studies on the quantification of TAG isomers in egg yolk lipids.

Several analytical methods for TAG isomers have been developed so far. Kuroda et al. found that TAG-Pis binding at least one saturated FA can be separated on a polymeric ODS column[11]. We reported that TAG-Pis binding two saturated FAs can be separated within a short period using a C28 column[12]. Furthermore, We revealed that resolution of TAG-E binding at least one saturated FA contained in natural fats and oils is possible using a recycle HPLC system equipped with a chiral column[13]. These methods can be applied to the separation and quantification of TAG isomers in egg yolk. In this study, the relative abundance of TAG isomers binding palmitic acids in egg yolk, immature egg, and chicken meat was examined because palmitic acid is a main FA in egg yolk and knowledge of its existing form in TAG would be important[1].

2 MATERIALS AND METHODS

2.1 Chemicals and materials

Standard TAG isomers (1,3-dipalmitoyl-2-linoleoyl-sn-glycerol (sn-PLP), 1,3-dipalmitoyl-3-linoleoyl-rape-glycerol (rape-PPL), 1,2-dipalmitoyl-3-linoleoyl-sn-glycerol (sn-PPL), 1,3-dioleoyl-2-palmitoyl-sn-glycerol (sn-OPO), 1-palmitoyl-2,3-dioleoyl-rape-glycerol (rape-POO), 1,2-dioleoyl-3-palmitoyl-sn-glycerol (sn-OOP), 1,3-dipalmitoyl-2-oleoyl-sn-glycerol (sn-POP), 1,2-dipalmitoyl-3-oleoyl-rape-glycerol (rape-POP), 1,2-dipalmitoyl-3-oleoyl-sn-glycerol (sn-PPO), and triundecanolate (C₁₁C₁₁C₁₁) were inhouse products of Tsukishima Foods Industry Co., Ltd. (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Egg and chicken breast meat were purchased at a supermarket in Tokyo, Japan. Immature egg was purchased from a poultry dealer, Tokyo, Japan.

2.2 Lipid extraction and fractionation of TAG

Lipids were extracted from each sample by the Bligh and Dyer method[14]. TAGs were fractionated from the respective extracted lipids by flash column chromatography using a hexane/ethyl acetate (9:1, v/v) mixture.

2.3 Relative abundance of TAG molecular species

All TAG samples were dissolved in acetone and adjusted it to 5% following the official method of JOCS[15]. 10 μL of the TAG sample solution was injected into an HPLC system ( Alliance e2695, Waters Corporation, Milford, MA) with a refractive index detector (RI 704, GL Science Inc., Tokyo, Japan) and a column with guard cartridge (Sunrise C28, 5 μm, 4.6 mm i.d. × 250 mm + 4.6 mm i.d. × 10 mm, Chro- maNik Technologies Inc., Osaka, Japan). The analytical conditions were as follows: mobile phase, acetone/acetonitrile = 60/40 (v/v); column temperature, 40°C; flow rate, 1.0 mL/min.

2.4 Analysis of TAG-PI

To obtain calibration curves for the quantification of TAG-PI, the sn-PLP/rape-PPL, sn-POP/rape-POO pair was adjusted to 50, 100, 200, and 400 ppm with 100 ppm C₁₁C₁₁C₁₁ as the internal standard (IS) in acetone. The sn-OPO/rape-POO pair was adjusted to the same concentration in acetone. All TAG samples were adjusted to a concentration of 1% in acetone, and 10 μL of each solution was injected into the HPLC system shown below. Calibration curves for each TAG-PI were prepared by plotting the concentration on the x-axis and the peak area/peak area ratio on the y-axis. Two sets of analytical conditions (shown in Sections 2.4.1 and 2.4.2) were employed for the quantification.
culation.

2.4.1 Analysis of sn-PLP/rac-PPL and sn-POP/rac-PPO

One was for the TAG consisting of two saturated FAs (SFAs) and one unsaturated FA (UFA). The HPLC system was Alliance e2695 (Waters Corporation) equipped with a suitable column (Sunrise C28, 5 μm, 4.6 mm i.d. × 250 mm + 4.6 mm i.d. × 10 mm, ChromaNik Technologies Inc.). The mobile phase, column temperature, and flow rate were acetone, 15°C, and 1.0 mL/min, respectively. This HPLC system was connected with an atmospheric pressure chemical ionization-mass spectrometer (APCI-MS) (Quattro micro API, Waters Corporation). The ionization mode, corona current, cone voltage, collision energy, source temperature, desolvation temperature, cone gas flow, desolvation gas flow, dwell time, inter-scan time, and data acquisition mode were APCI-positive, 3 μA, 20 V, 25 eV, 120°C, 450°C, 50 L/h, 200 L/h, 0.1 s, 0.1 s, and multiple reaction monitoring (MRM) mode, respectively. The precursor-to-product ion MRM transitions of sn-PLP/rac-PPL and sn-POP/rac-PPO were \( m/z \ 831.7[^{1}]\text{PPL} + [H]^+ > 551.5[^{1}]\text{PP}^+ \) and \( m/z \ 833.7[^{1}]\text{PPO} + [H]^+ > 551.5[^{1}]\text{PP}^+ \), respectively.

2.4.2 Analysis of sn-POP/rac-POO

A different set of analytical conditions were used for the TAG consisting of one SFA and two UFAs. The HPLC system was composed of a recycle pump (PUT12R, GL Sciences Inc.), an autosampler (GL-7420, GL Sciences Inc.), two automatic valves (VALVE UNIT 401, FLOM Co., Ltd., Tokyo, Japan), a detector (UV702, GL Sciences Inc.), a column oven (CO-705C, GL Sciences Inc.), and tandem jointed polymeric ODS columns (Inertsil ODS-P, 5 μm, 4.6 mm i.d. × 250 mm, GL Sciences Inc.). The mobile phase, column temperature, flow rate, and wave length were acetonitrile/2-propanol/hexane = 3/2/1 (v/v/v), 10°C, 1.0 mL/min, and 205 nm, respectively. This HPLC system was also connected with an APCI-MS (Quattro micro API, Waters Corporation) system. The ionization mode, corona current, cone voltage, collision energy, source temperature, desolvation temperature, cone gas flow, desolvation gas flow, dwell time, inter-scan time, and data acquisition mode were APCI-positive, 3 μA, 20 V, 25 eV, 120°C, 450°C, 50 L/h, 200 L/h, 0.1 s, 0.1 s, and MRM mode, respectively. The precursor-to-product ion MRM transition of sn-POP/rac-POO was \( m/z \ 876.8[^{1}]\text{POO} + [NH_{3}]^+ > 577.5[^{1}]\text{PO}^+ \).

2.5 Analysis of TAG-E

Before the analysis of TAG-E, the β-AAB type TAGs in each sample were collected using HPLC methods described in Section 2.4.1 and 2.4.2; then, the collected sample was dissolved in 2-propanol and adjusted to 10 ppm, and 20 μL of the sample solution was injected into a chiral HPLC system. The system was the same as that used for the separation of sn-POP/rac-POO shown in Section 2.4.2. A chiral column (CHIRALCEL OD-3R, 4.6 mm i.d. × 150 mm, Daicel Corporation, Tokyo, Japan) was used for the separation of TAG-Es. The mobile phase was methanol, and the flow rate was 0.5 mL/min. The MS conditions, too, were the same as those used for sn-PLP/rac-PPL and sn-POP/rac-PPO separation. The precursor-to-product ion MRM transitions for sn-LPP/sn-PPL, sn-OPP/sn-PPO, and sn-OPP/sn-POO were \( m/z \ 831.7[^{1}]\text{PPL} + [H]^+ > 551.5[^{1}]\text{PP}^+ \), \( m/z \ 833.7[^{1}]\text{PPO} + [H]^+ > 551.5[^{1}]\text{PP}^+ \), and \( m/z \ 859.8[^{1}]\text{POO} + [H]^+ > 603.5[^{1}]\text{OO}^+ \), respectively. The resolution and elution order of the pair of TAG-Es were confirmed using TAG-E standards. The analyses were carried out three times. The average of the TAG-E actual ratio was directly calculated from the peak area ratios in the MRM chromatograms.

3 RESULTS AND DISCUSSION

The relative abundance of the TAG molecular species was analyzed following the official method of JOC. The mixing ratio of acetone to acetonitrile (60:40, v/v) led to good separation performance and appropriate retention time. Previous studies have revealed a high abundance of TAG molecular species binding P, O, and linoleic acid (L) in egg yolk. The representative chromatogram for mature egg yolk is shown in Fig. 1. The abundance of the TAG molecular species is summarized in Table 1. All the analytical samples showed a high abundance of TAG molecular species such as POO, POL, OOL, and PPO (Table 1). The TAGs binding 2 kinds of FAs, specifically a combination of P and unsaturated FA, were selected as the target TAG. POO was the most abundant TAG molecular species and was thought to be a very important TAG molecular species in egg yolk. The PPO and PPL contents were also prominent (Table 1). OOL was one of the target TAG molecular species; however, TAG-Es consisting of only UFAs cannot be separated on CHIRALCEL OD-3R. Consequently, the distribution of TAG isomers in POO, PPL, and PPO was examined in this study.

The compositions of TAG-PI and TAG-E in POO, PPO, and PPL were analyzed using HPLC-APCI/MS/MS. The peaks in the chromatograms were identified using standard TAG isomers based on the retention time and MS spectra. The recycle HPLC system was used for the analysis of TAG-PIs consisting of 1 saturated FA and 2 unsaturated FAs, and all TAG-Es. The TAG-PI contents in each sample were quantified by the calibration curve shown in Table 2. In this study, the limit of detection (LOD) and quantification (LOQ) were evaluated using the rac-POO solution and calculated using the signal-to-noise ratio (s/n)\(^{19} \). The calculated and observed LOD (s/n = 3) and LOQ (s/n = 10) were 3 ppm and 10 ppm, respectively.

The representative TAG-PI chromatograms using standard TAGs and egg yolk TAG are illustrated in Fig. 1. The TAG-E chromatograms are also shown in Fig. 2. Further-
Fig. 1 MRM chromatogram of β-OPO and β-POO. (A) Standard TAG positional isomer pairs, (B) Egg yolk, (C) Immature egg yolk, (D) Chicken meat.

Table 1 Relative abundance of main TAG molecular species (abundance over 0.1) in egg yolk, immature egg yolk, and chicken meat.

<table>
<thead>
<tr>
<th></th>
<th>Egg yolk</th>
<th>Immature egg yolk</th>
<th>Chicken meat</th>
</tr>
</thead>
<tbody>
<tr>
<td>P_{3}OL</td>
<td>2.32 ± 0.02</td>
<td>3.15 ± 0.04</td>
<td>6.65 ± 0.18</td>
</tr>
<tr>
<td>P_{2}L</td>
<td>3.20 ± 0.04</td>
<td>3.40 ± 0.03</td>
<td>5.35 ± 0.07</td>
</tr>
<tr>
<td>OOL</td>
<td>6.06 ± 0.02</td>
<td>7.75 ± 0.02</td>
<td>12.35 ± 0.21</td>
</tr>
<tr>
<td>POL</td>
<td>22.83 ± 0.10</td>
<td>23.80 ± 0.15</td>
<td>17.20 ± 0.08</td>
</tr>
<tr>
<td>PPL</td>
<td>2.37 ± 0.03</td>
<td>1.96 ± 0.04</td>
<td>5.64 ± 0.09</td>
</tr>
<tr>
<td>OOO</td>
<td>5.78 ± 0.04</td>
<td>7.20 ± 0.02</td>
<td>8.22 ± 0.13</td>
</tr>
<tr>
<td>POO</td>
<td>34.30 ± 0.18</td>
<td>30.01 ± 0.07</td>
<td>18.45 ± 0.15</td>
</tr>
<tr>
<td>PPO</td>
<td>7.74 ± 0.10</td>
<td>6.39 ± 0.06</td>
<td>10.18 ± 0.33</td>
</tr>
<tr>
<td>PPP</td>
<td>0.15 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td>SOO</td>
<td>3.81 ± 0.18</td>
<td>4.45 ± 0.03</td>
<td>4.10 ± 0.02</td>
</tr>
<tr>
<td>PSO</td>
<td>7.05 ± 0.08</td>
<td>5.90 ± 0.04</td>
<td>4.10 ± 0.13</td>
</tr>
<tr>
<td>PPS</td>
<td>0.48 ± 0.03</td>
<td>0.36 ± 0.01</td>
<td>0.64 ± 0.05</td>
</tr>
</tbody>
</table>

(n = 3) (Mean ± SE, Area %)
more, the composition of TAG-PI and TAG-E is summarized in Table 3. From the data in the table, it is seen that P is mostly located at the sn-1 position in POO for all the samples. However, chicken meat also binds P at the sn-2 position.

### Table 2 Calibration curves for the respective isomers.

<table>
<thead>
<tr>
<th>Calibration curve (R)</th>
<th>Ratio of slopes</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-PLP y = 0.0073x (0.99)</td>
<td>β-PLP / β-PPL = 1.7</td>
</tr>
<tr>
<td>β-PPL y = 0.0044x (0.99)</td>
<td></td>
</tr>
<tr>
<td>β-OPO y = 80.3x (0.99)</td>
<td>β-POO / β-OPO = 1.2</td>
</tr>
<tr>
<td>β-POO y = 64.5x (0.99)</td>
<td></td>
</tr>
<tr>
<td>β-POP y = 0.0021x (0.99)</td>
<td>β-POP / β-PPO = 0.34</td>
</tr>
<tr>
<td>β-PPO y = 0.0061x (0.99)</td>
<td></td>
</tr>
</tbody>
</table>

R multiple correlation coefficient
and sn-3 positions, though egg yolk does not bind much P at these positions. PPL and PPO, namely, the TAGs consisting of 2 Ps and 1 UFA, in egg yolk and immature egg yolk showed a similar tendency that sn-OPP hardly exists. These differences would mean that the purpose for which the TAGs were constituted is different. It is thought that TAGs in egg yolk and milk fat have the same function: they act as the source of energy and nutrients for the development of organs in newborns. In the case of milk fat, the middle chain FA mainly locates at the sn-3 position of TAG\(^2\). It is reported that lingual lipase in babies specifically hydrolyzes FA at the sn-3 position\(^1, 4\) and that the FA is of middle chain type. Middle chain FAs can be effectively converted to energy because they are easily catabolized to acyl-CoA in the mitochondria of cells without binding to carnitine\(^2\). Egg yolk may also play the same role as milk fat and acts as a source of energy and nutrients to babies. FAs at the α position are more rapidly β-oxidized to produce energy as compared to those at the β-position\(^22\). Probably, P in eggs would play the same role as the middle chain FAs in milk fat because both are not functional FAs. Particularly, P in POO would be important because the sn-POO content was the highest and almost one-third of that in egg yolk TAG. Thus, Ps at the α position in egg yolk and immature egg yolk would be utilized quickly to produce energy in a closed system such as an egg. In contrast, Ps in TAG accumulated in white adipose tissue (WAT) would play a different role. The FAs in WAT are not converted to energy in the tissue. The TAG is first hydrolyzed by adipose triglyceride lipase (ATGL) to form one FA and one diacylglycerol molecule. The diacylglycerol is further hydrolyzed by hormone-sensitive lipase (HSL) to form one FA and one monooacylglycerol molecule. Finally, the monooacylglycerol is converted into FA and glycerol by monoacylglycerol lipase (MGL) in the final step of the lipolysis. The FAs are then released into the blood stream. Therefore, the TAG in WAT is hydrolyzed into 3 FAs and 1 glycerol by several kinds of lipase such as ATGL, HSL, and MGL\(^23, 24\). This is a multi-step process involving different lipases relating to digestion, such as lingual lipase, gastric lipase, and pancreatic lipase. For example, pancreatic lipase hydrolyzes TAG to form 2-monooacylglycerol and 2 FAs, and the 2-monooacylglycerol is not further hydrolyzed. A selectivity of lipase, probably lipoprotein lipase family toward egg yolk TAG existing in lipoprotein would be similar to lingual lipase that hydrolyze short and middle FA at sn-3 position of TAG. Lipase in egg would selectively hydrolyze P at the sn-1 position of the TAG and use it as the energy source. TAGs both in milk fat and egg yolk have the same function, namely, the TAG structure must be constituted to act as an energy source for newborns. The POO content of human milk fat is the highest; however, the binding position of P in the POO unit of human milk is mainly at sn-2, so that salt formation between calcium and free FA is prevented\(^25, 26\). This is because P is digested by pancreatic lipase in the digestive tract being absorbed into the newborn (infant). However, P in egg yolk TAG would be used as an energy source without going through the digestion process in the digestive tract; therefore, the location of P at the sn-1 position of the TAG would be beneficial for hydrolysis. The purpose for which POO is constituted is different between egg yolk TAG and milk fat TAG, though both serve as nutrients to give energy to newborns. In the case of the TAG consisting of 2 Ps and 1 UFA such as PPO and PPL, P can be present at all the binding positions in egg yolk, because UFA does not prefer to bind at the sn-1 position in egg yolk. Chicken meat contains sn-LPP and sn-OPP, which are none or minor TAG-E in egg yolk. This would be also ascribed to the difference of lipase discussed above.

4 CONCLUSION

The compositions of TAG-PI and TAG-E in egg yolk and chicken meat were examined in this study. It is not often conscious that TAG is an aggregation of many kinds of TAG-E’s. Many studies that have analyzed the FA composition at the sn-1, 2, and 3 positions of TAG in many kinds of fats and oils; even though the obtained information is valuable, the results do not grasp the essence of the study, namely, intact TAG molecules. The results of the present study reveal that the binding position of FA is affected by the FA combination in the TAG and that it affects the distribution of TAG-E’s. As mentioned before, the TAG structure affects the nutrient effect, e.g., comparison of the POO structures in egg yolk and milk fat. Furthermore, it has recently been reported that 1-oleoyl-2,3-dipalmitoyl-sn-glycerol (sn-OPP) and 1,2-dipalmitoyl-3-oleoyl-sn-glyc-
erol (sn-PPO) showed different crystal polymorphism in a mixture of sn-OPP and sn-PPO\(^27\). These facts indicate that understanding the composition of TAG-PI and TAG-E in fats and oils is very important from not only a nutritional point of view but also a physical point of view.

Conflict of interest
The authors declare no conflicts of interest.

References

Distribution of triacylglycerol isomers in egg yolk


