Formation Mechanisms and Edible Applications of Molecular Compound (MC) Forming Mixture Systems of Saturated-Unsaturated Mixed-Acid Triacylglycerols

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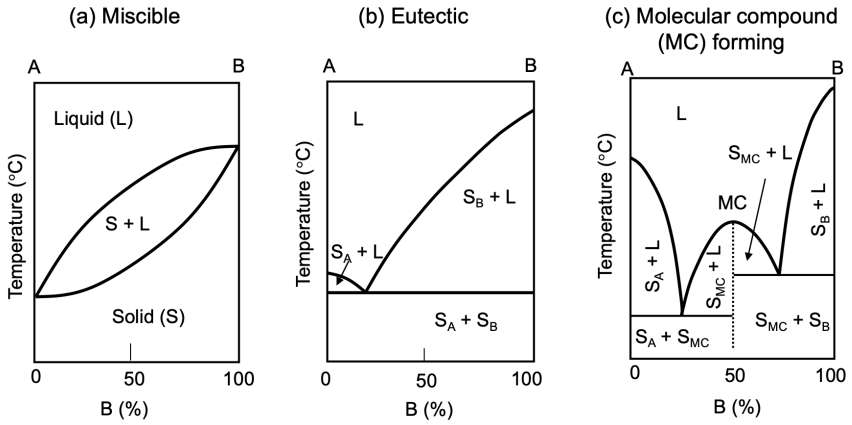
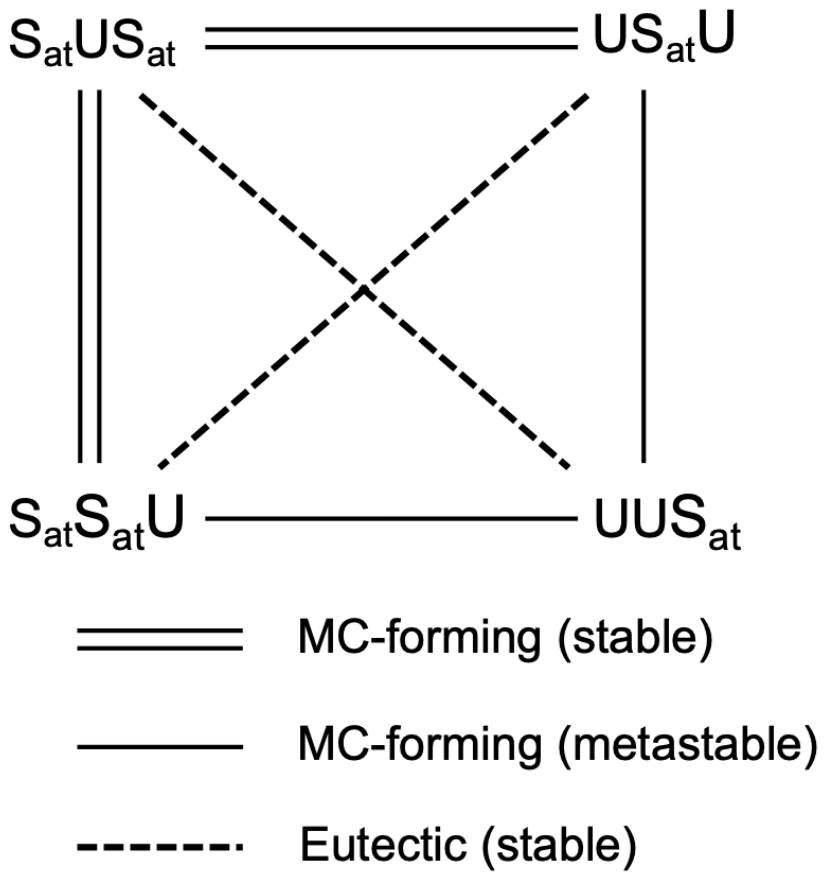
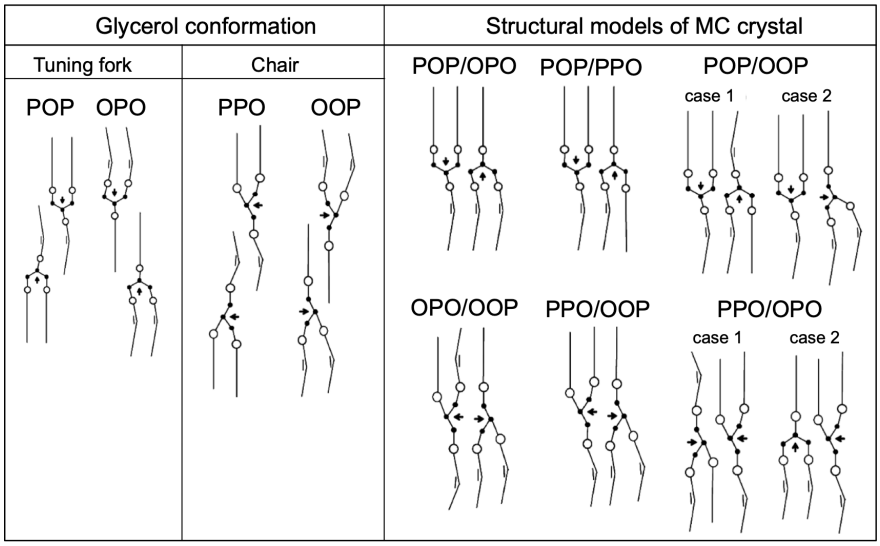
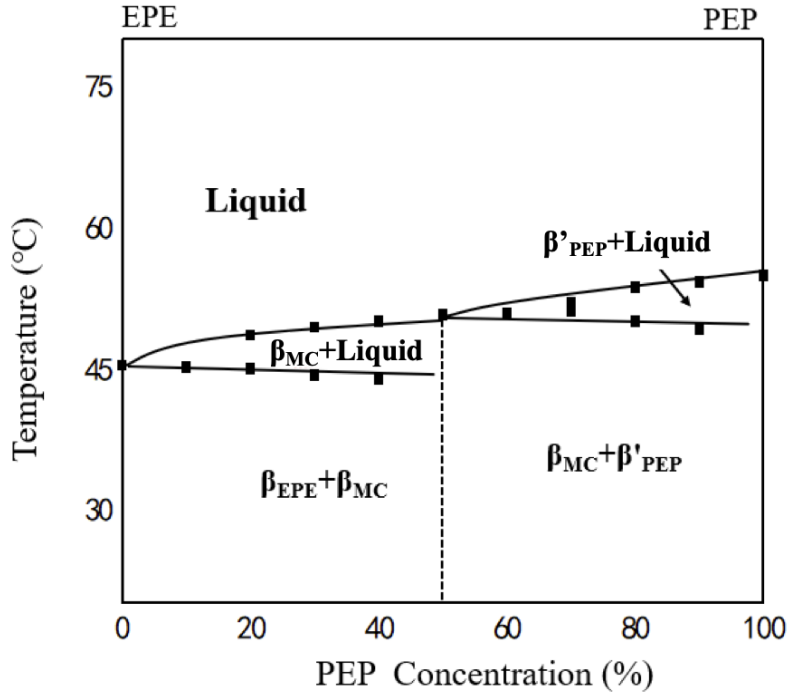
Abstract

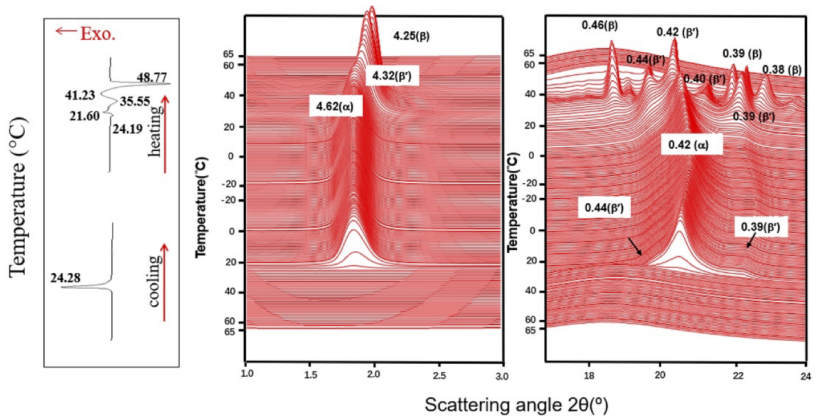
The triacylglycerols (TAGs) containing saturated (Sat) -unsaturated (U) fatty acid moieties (Sat-U mixed acid TAGs) are widely present in most natural fats and employed in many industrial applications. The mixing behavior of different Sat-U mixed acid TAGs acts important roles in the physicochemical properties TAG-based materials. Among the three main mixing states of miscible, eutectic and molecular compound (MC) forming mixtures, fundamental research has been conducted on the MC crystals formed by different Sat-U mixed acid TAGs to understand the structures, phase behavior and crystallization properties. This article reviews recent studies on the complex thermodynamic, kinetic and structural factors that affect the formation of MC crystals in binary and ternary mixtures of Sat-U mixed acid TAGs (SatUSat, SatSatU, USatU and UUSat) through specific molecular interactions among the component TAGs. Furthermore, the application of the MC-forming mixtures containing cacao butter to new types of cocoa butter alternative is reviewed.

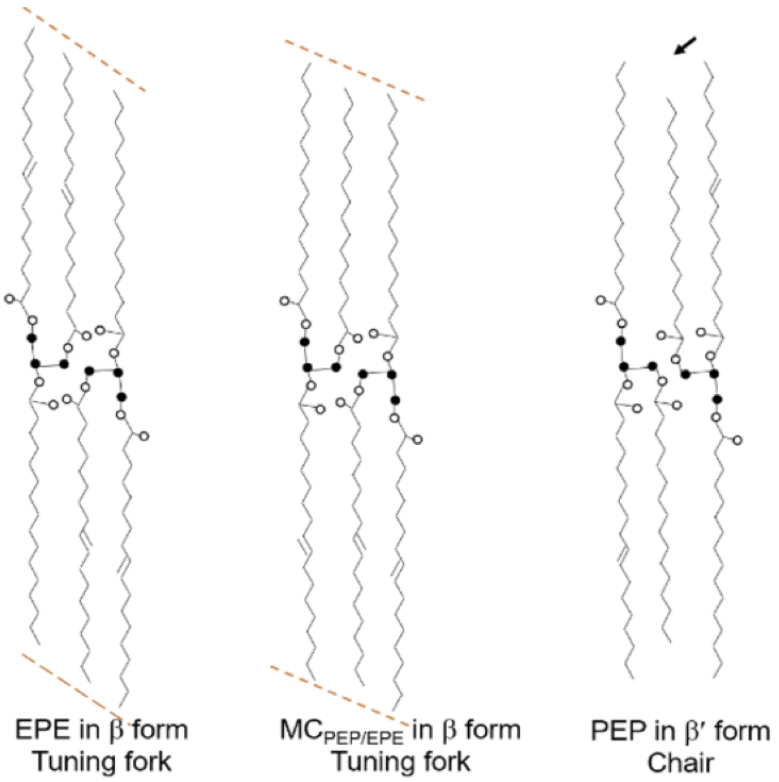




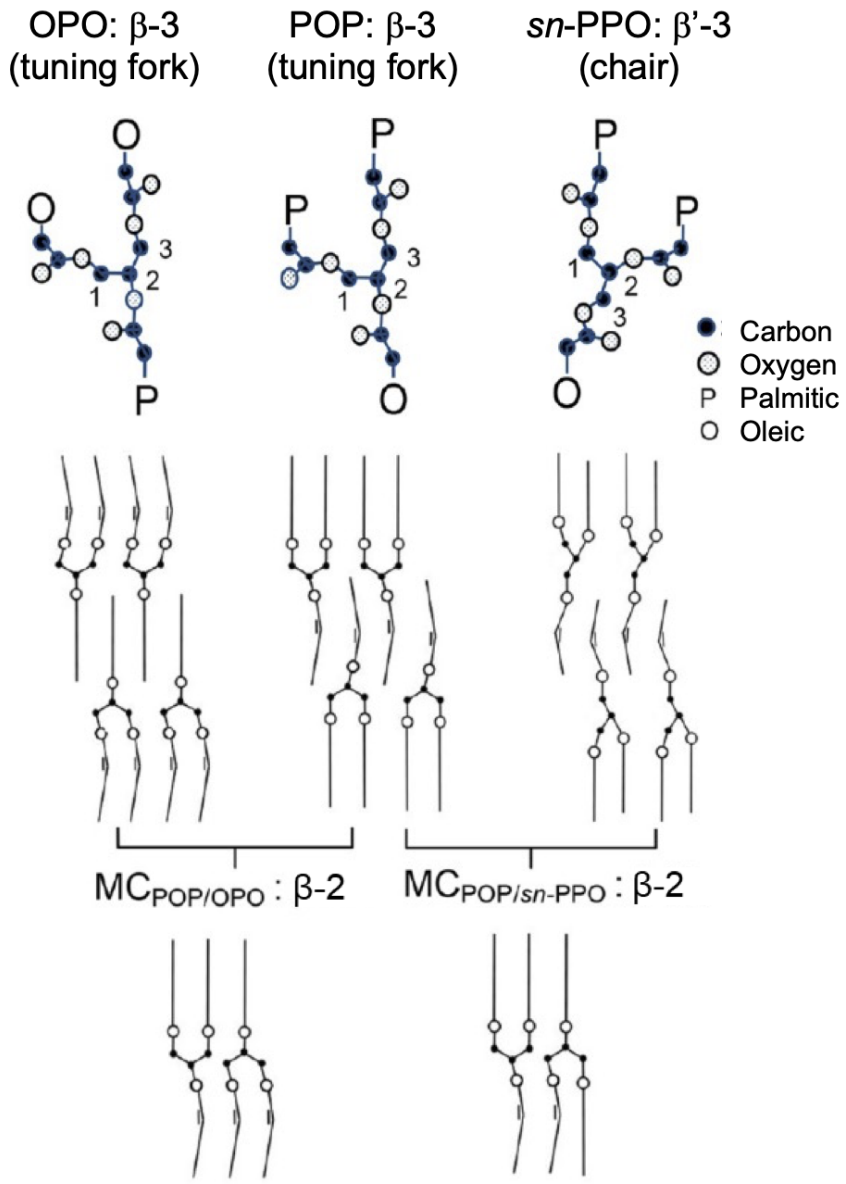


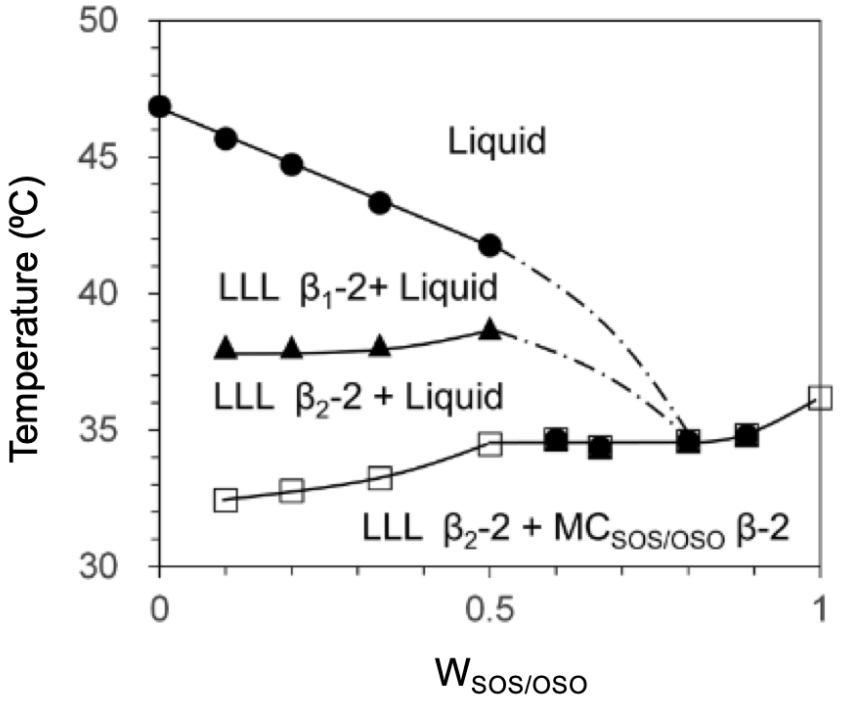


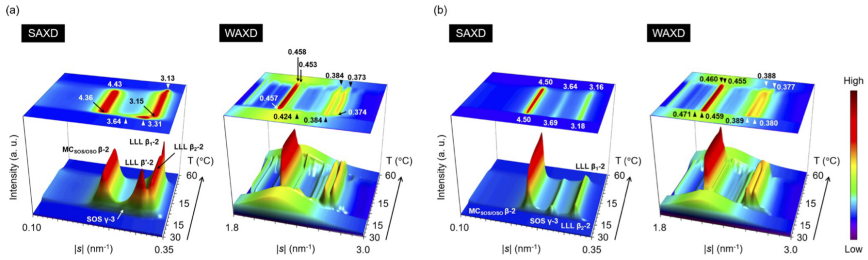


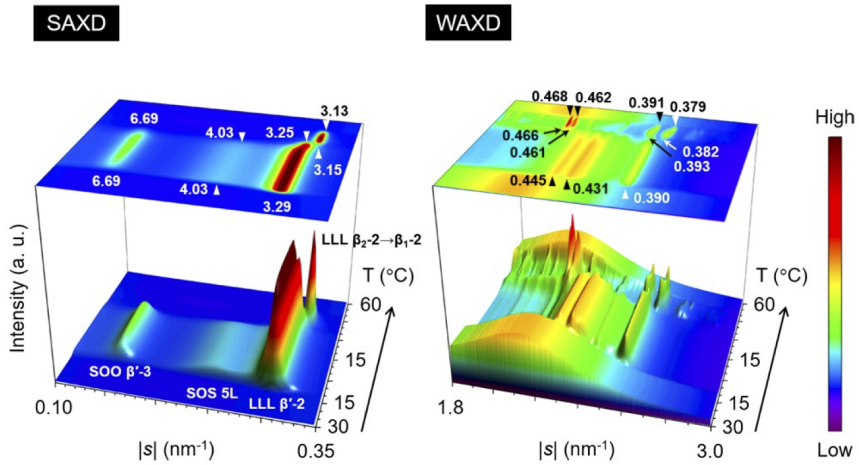


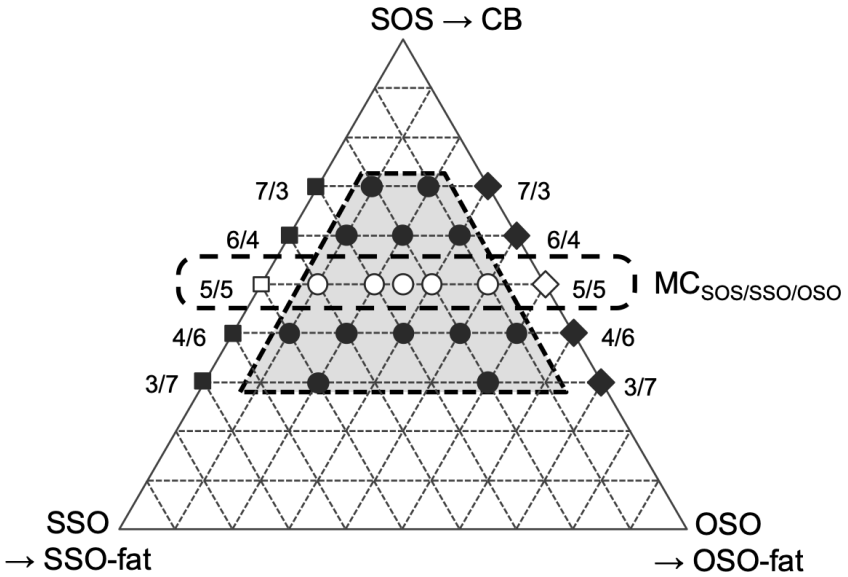
Cooling	Heating (2 ºC/min)
POP/OPO	
L $\xrightarrow{0.1;\ 0.5;\ 2\ ºC/min}$ β-2 (MC$_{POP/OPO}$)	→ L
POP/*rac*-PPO	
L $\xrightarrow{0.1\ ºC/min}$ β-2 (MC$_{POP/rac\text{-}PPO}$)	→ L
L $\xrightarrow{0.5;\ 2\ ºC/min}$ β'-2 (MC$_{POP/rac\text{-}PPO}$)	→ L → β-2 (MC$_{POP/rac\text{-}PPO}$) → L
POP/*sn*-PPO	
L $\xrightarrow{0.1;\ 0.5\ ºC/min}$ β'-3 (*sn*-PPO)	→ L
	β'-2 (MC$_{POP/sn\text{-}PPO}$) → L → β-2 (MC$_{POP/sn\text{-}PPO}$) → L
	β'-2 (POP) → L
L $\xrightarrow{2\ ºC/min}$ β'-3 (*sn*-PPO)	→ L
	α-2 (MC$_{POP/sn\text{-}PPO}$) → L → β'-2 (MC$_{POP/sn\text{-}PPO}$) → β-2 (MC$_{POP/sn\text{-}PPO}$) → L
	α-2 (POP) → L → β'-2 (POP → L
	α-2 (*sn*-PPO) → L

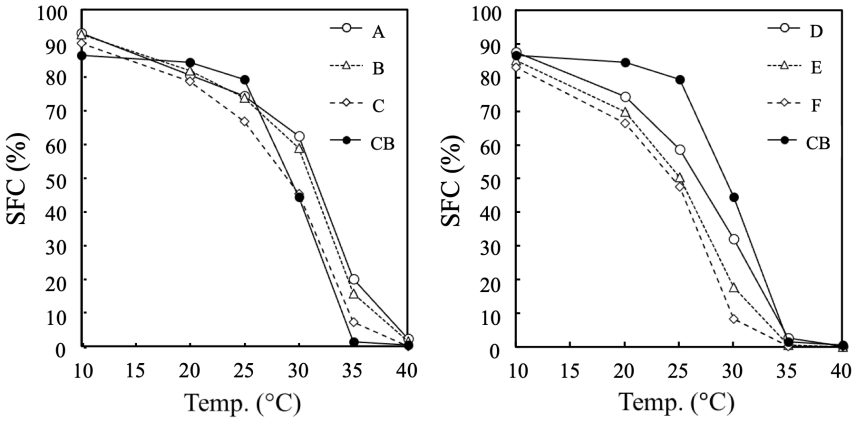












Formation Mechanisms and Edible Applications of Molecular Compound (MC) Forming Mixture Systems of Saturated-Unsaturated Mixed-Acid Triacylglycerols

Laura Bayés-García[1], Kiyotaka Sato[2], Lu Zhang[3], Shinichi Yoshikawa[4], Fumitoshi Kaneko[5], Yoshinori Yamamoto[6], Shimpei Watanabe[7], Ken Taguchi[8]

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Keywords Triacylglycerols, Mixing behavior, Molecular compound crystal, Cocoa butter equivalent, Cocoa butter substitute

Abstract

The triacylglycerols (TAGs) containing saturated (S_{at}) -unsaturated (U) fatty acid moieties (Sat-U mixed acid TAGs) are widely present in most natural fats and employed in many industrial applications. The mixing behavior of different Sat-U mixed acid TAGs acts important roles in the physicochemical properties TAG-based materials. Among the three main mixing states of miscible, eutectic and molecular compound (MC) forming mixtures, fundamental research has been conducted on the MC crystals formed by different Sat-U mixed acid TAGs to understand the structures, phase behavior and crystallization properties. This article reviews recent studies on the complex thermodynamic, kinetic and structural factors that affect the formation of MC crystals in binary and ternary mixtures of Sat-U mixed acid TAGs ($S_{at}US_{at}$, $S_{at}S_{at}U$, $US_{at}U$ and UUS_{at}) through specific molecular interactions among the component TAGs. Furthermore, the application of the MC-forming mixtures containing cacao butter to new types of cocoa butter alternative is reviewed.

41 # INTRODUCTION

42 The physicochemical properties (e.g., texture, rheology, melting behavior, spreadability,
43 appearance, *etc.*) of lipid-based products in the food, pharmaceutical and cosmetic industries are
44 largely determined by the polymorphic and mixing behavior of the constituent triacylglycerols (TAGs)
45 (Larsson et al., 2006, Floeter et al., 2018).

46 The polymorphic crystallization of TAGs, often based on complex simultaneous processes, and
47 the phase transformation phenomena are determined by many different factors, such as the chemical
48 nature of the fatty acid moieties in the TAG structures, and the crystallization conditions applied, which
49 can be tailored in many different ways by the application of external factors (Bayés-García et al.,
50 2015a), such as the use of dynamic thermal treatments (Bayés-García et al., 2013; Bayés-García et al.,
51 2016; Bayés-García et al., 2018), sonication (Martini, 2013; Chen et al., 2013; Ye et al., 2014; Lee et
52 al., 2015), shear (Sonwai et al., 2006; Mazzanti et al., 2011; Acevedo et al., 2012; Acevedo et al.,
53 2014), emulsification (Povey et al., 2014; Douaire et al., 2014), additives (Smith et al., 2011;
54 Yoshikawa et al., 2014; Bayés-García et al., 2022)). An in-depth understanding of the polymorphism
55 and mixing behavior of TAG components in binary, ternary and even more complex systems is then
56 required for the optimal control and design of industrial lipid crystallization procedures.

57 The mixing behavior of TAGs has been comprehensively reviewed by several authors (Floeter
58 et al., 2018; Zhang et al., 2018; Macridachis et al., 2020). Among all TAG species, those with
59 saturated-unsaturated mixed-acid compositions are widely present in most natural fats and used in
60 industrial applications. Therefore, extensive research has been carried out in their binary (Koyano et
61 al., 1992; Minato et al., 1997a; Minato et al., 1997b; Rousset et al., 1998; Takeuchi et al., 2002; Zhang
62 et al., 2007; Zhang et al., 2009; Ikeda et al., 2010; Mizobe et al., 2013; Ikeda et al., 2014; Bayés-García
63 et al., 2015b; Nakanishi et al., 2018; Cholakova et al., 2023; Alishevich et al., 2023; Wijarnprecha et
64 al., 2023), ternary (Koyano et al., 1993; Sasaki et al., 2012; Watanabe et al., 2018; Ghazani et al.,
65 2019a; Macridachis et al., 2021; Macridachis et al., 2022; Yoshikawa et al., 2022) and multicomponent

mixture systems (Bayés-García et al., 2017; Watanabe et al., 2021a). In parallel with the experimental studies on the TAG mixtures, theoretical studies have been carried out to model and predict the mixing behavior of different TAGs (Wesdorp et al., 2013; Seilert et al., 2021).

Although the number of molecules involved in complex end products is often on the order of several hundred (Myher et al., 1988; Gresti et al., 1993), the study on the solid-state miscibility on a smaller scale with a reduced number of TAG components, usually through binary or ternary mixtures, has been shown to provide highly valuable information that can be extrapolated to real fats, such as extra virgin olive oil (Bayés-Garcia et al., 2017), cocoa butter (Sasaki et al., 2012; Ghazani et al., 2019a; Ghazani et al., 2019b), cocoa butter and coconut oil (Joshi et al., 2020), palm oil (Gibon et al., 1996; Minato et al., 1996; Minato et al., 1997a; Lu et al., 2019; Gibon & Danthine, 2020), milk fat/palm oil/palm stearin mixtures (Mao et al., 2023) .

There are three main mixed states resulting from molecular interactions in binary systems of TAGs. In a miscible mixture (Figure 1a), a solid solution is formed at all the mixing ratios between the component TAGs with similar thermal stability and a high degree of isopolymorphism, as the integration of one crystal phase into the other does not cause any significant disturbance in crystal packing (Lusi, 2018). By contrast, dissimilar TAG components lead to eutectic behavior as shown in Figure 1b, where the eutectic composition and the degree of partial solid miscibility can be determined by, among other things, chain-length structures and melting behavior (Timms, 1984; Minato et al., 1996; Floeter et al., 2018; Cholakova, 2023).

Of particular interest is the third mixed state of the molecular compound (MC) formation (Figure 1c). This stoichiometric compound is formed only at well-defined compositions by specific molecular interactions between individual TAGs, resulting in unique structural and thermodynamic properties (see below). The wide range of possibilities given by chemical nature and processing conditions of the component TAGs may be further enhanced by the use of MC products due to their applicability in edible fat structuring. For example, the potential of MC crystals as partial replacers for *trans* or

91 saturated fats has already been demonstrated by increasing melting temperature, hardness and solid fat
92 content (Mykhaylyk et al., 2007).

93 In this review, a special emphasis has been given to the crucial role and potential applicability
94 of MC-forming mixture systems to modify of the physicochemical properties of lipid structures. It
95 should be noted that complex thermodynamic, kinetic and structural factors largely affect the
96 formation of MC in saturated-unsaturated-saturated ($S_{at}US_{at}$)/$US_{at}U$ and $S_{at}US_{at}$/$S_{at}S_{at}U$ systems
97 through specific molecular interactions among the component TAGs.

98 As a fundamental study to elucidate the formation mechanisms of MC, precise observation was
99 performed on the crystallization behavior of palmitic-oleic-based MC of 1,3-dipalmitoyl-2-oleoyl-
100 glycerol (POP)/1,3-dioleoyl-2-palmitoyl glycerol (OPO), POP/1,2-dipalmitoyl-3-oleoyl-*rac*-glycerol
101 (*rac*-PPO) and POP/1,2-dipalmitoyl-3-oleoyl-*sn*-glycerol (*sn*-PPO) when subjected to varied cooling
102 rates. By comparing the crystallization behavior of POP/*sn*-PPO and POP/*rac*-PPO systems, the effects
103 of glycerol structures and optical isomerization on MC crystal formation were also briefly described
104 (Bayés-García et al., 2023). The analysis of the influence of the *cis-trans* isomerization on MC
105 formation was performed by replacing *cis*-oleoyl to *trans*-elaidoyl (E) chains in the binary system of
106 1,3-dipalmitoyl-2-elaidoyl glycerol (PEP)/1,3-dielaidoyl-2-palmitoyl glycerol (EPE) (Zhang et al.,
107 2020).

108 The effects of MCs as polymorphic stabilizers of a third TAG component were discovered by
109 Yoshikawa et al., who observed the crystallization kinetics in ternary mixtures of 1,3-distearoyl-2-
110 oleoyl glycerol (SOS)/1,3-dioleoyl-2-stearoyl glycerol (OSO)/1,2,3-trilauroyl glycerol (LLL)
111 (Yoshikawa et al., 2022). It was found that the most stable β-form of LLL was activated by the presence
112 of β-crystals of $MC_{SOS/OSO}$. It was indicated that this work has a possibility to develop a new type of
113 cocoa butter substitute (CBS).

114 Until recently, the application of MC formation to the end food products has been rather limited
115 (Sibbald et al., 2016). However, systematic work was carried out to apply the MC-forming mixtures

to the development of cocoa butter equivalent (CBE) and cocoa butter substitute (CBS) formulations by forming the MC crystals in the ternary mixtures of cocoa butter, OSO, SSO and LLL fats (Watanabe et al., 2021; Watanabe et al., in submission). These studies may have indicated the high potential of the MC-forming TAG mixtures to produce the edible fats with novel functionality.

Molecular-level understanding of the formation mechanisms of MC crystals

Here briefly summarized are the main results of previous studies on the formation processes, structural properties and phase behavior of MC crystals.

It has been found that the following TAGs can form the MC crystals in their binary mixtures, in which the ratio of the component TAGs is always 50:50.

$S_{at}US_{at}$: Symmetric saturated and unsaturated TAGs such as POP, SOS, PEP

$S_{at}S_{at}U$: asymmetric saturated and unsaturated TAGs such as PPO (or OPP), SSO

$US_{at}U$: symmetric unsaturated and saturated TAGs such as OPO, OSO, EPE

UUS_{at}: asymmetric unsaturated and saturated TAGs such as OOS (or SOO) and OOP (or POO)

Figure 2 illustrates the combination of the TAGs, noting the MC-forming and eutectic mixtures. In the binary mixtures of $S_{at}US_{at}/S_{at}S_{at}U$ and $S_{at}US_{at}/US_{at}U$, the MC crystals are formed in the metastable α, β' and β polymorphs, as confirmed in the mixtures of SOS/OSO, SOS/SSO, POP/PPO, POP/PPO, PEP/EPE (see Introduction and section 3). However, the MC crystals are not formed and fully eutectic mixtures are formed in the combinations of $S_{at}US_{at}/UUS_{at}$ and $S_{at}S_{at}U/US_{at}U$. The former result was confirmed in the mixtures of POP/OOP (Zhang et al., 2007) and SOS/OOS (Zhang et al., 2009), and the latter result was confirmed in the mixture of PPO/OPO (Bayés-García et al., 2015b).

Interestingly, the MC crystals were formed in the mixtures of $US_{at}U/UUS_{at}$ and $S_{at}S_{at}U/UUS_{at}$ in their metastable states. For example, the MC crystals in the OPO/OOP and PPO/OOP mixtures occurred

only under metastable conditions and tended to separate into component TAGs to form eutectic mixture systems after 17 months of storage (Bayés-García et al., 2015b). These results were in contrast to those of previous studies on $S_{at}US_{at}/S_{at}S_{at}U$ and $S_{at}US_{at}/US_{at}U$ in which the MC crystals are thermodynamically stable.

The molecular understanding of the ability and inability to form the MC crystals is still open to question. The main key factors may be the stabilization of chain packing between unsaturated and saturated chains, π–π interactions between the double bonds in the unsaturated chains and stabilization of glycerol conformations.

Figure 3 illustrates the glycerol conformations and structural models of the MC crystals by taking palmitic and oleic chains as S_{at} and U chains, respectively. The glycerol conformations are tuning fork in POP and OPO, and chair in PPO and OOP, and all the TAGs form triple chain length in their stable polymorphic forms of β-3 in POP and OPO, and β'-3 in PPO and OOP (Bayés-García et al., 2015b, Floeter et al., 2018). Three problems may arise in the formation of the MC crystals of the double chain- length structure: the chain packing between palmitic and oleic chains, stacking of glycerol groups between the tuning fork and chair conformations, and the π–π interactions between the unsaturated chains.

In the case of $MC_{POP/OPO}$, the stacking of the glycerol groups may be easily accomplished, since both component TAGs form the tuning fork conformation. The chain-chain packing including the π–π interactions may be easiest in $MC_{POP/OPO}$, and thus the formation of $MC_{POP/OPO}$ can be easily done.

However, the stacking of the glycerol groups in $MC_{POP/PPO}$ may be difficult since POP and PPO exhibit the tuning fork and chair conformations, respectively. In addition, steric hindrance between the oleic and palmitic chains may be a disturbing factor in the formation of $MC_{POP/PPO}$. Nevertheless, the metastable and stable polymorphs are formed in $MC_{POP/PPO.}$ This suggests that the strong attractive interactions between palmitic chains may play dominant roles during the formation processes of $MC_{POP/PPO}$.

In the case of $MC_{OPO/OOP}$, the stacking of the tuning fork (OPO) and chair (OOP) glycerol conformations may not be easy, and the chain packing between the palmitic and oleic chains is rather unstable. Therefore, $MC_{OPO/OOP}$ is not thermodynamically stable. In the case of PPO/OOP, it seems that both the chain packing and

166 the stacking of the chair glycerol conformation of PPO and OOP may enable the formation of MC crystals, but
167 $MC_{PPO/OOP}$ is not thermodynamically stable. Finally, the structures of $MC_{POP/OOP}$ and $MC_{PPO/OPO}$ are unstable
168 because the stacking of the glycerol groups and the chain packing may not stabilize the MC crystals. To conclude,
169 the explanation of the formation of MC crystals at the molecular level seems to be incomplete except for
170 $MC_{POP/OPO}$ and $MC_{POP/PPO}$, and further research is needed.

171 The crystallization kinetics of MC depends on the TAG molecular structures as well as the rate
172 of cooling. For example, quite rapid cooling (> 40 ºC/min) of the binary mixture of POP/OPO resulted
173 in separate crystallization of POP and OPO in the first, while $MC_{POP/OPO}$ crystals started to form in
174 addition to the POP and OPO crystals with decreasing cooling rates (<30 ºC/min). The extent of the
175 $MC_{POP/OPO}$ crystals increased with decreasing cooling and heating rates after crystallization (Nakanishi
176 et al., 2018). This result indicates that the formation of MC is a kinetic process involving competitive
177 crystallization and structural rearrangement of the component TAGs.

178 Another interesting issue is the effect of racemization and optical isomerization of asymmetric
179 $S_{at}S_{at}U$ and UUS_{at} on the formation of MC when mixed with other TAGs. For example, the phase
180 behavior of POP/*rac*-PPO and OPO/*sn*-PPO is somewhat different in terms of the melting points of
181 $MC_{POP/rac\text{-}PPO}$ and $MC_{OPO/sn\text{-}PPO}$, although both mixtures form stable polymorph of the double chain
182 length structure (β-2). This may be related to the different crystallization kinetics of $MC_{POP/rac\text{-}PPO}$ and
183 $MC_{POP/sn\text{-}PPO}$ (see below).

184

185 **Formation of MC in binary mixtures of PEP/EPE**

186

187 Replacing the *cis* oleoyl chain in a TAG with a *trans* isomeric elaidoyl chain drastically changes
188 its physical and even nutritional properties. However, very little is known about the actual crystal
189 structures and phase behavior of elaidoyl-containing TAGs. To clarify the effect of *cis-trans*
190 isomerization on the crystallization behavior of TAGs, the phase behavior of binary mixtures of 1,3-

191 dipalmitoyl-2-elaidoyl-glycerol (PEP) and 1,3-dielaidoyl-2-palmitoyl-glycerol (EPE) was investigated
192 (Zhang et al., 2020). The binary mixtures of PEP and EPE were prepared at 10% intervals and
193 characterized by DSC, conventional powder X-ray diffraction, and synchrotron radiation X-ray
194 diffraction (SR-XRD). The effects of *cis-trans* isomerization on the crystallization behavior of
195 TAGs were examined by comparison with related *cis* and fully saturated counterparts.

196 The most stable polymorphic forms are β' for PEP, but β for EPE (Zhang et al., 2020). The
197 formation of MC crystals was first observed in the PEP/EPE mixture at a 50:50 ratio, as shown in the
198 phase diagram (Figure 4). The PEP-rich region exhibited a monotectic phase for β-form MC and
199 β'-form PEP, whereas the EPE-rich region exhibited a monotectic phase for β-form MC and β-
200 form EPE, which is quite similar to that observed for the POP/OPO (Minato et al. 1997).

201 The temperature dependence of the SR-XRD patterns and the DSC thermogram of PEP/EPE
202 at a 50:50 ratio taken during cooling and heating at a rate of 2 °C/min is shown in Figure 5. The
203 simultaneous crystallization of the β' form of $MC_{PEP/EPE}$ along with the α form was confirmed in
204 the WAXS spectra by the appearance of β' peaks of 0.44 nm and 0.39 nm and an α peak of 0.42 nm,
205 as indicated by the arrows. With increasing temperature, the α form transformed into the β' form
206 and then further transformed to the thermodynamically stable β form via solid-state
207 transformation. Then, the β form of MC melted at approximately 48 °C. In the SAXS spectra, the
208 solid-state transition of α → β' → β was confirmed by the shifting of the peak from 4.62 nm to 4.32
209 nm and then to 4.25 nm.

210 Figure 6 shows the structural model of $MC_{PEP/EPE}$ using the tuning fork conformation of
211 glycerol group, which allows the void (see arrow) at the methyl end of PEP in β′ form due to the
212 chain length mismatch between neighboring P and E chains to be filled by the acyl chain of EPE, as

213 indicated by the dotted line. It is worth noting that when the "O" in certain binary mixtures of palmitic-
214 oleic diacid TAGs such as POP and POO was replaced with "S", or even "E", the phase behavior
215 remained the same. For example, a eutectic phase was found in both the POP/*rac*-POO (Zhang et al.,
216 2007) and PSP/*rac*-PSS mixtures (Bhaggan et al., 2018), and the MC-forming mixture was formed in
217 the POP/*rac*-PPO (Minato et al. 1997) and PSP/*rac*-PPS mixtures (Boodhoo et al., 2009). The MC-
218 forming mixture was also found in the POP/OPO (Minato et al., 1997) and PEP/EPE mixtures (Zhang
219 et al., 2020), and thus it is speculated that MC is formed in the PSP/SPS mixture.
220

221 **Crystallization kinetics of MC crystals of POP/OPO and POP/PPO**

222

223 To elucidate the effects of glycerol structures on the kinetic properties of MC-forming mixture
224 phases, thermal analysis, X-ray diffraction and optical microscopy techniques were applied to the
225 binary mixtures of POP/OPO, POP/*rac*-PPO and POP/1,2-dipalmitoyl-3-oleoyl-*sn*-glycerol (*sn*-PPO)
226 (Bayés-García et al., 2023). All the three mixtures exhibited the MC-forming mixture at a 1:1 ratio.
227 The mixture samples were subjected to dynamic conditions of cooling at low and intermediate
228 rates of 0.1, 0.5 and 2 °C/min to complete crystallization and reheating at a constant rate of 2 °C/min,
229 while monitoring complex crystallization and polymorphic transition phenomena, as summarized in
230 Figure 7. The POP/OPO mixture formed only $MC_{POP/OPO}$ crystals with double chain-length structure
231 in its most stable β form when cooled under all the conditions analyzed, and they simply melted when
232 heated. The occurrence of $MC_{POP/OPO}$ β form was reported in both pure liquid (Minato et al., 1997b)
233 and *n*-dodecane solution systems (Ikeda et al., 2010).
234 Similarly, the most stable β form of $MC_{POP/rac\text{-}PPO}$ crystallized when cooled at 0.1 °C/min,
235 although a metastable β' form was detected at 0.5 and 2 °C/min, which transformed to β when heated.

236 By contrast, the POP/*sn*-PPO mixture showed significantly complex polymorphic events in all
237 the experimental conditions studied, showing the coexistence of $MC_{POP/sn\text{-}PPO}$ with polymorphs of pure
238 POP and *sn*-PPO component TAGs, even at the lowest cooling rate applied. In more detail, the 1:1
239 TAGs mixture crystallized into β' forms of *sn*-PPO (triple chain length), $MC_{POP/sn\text{-}PPO}$ (double chain
240 length) and POP (double chain length) at 0.1 and 0.5 °C/min. When heated, the metastable β' of
241 $MC_{POP/sn\text{-}PPO}$ transformed into its most stable β form before melting. At a higher cooling rate of
242 2 °C/min, the least stable polymorphs predominated, showing complicated simultaneous
243 crystallization processes of *sn*-PPO β' form (triple chain length) and α forms (double chain length) of
244 $MC_{POP/sn\text{-}PPO}$, POP and *sn*-PPO. Subsequent heating caused polymorphic transformations of α → β' →
245 β for $MC_{POP/sn\text{-}PPO}$, and α → L → β' for POP (see Figure 7).
246 Regarding the effects of optical isomerization in mixed systems of TAGs, extensive work had
247 previously been carried out by Craven and Lenki (Craven et al., 2013). Additionally, Mizobe et al.
248 analyzed the polymorphic structures of R-PPO, S-OPP and their mixtures, and concluded that the two
249 optical isomers had identical structural properties, whereas the polymorphic characteristics of R-PPO
250 and *rac*-PPO became different, with the latter corresponding to the 1:1 mixture of R-PPO/S-OPP
251 (Mizobe et al., 2013).
252 To understand the differences in the crystallization behavior of $MC_{POP/OPO}$ and $MC_{POP/sn\text{-}OPO}$, one
253 can pay attention to their structural models, which are mainly based on the glycerol structures of POP,
254 OPO and *sn*-PPO, as depicted in Figure 8. It can be assumed that the tuning fork glycerol conformation
255 is the most stable one for both POP and OPO β forms, since the palmitic and oleic acid chains are
256 located on different leaflets, favoring the packing of TAG molecules in triple chain-length structures
257 that avoid the steric hindrance between straight palmitic and bent oleic acid chains. By contrast, the
258 *sn*-PPO β' form may exhibit a chair-type glycerol conformation, which is also packed in a triple chain-
259 length structure for the same reasons explained above.

260 During the formation process of the MC crystals, the chain-length structures were changed from
261 triple to double, and close packing of glycerol groups and palmitic and oleic acid chains of neighboring
262 TAG molecules occurred. A higher ability of $MC_{POP/OPO}$ to form and stabilize in β form compared to
263 other MCs was observed, since the tuning fork-type glycerol conformation of the two TAG
264 components POP and OPO can be maintained in $MC_{POP/OPO,}$ resulting in a β-2 structure with palmitic
265 and oleic acid chains packed in separate leaflets. This ability was not observed in the POP/*sn*-PPO
266 mixture, as it did not form $MC_{POP/sn\text{-}PPO}$ alone, but it coexisted with single POP and *sn*-PPO TAG
267 components.

268 Polarized light microscopy data confirmed a higher crystallization rate for *sn*-PPO crystals
269 compared to other single TAGs or MCs, which may explain the separate crystallization when the
270 POP/*sn*-PPO mixture was cooled, and this may also be explained from a structural point of view.
271 According to our model (Figure 8), an extra molecular rearrangement may occur during the $MC_{POP/sn\text{-}}$
272 $_{PPO}$ formation, based on the change of the chair-type glycerol conformation of *sn*-PPO to the tuning
273 fork-type, leading to an MC with coexisting oleic and palmitic acid chains in the same leaflet.

274 Regarding the different crystallization behavior of $MC_{POP/sn\text{-}PPO}$ and $MC_{POP/rac\text{-}PPO}$, one may
275 consider the ability of R-PPO and S-OPP to form a stable molecular packing (or racemic compound),
276 which may interact with POP molecules to form a stable MC structure. However, further work may be
277 required to understand the mechanisms involved.

278

279 **Interactive polymorphic crystallization of $MC_{SOS/OSO}$ and LLL**

280

281 The effect of MC formation on the polymorphic crystallization of the other coexisting TAG was
282 examined using ternary TAG mixtures of LLL/SOS/OSO with a 1:1 weight ratio of SOS and OSO
283 (SOS/OSO = 1/1) (Yoshikawa et al., 2022).

Under a series of thermal conditions of cooling from the melt, isothermal holding, and heating, pure LLL without containing SOS and OSO formed β′ form crystals, which transformed to β form during the heating process after the crystallization. In 1992, it was found that the binary TAG mixture of SOS/OSO = 1/1 formed mainly β-form MC crystals of SOS and OSO ($MC_{SOS/OSO}$) during the cooling process (Koyano et al., 1992). Based on these results, the ternary mixtures of LLL/SOS/OSO/ at various mixing ratios of LLL and SOS/OSO=1/1 were investigated and the immiscible eutectic behavior was observed with retarded crystallization and lowered melting points of the formed crystals (Figure 9). Quite interesting results were found in the crystallization kinetics of two β forms of $MC_{SOS/OSO}$ and LLL; the β crystallization of $MC_{SOS/OSO}$ coincided with the β′ to β transformation or direct β melt-crystallization of LLL during the isothermal-holding process, as evident from the synchrotron radiation X-ray diffraction (SR-XRD) data shown in Figure 10. This coincidence strongly suggests the possibility that β crystallization of $MC_{SOS/OSO}$ triggered the β crystallization of LLL. As a result, the spherulitic crystals of LLL deformed and changed their size distribution with increasing concentration of SOS/OSO=1/1. These peculiar phenomena of the interrelationships between the crystallization of β forms of $MC_{SOS/OSO}$ and LLL have been defined as "interactive polymorphic crystallization" (Yoshikawa et al., 2022).

As a possible mechanism underlying the interactive polymorphic crystallization, epitaxial effects through triclinic-parallel ($T_{//}$) subcell matching between β crystals of LLL and $MC_{SOS/OSO}$ may occur in the same manner that β′ form seed crystals of tripalmitin or tristearin effectively accelerated β′ crystallization of coconut oil through orthorhombic-perpendicular ($O_{\perp}$) subcell matching (Mahisanunt et al., 2020). Another possible mechanism is martensitic transformation via cooperative displacement of atoms without diffusion in the crystal lattices, which can be initiated by the occurrence of local stress through mechanical or thermal stimulation, as indicated for petroselinic acid (Kaneko et al., 1997).

308 Elucidating the mechanism of interactive polymorphic crystallization requires further research,
309 such as SR-XRD experiments substituting the component TAGs (LLL, SOS, and OSO) with the other
310 corresponding TAGs (e.g., SSS, SSO, and SOO, respectively) and SR-XRD experiments using a
311 microbeam technique to analyze the local orientation of TAG molecules near the crystal-crystal
312 interfaces. For example, the SR-XRD data shown in Figure 11 indicate that the effect of facilitating β
313 crystallization of LLL in LLL/SOS/OSO was weakened by replacing OSO with rac-SOO, probably
314 because SOS and SOO crystallized separately in the less stable forms of SOS 5L (2L + 3L), in which
315 SOS formed in randomly packed double and triple chain-length structures (Mykhaylyk et al., 2007),
316 and SOO β′.

317 It may be worth noting that the study of LLL/SOS/OSO mixtures can be applied to solve the
318 problem of fat blooming in CBS-based compound coatings, which is partly caused by the polymorphic
319 transformation of lauric acid-based TAG crystals in CBS from β'-2 to β-2 (see below).

320

321 **Application of MC crystals to new types of cocoa butter equivalent (CBE) and cocoa butter**
322 **substitute (CBS)**

323

324 Cocoa butter (CB) is indispensable ingredient in chocolate, as it is responsible for the physical
325 properties such as hardness, texture, and melting behavior of chocolate products (Talbot, 2017).
326 However, CB is one of the most expensive ingredients of chocolate and its price is gradually
327 increasing due to several global issues such as low productivity of cacao due to climate change and
328 increasing demand for cacao products (Afoakwa, 2016). Therefore, the confectionery industry has
329 developed CB alternative fats (CBAs) with various functionalities to replace or blended with CB in
330 chocolate production.

CBAs can be classified into three groups based on their compositions: cocoa butter equivalent (CBE), cocoa butter replacer (CBR) and cocoa butter substitute (CBS) (Timms, 2003). Recently, two experimental studies on the physical properties and fat bloom stability of chocolate made with $S_{at}S_{at}U$ and $US_{at}U$ type fats forming MC crystals with CB revealed that these fats can be used as new types of CBA fats (Watanabe et al., 2021., Watanabe et al., in submission).

CBE

Systematic studies showed that the ternary mixtures of SOS, *rac*-SSO and OSO form MC crystals of stable β-2 form at a concentration of SOS of 50% with different concentrations of SSO and OSO (Watanabe et al., 2018). Figure 12 shows that the MC crystals with the stable β-2 polymorph are formed in a series of the ternary mixtures of SOS/SSO/OSO, in which the ratio of SOS/(SSO+OSO) was set to 50/50 and the ratio of SSO/OSO was varied. Based on this study, it was expected that the ternary mixtures can produce a new type of CBE with the stable polymorphic structure when SOS, SSO and OSO are replaced with CB, SSO-fat and OSO-fat. The SSO-fat and OSO-fat were prepared by full-hydrogenation, interesterification, and fractionation of canola oil high oleic sunflower oil (Watanabe et al., 2021). The physical properties and fat bloom stability of chocolates containing SSO-fat and OSO-fats, which were solidified by simple cooling without tempering process, were then evaluated (Watanabe et al., 2021).

It should be noted that in the chocolate fat phases, the final ratio of the three types of TAG, $S_{at}US_{at}/S_{at}S_{at}U/US_{at}U$, were 50/50/0–50/0/50. Figure 13 shows the temperature change in solid fat content (SFC) values of six fat blends and CB, where the concentrations (%) of $S_{at}US_{at}/S_{at}S_{at}U/US_{at}U$ in the fat blends are50/46/4(sample A), 50/40/10 (B), 50/30/20 (C), 50/20/30 (D), 50/10/40 (E), and 50/3/47 (F). The SFC measurement of ternary fat blends of CB, SSO-fat and OSO-fat showed sharp

354 melting profiles around body temperature. Similarly, in the hardness measurement chocolate samples
355 prepared with fat blends of $S_{at}US_{at}/S_{at}S_{at}U/US_{at}U$=50/30/20–50/20/30 showed almost the same
356 hardness as that of pure chocolate. In addition, no fat bloom formation was observed in dark chocolate
357 with the fat blends of $S_{at}US_{at}/S_{at}S_{at}U/US_{at}U$ of 50/20/30–50/0/50 during the one-year storage under
358 isothermal condition at 25 ˚C and thermal cycling condition between 20 and 30 ˚C. Overall, the CB,
359 SSO-fat, and OSO-fat blends can be used as a cocoa butter equivalent (CBE) without tempering
360 procedures.

361

362 **CBS**

363 The MC crystals made of CB and OSO-fat ($MC_{CB/OSO}$) were applied to compound chocolate
364 formulations by blending with lauric cocoa butter substitutes (CBS) (Watanabe et al., in submission).
365 Conventional CBSs have been produced by fractionation, hydrogenation and a combination of these
366 processes of lauric fats derived from palm kernel oil and other natural fats (Rossell, 1985). These fats
367 are composed of TAGs with lauric (L) and myristic (M) acid moieties including LLL, LLM, LMM,
368 and MMM which are crystallized into β′-2 form by simple cooling without tempering (Smith, 2012).
369 However, the CBS-based chocolate can potentially convert from the β′-2 to β-2 form, which leads to
370 fat bloom formation during long-term storage, because mono-acid TAGs such as LLL and MMM
371 have the potential to transform from β′-2 to the more stable β-2 form (β-tending; Koizumi et al.,
372 2022). In addition, cacao solids such as cacao powder and cacao liquor are often blended to improve
373 the flavour and taste of compound chocolate, resulting in more serious fat bloom due to the phase
374 separation of CBS and CB followed by polymorphic transformation of CB TAGs. This eutectic
375 effect makes it difficult to blend more than 5% cocoa butter in the compound chocolates (Laustsen,
376 1991).

377 As described in the previous section, studies on the polymorphic crystallization behavior of the
378 ternary mixture system of LLL/SOS/OSO with the SOS/OSO ratio of 1/1 have revealed that the
379 crystallization of β-2 form in $MC_{SOS/OSO}$ promoted the crystallization and polymorphic transformation
380 of β′-2 to β-2 in LLL (Yoshikawa et al., 2022). Based on these results, the physical properties and fat
381 bloom stability of lauric-based compound chocolate with $MC_{CB/OSO}$ were evaluated.

382 Table 1 shows six chocolate samples with the different relative concentrations (%) of CBS, OSO-
383 fat and CB investigated. The SFC studies showed that the fat blends of CBS and $MC_{CB/OSO}$ exhibited
384 immiscible eutectic behavior, as did the fat blends of CBS and CB. The results of SFC value,
385 crystallization rates and hardness of compound chocolate with $MC_{CB/OSO}$ suggested that the compound
386 chocolate is suitable for the chocolate production up to about 20% of $MC_{CB/OSO}$ in the fat phases.

387

388 Table 1. Relative concentrations (%) of CBS, CB and OSO-fat in total chocolate fat in eight
389 chocolate samples

Sample	CBS	OSO-fat	CB
A1	90.0	0.0	10.0
A2	85.0	0.0	15.0
A3	80.0	0.0	20.0
A4	75.0	0.0	25.0
B1	80.0	10.0	10.0
B2	70.0	15.0	15.0
B3	60.0	20.0	20.0
B4	50.0	25.0	25.0

390

391 In the fat bloom evaluation studies, while CBS/CB compound chocolate exhibited severe fat
392 bloom within a few weeks, the CBS/CB/OSO-fat compound chocolate showed no fat bloom under any
393 storage condition for 6 months (Table 2). In addition, the XRD studies revealed that no polymorphic
394 transformation occurred for the CBS/CB/OSO-fat blends during the 6 months storage, while the
395 polymorphic transformation from β′-2 to β-2 was observed for the fat blends of CBS/CB. The
396 polymorphic transformations of CB TAGs and β-tending TAG in CBS cause fat blooming in CBS-
397 based compound chocolate. Therefore, the mechanisms of fat blooming inhibition in compound
398 chocolate made of CBS/CB/OSO-fat summarized in Table 1 can be assumed that β-tending TAG
399 fractions in the CBS, such as LLL and MMM, may crystallize into β-form during the cooling process
400 via interactive polymorphic crystallization with $MC_{CB/OSO}$. However, further studies are required to
401 elucidate these mechanisms.
402
403 Table 2. Evaluation of fat bloom formation of eight dark chocolate samples after storage for 3 weeks
404 (3W) and 15 weeks (15W) under different thermal conditions, -; no fat bloom, +; slightly bloomed,
405 ++; fairly bloomed, +++: seriously bloomed

Chocolate sample	15˚C		20˚C		25˚C		15-25˚C	
	3W	15W	3W	15W	3W	15W	3W	15W
A1	+	+++	+	++	-	++	-	+
A2	+	+++	+	++	-	++	-	+
A3	+	+++	+	++	+	++	-	+
A4	+	+++	+	++	+	++	-	+
B1	-	-	-	-	-	-	-	-
B2	-	-	-	-	-	-	-	-
B3	-	-	-	-	-	-	-	-
B4	-	-	-	-	-	-	-	-

CONCLUSION

The study of the mixing behavior of principal TAGs and various natural and industrialized fats is of great importance, as it is one of the most informative and applicable studies to improve the physicochemical properties of lipid-based products using natural and technologically-produced lipid materials. Until recently, the phases of MC-forming mixture have been studied from a fundamental point of view using pure samples of saturated-unsaturated mixed-acid TAGs. However, the fat materials produced by full-hydrogenation, interesterification and fractionation of vegetable fats and oils have been used for the application of the MC crystals to end products such as fat spreads and confectionery fats. The physical properties of the MC crystals are different from those of the component TAGs and can improve the end products when applied; for example, the formation of MC crystals containing the mixed acid TAGs with oleic acid moiety can reduce the use of saturated fats (Sibbald et al., 2016), and the application of the MC crystals containing CB, OSO-fat, and SSO-fat to CBE and CBS improved the crystallization kinetics and fat bloom stability. It is expected to explore the molecular mechanisms of the ability and inability of MC formation, and the application of the MC crystals to other end products such as emulsified and aerated systems.

AUTHOR CONTRIBUTIONS

LB, KT, LZ, SY, FK, YY and SW performed the experiments and published their original articles, which are reviewed in this article. KS initiated and organized this work.

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Figure caption

Figure 1 Typical three binary mixing systems of triacylglycerols (TAGs). A and B: component TAGs, L; liquid phase, S_A, S_B and S_{MC}; solid phases of A, B and molecular compound (MC).

Figure 2 Combination of saturated (S_{at})-unsaturated (U) mixed-acid TAGs exhibiting MC-forming and eutectic binary mixtures.

Figure 3 Key factors affecting the formation of MC crystals in binary mixtures of TAGs containing palmitic (P) and oleic (O) acid moiety.

Figure 4 Mixing phase behavior of PEP and EPE.

Figure 5 DSC thermogram and SR-XRD patterns of PEP/EPE at a 50:50 ratio.

Figure 6 Possible mechanism of $MC_{PEP/EPE}$ formation.

455

456 **Figure 7** Summary of temperature-dependent polymorphic crystallization in the binary mixtures

457 of POP/OPO, POP/*rac*-PPO and POP/*sn*-PPO.

458

459 **Figure 8** Effects of chain packing and glycerol conformation on the formation of MC crystals.

460

461 **Figure 9** Phase behavior of LLL/SOS/OSO mixtures with different weight fractions of

462 SOS/OSO = 1/1 ($W_{SOS/OSO}$): melting of $MC_{SOS/OSO}$ β-2 (□), transformation β_2 -2 → β_1-2 of LLL (▲),

463 and melting of LLL β_1-2 (●).

464

465 **Figure 10** 3D surface plots and overhead projections of SR-XRD data on LLL/SOS/OSO =

466 2/1/1 (a) and 1/2/2 (b), taken during cooling at a rate of 2 °C/min, holding at 15 °C for 30 min, and

467 heating at a rate of 5 °C/min. Numbers hyphenated to Greek letters for crystal polymorphs represent

468 the chain-length structure. Unit: nm.

469

470 **Figure 11** 3D surface plots and overhead projections of SR-XRD data on LLL/SOS/*rac*-SOO =

471 2/1/1, taken during cooling at a rate of 2 °C/min, holding at 15 °C for 30 min, and heating at a rate of

472 5 °C/min. Numbers hyphenated to Greek letters for crystal polymorphs represent the chain-length

473 structure. Unit: nm.

474

475 **Figure 12** Phase diagram of ternary mixture systems of SOS/SSO/OSO. White symbols

476 represent MC crystals of β-2, black symbols represent eutectic mixtures of β-2 of MC crystals and

477 component TAG.

Figure 13 SFC profiles of CB and CB/SSO-fat/OSO-fat blends. Blending ratio of A–F are as follows. CB/SSO-fat/OSO-fat = (A) 50/50/0, (B) 50/40/10, (C) 50/30/20, (D) 50/20/30, (E) 50/10/40 and (F) 50/0/50.

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