Title: Profiling of regioisomeric triacylglycerols in edible oils by supercritical fluid chromatography/tandem mass spectrometry

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26 **Abstract:**

27 In this study, supercritical fluid chromatography (SFC) coupled with triple
28 quadrupole mass spectrometry was applied to the profiling of several regioisomeric
29 triacylglycerols (TAGs). SFC conditions (column, flow rate, modifier) were optimized
30 for the effective separation of TAGs. In the column test, a triacontyl (C30) silica gel
31 reversed-phase column was selected to separate TAG regioisomers. Multiple reaction
32 monitoring was used to selectively quantify each TAG. Then, the method was used to
33 perform detailed characterization of a diverse array of TAGs in palm and canola oils.
34 Seventy TAGs (C46:0 – C60:2) of these oils were successfully analyzed as a result, and
35 twenty isomeric TAG pairs were separated well. In particular, this method provided the
36 fast and high resolution separation of six regioisomeric TAG pairs (PPLn/PLnP,
37 PPL/PLP, PPO/POP, SPLn/SLnP, SPO/SOP, SSO/SOS—stearic acid (S, 18:0), oleic acid
38 (O, 18:1), linoleic acid (L, 18:2), linolenic acid (Ln, 18:3), palmitic acid (P, 16:0)) in a
39 short time (50 min) as compared to high performance liquid chromatography. We were
40 able to demonstrate the utility of this method for the analysis of regioisomeric TAGs in
41 edible oils.

42

43

44 **Keywords:**

45 Supercritical fluid chromatography, Regioisomers, Triacylglycerol, Triple quadrupole
46 mass spectrometry

47

48

49 **1. Introduction**

50 In lipidomics, the profiling of triacylglycerol (TAG) can be very challenging due
51 to the diversity and complexity of its structure [1,2]. The existence of various TAG
52 molecular species is determined by the composition and distribution of FAs at the
53 different stereochemical positions (*sn*-1, 2, and 3) on the glycerol backbone [3]. The
54 distribution of FA is not particularly random; rather, it is characteristic for vegetable
55 oils: saturated FAs occupy the *sn*-1/3 positions, and unsaturated FAs occupy the *sn*-2
56 position [4]. TAG is the major component of vegetable oils [4–7] and animal fats [8,9].
57 TAG structure determines the physical, chemical, and nutritional properties of edible
58 oils, thus determining the quality of oils using TAG profiling is critical [10,11]. Many
59 researchers have characterized the structure of a diverse array of TAGs in plants,
60 animals, and fish for these reasons [12–15].

61 Because there are numerous isomers for TAGs, different compositions of FAs can
62 have the same *m/z* ratio in mass spectrometric analysis. In particular, TAGs containing
63 two different acyl chains, A and B, can exist as two regioisomers, AAB and ABA,
64 depending on which chain occupies the *sn*-2 position [16,17]. Many studies have
65 reported on the analysis of TAG regioisomers by high performance liquid
66 chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS). Leskinen
67 *et al.* quantified four regioisomeric TAG pairs based on the preferential formation of
68 diacylglycerol (DAG) product ions by loss of *sn*-1/3 FAs from a TAG ion [18]. By way
69 of collision-induced dissociation (CID), the loss of *sn*-1/3 FAs is more favorable than
70 the loss of a *sn*-2 FA, which allows the composition of FAs in a TAG to be found based
71 on the observed product ions. The intensity of the DAG ions is also used to elucidate the
72 distribution of FAs in TAGs. In the specific case of a regioisomeric TAG pair that

73 follows the scheme, AAB and ABA, these TAGs produce the same product ions, $[AA]^+$
74 and $[AB]^+$, but they differ in their intensity ratio, denoted as $[AB]^+/[AA]^+$ [19]. There
75 was insufficient separation of the regioisomeric TAG pairs using the method by
76 Leskinen *et al.*; therefore, the formation of DAG ions was not clearly dependent on the
77 positional distribution of the FAs. Finally, it was demonstrated that the effective
78 separation is critical to the selective quantification of TAG regioisomers.

79 Two HPLC techniques, reversed-phase HPLC (RP-HPLC) [18–21] and silver ion
80 normal phase HPLC (Ag-HPLC) [22–24] have been widely applied to the separation of
81 TAG regioisomers. In RP-HPLC, the retention of TAGs is governed by the equivalent
82 carbon number ($ECN = CN - 2DB$) [25]. Under the optimized RP-HPLC system, most
83 TAGs were separated according to the ECN with the exception of regioisomers. The
84 partial separation of regioisomers was previously achieved with very long retention
85 times, which is not ideal for practical analysis [20]. In Ag-HPLC, TAGs are separated
86 according to the number and position of DBs. In a previous study, the separation of
87 regioisomeric TAG pairs containing up to seven DBs was performed in 90 min by Ag-
88 HPLC/MS [17]. Furthermore, the off-line 2D coupling of RP-HPLC and Ag-HPLC
89 provided chromatographic selectivity for various TAG regioisomers [26]. Finally, the
90 high resolution analysis of TAG regioisomers was achieved by using HPLC. However,
91 there still remains a need to develop a fast analytical method for TAG regioisomers, and
92 when there are a relatively large number of samples, a high-throughput system would be
93 required for the practical analysis.

94 In a previous study, the utility of supercritical fluid chromatography (SFC) was
95 demonstrated in the analysis of diverse lipids [27–29]. Thus, SFC/MS with monolith
96 octadecyl-silica (ODS) columns was applied to the analysis of TAGs in soybean [30].

97 As a result, several TAGs were effectively separated in a short analysis time (8 min)
98 compared to conventional HPLC. Though SFC was useful for high-throughput profiling,
99 it was not yet effective to separate and identify TAG isomers. Furthermore, the need to
100 develop a selective and sensitive quantification method using MS/MS still remains.
101 Therefore, the objective of this study is to develop a high-throughput and high-
102 resolution profiling method for TAG regioisomers by SFC/MS/MS.

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104

105 **2. Materials and methods**

106 **2.1. Materials**

107 Carbon dioxide (99.9% grade; Neriki Valve Co., Ltd, Amagasaki, Japan) was
108 used as the mobile phase. HPLC-grade methanol (Wako Pure Chemical Industries, Ltd.,
109 Osaka, Japan) containing 0.1% (w/w) ammonium formate (99.99%; Sigma–Aldrich
110 Japan K. K., Tokyo, Japan) was used as the modifier. The standard samples used in this
111 study were as follows: 1,2-distearoyl-3-oleoyl-glycerol (SSO), 1,3-distearoyl-2-oleoyl-
112 glycerol (SOS), 1-stearoyl-2-oleoyl-3-linoleoyl-glycerol (SOL), triolein (OOO), 1,2-
113 dioleoyl-3-linoleoyl-glycerol (OOL), 1,2-dilinoleoyl-3-oleoyl-glycerol (LLO), 1,3-
114 dilinoleoyl-2-oleoyl-glycerol (LOL), trilinolein (LLL), 1-stearoyl-2-oleoyl-3-palmitoyl-
115 glycerol (SOP), 1-stearoyl-2-linoleoyl-3-palmitoyl-glycerol (SLP), 1,2-dioleoyl-3-
116 palmitoyl-glycerol (OOP), 1-linoleoyl-2-oleoyl-3-palmitoyl-glycerol (LOP), 1,2-
117 dilinoleoyl-3-palmitoyl-glycerol (LLP), 1,2-dipalmitoyl-3-stearoyl-glycerol (PPS), 1,2-
118 dipalmitoyl-3-oleoyl-glycerol (PPO), 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), and
119 1,2,3-triheptadecenoyl-glycerol (MoMoMo), which were purchased from Larodan Fine
120 Chemicals AB (Malmö, Sweden). Incidentally, abbreviation of stearic acid (18:0), oleic

121 acid (18:1), linoleic acid (18:2), linolenic acid (18:3), margaroleic acid (17:1), and
122 palmitic acid (16:0) are S, O, L, Ln, Mo, and P, respectively.

123

124 **2.2. Sample preparation**

125 Each TAG standard was prepared by dissolving it in chloroform, and these
126 standard solutions were then stored at -30°C . Before use, each solution was diluted to
127 the desired concentration with chloroform. Furthermore, commercially obtained palm
128 and rapeseed oils were used for the analysis of biological samples. These oils were also
129 dissolved in chloroform, and triheptadecenoin (MoMoMo, 17:1–17:1–17:1) was used as
130 the internal standard (IS).

131

132 **2.3. Columns**

133 The columns used in this study were as follows: Inertsil ODS-4 column ($250 \times$
134 4.6 mm ID ; $5 \mu\text{m}$, GL Sciences Inc., Tokyo, Japan), Inertsil ODS-P column (250×4.6
135 mm ID ; $5 \mu\text{m}$, GL Sciences), Inertsil ODS-EP column ($250 \times 4.6 \text{ mm ID}$; $5 \mu\text{m}$, GL
136 Sciences), Inertsil ODS-SP column ($250 \times 4.6 \text{ mm ID}$; $5 \mu\text{m}$, GL Sciences),
137 InertSustain C18 column ($250 \times 4.6 \text{ mm ID}$; $5 \mu\text{m}$, GL Sciences), Sunrise C18 column
138 ($250 \times 4.6 \text{ mm ID}$; $5 \mu\text{m}$, ChromaNik Technologies Inc., Osaka, Japan), Sunrise C18-
139 SAC column ($250 \times 4.6 \text{ mm ID}$; $5 \mu\text{m}$, ChromaNik Technologies Inc.), Sunniest C18
140 column ($250 \times 4.6 \text{ mm ID}$; $5 \mu\text{m}$, ChromaNik Technologies Inc.), and YMC carotenoid
141 column ($250 \times 4.6 \text{ mm ID}$; $4 \mu\text{m}$, YMC Co., Ltd., Kyoto, Japan). The characteristics of
142 these columns were briefly described in Supplementary Table 1.

143

144

145 **2.4. SFC/triple quadrupole (QqQ) MS conditions**

146 SFC/QqQ MS analysis was performed using an Ultra Performance Convergence
147 Chromatography (Waters, Milford, MA, USA) and a Xevo TQ mass spectrometer
148 (Waters). The SFC and QqQ MS systems were controlled by MassLynx software. SFC
149 conditions were as follows: mobile phase, SCCO₂; modifier, methanol with 0.1% (w/w)
150 ammonium formate; outlet pressure, 1500 psi; initial inlet pressure, 2200 psi; column
151 temperature, 35°C. For each run, 5 µL of the sample was injected by the full sample
152 loop injection method. QqQ MS analysis was carried out in the positive ion mode of
153 electrospray ionization (ESI) under the following conditions: capillary voltage, 3000 V;
154 source temperature, 150°C; desolvation temperature, 350°C; cone gas flow rate, 50 L/h;
155 desolvation gas flow rate, 800 L/h; collision gas flow rate, 12 mL/h; MS collision
156 energy (CE), 20 V; extractor voltage, 3 V.

157

158

159 **3. Results and discussion**

160 **3.1. Construction of MRM transition for TAG profiling**

161 For the detailed characterization of numerous TAGs in edible oils, a highly
162 sensitive and highly selective detection method is required due to the complexity of the
163 TAG structure. The large number of FA combinations on the glycerol backbone makes
164 for a very diverse array of TAG compositions. The structures of isomeric TAGs that
165 have the same m/z ratio, despite their different compositions, are especially difficult to
166 determine using a single MS. When the regioisomeric TAG pairs are also mixed with
167 other isomers, the MS/MS spectrum data obtained from the fragmentation is essential to
168 analyze each TAG molecule.

For the selective detection of TAGs in this study, we applied multiple reaction monitoring (MRM), which is a non-scanning technique that can be performed on a QqQ MS. In the MRM of TAGs, two mass analyzers were used to monitor a particular product ion, DAG of a selected precursor ion, TAG. For the most effective detection, the MRM parameters, such as MRM transitions (precursor m/z (Q1) > product m/z (Q3)), cone voltage (CV), and MS/MS CE were optimized. First, the MRM transition was designed to analyze TAGs. In the positive mode of ESI, the ammoniated TAG ion ($[M+NH_4]^+$) was detected as the precursor m/z . In the CID-based fragmentation, TAG follows a specific pattern in its generation of product ions. With neutral losses of a FA from each TAG, the DAG ions ($[M+NH_4-RCOONH_4]^+$) were detected as the product ions of a particular m/z . In order to develop a TAG profiling method, 16 TAG standards (SSO, SOS, SLS, SOL, OOO, OOL, LLO, LOL, LLL, SOP, SLP, OOP, LOP, LLP, PPO, POP) were used, and MRM transitions for these TAGs were constructed (Supplementary Table 2). Based on the product ion scan, several product ions of a selected precursor ion were initially monitored (Supplementary Fig.1). In these data, the isomeric TAG pairs, SOL/OOO and SLP/OOP, produced different detectable product ions. For example, SOL produces three DAGs, $[SL]^+$, $[LO]^+$, and $[SO]^+$, as the product ions, whereas OOO produces only $[OO]^+$ as its product ion. This makes MRM a selective detection technique because a different product ion was selected as Q3 to construct the specific MRM transitions for each TAG molecule. For the MRM transition of OOO, $[OO]^+$ with a m/z ratio of 603.5 was used as the Q3. Of the three possible product ions of SOL, $[SL]^+$ shares the same m/z as $[OO]^+$ (603.5); therefore, it was more suitable to avoid using $[SL]^+$ as the Q3 in the MRM transition of SLO. Between the remaining product ions, the intensity of $[SO]^+$ should theoretically be greater than that

193 of [OL]⁺ according to the general pattern of neutral loss by CID, thus, [SO]⁺ with a *m/z*
194 ratio of 605.5 was used as the Q3 for the MRM transition of SLO. On the other hand,
195 the regioisomeric TAG pairs (SSO/SOS, LLO/LOL, PPO/POP) showed the same
196 product ions, but they differ in intensity. For example, SSO and SOS share the product
197 ions of [SS]⁺ and [SO]⁺, but the value of [SO]⁺/[SS]⁺ was greater for SOS than for SSO.
198 Hence, the exclusive use of MRM cannot sufficiently profile regioisomeric TAG pairs,
199 and effective separation is required.

200 Based on the product ion scan data, it was found that FAs with higher CNs and
201 DBs were more easily fragmented than FAs with lower CNs and DBs at the *sn*-1/3
202 positions. This was extensively studied in past [1–4,16,17], and in the data for SOL,
203 SLP, SOP, and LOP, the order of easily fragmented FAs was confirmed as follows: L <
204 O < S < P. Finally, we selected the product ion with the highest intensity to optimize the
205 MRM transition. We also tuned the method and instrumental parameters; for instance,
206 the CV was optimized by comparing the peak intensities and signal-to-noise ratios (S/N)
207 obtained from the programmed CVs in increments of 5 V for the range of 15–45 V.
208 Subsequently, the CE was also optimized using the same methods (data not shown).

209

210 **3.2. Optimization of SFC conditions to separate various TAGs**

211 Optimizing the separation conditions is critical to the development of a TAG
212 profiling methodology that uses SFC/QqQ MS. In particular, the priority is to select an
213 effective column for the separation of TAG isomers, including regioisomers. We had
214 found that an ODS column was able to separate diverse TAGs according to their CNs
215 and DBs in our previous study. The differences between ODS columns depend on the
216 following: carbon content, modification of stationary phase, type of particles, etc.

217 Therefore, various ODS columns were examined to discover an optimal column for
218 TAG profiling. Furthermore, a triacontyl (C30) bonded silica based RP column was also
219 examined because a previous paper reported on the utility of a high-number-of-carbons
220 column for the separation of TAG isomers [31].

221 We tested the separations of 16 TAG standards (SSO, SOS, SLS, SOL, OOO,
222 OOL, LLO, LOL, LLL, SOP, SLP, OOP, LOP, LLP, PPO, POP) using several ODS
223 columns and a C30 RP column (Supplementary Table 3). We particularly focused on
224 the separation of two isomeric pairs (SOL/OOO, SLP/OOP) and three regioisomeric
225 pairs (SSO/SOS, LLO/LOL, PPO/POP) for the column selection progress. In SFC, the
226 flow rate and modifier ratio significantly affect the separation of compounds. Generally,
227 a low flow rate and low modifier ratio provide high resolution with a relatively long
228 analysis time. The SFC conditions for examining several ODS columns were as follows:
229 flow rate, 1 mL/min; modifier ratio, 10% (v/v). All ODS columns used in this study
230 were effective for the separation of TAG standards that have different m/z ratios.
231 However, the Inertsil ODS-4, Inertsil ODS-EP, Inertsil ODS-SP, and Sunniest C18
232 columns did not sufficiently separate the TAG regioisomers, and they exhibited low
233 resolution for the separation of other isomers. The InertSustain C18, Sunrise C18, and
234 Sunrise C18-SAC columns were also unable to sufficiently separate the TAG
235 regioisomers, but these columns performed better than the aforementioned columns for
236 the separation of other isomers, including SOL, OOO, SLP, and OOP.

237 In another previous study, a polymeric ODS column was applied to the separation
238 of several TAG regioisomers [32]. The ODS groups of the polymeric ODS stationary
239 phase were more densely arranged than those of other ODS stationary phases, and the
240 polymeric ODS column can recognize steric differences in TAG isomers. PPO and POP

241 were especially separable using the recycled HPLC conditions with an Inertsil ODS-P
242 column and a run time of 110 min [33]. Therefore, we employed the Inertsil ODS-P
243 column in the separation of several TAG isomers. As a result, SSO, SOS, SOL, OOO,
244 SLP, and OOP were well separated as compared to other ODS columns. Though PPO
245 and POP were separated, the resolution was not sufficient for our purposes. Furthermore,
246 LLO and LOL were not separated by this column.

247 The SFC conditions using the C30 RP column were different from those for the
248 ODS columns. When the prior conditions (flow rate, 1 mL/min; modifier, 10% (v/v))
249 were used with an YMC carotenoid column, which is a C30 RP column, several TAGs
250 that have high CNs and low DBs eluted too late with broadened peak shape. Therefore,
251 optimization of the flow rate and modifier was required for TAG profiling using an
252 YMC carotenoid column. For the fast and effective separation of PPO and POP, the
253 initial flow rate and modifier were determined to be 2 mL/min and 20% (v/v),
254 respectively. The gradient conditions for TAG profiling were as follows: first, the initial
255 flow rate (2 mL/min) and initial modifier (20% (v/v)) were maintained for 15 min;
256 second, the flow rate was gradually increased to 3 mL/min over 10 min while the
257 modifier was maintained as 20% (v/v); third, the modifier was gradually increased to
258 30% (v/v) over 5 min, and the flow rate was maintained as 3 mL/min; fourth, these
259 conditions (flow rate, 3 mL/min; modifier, 30% (v/v)) were maintained for 80 min;
260 lastly, these conditions were gradually returned to the starting conditions (flow rate, 2
261 mL/min; modifier, 20% (v/v)) over the last 10 min, which makes the total run time 120
262 min. This analysis time was designed suitably for the profiling of diverse TAGs in
263 edible oils. By using these gradient conditions with the YMC carotenoid column, the 16
264 TAG standards were analyzed in 50 min (Fig. 1). As a result, several isomeric TAG

265 pairs, such as SSO/SOS, SOL/OOO, SLP/OOP, and PPO/POP, were well separated. In
266 particular, this method achieved a fast and high-resolution separation of PPO (RT: 20.44
267 min) and POP (RT: 19.06 min) as compared to other HPLC systems. Finally, the YMC
268 carotenoid column was established for the TAG profiling by SFC/QqQ MS.

269

270 3.3. Analysis of edible oils

271 We performed a detailed characterization of TAGs in palm and canola oils by
272 SFC/QqQ MS with a C30 RP column. Palm and canola oils are widely used throughout
273 the world, and the diverse TAG molecules in these oils were already identified in a
274 previous paper [12]. TAGs of these oils consist of several FAs, which include lignoceric
275 acid (Lg, 24:0), behenic acid (B, 22:0), arachidic acid (A, 20:0), gadoleic acid (G, 20:1),
276 stearic acid (S, 18:0), oleic acid (O, 18:1), linoleic acid (L, 18:2), linolenic acid (Ln,
277 18:3), margaric acid (Ma, 17:0), margaroleic acid (Mo, 17:1), palmitic acid (P, 16:0),
278 palmitoleic acid (Po, 16:1), and myristic acid (M, 14:0). In this study, the list of
279 previously identified TAGs was used to construct the MRM transitions for the profiling
280 of TAGs in palm and canola oils.

281 In these oils, there are various isomeric TAG pairs. For example, there are three
282 TAG (54:2) isomers, ALP, OGP, and SOO. For the analysis of these TAGs, it is critical
283 they are well separated in order to selectively detect them using specific MRM
284 transitions. Theoretically, $[AL]^+$, $[LP]^+$, and $[AP]^+$ can be obtained as the product ions
285 of ALP by CID. The intensities of $[AL]^+$ and $[LP]^+$ should be higher than that of $[AP]^+$
286 because the neutral losses of *sn*-1/3 FAs are more favorable than the loss of an *sn*-2 FA.
287 OGP has three DAG product ions ($[GO]^+$, $[OP]^+$, $[GP]^+$), and SOO has two DAG ions
288 ($[SO]^+$, $[OO]^+$). For the selective detection of these three TAG isomers in a mixture, the

specific MRM transition for each TAG molecule must be used. For the MRM transition of ALP, [AL]⁺ (m/z = 631.5) was used as the Q3. Furthermore, [SO]⁺ (m/z = 605.5) was used as the Q3 of SOO, and [OP]⁺ (m/z = 577.5) was used as the Q3 of OGP. The other DAG ions of OGP, [GO]⁺ (m/z = 631.5) and [GP]⁺ (m/z = 635.5) were not selected due to their equivalency to [AL]⁺ and [SO]⁺, respectively. Since each TAG isomer was analyzed using specific MRM transitions there were 72 MRM transitions that were applied to the detail profiling of TAGs in palm and canola oils (Supplementary Table 4).

For MRM, it is critical to successfully separate each TAG compound because the isotopic peaks of other TAGs can hinder precise quantification. For example, TAG (54:3) has one more DB and a lower m/z (by 2) than TAG (54:2). Theoretically, isotopes of TAG (54:3; m/z = 902.8) will be represented by m/z values of 903.8 and 904.8. Since TAG (54:2) also exhibits a peak at m/z = 904.8, it would be simultaneously detected with isotopic TAG (54:3) if their separation was not sufficient, and the quantification would not be reliable. Furthermore, the regioisomeric separation of TAGs is required for the quantification of each molecule because we already deduced that the differences in relative intensities ([AB]⁺/[AA]⁺) for the analysis of TAG regioisomers (AAB, ABA) were not sufficient.

The optimized gradient condition for SFC/QqQ MS with a C30 RP column was applied to separate diverse TAGs in palm and canola oils (Supplementary Fig.2). As a result, 70 TAGs (C46:0 – C60:2) were successfully analyzed in palm and canola oils (Table 1). Product ion scan was used to identify the composition and distribution of FAs in TAGs. The structure of TAGs was determined by the intensities of detected product ions. Relative ratios of individual DAG product ions in 70 TAGs were listed in Table 2. For the data analysis, it was necessary to distinguish between normal and isotopic peaks

313 within the respective sets of MRM data. For example, during the analysis of TAG (52:3)
 314 isomeric pairs, three MRM transitions (m/z 874 > m/z 573, m/z 874 > m/z 575, m/z 874
 315 > m/z 603) were used, and several peaks were detected in each set of MRM data (Fig.
 316 2A). We determined the reasonability of each peak based on their RT. As a result, four
 317 peaks of TAG (52:3) and two peaks of isotopic TAGs (52:4) were detected in these
 318 MRM data. The product ion scan was also applied to identify the structure of the four
 319 TAG (52:3) isomers (Fig. 2B). Finally, SPLn (RT: 23.33 min), SLnP (RT: 17.96 min),
 320 LOP (RT: 16.21 min), and OOPo (RT: 13.93 min) were successfully analyzed. This
 321 method allowed us to achieve the separation and identification of the following 20 TAG
 322 isomeric pairs: TAG (58:2), BOO and LgLP; TAG (56:2), BLP, AOO, and GOS; TAG
 323 (56:3), ALO and GOO; TAG (56:4), ALnO, ALL, and OGL; TAG (56:5), ALLn and
 324 GLL; TAG (54:1), AOP, SSO, and SOS; TAG (54:2), ALP, SOO, and OGP; TAG
 325 (54:3), SLO, GLP, and OOO; TAG (54:4), SOLn, SLL, and OOL; TAG (54:5), SLnL,
 326 OLnO, and OLL; TAG (54:6), SLnLn, OLnL, and LLL; TAG (54:7), LnOLn and
 327 LLLn; TAG (53:2), SLMa and OOMa; TAG (52:1), SPO and SOP; TAG (52:2), SLP
 328 and OOP; TAG (52:3), SPLn, SLnP, LOP, and OOPo; TAG (52:4), LnOP and LLP;
 329 TAG (50:1), PPO and POP; TAG (50:2), PPL, PLP, and OMO; and TAG (50:3), PPLn,
 330 PLnP, and OML. Of the analyzed TAGs, the detections of ALnO, ALLn, GLP, SLnL,
 331 and SLnLn were novel with respect to the previous report. Furthermore, the relative
 332 area (peak area/total peak area $\times$ 100%) of each TAG molecule was calculated for the
 333 detailed characterization of TAGs in palm and canola oils. The quantifications are based
 334 on triplicate analysis of the oil samples (n=3), and the values of percentages were
 335 described as mean $\pm$ SD. In order to examine the repeatability of analysis, the relative
 336 standard deviation (RSD (%)) of peak area was calculated (n=3). The RSD (%) was

different according to the TAG species. Almost TAGs have a high repeatability (RSD (%) < 10), whereas several low abundance TAGs showed a little low repeatability (RSD (%) < 25).

In particular, this method achieved the fast and high-resolution profiling of six TAG regioisomeric pairs within palm and canola oils. The RTs of TAG regioisomers were as follows: PLnP (RT: 16.61 min), PPLn (RT: 17.89 min), PLP (RT: 17.82 min), PPL (RT: 19.23 min), POP (RT: 20.37 min), PPO (RT: 21.85 min), SLnP (RT: 17.96 min), SPLn (RT: 23.33 min), SOP (RT: 28.06 min), SPO (RT: 30.88 min), SOS (RT: 39.78 min), and SSO (RT: 49.32 min) (Fig. 3). This utility of this method was clearly demonstrated for the effective separation of TAG regioisomers. During characterization, we found that the palm oil had six regioisomeric pairs (PPLn/PLnP, PPL/PLP, PPO/POP, SPLn/SLnP, SPO/SOP, SSO/SOS) and the canola oil had two pairs (PPL/PLP, SPLn/SLnP). This is the first report to profile six of the TAG regioisomeric pairs (PPLn/PLnP, PPL/PLP, PPO/POP, SPLn/SLnP, SPO/SOP, SSO/SOS) in palm and canola oils.

4. Conclusions

In this study, an effective analytical method was developed to profile TAGs, including regioisomers, *via* SFC/QqQ MS with a C30 RP column. This method achieved the high-resolution separation and selective detection of diverse TAGs in palm and canola oils, and 70 TAG molecular species (C46:0 – C60:2) were successfully identified. Furthermore, 20 pairs of TAG isomers were separated well enough to allow for the analysis of additional TAG molecules that were not previously reported.

361 Specifically, this was the first report on profiling of six TAG regioisomeric pairs
362 (PPLn/PLnP, PPL/PLP, PPO/POP, SPLn/SLnP, SPO/SOP, SSO/SOS) in edible oils.
363 Finally, this method demonstrated the utility of SFC for the high resolution separation
364 of TAG isomers to provide detailed characterizations of diverse TAGs. Furthermore, we
365 were able to demonstrate the applicability of SFC to the fast, high-resolution separation
366 of hydrophobic TAG regioisomers in edible oils, which rivals that of conventional
367 HPLC. Therefore, this SFC/QqQ MS technique designed to profile TAGs in a mixture
368 could be further adapted to lipidomics screening using quadrupole/time of flight MS.

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370

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493 **Figure legends**

494 **Fig. 1.** MRM chromatogram of 16 TAG standards that were separated by an YMC
495 carotenoid column (250 × 4.6 mm ID; 4 μm, YMC Co., Ltd.). (A) SSO, (B) SOS, (C)
496 SLS, (D) SOL, (E) OOO, (F) OOL, (G) LLO, (H) LOL, (I) LLL, (J) SOP, (K) SLP, (L)
497 OOP, (M) LOP, (N) LLP, (O) PPO, and (P) POP.

498

499 **Fig. 2.** (A) MRM chromatogram of TAG (52:3) and TAG (52:4) for the data analysis of
500 isomeric TAG (52:3) pairs: (a) SPLn (RT: 23.33 min), (b) SLnP (RT: 17.96 min), (c)
501 LOP (RT: 16.21 min), (d) OOPo (RT: 13.93 min). ●: the peak of isotopic TAG (54:4).
502 (B) The product ion scan data of (a) SPLn, (b) SLnP, (c) LOP, and (d) OOPo.

503

504 **Fig. 3.** MRM chromatogram of six regioisomeric TAG pairs ((A)PPLn/PLnP,
505 (B)PPL/PLP, (C) PPO/POP, (D) SPLn/SLnP, (E) SPO/SOP, (F) SSO/SOS) in palm and
506 canola oils. The RTs of each TAG are as following: (a) SOS (RT: 39.78 min), (b) SSO
507 (RT: 49.32 min), (c) SOP (RT: 28.06 min), (d) SPO (RT: 30.88 min), (e) SLnP (RT:
508 17.96 min), (f) SPLn (RT: 23.33 min), (g) POP (RT: 20.37 min), (h) PPO (RT: 21.85
509 min), (i) PLP (RT: 17.82 min), (j) PPL (RT: 19.23 min), (k) PLnP (RT: 16.61 min), and
510 (l) PPLn (RT: 17.89 min).

Table 1

TAG molecular species profiled by SFC/QqQ MS in palm and canola oils

No.	CN ^(a) :DB ^(b)	TAG molecular species		<i>m/z</i> ([M + NH ₄] ⁺)	tR ^(c) (min)	Relative peak area of each molecular species (%) ^(d)	
						Palm oil	Canola oil
1	60:2	24:0–18:1–18:1	LgOO	988.8	100.94	0.0097 ± 0.0011	0.039 ± 0.002
2	60:3	24:0–18:1–18:2	LgOL	986.8	81.28	0.0013 ± 0.0003	0.016 ± 0.001
3	60:4	24:0–18:2–18:2	LgLL	984.8	68.63	0.0007 ± 0.0001	0.0056 ± 0.0003
4	60:5	24:0–18:3–18:2	LgLnL	982.8	65.57	N.D. ^(e)	0.002 ± 0.0004
5	58:2	22:0–18:1–18:1	BOO	960.8	48.97	0.013 ± 0.001	0.12 ± 0.008
6		24:0–18:2–16:0	LgLP	960.8	34.03	N.D.	0.0029 ± 0.0005
7	58:3	22:0–18:1–18:2	BOL	958.8	42.74	0.003 ± 0.0002	0.052 ± 0.001
8	58:4	22:0–18:2–18:2	BLL	956.8	38.23	0.00077 ± 0.00021	0.019 ± 0.001
9	58:5	22:0–18:3–18:2	BLnL	954.8	36.95	N.D.	0.0076 ± 0.0011
10	56:1	22:0–18:1–16:0	BOP	934.8	73.78	0.014 ± 0.0008	0.0056 ± 0.0009
11	56:2	22:0–18:2–16:0	BLP	932.8	55.78	0.0043 ± 0.0005	0.0048 ± 0.0005
12		20:0–18:1–18:1	AOO	932.8	32.37	0.11 ± 0.008	0.35 ± 0.02
13		20:1–18:1–18:0	GOS	932.8	26.01	N.D.	0.0015 ± 0.0001
14	56:3	20:0–18:2–18:1	ALO	930.8	29.16	0.026 ± 0.004	0.14 ± 0.01
15		20:1–18:1–18:1	GOO	930.8	21.28	0.039 ± 0.007	0.71 ± 0.01
16	56:4	20:0–18:1–18:3	AOLn	928.8	28.02	N.D.	0.055 ± 0.002
17		20:0–18:2–18:2	ALL	928.8	26.43	0.011 ± 0.002	0.056 ± 0.009
18		18:1–20:1–18:2	OGL	928.8	19.18	0.016 ± 0.004	0.51 ± 0.06
19	56:5	20:0–18:2–18:3	ALLn	926.8	24.97	N.D.	0.024 ± 0.002
20		20:1–18:2–18:2	GLL	926.8	17.14	0.006 ± 0.001	0.15 ± 0.002
21	54:1	18:0–18:0–18:1	SSO	906.8	49.32	0.014 ± 0.003	N.D.
22		20:0–18:1–16:0	AOP	906.8	41.12	0.11 ± 0.007	0.016 ± 0.001
23		18:0–18:1–18:0	SOS	906.8	39.78	0.14 ± 0.009	0.043 ± 0.003
24	54:2	20:0–18:2–16:0	ALP	904.8	34.57	0.035 ± 0.002	0.012 ± 0.002
25		18:0–18:1–18:1	SOO	904.8	24.07	1.97 ± 0.34	1.43 ± 0.28
26		18:1–20:1–16:0	OGP	904.8	22.42	0.084 ± 0.016	0.044 ± 0.009
27	54:3	18:0–18:2–18:1	SLO	902.8	21.66	0.5 ± 0.07	0.79 ± 0.05
28		20:1–18:2–16:0	GLP	902.8	20.13	0.027 ± 0.006	N.D.
29		18:1–18:1–18:1	OOO	902.8	17.27	6.52 ± 1.02	43.5 ± 2.7
30	54:4	18:0–18:1–18:3	SOLn	900.8	20.39	0.014 ± 0.003	0.19 ± 0.01
31		18:0–18:2–18:2	SLL	900.8	19.18	0.18 ± 0.03	0.22 ± 0.02
32		18:1–18:1–18:2	OOL	900.8	15.23	2.23 ± 0.47	21.9 ± 2.7
33	54:5	18:0–18:3–18:2	SLnL	898.8	18.13	0.0082 ± 0.0018	0.096 ± 0.009
34		18:1–18:3–18:1	OLnO	898.8	14.17	0.051 ± 0.001	5.6 ± 0.8
35		18:1–18:2–18:2	OLL	898.8	13.36	0.51 ± 0.06	7.2 ± 0.6
36	54:6	18:0–18:3–18:3	SLnLn	896.8	16.78	N.D.	0.039 ± 0.003
37		18:1–18:3–18:2	OLnL	896.8	12.49	0.019 ± 0.004	2.6 ± 0.2
38		18:2–18:2–18:2	LLL	896.8	11.88	0.074 ± 0.003	0.89 ± 0.16
39	54:7	18:3–18:1–18:3	LnOLn	894.8	11.55	0.0071 ± 0.0019	1.9 ± 0.2
40		18:2–18:2–18:3	LLLn	894.8	10.94	0.0049 ± 0.0003	0.88 ± 0.14
41	54:8	18:3–18:2–18:3	LnLLn	892.8	10.27	0.0014 ± 0.0002	0.4 ± 0.03
42	54:9	18:3–18:3–18:3	LnLnLn	890.8	9.53	0.00022 ± 0.00002	0.13 ± 0.01
43	53:2	18:0–18:2–17:0	SLMa	890.8	21.01	0.032 ± 0.003	0.031 ± 0.001
44		18:1–18:1–17:0	OOMa	890.8	18.61	0.0044 ± 0.0005	0.0024 ± 0.0001
45	53:5	18:2–18:2–17:1	LLMo	884.8	18.33	0.00057 ± 0.00003	0.00053 ± 0.00011
46	52:1	18:0–16:0–18:1	SPO	878.8	30.88	0.17 ± 0.01	N.D.
47		18:0–18:1–16:0	SOP	878.8	28.06	2.02 ± 0.07	0.078 ± 0.004
48	52:2	18:0–18:2–16:0	SLP	876.8	24.37	0.72 ± 0.06	0.06 ± 0.01
49		18:1–18:1–16:0	OOP	876.8	18.26	25 ± 0.7	4.5 ± 0.1
50	52:3	18:0–16:0–18:3	SPLn	874.8	23.33	0.006 ± 0.0008	0.019 ± 0.001
51		18:0–18:3–16:0	SLnP	874.8	17.96	0.0057 ± 0.0003	0.0055 ± 0.0006
52		18:2–18:1–16:0	LOP	874.8	16.21	6.03 ± 0.25	2.2 ± 0.2
53		18:1–18:1–16:1	OOPo	874.8	13.93	0.042 ± 0.005	0.17 ± 0.02
54	52:4	18:3–18:1–16:0	LnOP	872.8	15.14	0.15 ± 0.01	0.74 ± 0.08
55		18:2–18:2–16:0	LLP	872.8	14.13	2.02 ± 0.13	0.87 ± 0.18
56	52:5	18:3–18:2–16:0	LnLP	870.8	13.12	0.053 ± 0.004	0.34 ± 0.06
57	52:6	18:3–18:3–16:0	LnLnP	868.8	12.18	0.0016 ± 0.0001	0.14 ± 0.006
58	51:1	18:1–17:0–16:0	OMaP	864.8	23.93	0.067 ± 0.004	0.0024 ± 0.0001
59	50:1	16:0–16:0–18:1	PPO	850.8	21.85	2.58 ± 0.58	N.D.
60		16:0–18:1–16:0	POP	850.8	20.37	28.7 ± 2.1	0.26 ± 0.06
61	50:2	16:0–16:0–18:2	PPL	848.8	19.23	0.68 ± 0.1	0.0012 ± 0.0003
62		16:0–18:2–16:0	PLP	848.8	17.82	9.1 ± 0.5	0.21 ± 0.03

63		18:1–14:0–18:1	OMO	848.8	13.86	0.39 ± 0.035	0.039 ± 0.001
64	50:3	16:0–16:0–18:3	PPLn	846.8	17.89	0.035 ± 0.002	N.D.
65		16:0–18:3–16:0	PLnP	846.8	16.61	0.074 ± 0.006	0.06 ± 0.01
66		18:1–14:0–18:2	OML	846.8	12.12	0.14 ± 0.03	0.034 ± 0.003
67	48:0	16:0–16:0–16:0	PPP	824.8	30.98	7.5 ± 0.07	N.D.
68	48:1	16:0–18:1–14:0	POM	822.8	15.14	1.04 ± 0.04	0.0032 ± 0.0003
69	48:2	16:0–18:2–14:0	PLM	820.8	13.19	0.31 ± 0.02	0.0032 ± 0.0008
70	46:0	16:0–16:0–14:0	PPM	796.8	20.91	0.32 ± 0.004	N.D.

a) CN; carbon number

b) DB; double bond

c) Average retention time (n=3)

d) Percentage of each molecular species to each polar lipid (peak area/total peak area), the values of percentages are means $\pm$ SD, (n=3)

e) N.D.; not detected

Table 2

Relative ratios of individual DAG product ions of TAGs in palm and canola oils

No	TAG	Ratio of DAG product ions	No	TAG	Ratio of DAG product ions
1	LgOO	$[\text{LgO}]^+ / [\text{OO}]^+ = 100/49$	36	SLnLn	$[\text{SLn}]^+ / [\text{LnLn}]^+ = 100/71$
2	LgOL	$[\text{OL}]^+ / [\text{LgO}]^+ / [\text{LgL}]^+ = 100/52/33$	37	OLnL	$[\text{LLn}]^+ / [\text{OLn}]^+ / [\text{OL}]^+ = 100/84/34$
3	LgLL	$[\text{LL}]^+ / [\text{LgL}]^+ = 100/51$	38	LLL	$[\text{LL}]^+ = 100$
4	LgLnL	$[\text{LLn}]^+ / [\text{LgLn}]^+ / [\text{LgL}]^+ = 100/45/40$	39	LnOLn	$[\text{OLn}]^+ / [\text{LnLn}]^+ = 100/39$
5	BOO	$[\text{BO}]^+ / [\text{OO}]^+ = 100/23$	40	LLLn	$[\text{LLn}]^+ / [\text{LL}]^+ = 100/45$
6	LgLP	$[\text{LgL}]^+ / [\text{LP}]^+ / [\text{LgP}]^+ = 100/11/17$	41	LnLLn	$[\text{LLn}]^+ / [\text{LnLn}]^+ = 100/46$
7	BOL	$[\text{OL}]^+ / [\text{BO}]^+ / [\text{BL}]^+ = 100/76/72$	42	LnLnLn	$[\text{LnLn}]^+ = 100$
8	BLL	$[\text{LL}]^+ / [\text{BL}]^+ = 100/66$	43	SLMa	$[\text{LMa}]^+ / [\text{SMa}]^+ / [\text{SL}]^+ = 100/60/48$
9	BLnL	$[\text{BLn}]^+ / [\text{LLn}]^+ / [\text{BL}]^+ = 100/40/22$	44	OOMa	$[\text{OMa}]^+ / [\text{OO}]^+ = 100/86$
10	BOP	$[\text{BO}]^+ / [\text{OP}]^+ / [\text{BP}]^+ = 100/69/39$	45	LLMo	$[\text{LMo}]^+ / [\text{LL}]^+ = 100/47$
11	BLP	$[\text{BL}]^+ / [\text{LP}]^+ / [\text{BP}]^+ = 100/92/52$	46	SPO	$[\text{SP}]^+ / [\text{OP}]^+ / [\text{SO}]^+ = 100/98/94$
12	AOO	$[\text{AO}]^+ / [\text{OO}]^+ = 100/76$	47	SOP	$[\text{SO}]^+ / [\text{OP}]^+ / [\text{SP}]^+ = 100/89/53$
13	GOS	$[\text{GO}]^+ / [\text{SO}]^+ / [\text{GS}]^+ = 100/25/23$	48	SLP	$[\text{LP}]^+ / [\text{SL}]^+ / [\text{SP}]^+ = 100/79/65$
14	ALO	$[\text{AL}]^+ / [\text{OL}]^+ / [\text{AO}]^+ = 100/57/51$	49	OOP	$[\text{OP}]^+ / [\text{OO}]^+ = 100/49$
15	GOO	$[\text{GO}]^+ / [\text{OO}]^+ = 100/64$	50	SPLn	$[\text{SP}]^+ / [\text{LnP}]^+ / [\text{SLn}]^+ = 100/60/59$
16	ALnO	$[\text{OLn}]^+ / [\text{ALn}]^+ / [\text{AO}]^+ = 100/57/33$	51	SLnP	$[\text{SLn}]^+ / [\text{LnP}]^+ / [\text{SP}]^+ = 100/12/9$
17	ALL	$[\text{LL}]^+ / [\text{AL}]^+ = 100/86$	52	LOP	$[\text{OL}]^+ / [\text{OP}]^+ / [\text{LP}]^+ = 100/96/83$
18	OGL	$[\text{GL}]^+ / [\text{GO}]^+ / [\text{OL}]^+ = 100/70/48$	53	OOPo	$[\text{OPo}]^+ / [\text{OO}]^+ = 100/38$
19	ALLn	$[\text{AL}]^+ / [\text{LLn}]^+ / [\text{ALn}]^+ = 100/84/68$	54	LnOP	$[\text{OLn}]^+ / [\text{OP}]^+ / [\text{LnP}]^+ = 100/69/69$
20	GLL	$[\text{LL}]^+ / [\text{GL}]^+ = 100/45$	55	LLP	$[\text{LP}]^+ / [\text{LL}]^+ = 100/50$
21	SSO	$[\text{SS}]^+ / [\text{SO}]^+ = 100/79$	56	LnLP	$[\text{LLn}]^+ / [\text{LP}]^+ / [\text{LnP}]^+ = 100/82/65$
22	AOP	$[\text{AO}]^+ / [\text{OP}]^+ / [\text{AP}]^+ = 100/88/62$	57	LnLnP	$[\text{LnP}]^+ / [\text{LnLn}]^+ = 100/53$
23	SOS	$[\text{SO}]^+ / [\text{SS}]^+ = 100/30$	58	OMaP	$[\text{MaP}]^+ / [\text{OMa}]^+ / [\text{OP}]^+ = 100/81/55$
24	ALP	$[\text{LP}]^+ / [\text{AL}]^+ / [\text{AP}]^+ = 100/56/44$	59	PPO	$[\text{OP}]^+ / [\text{PP}]^+ = 100/68$
25	SOO	$[\text{SO}]^+ / [\text{OO}]^+ = 100/72$	60	POP	$[\text{OP}]^+ / [\text{PP}]^+ = 100/38$
26	OGP	$[\text{GP}]^+ / [\text{GO}]^+ / [\text{OP}]^+ = 100/62/48$	61	PPL	$[\text{LP}]^+ / [\text{PP}]^+ = 100/61$
27	SLO	$[\text{SL}]^+ / [\text{OL}]^+ / [\text{SO}]^+ = 100/87/69$	62	PLP	$[\text{LP}]^+ / [\text{PP}]^+ = 100/39$
28	GLP	$[\text{GL}]^+ / [\text{LP}]^+ / [\text{GP}]^+ = 100/80/52$	63	OMO	$[\text{OM}]^+ / [\text{OO}]^+ = 100/31$
29	OOO	$[\text{OO}]^+ = 100$	64	PPLn	$[\text{LnP}]^+ / [\text{PP}]^+ = 100/43$
30	SOLn	$[\text{SO}]^+ / [\text{SLn}]^+ / [\text{OLn}]^+ = 100/79/72$	65	PLnP	$[\text{LnP}]^+ / [\text{PP}]^+ = 100/28$
31	SLL	$[\text{SL}]^+ / [\text{LL}]^+ = 100/47$	66	OML	$[\text{OM}]^+ / [\text{LM}]^+ / [\text{OL}]^+ = 100/56/38$
32	OOL	$[\text{OL}]^+ / [\text{OO}]^+ = 100/54$	67	PPP	$[\text{PP}]^+ = 100$
33	SLnL	$[\text{SLn}]^+ / [\text{SL}]^+ / [\text{LLn}]^+ = 100/68/19$	68	POM	$[\text{OP}]^+ / [\text{OM}]^+ / [\text{PM}]^+ = 100/100/82$
34	OLnO	$[\text{OLn}]^+ / [\text{OO}]^+ = 100/14$	69	PLM	$[\text{LP}]^+ / [\text{LM}]^+ / [\text{PM}]^+ = 100/32/20$
35	OLL	$[\text{OL}]^+ / [\text{LL}]^+ = 100/46$	70	PPM	$[\text{PM}]^+ / [\text{PP}]^+ = 100/58$

Figure 1

Figure 2

Figure 3