



Title	Formation of Acrolein in the Autoxidation of Triacylglycerols with Different Fatty Acid Compositions
Author(s)	Shibata, Ako; Uemura, Mariko; Hosokawa, Masashi; Miyashita, Kazuo
Citation	Journal of the American oil chemists society, 92(11-12), 1661-1670 https://doi.org/10.1007/s11746-015-2732-2
Issue Date	2015-12
Doc URL	http://hdl.handle.net/2115/63706
Rights	The final publication is available at www.springerlink.com
Type	article (author version)
File Information	Miyashita.pdf



[Instructions for use](#)

1 **Formation of Acrolein in the Autoxidation of Triacylglycerols with Different Fatty** 2 **Acid Compositions**

3
4 Ako Shibata, Mariko Uemura, Masashi Hosokawa, and Kazuo Miyashita*

5
6 *Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido*
7 *041-8611, Japan*

8
9 *Corresponding author: Tel.: +81 138 408804; fax: +81 138 408804

10 E-mail address: kmiya@fish.hokudai.ac.jp

11
12 **Abstract:** Fish, echium, linseed, and soybean oil triacylglycerols (TAGs) were oxidized
13 at 50 or 60°C to determine the effect of the polyunsaturated fatty acid composition on
14 the volatile product formation. The analysis of the oxygen consumption and total
15 volatile formation demonstrated that the soybean oil TAG had the highest oxidative
16 stability followed by linseed, echium, and fish oil TAGs. Our results were in agreement
17 with the expected average number of bis-allylic positions of each TAG. Higher
18 quantities of acrolein (2-propenal) and propanal were detected using the static
19 headspace gas chromatography method at the early stages of oxidation of echium and
20 fish oil TAGs; however, a considerable amount of propanal and only a small amount of
21 acrolein were found in the oxidized linseed oil TAG. The peak area ratio of acrolein to
22 propanal were 0.115, 0.569, and 2.554 after the 8 hr oxidation of linseed, echium, and
23 fish oil TAG, respectively, suggesting the preferential formation of acrolein, especially
24 during the fish oil TAG oxidation. The acrolein quickly increased during the first stage
25 of oxidation, but afterward, it either did not change or slightly decreased during the fish
26 oil oxidation. Because fish oil induces flavor deterioration from the very early stage of
27 the oxidation, the acrolein formation observed in the present study may be important for
28 fish oil deterioration.

29
30 **Keywords:** fish oil oxidation · flavor deterioration · volatiles · acrolein · oxidative
31 stability

32 33 34 **Introduction**

35
36 Eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are

37 typical n-3 polyunsaturated fatty acids (PUFAs) found in fish oils. These two long chain
38 PUFAs have been demonstrated to cause significant biochemical and physiological
39 changes in the body that primarily exhibit a positive influence on human nutrition and
40 health [1-5]. However, the development of fishy and metallic off-flavors that are often
41 found in fish oils rich in EPA and DHA dissuades people from consuming them. These
42 undesirable flavors mainly come from the oxidation of EPA and DHA in the fish oils.
43 The first step of the lipid oxidation is the abstraction of the bis-allylic hydrogen radical
44 from the substrate PUFA to form a lipid-free radical. The radical then reacts with
45 oxygen to form hydroperoxides. Thus, the level of oxidative deterioration in fish oil is
46 commonly assessed by measuring the hydroperoxides. However, EPA- and
47 DHA-hydroperoxides are very easily decomposed into volatile secondary oxidation
48 products that are responsible for the undesirable flavors in fish oils. Thus, it is possible
49 to have fish oils with very low hydroperoxide levels that also have an undesirable taste.
50 The rapid formation of volatile aldehydes is the most serious challenge that limits the
51 addition of fish oil to general food products.

52 The determination of secondary products, such as polymers, using the anisidine
53 value, the 2-thiobarbituric acid value, sensory analysis, and gas chromatographic (GC)
54 methods for volatile compounds is more important in evaluating the oxidative
55 deterioration of fish oil that contains EPA and DHA. Of the methods, the best technique
56 for determination of the volatile oxidation products is using GC. Volatile compounds
57 that are suitable for the GC determinations are primarily aldehydes and hydrocarbons.
58 Jacobsen [6] conclusively showed that there was no correlation between the peroxide
59 value, a typical value used to evaluate the oxidative stability of lipids, and the taste
60 panel response for fish oil-enriched spreads. However, the data on volatile compounds
61 obtained by the headspace methods using GC was correlated well with the sensory data.
62 Many types of volatile compounds that contain hydrocarbons, such as vinyl alcohols,
63 alkenals, alkadienals, alkatrienals, and vinyl ketones, have been identified in fish oil
64 oxidation [7-13]. However, the sensory impact of individual or combinations of volatile
65 oxidation compounds in food systems containing fish oil has yet to be established.
66 Hence, further research is needed to use this method to evaluate fish oil deterioration.

67 Because of their high threshold, hydrocarbons and vinyl alcohols will be relatively
68 insignificant in undesirable fishy flavors. However, different carbonyl compounds that
69 contain alkenals, alkadienals, alkatrienals, and vinyl ketones with a low threshold have
70 been demonstrated to be potent odorants that are responsible for the flavors, but none of
71 the separated volatiles imparted a fishy off-flavor [8,13]. Typical GC analyses that are
72 used to detect the volatile compounds from oxidized lipids include the direct injection

73 method, the dynamic headspace method and the static head space method, respectively.
74 Recently, the dynamic headspace method with a solid-phase microextraction (SPME)
75 fiber has been the most used [14-18]. Snyder *et al.* [19] have compared the three GC
76 methods for the analysis of volatile compounds from oxidized soybean oil. They found
77 that the proportions of 2,4-decadienal and 2,4-heptadienal in the direct injection and
78 dynamic headspace techniques were much higher than those found in the static
79 headspace method. However, the static head space method produced larger proportions
80 of 2-heptenal, pentane, and propanal. Additionally, more low molecular compounds
81 were found using the static head space method, including acrolein (2-propenal), at the
82 early stage of the oxidation. Acrolein is approximately 100 times more reactive than
83 4-hydroxy-2-nonenal, a well-known toxic lipid oxidation compound [20-22]. Acrolein
84 has also been known to produce undesirable and irritating odors with an odor threshold
85 of 3.6 ppb [23].

86 There have been several possible pathways for acrolein formation: the enzymatic
87 oxidation of spermine in biological systems [24], the oxidative homolytic fission of C-O
88 bonds of glycerol after the hydrolysis of triacylglycerol (TAG) during oxidation [10],
89 and cleavage of hydroperoxides of polyunsaturated fatty acids [23]. Endo *et al.* [23]
90 have demonstrated that the acrolein formation levels in thermally oxidized vegetable
91 oils increased with an increasing α -linolenate content in the oils, but the linoleate
92 content did not correlate with the acrolein formation. Hirayama *et al.* [25] found that
93 more acrolein was formed during the oxidation of methyl α -linolenate than during the
94 oxidation of methyl linoleate. These results suggest that the acrolein formation may
95 have a huge impact on the oxidative deterioration of lipids that have a high level of
96 unsaturated fatty acids, such as α -linolenic acid (18:3n-3), EPA (20:5n-3), and DHA
97 (22:6n-3). Thus, we report the volatile compounds formed during the autoxidation of
98 four types of TAGs with different fatty acid compositions, giving specific attention to
99 the acrolein formation.

100

101 **Methods**

102

103 **Standards and Substrate Lipids**

104

105 The silica gel (BW-60F) for the column chromatography was purchased from Fuji
106 Sylysia Chem. Ltd., Kasugai, Aichi, Japan. The activated carbon and Celite (545 RVS)
107 were products of Nacalai Tesque Inc., Kyoto Japan. The soybean oil and linseed oil
108 were purchased from Wako Pure Chemical Ind., Osaka and Summit Oil Mill Co. Ltd.,

109 Chiba, Japan, respectively. The echium oil and fish oil was kindly donated by De Wit
110 Special Oil Co., The Netherlands. Two kinds of fish oil, DHA concentrated oil
111 (DHA-55) and EPA concentrated oil (EPA-28MN) were gifts from Maruha Nichiro Co.,
112 Tsukuba, Japan. Both oils were mixed in equal parts and used as fish oil. Triolein
113 (purity>99%) was obtained from Wako Pure Chemical Ind. All the other chemicals and
114 solvents were of analytical grade.

115

116 **Lipid Substrate Purification**

117

118 Each oil (*ca.* 25 g) was passed through a column (50 cm x 4 cm i.d.) packed with a
119 *n*-hexane slurry mixture of activated carbon (100 g) and Celite (100 g) to remove the
120 tocopherols and pigments by eluting with *n*-hexane (1200 mL). The obtained oil (*ca.* 10
121 g) was refined using a silicic acid column (50 cm x 4 cm i.d.) packed with a *n*-hexane
122 slurry of Silica gel BW-60F (200 g) by eluting with *n*-hexane (200 mL) and a mixture of
123 *n*-hexane-diethyl ether (98:2 (200 mL) and 90:10 (1200 mL), v/v). The fraction eluted
124 with the *n*-hexane-diethyl ether (90:10) was composed of TAG and showed no other
125 impurities, such as free fatty acids, monoacylglycerols, or diacylglycerols, via thin-layer
126 chromatographic analysis using a 0.25 mm silica gel plate (Silica gel 60G; Merck)
127 developed with *n*-hexane/diethyl ether/acetic acid (70:30:1, v/v/v). The TAG spot was
128 detected with iodine vapor or 60% aqueous sulfuric acid charring. The identification of
129 the spot was performed using a standard TAG (triolein). The peroxide value of the
130 purified TAG was less than 1.0 meq/kg as determined by the AOCS Official Method
131 [26]. Few peroxides and tocopherols were also detected in the TAG via HPLC analysis
132 [27]. The fatty acid composition of the TAG was determined using gas chromatography
133 (GC) after conversion of the fatty acyl groups in the lipid to their methyl esters by
134 transesterification using sodium methoxide (CH₃ONa) as the catalyst [27]. The GC
135 analysis was performed on a Shimadzu GC-14B (Shimadzu Corporation, Kyoto, Japan)
136 equipped with a flame-ionization detector and a capillary column (Omegawax-320; 30
137 m x 0.32 mm i.d.; Supelco, Bellefonte, PA). The detector, injector, and column
138 temperatures were 260, 250, and 200°C, respectively. The carrier gas was helium, and it
139 had a flow rate of 50 kPa. The fatty acid content was expressed as a weight percentage
140 of the total fatty acids.

141

142 **Oxidation and Analysis**

143

144 Each 300 mg TAG sample was placed in a 20 mL aluminum sealed vial with a

145 butyl-gum septum (GL Science, Tokyo, Japan) and then incubated at 50°C or 60°C in
146 the dark. Before the incubation, the level of oxygen in the headspace gas of the vial was
147 estimated using a GC (Shimadzu GC-14B) [28]. The GC was equipped with a thermal
148 conductivity detector and a stainless steel column (3 m x 3.0 mm i.d.) packed with a
149 molecular Sieve 5A (GL Science). The temperatures at the injection port, detector port
150 and column oven were 120°C, 120°C and 70°C, respectively. The helium flow was 50
151 kPa. More than three separate vials containing similar samples were prepared and
152 incubated. A small portion (20 µL) of the headspace gas was taken from each vial using
153 a microsyringe through the butyl gum septum at selected times during the oxidation.
154 The decrease (%) in the oxygen was calculated from the changes in the oxygen to
155 nitrogen ratio compared with the ratio before incubation. Each data value at different
156 oxidation times of the different samples was expressed as the mean±SD (n=3).

157 The oxidation of the sample was also monitored via GC analysis of the volatile
158 compounds. A TAG sample of 300 mg was sealed in a 20 mL clean vial and incubated at
159 50°C or 60°C in the dark. For the static headspace GC analysis, after a definite time of
160 incubation, the sample vial was transferred into the HS-20 headspace auto-sampler
161 (Shimadzu Corporation) of the GC apparatus. The headspace gas in the vial was
162 automatically pressurized at 60°C for 2 min and then immediately injected through a
163 loop into the GC (Shimadzu GC-2014AFSC) equipped with a HP-1 capillary column
164 (50-m length, 0.32 mm i.d. and 1.05-µm film thickness; Agilent Technologies, CA,
165 USA) and a flame ionization detector. An initial oven temperature of 40°C for 5 min
166 was used, followed by a rate of 3°C/min to 70°C and then by a rate of 20°C/min to
167 200 °C, and finally, the temperature was held at 200°C for 4 min. Both the injection port
168 and the flame ionization detector were set at 250°C. Three replicate measurements of
169 each stored sample were performed, and the data were expressed as the mean±SD
170 (n=3).

171

172 **GC-MS Analysis**

173

174 The identities of the volatile compounds were obtained using solid phase
175 micro-extraction (SPME) and gas chromatography-mass spectrometry (GC-MS). The
176 volatiles were collected from the sealed vials containing different TAG samples after
177 incubation using a 50/30 µm DVB/CAR/PDMS SPME fiber (Supelco, Bellafonte, PA,
178 USA). The SPME fiber was exposed to the headspace for 5 min at room temperature
179 under the same conditions. The absorbed volatiles were then desorbed in the injection
180 port of the gas chromatograph GC-2010 equipped with a Model GCMS-QP2010 Ultra

181 mass spectrometer (Shimadzu Corporation). The GC conditions were the same as
182 described above. The mass spectrometer was operated in the electron impact ionization
183 mode (70 eV). The identification of the volatile compounds was performed by
184 comparison with the mass spectra from the NIST Standard Reference Database and by
185 injection of authentic standards. Acrolein peak was identified from parent ion (m/z 56)
186 and other target ions (m/z 55 and 27). In addition to GC-MS analysis, authentic sample
187 of acrolein was used for the identification of the GC peak. GC was carried out using two
188 different columns, namely HP-1 and DB-35.

189

190 **Results and Discussion**

191

192 **Comparison of the oxidative stability of different TAGs**

193

194 According to the traditional oxidation mechanism, the rate-limiting step in the reaction
195 is abstraction of the hydrogen atom that occurs at the bis-allylic position
196 ($\text{CH}=\text{CH}-\underline{\text{CH}_2}-\text{CH}=\text{CH}$) of the PUFA; therefore, the oxidative stability of
197 polyunsaturated lipids increase with a decreasing number of bis-allylic positions [29].
198 The average number of bis-allylic positions per each TAG molecule was obtained from
199 the mol concentration of the PUFA composing the TAG and the number of bis-allylic
200 position(s) of the PUFA. The mol concentration of the PUFA was computed using the
201 weight % of the PUFA (Table 1) and the molecular weight of each PUFA. Thus, the
202 average number of bis-allylic positions per molecule of soybean, linseed, echium, and
203 fish oil TAGs was calculated to be 0.613, 1.103, 1.287, and 2.187, respectively.

204 When the oxidative stability of the four types of TAGs at 50°C was compared by
205 measuring the decrease in the oxygen concentration (Fig. 1A), the oxidative stability
206 was found to be the highest for the soybean oil TAG, followed by the linseed and
207 echium oil TAGs, respectively. This order was in agreement with that expected from the
208 average number of bis-allylic positions. However, there was little difference in the rate
209 decrease of the echium oil TAG and the fish oil TAG, even though the average number
210 (2.187) of positions in the fish oil TAG was much higher than that (1.287) of the echium
211 oil TAG. The highest oxidative stability of the soybean oil TAG was also confirmed
212 using GC volatile analysis (Fig. 1B and 1C). Although the total peak area of the
213 volatiles increased with the incubation time for all the TAG samples, the soybean oil
214 TAG required several incubation times to provide a rapid increase in the total volatiles.

215

216 **Volatile Compound Analyses Using the Static Headspace Method**

217

218 Volatile oxidation products are directly responsible for or serve as markers for the flavor
219 deterioration in oxidized lipids. Specifically, in the oxidation of n-3 PUFA-containing
220 lipids, such as fish oil, the off-odors are formed at the very early stages of oxidation.
221 Because volatile oxidation products sometimes have a strong impact on the flavor at
222 extremely low concentrations, often below 1 ppm, much research has been performed
223 on the key volatile compounds that are responsible for the fish oil flavor deterioration.

224 Although early studies recognized that trimethylamine and compounds from
225 oxidizing PUFA were involved in the production of the distinct fishy flavors in marine
226 foods, a trimethylamine-like fishy odor was only apparent at a high concentration of
227 trimethylamine in the fish oils [8]. The major contributors to off-flavors in fish oil are
228 short-chain saturated and unsaturated aldehydes and ketones that have a greasy, oily,
229 green grassy or green plant-like odor [7]. Among the numerous carbonyl compounds
230 identified in the oxidizing fish lipids, 1-penten-3-one and 2,4-heptadienal have been
231 reported as the flavor deterioration indicators of oxidizing fish oil [11]. Additionally,
232 4-heptenal, 2,4-heptadienal, 2,6-nonadienal, 2,4,7-decatrienal, 1-pentene-3-one,
233 1-octen-3-one, and 1,5-octadien-3-one have also been demonstrated as major off-flavor
234 contributors [8,12,13].

235 Some of these aldehydes and ketones were also detected in the oxidations of four
236 types of TAGs that are studied here. The GC analysis of oxidized soybean, linseed,
237 echium, and fish oil TAGs at 60°C are shown in Fig. 2A, 2B, 2C, and 2D, respectively.
238 Although the same volatile compounds are found on each chromatogram (Fig. 2), the
239 GC profiles are different because of the different fatty acid composition of each TAG
240 (Table 1). For example, in the soybean oil TAG, the volatile compounds were relatively
241 small before 90 hr of incubation. After 198 hr of oxidation, pentane (3) was found in the
242 largest quantity followed by hexanal (5), propanal (2), and acrolein, respectively.
243 However, even after 8 hr of incubation, acrolein (1) and propanal (2) were found in the
244 oxidations of echium and fish oil TAGs (Fig. 2C and 2D, respectively), while the
245 acrolein (1) level was found to be low in the oxidation of linseed oil TAG (Fig. 2B). The
246 formation of acrolein and propanal was also found in the early stages of oxidation of the
247 echium and the fish oil TAGs at 50°C (data not shown). The peak area ratios of acrolein
248 to propanal were 0.115, 0.569, and 2.554 for the 8 hr oxidation of linseed, echium, and
249 fish oil TAG, respectively (Fig. 2), suggesting the preferential formation of acrolein,
250 especially during the fish oil TAG oxidation.

251 Acrolein is well-known as a representative undesirable volatile aldehyde found in
252 frying oils [30]. It provides undesirable and irritating odors [23]. Moreover, acrolein has

253 a harmful influence with a LD50 (oral) of 82 mg/kg [30]. It may cause eye, nasal, and
254 respiratory tract irritations, membrane damage, mitochondrial dysfunction, myelin
255 disruption in the nervous system, and it may induce epithelial cell injury [20-22].
256 Although acrolein can be formed in frying oils and in autoxidized lipids [19-22], a little
257 attention has been given to the formation of acrolein in autoxidized unsaturated lipids.
258 Based on the strong impact of acrolein on flavor deterioration and toxicity, more
259 attention should be given to acrolein formed in the lipid autoxidation because it is one
260 of the key volatile compounds that can be used to predict the sensory quality of
261 unsaturated lipids that are susceptible to oxidative degradation, such as fish oil.

262

263 **Changes in the Quantities of the Volatile Compounds During the TAG Oxidations**

264

265 The quantitative analysis of the major volatile compounds using the static headspace
266 GC method is shown in Fig. 3 and Fig. 4. The amounts of the volatile compounds are
267 expressed as peak intensities. All of the volatile compounds except for acrolein
268 increased with increasing incubation time. Acrolein was the most abundant volatile in
269 the fish oil TAG (Fig. 3D). It quickly increased, but then it stayed constant or slightly
270 decreased. The other main volatiles detected in the fish oil oxidation were propanal and
271 1-pentene-3-ol (Fig. 3D). The relative concentration of 1-pentene-3-ol was also
272 significant in the oxidized linseed (Fig. 3B) and echium (Fig. 3C) oil TAGs. Acrolein
273 was detected at a similar level to that of the 1-pentene-3-ol peak in the linseed and
274 echium oil TAGs. The major volatiles found in the oxidations of both TAGs were
275 pentane and propanal. These volatiles may be produced by the decomposition of the
276 monohydroperoxides from the n-3 PUFAs, such as 18:3n-3 and 18:4n-3 (Table 1) [19].
277 The oxidized soybean oil produced only small amounts of volatiles at the early stage of
278 the oxidation, but after 100 hr of incubation, the pentane rapidly increased (Fig. 3A).
279 Hexanal, an indicator of n-6 PUFA oxidation, steadily increased. Pentane and hexanal
280 are known to form from the 13-monohydroperoxide of linoleate that is abundant in
281 soybean oil TAGs (Table 1) [31].

282 Fig. 4 displays the increase in the other major volatiles produced during the
283 oxidation of the four types of TAGs. Although these volatile compound levels were
284 relatively lower than those shown in Fig. 3, the characteristic difference was found in
285 the rate increase of each volatile between the different TAGs. During the oxidation of
286 the soybean oil TAG (Fig. 4A), the rate increases of heptane, octane, and pentanal were
287 relatively higher than the other volatiles. However, during the oxidations of the linseed
288 (Fig. 4B), echium (Fig. 4C), and fish oil (Fig. 4D) TAGs, 2-pentenal and 2-butenal were

289 found in much larger relative quantities than the other volatiles.

290 The relative volatile composition changes according to the GC method used. A
291 relatively higher proportion of the low molecular weight volatiles may be found using
292 the static headspace method than those found by other GC volatile analysis methods,
293 including the SPME method [19]. The low molecular weight volatiles, such as acrolein,
294 propanal, pentane, 1-pentene-3-ol and 2-pentenal, can be found in the greatest
295 proportion at a high vapor pressure during the equilibration of the headspace gas in the
296 static headspace method. However, there is a limitation in the analysis of several
297 characteristic compounds, such as 2,4-decadienal and 2,4-heptadienal that originated
298 from the n-6 and n-3 PUFA hydroperoxides, when using the static headspace method
299 [9]. By operating at relatively lower temperatures (as low as 60°C), the dynamic
300 headspace method may measure the actual oxidation products present in the oils without
301 thermal decomposition of the flavor precursors (17). Another advantage of this method
302 is that it permits component enhancement to reveal the minor components that have a
303 significant flavor. However, the lower-boiling compounds may be lost during the
304 purging cycle in the SPME method, while other components, such as heptadienal and
305 decadienal, can be concentrated in the trap. Thus, using the dynamic headspace method,
306 the low molecular weight or low boiling volatile compounds, such as acrolein, propanal
307 and pentane, are found in much less relative quantities than those found using the static
308 headspace method operated at 60°C.

309 Many types of volatiles have been reported from the fish oil oxidation using
310 different types of analytical methods [8,13-18]. Each volatile detected from the oxidized
311 fish oil may have some responsible role for the oxidative deterioration; however, the
312 most notable volatiles are those formed during an early stage of the oxidation. This is
313 because fish oil induces oxidative deterioration from a very early stage of the oxidation.
314 Acrolein that was found in the present experiments may play an important role in fish
315 oil deterioration. As shown in Fig. 2B, 2C, and 2D, acrolein was detected in linseed,
316 echium, and fish oil TAGs, respectively, at the early stages of oxidation. Specifically,
317 the highest relative quantity of acrolein was found in fish oil TAG, followed by the
318 echium and linseed oil TAGs, respectively. Therefore, acrolein may have a large impact
319 on fish oil deterioration.

320 Prior studies have found higher levels of acrolein from the oxidation of TAGs that
321 are more abundant in α -linolenate than in linoleate or oleate [23]. When different types
322 of oils were oxidized at 60°C for 15 days and the volatiles were analyzed via the SPME
323 method, the highest level of acrolein was found in the cod liver oil, followed by the fish,
324 flaxseed, walnut, soybean, the low α -linolenic soybean, and the partially hydrogenated

325 vegetable oils, respectively [18]. Acrolein was also found in the oxidation of methyl
326 oleate, linoleate, and α -linolenate at 100°C [25] and 180°C [23]. Among the oxidation,
327 the level of acrolein was greatest in methyl linolenate. Relatively smaller amount of
328 acrolein was also found from methyl linoleate oxidation, but not or a little from methyl
329 oleate. As shown in Fig. 2, acrolein was formed with propanal from the very early stage
330 of oxidations of fish, echium, and linseed oil TAGs. In addition, the peak area ratio of
331 acrolein to propanal on the GC was the much highest in fish oil TAG, being followed by
332 echium oil TAG and linseed oil TAG, respectively. Therefore, more acrolein could be
333 produced in the lipid oxidation when the lipid contains more unsaturated fatty acids,
334 such as EPA and DHA.

335 Most probably, acrolein found in the early stage of fish oil oxidation is formed
336 during the decomposition of the oxidation products from EPA and DHA. Endo *et al.*
337 [23] have suggested the formation of acrolein by the cleavage of dihydroperoxides and
338 hydroperoxy epidioxides, since these secondary oxidation products are known to be
339 more readily produced from PUFAs having more double bonds [32]. Although the
340 formation mechanism of acrolein from EPA and DHA has not yet been made clear, it
341 might be quickly and continuously produced after propanal formation as shown in Fig.
342 5. Fig. 4 showed the different behavior of acrolein formation as compared with those of
343 other volatile compounds. Acrolein increased in an early stage of oxidation, but
344 thereafter, gradually decreased, while other volatile compounds did not decrease during
345 the experimental period. This may be due to the higher reaction activity of acrolein
346 (33,34). Acrolein would be quickly oxidized and/or decomposed during the incubation.

347

348 **Conclusions**

349

350 The present study revealed the preferential formation of acrolein (2-propanal) at the
351 very early stage of fish oil oxidation. Acrolein was most abundant volatile formed
352 during the fish oil oxidation, while other compounds such as propanal, pentane, and
353 hexanal were detected as major volatiles in echium, linseed, and soybean oil TAGs.
354 Thus, acrolein may act as a key compound having a strong impact on flavor
355 deterioration of fish oil, especially at early stage of the oxidation.

356 GC methods are the best technique for determination of the volatile oxidation
357 products formed during the lipid oxidation. Among them a dynamic headspace method
358 with SPME fiber is often used for this purpose. The dynamic headspace method has the
359 advantage of using lower temperatures and permitting enhancement of trace compounds
360 in complex mixtures of a wide range of volatile compounds. However, the

361 lower-boiling compounds such as acrolein may be lost during the purging cycle in the
362 SPME method. The present study indicated that static headspace method will be better
363 to detect acrolein. A judicious use of several GC techniques is necessary to evaluate
364 volatiles during the analysis of volatiles from lipid oxidation precursors.

365

366 **Acknowledgement** This work was supported by “Scientific technique research
367 promotion program from agriculture, forestry, fisheries and food industry” from
368 Ministry of Agriculture, Forestry and Fisheries in Japan.

369

370 **Abbreviations**

371 DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GC, gas chromatography;
372 GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid
373 chromatography; PUFA, polyunsaturated fatty acid; SPM, sphingomyelin; TAG,
374 triacylglycerol;

375

376 **References**

- 377 1. Kinsella JE (1986) Food components with potential therapeutic benefits: the n-3
378 polyunsaturated fatty acids of fish oils. *Food Technol* 40:89-146
- 379 2. Narayan B, Miyashita K, Hosokawa M (2006) Physiological effects of
380 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)-a review. *Food Rev*
381 *Inter* 22:291-307
- 382 3. Allayee H, Roth N, Hodis HN (2009) Polyunsaturated fatty acids and cardiovascular
383 disease: implications for nutrigenetics. *J Nutrigenet Nutrigenomics* 2:140-148
- 384 4. Russ, GL (2010) Dietary n-6 and n-3 polyunsaturated fatty acids: from biochemistry
385 to clinical implications in cardiovascular prevention. *Biochem Pharmacol*
386 235:937-946
- 387 5. Taneja A, Singh H (2012) Challenges for the delivery of long-chain n-3 fatty acids
388 in functional foods. *Annu Rev Food Sci Technol* 3:105-123
- 389 6. Jacobsen C (1989) Sensory impact of lipid oxidation in complex food systems,
390 *Fett/Lipid* 101:484-492
- 391 7. Hsieh TCY, Williams SS, Vejaphan W, Meyers SP (1989) Characterization of
392 volatile components of menhaden fish (*Brevoortia tyrannus*) oil. *J Am Oil Chem*
393 *Soc* 66:114-117
- 394 8. Karahadian C, Lindsay RC (1989) Evaluation of compounds contributing
395 characterizing fishy flavors in fish oils. *J Am Oil Chem Soc* 66:953-960
- 396 9. Frankel EN (1993) Formation of headspace volatiles by thermal decomposition of

- oxidized fish oils vs. oxidized vegetable oils. J Am Oil Chem Soc 70:767-772
- 398 10. Horiuchi M, Umamo K, Shibamoto T (1998) Analysis of volatile compounds formed
399 from fish oil heated with cysteine and trimethylamine oxide. J Agric Food Chem
400 46:5232-5237
- 401 11. Aidos I, Jacobsen C, Jensen B, Luten JB, van der Padt A, Boom RM (2002) Volatile
402 oxidation products formed in crude herring oil under accelerated oxidative
403 conditions. Eur J Lipid Sci Technol 104:808-818
- 404 12. Venkateshwarlu G, Let MB, Meyer AS, Jacobsen C (2004) Chemical and
405 olfactometric characterization of volatile flavor compounds in a fish oil enriched
406 milk emulsion. J Agric Food Chem 52:311-317
- 407 13. Venkateshwarlu G, Let MB, Meyer AS, Jacobsen C (2004) Modeling the sensory
408 impact of defined combinations of volatile lipid oxidation products on fishy and
409 metallic off-flavors. J Agric Food Chem 52:1635-1641
- 410 14. Dehaut A, Himber C, Mulak V, Grard T, Krzewinski F, Le Fur B, Duflos G (2014)
411 Evolution of volatile compounds and biogenic amines throughout the shelf life of
412 marinated and salted anchovies (*Engraulis encrasicolus*). J Agric Food Chem
413 62:8014-8022
- 414 15. Sae-leaw T, Benjakul S (2014) Fatty acid composition, lipid oxidation, and fishy
415 odour development in seabass (*Lates calcarifer*) skin during iced storage. Eur J
416 Lipid Sci Tech 116:885-894
- 417 16. Sae-leaw T, Benjakul S (2014) Oxidative stability of lipids from hepatopancreas of
418 Pacific white shrimp (*Litopenaeus vannamei*) as affected by essential oils
419 incorporation. Eur J Lipid Sci Tech 116:987-995
- 420 17. Serra A, Buccioni A, Rodriguez-Estrada MT, Conte G, Cappucci A, Mele M (2014)
421 Fatty acid composition, oxidation status and volatile organic compounds in
422 "Colonnata" lard from Large White or Cinta Senese pigs as affected by curing time.
423 Meat Sci 97:504-512
- 424 18. Gómez-Cortés P, Sacks GL, Brenna JT (2015) Quantitative analysis of volatiles in
425 edible oils following accelerated oxidation using broad spectrum isotope standards.
426 Food Chem 174:310-318
- 427 19. Snyder JM, Frankel EN, Selke E, Warner K (1988) Comparison of gas
428 chromatographic methods for volatile lipid oxidation compounds in soybean oil. J
429 Am Oil Chem Soc 65:1617-1620
- 430 20. Shibamoto T (2006) Analytical methods for trace levels of reactive carbonyl
431 compounds formed in lipid peroxidation systems. J Pharm Biomed Anal 41:12-25
- 432 21. Singh M, Nam DT, Arseneault M, Ramassamy C (2010) Role of by-products of

- 433 lipid oxidation in Alzheimer's disease brain: a focus on acrolein. *J Alzheimers Dis*
434 21:741-756
- 435 22. Moghe A, Ghare S, Lamoreau B, Mohammad M, Barve S, McClain C, Joshi-Barve
436 S (2015) Molecular mechanisms of acrolein toxicity: relevance to human disease.
437 *Toxicol Sci* 143:242-255
- 438 23. Endo Y, Hayashi C, Yamanaka T, Takayose K, Yamaoka M, Tsuno T, Nakajima S
439 (2013) Linolenic acid as the main source of acrolein formed during heating of
440 vegetable oils. *J Am Oil Chem Soc* 90:959-964
- 441 24. Pegg AE (2013) Toxicity of polyamines and their metabolic products. *Chem Res*
442 *Toxicol*, 26:1782-1800
- 443 25. Hirayama T, Yamaguchi M, Nakata T, Okumura M, Yamasaki T, Watanabe T,
444 Fukui S (1989) Formation of acrolein by the autoxidation of unsaturated fatty acid
445 methyl esters. *Eisei Kagaku* 35:303-306 (Japanese)
- 446 26. American Oil Chemists' Society (AOCS) (1998) Official methods and
447 recommended practices. Cd 8-53. AOCS, Champaign
- 448 27. Shimajiri J, Shiota M, Hosokawa M, Miyashita K (2013) Synergistic antioxidant
449 activity of milk sphingomyeline and its sphingoid base with α -tocopherol on fish oil
450 triacylglycerol. *J Agric Food Chem* 61:7969-7975
- 451 28. Cho S-Y, Miyashita K, Miyazawa T, Fujimoto K, Kaneda T (1987) Autoxidation of
452 ethyl eicosapentaenoate and docosahexaenoate. *J Am Oil Chem Soc* 64:876-879
- 453 29. Miyashita K (2014) Paradox of omega-3 PUFA oxidation. *Eur J Lipid Sci Tech*
454 116:1268-1279
- 455 30. Takeoka G, Perrino C, Buttery R (1996) Volatile constituents of used frying oils. *J*
456 *Agric Food Chem* 44:654-660
- 457 31. Frankel EN, Neff WE, Selke E (1981) Analysis of autoxidized fats by gas
458 chromatography-mass spectrometry: VII. volatile thermal decomposition products
459 of pure hydroperoxides from autoxidized and photosensitized oxidized methyl
460 oleate, linoleate and linolenate. *Lipids* 16:279-285
- 461 32. Porter NA, Caldwell SE, Mills KA (1995) Mechanisms of free radical oxidation of
462 unsaturated lipids. *Lipids* 30:277-290
- 463 33. Jan F, Stevens JF, Maier CS (2008) Acrolein: sources, metabolism, and
464 biomolecular interactions relevant to human health and disease. *Mol Nutr Food Res*
465 52:7-25.
- 466 34. LoPachin RM, Terrence Gavin T (2014) Molecular mechanisms of aldehyde
467 toxicity: a chemical perspective. *Chem Res Toxicol* 27:1081-1091.
- 468

469 Figure legends

470

471 Figure 1. Oxidative stability of soybean oil TAG (open circle), linseed oil TAG (open
472 square), echium oil TAG (open triangle), and fish oil TAG (open diamond). The stability
473 was analyzed by measuring the decrease in oxygen (A) and the increase in total volatiles
474 (B) and (C) in a head space gas of the vial. Oxidation was done at 50°C (A) and (B) or
475 60°C (C) in the dark. The data value was expressed as the mean \pm SD of three separate
476 experiments.

477

478 Figure 2. Representative GC of volatile compounds from oxidized soybean (A),
479 linseed (B), echium (C), and fish (D) oil TAGs after 8, 16, 90, and 198 hr incubation at
480 60°C. Volatile compounds from the oxidized TAG was analyzed by static headspace
481 GC method and identified by GC-MS method. Major volatiles: (1), acrolein; (2),
482 propanal; (3), pentane; (4), 1-penten-3-ol; (5), hexanal.

483

484 Figure 3. Increase in major volatile compounds during the oxidation of soybean (A),
485 linseed (B), echium (C), and fish (D) oil TAGs during the incubation at 60°C in the dark.
486 Major volatiles: acrolein (open circle;), propanal (open triangle), pentane (open square),
487 1-penten-3-ol (open diamond), and hexanal (solid circle). The data value was expressed
488 as the mean \pm SD of three separate experiments.

489

490 Figure 4. Increase in other major volatile compounds during the oxidation of soybean
491 (A), linseed (B), echium (C), and fish (D) oil TAGs during the incubation at 60°C in the
492 dark. Major volatiles: 2-butenal (open circle), pentanal (solid square), 2-pentenal (open
493 triangle), heptane (open diamond), octane (solid circle, 2-heptenal (open square), and
494 2-octenal (solid triangle). The data value was expressed as the mean \pm SD of three
495 separate experiments.

496

497 Figure 5. Proposed mechanism for the formation of acrolein from EPA and DHA.

Table 1. Composition (weight %) of Major Fatty Acids of Substrate TAG

Fatty acid	Soybean	Linseed	Echium	Fish
14:0	0.06±0.00	0.04±0.00	0.03±0.00	4.70±0.35
16:0	10.41±0.09	5.65±0.14	7.65±0.31	11.40±0.48
18:0	3.90±0.03	3.61±0.03	3.61±0.09	3.27±0.02
16:1n-7	0.10±0.00	0.08±0.00	0.15±0.00	3.51±0.16
18:1n-9	26.07±0.07	25.67±0.13	17.25±0.34	6.43±0.03
18:1n-7	1.41±0.01	0.84±0.03	0.59±0.01	1.79±0.02
20:1n-9	0.27±0.00	0.19±0.01	0.93±0.07	1.62±0.05
18:2n-6	51.41±0.01	16.92±0.02	16.15±0.18	0.54±0.00
18:3n-6	-	-	11.58±0.20	0.35±0.00
18:3n-3	4.58±0.01	43.92±0.13	29.28±0.11	0.24±0.00
18:4n-3	-	-	11.35±0.29	1.76±0.03
20:4n-6	-	-	-	2.67±0.05
20:5n-3	-	-	-	15.70±0.19
22:5n-3	-	-	-	2.90±0.09
22:6n-3	-	-	-	27.80±0.80
24:1n-9	-	-	-	0.55±0.02

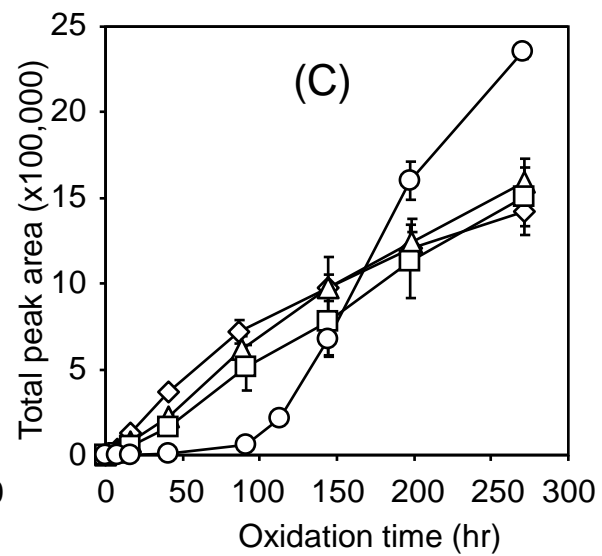
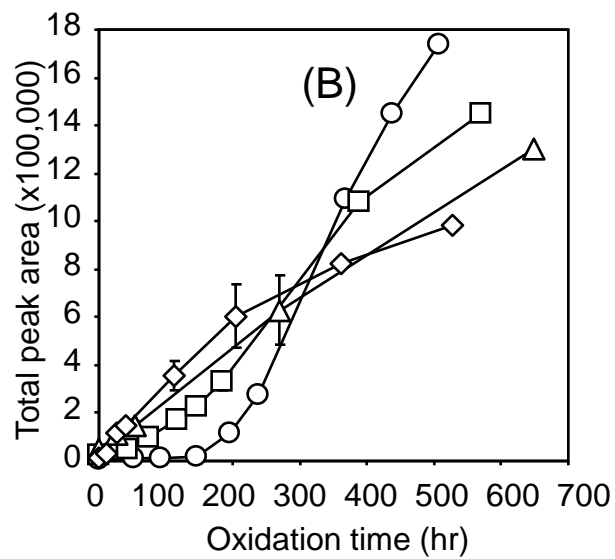
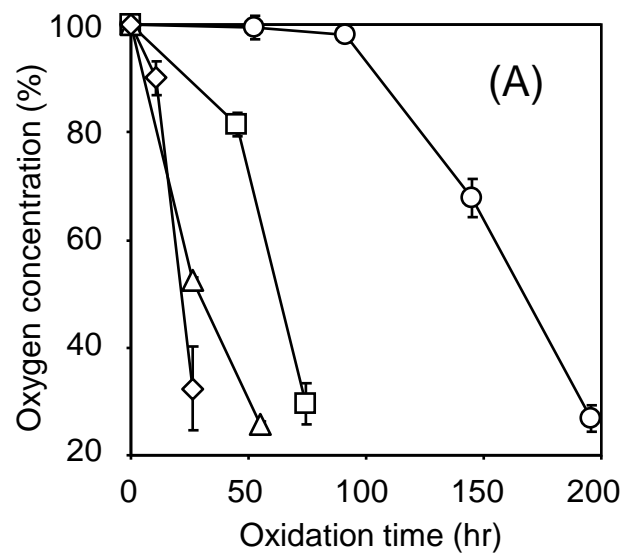


Figure 1

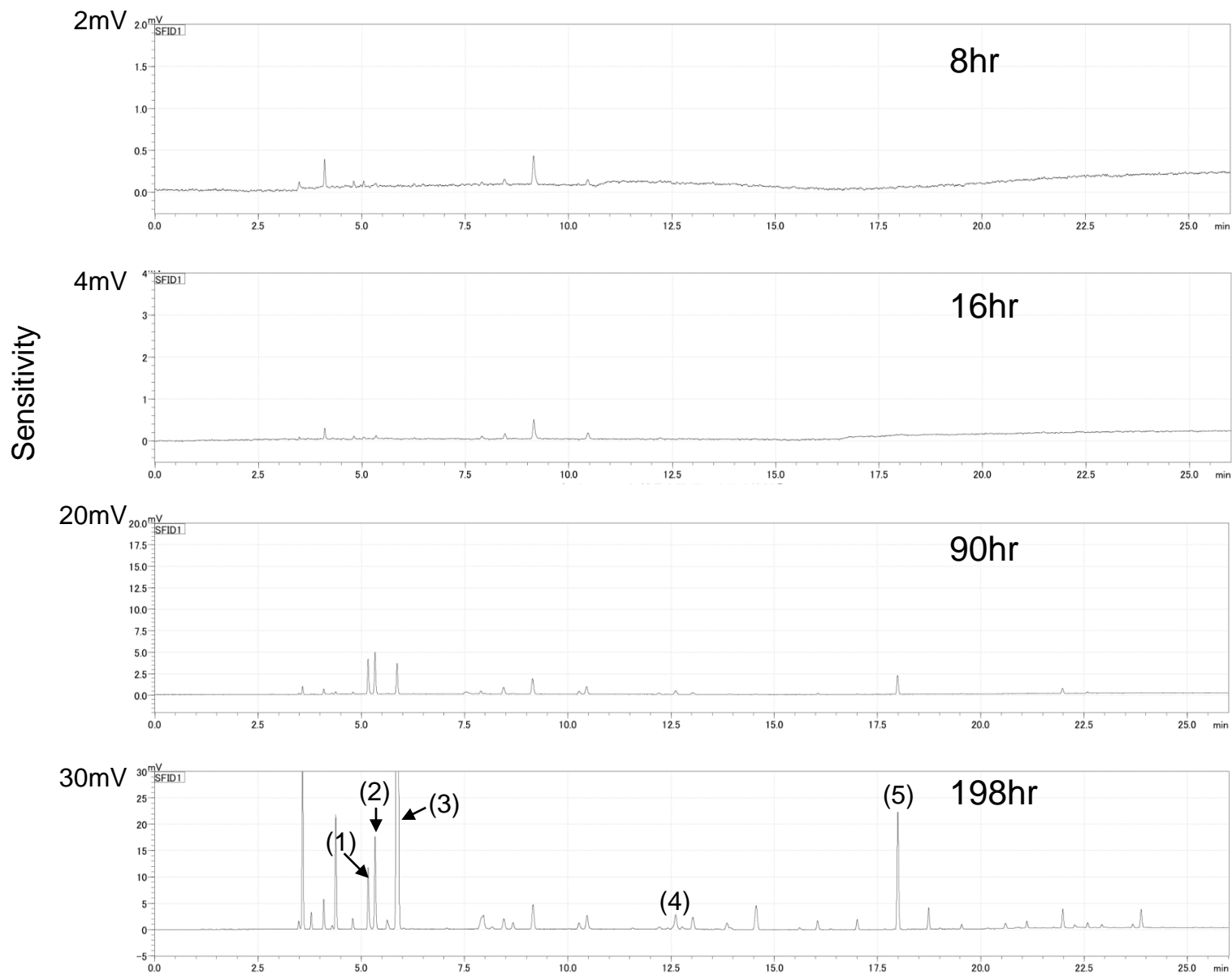


Figure 2 (A)

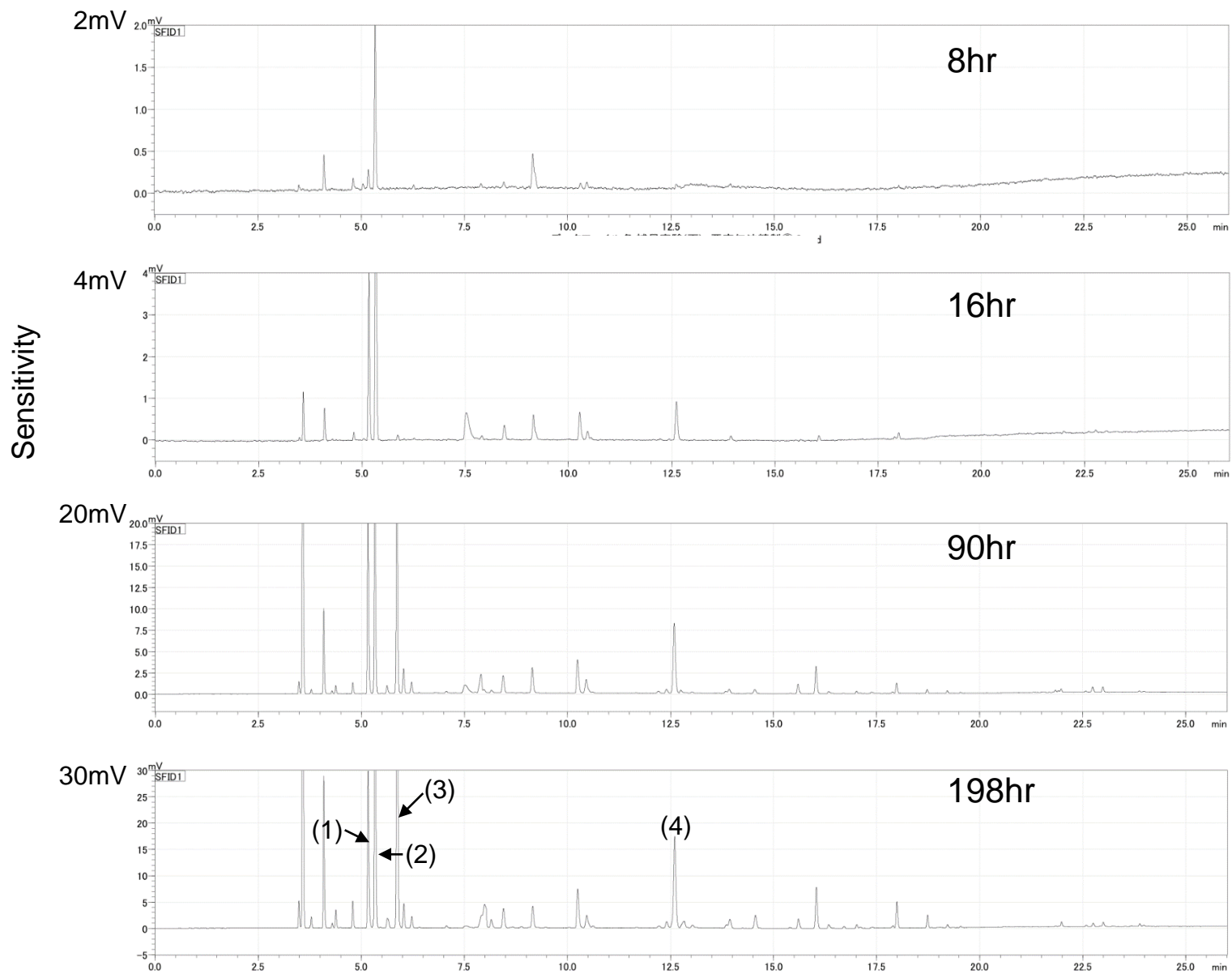


Figure 2 (B)

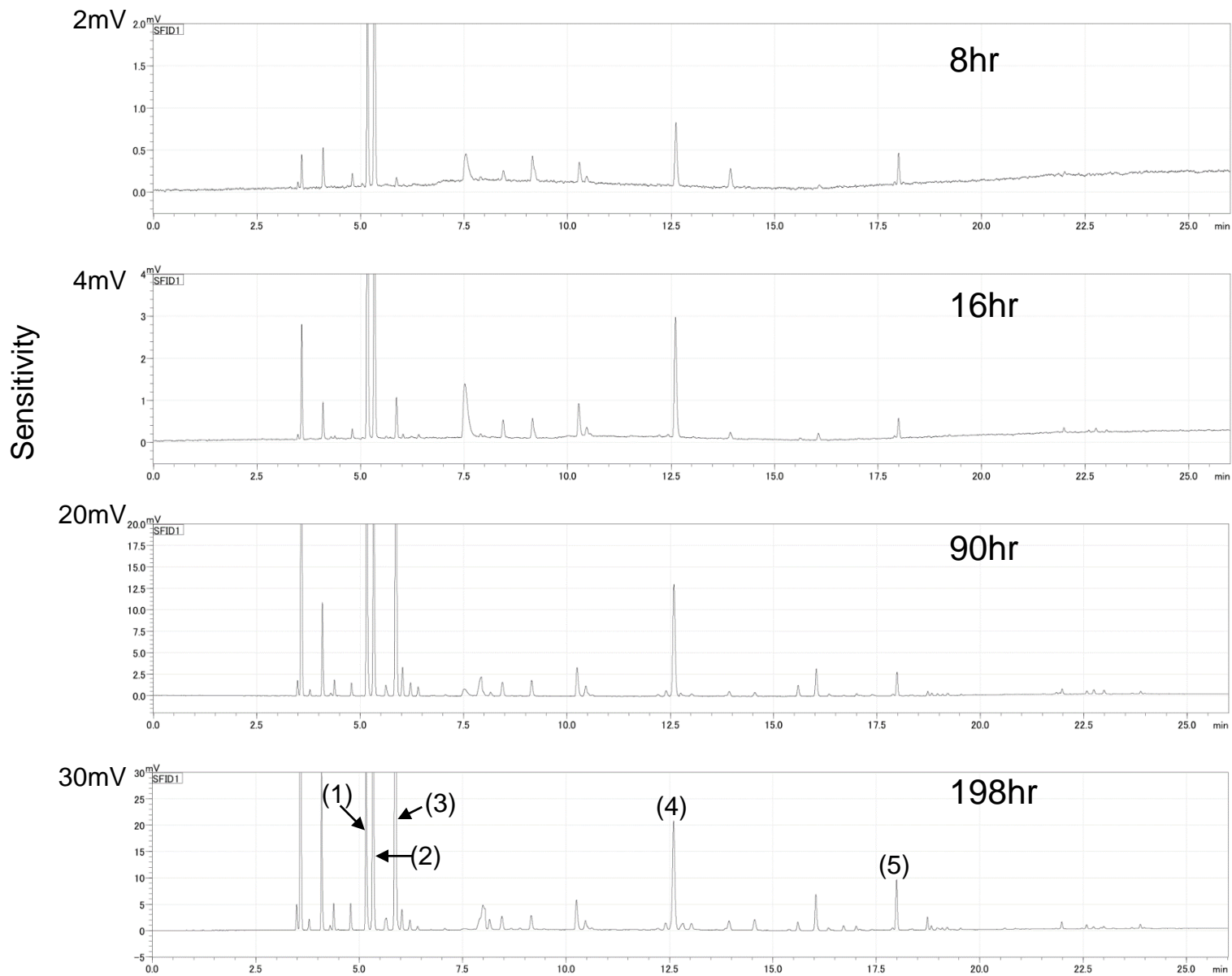


Figure 2 (C)

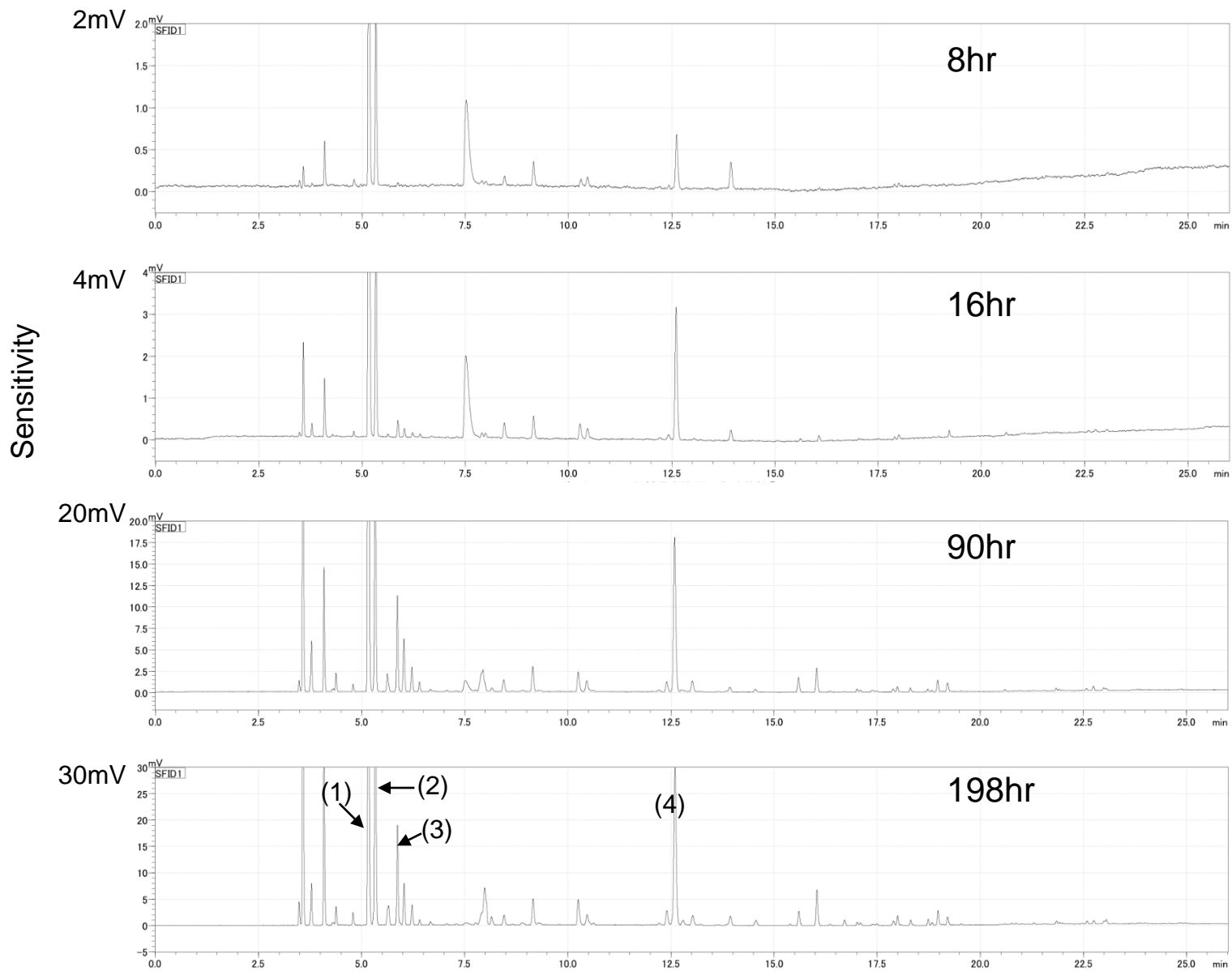


Figure 2 (D)

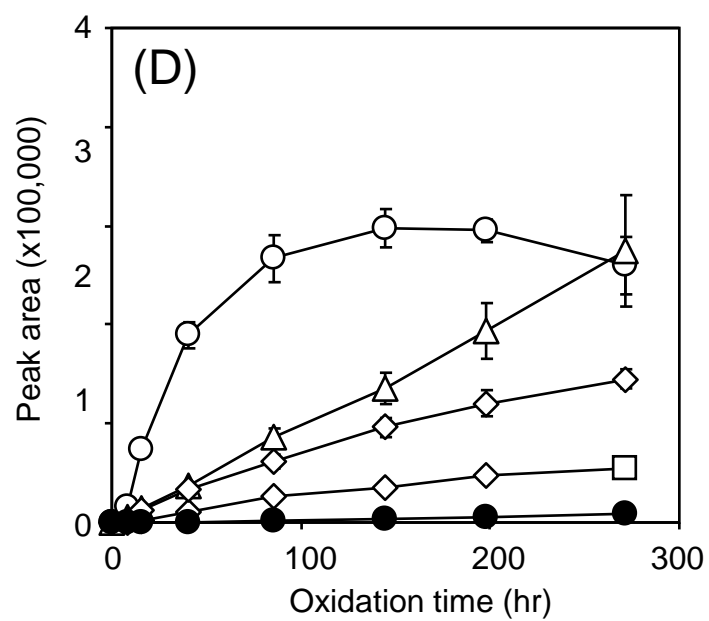
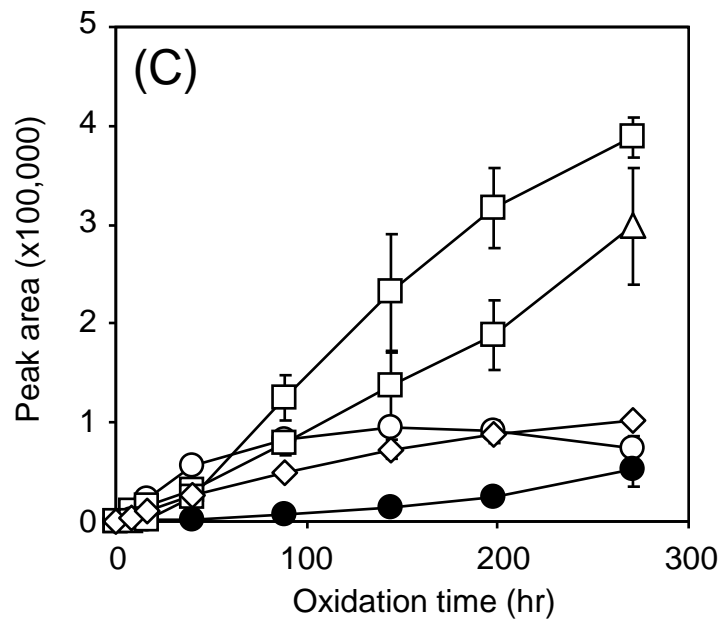
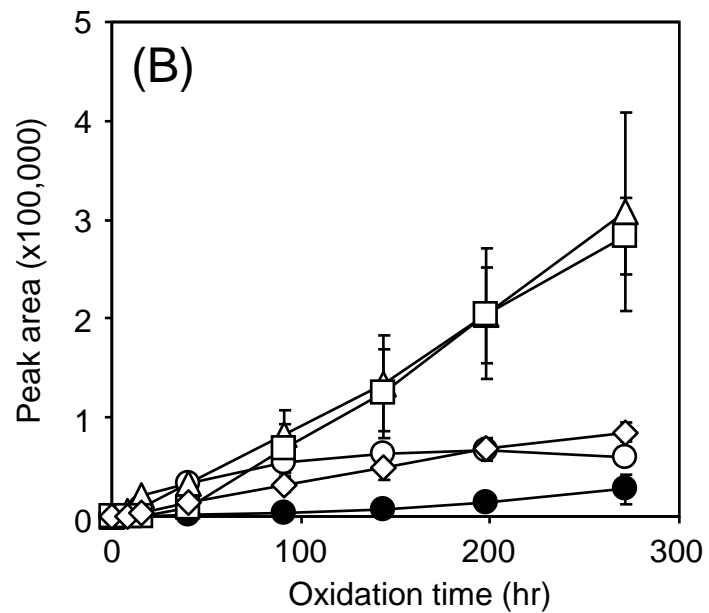
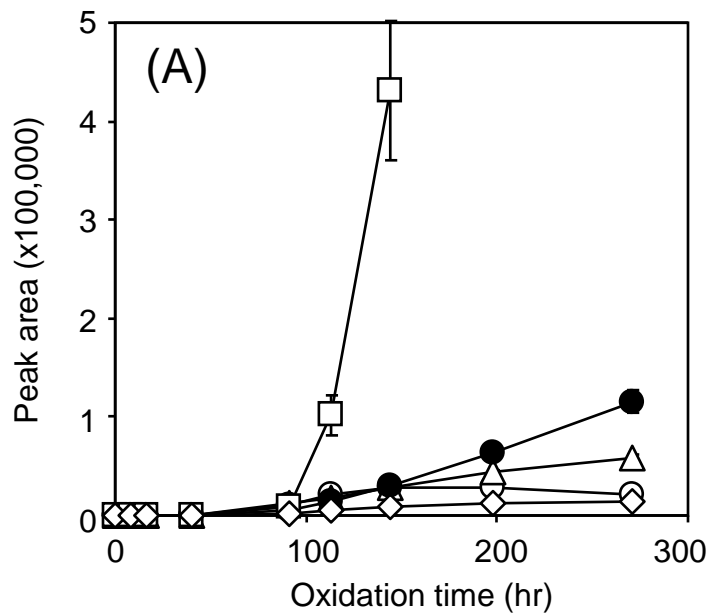


Figure 3

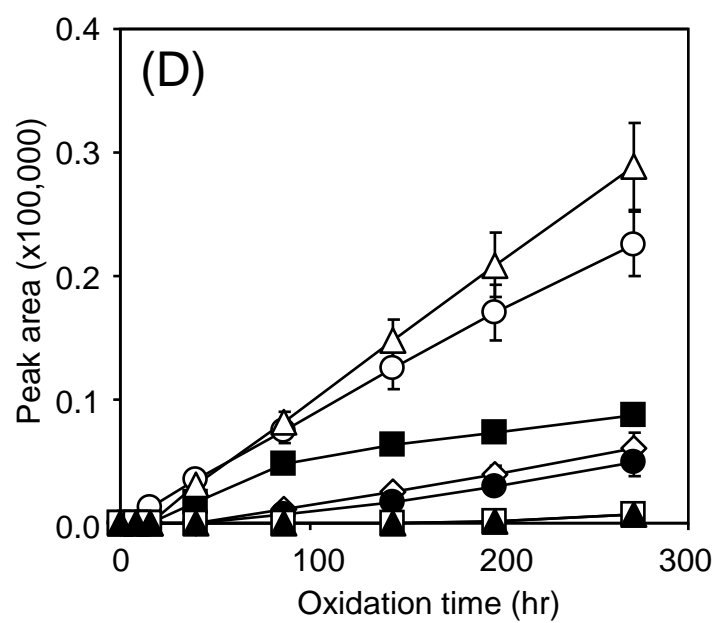
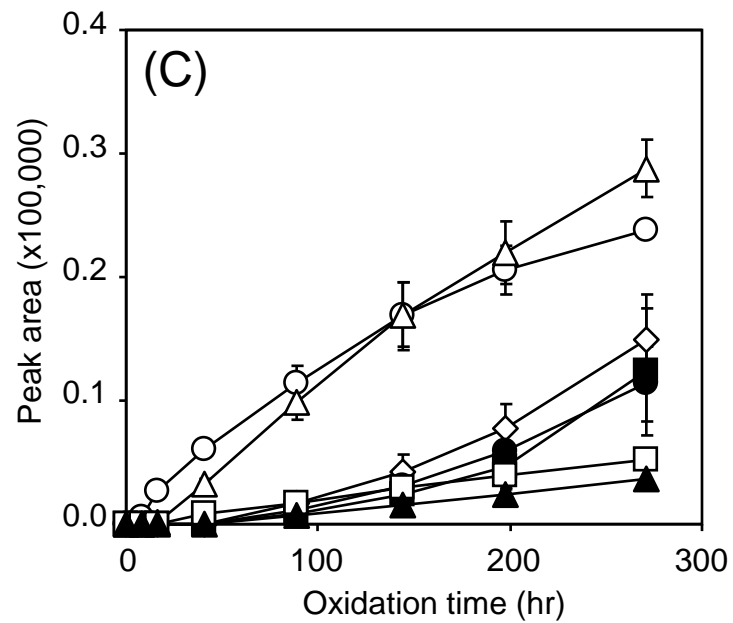
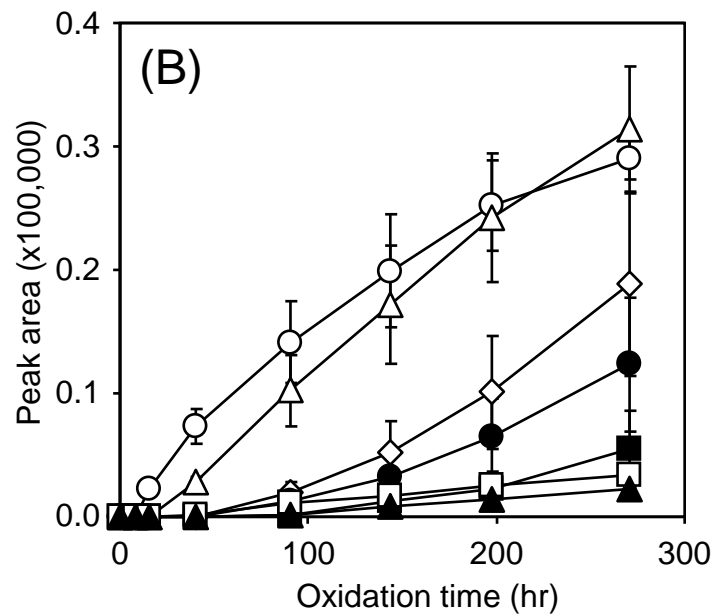
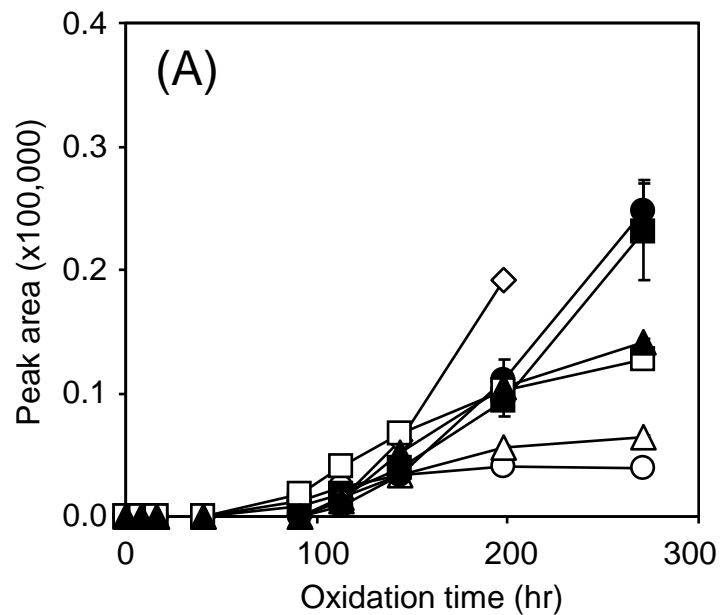


Figure 4

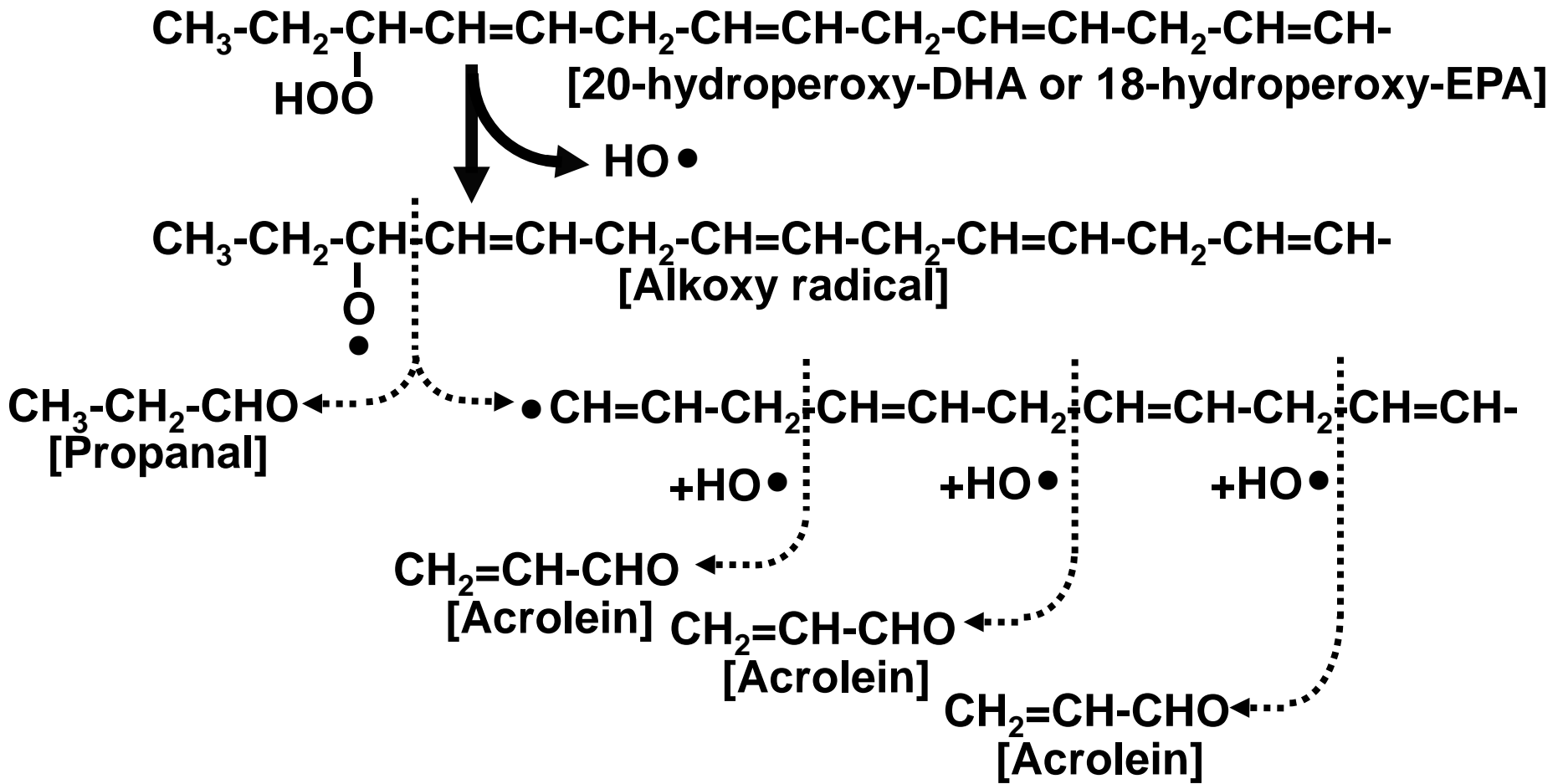


Figure 5