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New analytical evidence of discontinuous oxidation in dried microencapsulated lipids

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Abstract

Formation of hydroperoxy-, keto- and hydroxy- dienes was studied at 40 ºC in fatty acid methyl esters (FAME) microencapsulated in a dairy-like matrix formed by lactose and sodium caseinate. The FAME were obtained from conventional sunflower oil and the microcapsules were prepared by freeze-drying of an oil-in-water emulsion. For comparative purposes a neat sample of FAME was also tested. Results showed that for a given content of hydroperoxydienes much more elevated amounts of secondary products were detected in the microencapsulated sample compared to the neat sample of FAME. The contents of keto- and hydroxy- dienes found in the microencapsulated FAME ranged as a whole between 6 and 31 wt% of the analyzed compounds, while the neat sample showed values lower than 1.5%. Along with the fact that relatively higher contents of polymers were also found in the encapsulated sample, these results can be attributed to lipid droplets with very different oxidation states. On the one hand, the extract would be formed from droplets in early stages of oxidation containing hydroperoxides and very low contents of secondary products and, on the other, from droplets in advanced stages with decreased hydroperoxides and substantial contents of secondary products. Unlike the neat sample, hydroxydienes formed at significantly higher amounts than ketodienes in the microencapsulated FAME, suggesting a possible chemical role of the encapsulation matrix.

Key-words: Hydroperoxides, ketodienes, hydroxydienes, autoxidation, microencapsulation

1 Introduction

Dehydration of oil-in-water emulsions containing proteins and/or carbohydrates is a common process in the manufacture of food powders. Today there are a number of formulated food emulsions that are dehydrated such as infant formulae and emulsions containing polyunsaturated lipids or oils as a carrier of flavours, vitamins and other components [1-3]. The oil in these products is encapsulated as a disperse phase in the inner of solid particles that act as a physical barrier protecting it against oxidation.

Evaluation and control of lipid oxidation in microencapsulated lipids is of great significance because it results in the development of objectionable flavors and formation of compounds that may be detrimental to health [4]. The oxidation in these products is quite complex because droplets may oxidize at different rates and have very different oxidation states when the lipid extract is analyzed. Oxidized droplets may occur in the presence of others well protected by the encapsulation matrix. Even when the global content of oxidation products is low due to the oxidation of a minor fraction, complex mixtures of primary, secondary and even advanced oxidation compounds may be detected in quite different proportions to that of a sample of oil in continuous phase with the same total content of oxidation products [5]. It is therefore evident that evaluation of oxidation in such cases would not be possible with the methods of analysis normally applied in labs of quality control, based upon analytical indices such as the peroxide value.

In previous studies carried out in our lab, quantitative analysis of the main oxidation products of linoleic acid, i.e. hydroperoxy-, keto- and hydroxy- dienes, has been developed to analyze directly oxidized samples of fatty acid methyl esters (FAME) derived from vegetable oils. The separation and detection of analytes was based upon the NP-HPLC-UV analysis proposed by Hopia, Huang, & Frankel [6] to evaluate

48 oxidized samples of methyl linoleate. The conjugated diene structure of methyl linoleate
49 hydroperoxides and hydroxides can be readily detected by absorption of UV light at
50 232-234 nm, depending on the polarity of the solvent, while that of methyl linoleate
51 ketones are detected at 268-270 nm [7-8]. The method was then adapted to the analysis
52 of real samples of vegetable oils by applying an adequate derivatization step to
53 transform the oxidized triacylglycerol (TAG) molecules into FAME [9].

54 Compared to the analytical indices commonly applied to evaluate lipid oxidation, the
55 main advantage of this method is that allows quantitation of compounds and the
56 simultaneous determination of primary oxidation compounds, hydroperoxides, and their
57 principal secondary products, keto- and hydroxy- compounds, in low amounts. In this
58 regard, the quantification of lipid oxidation products in foods constitutes a starting point
59 to determine if the contents in the diet are sufficient to have a role in a variety of chronic
60 diseases, such as cardiovascular diseases and cancer. Oxidized lipids are known to be
61 involved in such diseases, but the real contribution of lipids coming from the diet is
62 unknown because the contents of oxidation products in foods have not been established
63 due to their analytical complexity [10].

64 The method has only been applied to the analysis of pure FAME, FAME obtained from
65 oils, and vegetable oils that were all oxidized in continuous phase [6-9, 11-12].

66 In this work we study the quantitative formation of hydroperoxy-, keto- and hydroxy-
67 dienes in FAME microencapsulated in a dairy-like matrix formed by lactose and sodium
68 caseinate [5]. The FAME were obtained from conventional sunflower oil and the
69 microcapsules were prepared by freeze-drying of an oil-in-water emulsion containing 10
70 wt% FAME, 10 wt% sodium caseinate, 10 wt% lactose and 70 wt% water. The water
71 activity and the glass transition temperature were measured along the oxidation study to
72 examine possible changes of the particle structure. Although the microencapsulated

sample under study can be considered a model system of microencapsulated oils or even of powdered infant formulae, it should be noted that ethyl esters of polyunsaturated fatty acids are microencapsulated and marketed as dietetic supplements or ingredients to supplement foods with functional lipids. The analyses were performed in the free and encapsulated lipid fractions of the microencapsulated FAME. For comparative purposes formation of the compounds was also studied in neat FAME. In addition to the HPLC method, the analysis of polymers by high-performance size-exclusion chromatography with refractive-index detection (HPSEC-RI) was also performed to evaluate advanced oxidation products.

2 Materials and Methods

Chemicals

Sodium caseinate and D-lactose monohydrate were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). Diethyl ether stabilized with 1% v/v ethanol (Super purity solvent, HPLC grade) was purchased from Romil, LTD (Cambridge, UK) and n-heptane (99% purity, HPLC grade) from Carlo Erba Reactifs-SDS (Val de Reuil, France). Both HPLC solvents were used as received.

Samples

FAME were obtained from refined high linoleic sunflower oil by transmethylation with KOH in methanol according to a previous report [11]. The oil was supplied by Koipe S.A. (Andújar, Jaén, Spain). The fatty acid composition was determined to be 6.7% C16:0, 0.2% C16:1, 3.6% C18:0, 33.0% C18:1, 55.2% C18:2 and 1.3% others.

The FAME were encapsulated in a matrix of sodium caseinate and D-lactose according to a previous report [13]. An o/w emulsion was prepared from the FAME and a solution containing the encapsulating components. The weight composition of the emulsion was 10% FAME, 10% sodium caseinate, 10% D-lactose and 70% water. A coarse emulsion was prepared in an Ultraturrax DI-25 (IKA, Germany) by applying 454 g for 2 min, 641 g for 2 min and 1294 g for 1 min. The emulsion was refined in an EmulsiFlex-C5 (Avestin Inc., Canada) high pressure homogenizer by applying 70 MPa and two passes. The emulsion was frozen -32 °C for 24 h and freeze-dried in a lab-scale Heto FD3 freeze-dryer (Allerød, Denmark) for 48 h. Finally, in batches of 20 g the dried emulsion was ground in a domestic electronic coffee grinder of 400 mL volume for 10 s at interval of 5 s and a homogenous powder product was obtained.

106 Oxidation conditions

107 A neat sample of FAME was oxidized in a beaker at 40 ºC in the dark by using an oven
108 with continuous air circulation. The surface-to-volume ratio of the FAME sample was
109 as high as 5 cm^{-1} so that oxidation was developed under non-limited oxygen conditions.
110 Three aliquots of 100 mg each were sampled periodically in a period of 7 days.

111 Independent 10-g samples of dried microencapsulated FAME were oxidized at 40 ºC in
112 closed amber bottles of 250 mL. An assay tube with 10 mL of a saturated solution of
113 potassium acetate was placed into each bottle to create an atmosphere of 23% relative
114 humidity [14]. Periodic samplings were performed in a period of 12 days. Three bottles
115 were drawn from the oven in each sampling and the samples were kept at -25ºC until
116 analysis.

117 Analyses of intact dried microencapsulated samples

118 Water activity

119 The water activity of the microencapsulated samples was measured using a PawKit
120 hygrometer (Decagon Devices Inc., Pullman, WA, USA).

121 Glass transition temperature

122 The glass transition temperature (T_g) of the microencapsulated FAME was determined
123 using a DSC Q 2000 calorimeter (TA Instruments, New Castle, DE, USA) according to
124 a previous report [13].

125 Lipid extraction from the microencapsulated samples

126 The free lipid fraction was extracted according to Sankarikutty, Sreekumar, Narayanan,
127 & Mathew [15]. A volume of 100 mL of n-hexane was added to 10-g sample and
128 stirring was applied at room temperature for 15 min. The solvent was filtered through a
129 filter paper and evaporated in a rotary evaporator at 40 ºC. The extract was dried to
130 constant weight using a stream of nitrogen. The treated powder was left in a fume
131 cupboard until the complete loss of trace hexane.

132 The encapsulated lipid fraction was extracted with dichloromethane and methanol (2:1,
133 v/v) in the absence of water according to a previous report [16]. In brief, a 75 mL
134 volume of dichloromethane followed by a 35 mL volume of methanol was added to the
135 sample. The mixture was vigorously stirred in a magnetic stirrer at room temperature for
136 30 min. The solvent was filtered through a filter paper and evaporated at 40 °C in a
137 rotary evaporator. The extract was finally dried to constant weight using a stream of
138 nitrogen.

139 Analysis of FAME

140 Analysis by HPLC

141 The main oxidation products of methyl linoleate, i.e. hydroperoxy-, keto- and hydroxy-
142 dienes, were determined quantitatively by an HPLC-UV method developed by our lab
143 [7]. A Waters 600 HPLC chromatograph (Waters Corp., Milford, MA, USA) equiped
144 with a 600 Waters pump, a Rheodyne injector valve (20-µL sample loop), a silica
145 HPLC column (LiChrospher® Si 60, 250 mm x 4 mm i.d., 5 µm particle size) (Merck,
146 Darmstadt, Germany), an HP 1050 Series variable wavelength UV detector (8 mm path
147 length) (Agilent Technologies Inc., Palo Alto, CA) and a 600 Waters controller was
148 used. The separation of analytes was performed in isocratic elution using n-

149 heptane:diethyl ether (82:18, v/v) with a flow rate of 1 mL/min. Ethanol present in the
150 diethyl ether as a stabilizer was not removed. Hydroperoxy- and hydroxy- dienes were
151 recorded by the UV detector at 234 nm, while ketodienes were at 268 nm. The FAME
152 samples both neat and extracted were dissolved in n-hexane in a range of 1-50 mg/mL
153 prior to analysis. Quantitative data were obtained by applying response factors reported
154 elsewhere [7].

155 Analysis of polymers

156 Analysis of polymers was performed according to IUPAC standard method 2.508 [17]. An
157 HPSEC chromatograph equipped with a Rheodyne 7725i injector with a 10-μL sample
158 loop, a Knauer 120 HPLC pump (Knauer, Berlin, Germany) and a Merck L-7490
159 refractive index detector (Merck, Darmstadt, Germany) was used. The separation was
160 performed on two 100 and 500 Å Ultrastyragel columns (25 cm x 0.77 cm i.d.) packed
161 with porous, highly cross-linked styrene-divinylbenzene copolymers (5 μm) (Agilent
162 Technologies, Palo Alto, CA) connected in series by using tetrahydrofuran as the
163 mobile phase at a flow rate of 1 mL/min.

164 Statistical analysis

165 Analytical determinations were carried out in triplicate and results were presented as
166 mean values. Comparisons were made by the Student's *t* test in Microsoft Excel 2010
167 (Microsoft Corporation, Redmond, WA, USA). Significance was defined at $p < 0.05$.

168

3 Results and discussion

The amounts of the free and encapsulated lipid fractions extracted, the water activity and the glass transition temperature (T_g) of the microencapsulated samples showed no substantial changes along the oxidation study. The amounts of lipids were 6-9 g/100 g sample for the free lipid fraction and 15-19 g/100 g of sample devoid of free oil for the encapsulated fraction. The water activity showed values of 0.20-0.27 and the T_g of 45-47 ºC. It can be said that the structure of the powder particles remained without significant changes that resulted in changes in the amounts of the free and encapsulated lipid fractions during the study. The fact that the oxidation temperature was lower than the T_g in just 5-7 ºC seemed to be sufficient so as not to occur such structural modifications [18]. Structural changes of the microencapsulation matrix can give rise to changes in the lipid distribution. Thus, an increase in the content of the free lipid fraction due to lactose crystallization is a well-known phenomenon in milk powder and dairy-like microencapsulated lipids [19, 20]. Absorption of water, storage temperature and storage time decrease the viscosity of the amorphous matrix allowing reorganization of its molecules [18, 19].

HPLC analysis showed that the chromatograms of the lipid extracts of the microencapsulated FAME were not different from those of the neat sample in terms of occurrence of new peaks or changes in the distribution of isomers in each group of compounds (**Fig. 1**).

As expected, oxidation was quicker in the continuous system. The hydroperoxides showed a period of slow formation followed by another of acceleration in both systems. Keto- and hydroxy- dienes displayed a similar pattern to that of hydroperoxides (**Fig. 2**). The substantial increase of hydroperoxides occurred at 4 days in the neat sample and at

8 days in both the free and encapsulated fractions of the microencapsulated sample. The formation of hydroperoxides in the accelerated period was however slower in the encapsulated fraction compared to both the free fraction and the neat sample, indicating clearly lower availability of oxygen. These results are in agreement with previous studies on oils microencapsulated in the same matrix as the one used in the present study [5, 14]. It was observed that in these products when an oil sample is stripped of its naturally occurring antioxidants and so unprotected the diffusion of oxygen through the matrix becomes the determining factor of the oxidation rate in the encapsulated fraction.

Formation of the main secondary oxidation products of methyl linoleate in the microencapsulated sample was quite different compared to the neat FAME. Both lipid fractions of the powder sample showed significantly much more elevated amounts of secondary products for a given content of hydroperoxydienes (**Fig. 3**). Whereas in the neat sample the relative contents of keto- and hydroxy- dienes as a whole were not higher than 1.5 wt% of the total compounds analyzed by HPLC, i.e. hydroperoxy-, keto- and hydroxy- dienes, the lipid fractions of the microencapsulated FAME showed values between 6 and 31 wt%. In addition, unlike the neat sample and other samples of neat FAME studied at different conditions elsewhere [7, 8], hydroxydienes formed at significantly higher amounts than ketodienes in the microencapsulated FAME.

The larger contents of degradation products from hydroperoxides found in the microencapsulated sample can be attributed to the discontinuous nature of oxidation in a product where the lipids constitute a disperse phase [5]. The extracts would come from lipid droplets with very different extents of oxidation. On the one hand, the lipid fraction would be formed from droplets relatively protected by the matrix, in early stages of oxidation and so with hydroperoxides and very low contents of secondary

products and, on the other, from droplets in advanced stages in which hydroperoxides decompose more quickly than are formed generating substantial contents of secondary oxidation products. This hypothesis was supported by the analysis of polymers. Likewise, for a given concentration of hydroperoxides the contents of polymers were also more elevated in the encapsulated lipid fraction than in the neat FAME sample (**Fig. 4**), indicating formation of advanced oxidation compounds in certain droplets. The free lipid fraction exhibited an intermediate situation between the continuous system and the encapsulated fraction (**Fig. 3-4**) and therefore also showed lipid droplets oxidizing at different rates. In previous studies, however, the free lipid fraction of dried microencapsulated oils showed an oxidative pattern similar to that of oils in continuous phase [5, 14]. Unlike the encapsulated fraction, most of the free oil comes from discrete discontinuous deposits localized on the surface of the powder particles, constituting the surface oil [20, 21]. However, part of the free oil is also formed from droplets localized in the vicinity of the particle surface that are accessible to the extraction solvent. These keep their structure and are completely surrounded by the encapsulation matrix. This more internal subfraction may oxidize following a pattern characteristic of a discontinuous phase.

Compared to the free fraction, polymerization in the encapsulated FAME occurred at early stages of oxidation (**Fig. 4**). This early polymerization is in agreement with results of previous studies [14]. Even though the total content of the oxidation products increased much more quickly in the free fraction of dried microencapsulated oils along storage, polymerization was detected at quite earlier stages in the encapsulated oil. It was suggested that a subfraction of the encapsulated oil was very susceptible to lipid oxidation and oxidized much more quickly than the free oil.

241 With regard to the higher contents of hydroxy- than keto- dienes found in the lipid
242 extracts, the results suggest that these differences could be related to an effect of the
243 encapsulation matrix, acting as a potent donor of hydrogen or as a reducing agent of
244 hydroperoxides. Due to its emulsifying properties and so its elevated concentration in
245 the interface oil-matrix, it seems reasonable to think that sodium caseinate could be
246 involved in such a hypothetical chemical effect of the encapsulation wall. In this regard,
247 caseins have been reported to show both antioxidant and free radical scavenging
248 activities in liquid systems [22]. Various types of bovine caseins inhibited oxidation of
249 linoleic acid in emulsion. The oxidation was induced with the radical initiator 2,2'-
250 azobis(2-amidinopropane) (AAPH) and inhibition was measured by oxygen
251 consumption. The authors suggested that caseins are preferred targets for free radical
252 intermediates over the lipid substrate. The same authors found that caseins were also
253 capable of quenching the nitrogen-centered radical 1,1-diphenyl-2-picryl-hydrazyl
254 (DPPH), showing free radical scavenging properties. Hidalgo et al. [23] have also
255 observed radical scavenging activity on DPPH in hydrolysates of bovine sodium
256 caseinate, showing antioxidant properties of its peptides. The amino acids tyrosine,
257 phenylalanine, tryptophan, proline, methionine, lysine and histidine have been reported
258 to be antioxidative [24]. Therefore, there are evidences on the capability of caseins to
259 donate hydrogen radicals. This hypothetical effect of the matrix did not seem to alter the
260 relative distributions of the isomers of hydroperoxydienes (**Fig. 1**), nor that of their
261 derived compounds, keto- and hydroxy- dienes (not shown). The chromatograms
262 showed a pattern characteristic of uninhibited oxidation. The isomerization of the forms
263 *cis*,*trans* or *trans*,*cis* to *trans*,*trans* was not inhibited by a potent hydrogen donor [25]
264 and the compounds with a *trans*,*trans* configuration were somewhat more abundant as a
265 result [7].

The higher contents of hydroxy- compared to keto- dienes in the microencapsulated FAME could also be explained by a direct reduction of hydroperoxides to hydroxides. In fact, hydroxydienes were detected in both lipid fractions at early stages of oxidation (**Fig. 3**). In this respect, methionine and cysteine residues in proteins, including casein, have shown peroxide scavenging activities in aqueous systems and it has been suggested that lipid hydroperoxides can be transformed into the corresponding hydroxides through a two-electron reduction [26-29]. In contrast to the hypothetical role of the protein acting as a potent hydrogen donor, formation of hydroxides by direct reduction of hydroperoxides would explain the fact that the geometrical isomers of hydroperoxydienes and those of their derived compounds were those characteristic of uninhibited oxidation. In addition, such formation of hydroxides is not in contrast to the assumption that the larger contents of degradation products from hydroperoxides found in the microencapsulated sample can be attributed to the discontinuous nature of lipid oxidation in these systems. Reduction of hydroperoxides to relatively more stable compounds would not give rise to an increase in the global oxidation rate nor in the formation of compounds of advanced oxidation, i.e. polymers.

Conclusions

The results of this study evidence the importance of applying complementary analytical methods to those assessing the primary oxidation products only to evaluate lipid oxidation in dried microencapsulated lipids or other foods in which the lipids constitute a disperse phase. Substantial formation of secondary oxidation products and even polymerization compounds can occur even when the level of global oxidation is low. The oxidative degradation of certain droplets or minor lipid fractions could help explain the development of rancidity in foods presenting quite low contents of hydroperoxides.

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Figure legends

Fig. 1. HPLC chromatograms registered at 234 and 268 nm of oxidized neat FAME (**a**) and the free (**b**) and encapsulated (**c**) fractions of oxidized microencapsulated FAME.

Fig. 2. Formation of hydroperoxy-, keto- and hydroxy- dienes in neat FAME (circles) and the free (squares) and encapsulated (triangles) fractions of microencapsulated FAME. Error bars express standard deviation.

Fig. 3. Relationships of keto- and hydroxy- with hydroperoxy- dienes in neat FAME (circles) and the free (squares) and encapsulated (triangles) fractions of microencapsulated FAME. Error bars express standard deviation.

Fig. 4. Relationship between polymers and hydroperoxydienes in neat FAME (circles) and the free (squares) and encapsulated (triangles) fractions of microencapsulated FAME. Error bars express standard deviation. Error bars express standard deviation.

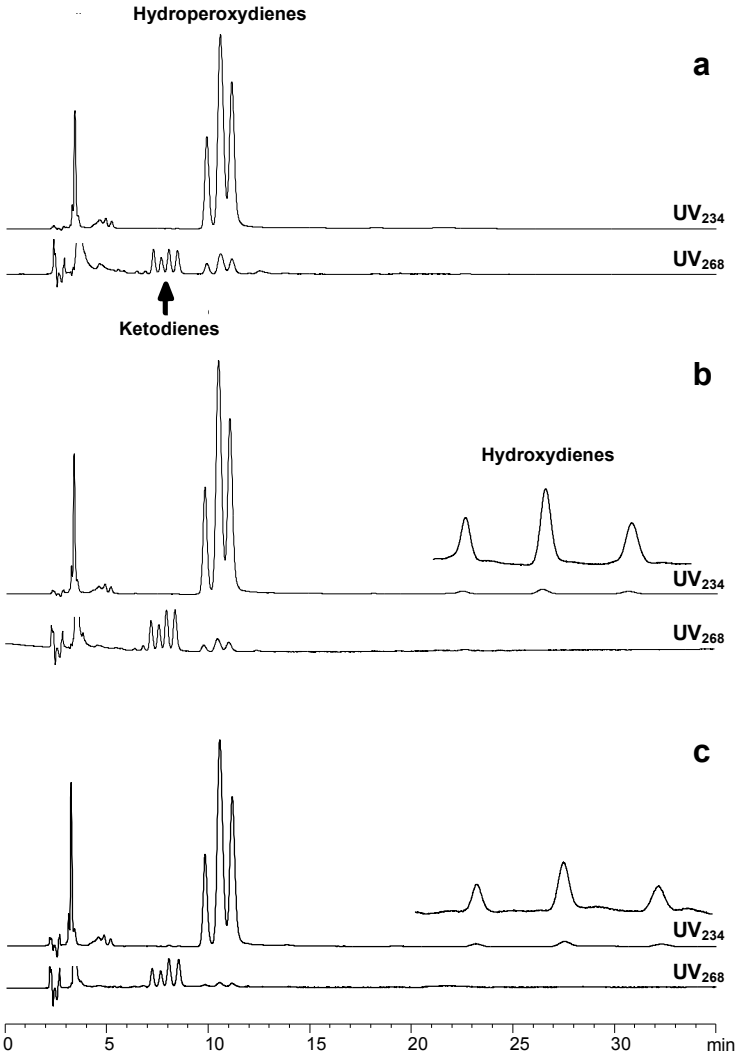
Hydroperoxydienes
a
UV234
UV268
Ketodienes
b
Hydroxydienes
UV234
UV268
c
UV234
UV268
0
5
10
15
20
25
30
min

Figure 1

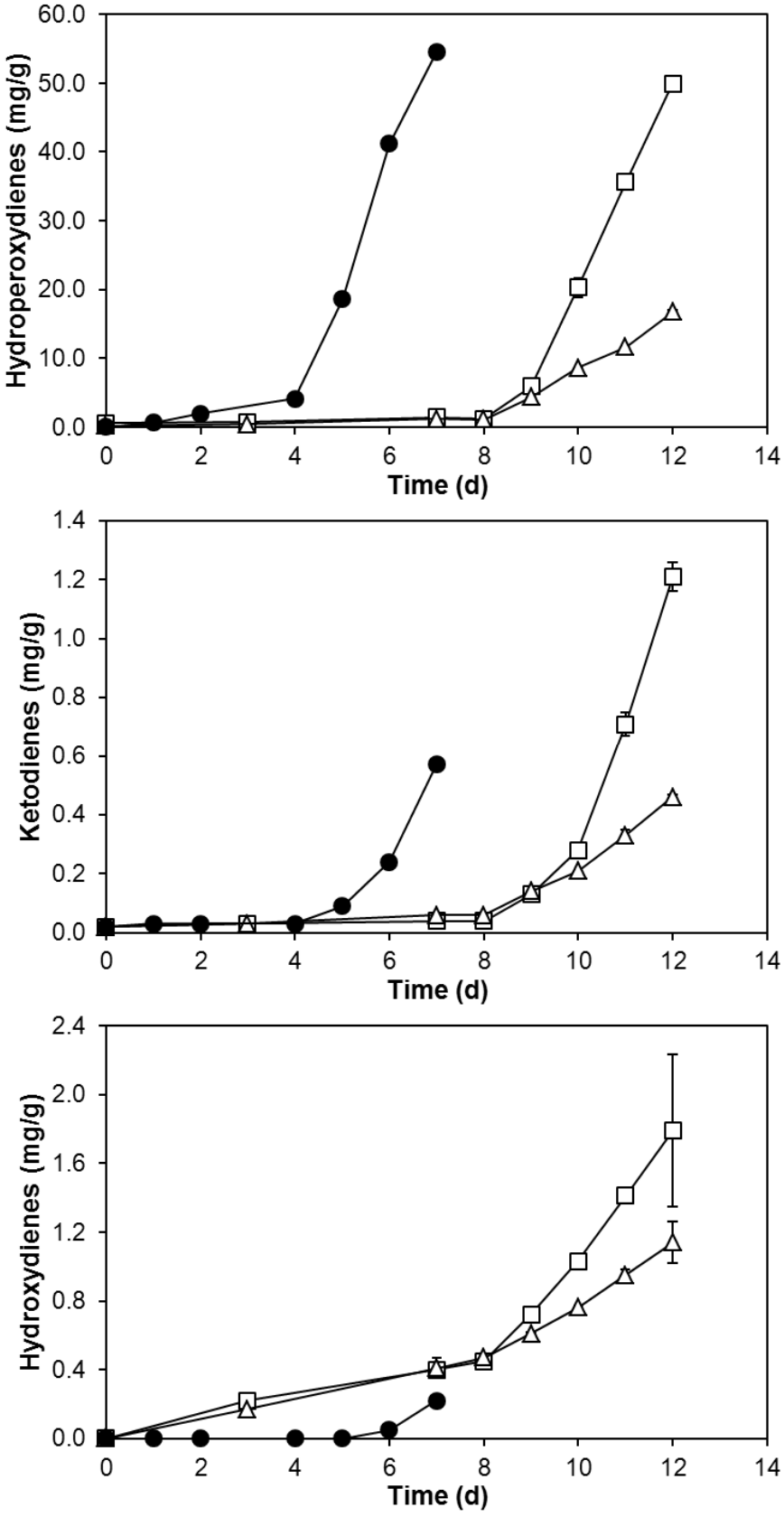
Hydroperoxydienes (mg/g)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
2
4
6
8
10
12
14
Time (d)
Ketodienes (mg/g)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
Time (d)
Hydroxydienes (mg/g)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0
2
4
6
8
10
12
14
Time (d)

Figure 2

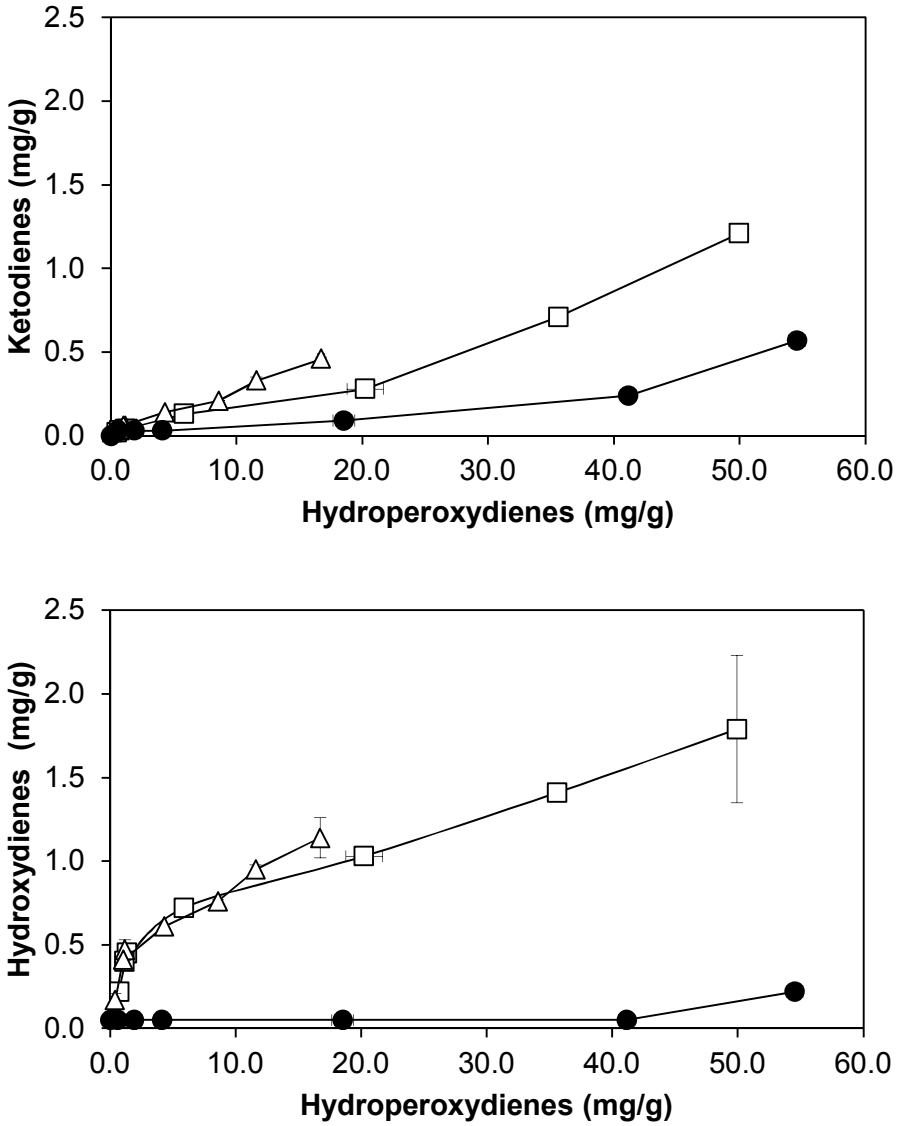
Ketodienes (mg/g)
Hydroperoxydienes (mg/g)
Hydroxydienes (mg/g)
Hydroperoxydienes (mg/g)
0.0
0.5
1.0
1.5
2.0
2.5
0.0
10.0
20.0
30.0
40.0
50.0
60.0

Figure 3

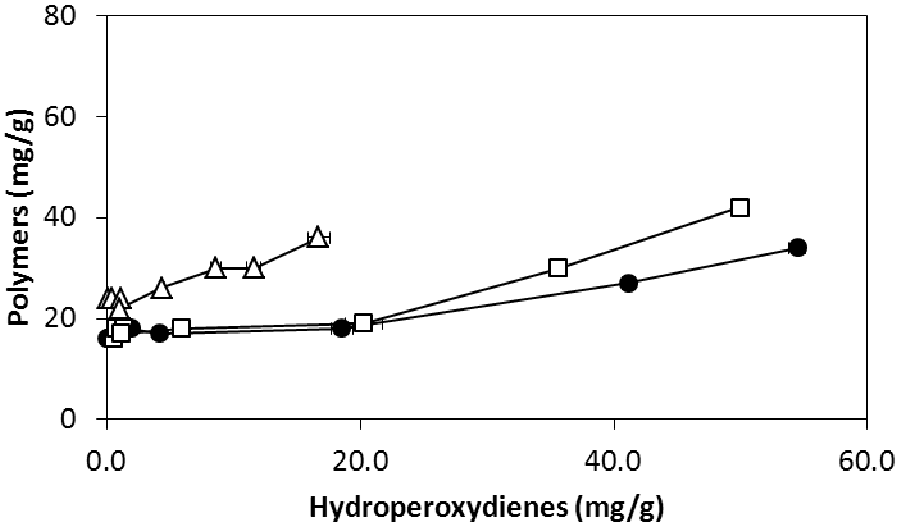
80
60
40
20
0
Polymers (mg/g)
0.0
20.0
40.0
60.0
Hydroperoxydienes (mg/g)

Figure 4