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Antioxidant activity of alkyl hydroxytyrosyl ethers in unsaturated lipids

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1 **ABSTRACT**

2 Antioxidant activity of ethyl and octyl hydroxytyrosyl ethers on lipids was
3 determined using Rancimat and open cup methods, at high temperature and 50 ºC,
4 respectively. The effect of the unsaturation of the matrix was evaluated using
5 sunflower, soya, and fish refined oils. The antioxidant activities of alkyl hydroxytyrosyl
6 ethers (HTy ethers), hydroxytyrosyl esters, and free hydroxytyrosol are similar and well
7 above than α-tocopherol at the same millimolar concentration. The relationship
8 between the induction period and HTy ethers concentration is a sigmoidal curve, being
9 necessary an accurate concentration of HTy ethers to achieve the maximum activity, as
10 higher as greater is the matrix unsaturation. The presence of tocopherols in the
11 commercial oils affects the antioxidant effect of HTy ethers. Thus, the addition of low
12 HTy ether concentration results in a positive effect whereas the effect of the addition
13 of high amounts of ethers is slightly lesser than that due to only phenol. The addition
14 of HTy ethers to commercial refined oils increases oil stability and preserves
15 tocopherols and polyunsaturated fatty acids from oxidation, maintaining their
16 nutritional properties for longer.

17

18 **KEYWORDS:** *hydroxytyrosol; lipophilic ether derivatives; antioxidant activity;*
19 *unsaturated edible oils*

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21 **1. INTRODUCTION**

22 For many years it is well known that 2-(3,4-dihydroxyphenyl)ethanol
23 (hydroxytyrosol, HTy, **1**) (Figure 1) and its ester derivatives are the main responsible for
24 the high oxidative stability of olive oils.[1] These esters are mainly hydroxytyrosyl acetate
25 (**2a**), aldehydic forms of oleuropein aglycone, and dialdehydic form of
26 decarboxymethyl oleuropein aglycone.[2-4]

27 It has been also shown that HTy and derivatives containing the *ortho*-cathecol
28 moiety have higher antioxidant activity than other phenols commonly used during the
29 conservation of oils and fats, such as butylated hydroxytoluene (BHT) or α-
30 tocopherol.[5,6] Besides, HTy and its acetate shows widely-ranged biological activities as
31 well as health and disease prevention effects in nutritional, cardiovascular,
32 neuroprotective, oxidative, cancerous and immunological aspects, among others.[7-12]

33 The food industry is undergoing new pressures due to the widespread concern
34 about the use of synthetic additives. Furthermore, there is a growing demand for new
35 antioxidants of natural origin or obtained by simple modification of natural products,
36 showing functional properties and without extraneous odor or taste. Besides, the
37 affordable recovery of hydroxytyrosol from olive oil waste water,[13-16] have focused
38 attention on the use of HTy as an alternative to synthetic antioxidants[17]. In fact, HTy
39 has already been used as an additive in tomato juice[18] and fish products.[19]

40 However, the highly polar nature of HTy reduces its solubility in lipids and thus
41 an effort in the synthesis of hydroxytyrosyl derivatives with a better
42 hydrophilic/lipophilic balance has been carried out for their possible use in the
43 protection of fatty foods against oxidation. In this way, the syntheses of isochromans[20-
44 23] and lipophilic esters of the alcoholic group have been published.[24-26] The
45 hydroxytyrosyl esters of fatty acids have shown similar antioxidant activity with
46 respect to hydroxytyrosol itself.[25,26] The acetylation of the primary alcoholic group of
47 HTy significantly increases its transport across the small intestinal epithelial barrier
48 enhancing its bioavailability. Nevertheless, certain lability of the ester function after
49 ingest has been found, since it was largely transformed into free HTy, in the intestinal
50 tract.[27]

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51 To avoid this hydrolysis process, a series of lipophilic alkyl hydroxytyrosyl ethers
52 (HTy ethers, **3**) (Figure 1) with linear alkyl side chains of variable length have been
53 synthesized from HTy.[28] The antioxidant activities of these compounds were evaluated
54 by several methods,[29] and again, a similar behavior with respect to HTy was found.
55 Moreover, these HTy ethers derivatives are rapidly absorbed through the intestinal
56 tract, and partially metabolized by Caco-2/TC7 cell monolayers, in keeping with their
57 lipophilic nature.[30] Studies with human hepatoma HepG2-cells indicate that HTy ethers
58 are extensively metabolized by the liver, being their metabolic rate higher in the more
59 lipophilic compounds.[31]

60 Concerning biological activities, HTy ethers inhibit platelet aggregation in a
61 concentration-dependent manner, although no linear relationship between this effect
62 and the alkyl chain length was found.[32] The effects were greater than that of free HTy
63 and a maximum effect was observed in the C4–C6 range.[32,33] In addition, HTy ethers
64 showed neuroprotective, cytoprotective, and antioxidant effects in rat brain slices
65 subjected to a hypoxia-reoxygenation model, being the maximum effect in the range
66 C4-C8 of the alkyl chain length.[34,35] Finally, the selective cytotoxic activity of HTy and
67 their ethers derivatives against A549 lung cancer cells and MRC5 nonmalignant lung
68 fibroblasts was recently investigated. As a result, the C12 derivative showed the
69 highest selective cytotoxicity as compared with free HTy.[36]

70 In this work, the effect of the unsaturation of lipid matrices (from sunflower,
71 soya, and fish oils) on the antioxidant activity of alkyl hydroxytyrosyl ethers is studied
72 by the Rancimat method. The protective effect of the addition of these ethers to
73 refined sunflower, soya and fish oils during their storage at 50 ^{o}C also is accomplished,
74 in order to know their availability as additive antioxidants for commercial fats and oils.
75 For this purpose, sunflower and soya oils, with similar unsaturation level but different
76 fatty acid composition, and fish oil with high unsaturation level have been chosen.
77 Primary and secondary oil oxidations have been determined by peroxide and anisidine
78 values, respectively.

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81 **2. MATERIALS AND METHODS**

82 **2.1. Materials**

83 All solvents and reagents were of analytical grade unless otherwise stated. HTy
84 was recovered with 95% purity from olive oil wastewaters by a procedure protect
85 under patent.[13] HTy acetate and octanoate (**2a** and **2b**) were obtained from free HTy
86 according to the procedure previously described[24,25] using ethyl acetate and methyl
87 octanoate as acylating agents in presence of *p*-toluensulfonic acid as catalyst. Ethyl and
88 octyl HTy ethers (**3a** and **3b**) were obtained from the 3,4-dibenzyl derivative of HTy by
89 alkylation of the primary alcoholic group with ethyl and octyl iodides, respectively, and
90 further transformed into the corresponding HTy ethers by hydrogenolytic cleavage of
91 the protecting benzyl groups.[28] α-Tocopherol and 3-(4-hydroxyphenyl)-1-propanol
92 were from Aldrich (Steinheim, Germany).

93 **2.2. Oils and Lipid Matrices**

94 Refined sunflower and soya oils were obtained from refining industries. Refined
95 fish oil was Menhaden fish oil without tocopherols from Sigma (Madrid, Spain). Lipid
96 matrices were obtained from refined oils by purification through alumina according to
97 the "free solvent" procedure.[37] Briefly, 200 g of oil were poured into a glass
98 chromatography column (45 x 3 cm id) packed with 100 g of alumina activated at 200
99 ^{o}C during 3 h. The purified matrices free of antioxidants were stored at –18 ^{o}C under
100 nitrogen. The fatty acid composition of lipid matrices obtained by purification of
101 sunflower, soya and fish oils are shown in Table 1.

102 **2.3. Oil Storage at 50 ^{o}C**

103 For oil storage studies, refined sunflower, soya, and fish oils were used. Spiked
104 oils samples were prepared by addition of adequate amounts of HTy ethers solutions
105 in methanol and of α-tocopherol in hexane. Solvents were evaporated in a rotary
106 evaporator under reduced pressure at room temperature. Aliquots (2.5 g) of each lot
107 were poured into open 3.5 mL-volume glass tubes and then, put into an air-forced
108 oven maintained at 50±1 ^{o}C.

109 **2.4. Oil Oxidation by Rancimat**

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110 A) Purified matrices from sunflower, soya, and fish oils were spiked with 0.5 mM of
111 free HTy, HTy esters (acetate, **2a**, and octanoate, **2b**), and HTy ethers (ethyl, **3a**, and
112 octyl, **3b**). Samples with sunflower and soya as matrices were subjected to oxidation in
113 a Rancimat apparatus at 100±1 ºC. The samples containing fish oils were subjected to a
114 80±1 ºC and 50±1 ºC because at 100 ºC they are altered in a very short period of time (<
115 1h) being not possible to determine the differences between the treatments.

116 B) A set of samples were prepared by fortifying the purified matrices of sunflower,
117 soya and fish oils with increasing amounts of ethyl and octyl HTy ether, (**3a**, **3b**), with
118 and without 600 ppm of α-tocopherol. The samples were subjected to the action of
119 the oxygen at the temperatures fixed in the epigraph 2.4.A. These samples were
120 compared with the matrix fortified with α-tocopherol only.

121 For each time sampling from sections 2.3 and 2.4 three tubes were taken and
122 analyzed.

123 **2.5. Analytical Methods**

124 *Determination of Fatty Acid Composition.* The fatty acid composition of the oils was
125 determined by capillary GC analysis of the methyl esters obtained by
126 transesterification of the oils with KOH in methanol at room temperature.[38]

127 *Oxidative Stability by the Rancimat Method.* The oxidative stability of lipid matrices
128 was evaluated by an accelerated test using a Rancimat apparatus (Model 743,
129 Metrohm Co. Basel, Switzerland). A flow of air (15 $L \cdot h^{-1}$) was bubbled successively
130 through the heated oil and cold water. In this process, the volatile oxidation products
131 were stripped from the oil and dissolved in the water, increasing the water
132 conductivity. The time taken until a sharp increase of conductivity occurs is termed
133 induction time (IT) and is expressed in hours.

134 *Iodine value.* It was determined by the Wijs' method.[39] A solution of oil in cyclohexane-
135 glacial acetic acid (1:1) reacts with the Wijs regent (iodine monochloride in acetic acid).
136 After a specified time, potassium iodide and water are added and the liberated iodine
137 titrated with sodium thiosulfate.

138 *Anisidine Value*. A solution of oil in isooctane reacts with a solution of *p*-anisidine in
139 glacial acetic acid (0.25% w/v), and the absorbance at 350 nm is determined.[40]

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Peroxide Value. A solution of oil in chloroform is mixed with glacial acetic acid and, then, a solution of aqueous potassium iodide is added. The liberated iodine is titrated with a standardized sodium thiosulphate solution (0.002 N) using a starch solution as indicator.[41]

Tocopherols. A solution of oil in hexane is analyzed by NP-HPLC-FL on silica ~~gel~~ column using propan-2-ol in hexane (1% v/v) as mobile phase, and a fluorescence detector with excitation wavelength at 290 nm and emission at 330 nm.[42]

Determination of HTy ethers in Oils. For the determination of HTy ethers in lipids, a method based on the determination of phenolic compounds in olive oil by SPE and RP-HPLC-UV analysis were developed.[4] Due to alkyl hydroxytyrosyl ethers are more apolar than HTy derivatives present in olive oil, SPE-diol cartridges were substituted by SPE-NH_2; in this way, ethers were more retained and cartridges washed with more polar solvents. As internal standard, 3-(4-hydroxyphenyl)-1-propanol was used instead of 3,4-dihydroxyphenylacetic acid because of the latter is retained in the amino cartridge. For HPLC analysis, a binary mixture of water with 1% of phosphoric acid and methanol in gradient mode was employed, obtaining a good separation of ethyl and octyl HTy ethers. The analytical procedure is as follows: A SPE-NH_2 cartridge of 500 mg and 3 mL of volume (Supelclean, Supelco, Bellefonte, USA) is activated with 6 mL of methanol and subsequently with 6 mL of hexane. In a 25-mL flask, the oil sample (1 g) is weighted and 0.5 mL of a standard solution (0.5 mM) of 3-(4-hydroxyphenyl)-1-propanol in methanol is added. The mixture is evaporated at reduced pressure and the residue dissolved in 6 mL of hexane. This solution is passed through the SPE-NH_2 cartridge with the aid of vacuum. The flask containing the sample is washed twice with 3 mL of hexane, and the washings are also poured into the cartridge. Once the washings have been introduced into the cartridge, the flask is cleaned with 4 mL of the admixture hexane/dichloromethane 95:5 (v/v) followed by two portions of 6 mL of hexane/diethyl ether 70:30 (v/v), and the solutions are passed consecutively through the cartridge. Then, a 10-mL conic tube is placed under the cartridge to recover the elution with 5 mL of chloroform/methanol 2:1 (v/v). The obtained solution containing HTy ethers is evaporated with the aid of a nitrogen stream. The residue is redissolved in 0.5 mL of ethanol and filtered with a 0.45 µm PTFE membrane filter. An aliquot of 20

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171 µL is injected in the liquid chromatograph. The HPLC analysis was carried out on an
172 Agilent 1100 liquid chromatographic system (Agilent, Stockport, UK), equipped with a
173 RP-C18 column (Lichrospher 100RP-18, 4.0 mm of i.d. x 250 mm length, particle size 5
174 µm, Merck, Darmstadt, Germany), using a binary admixture of water/phosphoric acid
175 (99.5:0.5, v/v) (solvent A) and methanol (solvent B) as mobile phase, at a flow rate of
176 0.8 $mL \cdot min^{-1}$, and 30 ºC. Solvent gradient changed according to the following
177 conditions: from 95% A to 50% A in 5 min; 25% A in 5 min; 20% A in 5 min; 5% A in 5
178 min; and 0% A in 5 min, followed by 5 min of maintenance and 95% A in 5 min.
179 Chromatograms were acquired at 280 nm. Calibration curves were determined using
180 solutions of each ether at concentrations 0.1, 0.25, 0.5, and 1.0 mM, and containing
181 internal standard at 0.5 mM. For the calculation of the recovery, sunflower oil matrices
182 with 0.2, 0.5, 1.0 and 5 mM of the HTy ethers were analyzed by triplicate using the
183 method above described.

184 All results are expressed as mean value (n=3) ± standard deviation (SD) and the
185 program SPSS Statistic v22.0 has been used for the study of the results.

186

187 **3. RESULTS AND DISCUSSION**

188 **3.1. Unsaturation rate of matrices**

189 Iodine values of the matrices were similar for sunflower and soybean oils and
190 rather higher for fish oil (Table 1).

191 **3.2. Influence of the Matrix on the Antioxidant Activity of HTy Derivatives**

192 For testing the effect of the matrix unsaturation, purified matrices from
193 sunflower, soya, and fish oils were spiked with the phenolic compounds assayed [free
194 HTy, HTy esters (**2a**, and **2b**), and HTy ethers (**3a**, and **3b**)]. The samples were subjected
195 to alteration at high temperature in a Rancimat apparatus. As expected, in each
196 different matrix subjected to oxidation, HTy derivatives showed similar antioxidant
197 activity per millimole of substance (Table 2).

198 In the assays, the matrices spiked with increasing amounts of **3a** and **3b**, with
199 and without 600 ppm of α-tocopherol, were compared with those containing α-

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200 tocopherol only. It can be seen (Figures 2-4) that the antioxidant effect of HTy ethers
201 has a sigmoidal behavior with the increase of concentration as previously observed in
202 sunflower oil.[29] Nevertheless, the maximum antioxidant activity is reached at different
203 phenol concentration in each matrix. Thus, the values are 2.5 mM in sunflower, 4 mM
204 in soya, and 15 mM in fish. These results indicate that antioxidant effect does not only
205 depend of the unsaturation rate but is also affected by the nature of the fatty acids. In
206 all the cases, the activity of hydroxytyrosyl ethers is higher than that of α-tocopherol at
207 any concentration.

208 As refined vegetable oils commercially available contain tocopherols, their
209 effect on the antioxidant activity of HTy ethers has also been studied in matrices
210 spiked with 600 ppm (1.4 mM) of α-tocopherol together with the phenols. Results
211 (Figures 2-4) showed opposite effects depending on the phenol concentration. Thus,
212 an increase of stability is observed at low concentrations of phenol, whereas a
213 decrease occurs at high ones, in comparison with the phenol only. Similar facts were
214 also observed in olive oil spiked with HTy.[6] The negative effect of tocopherol happens
215 from about 0.7, 0.7, and 0.1 mM of phenols for sunflower, soya, and fish oils,
216 respectively. Consequently, although the antioxidant effect of HTy ethers addition to
217 commercial oils is always positive, its magnitude will depend on the concentration of
218 both antioxidants and the unsaturation of the lipid matrix.

219

220 **3.3. Evolution of Oil Quality Parameters in Sunflower and Soya Oils Spiked with HTy**
221 **ethers, at 50 ^{o}C in open cups**

222 Once it has been studied the antioxidant effect of HTy ethers in lipid matrices at
223 high temperature, the evolution of oil quality parameters have been tested in refined
224 sunflower and soya oils. These commercial oils were spiked with low concentration
225 (0.5 mM) of ethyl (**3a**) and octyl (**3b**) hydroxytyrosyl ethers, and maintained at 50 ^{o}C in
226 open cups, kept out of light. Using this concentration of phenolic compounds, the
227 tocopherols have a slight positive effect as above mentioned (see Figures 2-3).

228 Anisidine value has been the parameter used to evaluate the oxidation level of
229 oils since alken-2-als are produced in the secondary oxidation of fatty chains. In

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230 sunflower oil containing 869 and 44 ppm of α- and γ- tocopherols, respectively,
231 without any HTy derivative, a slight increase of anisidine value is observed at 45 days
232 of storage, and a very sharp rise at 60 days (Figure 5). However, the presence of 0.5
233 mM HTy ethers maintains a low level of oxidation until 60 days. Alike, in soya oil
234 containing 145, 842, and 252 ppm of α-, γ-, and δ-tocopherols, respectively, the slight
235 increase of anisidine value is observed until 60 days of storage, and the sharp rise
236 occurred at 75 days, while the presence of 0.5 mM HTy ethers keeps a low level of
237 oxidation during 60 days (Figure 6). The anisidine value lower in soya than in sunflower
238 oils could be attributed to the higher tocopherol concentrations and the major activity
239 of γ- and δ-forms.[42] The behavior of ethyl and octyl ethers was similar in both oils.

240 **3.4. Evolution of Quality Parameters in Fish Oil Spiked with Ethyl Hydroxytyrosyl**
241 **Ether, at 50 °C in open cups**

242 From Figure 4**,** it is deduced that high concentrations of α-tocopherol are
243 necessary to produce a noticeable antioxidant effect in fish oil and are similar for 4.5
244 mM of α-tocopherol and 1 mM of hydroxytyrosyl ethers. Consequently, refined fish
245 oils spiked with these antioxidants and the mixture of both were maintained in open
246 cups at 50 °C and the oxidation parameters evaluated in comparison with non spiked
247 oil. From the first week, the peroxide value (PV) showed a great increase in all samples,
248 and differences among them at each time were small (Figure 7). These results indicate
249 that primary oxidation is scarcely affected by the presence of antioxidants at these
250 concentrations.

251 On the other hand, in unprotected oil the anisidine value increases rapidly from
252 the first week, while the addition of 1 mM of ethyl hydroxytyrosyl ether or 4.5 mM of
253 α-tocopherol allow to maintain low levels for the anisidine value until the fourth week
254 (Figure 8). The joint addition of both antioxidants to fish oil has an additional positive
255 effect on the anisidine value from the fifth week. These facts would suggest that low
256 concentrations of HTy ethers prevent secondary oxidation like higher concentrations
257 of α-tocopherol does, and that the admixture of both antioxidants is lightly more
258 effective only after long storage times.

259 Fatty acid compositions of fish oil and fish oil spiked with antioxidants along the
260 induction period are shown in Table 3. After 30 days of storage, fish oil showed a

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261 significant decrease of polyunsaturated acids (mainly EPA, 20:5 and DHA, 22:6), while
262 in all the spiked matrices only a small diminution was observed. After 45 days of
263 storage, all of the oils showed a decrease of polyunsaturated fatty acid, being the
264 decrease similar in the tree spiked matrices and lesser than in unprotected oil. These
265 results are in agreement with those of anisidine value since low concentration of the
266 ethyl hydroxytyrosyl ether has similar activity than high one of α-tocopherol.

267 **3.5. Evolution of the Concentration of Tocopherols during Commercial Oils Oxidation**
268 **at 50 ºC**

269 The evolution of tocopherols in commercial refined oils during the storage at 50
270 ºC is affected by the presence of hydroxytyrosyl ethers. In refined sunflower and soya
271 oils spiked with 0.5 mM of hydroxytyrosyl ethers, the concentrations of tocopherols
272 decreased more slowly than in unspiked oils. Thus, after 8 weeks, α- and γ-tocopherols
273 disappeared in sunflower oil (Figure 9), whereas in soya oil, α-tocopherol disappeared,
274 γ- diminished, and δ-tocopherol remained virtually intact (Figures 10). Differences
275 between ethyl and octyl ether effects were negligible. The evolution of α-tocopherol in
276 two fish oil samples, one spiked with 4.5 mM of α-tocopherol only and other with the
277 admixture of α-tocopherol (4.5 mM) with ethyl hydroxytyrosyl ether (1 mM), resulted
278 in a more rapid diminution that in the sample containing only tocopherol during the
279 first week. A decrease, at similar rate, along the second and third weeks down to
280 negligible concentration at the same time than the sample containing both
281 antioxidants (Figure 11). Therefore, in all matrices, the HTy ethers perform a protective
282 effect on tocopherol contents.

283 **3.6. Evolution of HTy ethers during Oxidation at 50 ºC**

284 Using the analytical procedure above described, recoveries higher than 85%
285 and good chromatographic isolation of HTy ethers were obtained.

286 In refined sunflower and soya oils spiked with 0.5 mM of ethyl and octyl
287 hydroxytyrosyl ethers, stored at 50 ºC, the concentrations of alkyl ethers decrease over
288 the time, being negligible after 60 days. At this time, sunflower oil does not contain
289 tocopherols (Figure 9), and anisidine value increases quickly (Figure 5). In soya oil,
290 significant amounts of tocopherols remain (Figure 10) and anisidine value increases

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291 more slowly (Figure 6). In refined fish oil spiked with 1 mM ethyl hydroxytyrosyl ether
292 and its admixture with 5 mM α-tocopherol, the phenol concentration decreases at the
293 same rate during 30 days of storage at 50 ºC. At this time, significant amount of α-
294 tocopherol remains in the oil spiked with the mixture (Figure 11) justifying the
295 difference of anisidine value between this sample and the oil spiked only with ethyl
296 hydroxytyrosyl ether (Figure 8).

297 To sum up, hydroxytyrosyl ethers show high antioxidant activity when it is
298 added to commercial oils and the concentration needed to obtain the maximum effect
299 depends on the unsaturation level, type of polyunsaturated fatty acid, and tocopherols
300 contents of the lipid matrix. Small amounts of HTy ethers perform a protective effect
301 on the tocopherols oil content and allow prolong the lifetime of these commodities. In
302 the case of fish oil, the addition of low concentrations of HTy ethers together with high
303 concentrations of α-tocopherol produces only a slight increase of stability compared
304 with the single phenol.

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308 **NOTES**

309 The authors declare no competing financial interest.

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TABLES

Table 1. Fatty acid composition and Iodine Value (IV) of glyceridic matrices used in Rancimat test.

Fatty acid composition (% on total FAME)			
	Sunflower	Soybean	Fish
C14:0	n.d.	n.d.	8.4±0.1
C16:0	5.8±0.1	10.8±0.1	19.2±0.4
C16:1	0.1±0.0	0.1±0.0	11.9±0.3
C18:0	3.7±0.1	4.2±0.1	3.6±0.2
C18:1	27.9±0.5	27.3±0.5	10.5±0.3
C18:2	62.4±0.7	51.7±0.7	1.8±0.0
C18:3	0.1±0.0	5.9±0.1	1.9±0.0
C18:4	n.d	n.d.	3.9±0.1
C20:4	n.d.	n.d.	1.9±0.0
C20:5	n.d.	n.d.	16.3±0.3
C22:5	n.d.	n.d.	2.8±0.1
C22:6	n.d.	n.d.	17.8±0.5
IV	130.9±3.5	127.3±4.1	158.1±4.2

n.d.: not detected
Data are expressed as mean (n=3) ±SD
FAME: Fatty acid methyl esters

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Table 2. Induction times (IT) of glyceridic matrices spiked with 0.5 mM of HTy and its derivatives, obtained by the Rancimat method.

	Induction Time (hours)			
Purified matrix	**Sunflower**	**Soya**	**Fish**	**Fish**
Temperature	**100 ºC**	**100 ºC**	**80 ºC**	**50 ºC**
Without phenol	1.4±0.1	1.3±0.1	2.0±0.1	6.4±0.1
Hydroxytyrosol (1)	13.1±0.3	9.4±0.2	3.1±0.1	36.0±0.4
HTy Acetate (2a)	12.1±0.2	8.4±0.1	2.8±0.1	35.2±0.4
HTy Octanoate (2b)	11.5±0.1	7.8±0.1	2.7±0.1	35.5±0.4
Ethyl HTy Ether (3a)	14.3±0.3	10.0±0.1	3.6±0.1	41.7±0.4
Octyl HTy Ether (3b)	13.5±0.2	8.9±0.1	3.4±0.1	39.1±0.3

Data are expressed as means (n=3) ±SD

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Table 3. Fatty acid composition of fish oil spiked with 1 mM ethyl hydroxytyrosyl ether (**3a**), 4.5 mM α-tocopherol (α-T) and mixture of both after 30 and 45 days of storage at 50 °C in open cups.

	Initial	Fish oil (FO)		FO + Ethyl HTy Ether 3a (1 mM)		FO + α-T (4.5 mM)		FO + Ethyl HTy Ether 3a (1 mM) + α-T (4.5 mM)	
Storage time (d)	**0**	**30**	**45**	**30**	**45**	**30**	**45**	**30**	**45**
14:0	8.4±0.1	10.1±0.1	11.2±0.1	8.6±0.1	9.7±0.1	8.8±0.1	8.2±0.1	8.9±0.1	10.1±0.1
16:0	19.2±0.4	22.0±0.3	25.2±0.4	19.9±0.1	23.1±0.2	20.0±0.1	19.0±0.4	19.8±0.2	23.2±0.3
16:1	11.9±0.3	13.7±0.2	15.0±0.2	12.4±0.1	13.9±0.2	12.4±0.4	11.4±0.1	12.5±0.1	13.9±0.1
18:0	3.6±0.2	4.0±0.1	4.7±0.1	3.7±0.1	4.3±0.1	3.8±0.1	3.4±0.1	3.9±0.1	4.2±0.1
18:1	10.5±0.3	12.2±0.2	13.3±0.2	10.6±0.1	12.3±0.1	10.3±0.1	10.3±0.2	10.5±0.1	12.3±0.1
18:2	1.8±0.0	2.0±0.1	1.9±0.0	1.8±0.0	1.9±0.0	1.9±0.0	1.6±0.0	1.9±0.0	1.9±0.0
18:3	1.9±0.0	1.9±0.0	1.7±0.0	1.8±0.0	1.8±0.0	1.7±0.0	1.4±0.0	1.9±0.0	1.7±0.0
18:4	3.9±0.1	3.5±0.1	2.9±0.1	3.7±0.1	3.4±0.1	3.7±0.1	2.7±0.1	3.6±0.04	3.4±0.1
20:4	1.9±0.0	1.7±0.0	1.6±0.0	1.8±0.0	1.7±0.0	1.7±0.0	1.4±0.0	1.7±0.0	1.7±0.0
20:5	16.3±0.3	12.4±0.1	10.7±0.1	15.8±0.1	12.7±0.1	15.8±0.1	10.3±0.1	15.9±0.1	12.6±0.1
22:5	2.8±0.1	2.4±0.1	1.9±0.0	2.7±0.1	2.3±0.1	2.6±0.1	2.0±0.1	2.5±0.1	2.2±0.0
22:6	17.8±0.5	14.1±0.3	9.9±0.1	17.2±0.3	12.9±0.1	17.6±0.4	10.6±0.1	16.8±0.2	12.8±0.1

* Data, expressed as % on total FAME, are means (n=3) ±SD

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FIGURE CAPTIONS

Figure 1. Chemical structures of hydroxytyrosol (HTy), hydroxytyrosyl esters (HTy esters), and hydroxytyrosyl ethers (HTy ethers).

Figure 2. Induction times (IT) of sunflower oil matrices spiked with alkyl hydroxytyrosyl ethers and α-tocopherol determined by the Rancimat method at 100 ^{o}C.

Figure 3. Induction times (IT) of soya oil matrices spiked with alkyl hydroxytyrosyl ethers and α-tocopherol determined by the Rancimat method at 100 ^{o}C.

Figure 4. Induction times (IT) of fish oil matrices spiked with alkyl hydroxytyrosyl ethers and α-tocopherol determined by the Rancimat method at 80 ^{o}C.

Figure 5. Evolution of anisidine value of refined sunflower oil (containing 869 ppm of α- and 44 ppm of γ-tocopherols) spiked with 0.5 mM of ethyl and octyl hydroxytyrosyl ethers, maintained at 50 ^{o}C in open cups.

Figure 6. Evolution of anisidine value of refined soya oil (containing 842 ppm of γ-, 252 ppm of δ- and 145 ppm of α-tocopherols) spiked with 0.5 mM of ethyl and octyl hydroxytyrosyl ethers, maintained at 50 ^{o}C in open cups.

Figure 7. Evolution of peroxide value (PV) in refined fish oil spiked with 1 mM ethyl hydroxytyrosyl ether and the mixture with 4.5 mM of α-tocopherol maintained at 50 ^{o}C in open cups.

Figure 8. Evolution of anisidine value in matrices of fish oil, spiked with 1 mM ethyl hydroxytyrosyl ether, 4.5 mM α-tocopherol, and the mixture of both, maintained at 50 ^{o}C in open cups.

Figure 9. Evolution of tocopherols in refined sunflower oil (containing 869 ppm of α- and 44 ppm of γ-tocopherols) spiked with 0.5 mM of ethyl and octyl hydroxytyrosyl ethers maintained at 50 ^{o}C in open cups.

Figure 10. Evolution of tocopherols in refined soya oil (containing 145 ppm α-, 842 ppm γ- and 252 ppm δ- tocopherols) spiked with 0.5 mM of ethyl and octyl hydroxytyrosyl ethers maintained at 50 ^{o}C in open cups.

Figure 11. Evolution of tocopherols in refined fish oil spiked with 4.5 mM α-tocopherol and the mixture 1 mM of ethyl hydroxytyrosyl ether and 4.5 mM of α-tocopherol, maintained at 50 ^{o}C in open cups.

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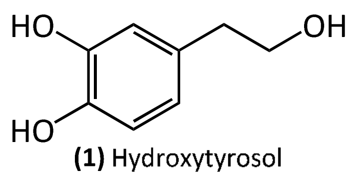
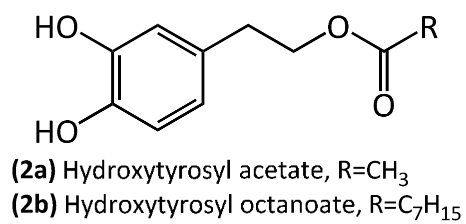
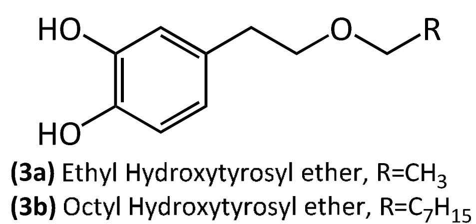
Figure 1



(1) Hydroxytyrosol



(2a) Hydroxytyrosyl acetate, R=CH_3
(2b) Hydroxytyrosyl octanoate, R=C_7H_{15}



(3a) Ethyl Hydroxytyrosyl ether, R=CH_3
(3b) Octyl Hydroxytyrosyl ether, R=C_7H_{15}

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Figure 2

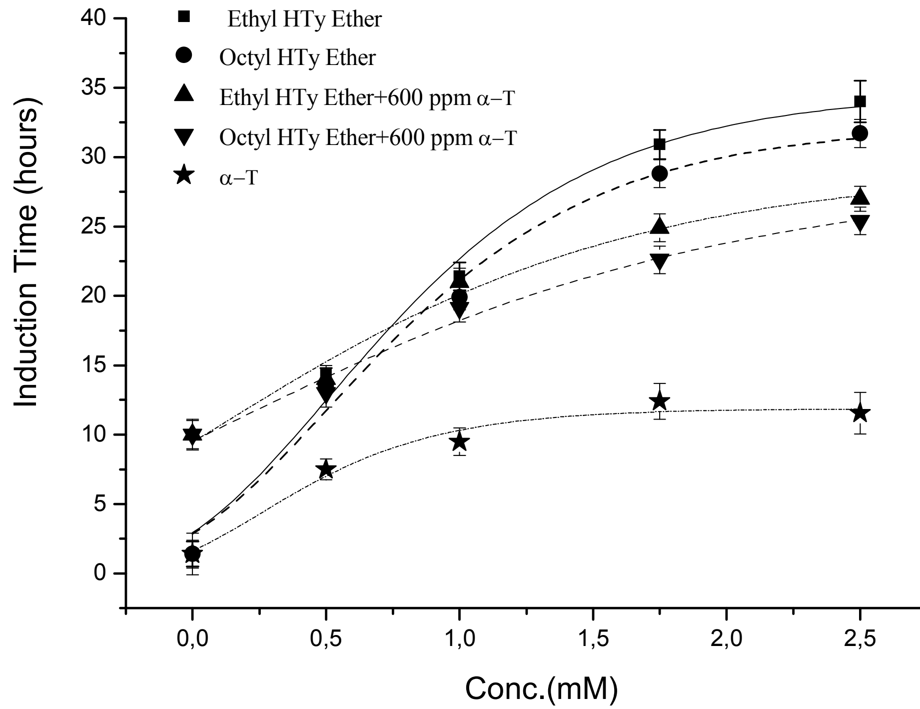

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Figure 3

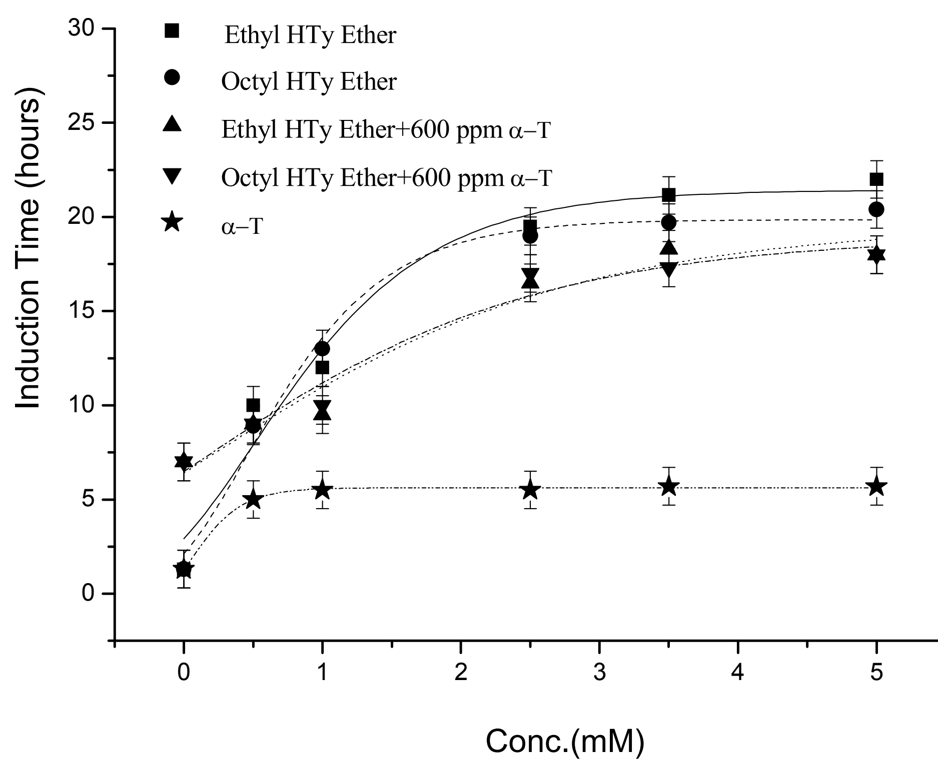

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Figure 4

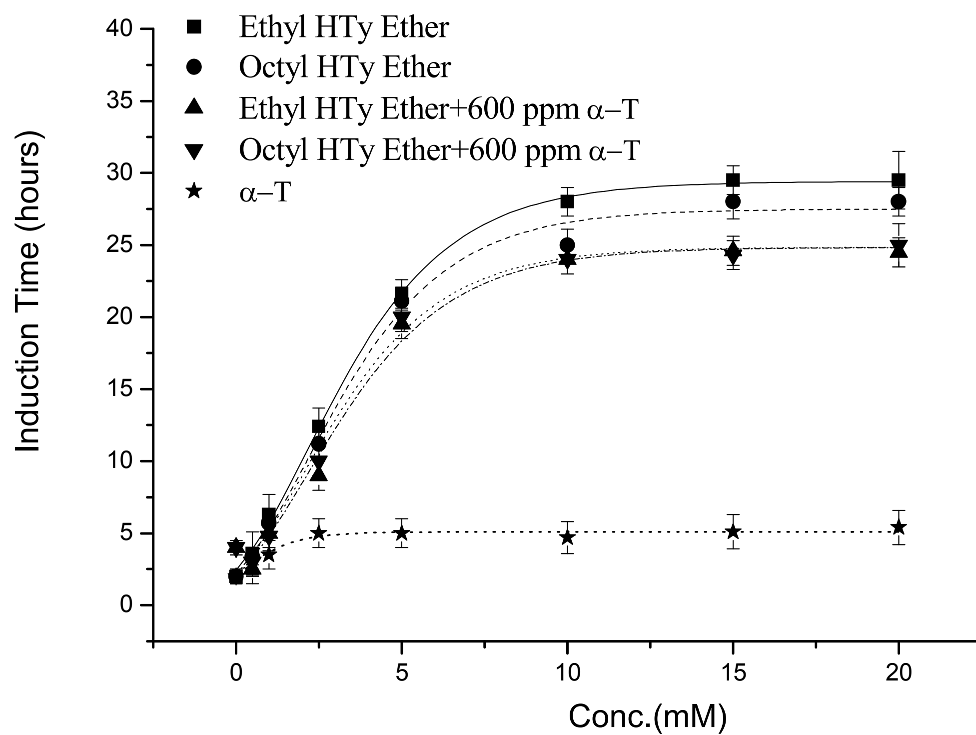

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Figure 5

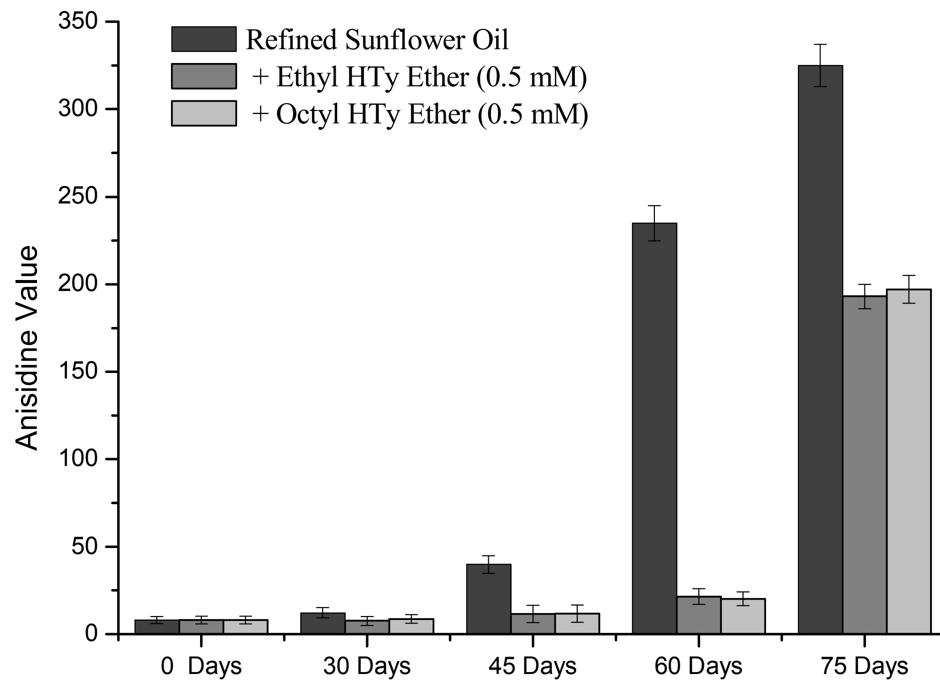

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Figure 6

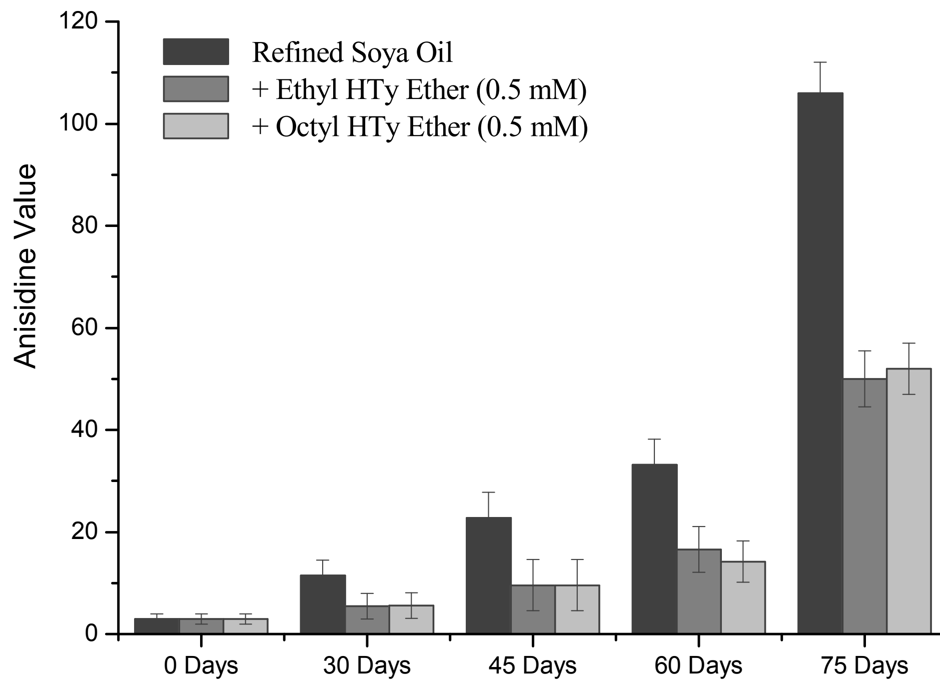

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Figure 7

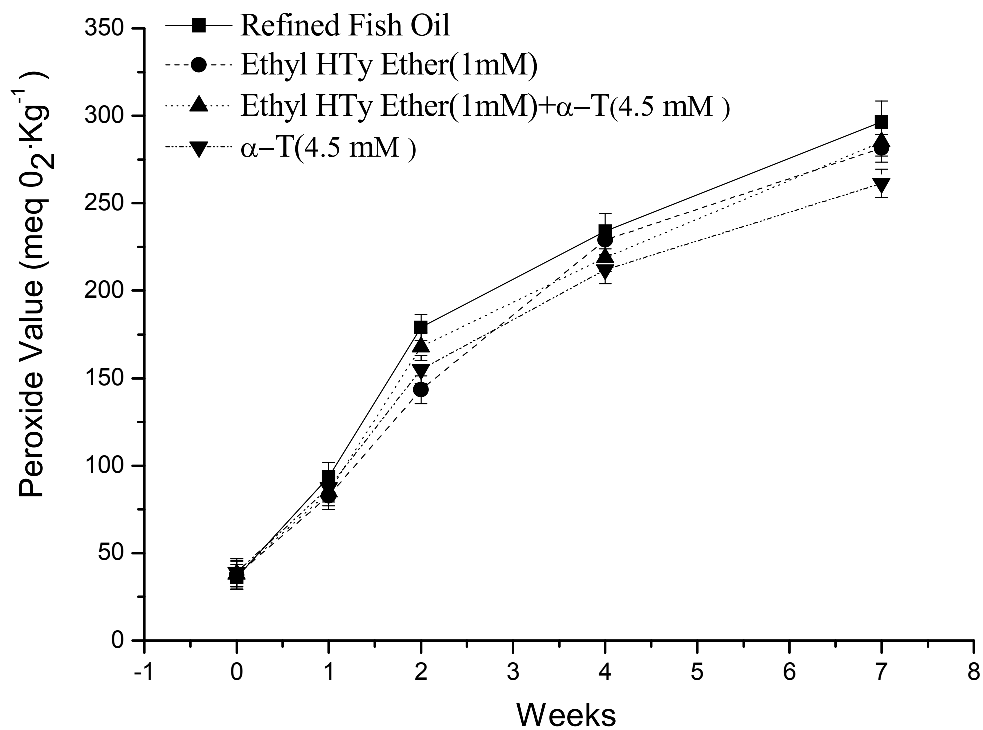

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Figure 8

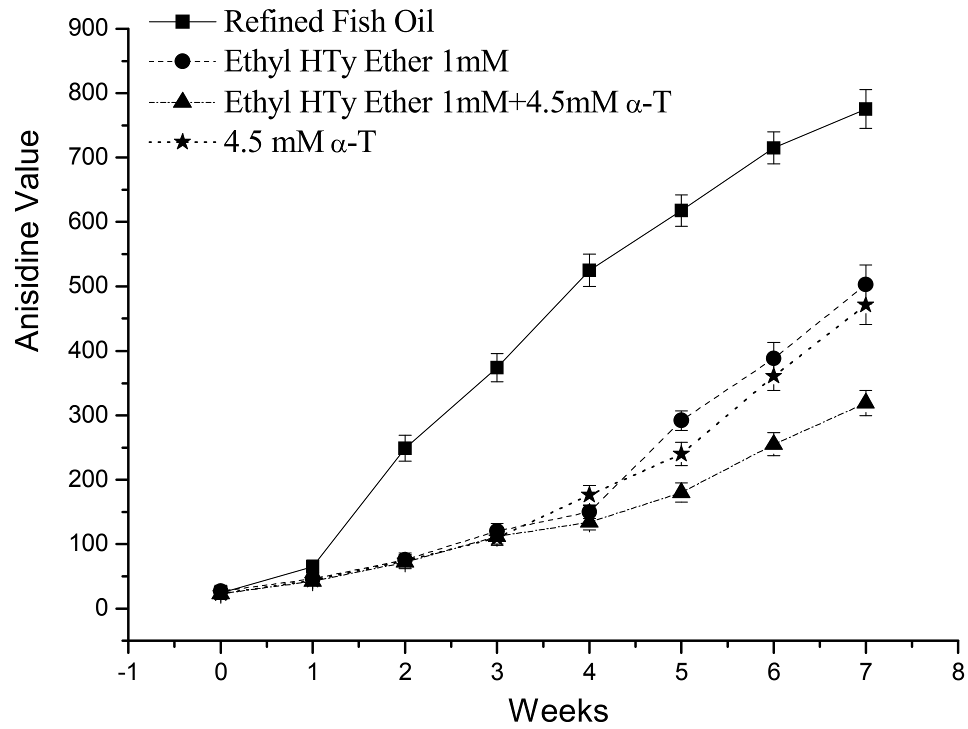

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Figure 9

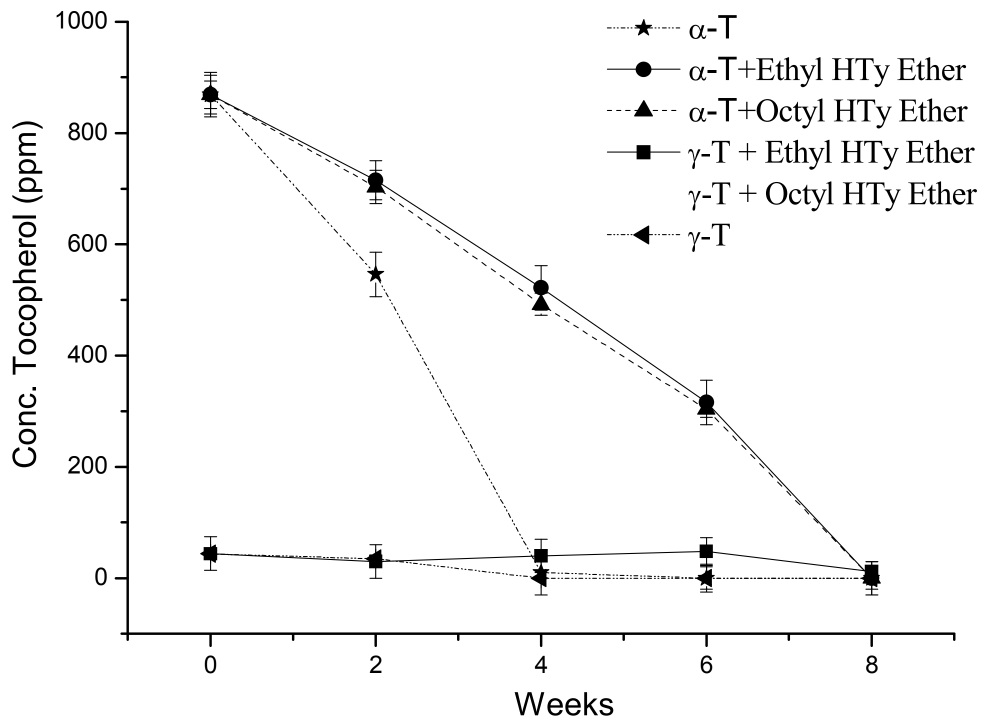

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Figure 10

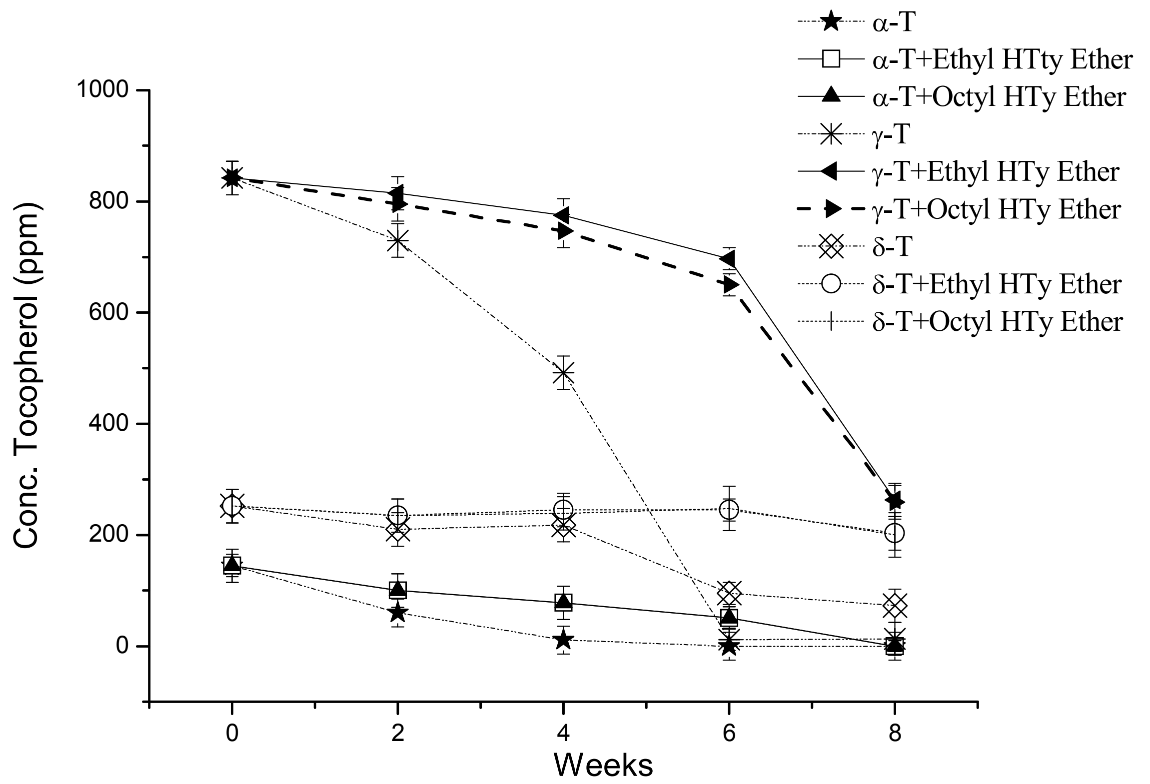

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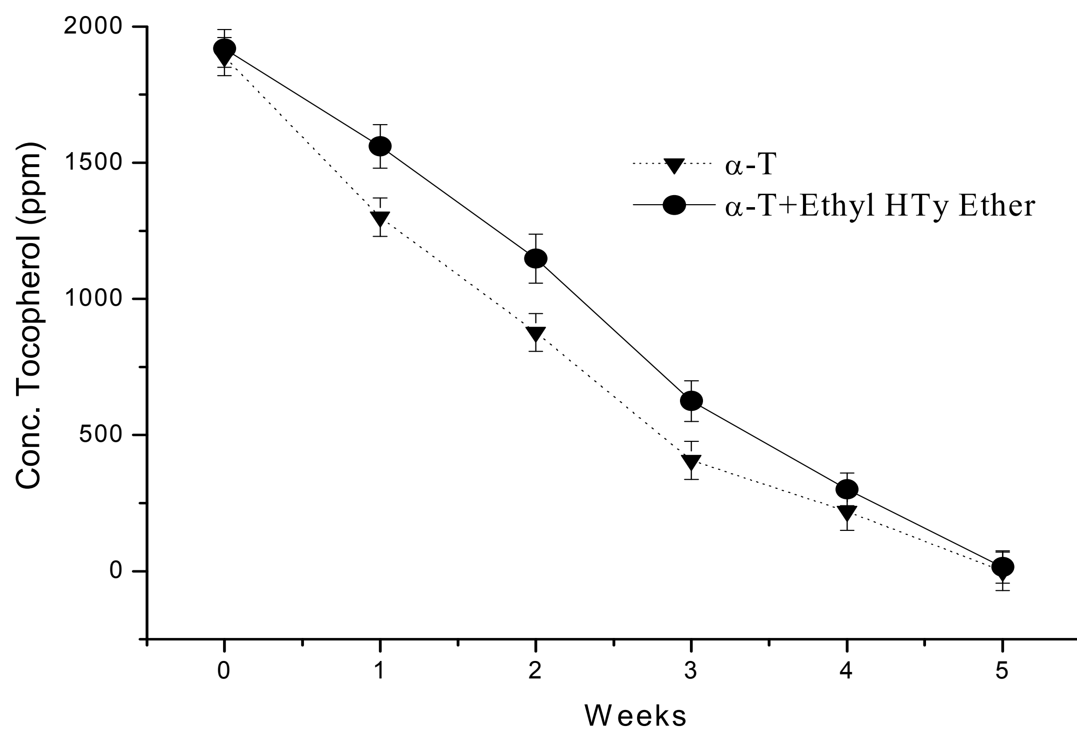
Figure 11



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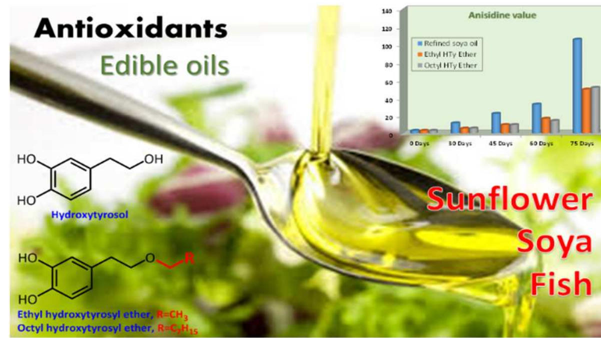
Graphic for table of contents



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