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1 **Synergic Antioxidant Activity of γ -Terpinene with Phenols and Polyphenols**
2 **Enabled by Hydroperoxyl Radicals**

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27 **Abstract**

28 Antioxidant interactions of γ -terpinene with α -tocopherol mimic 2,2,5,7,8-pentamethyl-6-
29 chromanol (PMHC) and caffeic acid phenethyl ester (CAPE), used as models, respectively, of mono-
30 and poly-phenols were demonstrated by differential oximetry during the inhibited autoxidation of
31 model substrates: stripped sunflower oil, squalene, and styrene. With all substrates, γ -terpinene acts
32 synergistically regenerating the chain-breaking antioxidants PMHC and CAPE from their radicals,
33 via the formation of hydroperoxyl radicals. The stoichiometric factors for mixtures PMHC/ γ -
34 terpinene and CAPE/ γ -terpinene increased with γ -terpinene concentration, while rate constants for
35 radical-trapping were unchanged by γ -terpinene, being 3.1×10^6 and $4.8 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ for PMHC and
36 CAPE in chlorobenzene (30°C). Using 3,5-di-*tert*-butylcatechol and 3,5-di-*tert*-butyl-1,2-
37 benzoquinone we demonstrate that γ -terpinene can reduce quinones to catechols enabling their
38 antioxidant activity. The different synergy mechanism of γ -terpinene with mono- and poly-phenolic
39 antioxidants is discussed and its relevance is proven in homogenous lipids using natural α -tocopherol
40 and hydroxytyrosol as antioxidants, calling for further studies in heterogenous food products.

41

42

43 **Keywords:** γ -terpinene; α -tocopherol; caffeic acid; hydroxytyrosol; synergy; quinones' regeneration;
44 stripped sunflower oil; squalene.

45

46

47 Chemical compounds studied in this article: γ -Terpinene (PubChem CID 7461); Squalene
48 (PubChem CID 638072); 2,2,5,7,8-Pentamethyl-6-chromanol (PubChem CID 99479); Caffeic acid
49 phenethyl ester (PubChem CID 5281787); Styrene (PubChem CID 7501); 3,5-Di-*tert*-butylcatechol
50 (PubChem CID 66099); 3,5-di-*tert*-butyl-1,2-benzoquinone (PubChem CID 24849680); Sunflower
51 seed oil (PubChem SID 404771784); Vitamin E (*d*- α -tocopherol; PubChem CID 14985);
52 Hydroxytyrosol (PubChem CID 82755).

53 **1. Introduction**

54 Plant essential oils have been shown to possess a wealth of biological effects, which have often
55 been associated to a purported antioxidant activity (Bakkali, Averbeck, Averbeck, & Idaomar, 2008).
56 Most of them are classified as Generally Recognized as Safe (GRAS) by the US Food and Drug
57 Administration, and are also studied as potential alternatives to synthetic antioxidants in the food
58 industry (Pateiro et al., 2018). Their antioxidant activities clearly depend on their composition,
59 particularly on the concentration of phenolic components, which have chain-breaking antioxidant
60 activity (Amorati & Valgimigli, 2018). On the other hand, recent work from our group has
61 demonstrated that different mechanisms are responsible for the antioxidant behaviour of some non-
62 phenolic components (Baschieri, Ajvazi, Tonfack, Valgimigli & Amorati, 2017).

63 Among such components, γ -terpinene, a pre-aromatic terpene, is widely present in essential oils
64 of many medicinal and aromatic plants, like citrus (Barboni, Luro, Chiaramonti, Desjobert, Muselli,
65 & Costa, 2009), savory, thyme (De Lisi, Tedone, Montesano, Sarli, & Negro, 2011), juniperus,
66 oregano (Bendahou et al. 2008), and others. It has been reported to possess not only anti-inflammatory
67 but also antioxidant activity (Ramalho, Oliveira, Lima, Bezerra-Santos, & Piuvezam, 2015). The
68 antioxidant mechanism of γ -terpinene was first clarified by Foti and Ingold (2003). However, little is
69 known on the possible interplay of the antioxidant activity of γ -terpinene with that of phenolic
70 antioxidants, be them found in the same plant or added to food products to aid their preservation.

71 Phenolic compounds, including many flavonoids, are abundant in plants (Dimitros 2006). They
72 possess ideal structure as antioxidants and are well-known to inhibit or stop the autoxidation of lipids
73 (Maqsood, Benjakul, Abushelaibi, & Alam, 2014; Matera et al. 2013). Although phenolic
74 antioxidants at a low concentration can provide an effective protection to lipids, they do not work
75 perfectly for long-term protection (Choe & Min, 2009). Interestingly, research in food preservation
76 has shown that essential oils can extend the shelf life of polyphenols-rich food like berries and that
77 they reduce the decay on storage of naturally contained flavonoids (Jin, Wu, Xu, Wang, Wang, &
78 Zheng, 2012). This apparently suggests a cooperative effect among phenolic antioxidants and

79 essential oils components, although the mechanism is not clarified in its molecular basis. Synergism
80 among antioxidants is certainly one of the most prominent strategies in modern antioxidant research
81 (Johansson, Shanks, Engman, Amorati, Pedulli, & Valgimigli, 2010; Valgimigli et al., 2013), beside
82 being the the primary strategy set up by nature (Niki, Saito, Kawakami, & Kamiya, 1984).

83 With those thoughts in mind, our hypothesis was that there might be synergic antioxidant effect
84 between γ -terpinene and phenolic antioxidants. The present investigation aims to provide an insight
85 into the occurrence and mechanism of such a synergic effect, and its potential application in food
86 chemistry. α -Tocopherol is perhaps the most important lipid-soluble phenolic antioxidant in nature
87 (Niki et al, 1984), hence we chose its close mimic PMHC (2,2,5,7,8-pentamethyl-6-chromanol), **1H**,
88 as a model monophenolic antioxidant in our investigation, since it has identical core structure and
89 reactivity compared to the natural counterpart (it differs only by truncation of the lipophilic tail, Fig.
90 1) and can be obtained in high purity (Baschieri, Pizzol, Guo, Amorati, & Valgimigli, 2019b). Caffeic
91 acid is ubiquitous in plants (Meinhart et al. 2019) and it is a very effective catechol-type antioxidant
92 (Chen & Ho, 1997; Markovic' & Tošovic, 2016) which was chosen as the model structure for
93 polyphenols. Its lipophilic phenetyl ester CAPE (**2H₂**) was selected in our experiments to study the
94 antioxidant interaction of polyphenols with γ -terpinene (Fig. 1).

95 In the present work, we studied the behaviour of monophenolic **1H** and polyphenolic **2H₂**
96 combined with γ -terpinene in the inhibition of the autoxidation of sunflower oil and squalene by
97 differential oximetry. This is a direct method for monitoring the kinetics of oxygen consumption
98 during the inhibited autoxidation of a reference substrate, which was demonstrated to be the golden
99 standard in antioxidant testing (Amorati, Baschieri, Morroni, Gambino, & Valgimigli, 2016; Amorati
100 & Valgimigli, 2015; 2018). The choice of sunflower oil and squalene as model oxidizable substrates
101 was based on their importance as dietary lipids and on their structural differences (see Fig. 1), so to
102 comprise the variability encountered in food products. To prove that results are of general relevance
103 and are not dependend on the substrate, we extended the investigation to styrene, since it is the best
104 known reference oxidizable substrate in antioxidant testing (Johansson et al., 2010; Amorati &

105 Valgimigli, 2015; 2018). In order to rationalize the mechanism of antioxidant interaction between γ -
106 terpinene and polyphenolic antioxidants we also performed experiments with well established
107 (Amorati, Valgimigli, Panzella, Napolitano, & d'Ischia, 2013) 3,5-di-*tert*-butylcatechol (DTBC, **3H₂**)
108 and its oxidized product 3,5-di-*tert*-butyl-*o*-benzoquinone (DTBQ, **3**) as a model catechol/quinone
109 redox couple.

110 <Fig. 1 about here>

111 **2. Materials and Methods**

112 *2.1. Materials*

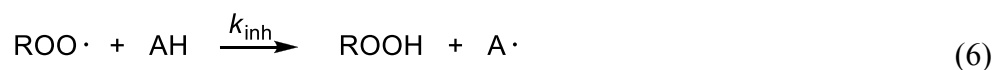
113 All chemicals and solvents were commercially available (Aldrich-Fluka-Sigma-Merck, Milan, Italy).
114 2,2'-Azobis(isobutyronitrile) (AIBN) was recrystallized from methanol. 2,2,5,7,8-Pentamethyl-6-
115 chromanol (PMHC, **1H**) was recrystallized from hexane. Caffeic acid phenethyl ester (CAPE, **2H₂**),
116 3,5-di-*tert*-butylcatechol (DTBC, **3H₂**), and 3,5-di-*tert*-butyl-*o*-benzoquinone (DTBQ, **3**) were used
117 as received. Squalene ($\geq 98\%$), styrene ($\geq 99\%$) and γ -terpinene (97%) were percolated twice through
118 activated basic alumina and once through silica to remove impurities and traces of hydroperoxides.
119 Stripped Sunflower Oil (SSO) was prepared from food-grade sunflower oil (*Helianthus annuus* Seed
120 Oil) purchased from a local market by purification as described in previous work to remove all
121 naturally occurring antioxidants (Baschieri et al. 2019b). All solutions were in chlorobenzene (99.9%
122 HPLC grade), unless otherwise noted.

123 *2.2. Determination of antioxidant activity based on inhibited autoxidation*

124 Hydrocarbon autoxidation is a free-radical chain reaction described by eqs 1-4, and it causes oxygen
125 consumption at a constant rate when it is initiated with a constant rate R_i , under controlled conditions
126 in the absence of inhibitors, as described in eq. 5, where k_p and $2k_t$ are, respectively, the rate constant
127 for chain propagation and termination of the oxidizable substrate. Oxygen consumption is instead
128 slowed down or delayed by an inhibition time τ when an antioxidant (AH) is breaking the radical
129 chain, competing with propagation (eqs. 6, 7), depending on the efficacy of the antioxidant (Amorati,
130 Pedulli, & Valgimigli, 2011, Amorati & Valgimigli, 2015).



$$-\frac{d[O_2]}{dt} = \frac{k_p [RH] \sqrt{R_i}}{\sqrt{2k_t}} + R_i \quad (5)$$



138 Efficacy of antioxidants was studied by measuring the kinetics of oxygen consumption during the
 139 autoxidation of a reference substrate, both in the presence and in the absence of antioxidants in a
 140 closed system. A two-channel oxygen uptake apparatus developed in our laboratory, based on a
 141 Validyne (Northridge, CA, USA) DP 15 differential pressure transducer, was used to record the
 142 consumption of the oxygen (Lucarini, Pedulli, Valgimigli, Amorati, Minisci, 2001, Baschieri et al.
 143 2017; 2019b). All the autoxidation experiments were initiated by the thermal decomposition of AIBN
 144 at 30 °C, in chlorobenzene (PhCl). In a typical experiment, an air-saturated solution of the oxidizable
 145 substrate containing AIBN (0.025 M) in PhCl (sample) is equilibrated at 30 °C with an identical
 146 (reference) solution containing an excess of PMHC (25 mM) so to block any radical chain. After
 147 reaching a constant O₂ consumption in the sample, a stock solution of antioxidant in PhCl (1 mM) is
 148 injected in the sample flask. From the plot of oxygen consumption, it was possible to calculate the
 149 inhibition rate constant (k_{inh}) and the stoichiometric factor (n) from eqs. 8 and 9, using the already
 150 known rate constants k_p and $2k_t$ of the chosen substrates (Amorati, Valgimigli, Panzella, Napolitano,
 151 & d'Ischia, 2013; Valgimigli et al. 2013; Amorati et al. 2016).

$$-\frac{d[\text{O}_2]}{dt} = \frac{k_p}{nk_{inh}} \frac{[\text{RH}]R_i}{[\text{AH}]} + R_i \quad (8)$$

$$R_i = n [\text{AH}] / \tau \quad (9)$$

152

153

154 The initiation rate R_i was determined in matched preliminary experiments by the inhibitor method,
 155 using PMHC as a reference antioxidant and equation 9, where τ is the length of the inhibition time.

156 The length τ could also be used to compare the antioxidant activity directly, the longer of the duration,
 157 the bigger of the activity (Amorati, Valgimigli, Dinér, Bakhtiari, Saeedi, & Engman, 2013; Amorati
 158 & Valgimigli, 2015).

159 2.3 UV-Vis Spectroscopy

160 Spectra were recorded at 30°C in a Thermo Scientific (Milan, Italy) Biomate 5 coupled with a Heto
 161 DBT Hetotherm (Birkerød, Denmark) thermostating water circulator for temperature control.
 162 Kinetics of formation and decay of the quinone **3** were monitored at 400 nm in PhCl containing 0.025
 163 M AIBN so to match the rate of radical generation R_i that was set during autoxidations, in presence
 164 and absence of γ -terpinene.

165 2.4. Statistical Analysis

166 Each inhibition rate constant (k_{inh}) and stoichiometric factor (n) is expressed as an average \pm standard
 167 deviation (SD) from at least three independent kinetic measurements.

168

169 3. Results and Discussion

170 3.1. Inhibition of the autoxidation of natural lipids by phenolic antioxidants with γ -terpinene

171 Sunflower oil is a prototypical natural oxidizable dietary lipid, due to its modest price, high
 172 availability in the food industry and facile oxidation (Guillen & Goicoechea, 2008; Smith, King, &
 173 Min, 2007). Stripped sunflower oil (SSO) was obtained by removing natural phenolic components
 174 and carotenoids that would impair its oxidation, and it was used as model oxidizable substrate in this

175 study, because its autoxidation had been kinetically calibrated for qualitative antioxidants test in
176 previous work (Baschieri et al., 2019b).

177 Squalene, a triterpenic polyunsaturated hydrocarbon which has attracted a lot of research interest
178 because of its benefit to human health, was chosen as another natural lipidic oxidizable substrate in
179 this study. It is ubiquitous in plants and abundant in vegetable oils (Baschieri et al., 2019b), it is
180 present also in all animals and in humans, being the precursor of sterols including cholesterol (Reddy
181 & Couvreur, 2009). Moreover, it is used as a diet supplement due to its numerous beneficial properties
182 (Reddy & Couvreur, 2009).

183 The antioxidant behavior of γ -terpinene was investigated in the controlled autoxidation of both
184 SSO and squalene. The analogue of α -tocopherol PMHC (**1H**), and the lipid-soluble phenethyl ester
185 derivative of caffeic acid (CAPE, **2H₂**) were also used as antioxidants in matched experiments with
186 the same lipid substrates. Results summarized in Fig. 2 showed that γ -terpinene alone, in the
187 millimolar range, could only slow down the lipid autoxidation by a modest margin, while, as expected,
188 the mono- and poly-phenolic antioxidants (**1H** and **2H₂**) showed neat inhibition of the autoxidation
189 already at micromolar concentrations. Most interesting, when γ -terpinene and each of the phenolic
190 antioxidants were used together in the protection of either SSO or squalene, the antioxidant activity
191 toward both lipids was greatly enhanced as compared to the use of **1H** or **2H₂** alone, being clearly
192 higher than the sum of the contributions of each phenolic antioxidant and γ -terpinene, *i.e.* the
193 combination showed clear synergic antioxidant effect.

194 <Fig. 2 about here>

195 In oxygen-uptake kinetics during autoxidation, when a clear inhibited period is observed as in
196 Fig. 2 (A,C,D), the slope on the inhibited period is inversely related to the rate constant for peroxy
197 radical trapping by the antioxidant (see eq. 8), while its duration (τ) depends on the concentration of
198 the antioxidant and the stoichiometric factor n , *i.e.* the number of radicals trapped by each molecule
199 of antioxidant according to eq. 9. An inspection of Fig. 2 shows that while γ -terpinene produced no
200 neat inhibition period, and both **1H** or **2H₂** did so, the combination of either **1H** or **2H₂** with γ -

201 terpinene did not significantly change the slope of the inhibited period as compared to that produced
202 by each of the phenols alone, but it extended its duration in a dose-dependent fashion (see inserts in
203 Figure 2A-D). This kinetic behavior would indicate, that γ -terpinene acts as the co-antioxidant which
204 regenerates the main antioxidant, **1H** or **2H₂**, as it is consumed during the autoxidation, similarly to
205 the behaviour previously observed and discussed for other co-antioxidant couples (Amorati, Ferroni,
206 Lucarini, Pedulli., & Valgimigli, 2002; Valgimigli et al. 2013)

207 Such a synergic antioxidant behavior was observed both with SSO and squalene as the
208 oxidizable substrates, despite the structural differences, indicating that the synergy between γ -
209 terpinene and phenols might be a general property, not related to the oxidizing substrate. To confirm
210 this hypothesis we further investigated the synergy in the autoxidation of styrene initiated by AIBN.
211 Indeed, styrene is by far the best known reference oxidizable substrate to test antioxidants (Johansson
212 et al. 2010; Amorati & Valgimigli 2015) and its clearly defined rate constants for chain propagation
213 ($k_p = 41 \text{ M}^{-1}\text{s}^{-1}$ at 30°C) and chain termination ($2k_t = 4.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ at 30°C) allow quantitative
214 evaluation of synergy, as well as representing a robust model to achieve mechanistic understanding
215 (Matera et al. 2015).

216 Results were qualitatively superimposable to those obtained with SSO and squalene as the
217 oxidizable substrates (see Appendix, Fig. S1). From the oxygen-uptake plots, the apparent n value of
218 the mixtures was calculated according to equation 9, where R_i is the rate of radical initiation produced
219 by AIBN. According to the well-known chain-breaking antioxidant mechanism, each molecule of
220 phenol or catechol inactivates two ROO• (see eqs. 6, 7). Hence, the stoichiometric factors of both **1H**
221 and **2H₂**, have the theoretical value $n = 2$. The apparent n value obtained for the phenolic antioxidants
222 when mixed with different amounts of γ -terpinene, shown in Table 1, were always larger than 2, and
223 increased with the concentration of γ -terpinene. Moreover, there was no major difference between
224 the rate of oxygen consumption recorded in the presence or absence of γ -terpinene, hence both for
225 **1H** and **2H₂** the apparent rate constants for inhibition k_{inh} (calculated from eq. 8) was not significantly
226 different in the presence/absence of γ -terpinene (see Table 1), which supports the role γ -terpinene

227 only as the co-antioxidant, able to afford regeneration of the main phenolic antioxidants, similarly to
 228 what was observed using SSO or squalene as the oxidizable substrates. It is worth noting that the
 229 rate constant k_{inh} measured here for **1H** is in excellent agreement with previous literature (Valgimigli
 230 et al. 2013), while that determined for **2H₂** is reported for the first time but is in line with that of other
 231 catechol-type antioxidants (Amorati, Valgimigli, Panzella, Napolitano, & d'Ischia, 2013), which
 232 stands for the reliability of our current kinetic measurements.

233

234 **Table 1.** Apparent stoichiometric factors (n) and inhibition rate constant (k_{inh}) of PMHC (**1H**) and
 235 CAPE (**2H₂**) when mixed with different amounts of γ -terpinene during the autoxidation of styrene
 236 initiated by AIBN at 30°.

Synergic combination	[antiox] (μ M)	[γ -terpinene] (mM)	τ (s)	n^a	$-(d[O_2]/dt)_{inh}^1$ ($M s^{-1}$)	app. k_{inh}^a ($10^5 M^{-1} s^{-1}$)
PMHC/ γ -terpinene	2.5	0	1836	2.0 \pm 0.1	3.1×10^{-8}	31.3 \pm 3.5
		7.83	2372	2.6 \pm 0.2	3.5×10^{-8}	28.1 \pm 4.1
		15.65	2751	3.0 \pm 0.2	3.7×10^{-8}	26.2 \pm 5.8
		31.30	3396	3.7 \pm 0.3	3.0×10^{-8}	32.0 \pm 3.9
CAPE/ γ -terpinene	2.5	0	1150	2.0 \pm 0.1	2.0×10^{-7}	4.8 \pm 1.1
		7.83	1650	2.9 \pm 0.2	1.8×10^{-7}	5.5 \pm 0.9
		15.65	1914	3.3 \pm 0.2	1.8×10^{-7}	5.5 \pm 0.7
		23.48	2022	3.6 \pm 0.3	2.1×10^{-7}	4.5 \pm 1.3

237 All values are average from at least 3 independent measurements. ^a Errors for n and k_{inh} represent \pm
 238 SD.

239

240 3.2. Exploration of the mechanism behind the synergy

241 Phenolic antioxidants can retard or block the oxidation of lipids by scavenging chain-carrying
 242 peroxy radicals. Both monophenolic antioxidants like α -tocopherol or **1H** and polyphenolic like
 243 caffeic acid or **2H₂** have a stoichiometric factor $n = 2$, although they possess different number of
 244 active OH groups. The first step of their antioxidant mechanism is similar: a formal H-atom transfer
 245 to a ROO \cdot . The difference is in the second step. For monophenolic antioxidants like **1H**, the phenoxyl

246 radical resulting from the first step will trap a second ROO• by addition to the aromatic ring. Instead,
247 the phenoxyl radical resulting from a catechol is a semiquinone and can donate another hydrogen
248 atom to ROO• to yield the corresponding quinone, as illustrated in Fig. 3 (Amorati, Valgimigli,
249 Panzella, Napolitano, & d'Ischia, 2013; Matera et al., 2015).

250 A synergetic effect between antioxidants based on the regeneration of a more effective
251 antioxidant by a less effective synergist occurs mostly when one antioxidant has a higher reduction
252 potential than the other (Valgimigli, Lucarini, Pedulli, & Ingold, 1997; Pedrielli & Skibsted, 2002;
253 Johansson et al. 2010), or when the radical formed from one antioxidant can be reduced by H-atom
254 transfer from another antioxidant having a weaker X-H bond in the active site (Amorati et al., 2002;
255 Valgimigli et al., 2013). A typical example is the long established synergy between α -tocopherol and
256 ascorbic acid, in which ascorbic acid regenerates α -tocopherol by transferring a hydrogen to α -
257 tocopheroxyl radical (Niki et al. 1984). The synergism between α -tocopherol and other co-
258 antioxidants was also investigated (Amorati et al. 2002, Pedrielli & Skibsted, 2002; Thiyam,
259 Stöckmann, & Schwarz, 2006; Amorati, Valgimigli, Dinér, Bakhtiari, Saedi, & Engman, 2013). In
260 general, synergism between phenol-type antioxidants was attributed to a similar mechanism in which
261 the fastest antioxidant reacts first with chain-carrying peroxy radicals to yield the corresponding
262 phenoxyl radical that is reduced back by formal H-atom transfer from the co-antioxidant (Valgimigli
263 et al. 2013).

264 <Fig. 3 about here>

265 In this scenario, the synergic contribution of γ -terpinene is less obvious. The antioxidant
266 mechanism of γ -terpinene was disclosed by Foti and Ingold (2003) who proposed that the addition of
267 γ -terpinene in the peroxidation of lipids would change the propagation chain-carrier from ROO• to
268 HOO• (hydroperoxyl radical) since γ -terpinene itself is rapidly attacked by ROO• and releases HOO•.
269 Hydroperoxyl radicals, HOO•, can both propagate the oxidation and be quenched by another HOO•
270 or by ROO• (self-termination or cross-termination). Since the self termination of HOO• and its cross
271 termination with ROO• is much faster than the self-termination of ROO•, the overall termination

272 efficiency would increase in the presence of γ -terpinene, justifying its antioxidant behavior (Fig. 3A).
273 This termination-enhancing antioxidant activity is common to other terpenoids and is expected to
274 bring only limited contribution to the overall antioxidant activity (Baschieri et al. 2017).

275 Recently, some of us showed that $\text{HOO}\cdot$ could reduce phenoxyl radical to their parent phenol
276 because of the extremely low Bond Dissociation Enthalpy (BDE) of $\text{H-OO}\cdot$ (45 kcal/mol) (Baschieri,
277 Valgimigli, Gabbanini, DiLabio, Romero-Montalvo, & Amorati, 2018; Cedrowski, Litwinienko,
278 Baschieri, & Amorati, 2016), which is lower than the O-H BDE of phenolic antioxidants (typically
279 72 to 82 kcal/mol) (Warren, Tronic & Mayer, 2010).

280 The regeneration of the starting phenol from its phenoxyl radical by $\text{HOO}\cdot$ generated from γ -
281 terpinene nicely explains the synergy with monophenols like PMHC **1H** as depicted in Fig. 3B;
282 however, in the case of catechols, in principle regeneration could occur both by 1-electron reduction
283 of the semiquinone radical and by (stepwise) 2-electron reduction of the quinone as depicted in Fig.
284 3C. To achieve a better understanding of the regeneration mechanism of phenolic antioxidants by γ -
285 terpinene, matched sets of experiments were conducted by injecting γ -terpinene into the styrene
286 autoxidation system at the beginning of the experiment and/or after the phenolic antioxidant was
287 consumed, *i.e.* at the time when the substrate starts to oxidize again. PMHC **1H** was again used as the
288 prototype of monophenolic antioxidants, while 2,5-di-*tert*-butyl catechol (DTBC, **3H₂**) was used as
289 model for polyphenols, since it is a better established catechol-type antioxidant (Amorati, Valgimigli,
290 Panzella, Napolitano & d'Ischia, 2013) and its corresponding quinone (DTBQ, **3**) is also
291 commercially available and stable in solution.

292 As shown in Fig. 4A, injecting γ -terpinene into the sample after the inhibition by **1H** had ended,
293 meaning that **1H** had been completely oxidized, could not restart the inhibition; instead if γ -terpinene
294 was injected before **1H**, the subsequent injection of **1H** gave an inhibition period much longer than
295 that observed without prior injection of γ -terpinene (see Appendix, Fig S2). This means that the final
296 oxidized products of **1H** could not be regenerated by γ -terpinene, while only the intermediate
297 phenoxyl radical can be efficiently regenerated. This confirms our suggested mechanism depicted in

298 Fig. 3B. On the other hand, using the catechol **3H₂** instead of **1H**, injection of γ -terpinene caused the
299 reboot of a new inhibition period when injected after the complete consumption of **3H₂**, i.e. when
300 inhibition by **3H₂** had terminated and the autoxidation was running again uninhibited (Fig. 4B). This
301 implies that the quinone (**3**) formed as the final oxidized products of the catechol can be reduced back
302 to the starting antioxidant (see Fig. 3C).

303 <Fig. 4 about here>

304 Quinones are easily formed by the oxidation of related catechols, and they are generally expected
305 to be the main final oxidized product when catechols behave as antioxidants. This was also confirmed
306 in our experimental settings by monitoring the growth of UV absorbance of *ortho*-quinone **3** at 400nm
307 during the autoxidation of styrene inhibited by **3H₂** (Figure 5A). To confirm our hypothesis,
308 autoxidation experiments were performed using the oxidized quinone DTBQ **3** as the antioxidant.
309 While the injection of **3** alone in autoxidizing styrene did not produce any inhibition (Fig. 5B)
310 subsequent injection of γ -terpinene caused the appearance of a long inhibition period, confirming
311 that the quinone **3** could be regenerated by γ -terpinene to its starting catechol. This was also confirmed
312 by parallel experiments in which the time-course of the concentration of **3** was monitored by UV
313 spectroscopy at 400 nm during autoxidations inhibited by **3** and γ -terpinene: the UV signal of **3**
314 progressively declined during the autoxidation supporting its reduction to **3H₂** (see Appendix, Fig.
315 S3). Additional experiments with co-injection of the the quinone and γ -terpinene in different order
316 confirmed this conclusion (see Appendix, Fig. S4).

317 <Fig. 5 about here>

318 We attribute the reduction of **3** (and other quinones) by γ -terpinene during the autoxidation to
319 the release of HOO•, which would act as the reducing agent. Although this reducing behavior might
320 be counterintuitive for a reputedly oxidizing radical, it is supported by previous solid evidence that
321 it rapidly reduces both phenoxyl radicals (Cedrowski et al., 2016) and nitroxides (Bascieri et al.,
322 2018). However, to explain the reduction of quinones by γ -terpinene other mechanistic possibilities

323 could also have a role, like those recently disclosed to explain the synthesis of catechols using
324 methylcyclohexadiene as the reducing agent (Baschieri, Amorati, Valgimigli, & Sambri, 2019a).

325

326 *3.3 Relevance of the interaction between quinones and γ -terpinene in food science*

327 The chemistry disclosed in the previous section concerning the possibility for γ -terpinene to enable
328 the antioxidant behavior of quinones by reduction to the parent catechols appears particularly
329 important in the protection of food products, since quinones are often abundant in vegetable tissues,
330 also resulting from air oxidation of the parent polyphenols. This suggests that γ -terpinene could afford
331 unusually effective protection owing to its synergic interplay with such products. On the other hand,
332 it is clear from Fig. 4 and 5 that the redox cycling of the quinones to the parent catechols, then back
333 to quinones and so on, is not fully efficient and either redox species might be consumed in side
334 reactions, since the inhibition is not infinite or limited only by the consumption of γ -terpinene. Clearly,
335 further studies would be necessary to fully rationalize the reasons for imperfect redox cycling;
336 however, in current investigation our interest focused on assessing its relevance in food chemistry.
337 Therefore, we switched back to SSO and squalene as relevant dietary lipids and tested the antioxidant
338 protection of quinone **3** in combination with γ -terpinene. With SSO **3** afforded no protection when
339 used alone; however, subsequent addition of γ -terpinene enabled its antioxidant activity (Fig. 5C),
340 and co-addition of **3** and γ -terpinene at the beginning of the autoxidation afforded even higher
341 antioxidant protection (see Appendix, Fig. S4). Similarly, co-addition of **3** and γ -terpinene afforded
342 full protection of squalene (Fig. 5D). Interestingly, with squalene even **3** alone afforded some
343 antioxidant protection (see Appendix) possibly due to the release of HOO• radical as a side event
344 during the autoxidation of squalene, as previously observed (Baschieri et al. 2019). This aspect would
345 certainly deserve further investigation. However, its combination with γ -terpinene resulted in much
346 enhanced antioxidant activity (see Appendix). Overall, current results demonstrate the effectiveness
347 hence the relevance of the redox interplay between quinones and γ -terpinene in protecting food

348 products, and complete the rationale explaining the synergy between γ -terpinene and both mono-and
349 poly-phenolic antioxidants in this respect.

350

351 **4. Conclusions**

352 In conclusion, γ -terpinene can enhance the protection of natural lipids or other oxidizable
353 substrates operated by phenolic antioxidants. The synergic mechanism is based on the sacrificial
354 oxidation γ -terpinene with release of $\text{HOO}\cdot$ during the autoxidation. The exact consequence of such
355 chain-transfer process then depends on the structure of the phenolic antioxidants. With monophenolic
356 antioxidants like tocopherol, $\text{HOO}\cdot$ could reduce the the phenoxyl radical formed upon trapping
357 chain-carrying radicals ($\text{ROO}\cdot$) thereby regenerating the starting antioxidant. No synergy is however
358 displayed when the phenol has been fully oxidized to the final oxidation products, meaning that the
359 phenol and γ -terpinene need to be simultaneously present in the system. On the other hand, with
360 polyphenolic catechol antioxidants regeneration to the starting catechol can occur both from the
361 phenoxyl (semiquinone) radicals and from the final oxidized quinone, expanding the usefulness of
362 this synergic antioxidant chemistry. In both cases synergy occurs via establishing a catalytic cycle in
363 which γ -terpinene acts as the sacrificial reductant. Since phenols and polyphenols are normally co-
364 existing with terpinene or structurally related terpenes in vegetable extracts and food products, the
365 synergic activity disclosed here is likely to have major significance and it can be exploited in rational
366 strategies for antioxidant food protection. Additionally, it helps explain the purported beneficial effect
367 of flavonoids even after they have been oxidized due to prolonged storage of food of vegetable
368 products.

369 Lipids are essential cell membrane constituents (Lingwood & Simons, 2010) and key
370 components in food. Their non-enzymatic oxidation causes deterioration of food flavour, color,
371 texture and nutritional value (Falowo, Fayemi, & Muchenje, 2014), beside the formation of toxic off-
372 products like 4-hydroxynonenal and other electrophilic carbonyl compounds (Guillen & Goicoechea,
373 2008). Thus, great research effort in food science has been devoted to protecting lipids from oxidation

374 by antioxidants, especially those obtained from natural sources (Falowo, et al. 2014, Amorati &
375 Valgimigli, 2018). We believe that the chemistry disclosed in this work brings relevant contribution
376 in this respect.

377

378 **Declaration of interest**

379 The authors declare no competing financial interest.

380

381 **Acknowledgments**

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384

385 **Appendix A. Supplementary data**

386 Supplementary data associated with this article can be found, in the online version, at ...

387

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Figure captions

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526 **Fig. 1.** Oxidizable substrates and antioxidants investigated in this study

527
528 **Fig 2.** Oxygen consumption during the autoxidation of SSO (A-B), and squalene (C-D). Each panel
529 displays the curves recorded for the autoxidation of the substrates without inhibitors (dotted lines),
530 and in the presence of 15.65 mM γ -terpinene (dashed lines). In panels A and C grey plots represent
531 the inhibition by PMHC **1H** (2.5 μ M) alone, while black curves represent inhibition by the mixture
532 of **1H** (2.5 μ M) with γ -terpinene (15.65 mM). In panels B and D grey plots represent inhibition by
533 CAPE **2H₂** (2.5 μ M) alone, and black curves inhibition by the mixture of **2H₂** (2.5 μ M) with γ -
534 terpinene (15.65 mM). Inserts: plots of the experimental inhibition periods τ vs the concentration γ -
535 terpinene for different mixtures **1H** or **2H₂** (2.5 μ M) / γ -terpinene.

536
537 **Fig. 3.** Mechanism of the antioxidant activity of (A) γ -terpinene alone (after Foti & Ingold, 2003) and
538 of the antioxidant synergy between (B) γ -terpinene and monophenolic antioxidants (using **1H** as
539 model compound) and (C) γ -terpinene and polyphenolic antioxidants (using **2H₂** or **3H₂** as model
540 compound).

541
542 **Fig. 4.** Oxygen consumption measured during the autoxidation of styrene initiated by 0.025 M AIBN
543 at 30°C. (A) 1: uninhibited; 2: injection of **1H** (2.5 μ M); 3: injection of γ -terpinene (15.7 mM). (B)
544 1: uninhibited; 2: injection of **3H₂** (7.5 μ M); 3: injection of γ -terpinene (15.7 mM).

545
546 **Fig. 5.** (A) Growth of the UV signal at 400 nm due to quinone **3** during the autoxidation of styrene
547 initiated by 0.025 M AIBN at 30°C, inhibited by **3H₂** 67 μ M. (B) Oxygen consumption during the
548 autoxidation of styrene initiated by 0.025 M AIBN at 30°C in the absence of antioxidant (1), and
549 upon their addition: 2 = injection of DTBQ **3** (7.5 μ M); 3 = injection of γ -terpinene (7.83 mM). (C)
550 Oxygen consumption during the autoxidation of SSO initiated by 0.025 M AIBN at 30°C in the

551 absence of antioxidant (1), and upon their addition: 2 = injection of DTBQ **3** (7.5 μ M); 3 = injection
552 of γ -terpinene (7.83 mM). (D) Oxygen consumption during the autoxidation of squalene initiated by
553 0.025 M AIBN at 30°C in the absence of antioxidant (1), or in the presence of a mixture of DTBQ **3**
554 (7.5 μ M) and γ -terpinene (7.83 mM).

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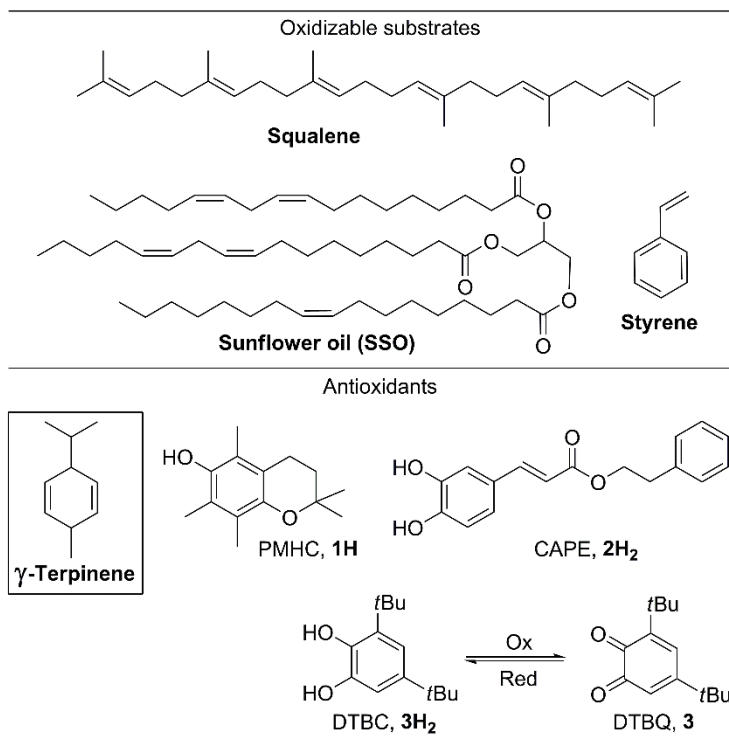
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585 **Fig. 1.** Oxidizable substrates and antioxidants investigated in this study

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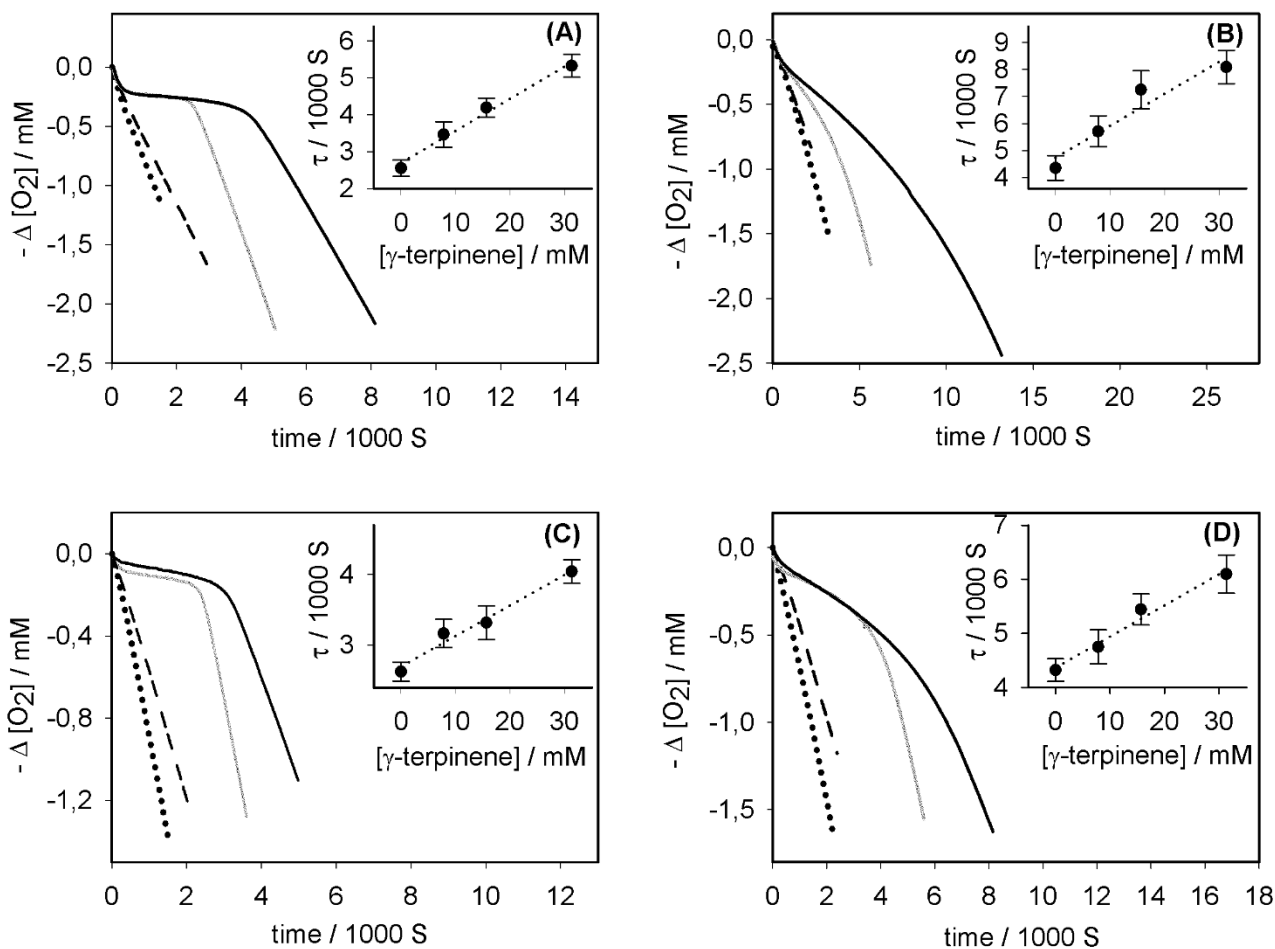
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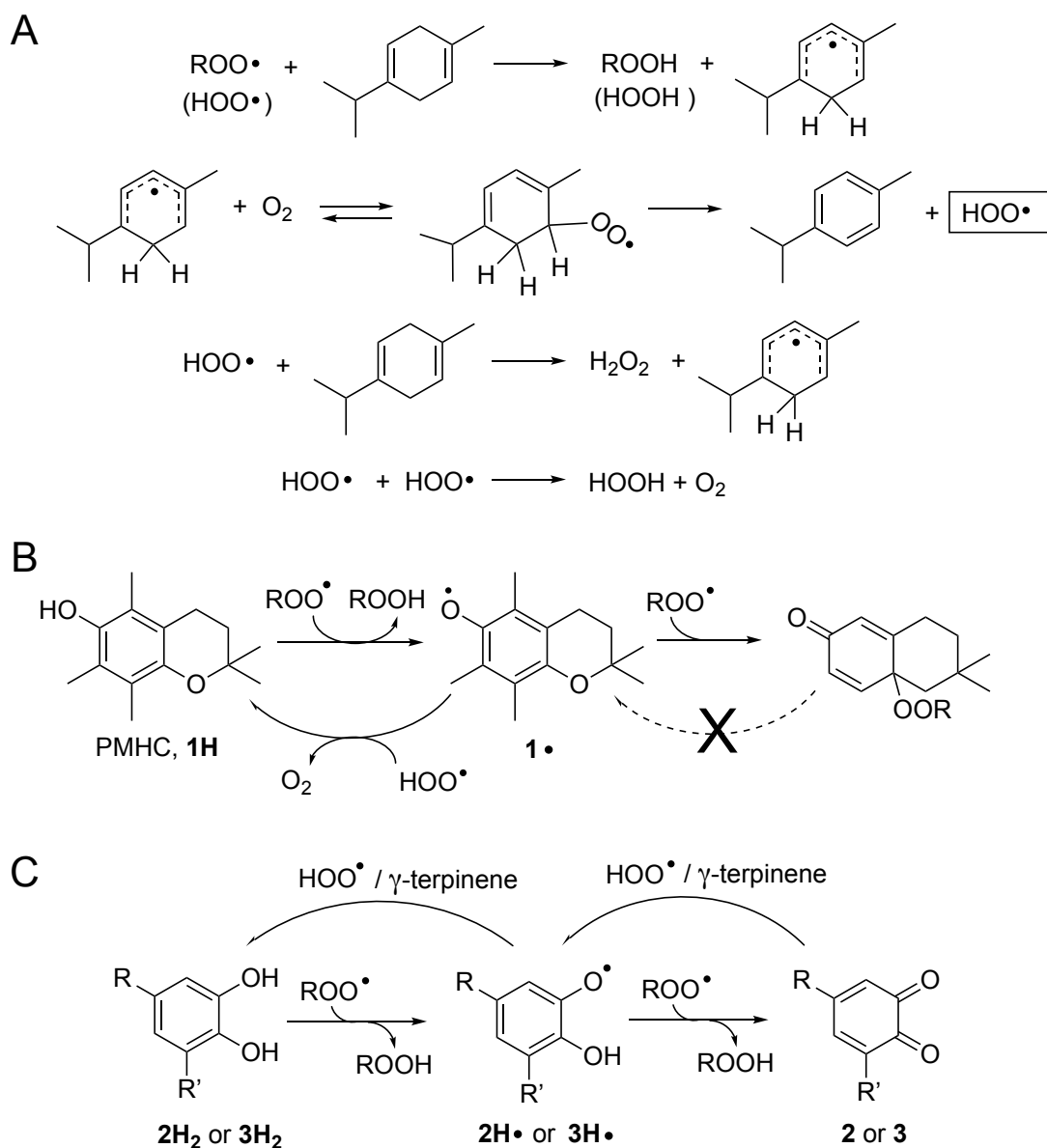
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 607 terpinene for different mixtures **1H** or **2H₂** (2.5 μ M) / γ -terpinene.

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615 **Fig. 3.** Mechanism of the antioxidant activity of (A) γ -terpinene alone (after Foti & Ingold, 2003) and

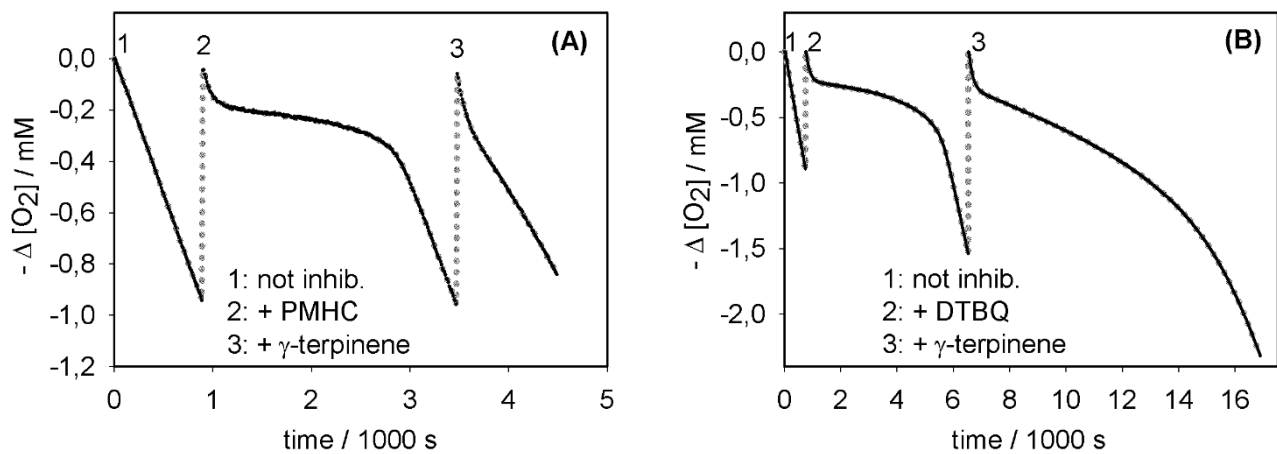
616 of the antioxidant synergy between (B) γ -terpinene and monophenolic antioxidants (using **1H** as

617 model compound) and (C) γ -terpinene and polyphenolic antioxidants (using **2H₂** or **3H₂** as model

618 compound).

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624 **Fig. 4.** Oxygen consumption measured during the autoxidation of styrene initiated by 0.025 M AIBN

625 at 30°C. (A) 1: uninhibited; 2: injection of **1H** (2.5 μM); 3: injection of γ -terpinene (15.7 mM). (B)

626 1: uninhibited; 2: injection of **3H₂** (7.5 μM); 3: injection of γ -terpinene (15.7 mM).

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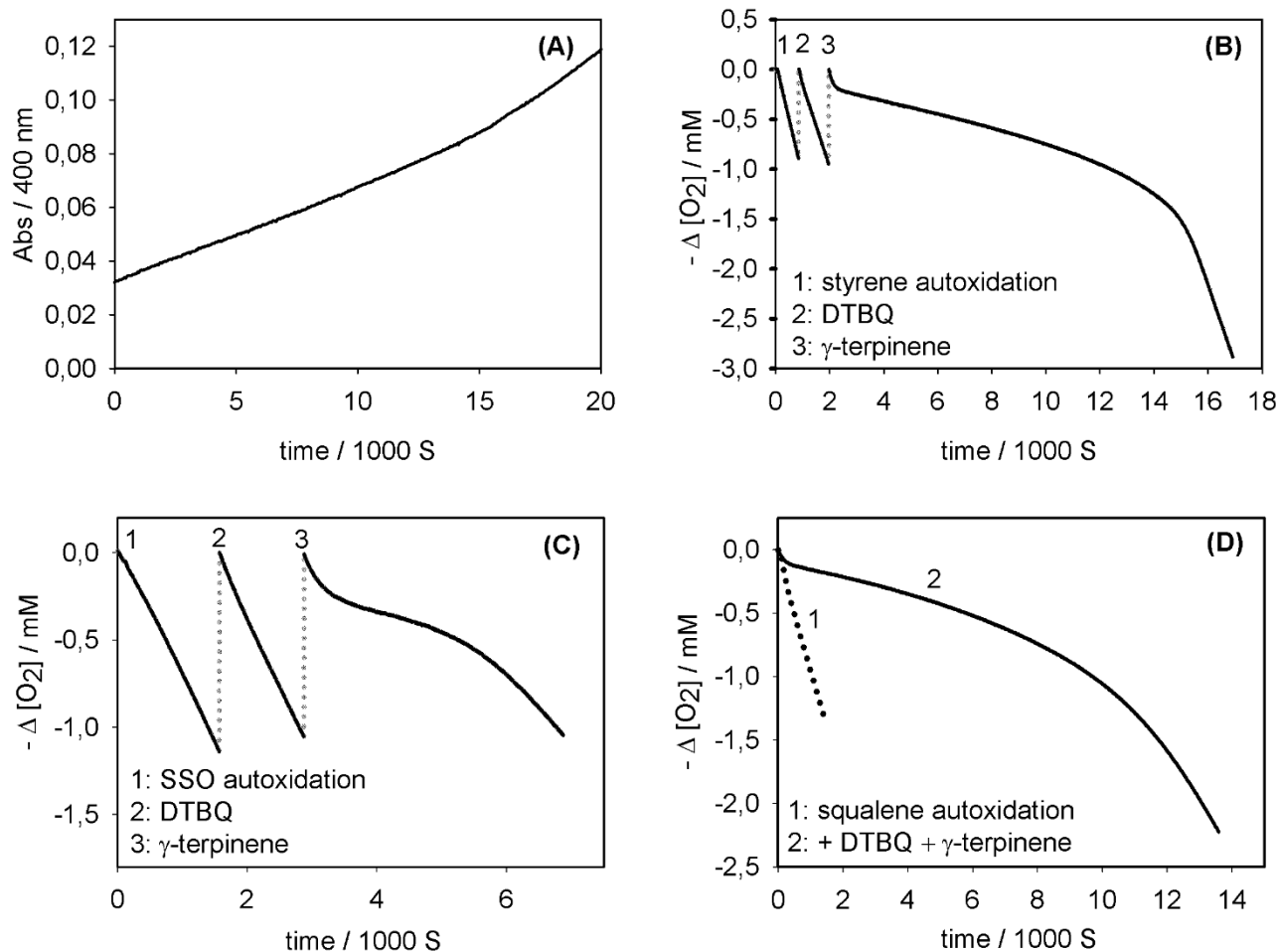
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640 **Fig. 5.** (A) Growth of the UV signal at 400 nm due to quinone **3** during the autoxidation of styrene

641 initiated by 0.025 M AIBN at 30°C, inhibited by **3H**₂ 67 μM. (B) Oxygen consumption during the

642 autoxidation of styrene initiated by 0.025 M AIBN at 30°C in the absence of antioxidant (1), and

643 upon their addition: 2 = injection of DTBQ **3** (7.5 μM); 3 = injection of γ-terpinene (7.83 mM).

644 Oxygen consumption during the autoxidation of SSO initiated by 0.025 M AIBN at 30°C in the

645 absence of antioxidant (1), and upon their addition: 2 = injection of DTBQ **3** (7.5 μM); 3 = injection

646 of γ-terpinene (7.83 mM). (D) Oxygen consumption during the autoxidation of squalene initiated by

647 0.025 M AIBN at 30°C in the absence of antioxidant (1), or in the presence of a mixture of DTBQ **3**

648 (7.5 μM) and γ-terpinene (7.83 mM).

649

Synergic Antioxidant Activity of γ -Terpinene with Phenols and Polyphenols Enabled by Hydroperoxyl Radicals

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Appendix A – Supplementary Material

Content

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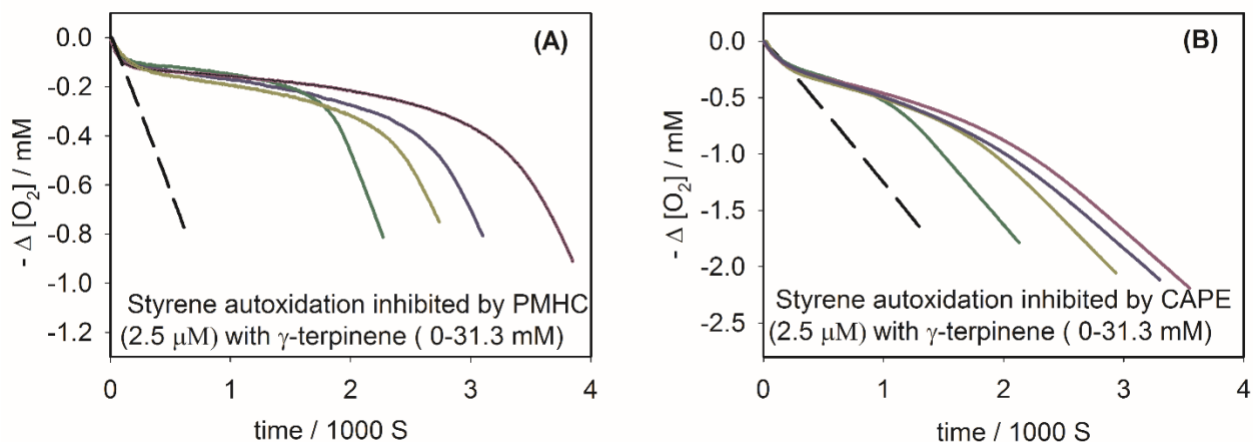


Fig. S1. Oxygen consumption measured during the autoxidation of styrene initiated by 0.025 M AIBN at 30°C in the absence of antioxidant (dashed line in both panels), and inhibited by (A) PMHC **1H** (2.5 μM) in the absence of γ -terpinene (green line), in the presence of γ -terpinene (7.83 mM - yellow line; 15.7 mM - blue line; 31.3 mM - purple line), or by (B) CAPE **2H₂** (2.5 μM) alone (green line) or in the presence of growing amounts of γ -terpinene (the same color coding as in panel A is used).

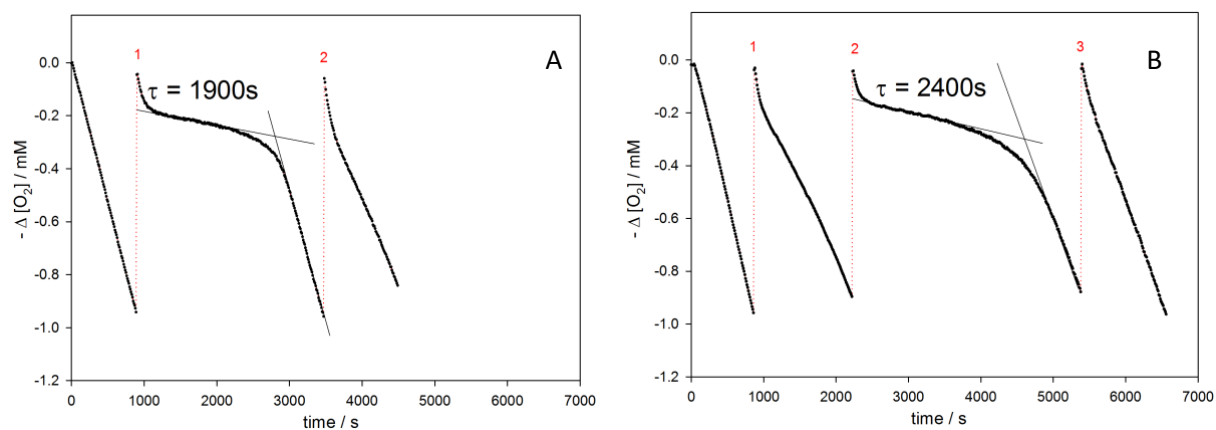


Fig. S2. Oxygen consumption during the autoxidation of styrene initiated by 0.025 M AIBN at 30°C without inhibitors and upon injection of antioxidants at the time points indicated by numbers: (A) 1 = injection of PMHC **1H** (2.5 μM), 2 = subsequent injection of γ -terpinene (20 mM); (B) 1 = injection of γ -terpinene (15.7 mM), 2 = subsequent injection of PMHC **1H** (2.5 μM), 3 = subsequent injection of γ -terpinene (15.7 mM). Note that inhibition period τ in panel B is larger than in panel A (where it corresponds to $n = 2.1$) despite the identical concentration of injected PMHC.

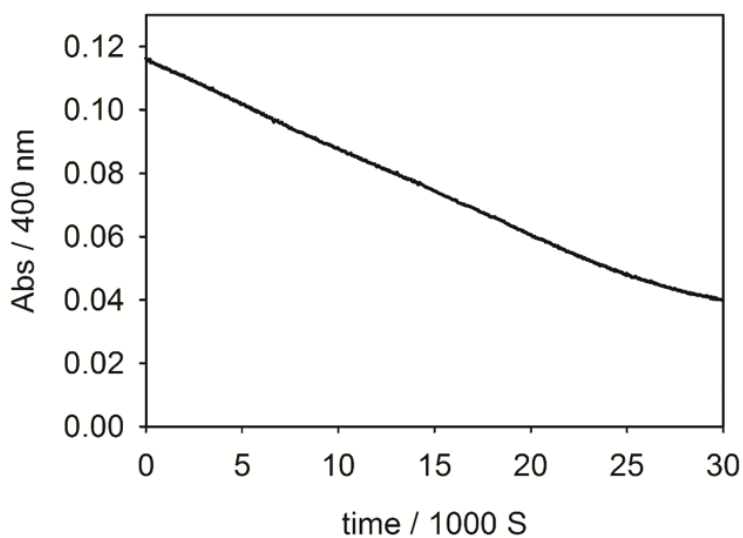


Fig. S3. Decay of the UV signal at 400 nm due to quinone **3** during the autoxidation of styrene initiated by 0.025 M AIBN at 30°C, inhibited by **3** (67 μ M) and γ -terpinene (15.7 mM).

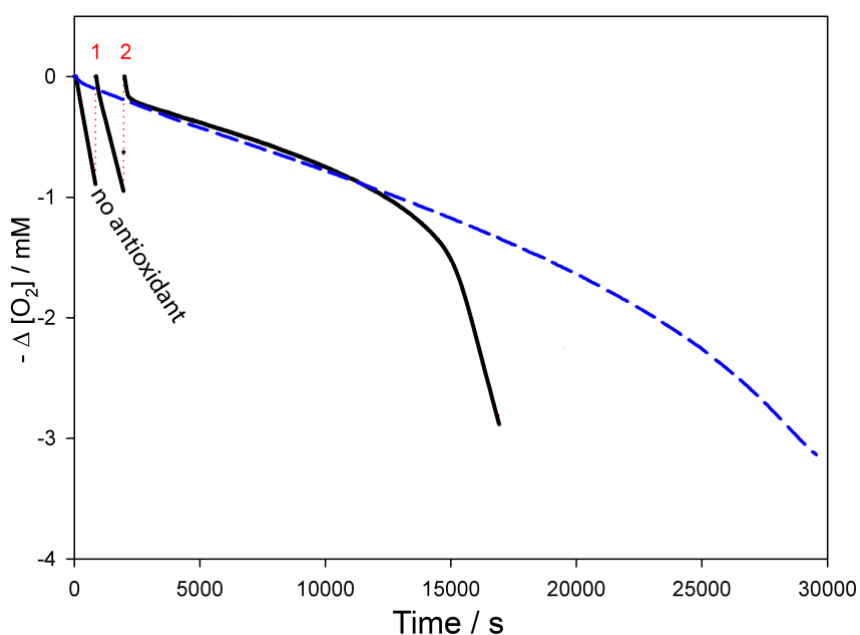


Fig. S4. Oxygen consumption measured during the autoxidation of SSO initiated by 0.025 M AIBN at 30°C in the absence of antioxidants and (1) upon injection of DTBQ **3** (7.5 μ M), (2) upon subsequent injection of γ -terpinene (15.7 mM). The blue dashed line represents the autoxidation under identical settings inhibited by a mixture of DTBQ **3** (7.5 μ M) and γ -terpinene (15.7 mM) injected together at the beginning of the autoxidation. Note that despite the identical concentration of

the antioxidants their effectiveness is higher if they are added together, possibly meaning that some side reaction is consuming **3** in the absence of γ -terpinene.

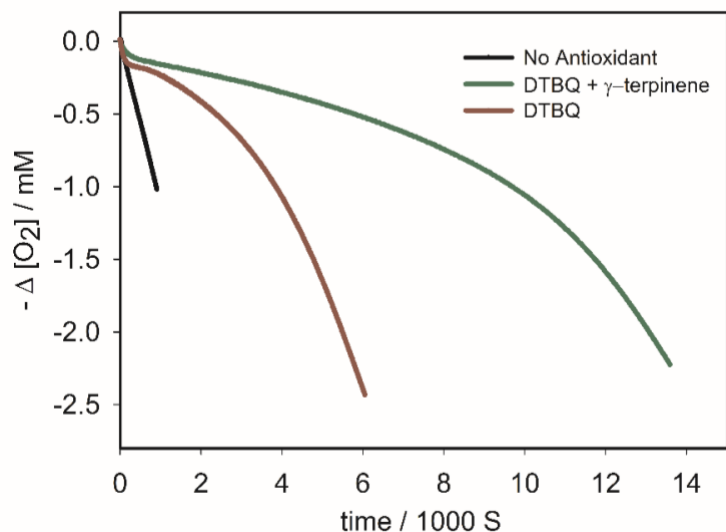


Fig. S5. Oxygen consumption measured during the autoxidation of squalene initiated by 0.025 M AIBN at 30°C in the absence of antioxidant (black line), inhibited by 2.5 μ M DTBQ **3** (red line), or inhibited by 2.5 μ M DTBQ **3** and 15.7 mM γ -terpinene (green line).

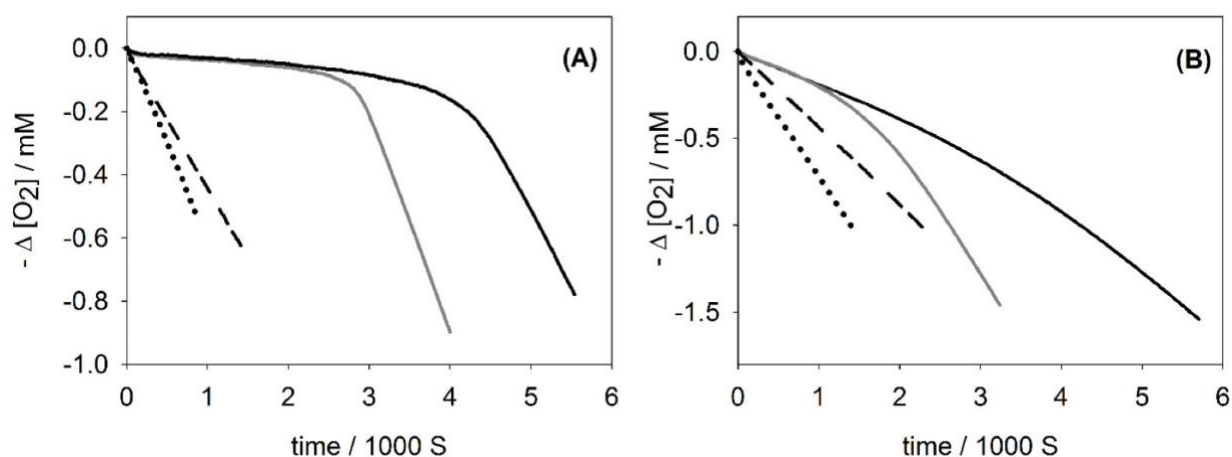


Fig. S6. Oxygen consumption measured during the autoxidation of sunflower oil initiated by 0.025 M AIBN at 30°C in the absence of antioxidants (dotted lines), inhibited by 15 mM γ -terpinene (dashed lines), or (A) inhibited by 3 μ M vitamin E (*d*- α -tocopherol, gray line) or by a mixture of 2.5 μ M vitamin E + 15 mM γ -terpinene (black line), (B) inhibited by 6 μ M hydroxytyrosol (gray line) or by a mixture of 6 μ M hydroxytyrosol + 15 mM γ -terpinene.