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

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Abstract

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

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

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

1. Introduction

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

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

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

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

With those thoughts in mind, our hypothesis was that there might be synergic antioxidant effect between γ-terpinene and phenolic antioxidants. The present investigation aims to provide an insight into the occurrence and mechanism of such a synergic effect, and its potential application in food chemistry. α-Tocophenol is perhaps the most important lipid-soluble phenolic antioxidant in nature (Niki et al, 1984), hence we chose its close mimic PMHC (2,2,5,7,8-pentamethyl-6-chromanol), **1H**, as a model monophenolic antioxidant in our investigation, since it has identical core structure and reactivity compared to the natural counterpart (it differs only by truncation of the lipophilic tail, Fig. 1) and can be obtained in high purity (Baschieri, Pizzol, Guo, Amorati, & Valgimigli, 2019b). Caffeic acid is ubiquitous in plants (Meinhart et al. 2019) and it is a very effective catechol-type antioxidant (Chen & Ho, 1997; Markovic´ & Tošovic, 2016) which was chosen as the model structure for polyphenols. Its lipophilic phenetyl ester CAPE (**$2H_2$**) was selected in our experiments to study the antioxidant interaction of polyphenols with γ-terpinene (Fig. 1).

In the present work, we studied the behaviour of monophenolic **1H** and polyphenolic **$2H_2$** combined with γ-terpinene in the inhibition of the autoxidation of sunflower oil and squalene by differential oximetry. This is a direct method for monitoring the kinetics of oxygen consumption during the inhibited autoxidation of a reference substrate, which was demonstrated to be the golden standard in antioxidant testing (Amorati, Baschieri, Morroni, Gambino, & Valgimigli, 2016; Amorati & Valgimigli, 2015; 2018). The choice of sunflower oil and squalene as model oxidizable substrates was based on their importance as dietary lipids and on their structural differences (see Fig. 1), so to comprise the variability encountered in food products. To prove that results are of general relevance and are not dependend on the substrate, we extended the investigation to styrene, since it is the best 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, **3H$_2$**)
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, **2H$_2$**),
116 3,5-di-*tert*-butylcatechol (DTBC, **3H$_2$**), and 3,5-di-*tert*-butyl-*o*-benzoquinone (DTBQ, **3**) were used
117 as received. Squalene (≥98%), styrene (≥99%) and γ-terpinene (97%) were percolated twice through
118 activated basic alumina and once therough 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).

131 $$\text{Initiator} \xrightarrow{R_i} \text{R}\cdot \quad (1)$$

132 $$\text{R}\cdot + \text{O}_2 \longrightarrow \text{ROO}\cdot \quad (2)$$

133 $$\text{ROO}\cdot + \text{RH} \xrightarrow{k_p} \text{ROOH} + \text{R}\cdot \quad (3)$$

134 $$2\ \text{ROO}\cdot \xrightarrow{2k_t} \text{products} \quad (4)$$

$$-\frac{d[\text{O}_2]}{dt} = \frac{k_p\,[\text{RH}]\sqrt{R_i}}{\sqrt{2\,k_t}} + R_i \quad (5)$$

135

136 $$\text{ROO}\cdot + \text{AH} \xrightarrow{k_{inh}} \text{ROOH} + \text{A}\cdot \quad (6)$$

137 $$\text{ROO}\cdot + \text{A}\cdot \longrightarrow \text{products} \quad (7)$$

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 O_2 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 consumtion, 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).

$$-\frac{d[O_2]}{dt} = \frac{k_p}{nk_{inh}}\frac{[RH]Ri}{[AH]} + R_i \quad (8)$$

$$R_i = n\,[AH] / \tau \quad (9)$$

The initiation rate R_i was determined in matched preliminary experiments by the inhibitor method, using PMHC as a reference antioxidant and equation 9, where τ is the length of the inhibition time. The length τ could also be used to compare the antioxidant activity directly, the longer of the duration, the bigger of the activity (Amorati, Valgimigli, Dinér, Bakhtiari, Saeedi, & Engman, 2013; Amorati & Valgimigli, 2015).

2.3 UV-Vis Spectroscopy

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

2.4. Statistical Analysis

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

3. Results and Discussion

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

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

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

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

The antioxidant behavior of γ-terpinene was investigated in the controlled autoxidation of both SSO and squalene. The analogue of α-tocopherol PMHC (**1H**), and the lipid-soluble phenethyl ester derivative of caffeic acid (CAPE, $\mathbf{2H_2}$) were also used as antioxidants in matched experiments with the same lipid substrates. Results summarized in Fig. 2 showed that γ-terpinene alone, in the millimolar range, could only slow down the lipid autoxidation by a modest margin, while, as expected, the mono- and poly-phenolic antioxidants (**1H** and $\mathbf{2H_2}$) showed neat inhibition of the autoxidation already at micromolar concentrations. Most interesting, when γ–terpinene and each of the phenolic antioxidants where used together in the protection of either SSO or squalene, the antioxidant activity toward both lipids was greatly enhanced as compared to the use of **1H** or $\mathbf{2H_2}$ alone, being clearly higher that the sum of the contributions of each phenolic antioxidant and γ–terpinene, *i.e.* the combination showed clear synergic antioxidant effect.

<Fig. 2 about here>

In oxygen-uptake kinetics during autoxidation, when a clear inhibited period is observed as in Fig. 2 (A,C,D), the slope on the inhibited period is inversely related to the rate constant for peroxyl radical trapping by the antioxidant (see eq. 8), while its duration (τ) depends on the concentration of the antioxidant and the stoichiometric factor *n*, *i.e.* the number of radicals trapped by each molecule of antioxidant according to eq. 9. An inspection of Fig. 2 shows that while γ–terpinene produced no neat inhibition period, and both **1H** or $\mathbf{2H_2}$ did so, the combination of either **1H** or $\mathbf{2H_2}$ with γ–

terpinene did not significantly change the slope of the inhibited period as compared to that produced by each of the phenols alone, but it extended its duration in a dose-dependent fashion (see inserts in Figure 2A-D). This kinetic behavior would indicate, that γ-terpinene acts as the co-antioxidant which regenerates the main antioxidant, **1H** or $\mathbf{2H_2}$, as it is consumed during the autoxidation, similarly to the behaviour previously observed and discussed for other co-antioxidant couples (Amorati, Ferroni, Lucarini, Pedulli., & Valgimigli, 2002; Valgimigli et al. 2013)

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

Results were qualitatively superimposable to those obtained with SSO and squalene as the oxidizable substrates (see Appendix, Fig. S1). From the oxygen-uptake plots, the apparent *n* value of the mixtures was calculated according to equation 9, where R_i is the rate of radial initiation produced by AIBN. According to the well-known chain-breaking antioxidant mechanism, each molecule of phenol or catechol inactivates two ROO• (see eqs. 6, 7). Hence, the stoichiometric factors of both **1H** and $\mathbf{2H_2}$, have the theoretical value $n = 2$. The apparent *n* value obtained for the phenolic antioxidants when mixed with different mounts of γ-terpinene, shown in Table 1, were always larger than 2, and increased with the concentration of γ-terpinene. Moreover, there was no major difference between the rate of oxygen consumption recorded in the presence or absence of γ-terpinene, hence both for **1H** and $\mathbf{2H_2}$ the apparent rate constants for inhibition k_{inh} (calculated from eq. 8) was not significantly different in the presence/absence of γ-terpinene (see Table 1), which supports the role γ-terpinene

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

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

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

All values are average from at least 3 independent measurements. [a] Errors for n and k_{inh} represent ± SD.

3.2. Exploration of the mechanism behind the synergy

Phenolic antioxidants can retard or block the oxidation of lipids by scavenging chain-carrying peroxyl radicals. Both monophenolic antioxidants like α-tocopherol or **1H** and polyphenolic like caffeic acid or **2H₂** have a stoichiometric factor n = 2, although they possess different number of active OH groups. The first step of their antioxidant mechanism is similar: a formal H-atom transfer to a ROO•. 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 transfering 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, Saeedi, & 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 peroxyl 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 attaked 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

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

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

The regeneration of the starting phenol from its phenoxyl radical by HOO• generated from γ-terpinene nicely explains the synergy with monophenols like PMHC **1H** as depicted in Fig. 3B; however, in the case of catechols, in principle regeneration could occur both by 1-electron reduction of the semiquinone radical and by (stepwise) 2-electron reduction of the quinone as depicted in Fig. 3C. To achieve a better understanding of the regeneration mechanism of phenolic antioxidants by γ-terpinene, matched sets of experiments were conducted by injecting γ-terpinene into the styrene autoxidation system at the beginning of the experiment and/or after the phenolic antioxidant was consumed, *i.e.* at the time when the substrate starts to oxidize again. PMHC **1H** was again used as the prototype of monophenolic antioxidants, while 2,5-di-*tert*-butyl catechol (DTBC, $\mathbf{3H_2}$) was used as model for polyphenols, since it is a better established catechol-type antioxidant (Amorati, Valgimigli, Panzella, Napolitano & d'Ischia, 2013) and its corresponding quinone (DTBQ, **3**) is also commercially available and stable in solution.

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

Fig. 3B. On the other hand, using the catechol **3H$_2$** instead of **1H**, injection of γ-terpinene caused the reboot of a new inhibition period when injected after the complete consumtion of **3H$_2$**, i.e. when inhibition by **3H$_2$** had terminated and the autoxidation was running again uninhibited (Fig. 4B). This implies that the quinone (**3**) formed as the final oxidized products of the catechol can be reduced back to the starting antioxidant (see Fig. 3C).

<Fig. 4 about here>

Quinones are easily formed by the oxidation of related catechols, and they are generally expected to be the main final oxidized product when catechols behave as antioxidants. This was also confirmed in our experimental settings by monitoring the growth of UV absorbance of *ortho*-quinone **3** at 400nm during the autoxidation of styrene inhibited by **3H$_2$** (Figure 5A). To confirm our hypothesis, autoxidation experiments were performed using the oxidized quinone DTBQ **3** as the antioxidant. While the injection of **3** alone in autoxidizing styrene did not produce any inhibition (Fig. 5B) subsequent injection of γ –terpinene caused the appearance of a long inhibition period, confirming that the quinone **3** could be regenerated by γ-terpinene to its starting catechol. This was also confirmed by parallel experiments in which the time-course of the concentration of **3** was monitored by UV spectroscopy at 400 nm during autoxidations inhibited by **3** and γ-terpinene: the UV signal of **3** progressively declined during the autoxidation supporting its reduction to **3H$_2$** (see Appendix, Fig. S3). Additional experiments with co-injection of the the quinone and γ-terpinene in different order confirmed this conclusion (see Appendix, Fig. S4).

<Fig. 5 about here>

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

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

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

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

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

Declaration of interest

The authors declare no competing financial interest.

Acknowledgments

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Appendix A. Supplementary data

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

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Figure captions

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

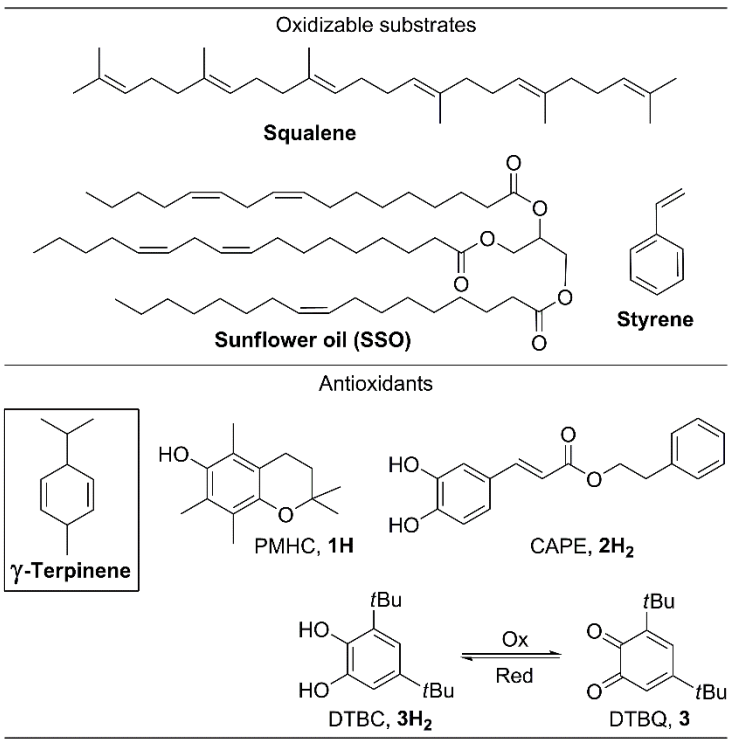
Fig 2. Oxygen consumption during the autoxidation of SSO (A-B), and squalene (C-D). Each panel displays the curves recorded for the autoxidation of the substrates without inhibitors (dotted lines), and in in the presence of 15.65 mM γ-terpinene (dashed lines). In panels A and C grey plots represent the inhibition by PMHC **1H** (2.5 μM) alone, while black curves represent inhibition by the mixture of **1H** (2.5 μM) with γ-terpinene (15.65 mM). In panels B and D grey plots represent inhibition by CAPE $\mathbf{2H_2}$ (2.5 μM) alone, and black curves inhibition by the mixture of $\mathbf{2H_2}$ (2.5 μM) with γ-terpinene (15.65 mM). Inserts: plots of the experimental inhibition periods τ *vs* the concentration γ-terpinene for different mixtures **1H** or $\mathbf{2H_2}$ (2.5 μM) / γ-terpinene.

Fig. 3. Mechanism of the antioxidant activity of (A) γ-terpinene alone (after Foti & Ingold, 2003) and of the antioxidant synergy between (B) γ-terpinene and monophenolic antioxidants (using **1H** as model compound) and (C) γ-terpinene and polyphenolic antioxidants (using $\mathbf{2H_2}$ or $\mathbf{3H_2}$ as model compound).

Fig. 4. Oxygen consumption measured during the autoxidation of styrene initiated by 0.025 M AIBN at 30°C. (A) 1: uninhibited; 2: injection of **1H** (2.5 μM); 3: injection of γ-terpinene (15.7 mM). (B) 1: uninhibited; 2: injection of $\mathbf{3H_2}$ (7.5 μM); 3: injection of γ-terpinene (15.7 mM).

Fig. 5. (A) Growth 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 $\mathbf{3H_2}$ 67 μM. (B) Oxygen consumption during the autoxidation of styrene initiated by 0.025 M AIBN at 30°C in the absence of antioxidant (1), and upon their addition: 2 = injection of DTBQ **3** (7.5 μM); 3 = injection of γ-terpinene (7.83 mM). (C) Oxygen consumption during the autoxidation of SSO initiated by 0.025 M AIBN at 30°C in the

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



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

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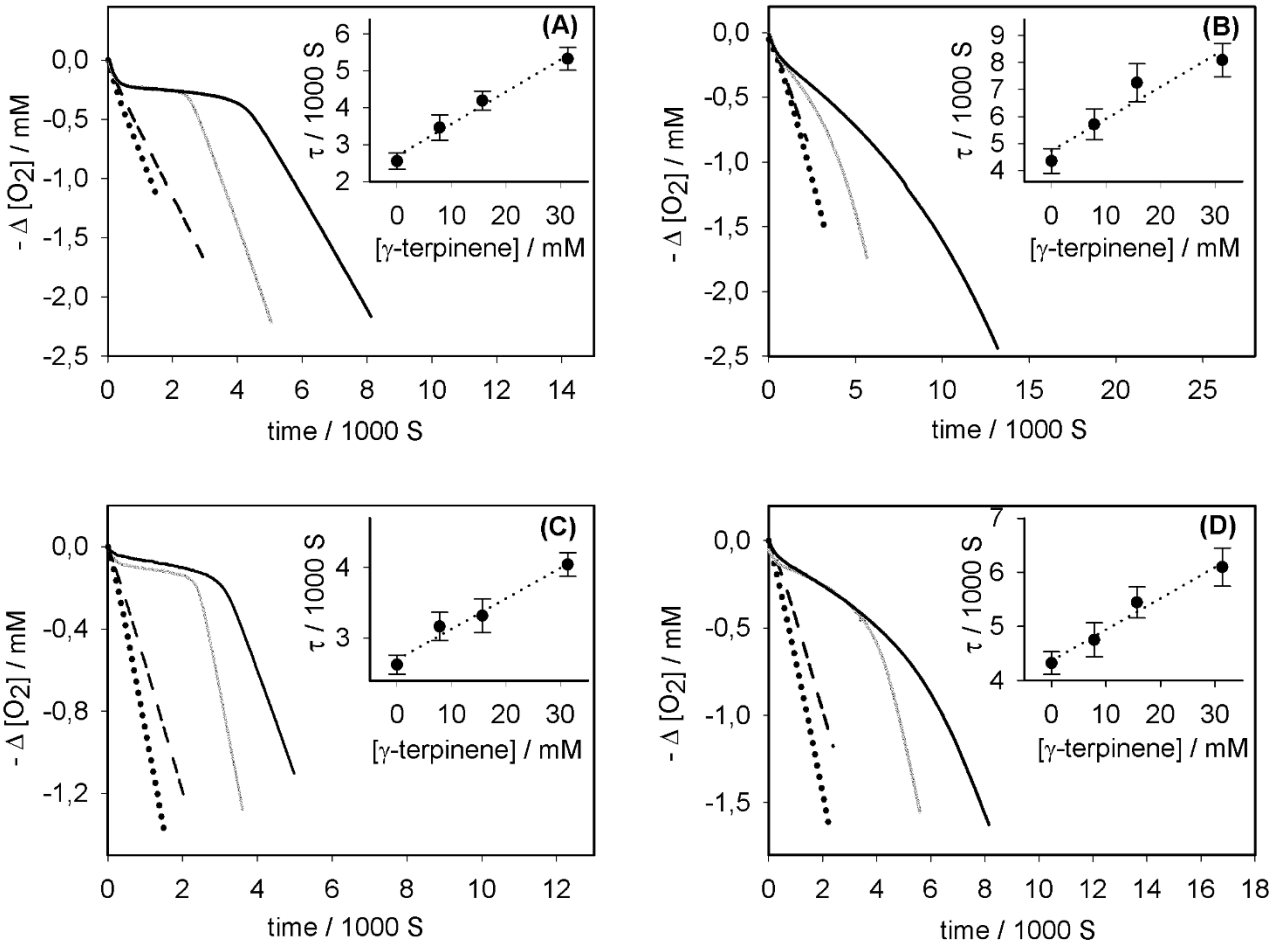

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600 **Fig 2.** Oxygen consumption during the autoxidation of SSO (A-B), and squalene (C-D). Each panel
601 displays the curves recorded for the autoxidation of the substrates without inhibitors (dotted lines),
602 and in in the presence of 15.65 mM γ-terpinene (dashed lines). In panels A and C grey plots represent
603 the inhibition by PMHC **1H** (2.5 μM) alone, while black curves represent inhibition by the mixture
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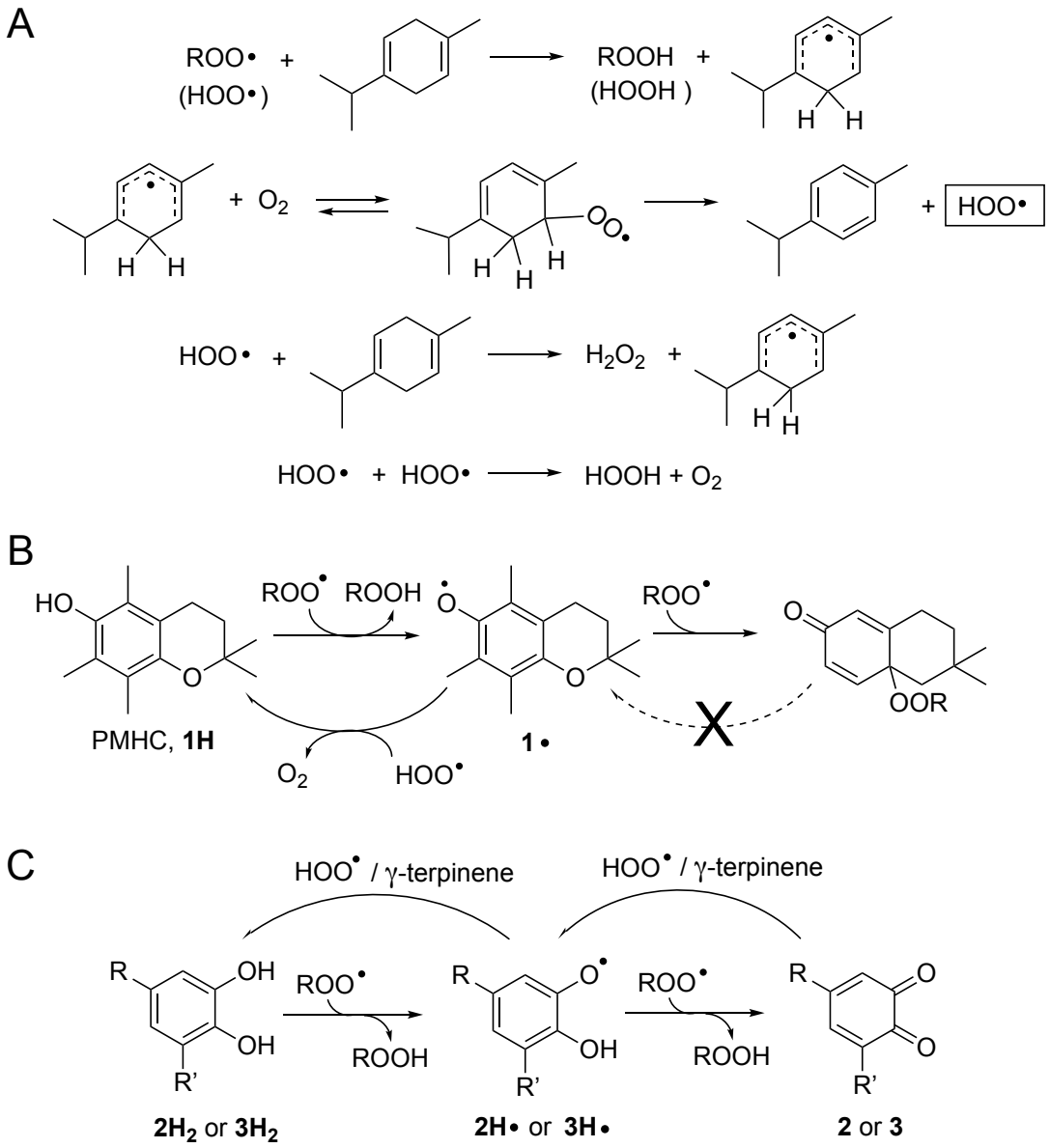

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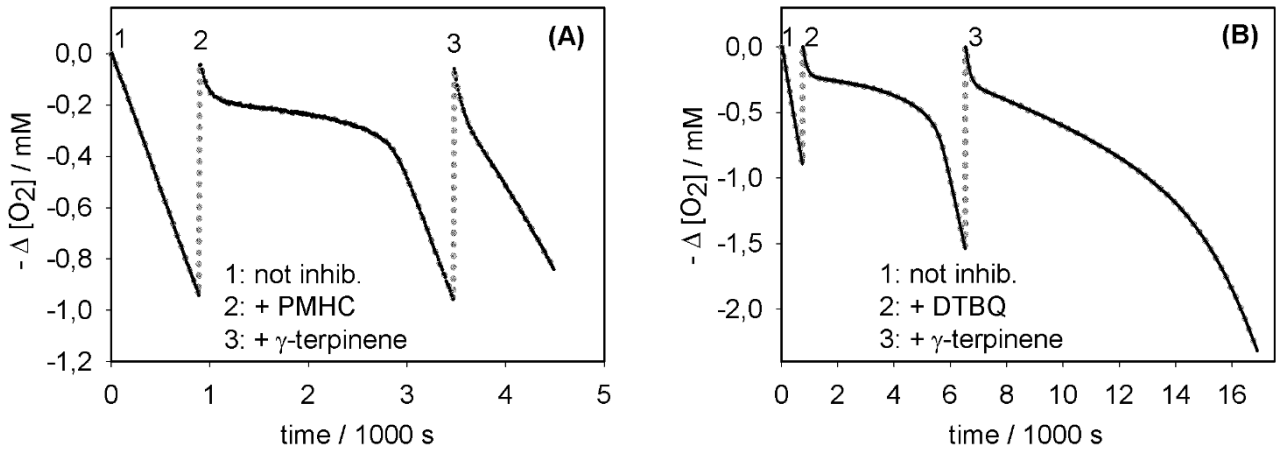

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624 **Fig. 4**. Oxygen consumption measured during the autoxidation of styrene initiated by 0.025 M AIBN

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 $\mathbf{3H_2}$ (7.5 μM); 3: injection of γ-terpinene (15.7 mM).

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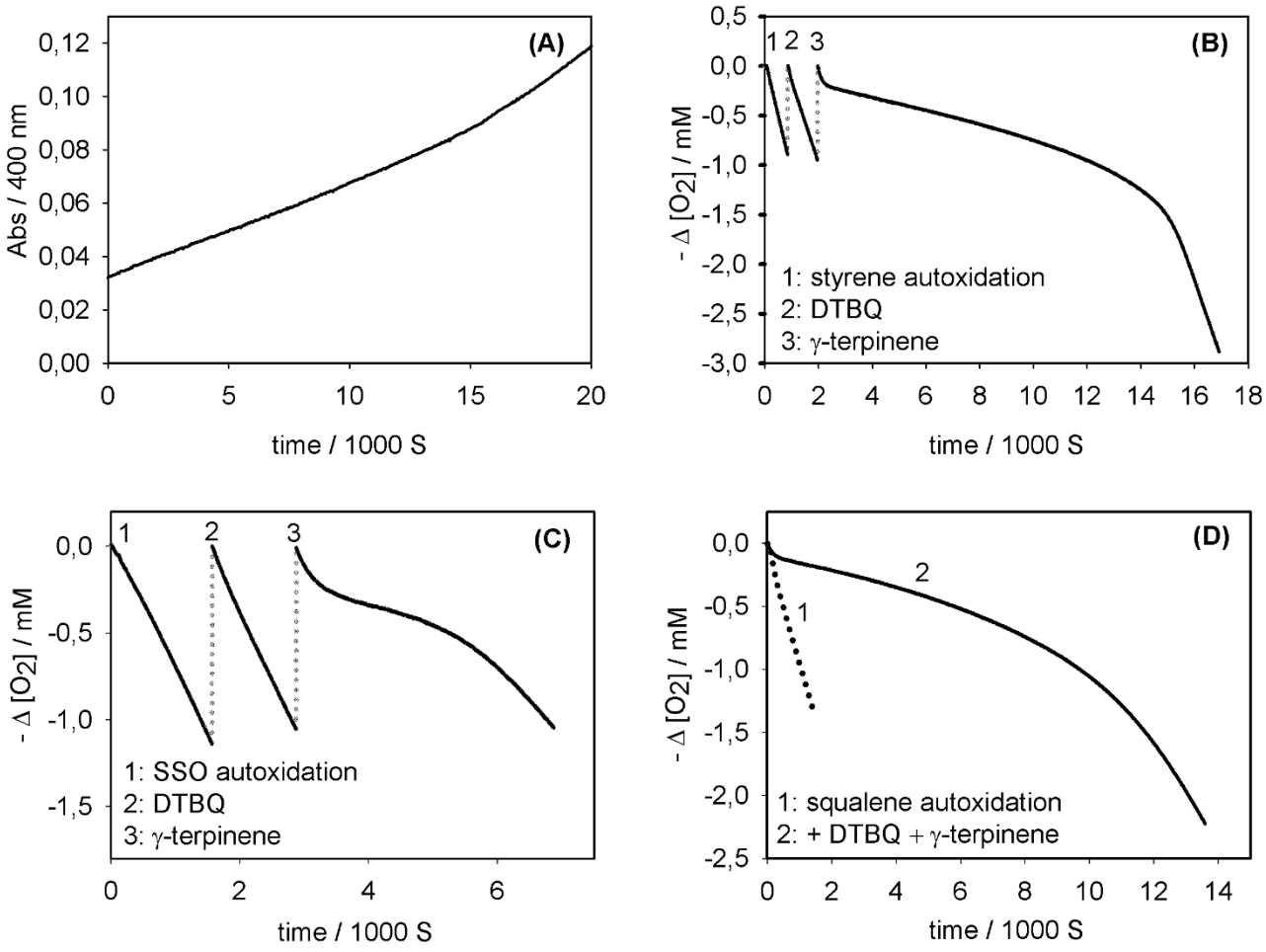

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640 **Fig. 5.** (A) Growth of the UV signal at 400 nm due to quinone **3** during the autoxidation of styrene
641 initiated by 0.025 M AIBN at 30°C, inhibited by **3H$_2$** 67 μM. (B) Oxygen consumption during the
642 autoxidation of styrene initiated 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). (C)
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).

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

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Appendix A – Supplementary Material

Content

Fig. S1.	Autoxidation of styrene inhibited by **1H** or **2H**$_2$ and γ-terpinene.	Page 2
Fig. S2.	Autoxidation of styrene inhibited by **1H** and γ-terpinene used in sequence.	Page 2
Fig. S3.	Decay of UV signal at 400 nm during the autoxidation of styrene inhibited by **3** and γ-terpinene.	Page 3
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Fig. S6.	Autoxidation of sunflower oil inhibited by vitamin E or hydroxytyrosol in the absence or presence of γ-terpinene.	Page 4

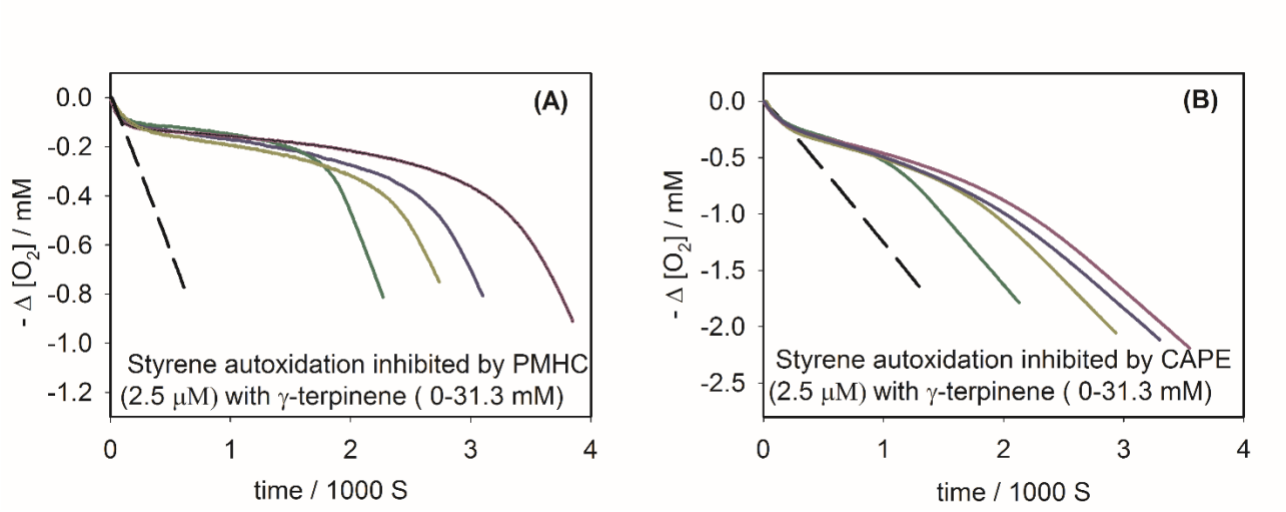

Fig. S1. Oxygen consumption measured during the autoxidation of styrene initiated by 0.025 M AIBN at 30°C in the absence of antioxidant (dashed line in both panels), and inhibited by (A) PMHC **1H** (2.5μM) in the absence of γ-terpinene (green line), in the presence of γ-terpinene (7.83mM-yellow line; 15.7mM-blue line; 31.3mM -purple line), or by (B) CAPE **2H**$_2$ (2.5μM) alone (green line) or in the presence of growing amounts of γ-terpinene (the same color coding as in panel A is used).

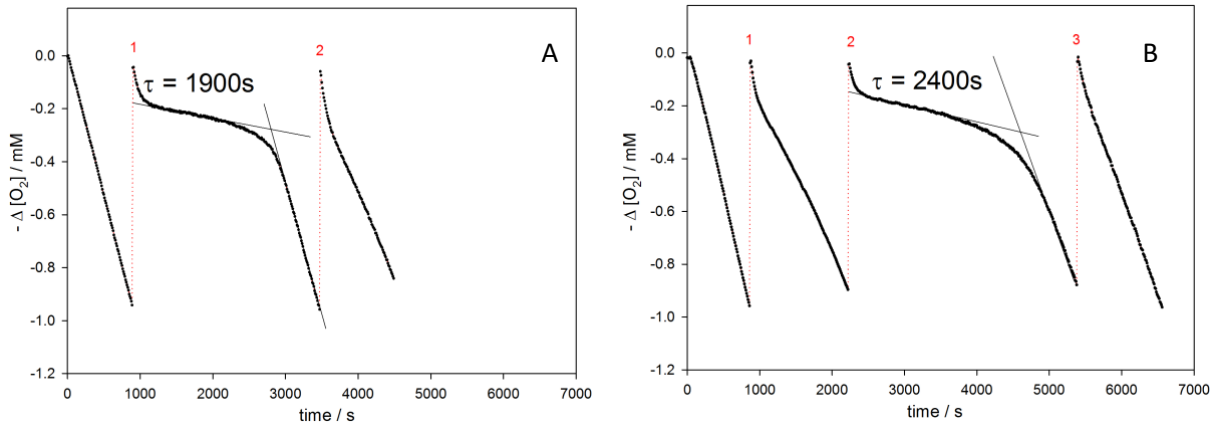

Fig. S2. Oxygen consumption during the autoxidation of styrene initiated by 0.025 M AIBN at 30° without inhibitors and upon injection of antioxidants at the time points indicated by numbers: (A) 1 = injection of PMHC 1H (2.5 μM), 2 = subsequent injection of γ-terpinene (20 mM); (B) 1 = injection of γ-terpinene (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.

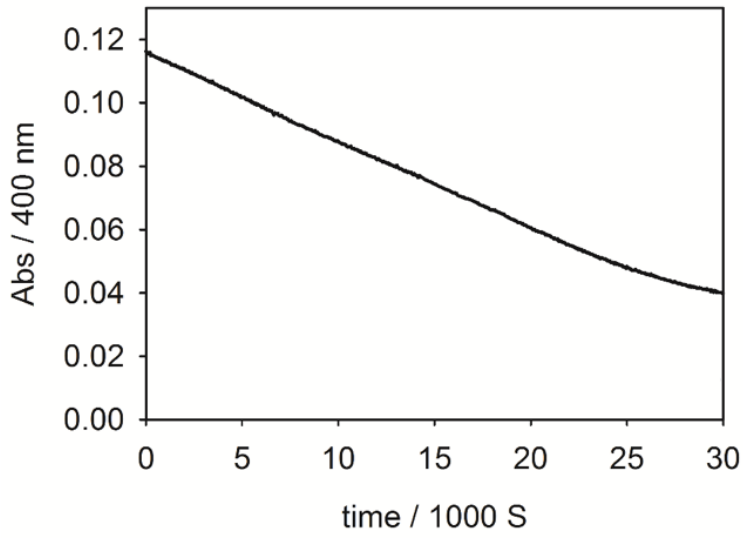

Fig. S3. Decay of the UV signal at 400 nm due to quinone **3** during the autoxidation of styrene initiated by 0.025 M AIBN at 30°C, inhibited by **3** (67 µM) and γ-terpinene (15.7 mM).

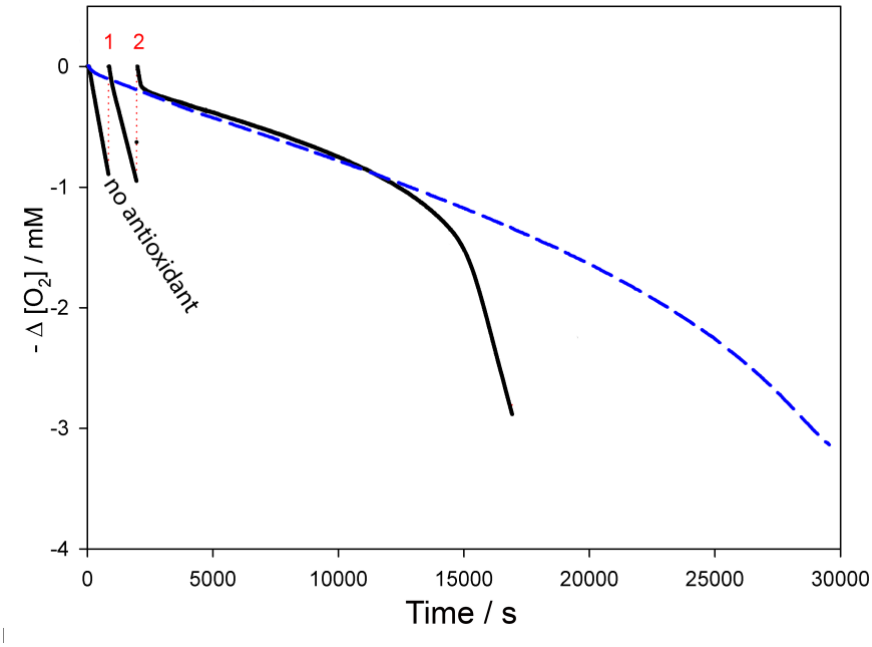

Fig. S4. Oxygen consumption measured during the autoxidation of SSO initiated by 0.025 M AIBN at 30°C in the absence of antioxidants and (1) upon injection of DTBQ **3** (7.5 µM), (2) upon subsequent injection of γ-terpinene (15.7 mM). The blue dashed line represents the autoxidation under identical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

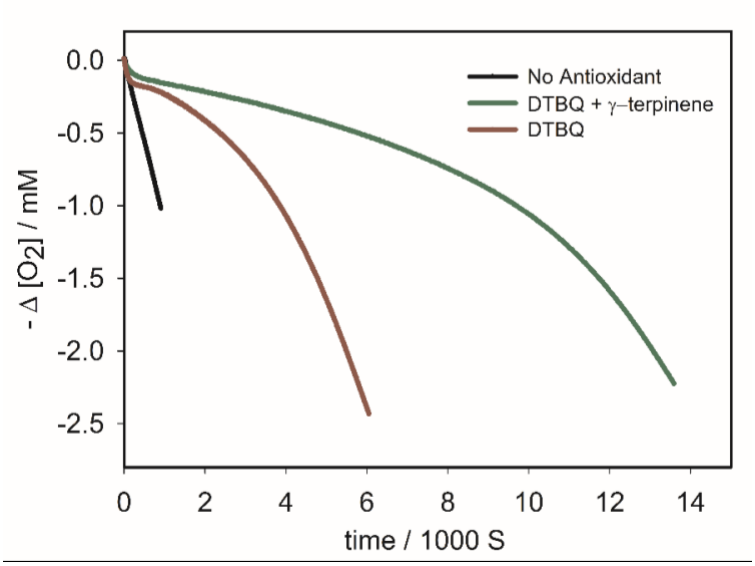

Fig. S5. Oxygen consumption measured during the autoxidation of squalene initiated by 0.025 M AIBN at 30°C in the absence of antioxidant (black line), inhibited by 2.5 µM DTBQ **3** (red line), or inhibited by 2.5 µM DTBQ **3** and 15.7 mM γ-terpinene (green line).

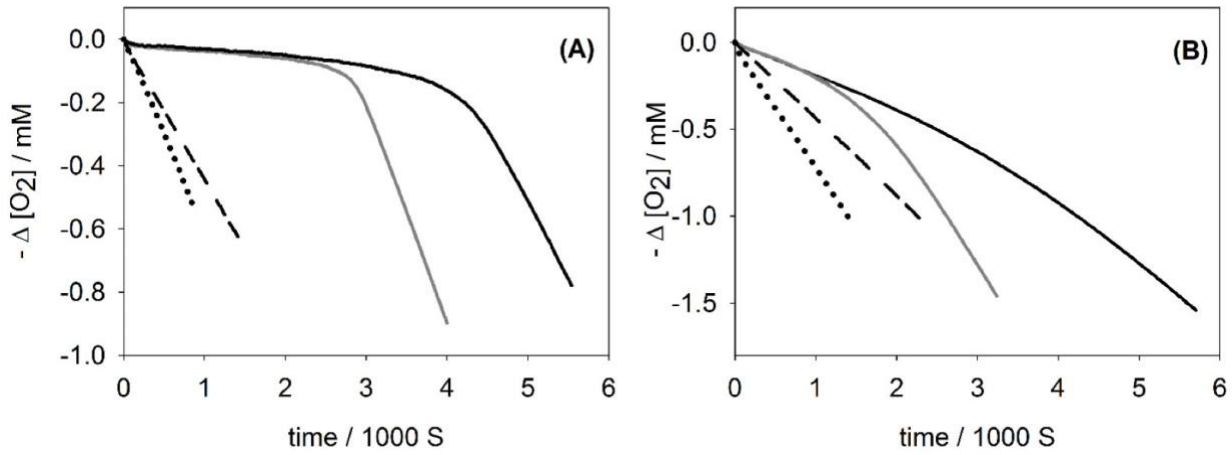

Fig. S6. Oxygen consumption measured during the autoxidation of sunflower oil initiated by 0.025 M AIBN at 30°C in the absence of antioxidants (dotted lines), inhibited by 15 mM γ-terpinene (dashed lines), or (A) inhibited by 3 µM vitamin E (*d*-α-tocopherol, gray line) or by a mixture of 2.5 µM vitamin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