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The paradoxical effect of extra-virgin olive oil on oxidative phenomena during *in vitro* co-digestion with meat

Serena Martini, Martina Cavalchi, Angela Conte, Davide Tagliazucchi*

Department of Life Sciences, University of Modena and Reggio Emilia, Via Amendola 2, 42100

Reggio Emilia, Italy

* Corresponding author. Tel.: +39-0522-522060; fax: +39-0522-522053

E-mail address: davide.tagliazucchi@unimore.it (D. Tagliazucchi)

1 **Abstract**

2 Extra-virgin olive oil is an integral part of the Mediterranean diet and its consumption has been
3 associated with a reduction risk of chronic diseases. Here we tested the potential of extra-virgin olive
4 oil to limit the oxidative phenomena during *in vitro* gastro-intestinal co-digestion with turkey breast
5 meat. The extra-virgin olive oil was particularly rich in oleuropein aglycone isomers, which
6 represented the 66.8% of total phenolic determined with MS/MS experiments. Meals supplemented
7 with extra-virgin olive oil equivocally affected lipid peroxidation. At low concentration (2.5% respect
8 to meat), a significant inhibition of lipid oxidation was observed, whereas lipid peroxidation was
9 greatly enhanced when the amount of extra-virgin olive oil was increased in the gastro-intestinal
10 system. The inhibitory effect observed at 2.5% extra-virgin olive oil was due to the antioxidant
11 properties of extra-virgin olive oil phenolic compounds. At high concentration, extra-virgin olive oil
12 phenolic compounds (especially hydroxytyrosol-derivative) behaved as pro-oxidants increasing the
13 generation of lipid hydroperoxides from meat. At the same time, the presence in the digestive system
14 of catalyzers from meat induced the peroxidation of extra-virgin olive oil fatty acids, which was
15 further intensified by the pro-oxidant activity of extra-virgin olive oil phenolic compounds. Our study
16 underlined the importance of the timing and amount of consumption of extra-virgin olive oil as well
17 as its phenolic composition in limiting the peroxidative phenomena on meat lipids during digestion.

18 **Keywords:** extra-virgin olive oil, mass spectrometry, Mediterranean diet, oleuropein, lipid
19 peroxidation, antioxidant activity, pro-oxidant activity

20 **1. Introduction**

21 The traditional Mediterranean diet is likely to be the ideal dietary pattern for the prevention of
22 digestive tract cancers and cardiovascular diseases (Barak & Fridman, 2017). The health benefits of
23 the Mediterranean diet have been associated with the high intake of vegetable foods rich in
24 phytochemicals such as fruits, vegetables, cereals, legumes, nuts and seeds, the moderate
25 consumption of fermented dairy products, fish, poultry and wine and the low intake of meats (Bach-
26 Faig et al., 2011). In addition, also the typical cooking procedures are especially effective in
27 guaranteeing the highest nutritional value in terms of phytochemicals bioavailability and
28 preservation of the raw materials (Pellegrini & Fogliano, 2017). Olive oil and especially extra-
29 virgin olive oil (EVOO) represented the typical fat of Mediterranean cuisine and showed unique
30 healthy features (Covas, 2007). Extra-virgin olive oil, produced by mechanically pressing ripe
31 olives, contains several bioactive and antioxidant components such as polyphenols, phytosterols and
32 vitamin E as well as monounsaturated fatty acids (Covas, 2007). Two recent studies published in
33 the PREDIMED project showed that a Mediterranean diet supplemented with extra-virgin olive oil
34 reduced the incidence of major cardiovascular events and cardiovascular mortality in a
35 Mediterranean population at high cardiovascular risk respect to a low-fat Mediterranean diet
36 (Estruch et al., 2013; Guasch-Ferré et al. 2014). Indeed, a randomized, crossover, controlled
37 trial suggested that daily consumption of high- and medium-polyphenol olive oil decreased
38 oxidative damage on lipids and reduced lipid cardiovascular risk factors respect to the consumption
39 of low-polyphenol olive oil (Covas et al., 2006).

40 The typical Western diet, instead, is characterized by high intake of fried foods, salty snacks, high-
41 fat dairy products, eggs and meat and low intake of plant-based foods. Although the results are still
42 controversial (Li et al., 2015), some studies have associated the Western dietary pattern with higher
43 risk of colorectal tumours and cardiovascular diseases (Kesse, Clavel-Chapelon, & Boutron-Ruault,
44 2006). In this context, a high intake of meat (especially red meat and processed meat) has been
45 associated with an increased risk of cancers, principally colorectal cancer, and cardiovascular

46 diseases (Ferguson, 2010; Micha, Wallace, & Mozaffarian, 2010). There is evidence that this risk
47 may not be caused by meat per se, but may reflect high-fat intake, and/or carcinogens generated
48 through various cooking and processing methods (Ferguson, 2010; Gorelik, Kanner, Schurr, &
49 Kohen, 2013). In particular, the oxidative phenomena involving polyunsaturated fatty acids
50 occurring during meat cooking and gastro-intestinal digestion can result in the formation of lipid
51 oxidation products, such as lipid hydroperoxides and small reactive compounds collectively known
52 as advanced lipoxidation end-products (ALEs), such as malondialdehyde and 4-hydroxy-2-nonenal,
53 which might adversely affect human health following their consumption (Papuc, Goran, Predescu,
54 & Nicorescu, 2016). Lipid hydroperoxides generated during meat cooking and especially during
55 gastro-intestinal digestion can be absorbed in the human gastro-intestinal tract, incorporated in
56 chylomicrons and, thus, trigger the onset and progression of atherosclerosis (Staprans, Rapp, Pan,
57 Kim, & Feingold, 1994). Lipid hydroperoxides can also induce oxidative stress damage and
58 antioxidant enzyme response in Caco-2 human colon cells participating in tissue injuries and in the
59 onset and progression of degenerative diseases in humans (Wijeratne & Cuppett, 2006). ALEs are
60 also considered highly cytotoxic, mutagenic and carcinogenic compounds, which can be also
61 involved in the progression of atherosclerosis (Papuc et al., 2016). Strategies to inhibit the
62 formation of lipid hydroperoxides and ALEs during gastro-intestinal digestion of meat could
63 mitigate these health risks. Kanner and co-workers showed that red wine polyphenols inhibited the
64 formation of lipid oxidation products during the *in vitro* gastric digestion of red meat and this
65 decrease was accompanied by a reduction in the absorption of ALEs in humans (Gorelik,
66 Ligumsky, Kohen, & Kanner, 2008a; Gorelik, Ligumsky, Kohen, & Kanner, 2008b). Other *in vitro*
67 and *in vivo* studies demonstrated that oxidation during digestion can be reduced when meat is
68 combined with other foods such as coffee or spices (Sirota, Gorelik, Harris, Kohen, & Kanner,
69 2013; Tagliazucchi, D., Verzelloni, E., & Conte, A., 2010; Van Hecke, Ho, Goethals, & De Smet,
70 2017).

71 The aim of this study was to elucidate whether extra-virgin olive oil could affect the oxidative
72 phenomena during co-digestion with grilled turkey meat employing a harmonized basic static
73 COST Action INFOGEST *in vitro* digestive model.

74 **2. Materials and methods**

75 **2.1. Materials**

76 All of the digestive enzymes (α -amylase from porcine pancreas, pepsin from porcine gastric mucosa
77 and pancreatin from porcine pancreas), phenolic standards and reagents for analytical determination
78 were obtained from Sigma-Aldrich (Milan, Italy). The mass spectrometry reagents and solvents for
79 phenolic compounds extraction were obtained from BioRad (Hercules, CA, USA). Turkey breast
80 meat (*pectoralis major*) and extra-virgin olive oil were purchased in a local supermarket (Reggio
81 Emilia, Italy).

83 **2.2. Extraction of phenolic compounds from extra-virgin olive oil**

84 Phenolic compounds from extra-virgin olive oil (EVOO) were extracted following the procedure
85 reported in Martini, Conte, & Tagliacruzchi (2017). Briefly, 15 grams of EVOO were mixed with 15
86 mL of a methanol/water/formic acid solution (70/28/2; v/v) and incubated for 120 minutes at 30°C
87 in a rotary wheel. After incubation, the mixtures were centrifuged at 3000g for 30 minutes at 4°C.
88 When extraction was completed, the samples were stored on freezer shelves at -20°C and allowed
89 to stand overnight for lipid precipitation and separation (Lentza-Rizos, Avramides, & Cherasco,
90 2001). After that, two fractions were obtained: the liquid supernatant (phenolic-rich fraction) and
91 the solid pellet (fat fraction). Both the fractions were stored at -20°C until analysis.

93 **2.3. Total phenolic content and antioxidant activity determination in extra-virgin olive oil** 94 ***phenolic-rich fraction***

95 Total phenolic content was determined on the EVOO phenolic-rich fraction using the Folin-
96 Ciocalteau assay and gallic acid as standard (Singleton, Orthofer, & Lamuela-Raventos, 1999). Data
97 were expressed as mg of gallic acid equivalent per 100 g of EVOO.

98 The total antioxidant properties of EVOO phenolic-rich fraction were analyzed by using five
99 different assays. The radical scavenging ability was assayed by using the ABTS assay according to

100 Re et al. (1999). For the determination of the Fe³⁺ reducing ability, a protocol based on the ferric
101 reducing/antioxidant power (FRAP) assay was utilized (Benzie & Strain, 1999). The capacity to
102 scavenge hydroxyl radical and superoxide anion were evaluated according to the methods reported
103 by Martini, Conte, & Tagliazucchi (2017). The results were expressed as μmol of trolox
104 equivalent/mmol of phenolic compounds. The Fe²⁺-chelation ability of EVOO phenolic-rich
105 fraction was evaluated by the ferrozine assay (Karama & Pegg, 2009).

106

107 ***2.4. Identification and quantification of phenolic compounds by liquid chromatography***
108 ***electrospray ionization ion trap mass spectrometer (LC-ESI-IT-MS)***

109 Phenolic-rich fraction of EVOO was analyzed on a HPLC Agilent 1200 Series system equipped
110 with a C18 column (HxSil C18 Reversed phase, 250×4.6 mm, 5 μm particle size, Hamilton
111 company, Reno, Nevada, USA) as reported in Mena, Cirlini, Tassotti, Herrlinger, Dall'Asta, & Del
112 Rio (2016). The mobile phase consisted of (A) H₂O/formic acid (99.9:0.1, v/v) and (B)
113 acetonitrile/formic acid (99.9:0.1, v/v). The gradient started at 1% B for 1 min then linearly ramped
114 up to 40% B in 13 min. The mobile phase composition was raised up to 99% B in 13 min and
115 maintained for 2 min in order to wash the column before returning to the initial condition. After
116 passing through the column, the eluate was split, and 0.3 mL/min was directed to an Agilent 6300
117 ion trap mass spectrometer. Negative ESI-MS parameters were the same as reported in Martini et al.
118 (2017). Identification of phenolic compounds in all samples was carried out using full scan, data-
119 dependent MS² scanning from *m/z* 100 to 1500 and selected reaction monitoring.

120 Phenolic compounds were quantified in hydroxytyrosol equivalents with the exception of luteolin
121 that was quantified as luteolin equivalents.

122

123 ***2.5. In vitro digestion of grilled turkey breast meat***

124 Turkey breast meat (average size of 10x15x0.4 cm) was cooked on a grill at 140°C for 5 min until
125 complete cooking was achieved. After cooking, the meat was cooled on ice and stored at -80°C

126 overnight. Frozen meat was then homogenized in a laboratory blender and divided in portions of 5
127 g. Grilled and homogenized turkey meat was *in vitro* digested following the protocol previously
128 developed within the COST Action INFOGEST (Minekus et al., 2014). Simulated salivary, gastric,
129 pancreatic and bile fluids were prepared according to Minekus et al. (2014). To simulate the oral
130 phase, 5 g of homogenized grilled turkey breast meat were mixed with 5 mL of simulated salivary
131 fluid containing 150 U/mL of porcine α -amylase and incubated for 5 min at 37°C in a rotating
132 wheel (10 rpm). The gastric phase was carried out by adding 10 mL of simulated gastric fluid to the
133 bolus. The pH was adjusted to 2.0 with HCl 6 mol/L and supplemented with porcine pepsin (2000
134 U/mL of simulated gastric fluid). The gastric bolus was then incubated for 120 min at 37°C in a
135 rotating wheel (10 rpm). The intestinal digestion was carried out by adding 10 mL of pancreatic
136 fluid and 5 mL of bile fluid to the gastric bolus, adjusting the pH to 7.0 and supplemented with
137 pancreatin. The chyme was further incubated for 120 min at 37°C in a rotating wheel (10 rpm). For
138 each digestion, aliquots were taken after 0 and 5 minutes of salivary digestion, after 30, 60, 90 and
139 120 minutes of gastric digestion and after 30, 60, 90 and 120 minutes of intestinal digestion. The
140 digested samples were immediately cooled on ice and frozen at -80°C for further analysis. The
141 digestions were performed in triplicate. In addition, a control digestion, which included only the
142 gastro-intestinal juices and enzymes and water in place of meat, was carried out to consider the
143 possible impact of the digestive enzymes and fluids in the subsequent analysis.

144

145 ***2.6. In vitro co-digestion of grilled turkey breast meat with extra-virgin olive oil, extra-virgin*** 146 ***olive oil phenolic-rich fraction or extra-virgin olive oil fat fraction***

147 In the co-digestion experiments EVOO was added to the grilled and homogenized turkey breast
148 meat in proportion of 2.5%, 5% and 10% respect to meat (w/w). After that, the *in vitro* digestions
149 were carried out as reported above. Specific control digestions, in which meat was replaced with
150 water, were carried out to check the possible impact of EVOO in the subsequent analysis.

151 Further experiments were carried out to gain more information about the effect of EVOO
152 polyphenols or fatty acids on the oxidative phenomena during *in vitro* co-digestion with meat.
153 These co-digestions were carried out as reported above but replacing EVOO with the corresponding
154 amount of EVOO phenolic-rich fraction or EVOO fat fraction.

155

156 ***2.7. Determination of lipid hydroperoxides***

157 Lipid hydroperoxides were extracted by 10-fold dilution in methanol HPLC grade under slow
158 stirring for 60 min (Tagliazucchi et al., 2010). After centrifugation at 3000g for 15 min at 4°C, the
159 hydroperoxides in the supernatants were determined with the FOX assay (Nourooz-Zadeh, 1999) at
160 560 nm adapted to a microplate reader. The FOX reagent contained 250 µmol/L of ammonium
161 ferrous sulfate, 100 µmol/L xylenol orange, 25 mmol/L H₂SO₄, and 4 mmol/L BHT in 90% (v/v)
162 methanol HPLC grade. For the assay, 60 µL of extracted sample were added to 140 µL of FOX
163 reagent and incubated for 30 minutes at room temperature. The hydroperoxides content was
164 expressed in nanomol H₂O₂ equivalents per g of meat.

165

166 ***2.8. Determination of advanced lipoxidation end-products***

167 Advanced lipoxidation end-products were quantified as thiobarbituric acid-reactive substances
168 (TBA-RS) on digested samples as reported by Buege & Aust (1978). Briefly, 80 µL of digested
169 sample was added to 200 µL of water, 120 µL of trichloroacetic acid 50% and 200 µL of
170 tiobarbituric acid solution (0.75% in 0.5 N HCl). The mixture was incubated for 30 min in boiling
171 water, cooled, and then centrifuged at 10000g for 5 min at 20°C. The TBA-RS in the supernatant
172 was determined at 532 nm, and the results were expressed as nanomol malondialdehyde (MDA)
173 equivalent per g of meat.

174

175 ***2.9. Statistics***

176 All data are presented as mean \pm SD for three replicates for each prepared sample. Univariate
177 analysis of variance (ANOVA) with Tukey's post-hoc test was applied using Graph Pad prism 6.0
178 (GraphPad Software, San Diego, CA, U.S.A.) when multiple comparisons were performed. The
179 differences were considered significant with $P < 0.05$.

180 3. Result

181 3.1. Total phenolic content, phenolic profile and antioxidant properties of extra-virgin olive oil

182 Extra-virgin olive oil (EVOO) was characterized for its content in total phenolic compounds,
183 individual phenolic compounds as well as for its antioxidant properties. The total amount of
184 phenolic compounds extracted from EVOO was 127.8 ± 2.5 mg of gallic acid equivalent/100 g of
185 EVOO. The phenolic profile of EVOO was investigated using a non-targeted procedure through
186 LC-ESI-MS/MS experiments. The mass spectrum data along with peak assignments and retention
187 time for the identified phenolic compounds are described in **Table 1**. This approach allowed the
188 tentative identification of 33 compounds (**Table 1**). The total amount of phenolic compounds
189 identified by MS/MS experiments was 98.9 ± 1.8 mg/100 g of EVOO, which represented the 77.4%
190 of total phenolic compounds determined with the Folin-Ciocalteu assay. Considering the
191 individual phenolic compounds, the EVOO used in our study was particularly rich in oleuropein
192 aglycone and ligstroside aglycone. The sum of the amount of the oleuropein aglycone isomers
193 represented the 51.7% and the 66.8% of total phenolic determined with the Folin-Ciocalteu assay
194 and the MS/MS experiments, respectively. Instead, the amount of ligstroside aglycone isomers was
195 the 21.6% and the 27.9% of total phenolic determined with the Folin-Ciocalteu assay and the
196 MS/MS experiments, respectively. In addition, two non-phenolic compounds, namely elenolic acid
197 (m/z 241; MS² ion fragments at m/z 209, 165, 139, 127, 121) and hydroxyelenolic acid (m/z 257;
198 MS² ion fragments at m/z 225, 195, 137) were identified in the EVOO phenolic-rich extract.
199 EVOO phenolic compounds were able to scavenge hydroxyl radical (569.5 ± 40.5 μ mol of trolox
200 equivalent/mmol of phenolic compounds), superoxide anion (2569.2 ± 194.7 μ mol of trolox
201 equivalent/mmol of phenolic compounds) and the ABTS organic nitro-radical (1218.3 ± 134.8 μ mol
202 of trolox equivalent/mmol of phenolic compounds). There was no evidence of chelation of Fe²⁺ by
203 EVOO phenolic-rich extract. Finally, EVOO phenolic compounds were able to reduce ferric iron to
204 ferrous iron (187.0 ± 17.4 μ mol of trolox equivalent/mmol of phenolic compounds).

205

206 **3.2. Effect of extra-virgin olive oil on lipid oxidation during co-digestion with turkey breast meat**

207 **Figure 1A** shows the cumulative amount of lipid hydroperoxides during *in vitro* digestion of turkey

208 breast meat and during co-digestion of turkey breast meat with different amounts of EVOO. In the

209 control digestion without meat, no reactivity with the FOX assay was detected (data not shown).

210 The level of lipid hydroperoxides measured during the digestion of turkey breast meat remained

211 constant during the 5 minutes of salivary digestion ($P>0.05$). After 30 minutes of the gastric

212 digestion, the level of lipid hydroperoxide significantly increased ($P<0.05$) and then remained

213 constant during the subsequent 90 minutes of gastric digestion (**Figure 1A**). The transition from

214 gastric to pancreatic treatment significantly increased the lipid hydroperoxides value from $406.1 \pm$

215 37.8 nmol H₂O₂/g of meat at the end of gastric digestion to 2127.1 ± 142.8 nmol H₂O₂/g of meat

216 after 30 min of pancreatic digestion ($P<0.001$) (**Figure 1A**). Subsequently, the amount of lipid

217 hydroperoxides decreased gradually reaching the value of 361.9 ± 52.4 nmol H₂O₂/g of meat after

218 120 min of the intestinal incubation. As reported in **Figure 1B**, the amount of TBA-RS tended to

219 increase significantly ($P<0.001$) during the first 60 minutes of gastric digestion of turkey breast

220 meat, after that it did not change significantly during the remaining time of digestion. In the control

221 digestion without meat, no reactivity with the TBA-RS assay was detected (data not shown).

222 When turkey breast meat was co-digested with 2.5% of EVOO we found a decrease in the amount

223 of generated lipid hydroperoxides both during gastric and pancreatic digestion (**Figure 1A**). At the

224 end of the gastric digestion, the amount of lipid hydroperoxides generated in presence of 2.5% of

225 EVOO was 162.7 ± 12.1 nmol H₂O₂/g of meat (inhibition of 59.9%) whereas at the end of the

226 pancreatic digestion the amount of lipid hydroperoxides dropped to a value near to zero (23.5 ± 7.0

227 nmol H₂O₂/g of meat, which resulted in a inhibition of 93.5%). The TBA-RS production during co-

228 digestion with 2.5% of EVOO was inhibited by 33.5% at the end of the gastric digestion and 34.4%

229 at the end of the pancreatic digestion (**Figure 1B**). Surprisingly, when the amount of EVOO was

230 increased in the digestive system we found an unexpected increase in the amount of lipid

231 hydroperoxides (**Figure 1A**) at all times of digestion. The increase in lipid hydroperoxides was

232 dependent on the concentration of EVOO (**Figure 2**). Whereas, the amount of generated TBA-RS
233 was higher in the sample co-digested with EVOO at 5% or 10% respect to turkey breast meat
234 ($P<0.05$) after 30 minutes of gastric digestion, but we did not find significant differences between
235 the digested turkey breast meat and the turkey breast meat co-digested with 5% or 10% of EVOO
236 during the remaining time of digestion (**Figure 1B** and **Figure 2**).

237

238 ***3.3. Effect of extra-virgin olive oil phenolic compounds on lipid oxidation during co-digestion*** 239 ***with turkey breast meat***

240 When turkey breast meat was co-digested with the EVOO phenolic-rich extract at the same
241 concentration as found in 2.5% EVOO, a strong inhibition in both lipid hydroperoxides and TBA-
242 RS formation was revealed (**Figure 3**). At the end of the digestion, the formation of lipid
243 hydroperoxide was totally inhibited whereas the inhibition recorded by the determination of TBA-
244 RS accumulation was 30.8%. Results were quite similar to the inhibition observed after co-
245 digestion of turkey breast meat with 2.5% EVOO. However, co-digestion of turkey breast meat with
246 EVOO phenolic-rich fraction at the same concentrations found in 5% and 10% EVOO determined
247 an increase in the concentration of lipid hydroperoxides, which was dependent on phenol
248 concentration (**Figure 3**). The increase in lipid hydroperoxides concentration due to 5% and 10%
249 EVOO phenolic-rich fraction was lower respect to the increase observed in presence of 5% and
250 10% EVOO. On the contrary, TBA-RS production was inhibited by the addition of phenolic-rich
251 fraction to turkey breast meat at the same concentrations found in 5% and 10% EVOO (**Figure 3**).

252

253 ***3.3. Effect of extra-virgin olive oil fat fraction on lipid oxidation during co-digestion with turkey*** 254 ***breast meat***

255 To demonstrate a possible involvement of EVOO triglycerides in the enhancement of the lipid
256 peroxidation observed at high EVOO concentrations, 10% EVOO was *in vitro* digested without
257 meat. As reported in **Figure 4A**, *in vitro* digestion of 10% EVOO resulted in a lipid hydroperoxides

258 amount at the end of the digestion of 94.15 ± 1.3 nmol H₂O₂/g of meat which was not significantly
259 different with the value measured at the beginning of the digestion (102.70 ± 11.5 nmol H₂O₂/g of
260 meat). No TBA-RS formation was recorded during the digestion of EVOO alone (**Figure 4B**).
261 However, when EVOO fat fraction was co-digested with meat, an increased formation of lipid
262 hydroperoxides and TBA-RS respect to the digestion of meat alone was observed (**Figure 4A** and
263 **B**).

264 4. Discussion

265 Lipid peroxidation of poly-unsaturated fatty acids is an oxidative phenomenon, which ultimately
266 may result in the formation of toxic compounds. A large number of studies have suggested a link
267 between products of lipid peroxidation, such as lipid hydroperoxides and lipid oxidation end-
268 products and various health conditions including atherosclerosis, neurodegenerative diseases, and
269 cancer. Meat, which contains high concentrations of iron catalysers and poly-unsaturated fatty acids
270 such as linoleic, linolenic, arachidonic, and docosahexaenoic acids, is particularly sensitive to lipid
271 oxidation (Tirosh, Shpaizer, & Kanner, 2015). Lipid hydroperoxides and lipid oxidation end-
272 products may be already present in meat but, more interestingly, they may be generated during its
273 gastro-intestinal digestion (Kanner & Lapidot, 2001). In addition, lipid peroxidation proceeds
274 rapidly when the raw meat structure is disrupted such as after cooking and mastication (Papuc et al.,
275 2017). The main pathways to free radical chain reaction initiation in lipid peroxidation during
276 gastro-intestinal digestion of meat involves the formation of the hydroxyl radical (HO•) through
277 Fenton reaction, the production of perhydroxy radical (HOO•) via generation of superoxide anion
278 ($O_2^{\bullet-}$) by reaction between ferrous iron with dissolved oxygen and the generation of
279 perferrylmyoglobin-containing peptides (**Figure 5**) (Carlsen & Skibsted, 2004; Oueslati, de La
280 Pomélie, Santé-Lhoutellier, & Gatellier, 2016; Papuc et al., 2017). All of these reactive species are
281 able to abstract a hydrogen from lipids generating a fatty acyl radical (L•), which in turn reacts with
282 dissolved oxygen to form a hydroperoxyl radical (LOO•). The resulting radical can abstract a
283 hydrogen atom from another unsaturated fatty acyl group (LH) and produce a new fatty acyl radical
284 (L•) and a lipid hydroperoxide (LOOH). In the presence of ferrous iron (Fe^{2+}), lipid hydroperoxide
285 can decompose to an alkoxy radical (LO•), which can undergo cleavage giving rise to a huge range
286 of volatile and non-volatile compounds, collectively known as advanced lipoxidation end-products
287 (Papuc et al., 2017) (**Figure 5**).

288 Perhydroxy and hydroxyl radicals can be easily formed in the gastric milieu in the presence of
289 dissolved oxygen and ferrous iron (Oueslati et al., 2016). Ferrous iron is endogenously present in

290 meat and can be released from meat itself after cooking and mastication (Kanner & Lapidot, 2001
291 Lombardi-Boccia, Martinez-Dominguez, & Aguzzi, 2002). Oxygen can be already present in a low
292 amount in the gastric fluid and can be released from meat following mastication (Kanner & Lapidot,
293 2001). Ferrous iron can generate $O_2^{\bullet-}$ from dissolved oxygen (**Figure 5**). At low pH such as found
294 in the gastric medium $O_2^{\bullet-}$ forms HOO^{\bullet} , which can initiate lipid peroxidation. Indeed, in acidic
295 medium, an important fraction of $O_2^{\bullet-}$ can disproportionate into hydrogen peroxide (H_2O_2) and
296 oxygen (Oueslati et al., 2016). The formation of HO^{\bullet} is possible by H_2O_2 decomposition, catalysed
297 by ferrous iron (Fenton reactions), or by H_2O_2 reaction with $O_2^{\bullet-}$ (Haber–Weiss reaction) (Papuc et
298 al., 2017). Harel & Kanner (1985) estimated the production of H_2O_2 in ground turkey muscle at
299 0.045 mM/h at 37°C and at pH 5.6. Oueslati et al. (2016) demonstrated that under gastric conditions
300 and in presence of oxidants (a mixture of ferrous iron and H_2O_2), $O_2^{\bullet-}/HOO^{\bullet}$ were detected in
301 higher quantity than HO^{\bullet} . Increasing the pH from 3.5 to 6.5 hardly affected the kinetics of free
302 radical production. Tagliazucchi et al. (2010) found that the complete chelation of ferrous iron by
303 EDTA decreased the lipid peroxidation during gastric digestion of turkey breast meat by about 70%
304 suggesting that Fenton chemistry is the most important factor in initiating turkey breast meat lipid
305 peroxidation during gastric digestion. The lower contribution of perferrylmyoglobin-mediated
306 peroxidation in turkey breast meat can be due to the low heme-iron content of turkey breast meat
307 (Lombardi-Boccia et al., 2002). In other meat-types, the relative contribution of free iron and heme-
308 iron in initiating lipid peroxidation can be different depending on their concentration.

309 In our gastro-intestinal system, the level of lipid hydroperoxides increased during gastric digestion
310 of turkey breast meat by 3.7-fold. According to the kinetics of formation of $O_2^{\bullet-}/HOO^{\bullet}$ and HO^{\bullet}
311 under gastric conditions (Oueslati et al. 2016), the greatest increase was found in the first 30
312 minutes of digestion (3-fold increase respect to time zero). The increase in lipid hydroperoxides
313 during gastric digestion was followed by an increase of 3.2-fold (respect to time zero) in the amount
314 of TBA-RS. The transition in the intestinal fluids caused an immediate 5.2-fold increase in the level
315 of lipid hydroperoxides respect to the end of the gastric digestion. This increase could be a

316 consequence of the emulsification and micelization of meat fatty acids by bile salts. Previous work
317 showed that lipid oxidation occurs faster in water-in-oil emulsion than in bulk oil or dispersion
318 without emulsifier (Berton-Carabin, Ropers, & Genot, 2014). The causes can be related to different
319 aspects. Firstly, the creation of interfacial area between the fat and the aqueous phase may favour
320 the contacts between oxidants and oxygen, dissolved in the aqueous phase, and fatty acids (Berton-
321 Carabin et al., 2014). Another cause can be attributed to the solubilisation of already formed lipid
322 hydroperoxides in the micelles, which in turn may promote the oxidative reaction in the micelles
323 itself (Donnelly, Decker, & McClements, 1998). In addition, it has been proved that hydrophobic
324 bile acids, in presence of iron, enhanced lipid peroxidation of arachidonic acid (Sreejayan & von
325 Ritter, 1998). Further incubation in simulated intestinal fluid resulted in the disappearance of lipid
326 hydroperoxides. Rodríguez-Malaver, Leake, & Rice-Evans (1997) found that at pH 7.4 (such as
327 found in the intestinal fluid) copper-induced formation of lipid hydroperoxides in LDL was more
328 rapid respect to pH 5 and that lipid hydroperoxides gradually declined towards zero. Therefore,
329 neutral or slightly alkaline pH values seem to enhance the decomposition of lipid hydroperoxides.
330 The increased production of lipid hydroperoxides in the intestinal fluid and their rapid
331 decomposition did not result in an increased production of TBA-RS.

332 Meals supplemented with EVOO may equivocally affect lipid peroxidation during gastro-intestinal
333 digestion, as found in this study. At low realistic concentration of 2.5% of EVOO, respect to meat
334 (w/w), a significant inhibition of lipid oxidation was observed, whereas lipid peroxidation was
335 greatly enhanced when the EVOO amount was increased in the gastro-intestinal system. *In vitro*
336 digestion of turkey breast meat in presence of EVOO-phenolic rich fraction at the same
337 concentration as found in 2.5% EVOO resulted in a decrease in turkey breast meat lipid
338 peroxidation similar to that observed during co-digestion of turkey breast meat and 2.5% EVOO
339 (**Figures 2 and 3**). This effect was due to the antioxidant properties of EVOO phenolic compounds.
340 EVOO used in this study contained 127.8 mg/100g of total phenolic compounds. Wide ranges
341 (from 10 to 140 mg/100g) have been previously reported for the concentration of total phenolic

342 compounds in EVOO (Del Carlo et al., 2004; Samaniego Sánchez et al., 2007). Furthermore, we
343 demonstrated that EVOO phenolic compounds were efficient scavengers of free radicals. Our
344 findings suggest that EVOO phenolic compounds may act at different levels (**Figure 5**). In the first
345 step of the reaction, they may scavenge superoxide anion ($O_2^{\bullet-}/HOO^{\bullet}$) and hydroxyl radical (HO^{\bullet})
346 preventing the initiation step of the reaction. Indeed, EVOO phenolic compounds may act as radical
347 scavengers by reacting directly with hydroperoxyl (LOO^{\bullet}) or alkoxy (LO^{\bullet}) radical, preventing the
348 propagation step of the reaction. EVOO phenolic compounds could act by donating a hydrogen
349 atom to the LOO^{\bullet} or LO^{\bullet} radicals as suggested by other authors (Saija et al., 1998). There was no
350 evidence of chelation of Fe^{2+} by EVOO phenolic-rich extract, suggesting that iron sequestering is
351 not a mechanism of lipid peroxidation prevention by EVOO phenolic compounds. EVOO phenolic
352 compounds exerted an ambiguous pro- or antioxidant effect, depending on their concentration. In
353 fact, *in vitro* co-digestion of meat and EVOO phenolic-rich fraction at 5% and 10% showed an
354 increased lipid hydroperoxide production (**Figure 3**). A hypothesized reaction mechanism
355 explaining this pro-oxidant outcome of the EVOO phenolic compounds is proposed in **Figure 5**.
356 This study provides evidence that phenolic compounds are able to reduce Fe^{3+} to Fe^{2+} , thereby
357 stimulating the Fenton and Haber–Weiss reactions and the formation of $O_2^{\bullet-}$, HOO^{\bullet} , H_2O_2 and HO^{\bullet} .
358 Indeed, phenolic compounds containing a catechol moiety such as hydroxytyrosol and derivative
359 (oleuropein aglycone) may generate both $O_2^{\bullet-}$ and H_2O_2 in presence of Fe^{3+} and dissolved
360 oxygen (Eghbaliferiz & Iranshashi, 2016; Fabiani, Fuccelli, Pieravanti, De Bartolomeo, & Morozzi,
361 2009) (**Figure 6**). It can be speculated that, at high EVOO phenolic compounds concentration, the
362 oxidative stress induced by iron re-cycling and $O_2^{\bullet-}$ and H_2O_2 produced by phenolic compounds
363 exceed their $O_2^{\bullet-}/HOO^{\bullet}$ and HO^{\bullet} scavenging properties resulting in an increased production of
364 lipid hydroperoxides. It is however not clear why this enhanced formation was not observed for
365 TBA-RS during the turkey breast meat digestion in presence of high EVOO phenolic compounds
366 concentration. It is possible that, at high concentration, EVOO phenolic compounds may still

367 effectively scavenge LOO• and LO• radicals preventing the decomposition of lipid hydroperoxides
368 in aldehydic TBA reactive compounds.

369 Our results differ from Kuffa, Piesbe, Krueger, Reed, & Richards (2009) who found that the
370 addition of low concentrations of grape seed extract during simulated gastric digestion of high-fat
371 turkey meat had a pro-oxidant effect, while higher concentrations exerted an antioxidant effect.
372 Similarly, Van Hecke et al. (2016) found that, during digestion of high-fat beef, phenolic acids
373 displayed either pro-oxidant or antioxidant behaviour at lower and higher doses, respectively;
374 whereas ascorbic acid was pro-oxidant at all doses. However, previous studies found that increasing
375 concentrations of caffeic and chlorogenic acids as well as rutin and quercetin stimulated the
376 formation of HO• and O₂•⁻/HOO• in a reaction mixture containing H₂O₂ and Fe³⁺ (Oueslati et al.,
377 2016). Indeed, Tirosh et al. (2015) established that vitamin E behaved as an antioxidant or pro-
378 oxidant depending on the concentration of un-saturated fatty acids. Probably, the balance between
379 the pro-oxidative and antioxidative activity of phenolic compounds on lipid oxidation during gastro-
380 intestinal digestion is dependent on the type and concentration of fatty acids, the type of iron
381 catalyzers, and type and amounts of phenolic compounds.

382 Since the pro-oxidant effect of polyphenols only partially explained the enhanced formation of lipid
383 hydroperoxides during the digestion of turkey breast meat in presence of EVOO, we decided to
384 study the involvement of EVOO fatty acids. *In vitro* digestion of 10% EVOO without meat did not
385 result in the production of lipid hydroperoxides. On the contrary, as shown in **Figure 4**, when the
386 fat fraction of EVOO was digested with turkey breast meat, at the same concentration as found in
387 10% EVOO, a significant increase in the amount of lipid hydroperoxides and TBA-RS was
388 observed respect to the turkey meat sample. These observations indicated that, the presence of meat
389 catalyzers promoted the peroxidation of EVOO lipids that, otherwise, did not occur during *in vitro*
390 digestion of EVOO alone.

391 Based on our results, we believe that the pro-oxidant effect of EVOO at high concentration was a
392 consequence of the interaction between the different variables in the system. EVOO phenolic

393 compounds behaved as pro-oxidants increasing the generation of lipid hydroperoxides from meat
394 (**Figure 3**). At the same time, the presence in the digestive system of catalyzers from meat induced
395 the peroxidation of EVOO fatty acids, which was further intensified by the pro-oxidant activity of
396 EVOO phenolic compounds (**Figure 4**).

397 In a previous study, Tirosh et al. (2015) found that the addition of olive oil to turkey red meat
398 decreased the entity of meat lipid peroxidation in a concentration-dependent manner. At the same
399 concentration that enhanced lipid peroxidation in our system (i.e. 5% and 10% EVOO), olive oil
400 exhibited inhibitory effect on lipid peroxidation in Tirosh et al. (2015). The different results could
401 arise from differences in the type of meat (red vs white) used in the studies which reflected different
402 mechanisms of initiation of lipid peroxidation. In turkey breast (white) meat, Fenton and Haber-
403 Weiss chemistry represented the major initiators of lipid peroxidation during gastro-intestinal
404 digestion, whereas in turkey red meat met-myoglobin had a predominant role in lipid peroxidation
405 initiation (Kanner & Lapidot, 2001; Tagliazucchi et al., 2010). Indeed, also the phenolic
406 composition of EVOO may have influenced the results. Our EVOO was rich in hydroxytyrosol
407 derivative (such as oleuropein aglycone, which represented more than 60% of EVOO phenolics,
408 **Table 1**) which are prone to exert pro-oxidant effects. Vice versa, tyrosol and its derivative (such as
409 ligstroside aglycone) are not pro-oxidant (Fabiani et al., 2009). It is possible that EVOO rich in
410 tyrosol-derivative and poor in hydroxytyrosol-derivative do not exert pro-oxidant effect during meat
411 lipid oxidation. Thus, the specific composition of different EVOO preparations and meat but also
412 the different heating treatments of the meat might contribute to explaining some contrasting
413 literature findings.

414 **5. Conclusions**

415 This study provided evidence of a possible paradoxical effect of EVOO on lipid peroxidation during
416 digestion of turkey breast meat. At the lowest tested concentration of 2.5%, EVOO addition to
417 turkey breast meat resulted in an inhibition of the generation of lipid hydroperoxides and TBA-RS
418 during gastro-intestinal digestion. This effect was ascribed to the phenolic content of EVOO.

419 However, at higher concentration EVOO enhanced the formation of lipid hydroperoxides (but not
420 of TBA-RS) during co-digestion with turkey breast meat. This effect was attributed to the ability of
421 meat catalyzers to promote the peroxidation of the EVOO fat fraction and to the pro-oxidant
422 behaviour of EVOO phenolic compounds. The balance between the enhancing effect of EVOO fat
423 and the inhibitory effect of EVOO phenolic compounds on TBA-RS production may help to explain
424 the results obtained with the TBA-RS assay when turkey breast meat was co-digested with EVOO.

425 The EVOO tested in this study was particularly rich in hydroxytyrosol-derivative that are able to
426 increase the oxidative stress during *in vitro* gastro-intestinal digestion as a consequence of the
427 presence of a catechol group. Since the phenolic composition of EVOO is greatly variable
428 depending on the cultivar and agro-climatic factors (such as growing, harvesting time, seasonal
429 variability), it is plausible that different EVOO with different phenolic composition (i.e. high in
430 tyrosol-derivative and low in hydroxytyrosol-derivative) may have a different impact on oxidative
431 phenomena on lipids. Therefore, it is of paramount importance to study the phenolic composition of
432 antioxidant-rich foods used in this type of study to better understand their impact on lipid
433 peroxidation during the digestion of meat. Based on our findings, we therefore recommend that
434 future studies investigate the phenolic profile of the tested antioxidant-rich foods, since some pro-
435 oxidant effects were observed which depend on the amount and type of phenolic compounds.
436 Indeed, our study underlined the importance of the timing and amount of consumption of EVOO in
437 limiting the peroxidative phenomena on meat lipids, which have been partially associated with the
438 potential adverse effects of meat consumption on human health.

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Figure captions

Figure 1. Turkey breast meat lipid peroxidation as affected by extra-virgin olive oil during *in vitro* gastro-intestinal digestion. (A) Changes in lipid hydroperoxides concentration. (B) Changes in advanced lipoxidation end-products measured as TBA-RS and expressed as nmol malondialdehyde (MDA)/g of meat. (○) Meat alone, (□) meat with 2.5% extra-virgin olive oil (w/w), (■) meat with 5% extra-virgin olive oil (w/w), (▒) meat with 10% extra-virgin olive oil (w/w).

Figure 2. Effect of extra-virgin olive oil concentration on the amount of lipid hydroperoxides and advanced lipoxidation end-products measured at the end of the gastro-intestinal digestion of turkey breast meat. Lipid hydroperoxides (■) were expressed as nmol H₂O₂/g of meat (left y-axis) whereas advanced lipoxidation end-products (●) were measured as TBA-RS and expressed as nmol malondialdehyde (MDA)/g of meat (right y-axis). EVOO: extra-virgin olive oil.

Figure 3. Effect of extra-virgin olive oil phenolic compounds concentration on the amount of lipid hydroperoxides and advanced lipoxidation end-products measured at the end of the gastro-intestinal digestion of turkey breast meat. Lipid hydroperoxides (■) were expressed as nmol H₂O₂/g of meat (left y-axis) whereas advanced lipoxidation end-products (●) were measured as TBA-RS and expressed as nmol malondialdehyde (MDA)/g of meat (right y-axis). Left y-axis shows that EVOO phenolic-rich fraction at low concentration (2.5% w/w respect to meat) strongly inhibited the formation of lipid hydroperoxide at the end of the digestion, whereas at high concentration (5% and 10% w/w respect to meat) exerted a pro-oxidant effect leading to an increase in lipid hydroperoxide concentration. Differently, the right y-axis shows that EVOO phenolic-rich fraction generally inhibited the production of TBA-RS at each concentration. EVOO: extra-virgin olive oil.

Figure 4. Lipid oxidation during *in vitro* digestion of 10% extra-virgin olive oil alone and during the co-digestion of 10% extra-virgin olive oil fat fraction and meat. (A) Figure 4A describes the variations in the levels of lipid hydroperoxides at the end of the gastro-intestinal

digestion expressed as nmol H₂O₂/g of meat. (B) Figure 4B describes the variations in the levels of advanced lipoxidation end-products measured as TBA-RS at the end of the gastro-intestinal digestion expressed as nmol malondialdehyde (MDA)/g of meat. () Meat alone, (□) meat with 10% extra-virgin olive oil (w/w), () 10% extra-virgin olive oil alone (w/w), () meat with 10% extra-virgin olive oil fat fraction (w/w), () meat with 10% extra-virgin olive oil phenolic-rich fraction (w/w). Results showed how the EVOO fat fraction interacted with meat catalysers leading to an increased production of lipid hydroperoxides and TBA-RS. Different letters indicate that the values are significantly different ($P < 0.05$).

Figure 5. Proposed underlying mechanism explaining the anti- or pro-oxidant effect of extra-virgin olive oil phenolic compounds during gastro-intestinal digestion of turkey breast meat.

At low concentration, extra-virgin olive oil phenolic compounds act as antioxidant inhibiting meat lipid peroxidation (phenolic compounds in light-grey boxes). Extra-virgin olive oil phenolic compounds may act at different levels. In the first step of the reaction, they may scavenge superoxide anion ($O_2^{\bullet-}/HOO^{\bullet}$) and hydroxyl radical (HO^{\bullet}) preventing the initiation step of the reaction. Indeed, extra-virgin olive oil phenolic compounds may act as radical scavengers by reacting directly with hydroperoxyl (LOO^{\bullet}) or alkoxy (LO^{\bullet}) radical, preventing the propagation step of the reaction. At high concentration, extra-virgin olive oil phenolic compounds act as pro-oxidant enhancing meat lipid peroxidation (phenolic compounds in black boxes). Extra-virgin olive oil phenolic compounds are able to reduce Fe^{3+} to Fe^{2+} , thereby stimulating the formation of $O_2^{\bullet-}$, HOO^{\bullet} , H_2O_2 and HO^{\bullet} and increasing the oxidative stress in the gastro-intestinal milieu. Indeed, phenolic compounds containing a catechol moiety such as hydroxytyrosol and derivative (oleuropein) may generate, in presence of Fe^{3+} and dissolved oxygen, both $O_2^{\bullet-}$ and H_2O_2 (see Figure 6). R^1 may indicate either a hydrogen (tyrosol) or a hydroxyl group (hydroxytyrosol). R^2 may indicate either a hydroxyl group (tyrosol or hydroxytyrosol) or an elenolic acid group (ligstroside aglycone or oleuropein aglycone).

Figure 6. Production of $O_2^{\bullet-}$ and H_2O_2 by phenolic compounds containing a catechol moiety in presence of Fe^{3+} . R^2 may indicate either a hydroxyl group (hydroxytyrosol) or an elenolic acid group (oleuropein aglycone).

Table 1. Mass spectra and quantitative data for phenolic compounds identified in the extra-virgin olive oil phenolic-rich extract. Values represent means \pm standard deviation of triplicate determination^a.

Peak	Rt (min)	Compound	[M-H] ⁻ (m/z)	MS ² ion fragments (m/z)	mg/100 g EVOO
1	10.6	Tyrosol	137	119	< LOQ
2	11.4	Tyrosol	137	119	< LOQ
3	6.7	Vanillin	151	123	< LOQ
4	13.1	Vanillin	151	123	< LOQ
5	13.6	Vanillin	151	123	< LOQ
6	10.0	Hydroxytyrosol	153	123	0.46 \pm 0.01
7	12.7	Dihydroxytyrosol	169	151	< LOQ
8	18.4	Luteolin	285	241, 175, 199, 133	0.15 \pm 0.01
9	17.1	Decarbossimethyl-oleuropein aglycone (3,4-DHPEA-EDA)	319	195, 165	0.05 \pm 0.01
10	17.4	Decarbossimethyl-oleuropein aglycone (3,4-DHPEA-EDA)	319	195, 165	0.13 \pm 0.01
11	16.8	Hydroxymethyl-decarbossimethyl-ligstroside aglycone (Hydroxymethyl-p-HPEA-EDA)	333	301, 213, 181	0.33 \pm 0.02
12	17.2	Hydroxymethyl-decarbossimethyl-ligstroside aglycone (Hydroxymethyl-p-HPEA-EDA)	333	301, 213, 181	0.28 \pm 0.01
13	16.5	Hydroxy-decarbossimethyl-oleuropein aglycone (Hydroxy-3,4-DHPEA-EDA)	335	199, 153, 181	0.57 \pm 0.01
14	16.8	Ligstroside aglycone (p-HPEA-EA)	361	294, 259, 223	1.34 \pm 0.18
15	17.5	Ligstroside aglycone (p-HPEA-EA)	361	294, 259, 223	1.69 \pm 0.12
16	18.3	Ligstroside aglycone (p-HPEA-EA)	361	294, 259, 223	2.44 \pm 0.17
17	18.8	Ligstroside aglycone (p-HPEA-EA)	361	294, 259, 223	5.22 \pm 0.22
18	19.5	Ligstroside aglycone (p-HPEA-EA)	361	294, 259, 223	1.86 \pm 0.08
19	20.1	Ligstroside aglycone (p-HPEA-EA)	361	294, 259, 223	1.57 \pm 0.05
20	20.7	Ligstroside aglycone (p-HPEA-EA)	361	294, 259, 223	3.12 \pm 0.15
21	21.7	Ligstroside aglycone (p-HPEA-EA)	361	294, 259, 223	10.33 \pm 0.14

22	15.2	Oleuropein aglycone (3,4-DHPEA-EA)	377	241, 307, 275, 345, 139	1.96 ± 0.14
23	16.6	Oleuropein aglycone (3,4-DHPEA-EA)	377	241, 307, 275, 345, 139	4.07 ± 0.22
24	17.1	Oleuropein aglycone (3,4-DHPEA-EA)	377	241, 307, 275, 345, 139	4.43 ± 0.15
25	18.1	Oleuropein aglycone (3,4-DHPEA-EA)	377	241, 307, 275, 345, 139	6.86 ± 1.60
26	18.9	Oleuropein aglycone (3,4-DHPEA-EA)	377	241, 307, 275, 345, 139	8.22 ± 0.39
27	19.4	Oleuropein aglycone (3,4-DHPEA-EA)	377	241, 307, 275, 345, 139	6.60 ± 0.11
28	20.3	Oleuropein aglycone (3,4-DHPEA-EA)	377	241, 307, 275, 345, 139	33.90 ± 0.53
29	13.8	Dihydroxy-ligstroside aglycone (Dihydroxy-p-HPEA-EA)	393	361, 257, 195, 151	0.06 ± 0.02
30	15.9	Dihydroxy-ligstroside aglycone (Dihydroxy-p-HPEA-EA)	393	361, 257, 195, 151	1.25 ± 0.19
31	16.6	Dihydroxy-ligstroside aglycone (Dihydroxy-p-HPEA-EA)	393	361, 257, 195, 151	0.54 ± 0.09
32	17.1	Dihydroxy-ligstroside aglycone (Dihydroxy-p-HPEA-EA)	393	361, 257, 195, 151	0.37 ± 0.02
33	17.5	Dihydroxy-ligstroside aglycone (Dihydroxy-p-HPEA-EA)	393	361, 257, 195, 151	1.12 ± 0.10
Total phenolic compounds					98.93 ± 1.84

^aQuantified as hydroxytyrosol equivalent with the exception of luteolin which was quantified as luteolin equivalent











