1   # Contribution of Galloylation and Polymerization to the

2   # Antioxidant Activity of Polyphenols in Fish Lipid Systems

3   Jacobo Iglesias*, Manuel Pazos, Salomé Lois and Isabel Medina

4   Instituto de Investigaciones Marinas (CSIC), Eduardo Cabello 6, E-36208 Vigo, Spain,

5

6   Running title header: Influence of polyphenol structure on fish lipid oxidation

7

8

9   *Author to whom correspondence should be addressed [e-mail: jacobo@iim.csic.es; telephone +34

10   986 231930; fax +34 986 292762].

11

Abstract

Polyphenolic fractions extracted from pine (*Pinus pinaster*) bark, grape (*Vitis vinifera*) pomace and witch hazel (*Hamamelis virginiana*) bark were selected for investigating the influence of the number of phenolic units, polymerization, and the content of esterified galloyl residues, galloylation, on their efficacy for inhibiting lipid oxidation in fish lipid enriched foodstuffs. Experiments carried out with non-galloylated pine bark fractions with different polymerization degrees, demonstrated that the number of catechin residues per molecule modules their reducing and chelating properties in solution. In real food systems as bulk fish oil and fish oil-in-water emulsions the efficacy against lipid oxidation was highly dependent on the physical location of the antioxidant at the oxidative sensitive sites. The lowest polymerized fractions were the most efficient in bulk fish oil samples, while proanthocyanidins with an intermediate polymerization degree showed the highest activity in fish oil-in-water emulsions. Galloylation did not influence antioxidant effectiveness of proanthocyanidins in bulk fish oils. The presence of galloyl groups favored the antioxidant activity of the polyphenols in emulsions, although results indicated that a high degree of galloylation did not improve significantly the activity found with medium galloylated proanthocyanidins. The results obtained in this research provide useful information about the relationship between structure and antioxidant activity in order to design antioxidant additives with application in fish oil-enriched functional foods.

Keywords: lipid oxidation, proanthocyanidins, polymerization, galloylation, fish oil, fish oil emulsion

34

Introduction

Seafood is very susceptible to suffer lipid oxidation, which leads to the development of rancidity and the reduction of shelf-life and nutritive value. The delay of this process is important for the entire production and commercialization chain of this type of foodstuffs and the addition of antioxidant substances is one of the most widely strategies for preventing or retarding lipid deterioration (1). The adverse toxicological reports of many synthetic compounds and the consumer interest on natural food additives have caused in recent years an enormous demand of natural bioactive substances with food antioxidant activity.

Polyphenolic extracts from natural sources are very interesting additives because of their natural origin and efficiency for inhibiting lipid oxidation processes. Mechanistically, the antioxidant behavior of phenolics has been attributed to their free radical-scavenging activity (2, 3), capacity to protect or recycle endogenous antioxidants (4, 5) and ability to chelate redox-active metals (6). Among polyphenolics, the family of flavan-3-ols, together with their related oligomers, proanthocyanidins (Fig. 1) is extensively distributed in nature (7). The importance of the polymerization degree (number of polyphenolic units) and the percentage of galloylation (content of esterified galloyl groups) on their antioxidant activity has been evidenced in several *in vitro* assays (8, 9), in low density lipoproteins (10) or human colon cancer cells (11). Oligomeric procyanidins (roughly two to seven residues) have been considered more efficient than monomers but high degrees of polymerization have been also associated to mucosal irritability and astringency effects. The increment of hydroxylic groups in the pyrogallol moiety provides more hydrogen atoms or electrons than the catechol group and affects the redox potential and the metal-chelation capacity of polyphenols (1). Variation on polymerization and galloylation can also affect the polarity of polyphenols, influencing their location on the oxidation sensitive sites of foods in which lipids can be found on different compartments.

59 The use of polyphenols coming from agricultural and forestry by-products provides additional

60 advantages as the waste recycling and the low cost of the raw material. Pine (*Pinus pinaster*) bark,

61 grape (*Vitis vinifera*) pomace and witch hazel (*Hamamelis virginiana*) bark are residues from the

62 wood and wine industries, which contain a great variety of phenolic substances (11, 12). In recent

63 studies, the extraction, fractionation and characterization of the polyphenolic content of these

64 residues showed a high content of proanthocyanidins with different polymerization degrees. Grape

65 fractions presented gallate esters in their structure (Fig. 1), while no galloylation was detected in the

66 pine extracts (11). Witch hazel extracts showed a low content of oligomeric proanthocyanidins but a

67 high content of hydrolysable tannins based on gallate esters (Fig. 1) (13).

68 The aim of this investigation was to study the influence of polymerization and galloylation on the

69 activity of polyphenols for inhibiting oxidation of fish lipid systems. For this purpose, polyphenolic

70 fractions extracted from pine bark, grape pomace and witch hazel bark, with different compositions,

71 were tested as antioxidants in bulk oil and oil-in-water emulsions. Results were explained

72 considering the structural moieties of the phenolic compounds that have a significant influence on

73 their reducing and chelating activities and polarity.

74 **Materials and Methods**

75 **Materials.** High quality fish liver oil from cod (*Gadus morhua*) was purchased from Fluka (New-

76 Ulm, Switzerland). Cod liver oil was flavorless and odorless and had a peroxide value of 0.5 meq.

77 oxygen/kg. Soybean lecithin (40% L-α-phosphatidylcholine, Sigma, St. Louis, MO) was used as

78 emulsifying agent. Ferrozine, $FeCl_2.4H_2O$ and $FeCl_3 \cdot 6H_2O$ were also supplied by Sigma. 2,4,6-

79 Tri(2-pyridyl)-s-triazine (TPTZ) was obtained from Fluka (New-Ulm, Swizerland). All chemicals

80 and solvents used were either analytical or HPLC grade (Ridel-Haën, Seelze, Germany).

81 **Polyphenolic fractions.** Phenolic fractions from pine (*Pinus pinaster*) bark (IP, IIP, IIIP, IVP,

82 VP, VIP, VIIIP, IXP, XP and XIP), grape (*Vitis vinifera*) pomace (IVG, VIG and VIIIG) and witch

83 hazel (*Hamamelis virginiana*) bark (IVH, VIH and VIIIH) were prepared by fractionation of

4

84 polyphenolic extracts soluble in both ethyl acetate and water according to Torres and Bobet (14),

85 and Touriño et al. (11, 12) following the general procedure shown in Scheme 1. Briefly, the crude

86 extract contained mainly flavanol (catechin) monomers, flavanol oligomers (proanthocyanidins) and

87 monomeric glycosylated flavonols. It was then separated into a set of fractions differing in

88 composition and proanthocyanidin structure (Scheme 1, Fig. 1 and Table 1). The RP-HPLC step

89 fractionated the components on the basis of their hydrophobic interactions with the stationary phase,

90 which is not always related to the size of the flavonoids. Chromatography on Toyopearl separated

91 the components mainly by size: monomers from oligomers. The average molecular weight and

92 composition (mean degree of polymerization, mDP, and percentage of galloylation) (Table 1) were

93 estimated by HPLC analysis after depolymerization with cysteamine (13). Homologous fractions

94 extracted from pine bark and grape pomace are mainly composed by proanthocyanidins with similar

95 polymerization degrees but they differed in the percentage of galloylation, since grape fractions

96 showed medium galloylation and esterified galloyl groups were absent in pine polyphenols (Table

97 1). Fractions II and III from pine and grape also contained monomeric glycosylated flavonols.

98 *Hamamelis virginiana* fractions were composed by > 80% of hydrolyzable tannins and 20% of

99 proanthocyanidins (15), fundamentally hamamelitannin, methyl gallate, and galloyl glucoses with

100 2−10 galloyl moieties (Fig. 1). Such composition leads to a high galloylation level superior than

101 100% since hydrolizable tannins contain several galloyl residues per each molecule in their

102 structure (Fig.1). A more detailed description of the composition of each fraction has been

103 previously reported (11, 12, 14).

104 **Reducing Power of the Phenolic Compounds.** FRAP (Ferric Reducing Antioxidant

105 Power) method was used by adaptation of the procedure of Benzie and Strain (16). The FRAP

106 reagent was prepared daily by mixing acetate buffer 300 mM (pH 3.6), TPTZ 10 mM and ferric

107 chloride 20 mM, in the ratio 10:1:1, respectively. TPTZ solution was prepared in HCl 40 mM. 1.5

108 mL of FRAP reagent were incubated for 10 min at 37 ºC. Then, 150 μL of water and 50 μL of

109 phenolic solution (0.2-4 mg/L) were added and the absorbance was measured at 593 nm after 4 min.

110 The standard curve was built with ferrous chloride. FRAP values were calculated for increasing

111 molar concentrations of each antioxidant and a linear relation was obtained. The slope provided the

112 number of moles of ferric chloride which were reduced by 1 mol of antioxidant. Since only 1

113 electron is required to reduce each Fe^{3+} atom, the obtained value provided the number of electrons

114 donated for each antioxidant molecule.

115 **Chelating Activity of the antioxidants.** The capacity of the polyphenolic fractions for

116 chelating ferrous iron was determined using an adaptation of Kolayli et al. procedure (17). A total

117 of 0.2 mL of polyphenolic solution was mixed with 1.2 mL of 0.12 M KCl, 5 mM L-histidine

118 solution (pH 6.8), and 0.2 mL of 0.2 mM ferrous chloride. Then, 0.4 mL of 1 mM ferrozine was

119 added, and the samples were incubated at room temperature for 10 min. The absorbance was

120 measured at 560 nm, and the chelating capacity was expressed as the percentage of ferrous iron

121 chelated by 0.2 mM of phenolic compound.

122 **Polarity of the antioxidants.** The polarity of phenolics was determined by their partition

123 between aqueous and oily phases according to Huang et al. (18) and Pazos et al. (19). Briefly, 1 mL

124 of fish oil and 1 mL of water containing antioxidants were well mixed and centrifuged. The

125 phenolic content in the aqueous phase before and after mixing was quantified by the Folin–

126 Ciocalteu method (20), and the amount of antioxidant in the oily phase was calculated as the

127 difference between the total amount of antioxidant in water before mixing and the amount after

128 mixing with oil. Data were showed as the percentage of the antioxidant in the oily phase and

129 calculated as: $(W_O/W_T) \times 100$ where W_O is the amount of antioxidant in the oily phase and W_T is the

130 total amount of antioxidant.

131 **Oxidation of fish oil and fish oil-in-water emulsions.** Samples of fish oils (5 g) were

132 introduced into 50-mL Erlenmeyer flasks, and the polyphenolic fractions were added at

133 concentration of 100 μg/g. For each system, triplicate samples were prepared and subjected to

134 oxidation at 40 °C. Oxidative stability was followed by measuring conjugated diene and triene

hydroperoxides in duplicate. Oil-in-water emulsions, containing 1% lecithin and 10% fish oil, were prepared as previously described (18). Briefly, lecithin and fish oil were homogenized with distilled water and emulsified by sonicating for a total of 10 min in an ice bath (Selecta, Barcelona, Spain). 5 mL of emulsion were introduced into 50 mL Erlenmeyer flasks and the different antioxidants were added at 100 µg/g. For each system, triplicate samples were prepared and subjected to incubation at 35 °C. Lipid oxidation was followed by the measurement of conjugated diene hydroperoxides, the measurement of fluorescence compounds or the formation of volatiles. Triplicate samples were taken at different sampling times. Induction oxidation periods were calculated as the value in days corresponding to the intersection point of the tangents in the initiation and propagation steps of the oxidation curve.

Conjugated diene and triene hydroperoxides. Conjugated dienes and trienes, expressed in millimoles of hydroperoxide per kilogram of oil, were determined by using the method reported by Huang et al. (21). Briefly, about 100 mg of fish oil samples and emulsion samples were dissolved in hexane and ethanol, respectively. Absorbance was measured at 234 and 268 nm for determining the conjugated diene and triene hydroperoxides respectively (UV–Vis Spectrophotometer Perkin–Elmer).

Measurement of fluorescence compounds. 100 mg of emulsion samples were dissolved in ethanol. Fluorescence was measured at 345/416 and 393/463 nm (Perkin–Elmer LS 3B) and standardized with a quinine sulphate solution (1 µg/mL in 0.05 M H_2SO_4) according to the procedure described by Pazos et al. (22).

Volatile analysis in fish oil-in-water emulsions. Volatiles were extracted from fish oil-in-water emulsions by headspace solid-phase microextraction (HS-SPME) and analyzed by gas chromatography-mass spectrometry according to Iglesias et al. (23). Briefly, 0.5 mL of emulsion were introduced in a HS-SPME vial fitted with a silicone septum and exposed to a CAR-PDMS fibre (75 µm Carboxen/polydimethylsiloxane coating, Supelco, Bellefonte, PA) during 30 min at 60

160 ºC. The fibre was then removed from the vial and inserted into the GC injection port for desorption

161 during 10 min to 300 ºC. Determination of volatiles was performed by the method of internal

162 standard using 3-methyl-3-buten-1-ol. GC–MS analysis was performed in a Thermo Finnigan

163 ThermoQuest (San Jose, CA) gas chromatograph equipped with a split/splitless injector and

164 coupled with a Trace quadrupole mass detector (Thermo Finnigan ThermoQuest, San Jose, CA).

165 **Sensory analysis**. Sensory analysis was evaluated by an expert panel formed by four trained

166 specialists in descriptive analysis of fishy off-flavors, in a room designed for the purpose. Samples

167 were placed at room temperature during 10 minutes before analysis. Panelists were concentrated on

168 detecting rancidity odors (24).

169 **Statistical analysis.** Analyses were performed by triplicate and the data were compared by one-

170 way analysis of variance (ANOVA) and the least-squares difference method (25). The means were

171 compared by the least squares difference method with Statistica 6.0 program (Statsoft, Tulsa,

172 Oklahoma).

173

Results

Several fractions extracted from pine bark were chosen for studying the relationship between the antioxidant activity and the polymerization degree in fish lipid systems (Table 1). Pine fractions with different polymerization constitute an excellent model for evaluating this factor since are composed by oligomeric catechins with different size devoid of gallate esters (11). Grape proanthocyanidins have a low-medium galloylation percentage; therefore the presence of galloylated residues in the proanthocyanidin moiety was addressed by comparing the activity found in some homologous fractions extracted from pine bark and grape pomace (IVG, VIG and VIIIG, Table 1). Finally, the effect of the galloylation was assured by testing fractions coming from witch hazel (IVH, VIH and VIIIH, Table 1) having a high content of galloyl groups.

Relationship between polyphenolic structure and physicochemical characters.

The capacity to donate electrons (reducing capacity) of the different proanthocyanidins was estimated through the FRAP assay. Values obtained for the non-galloylated fractions (pine fractions) indicated that the number of donated electrons per molecule was increased with the number of catechin monomers. According to this, the maximum value of this parameter (6.1) was attributed to the highest polymerized fraction, XIP, with mDP=3.4 (Table 1). Data were submitted to statistical analysis and a significant correlation between polymerisation and the number of donated electrons was then detected (R^2 0.91).

The iron-chelating capacity test measures the ability of antioxidants to compete with ferrozine in chelating ferrous ions and therefore, measure the ability of the different proanthocyanidins to inactivate one of the most important metallic catalyst of lipid oxidation. The plots of iron-chelating capacity as a function of IIP, IIIP, IVP, VP and VIIIP concentration are shown in Fig. 2A. Clear differences on the chelating behavior were observed depending on the size of the proanthocyanidin. Table 1 illustrates such differences showing the percentage of ferrous iron chelated by 0.2 mM of phenolic compound. In agreement with data obtained for the reducing ability, the results obtained for the different pine fractions showed a direct relationship between this capacity and the size of the

proanthocyanidin. A significant correlation between size and chelating ability was also found (R^2

0.92).

The effect of galloylation on the *in vitro* reducing and chelating properties was checked by comparing homologous fractions extracted from pine bark and grape pomace. Table 1 shows the capacity to donate electrons and chelating ability for some grape fractions (IVG, VIG and VIIIG), in order to compare their values with those corresponding from pine. As regards to the reducing ability, data indicated that grape fractions showed higher capacity to donate electrons than their corresponding non-galloylated fractions, especially in the case of the most galloylated grape fraction, VIIIG. Additionally, the highly galloylated fractions from which hazel, IVH, VIH and VIIIH, showed a clear higher reducing ability than medium galloylated grape fractions.

The comparison of the non-galloylated fractions IVP, VIP and VIIIP and their galloylated counterparts from grape pomace demonstrated a positive influence of the esterified galloyl groups on the chelating ability (Fig. 2B). Fractions IVG, VIG and VIIIG showed very similar values of this property (94.2, 87.0 and 95.5), which were higher than their homologous fractions extracted from pine bark, (77.4, 75.5 and 82.6). The highly galloylated fractions, IVH, VIH and VIIIH, exhibited similar ability to chelate ferrous ions than their equivalent medium galloylated grape fractions (92.8, 86.2 and 93.4).

One important aspect in the antioxidant efficiency of proanthocyanidins in lipid systems is related to their location at the oxidative sensitive sites, which is highly dependent on their solubility in lipid and water. Table 1 shows the partition coefficients between fish oil and water for every fraction tested in this study. Pine fractions IVP, VP, VIP, VIIIP, IXP, XP and XIP, with a similar composition (monomers and oligomers of flavanols), showed an increase in the oily solubility related to growing number of phenolic residues (Table 1). The increment of the number of phenolic monomers holding apolar aromatic rings could explain this observation. As regards to galloylation, grape galloylated fractions showed a higher distribution in the oily phase compared to their pine homologous. The increment in the number of apolar aromatic rings forming part of the gallate

10

group could explain this finding. Finally, fractions IVH, VIH and VIIIH from witch hazel bark,
showed the highest distribution in the oily phase. Consequently, polymerization and galloylation
confer apolar character to the polyphenols, favoring their partition to oily phases.

Influence of the polymerization on the antioxidant activity of polyphenols in bulk fish oil and fish oil-in-water emulsions.

Fig. 3 illustrates the effect of polymerization on the generation of conjugated dienes and trienes in bulk fish oils. Fish oils were supplemented with IXP (polymerization: 1.9), XP (polymerization: 2.2) and XIP (polymerization: 3.4). Data obtained indicated that the rate of oxidation and the amount of oxidation products formed was lower in IXP samples. Pine fractions with higher polymerization degrees were not active for inhibiting lipid oxidation in fish oils. These results were in agreement with sensory detection of off-flavors (Table 2). Incipient rancid odors were detected in control oils and oil samples supplemented with fractions XP and XIP by the six day of storage, and in samples with fraction IXP by the seven day. Table 3 illustrates the percentage of inhibition on the formation of the lipid oxidation products in bulk oils supplemented with different pine fractions. Only fraction IXP, with the lower polymerization degree, showed a significant inhibition on the formation of lipid oxidation products.

The addition of 100 μg/g of pine fractions to fish oil-in-water emulsions significantly inhibited the development of oxidation (Fig. 3). The comparison among fractions IXP, XP and XIP illustrates the significant effect of polymerization. Table 4 exemplifies such effect expressed as the percentage of inhibition on the formation of lipid oxidation products, conjugated hydroperoxides and fluorescent compounds. Low antioxidant activity was detected for the less polymerized fraction IXP. Fraction XP, with an intermediate polymerization degree, inhibited the formation of conjugated dienes and trienes and fluorescence compounds in a 53%, 38% and 54%, respectively after 7 days of storage. In the same period, fraction XIP also inhibited the formation of conjugated dienes and trienes and fluorescent compounds (22%, 25% and 43%, respectively) but showing lower activity than fraction

251 XP. Fractions IVP and VIIIP also showed lower values of inhibition than those corresponding to

252 XP. Therefore, an optimum polymerization degree seems to occur round mDP values of 2.2.

253 Results obtained in both experiments did not evidence a positive trend between the polymerization

254 degree of the proanthocyanidins and their antioxidant activity in both, fish oil and fish oil

255 emulsions. The less polymerized proanthocyanidins were more efficient for preventing lipid

256 oxidation in bulk fish oil samples, whereas medium-high polymerized fractions were the most

257 active to delay lipid oxidation in fish oil emulsions.

Influence of the galloylation on the antioxidant activity of polyphenols in bulk fish oil and fish oil-in-water emulsions.

258 **Influence of the galloylation on the antioxidant activity of polyphenols in bulk**

259 **fish oil and fish oil-in-water emulsions.**

260 The comparison between the effectiveness for inhibiting lipid oxidation of bulk fish oils of grape

261 and pine homologous fractions demonstrated that galloylation did not improve the antioxidant effect

262 of proanthocyanidins (Fig. 4 and Table 3). The values of inhibition for the formation of conjugated

263 dienes and trienes showed the lack of efficiency of any grape galloylated proanthocyanidins for

264 depleting lipid oxidation. The presence of galloylated residues did not improve the null antioxidant

265 effectiveness detected for pine fractions having polymerization degrees higher than 2. Fraction IXG

266 (mDP = 2.0) did not show any antioxidant activity since the inhibition values obtained in the

267 formation of lipid oxidation products were very low (Table 3). Even more, galloylation seems to

268 induce prooxidant effects on bulk oils as can be observed from the negative values given in Table 3.

269 Fig. 4 illustrates the data comparing fraction IVP versus IVG and VIIIP versus VIIIG. Data

270 demonstrated the absence of antioxidant activity for any proanthocyanidinic fraction evaluated in

271 this experiment. Conversely, a significant prooxidant activity was observed in samples

272 supplemented with the galloylated fractions. The sensory analysis confirmed these results since

273 rancid off-odors were detected in all samples after 6 days of storage (Table 2). Therefore, the

274 presence of galloyl residues in the grape fractions was not able to enhance the antioxidant activity

275 of proanthocyanidins in bulk fish oils.

276 The presence of galloyl groups significantly favored the antioxidant behavior of proanthocyanidins

277   in fish oil-in-water emulsions (Table 4). Grape galloylated fractions showed higher inhibition

278   values than their corresponding pine non-galloylated fractions. Figure 4 summarizes the data

279   comparing fraction IVP versus IVG and VIIIP versus VIIIG. Control samples showed shorter

280   induction periods of formation of both conjugated dienes (3 days) and fluorescent compounds (4

281   days) than the samples supplemented with proanthocyanidins. Emulsions supplemented with

282   fraction VIIIP exhibited a significant increment of conjugated dienes and fluorescent compounds by

283   the fifth and sixth day of storage respectively, whereas the induction periods in the remainder

284   samples was over 6 and 7 days. These induction periods were in accordance with the sensory

285   detection of rancid off-flavors (Table 2). The amount of conjugated dienes and fluorescent

286   compounds formed resulted significantly lower in the presence of galloylated fractions from grape,

287   IVG and VIIIG, than in the case of their non-galloylated counterparts from pine. Therefore, the

288   galloylated fractions were more effective for inhibiting oxidation in fish oil-in-water emulsions than

289   their counterparts without galloyl residues. It is interesting to note that fraction VIIIG, having the

290   higher galloylation degree 0.34 (Table 1), was generally the grape fraction with lower antioxidant

291   effectiveness in fish oil emulsions.

292   The antioxidant activity of the low-medium galloylated IVG fraction was compared with the highly

293   galloylated fraction IVH, extracted from witch hazel (Fig. 5, Table 5). Rancid off-flavors were

294   detected by the fourth day in the control samples, while in the remaining ones off-odors were not

295   perceived until the fifth day of storage. Incipient rancidity was detected in IVG and IVH by the

296   sixth day. The analysis of lipid oxidation products confirmed these results. The formation of

297   conjugated dienes and volatiles indicated very similar induction periods for all samples, but the

298   generation rate of these lipid oxidation products and the amount formed was found to be

299   significantly different for each sample (Fig. 5). Fraction IVG was the most effective and inhibited

300   significantly the formation of conjugated dienes and volatiles (Table 5). Fraction IVH showed an

301   intermediate antioxidant inhibition with values lower than those of IVG. Consequently, the presence

302   of galloyl residues favors the antioxidant activity of the polyphenols in emulsions, although the

303 antioxidant performance of polyphenols with low-medium galloylation was not apparently

304 improved by highly galloylated polyphenols. These results confirm the previous finding described

305 in the above experiments related to fraction VIIIG.

Discussion

307 The present study revealed a direct relationship between the size of polyphenols and an

308 improvement in their reducing and iron-chelating properties. *In vitro* experiments carried out with

309 pine fractions demonstrated that the most polymerized proanthocyanidins provide the major

310 reducing and chelating capacities determined through the FRAP and ferrous-chelating assays. The

311 strong capacity of high polymerized polyphenols to donate electrons and chelate ferrous can be

312 attributed the presence of more hydroxyl groups contained in their structure (1). Our results

313 reinforce those reported by Touriño et al. (11) who found a slight positive correlation between the

314 degree of polymerization and the antiradical-scavenging properties against DPPH radical. Steric

315 impediments resulting from the incorporation of additional monomers and the relative high volume

316 of the DPPH radical may restrict the accessibility of these radicals to the active hydroxyl groups of

317 highly polymerized polyphenols. This fact seems to limit the application of DPPH assay to low-

318 medium polymerized polyphenols. Steric interactions could also explain the lack of correlation

319 between the electronic transference of the organic Tri(2,4,6-trichloro-3,5-dinitrophenyl)methyl

320 (HNTTN) radical and the size of proanthocyanidins (11). The assays to evaluate reducing (FRAP)

321 and metal-chelation capacities (ferrous-ferrozine) probably enclosed lower steric impediments since

322 they are based on the electronic donation or chelation of ferrous ions, with relative small size.

323 In spite of the improvement observed for the *in vitro* reducing and chelating capacities, with the

324 number of phenolic residues, such direct relation was not observed in bulk fish oils neither in fish

325 oil in-water emulsions. The efficacy of the pine proanthocyanidins in bulk fish oil was negatively

326 related to the polymerization degree. This result is in agreement with a preliminary study in which

327 galloylated monomers extracted from grape pomace were more efficient than oligomers for

328 inhibiting oxidation of bulk fish oils (22). According to the polar paradox hypothesis, hydrophilic

14

329 antioxidants are more effective in bulk oil systems since are positioned at the oil-air interface where

330 lipids are exposed to oxygen (1). Polymerization diminishes the hydrophility of the

331 proanthocyanidins, and as a consequence, the most polymerized are more diluted in the oily phase

332 and their antioxidant activity in bulk fish oil results weak. These results are in agreement with

333 previous investigations that showed a decrease of the antioxidant activity in bulk fish oil by

334 increasing the size of the alkyl chain of hydroxytyrosol fatty acid esters, and therefore, increasing

335 lipophilicity (26).

336 Different tendency was observed when pine fractions were tested in fish oil-in water emulsions. The

337 maximum inhibition was achieved with an intermediate number of polyphenolic residues, situated

338 between 2 and 3, according to previous results suggested by Pazos et al. (22). The potential of

339 phenolics to behave as effective antioxidants in lipid biphasic matrixes appears to be also dictated

340 by their location at the lipid interfaces where their efficacy is higher. Medina et al. (26) have

341 revealed that the presence of esterified aliphatic chains with low-medium length (2-10 carbons)

342 enhances the antioxidant activity of hydroxytyrosol by favoring its location in the water-oil

343 interfaces. Proanthocyanidins are a special case as regards to their situation in biphasic systems,

344 since they might be able to promote both hydrophobic and hydrophilic interactions due to their

345 amphipathic structure with polar hydroxyl groups and lipophilic aromatic rings. Consequently, they

346 can act as a surfactant, and such property favors their location in the water-oil interface. Saint-Cricq

347 De Gaulejac et al. (27) have suggested that the largest proanthocyanidins possess a reduced

348 accessibility for the –OH groups, which could diminish their tendency to be located in the lipid-

349 water interface.

350 Galloylation seems to play an important role in the antioxidant efficacy of the polyphenolic

351 fractions in fish oil-in-water emulsions. Galloylated fractions from grape and witch hazel bark

352 showed higher antioxidant activity than their non-galloylated counterparts extracted from pine bark.

353 The positive influence of the galloylation on the antioxidant activity is consistent with the fact that

354 the pyrogallol moiety provides of additional hydroxyl groups with radical-scavenging and chelating

15

ability (22). Accordingly, the polyphenolic fractions with elevated galloylation exhibited strong electronic donation power and chelating capacity (Table 1). The π-π stacking arrangement between the aromatic gallate and the catechol B ring could cause an additional stabilization of the respective phenoxyl radical by delocalization of the unpaired electron between these π orbitals as has been previously demonstrated by Freitas et al. (28).

As regards to the location at the oxidative sensitive sites of the emulsion, the "polar paradox" establishes that the lower polarity of the galloylated proanthocyanidins (Table 1) facilitates their location in the lipid-water interfaces, and then improving their antioxidant efficacy (1). In the case of bulk fish oils, an increase of galloyl residues, increasing solubility in the oily phase, results in decreased antioxidant activity.

The efficacy of the IVH fraction was slightly lower than that of IVG when both were compared in fish oil-in-water emulsions. The high content in hydrolyzable tannins with esterified gallate groups in their structure, as hamamelitannin or different galloylated glucoses (15), provides to these fractions an important content of pyrogallol moieties (Fig. 1). Therefore, our results reveal that a medium-low galloylation confers optimum antioxidant activity to the polyphenols in fish-oil-emulsions, and that is not improved by increasing the content of galloylated residues.

The present investigation describes how the reducing, iron-chelating and lipophilic properties of the polyphenols are modulated by structural factors as polymerization and galloylation. As a consequence, the impact of both parameters on the antioxidant efficacy was different when they were added to bulk fish oil or fish oil enriched emulsions. The results here exposed provide useful insights into the polyphenolic structure/activity relationship. Therefore, this study could help in the rational design of antioxidants for getting a final additive with optimal polymerization and galloylation degrees and a convenient cost.

384   **References**

385   1. Frankel, E.N. *Lipid Oxidation.* The Oily Press LTD: West Ferry, Dundee, Scotland, 1998.

386   2. Morello, J.; Vuorela, S.; Romero, M.; Motilva, M.; Heinonen, M. Antioxidant Activity of Olive
387   Pulp and Olive Oil Phenolic Compounds of the Arbequina Cultivar. *J. Agric. Food Chem.* 2005, *53,*
388   2002-2008.

389   3. Andersen, M. L.; Skibsted, L. H., Lipid oxidation, antioxidants, and spin trapping. In *ACS*
390   *Symposium Series*, 2007; Vol. 956, pp 106-117

391   4. Zhou, B.; Wu, L.-.; Yang, L.; Liu, Z.-. Evidence for α-tocopherol regeneration reaction of green
392   tea polyphenols in SDS micelles. *Free Radic. Biol. Med.* 2005, *38,* 78-84.

393   5. Iglesias, J.; Pazos, M.; Andersen, M.L.; Skibsted, L.H.; Medina, I. Caffeic Acid as Antioxidant in
394   Fish Muscle: Mechanism of Synergism with Endogenous Ascorbic Acid and α-Tocopherol. *J.*
395   *Agric. Food Chem.* 2009, *57,* 675-681.

396   6. Khokhar, S.; Owusu Apenten, R.K. Iron binding characteristics of phenolic compounds: some
397   tentative structure–activity relations. *Food Chem.* 2003, *81,* 133-140.

398   7. Moure, A.; Cruz, J.M.; Franco, D.; Domínguez, J.M.; Sineiro, J.; Domínguez, H.; José Núñez,
399   M.; Parajó, J.C. Natural antioxidants from residual sources. *Food Chem.* 2001, *72,* 145-171.

400   8. Plumb, G.W.; De Pascual-Teresa, S.; Santos-Buelga, C.; Cheynier, V.; Williamson, G.
401   Antioxidant properties of catechins and proanthocyanidins: Effect of polymerisation, galloylation
402   and glycosylation. *Free Radic. Res.* 1998, *29,* 351-358.

403   9. Rosch, D.; Mugge, C.; Fogliano, V.; Kroh, L.W. Antioxidant Oligomeric Proanthocyanidins
404   from Sea Buckthorn (*Hippophae rhamnoides*) Pomace. *J. Agric. Food Chem.* 2004, *52,* 6712-6718.

405   10. Lourenço, C.F.; Gago, B.; Barbosa, R.M.; De Freitas, V.; Laranjinha, J. LDL isolated from
406   plasma-loaded red wine procyanidins resist lipid oxidation and tocopherol depletion. *J. Agric. Food*
407   *Chem.* 2008, *56,* 3798-3804.

408   11. Touriño, S.; Selga, A.; Jiménez, A.; Juliá, L.; Lozano, C.; Lizárraga, D.; Cascante, M.; Torres,
409   J.L. Procyanidin fractions from pine (*Pinus pinaster*) bark: Radical scavenging power in solution,
410   antioxidant activity in emulsion, and Antiproliferative effect in melanoma cells. *J. Agric. Food*
411   *Chem.* 2005, *53,* 4728-4735.

412 12. Touriño, S.; Lizárraga, D.; Carreras, A.; Lorenzo, S.; Ugartondo, V.; Mitjans, M.; Vinardell,
413 M.P.; Julía, L.; Cascante, M.; Torres, J.L. Highly galloylated tannin fractions from witch hazel
414 (*Hamamelis virginiana*) bark: Electron transfer capacity, *in vitro* antioxidant activity, and effects on
415 skin-related cells. *Chem. Res. Toxicol.* 2008, *21,* 696-704.

416 13. Torres, J.; Selga, A. Procyanidin size and composition by thiolysis with cysteamine
417 hydrochloride and chromatography. *Chromatographia* 2003, *57,* 441-445.

418 14. Torres, J.; Bobet, R. New flavanol derivatives from grape (*Vitis vinifera*) byproducts.
419 Antioxidant aminoethylthio-flavan-3-ol conjugates from a polymeric waste fraction used as a
420 source of flavanols. *J. Agric. Food Chem.* 2001, *49,* 4627-4634.

421 15. González, M. J.; Torres, J.L.; Medina, I. Impact of thermal processing on the activity of
422 gallotannins and condensed tannins from Hamamelis virginiana used as functional ingredients in
423 seafood. *J. Agric. Food Chem.* 2010, *58,* 4274-4283.

424 16. Benzie, I.F.F.; Strain, J.J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of
425 "Antioxidant Power": The FRAP Assay. *Anal. Biochem.* 1996, *239,* 70-76.

426 17. Kolayli, S.; Ocak, M.; Küçük, M.; Abbasoglu, R. Does caffeine bind to metal ions? *Food Chem.*
427 2004, *84,* 383-388.

428 18. Huang, S.; Hopia, A.; Schwarz, K.; Frankel, E.N.; German, J.B. Antioxidant Activity of α-
429 Tocopherol and Trolox in Different Lipid Substrates: Bulk Oils vs Oil-in-Water Emulsions. *J.*
430 *Agric. Food Chem.* 1996, *44,* 444-452.

431 19. Pazos, M.; Alonso, A.; Fernández-Bolaños, J.; Torres, J.L.; Medina, I. Physicochemical
432 properties of natural phenolics from grapes and olive oil byproducts and their antioxidant activity in
433 frozen horse mackerel fillets. *J. Agric. Food Chem.* 2006, *54,* 366-373.

434 20. Singleton, V.L.; Rossi, J.A. Jr. Colorimetry of Total Phenolics with Phosphomolybdic-
435 Phosphotungstic Acid Reagents. *Am. J. Enol. Vitic.* 1965, *16,* 144-158.

436 21. Huang, S.; Frankel, E.N.; Schwarz, K.; Aeschbach, R.; German, J.B. Antioxidant Activity of
437 Carnosic Acid and Methyl Carnosate in Bulk Oils and Oil-in-Water Emulsions. *J. Agric. Food*
438 *Chem.* 1996, *44,* 2951-2956.

439 22. Pazos, M.; Gallardo, J.M.; Torres, J.L.; Medina, I. Activity of grape polyphenols as inhibitors of
440 the oxidation of fish lipids and frozen fish muscle. *Food Chem.* 2005, *92,* 547-557.

441 23. Iglesias, J.; Lois, S.; Medina, I. Development of a solid-phase microextraction method for
442 determination of volatile oxidation compounds in fish oil emulsions. *Journal of Chromatography A,*
443 2007, *1163,* 277-287.

444 24. Richards, M. P.; Kelleher, S. D.; Hultin, H. O. Effect of washing with or without antioxidants
445 on quality retention of mackerel fillets during refrigerated and frozen storage. *J. Agric. Food Chem.*
446 1998, *46,* 4363-4371.

447 25. Sokal, R.; Rohlf, F. *Biometry.* Freeman and Company: San Francisco, CA, 1981; pp. 859.

448 26. Medina, I.; Lois, S.; Alcántara, D.; Lucas, R.; Morales, J.C. Effect of Lipophilization of
449 Hydroxytyrosol on Its Antioxidant Activity in Fish Oils and Fish Oil-in-Water Emulsions. *J. Agric.*
450 *Food Chem.* 2009, *57,* 9773-9779.

451 27. De Gaulejac, N.S.; Provost, C.; Vivas, N. Comparative study of polyphenol scavenging
452 activities assessed by different methods. *J. Agric. Food Chem.* 1999, *47,* 425-431.

453 28. De Freitas, V. A.; Glories, Y., Laguerre, M. Incidence of Molecular Structure in Oxidation of
454 Grape Seed Procyanidins. *J. Agric. Food Chem.* 1998, *46,* 376-382.

Figure captions

Scheme 1: Preparation of the different fractions from pine, grape and witch hazel residues.

Figure 1: Structures of hydrolyzable and condensed tannins

Figure 2: Chelating capacity of the different pine fractions (A) and comparison between pine and grape homologous (B).

Figure 3: Formation of conjugated diene and triene hydroperoxides in fish oil and fish oil in water emulsions supplemented with pine procyanidinic fractions with different polymerization degrees.

Figure 4: Comparative formation of lipid oxidation products in fish oil and fish oil in water emulsions supplemented with pine and grape procyanidins.

Figure 5: Comparative formation of lipid oxidation products in fish oil in water emulsions supplemented with grape and witch hazel phenolics.

Scheme1

Grape/Pine/Wich Hazel by-product

EtOH 70%

Crude extract

AcOEt

Total extract

RP-HPLC, 12% CH₃CN → RP-HPLC, 24% CH₃CN

I
Monomers+oligomers
flavanols

Toyopearl HW-40F
MeOH | Acetone/water (3:2)

IX
dimers
flavanols

X
oligomers
flavanols

XI
polymers
flavanols

II
Monomers flavonols+
polymers flavanols

Toyopearl HW-40F
MeOH | Acetone/water (3:2)

V
monomers
flavanols

VI
oligomers
flavanols

Toyopearl HW-40F
MeOH | Acetone/water (3:2)

III
monomers
flavanols
+
flavonols

IV
oligomers
polymers
flavanols

Toyopearl HW-40F
MeOH | Acetone/water (3:2)

VII
monomers
flavonols

VIII
polymers
flavanols

Figure 1

Condensed tannins
(100% in pine and grape fractions, 20% in witch hazel fractions)

Hydrolyzable tannins
(0% in pine and grape fractions, 80% in witch hazel fractions)

Monomeric catechins

R_1=H, R_2=OH, R_3=H (+)-Catechin
R_1=OH, R_2=H, R_3=H (-)-Epicatechin
R_1=H, R_2=OH, R_3=OH (+)-Gallocatechin
R_1=OH, R_2=H, R_3=OH (-)-Epigallocatechin

Polymeric catechins (arrows indicated possible polymerization positions)

R_1=H (Procyanidins) or OH (prodelphinidins)

R_2=OH or Gallate group

(Just in grape and witch hazel fractions)

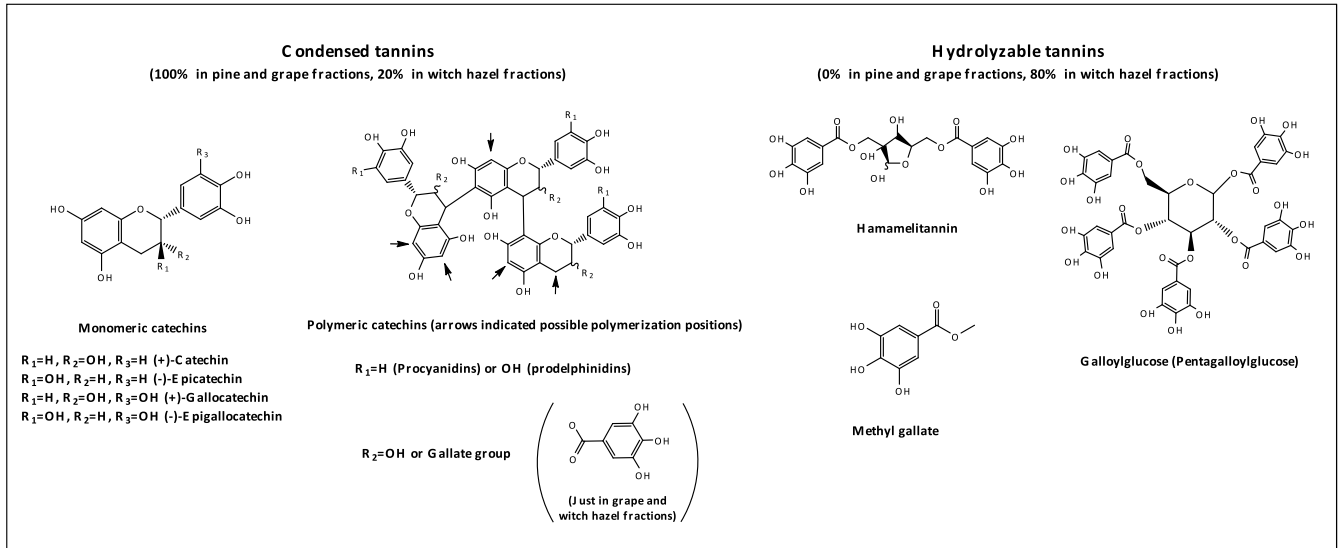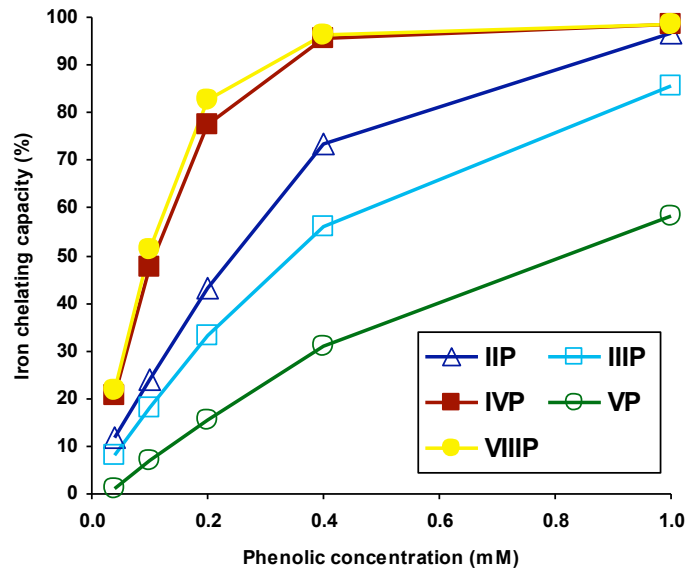**Hamamelitannin**

Methyl gallate

Galloylglucose (Pentagalloylglucose)
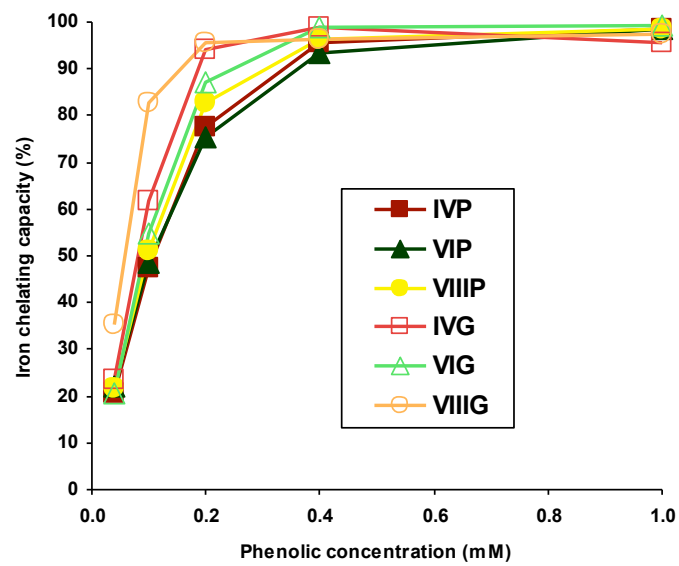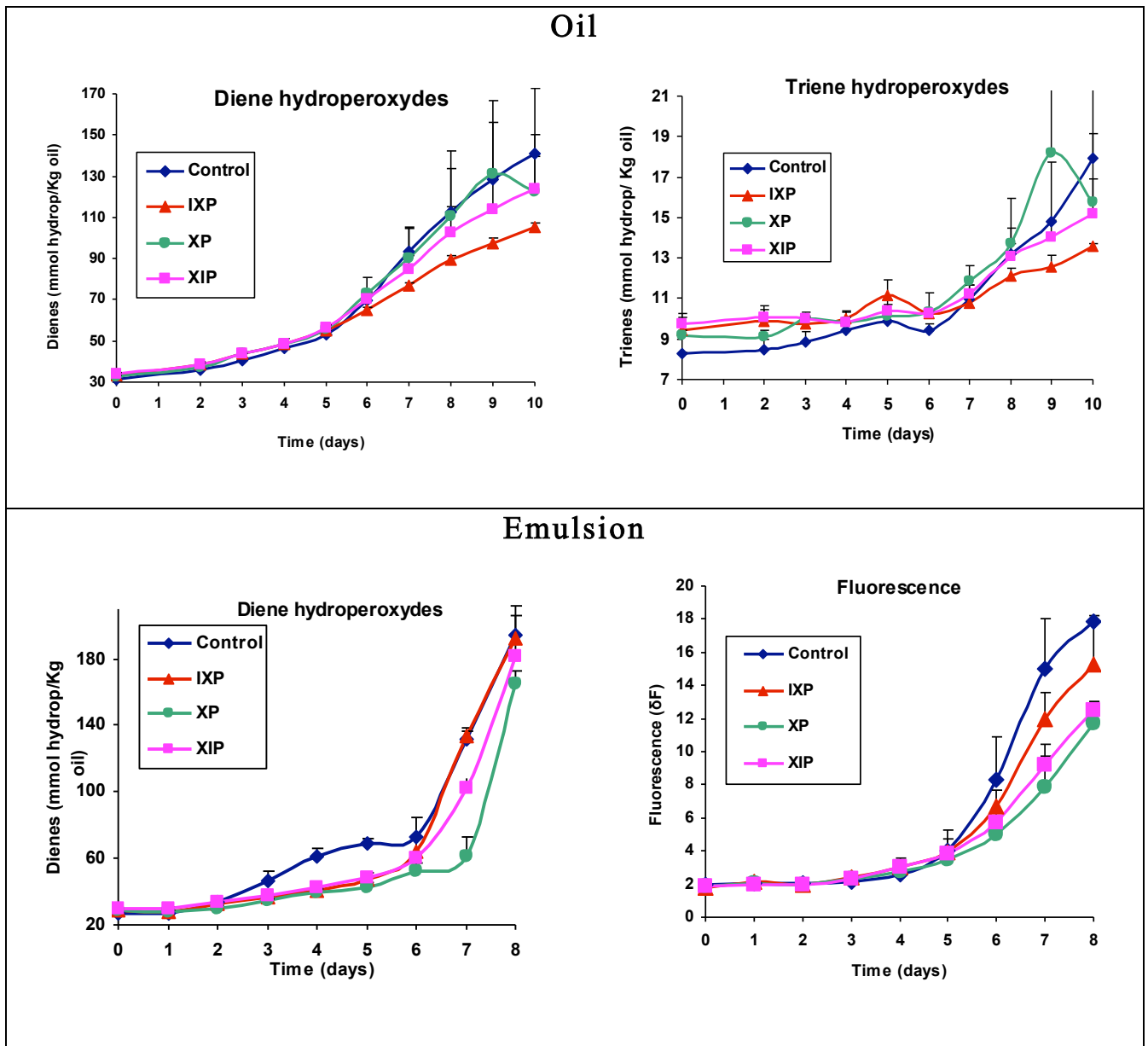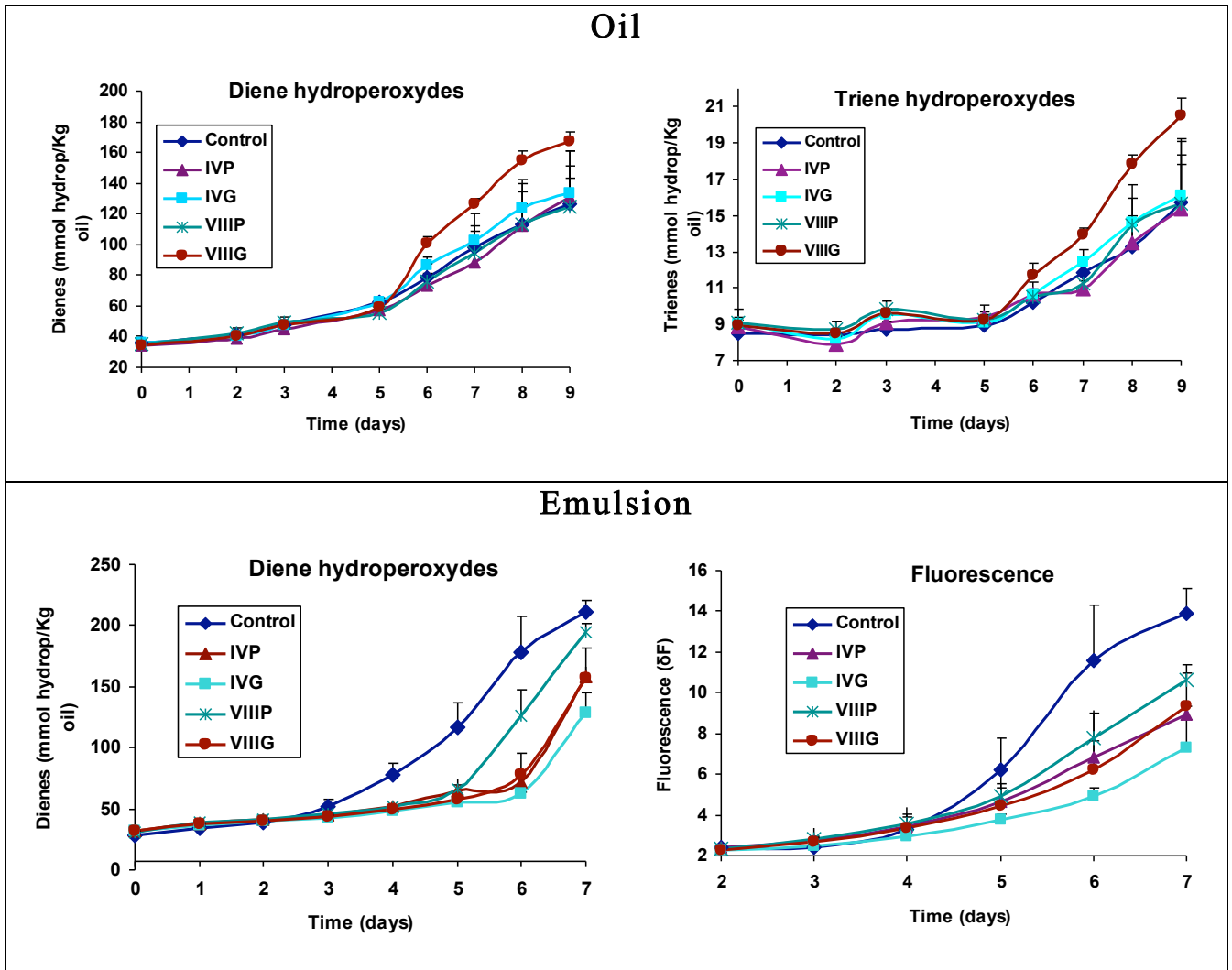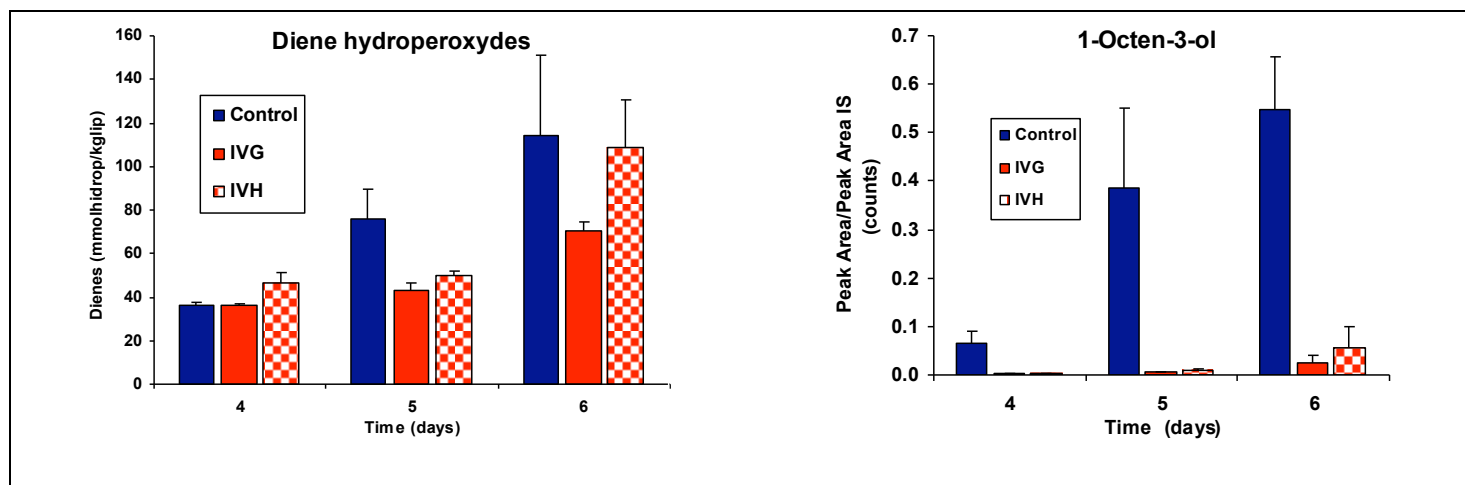
Figure 2

A

B

Figure 3

Figure 4

Figure 5

Tables

Table 1: Composition and characterization of the different fractions.

Fraction	mDP [b]	Galloylation (mol of gallate/mol of polyphenol)[a]	Reducing capacity (Number of donated electons/mol)	Chelating ability (%)	Partition coefficient (% in oil phase)
IP	1.9	0.00	3.3 ± 0.2	49.4 ± 0.0	10.6 ± 0.6
IIP	2.9	0.00	5.6 ± 0.0	70.0 ± 0.4	42.0 ± 1.1
IIIP	1.0	0.00	3.1 ± 0.1	33.1 ± 0.2	17.7 ± 1.6
IVP	2.9	0.00	5.6 ± 0.0	77.4 ± 0.5	18.5 ± 3.0
VP	1.0	0.00	2.0 ± 0.2	15.6 ± 0.3	8.1 ± 2.0
VIP	2.7	0.00	5.9 ± 0.0	75.5 ± 0.1	9.8 ± 1.5
VIIIP	3.0	0.00	5.4 ± 0.2	82.6 ± 0.7	16.4 ± 0.4
IXP	1.9	0.00	4.4 ± 0.1	44.1 ± 0.1	6.1 ± 0.4
XP	2.2	0.00	4.0 ± 0.0	58.1 ± 0.5	8.1 ± 0.2
XIP	3.4	0.00	6.1 ± 0.2	88.3 ± 0.1	18.5 ± 2.2
IVG	2.7	0.25	5.8 ± 0.1	94.2 ± 0.3	24.7 ± 2.0
VIG	2.4	0.16	6.0 ± 0.0	87.0 ± 0.4	10.1 ± 1.4
VIIIG	3.4	0.34	6.3 ± 0.1	95.5 ± 0.1	27.0 ± 1.5
IVH [a]	1.6	>100 [c]	6.1 ± 0.0	92.8 ± 3.6	29.9 ± 2.5
VIH [a]	2.6	>100 [c]	7.0 ± 0.2	86.2 ± 1.4	24.3 ± 2.4
VIIIH [a]	1.1	>100 [c]	8.0 ± 0.3	93.4 ± 3.3	41.3 ± 4.1

[a] Hamamelis fractions were composed by > 80% of hydrolizable tannins.
[b] Mean degree of polymerization of the proanthocyanidinic fraction.
[c] Percentage of galloylation adds up the contributions of both condensed and hydrolyzable tannins.

Table 2: Development of rancid odors during storage of fish oil and fish oil enriched real samples supplemented with different proanthocyanidinic fractions.

		POLYMERIZATION					
	DAY	3	4	5	6	7	8
OIL	Control	Not rancid odor	Incipient rancid odor	Incipient rancid odor	Rancid odor	Rancid odor	Rancid odor
OIL	IXP	Not rancid odor	Not rancid odor	Not rancid odor	Not rancid odor	Incipient rancid odor	Incipient rancid odor
OIL	XP	Not rancid odor	Not rancid odor	Not rancid odor	Incipient rancid odor	Rancid odor	Rancid odor
OIL	XIP	Not rancid odor	Not rancid odor	Not rancid odor	Incipient rancid odor	Incipient rancid odor	Rancid odor
EMULSION	Control	Incipient rancid odor	Rancid odor	Rancid odor	Rancid odor	Rancid odor	Rancid odor
EMULSION	IXP	Not rancid odor	Incipient rancid odor	Rancid odor	Rancid odor	Rancid odor	Rancid odor
EMULSION	XP	Not rancid odor	Not rancid odor	Incipient rancid odor	Rancid odor	Rancid odor	Rancid odor
EMULSION	XIP	Not rancid odor	Not rancid odor	Incipient rancid odor	Rancid odor	Rancid odor	Rancid odor
		GALLOYLATION					
	Day	3	4	5	6	7	8
OIL	Control	Not rancid odor	-	Not rancid odor	Rancid odor	Rancid odor	Rancid odor
OIL	IVP	Not rancid odor	-	Not rancid odor	Rancid odor	Rancid odor	Rancid odor
OIL	IVG	Not rancid odor	-	Not rancid odor	Rancid odor	Rancid odor	Rancid odor
OIL	VIIIP	Not rancid odor	-	Not rancid odor	Rancid odor	Rancid odor	Rancid odor
OIL	VIIIG	Not rancid odor	-	Incipient rancid odor	Rancid odor	Rancid odor	Rancid odor
EMULSION	Control	Incipient rancid odor	Rancid odor	Rancid odor	Rancid odor	Rancid odor	-
EMULSION	IVP	Not rancid odor	Not rancid odor	Not rancid odor	Incipient rancid odor	Rancid odor	-
EMULSION	IVG	Not rancid odor	Not rancid odor	Not rancid odor	Incipient rancid odor	Rancid odor	-
EMULSION	VIIIP	Not rancid odor	Not rancid odor	Incipient rancid odor	Rancid odor	Rancid odor	-
EMULSION	VIIIG	Not rancid odor	Not rancid odor	Not rancid odor	Incipient rancid odor	Rancid odor	-

Table 3: Inhibition[1] on the formation lipid oxidation products in bulk fish oils by homologous fractions with different polymerization degrees extracted from pine bark and grape pomace (mean±sd)[2].

DAY 8	Fish oil			
	Conjugated dienes (%)		Conjugated trienes (%)	
Fraction	PINE	GRAPE	PINE	GRAPE
IV	1.2 ± 9.5^a	-9.5 ± 14.2^b	-1.7 ± 1.2^a	-9.9 ± 10.3^b
VIII	0.2 ± 5.8^a	-37.0 ± 5.1^a	-2.1 ± 1.6^a	-34.1 ± 4.0^a
IX	20.3 ± 1.4^b	3.8 ± 11.7^b	7.9 ± 2.9^b	0.6 ± 6.5^b
X	2.0 ± 3.2^a	6.9 ± 4.1^b	-3.9 ± 0.8^a	-3.8 ± 2.5^b
XI	8.8 ± 2.8^a	10.3 ± 8.2^b	0.9 ± 5.0^a	-2.1 ± 0.6^b

[1] % Inhibition = [(C-S)/C] X 100 where C = oxidation product formed in control and S = oxidation product formed in sample. [2]Values in each column with the same superscript letter were not significantly different. $P < 0.05$.

Table 4: Inhibition[1] on the formation lipid oxidation products in fish oil-in-water emulsions by homologous fractions with different polymerization degrees extracted from pine bark and grape pomace (mean±sd)[2].

DAY 7	Fish oil emulsion					
	Conjugated dienes (%)		Conjugated trienes (%)		Fluorescent compounds (%)	
Fraction	PINE	GRAPE	PINE	GRAPE	PINE	GRAPE
IV	29.1 ± 3.8^c	45.3 ± 7.7^c	33.2 ± 12.1^b	62.3 ± 2.8^c	35.6 ± 0.1^b	47.7 ± 2.1^b
VIII	9.0 ± 3.1^b	29.4 ± 6.5^a	25.4 ± 2.7^b	47.0 ± 5.7^a	32.7 ± 1.6^b	23.5 ± 0.7^a
IX	-1.6 ± 2.2^a	40.2 ± 1.8^b	3.8 ± 2.6^a	39.5 ± 0.5^b	20.6 ± 11.1^a	39.8 ± 12.8^b
X	53.3 ± 8.7^c	54.0 ± 6.4^c	38.4 ± 8.3^c	48.5 ± 5.3^d	54.1 ± 6.1^d	58.8 ± 11.8^c
XI	22.3 ± 4.1^b	57.9 ± 4.8^{bc}	24.7 ± 3.9^b	49.4 ± 3.8^{cd}	43.5 ± 1.2^c	61.9 ± 13.7^c

[1] % Inhibition = [(C-S)/C] X 100 where C = oxidation product formed in control and S = oxidation product formed in sample. [2] Values in each column with the same superscript letter were not significantly different ($p < 0.05$).

Table 5: Inhibition[1] on the formation lipid oxidation products on fish oil in water emulsions by the IV fractions extracted from grape pomace and witch hazel bark (mean±sd)[2].

	Conjugated dienes (%)		1-Octen-3-ol (%)	
	Day 5	Day 6	Day 5	Day 6
IVG	42.8 ± 4.0^{b}	38.6 ± 4.1^{b}	92.2 ± 0.6^{b}	83.1 ± 4.2^{b}
IVH	33.8 ± 2.1^{a}	19.8 ± 8.1^{a}	89.4 ± 1.4^{a}	63.7 ± 5.6^{a}

[1] % Inhibition = $[(C-S)/C]$ X 100 where C = oxidation product formed in control and S = oxidation product formed in sample. [2]Values in each column with the same superscript letter were not significantly different ($p < 0.01$).