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Activity of grape polyphenols as inhibitors of the oxidation of fish lipids and frozen fish muscle

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Abstract

Grape phenolic compounds were obtained from grape (*Vitis vinifera*) pomace by pressing and posterior maceration. Phenolics resulting of the crude grape extract were fractionated by preparative chromatography. The ability of fractions, containing flavanol monomers, oligomers (procyanidins) and glycosylated flavonols, for inhibiting oxidation of fish lipids was determined. The tests were conducted in fish oils, fish oil-in-water emulsions and in fatty fish species such as mackerel during frozen storage. Partitioning coefficients of grape phenolics showed high solubility in the aqueous media and low in oily media. The results suggest that an optimal combination of procyanidin, degree of polymerization and percentage galloylation may be related to the highest antioxidant efficacy of grape polyphenols in the different systems tested. Monomers were more effective in oily systems. Flavanol oligomers were the most potent inhibitors of oxidation in emulsions and in frozen fish muscle.

Keywords: Fish lipids; Frozen fish muscle; Emulsions; Lipid oxidation; Antioxidants; Grape flavonoids

1. Introduction

Marine lipids have a high content of polyunsaturated fatty acids (PUFA), in particular, eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3) (Ackman, 1999; Pigott & Tucker, 1987). Degradation of PUFA by autooxidation during storage and processing of fish oils and fatty fish, easily leads to the formation of volatiles associated with rancidity (Flick & Martin, 1992; Hsieh & Kinsella, 1989). The rate and degree of the reaction are much dependent upon the fish species, the presence or absence of activators and inhibitors and the treatment or storage (Petillo, Hultin, Krzynowek, & Autio, 1998; Undeland, Ekstrand, & Linggnert, 1998). Rancid off-flavors are still the main objections in the production and commercialization of fish and foodstuffs containing fish oils (Fujimoto, 1993; Jacobsen et al., 1999).

Technological approaches such as glazing with a thin layer of ice or a washing step for eliminating pro-oxidants have demonstrated to retard the rate of oxidation on fatty fish (Richards, Kellerher, & Hultin, 1998; Simeonidou, Govaris, & Vareltzis, 1997). The use of antioxidants is emerging as an effective methodology for controlling rancidity in oils and food (Boyd, Green, Giesbrecht, & King, 1993; Frankel, 1998). Metal chelators or reducing agents have been widely employed in model systems of fish muscle (Richards et al., 1998). Consumers and food industry request natural antioxidants substituting those synthetics (Löliger, 1983). Natural phenolic compounds proved to be effective in preventing rancidity of many lipid systems, in particular fish oils (Medina, Satué-Gracia, German, & Frankel, 1999; Ramanathan & Das, 1992) and minced fish muscle

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or surimi (Fagbenro & Jauncey, 1994; Ikawa, 1998). The activity of these compounds is often difficult to predict, because of the different mechanisms involved in the antioxidant effectiveness (Frankel, 1998).

Grape polyphenolics are well-known antioxidants (Natella, Belelli, Gentili, Ursini, & Scaccini, 2002; Torres et al., 2002). Grape skins and seeds are rich source of these compounds including flavonoids with different degree of polymerization known as proanthocyanidins (Souquet, Cheynier, Brossaud, & Moutounet, 1996). The antioxidant activity of grape polyphenols may depend on the degree of polymerization (Blazsó, Gábor, & Rohdewald, 1997; Yamaguchi, Yoshimura, Nakazawa, & Ariga, 1999). Oligomers (roughly 2–7 residues) are considered more efficient than monomers. Materials of higher degree of polymerization are also active but may be mucosal irritant and show astringency effects (Bravo, 1998).

This work aimed to study the antioxidant activity of grape flavonoids obtained from wine industry byproducts in food systems containing fish oils and frozen fatty fish. Mackerel (*Scomber scombrus*), an under-utilized fatty fish species, has been selected due to the high interest in stabilizing its quality and getting high-value-added products.

Different monomeric and oligomeric procyanidin mixtures were tested as antioxidants in frozen mackerel, fish oils and fish oil-in-water emulsions. The rate of oxidation was monitored by the formation of hydroperoxides and aldehydes together with fluorescent compounds arising from protein-oxidized lipid interactions. The protection on $n-3$ PUFA was also tested. Antioxidant activity was discussed on the basis of the effectiveness in inhibiting oxidation products and in relation to molecular structure of the fractions, solubility, partitioning and adsorption.

2. Materials and methods

2.1. Materials

Fresh Atlantic mackerel (*S. scombrus*) was supplied by a local market. A high quality fish oil was purchased by Fluka (New-Ulm, Swizerland). It was flavorless and odorless, had a peroxide value of 0.5 meq/kg and contained Vit. A = 1440 IU/g and Vit D3 = 150 IU/g. It was composed by: 5.8% of 14:0; 11.9% of 16:0; 6.7% of 16:1 ω7; 1.8% of 18:0; 2.8% of 18:1 ω7; 11.6% of 18:1 ω9; 1.9% of 18:2 ω6; 3.7% of 18:3 ω3; 7.4% of 20:1 ω9; 1.3% of 20:4 ω3; 0.6% of 20:4 ω6; 14.9% of 20:5 ω3; 8.8% of 22:1 ω11; 2.8% of 22:5 ω3 and 17.9% of 22:6 ω3. Soybean lecithin (40% L-α-phosphatidylcholine, Sigma, St. Louis, MO) was used as emulsifying agent. The Folin-Ciocalteu reagent, gallic acid, and propyl gallate were obtained from Sigma. All chemicals and solvents used were either analytical or HPLC grade (Ridel-Haën, Seelze, Germany).

Grape flavonoids was obtaining as a byproduct from pressing destemmed Parellada grapes (Vitis vinifera) and consisted of skins, seeds, and a small amount of stems. This byproduct was collected in the month of October during the 1998 harvest, cooled immediately after pressing, and frozen.

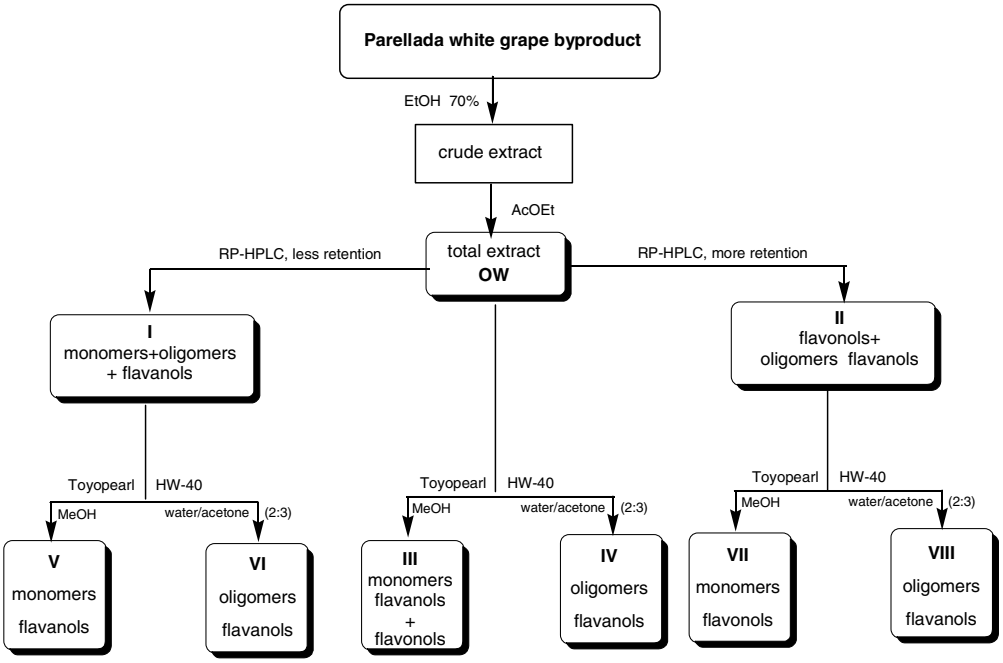
2.2. Isolation of grape phenolics

The isolation of the phenolic fraction OW and its fractionation were performed according to Torres and Bobet (2001) and Torres et al. (2002). OW contained mainly flavanol (catechin) monomers, flavanol oligomers (procyanidins) and monomeric glycosylated flavonols. The total extract OW was separated into a set of fractions differing in composition and procyanidin structure (Scheme 1, Fig. 1 and Table 1). The size and composition (mean degree of polymerisation, percentage galloylation) of the procyanidins within the fractions were estimated from HPLC analysis after depolymerization with cysteamine (Torres & Selga, 2003). The RP-HPLC step fractionated the components on the basis of their hydrophobic interactions with the stationary phase, which is not always related to the size of the flavonoids. Chromatography on Toyopearl separated the components mainly by size: monomers from oligomers. Thus, direct Toyopearl on OW yielded a mixture of monomers III and a mixture of procyanidins IV showing the broadest range of sizes and galloylation among the fractions tested. RP-HPLC yielded a mixture (I) of monomeric catechins and small oligomers (mostly dimeric procyanidins) and a mixture (II) of monomeric flavonols and bulkier procyanidins. Toyopearl chromatography on I and II allowed the discrimination between monomeric catechins (V) and monomeric flavonols (VII) and yielded two procyanidin fractions (VI, VIII) with significantly different mean degree of polymerization and percentage galloylation.

Moreover, fractions were subjected to analytical RP-HPLC to compare their hydrophobic characteristics (Torres et al., 2002). The antiradical power of the fractions was evaluated by the DPPH method (Blois, 1958), as previously described (Torres et al., 2002).

2.3. Preparation of emulsions

Oil-in-water emulsions containing 1% lecithin and 10% fish oil were prepared in water as previously described (Huang et al., 1996b). 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, Spain). Ten millilitres of emulsions were introduced into 50-mL Erlenmeyer flasks and the different antioxidants were added at 0.01% w/w.



Scheme 1.

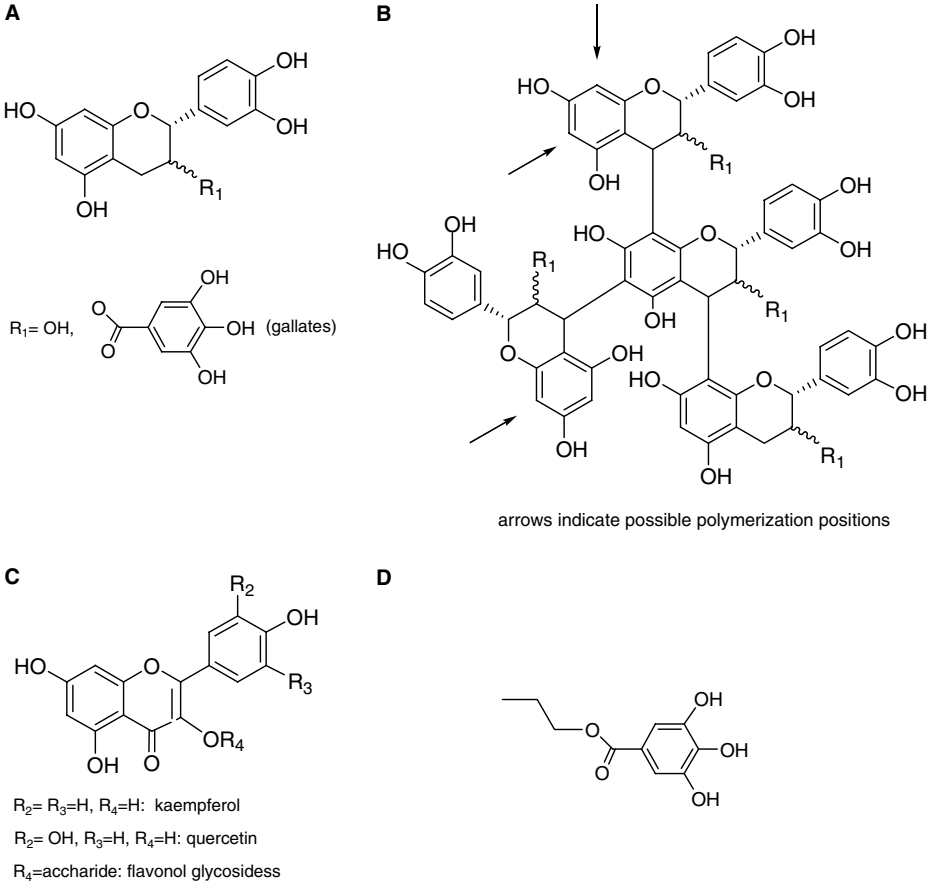


Fig. 1. Molecular structures of grape flavonoids found in Parellada grape byproducts.

Table 1
Mean degree of polymerization, mean molecular weight, percentage galloylation and antiradical power of grape phenolic fractions

Fraction	Mean degree of polymerization	Mean molecular weight	Galloylation (%)	Antiradical power[a]	Estimated molar concentration at 0.01% weight
Total extract (OW)	1.7	552	15	19	1.81×10^{-5}
Monomers and oligomers flavanols (I)	1.4	422	7	26	2.36×10^{-5}
Oligomers polymers flavanols (IV)	2.7	880	25	27	1.14×10^{-5}
Monomers flavanols (V)	1.0	290	<1	20	3.44×10^{-5}
Oligomers flavanols (VI)	2.4	751	16	24	1.33×10^{-5}
Monomers flavonols (VII)	1.0	302	0	7	3.31×10^{-5}
Polymers flavanols (VIII)	3.4	1160	34	27	0.86×10^{-5}
Propyl gallate	1.0	212			4.72×10^{-5}

[a] $(1/ED_{50}) \times 10^3$, were ED_{50} was the amount of fraction (μg) able to consume half the amount (μg) of DPPH free radical divided by the initial concentration of DPPH. $SD < 2$, $n = 3$.

2.4. Frozen storage of mackerel

Fish was deboned, eviscerated and the white muscle was separated and minced. Antioxidants were added at concentration of 0.01% w/w. Portions of 10 g of fish muscle were placed into 50-mL Erlenmeyer flasks and stored at −10 °C to ensure oxidation in all samples within 5–6 months.

2.5. Oxidation procedure

Samples of fish oils (5 g) were introduced into 50-mL Erlenmeyer flasks and exposed to oxidation while shaking at 40 °C. Antioxidants were added at concentration of 0.01% w/w. For each antioxidant and control, triplicate samples were prepared and subjected to oxidation. Oxidative stability was followed by measuring conjugated diene and triene hydroperoxides in duplicate. The experiment was repeated twice.

Samples of fish oil-in-water emulsions were oxidized during shaking at 30 °C. For each antioxidant and control, triplicate samples were prepared and subjected to oxidation. Oxidative stability was evaluated by measuring conjugated diene and triene hydroperoxides and fluorescence compounds in duplicate. The experiment was repeated twice.

Duplicate samples of frozen muscle were taken regularly during six months, and oxidation was followed by measuring peroxide value, conjugated diene and triene hydroperoxides, TBA-index, fluorescence compounds and degradation of PUFA in duplicate. The experiment was repeated twice.

Inhibition of oxidation was calculated during the propagation period of controls according to Frankel (1998). Induction periods were calculated as the time (in days) required for a sudden change in the rate of oxidation by the method of tangents to the two parts of the kinetic curve (Alaiz, Hidalgo, & Zamora, 1997; Frankel, 1998). The effectiveness of each antioxidant was evaluated by comparing the inhibition of oxidation, the induction periods of oxidation and the maximal amount of oxidation products formed during the experiments. Propyl gallate was used as synthetic control in all systems.

2.6. Lipid extraction

Lipids were extracted from mackerel muscle (Bligh & Dyer, 1959). Lipid content was determined gravimetrically in duplicate and expressed as percentage wet weight (Herbes & Allen, 1983).

2.7. Lipid oxidation analysis

2.7.1. Peroxide value

Peroxide value of fish muscle was determined by the ferric thiocyanate method (Chapman & McKay, 1949) and was expressed as mmol oxygen/kg lipid. Analyses were performed in duplicate.

2.7.2. Conjugated diene and triene hydroperoxides

About 100 mg of fish oil samples and emulsion samples were dissolved in hexane and in ethanol, respectively. Absorbance was measured at 234 and 268 nm (UV–Vis Spectrophotometer Perkin–Elmer) and calculated as mmol hydroperoxydes/kg of oil as described previously (Huang, Frankel, Schwarz, Aeschbach, & German, 1996a). As for fish muscle, conjugated hydroperoxides were measured from the organic phase resulting from the Bligh and Dyer extraction (1959) by solving 100 mg of lipids in hexane and measuring absorbance as above described.

2.7.3. TBA index

The thiobarbituric acid index (TBA-i) (mg malonaldehyde/Kg muscle) was determined according to Vyncke (1970).

2.7.4. Measurement of fluorescence compounds

About 100 mg of emulsion samples were dissolved in ethanol. Fluorescence was measured at 345/416 and 393/

463 nm (Perkin–Elmer LS 3B) and was standardized with a quinine sulphate solution (1 μg/mL in 0.05 M H_2SO_4) according to the procedure described by Nielsen, Finto, and Hurrell (1995). Fluorescence of fish muscle was measured directly from the aqueous phase of the resulting Bligh and Dyer extraction. The relative fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the sample fluorescence at each excitation/emission maximum, and F_{st} is the corresponding fluorescence intensity of a quinine sulfate solution (1 μg/mL in 0.05 M H_2SO_4) at the corresponding wavelength. The fluorescence shift (δF) was calculated as the ratio between both RF values: $\delta F = RF_{393/463\ nm}/RF_{345/416\ nm}$.

2.7.5. Fatty acid analysis

Lipids extracted from mackerel muscle were methylized (Lepage & Roy, 1986) and analyzed by gas-chromatography (Christie, 1982).

2.8. Determination of partitioning coefficients between oil and water

The partition coefficient between oil and water for each antioxidant was calculated according to Huang et al. (1996a). Briefly, 1mL of fish oil and 1 mL of water containing antioxidants were well mixed and centrifuged. The antioxidant concentrations in the aqueous phase before and after mixing were quantified by the Folin–Ciocalteu method (Singleton & Rossi, 1965) and expressed as gallic acid, quercetin and catechin equivalents. The amount of antioxidant in the oily phase was calculated as the difference between the total amount of antioxidant in water before mixing and the amount after mixing with oil. Coefficients were calculated as: V_W/V_O. (W_O/W_W) where V_W is the volume of water, V_O is the volume of oil, W_O is the amount of antioxidant in the oily phase and W_W is the amount of the antioxidant in the aqueous phase.

2.9. Measure of adsorption of antioxidants onto fish muscle

Mackerel minced muscle (1 g) was mixed with 1 mL of water containing antioxidants and centrifuged. The antioxidant concentrations in the aqueous phase before and after fish muscle addition were determined by the Folin–Ciocalteu method (Singleton & Rossi, 1965). Fish proteins were removed by precipitation with freeze ammonium sulfate before quantification. Samples with no antioxidants were used as blanks. Ammonium sulfate had no effect on grape phenolics and propyl gallate. The adsorption onto fish muscle was calculated as the total antioxidant amount – the antioxidant amount in the aqueous phase after muscle mixing.

2.10. Statistical analysis

The data were compared by one-way analysis of variance (ANOVA) (Sokal & Rohlf, 1981), and the means were compared by a least squares difference method with Statistica 6.0 program (Statsoft, Tulsa, Oklahoma).

3. Results

3.1. Characterization of the flavonoid fractions by their degree of polymerization and percentage galloylation, and antiradical activity

Total extract OW obtained from Parellada grape byproducts contained mainly catechins (flavanols), both monomeric and oligomeric, and glycosylated flavonols. The fractions differ in their mean degree of polymerization, percentage galloylation and antiradical power by the DPPH assay (Table 1). Oligomers showed numerical higher antiradical power than monomers. Monomeric flavonols were less potent than monomeric flavanols. OW showed an intermediate antiradical power. Propyl gallate and grape monomers (Fractions V and VII) were employed in higher molar concentrations than polymers and oligomers.

3.2. Inhibition of lipid oxidation in fish oil

The fish oil employed had a high content of PUFA (around 43%) and was hereby highly susceptible to oxidation. All grape phenolic extracts showed significant antioxidant efficiency for retarding the induction periods of oxidation. The induction periods (days) of conjugated dienes were: control = 2.8; OW = 3.2; I = 4.2; IV = 3.7; V = 4.9; VI = 4.2; VII = 4.4; VIII = 3.2 and propyl gallate = non detected. The induction periods (in days) of triene conjugated formation were: control = 3.9; OW = 4.1; I = 5.0; IV = 5.1; V = 5.3; VI = 5.0; VII = 5.3; VIII = 5.7 and propyl gallate = 6.0. Table 2 shows

Table 2
Inhibition by 0.01% phenolics on the formation of hydroperoxydes in fish oils at 40 °C (mean ± sd)[A,B]

Phenolic antioxidants	Conjugated dienes (day-5)	Conjugated trienes (day-6)
Control	0.0 ± 0.3^{a}	0.2 ± 1.1^{a}
OW	30.6 ± 0.2^{c}	17.2 ± 1.5^{b}
I	43.5 ± 5.6^{d}	38.5 ± 3.9^{c}
IV	20.2 ± 2.3^{b}	41.9 ± 2.9^{c}
V	61.6 ± 2.3^{e}	57.6 ± 5.9^{d}
VI	44.7 ± 3.9^{d}	46.2 ± 6.6^{cd}
VII	43.2 ± 4.1^{d}	36.6 ± 6.9^{c}
VIII	15.3 ± 0.7^{b}	46.3 ± 2.3^{cd}
Propyl gallate	69.3 ± 1.7^{f}	67.8 ± 1.7^{e}

[A] Percentage inhibition = $[(C - S)/C] \times 100$ where C = oxidation product formed in control; S = oxidation product formed in sample.

[B] Values in each column with the same superscript letter were not significantly different ($p < 0.01$).

the percent of inhibition on the formation of hydroperoxides. These data confirm a significant antioxidant activity of grape phenolics and propyl gallate in fish oils. The synthetic antioxidant was more effective for inhibiting the formation of hydroperoxides, both conjugated dienes and trienes, than grape flavonoids. Grape monomers were more effective than grape oligomers. Monomeric flavanols (V) were more effective in fish oils than monomeric flavonols (VII).

3.3. Inhibition of lipid oxidation in fish oil-in water emulsion

Fig. 2 shows the formation of hydroperoxides and fluorescent compounds in fish oil-in-water emulsions.

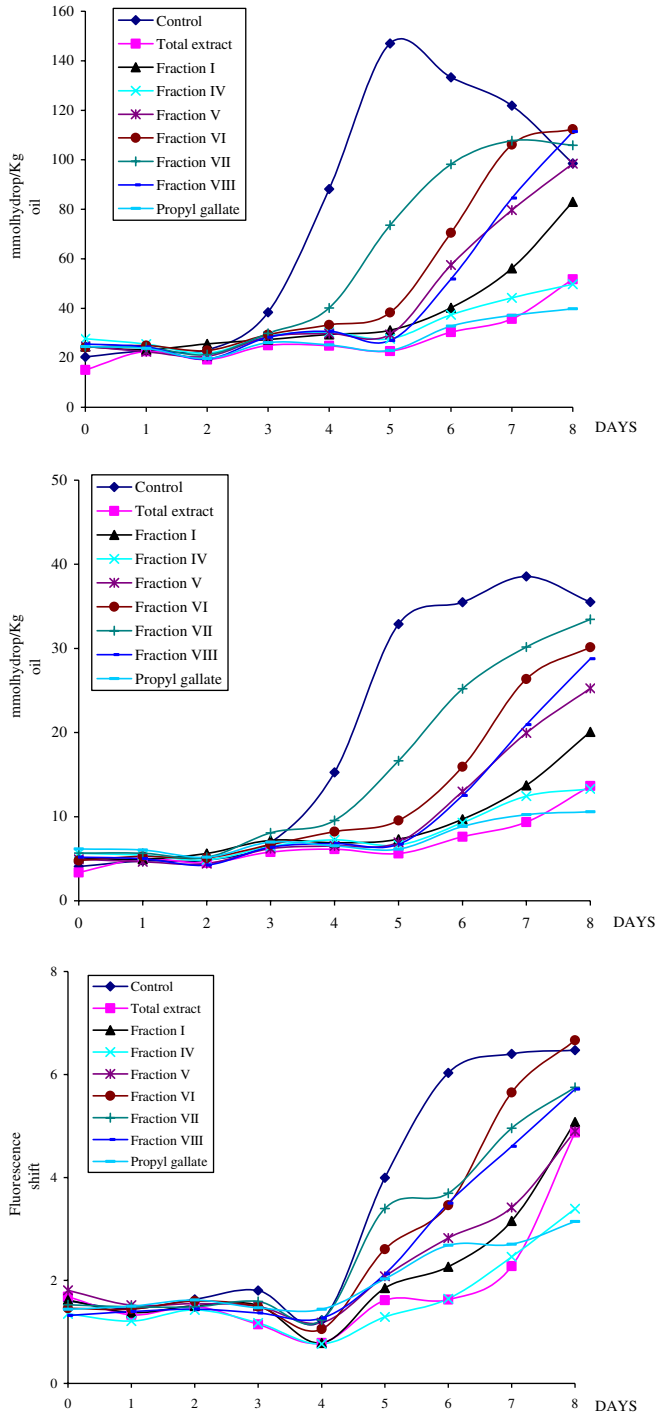


Fig. 2. Formation of conjugated dienes (a); conjugated trienes (b); fluorescent compounds (c) in fish oil-in-water emulsions treated with grape-phenolics and propyl gallate during oxidation at 30 °C.

Table 3
Inhibition by 0.01% phenolics on the formation of hydroperoxydes and fluorescent compounds in fish oils-in water emulsions at 30 °C (mean ± sd)[A,B]

Phenolic antioxidants	Conjugated dienes		Conjugated trienes		Fluorescent compounds	
	(Day-4)	Day 7	(Day-5)	Day 8	(Day-6)	Day 8
Control	0.0 ± 0.2[a]	0.7 ± 0.5[a]	0.1 ± 0.1[a]	0.1 ± 0.1[a]	0.2 ± 0.4[a]	0.0 ± 0.3[a]
OW	71.8 ± 4.5[c]	70.7 ± 2.1[e]	82.9 ± 0.19[c]	61.6 ± 2.1[e]	73.0 ± 8.8[cd]	24.7 ± 2.1[b]
I	66.9 ± 1.6bc	53.9 ± 2.8[d]	77.8 ± 4.8[c]	55.1 ± 4.1[d]	62.5 ± 7.8[c]	21.6 2.1[b]
IV	65.7 ± 0.6[b]	63.7 ± 1.6[e]	79.9 ± 3.4[c]	62.5 ± 7.8[ef]	72.7 ± 2.1[d]	47.6 ± 2.1[c]
V	66.2 ± 4.0[bc]	49.9 ± 3.4[d]	78.9 ± 6.1[c]	39.1 ± 4.2[c]	62.5 ± 2.1[c]	24.3 ± 2.1[b]
VI	62.2 ± 2.8[b]	12.9 ± 1.5[b]	71.0 ± 0.9[b]	15.1 ± 3.3[b]	42.7 ± 12.9[b]	−3.0 ± 2.9[a]
VII	64.6 ± 5.7[bc]	11.6 ± 4.9[b]	67.8 ± 1.5[b]	17.9 ± 6.7[b]	38.8 ± 5.3[b]	18.4 ± 1.4[b]
VIII	65.2 ± 0.6[b]	30.7 ± 9.7[c]	79.6 ± 6.4[c]	19.0 ± 5.1[b]	41.9 ± 2.1[b]	2.5 ± 0.4[a]
Propyl gallate	71.4 ± 2.1[c]	69.5 ± 5.5[e]	81.3 ± 2.1[c]	70.2 ± 2.1[f]	55.6 ± 3.9[bc]	48.6 ± 6.8[c]

[A] Percentage inhibition = $[(C - S)/C] \times 100$ where C = oxidation product formed in control; S = oxidation product formed in sample.
[B] Values in each column with the same superscript letter were not significantly different ($p < 0.01$).

The comparison of the induction periods and rate of oxidation showed that all grape fractions and propyl gallate were active against oxidation. The rate of oxidation and the amount of oxidation products formed differed among antioxidants. The induction periods for conjugated diene formation (in days) were: control = 2.7; OW = 7.0; I = 4.8; IV = 6.0; V = 4.1; VI = 4.0; VII = 3.0; VIII = 4.5 and propyl gallate = 7.7. The induction periods for conjugated triene formation (in days) were: control = 3.3; OW = 6.2; I = 6.0; IV = 6.0; V = 5.4; VI = 5.2; VII = 4.2; VIII = 5.5 and propyl gallate = 6.1. The induction periods for fluorescence formation (in days) were: control = 4.2; OW = 6.0; I = 5.0; IV = 5.9; V = 5.3; VI = 4.5; VII = 4.3; VIII = 4.6 and propyl gallate = 5.2.

Considering these data and data of inhibitory activity (Table 3), propyl gallate, and the total extract (OW) and fraction IV containing oligomeric flavanols with intermediate degree of polymerization and percentage galloylation, were the most efficient antioxidants. Monomers were less effective in this system than in bulk fish oil compared with polymers. Monomeric flavonols (VII) were the least efficient.

3.4. Inhibition of lipid oxidation in mackerel frozen muscle

Fig. 3 depicts the formation of peroxides and TBA-index obtained in minced mackerel after six months of frozen storage. The induction periods of formation of peroxides and aldehydes were significantly retarded in samples treated with antioxidants: control = 22 and 29 days; OW = 26 and 34 days; IV = 30 and 35 days; V = 32 and 37 days; VIII = 26 and 34 days and propyl gallate = 34 and 37 days. These data, the amount of oxidation products formed and percent of inhibition (Table 4), demonstrated that all flavonoid fractions and propyl gallate were effective for retarding oxidation during frozen storage. The inhibition of formation of hydroperoxides was higher than the inhibition of formation of volatiles. Frozen mackerel treated with propyl gallate showed the largest induction periods of oxidation and the lowest rate and amount of oxidation products formed except for fluorescent compounds. Grape phenolic fractions showed different effectiveness for decreasing the rate of oxidation and the amount of oxidation products formed. Fractions IV and V were the most efficient, and VIII gave the poorest results.

Significant degradation of $n-3$ PUFA was observed in controls after 10 weeks of freezer storage (Table 5). Upon extended freezer storage a significant protection of $n-3$ PUFA by grape fractions and propyl gallate was observed. There were not significant differences among tested antioxidants.

3.5. Partitioning coefficients between oil and water

Grape flavonoids were amply found in the aqueous phase and propyl gallate was more partitioned toward the oily phase. Partitioning coefficients between oil and water were: OW = 0.11 ± 0.01; IV = 0.12 ± 0.01; V = 0.19 ± 0.08; VII = 0.36 ± 0.03; VIII = 0.28 ± 0.05; propyl gallate = 0.83 ± 0.01. Fractions OW and IV were more polar than fractions V, VII and VIII. Propyl gallate was less polar than grape phenolics.

The hydrophobic character of grape flavonoids was also deduced by their behavior in RP-HPLC. The higher retention on analytical RP-HPLC of fractions VII and VIII showed that their components were able to establish stronger hydrophobic interactions than those of fractions V and VI (Scheme 1). The retention on analytical RP-HPLC of components of fraction IV demonstrated that they were fairly hydrophobic.

3.6. Adsorption of antioxidants onto fish muscle

In fish muscle, antioxidants were highly adsorbed on muscle and they were not detected in the aqueous phases after addition and mixing of minced muscle.

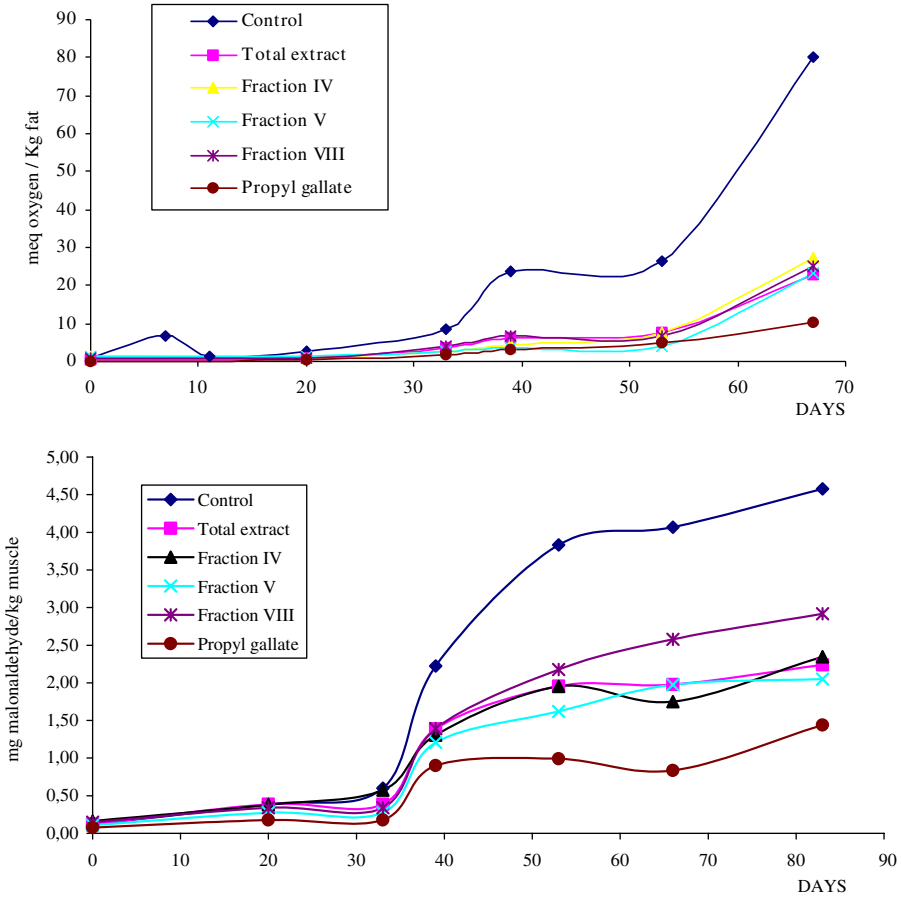


Fig. 3. Formation of peroxides (a); aldehydes (b) in mackerel minced muscle during frozen storage at −10 °C.

Table 4
Inhibition by 0.01% Phenolics on the formation of hydroperoxydes, aldehydes, conjugated dienes and trienes, and fluorescent compounds in mackerel minced muscle during frozen storage at −10 °C (mean ± sd)[A,B]

Phenolic antioxidants	Peroxides		Conjugated dienes		Conjugated trienes		Aldehydes		Fluorescent compounds
	Day 39	Day 67	Day 53	Day 83	Day 53	Day 83	Day 39	Day 83	Day 83
Control	0.0 ± 0.1^{a}	0.5 ± 1.1^{a}	0.0 ± 0.4^{a}	0.0 ± 0.1^{a}	0.2 ± 0.1^{a}	0.3 ± 0.2^{a}	0.6 ± 0.4^{a}	0.0 ± 0.1^{a}	0.6 ± 0.1^{a}
OW	70.7 ± 7.2^{b}	70.4 ± 3.3^{b}	63.8 ± 1.3^{c}	25.6 ± 0.2^{b}	72.4 ± 5.8^{b}	17.4 ± 2.5^{b}	17.5 ± 1.4^{b}	45.5 ± 2.3^{c}	100.0 ± 4.1^{c}
IV	85.2 ± 5.1^{bc}	66.2 ± 4.3^{b}	113.9 ± 4.2^{e}	54.7 ± 10.5^{c}	95.1 ± 2.7^{c}	32.1 ± 6.5^{c}	25.6 ± 5.1^{c}	43.8 ± 1.5^{c}	93.3 ± 9.4^{c}
V	89.7 ± 2.9^{c}	71.3 ± 8.3^{b}	89.6 ± 10.3^{d}	62.4 ± 2.4^{c}	86.2 ± 10.3^{bc}	76.2 ± 3.9^{d}	28.7 ± 4.7^{c}	50.1 ± 5.8^{c}	101.2 ± 7.2^{c}
VIII	69.7 ± 1.9^{b}	68.2 ± 0.1^{b}	46.4 ± 3.8^{b}	−6.5 ± 7.4^{a}	67.8 ± 10.3^{b}	19.6 ± 1.9^{b}	19.0 ± 2.7^{b}	28.8 ± 1.7^{b}	100 ± 7.2^{c}
Propyl gallate	83.9 ± 0.5^{bc}	86.5 ± 3.7^{c}	79.3 ± 7.7^{d}	53.6 ± 6.3^{c}	108.0 ± 3.5^{d}	100.3 ± 12.3^{e}	46.4 ± 0.9^{d}	65.0 ± 3.2^{d}	75.4 ± 3.4^{b}

[A] Percentage inhibition = $[(C - S)/C] \times 100$ where C = oxidation product formed in control; S = oxidation product formed in sample.
[B] Values in each column with the same superscript letter were not significantly different ($p < 0.01$).

4. Discussion

Oxidation in the different systems containing fish lipids: bulk oils, oil-in-water emulsions and frozen fish muscle was retarded by the flavonoid components obtained from grape byproducts. The inhibition of oxidation in mackerel muscle by natural grape components is a significant result from this paper, due to the economic importance of prolonging the quality of fatty fish during storage.

The grape flavanoids obtained from byproducts resulting from viticulture (Parellada grape) differ substantially in their molecular structure and antiradical activity in vitro (Table 1). They differ in the number of phenolic residues and the number and position of hydroxylic groups. Oligomers and monomers showed different DPPH free radical scavenging efficiency, with oligomers being more efficient than monomers. Flavanol monomers showed higher antiradical power than flavonol monomers. This is in agreement with the observa-

Table 5
Concentration of 22:6 $n-3$ (mg/g lipid) in mackerel frozen muscle treated with grape phenolics and propyl gallate (mean ± sd)[A]

Sample	Week 0	Week 10	Week 12	Week 17–26
Control	24.9 ± 0.7^{a}	24.1 ± 0.5^{a}	$22.6\ 0.2^{a}$	19.5 ± 0.5^{a}
OW	25.1 ± 0.5^{a}	23.3 ± 0.3^{a}	24.6 ± 0.4^{b}	22.0 ± 0.8^{b}
IV	24.4 ± 0.6^{a}	24.0 ± 0.7^{a}	25.2 ± 0.3^{b}	21.9 ± 1.5^{ab}
V	23.7 ± 0.5^{a}	23.7 ± 0.6^{a}	25.9 ± 0.2^{b}	21.4 ± 1.1^{ab}
VIII	24.1 ± 0.8^{a}	23.1 ± 0.3^{a}	24.4 ± 0.1^{b}	22.1 ± 2.1^{ab}
Propyl gallate	26.4 ± 0.8^{a}	25.1 ± 0.5^{a}	24.06 ± 0.7^{b}	22.6 ± 0.1^{b}

[A] Values in each column with the same superscript letter were not significantly different ($p < 0.01$).

tion that glycosylated flavonols are less efficient scanvengers than the corresponding aglycones (Rice-Evans, Miller, & Paganga, 1996). Hereby, the DPPH-potency of the total extract OW was intermediate, probably due to the presence of less efficient glycosylated flavonols (Torres et al., 2002). As regards to hydroxylic groups, different studies have demonstrated that the pyrogallol moiety provides more hydrogen atoms or electrons than the catechol group (Bors, Michel, & Stettmaier, 2000). Therefore, antiradical activity of phenolics should increase with the percentage galloylation (Torres et al., 2002) affecting the redox potential and the metal-chelation capacity (Frankel, 1998).

However, the efficiency detected in fish lipids did not show a direct relationship with the number of phenolic residues and the galloylation. Monomers were more efficient than oligomers in fish oils. Fraction VIII with the highest galloylation percentage was not the most active in emulsions and frozen fish muscle. Other features must be also involved in the overall antioxidant efficiency in heterogeneous fish products. Partitioning coefficients and the RP-HPLC retention revealed that the components of grape fractions showed different physical properties such as lipophilicity, solubility and partition.

In bulk fish oils, propyl gallate and monomeric flavanols showed the highest efficiency. A previous study in bulk corn oil showed a higher effectiveness of monomers flavanols than polymers (Torres et al., 2002). The degree of polymerization and percentage galloylation of procyanidins did not show any relation to the activity in bulk oils: fractions IV, VIII, and OW showed the same efficiency. Glycosylated flavonol monomers (fraction VII) were less efficient than the non-galloylated monomeric flavanols (V, mostly catechin) accordingly with their antiradical power.

In oil-in-water emulsions, grape oligomers were more efficient than monomers. Propyl gallate was also highly effective in emulsions in agreement with the observation that hydrophobic compounds are efficient in emulsions because they are largely accumulated in the oily/aqueous interface (Frankel, 1998). However, hydrophilic antioxidants are less active since they are diluted in the water phase. Interestingly, the efficient fractions OW and IV were more polar than propyl gallate and most of the other fractions, as judged by their partition coefficients but they showed the highest activity in emulsions. While these fractions are highly water soluble and consequently not likely to be located at the oil-water interface, most of their components are able to establish hydrophobic interactions as showed by their retention in RP-HPLC. Their effectiveness may be related to a capacity of procyanidins to establish hydrophobic and/or hydrophilic interactions, depending on the environment, as already suggested (Torres et al., 2002). They have hydrophobic cores with hydrophilic hydroxyl groups and may expose both or any of the two regions, resulting in surfactant-like accumulation active water–oil interfaces.

With regard to the molecular structure, fraction IV with an intermediate polymerization degree and galloylation percentage (2.7% and 25%, respectively), is more effective than fractions VIII and VI. Apparently, the antioxidant efficacy is not directly related to any of the structural parameters measured. The results suggest that there may be an optimal combination of both degree of polymerization and percentage galloylation in relation to the antioxidative efficiency of procyanidins in fish oil-in-water emulsions. It has recently suggested that galloylation might be less significant than procyanidin size (Torres et al., 2002). Further studies with fractions of fixed degree of polymerization and different galloylation should help clarify this point.

As also observed in the emulsion, fraction IV was the most effective in inhibiting oxidation in mackerel muscle during frozen storage. The total extract, OW, showed significant efficiency as well. According to the data found in fish oil emulsions, both fractions were active at lower molar concentrations than those of the synthetic antioxidant and monomeric flavanols, fraction V. There was no difference in the prevention of PUFA degradation among antioxidants during the period of frozen storage.

Grape flavonoids were totally adsorbed within the fish muscle. This is an important result considering development of technological strategies to minimize oxidation, e.g. immersion or glazing for whole fish or fillets into antioxidants solutions. By using an aqueous solution of grape phenolics, the compounds will migrate from water to the flesh surface. Propyl gallate was also highly adsorbed within the fish muscle. However, propyl gallate is not soluble in aqueous solutions and will consequently not be suitable for immersion techniques.

5. Conclusions

In oils, propyl gallate was much better antioxidant than grape flavonoids. In emulsions, grape polyphenolic fractions were as effective as propyl gallate. Monomers

were more effective in oily systems than polymers. However, flavanol oligomers were the most potent inhibitors of oxidation in emulsions and in frozen fish muscle. In both oils and emulsions, monomeric flavanols were more effective than monomeric glycosylated flavonols.

The effectiveness of inhibiting oxidation in fish oils seems to be more dependent on the physico-chemical properties rather than the intrinsic redox capacity of the antioxidant, related to the number of orto-dihydroxy groups and gallates. To test this hypothesis, further studies with more structurally diverse fractions will have to be addressed. The results also suggest that an optimal combination of procyanidin degree of polymerization and percentage galloylation may be related to the highest antioxidant efficacy of grape polyphenols in emulsions and in frozen fish muscle.

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