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ORIGINAL PAPER

Manuel Pazos · Ma Jesús González · José Manuel Gallardo · Josep Lluís Torres · Isabel Medina

Preservation of the endogenous antioxidant system of fish muscle by grape polyphenols during frozen storage

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Abstract The capacity of a phenolic extract, OW, obtained from grape (*Vitis vinifera*) by-products, and of a purified fraction of procyanidins from OW, fraction IV, for preservation of endogenous antioxidants of fatty fish was investigated during frozen storage. They were used in muscle concentrations of 0.01% (w/w). Grape polyphenols were compared with propyl gallate, a synthetic antioxidant. The exogenous compounds were added to minced mackerel (*Scomber scombrus*) muscle and horse mackerel (*Trauchurus trauchurus*) fillets, before freezing at −10 °C. The results demonstrated that grape polyphenols and propyl gallate inhibit the depletion of endogenous α-tocopherol, ubiquinone-10 and total glutathione. Grape polyphenols and propyl gallate showed similar efficiency for preservation of ubiquinone, in both minced and filleted muscle, and total glutathione, in minced muscle. Total glutathione in the fillets was better maintained by propyl gallate than grape polyphenols. The endogenous antioxidant more efficiently preserved by grape polyphenols and propyl gallate was α-tocopherol. Its loss elapsed faster in the order control> OW>fraction IV>propyl gallate. The depletion of α-tocopherol was highly correlated with the evolution of lipid oxidation. The development of lipid oxidation was repressed, while the concentration of α-tocopherol was not reduced up to critical levels.

Keywords Endogenous antioxidant system · Frozen fish muscle · Oxidation · Grape polyphenols

M. Pazos (✉) · M. J. González · J. M. Gallardo · I. Medina
Instituto de Investigaciones Marinas del CSIC,
Eduardo Cabello 6, 36208 Vigo, Spain
e-mail: mpazos@iim.csic.es
Tel.: +34-986-231930
Fax: +34-986-292762

J. L. Torres
Instituto de Investigaciones Químicas y Ambientales de Barcelona, IIQAB-CSIC,
Jordi Girona 18–26, 08034 Barcelona, Spain

Introduction

Fatty fish is an important type of nutritional seafood particularly owing to the high concentration of polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (20:5ω-3) and docosahexaenoic acid (22:6ω-3) [1]. The content of unsaturated lipids gives an important functionality to fatty fish related to well-known effects on cardiovascular diseases and arteriosclerosis [2] but also leads to important loss of quality associated with rancidity. Rancidity still continues to be the main problem in the production and commercialisation of fatty fish. During storage and processing of fatty fish, off-flavours are easily produced by oxidation of PUFA [3].

In vivo, fatty fish contains an antioxidant system that stabilises its high content of unsaturated lipids. The endogenous antioxidant system includes compounds that are able to scavenge free radicals and enzymes, which remove reactive oxygen species, such as radical superoxide, hydrogen peroxide and lipid peroxides [4]. The major lipophilic antioxidants in fish are α-tocopherol, ubiquinone and carotenoids. Glutathione and ascorbate are hydrophilic compounds with antioxidant potential [5]. In post mortem conditions, those endogenous antioxidants are consumed sequentially and some studies have related their loss with oxidation development [4, 6, 7].

Some natural polyphenols are effective in preventing rancidity of fish muscle [8–10]. Among these antioxidants, grape oligomeric catechins (procyanidins) [11] are highly effective in delaying lipid oxidation in minced fatty fish muscle during frozen storage [12]. Some of these grape compounds are capable of inducing apoptosis in cancer cells and are powerful antiradical agents [13]. Therefore, they have a potential interest as food supplements. Recent studies have also demonstrated that natural flavonoids can regenerate and protect α-tocopherol in homogenous methyl linoleate systems [14] and in human low-density lipoproteins [15].

The aim of this work was to study the possible protective effect of grape procyanidins on endogenous antioxidants of fatty fish during frozen storage. The exper-

iments were carried out on minced mackerel and on horse mackerel fillets. α-Tocopherol, ubiquinone-10 and total glutathione were the endogenous antioxidants studied. Their depletion was correlated with lipid oxidation. This study contributes to better knowledge of the antioxidant mechanism of grape proyanidins in frozen fatty fish and reinforces their use as functional ingredients.

Materials and methods

Materials

Fresh Atlantic mackerel (*Scomber scombrus*) and horse mackerel *(Trauchurus trauchurus)* were supplied by a local market. The Folin–Ciocalteu reagent and propyl gallate were obtained from Sigma (St. Louis, MO, USA). All chemicals and solvents used were either analytical or high-performance liquid chromatography (HPLC) grade (Riedel de-Haën, Seelze, Germany). The raw material for obtaining grape flavonoids was the by-product from pressing destemmed Parellada grapes (*Vitis vinifera*) and consisted of skins, seeds, and a small amount of stems.

Isolation and characterisation of grape phenols

Two grape extracts were employed in this study. Total extract OW contained mainly flavanol (catechins) monomers, flavanol oligomers (procyanidins) and monomeric glycosylated flavonols. Fraction IV, a purified fraction from OW, was composed of a mixture of procyanidins with a mean degree of polymerisation and a percentage of galloylation of 2.7 and 25%, respectively. The phenolic extract OW from grape by-products was obtained according to the method of Torres et al. [16]. Chromatography of Toyopearl HW-40 of OW was used for isolating fraction IV [17]. Both extracts were effective in stabilising fish oil systems and muscle in a previous study [12].

Frozen storage of minced fish muscle and fish fillets

Fresh mackerel were deboned and the light muscle was separated and minced. Skin-on fillets from fresh horse mackerel (20–25 g) were obtained. OW, fraction IV and propyl gallate, used as a synthetic control, were added in an aqueous solution. The final concentration of exogenous antioxidants was 0.01% (w/w). In control samples, a similar amount of distilled water was added. Portions of minced muscle (10 g) were placed into 50-ml Erlenmeyer flasks and stored at −10 °C. The fillets were packed in plastic bags and stored at −10 °C. Duplicate and triplicate samples were taken at different times from minced muscle and fillets, respectively. The samples were thawed at room temperature 1 h before of analysis.

Sensory analysis

A professional sensory panel, composed of 3–4 people, classified the raw odour as fresh, without fresh odour, low intensity of rancid odour and high intensity of rancid odour [18]. A quantification of raw odour was made by assigning a score from 0 to 3. So, a score of 0 indicated freshness and a score of 3 indicated a high intensity of rancid odour.

Extraction and analysis of α-tocopherol and ubiquinone-10

Lipid-soluble antioxidants were extracted by adaptation of the method of Burton et al. [19]. Minced muscle (1 g) was homogenised in 3 ml of chilled 5 mM sodium phosphate buffer, pH 8.0. A 4 ml aliquot of 0.1 M sodium dodecyl sulfate was added to the homogenate and the mixture was vigorously shaken for 1 min. Then, absolute ethanol (8 ml) was added and the mixture was shaken for 1 min. Hexane (2 ml) was then added and the mixture was shaken for 1 min. After a brief chilled centrifugation, the top hexane phase was recovered and the aqueous phase was washed with hexane (1 ml). The hexane layer was dried under a stream of nitrogen. Lipid-soluble antioxidants were extracted twice from the oily drops that remained with methanol (1 ml). Finally, methanol was evaporated under a stream of nitrogen and absolute ethanol (300 μl) was added. Antioxidants were quantified by HPLC as described Cabrini et al. [20].

Extraction and analysis of total glutathione and thiobarbituric acid reactive substances

A modification of the method by Petillo et al. [4] was used as the extraction procedure. Two grams of muscle was homogenised with chilled 5% 5-sulfosalicylid acid (10 ml). Reduced glutathione and oxidised glutathione were measured together as total glutathione using an enzymatic assay based on glutathione reductase [21]. Reduced glutathione was used as a standard. The thiobarbituric acid reactive substances (TBARS) content (μmol of malonaldehyde per kilogram of muscle) was determined according to the method of Vyncke [22].

Lipid extraction

Lipids were extracted from muscle by the Bligh and Dyer method [23]. The lipid content was determined gravimetrically [24].

Peroxide value

The peroxide value of fish muscle was determined by the ferric thiocyanate method [25] and was expressed as milliequivalents of oxygen per kilogram of lipid.

Data collection

Each experiment was repeated twice. All analyses were performed in duplicate. Comparison of means and correlations were performed by a least-squares difference method [26].

Results

Possible protection of endogenous antioxidants in mackerel minced muscle

The control fish suffered a strong depletion of α-tocopherol between days 25 and 33, and contained only 14% of the initial α-tocopherol level at day 33 (Fig. 1a). However, OW, fraction IV and propyl gallate delayed significantly the loss of α-tocopherol. With these treatments the depletion of α-tocopherol also started between days 25 and 33, but the rate of depletion was significantly higher for the control than for samples with added antioxidants. The rate of α-tocopherol degradation followed the order control>OW>IV>propyl gallate. At day 83, OW, fraction IV and propyl gallate preserved 30, 39 and 55%, respectively, of the initial α-tocopherol level.

The levels of ubiquinone-10 decreased more slowly than those of α-tocopherol (Fig. 1b). The control main-

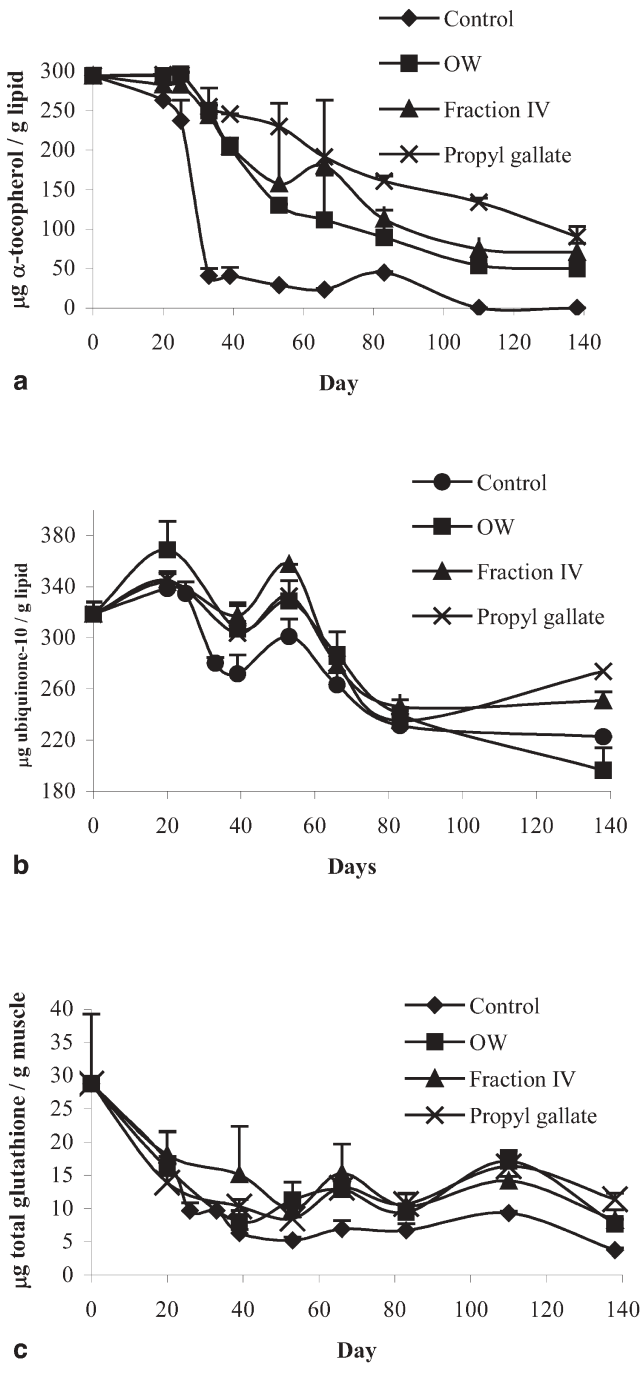

Fig. 1 Levels of α-tocopherol (**a**), ubiquinone-10 (**b**) and total glutathione (**c**) in mackerel minced muscle with and without addition of exogenous compounds during storage at −10 °C

tained about 70% of the original ubiquinone-10 level at day 138. OW, fraction IV and propyl gallate preserved significantly this lipophilic antioxidant, maintaining its levels above those than in the controls. After 138 days, fraction IV and propyl gallate were the most active antioxidants for preservation of ubiquinone.

Total glutathione was rapidly degraded in the controls and the samples treated with exogenous antioxidants. A reduction of 40–50% of total glutathione was observed during the first 20 days (Fig. 1c). After this period, all samples with exogenous antioxidants had higher total glutathione levels than the controls. So, at day 66, OW, fraction IV and propyl gallate preserved about 50% of the original total glutathione, against the 24% that was left in the controls.

Possible protection of endogenous antioxidants in horse mackerel fillets

In minced fish muscle, for preservation of endogenous antioxidants OW showed lower activity than or similar activity as fraction IV. Therefore, it was not tested in fish fillets. The level of α-tocopherol in the control fillets decreased significantly during the first 20 days (Fig. 2a). In contrast, fillets treated with fraction IV maintained 55% of the initial α-tocopherol level at day 33 and 38% at day 47. Propyl gallate also preserved over 85% of the original α-tocopherol level after 68 days at −10 °C.

The levels of ubiquinone-10 were conserved almost intact in the control fillets and in fillets treated with exogenous antioxidants after 47 days at −10 °C (Fig. 2b). There was a significant preservation of ubiquinone in samples treated with fraction IV and propyl gallate after 118 days. At this time, the control fillets decreased their initial ubiquinone levels up to 40% and fillets with fraction IV and propyl gallate still had about 75% of the original levels.

The control fillets lost over 60% of the initial glutathione level during the first 33 days at −10 ° (Fig. 2c). Fraction IV and propyl gallate were efficient in delaying the degradation of total glutathione. They conserved over 70% of the initial level at day 47, against 40% that was left in the controls. Propyl gallate showed higher effectiveness for preserving the glutathione level than fraction IV at day 68. After that, the levels of glutathione in the control fillets and in the fillets treated with both exogenous antioxidants were not significantly different.

Correlation between the preservation of endogenous antioxidants and lipid oxidation

The kinetics of peroxide and aldehyde formation was compared with the depletion of endogenous antioxidants. The data for minced muscle (Table 1, Fig. 1) indicated that α-tocopherol degradation was highly correlated with the increase of peroxides and TBARS (R^2=0.998 and R^2=0.893, respectively). With regards to glutathione, there was no correlation between lipid oxidation and levels of glutathione, owing to an extensive loss of glutathione during the first 20 days (Fig. 1c), before the formation of oxidation products. Ubiquinone was not correlated with the development of lipid oxidation.

A high inhibition of peroxides and off-flavour formation was observed by the use of fraction IV and propyl gallate in horse mackerel fillets (Table 2). The rates of odour and peroxide formation and α-tocopherol depletion

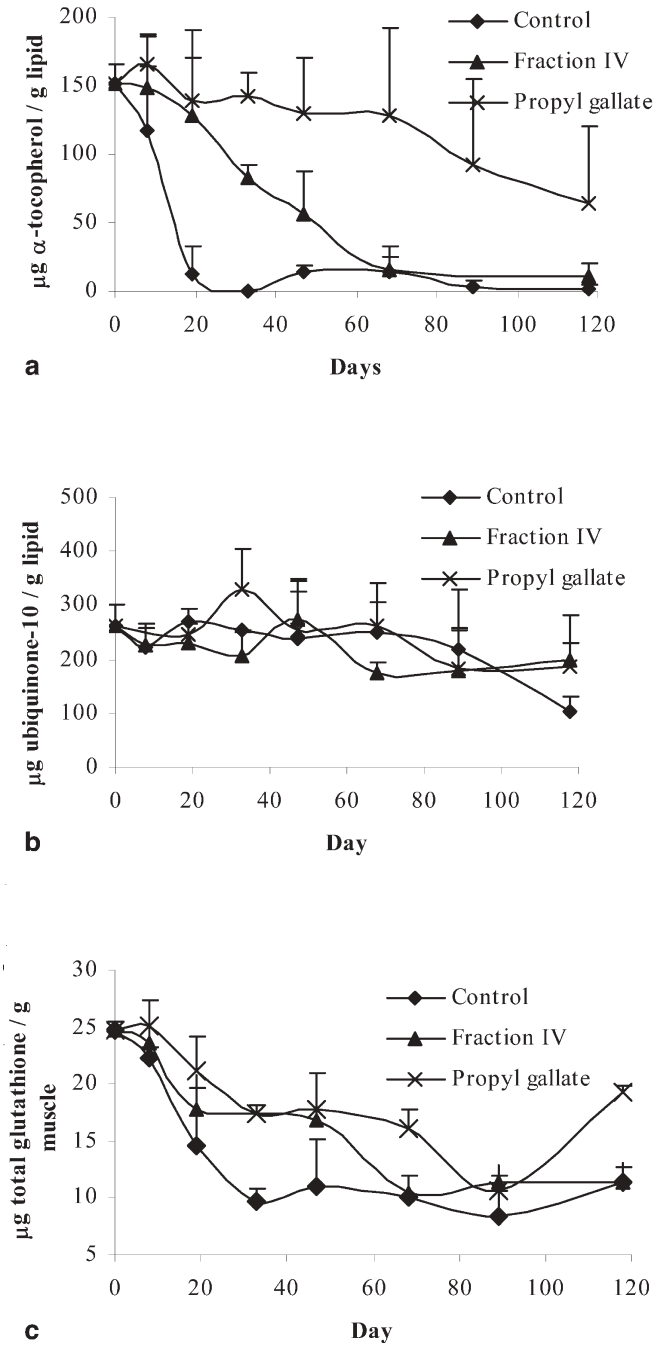

Fig. 2 Levels of α-tocopherol (**a**), ubiquinone-10 (**b**) and total glutathione (**c**) in horse mackerel fillets with and without addition of exogenous compounds during storage at −10 °C

were highly correlated (R^2=0.997 and R^2=0.999, respectively). The rates of glutathione depletion correlated to a lesser extent with peroxide and odour formation (R^2=0.916 and R^2=0.859, respectively). Ubiquinone was not correlated with lipid oxidation.

Discussion

The data of frozen minced muscle and fillets showed that the development of lipid oxidation is strongly related to the levels of endogenous α-tocopherol. Peroxides, TBARS and off-flavours were inhibited, while the α-tocopherol concentration did not fall below a critical level. When α-tocopherol had almost totally disappeared, faster formation of these oxidation products was observed. In a previous work, Erickson [7] also observed that the consumption of α-tocopherol coincided with the increase of lipid oxidation products. Total glutathione, which is formed mainly by reduced glutathione [6], of minced muscle and fillets decreased in the early stages of fish storage, while a good sensory score was still maintained. This finding was in agreement with previous observations [4, 6]. The "pecking order" [27], based on the one-electron reduction potential, also predicts this faster depletion of reduced glutathione than α-tocopherol in oxidative processes. Ubiquinone-10 was stabler than glutathione and α-tocopherol, and it was maintained over the initial levels for at least 70 days in both minced and filleted muscle. Ubiquinone-10 is the oxidised form of coenzyme Q and their reduced species, ubiquinol and semiubiquinone, are the actual substances with antioxidant activity. Ubiquinol could act as an antioxidant by direct reaction with oxygen radicals or by means of regeneration of α-tocopherol from α-tocoferoxil radical [27]. But, its very low concentrations in light and dark fish muscle [4] suggest a minor role of ubiquinol in the antioxidative process of fish muscle.

The data of this work indicated that the preservation of gluthatione and ubiquinone by grape polyphenols should not have a significant relevance for stabilising fatty fish muscle. However, the stabilisation of frozen fatty fish by grape procyanidins could be related to the protection of endogenous α-tocopherol. α-Tocopherol seems to be one of the last defences of fish muscle to avoid oxidation. Its reduction below a critical level would lead to lipid oxidation in both controls and samples with exogenous antioxidants. The mechanism involved in α-tocopherol preservation can be related to the capacity of polyphenols for restraining peroxide formation and/or for regenerating α-tocopherol. Some polyphenols, like catechin, epicatechin and quercetin, are able to regenerate α-tocopherol in human lipoproteins and linoleic systems [14, 15].

The activity of grape procyanidins and propyl gallate for preservation of endogenous antioxidants was not similar in all systems. Grape polyphenols and propyl gallate showed a similar efficiency for conservation of ubiquinone, in both minced and filleted muscle. They also showed similar activity for preservation of total glutathione in minced muscle. However, total glutathione was better preserved by propyl gallate in fillets and propyl gallate was also the most active for inhibiting α-tocopherol degradation in minced muscle and fillets.

In the evaluation of the effectiveness of these exogenous compounds, it should be considered that propyl gallate was employed in higher molar concentration. The

Table 1 Formation of peroxides and aldehydes in minced mackerel muscle without and with exogenous antioxidative treatment during frozen storage at −10 °C

Days	Hydroperoxides (mEq oxygen/kg lipid)				Aldehydes (μmol malonaldehyde/kg muscle)			
	Control	OW	Fraction IV	Propyl gallate	Control	OW	Fraction IV	Propyl gallate
0	0.8±0.3	0.6±0.0	1.1±0.0	0.9±0.0	2.0±0.2	1.6±0.3	2.1±0.2	1.1±0.2
25	2.9±0.0	1.0±0.1	0.9±0.4	0.5±0.1	5.6±0.7	5.4±1.2	5.0±0.9	2.2±1.2
33	8.4±3.6	3.8±0.8	2.6±1.7	1.7±0.3	8.1±1.2	5.5±1.0	4.4±0.8	2.7±0.7
39	24.0±3.0	6.1±0.1	4.8±3.1	3.3±0.1	31.2±1.0	19.6±0.3	18.2±1.0	12.1±0.2

Table 2 Formation of peroxides and odour sensory scores in horse mackerel filleted muscle, without and with exogenous antioxidative treatment, during frozen storage at −10 °C

Days	Peroxides (mEq oxygen/kg lipid)			Sensory scores of raw odour		
	Control	Fraction IV	Propyl gallate	Control	Fraction IV	Propyl gallate
0	0.9±0.2	0.9±0.2	0.9±0.2	0.00	0.00	0.00
8	1.0±0.4	1.4±0.5	0.5±0.1	0.00	0.00	0.00
19	12.1±4.5	5.8±1.3	0.3±0.1	2.00	1.00	0.50
33	23.0±6.3	9.2±4.5	3.12±0.4	2.50	1.00	0.75
47	19.6±7.0	8.4±2.3	4.6±1.0	1.75	1.50	1.00
68	22.6±7.6	23.8±6.7	2.9±0.8	1.75	3.00	1.25
89	30.8±6.1		13.9±5.3	2.25		1.25
118	26.5±7.0	31.7±3.5	11.6±3.5	2.00	2.25	1.50

estimated molar concentrations of propyl gallate, OW and fraction IV were 0.47, 0.18 and 0.11 mmol/kg muscle, respectively. Additionally, the molecular structure can play a significant role. Propyl gallate has a smaller molecular size and it is more lipophyllic than OW and fraction IV [12].

Fraction IV was more effective for prevention of lipid oxidation and α-tocopherol depletion than OW. This finding can be attributed to the composition of fraction IV, which is richer in procyanidins. Monomers probably decreased the overall activity of OW according to the suggestion that polymers are more efficient antioxidants than monomers [12]. Additionally, the high activity of procyanidins of fraction IV can also be attributed to the appropriate combination of both the degree of polymerisation and the percentage of galloylation. Studies performed with procyanidins with different degrees of polymerisation and galloylation showed that procyanidins contained in fraction IV were the most active antioxidants in lipid emulsions and frozen fatty fish [12].

In conclusion, the preservation of α-tocopherol by grape polyphenols seems to be a relevant aim for increasing the oxidative stability of fatty fish. α-Tocopherol, which is probable the last endogenous barrier against lipid oxidation in fish muscle, was specially preserved by these exogenous compounds. So, effective treatments for inhibiting lipid oxidation in fish foodstuffs should be obtained by the application of natural compounds with suitable redox potential able to stabilise and/or regenerate intact α-tocopherol. Work is now in progress to establish synergisms between grape fractions and the endogenous antioxidant system in fish tissues. Grape procyanidins were able to stabilise frozen fatty fish and preserve an important compound as vitamin E. They have also shown functional properties [13]. Therefore, grape procyanidins can be a potential and attractive food additive.

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