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Effect of grape antioxidant dietary fibre on the prevention of lipid oxidation in minced fish: Evaluation by different methodologies

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Abstract

The effect of grape antioxidant dietary fibre (GADF) addition to minced fish muscle (MFM) on lipid stability during frozen storage (6 months) was studied. Concentrations of 0%, 2%, and 4% GADF were added to MFM samples. Analyses were carried out immediately after preparation of samples and during and after storage at −20 °C. GADF was characterized in terms of dietary fibre, total polyphenols and antioxidant capacity, and multifunctional antioxidant assays were carried out on all the MFM samples. The addition of red grape fibre considerably delayed lipid oxidation in minced horse mackerel muscle during the first 3 months of frozen storage.

Keywords: Antioxidant dietary fibre; Grape polyphenols; Frozen storage; Lipid oxidation

1. Introduction

Seafoods possess high nutritional value, and also functional properties, thanks to their readily-digested protein. As such, they are a good source of vitamins and minerals, and fatty fish furthermore contain high concentrations of polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Ackman, 1999). However, because of this high unsaturated lipid content, fish products are very susceptible to loss of quality through lipid oxidation. The onset of rancidity is fast, particularly in fatty and semi-fatty species like horse mackerel (*Trachurus trachurus*), in whose muscle large amounts of haemoglobin (a well-known activator of lipid oxidation) and lipids coexist (Richards & Hultin, 2002).

The use of antioxidants is an effective way to minimize or prevent lipid oxidation in food products (Boyd, Green, Giesbrecht, & King, 1993; Frankel, 1998), retarding the formation of toxic oxidation products, maintaining nutritional quality and prolonging the shelf life of foods (Jadhav, Nimbalkar, Kulkarni, & Madhavi, 1996). Synthetic antioxidants have been widely used to retard lipid oxidation in foods (Ahmad, 1996), but nowadays there is growing interest in finding naturally occurring antioxidants for use in foods (Löliger, 1983), and for possible in vivo use. There has been increasing interest in identifying plant extracts, which minimize or retard lipid oxidation in lipid-based food products; for example, some natural phenolic compounds, that are effective in preventing rancidity in many lipid systems like fish muscle (Ikawa, 1998; Medina, Satué-García, German, & Frankel, 1999; Medina et al., 2003).

Recent research has stressed the importance of vinification by-products as plant materials that are particularly rich in a wide range of polyphenols (Alonso, Guillén, Barroso, Puertas, & García, 2002; Torres et al., 2002). Grape by-products contain significant amounts of phenolic compounds, mostly flavonoids (Escribano-Bailón, Guerra, Rivas-Gonzalo, & Santos-Buelga, 1995; Mazza, 1995), which have been reported to have beneficial affects on lipid metabolism (Teissedre, Frankel, Waterhouse, Peteg, & German, 1996). There are many references in the literature to the composition and antioxidant properties of grape polyphenols (González-Paramás, Estebam-Ruano, Santos-Buelga, Pascual-Teresa, & Rivas-Gonzalo, 2004; Yilmaz & Toledo, 2004), and two studies were recently published on the effectiveness

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of grape polyphenols in delaying lipid oxidation in minced fatty fish muscle during frozen storage (Pazos, Gallardo, Torres, & Medina, 2005a; Pazos, González, Gallardo, & Torres, 2005b). It has also been reported that many of the anthocyanidins/anthocyanins, flavonoids and phenolic acids have antioxidant activities similar to synthetic antioxidants such as BHA and BHT (Fukumozo & Mazza, 2000).

Wine by-products are also rich in dietary fibre (DF) (Bravo & Saura-Calixto, 1998). It was recently proposed to define antioxidant dietary fibre as a natural product that combines the beneficial effects of dietary fibre and natural antioxidants, such as polyphenol compounds (Saura-Calixto, 1998). Dietary fibre can also be an effective tool in seafood processing for improving functional properties, such as water binding, gelling, etc.

The object of this work was to study the effect of grape antioxidant dietary fibre (GADF) addition to minced fish muscle (MFM) on lipid stability during frozen storage. Horse mackerel (*T. trachurus*), an under-utilized semi-fatty fish species, was selected, due to interest in regulating its quality and developing high-added-value products. Different methodologies were assessed in order to measure the multifunctional antioxidant activity of GADF on MFM during storage and to compare the results produced by different methods.

2. Materials and methods

2.1. Preparation of fish and samples

Minced muscle was prepared from ice-stored horse mackerel (*T. trachurus*) fillets. Individuals were filleted without removing the skin by a local seafood company and transported to the pilot plant. The muscle was extracted using a Baader model 694 deboner machine (Lübeck, Germany) equipped with a drum with 3 mm holes. Red grape pomace (peels and seeds) (*Vitis vinifera* var. undetermined) obtained from a winery (Vinícola de Castilla, S.A., Manzanares, Ciudad Real, Spain) was processed following a patented procedure (Saura-Calixto & Larrauri, 1997), freeze-dried, milled to a particle size less than 0.5 mm and stored at −20 °C until analysis. The final product, named grape antioxidant dietary fibre (GADF), was mixed into the minced fish muscle (MFM). Concentrations of 0, 2, and 4% GADF [(0-GADF), (2-GADF), and (4-GADF), respectively] were added to MFM samples, which were then stored at −20 °C for 6 months. The procedure was as follows: the fish was mixed in a mixing machine model RM-20 (Mainca, Granollers, Spain). The GADF was dispersed in cold water (as per formulation) and added to the MFM. The mixing time was standardized to 6 min (the final temperature was below 6 °C in all cases). In all lots moisture was adjusted to 76%, as in the original muscle. The samples were placed on 21.5 × 15 × 3.5 cm aluminium trays and then frozen in a Saubre "Benjamin" model horizontal plate freezer (Hanst-Moller, Germany) to −20 °C. The samples were then filleted, packed in bags and stored at −20 °C. GADF was characterized in terms of DF, total polyphenols and antioxidant capacity, and multifunctional antioxidant assays were tested on all the MFM samples: 0-GADF, 2-GADF, and 4-GADF. Analyses were performed immediately after preparation of samples and during and after storage at −20 °C.

2.2. Chemicals

Stable free radical DPPH· (2,2-diphenyl-1-picrylhydrazyl) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E, were from Aldrich Chemical Co. (St. Louis, MO, USA). TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) was from Fluka Chemical (Madrid, Spain). 1,1,3,3,-Tetraethoxypropane was from Sigma Chemical Co. (St. Louis, MO, USA). Thiobarbituric acid was from Merck (Germany); $FeCl_3.6H_2O$, acetone, ethanol, hexane, and methanol were from Panreac Química S.A. (Madrid, Spain). All reagents were of analytical grade.

2.3. Chemical analysis

2.3.1. Dietary fibre

The AOAC enzymatic-gravimetric method (Prosky, Asp, Schweizer, Devries, & Furda, 1988) was modified in our laboratory: dialysis against water was used instead of ethanol precipitation of soluble dietary fibre (sDF) (Mañas & Saura-Calixto, 1995) for DF analysis. After enzymatic hydrolysis of digestible components, insoluble DF (iDF) and soluble DF (sDF) fractions were separated and chemically hydrolyzed. IDF fractions were hydrolysed with 12 M sulphuric acid (30 °C, 1 h) and then diluted to 1 M sulphuric acid (100 °C, 90 min.). The remaining residues were gravimetrically quantified as Klason lignin (KL) after drying at 105 °C to constant weight. sDF dialysates were hydrolysed with 1 M sulphuric acid (100 °C, 90 min). Constituent neutral sugars (NS) and uronic acids (UA) were quantified in the hydrolysates. NS were quantified by gas cromatography (Jimenez-Escrig, Rincon, Pulido, & Saura-Calixto, 2001). UA were quantified spectrophotometrically by the Scott method (Scott, 1979) using galacturonic acid as standard. iDF was calculated as (NS + UA + KL), and sDF as (NS + UA).

2.3.2. Extraction of phenolics

Ground freeze-dried GADF sample (1 g) was placed in a test tube; 40 ml methanol/water (50:50) was added, plus HCl, to obtain a final pH of 2.0. The solution was thoroughly shaken at room temperature for 1 h and centrifuged at 2500*g* for 10 min, and the supernatant was recovered. Forty millilitres of acetone/water (70:30) were added to the residue, and shaking and centrifugation were repeated. Both extracts were mixed. This procedure has been used by our group and is described elsewhere (Jimenez-Escrig et al., 2001). Extractions were performed in triplicate and used to calculate the total phenolics content and the antioxidant

capacity. The total phenolics in the extracts were determined spectrophotometrically, according to the Folin–Ciocalteu procedure (Singleton, Orthofer, & Lamuela-Raventós, 1999), using gallic acid as standard (concentration range 5–25 mg per 100 ml), and the results were expressed as gallic acid equivalents (GAE).

2.4. Lipid damage measurements

2.4.1. DPPH· assay

The antioxidant activity of the GADF plus MFM was measured in terms of radical scavenging activity, according to the DPPH· method (Sanchez-Moreno, Larrauri, & Saura-Calixto, 1998). Briefly, an aliquot of sample extract at different concentrations was added to a DPPH· solution, and the absorbance at 515 nm was measured until the reaction reached a plateau. A calibration curve at 515 nm was plotted with DPPH· to calculate the remaining DPPH· concentration in the reaction medium. The parameter EC_{50}, which reflects the depletion of DPPH· free-radical to 50%, was expressed in terms of grams of GADF (dry matter) plus MFM equivalent per gram of DPPH· in the reaction medium. The lower value the higher antioxidant activity.

2.4.2. FRAP assay

The reducing power of GADF plus MFM was estimated, following the procedure described by Benzie and Strain (1996), with some modifications introduced in our laboratory (Jiménez-Escrig, Dragsted, Daneshvar, Pulido, & Saura-Calixto, 2003). Briefly, 900 μl of FRAP reagent, freshly prepared and warmed at 37 °C, was mixed with 90 μl distilled water and either 30 μl of test sample or standard or appropriate reagent blank. The FRAP reagent contained 2.5 ml of a 10 mM TPTZ solution in 40 mM HCl, plus 2.5 ml of 20 mM $FeCl_3$-$6H_2O$, plus 25 ml 0.3 mM acetate buffer at pH 3.6. Readings at the absorption maximum (595 nm) were taken every 15 s using a Beckman DU-640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA) equipped with a thermostatic autocell holder. Temperature was maintained at 37 °C. Readings were taken at 30 min for calculation of FRAP values. Methanolic solutions of known Trolox concentrations were used for calibration. The antioxidant results were expressed as μmol equivalents of Trolox per gram of GADF plus MFM (dry matter).

2.4.3. Measurement of conjugated diene and triene hydroperoxides

Lipids were extracted from mackerel muscle (Bligh & Dyer, 1959) and the lipid content was determined gravimetrically in triplicate (Herbes & Allen, 1983). Conjugated hydroperoxides were measured from fish oil samples dissolved in hexane, and absorbance was measured at 234 nm and 268 nm. Concentrations of hydroperoxides were calculated as mmol of hydroperoxides per kilogram of oil as described by Frankel, Huang, Kanner, and Bruce-German (1994).

2.4.4. Thiobarbituric acid index (TBA-i)

TBA-i was determined according to Vynke (1970) on a 5% trichloracetic acid extract of the restructured fish muscle. Results were expressed as mg malondialdehyde per kilogram of sample. The spectrophotometer used was a Perkin–Elmer Lambda 15 UV/Vis spectrophotometer.

2.4.5. Antioxidant effectiveness

In both instances, conjugated hydroperoxides and thiobarbituric acid index, the antioxidant effectiveness was calculated as per cent inhibition of oxidation (%*I*), as described by Frankel (1998): $\%I = (c - s/c)*100$, where c = high increment of 0-GADF in the experiment and s = increment of sample with added GADF at the same time. High levels of %I indicate greater antioxidant effectiveness.

2.5. Statistical analyses

Tukey's test ($p < 0.05$) was used to determine the differences in the mean values. The Statgraphics Plus 2.1 program was used for this.

3. Results and discussion

3.1. Characterization of grape antioxidant dietary fibre

The chief characteristics of this natural product are that it is rich in both DF and polyphenolic compounds and also exhibits relatively high antioxidant activity (ferric reducing ability and radical scavenging capacity) as measured in vitro (Table 1). It is worth noting that the sDF content of this GADF is relatively high, in comparison with total DF content. The physical and chemical characteristics of the DF fractions will dictate the specific local response in the gut and the associated systemic reactions. sDF is distinguished by its ability to form viscous gels in the intestinal tracts. iDF does not exhibit viscosity but instead is characterized by faecal-bulking capacity. Both forms of fibre share the ability to bind water or mineral cations and can be used by the colonic microflora as a fermentable substrate (Schieber, Stintzing, & Carle, 2001).

Table 1
Proximate composition of grape antioxidant dietary fibre (GADF) (gram per 100 g of d.m.)[a]

Protein	8.08 ± 0.4
Fat	9.44 ± 0.1
Ash	2.43 ± 0.1
Extractable polyphenols	5.63 ± 0.2
Insoluble dietary fibre	53.21 ± 3.4
Soluble dietary fibre	20.78 ± 0.76
DPPH·	153 ± 9
FRAP	525 ± 28

Radical scavenging capacity (DPPH·), expressed as $CE_{50\%}$ (gram GADF (d.m.)/g DPPH·) and ferric reducing ability (FRAP), expressed as μmol of Trolox equivalents per gram of GADF (d.m.). d.m.: dry matter.

[a] Values are means ± standard deviation of three replicate determinations.

3.2. Antioxidant assays

A multifunctional methodology was used to assess how well GADF protected MFM against oxidation during storage (6 months) at −20 °C. Some authors distinguish between the antioxidant capacity and the reactivity (Roginsky & Lissi, 2005) of polyphenols. As they describe them, while the antioxidant capacity gives information about the duration of antioxidative action (ability to retard oxidative degradation), the reactivity characterizes the starting dynamics of antioxidation at certain concentrations of an antioxidant or an antioxidant mixture (determined by the reactivity of an antioxidant to active free radicals in the corresponding reaction). For this reason two kinds of analytical methods were used: indirect methods to examine the ability of GADF in MFM to scavenge some free radicals (DPPH·) and the ferric reducing ability of plasma; and direct methods entailing studies of chain peroxidation and the effect of a tested food containing antioxidants on the oxidative degradation of a tested system (formation of conjugated hydroperoxides and aldehydes).

The ferric ion reducing antioxidant power (FRAP) is a method based on a single electron transfer reaction between an oxidant and antioxidant, i.e., phenol antioxidants are oxidized by the oxidant Fe (III). As a result a single electron is transferred from the antioxidant molecule to the oxidant. The standard redox potential of Fe (III)/Fe(II) is 0.77 V; any compound with lower redox potential can theoretically reduce Fe(III) to Fe(II) (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002). At the end of the third and the sixth months of storage, the ability to reduce Fe(III) to Fe(II) was greater in both 4-GADF and 2-GADF samples than in MFM without GADF (Table 2). Effectiveness was greatest (225%) at the end of the third month in the 4-GADF sample. The same results were found for radical scavenging activity (DPPH·), in which antioxidant capacity at the end of the third month of storage was higher in both MFMs with GADF than in the MFM without GADF (Table 3). According to the DPPH· assay, 4-GADF provided greater protection than 2-GADF; this assay may be used to indicate the scavenging effect of the polyphenols in the fish matrix. A high decrease in antioxidant activity during the first 3 months is shown in both assays, it seems that a major part of the antioxidants from GADF were consumed in preventing lipid oxidation of the MFM sample. No major changes was observed after 90 days of storage in all the samples.

By continuous monitoring during frozen storage of conjugated hydroperoxides and aldehydes, products formed during lipid oxidation, the process can be observed in close detail. Fig. 1 depicts the formation of conjugated dienes (a) and trienes (b) in minced horse mackerel after 6 months of frozen storage. During frozen storage, the samples with dietary fibre added (2-GADF and 4-GADF) exhibited less formation of dienes than the control lot (0-GADF) until 90 days and less formation of trienes. The rate of oxidation inhibition (Table 4) when the control increments were highest (at 30 days of storage) was 26.42% for the lot with 2% GADF and 62.34% for the lot with 4% GADF added. From these values it would seem that GADF protects the initial formation of oxidation compounds at 30 days of frozen storage.

Table 2
Ferric reduction ability (FRAP) of minced fish muscle (MFM) with added GADF during 6 months of freeze storage (−20 °C)[A]

Sample	Day 0	Day 90	Day 180
0-GADF	27 ± 3.1a	4 ± 0.2a	3.4 ± 0.5a
2-GADF	38 ± 3.8b	6 ± 0.6b	5.3 ± 0.8b
4-GADF	53 ± 3.2c	13 ± 1.1c	8.1 ± 0.8c

Different letters in the same column indicate significant difference ($p < 0.05$).
[A] Values are means ± standard deviation of three replicate determinations.

Table 3
Radical scavenging capacity (DPPH·) expressed as $CE_{50\%}$ (gram GADF (d.m.)/g DPPH·) of MFM with added GADF during 3 months of freeze storage (−20 °C)[A]

Sample	Day 0	Day 90
0-GADF	12.12 ± 1.1a	350 ± 17a
2-GADF	10.96 ± 0.2b	193 ± 12b
4-GADF	7.10 ± 0.5c	156 ± 9.8c

d.m.: dry matter. Different letters in the same column indicate significant difference ($p < 0.05$).
[A] Values are mean ± standard deviation of three replicate determinations.

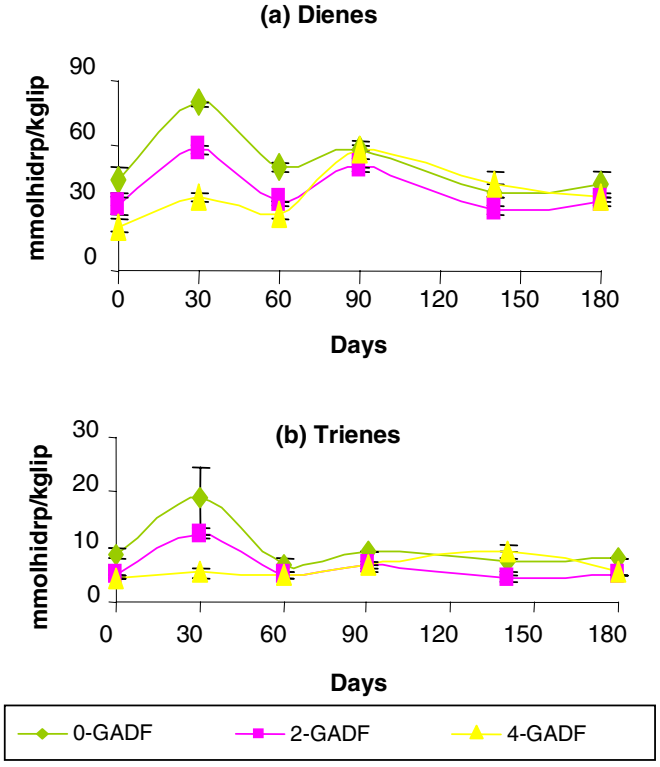

Fig. 1. Formation of conjugated dienes (a) and trienes (b) in samples of minced fish muscle added with grape antioxidant dietary fibre during frozen storage at −20 °C.

Table 4
Percentage of inhibition[A] of formation of conjugated dienes and trienes and thiobarbituric index (TBA-i) in minced fish samples (means ± SD)[B]

Sample	DIENES (mmol hydroperoxide per kg lipid) Day 30	TRIENES (mmol hydroperoxide per kg lipid) Day 30	i-TBA (mg MDA per kg muscle) Day 90
0-GADF	0.00 ± 8.96a	0.00 ± 6.32a	0.00 ± 4.49a
2-GADF	26.42 ± 0.08b	32.16 ± 4.25b	57.28 ± 9.17b
4-GADF	62.34 ± 1.02c	89.43 ± 7.79c	54.13 ± 12.16b

[A] $\%I = (c - s/c)*100$, where c = high increment of 0-GADF in the experiment and s = increment of sample with added GADF at the same time.
[B] Different letters in the same column indicate significant differences ($P < 0.05$).

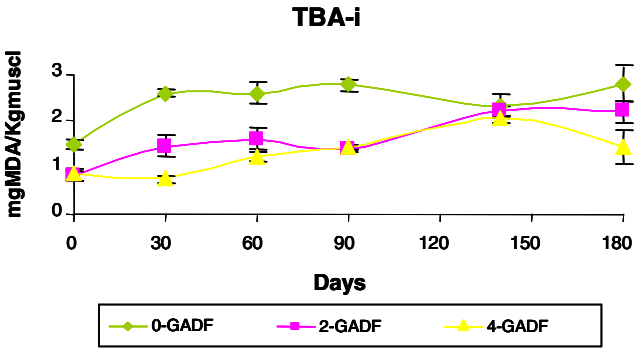

Fig. 2. Formation of aldehydes in samples of minced fish muscle added with grape antioxidant dietary fibre during frozen storage at −20 °C.

TBA-index values were lower in the samples with dietary fibre added for most of the storage period (Fig. 2). The rate of inhibition at 90 days of frozen storage (higher control values of TBA-index) was 57.28% for the samples with 2% added fibre and 54.13% for samples with 4% added fibre (Table 4). The antioxidant action was not significantly different for the two levels of dietary fibre at this time of frozen storage. There was less inhibition of conjugated hydroperoxide formation than of aldehyde formation; the same effect has been described in the case of tocopherols, which can increase primary lipid oxidation products, by donating hydrogen to a peroxyl radical, to form a lipid hydroperoxide, while simultaneously decreasing formation of low molecular weight volatile secondary compounds of oxidation (Decker, Warner, Richards, & Shahidi, 2005).

The grape flavonoids in GADF have considerable potential as antioxidants, based on the combined actions of non-extractable polyphenols (NEPP) (polymeric proanthocyanidins and high molecular weight hydrolysable tannins) and extractable polyphenols (EPP) (anthocyanins, flavonols, flavan-3-ols and phenolic acids), both of which show some promise in the fields of nutrition and health. Use of EPPs as antioxidants has been widely reported (Macheix, Sapis, & Fleuriet, 1991; Escribano-Bailón et al., 1995). Hagerman, Rield, and Jones (1998) reported that NEPPs were 15–30 times more effective at quenching peroxyl radicals than simple phenols. Flavonoids are able to scavenge the radicals of hydroxyl, peroxyl, superoxide and nitric oxide (as shown in Table 3). Besides free radical scavenging activities, flavonoids possess metal chelating properties (Bors, Michel, & Stettmeier, 2000; Yusuf & Romeo, 2004), which are very important in preventing the development of rancidity in fish (Ramanathan & Das, 1993; Richards & Hultin, 2002). Pazos et al. (2005a) have reported that an optimal combination of degree of procyanidin polymerization and percentage of galloylation, as in GADF, may help to account for the high antioxidant efficacy of grape polyphenols in frozen fish muscle. Pazos et al. (2005b) also suggested that grape procyanidins are able to stabilize frozen fatty fish and preserve vitamin E (an endogenous antioxidant of fish). These results are consistent with our own findings of high GADF antioxidant capacity during frozen storage in a matrix made of minced muscle. We found high antiradical activity in the samples with GADF, but this was not always accompanied by high antioxidant activity in foods. This study shows that antiradical activity (measured as H-donating activity) in samples with GADF and antioxidant capacity, retarding the formation of degradation products from lipids in MFM, are correlated. The results suggest that GADF possesses notable antioxidant properties and is able to significantly inhibit the development of rancidity in frozen fish muscle during the first 3 months. All methodologies produced similar results, which suggests that methods of this kind are valid for assessing the antioxidant capacity of GADF added to frozen minced muscle.

As reported above, GADF is a good antioxidant which could not only preserve MFM from oxidation during storage but could also produce health benefits for the consumer, thanks to its bioactive compound and DF content. It should be noted in this connection that it in vitro activities can only be considered potentially relevant in biological systems and that in vivo activities also depend on bioavailability and biotransformation. It is known that polyphenols such as gallic acid, caffeic acid, malvidin, catechin, and rutin, which are contained in red grape, can be partly bioavailable (Manach, Wiliamson, Morand, Scalbert, & Remesy, 2005), and consequently their in vivo activities could be significant.

4. Conclusions

The addition of red grape fibre considerably inhibits oxidation in horse mackerel minced muscle during the first 3 months of frozen storage. These results indicate that GADF could be used as an ingredient to prevent oxidation in minced fish during frozen storage. The reason for this effect may be either the chelating action of fibre on some prooxidant metals or the action of polyphenols associated with dietary fibre.

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