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Oxidation stability of muscle with quercetin and rosemary during thermal and high-pressure gelation

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Abstract

The antioxidant activity of quercetin and rosemary extracts were studied in minced fish and after subsequent treatments to form gels. It was deduced from FRAP (reducing capacity) and DPPH· (antiradical scavenging) results that both extracts showed antioxidant capacity after processing. The rosemary extract was more effective at protecting from lipid oxidation; whereas protein oxidation was prevented by both antioxidants, although quercetin was the most efficient antioxidant in those batches subjected to thermal treatment for gel formation (conventional and microwave).

Keywords: Antioxidant activity; Protein and lipid oxidation; Rosemary; Quercetin

1. Introduction

There has been an increasing interest in the consumption of restructured minced products in recent years. However, several factors may alter their quality during processing. One of the main factors influencing seafood stability is the oxidation of the different muscle components, due to either the storage conditions or the processing to which minced fish is subjected to obtain and guarantee a safe final product.

In this sense, rapid lipid oxidation is frequently observed and a decline in acceptability has been associated with a rise in 2-thiobarbituric acid (TBA) values. However, there are few references about the oxidation of proteins. Formation of carbonyl groups in proteins has been widely used as a measure of oxidation (Srinivasan & Hultin, 1995). Protein oxidation may occur more rapidly than lipid oxidation in biological systems such as muscle (Davies & Golberg, 1987; Srinivasan & Hultin, 1995), since protein is within the aqueous phase where many radicals are formed (Soyer & Hultin, 2000). Davies (1986) and Srinivasan and Hultin (1995) described the sequence of changes in proteins as the following. Firstly, free radicals react with side chains of proteins, producing protein free radicals. Secondly, these free radicals may react with molecular oxygen to form peroxy radicals, which in turn can capture hydrogen from another molecule yielding hydrogen hydroperoxides. Finally, these protein hydroperoxides may break down, one of the resulting products being the carbonyl groups. Furthermore, protein radicals could react with susceptible lipids to enhance the rate of lipid oxidation, although they could also serve to scavenge free radicals and thus show an antioxidant activity with respect to the lipids (Soyer & Hultin, 2000).

Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), and α-tocopherol have been used to prevent lipid oxidation

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in raw and cooked mince (Montero, Gómez-Guillén, & Borderías, 1996; Weilmeier & Regestein, 2004). However, the search for antioxidants from natural sources for controlling oxidation is receiving considerable attention. Synthetic antioxidants have been widely used in the restructured mince industry (fish and meat), but consumer concern about safety was led this industry to seek natural sources of antioxidants.

The application of plant extracts to prevent fish oxidative rancidity has been studied in certain fish products like fillets (Weilmeier & Regestein, 2004; Aubourg et al., 2004), mince gels (Pérez-Mateos, Gómez-Guillén, Hurtado, Solas, & Montero, 2002) and emulsions (Frankel, Huang, & Aeschbach, 1996). Processing for mince extraction, preparation of batter and cooking, make necessary the addition of antioxidants to avoid oxidative rancidity. Regarding this subject, quercetin and rosemary extracts are some of the most used natural antioxidants.

It is important to consider the bio-availability of these antioxidants in the final product. Several in vitro techniques are used for this purpose. Two of the most widely used techniques are the measure of the antioxidant activity by FRAP and DPPH$^{\cdot}$ assays. FRAP (the ferric reducing/antioxidant power) assay, measures the reducing ability of the antioxidant (Benzie & Strain, 1996); whereas DPPH$^{\cdot}$ determination evaluates the antioxidant ability to scanvege a free synthetic radical (2,2-diphenyl-1-picryhydrazyl DPPH$^{\cdot}$) (Brand-Williams, Cuvelier, & Berset, 1995; Sánchez-Moreno, Larrauri, & Saura-Calixto, 1998).

This study investigated the in vitro bio-availability and the effects of quercetin and rosemary extracts on lipid and protein oxidation in mince fish muscle, as well as during subsequent treatments such as homogenization to form a batter and gelation induced by both heat (conventional and microwave) and high pressure.

2. Materials and methods

2.1. Sample preparation

Atlantic mackerel (*Scomber scombrus*) used in this study were caught on the Cantabrian coast and kept at 4 °C for 48 h. Fish (14 kg) were headed, gutted and washed. Skin and bones were removed with a deboning machine with a pore of 3 mm (Baader 694, Lübeck, Germany).

Proximate analyses of mince were performed according to Association of Official Analytical Chemists procedures (1984): moisture (method 24003), ash (method 1821), and protein (method 24024). Crude fat was determined following the method of Bligh and Dyer (1959). Proximate analyses were: total protein 18.0% ± 0.53, moisture 76.9% ± 0.16, total fat 3.78% ± 0.09, and ash 0.96% ± 0.01.

The mince was divided in three batches: minced muscle (M), mince muscle + 0.3% of quercetin extract (MQ) and mince muscle plus 0.6% of rosemary extract (MR). The natural antioxidants, quercetin and rosemary extract (Altaquímica. Barcelona, Spain), were added in these proportions to achieve antioxidant activity according to preliminary trials. Both ingredients were blended to a homogeneous distribution.

The batch consisting of minced muscle was divided in four sub-batches. Three of them were placed in a refrigerated vacuum homogenizer (Stephan UM5, Stephan u. Söhne GmbH & Co., Germany), and ground for 1 min at high speed sodium chloride (2% w/w) (Panreac, Montplet & Esteban S.A., Barcelona, Spain) was added and homogenized for 3 min at slow speed. Quercetin extract (0.3%) was added to the first sub-batch (sol Q) with crushed ice to give the required final moisture (77%), whereas rosemary extract (0.6%) was added to the second one (sol R). No antioxidant extract was added to the third sub-batch (sol). The homogenate was beaten slowly for 5 min under vacuum, with the temperature being maintained below 10 °C.

Protein, fat and moisture of sol and gel formulation were calculated from the proximate analyses carried out on the mince. Mince + sodium chloride (sol and gel formulation) were 77.5% water, 16.6% protein and 3.39% fat; sol and gel formulations with quercetin were 77.3% water, 16.3% protein and 3.31% fat; and sol and gel formulation with rosemary were 77.3% water, 16.0% protein and 3.25% fat.

2.2. Gel forming treatments

Homogenates with sodium chloride were put into flexible plastic casing (Krehalon Soplaril, Barcelona, Spain) of 40 μm thickness and 3.5 cm diameter. Conventional thermal treatment was used for gel formation (90 °C/50 min) (TQ; TR). High pressure treatments were performed in a high pressure pilot unit (ACB 665, Gec Alsthom, Nantes, France), where the temperature of immersion medium (distilled water) was controlled via a thermocouple with programmed thermostatization equipment (model IA/2230 AC, INMASA, Barcelona, Spain). The pressure was increased by 2.5 MPa/s. The high pressure treatments applied were 300 MPa/25 °C/15 min (P25Q, P25R) and 300 MPa/5–7 °C/15 min (P7Q, P7R). A microwave treatment was also tested to obtain the gels, consisting of 700 W during 90 s (45 s on each side) (mwQ, mwR); where water was used to cover the bottom of the plate.

2.3. Antioxidant activity

The ferric reducing/antioxidant power (FRAP) assay was used as a measure of the reducing ability of gels following the method Benzie and Strain (1996). It is based on the increase in absorbance at 595 nm due to the for-

mation of the complex tripiridiltriazine (TPTZ)-Fe(II) in the presence of tissue reducing agents. Absorbance was read at 4 and 30 min. The parameter equivalent concentration 1 or EC_1 was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mmol/l $FeSO_4 \cdot 7H_2O$. EC_1 was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mmol/l concentration of Fe(II) solution, determined using the corresponding regression equation.

2.4. Free radical scavenging measurement

The anti-radical capacity of the sample extracts and pure compounds (quercetin and rosemary), was estimated according to the procedure reported by Brand-Williams et al. (1995), slightly modified by Sánchez-Moreno et al. (1998). Samples were thawed and the extracts were obtained by homogenizing 10 g of each one with 50 ml of methanol (Omni-mixer, Type OM, Ivan Sorvall, Inc., Norwalk Conn., USA) during 2 min (setting 6) in a bath containing water and ice. Afterwards, the extracts were filtered under vacuum. The data is reported as EC_{50}, which is the concentration of antioxidant required for 50% scavenging of DPPH· radical. The specified time (T_{EC50}) is the time needed to reach a steady state at the concentration corresponding to EC_{50}.

2.5. Protein oxidation

Determination of *carbonil radical* was performed following the method described by Srinivasan and Hultin (1995). Results were expressed as nmol per mg protein.

2.6. Lipid oxidation

TBA index (thiobarbituric acid) was determined following the method of Vyncke (1970), incubating at 90 °C for 40 min. Results were expressed as μmol malonaldehyde per 100 g of sample (muscle or gel, respectively).

2.7. Statistical analysis

Two-way analysis of variance was run. The computer program used was the Statgraphics Plus (Rockville, MD, USA) statistical program. Pairwise comparison of the differences between means was performed using Duncan's test with confidence intervals set for a level of significance of $p \leqslant 0.05$.

3. Results and discussion

Preliminary trials showed that it was necessary to use rosemary extract at twice the concentration of quercetin in order to obtain a similar range of antioxidant activity. In the present study, the antioxidant capacity of different batches of minced muscle, sol and gels containing quercetin (0.3%) and rosemary (0.6%) extracts, measured by FRAP, is shown in Fig. 1. The samples which contained quercetin gave higher FRAP values corresponding to, compared to those including rosemary, despite the latter being at twice the concentration. Mince muscle (M) and mince muscle homogenized with NaCl (2%) to form a batter (sol) did not show any reducing activity. However, both muscle and sol containing either quercetin or rosemary showed this activity, which was considerably higher ($p \leqslant 0.05$) in those samples with quercetin. The sol with quercetin (sol Q) showed a significantly lower activity than muscle with quercetin (MQ), when FRAP index was measured after 30 min reaction. This was probably due to the prooxidant effect of NaCl solubilized in the muscle, as has been previously described (Andersen & Skibsted, 1991; Kanner, Harrel, & Jafe, 1991). No decrease was found in the reducing ability when sol with antioxidants was subjected to gelation by conventional heat treatment. Furthermore, a slight but significant increase was found in FRAP values ($p \leqslant 0.05$) corresponding to gels induced by high pressure at 7 °C and, especially, at 25 °C, containing both quercetin and rosemary. It seems that high pressure as a treatment for gel induction may promote antioxidant activity. This could be due to either a direct or indirect effect on antioxidants (rosemary and quercetin). High pressure may lead to a reduction of the interactions between these antioxidants and muscle compounds, giving a higher availability of the antioxidant molecules to hinder oxidation phenomena. Furthermore, it is known that high pressure itself has a lipid prooxidant effect, which may be diminished by the addition of certain antioxidants like rosemary extract (Pérez-Mateos et al., 2002). Microwave treatments led to similar FRAP values to those found in the case of batters and gels

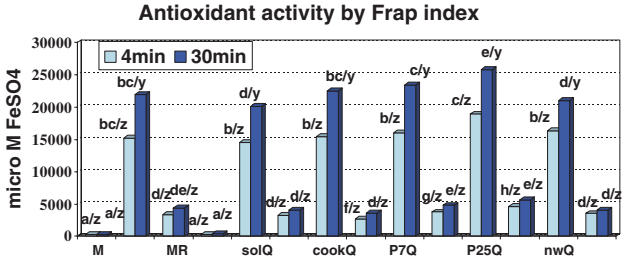

Fig. 1. Total antioxidant activity by FRAP of different samples: mince (M), mince with antioxidant (MQ and MR), batter or sol, sol with antioxidant (sol Q and sol R), gel induced at 90 °C (TQ and TR), gel induced at 300 MPa of high pressure/7 °C (P7Q and P7 R), gel induced at 300 MPa of high pressure/25 °C (P25Q and P25R) and gel induced by microwave (mwQ and mwR). Different letters (*a*, *b*, *c*) indicate significant differences ($p \leqslant 0.05$) among samples, different letters (*z*, *y*, *x*) indicate significant differences ($p \leqslant 0.05$) between times of measurement for the same sample.

obtained by conventional heat treatment for both antioxidants. The pure quercetin extract at 0.3% showed a similar reducing ability to that described for MQ, while pure rosemary extract at 0.6% showed about six times higher ability than MR. The loss of activity observed for rosemary extract in muscle and gel could be due to its role in lipid oxidation, which consequently involves a reduction in the bio-availability of this antioxidant. It has been reported that rosemary extract reduces the lipid oxidation in gels induced by heat and high pressure (Pérez-Mateos et al., 2002).

It was observed that the reducing capacity was not stable with reaction time, but tended to increase from 4 min up to 30 min in all the batches. This increase was about 20% in samples including rosemary and 35–40% in those containing quercetin. These results are in accordance with data reported by Benzie and Strain (1996) for pure extracts of phenolic compounds. Considering these results, the increase observed in the reducing ability with reaction time could be considered as another parameter to define the antioxidant capacity of a compound.

The antioxidant activity measured by the scavenging of the synthetic radical (2,2-diphenyl-1-picryhydrazyl DPPH$^{\cdot}$) is shown in Table 1. EC_{50} is the concentration of antioxidant required for 50% scavenging of DPPH$^{\cdot}$ radical in a period of time T_{EC50}. Short times and low concentrations are important to define good antioxidant activity. For quercetin and rosemary pure extracts, EC_{50} was 0.12 and 0.9 mg/ml, whereas T_{EC50} was 4.5 and 7.5 min, respectively, indicating the higher in vitro antioxidant effect of the quercetin extract. Regarding this subject, it could be considered that quercetin and rosemary extracts show a medium standard antioxidant activity, given that concentration is quite low but time is not according with Sánchez-Moreno et al. (1998). These authors classified the kinetic behaviour of different antioxidant compounds according to T_{EC50} values. Ascorbic acid was an example of rapid kinetic behaviour since the time needed to achieve a steady state (T_{EC50}) was less than 5 min. α-Tocopherol was classified as intermediate, with a T_{EC50} value within the interval 5–30 min, and rutin was an example of slow kinetic behaviour with higher T_{EC50} values.

Table 1
Total antioxidant activity by DPPH$^{\cdot}$ of different samples mince (M), mince with antioxidant (MQ and MR), batter or sol, sol with antioxidant (sol Q and sol R), gel induced at 90 °C (TQ and TR), gel induced at 300 MPa of high pressure/7 °C (P7Q and P7R), gel induced at 300 MPa of high pressure/25 °C (P25Q and P25R) and gel induced by microwave (μwQ and μwR)

	EC_{50} (mg/ml)	T_{EC50} (min)
M	nd	
MQ	0.24	5.0
MR	1.2	3.8
Sol	nd	
Sol Q	0.2	22
Sol R	1.05	3.3
TQ	0.26	3.6
TR	1.8	2.9
P7Q	0.7	6.6
P7R	1.4	1.8
P25Q	0.29	3.3
P25R	1.6	2.8
MwQ	0.15	9.0
MwR	0.6	6.25

nd: not detectable.

When quercetin and rosemary extracts were blended with muscle (MQ and MR), they lost efficiency and the EC_{50} noticeably increased, being higher in the case of MR. However, T_{EC50} values were similar for both, MQ and MR. The addition of salt did not involve any change in EC_{50}, and sol R and sol Q showed similar values to those described for MR and MQ, respectively. Nevertheless, sol Q showed higher T_{EC50} values than MQ. The loss of antioxidant activity observed with respect to pure extracts was probably due to the interaction between antioxidant molecules and mince or sol constituents, respectively. The antioxidant capacity in gels induced by conventional heat treatment was similar to that found for both, mince muscle enriched with antioxidants and gels induced by high pressure applied at moderate temperature (25 °C). However, the gels induced by high pressure in cold conditions (7 °C) containing quercetin showed increased EC_{50} values and thus, lower scavenging antioxidant capacity. On the other hand, those gels obtained by microwave treatment showed the best antioxidant capacity, which were similar to that found for pure rosemary and quercetin extracts. It seems that the short time needed to obtain the microwave gels promotes a rapid protein–protein interaction and, thus, is not enough to establish any bonds between muscle protein and antioxidant molecules, keeping the latter more bio-available.

Although T_{EC50} values were lower in pure quercetin extracts than in rosemary extracts, the opposite behaviour was observed when these antioxidants were added to mince, sol or gel, with the lowest T_{EC50} values in samples including rosemary. Nevertheless, according to the antioxidant kinetic classification defined by Sánchez-Moreno et al. (1998), an intermediate kinetic behaviour was found in most cases.

The mechanism of protection given by an antioxidant was postulated to occur at the initial stage and more effectively during the propagation stage of oxidation in the case of peroxy radical (ROO$^{\cdot}$) scavengers such as phenolic compounds. The peroxy radicals formed are intercepted or inhibited by a free radical acceptor (phenolic structure), which stops the chain reaction as a consequence (Basaga, Tekkaya, & Acikel, 1997).

Since the antioxidants used in the present study were chiefly phenol based compounds, the determination of radical scavenging capacity should have been an ade-

quate method to evaluate the antioxidant ability of the pure rosemary and quercetin extracts. However, it is possible that some interactions with lipids and proteins take place when these antioxidants are blended with mince muscle and, as a result of this, they partially loose their scavenger capacity.

The reducing ability of these polyphenols (quercetin and rosemary) seems to be a more important factor to dictate the antioxidant activity than the free-radical-scavenging capacity, given that differences were less noticeable in the latter. Each method mentioned above measures the ability of the antioxidants in different steps of the oxidative chain, and thus the mechanism of antioxidative action is different. The ability of monomeric phenolic compounds as antioxidants depends on both the degree of hydroxylation and the extent of conjugation (Hodnick, Milosevljevic, Nelson, & Pardini, 1988). However, there are no methods to evaluate the remaining antioxidant activity in a substrate like mince fish and the products obtained after gelation. Regarding this subject, it is necessary to take into account possible interactions between antioxidant molecules and mince compounds that influence the bio-availability of the antioxidants. Quercetin extract seems to be more bioavailable than rosemary extract when included into a fish gel matrix.

The carbonyl groups content, measured as an index of protein oxidation, is shown in Fig. 2. The addition of quercetin to mince muscle slightly increased ($p \leqslant 0.05$) the carbonyl groups content. This result seems to be in conflict with the antioxidant properties of the quercetin extract. It could be possible that, as a consequence of the interactions that may take place between quercetin and protein, sites arose in proteins that were new susceptible to oxidation. The solubilization of protein with sodium chloride gave rise to a noticeable increase in carbonyl groups, despite a lower content of protein (1.5%). This effect has been previously reported in other studies (Karastogiannidou, 1999). The native structure of the protein is often the most stable conformation, and a chemical change in the side groups may probably lead to a partial loss of stability (Hultin, 1986). Both extracts, quercetin and especially rosemary, acted as antioxidants and decreased the carbonyl groups content in sol samples. No differences in carbonyl groups content were found between gels induced by conventional heat or microwave treatment, respectively. However, the presence of quercetin extract gave rise to noticeably lower levels of carbonyl groups. Furthermore, it seems that high pressure treatment promoted the formation of carbonyl groups in spite of the addition of rosemary or quercetin extract, although levels were higher in those gels including rosemary, mainly when high pressure was applied at moderate temperature (25 °C). The high pressure induced protein oxidation was more than thermal treatments. Quercetin extract seems to be more effective when the gel networks are formed by thermal treatments rather than by high pressure treatment.

Formation of thiobarbituric acid reactive substances (TBARS) is shown in Fig. 3. The addition of antioxidant extracts to mince muscle did not substantially modify the lipid oxidation level, probably because a slight blending did not induce oxidation. However, the homogenization of minced muscle with salt caused a twofold increase in lipid oxidation ($p \leqslant 0.05$), despite the fat content in M being slightly lower than in sol. It is known that sodium chloride has a prooxidant effect, speeding up the formation of TBARS (Karastogiannidou, 1999). The inclusion of antioxidants in the formula led to a decrease in TBARS values ($p \leqslant 0.05$), especially in case of rosemary. Conventional thermal treatment significantly decreased the oxidation level found in sol and mince samples ($p \leqslant 0.05$), probably because there was a slight variation in formula, mainly in protein content (2.17% lower). In addition, lipids may interact

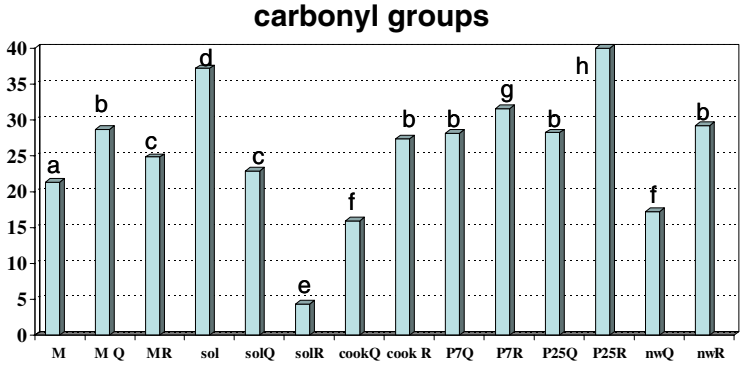

Fig. 2. Carbonyl groups content (mol/g protein) of different samples: mince (M), mince with antioxidant (MQ and MR), batter or sol, sol with antioxidants (sol Q and sol R), gel induced at 90 °C (TQ and TR), gel induced at 300 MPa of high pressure/ 7 °C (P7Q and P7R), gel induced at 300 MPa of high pressure/25 °C (P25Q and P25R) and gel induced by microwave (mwQ and mwR). Different letters (*a*, *b*, *c*) indicate significant differences ($p \leqslant 0.05$) among samples.

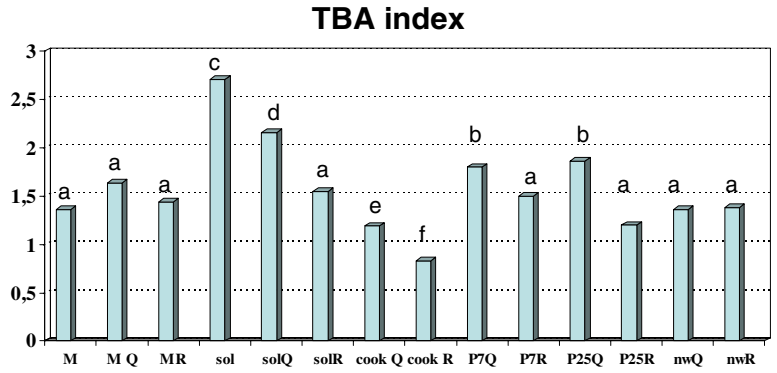

Fig. 3. Thiobarbituric acid (TBA) index of different samples: mince (M), mince with antioxidant (MQ and MR), batter or sol, sol with antioxidant (sol Q and sol R), gel induced at 90 °C (TQ and TR), gel induced at 300 MPa of high pressure/7 °C (P7Q and P7R), gel induced at 300 MPa of high pressure/25 °C (P25Q and P25R) and gel induced by microwave (mwQ and mwR). Different letters (*a*, *b*, *c*) indicate significant differences ($p \leqslant 0.05$) among samples.

covalently with proteins upon gelling, leading to a considerable reduction of the amount of available TBA reactive substances. The high pressure gave rise to similar values at 7 and 25 °C, respectively, for rosemary and quercetin, although rosemary was shown to be more effective than quercetin extract. TBARS values for gels induced by microwave treatment including both quercetin and rosemary were similar to those described for gels induced by high pressure containing rosemary.

In general, the effectiveness of rosemary to prevent lipid oxidation was higher than that shown by quercetin although it should be taken into account that the former was added in double the amount. Rosemary gave rise to a lower antioxidant activity measured by FRAP when added to mince muscle, sol and gels, although the pure rosemary extract presented higher FRAP values than pure quercetin. Thus, it seems that rosemary may interact with lipids to a higher degree, preventing their oxidation. Quercetin and rosemary extracts remained partially bio-available in the final gels. Rosemary gave rise to a higher protection against lipid oxidation in gels induced by heat and high pressure treatment, meanwhile quercetin seemed to be the most effective against oxidation of proteins, mainly in gels induced by conventional heat treatment.

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