Caffeic Acid as Antioxidant in Fish Muscle: Mechanism of Synergism with Endogenous Ascorbic Acid and α-Tocopherol

Running title header: Antioxidative mechanism of caffeic acid in fish muscle

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ABSTRACT

In an emulsion of corn oil in water with the addition of caffeic acid (Caf-OH) and α-tocopherol (α-TOH), Caf-OH was found very active in delaying lipid oxidation without affecting significantly kinetics for α-TOH degradation. In contrast, Caf-OH addition to fish muscle retarded both degradation of endogenous α-TOH and propagation of lipid oxidation, measured by peroxide value (PV) and thiobarbituric acid reactive substances (TBARS), with increasing effect with increasing Caf-OH addition (55.5-555.1 μmol/kg). Electron spin resonance (ESR) spectroscopy confirmed a higher capacity of Caf-OH to regenerate α-TOH via reduction of the α-tocopheroxyl radical compared to other cinnamic acid derivatives (*o*-coumaric, ferulic and chlorogenic acids). Degradation of endogenous ascorbate ($AscH^-$) was accelerated at higher concentration of Caf-OH in fish tissue, suggesting a role of $AscH^-$ in regeneration of Caf-OH. These results indicate that the antioxidant mechanism of Caf-OH implies the protection of endogenous α-TOH localized in tissue membranes where lipid oxidation is initiated and, at the same time, the Caf-OH regeneration by the endogenous $AscH^-$. These combined effects result in a stronger antioxidant protection against lipid oxidation by favoring, as a final point, the protection of α-TOH, which is suggested as the last defense of fish muscle against lipid oxidation.

Keywords: Lipid oxidation, α-tocopherol, ascorbate, caffeic acid, hydroxycinnamic acids, synergism, muscle tissue

INTRODUCTION

Lipid oxidation is a critical factor in the loss of quality and nutritional value during the processing and storage of muscle-based foods, in particular, fish muscle (*1*). Fish muscle contains high amounts of unsaturated fatty acids, which are predisposed to suffer decomposition via oxidative reactions (*2*). Several investigations have also related the high susceptibility of fish muscle to undergo lipid oxidation with the content and pro-oxidant activity of fish hemoglobins (*3, 4*).

In the last years, the addition of natural substances with antioxidant properties is an emerging strategy for protecting biological systems and foodstuffs from oxidative damage. Tea catechins (*5*), grape proanthocyanidins (*6*), rosemary extracts (*7*) and hydroxytyrosol extracted from olive oil (*8*) have demonstrated elevated potential to inhibit lipid oxidation in fish muscle-based food products. Among the natural substances, hydroxycinnamic acids, including caffeic (Caf-OH), ferulic, *o*-coumaric or chlorogenic acids have also attracted considerable attention as food antioxidant additives due to their potential biological and antioxidant activities (*9*). Caf-OH is found naturally in various agricultural products such as seeds, fruits, tubers and herbaceous parts of many vegetable species (*10*). In previous investigations, we have reported the efficiency of Caf-OH to protect refrigerated fish muscle against lipid oxidation (*11*), resulting that antioxidant activity the highest among hydroxycinnamic acids (*12*).

Caf-OH, in general *o*-dihydroxy phenolics, can protect lipids from oxidation by at least two well-described mechanisms: i) scavenging free radicals as a primary antioxidant and ii) chelating active transition metals to form inactive metallic complexes (*2*). Additionally, some phenolic compounds can establish cooperative redox interactions with

the endogenous antioxidant substances, which reinforce synergistically the resistance of the system to suffer oxidative damage. It is well documented that the α-tocopheroxyl radical formed during the antioxidant action of α-tocopherol (α-TOH), can be reduced by ascorbic acid ($AscH^-$) (*13*). Green tea catechins have also demonstrated capacity to repair α-TOH in low-density lipoproteins (*14*) and SDS micelles (*15*). Previous investigations also suggested a regenerative activity of Caf-OH on α-TOH in low-density lipoproteins, while in turn, Caf-OH is repaired by $AscH^-$ (*10, 16*).

The present investigation was aimed to get better knowledge of the antioxidant mechanism of Caf-OH in muscle tissues by evaluating the redox cycles of Caf-OH with important endogenous antioxidant substances of tissues, α-TOH and $AscH^-$. For this purpose, the interaction of Caf-OH with α-TOH has been initially studied in a simple model system of oil-in-water emulsions since this model has shown similar behavior to muscle tissues in different experiments conceived to test antioxidant activity (*17*). After this, the lipid oxidation and the consumption rate of the endogenous α-TOH and $AscH^-$ were studied in a fish muscle tissue. The capacity of Caf-OH and structurally-related compounds (**Figure 1**) for regenerating endogenous α-TOH was further investigated by using Electronic Spin Resonance (ESR) spectroscopy, and by evaluating the capacity of phenolic compounds to reduce α-tocopheroxyl radicals in hexane, a aprotic homogenous system, and in sodium dodecyl sulfate (SDS) micelles, a model system that mimics cellular membranes.

MATERIALS AND METHODS

Chemicals. Ascorbic acid, DL-all-rac-α-tocopherol, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), sodium dodecyl sulfate (SDS), *o*-coumaric acid, ferulic acid, caffeic acid, chlorogenic acid, 4,5-dimethyl-o-phenilenediamine (DMPD), soybean lecithin (40% L-α-phosphatidylcholine) and streptomycin sulfate were purchased from Sigma (Steinheim, Germany). Propyl gallate was acquired from Merck (Darmstadt, Germany), 2,2,6,6,-Tetramethylpiperidine-1-oxyl (TEMPO) (98%) was obtained from Fluka (Buchs, Switzerland). Stripped corn oil without α-TOH was acquired from Acros Organics (Pittsburgh, PA, USA). All chemicals were of analytical grade and the water was purified using a Milli-Q system (Millipore, Billerica, Massachusetts).

Experiments in oil-in-water emulsions. An amount of 50 g of oil-in-water emulsions, containing 1% lecithin and 10% stripped corn oil, were prepared as previously described (*18*). α-TOH was incorpored by adding 200 μL of a 10,000 μg α-TOH/mL methanolic solution into the water phase previous adding the lecithin and corn oil. The emulsion (10 mL) was introduced into 50-mL Erlenmeyer flasks and oxidation was initiated by adding 1 mM of the free radical generator AAPH (100 μL of a 100 mM solution) and were incubated during the entire monitoring period at 35 ºC. After the decay of α-TOH reached the 60% of the initial levels (19.3 hours), 100 μL of a 20 mM methanolic solution of Caf-OH was added, in order to achieve the possible regeneration of α-TOH by the hydroxycinnamic acid. This starting point was chosen since in a recent work by Pazos et al. the regeneration of the endogenous antioxidant by Caf-OH has been observed after the depletion of the 40% of the initial levels in fish muscle samples (*11*). Lipid oxidation was monitored by the measurement of conjugated diene hydroperoxides, expressed in mmol of hydroperoxide per g of oil, by using the method reported by Huang

et al. (*19*). The consumption rate of α-TOH was monitored according to an extraction procedure and analysis previously described (*20*).

Fish tissue model system. Two batches of horse mackerel (*Trachurus trachurus*) caught at the Galician platform were used within the first 24 h of sacrifice. For each experiment, 8 kg (20-24 different fish) were deboned and eviscerated. The white muscle was separated and passed through a Kenwood mincer (Kenwood Mfg Co. Ltd., Woking, England) fitted with an 8-mm diameter hole size mincing screen. It was supplemented with Streptomycin sulfate (200 μg/g) for inhibiting microbial growth. Caffeic acid was added at different concentrations between 0 (control) and 555.1 μmol/kg. Portions of 8 g of fish muscle were placed into 50-mL Erlenmeyer flasks and stored at 4 °C on ice during 12 days. Duplicate samples were taken and analyzed at different sampling times.

Lipid extraction. Lipids were extracted from fish muscle according to Bligh and Dyer (*21*) and quantified gravimetrically.

Determination of primary products of lipid oxidation. Peroxide value index (PV) of fish muscle was determined by the ferric thiocyanate method (*22*) and was expressed as meq. oxygen / kg lipid.

Determination of secondary products of lipid oxidation. The thiobarbituric acid reactive substances index (TBARS) was determined according to Vyncke (*23*) and was expressed as mmol malonaldehyde/kg muscle.

Determination of α-tocopherol. α-TOH was extracted from oil-in-water emulsions and fish muscle by adaptation of the procedure of Burton et al. (*24*) as described by Pazos et al. (*20*). The analysis of α-TOH was performed by HPLC-DAD according to Cabrini et al. (*25*).

Determination of Ascorbate. $AscH^-$ was acidically extracted from the minced fish muscle, oxidized, derivatized with DMPD and analyzed by HPLC coupled to a fluorescence detector according to Iglesias et al. (*26*).

ESR Experiments to Evaluate the Capacity to Reduce α-Tocopheroxyl Radicals by Caffeic Acid and other Hydroxycinnamic Acid Derivatives. The behavior of caffeic acid and other hydroxycinnamic acid derivatives (*o*-coumaric, ferulic and chlorogenic acids) to regenerate α-TOH from α-tocopheroxyl radical was evaluated in two different environments, a homogenous solution in hexane and a membrane model system consisting of sodium dodecyl sulfate (SDS)-micelles. The regenerative capacity of those natural phenolic compounds was also compared with a synthetic phenolic antioxidant, propyl gallate. α-Tocopheroxyl radical was generated by the chemical reaction of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) with α-TOH. Subsequent, it was quantified by Electronic Spin Resonance (ESR) spectroscopy. Experiments in hexane solution were carried out as described in a previous paper (*27*). Briefly, α-tocopheroxyl radical was generated directly in an ESR quartz capillary tube with an internal diameter of 4.2 mm (Wilmad, Buena, NJ, USA) by mixing N_2-saturated hexane solutions of α-TOH and DPPH radical. After 30 sec, phenolics were added in the same molar concentration (530 μM) in ethanol solution, and substituted by ethanol in control samples. The final concentration of α-TOH and DPPH radical in the hexane system was 2.0 and 0.013 mM, respectively. The reaction mixture was homogenized by bubbling N_2 for 40 sec. ESR spectra were recorded on a Jeol Jes-FR30 ESR spectrometer (JEOL Ltd., Tokyo, Japan) at room temperature after 1 min of DPPH addition, under the following ESR settings:

microwave power, 4 mW; sweep width, 50 G; sweep time, 2 min; modulation amplitude, 3.2 G; time constant 0.3 s.

A micellar solution of 200 mM sodium dodecyl sulfate (SDS) was prepared in 50 mM phosphate buffer, pH 6.8. α-TOH was dispersed in SDS micelles to a final concentration of 2 mM. α-Tocopheroxyl radicals were generated by mixing 1.8 mL of N_2-saturated SDS micelles with 0.1 mL of DPPH in ethanol. After 30 sec, phenolic compounds were incorporated to the micellar system in water:acetonitrile (5:1) solution, and substituted by water:acetonitrile (5:1) in control samples. Final concentrations of α-TOH and DPPH radical were 1.8 and 0.025 mM, respectively. The reaction mixture was homogenized by bubbling N_2 for 40 sec. ESR spectra were recorded at room temperature on a Jeol Jes-FR30 ESR spectrometer (JEOL Ltd., Tokyo, Japan) after 1 min of DPPH addition. ESR parameters were as above mentioned.

The ratio between the peak-to-peak amplitude of α-tocopheroxyl radical and the Mn(II)-marker attached to the cavity of the spectrometer was used as a relative signal intensity of α-tocopheroxyl radical, as previously described (*27*). The number of moles of α-tocopheroxyl radical reduced per mole of phenolic compound was estimated by the slopes of the linear regressions between concentrations of reduced α-tocopheroxyl radical and phenolic concentrations. The concentration of α-tocopheroxyl radical was calculated relating the total double integrated area of the α-tocopheroxyl radical signal to the total signal-area corresponding to a known concentration of TEMPO radical. The area of signals was integrated by using the Bruker WinEPR software, whereas the simulation and fitting of the ESR spectra were performed using the PEST WinSIM program (*28*).

Statistical analysis. The experiments were performed twice and data are reported as mean ± standard deviation of three replicates. The data were analyzed by one-way analysis of variance (ANOVA) and the least-squares difference method. Statistical analyses were performed with the software Statistica 6.0.

RESULTS

Interaction between Caf-OH and α-TOH in Oil-in-water Emulsions. The interaction of Caf-OH with α-TOH was initially investigated in oil-in-water emulsions. The addition of Caf-OH after 19.3 hours of initiating oxidation, significantly retarded the propagation of lipid oxidation since the formation of conjugated dienes peroxides was actively inhibited in comparison with control samples (**Figure 2A**). A strong generation of conjugated dienes was observed after approximately 70 hours in emulsions without Caf-OH, whereas no significant formation of those oxidation products was detected at the end of the experiment in samples with supplemented Caf-OH. The hydroxycinnamic acid was added once α-TOH decrease up to the 60 % of the initial levels (after 19.3 hours) in order to achieve its possible regeneration. However, α-TOH exhibited an almost-linear decay and no significant differences were observed between controls and samples with supplemented Caf-OH (**Figure 2B**).

Inhibitory Effect of Caf-OH on Lipid Oxidation in Fish Muscle. The antioxidant efficiency of different concentrations of Caf-OH (0-555.1 μmol/kg) was tested in minced fish muscle during refrigerated storage. In this experiment, the rate of lipid oxidation was evaluated by means of the peroxide value and TBARS index (**Figure 3**). The increment of peroxide value and TBARS index was shown to be significantly faster in the controls

than in the samples supplemented with Caf-OH. Therefore, the addition of Caf-OH was effective to inhibit the lipid oxidation, and the antioxidant behavior in fish muscle was positively related with an increasing concentration of Caf-OH. The induction periods of PV and TBARS were 2 days for the control and 3, 9, 11 and 12 days for the different samples with increasing concentrations of Caf-OH.

Interaction between Caf-OH and Endogenous α-TOH and $AscH^-$ in Fish Muscle. The same samples used for evaluating lipid oxidation were tested for monitoring the concentration of the endogenous antioxidants α-TOH and $AscH^-$. Caf-OH was found to be an efficient protective agent for retarding the depletion of α-TOH since the consumption rate of this compound was faster in the control than in fish muscle with supplemented Caf-OH (**Figure 4A**). The levels of α-TOH in the control samples were maintained during the first day of experiment and after this, a strong depletion was observed until the complete reduction at fifth day. Muscle tissue with 55.5 μmol/kg of Caf-OH maintained the initial values of α-TOH during approximately 3 days and, after this, decreased until the fifth day where a 7.2 % of the initial values were achieved. The levels of α-TOH were maintained until the day 9 in the samples with 138.8 μmol/kg of Caf-OH, until day 11 with 277.6 μmol/kg and finally no significant depletion was achieved in the 12 days of the experiment in muscle tissues supplemented with 555.1 μmol/kg.

The behavior of endogenous $AscH^-$ was drastically different from that of α-TOH. The kinetics of $AscH^-$ showed differences between the control and tissues supplemented with Caf-OH, but the protective effect on α-TOH was not observed on $AscH^-$ (**Figure 4B**). The initial values of $AscH^-$ were maintained during 1 day, and after this, different

depletion rates were observed depending on the amount of Caf-OH added. Control and fish muscle with the lowest Caf-OH concentrations (55.5 and 138.8 μmol/kg) were found to maintain better the levels of endogenous $AscH^-$. Muscle tissues supplemented with 555.1 μmol/kg of Caf-OH exhibited the fastest depletion for $AscH^-$, while samples with 277.6 μmol/kg of Caf-OH showed an intermediate depletion of $AscH^-$. Therefore, the consumption of the endogenous $AscH^-$ in fish muscle was faster in samples with the added Caf-OH, and the rate of depletion was directly related with the concentration of the exogenous antioxidant.

Reduction of α-Tocopheroxyl Radicals by Caffeic and other Hydroxycinnamic Acids in Homogenous Hexane Systems. The capacity of Caf-OH and related hydroxycinnamic acids to regenerate α-TOH via reduction of α-tocopheroxyl radicals was initially evaluated in a simply homogenous system in hexane. The synthetic phenolic propyl gallate was also investigated since previous studies showed a comparable antioxidant activity with Caf-OH in fish muscle (*12*). Caf-OH was found to be active in reducing α-tocopheroxyl radicals at concentration of 530 μM (**Figure 5A**). Propyl gallate was also effective at the same concentration, being significantly better than Caf-OH ($p<0.05$). In contrast, the structurally-related hydroxycinnamic acids, *o*-coumaric, ferulic and chlorogenic, did not have a significant effect on α-tocopheroxyl radical at the same phenolic concentration. Increasing the phenolic level up to 1060 μM, ferulic and chlorogenic acids were found to reduce α-tocopheroxyl radical, whereas *o*-coumaric acid was still not effective (**Figure 5B**). The present results indicated the corresponding efficacy for hydroxycinnamic acids to regenerate α-TOH from α-tocopheroxyl radical in

a homogenous hexane system: caffeic acid > chlorogenic acid > ferulic acid > *o*-coumaric acid.

The reduction of α-tocopheroxyl radicals displayed a phenolic concentration-dependence for caffeic acid and propyl gallate, and such concentration-dependence was found to be linear ($R^2 < 0.97$) at the phenolic concentration range studied (**Table 1**). The slope of the linear regressions was used to estimate the amount of α-tocopheroxyl radicals reduced per mol of phenolic compound. Thus, one mol of propyl gallate reduced approximately 3.7×10^{-4} mol of α-tocopheroxyl radical, which is more than twice of the corresponding α-tocopheroxyl radical reduced by caffeic acid, 1.4×10^{-4} mol.

Reduction of α-Tocopheroxyl Radicals by Caffeic and other Hydroxycinnamic Acids in SDS micelles. The capacity of Caf-OH and structurally-related hydroxycinnamic acid to reduce α-tocopheroxyl radicals was also evaluated in a micellar system of sodium docecyl sulfate (SDS) micelles containing α-TOH because of the structural similarities of that system with phospholipids, which are considered the structural components in cellular membranes (*29, 30*). As in cellular membranes, SDS-micelles present a polar-head negatively charged (sulfate groups in SDS) that is orientated to the external part in close contact with the aqueous medium, and the non-polar tails (dodecyl groups in SDS) lie mainly in the internal core of micelle. α-TOH localized in either SDS-micelles or cellular membranes should have its phenolic ring oriented to the surface and the saturated phytyl ($C_{16}H_{33}$) side chain to the inner part.

Caf-OH at concentration of 400 μM was found to be the most effective to reduce α-tocopheroxyl radicals in SDS micelles, followed in decreasing order by ferulic acid ≈ chlorogenic acid (**Figure 6**). *o*-Coumaric acid did not show significantly differences with

control samples, and therefore that hydroxycinnamic acid was not found active in reducing α-tocopheroxyl radicals at the same concentration. The regenerated α-tocopheroxyl radical and phenolic concentration exhibited an approximately linear relationship for caffeic acid and propyl gallate ($R^2 > 0.88$), and then, the scope of the linear regression was used to estimate the mol of α-tocopheroxyl radicals reduced by mole of phenolic (**Table 1**). The results indicated that propyl gallate is approximately 10 times more effective in regenerating α-tocopheroxyl radicals to α-TOH in SDS micelles, since propyl gallate and caffeic acid reduced respectively 2.17×10^{-2} and 1.83×10^{-3} mol of α-tocopheroxyl radicals by mol of phenolic.

DISCUSSION

α-Tocopherol (α-TOH) is recognized as the most important lipid-soluble, chain-breaking antioxidant in animal tissues (*2*). Recent works suggest that α-TOH is one of the last antioxidant defenses in fish muscle tissue and its decrease below a critical concentration leads to lipid peroxidation (*11, 20*). Additionally, the α-TOH consumption measured in red blood cells as ratio between the levels of α-TOH oxidation product α-tocopherolquinone and α-TOH, has been used to assess the antioxidant status of humans (*31*) and to test the oxidative potency of prooxidants in red cell membranes (*32*). The present paper demonstrates the efficiency of Caf-OH in prolonging the preservation of the endogenous α-TOH in fish muscle tissue during chilling storage. Supplementation with increasing levels of Caf-OH put into effect a longer preservation of the endogenous α-TOH in fish muscle.

This protective capacity of Caf-OH against α-TOH consumption could be explained by three different pathways: (i) inhibition of attack by lipid peroxidation-derived radicals

(peroxyl, alkoxyl and alkyl radicals); (ii) regeneration of α-TOH by reduction from α-tocopheroxyl radical; and (iii) influence on α-TOH metabolism. Several investigations demonstrated the ability of Caf-OH and other *o*-dihydroxy (catechol)-containing phenolics to increase α-TOH levels in plasma and lipoproteins by dietary supplementation (*33*). However, the modulation of α-TOH metabolism by phenolic compounds does not seem to be a reliable mechanism since the catechol-containing phenolics did not interfere on α-TOH metabolism in hepatocyte cultures (*34*). On the other hand, Caf-OH is a well-known scavenger of lipid peroxidation-derived radicals due to its lateral double bond conjugated with the catechol ring proportionates an extensive electron delocalization that increases the stability of the phenolic *o*-semiquinone radical, and consequently, antioxidant activity. A higher activity scavenging AAPH-derived peroxyl radicals was observed by caffeic acid in comparison with monohydroxycinnamic acid derivatives, ferulic acid and *o*-coumaric acid (*35*). Similar antioxidant efficiency (caffeic acid > ferulic acid ≥ coumaric acid) were exhibited by these hydroxycinnamic acids in LDL exposed to AAPH (*36*), bulk methyl linoleate stressed at 40 ºC (*37*) and fish muscle tissue (*12*). Our data demonstrate a good correlation between the antioxidant activity attributed to Caf-OH in fish muscle tissue and the protection of the endogenous α-TOH levels, given that the most effective Caf-OH concentration for preserving α-TOH from consumption was found to be also the most active to delay lipid peroxidation. According to these abilities to trap lipid peroxidation-derived radicals and the preservation of α-TOH via minimizing its attack by free radical species, Caf-OH seems the most effective hydroxycinnamic acid derivative in protecting α-TOH from consumption.

The reduction of α-tocopheroxyl radicals by phenolic compounds (ArOH) can take place through an electron transfer or hydrogen-atom transfer mechanism. Both pathways give the same net result, α-TO$^{\cdot}$ + ArOH → α-TOH + ArO$^{\cdot}$, but the H-atom transfer is a one-step process favored in protic solvents whereas the electron transfer mechanism is a two-steps process more typical in aprotic mediums. The redox potentials and bond dissociation enthalpies (BDE) of the O-H bond are the physicochemical parameters that control the thermodynamic ability for electron transfer and H-atom transfer of phenolic compounds, respectively. The present paper reports a higher activity of Caf-OH to reduce α-tocopheroxyl radical in either an aprotic medium (hexane) or aqueous SDS-micelles. Ferulic and chlorogenic acids showed intermediary activity, whereas *o*-coumaric acid was not able to reduce α-tocopheroxyl radicals even when used at a very high molar ratio (coumaric:α-tocopheroxyl radical ≈ 1507:1). These results are in agreement with the lower one-electron reduction potential reported for Caf-OH, an intermediate reduction potential for ferulic acid and a higher potential for *o*-coumaric (*35*), that suggest a more elevated capacity to donate electrons by caffeic acid, followed in decreasing order by ferulic acid and *o*-coumaric. The BDE of the O-H bond point in the same direction given that those increase in the order caffeic < ferulic < *o*-coumaric, and lower BDE favor the H-transfer processes (*35*). The reduction of the BDE for O-H bond with the presence of a second hydroxyl group at ortho position, as caffeic acid structure includes, is well-described. Previous investigations have also shown that the phenolics with lower BDE for O-H bond exhibit a higher capacity to reduce α-tocopheroxyl radical in hexane (*27*).

Our data in fish muscle showed that samples supplemented with higher concentration of Caf-OH suffered a faster consumption of the levels of $AscH^{-}$.

Presumably, this fact is a consequence of Caf-OH regeneration promoted by $AscH^-$, analogous to the observed regeneration of α-TOH by $AscH^-$ in biological systems (*13*). According to Buettner et al. (*38*), the low reduction potential of $AscH^-$ (E=0.28 V) enables it to repair oxidizing free radicals with greater reduction potential, including the exogenous Caf-OH (E=0.54 V), and consequently the reaction is thermodynamically feasible. This feature has been observed for the regeneration of quercetin and other flavonoids by $AscH^-$ (*39*). The product of this reaction is a very stable ascorbyl radical ($AscH^{\cdot-}$) that is eliminated in the aqueous phase by disproportionation (*40*) or reductase activity (*41*). The decay of this radical facilitates the protective effect of $AscH^-$ over Caf-OH since the direct reaction between $AscH^-$ and Caf-$O^{\cdot}$ is kinetically pulled. The reduction of caffeic acid quinone by $AscH^-$ for obtaining the Caf-$O^{\cdot}$ and dehydroascorbate (Asc^-) (Caf=O + $AscH^{\cdot-}$ → Caf-$O^{\cdot}$ + Asc^-) and the subsequent radical recombination involving Caf-$O^{\cdot}$ and $AscH^{\cdot-}$ (Caf-$O^{\cdot}$ + $AscH^{\cdot-}$ + H^+→ Caf-OH + Asc^-) is the other possible electron transfer reaction between Caf-OH and $AscH^-$ (*16*).

REDOX cycles can be limited by the distribution of molecules since an effective contact among those is critical to establish redox interactions. The phenolic nature of Caf-OH supplies an advantage for facilitating its localization at the phospholipid:water interface of biological membranes. α-TOH is a lipophilic endogenous antioxidant that is mostly located in the outer monolayer with the chromanol ring oriented to the aqueous phase, while the hydrophilic $AscH^-$ is localized in the water phase. Therefore, this localization of Caf-OH at the phospholipid:water interface makes reliable both interactions with α-TOH and $AscH^-$ in muscle tissues (**Figure 7**). In the oil-in-water emulsions employed in the present study, however, α-TOH is mainly distributed in the

oil droplets, which are surrounded by an emulsifier of phospholipidic nature. Therefore, the interaction between Caf-OH and α-TOH should be more difficult in the oil-in-water emulsions, and that could be the explanation of reduced protection of Caf-OH over the α-TOH depletion. However, Caf-OH supplementation was able to retard lipid oxidation in oil-in-water emulsions, which is in accordance with an important location of Caf-OH at phospholipid-water interface. Previous investigations have also indicated the effectiveness of Caf-OH to delay lipid oxidation in oil-in-water emulsions, but Caf-OH was found to promote lipid oxidation at pH 3 due probably to its capacity to reduced Fe(III) to the prooxidant Fe(II) (*42*).

In summary, Caf-OH was able to retard both endogenous α-TOH consumption and lipid oxidation propagation in fish muscle tissue, and this protection was positively correlated with increasing concentrations of Caf-OH. In contrast, the consumption of endogenous $AscH^-$ was accelerated by supplementing higher amounts of Caf-OH in fish tissue, which is in concordance with a recycling role of $AscH^-$ on supplemented Caf-OH. Electronic Spin Resonance (ESR) experiences demonstrated the higher capacity of Caf-OH to regenerate α-TOH via reduction of α-tocopheroxyl radical in comparison to other hydroxycinnamic acids (*o*-coumaric, ferulic and chlorogenic acids). These redox interactions (**Figure 7**) found between Caf-OH and endogenous α-TOH and $AscH^-$ in muscle tissues, favor as a final point the protection of α-TOH that is localized at the active places for oxidation, tissue membranes. Such dynamic cooperation with endogenous antioxidants seems to be an important mechanism in the antioxidant role of Caf-OH in muscle tissues.

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FIGURE CAPTIONS

Figure 1. Chemical structures of the phenolic acids studied.

Figure 2. Effect of Caf-OH to inhibit the formation of lipid hydroperoxides (A) and the consumption of α-TOH (B) in oil-in-water emulsions.

Figure 3. Efficiency of different concentrations of Caf-OH to inhibit formation of lipid hydroperoxides (A) and TBARS (B) in fish muscle tissue.

Figure 4. Effect of different concentrations of Caf-OH on the endogenous α-TOH (A) and ascorbic acid (B) from fish muscle tissue.

Figure 5. Capacity to reduce α-tocopheroxyl radical in a homogenous hexane system by Caf-OH, other hydroxycinnamic acid derivatives (*o*-coumaric, ferulic and chlorogenic acids) and the synthetic phenolic propyl gallate. α-Tocopheroxyl radical was monitored by ESR spectroscopy in the presence of 530 μM (A) or 1060 μM (B) of phenolics.

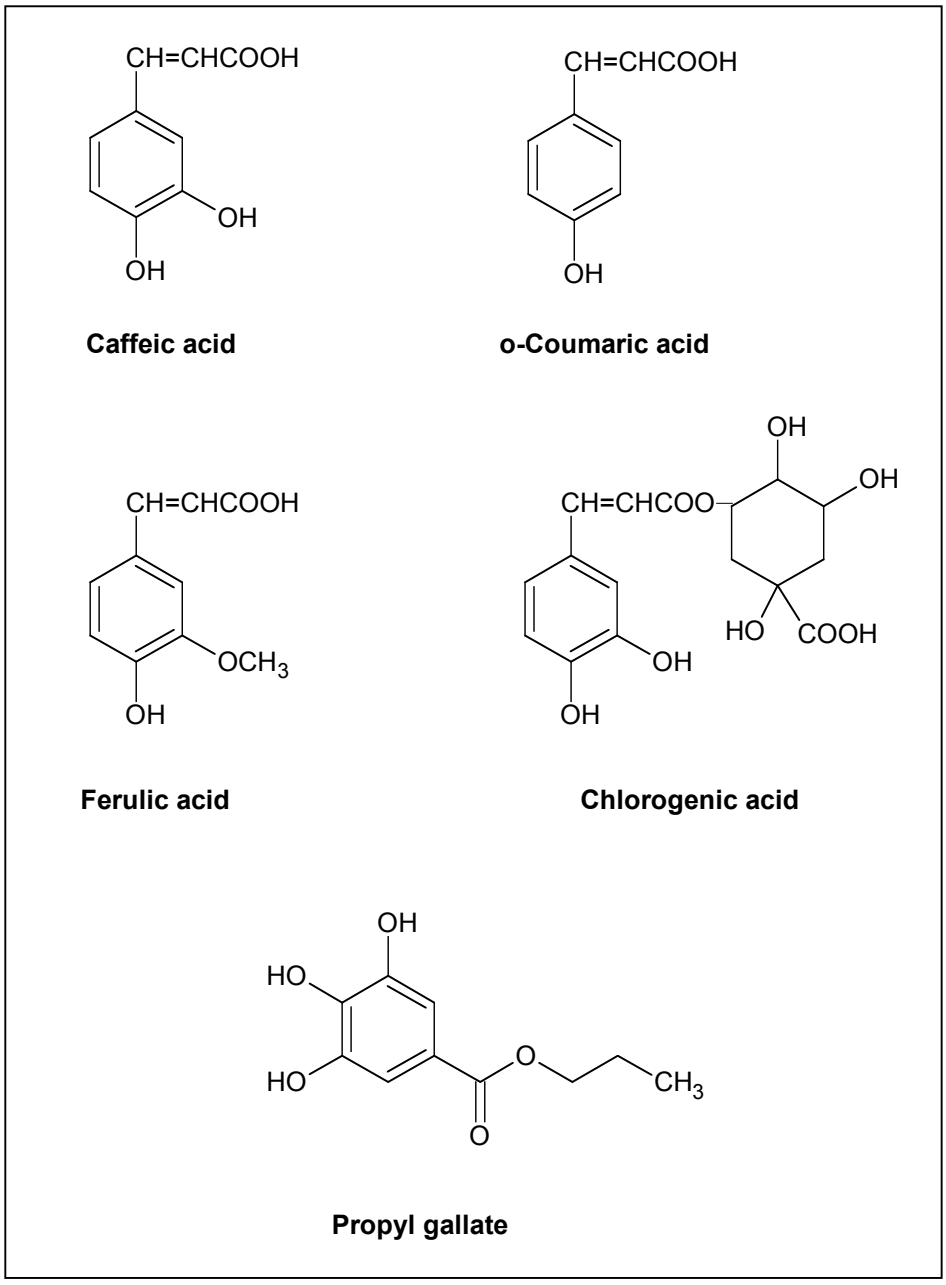
Figure 6. Capacity to reduce α-tocopheroxyl radical in SDS micelles by Caf-OH, other hydroxycinnamic acid derivatives (*o*-coumaric, ferulic and chlorogenic acids) and the synthetic phenolic propyl gallate. α-Tocopheroxyl radical was monitored by ESR spectroscopy in the presence phenolics at 400 μM.

Figure 7. Proposed redox cycles between exogenous Caf-OH and endogeneous α-TOH and ascorbic acid in muscle tissues.

Table 1. Moles of α-tocopheroxyl radical reduced per mol of phenolic, determined as the slope of the linear regression found between the concentration of reduced α-tocopheroxyl radical and phenolic concentration.

		Tocopheroxyl radical regenerated (μM)	Phenolic concentration (μM)	R^2	Slope (moles tocopheroxyl radical reduced /mol phenolic)
Hexane	Caffeic acid	0.078-0.366	100-2100	0.97	1.4×10^{-4}
	Propyl gallate	0.112-0.410	20-800	0.97	3.7×10^{-4}
SDS micelles	Caffeic acid	0.679-2.804	60-1200	0.91	1.83×10^{-3}
	Propyl gallate	0.866-3.189	5-100	0.89	2.17×10^{-2}

1 Figure 1



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1 Figure 2

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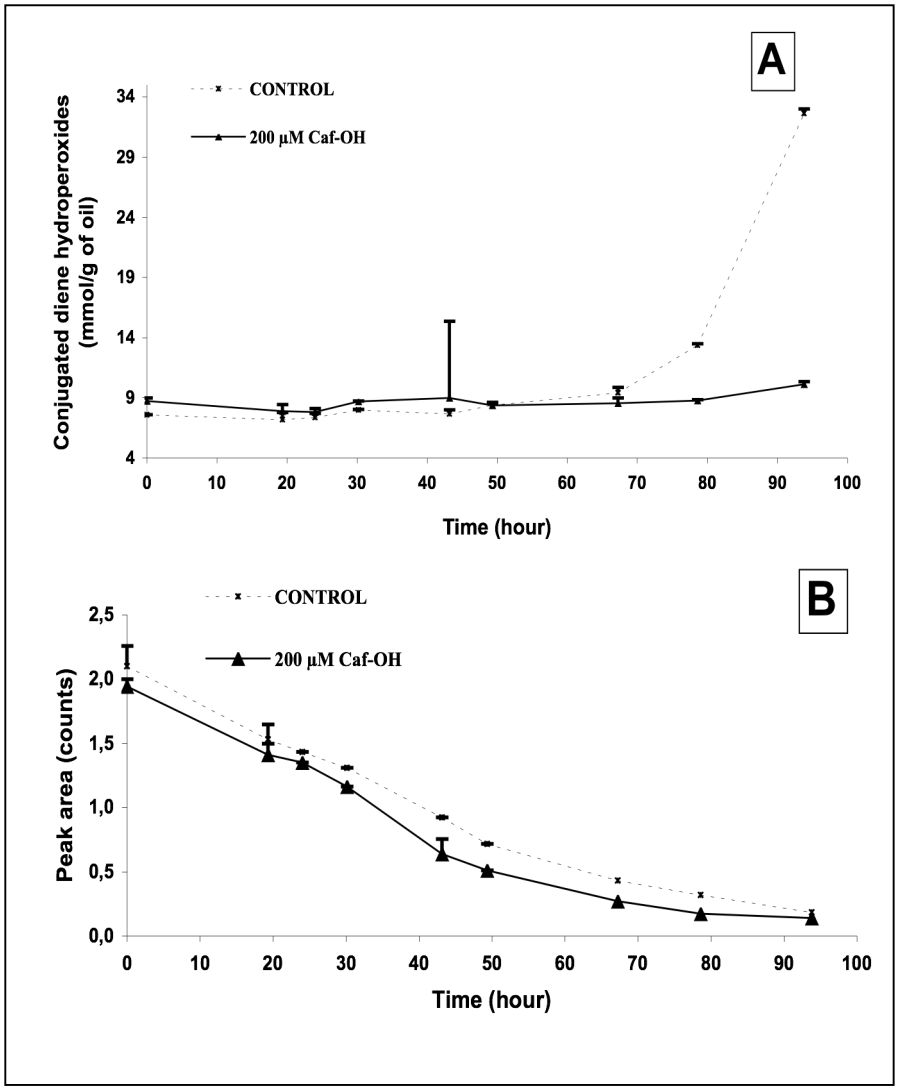

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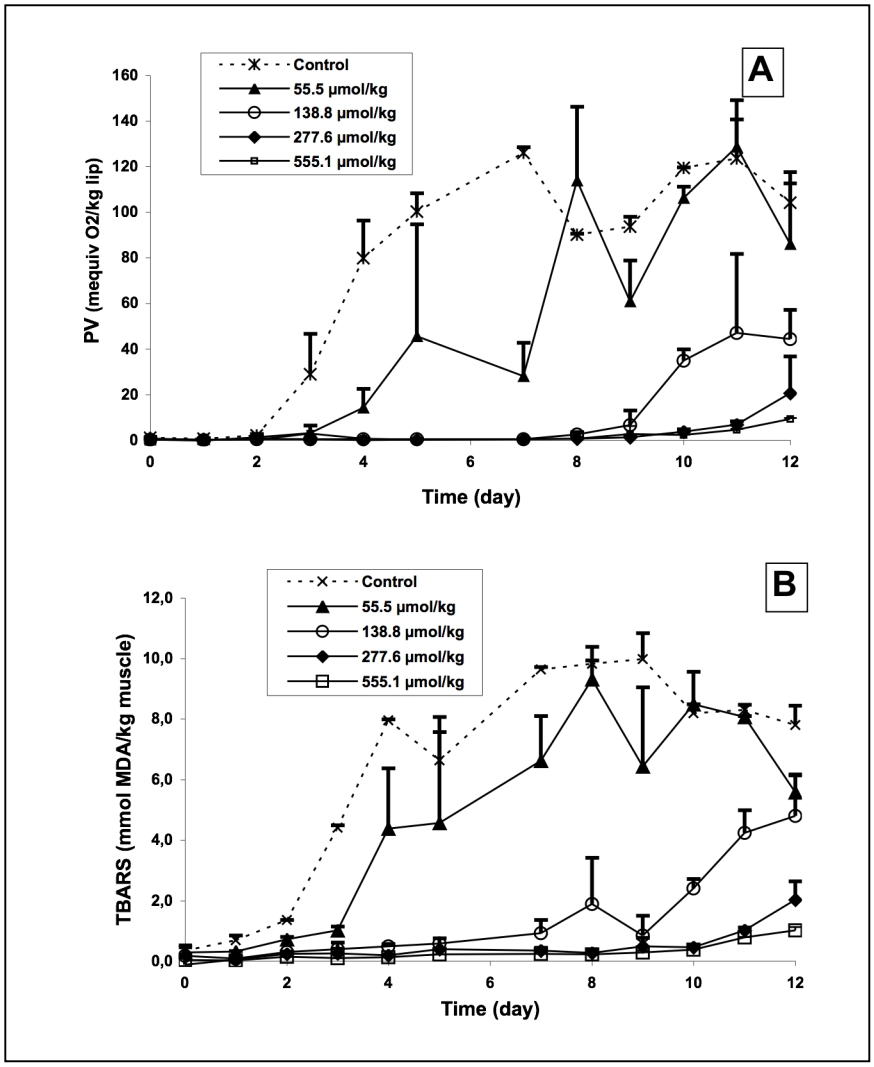

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1 Figure 4

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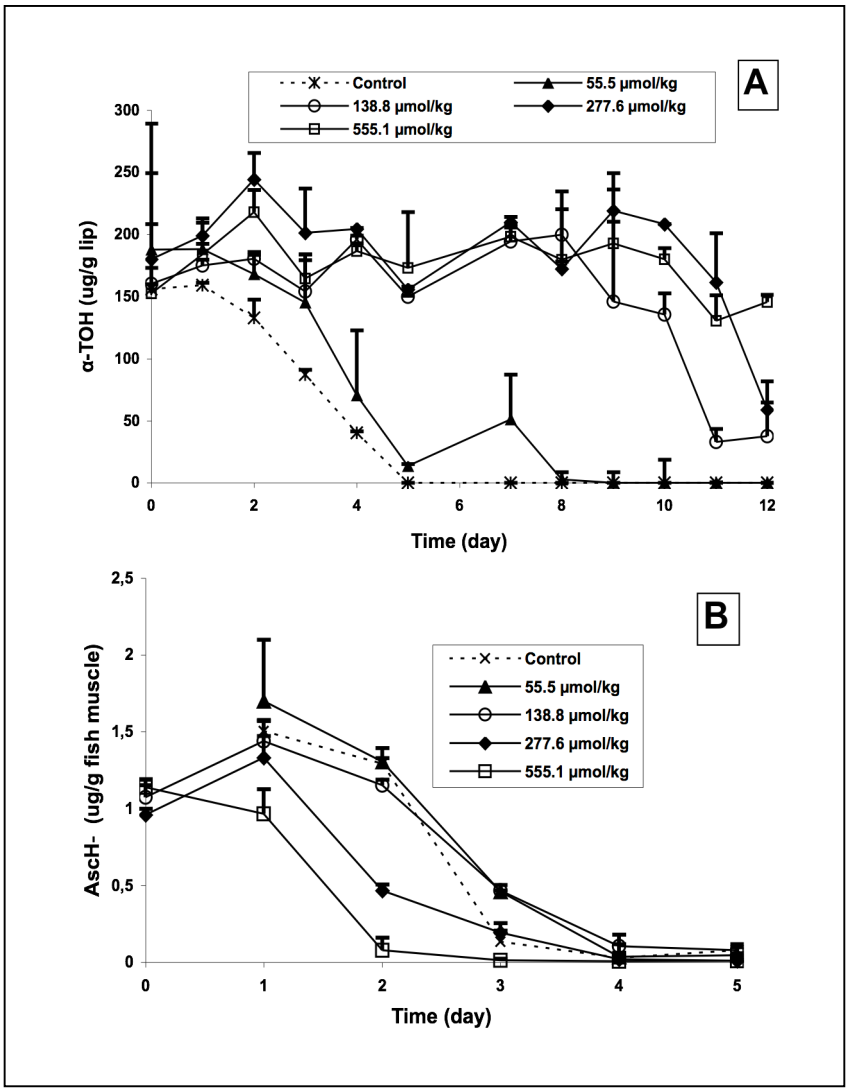

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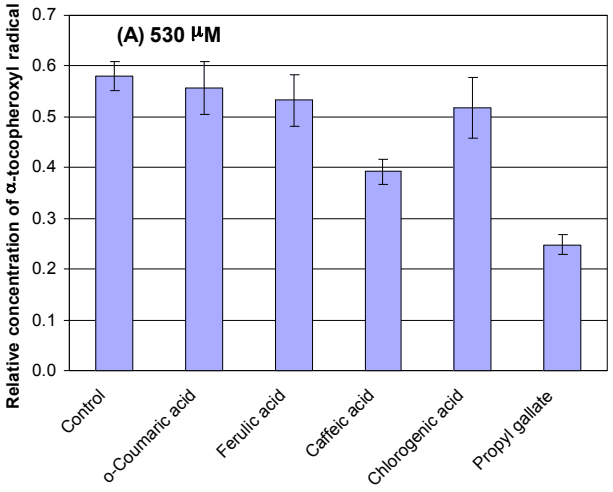

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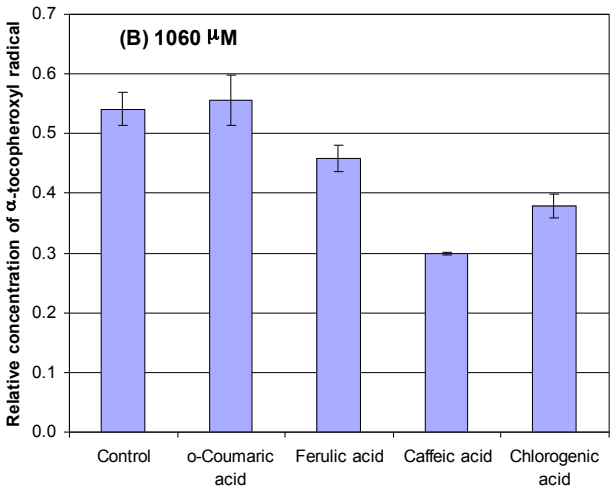

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1 Figure 6

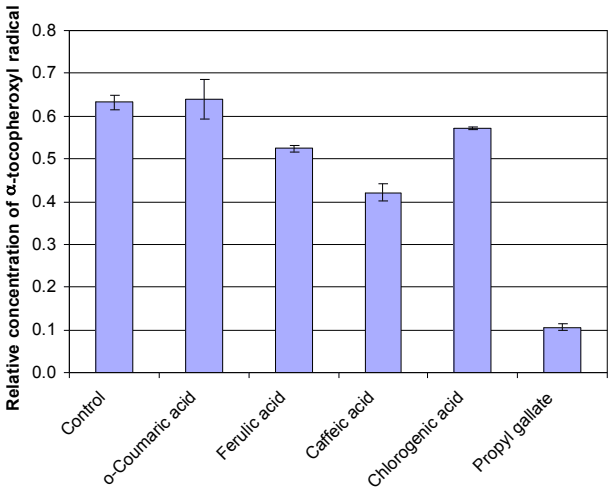

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Figure 7