DOI:10.1021/jf903757h

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Effect of Phenolic Compounds on the Formation of α-Aminoadipic and γ-Glutamic Semialdehydes from Myofibrillar Proteins Oxidized by Copper, Iron, and Myoglobin

MARIO ESTÉVEZ*,† AND MARINA HEINONEN

Department of Food and Environmental Sciences, Food Chemistry, University of Helsinki, Helsinki 00014, Finland. † Current address: Department of Animal Production and Food Science, Food Technology, University of Extremadura 10071, Cáceres, Spain.

The effect of selected phenolic compounds, namely, gallic acid, chlorogenic acid, genistein, catechin, cyanidin-3-glucoside and rutin, on the formation of specific protein carbonyls, α-aminoadipic and γ-glutamic semialdehydes (AAS and GGS, respectively), from oxidized myofibrillar proteins, was studied in the present article. Suspensions containing myofibrillar proteins (20 mg/mL) and the aforementioned phenolic compounds (1 mM) were oxidized (37 °C for 20 days) in the presence of copper acetate, iron ($FeCl_3$), or myoglobin (10 μM) in combination with 1 mM H_2O_2 and analyzed for AAS and GGS using liquid chromatography−electrospray ionization−mass spectrometry (LC-ESI-MS). Suspensions with added α-tocopherol (1 mM) and a control group (with no phenolic compound) were also considered. In the presence of copper, the α-tocopherol and most phenolic compounds significantly inhibited the formation of AAS and GGS. In iron- and myoglobin-oxidized suspensions, however, some of those phenolic compounds (i.e., chlorogenic acid and genistein) promoted the formation of the semialdehydes. Besides the influence of the oxidation promoters, the overall effect of plant phenolics on protein oxidation is likely affected by the chemical structure of the phenolics and the result of the interactions between these compounds and myofibrillar proteins. Plausible mechanisms for the antioxidant and pro-oxidant effects of plant phenolics on myofibrillar proteins are proposed in the present article. This study highlights the complexity of redox reactions between plant phenolics and oxidizing myofibrillar proteins.

KEYWORDS: Oxidation; myofibrillar proteins; semialdehydes; DNPH-method; LC-ESI-MS; phenolic compounds

INTRODUCTION

The role of food proteins as targets for reactive-oxygen species (ROS) has been ignored for decades. Protein oxidation is, currently, one of the most relevant and innovative issues of study within the food chemistry field. Lately, numerous studies have focused on the occurrence and consequences of protein oxidation in meat and meat products (*1*−*5*). Nevertheless, further studies on this issue are required as the precise mechanisms involved in the oxidative degradation of muscle proteins and the fate of particular amino acids during handling, processing, and storage of muscle foods are poorly understood. In order to comprehend these complex mechanisms in muscle foods, it is essential to fully understand the basic chemistry of protein oxidation and to employ highly sensitive and specific methodologies. Recently, an advanced technique for the accurate detection of particular protein oxidation products, α-aminoadipic and γ-glutamic acid semialdehydes, (AAS and GGS, respectively) has been developed by using liquid chromatography−electrospray ionization−mass spectrometry (LC-ESI-MS) (*6*). AAS is the main oxidation product from lysine, while GGS derives from the oxidative degradation of arginine and proline (*7*). These compounds have been highlighted as reliable protein oxidation markers (*6*) and have been found in considerably high levels in different meat products (*5*). Nevertheless, the mechanisms involved in the formation of these semialdehydes in muscle foods are poorly understood. Both semialdehydes are known to be formed in the presence of glucose, various transition metals, and hydrogen peroxide (*6*−*8*). The metal-catalyzed oxidation of proteins as well as the Maillard reaction might be the main formation routes of AAS and GGS in biological samples (*7*, *8*). In meat systems, myoglobin (Mb) and nonheme iron play a major role in generating ROS and, therefore, in promoting lipid oxidation (*9*). However, the ability of these oxidation promoters to initiate the oxidation of myofibrillar proteins and promote the formation of these specific protein oxidation products is unknown.

Plant phenolics are bioactive compounds of increasing interest among food technologists and consumers. Flavonoids and phenolic acids are known to display antioxidant, anti-inflammatory, and antimicrobial properties (*10*). Diets enriched in flavonoid-containing foods are associated with reduced incidence of various cancer types, with the flavonoids being considered to play a

*Corresponding author. Phone: +34 927257122. Fax: +34 927257110. E-mail: mariovet@unex.es.

 Published on Web 03/02/2010

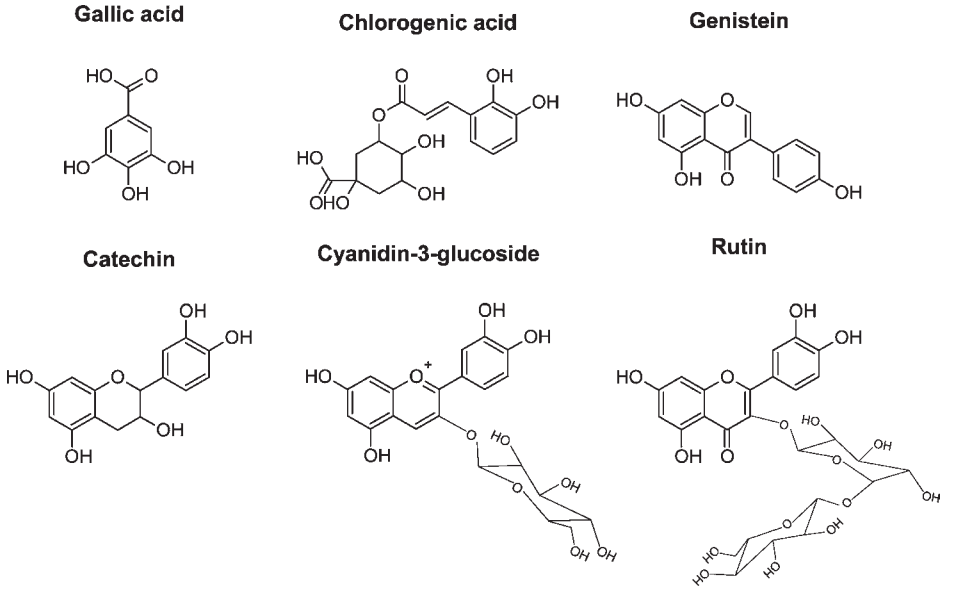

Figure 1. Chemical structures of phenolic compounds tested in the present study.

relevant health-protecting role (*10*). The belief that these natural substances provide an enhanced healthy value to functional food products has led to a considerable increase of health claims in foods containing phenolic-rich plant materials. However, these compounds can also display, under particular circumstances, pro-oxidant effects in food systems. As a result of that pro-oxidant action, phenolic compounds and other bioactive compounds (i.e., vitamins) have been found to exhibit, under particular circumstances, harmful effects leading to an increased risk of suffering coronary heart diseases, stroke, and cancer (*10*). The protective role of phenolic compounds against myofibrillar protein oxidation has been scarcely studied. In a previous paper, we reported the effect of selected phenolic compounds on protein oxidation assessed by means of fluorescence spectroscopy (*11*). Whereas both antioxidant and pro-oxidant effects were reported in that paper, the specific chemical mechanisms of interaction between phenolic compounds, myofibrillar proteins, and other components of the oxidative reactions (i.e., metals) remain unknown. The recently developed LC-ESI-MS method for the detection of AAS and GGS in oxidized myofibrillar proteins enables the study of the chemical insight of these interactions and mechanisms. The present study is devoted to studying the effect of selected phenolic compounds, namely, gallic acid, chlorogenic acid, genistein, catechin, cyanidin-3-glucoside, and rutin, on the formation of AAS and GGS from myofibrillar proteins oxidized in the presence of copper (Cu^{2+}), iron (Fe^{3+}), and myoglobin.

MATERIALS AND METHODS

Material. Cyanidin-3-glucoside, caffeoylquinic acid (chlorogenic acid), catechin, rutin, and genistein were purchased from Extrasynthese (Lyon, France), gallic acid was obtained from Sigma Aldrich (Steinheim, Germany), and α-tocopherol was from Merck (Darmstadt, Germany). The structures of the phenolic compounds used in the present study are shown in **Figure 1**. Myoglobin from equine skeletal muscle was purchased from Sigma Aldrich (Steinheim, Germany). All other chemicals were supplied by J.T. Baker (Deventer, Holland), Riedel de-Häen (Seelze, Germany), and Sigma Aldrich (Steinheim, Germany). All chemicals were of analytical grade except for HPLC-grade methanol. The water used was purified by passage through a Milli-Q system (Millipore Corp., Bedford, MA). Porcine *longissimus dorsi* muscle was purchased in local supermarkets in Helsinki. Myofibrillar proteins were isolated and purified from porcine muscles according to a method described elsewhere (*12*). Egg shell membrane was isolated from fresh white leghorn hen eggs, thoroughly washed with distilled water, cut into small pieces (5 mm × 5 mm), and finally dried with filter paper before use.

Preparation and in Vitro Oxidation of Protein Suspensions. Myofibrillar proteins were suspended (20 mg/mL) in 15 mM PIPES buffer (pH 6) containing 0.6 M sodium chloride. Eight different suspensions (30 mL) were prepared depending on the addition of selected phenolic compounds (1 mM), gallic acid, chlorogenic acid, genistein, catechin, cyanidin-3-glucoside, and rutin, including suspensions with α-tocopherol and a control group (with no phenolic compound). All suspensions were prepared in triplicate in sealed vials and oxidized using three different oxidation promoters, copper acetate, iron ($FeCl_3$), or myoglobin (10 μM), in combination with 1 mM H_2O_2. During the in vitro oxidation, we stirred suspensions constantly and kept them in an oven at 37 °C for 20 days. Sampling was carried out at days 1, 4, 10, 15, and 20 for analyses.

Determination of Total Carbonyls by the DNPH Method. Total protein carbonyls were quantified in protein suspensions at sampling times according to the method described by Butterfield and Stadtman (*13*) with minor modifications. An aliquot (100 μL) of protein suspensions was dispensed in 2 mL Eppendorf tubes. Proteins were precipitated by cold 10% TCA (1 mL) and subsequent centrifugation for 5 min at 5000 rpm. One pellet was treated with 1 mL of 2 M HCl (protein concentration measurement) and the other with an equal volume of 0.2% (w/v) DNPH in 2 M HCl (carbonyl concentration measurement). Both samples were incubated for 1 h at room temperature. Afterward, samples were precipitated by 10% TCA (0.8 mL) and washed twice with 1 mL of ethanol/ethyl acetate (1:1, v/v) to remove excess DNPH. The pellets were then dissolved in 2 mL of 6 M guanidine HCl in 20 mM sodium phosphate buffer at pH 6.5, stirred, and centrifuged for 2 min at 5000 rpm to remove insoluble fragments. Protein concentration was calculated from absorption at 280 nm using BSA as the standard. The amount of carbonyls was expressed as nmol of carbonyl per mg of protein using an absorption coefficient of 21.0 nM^{-1} cm^{-1} at 370 nm for protein hydrazones. The overall effect of added phenolics and α-tocopherol on the total amount of protein carbonyls was calculated as follows: $[(C_{15} - T_{15})/C_{15}] \times 100$, where C_{15} is the total carbonyl content in the control sample at day 15, and T_{15} is the carbonyl content in the treated sample at day 15. Positive values ($C_{15} > T_{15}$) should be interpreted as percent inhibition against the formation of protein carbonyls, while negative values ($C_{15} < T_{15}$) denote pro-oxidant activity and should be interpreted as percent promotion in the formation of protein carbonyls.

Synthesis of AAS-ABA and GGS-ABA. *N*-Acetyl-L-AAS and *N*-acetyl-L-GGS were synthesized from *N*α-acetyl-L-lysine and *N*α-acetyl-L-ornithine using lysyl oxidase activity from egg shell membrane following the procedure described by Akagawa et al. (*14*). Briefly, 10 mM *N*α-acetyl-L-lysine and 10 mM *N*α-acetyl-L-ornithine were independently incubated with constant stirring with 5 g of egg shell membrane in 50 mL of 20 mM

sodium phosphate buffer (pH 9.0) at 37 °C for 24 h. The egg shell membrane was then removed by centrifugation and the pH of the solution adjusted to 6.0 using 1 M HCl. The resulting aldehydes were reductively aminated with 3 mmol *p*-aminobenzoic acid (ABA) in the presence of 4.5 mmol sodium cyanoborohydride ($NaCNBH_3$) at 37 °C for 2 h with stirring. Then, ABA derivatives were hydrolyzed by 50 mL of 12 M HCl at 110 °C for 10 h. The hydrolysates were evaporated at 40 °C in vacuo to dryness. The resulting AAS-ABA and GGS-ABA were purified by using silica gel column chromatography and ethyl acetate/acetic acid/water (20:2:1, v/v/v) as the elution solvent.

Derivatization and Acid Hydrolysis of Oxidized Proteins. Oxidized proteins were prepared for LC analysis according to the procedure described by Akagawa et al. (*14*, *15*) with minor modifications as described in Estévez et al. (*6*). At sampling times, an aliquot (200 μL) of protein suspension was dispensed in 2 mL Eppendorf tubes. Proteins were precipitated with 2 mL of cold 10% TCA and subsequent centrifugation at 2000 rpm for 30 min. The resulting pellets were treated again with 2 mL of cold 5% TCA and proteins precipitated after centrifugation at 5000 rpm for 5 min. Pellets were then treated with 0.5 mL of 250 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer at pH 6.0 containing 1% sodium dodecyl sulfate (SDS) and 1 mM diethylenetriaminepentaacetic acid (DTPA), 0.5 mL of 50 mM ABA in 250 mM MES buffer at pH 6.0 and 0.25 mL of 100 mM $NaCNBH_3$ in 250 mM MES buffer at pH 6.0. The derivatization was completed by allowing the mixture to react for 90 min, while tubes were immersed in a water bath at 37 °C and stirred regularly. All solutions employed for the derivatization procedure were freshly made at sampling days. The derivatization reaction was stopped by adding 0.5 mL of cold 50% TCA followed by centrifugation at 5000 rpm for 5 min. Pellets were then washed twice with 1 mL of 10% TCA and 1 mL of ethanol-diethyl ether (1:1, v/v). Centrifugations at 5000 rpm for 5 min were performed after each washing step. Protein hydrolysis was performed at 110 °C for 18 h in the presence of 6 M HCl. Hydrolysates were finally dried in vacuo at 40 °C using a rotaevaporator. Hydrolysates were finally reconstituted with 200 μL of Milli-Q water and filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 μm pore size, Pall Corporation, USA) for LC analysis.

Detection of AAS-ABA and GGS-ABA by LC-ESI-MS. Derivatized semialdehydes were subjected to LC separation and MS analysis in accordance with Estévez et al. (*6*). Samples (2 μL) were injected into an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a Luna reversed-phase (RP) column (5 μm C_{18} II column, 150 × 1.00 mm i.d., Phenomenex Torrance, CA, USA) eluted at a flow rate of 50 μL/min with isocratic water/2.5% acetic acid (solvent A; 95%) and methanol/2.5% acetic acid (solvent B; 5%). The column was operated at a constant temperature of 30 °C. Standards (0.2 μL) were run and analyzed under the same conditions.

Mass spectrometric analysis was carried out in the positive ion mode on an Esquire-LC quadrupole ion trap mass spectrometer equipped with an ESI interface (Bruker Daltonics, Bremen, Germany) and LC-MSD Trap software, version 5.2 (Bruker Daltonics). MS^n methods were carried out for identification purposes and optimized for AAS-ABA and GGS-ABA. Capillary voltage was 3500 V, capillary exit offset 25 V, and skimmer potential 15 V, and the trap drive value was 36. Conventional ESI−MS data were recorded using a scan range of 100−700 *m*/*z*. Nebulizer (nitrogen) pressure was 50 psi, dry gas (nitrogen) flow 8 L/min, and dry temperature 300 °C. MS^2 and MS^3 were subsequently carried out for molecules of interest and recorded using helium (99.996%) as the collision gas. The peaks corresponding to the protonated AAS-ABA and GGS-ABA were manually integrated from selected ion chromatograms and the resulting areas used as arbitrary indicators of the abundance of both semialdehydes. Results are expressed as arbitrary area units (AU). The overall effect of added phenolics and α-tocopherol on the formation of semialdehydes was calculated as aforementioned for the total amount of protein carbonyls.

Data Analysis. All types of suspensions were made in duplicate in two independent experiments, and all analyses were made in triplicate (n = 6). Data obtained from instrumental analysis were used as variables and computed in an analysis of variance using SPSS for Windows ver. 6.1 in order to detect differences between protein suspensions for the formation of protein hydrazones and protein semialdehydes. Tukey's tests were performed as posthoc analysis in order to compare the means derived from suspensions with different added phenolics and oxidation promoters. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

The MS signal for AAS and GGS increased in protein suspensions throughout the oxidation assay (**Figure 2**). The present results suggest that lysine, proline, and/or arginine from myofibrillar proteins are oxidized in the presence of Cu^{2+}, Fe^{3+}, and Mb in combination with H_2O_2 to yield AAS and GGS. The curves obtained for the progress of the semialdehydes during the oxidation assay (**Figure 2A** and **B**) are fairly comparable with that obtained for the total amount of protein carbonyls using the DNPH method (**Figure 2C**). AAS and GGS have been highlighted as the main protein carbonyls in biological samples and comprise around 70% of total carbonyl compounds in BSA subjected to metal catalyzed oxidation (*7*). The results obtained in this study, including the high and significant correlations found between DNPH measurements and GGS (0.94; $p < 0.001$) and AAS (0.95; $p < 0.001$), support the fact that both semialdehydes could be highly representative of the total amount of carbonyl compounds formed during metal catalyzed oxidation of myofibrillar proteins. The consistency between the results from the DNPH method and the LC-MS data was not observed by Armenteros et al. (*5*) who observed an overestimation of the total carbonyl content by the DNPH method in meat products with high lipid content. The evolution of the protein carbonyls and both semialdehydes in protein suspensions is consistent with the trends observed in a previous study (*6*). The highest concentration of protein oxidation products in suspensions was found at day 15, while a noticeable decrease was observed after that point. The involvement of the semialdehydes in further reactions would explain the loss of semialdehydes by the end of the pro-oxidant storage. In fact, recent scientific data supports the Strecker-type reaction among the carbonyl group from AAS and GGS and free amines (*16*).

Copper, Iron, and Myoglobin as Promoters of Protein Carbonyl Formation. Copper (Cu^{2+}), iron (Fe^{3+}), and Mb displayed different abilities to promote the oxidation of myofibrillar proteins. Suspensions oxidized with Cu^{2+} had significantly higher amounts of AAS, GGS, and total protein carbonyls than suspensions oxidized with Mb or Fe^{3+} (**Figure 2**). Suspensions with added Mb had significantly higher amounts of GGS and total protein carbonyls than those oxidized in the presence of Fe^{3+}. In general, Cu^{2+} displayed the most intense pro-oxidant action toward the formation of carbonyl compounds from myofibrillar proteins followed by Mb and Fe^{3+}. Copper ions have been previously described as efficient initiators of protein oxidation in live tissues and food proteins (*15*, *17*, *18*). The pro-oxidant effect of Cu^{2+} on food proteins has been principally regarded in the promotion of tryptophan depletion and gain of fluorescent Schiff bases (*12*, *18*). According to the results from the present study, Cu^{2+} also plays a relevant role as promoter of protein carbonyl formation. Reactive-oxygen species (ROS) formed from the reaction among metal ions and oxygen and/or H_2O_2 would initiate the oxidative degradation of amino acids (**Figure 3**). The formation of AAS and GGS involves an eventual oxidative deamination of the amino acid residues in the presence of metal ions such as Cu^{2+} and Fe^{3+} (*19*). The resulting reduced forms of these metal ions, Cu^{+} and Fe^{2+}, respectively, could have reacted with H_2O_2 to generate hydroxyl radicals through the Fenton reaction. The hydroxyl radicals perpetuate the oxidative reactions by abstracting a susceptible hydrogen atom from a neighboring amino acid side chain (**Figure 3**). As a result, the combination of transition metals with H_2O_2 results in a highly effective pro-oxidant system for generating protein semialdehydes. According

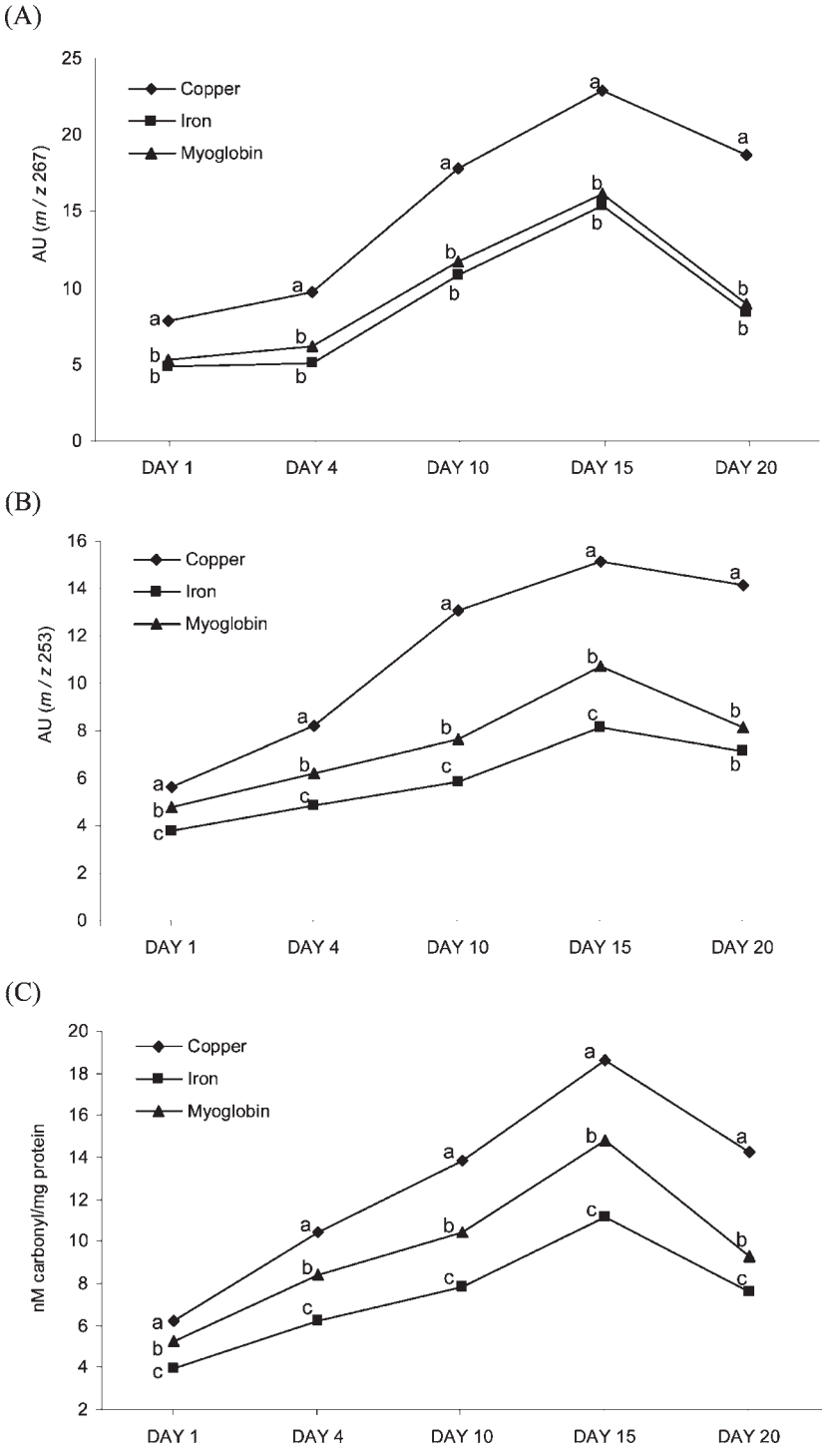

Figure 2. Progress of AAS (**A**), GGS (**B**), and total protein carbonyls (**C**) during in vitro oxidation of myofibrillar proteins using copper, iron, and myoglobin. Different letters (a−c) on the same day of the oxidation assay denote statistical differences between oxidation promoters ($P < 0.05$).

to the results obtained, copper ions are more efficient than their iron counterparts in promoting the formation of AAS and GGS. In contrast to iron, copper is able to bind to specific binding sites in proteins such as collagen, leading to enhanced pro-oxidant activity (*20*). Hawkins and Davies (*20*) ascribed to this mechanism the higher and more specific pro-oxidant effect of Cu^{2+}/H_2O_2 on collagen compared to that exerted by Fe^{2+}/H_2O_2. Knott et al. (*21*) referred to this mechanism to explain the pro-oxidant effect of copper ions toward lipids and proteins in low-density lipoproteins. As described for collagen and lipoproteins, it is plausible to consider that copper ions could have found binding sites in myofibrillar proteins from which the ions exerted an enhanced pro-oxidant action. Whereas this proposed mechanism requires further confirmation, it would explain the higher amounts of protein carbonyls in suspensions oxidized in the presence of Cu^{2+}/H_2O_2 compared to those oxidized by Fe^{2+}/H_2O_2. However, copper ions are minor components of muscles, and hence, the relative importance of these transition metals as oxidation promoters in meat products might not be as relevant as the role played by iron and Mb. In fact, heme and nonheme iron have been traditionally considered the main lipid oxidation promoters in meat systems (*22*). The pro-oxidant mechanism exerted by Mb in the present experiments may be different from those described for metal ions. In the presence of H_2O_2, Mb forms hypervalent

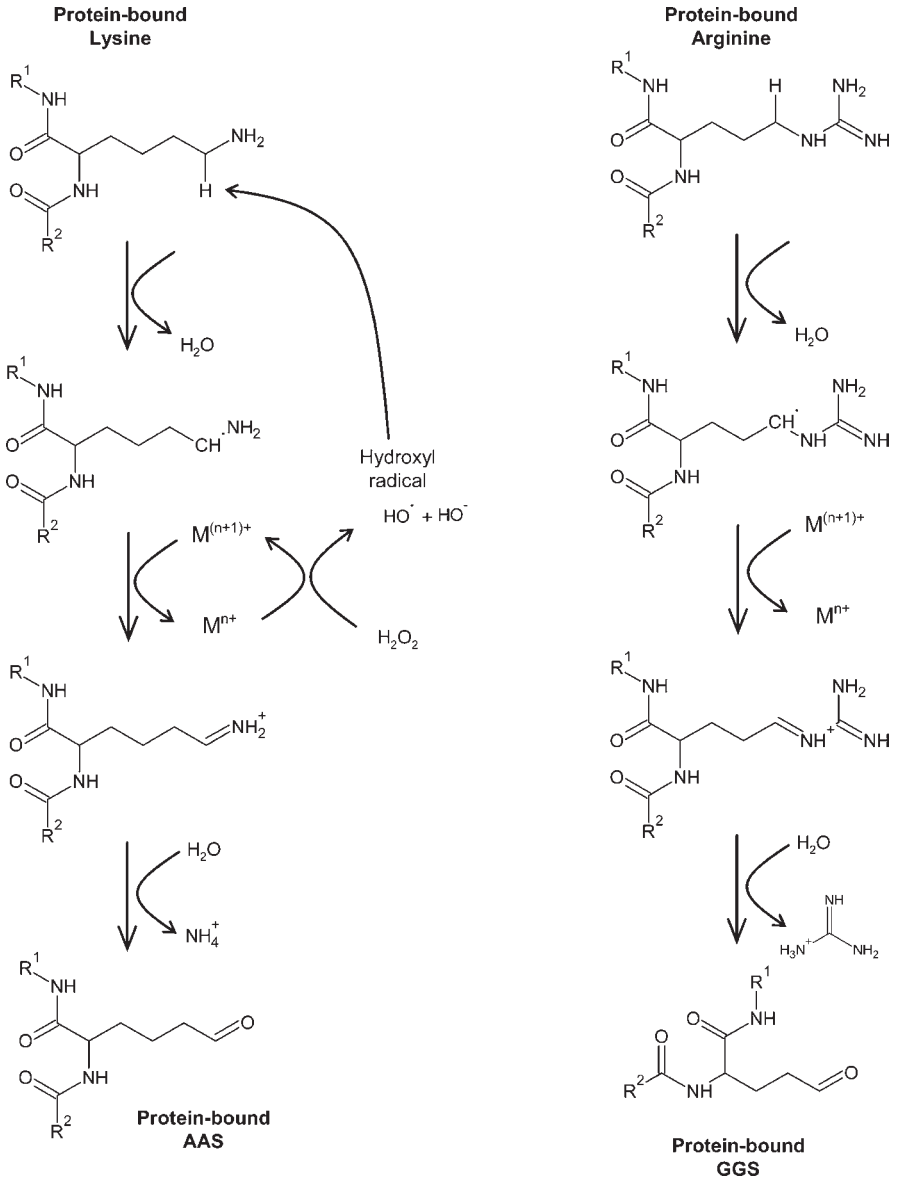

Figure 3. Formation of AAS and GGS from protein-bound lysine and arginine by metal ions (M) and hydrogen peroxide.

species such as ferrylmyoglobin (MbFe(IV)=O) which have been found to initiate lipid and protein oxidation (*23*−*25*). H_2O_2-activated Mb is known to promote the formation of disulfide and dityrosines cross-links (*24*, *25*). The present results demonstrate that this oxidation system is also involved in the formation of the specific protein carbonyls AAS and GGS from myofibrillar proteins. The pro-oxidant action exerted by Mb/H_2O_2 was more efficient than that displayed by Fe^{3+}/H_2O_2. This observation is consistent with that of authors who reported that the pro-oxidative activity of Mb in muscle lipids is higher than that of nonheme iron (*22*, *26*). However, these results contrast with those recently reported by Ganhão et al. (*27*) who studied the formation of AAS and GGS in cooked and chilled burger patties. These authors proposed that the intense formation of protein semialdehydes during chill storage of cooked patties was mainly caused by the nonheme iron released from the heme molecule during cooking. However, the effect displayed by iron and Mb in the present model systems might not directly reflect the relative contribution of each pro-oxidant factor toward protein oxidation in more complex muscle foods. Whereas the relative contribution of each iron form to protein oxidation in meat systems might remain indefinite, both heme and nonheme iron might be highly responsible for the formation of protein semialdehydes in meat systems.

Effect of Phenolic Compounds on the Formation of Protein Carbonyls. The effects of added phenolic compounds and α-tocopherol on the formation of AAS, GGS, and total protein carbonyls from myofibrillar proteins are shown in **Tables 1**, **2**, and **3**, respectively. Both antioxidant and pro-oxidant effects were observed with these effects being largely affected by the oxidizing agents, Cu^{2+}, Fe^{3+}, and Mb.

In suspensions oxidized by Cu^{2+}, α-tocopherol and most phenolic compounds clearly inhibited the formation of AAS (14−64%), GGS (14−75%), and the total protein carbonyls (13−46%) to different extents. In general, results from the DNPH method were in agreement with those obtained from the LC-MS analysis of protein semialdehydes. The antioxidant activity of plant phenolics is mainly derived from their ability to act as metal chelators and radical scavengers, and it is generally accepted that hydroxyl groups attached to phenolic rings play a major role in the antioxidant mechanism (*10*). Hence, plant phenolics could have inhibited the oxidative degradation of myofibrillar proteins in the present assay by hindering the pro-oxidant action of Cu^{2+} through its chelation. The above-mentioned mechanism could be

Table 1. Effect of Selected Phenolic Compounds and Tocopherol on the Formation of AAS at Day 15 of the Oxidation Assay (Mean ± Standard Deviation)[a]

	copper		iron		myoglobin	
	mean	SD	mean	SD	mean	SD
gallic acid	−17.22 f,z	3.66	−26.62 d,y	3.88	13.66 b,x	3.80
cyanidin-3-glucoside	28.56 c,x	6.34	20.03 c,y	3.32	−27.08 de,z	6.71
catechin	14.21 e,x	2.07	14.73 c,x	1.96	−33.37 e,y	5.14
chlorogenic acid	32.39 bc,x	4.50	−30.13 d,z	4.31	−20.15 d,y	3.38
genistein	22.77 d,x	4.10	−53.75 e,z	8.00	−10.58 c,y	2.20
rutin	35.45 b,x	3.59	27.12 b,y	6.29	−26.23 de,z	6.53
tocopherol	64.93 a,x	5.26	45.25 a,y	6.66	39.03 a,y	9.30

[a] Positive values denote antioxidant activity (percent inhibition against AAS formation), while negative values denote pro-oxidant activity (percent promotion of AAS formation). Different lowercase letters (a−f) within a column denote statistical differences between phenolics ($P < 0.05$). Different lowercase letters (x−z) within a row denote statistical differences between oxidation promoters ($P < 0.05$).

Table 2. Effect of Selected Phenolic Compounds and Tocopherol on the Formation of GGS at Day 15 of the Oxidation Assay (Mean ± Standard Deviation)[a]

	copper		iron		myoglobin	
	mean	SD	mean	SD	mean	SD
gallic acid	−11.51 f,y	2.20	−19.13 d,z	4.72	10.04 b,x	2.93
cyanidin-3-glucoside	21.32 d,x	3.71	22.28 c,x	4.32	−42.02 d,y	10.64
catechin	13.90 e,y	2.50	30.03 b,x	3.31	−42.63 d,z	7.52
chlorogenic acid	26.49 c,x	4.80	−32.90 e,z	6.48	−19.83 c,y	3.82
genistein	19.81 d,x	4.69	−65.69 f,z	5.82	−16.66 c,y	2.79
rutin	34.47 b,x	4.77	23.91 bc,y	3.71	−16.11 c,z	4.21
tocopherol	74.87 a,x	4.42	58.00 a,y	5.04	38.44 a,z	4.77

[a] Positive values denote antioxidant activity (percent inhibition against GGS formation), while negative values denote pro-oxidant activity (percent promotion of GGS formation). Different lowercase letters (a−f) within a column denote statistical differences between phenolics ($P < 0.05$). Different lowercase letters (x−z) within a row denote statistical differences between oxidation promoters ($P < 0.05$).

Table 3. Effect of Selected Phenolic Compounds and Tocopherol on the Formation of Total Protein Carbonyls at Day 15 of the Oxidation Assay (Mean ± Standard Deviation)[a]

	copper		iron		myoglobin	
	mean	SD	mean	SD	mean	SD
gallic acid	−10.71 d,y	2.06	−12.90 d,y	2.02	16.00 a,x	2.46
cyanidin-3-glucoside	14.09 c,y	2.41	23.14 b,x	2.48	−16.57 b,z	2.07
catechin	13.34 c,x	0.91	10.85 c,x	2.54	−31.99 d,y	4.08
chlorogenic acid	25.90 b,x	4.44	−22.39 e,y	5.12	−25.97 c,y	2.32
genistein	14.62 c,x	1.90	−41.47 f,z	8.35	−10.86 b,y	2.06
rutin	26.31 b,y	2.35	33.13 a,x	3.13	−14.13 b,z	4.69
tocopherol	45.67 a,x	4.56	34.74 a,y	3.13	19.28 a,z	2.89

[a] Positive values denote antioxidant activity (percent inhibition against protein carbonyl formation), while negative values denote pro-oxidant activity (percent promotion of protein carbonyl formation). Different lowercase letters (a−f) within a column denote statistical differences between phenolics ($P < 0.05$). Different lowercase letters (x−z) within a row denote statistical differences between oxidation promoters ($P < 0.05$).

also applied to phenolics which displayed antioxidant activity in suspensions with added Fe^{3+}. In addition, plant phenolics could have contributed to protect myofibrillar proteins by scavenging ROS eventually derived from the Fenton reaction between metal ions and H_2O_2. Among phenolic compounds, rutin and chlorogenic acid displayed the highest percent inhibition against the formation of total protein carbonyls, and similar results were obtained for the semialdehydes. Cyanidin-3-glucoside and genistein displayed clear inhibitory effects against the formation of protein semialdehydes, whereas catechin showed in general significantly lower percent inhibitions. In suspensions oxidized by Cu^{2+} /H_2O_2, gallic acid displayed an overall pro-oxidant effect. These results contrast with those obtained in a previous paper devoted to evaluating the effect of similar plant phenolics against the oxidation of myofibrillar proteins in oil-in-water emulsions (*11*). In that study, cyanidin-3-glucoside, gallic acid, and genistein inhibited lipid and protein oxidation to a higher extent than the other tested phenolics. The variation between both studies in terms of the model system employed (oil-in-water emulsion vs protein suspension), oxidation conditions (presence vs absence of ascorbate), and protein oxidation measurements (fluorescent Schiff bases vs specific protein carbonyls) would explain the lack of consistency between the results. This observation reflects the complexity of the chemistry behind the antioxidant action of plant phenolics. In fact, the overall effect displayed by a phenolic compound in food and biological systems is known to be affected by a variety of factors including the composition and characteristics of the substrate, the stage and intensity of the oxidative reactions, and the localization of the phenolics (*28*).

As mentioned before, the effect of plant phenolics was largely affected by the oxidizing agent added to protein suspensions. The effect of certain phenolic compounds shifted from overall antioxidant to overall pro-oxidant when added to suspensions oxidized in the presence of Fe^{3+} and Mb. Whereas chlorogenic acid and genistein showed inhibitory effects against carbonyl formation in suspensions with added Cu^{2+}, these compounds exerted pro-oxidant actions in suspensions with added Fe^{3+}. In this system, gallic acid, chlorogenic acid, and genistein promoted the formation of AAS (27−54%), GGS (19−66%), and total protein carbonyls (13−41%). In suspensions oxidized in the presence of Mb, all phenolic compounds displayed overall pro-oxidant effects. Previous studies carried out by Akagawa and Suyama (*29*) and us (*11*) reported pro-oxidant actions for some of the aforementioned compounds. Whereas the pro-oxidant effect of phenolics has been profusely documented, the exact mechanisms are still to be fully clarified. Recently, Akagawa et al. (*30*) reported that phenolics undergo an auto-oxidation process in the presence of transition metals leading to the formation of the corresponding quinones which are able to promote lipid and protein oxidation. Furthermore, the same authors highlighted that those quinone structures are cofactors in several lysyl-oxidase enzymes and themselves might be able to display amine-oxidase activities. According to this proposal, quinone forms of plant phenolics could catalyze the oxidative deamination of susceptible amino acids to form the corresponding semialdehydes. In the present experiments, the conversion of certain phenolic compounds into their equivalent quinone forms could have triggered their ability to exert the oxidative degradation of lysine, proline, and arginine side chains and, hence, promote the formation of AAS and GGS. This likely mechanism is depicted in **Figure 4** and proposed as a plausible hypothesis. The activation of the pro-oxidant potential of plant phenolics by transition metals, described as metal-initiated pro-oxidant activity, requires the preceding formation on the quinone form and depends upon a number of factors including the chemical structure of the plant phenolics and the ability of the metal ions to induce such chemical conversion (*29*, *30*). Both factors could have been influential in the present experiments. Supporting the results obtained in

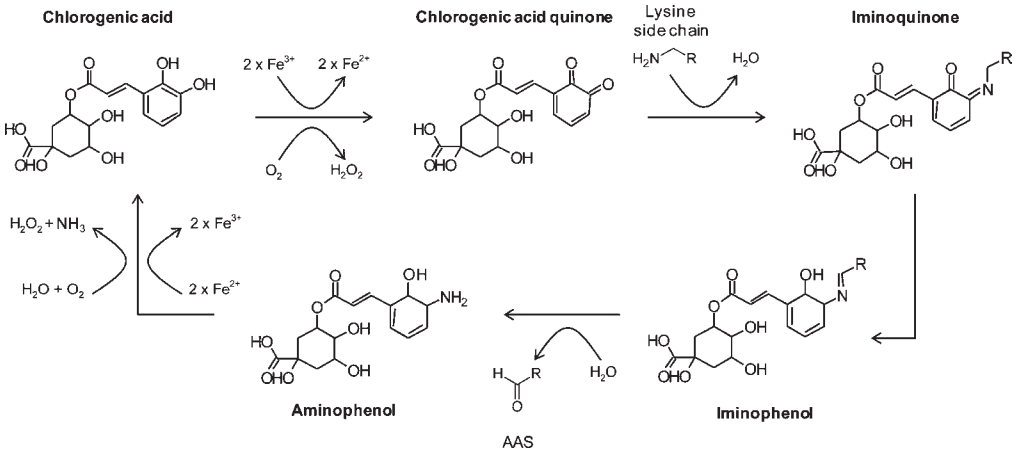

Figure 4. Metal-initiated pro-oxidant action of phenolic compounds on a lysine residue for the formation of AAS through the amine oxidase-like activity (for illustration purposes, the auto-oxidation of chlorogenic acid in the presence of Fe^{3+} is proposed).

suspensions with added Fe^{3+}, Rietjens et al. (*31*) reported that phenolics with a monohydroxy configuration in the B-ring (i.e., genistein) are more prone to undergo auto-oxidation than the 3′-4′ dihydroxy configuration (found in catechin and cyanidin-3-glucoside) and that the resulting quinone structures show particularly intense pro-oxidant potential. Additionally, certain oxidizing agents, namely, Fe^{3+} and Mb, could have been more active than Cu^{2+} at converting the antioxidant hydroxyl forms into the corresponding pro-oxidant quinone structures as several phenolics displayed overall antioxidant effects in Cu^{2+}-oxidized suspensions and overall pro-oxidant effects in suspensions oxidized by Fe^{3+} and Mb. In this sense, H_2O_2-activated Mb is known to be able to oxidize a large variety of flavonoids into their corresponding quinone forms, and rutin is particularly sensitive to this reaction (*32*). This is in good agreement with results obtained in the present study as rutin only displayed an overall pro-oxidant effect in suspensions oxidized by Mb/H_2O_2.

It is plausible that phenolic compounds concurrently display both antioxidant and pro-oxidant actions as these compounds might be present in protein suspensions in both hydroxyl and quinone forms. The overall effect displayed by plant phenolics might be the result of the balance between both actions which is dependent on the activation effect of transition metals and Mb and their own susceptibility to undergo auto-oxidation. Like this, overall antioxidant effects displayed by certain phenolic compounds such as genistein or chlorogenic acid in suspensions with added Cu^{2+} would reflect that most phenolic molecules were in the hydroxyl form which exerted antioxidant actions. The conversion of the aforementioned phenolics into the corresponding quinone forms would explain the overall pro-oxidant effects displayed by genistein and chlorogenic acid in suspensions with added Fe^{3+} and Mb, whereas further experiments would confirm the precise mechanism.

In all types of suspensions, α-tocopherol displayed the most intense antioxidant activity against protein carbonyl gain which is in agreement with results from previous studies (*11*, *33*). Viljanen et al. (*33*) reported that α-tocopherol inhibited lipid and protein oxidation in whey protein emulsions to a higher extent than berry phenolics. Similarly, α-tocopherol was more efficient than selected phenolic compounds at inhibiting tryptophan depletion in oxidized myofibrillar proteins (*11*). Apparently, the antioxidant action of α-tocopherol was also affected by the addition of different oxidation promoters with this effect being similar to that displayed by plant phenolics. The addition of Fe^{3+} and Mb diminished the effect of α-tocopherol, although this compound never displayed pro-oxidant effects.

In summary, the present results highlight the complexity of the mechanisms involved in the interactions between myofibrillar proteins, plant phenolics, and oxidizing agents. The consistency between the results obtained by the DNPH and the LC-MS methods highlight the convenient usage of the former for assessing the overall oxidation status of the proteins in a particular food, whereas the LC-MS method should be applied whenever a more in-depth study of the protein oxidation mechanisms is required. Phenolic compounds can display, as redox-active compounds, antioxidant and pro-oxidant effects on myofibrillar proteins. Copper, iron, and myoglobin could promote the formation of AAS and GGS from myofibrillar proteins by exerting, themselves, a variety of pro-oxidant actions and by activating the pro-oxidant activity of phenolic compounds. The usage of phenolic compounds and phenolic-rich materials as functional ingredients in muscle foods should be administrated carefully since these substances could contribute pro-oxidant effects with likely unpleasant consequences in meat quality and the consumer's health.

ACKNOWLEDGMENT

We acknowledge the technical support from Miikka Olin, Kirsti Risunen, and Maija Ylinen.

LITERATURE CITED

(1) Estévez, M.; Ventanas, S.; Cava, R. Protein oxidation in frankfurters with increasing levels of added rosemary essential oil: effect on colour and texture deterioration. *J. Food Sci.* **2005**, *70*, 427–432.

(2) Ventanas, S.; Estévez, M.; Tejeda, J. F.; Ruiz, J. Protein and lipid oxidation in *longissimus dorsi* and dry cured loin from Iberian pigs as affected by crossbreeding and diet. *Meat Sci.* **2006**, *72*, 647–655.

(3) Ventanas, S.; Ventanas, J.; Tovar, J.; García, C.; Estévez, M. Extensive feeding versus oleic acid and tocopherol enriched mixed diets for the production of Iberian dry-cured hams: effect on chemical composition, oxidative status and sensory traits. *Meat Sci.* **2007**, *77*, 246–256.

(4) Lund, M. N.; Lametsch, R.; Hviid, M. S.; Jensen, O. N.; Skibsted, L. H. High-oxygen packaging atmosphere influences protein oxidation and tenderness of porcine longissimus dorsi during chill storage. *Meat Sci.* **2007**, *77*, 295–303.

(5) Armenteros, M.; Heinonen, M.; Ollilainen, V.; Toldrá, F.; Estévez, M. Analysis of protein carbonyls in meat products by using the DNPH method, fluorescence spectroscopy and liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). *Meat Sci.* **2009**, *83*, 104–112.

(6) Estévez, M.; Ollilainen, V.; Heinonen, M. Analysis of protein oxidation markers - α-aminoadipic and γ-glutamic semialdehydes - in

food proteins by using LC-ESI-multi-stage tandem MS. *J. Agric. Food Chem.* **2009**, *57*, 3901–3910.
(7) Requena, J. R.; Chao, C.-C.; Levine, R. L.; Stadtman, E. R. Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proc. Nat. Acad. Sci. U.S.A.* **2001**, *98*, 69–74.
(8) Akagawa, K.; Sasaki, D.; Kurota, Y.; Suyama, K. Formation of α-aminoadipic and γ-glutamic semialdehydes in proteins by the Maillard reaction. *Ann. N.Y. Acad. Sci.* **2005**, *1043*, 129–134.
(9) Love, J. D.; Pearson, A. M. Metmyoglobin and nonheme iron as prooxidants in cooked meat. *J. Agric. Food Chem.* **1974**, *22*, 1032–1034.
(10) Bravo, L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, *56*, 317–333.
(11) Estévez, M.; Kylli, P.; Puolanne, E.; Kivikari, R.; Heinonen, M. Oxidation of skeletal muscle myofibrillar proteins in oil-in-water emulsions: interaction with lipids and effect of selected phenolic compounds. *J. Agric. Food Chem.* **2008**, *56*, 10933–10940.
(12) Estévez, M.; Kylli, P.; Puolanne, E.; Kivikari, R.; Heinonen, M. Fluorescence spectroscopy as a novel approach for the assessment of myofibrillar protein oxidation in oil-in-water emulsions. *Meat Sci.* **2008**, *80*, 1290–1296.
(13) Butterfield, D. A.; Stadtman, E. R. Protein oxidation processes in aging brain. *Adv. Cell Aging Gerontol.* **1997**, *2*, 161–191.
(14) Akagawa, M.; Sasaki, D.; Ishii, Y.; Kurota, Y.; Yotsu-Yamashita, M.; Uchida, K.; Suyuma, K. New methods for the quantitative determination of mayor protein carbonyls, α-aminoadipic and γ-glutamic semialdehydes: Investigation of the formation mechanism and chemical nature in vitro and in vivo. *Chem. Res. Toxicol.* **2006**, *19*, 1059–1065.
(15) Akagawa, M.; Sasaki, T.; Suyama, K. Oxidative deamination of lysine residue in plasma protein of diabetic rats. *Eur. J. Biochem.* **2002**, *269*, 5451–5458.
(16) Estévez, M.; Ventanas, S.; Heinonen, M. Headspace volatiles in oxidized food proteins. *Proc. 55th Int. Con. Meat Sci. Technol.* **2009**, 347–35.
(17) Giessauf, A.; Steiner, E.; Esterbauer, H. Early destruction of tryptophan residues of apolipoprotein B is a vitamin E independent process during copper-mediated oxidation of LDL. *Biochim. Biophys. Acta* **1995**, *1256*, 221–232.
(18) Viljanen, K.; Kivikari, R.; Heinonen, M. Protein-lipid interactions during liposome oxidation with added anthocyanin and other phenolic compounds. *J. Agric. Food Chem.* **2004**, *52*, 1104–1111.
(19) Stadtman, E. R. Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radical Biol. Med.* **1990**, *9*, 315–325.
(20) Hawkins, C. L.; Davies, M. J. Oxidative damage to collagen and related substrates by metal ion/hydrogen peroxide systems: random attack or site-specific damage? *Biochim. Biophys. Acta* **1997**, *1360*, 84–96.
(21) Knott, H. M.; Baoutina, A.; Davies, M. J.; Dean, R. T. Comparative time-courses of copper-ion-mediated protein and lipid oxidation in low density lipoprotein. *ABB* **2002**, *400*, 223–232.
(22) Monahan, F. J.; Crackel, R. L.; Gray, J. I.; Buckley, D. J.; Morrissey, P. A. Catalysis of lipid oxidation in muscle model systems by haem and inorganic iron. *Meat Sci.* **1993**, *34*, 95–106.
(23) Baron, C. P.; Andersen, H. J. Myoglobin-induced lipid oxidation. A review. *J. Agric. Food Chem.* **2002**, *50*, 3887–3897.
(24) Frederiksen, A. M.; Lund, M. N.; Andersen, M. L.; Skibsted, L. H. Oxidation of porcine myosin by hypervalent myoglobin: the role of thiol groups. *J. Agric. Food Chem.* **2008**, *56*, 3297–3304.
(25) Ostdal, H.; Davies, M. J.; Andersen, H. J. Reaction between protein radicals and other biomolecules. *Free Radical Biol. Med.* **2002**, *33*, 201–209.
(26) Johns, A. M.; Birkinshaw, L. H.; Ledward, D. A. Catalysts of lipid oxidation in meat-products. *Meat Sci.* **1989**, *25*, 209–220.
(27) Ganhão, R.; Morcuende, D.; Estévez, M. Tryptophan depletion and formation of α-aminoadipic and γ-glutamic semialdehydes in porcine burger patties with added phenolic-rich fruit extracts. *J. Agric. Food Chem.* [Online early access]. DOI: 10.1021/jf903356m. Published Online: Feb 19, **2010**.
(28) Frankel, E. N.; Meyer, A. S. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants: review. *J. Sci. Food Agric.* **2000**, *80*, 1925–1941.
(29) Akagawa, M.; Suyama, K. Aminooxidase-like activity of polyphenols. Mechanism and properties. *Eur. J. Biochem.* **2001**, *268*, 1953–1963.
(30) Akagawa, M.; Shigemitsu, T.; Suyama, K. Oxidative deamination of benzylamine and lysine residue in bovine serum albumin by green tea, black tea, and coffee. *J. Agric. Food Chem.* **2005**, *53*, 8019–8024.
(31) Rietjens, I.; Boersma, M.; de Haan, L.; Spenkelink, B.; Awad, H.; Cnubben, N.; van Zanden, J. J.; van der Woude, H; Alink, G. M.; Koeman, J. H. The pro-oxidant chemistry of the natural antioxidants vitamin C, vitamin E, carotenoids and flavonoids. *Environ. Toxicol. Pharmacol.* **2002**, *11*, 321–333.
(32) Jorgensen, L. V.; Skibsted, L. H. Flavonoid deactivation of ferrylmyoglobin in relation to ease of oxidation as determined by cyclic voltammetry. *Free Radical Res.* **1998**, *28*, 335–351.
(33) Viljanen, K.; Kylli, P.; Hubbermann, E-.M.; Schwarz, K.; Heinonen, M. Anthocyanin antioxidant activity and partition behavior in whey protein activity. *J. Agric. Food Chem.* **2005**, *53*, 2022–2027.

Received for review October 27, 2009. Revised manuscript received February 11, 2010. Accepted February 20, 2010. M.E. thanks the European Community for the economical support from the Marie Curie Intra-European Fellowship.