Tryptophan Depletion and Formation of α-Aminoadipic and γ-Glutamic Semialdehydes in Porcine Burger Patties with Added Phenolic-Rich Fruit Extracts

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The effect of added fruit extracts on the oxidation of muscle proteins in porcine burger patties subjected to cooking and chill storage was studied. Extracts from arbutus berries (*Arbutus unedo* L., AU), common hawthorns (*Crataegus monogyna* L., CM), dog roses (*Rosa canina* L., RC), and elm-leaf blackberries (*Rubus ulmifolius* Schott, RU) were prepared, characterized, added to burger patties (3% of total weight), and evaluated as inhibitors of protein oxidation. Negative (no added extract, C) and positive control (added quercetin, 230 mg/kg, Q) groups were also included in the design. Protein oxidation was assessed by means of tryptophan loss using fluorescence spectroscopy (FS) and formation of the specific protein carbonyls α-aminoadipic (AAS) and γ-glutamic semialdehyde (GGS) using liquid chromatography and mass spectroscopy (LC−MS). Both advanced methodologies (FS and LC−MS) were found to be reliable and specific protein oxidation measurements that allow us to gain chemical insight into protein oxidation. The mechanisms likely involved in the oxidative reactions affecting proteins during cooking and storage of burger patties are profusely discussed. Phenolic-rich fruit extracts protected tryptophan residues against oxidation and inhibited the formation of both semialdehydes in burger patties during cooking and subsequent chill storage. In general, RC, RU, and AU were the most effective inhibitors of protein oxidation, with this effect being more intense than that of pure polyphenols like quercetin. These fruit extracts could be considered functional ingredients as their antioxidant actions contribute to the enhancement of the nutritional value of the meat products.

KEYWORDS: Protein oxidation; burger patties; protein carbonyls; tryptophan fluorescence; fruit extracts; phenolic compounds; antioxidant action

INTRODUCTION

The occurrence of oxidative reactions in muscle foods is a major cause of food deterioration because it leads to an undesirable loss of sensory and nutritional value (*1*). Whereas the factors, mechanisms, and consequences of lipid oxidation with respect to meat quality have been profusely studied and clearly established, numerous aspects regarding the oxidation of muscle proteins require further clarification. Amino acids are oxidized in the presence of reactive oxygen species (ROS), leading to the formation of a large variety of products, including carbonyl compounds, cross-links, and other side chain modifications (*2*). Recent studies have investigated the susceptibility of myofibrillar proteins to oxidation and the effect of oxidizing lipids and selected phenolic compounds on the oxidative degradation of muscle proteins (*3−6*). The technological properties of myofibrillar proteins as well as particular meat quality traits such as color and texture are seriously affected as a result of the oxidative reactions (*7−9*). Protein oxidation has been widely assessed in muscle foods by measuring carbonyl formation using the dinitrophenylhydrazine (DNPH) method [cf. reviewed by Estévez et al. (*10*)]. However, the aforementioned method provides a general oxidation index, while the specific structures and formation pathways of carbonyl compounds in oxidized myofibrillar proteins remain unknown. Lately, α-aminoadipic and γ-glutamic semialdehydes (AAS and GGS, respectively) have been originally detected in oxidized myofibrils and other food proteins using liquid chromatography and mass spectrometry (LC−MS) (**Figure 1**) (*11*). These carbonyl compounds were highlighted as specific and reliable protein oxidation indicators, although they have not yet been used as such in real meat products. Another innovative technique, namely fluorescence spectroscopy (FS), has been successfully employed for assessing the loss of tryptophan and the formation of Schiff bases in model systems and meat products (*3*, *12*).

The oxidative degradation of myofibrillar proteins could be inhibited by using antioxidants. Plant polyphenols have become highly popular among food chemists because their mechanisms of action go beyond the inhibition of oxidative reactions. Numerous studies strongly support a role for polyphenols in the prevention

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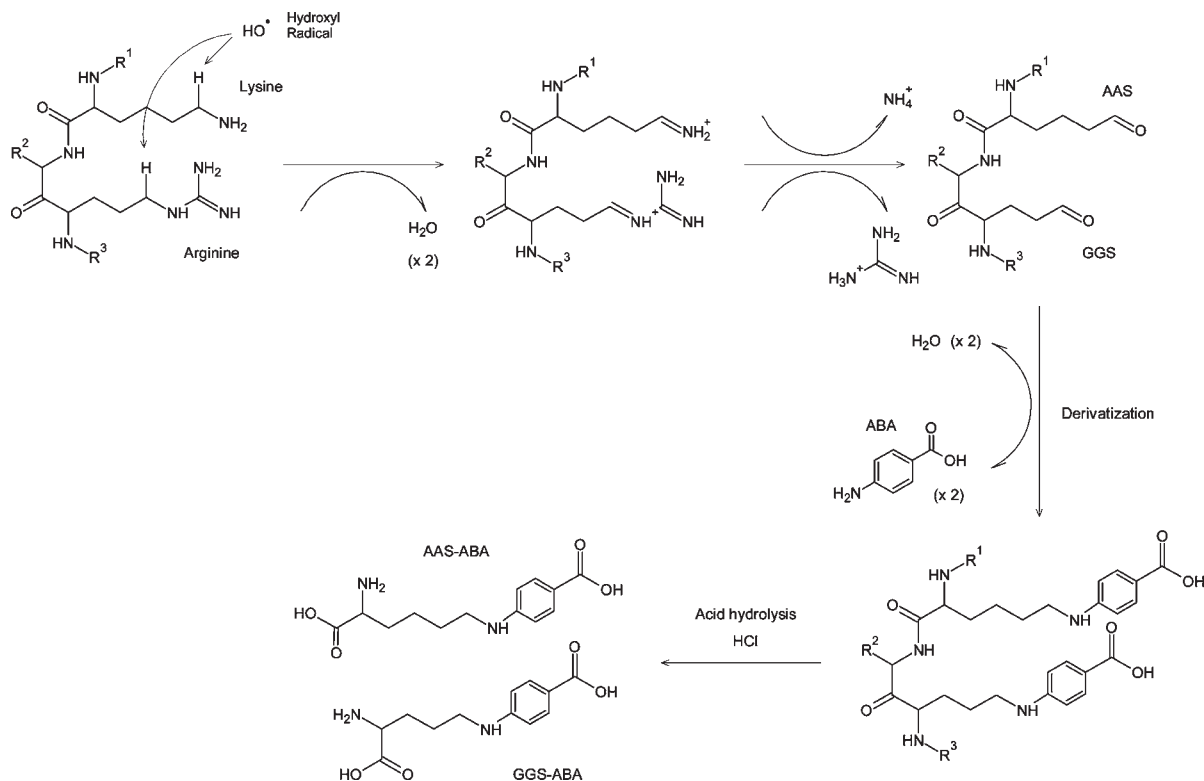*To whom correspondence should be addressed. E-mail: mariovet@unex.es.

Figure 1. Formation of α-aminoadipic (AAS) and γ-glutamic acid semialdehydes (GGS) and subsequent derivatization with *p*-aminobenzoic acid (ABA) for LC−MS analysis.

of degenerative diseases, particularly cardiovascular diseases and cancers [cf. reviewed by Scalbert et al. (*13*)]. A variety of plant materials and phenolic-enriched extracts have been reported to be effective at inhibiting tryptophan degradation and the formation of protein carbonyls in meat products (*7, 14, 15*). Other studies, which aimed to shed light on the chemical insight of the complex interaction mechanisms between proteins and plant phenolics, reported that certain plant phenolics display antioxidant or pro-oxidant effects depending on their structure, their concentration, and other environmental conditions (*4, 16, 17*). The Mediterranean forest from southwestern Spain offers a large variety of wild fruits and berries such as arbutus berries (*Arbutus unedo* L., AU), common hawthorns (*Crataegus monogyna* L., CM), the dog rose (*Rosa canina* L., RC), and elm-leaf blackberries (*Rubus ulmifolius* Schott, RU). Whereas these fruits are popular in traditional cuisine and folk medicine, scientific information about their antioxidant potential is scarce. According to some preliminary studies (*18*), these fruits contain large amounts of polyphenols and show intense antioxidant activity against DPPH and ABTS radicals. However, the effect of these fruits or their extracts on the oxidative stability of food proteins as well as the suitability of using these fruits as functional ingredients in meat products is unknown. This study aims to investigate the effect of selected fruit extracts on the oxidation of proteins in porcine burger patties by means of tryptophan loss using FS and formation of AAS and GGS using LC−MS. The routine DNPH method is also applied for comparative purposes.

MATERIALS AND METHODS

Chemicals. All chemicals and reagents used for this work were purchased from Panreac (Panreac Química, S. A., Barcelona, Spain), Merck (Merk, Darmstadt, Germany), and Sigma Chemicals (Sigma-Aldrich, Steinheim, Germany).

Fruits. Samples of strawberry tree (*Arbutus unedo* L.), common haw-thorn (*Crataegus monogyna* L.), dog rose (*Rosa canina* L.), and elm-leaf

blackberry (*Rubus ulmifolius* Schott) cultivars were collected from the Cáceres region of Spain (altitude of 450 m) during the summer and autumn of 2007. After being harvested by hand, the samples were immediately transferred to the laboratory, cleaned, and sorted to eliminate damaged and shrivelled fruits and processed for the extraction of phenolic compounds.

Extraction of Fruit Phenolics. Fruits (30 g), including peel and pulp, were cut into pieces, while the seeds were carefully removed. Fruit was ground using a domestic electric mill, dispensed in a falcon tube, and homogenized with 10 volumes (w/v) of absolute ethanol using an Ultra-Turrax homogenizer. The homogenates were centrifuged at 2600*g* for 10 min at 6 °C. The supernatants were collected, and the residue was re-extracted once more following the procedure previously described. The two supernatants were combined, evaporated using a rotoevaporator, and redissolved using 250 g of distilled water. Water solutions from each fruit were prepared twice and refrigerated until they were used for the manufacture of each of the two sets of burger patties as detailed below (< 24 h). No insoluble fragments or residues were observed in the water solutions.

Total Phenolic Content and Antioxidant Activity of Fruit Extracts. The total phenolic content (TPC) of fruit extracts was determined following the Folin-Ciocalteu method (*19*) with minor modifications as described by Ganhão et al. (*18*). Briefly, an aliquot of extract and another aliquot of water (total volume of 0.5 mL) were mixed with 2.5 mL of 1:10 diluted Folin-Ciocalteu's phenol reagent, followed by 2 mL of 7.5% (w/v) sodium carbonate. The mixture was shaken and allowed to stand for 60 min at room temperature in the dark. The absorbance of the resulting bluish color was measured at 740 nm using a spectrophotometer. Phenolic content was estimated from a standard curve of gallic acid, and results are expressed as milligrams of gallic acid equivalents (GAE) per 100 g of fresh fruit.

The antioxidant activity of fruit extracts was evaluated by using the 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) assay (*20*). Briefly, aliquots of each fruit extract at different concentrations were mixed with 1950 μL of a DPPH solution (6×10^{-5} M) in methanol. The reaction mixture was stirred and left to stand at room temperature in the dark for 90 min. The decrease in absorbance was measured at 517 nm using methanol as a blank. A calibration curve at 517 nm was plotted with DPPH to calculate

Article

J. Agric. Food Chem., Vol. 58, No. 6, 2010 **3543**

the remaining DPPH concentration in the reaction environments. The antioxidant activity of fruit extracts against the DPPH radical is expressed as the amount of fruit necessary to decrease the initial DPPH concentration by 50% (EC_{50}).

The activity of fruit extracts against the 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical (*21*) was calculated as Trolox equivalent antioxidant capacity (TEAC) as follows. The ABTS radical cation ($ABTS^{\bullet+}$) solution was generated by the reaction of 7 mM ABTS and 2.45 mM potassium persulfate (in equal quantities), after incubation at room temperature in the dark for 15 h. The freshly made $ABTS^{\bullet+}$ solution was then diluted with ethanol to yield an absorbance of 0.70 ± 0.04 at 734 nm. A 10 μL aliquot of the fruit extracts was added to 1 mL of the $ABTS^{\bullet+}$ solution and mixed thoroughly. The reaction mixture was allowed to stand at room temperature in the dark for 6 min, and the absorbance at 734 nm was immediately recorded. A standard curve was obtained by using a Trolox standard solution at various concentrations (ranging from 0 to 2.0 mM) in 80% ethanol. Results were expressed as micromolar Trolox equivalents per gram of fruit.

Manufacture of Porcine Burgers. Six types of porcine burger patties were prepared depending on the addition of different fruit extracts (AU, CM, RC, and RU), including negative (no added extract, C) and positive control (added quercetin, 230 mg/kg, Q) groups. The concentration of quercetin is equivalent in GAE to the average of TPC of the fruit extracts employed for the manufacture of the burger patties. In the basic formulation, the ingredients per kilogram of patty were as follows: 725 g of meat (porcine *longissimus dorsi* muscle), 250 g of distilled water, and 25 g of sodium chloride. In the formulation of the treated patties, the 250 g of distilled water was replaced with 250 g of a water solution containing the corresponding fruit extracts or the quercetin. All ingredients were minced in a cutter until a homogeneous raw batter was obtained. Twelve burger patties per batch were prepared in two independent manufacturing processes (eight patties per batch each time). Burger patties were shaped using a conventional burger maker (100 g/patty), to give average dimensions of 10 cm (diameter) and 1 cm (thickness). From the total of 12 patties per batch, four of them (raw patties) were set aside and eventually frozen (−80 °C) until they were required for analyses (<2 weeks) while the other eight were subjected to a cooking procedure. Preliminary cooking trials were performed to establish the cooking conditions required to achieve a meat core temperature of 73 °C. Patties were placed on trays and cooked at 170 °C for 18 min in a forced-air oven. Four cooked patties per batch were set aside and eventually frozen (−80 °C) until they were required for analyses, while the remaining four were subsequently stored for 12 days at 2 °C in a refrigerator under white fluorescent light (620 lx), simulating retail display conditions. Subsequent to the refrigerated storage, cooked and chilled samples were also frozen (−80 °C) until they were required for analyses. Samples corresponding to raw, cooked, and cooked and chilled burgers (four patties per batch and processing treatment) were analyzed for tryptophan fluorescence and carbonyl gain.

Tryptophan Fluorescence Measurements. The natural fluorescence of tryptophan was assessed by using fluorescence spectroscopy (*3*). Burger patties (1 g) were ground and homogenized in a 1:10 (w/v) ratio in 10 mL of 20 mM sodium phosphate buffer (pH 6.5) containing 0.6 M NaCl using an Ultra-Turrax homogenizer for 30 s. The water homogenates were filtered through gauze to remove insoluble extract which was then dispensed in a 4 mL quartz spectrofluorometer cell. Emission spectra of tryptophan were recorded from 300 to 500 nm with the excitation wavelength established at 283 nm (LS 55 Perkin-Elmer luminescence spectrometer). The excitation and emission slit widths were set to 10 nm, whereas data were collected at 500 nm/min. The results obtained were expressed as fluorescence intensity units emitted by protein oxidation products at 360 nm. These values were corrected according to the protein content of burgers via multiplication by a correction factor ($C_f = P_t/P_p$, where P_t is the total average of the protein contents from all burgers and P_p is the mean of the protein content from each burger). The percent inhibition of fruit extracts against loss of tryptophan was calculated as follows: $100 - [100(T_1 - T_{12})/(C_1 - C_{12})]$, where *T* and *C* are the fluorescence values of the treated and control samples, respectively, and subscripts represents raw (1) and cooked and chilled samples (12).

Determination of Total Carbonyl Content by the DNPH Method. Protein oxidation, as measured by the total carbonyl content, was evaluated by derivatization with DNPH according to the method described

by Oliver et al. (*22*) with slight modifications. Burger patties (1 g) were minced and then homogenized in a 1:10 (w/v) ratio with 10 mL of 0.6 M NaCl and 20 mM sodium phosphate buffer (pH 6.5) using an Ultra-Turrax homogenizer for 30 s. The homogenates were divided in two equal aliquots of 0.2 M and dispensed in 2 mL Eppendorf tubes. Proteins were precipitated with cold 10% trichloroacetic acid (TCA) (1 mL) and subsequent centrifugation for 5 min at 4200*g*. 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 with 10% TCA (1 mL) and washed three times with 1 mL of an ethanol/ethyl acetate mixture (1:1, v/v) to remove excess DNPH. The pellets were then dissolved in 1.5 mL of 6 M guanidine hydrochloride in 20 mM sodium phosphate buffer (pH 6.5), stirred, and centrifuged for 2 min at 4200*g* to remove insoluble fragments. The protein concentration was calculated from absorption at 280 nm using BSA as a standard. The amount of carbonyls was expressed as nanomoles of carbonyl per milligram of protein using an absorption coefficient of 21.0 nM^{-1} cm^{-1} at 370 nm for protein hydrazones. The percent inhibition of fruit extracts against carbonyl gain was calculated as follows: $100 - [100(T_{12} - T_1)/(C_{12} - C_1)]$, where *T* and *C* are the carbonyl contents of the treated and control samples, respectively, and subscripts represent raw (1) and cooked and chilled samples (12).

Analysis of AAS and GGS by LC−ESI-MS. Standard AAS and GGS were synthesized in vitro from *N*α-acetyl-L-lysine and *N*α-acetyl-L-ornithine using lysyl oxidase activity from egg shell membranes following the procedure described by Akagawa et al. (*23*). AAS and GGS were analyzed in meat products following a derivatization procedure (**Figure 1**) and using a LC−ESI-MS technique described by Akagawa et al. (*23*) and Estévez et al. (*11*), respectively. Meat samples were cut, minced, and subsequently homogenized in a 1:10 (w/v) ratio in 10 mM phosphate buffer containing 0.6 M NaCl using an Ultra-Turrax homogenizer for 30 s. Aliquots (150 μL) were dispensed in Eppendorf tubes, precipitated with 2 mL of 10% TCA, and centrifuged at 670*g* for 30 min. The supernatants were removed and the resulting pellets treated with 2 mL of 5% TCA and subsequently centrifuged at 4200*g* for 5 min. Then, protein carbonyls were derivatized with 500 μL of 250 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer containing 1% sodium dodecyl sulfate (SDS) and 1 mM diethylenetriaminepentaacetic acid (DTPA), 500 μL of 250 mM MES buffer containing 50 mM *p*-aminobenzoic acid (ABA), and 250 mM MES buffer containing 100 mM sodium cyanoborohydride ($NaCNBH_3$). The mixture was incubated at 37 °C for 1 h while immersed in a water bath and stirred regularly. Afterward, the protein was again precipitated by the addition of 500 μL of 50% TCA and centrifuged for 10 min at 16770*g*. The supernatants were removed and the pellets precipitated with 1 mL of 10% TCA and then washed twice with 1 mL of a 1:1 (v/v) ethanol/diethyl ether mixture, shaken, and centrifuged for 5 min at 16770*g*. Then, the precipitates were hydrolyzed with 6 N HCl at 110 °C for 18 h. The protein hydrolysates were dried using a rotatory evaporator at 40 °C, and finally, the dried extracts were redissolved in 200 μL of Milli-Q water. Samples (4 μL) were injected into a Hewlett-Packard (Avondale, PA) series 1100 LC instrument coupled to a mass spectrometer detector (MSD) equipped with a Luna reversed-phase (RP) column [5 μm C_{18} II column, 150 mm × 1.00 mm (inside diameter); Phenomenex, Torrance, CA] that eluted at a flow rate of 50 μL/min with an isocratic water/2.5% acetic acid mixture (solvent A, 95%) and a methanol/2.5% acetic acid mixture (solvent B, 5%). The column was operated at a constant temperature of 30 °C. MS analysis was conducted in the positive 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). The capillary voltage was 3500 V, the capillary exit offset 25 V, the skimmer potential 15 V, and the trap drive value 36. Conventional ESI-MS data were recorded using a scan range of *m/z* 100−700. The nebulizer (nitrogen) pressure was 50 psi, the dry gas (nitrogen) flow 8 L/min, and the desolvation temperature 300 °C. Identification of both semialdehydes was confirmed by positive matches of retention time, mass spectra, and fragmentation pattern with those of the standard compounds. The peaks corresponding to the molecular ions of AAS-ABA (*m/z* 267) and GGS-ABA (*m/z* 253) were manually integrated from extracted ion chromatograms (EIC) and the resulting areas (area units, AU) used as arbitrary indicators of the abundance of

3544 *J. Agric. Food Chem.,* Vol. 58, No. 6, 2010

Ganhão et al.

Table 1. Total Phenolic Content (TPC) and Antioxidant Activity of Ethanolic Fruit Extracts against DPPH and ABTS Radicals[a]

	TPC[b]		DPPH[c]		ABTS[d]	
	mean	standard deviation	mean	standard deviation	mean	standard deviation
RC	1175 b	222	0.63 a	0.11	94 b	14
CM	2068 a	457	0.42 a	0.16	135 a	11
RU	493 c	73	0.64 a	0.07	44 c	16
AU	428 c	61	0.83 b	0.16	50 c	11

[a] Different letters a−c within a column denote statistical differences between means from different fruit extracts ($p < 0.05$). [b] Results are expressed as milligrams of GAE per 100 g of fruit fresh matter. [c] Results are expressed as EC_{50} values. [d] Results are expressed as micromolar TEAC per gram of fruit fresh matter.

both semialdehydes. Results were corrected considering the total protein content from each meat product, as described above. The percent inhibition of fruit extracts against the formation of semialdehydes was calculated as previously described for total carbonyl gain.

Data Analysis. The TPC and the in vitro DPPH and TEAC assays were performed in triplicate with each fruit extract (two extractions per fruit by three analyses; $n = 6$ per fruit). Four burger patties per batch and per processing treatment were produced and used as experimental units. All analyses were performed in triplicate for each burger patty (four burger patties by three analyses; $n = 12$ per batch and processing treatment). Data obtained from statistical analyses were used as variables and computed via an analysis of variance using SPSS for Windows version 6.1 to study the effect of the added fruit extracts. Pearson correlations were also calculated to establish relationships between parameters. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Total Phenolics and Antioxidant Activity of Fruit Extracts. The amount of total phenolics ranged from 428 to 2068 mg of GAE/100 g of fruit, with extracts from CM having significantly larger amounts of phenolics than RC, RU, and AU (**Table 1**). Consistently, CM exhibited the most intense scavenging activity against both DPPH and ABTS radicals. More detailed information about the composition and characteristics of the fruits evaluated in this study has recently been published (*18*).

Effect of Fruit Extracts on Tryptophan Loss. The fluorescence of tryptophan residues was measured in water extracts from raw, cooked, and cooked and chilled burger patties (**Table 2**). The presence of the aromatic ring in tryptophan residues is responsible for the natural fluorescence emitted by this amino acid at ∼360 nm when it is excited at 280 nm. The fluorescence emitted by extracts from raw samples ranged from 290 to 317 nm, and no significant differences were found between groups. Tryptophan fluorescence has been shown to have good correlations with tryptophan concentration (*24*). As expected, the addition of phenolic-enriched fruit extracts did not affect the initial concentration of tryptophan in raw samples, and phenolic compounds did not interfere with tryptophan fluorescence measurements. Processing technologies applied to burger patties (cooking and chilling) significantly weakened the emission of tryptophan fluorescence in control samples. The decrease in the intrinsic fluorescence of tryptophan is explained by physicochemical changes in proteins, including those derived from oxidative stress (*25*). Therefore, the decrease in tryptophan fluorescence may be due to the oxidative degradation of tryptophan and its conversion into radicals and other oxidation products such as kynurenines (*25, 26*). In this study, the oxidation of tryptophan was very likely initiated by reactive oxygen species (ROS) formed as a result of the high temperatures during cooking (13% tryptophan loss) as heat and oxygen promote tryptophan oxidation (*27*). The mincing process and the cooking process cause the

Table 2. Tryptophan Fluorescence[a] in Raw, Cooked, and Cooked and Chilled Burger Patties with Added Fruit Extracts and Quercetin[b]

	raw		cooked		cooked and chilled	
	mean	standard deviation	mean	standard deviation	mean	standard deviation
C	317 x	39	277 y	34	217 c,z	26
RC	290	17	270	38	281 a	16
CM	302	36	275	20	281 a	31
RU	309	25	283	22	273 a	22
AU	310 x	21	277 x	21	245 b,y	22
Q	310	28	268	23	251 b	39

[a] Results are expressed as fluorescence intensity units. The linearity ($R^2 = 0.9923$; $p < 0.05$) between protein concentration and tryptophan fluorescence was statistically significant ($A_{360} = 25123c + 28812$, with c being the protein concentration in the meat extract). [b] Different letters a−c within a column of the same processing treatment denote statistical differences between means from different fruit extracts ($p < 0.05$). Different letters x−z within a row of the same fruit extract denote statistical differences between means from different treatments ($p < 0.05$).

disruption of muscle tissues and the release of iron from the heme molecule, enhancing the susceptibility of meat lipids and proteins to oxidation during the subsequent refrigerated storage (22% tryptophan loss). As supported by recent studies (*28*), the presence of non-heme iron and oxidizing lipids promotes the oxidative degradation of tryptophan residues and, hence, the loss of its natural fluorescence. Fluorescence spectroscopy has been recently proposed to be a simple, fast, and solvent-free procedure for assessing protein oxidation in food proteins by means of carbonyl gain and tryptophan loss (*3*). However, the suitability of using this technique for assessing tryptophan oxidation in real muscle foods remained unclear. This study originally highlights the fact that fluorescence spectroscopy enables the assessment of protein oxidation in muscle foods through a highly specific and reliable measurement.

Phenolic-rich fruit extracts protected tryptophan residues against oxidation during cooking as no significant losses of tryptophan fluorescence were observed for burger patties with added fruit extracts. Moreover, the emission of tryptophan fluorescence in burger patties treated with RC, CM, RU, and Q remained invariable during the subsequent refrigerated storage, reflecting the fact that a protecting effect of those fruit extracts against tryptophan oxidation occurred beyond cooking. The fluorescence emitted by the water extracts from cooked and chilled burger patties reflects the tryptophan remaining in the samples after application of the technological processes. Cooked and chilled burger patties with added RC, CM, and RU extracts displayed a significantly more intense fluorescence than the control counterparts, whereas samples with added AU and Q displayed intermediate values. Consistently, the effectiveness of fruit extracts as inhibitors of tryptophan depletion was as follows: RC > CM > RU > AU = Q. The fruit extracts used in this study comprise a variety of phenolic compounds with proven antioxidant potential (*18*). In agreement with these results, RC had larger amounts of total phenolic compounds and displayed a more intense in vitro antioxidant activity against the ABTS radical than RU and AU. RC extracts were more effective against tryptophan depletion than CM extracts, whereas the latter displayed a more intense antioxidant activity in vitro. RC is known to contain considerably high levels of ascorbic acid (*29*) and glucoside proanthocyanidins such as cyanidin 3-glucoside (*30*). Estévez et al. (*4*) reported an intense antioxidant effect of cyanidin 3-glucoside on myofibrillar proteins. In comparison with other selected phenolic compounds, this anthocyanin was more effective in hindering the formation of protein carbonyls and the depletion of tryptophan in oil-in-water emulsions. More specifically,

Article

J. Agric. Food Chem., Vol. 58, No. 6, 2010   **3545**

Salminen et al. (*28*) found that cyanidin 3-glucoside successfully inhibits the oxidative degradation of tryptophan into the corresponding oxidation products. The considerably high percent inhibitions exhibited by CM extracts against tryptophan depletion should be also attributed to the high concentration of phenolic compounds in this fruit, including proanthocyanidins, anthocyanins, catechins, and chlorogenic acid (*31*). RU also contains large amounts of phenolic acids and glucoside flavonols (*32*). The protective role of some of these phenolic compounds on myofibrillar proteins has been previously reported (*4,5*). The stability of tryptophan in processed foods is of major concern because the amino acid is involved in a large variety of biological functions and is a precursor for a series of metabolic reactions (*1*). Additionally, tryptophan oxidation products such as kynurenines and particularly nitroso compounds and carbolines formed during boiling or grilling of meat products exhibit mutagenic and carcinogenic potential (*33*). Therefore, the loss of tryptophan during meat processing might have a great impact on consumers in terms of nutritional value and health risks. In this sense, the addition of the fruit extracts studied here considerably enhances the nutritional value and safety of porcine burger patties subjected to cooking and chilling treatments.

Effect of Fruit Extracts on the Formation of Protein Semialdehydes. The formation of carbonyl compounds is one of the most remarkable changes taking place during the oxidation of food proteins. Some major amino acids such as lysine, histidine, proline, and arginine yield carbonyl compounds, and therefore, the concentration of such compounds is a meaningful indicator of the oxidative status of muscle proteins. Raw, cooked, and cooked and chilled burger patties were analyzed for the amount of total protein carbonyls using the DNPH method (**Table 3**). The total amount of carbonyl compounds increased significantly in control burger patties after cooking. The carbonyl gain in cooked burger patties increased to a greater extent during the subsequent refrigerated storage. The DNPH method is a widespread procedure for quantifying the total amount of protein carbonyls in meat products (*10*). However, this method provides no information about the precise chemical structures of the carbonyl compounds, and therefore, the oxidation mechanisms are missing. Recently, Estévez et al. (*11*) detected, for the first time in oxidized myofibrillar proteins, two specific protein carbonyls, the γ-glutamic semialdehyde and the α-aminoadipic semialdehyde (GGS and AAS, respectively). Until now (**Tables 4** and **5**), these specific protein oxidation products have never been used as indicators of protein oxidation in processed muscle foods. In agreement with the results from the DNPH method, the relative concentration of both semialdehydes increased in burger patties after cooking and considerably more during the following chilled storage. The factors and oxidation mechanisms involved in the formation of these semialdehydes have been established (*34*) and are shown in **Figure 1**. AAS is a specific oxidation product from lysine, whereas GGS is derived from the oxidative degradation of proline and arginine. The consistency between the DNPH method and the LC−MS results, supported by significant and positive correlations (**Table 6**), was expected as GGS and AAS are considered to account for $\sim$70% of the total amount of protein carbonyls (*34*). In contrast to the DNPH method, however, the detection of the semialdehydes enables the study of the chemical insight of the mechanisms involved in the oxidative degradation of the muscle proteins from the burger patties. AAS and GGS are

Table 3. Protein Hydrazones (nanomolar hydrazones per milligram of protein) in Raw, Cooked, and Cooked and Chilled Burger Patties with Added Fruit Extracts and Quercetin[a]

	raw		cooked		cooked and chilled	
	mean	standard deviation	mean	standard deviation	mean	standard deviation
C	1.65 z	0.38	3.68 a,y	0.54	9.52 a,x	2.05
RC	2.22 y	0.29	2.58 b,y	0.90	4.21 b,x	1.19
CM	1.93 y	0.71	2.56 b,xy	0.67	3.97 b,x	1.54
RU	1.94 y	0.25	2.14 b,xy	0.43	3.23 b,x	1.08
AU	2.35 y	0.53	2.60 b,y	0.62	4.29 b,x	1.44
Q	2.21 y	0.37	2.17 b,y	0.53	3.73 b,x	1.33

[a] Different letters a−c within a column of the same processing treatment denote a statistical difference between means from different fruit extracts ($p < 0.05$). Different letters x−z within a row of the same fruit extract denote statistical differences between means from different treatments ($p < 0.05$).

Table 4. α-Aminoadipic Semialdehyde[a] (AU) in Raw, Cooked, and Cooked and Chilled Burger Patties with Added Fruit Extracts and Quercetin[b]

	raw		cooked		cooked and chilled	
	mean	standard deviation	mean	standard deviation	mean	standard deviation
C	0.00 z	—	1.62 a,y	0.16	6.04 a,x	0.43
RC	0.00 z	—	0.56 c,y	0.07	1.09 c,x	0.13
CM	0.00 z	—	0.51 c,y	0.07	2.08 b,x	0.33
RU	0.00 z	—	1.09 b,y	0.14	1.58 bc,x	0.30
AU	0.00 z	—	1.04 b,y	0.11	1.78 b,x	0.26
Q	0.00 z	—	1.01 b,y	0.15	1.71 b,x	0.32

[a] Mean values of 0.00 denote that the amount of semialdehyde was below the detection limit. [b] Different letters a−c within a column of the same processing treatment denote a statistical difference between means from different fruit extracts ($p < 0.05$). Different letters x−z within a row of the same fruit extract denote statistical differences between means from different treatments ($p < 0.05$).

Table 6. Pearson Correlations[a] between Measured Parameters

	AAS	GGS	TRYP
DNPH	0.87[b]	0.91[b]	−0.57[c]
AAS	—	0.91[b]	−0.61[c]
GGS	—	—	−0.61[c]

[a] Data from 72 patties employed for calculation of Pearson correlations (four patties per batch and processing technology by six batches by three processing technologies). [b] $p < 0.001$. [c] $p < 0.01$.

Table 5. γ-Glutamic Acid Semialdehyde (AU) in Raw, Cooked, and Cooked and Chilled Burger Patties with Added Fruit Extracts and Quercetin[a]

	raw		cooked		cooked and chilled	
	mean	standard deviation	mean	standard deviation	mean	standard deviation
C	2.11 a,z	0.15	5.94 a,y	0.88	11.34 a,x	1.14
RC	1.75 b,z	0.18	3.65 b,y	0.31	5.68 bc,x	0.77
CM	1.65 b,z	0.23	4.05 b,y	0.30	6.87 b,x	0.72
RU	1.23 c,z	0.18	3.43 b,y	0.32	4.96 c,x	0.43
AU	1.29 c,z	0.14	3.94 b,y	0.44	6.72 b,x	0.72
Q	1.45 bc,z	0.19	3.41 b,y	0.44	6.79 b,x	0.53

[a] Different letters a−c within a column of the same processing treatment denote a statistical difference between means from different fruit extracts ($p < 0.05$). Different letters x−z within a row of the same fruit extract denote statistical differences between means from different treatments ($p < 0.05$).

known to be formed in vitro in the presence of transition metals alone or in combination with hydrogen peroxide (*11*, *34*). In this experiment, the oxidative degradation of the amino acids could have been initiated by ROS formed as a result of the reaction between non-heme iron and oxygen. The high temperatures reached during burger cooking enhanced the formation of ROS and, hence, the oxidative degradation of the amino acids. In meat systems, however, oxidizing lipids should play a major role in protein oxidation. It is plausible to consider that hydroperoxides and acyl radicals formed during thermal decomposition of unsaturated fatty acids strongly contributed to the degradation of susceptible amino acids. The timely interaction between lipids and proteins during oxidative reactions has been profusely described in various model systems (*3*, *4*, *16*) and also in meat products (*7*, *14*, *35*). According to the carbonyl measurements, the susceptibility of proteins from burger patties to oxidation increased after cooking during the subsequent refrigerated storage. The acceleration of oxidative reactions in cooked meats has been described previously for lipid oxidation (*36*, *37*) and might be caused by a number of factors, including the disruption of cellular compartmentalization and exposure of membrane lipids to a pro-oxidative environment and release of catalytic free iron from myoglobin, among others. Certainly, iron might be mainly responsible for the enhanced formation of semialdehydes during the chilling storage of the cooked patties as the release of iron from the heme molecule during thermal treatment of meat enhances its ability to promote oxidative reactions (*38*). Besides the ability of this metal to yield ROS through the Fenton and Haber-Weiss reactions (*1*), iron could be mainly responsible for the deamination of the original amino acid which triggers the eventual formation of the corresponding semialdehyde (*31*).

Phenolic-rich extracts of selected fruits significantly diminished the carbonyl gain in burger patties during cooking and chilling and specifically inhibited the formation of both semialdehydes. Compared to the control counterparts, cooked and cooked and chilled burger patties treated with fruit extracts had significantly smaller amounts of total carbonyls, GGS, and AAS. According to the results obtained from the DNPH method, all fruit extracts and quercetin exhibited equivalent effectiveness against the formation of protein carbonyls. The LC–MS results found, however, significant differences between treated samples for AAS and GGS gain. In a manner consistent with the results from the tryptophan depletion, cooked patties with added RC and CM had significantly smaller amounts of GGS than cooked patties with added RU, AU, and Q. After being refrigerated for 12 days, patties with added RC remained the least oxidized samples. RU was significantly more effective against the formation of AAS than CM, AU, and Q, whereas RC exhibited an intermediate position. Taking into account the percent inhibitions calculated for each fruit extract (**Figure 2**), RC was the most efficient fruit at diminishing the formation of the semialdehydes, followed by RU and the remaining fruit extracts. Whereas there are no scientific data about the effect of these particular fruits on the formation of protein carbonyls, several previous papers have illustrated the interactions between their phenolic components and muscle proteins. Phenolic diterpenes (*7*), certain phenolic acids (*15*), flavonols (*14*), and tea catechins (*39*) have been found to inhibit protein oxidation in meat products as measured by the DNPH method. Estévez et al. (*4*) reported inhibitory effects of gallic acid, cyanidin 3-glucoside, and genistein against the formation of protein carbonyls in myofibrillar proteins using FS. More recently, the same authors (*5*) have proposed plausible antioxidant mechanisms of several of the aforementioned phenolic compounds against the formation of semialdehydes GGS and AAS. Fruit phenolics could have acted as scavengers of iron- and
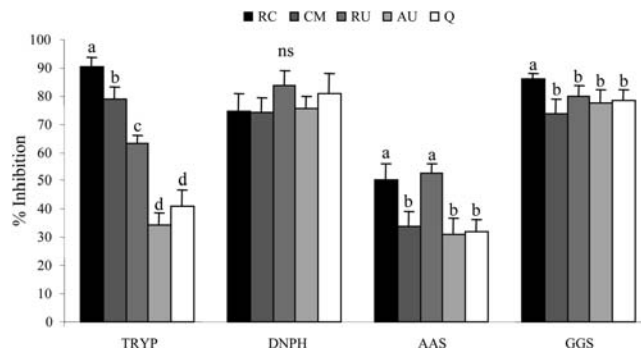

Figure 2. Percent inhibition of fruit extracts vs tryptophan degradation (TRYP) and vs formation of protein hydrazones, AAS, and GGS. Significant differences ($p < 0.05$) between fruit extracts within a measured parameter are denoted by different letters (a–d). ns indicates nonsignificant differences.

lipid-mediated ROS which very likely are main initiators of amino acid degradation. In addition, phenolics could have acted as chelators of non-heme iron which leads to an inhibition of its pro-oxidant actions. Both mechanisms are responsible for the antioxidant actions of the phenolics naturally present in the tested fruits (*40*). The chelating activity of fruit phenolics would be greatly significant during the refrigerated storage of cooked meats when non-heme iron is probably playing a major role as a promoter of semialdehyde formation. The formation of GGS and AAS lowers the nutritional value of muscle foods as it involves the loss of essential amino acids. Additionally, protein semialdehydes might have an impact on meat quality as these semialdehydes are known to participate in the formation of cross-links (*34*) that are responsible for an increased hardness in meat products (*7*). As a result, fruit extracts might provide enhanced nutritional and quality traits to meat products through their antioxidant actions against carbonyl gain.

It is particularly remarkable that fruit extracts tested in this study generally exhibited higher percent inhibitions against carbonyl gain and tryptophan depletion than the pure phenolic compound, quercetin, used as a positive control. In fact, the percent inhibitions reported here are considerably higher than those found in previous papers where particular plant phenolics were evaluated as inhibitors of tryptophan loss and carbonyl gain in food proteins. For example, cyanidin 3-glucoside, a major phenolic component of RC, displayed moderate percent inhibitions against tryptophan loss (15–30%) (*28*) and carbonyl gain (19–31%) (*2*) compared to the considerably high percents displayed by the RC extract in this work (50–90%). The results from the previous papers and the results presented here are comparable as long as the concentrations of total phenolic compounds in the systems are equivalent among studies ($\sim$100 μM). Plant phenolics are known as redox-active compounds that display antioxidant and pro-oxidant actions, depending on their concentration and the presence of other redox-active compounds (*41*). Estévez and Heinonen (*5*) recently suggested that both actions might take place on myofibrillar proteins at the same time to different extents. Therefore, the overall effect (antioxidant or pro-oxidant) displayed by plant phenolics is largely determined by the result of the balance between the antioxidant and pro-oxidant actions. It is plausible to consider that fruit extracts will usually result in an overall intense antioxidant effect when added to a particular food, as the complex mixture of phenolic compounds naturally present in the fruit at specific concentrations is meant to prevent oxidative reactions in living plant tissues. In contrast, the overall effect of one particular phenolic in a particular meat product is mostly

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J. Agric. Food Chem., Vol. 58, No. 6, 2010 **3547**

unpredictable as its behavior might be affected by a number of factors that are difficult to control.

In conclusion, using extracts from RC, CM, RU, and AU results in a successful strategy for inhibiting the oxidative degradation of muscle proteins during processing and storage of burger patties. As a consequence, these fruit extracts could be considered functional ingredients as their antioxidant actions contribute to the enhancement of the nutritional value of the meat products. The assessment of tryptophan depletion by using fluorescence spectroscopy and the detection of GGS and AAS by LC−MS are specific and reliable protein oxidation measurements. The significant and negative correlations between both measurements illustrate the timely consistency between both techniques. By using these advanced methodologies, upcoming studies should shed light on the impact of protein oxidation on particular meat quality traits and the potential usage of plant phenolics as inhibitors of these oxidative reactions.

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