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Formation of Lysine-Derived Oxidation Products and Loss of Tryptophan during Processing of Porcine Patties with Added Avocado Byproducts

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ABSTRACT: The effects of the addition of avocado oil and a phenolic-rich avocado peel extract on protein oxidation were studied in porcine patties subjected to cooking and chilled storage. Protein oxidation was assessed by means of tryptophan loss and the formation of specific lysine oxidation products, such as α-aminoadipic semialdehyde (AAS), α-aminoadipic acid (AAA), and Schiff bases. In the present paper, quantitative data of AAA are reported for the first time on a food matrix. The addition of the avocado extract inhibited the formation of AAS, AAA, and Schiff bases in patties during cooking and subsequent chilled storage. The antioxidant effect may respond to the protecting effect of phenolic compounds, mainly procyanidins, found on the avocado extract. Apparently, the combination of both strategies (back-fat replacement and addition of avocado extract) does not lead to an enhanced advantage on the oxidative stability of the product. The novel methodologies used in the present study enable a better comprehension of the mechanisms and consequences of protein oxidation in food systems.

KEYWORDS: *avocado phenolics, lysine oxidation, α-aminoadipic semialdehyde, α-aminoadipic acid, tryptophan fluorescence, Schiff bases*

INTRODUCTION

Protein oxidation in living tissues has been extensively studied as it plays an essential role in the pathogenesis of relevant degenerative diseases. However, in meat and in muscle foods, the onset of protein oxidation and its consequences on the final product quality are poorly documented. However, it is known that muscle proteins are readily oxidized upon slaughter and during cold storage, processing, and cooking.[1] As a result, significant chemical changes occur in proteins such as tryptophan loss, increases in carbonyls, and the formation of inter- and intraprotein cross-links,[1] leading to important changes in protein composition or modifications on the primary and tertiary structures of proteins. Therefore, the technological, sensory, and nutritional quality of meat products may be impaired.[2−4] Regardless of the recent advances in the understanding of protein oxidation mechanisms,[5] many challenges are still waiting to be accomplished in this field. The current lack of knowledge is mainly due to the lack of precise methodologies to assess the formation of specific protein oxidation products, as well as the loss of some particular amino acids. Recently, with the purpose of developing specific and reliable techniques to assess protein oxidation in meat products, fluorescence spectroscopy was introduced to assess the loss of tryptophan in myofibrillar proteins and complex meat systems.[6−8] Meanwhile, the formation of specific carbonyls such as the α-aminoadipic (AAS) and the γ-glutamic (GGS) semialdehydes generated by the oxidation of basic amino acids through metal-catalyzed reactions has been quantified by fluorescent high-performance liquid chromatography (HPLC-FLD).[9] Both carbonyls (AAS and GGS) have been identified as reliable protein oxidation markers in meat products such as raw and cooked patties, cooked sausages, and fermented meats.[7,8,10] Recently, it has been described that AAS and GGS are not final oxidation products as the carbonyl moiety is still highly reactive and may be involved in several reactions. Thus, the carbonyl group from these semialdehydes may undergo a further oxidative degradation by which the aldehyde moiety is oxidized into a carboxylic acid.[11] In particular, the ongoing oxidation of AAS under intense oxidative conditions (i.e., induced by hydroxyl radical-generating systems) would lead to the formation of a stable end-product: α-aminoadipic acid (AAA) (Figure 1). In cell cultures and model systems, AAA has been identified as an oxidation product of lysine and has been highlighted as a more reliable marker of protein oxidation than its carbonyl precursor, the AAS.[11] However, AAA has never been identified in a food matrix. In addition, the carbonyl moiety of an AAS residue may react with an ε-amino group from a neighboring protein-bound lysine or arginine, as well as with other AAS residues to form a covalent bond via Schiff bases and aldol condensation structures, respectively (Figure 1).[5] Schiff bases are well-known in muscle foods, and many studies have attributed the formation of these condensation structures to the reaction between carbonyls generated by lipid oxidation and the amino groups of amino acids.[12−15] Whereas Xiong,[16] among others, has suggested the potential implication of protein carbonyls in the formation of protein cross-links via Schiff base formation, the likely contribution of specific protein carbonyls such as AAS

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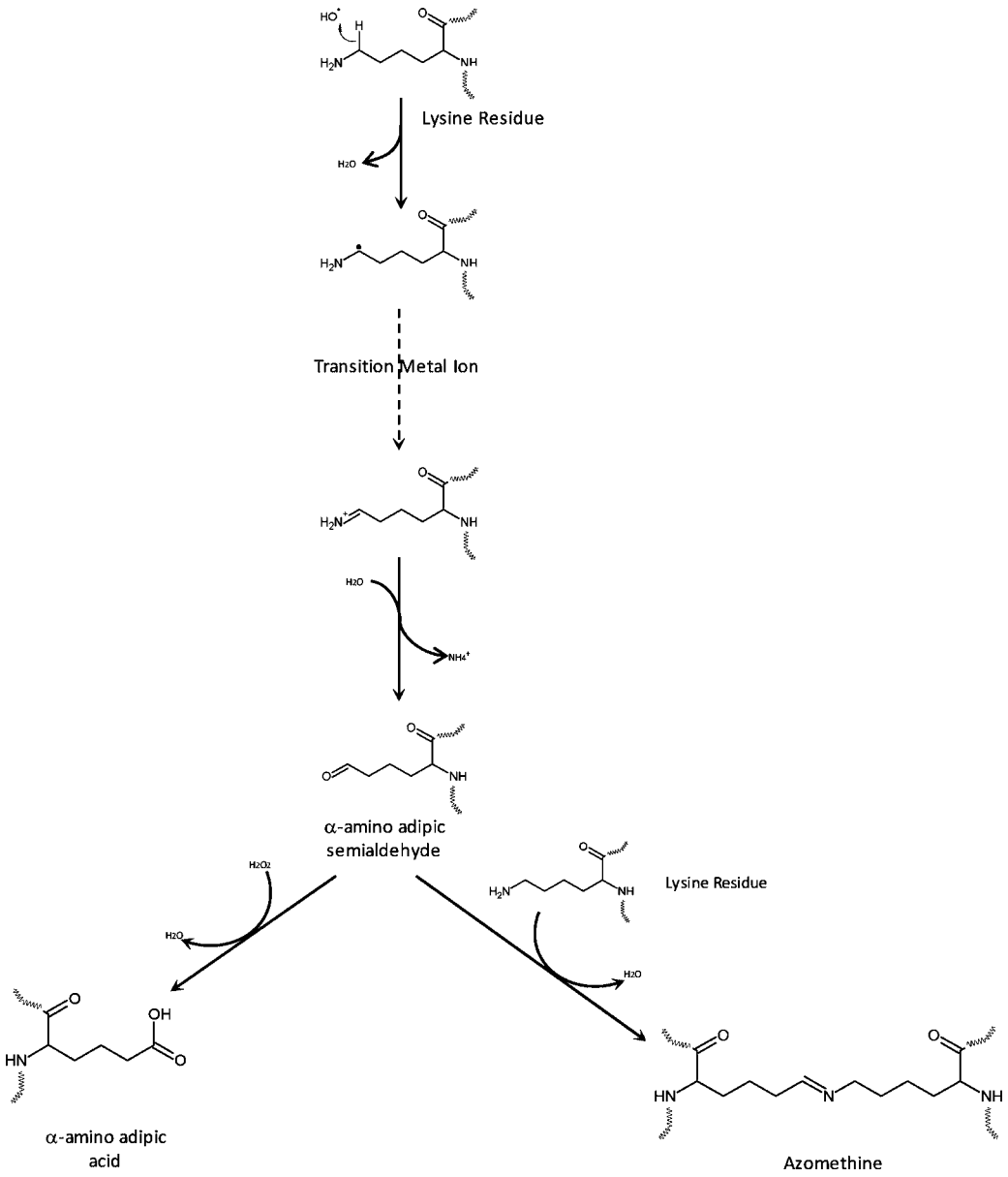

Figure 1. Metal-catalyzed oxidation of lysine to yield α-aminoadipic semialdehyde (AAS) and subsequent formation of α-aminoadipic acid and an azomethine structure upon reaction with a lysine residue.

to the formation of Schiff bases has never been considered in food matrices.

To avoid the potential negative impact of protein oxidation in muscle foods, it is necessary to develop effective antioxidant strategies. An interesting option in the meat-processing industry is the use of phytochemicals owing to their natural origin and additional bioactive effects.[17] Besides, a variety of plant materials and phenolic-enriched extracts have been reported to inhibit protein oxidation.[18−20] In particular, phenolic compounds from fruits have been described as effective against the formation of AAS and GGS and the loss of tryptophan.[7] Avocado (*Persea americana* Mill.) has received considerable interest due to not only its high nutritional value and reported health benefits[21] but also the antioxidant capacity of the phenolic compounds extracted from the peel and seed of the avocado fruit.[21,22] In addition, avocado has a high oil content with considerable amounts of oleic acid,[23] and avocado has therefore been proclaimed as a successful porcine back-fat replacement in meat products.[24] By using this strategy, the amount of monounsaturated fatty acids increases at the expense of saturated and polyunsaturated fatty acids, leading to an enhancement of the oxidative stability and nutritional value of the final product.[24] Nevertheless, the impact of avocado phenolics and avocado oil on the specific chemical modification caused in proteins as a result of oxidative reactions is unknown.

The present study was devoted to the analysis of the loss of tryptophan and the oxidation of lysine in patties subjected to cooking and chilling processes by detecting and quantifying, for the first time, specific lysine-derived oxidation products, including AAA and Schiff bases. The effect of the substitution of porcine back-fat with avocado oil and the addition of phenolic-rich avocado extracts on these chemical modifications is also assessed.

MATERIALS AND METHODS

Chemicals. All chemicals were supplied from Panreac (Panreac Química, S.A., Barcelona, Spain), Merck (Merck, Darmstadt, Germany), and Sigma Chemicals (Sigma-Aldrich, Steinheim, Germany). Extraction solvents were compatible for industrial food use.

Materials. 'Hass' avocado (*P. americana* Mill.) fruit was bought from a local supermarket in Madrid (Spain) and maintained at room temperature until full ripeness. Peels were manually separated from avocados and frozen (−80 °C) until chemical analysis, and extractions were carried out.

Avocado oil was obtained from a supermarket in Mexico City (Mexico) and kept under refrigeration (+4 °C) prior to analysis and the manufacture of patties.

Porcine longissimus dorsi muscle and porcine back-fat belonged to industrial genotypes slaughtered in a local slaughterhouse in Cáceres (Spain). The day after slaughter, the meat was freed from visible fat, whereas the back-fat was cleaned and freed from the skin. Raw materials were immediately chopped into pieces (2 cm^3), frozen (−18 °C, 24 h), and used as such for the manufacture of the patties.

Avocado Extracts. Phenolic-rich avocado extracts were obtained from 10 g of peel from 'Hass' avocado treated twice with 30 mL of acetone/water (70:30 v/v), evaporated, and redissolved in 50 g of distilled water. Further details about the procedure followed for the extraction of avocado phenolics can be found elsewhere.[21]

α- and γ-Tocopherol Quantification. The quantification of α- and γ-tocopherol was made using a HPLC-FLD method as described by Rodriguez-Carpena et al.[25] For quantification purposes, standard curves were prepared using standards of both α- and γ-tocopherol supplied by Sigma-Aldrich.

Total Chlorophyll Content. The total chlorophyll content was measured according to the standard analytical method of the AOCS.[26] This method is used to determine mg/kg of chlorophyll-related pigments (predominantly pheophytin *a*) in oils from spectrophotometric absorption measurements at 630 and 710 nm.

Fatty Acid Profile. Fatty acid methyl esters (FAMEs) were analyzed by gas chromatography following the method described by Rodriguez-Carpena et al.[25] Tridecanoic acid was used as internal standard. Results were expressed as grams per 100 g of detected FAMEs.

Total Phenolic Content (TPC) on the Avocado Oil and the Avocado Extract. The TPC of the avocado materials was determined following the Folin−Ciocalteu method,[21] which is based on spectrophotometric absorption measurements at 765 nm. Phenolic content was calculated from a standard curve of caffeic acid and gallic acid for the avocado oil and the avocado extract, respectively. Results were expressed as milligrams of caffeic acid equivalents (CAE) per kilogram of oil, for the avocado oil, and as milligram gallic acid equivalents (GAE) per 100 g of dry matter, in the case of the avocado extract.

UPLC Analysis of Phenolic Compounds. A Waters Acquity UPLC coupled to a PDA and a FLD detector was used for phenolic analyses (Waters, Milford, MA, USA), in accordance with the method described by Rodriguez-Carpena et al.[21] The column (Waters HSS T3 C18, 2.1 mm × 150 mm × 1.8 μm) was heated to 40 °C, and the autosampler tray was cooled to 4 °C. Samples were eluted over a gradient from 0% (0.5% formic acid) to 40% acetonitrile (0.5% formic acid) over 28 min at a rate of 0.5 mL/min. Using the PDA detector, hydroxybenzoic acids (OH-B) were quantified as gallic acid equivalents at 280 nm, hydroxycinnamic acids (OH-C) as chlorogenic acid equivalents at 320 nm, and flavonols as rutin equivalents at 365 nm. Catechins and proanthocyanidins were detected by FLD by setting the excitation and emission wavelengths at 280 and 325 nm, respectively, and quantified as (β)-catechin equivalents. Upon quantification following the previously described procedure, all subclasses of phenolic compounds were expressed as milligrams per 100 g of dry matter.

CUPRAC Assay. The antioxidant capacity assay was carried out utilizing the copper(II)−neocuproine (CUPRAC) reagent as the chromogenic oxidizing agent as described by Rodriguez-Carpena et al.[21] Spectrophotometric absorption measurements were made at 450 nm against a blank reagent. Calibration curves (absorbance vs concentration) of each antioxidant were constructed at various concentrations using Trolox standard solution (ranging from 0.25 to 2 mmol) in ethanol. Results were calculated in terms of Trolox equivalent antioxidant capacity (TEAC) and expressed as millimole Trolox equivalents per gram of fresh matter.

DPPH Assay. The DPPH assay reported by Rodriguez-Carpena et al.[21] was employed for the measurement of the antioxidant activity of extracts using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, and the absorbance at 517 nm was recorded. A standard curve was obtained using a Trolox standard solution at various concentrations (ranging from 0.25 to 2 mmol) in methanol. Results were calculated as TEAC and expressed as millimole Trolox equivalents per gram of fresh matter.

Manufacture of Porcine Patties. Depending on the partial replacement of porcine back-fat by avocado oil [porcine (P) vs avocado (A)] and the addition of avocado peel extract [control (C) vs treated (T)], four types of patties were prepared, namely, PC, PT, AC, and AT. Ingredients per kilogram of porcine patty (PC) were as follows: 700 g of porcine longissimus dorsi muscle, 180 g of distilled water, 100 g of pork back-fat, and 20 g of NaCl. For the elaboration of patties treated with avocado oil (A), 50 g of pork back-fat was replaced by 50 g of avocado oil per kilogram of patty. In the treated patties (T) 50 g of the distilled water was replaced by 50 g of a water solution extract from the peel avocado. The dose of oil and avocado extract was set in accordance with preliminary studies that guarantee the sensory and technological feasibility of the present experimental design. All ingredients were minced in a cutter until a homogeneous raw batter was obtained. Patties were shaped using a conventional patty maker (100 g/patty), to give average dimensions of 10 cm (diameter) × 1 cm (thickness). Eighteen patties per group were produced in two independent manufacturing processes (9 patties per group each time). Depending on the processing treatment applied, the 18 patties were divided into three different groups: raw (R), cooked (CO), and cooked and chilled (CC) patties (n = 6 per oil treatment and processing treatment). R patties were frozen (−80 °C) the day of manufacture until the analytical experiments. CO and CC patties were cooked at 170 °C for 18 min in a forced-air oven and allowed to cool at room temperature. CC patties were, upon cooking, stored for 15 days at 5 °C under white fluorescent light (1620 lx). CO and CC samples were also frozen (−80 °C) until required for analysis.

Proximate Composition of Patties. Moisture, protein, and total lipid contents were determined using official methods.[26] The method of Folch et al.[27] was used for determining fat content in patties. The cooking loss of patties was calculated as follows: cooking loss = $[(W_b - W_a)/W_b] \times 100$ where W_b and W_a are the weights of the patties before and after cooking, respectively.

Fluorescence Measurements of Tryptophan and Schiff Base Structures. The natural fluorescence of tryptophan and the emission of fluorescence by protein oxidation products (Schiff base structures) were assessed by using fluorescence spectroscopy.[6] 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) using an Ultra-Turrax homogenizer for 30 s. The water homogenates were filtered through gauze to remove insoluble particles. A 1 mL aliquot of the homogenates was redissolved in 20 mL of the 20 mM sodium phosphate buffer and then dispensed in a 4 mL quartz spectrofluorometer cell. Emission spectra of tryptophan were recorded from 300 to 400 nm with the excitation wavelength established at 283 nm (LS 55 Perkin-Elmer luminescence spectrometer). Emission spectra of Schiff bases were recorded from 400 to 500 nm with the excitation wavelength set at 350 nm. For both measurements, excitation and emission slit widths were set at 10 nm and data were collected at 500 nm per minute in both measurements. Tryptophan content was calculated from a standard curve of *N*-acetyl-L-tryptophan amide (NATA). The linearity (R^2 = 0.9955; $p < 0.05$) between NATA concentration (ranged from 0.1 to 0.5 μM) and fluorescence intensity was statistically significant. Results were expressed as milligrams NATA equivalents per 100 g of sample. The content of Schiff base structures was expressed as fluorescence

Table 1. Composition (Mean ± Standard Deviation) of Experimental Raw Patties[a]

	porcine patties		avocado patties				
	control	treated	control	treated	R	T	RT
α-tocopherol[b]	0.34 x ± 0.08	0.37 x ± 0.01	1.08 y ± 0.01	1.07 y ± 0.01	<0.001	0.214	0.523
γ-tocopherol[b]	0.02 x ± 0.00	0.02 x ± 0.00	0.06 y ± 0.01	0.06 y ± 0.01	<0.001	0.350	0.786
chlorophylls[b]	LOD[c]	0.07 x ± 0.02	0.27 y ± 0.04	0.33 z ± 0.03	<0.001	0.125	0.090
% fatty acids[d]							
C12:0	0.04 y ± 0.01	0.05 y ± 0.01	0.02 x ± 0.01	0.03 x ± 0.01	0.031	0.621	0.399
C14:0	0.98 y ± 0.05	1.02 y ± 0.03	0.51 x ± 0.04	0.56 x ± 0.02	<0.001	0.426	0.423
C16:0	22.10 y ± 0.71	22.02 y ± 0.82	16.77 x ± 0.44	17.50 x ± 0.30	<0.001	0.544	0.259
C16:1 (n-7)	2.26 ± 0.30	1.96 ± 0.14	2.80 ± 0.22	2.75 ± 0.03	0.512	0.652	0.555
C17:0	0.37 y ± 0.03	0.32 y ± 0.05	0.19 x ± 0.01	0.18 x ± 0.01	<0.001	0.624	0.669
C17:1 (n-7)	0.34 y ± 0.05	0.28 y ± 0.04	0.20 x ± 0.02	0.17 x ± 0.01	<0.001	0.189	0.899
C18:0	11.93 y ± 1.37	12.57 y ± 1.23	6.71 x ± 0.70	7.06 x ± 0.24	<0.001	0.201	0.650
C18:1 (n-9)	39.81 x ± 1.56	39.27 x ± 1.48	48.62 y ± 0.65	47.94 y ± 0.59	<0.001	0.711	0.642
18:1 (n-7)	3.64 ± 0.43	3.32 ± 0.17	4.10 ± 0.23	3.87 ± 0.10	0.022	0.255	0.423
C18:2 (n-6)	12.41 x ± 0.41	12.66 x ± 0.12	15.51 y ± 0.18	15.67 y ± 0.29	<0.001	0.441	0.710
C18:3 (n-6)	0.13 x ± 0.06	0.24 y ± 0.13	0.13 x ± 0.06	0.16 x ± 0.06	0.038	0.222	0.025
C18:3 (n-3)	0.94 ± 0.19	0.88 ± 0.15	0.89 ± 0.12	0.83 ± 0.08	0.089	0.236	0.521
C20:0	0.58 xy ± 0.27	0.71 y ± 0.21	0.52 xy ± 0.13	0.45 x ± 0.20	<0.001	0.332	0.099
C20:1 (n-9)	1.39 y ± 0.29	1.61 y ± 0.31	1.07 x ± 0.17	0.96 x ± 0.15	0.045	0.536	0.225
C20:2 (n-6)	0.83 y ± 0.22	0.95 y ± 0.15	0.52 x ± 0.08	0.49 x ± 0.11	<0.001	0.362	0.269
C20:3 (n-6)	0.43 y ± 0.27	0.59 z ± 0.16	0.36 xy ± 0.15	0.29 x ± 0.10	0.012	0.630	0.054
C20:4 (n-6)	1.02 y ± 0.43	1.02 y ± 0.14	0.72 x ± 0.11	0.66 x ± 0.15	0.023	0.584	0.365
C20:3 (n-3)	0.52 y ± 0.34	0.51 y ± 0.14	0.34 x ± 0.06	0.36 x ± 0.15	<0.001	0.754	0.856
C22:6 (n-3)	0.09 y ± 0.04	0.04 x ± 0.02	0.03 x ± 0.01	0.06 xy ± 0.02	0.212	0.584	0.035

[a]R, effect of the replacement of porcine fat by avocado oil in patties; T, effect of the treatment with an avocado extract; RT, interaction between the aforementioned effects. Different letters (x–y) within a row of the same processing treatment denote statistical differences between means from different type of patties ($p < 0.05$). [b]Results are expressed as mg/100 g sample. [c]LOD denotes that the amount of the chlorophyll was below the detection limit. [d]Results are expressed as percent of total fatty acids analyzed.

intensity units emitted at 460 nm. These values were corrected according to the protein content of patties 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 patties and P_p is the mean of the protein content in each kind of patty).

HPLC-FLD Analysis of AAS. Samples (5 mg of protein) were derivatized with 50 mM aminobenzoic acid (ABA) and subsequently hydrolyzed with 6 N HCl according to the procedure described by Utrera et al.[9] Hydrosylates were dried in vacuo, reconstituted with 200 μL of Milli-Q water, and filtered through a PVDF syringe filter. Samples were injected in a HPLC using a Cosmosil 5C18-AR-II RP-HPLC column (5 μm, 150 × 4.6 mm) and a guard column (10 × 4.6 mm) filled with the same material. The Shimadzu "Prominence" HPLC apparatus (Shimadzu Corp., Kyoto, Japan) was equipped with a quaternary solvent delivery system (LC-20AD), a DGU-20AS online degasser, an SIL-20A autosampler, an RF-10A XL fluorescence detector, and a CBM-20A system controller. Fifty millimolar sodium acetate buffer (pH 5.4, eluent A) and acetonitrile (ACN, eluent B) were used as eluents. A low-pressure gradient program was used, varying eluent B concentration from 0% (min 0) to 8% (min 20). The injection volume was 1 μL, the flow rate was kept at 1 mm/min, and the temperature of the column was maintained constant at 30 °C. Excitation and emission wavelengths were set at 283 and 350 nm, respectively. Identification of the derivatized semialdehyde in the FLD chromatograms was carried out by comparing their retention times (t_R) with those from a standard compound,[9] injected and analyzed under the above-mentioned conditions. The peaks corresponding to AAS–ABA were manually integrated from FLD chromatograms and the resulting areas plotted against an ABA standard curve (ranging from 0.1 to 0.5 mM). Regression coefficients >0.99 were obtained. The estimation of the quantities of AAS–ABA through an ABA standard curve was accomplished by assuming that the fluorescence emitted by 1 mol of ABA is equivalent to that emitted by 1 mol of derivatized protein carbonyl. Results are expressed as nanomoles of AAS per milligram of protein.

HPLC-FLD Analysis of AAA. Patties (1 g) were minced and then homogenized 1:10 (w/v) in a 6 M NaCl/20 mM sodium phosphate buffer (pH 6.5) using an Ultraturrax homogenizer for 30 s. Depending on the sample, the final meat homogenates contained from 0.015 to 0.025 g/mL protein. An aliquot (400 μL) was dispensed in 2 mL screw-capped eppendorf tubes. Proteins were precipitated with 1.6 mL of cold 10% TCA and subsequent centrifugation at 10000 rpm for 5 min. Protein hydrolysis was carried out at 110 °C for 18 h in the presence of 6 M HCl. Hydrolysates were finally dried in a vacuum centrifuge using a Savant speed-vac concentrator and finally reconstituted with 150 μL of 200 mM tetraborate buffer (pH 8.5). The derivatization procedure involved the addition of 50 μL of 0.2 mM 9-fluorenylmethyl chloroformate (FMoc) dissolved in acetonitrile. The tube was agitated and the derivatization allowed to proceed for 90 s; 500 mL of heptane was added and again vortex-mixed for 1 min to stop the reaction. The upper phase was discarded by aspiration followed by second and third extractions according to the same process. The lower phase was filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 μm pore size, Pall Corp., USA) for HPLC analysis. An aliquot (1 μL) from the reconstituted protein hydrosilates was injected and analyzed in the above-mentioned HPLC equipment using a Zorbax Eclipse AAA column (3.5 μm, 4.6 mm × 150 mm) and a guard column (10 × 4.6 mm) filled with the same material. Eluent A was 20 mM ammonium acetate (pH 6.5) and 15% methanol, and eluent B was 90% acetonitrile. The flow rate was constant at 1.0 mL/min, and the column was maintained at 35 °C. The gradient profile was as follows: 0–1.5 min, 12% B; 1.5–2.0 min, 12–18% B; 2.0–9.0 min, 18% B; 9.0–9.5 min, 18–25% B; 9.5–12.5 min, 25% B; 12.5–13.0 min, 25–30% B; 13.0–16.0 min, 30% B; 16.0–17.0 min, 30–40% B; 17.0–20.0 min, 40% B; 20.0–22.0 min, 40–50% B; 22.0–23.0 min, 50% B; 23.0–24.0 min, 50–99% B. Excitation and emission wavelengths were set at 263 and 313 nm, respectively. Identification of the derivatized aminoadipic acid in the FLD chromatograms was carried out by comparing their retention times (t_Rt with those from a standard

Table 2. Tryptophan Fluorescence (Mean ± Standard Deviation) in Experimental Raw, Cooked, and Cooked and Chilled Patties[a]

	porcine patties		avocado patties				
	control	treated	control	treated	R	T	RT
raw	0.77 by ± 0.04	0.63 bx ± 0.06	0.67 bx ± 0.05	0.68 bx ± 0.02	0.333	0.009	0.006
cooked	0.20 ay ± 0.02	0.12 ax ± 0.03	0.14 ax ± 0.02	0.13 ax ± 0.02	0.016	<0.001	0.006
cooked and chilled	0.14 ax ± 0.05	0.10 ax ± 0.03	0.12 ax ± 0.02	0.10 ax ± 0.02	0.386	0.053	0.761

[a]R, effect of the replacement of porcine fat by avocado oil in patties; T, effect of the treatment with an avocado extract; RT, interaction between the aforementioned effects. Different letters (a–b) within a column of the same type of patty denote statistical differences between means from different processing treatments ($p < 0.05$). Different letters (x–y) within a row of the same processing treatment denote statistical differences between means from different type of patties ($p < 0.05$). Results are expressed as mg NATA Equivalents/100 gr sample.

compound injected and analyzed in the above-mentioned conditions. The peaks corresponding to AAA–FMoc were manually integrated from FLD chromatograms and the resulting areas plotted against an AAA–FMoc standard curve with known amounts (ranging from 5 to 0.4 pM). Regression coefficients >0.98 were obtained.

Analysis of Hexanal by GC-MS. Hexanal was used an indicator of lipid oxidation and analyzed from the headspace of R, CO, and CC patties by using solid-phase microextraction (SPME) and gas chromatography–mass spectrometry (GC-MS) following the method described by Estévez et al.[28] Hexanal was positively identified by comparing its mass spectra and retention time with those displayed by the standard compound (Sigma-Aldrich). The result from the hexanal analysis was provided in arbitrary area units (AAU).

Statistical Analysis. All data were expressed as the mean ± standard deviation. The effect of the processing treatment applied to the patties was analyzed by one-way analyses of variance (ANOVA) and Tukey's tests, in all measurements. To analyze the effect of the replacement of porcine fat by avocado oil in patties (R), the effect of the treatment with an avocado extract (T), and the effect of its interaction (RT), a two-way ANOVA was used. For the assessment of the relationships between the different protein oxidation measurements, Pearson's correlation coefficients were calculated. Data were analyzed using the mixed procedure of SPSS for Windows (v. 15.0). Differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Characterization of the Avocado Oil. The avocado oil showed a TPC of 12.75 mg of caffeic acid equivalent/kg oil and high chlorophyll content (65.49 mg chlorophyll/kg oil). With regard to the fatty acids profile, high amounts of oleic, linoleic, and palmitic acids were found (57.44, 18.7, and 12.87%, respectively). Considerable amounts of α- and γ-tocopherol were also detected, 9.04 and 1.78 mg/100 g oil, respectively. Further details are found elsewhere.[25]

Characterization of the Avocado Extract. The avocado extract had a high TPC content (6082 ± 863 mg GAE/100 g dry matter). Catechins (237.8 ± 4.2 mg/100 g dry matter), hydroxycinnamic acids (282.7 ± 6.9 mg/100 g dry matter), flavonols (2.5 ± 0.7 mg/100 g dry matter), and procyanidins (4592.0 ± 129.4 mg/100 g dry matter) were found to be the major phenolic constituents of the avocado extracts. The avocado extract exhibited an intense scavenging activity against both CUPRAC and DPPH radicals (275.36 ± 59.09 and 130.26 ± 36.80 mmol Trolox/g fresh matter, respectively). More detailed information about the composition and characteristics of the avocado extract evaluated in this study has recently been published elsewhere.[21]

Characterization of the Patties. R patties (moisture, 71.5%; protein, 17.3%; fat, 10.0%) and CO patties (moisture, 62.5; protein, 22.1%; fat, 14.3%) from all groups and treatments had similar proximate compositions and cooking losses (21.7%). As expected, the avocado oil contributed large amounts of tocopherols and chlorophylls to A patties (Table 1). The addition of the avocado extract was not found to affect the composition of the patties in tocopherols and chlorophylls as the peel from avocado is mostly rich in phenolic compounds, as aforementioned. The replacement of porcine back-fat by avocado oil had a significant impact on the fatty acid profile of the patties. A patties had significantly lower percents of saturated fatty acids such as palmitic (C16:0) and stearic (C18:0) acids and significantly higher percents of mono- and polyunsaturated fatty acids such as oleic (C18:1) and linoleic (C18:2) acids. Treating the patties with the phenolic-rich avocado extract had no influence on their fatty acid composition.

Tryptophan Loss. Tryptophan content was determined in raw, cooked, and cooked and chilled patties (Table 2) by measuring the fluorescence emitted by this amino acid. The decrease of tryptophan fluorescence has been attributed to the oxidative degradation of tryptophan and its conversion into radicals.[29] As expected, the technological process applied to the PC patties, cooking and the following refrigerated storage, caused a significant decrease in the amount of tryptophan (Table 2). Oxidation of tryptophan was very likely initiated by reactive oxygen species (ROS) formed during cooking as a result of the cleavage of existing hydroperoxides. This initiation may have been promoted by the release of highly catalytic iron from the heme molecule and the disruption of the muscle tissues caused by high temperatures. Specifically, hydroxyl radicals, generated mainly by reaction of transition metals with hydrogen peroxide, and superoxide radicals, produced by autoxidation of myoglobin, are known to react with aromatic rings.[30] Additionally, the presence of oxidizing lipids is considered to contribute to the oxidative degradation of tryptophan residues.[7] These results are in accordance with results obtained when bovine meat is heated to a temperature above 60 °C.[30]

The addition of both avocado oil and phenolic extracts caused a slight but significant reduction on the tryptophan quantity in raw AC and AT patties (0.63–0.68 vs 0.77 mg NATA equiv/100 g sample). The impact of the processing technologies previously described for PC patties was also observed in patties treated with avocado byproducts. The use of avocado oil as back-fat replacement and the addition of the avocado extract were not found to have a clear beneficial effect against tryptophan loss on porcine meat patties under the conditions applied in the present study. Whereas the avocado phenolics displayed high antioxidant in vitro activity, plant phenolics display complex and sometimes contradictory antioxidant or pro-oxidant effects on food proteins.[6,31] Moreover, tryptophan is one of the most susceptible amino acid residues to free radical attack.[32] The results obtained for

Table 3. α-Aminoadipic Semialdehyde (Mean ± Standard Deviation) in Experimental Raw, Cooked, and Cooked and Chilled Patties[a]

	porcine patties		avocado patties				
	control	treated	control	treated	R	T	RT
raw	0.27 ax ± 0.04	0.24 ax ± 0.04	0.26 ax ± 0.05	0.22 ax ± 0.04	0.558	0.017	0.500
cooked	0.76 by ± 0.16	0.52 bx ± 0.09	0.60 bx ± 0.08	0.53 bx ± 0.10	0.042	0.003	0.092
cooked and chilled	1.09 cy ± 0.22	0.68 cx ± 0.10	0.70 bx ± 0.11	0.63 bx ± 0.10	<0.001	<0.001	0.004

[a]R, effect of the replacement of porcine fat by avocado oil in patties; T, effect of the treatment with an avocado extract; RT, interaction between the aforementioned effects. Different letters (a–c) within a column of the same type of patty denote statistical differences between means from different processing treatments ($p < 0.05$). Different letters (x–y) within a row of the same processing treatment denote statistical differences between means from different type of patties ($p < 0.05$). Results are expressed as nmol/mg protein.

the avocado phenolics contrast with the intense protective effects displayed by phenolic-rich Mediterranean fruits against the oxidation of tryptophan in porcine patties subjected to similar cooking and chilling procedures.[7]

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 tryptophan loss and Schiff base formation.[6,7] This study highlights the fact that fluorescence spectroscopy enables the assessment of protein oxidation and originally applies a reliable method to quantify the loss of one essential amino acid in muscle foods. The oxidation of tryptophan in foods holds deep scientific and technological meaning as this amino acid is involved in a large variety of biological functions and is a precursor for a series of metabolic reactions and because tryptophan oxidation products such as kynurenines exhibit mutagenic and carcinogenic potential.[28]

Formation of α-Aminoadipic Semialdehyde. R, CO, and CC patties were analyzed for the amount of total AAS (Table 3). The technological process applied to patties caused successive increases in the amount of AAS in PC patties in the following order: R < CO < CC. These results demonstrate that lysine is oxidatively modified during meat cooking and the subsequent chilled storage to yield AAS. AAS is formed as a result of the oxidative deamination of the side chains from lysine residues in the presence of ROS and transition metals such as iron.[5] An effect of high temperature-driven effects on the formation of ROS is suspected. ROS may have promoted the formation of AAS up to the levels found in raw PC patties. Cooking also increased the susceptibility of meat proteins to undergo further carbonylation as the increase of AAS during the following chilled storage was even more intense. This effect could be attributed to the disruption of cellular compartmentalization and exposure of membrane lipids, creating a strong oxidative environment.[7]

A significant reduction in the amount of AAS was observed in AC patties compared to the PC counterparts. These results show that the back-fat replacement by avocado oil inhibited the formation of AAS in CO and CC patties. It is plausible to consider that the addition of avocado oil contributed to the inhibition of protein oxidation through the antioxidant activity of the natural components of avocado oil such as tocopherols, chlorophylls, and polyphenols.[21] Previous studied confirmed the antioxidant effect of tocopherols and selected phenolic compounds against the formation of AAS in myofibrillar proteins.[31] According to the present results, the antioxidant capacity of these compounds was enough to counterbalance the potential oxidative instability derived from the increased amount of unsaturated fatty acids provided by the avocado oil in the AC patties. In a previous study, the back-fat replacement by avocado oil inhibited lipid oxidation in similar meat systems.[22]

The addition of the phenolic-rich avocado extracts had also a protecting effect against the carbonylation occurring during cooking and chilling of PT patties. In a previous study, the addition of extracts from phenolic-rich wild fruits to cooked and chilled porcine patties led to a similar antioxidant protection.[7] The antioxidant effect of the avocado extract could be ascribed to its high content of catechins, procyanidins, and phenolic acids.[21] According to our estimation, the avocado extract contributed 600 GAE per kilogram of patty. These compounds may behave as antioxidants on proteins, owing to their ability to scavenge radical species and/or chelate transition metals such as nonheme iron.[6] This transition metal is known to play a relevant role as promoter of protein carbonylation in meat systems.[5] Protein carbonylation is regarded as a harmful expression of the oxidative damage to meat proteins owing to its influence on the spoilage of nutritional, technological, and sensory properties of meat systems.[5] The addition of avocado oil and extracts reduces the formation of specific protein carbonyls in patties and may contribute to diminish the undesirable effects of such chemical changes in these food products.

In contrast to the results in PT patties, no significant effect of the avocado extract was detected in the formation of AAS in AT patties. Therefore, the results obtained in the present work would advise against using both strategies (fat replacement and avocado extract) at the same time as it does not lead to an enhanced antioxidant effect against the formation of AAS. It is important to highlight that the oxidation levels in AC patties are significantly lower than those in PC patties. Plausibly, phenolics from avocado peel extracts could have exerted, as radical scavengers, their antioxidant actions to a higher extent in the system with higher oxidative instability (porcine patties). The already reduced oxidation levels in AC patties as a result of the addition of the avocado oil could explain the apparent less intense antioxidant activity of the phenolic-rich avocado extracts in AT patties. In fact, it is intriguing that the avocado oil contributed high levels of natural antioxidants to AC patties (phenolic compounds, chlorophylls, and tocopherols). The addition of extra phenolic compounds from the avocado peel extract in AT patties may have led to interactions between all of these redox-active compounds. The interactions between redox-active compounds such as tocopherols, polyphenols, and ascorbic acid are known to take place in food matrices, and the outcome of those complex interactions is, in most cases, unexpected.[17,31] Under the conditions of the present experiment, these interactions did not lead to an enhanced effect. Finally, the differences between C and A patties in terms of lipid composition could have affected the overall effect exerted

Table 4. α-Aminoadipic Acid (Mean ± Standard Deviation) in Experimental Raw, Cooked, and Cooked and Chilled Patties[a]

	porcine patties		avocado patties				
	control	treated	control	treated	R	T	RT
raw	LOD[b] a	LOD	LOD a	LOD	*	*	*
cooked	0.56 by ± 0.08	LOD x	0.76 bz ± 0.10	LOD x	0.007	<0.001	0.007
cooked and chilled	0.84 cy ± 0.06	LOD x	0.89 cy ± 0.12	LOD x	0.426	<0.001	0.426

[a]R, effect of the replacement of porcine fat by avocado oil in patties; T, effect of the treatment with an avocado extract; RT, interaction between the aforementioned effects. Different letters (a–c) within a column of the same type of patty denote statistical differences between means from different processing treatments ($p < 0.05$). Different letters (x–y) within a row of the same processing treatment denote statistical differences between means from different type of patties ($p < 0.05$). Results are expressed as picomol/mg protein. [b]LOD denotes that the amount of the acid was below the detection limit.

Table 5. Schiff Base Structures (Mean ± Standard Deviation) in Experimental Raw, Cooked, and Cooked and Chilled Patties[a]

	porcine patties		avocado patties				
	control	treated	control	treated	R	T	RT
raw	23.7 ax ± 4.0	156.9 bz ± 44.0	LOD[b] aw	115.2 ay ± 24.5	0.008	<0.001	0.325
cooked	154.8 bz ± 27.5	55.0 ax ± 13.4	90.5 by ± 11.2	80.2 axy ± 23.5	0.043	<0.001	<0.001
cooked and chilled	169.0 by ± 11.0	71.9 ax ± 16.4	213.1 cy ± 63.8	93.1 ax ± 12.3	0.117	<0.001	0.895

[a]R, effect of the replacement of porcine fat by avocado oil in patties; T, effect of the treatment with an avocado extract; RT, interaction between the aforementioned effects. Different letters (a–c) within a column of the same type of patty denote statistical differences between means from different processing treatments ($p < 0.05$). Different letters (w–z) within a row of the same processing treatment denote statistical differences between means from different type of patties ($p < 0.05$). Results are expressed as fluorescence intensity units. [b]LOD denotes that the amount of the Schiff base was below the detection limit.

by avocado phenolics. The redox actions of phenolic compounds depend on multiple factors including the amount, composition, and susceptibility of the lipids in the system to oxidative reactions.[17] These factors influence the location, distribution, redox behavior, and, eventually, the antioxidant action of phenolic compounds.[17]

To establish links between the different protein oxidation measurements, Pearson correlation coefficients were determined (Table 7). A negative and significant correlation was observed between the formation of AAS and the loss of tryptophan, highlighting the connection between both manifestations of protein oxidation.[5]

Formation of α-Aminoadipic Acid. AAA was quantified by HPLC-FLD in R, CO, and CC patties (Table 4). AAA was not detected in the raw PC patties. Meanwhile, a significant successive increase in the amount of AAA was observed in CO and CC PC and AC patties. The good agreement between these results and the AAS results is manifested as a significant positive correlation between both lysine oxidation products (Table 7). These results are in concordance with the results reported by Sell et al.[11] on oxidized culture cells, in which the AAA was identified as a further oxidation product of lysine, via the formation of AAS. It is worth noting that the formation of AAA from AAS requires the presence of oxygen and an oxidizing agent, such as hydrogen peroxide (H_2O_2), naturally present in meat (Figure 1).[5]

No significant effect of the back-fat replacement with avocado oil was observed on the formation of AAA in porcine patties. However, the addition of the avocado extract kept the levels of AAA below quantification limits. This observation suggests that the most abundant and redox-active phenolic components of avocado extracts, namely, procyanidins,[33] had higher protective effect against AAA formation than those present in the avocado oil, namely, tocopherols and chlorophyll pigments. The limited antioxidant action of chlorophylls against the formation of AAA could be ascribed to the fact that these phytochemicals display a low rate of hydroperoxides decomposition,[34] directly implicated in the formation of AAA from AAS. Phenolic components from avocado oils may be efficient at scavenging radicals produced at initial stages of the oxidative reactions but display certain limitations under the further and more intense pro-oxidant conditions at which AAA is formed.

The contradictory effect of the same antioxidant strategy against different protein oxidation manifestations highlights the complex and diverse mechanisms governing protein oxidation and the specificity of the phenolics' action on oxidizing proteins. The present results suggest that the mechanisms involved in the efficient antioxidant actions of avocado phenolics against lysine oxidation are not applicable in protecttion against the early oxidation of tryptophan residues. Likewise, phytochemicals from the avocado oil such as chlorophylls are efficient at inhibiting the formation of AAS, whereas no significant effect is observed against the eventual formation of AAA. Hence, the assessment of protein oxidation in foods using various measurements is highly recommended in future studies to obtain an accurate overall picture of the oxidative damage to food proteins and the actual effectiveness of particular antioxidant compounds.

Specific protein oxidation products, such AAS and AAA, are reliable protein oxidation markers. Furthermore, they are responsible for important modifications of the native structure and/or integrity of muscle proteins affecting meat quality including texture traits, water-holding capacity, and some other technological properties.[5] It is worth noting that the formation of AAA from the original amino acid residue (lysine) involves the loss of a protonable amino group and the formation, in its place, of a carboxylic moiety. The modification of the electric arrangement of the proteins, their tertiary conformation, and loss of functionality may be among the plausible effects of this severe chemical modification on meat proteins.[5] Antioxidant strategies are needed to minimize these unpleasant oxidative-driven effects. Hence, the use of the avocado extract is a reliable strategy to retard the formation of protein oxidation carbonyls. The present paper provides, for the first time, quantitative data

Table 6. Hexanal Contents (Mean ± Standard Deviation) in Experimental Raw, Cooked, and Cooked and Chilled Patties[a]

	porcine patties		avocado patties				
	control	treated	control	treated	R	T	RT
raw	0.39 a ± 0.10	0.49 a ± 0.18	0.44 a ± 0.12	0.38 a ± 0.09	0.230	0.158	0.225
cooked	130.28 bz ± 21.12	99.97 by ± 14.31	72.69 bx ± 16.21	69.56 bx ± 12.69	<0.001	<0.001	0.030
cooked and chilled	354.33 cy ± 46.93	253.41 cx ± 44.91	236.69 cx ± 39.54	230.85 cx ± 41.87	<0.001	0.003	0.011

[a]R, effect of the replacement of porcine fat by avocado oil in patties; T, effect of the treatment with an avocado extract; RT, interaction between the aforementioned effects. Different letters (a–c) within a column of the same type of patty denote statistical differences between means from different processing treatments ($p < 0.05$). Different letters (w–z) within a row of the same processing treatment denote statistical differences between means from different type of patties ($p < 0.05$). Results are expressed as area units × 10^6.

on the amount of AAA in meat systems, proving that HPLC-FLD enables a specific and accurate assessment of this compound.

Formation of Schiff Base Structures. AAS generated from lysine oxidation may be involved in cross-linking with amino groups from the side chain of proteins via Schiff base formation. These compounds are conjugated fluorophores and were detected by recording fluorescence at 460 nm when excited at 350 nm (Table 5). The formation of Schiff bases was promoted by cooking and subsequent chilled storage, as significant increases in CO and CC PC patties were observed. The formation of these condensation products depends on carbonyl production and also on the availability of amino groups in protein side chains.[35] The increases of these fluorescent products are consistent with those previously reported for AAS in PC patties. With this fact taken into consideration, the formation of Schiff bases could be at least partially attributed to the reaction of AAS with amino groups of proteins. Lipid-derived carbonyls may also be involved in the formation of Schiff bases as reported by Chelh et al.[35] Unfortunately, the current technique does not allow establishing the precise contribution of each route to the formation of these fluorescent products.

The back-fat replacement by avocado oil produced a protective effect against the formation of Schiff bases during the manufacture and cooking of patties, because the amount of Schiff bases was significantly lower in the raw and cooked AC patties than in the PC counterparts. However, no effect was observed on the subsequent chilled storage. Therefore, it is reasonable to consider that lipid oxidation aldehydes formed in AC patties during heat treatment could interact also with proteins contributing to yield higher quantities of Schiff bases. The same tendency was found in cooked rhea meat stored for 28 days under vacuum-packaging.[36]

The addition of the avocado extract caused an apparent increase of fluorescent products in raw PT and AT patties. This increase may be attributed to the natural fluorescence emitted by the compounds found in the avocado extract, such as procyanidins and flavonols. The loss of the natural fluorescence of procyanidins and flavonols upon heating could explain the lack of interferences caused by these compounds in CO and CC patties. In fact, the avocado extract had a protective effect against the formation of fluorescent oxidation products in PT and AT patties during cooking and chilled storage.

The addition of the avocado extract is a successful strategy for inhibiting the formation of Schiff bases during the processing and storage of patties. It is of interest to minimize the formation of Schiff bases as these compounds involve progressive conjugation and/or cross-linking, leading to protein denaturation, polymerization, aggregation insolubilization, and impaired functionality.[5,16] Previous studies have linked massive carbonylation and formation of cross-linking to the loss of water-holding capacity in meat subjected to frozen storage[37] and to textural problems in meat emulsions.[7,18] Another adverse effect generated by protein aggregation is the decrease of protein susceptibility to digestive proteases, with a negative impact on the nutritional value of meat products.[30]

Intriguingly, fluorescence spectroscopy is generally used to quantify Schiff bases as a measurement of lipid oxidation but not to assess protein oxidation or the efficiency of antioxidant strategies against these chemical modifications.[13,30,35,36] The present work demonstrates that this method is a suitable and easy way to assess protein oxidation and the protective effect of antioxidant strategies. A positive and significant correlation was also found between the formation of AAA and Schiff base formation, reflecting the fact that both are secondary oxidation products of lysine.

Lipid–Protein Oxidation Interactions. Samples from the present study were also analyzed for hexanal content to shed light on the likely interactions between lipids and proteins during oxidative reactions. As displayed in Table 6, the hexanal

Table 7. Pearson Correlations between Measured Parameters[a]

	AAS	AAA	TRYP
hexanal	0.81***	0.58*	0.69**
Schiff base	0.48**	0.62**	−0.30*
AAS		0.65**	−0.75**
AAA			−0.39*

[a]Data from $n = 72$ patties employed for calculation of Pearson correlations. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

levels increased significantly in P and A patties as a consequence of cooking and subsequent refrigerated storage. In agreement with the protein oxidation measurements, the replacement of back-fat with avocado oil increased the oxidative stability of the lipids from the patties despite the increase in fatty acid unsaturation. As aforementioned, the incorporation of antioxidant compounds from avocado oil to the patties, namely, phenolics, chlorophylls, and tocopherols, may compensate for the significant increase of PUFA in A patties compared to C patties. Likewise, the phenolic-rich avocado extract inhibited the formation of hexanal in PC patties. Consistent with AAS results, the effect of such avocado extract in A patties was not significant. The reliability between the formation of hexanal and in particular lysine-derived oxidation products such as AAS highlights the timely interaction between lipid and protein oxidation and is supported by significant and positive correlations ($r = 0.81$; $p < 0.001$). The potential connection between proteins and oxidizing lipids has been a recurring topic of interest for food scientists. Recent studies support the

proposal that primary and secondary lipid oxidation products may affect food proteins, leading to the oxidative deterioration of the latter, including the formation of proteins carbonyls such as AAS.[5] The present results suggest that avocado phenolics may have protected proteins against oxidation by two mechanisms: (i) directly, by inhibiting the oxidation of particular amino acid residues such as tryptophan and lysine; and (ii) indirectly, by inhibiting lipid oxidation and, hence, hindering the potential pro-oxidant effect of oxidizing lipids on meat proteins.

In conclusion, the methodology employed enables the analysis of specific protein oxidation products never before described in food systems. The assessment of such specific oxidation markers allows a better understanding of the protein oxidation mechanisms and the potential consequences in food systems. The efficiency of the strategies against protein oxidation depends on the protein target and oxidation route, which highlights the specificity of the antioxidant actions of avocado phytochemicals. Even though avocado byproducts were not able to inhibit all chemical changes induced by protein oxidation, phenolic-rich avocado extracts could be recommended as functional ingredients as they contribute to enhance the nutritional and technological value of the meat products through their antioxidant actions. Further studies are required to fully comprehend the exact interaction mechanisms of avocado phenolics with meat proteins to develop more efficient antioxidant strategies using these phytochemicals.

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