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ORIGINAL PAPER

Formation of N^{ϵ}-carboxymethyllysine and N^{ϵ}-carboxyethyllysine in ground beef during heating as affected by fat, nitrite and erythorbate

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Abstract Dietary advanced glycation endproducts (AGEs), such as N^{ϵ}-carboxymethyllysine (CML) and N^{ϵ}-carboxyethyllysine (CEL), may promote aging and increase the risk for development of various chronic diseases. The effects of beef fat (8.3 – 32.4 %), sodium nitrite (50 – 150 mg/kg) and sodium erythorbate (100 – 1000 mg/kg) contents on the formation of free and protein-bound AGEs including CML and CEL in ground beef during heating (100 °C, 0 – 60 min) were investigated. The proportion of free AGEs accounted for 1.1 – 8.7 % of the sum of free and protein-bound AGEs, and decreased as the severity of the heat treatments increased. The amounts of free CML and CEL formed in ground beef (based upon protein weight) during heating were not affected by the levels of beef fat or sodium nitrite, but slightly decreased with the addition of 100 – 400 mg/kg erythorbate. The formation of protein-bound CML and CEL during heating was promoted with the addition of beef fat and erythorbate, but inhibited by nitrite. The overall effects of fat, nitrite and erythorbate on AGEs formation were relatively small compared to that of heat treatments. This study provides a greater understanding of the compositional factors involved in the formation of AGEs in complex foods during heating, which may help the optimization of thermal processes so that lower levels of AGEs are formed during food production operation.

Keywords Beef · Advanced glycation endproducts · Thermal process · Nitrite · Erythorbate · Fat

Introduction

Advanced glycation endproducts (AGEs) in foods have been linked to accelerating aging and increasing risks for diabetes, kidney diseases and other health problems [1]. Typical AGEs in foods include N^{ϵ}-carboxymethyllysine (CML), N^{ϵ}-carboxyethyllysine (CEL), pyrraline, and pentosidine, while CML is the most widely studied one. The amounts of AGEs in food could be greatly increased by heat treatments, depending on the severity and nature of any thermal process that may be applied to the food [2–4]. Food composition could also affect the levels of AGEs. Food macronutrients including proteins, carbohydrates and lipids serve as reactants for the Maillard reaction and lipid peroxidation, two reactions that promote AGEs formation in foods [5, 6]. Foods with high levels of proteins normally contain higher amounts of AGEs than lower protein foods, specifically foods with high levels of lysine, arginine or cysteine. Therefore it is anticipated that muscle foods would have higher concentrations of AGEs than fruits and vegetables on a weight basis [3]. The amounts of AGEs are high in foods with high polyunsaturated fatty acids content [6–8], suggesting that lipid peroxidation plays an important role in AGE formation; however, there are few reports on the levels of AGEs as affected by the animal fat content naturally

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present in meat. Other food components such as antioxidants and pro-oxidants, may inhibit or promote the formation of AGEs in foods during processing and storage [9].

Nitrite and erythorbate (D-isoascorbate) are widely used in processed meat products. The major function of nitrite or nitrous acid in meat is to provide a stable red or pink color through the formation of NO which reacts with myoglobin to form nitrosylmyoglobin. Nitrite can also act as an antioxidant, inhibit the growth of some microorganisms, and provide a pleasant cured flavor [10]. Erythorbate is the stereoisomer of ascorbate, and these two compounds are used interchangeably in meat to facilitate a fast formation and then retention of a red or pink color, prevent the formation of carcinogenic nitrosamine, and reduce lipid oxidation [11–13]. Fleenor et al. [14] showed that nitrite could reduce oxidative stress-induced AGEs formation in live mice, but the effect of nitrite on AGEs in food or model systems is unclear. The role of ascorbate or ascorbic acid on AGEs formation in foods is also uncertain, since study results from different research groups varied greatly [15–18]. Ascorbate or ascorbic acid could inhibit glycation reactions in bovine albumin-glucose/fructose model systems [15], but had little or no effect on the formation of CML in saccharide-lysine systems during microwave heating [16]. Ascorbate could also serve as a reactant to form CML and CEL [17, 18], and thus promote AGEs formation. Although nitrite and erythorbate are among the most commonly used additives in meat products, to the best of our knowledge, there is no systematic study on the effects of these compounds on the formation of AGEs in meat or other food products, specifically during heating.

The majority of reported studies about the formation of AGEs in food have been focused on protein-bound (protein glycation adducts) CML or the sum of free (glycated amino acids) and protein-bound CML. There are very limited published data on other important AGEs such as CEL [2, 19], and there is especially a lack of study on free AGEs in muscle foods. Since different types (e.g. CML vs. CEL) and forms (free vs. protein-bound) of AGEs in food may have different bioavailability and physiological effect [5, 19], it is important to quantify each type of free and protein-bound AGEs separately.

The objective of this study was to investigate the formation of free and protein-bound CML and CEL in ground beef during heating as affected by beef fat content (8–32 %), sodium nitrite (50–150 mg/kg), and sodium erythorbate (100–1000 mg/kg). A validated HPLC–MS/MS method instead of the most commonly used immunoassay was employed for much more accurate quantification of AGEs in ground beef [20], although the chromatographic method is quite costly and requires 2–3 days for the sample preparation. This study provides a greater understanding of the compositional factors involved in the formation of AGEs in complex foods during heating, which may help the optimization of thermal processes so that lower levels of AGEs are formed during food production operation.

Materials and methods

Reagents

CML (98 %), CEL (98 %) and d_4-CML (98 %) were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada), and sodium erythorbate was from J&K Scientific Ltd. (Shanghai, China). HPLC grade normal hexane, methanol, chloroform, formic acid and ammonium acetate, as well as analytical grade trichloroacetic acid, hydrochloric acid, sodium nitrite, sodium hydroxide, sodium borohydride, sodium tetraborate decahydrate and boric acid were purchased from Sinopharm (Shanghai, China). Standard solutions including AGEs standard mixture (CML 300 μg/L, CEL 300 μg/L, and d_4-CML 400 μg/L) and the internal standard (d_4-CML 8 mg/L) in methanol–water (80:20, v/v), as well as sodium erythorbate water solution (100 mg/mL) and sodium nitrite water solution (25 mg/mL) were prepared right before each experiment.

Preparation of ground beef samples

About 4 kg of ground beef (Khorchin, Shanghai, China) was mixed in a blender (8010s, Waring, Inc, Torrington, Connecticut, USA) at low speed three times with about 10 s each time. The ground beef was then sealed in plastic bags (about 500 g/bag), stored at −80 °C, and thawed overnight in a refrigerator (about 4 °C) before further testing.

To evaluate the effects of sodium nitrite and sodium erythorbate on the formation of AGEs, 240–720 μL of sodium nitrite solution (25 mg/mL) or 120–1200 μL of sodium erythorbate solution (100 mg/mL) was added to 120 g of ground beef to obtain beef samples with different amounts of sodium nitrite (50, 100 and 150 mg/kg) or sodium erythorbate (100, 400 and 1000 mg/kg).

To investigate the influence of beef fat content on the formation of AGEs, two levels of beef fat were added into ground beef (15:85, 30:70 w/w). The moisture, fat and protein contents in ground beef and beef fat were determined in triplicate using AOAC methods [4, 21].

Heat treatments

Ground beef (ca. 13.0 g) was sealed in a cylindrical aluminum cell, heated at 100 °C for 5, 10, 30, or 60 min in boiling water, and rapidly cooled down in ice-water [4, 22]. The heat treated sample was mixed well with a mortar and pestle prior to AGEs analysis [4]. Each thermal treatment was

repeated 3–4 times on different days and duplicated extracts for AGEs were conducted for each heat treated sample.

Sample preparation for analyses of free N^{ε}-carboxymethyllysine and N^{ε}-carboxyethyllysine

Sample preparation for free AGEs analyses was optimized based upon a water extraction method [19, 23]. About 1.0000 g of raw or cooked beef was mixed well with 10 mL pre-cooled 2% (v:v) trichloroacetic acid aqueous solution and 100 µL d_4-CML (8 mg/L), and then homogenized (F6/10, Superfine Homogenizers, Fluko Equipment Ltd., Shanghai, China; 15,000 rpm) for three times at about 8 s each time. After standing for 20 min at 4 °C, the mixture was centrifuged (TDL-5-A, Shanghai Anting Scientific Instrument Factory, Shanghai, China) at 5000 rpm (4,472 g) for 20 min to precipitate protein. The supernatant was then vigorously mixed with the same volume of n-hexane, and further centrifuged for 10 min to remove fat and precipitate the residual protein. Following this, the target compounds in aqueous layer were recovered and further cleaned up with an MCX cartridge (60 mg/3mL, Shanghai ANPEL Scientific instrument Co., Ltd, Shanghai, China), dried under nitrogen with an evaporator (DC12H, Shanghai ANPEL Scientific Instrument Co., Ltd, Shanghai, China) in a water bath of 60 °C, reconstituted in methanol–water (80:20, v:v), and filtered through a 0.22 µm filter [23].

Sample preparation for analyses of protein-bound N^{ε}-carboxymethyllysine and N^{ε}-carboxyethyllysine

Sample preparation for determination of protein-bound CML and CEL was conducted using an acid hydrolysis method combined with solid phase extraction [23–25], and followed the procedure described in our previous study [23]. In brief, ground beef sample was reduced with sodium borohydride for 8 h, mixed with methanol-chloroform (1:2, v:v) and centrifuged to remove fat and precipitate protein. Following this, the protein was hydrolyzed in HCl at 110 °C for 24 h, spiked with internal standard, vacuum dried and reconstituted in water. The target compounds were further purified with an MCX cartridge, dried under nitrogen at 60 °C, reconstituted in methanol–water, and filtered through a 0.22 µm filter [23].

HPLC-MS/MS analysis

A Waters 2695 HPLC system (Waters Inc., Milford, MA, USA) equipped with an Atlantis hydrophilic interaction liquid chromatography (HILIC) silica column (150 mm × 2.1 mm, 3 µm; Waters Inc.) and a Waters Quattro Micro triple-quadrupole tandem mass spectrometer (MS/MS) (Waters Inc., Milford, MA, USA) were used to determine the amounts of free and protein-bound CML and CEL in sample extracts. The experimental procedure as well as the parameters used for HPLC and mass spectrometer were based upon our previous study [4, 23].

Statistical analysis

One-way analysis of variance was conducted (SPSS Version 19, IBM Corp. Armonk, NY) to evaluate whether there was significant difference ($p < 0.05$) in the average level of each tested AGE among ground beef samples varied in fat, nitrite, or erythorbate concentration.

Results and discussion

Ground beef composition

The base ground beef contained 69.3% water, 8.3% fat, and 21.5% protein; beef fat contained 9.1% water, 88.7% fat, and 2.5% protein. The composition of the formulated ground beef with 15% beef fat added was calculated to be: 60.3% water, 20.4% fat and 18.7% protein; and that with 30% fat added included: 51.2% water, 32.4% fat, and 15.8% protein.

N^{ε}-carboxymethyllysine and N^{ε}-carboxyethyllysine in raw and heat treated beef

The sensitivity and recovery for analyses of free CML and CEL [23] as well as protein-bound CML and CEL [4] in ground beef with the HPLC-MS/MS method were reported in our previous studies.

The majority of AGEs in raw beef (8.3% fat, 21.5% protein) existed as protein adducts, and the sum of free CML and free CEL only accounted for about 8.7% of the sum of free and protein-bound AGEs (Fig. 1). The average levels of free CML and free CEL in raw beef were 0.34 mg/kg (±0.05 mg/kg) and 0.44 mg/kg (±0.10 mg/kg), respectively; while the levels of protein-bound CML and CEL in raw beef were 4.31 ± 1.09 mg/kg and 4.66 ± 1.12 mg/kg, respectively. The majority of lysine (the amino acid required for the formation of CML and CEL in meat) is incorporated into peptide chains instead of existing as free amino acid in beef, which may lead to a much higher level of protein-bound CML or CEL than either free CML or CEL.

Heating caused large increases in protein-bound AGEs, with much less of an effect on the amounts of free AGEs in ground beef. For example, after heating for 5 min at 100 °C, the amounts of both protein-bound CML and CEL in beef increased 158% and 172%, respectively; while after 60 min of heating, an average 10-fold increase in protein-bound CML and sixfold increase in protein-bound CEL

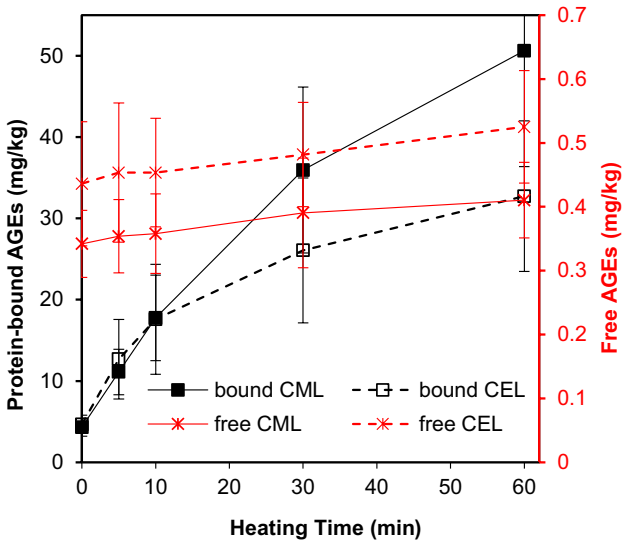

Fig. 1 The amounts of free and protein-bound N^{ε}-carboxymethyllysine (CML) and N^{ε}-carboxyethyllysine (CEL) in ground beef (8.3 % fat, 21.5 % protein) formed during heating. The data were mean of ten replicate and the vertical bars were standard deviation

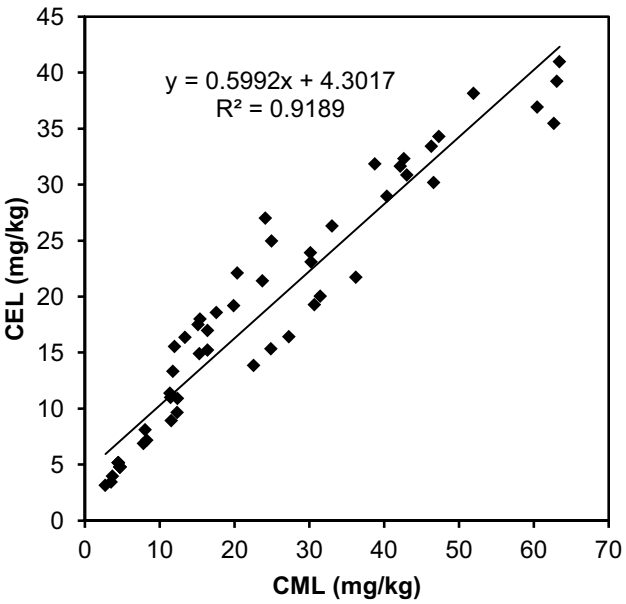

Fig. 2 The concentrations of protein-bound N^{ε}-carboxymethyllysine (CML) versus (N^{ε}-carboxyethyllysine) CEL in raw and heat treated ground beef

were observed. By comparison, there was only about a 20 % increase in free CML or CEL following a 60 min heat treatment. Since heat treatments had much greater impact on the levels of protein-bound AGEs than free AGEs, the percentage of free AGEs relative to the total AGEs in ground beef decreased with heating time and only accounted for 1.1 % of total tested AGEs after 60 min of heating. Very few studies have examined both free and protein-bound AGEs in foods [2, 19, 23], and no explanation has been proposed on how heating affects their formation differently. We propose that although the formations of free CML or CEL (lysine as one of the reactants) and protein-bound CML or CEL (lysine residues in peptides as reactant) follow similar pathways through the Maillard reaction and lipid peroxidation [5, 6], the α-amino group and α-carboxylic acid group in addition to the ε-amino group in lysine molecule make it quite reactive in participating various reactions other than those leading to the formation of AGEs; therefore, heating did not promote the formation of free CML or CEL in ground beef as much as that for protein-bound CML or CEL.

The amounts of protein-bound CML and CEL in raw beef were quite similar, and the formation rates of these protein-bound AGEs during the first 10 min of heating were also similar (Fig. 1). However, as the heating time was increased to 30 or 60 min, the formation rate of CML was faster than that for CEL, resulting in 20 % more CML than CEL in beef after heating for 60 min. The amounts of protein-bound CML was linearly correlated with CEL for all samples including ground beef with added fat, nitrite or erythorbate (Fig. 2, $R^2 = 0.919$). Similar correlation was found in an earlier study [4] ($R^2 = 0.920$) for ground beef heated at various temperatures (65–100 °C) and times (0–60 min). However, no obvious linear or non-linear relationship was found between the amounts of free CML and free CEL in this study.

Effects of fat content on the levels of N^{ε}-carboxymethyllysine and N^{ε}-carboxyethyllysine

Table 1 shows the effects of fat content on the levels of free and protein-bound CML and CEL in heat treated ground beef. Based upon protein weight, the amounts of free CML and CEL in ground beef formed during heating were not affected by the beef fat content. The slightly higher content of free CEL in heat treated beef with 32.4 % fat was due to the initial higher level of free CEL in that sample of raw beef (2.02 ± 0.26 mg/kg protein) compared to the raw beef samples containing 8.3 % fat (CEL:1.68 ± 0.12 mg/kg protein) and 20.4 % fat (CEL:1.79 ± 0.03 mg/kg protein). For protein-bound CML and CEL, ground beef containing higher fat in general led to a somewhat higher levels of AGEs (based upon protein-weight) formed during heating, although after the same heat treatment, the levels of protein-bound CML or CEL in ground beef varying in fat content were very likely not significant different. As indicated in several other studies, the formation of AGEs in foods is mainly caused by glycation tied to the Maillard reaction, with lipid peroxidation also contributing to the formation of AGEs; AGEs was higher in foods with a greater proportion of unsaturated fat [6–8]. Since beef fat used here has a relatively high proportion of saturated fatty acids than

Table 1 Effects of fat content on the levels of free and protein-bound N^{ε}-carboxymethyllysine (CML) and N^{ε}-carboxyethyllysine (CEL) in heat treated (100 °C, 0–60 min) ground beef ($n = 3$, based upon mg/kg sample weight and mg/kg protein weight)

Heating time (min)	Free AGEs (mg/kg)		Bound AGEs (mg/kg)		Free AGEs (mg/kg protein)		Bound AGEs (mg/kg protein)	
	CML	CEL	CML	CEL	CML	CEL	CML	CEL
8.3% Fat								
Raw	0.35 ± 0.02	0.34 ± 0.03	3.68 ± 0.42	3.98 ± 1.16	1.61 ± 0.08	1.68 ± 0.12	17.14 ± 1.96	18.52 ± 5.39
5	0.38 ± 0.07	0.37 ± 0.05	8.04 ± 0.28	8.11 ± 1.27	1.75 ± 0.33	1.67 ± 0.23	37.40 ± 1.28[a]	37.71 ± 5.91
10	0.39 ± 0.08	0.39 ± 0.02	12.37 ± 1.75	10.91 ± 2.03	1.81 ± 0.36	1.80 ± 0.10	57.55 ± 8.15[a]	50.76 ± 9.45
30	0.44 ± 0.09	0.43 ± 0.06	27.26 ± 9.63	16.43 ± 2.79	2.00 ± 0.43	1.99 ± 0.29	126.80 ± 44.81	76.41 ± 12.96
60	0.47 ± 0.04[a]	0.46 ± 0.04[a]	36.20 ± 10.41	21.73 ± 3.46	2.17 ± 0.17	2.11 ± 0.19	168.38 ± 48.43	101.06 ± 16.10[a]
20.4% Fat								
Raw	0.34 ± 0.03	0.33 ± 0.01	3.50 ± 0.71	3.45 ± 0.71	1.83 ± 0.15	1.79 ± 0.03	18.71 ± 3.81	18.47 ± 3.79
5	0.33 ± 0.05	0.33 ± 0.05	8.24 ± 0.36	7.18 ± 0.68	1.78 ± 0.25	1.75 ± 0.26	44.05 ± 1.91[b]	38.42 ± 3.64
10	0.35 ± 0.04	0.35 ± 0.02	12.31 ± 0.62	9.65 ± 0.83	1.86 ± 0.23	1.86 ± 0.09	65.85 ± 3.33[a,b]	51.59 ± 4.43
30	0.34 ± 0.06	0.38 ± 0.03	24.85 ± 6.05	15.34 ± 1.56	1.84 ± 0.32	2.02 ± 0.13	132.87 ± 32.34	82.02 ± 8.37
60	0.38 ± 0.06[b]	0.42 ± 0.04[a,b]	31.45 ± 4.65	20.05 ± 1.19	2.01 ± 0.30	2.22 ± 0.19	168.17 ± 24.86	107.22 ± 6.34[a,b]
32.4% Fat								
Raw	0.28 ± 0.05	0.32 ± 0.04	2.73 ± 0.20	3.15 ± 0.53	1.79 ± 0.32	2.02 ± 0.26	17.25 ± 1.29	19.93 ± 3.37
5	0.28 ± 0.05	0.33 ± 0.03	7.81 ± 0.35	6.89 ± 0.87	1.77 ± 0.31	2.09 ± 0.18	49.44 ± 2.20[c]	43.59 ± 5.52
10	0.29 ± 0.04	0.34 ± 0.04	11.53 ± 0.68	8.92 ± 0.69	1.84 ± 0.26	2.16 ± 0.27	72.99 ± 4.32[b]	56.48 ± 4.34
30	0.31 ± 0.05	0.36 ± 0.03	22.54 ± 2.13	13.85 ± 0.51	1.94 ± 0.30	2.27 ± 0.18	142.64 ± 13.46	87.65 ± 3.24
60	0.34 ± 0.05[b]	0.37 ± 0.04[b]	30.67 ± 1.61	19.28 ± 0.86	2.13 ± 0.31	2.35 ± 0.23	194.13 ± 10.17	122.02 ± 5.46[b]

[a,b,c]The average levels of AGEs with different letters were significant different ($p < 0.05$) among samples with different fat content but subjected to the same heat treatment. Data are presented as mean ± standard deviation of triplicate treatments and duplicate analyses for AGEs conducted for each treatment

Table 2 Effects of sodium nitrite and heat treatments (100 °C, 0–60 min) on the levels of free and protein-bound N^{ε}-carboxymethyllysine (CML) and N^{ε}-carboxyethyllysine (CEL) in ground beef ($n = 3$, based upon mg/kg sample weight)

Sodium nitrite (mg/kg)	Heating time (min)	Free AGEs (mg/kg)		Bound AGEs (mg/kg)	
		CML	CEL	CML	CEL
Raw meat		0.39 ± 0.05	0.39 ± 0.02	4.46 ± 1.50	5.18 ± 1.00
0 (control)	5	0.39 ± 0.03	0.39 ± 004	13.35 ± 2.52	16.38 ± 4.11[a]
	10	0.38 ± 0.06	0.42 ± 0.08	20.36 ± 4.39	22.12 ± 5.25[a]
	30	0.43 ± 0.07	0.44 ± 0.04	38.76 ± 8.37	31.85 ± 6.33[a]
	60	0.41 ± 0.05	0.49 ± 0.08	51.94 ± 5.69	38.16 ± 4.5[a]
50	5	0.36 ± 0.07	0.41 ± 0.07	11.95 ± 1.87	15.55 ± 3.25[a]
	10	0.37 ± 0.02	0.42 ± 0.04	17.57 ± 2.72	18.57 ± 1.34[a,b]
	30	0.40 ± 0.06	0.42 ± 0.03	30.16 ± 4.82	23.91 ± 3.54[b]
	60	0.38 ± 0.02	0.46 ± 0.06	43.02 ± 7.07	30.85 ± 2.98[b]
100	5	0.35 ± 0.02	0.36 ± 0.05	11.43 ± 2.00	11.01 ± 0.82[b]
	10	0.37 ± 0.02	0.38 ± 0.05	16.40 ± 0.94	15.23 ± 1.70[b]
	30	0.38 ± 0.05	0.45 ± 0.05	30.24 ± 5.32	23.10 ± 1.16[b]
	60	0.38 ± 0.02	0.45 ± 0.08	42.14 ± 4.77	31.65 ± 2.47[b]
150	5	0.34 ± 0.03	0.40 ± 0.04	11.36 ± 1.26	11.39 ± 0.27[b]
	10	0.36 ± 0.03	0.39 ± 0.03	16.38 ± 2.17	16.98 ± 1.42[a,b]
	30	0.39 ± 0.03	0.43 ± 0.05	33.04 ± 4.81	26.32 ± 2.21[a,b]
	60	0.39 ± 0.04	0.48 ± 0.06	42.65 ± 7.41	32.32 ± 0.89[b]

[a,b]The average levels of AGEs with different letters were significant different ($p < 0.05$) among samples with different levels of nitrite added but subjected to the same heat treatment. Data are presented as mean ± standard deviation of triplicate treatments and duplicate analyses for AGEs conducted for each treatment

the vegetable oils or fatty acids used in other studies [6–8], the degree of lipid peroxidation during heating would have occurred to a less extent in our study with an anticipated lower amount of AGEs formed compared to what had been observed in the studies cited here.

In our previous study [23], we found that fat content in pork and beef cuts from different animal parts and companies had little or no effect on the amounts of protein-bound CML and CEL formed during commercial sterilization (121 °C, 10 min). This study based on ground beef added with different levels of beef fat confirmed that the formation of CML and CEL during heating were not or slightly affected by beef fat content. The finding that the amounts of free CML and CEL formed during heating were not affected by beef fat content in ground beef is significant, filling the avoid of understanding of the free AGEs formation in muscle foods during heating.

When the data were analyzed based on sample weight instead of protein weight, a different trend was observed. The levels of free and protein-bound CML and CEL in beef containing a higher fat content (for both raw and heat treated samples) were generally lower than that in ground beef with a lower fat content on a sample weight basis (Table 1). This was probably because that the ground beef with a higher fat content had proportionally less lysine and lysine residue by weight, and therefore lower concentrations of reactants directly responsible for CML and CEL formation. The Maillard reaction likely played a more important role than lipid peroxidation in AGEs formation in meat, although both reactions could produce glyoxal or methylglyoxal which react with lysine and lysine residue to form CML or CEL [5, 6].

Effects of sodium nitrite

Sodium nitrite did not affect the levels of free CML and CEL in heat treated ground beef; however, nitrite inhibited protein-bound AGEs formation, particularly protein-bound CEL (Table 2). The addition of sodium nitrite resulted in an average 10% (50 ppm nitrite, 5 min heating) to 22% decrease in protein-bound CML (50 or 100 ppm nitrite, 30 min heating) depending upon the heating time and the nitrite level. Changing the nitrite concentration from 50 to 150 ppm had little effect on protein-bound CML formation. However, nitrite inhibition on protein-bound CEL formation was concentration dependent. At 50 mg/kg nitrite, less inhibition was observed in ground beef heated for 5–10 min, but with longer heating (30–60 min) results were similar to those at higher nitrite levels. The addition of 100 mg/kg nitrite resulted in a significant decrease (17–33%) of protein-bound CEL in all heat treated ground beef samples compared to the control, and was slightly more effective

Table 3 Effects of sodium erythorbate and heat treatments (100 °C, 0–60 min) on the levels of free and protein-bound N^{ε}-carboxymethyllysine (CML) and N^{ε}-carboxyethyllysine (CEL) in ground beef ($n = 4$, based upon mg/kg sample weight)

Erythorbate (mg/kg)	Heating time (min)	Free AGEs (mg/kg)		Bound AGEs (mg/kg)	
		CML	CEL	CML	CEL
Raw meat		0.30 ± 0.03	0.52 ± 0.10	4.68 ± 1.16	4.79 ± 1.20
0 (control)	5	0.31 ± 0.04	0.57 ± 0.07[a]	11.75 ± 2.03	13.33 ± 4.97
	10	0.31 ± 0.03	0.52 ± 0.07	19.87 ± 5.15	19.21 ± 6.88
	30	0.32 ± 0.05	0.55 ± 0.08	40.34 ± 9.74	28.98 ± 8.29
	60	0.36 ± 0.02	0.60 ± 0.06[a]	60.44 ± 13.31	36.93 ± 7.85
100	5	0.27 ± 0.06	0.48 ± 0.07[b]	15.31 ± 2.55	14.90 ± 2.35
	10	0.29 ± 0.04	0.46 ± 0.03	23.72 ± 8.34	21.41 ± 4.04
	30	0.28 ± 0.05	0.49 ± 0.04	46.63 ± 14.64	30.20 ± 4.62
	60	0.28 ± 0.07	0.49 ± 0.05[b]	62.69 ± 20.97	35.47 ± 3.09
400	5	0.24 ± 0.07	0.44 ± 0.02[b]	15.09 ± 4.09	17.51 ± 4.27
	10	0.27 ± 0.07	0.46 ± 0.08	24.13 ± 7.69	27.03 ± 6.54
	30	0.28 ± 0.07	0.48 ± 0.06	46.31 ± 17.29	33.42 ± 7.96
	60	0.29 ± 0.07	0.55 ± 0.07[a,b]	63.43 ± 21.28	41.00 ± 9.52
1000	5	0.33 ± 0.10	0.49 ± 0.06[a,b]	15.36 ± 3.89	17.99 ± 4.60
	10	0.32 ± 0.03	0.47 ± 0.06	24.95 ± 6.57	24.96 ± 6.22
	30	0.31 ± 0.05	0.52 ± 0.04	47.28 ± 15.66	34.31 ± 9.77
	60	0.36 ± 0.05	0.62 ± 0.09[a]	63.08 ± 22.60	39.25 ± 8.70

[a,b]The average levels of AGEs with different letters were significant different ($p < 0.05$) among samples with different levels of erythorbate added but subjected to the same heat treatment. Data are presented as mean ± standard deviation of quadruplicate treatments and duplicate analyses for AGEs conducted for each treatment

than that of 150 mg/kg on reducing the levels of protein-bound CEL.

Nitrite or nitrous acid in meat can form NO and NO_2 [10]. NO further reacts with myoglobin to form nitrosylmyoglobin; NO_2 reacts with water to form HNO_2 and HNO_3. Nitrite in meat can function as an antioxidant not only because of NO and HNO_2, which can be easily oxidized [10]. As pointed out by Morrissey and Tichivangana [26], nitrosylmyoglobin and nitrite can form stable complex with iron, preventing the release of heme iron and inhibiting the catalytic activity of free iron for oxidation. The antioxidant activity of nitrite may slow down the oxidative processes during AGEs formation, and thus reduce the amounts of AGEs formed during heating [9].

Effects of sodium erythorbate

The effects of sodium erythorbate on free and protein-bound CML and CEL were quite different (Table 3). The addition of 100 and 400 mg/kg of erythorbate resulted in slight decrease in both free CML and CEL compared to the control, while adding 1000 mg/kg erythorbate did not affect the levels of free AGEs. A somewhat increase (although not significantly different) in protein-bound CML and CEL during heating was observed when sodium erythorbate was added. The effects of erythorbate on the amount of free and protein-bound AGEs showed different trends and were dependent of erythorbate concentration. This may be tied to the multiple roles erythorbate played as an antioxidant, pro-oxidant and reactant with lysine or lysine residue to form CML and CEL in complex muscle food systems.

As a stereoisomer of ascorbate, erythorbate has similar functionalities as ascorbate [13, 27]. In particular, erythorbate can also function as an antioxidant or pro-oxidant depending on its concentration, presence of metal ions (such as Cu^{2+}, Fe^{3+}), pH and other factors; while the dual-role of erythorbate may lead to opposite effects on AGEs formation as antioxidants in general may inhibit the formation of AGEs and pro-oxidants promote their formation [28–30]. What is more, unlike other antioxidants or pro-oxidants, ascorbate and erythorbate are reducing carbohydrates which can react with lysine and lysine residue, leading to the formation of CML and CEL [17, 18, 31]. The study of Dunn et al. [17] showed the formation of CML due to the reactions between ascorbate and lysine. They proposed that ascorbic acid was oxidized to dehydroascorbic acid (DHA), degraded to threose which reacted with lysine to form Schiff base, followed by Amadori rearrangement and oxidative cleavage to form CML. Smuda and Glomb [31] proposed a degradation pathway of ascorbic acid that leading to the formation of methylglyoxal, a precursor of CEL as follows. The oxidized form of ascorbic acid, DHA was hydrolyzed to

2,3-diketogluconic acid (DKG), which underwent isomerization to form 2,4-DKG, 3,4-DKG, and 3,5-DKG. From 3,5-DKG, glyceric aldehyde was formed through β-dicarbonyl cleavage, followed by the formation of methylglyoxal after elimination of water.

Conclusions

The proportion of free CML and CEL accounted for 1.1–8.7% of the sum of free and bound CML and CEL, and decreased as the severity of heat treatments increased. The amounts of free CML and CEL in ground beef (based upon protein weight) formed during heating were not affected by beef fat content and sodium nitrite, and 60 min of heat treatment at 100 °C resulted in no more than 20% increase in free CML or CEL. However, heating resulted in much significant change in the amounts of protein-bound AGEs in ground beef, as much as 6–10-fold increase in protein-bound CML and CEL after 60 min of heat treatment. The effects of sodium erythorbate on the formation of CML and CEL were complicated depending upon the amount of erythorbate added and the forms of AGEs tested. Although beef fat content, nitrite and erythorbate may inhibit or promote the formation of CML and CEL in ground beef during heating, the severity of heat treatment (such as temperature and time) had a much greater impact than these three single factors on AGEs formation.

The formation of AGEs would also be affected by other compounds or ingredients such as sugars, salts, alcohol (e.g. cooking wine), and acids (e.g. vinegar and lemon juice) in a processed meat; the effects of these compounds or ingredients on AGEs formation during manufacture or retail/consumer preparation of processed meat are still waiting to be explored. In addition, the combined effects of various ingredients, such as combination of different levels of nitrite, erythorbate and salt, as well as the effects of storage before (such as cured meat stored at different temperatures for different periods of time) and after heating remain unknown. The effects of three single factors on the formation of AGEs reported in this study serve as a foundation for further studies that will improve our understanding of the combined effects of different components in a food formulation on the AGEs formation during heating.

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