1   **Formation of free and protein-bound carboxymethyllysine and carboxyethyllysine in**
2   **meats during commercial sterilization**

3   XiaohuaSun^a, JumingTang^b, Jing Wang^a, Barbara A Rasco^{*c}, KeqiangLai^a, Yiqun Huang^{*a,c}

4   ^aCollege of Food Science and Technology, Shanghai Ocean University, No. 999 HuchengHuan
5   Road, LinGang New City, Shanghai, China 201306.

6   ^b Department of Biological System Engineering, WashingtonStateUniversity, Pullman,
7   WA99164-6120, USA.

8   ^cSchool of Food Science, WashingtonStateUniversity, Pullman, WA99164-6376, USA.

9   *Corresponding authors:

10   (1-509)335-9447(phone), (1-509)335-4815(fax), rasco@wsu.edu (B. A. Rasco);
11   (86-21)6190-0370(phone), (86-21)6190-03659 (fax), yiqunh@hotmail.com (Y. Huang).

12   **Abstract**

13   The effect of commercial sterilization treatments on the levels of advanced
14   glycationendproducts (AGEs) in meats was investigated. The amounts of both free and
15   protein-bound N^ε-carboxymethyllysine (CML) and N^ε-carboxyethyllysine (CEL) in beef
16   (rump, ribeye, short plate), pork (hind leg, tenderloin, belly), and chicken (chicken breasts,
17   drumsticks) were determined using an HPLC–MS/MS method. Beef and pork had a small
18   proportion (raw < 15%; sterilized < 8%) of free AGEs compared to the total AGEs, but raw
19   chicken breasts had very high levels of free CEL (7.12±9.98 mg/kg; *n*=13) with large
20   biological variation compared to pork (0.19±0.09 mg/kg; *n*=9) and beef (0.44±0.19 mg/kg;
21   *n*=9). Commercialsterilization (121°C for 10 min) did not significantly affect the amounts of
22   free CML or CEL, but led to about 0.6- to 3.6-fold increase of protein-bound CML and CEL.
23   The amounts of protein and fat content in beef or pork had very little effect on the formation
24   ofprotein-bound AGEs during sterilization process.

25   **Keywords:** Advanced glycationendproduct; carboxymethyllysine; carboxyethyllysine; beef;
26   pork; chicken

1. Introduction

Meat is a rich source of advanced glycationendproducts (AGEs) compared to other foods, such as fruits and vegetables. AGEs may promote aging, oxidative stress and inflammation and have been tied to increased risks for diabetes, cardiovascular diseases and pancreatic cancer, although the role of these compounds in the diet and relation to diseases has not yet been clearly established (Nguyen, 2006; Ames, 2007; Uribarri et al., 2010; Sun et al., 2015; Jiao et al., 2015). Glycation is a non-enzymatic modification of proteins or protein derivatives by reducing sugars or sugar derivatives (Rabbani & Thornalley, 2012). AGEs in foods could be formed through the Maillard reaction and lipid oxidation (Fu et al., 1996; Rabbani & Thornalley, 2012), and the levels of AGEs in foods depend upon various factors such as the type of food, food composition, and cooking or processing method (Hull, Woodside, Ames, & Cuskelly, 2012; Chen & Smith, 2015). High protein and/or high fat foods, such as meats, generally contain relatively high levels of AGEs, and heat treatments promote AGEs formation (Goldberg et al., 2004; Uribarri et al., 2010; Sun et al., 2015).

Many different types of AGEs have been found in foods, which were mainly tied to the glycation of lysine, arginine and cysteine residues (Rabbani & Thornalley, 2012), but lysine derived AGE N^ε-carboxymethyllysine (CML) is the most widely studied one and usually used as a marker for AGEs in foods (Goldberg et al., 2004; Uribarri et al., 2010; Hull et al., 2010). Quantification of CML in a large variety of foods prepared with different cooking methods including boiling, frying, roasting/oven-baking, broiling has been reported. For example, Goldberg et al. (2004) used enzyme-linked immunosorbent assay (ELISA) to

determine the amount CML in 250 foods typically consumed by a multiethnic urban population. Uribarri et al.(2010)quantifiedCML in 549 uncooked or cooked foods typically consumed by a Northeastern Americanurban population with ELISA.Takeuchi et al. (2015) applied ELISA to determine glucose-derived AGEs, fructose-derived AGEs, glyceraldehyde-derived AGEs and CML in 1650 beverages and foods commonly consumed in Japan. Hull et al. (2012) employed a more sophisticated instrumental method, ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS), to determine CML in 256 foods commonly consumed in a Northern Irish population,and reported their data in four commonly used forms (such as mg/100 g, and mmol/mol lysine) instead of expressing the data in unitslimited by the use of ELISA methods.The majority of studies on AGEs have been focused on thelevels of CMLin foods as affected by the culinary techniques, such as baking or broiling versus boiling, breading versus non-breading, and addition of sauces or not before the cooking (Chao, Hsu,& Yin, 2009; Hull et al., 2010; Chen & Smith, 2015). There are very few publications on the formation of AGEs in foods during thermal treatments that simulated pasteurization or commercial sterilization conditions commonly used in the food industry (Ahmed et al., 2005; Zhang, Huang, Xiao,& Mitchell, 2011; Sun et al., 2015). In particular, there is no reported study (to the best of our knowledge) on the AGEs formation in meats duringcommercial sterilization, a process commonly employing heat to destroy spores of the pathogenic*Clostridium botulinum*.

The purpose of this study was to investigate the effects of commercial sterilization on the levels of CML and N^ε-carboxyethyllysine (CEL) in beef (rump, ribeye, short plate), pork

69 (hind leg, tenderloin, belly), and chicken (chicken breasts, drumsticks). Since the
70 bioavailability and physiological effects of protein-bound AGEs (protein glycation adducts)
71 and free AGEs (glycated amino acids) are most likely different (Ahmed et al. 2005; Rabbani
72 & Thornalley, 2012), both protein-bound and free CML and CEL in meats were quantified
73 with a validated HPLC–MS/MS method that allows for a more accurate quantification of
74 these AGEs compared to the commonly used immunoassay (Scheijen et al., 2016). What is
75 more, for each type of meat cut, samples produced by three different companies were used to
76 increase the range of variability due to different sample sources.

77 **2. Materials and methods**

78 *2.1. Reagents*

79 AGEs including CML (98%), CEL (98%) and d₄-CML (98%) were purchased from
80 Toronto Research Chemicals Inc. (Toronto, Ontario, Canada) and all other chemicals were
81 from Sinopharm (Shanghai, China). Normal hexane, methanol, chloroform, formic acid and
82 ammonium acetate were HPLC grade, and the others were analytical grade. Methanol-water
83 (80:20, v/v) was used as solvent to prepare AGEs standard solutions including AGEs standard
84 mixture (CML 300 µg/L, CEL 300 µg/L, and d₄-CML 400 µg/L) and the internal standard
85 (d₄-CML 8 mg/L).

86 *2.2. Preparation of beef, pork and chicken samples*

87   Fresh(keep refrigerated, never frozen)meatsincluding beef (rump, ribeye, short plate),
88   pork (hind leg, tenderloin, belly) and chicken (breasts, drumsticks) were purchased from
89   aMetro AG storein Shanghai, China. For each type of meat cut, meatsmanufacturedfrom
90   three different companies were collected. About 400 g of meat wascut into small pieces, and
91   mixed in a blender (8010 s, Waring Inc., Torrington, Connecticut, USA) at low speed 4 times
92   for about 10 s each time.About half of the minced meat was usedfor AGEs analyses and
93   sterilization, and the other half wasstored at -80°C before being used fordetermination
94   ofwater, fat and protein content(Sun et al., 2015).

95   To mimiccommercial sterilization process, ground meat was filledin an aluminum
96   cylindrical cell (diameter, 50 mm; height 5 mm) (Kong, Tang, Rasco, Crapo, & Smiley, 2007;
97   Sun et al., 2015), sealed and heatedat 121°C for 10 min in an oil bath (HAAKE PC 300-S7;
98   Thermo Fisher Scientific Inc.,WalthamMA) with dimethyl silicone (Sinopharm,
99   Shanghai,China) as the heating media.The small custom-design test cell allowed for rapid
100   heat transfer and relatively good heating uniformity for a sample sealed insidethe cell (Kong
101   et al., 2007).The come-up time (time required for the cold spot to reach 121°C) for the meats
102   in the test cells were about 4–5.5 min, and the use of 10 min of heating time was to obtain F_0
103   of 4 min and above as required for sterilizing canned foods with a sufficient safety margin
104   (Heinz &Hautzinger, 2007). Following the heat treatment, the cell was immediately
105   immersed into ice-water to rapidly cool down the sample.

106   2.3. Sample preparation for analyses offreeAGEs

107   A water extraction method based upon the studies of Zhang et al. (2011) and Hegele,
108   Buetler, and Delatour (2008) with modification was used to prepare samples for free AGEs
109   analysis. Instead of using nonafluoropentanoic acid (Zhang et al., 2011; Hegele et al., 2008),
110   trichloroacetic acid was used in this study to precipitate proteins to avoid possible
111   deterioration of column (Schettgen et al., 2007; Sun et al., 2015). The detailed preparation
112   method was as follows. First, a mixture of *ca.* 0.5000–1.0000 g raw or sterile meat (beef, pork
113   and chicken), pre-cooled trichloroacetic acid (2% v/v, 10 mL) and d₄-CML (100 µL, 8 mg/L)
114   was homogenized (F6/10, Superfine Homogenizers, Fluko Equipment Ltd., Shanghai, China;
115   15000 rpm) for about 30 s in an ice-water bath, and then centrifuged (TDL-5-A, Shanghai
116   Anting Scientific Instrument Factory, Shanghai, China) at 5,000 rpm for 20 min to precipitate
117   protein. Next, the supernatant was mixed well with 10 mL of n-hexane, and centrifuged again
118   at 5,000 rpm for 10 min to defat and to precipitate the residual proteins. Following this, 5 mL
119   of the recovered aqueous layer was loaded onto a pre-activated MCX column (60 mg/3 mL,
120   Shanghai ANPEL Scientific Instrument Co., Ltd, Shanghai, China). The column was washed
121   with 3 mL water and 3 mL methanol in sequence. Finally, the target compounds were eluted
122   with 5 mL 5% ammonia in methanol, dried in nitrogen using a nitrogen evaporator (DC12H,
123   Shanghai ANPEL Scientific Instrument Co., Ltd, Shanghai, China) at 60 °C, reconstituted
124   with 1–2 mL methanol-water (80:20, v:v), and filtered through a 0.22 µm filter prior
125   to HPLC–MS/MS analysis. Since the levels of free AGEs in different meats may vary greatly,
126   the amounts of meat sample and solvent used to extract free AGEs were modified based upon
127   the results of our preliminary experiments so that the amounts of free CML and CEL in the

128 final extract were within the linear range of 20–1500 µg/L for their quantification with
129 the HPLC–MS/MS method. Triplicate experiments were conducted.

130 *2.4. Sample preparation for analysis of protein-bound AGEs*

131 A modified acid hydrolysis method was employed to prepare sample for protein-bound
132 AGEs analysis (Assar, Moloney, Lima, Magee, & Ames, 2009; Niquet-Léridon & Tessier,
133 2011), which was described in detail in our previous study (Sun et al., 2015). In brief, a meat
134 sample was incubated with borate buffer (0.2 M, pH 9.2) and sodium borohydride for 8 h,
135 mixed with methanol-chloroform (1:2, v:v) and centrifuged to defat and precipitate proteins.
136 Next, the precipitated proteins were hydrolyzed in hydrochloric acid (HCl) at 110 °C for 24 h.
137 Following this, the diluted protein hydrolysate was spiked with d₄-CML, dried, and
138 reconstituted in water. The sample solution was further cleaned up with an MCX cartridge
139 and 0.22 µm filter. The dilution factor for protein hydrolysate varied depended upon the type
140 of meat analyzed to ensure that the amounts of AGEs in the diluted hydrolysates of both the
141 raw and sterilized meat were within the linear range of 20–1500 µg/L for HPLC–MS/MS
142 analysis. The extraction of each sample was repeated three times.

143 *2.5. HPLC–MS/MS analysis*

144 The amounts of AGEs in meat extracts were determined with a Waters 2695 HPLC
145 system (Waters Corp., Milford, MA, USA) and a Waters Quattro Micro triple-quadrupole

tandem mass spectrometer (MS/MS). An Atlantis hydrophilic interaction liquid chromatography (HILIC) silica column (150 mm×2.1 mm, 3 µm; Waters Corp.) was used in the HPLC system. The collision energy was 20 eV, and cone voltage was 20 V for CEL determination. The product ions at m/z 130 and at m/z 84 were used for quantification and confirmation of CML or CEL, respectively. All other experimental procedures (except for the concentration of AGEs standard mixture and the internal standard) were the same as our previous study (Sun et al., 2015).

The recovery rates for AGEs in each type of meats were determined through spiking ground meat or protein hydrolysates of meats (for protein-bound AGEs) with CML or CEL standard at three different levels (free CML or CEL: 200, 500, 1000 µg/kg; protein-bound CML or CEL in pork or chicken: 100, 500, 1000 µg/kg; protein-bound CML in beef: 390, 1950, 3900 µg/kg; protein-bound CEL in beef: 310, 1550, 3100 µg/kg) and the internal standard d₄-CML (400 µg/L) before the extraction. Ground meat with only d₄-CML added was used as a blank. Each treatment was repeated six times. The calculation of CML or CEL in meat was based on the ratio of its response factor to that of the internal standard (Sun et al. 2015).

2.6. Statistical analysis

Single factor analysis of variance (Excel 2013; Microsoft Corp., Redmond, WA, USA) was applied to determine whether there was significant difference ($p < 0.05$) in AGEs

between different types of raw or sterile meats (such as raw pork versus raw beef), as well as between the raw and sterile meats of the same kind (such as raw versus sterile pork).

3. Results and discussion

3.1. Water, fat and protein contents in beef, pork and chicken

Beef contained 58.9–75.0% water, 0.4–17.1% fat and 16.5–22.2% protein (**Table 1**) (all data presented in the paper were based upon sample weight unless otherwise specified). Fat content varied greatly among the beef samples. Rump meat had the least fat ($\leq 1\%$), while ribeye and short plate had an average fat content of 12.2% and 10.9%, respectively. The amount of water (44.6–75.9%), fat (0.4–39.0%) and protein (13.0–22.4%) in pork from different cuts also varied greatly. Pork belly had the highest fat (27.3–39.0%), and the lowest water (44.6–51.9%) and protein (13.0–14.8%) content. The meat cuts from the hind leg and tenderloin had much low fat content compared to pork belly meat: 4 out of 6 samples had less than 1% fat, and the other two samples contained 8.2% (hind leg) and 1.8% (tenderloin) of fat, respectively. Chicken breasts and drumsticks had relatively low fat (0.3–9.8%) with 70.4–75.4% of water and 18.6–24.2% of protein.

3.2. Recovery of AGEs in beef, pork and chicken for their analyses with HPLC–MS/MS

The limit of detection for CML was 4 $\mu\text{g/kg}$, and for CEL was 5 $\mu\text{g/kg}$ with the HPLC–MS/MS method, while the limit of quantification for CML and CEL were 12 and 15

µg/kg, respectively. **Table 2** shows the recovery of free and protein-bound AGEs in beef, pork, and chicken for the HPLC–MS/MS method. The average recovery of free CML were in the range of 96.4% (±6.4%) to 113.8% (±12.0%), and free CEL 82.9% (±7.7%) to 102.7% (±2.1%). The recovery of protein-bound CML ranged from 77.5% (±10.7%) to 112.6% (±10.6%), and protein-bound CEL from 81.0% (±11.1%) to 110.0% (±2.9%) depending on the spiked AGE level and the meat type.

3.3. Free AGEs in raw beef, pork and chicken

Raw pork had a significant ($p < 0.05$) lower amount of free CML (0.23 ± 0.13 mg/kg) and CEL (0.19 ± 0.09 mg/kg) compared to beef (CML 0.41 ± 0.14 mg/kg, CEL 0.44 ± 0.19 mg/kg) (**Table 3**). However, for both raw pork and beef, free AGEs (sum of free CML and CEL) only accounted for a small proportion of total AGEs (sum of free AGEs and protein-bound AGEs), 5.2–14.1% for pork and 5.7–14.3% for beef, respectively.

Unlike pork and beef, fresh chicken breasts had extremely high amount of free CEL (7.51 ± 1.40 mg/kg), while chicken drumsticks also had higher levels of free CEL (0.80 ± 0.21) than pork or beef (**Table 3**). Free AGEs accounted for 18.3–34.1% of total AGEs in raw drumsticks and 59.7–71.4% in raw chicken breasts. In particular, free CELs in raw chicken breasts accounted for as high as 57.2–68.6% of the total AGEs. Due to the unusual high amounts of free CEL found in the three tested chicken breasts, an additional 10 fresh chicken breasts varying in species or producers were purchased from the same Metro AG store and analyzed for their free CML and CEL contents. A large biological variation in the levels of

free AGEs was found in fresh chicken breasts ($n=13$), particularly for free CEL ranging from 0.91 mg/kg to 38.66 mg/kg (7.12 ± 9.98 mg/kg), although the distribution of free CML was in a relatively narrow range (0.21–1.39 mg/kg, 0.68 ± 0.36 mg/kg) (**Fig. 1a**). There is a lack of published data about free AGEs in muscle foods, and to the best of our knowledge, this study revealed for the first time a possibly high level of free CEL in muscle foods. Since free AGEs may have different physiological effects compared to protein-bound AGEs, it is important to quantify free and protein-bound AGEs separately (Zhang et al., 2011; Rabbani & Thornalley, 2012), particularly for samples like chicken breasts possibly containing very high levels of free AGEs.

3.4. Free AGEs in beef, pork and chicken as affected by commercial sterilization

The sterilization process had no obvious effect on the amount of free AGEs (**Table 3, Fig. 1b**), resulting in no significant change in the average amount of CML or CEL in sterile pork (CML 0.25 ± 0.15 mg/kg, CEL 0.19 ± 0.08 mg/kg; $n=9$), beef (CML 0.41 ± 0.11 mg/kg, CEL 0.45 ± 0.20 mg/kg; $n=9$), and chicken breasts (CML 0.71 ± 0.35 mg/kg, CEL 7.34 ± 10.61 mg/kg; $n=13$) compared to the amounts of free AGEs in their fresh meat counterparts previously discussed.

The studies by Zhang et al. (2011) and Ahmed et al. (2005) were among the very few publications reporting the levels of free AGEs in food items as affected by heating. Zhang et al. (2011) showed that the levels of free CML (raw, 0.37 ± 0.02 mg/kg; roasted, 0.47 ± 0.06 – 0.66 ± 0.04 mg/kg) in almonds increased about 50% after roasting (129–182 °C, 3.8–70 min),

and free CEL (raw, 0.42 ± 0.02 mg/kg; roasted, $0.57 \pm 0.05 - 1.16 \pm 0.08$ mg/kg) increased about 120%. Ahmed et al. (2005) reported that free CML (raw, 147 ± 2 nM; pasteurized, 214 ± 6 nM; 252 ± 14 nM) in milk increased by 46% due to pasteurization (63 °C, 30 min) and 71% due to sterilization (115 °C, 15 min), while no significant difference in free CEL (raw, 140 ± 4 nM; pasteurized, 147 ± 6 nM; 160 ± 4 nM) was found between the pasteurized and raw milk, but 14% increase in CEL was found in the sterilized milk. The results of this study revealed different effect of heat treatment on the formation of free AGEs from the studies by Zhang et al. (2011) and Ahmed et al. (2005), indicating that the formation of free AGEs during heating was strongly dependent upon the type of foods in addition to the severity of the heat treatment.

3.5. Protein-bound AGEs in fresh beef, pork and chicken

The amounts of both protein-bound CML and CEL in fresh pork and chicken were similar, but were significant less ($p < 0.05$) than that in raw beef (beef: CML 4.41 ± 0.72 , CEL 3.90 ± 1.13 mg/kg; pork: CML 2.90 ± 0.61 , CEL 1.65 ± 0.30 mg/kg; chicken: CML 2.47 ± 0.37 , CEL 1.32 ± 0.32 mg/kg) (**Table 3**). Except for one beef sample, all tested raw meat contained more protein-bound CML than CEL. The ratio of protein-bound CML to CEL in raw beef was 0.7–2.0, in pork was 1.1–2.8, and in chicken was 1.6–2.3.

For raw beef, pork or chicken, there was a great disparity in the level of protein-bound CML or CEL among experimental values for the same type of meat cuts from different company, and among different types of meat cuts from the same company. This indicates a

high amount of biological variation in meat. In addition, there was generally no significant difference in protein-bound CML or CEL between different cuts of raw meat (such as chicken breasts versus drumsticks), or between raw meat from different companies (such as three beef cuts from company-1 versus that from company-2). The only exception was that pork from one supplier (**# 4 in Table 3**) contained significant lower level of protein-bound CML compared to that from the other two companies.

3.6. Protein-bound AGEs in beef, pork and chicken as affected by commercial sterilization

The sterilization process led to an average of 196% (82–360%) increase in protein-bound CML and 203% (80–345%) increase in CEL in beef. Similarly, an average 218% (92–278%) increase in protein-bound CML and 190% (127–277%) increase in protein-bound CEL was found in chicken due to the sterilization. Unlike beef and chicken, protein-bound CML in pork in general formed faster than protein-bound CEL during sterilization as shown in an increase in the ratio of CML to CEL in sterile pork (CML/CEL:1.7–3.7) compared to that in raw pork (CML/CEL:1.1–2.8), and that the average increase of protein-bound CML (increased 224%, 163–318%) was much higher than protein-bound CEL (increased 122%, 60–243%). Similar to that in raw meats, the amounts of protein-bound CML and CEL in sterile chicken (CML 7.68 ± 1.22 mg/kg; CEL 3.72 ± 0.53 mg/kg) and in pork (CML 9.51 ± 2.85 , CEL 3.49 ± 0.50 mg/kg) were close, but significant lower ($p < 0.05$) than that in beef (CML 8.24 – 21.98 mg/kg, 13.01 ± 4.79 mg/kg; CEL 5.67 – 19.64 mg/kg, 11.70 ± 4.58 mg/kg). In addition, there did not appear to be direct relationship between the levels of protein-bound

265 AGEs in raw meat and in sterile meat, as raw meat containing high level of protein-bound
266 AGEs (such as the Rump #2, Hind leg #6 in **Table 3**) did not necessary result in sterile meat
267 with high level of protein-bound AGEs.

268 The amounts of protein-bound CML in commercial sterilized beef, pork and chicken
269 were in general lower than that of cooked meats reported by Chen and Smith (2015). The
270 CML in baked and broiled meats reported by Chen & Smith (2015) were as follows: beef
271 14.31 mg/kg, 21.84 mg/kg; pork 12.53 mg/kg, 20.35 mg/kg; chicken 13.58 mg/kg, 19.69
272 mg/kg(no CEL data was provided in the study). Although the internal temperature were
273 71–74 °C, an overall higher cooking temperature (177–232°C) and longer cooking time
274 (14–45 min) were used in the study of Chen and Smith (2015), which may contribute to the
275 higher CML contents.

277 *3.7. Protein-bound AGEs in raw and cooked meats as affected by protein and fat contents*

278 In general, a higher level of protein would suggest that a greater amount of lysine
279 residues would be available as reactant to form CML or CEL. Also, foods with higher levels
280 of fat are more susceptible to lipid oxidation contributing to AGEs formation. However, this
281 study indicated that a high protein or fat content is not necessary tied to higher levels of AGEs
282 in raw meat. As shown in **Fig. 2** and **Fig. 3**, there was no direct relationship between the
283 protein or fat content and the amount of protein-bound CML or CEL in raw meat. In addition,
284 a high protein or fat content in meat did not necessary lead to higher amounts of
285 protein-bound AGEs being formed during commercial sterilization (**Fig. 2 & 3**). Typically,

the beef cut #2 and #5 (**Fig. 2 a & b**) had relatively high protein content, but following sterilization, they had relatively low amount of protein-bound CML and CEL; while beef cut #4 (**Fig. 2 c & d**) had the highest amount of fat (based on protein weight), but following sterilization, its protein-bound CEL was the lowest and protein-bound CML was one of the four lowest among the 9 beef cuts tested.

Another typical example was pork belly meat (**Fig. 3**), which contained much higher fat ($32.0 \pm 6.2\%$) and less protein ($14.2 \pm 1.0\%$) than the meat from hind leg and tenderloin (fat, $2.0 \pm 3.1\%$; protein $21.3 \pm 1.4\%$). Based upon the sample weight, there was no significant difference in protein-bound CML or CEL between raw or sterilized pork belly meat and the other two pork cut meats (**Fig. 3a & b**). This could be possibly explained by the counteractive effects of higher fat and lower protein content in pork belly meat. A high fat content may promote AGEs formation, but this promoting effect may be offset by a lower level of proteins, particularly lower proportion of available lysine residues participating in the reactions forming CML and CEL in pork belly meats. To further examine the effect of fat content on AGEs formation, the amounts of AGEs in both raw and sterilized pork were analyzed based upon the protein weight (**Fig. 3c & d**). Except for protein-bound CEL in raw belly meat and tenderloin, there was no significant difference in protein-bound CML or CEL (based on protein weight) between belly meat and other two types of meat cuts for either raw or commercial sterilized pork. In particular, there was no significant difference in the amounts of protein-bound CML or CEL formed during sterilization process between pork belly meat and other types of pork cuts (increased in mg/kg protein, CML: belly, 20.1–51.2,

hind leg 19.8–50.2, tenderloin 19.4–43.9; CEL: belly, 6.6–19.9, hind leg 7.9–10.5, tenderloin 8.1–14.1); however, the average increase of protein-bound CML and CEL in pork belly were about 17–19% higher than in the other types of pork cuts, indicating a trend for higher AGEs formation in high fat pork belly, although probably because of the wide biological variation, no statistically significant difference was found.

Beef and pork fat consists of a high proportion of saturated fatty acids and is less susceptible to lipid oxidation compared to vegetable oils or fatty acids used in other studies (Fu et al., 1996; Lima, Assar, & Ames, 2010). The level of saturation of the fat may be more important than the total fat content when it comes to AGEs formation in raw and heated foods, and thus, the amount of fat in meat had little or no effect on the formation of AGEs in raw and processed meat products.

4. Conclusions

Raw beef and pork had a small proportion (< 15%) of free AGEs compared to the total AGEs, but raw chicken breasts had very high levels of free CELs, accounting for 57.2–68.6% of the total AGEs. Commercial sterilization did not significantly affect the amounts of free CML or CEL in beef, pork, or chicken, but led to about 0.6- to 3.6-fold increase in protein-bound CML and CEL. Raw and sterilized beef in general had significant higher levels of protein-bound AGEs than in pork and chicken. There was a high variation in the amount of free or protein-bound AGEs among different cuts of pork, beef or chicken. However, in general, there was no significant difference in protein-bound CML or CEL between

327 commercially sterilized meat from different cuts or from different companies. The amount of
328 protein and fat content in beef or pork had very little or no effect on the formation of
329 protein-bound AGEs during the sterilization process. The formation of AGEs in meats as
330 affected by commercial sterilization with various heating temperature and time should be
331 investigated in the future to be able to predict AGEs formation during thermal processing of
332 meat products at industrial level.

333 **Acknowledgements**

334 This study was funded by the USDA-NIFA 2011-68003-20096, Shanghai Ocean University
335 (A1-0209-14-0902-2) and the Washington State University Agricultural Research Center.

336   **References**

- 337   Ahmed, N., Mirshekar-Syahkal, B., Kennish, L., Karachalias, N., Babaei-Jadidi,
338   R., & Thornalley, P. J. (2005). Assay of advanced glycation endproducts in selected
339   beverages and food by liquid chromatography with tandem mass spectrometric detection.
340   *Molecular Nutrition & Food Research*, 49, 691–699.
- 341   Ames, J. M. (2007). Evidence against dietary advanced glycation endproducts being a risk to
342   human health. *Molecular Nutrition & Food Research*, 51, 1085–1090.
- 343   Assar, S. H., Moloney, C., Lima, M., Magee, R., & Ames, J. M. (2009). Determination of
344   N^ε-(carboxymethyl)lysine in food systems by ultra performance liquid
345   chromatography-mass spectrometry. *Amino Acids*, 36, 317–326.
- 346   Chao, P.-C., Hsu, C.-C., & Yin, M.-C. (2009). Analysis of glycative products in sauces and
347   sauce-treated foods. *Food Chemistry*, 113, 262–266.
- 348   Chen, G., Smith, J. C. (2015). Determination of advanced glycation endproducts in cooked
349   meat products. *Food Chemistry*, 168, 190–195.
- 350   Fu, M.-X., Requena, J. R., Jenkins, A. J., Lyons, T. J., Baynes, J. W., & Thorpe, S. R. (1996).
351   The advanced glycation end product, N^ε-(carboxymethyl)lysine, is a product of both
352   lipid peroxidation and glycoxidation reactions. *The Journal of Biological Chemistry*, 271,
353   9982–9986.

354 Goldberg, T., Cai, W., Peppas, M., Dardaine, V., Baliga, B. S., Uribarri, J., et al. (2004).
 355 Advanced glycoxidation end products in commonly consumed foods. *Journal of the*
 356 *American Dietetic Association*, 104, 1287–1291.

357 Hegele, J., Buetler, T., & Delatour, T. (2008). Comparative LC-MS/MS profiling of free and
 358 protein-bound early and advanced glycation-induced lysine modifications in dairy
 359 products. *Analytica Chimica Acta*, 617, 85–96.

360 Heinz, G., & Hautzinger, P. (2007). Meat Processing Technology for small- to medium-scale
 361 producers. Food and Agriculture Organization of the United Nations, Regional Office
 362 for Asia and the Pacific, Bangkok, Thailand, pp. 288–296.

363 Hull, G. L. J., Woodside, J. V., Ames, J. M., & Cuskelly, G. J. (2012).
 364 N^ε-(carboxymethyl)lysine content of foods commonly consumed in a Western style diet.
 365 *Food Chemistry*, 131, 170–174.

366 Jiao, L., Stolzenberg-Solomon, R., Zimmerman, T. P., Duan, Z., Chen, L., Kahle, L., et al.
 367 (2015). Dietary consumption of advanced glycation end products and pancreatic cancer
 368 in the prospective NIH-AARP diet and health study. *The American Journal of Clinical*
 369 *Nutrition*, 101, 126–34.

370 Kong, F., Tang, J., Rasco, B., Crapo, C., & Smiley, S. (2007). Quality changes of
 371 salmon (*Oncorhynchus gorbuscha*) muscle during thermal processing. *Journal of Food*
 372 *Science*, 72, S103–S111.

373 Lima, M., Assar, S. H., & Ames, J. M. (2010). Formation of N-epsilon-(carboxymethyl)
 374 lysine and loss of lysine in casein glucose-fatty acid model systems. *Journal of*
 375 *Agricultural and Food Chemistry*, 58, 1954–1958.

376 Niquet-Léridon, C., & Tessier, F. J. (2011). Quantification of N^ε-carboxymethyl-lysine in
 377 selected chocolate-flavoured drink mixes using high-performance liquid
 378 chromatography-linear ion trap tandem mass spectrometry. *Food Chemistry*, 126,
 379 655–663.

380 Nguyen, C. V. (2006). Toxicity of the AGEs generated from the Maillard reaction: On the
 381 relationship of food-AGEs and biological-AGEs. *Molecular Nutrition & Food*
 382 *Research*, 50, 1140–1149.

383 Rabbani, N., & Thornalley, P. J. (2012). Glycation research in amino acids: a place to call
 384 home. *Amino Acids*, 42, 1087–1096.

385 Scheijen, J. L. J. M., Clevers, E., Engelen, L., Dagnelie, P. C., Brouns, F., Stehouwer, C. D. A.,
 386 et al. (2016). Analysis of advanced glycation endproducts in selected food items by
 387 ultra-performance liquid chromatography tandem mass spectrometry: presentation of a
 388 dietary AGE database. *Food Chemistry*, 190, 1145–1150.

389 Schettgen, T., Tings, A., Brodowsky, C., Müller-Lux, A., Musiol, A., & Kraus, T. (2007).
 390 Simultaneous determination of the advanced glycation end product
 391 N^ε-carboxymethyllysine and its precursor, lysine, in exhaled breath condensate using

392 isotope-dilution–hydrophilic-interaction liquid chromatography coupled to tandem mass
 393 spectrometry. *Analytical and Bioanalytical Chemistry*, 387, 2783–2791.

394 Sun X, Tang J, Wang J, Rasco BA, Lai K, & Huang Y. (2015). Formation of advanced
 395 glycationendproducts in ground beef under pasteurisation conditions. *Food Chemistry*,
 396 172, 802–807.

397 Takeuchi, M.,Takino, J.,Furuno, S.,Shirai, H., Kawakami, M.,Muramatsu, M., et al.(2015).
 398 Assessment of the concentrations of various advanced glycation end-products in
 399 beverages and foods that are commonly consumed in Japan. *PloS One*, 10(3), e0118652.
 400 Doi: 10.1371/journal.pone.0118652 .

401 Uribarri, J., Woodruff, S., Goodman, S., Cai, W., Chen, X., Pyzik, R., et al. (2010).
 402 Advanced glycation end products in foods and a practical guide to their Reduction in the
 403 diet. *Journal of the American Dietetic Association*, 110, 911–916.

404 Zhang, G., Huang, G., Xiao, L., & Mitchell, A. E. (2011).Determination of advanced
 405 glycationendproducts by LC-MS/MS in raw and roasted almonds
 406 (*Prunusdulcis*).*Journal of Agricultural and Food Chemistry*, 59, 12037–12046.

407 **Figure Captions**

408 **Fig.1.** Free CML and CEL in (a) raw, and (b) commercial sterilized chicken breasts varying
409 in species and producers ($n=13$). The number 1–13 in x-axis represented chicken breasts
410 from different producers or species.

411 **Fig. 2.** Relationship of (a) protein and protein-bound CML, and (b) protein and protein-bound
412 CEL based on sample weight, as well as (c) fat and protein-bound CML, and (d) fat and
413 protein-bound CEL based on protein weight in raw and commercial sterilized beef. The
414 number 1–9 in x-axis represented beef cuts from different animal parts (1–3 rump; 4–6
415 ribeye; 7–9 short plate) and different companies (samples from 3 different companies were
416 grouped as: 1, 4, 7; 2, 5, 8; 3, 6, 9).

417 **Fig. 3.** Relationship of (a) protein and protein-bound CML, and (b) protein and protein-bound
418 CEL based on sample weight, as well as (c) fat and protein-bound CML, and (d) fat and
419 protein-bound CEL based on protein weight in raw and commercial sterilized pork. The
420 number 1–9 in x-axis represented pork cuts from different animal parts (1–3 hind leg; 4–6
421 tenderloin; 7–9 belly) and different companies (samples from 3 different companies were
422 grouped as: 1, 4, 7; 2, 5, 8; 3, 6, 9).

Table 1

Water, fat and protein contents in beef, pork, chicken from different meat cuts and companies
($n=3$).

Meat type ^a			Water (%)	Fat (%)	Protein (%)
Beef	Rump	1	75.0±0.1	0.7±0.1	22.0±0.6
		2	74.2±0.7	1.0±0.4	20.8±0.7
		3	74.8±0.2	0.4±0.1	22.2±0.6
	Ribeye	1	62.5±0.4	17.1±1.9	16.5±0.4
		2	69.7±0.1	5.3±0.5	20.5±1.5
		3	58.9±0.2	14.2±0.3	18.4±0.4
	Short plate	1	61.7±0.4	14.9±0.8	18.0±0.4
		2	62.8±0.9	10.8±0.6	19.0±0.2
		3	67.2±0.4	7.1±1.1	18.5±0.6
Pork	Hind leg	4	75.9±0.1	0.5±0.1	21.1±0.4
		5	69.5±0.5	8.2±2.2	18.8±0.6
		6	73.3±0.5	0.7±0.4	22.4±0.5
	Tenderloin	4	75.7±0.1	0.4±0.1	22.2±0.9
		5	74±0.4	0.6±0.1	20.9±0.6
		6	70.7±0.3	1.8±0.3	22.2±0.7
	Belly	4	51.9±1.1	29.8±0.8	14.8±0.2
		5	51.7±0.2	27.3±0.3	14.7±0.7
		6	44.6±0.3	39.0±0.7	13.0±0.2
Chicken	Breast	7	75.4±0.0	0.3±0.1	22.7±0.1
		8	74.4±0.1	0.3±0.0	24.2±0.1
		9	73.4±0.2	0.3±0.1	23.7±0.7
	Drumstick	7	74.4±0.2	1.8±0.1	18.9±0.2
		8	70.4±0.4	9.8±0.6	18.6±0.3
		9	71.4±0.7	5.6±1.2	19.2±0.1

^aThe number 1–9 represented nine different companies that produced the meat.

Table 2

Recovery of free and protein-bound AGEs in beef, pork and chicken for their analyses with HPLC–MS/MS ($n=6$).

AGEs	Recovery of free AGEs (%)			Recovery of protein-bound AGEs (%)		
	200 (µg/kg)	500 (µg/kg)	1000 (µg/kg)	100 (µg/kg)	500 (µg/kg)	1000 (µg/kg)
Beef						
CML	81.8–121.7	90.7–118.6	94.2–127.2	66.9–91.0 ^a	88.4–102.5 ^a	92.1–108.0 ^a
CEL	89.6–116.7	75.5–93.5	86.6–97.0	71.6–93.5 ^b	95.6–110.6 ^b	99.5–114.7 ^b
Pork						
CML	96.3–119.2	101.4–123.6	88.0–102.1	96.8–124.3	79.0–103.5	99.1–105.3
CEL	71.6–111.3	91.8–106.0	87.2–91.4	99.1–113.0	97.4–103.9	106.4–114.7
Chicken						
CML	81.6–123.2	93.9–105.6	100.2–103.9	88.3–122.3	96.9–120.2	89.5–103.2
CEL	75.9–96.1	94.1–105.6	99.0–104.9	90.7–120.6	93.7–111.9	97.7–104.6

^{a,b}The spiking levels of protein-bound CML in beef were 390, 1950, 3900 µg/kg , and protein-bound CEL were 310, 1550, 3100 µg/kg, respectively.

Table 3

Free and protein-bound AGEs in raw and commercial sterilized (121 °C, 10 min) beef, pork and chicken from different meat cuts and companies ($n=3$).

Meat type ^a		AGEs in raw meat (mg/kg) ^b				AGEs in cooked meat (121°C, 10min) (mg/kg) ^b			
		Free CML	Free CEL	Bound CML	Bound CEL	Free CML	Free CEL	Bound CML	Bound CEL
Beef									
Rump	1	0.34±0.01	0.22±0.01	3.62±0.31	5.60±0.35	0.38±0.02	0.21±0.02	10.66±0.52	19.64±0.70
	2	0.54±0.01	0.28±0.02	5.57±0.42	4.94±0.13	0.51±0.01	0.29±0.02	10.16±0.62	12.24±0.57
	3	0.32±0.02	0.23±0.03	3.97±0.62	3.44±0.38	0.35±0.01	0.24±0.01	18.26±1.82	15.30±0.64
Ribeye	1	0.34±0.01	0.41±0.01	3.82±0.19	2.39±0.06	0.37±0.01	0.42±0.03	8.44±0.59	5.67±0.32
	2	0.51±0.03	0.63±0.02	4.02±0.15	3.99±0.33	0.40±0.01	0.58±0.04	8.24±0.72	8.69±0.60
	3	0.25±0.09	0.39±0.13	4.55±0.64	4.45±0.43	0.32±0.01	0.45±0.04	11.67±0.54	14.00±0.04
Short plate	1	0.41±0.41	0.59±0.02	4.20±0.26	2.06±0.09	0.41±0.14	0.57±0.18	16.57±1.82	7.79±0.14
	2	0.67±0.03	0.74±0.04	4.31±0.60	4.15±0.16	0.66±0.01	0.84±0.04	11.13±0.64	7.48±0.40
	3	0.31±0.01	0.50±0.01	5.64±1.13	4.09±0.30	0.35±0.00	0.48±0.02	21.98±0.66	14.51±0.69
Pork									
Hind leg	4	0.14±0.01	0.10±0.01	2.35±0.05	2.02±0.13	0.17±0.01	0.13±0.01	6.52±0.20	3.77±0.12
	5	0.14±0.01	0.12±0.02	2.97±0.16	1.79±0.14	0.16±0.01	0.13±0.01	12.41±0.37	3.76±0.20
	6	0.40±0.01	0.14±0.01	3.43±0.01	1.95±0.15	0.42±0.01	0.14±0.00	10.49±0.19	3.72±0.28
Tenderloin	4	0.15±0.01	0.33±0.04	2.32±0.22	1.79±0.20	0.16±0.01	0.25±0.01	6.62±0.18	3.73±0.10
	5	0.13±0.01	0.14±0.01	3.11±0.15	1.21±0.07	0.12±0.00	0.17±0.01	12.28±0.18	4.15±0.48
	6	0.35±0.01	0.09±0.00	3.72±0.15	1.59±0.11	0.39±0.01	0.09±0.00	12.36±0.28	3.38±0.25
Belly	4	0.14±0.01	0.28±0.00	1.83±0.19	1.64±0.06	0.16±0.01	0.28±0.00	4.81±0.12	2.62±0.10
	5	0.18±0.01	0.25±0.01	3.09±0.16	1.69±0.13	0.17±0.00	0.24±0.00	10.18±0.30	2.81±0.37
	6	0.44±0.01	0.28±0.01	3.24±0.12	1.16±0.35	0.52±0.01	0.30±0.01	9.90±0.06	3.75±0.09
Chicken									
Breast	7	0.31±0.03	7.71±0.08	2.16±0.15	1.06±0.02	0.38±0.01	7.33±0.10	7.60±0.05	3.52±0.13
	8	0.27±0.03	6.02±0.10	2.67±0.07	1.57±0.13	0.35±0.04	5.66±0.08	9.09±0.27	3.99±0.21
	9	0.21±0.02	8.80±0.06	2.82±0.13	1.81±0.28	0.34±0.01	8.08±0.10	8.19±0.12	4.61±0.31
Drumstick	7	0.65±0.02	1.00±0.04	2.21±0.04	0.98±0.03	0.85±0.01	0.98±0.01	8.36±0.23	3.69±0.12
	8	0.53±0.01	0.81±0.04	2.06±0.33	1.16±0.07	0.78±0.08	0.95±0.01	7.29±0.37	3.43±0.12
	9	0.36±0.01	0.59±0.03	2.89±0.12	1.35±0.04	0.51±0.02	0.73±0.01	5.54±0.10	3.07±0.13

^aThe number 1–9 represented nine different companies that produced the meat.

^b The data were based upon sample weight.

Fig.1. Free CML and CEL in (a) raw, and (b) commercial sterilized chicken breasts varying in species and producers ($n=13$). The number 1–13 in x-axis represented chicken breasts from different producers or species.

Fig. 2. Relationship of (a) protein and protein-bound CML, and (b) protein and protein-bound CEL based on sample weight, as well as (c) fat and protein-bound CML, and (d) fat and protein-bound CEL based on protein weight in raw and commercial sterilized beef. The number 1–9 in x-axis represented beef cuts from different animal parts (1–3 rump; 4–6 ribeye; 7–9 short plate) and different companies (samples from 3 different companies were grouped as: 1, 4, 7; 2, 5, 8; 3, 6, 9).

Fig. 3. Relationship of (a) protein and protein-bound CML, and (b) protein and protein-bound CEL based on sample weight, as well as (c) fat and protein-bound CML, and (d) fat and protein-bound CEL based on protein weight in raw and commercial sterilized pork. The number 1–9 in x-axis represented pork cuts from different animal parts (1–3 hind leg; 4–6 tenderloin; 7–9 belly) and different companies (samples from 3 different companies were grouped as: 1, 4, 7; 2, 5, 8; 3, 6, 9).