Original Research Article

Free and protein-bound N^{ϵ} -carboxymethyllysine and N^{ϵ} -carboxyethyllysine in fish muscle: biological variation and effects of heat treatment

Lihong Niu^a, Xiaohua Sun^a, Juming Tang^b, Jing Wang^a, Barbara A Rasco^{c*}, Keqiang Lai^a, Yiqun Huang^{a,c*}

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

^b Department of Biological System Engineering, Washington State University, Pullman, WA99164-6120, USA.

^c School of Food Science, Washington State University, Pullman, WA99164-6376, USA.

*Corresponding authors:

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

Abstract

N^ε-Carboxymethyllysine (CML) and *N*^ε-carboxyethyllysine (CEL) are typical advanced glycation end-products (AGEs) found in foods, which have been linked to various health risks. Little is known about AGEs formation in fish muscle and the variability in AGE formation from one animal to another. In this study, free CML and CEL (glycated amino acids) and their protein-bound forms (protein glycation adducts) in fresh grass carp (*Ctenopharyngodon idellus*) and catfish (*Clarias leather*) muscle before and after heating (100 °C, 5, 10, 30 min) were determined by an HPLC-MS/MS method. High biological variation in CML and CEL levels was found between individual fish, particularly for CEL in catfish muscle [$n = 21$, free CEL 0.18–30.1(6.50 ± 7.19) mg/kg; protein-bound CEL 0.48–46.3 (3.08 ± 2.70) mg/kg]. Heating resulted in great increase of protein-bound CML (2.1–10.8 fold increase) and CEL (27%–242% increase) in fish muscle, but had little or no effect on free CML and CEL contents. Simple kinetic functions did not fit well for the formation rate of protein-bound AGEs during heating, although zero-order reaction fitted very well for some individual fish, which further indicated the complexity of AGEs formation and the strong impact of biological variation of individual fish on AGEs formation.

Keywords

Advanced glycation endproducts; Food Analysis; Food Composition;
Carboxymethyllysine; Carboxyethyllysine; Fish; Heating; Biological variation
Ctenopharyngodon idellus; *Clarias leather*

1. Introduction

Advanced glycation end-products (AGEs) is a group of relatively stable compounds resulted from non-enzymatic modifications of proteins or protein derivatives by reducing sugars or sugar derivatives (Rabbani and Thornalley, 2012). AGEs are found in the advanced stages of the Maillard reaction, a non-enzymatic reaction involving a series of complicated reactions between carbonyl groups of reducing sugars (glucose, fructose, lactose, etc.) and free amino groups of proteins, polypeptides, amino acids or nucleic acids (Rabbani and Thornalley, 2012; Singh et al., 2001). Some AGEs, such as *N*^ε-carboxymethyllysine (CML) and *N*^ε-carboxyethyllysine (CEL), can also form at the early stages of the Maillard reaction, and through lipid peroxidation (Ahmed et al., 1997; Fu et al., 1996; Nguyen et al., 2013; Rabbani and Thornalley, 2012). Therefore, CML and CEL can also be classified as advanced lipid peroxidation end-products. Although the majority of dietary AGEs may be degraded by gut microflora or rapidly excreted by the kidneys, there is still concern that AGEs may have a negative impact on human health (Ames, 2007). More recent studies indicate a possible link between dietary AGEs and inflammation, aging and increased oxidative stress, and increased risks for kidney diseases, diabetes, obesity, and cancers (Jiao et al., 2015; Nguyen, 2006; Poulsen et al., 2013; Uribarri et al., 2010).

The amounts of AGEs (predominantly CML) in various raw and cooked or processed foods have been reported, clearly indicating that the levels of AGEs in

foods vary depending on the type of food and cooking or processing methods (Hull et al., 2012; Sun et al., 2015, 2016a ; Uribarri et al., 2010; Zhang et al., 2011). In general, relatively high levels of AGEs were found in foods containing high protein and/or fat (such as meat) and in foods previously subjected to high cooking or processing temperature (such as breakfast cereals and grilled meat) (Hull et al., 2012; Sun et al., 2015, 2016a; Uribarri et al., 2010). However, the published data on the levels of AGEs in a specific muscle food may vary greatly. For example, the CML content in raw beef was reported as about 0.13 mg/kg in an unidentified cut of beef (Chao et al. 2009), 2.05 ± 0.40 mg/kg in rib round steak (Chen and Smith, 2015), 2.76–4.32 mg/kg in ground beef (Sun et al., 2015), and 3.62–5.64 mg/kg in rump, ribeye and short plate (Sun et al., 2016a). It is unclear whether this disparity in the CML content of beef was mainly due to the biological variation between individual animals or between different types of muscle tissues, the difference in postmortem storage and processing of the beef, or even due to the difference in CML quantification methods employed by separated research groups. In the past ten years, we have seen an increasing number of publications on AGEs in various foods as affected by heating, and food ingredients (such as sugars, fat) or additives (such as antioxidants), but there is still a lack of systematic study investigating the levels of AGEs in muscle foods and its rate of formation. This is the first study to examine biological variation and to determine the role this plays in AGEs formation.

Free AGEs (glycated amino acids) and protein-bound AGEs (protein glycation

adducts) may have different bioavailability and physiological effects (Ahmed et al., 2005; Rabbani and Thornalley, 2012). Yet, most of the published studies on dietary AGEs were focused on protein-bound AGEs or the mixture of free and protein-bound AGEs without differentiating between free and protein-bound forms, and there were only a few reported studies that included free AGEs in foods (Ahmed et al., 2005; Li et al., 2015; Sun et al., 2016a; 2016b; Zhang et al., 2011). The proportion of free and protein-bound AGEs in foods is associated with the type of food and the cooking/heating method used. For example, the amount of protein-bound AGEs was much higher than free AGEs in almond (Zhang et al., 2011), bovine milk (Ahmed et al., 2005), pork and beef (Sun et al., 2016a), but free AGEs could account for as much as 71% of the total AGEs in chicken breasts (Sun et al., 2016a). In addition, the levels of free AGEs and protein-bound AGEs in foods were affected by heat treatments differently. The formation of protein-bound AGEs in foods was greatly accelerated by heating, but this same effect was not seen for free AGEs, where heating had much less of an effect (Ahmed et al., 2015; Zhang et al., 2011) or even no effect (Sun et al., 2016a) on the levels of free AGEs in foods. Therefore, it is important to differentiate between free and protein-bound AGEs in foods.

The objectives of this study were to evaluate the degree of biological variation on the levels of AGEs (including CML and CEL) in raw fish muscle, and to understand the effects of heat treatments on the amounts of free and protein-bound AGEs in fish muscle. Both free and protein-bound CML and CEL in fish muscle were

quantified with a validated HPLC-MS/MS method, since this would provide more accurate results compared to the commonly used immunoassay (Scheijen et al., 2016).

2. Materials and methods

2.1. Reagents

Analytical grade trichloroacetic acid, chloroform, sodium borohydride, sodium borate, boric acid, sodium hydroxide, hydrochloric acid and HPLC grade hexane and methanol were purchased from Sinopharm (Shanghai, China). HPLC-grade formic acid and ammonium acetate were acquired from Sigma (St. Louis, MO). AGEs standards including d₄-CML, CML and CEL, were 98% purity and purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada).

Stock solutions of CML (400 µg/mL), CEL (300 µg/mL), and d₄-CML (100 µg/mL) were prepared by dissolving each of the chemicals in methanol-water solution (80:20, v:v), and stored at -20 °C. AGEs standard mixture (CML 300 ng/mL, CEL 300 ng/mL, and d₄-CML 400 ng/mL) and the internal standard d₄-CML (8 µg/mL) were prepared by diluting stock solutions right before being used for sample analysis.

2.2. Fish preparation

Live grass carp (*Ctenopharyngodon idellus*) (2.84 ± 0.33 kg; $n = 15$) and catfish (*Clarias leather*) (2.55 ± 0.41 kg; $n = 21$) were purchased from a local fishmonger in Shanghai, China. Both grass carp and catfish are widely cultivated fish species in the

world. Grass carp are herbivores, while *Clarias* spp. are voracious omnivores. Four different batches of grass carp were collected over a period of about three months and three batches of catfish were collected over a period of two months. The minimum weight of grass carp and catfish were 2.2 kg and 1.7 kg, respectively, so that there was adequate sample for later experiments.

Fish were killed by a hard blow to the head, gutted and scaled, and rinsed with water by the seller. The whole fish were buried in ice in a cooler and transported to the laboratory within 30 min after they were killed. Once the fish arrived at the laboratory, a steak of 10 cm width (573 ± 54 g) was cut off from each grass carp, using the insertion of the dorsal fin as the center line, or a steak of 10 cm width (471 ± 61 g) was cut off from each catfish, starting from the insertion of the dorsal fin towards the posterior (**Fig. 1**). The selection of these portions of grass carp or catfish for the experiments was based upon our preliminary experiments or previous studies (Cao et al., 2016), so that sample variance known to occur in different portions of a fish from anterior to posterior would be minimized. Immediately before heat treatments or prior to AGEs analysis, the white muscle above the lateral line on both sides of a fish steak was carefully removed, cut into small cubes and ground at low speed for 20 s and then high speed for 10 s in a homogenizer (8010 s; Waring, Inc., Torrington, CT). All samples were kept in sealed plastic bags and buried in ice until further use.

2.3. Heat treatments

Ground fish (12.10 ± 0.10 g) was sealed into a custom-design cylindrical aluminum cell which allows for rapid heat transfer (Kong et al., 2007), heated in boiling water for 5, 10, or 30 min, and immersed into an ice/water mixture to cool down rapidly. The heat treated meat and exudate were collected and ground in a mortar, and then immediately tested.

2.4. Analysis of protein, moisture, and fat contents in fish muscle

The protein, moisture, and fat contents in each raw fish were determined according to AOAC methods (AOAC, 2005). A Kjeldahl method (AOAC 928.08) with an automatic Kjeldahl apparatus (UDK 159; VELP Scientific, Usmate, MB, Italy) was applied to measure the nitrogen content in fish muscle, and a conversion factor of 6.25 was used to calculate protein content from the nitrogen content. The moisture content (AOAC 950.46) of fish muscle was determined using an oven drying method in which fish sample was heated at 105 °C until reaching a constant weight. To determine fat content (AOAC 991.36) in fish muscle, a solvent extraction method with an automatic Soxhlet solvent extractor (SZF-06; Shanghai Jiading Food and Oil Instrument, Shanghai, China) was applied.

2.5. Sample preparation for analysis of free CML and CEL

For analysis of free CML and CEL in raw and heat treated fish muscle, samples

were prepared as previously described (Sun et al., 2016a). This procedure mainly included the following steps: precipitating proteins in fish homogenate with trichloroacetic acid, defatting and precipitating residual protein with hexane, recovering analyte in aqueous layer, and the solution being further cleaned with an MCX column (60 mg/3 mL) and a hydrophilic PTFE syringe filter (13 mm × 0.22 µm). Both the MCX columns and syringe filters were purchased from Shanghai ANPEL Scientific Instrument, Ltd (Shanghai, China). The extraction process was repeated three times for each sample.

2.6. Sample preparation for analysis of protein-bound CML and CEL

The sample preparation for analysis of protein-bound CML and CEL in fish muscle was based upon the steps described in our previous publication (Sun et al., 2015). The method requires reducing AGEs in the sample with sodium borohydride for 8 h, defatting and precipitating proteins with methanol-chloroform, acid hydrolysis of proteins at 110 °C for 24 h, and further cleaning up with a MCX column and a 0.22 µm filter (Assar et al., 2009; Niquet-Léridon and Tessier, 2011). Triplicate analyses were conducted for each sample.

2.7. HPLC-MS/MS analysis

The analysis of AGEs was performed with a Waters 2695 HPLC system and a Waters Quattro Micro triple-quadrupole tandem mass spectrometer (Waters Corp.,

Milford, MA) operated in positive electrospray ionization (ESI) mode. The column used for the HPLC system was an Atlantis hydrophilic interaction liquid chromatography (HILIC) silica column (150 mm $\times$ 2.1 mm, 3 μ m; Waters Corp.) The column temperature (35 $^{\circ}$ C), binary mobile phase composition and flow rate for HPLC was described in detail in Sun et al. (2015). The experimental procedure and setting for mass spectrometric analysis were based upon the method described in Sun et al. (2015), with some modification in collision energy, cone voltage, and product ions used for confirmation of CEL (Sun et al., 2016a). AGEs standard mixture (CML 300 ng/mL, CEL 300 ng/mL, and d₄-CML 400 ng/mL) was used to calculate response factors of CML, CEL and d₄-CML each time right before analysis of a batch of fish muscle samples. The ratio of the response factor of CML or CEL to that of the d₄-CML was considered as a constant within a predetermined linear range, which, together with the known concentration of d₄-CML and its peak area, was used to calculate the level of CML or CEL in a fish muscle sample based on the peak area of CML or CEL.

The recovery rates for the CML and CEL tests were determined through spiking fish samples (for free AGEs) or fish protein hydrolysates (for protein-bound AGEs) with internal standard d₄-CML and CML or CEL standard at three different levels (200, 500, 1000 μ g/kg). In addition, samples spiked only with d₄-CML were used as the control, and the ratio of the response factor of CML or CEL to that of the d₄-CML was used to determine the initial amounts of CML and CEL in the samples which

were deducted from that of the spiked samples to calculate the recovery of CML or CEL (Sun et al. 2015). Recovery tests were repeated 6 times.

2.8. Statistical analysis

The amounts of AGEs in fish samples were reported as the average of triplicate measurements $\pm$ standard deviation of the measurements. One-way analysis of variance was performed (SPSS Version 19; IBM Corp. Armonk, NY) to see whether there were significant differences ($p < 0.05$) in the average levels of AGEs among raw and heat-treated fish muscle. In addition, a simple kinetic function, $dC/dt = KC^n$, was applied to evaluate the formation rate of AGEs during heating. In the equation, C is the concentration of CML or CEL in fish muscle at time t , K is a rate constant, and n is the order of reaction. The best fit of n ($n = 0, 1$, or 2), judged by the highest r^2 , was considered as the reaction order for the tested CML or CEL (Excel 2013; Microsoft Corp., Redmond, WA).

3. Results and discussion

3.1. LC-MS/MS chromatograms and recovery of AGEs in fish samples

Fig. 2 shows LC-MS/MS chromatograms of fish muscle spiked with internal standard d₄-CML obtained through multiple reaction monitoring in positive-ion mode. Retention times of CML, CEL and d₄-CML in a typical fish extract were 2.95 min, 3.14 min, and 3.26 min, respectively. Two major product ions including m/z 130 and

m/z 84 were observed for both CML and CEL, which were due to the collision induced dissociations of CML and CEL, resulting in the loss of fragments $-NHCH_2COOH$ (for CML), $-NHCH(CH_3)COOH$ (for CEL), and/or $-COOH$. These were consistent with other reported studies (Teerlink et al., 2004; Zhang et al., 2011). The transitions of m/z 219 $\rightarrow$ m/z 130 for CEL and m/z 205 $\rightarrow$ m/z 130 for CML were used for quantification, and transitions of m/z 219 $\rightarrow$ m/z 84 for CEL and m/z 205 $\rightarrow$ m/z 84 for CML were used for confirmation to improve the selectivity. The HPLC-MS/MS method for analysis of CML and CEL had high sensitivity with 4–5 $\mu\text{g/kg}$ limit of detection (signal-to-noise ratio = 3) and 12–15 $\mu\text{g/kg}$ limit of quantification (signal-to-noise ratio = 10) (Sun et al., 2015). Recoveries of free AGEs in grass carp and catfish were in the range of 101–122%, and for protein-bound AGEs 79–105% (Table 1).

3.2. Sample information

The grass carp white muscle used in this study contained 18.2–21.3% ($20.2 \pm 1.1\%$) proteins, 78.0–80.4% ($79.0 \pm 0.7\%$) moisture and 0.3–0.9% ($0.5 \pm 0.2\%$) fat (Table 2). All data were based upon sample weight unless specified otherwise. The catfish white muscle had 16.7–20.4% ($19.1 \pm 1.0\%$) protein, 72.3–77.8% ($74.6 \pm 1.6\%$) moisture, and 2.6–8.8% ($6.2 \pm 2.1\%$) fat, respectively (Table 3). The catfish muscle had a relatively high amount of fat and larger variation in moisture and fat contents compared to the grass carp.

3.3. Free and protein-bound CML and CEL in fish muscle

The AGEs levels in raw grass carp muscle ($n = 15$) were: free CML, 0.22–3.18 (1.29 ± 1.15) mg/kg; free CEL, 0.03–0.28 (0.16 ± 0.10) mg/kg; protein-bound CML, 0.66–2.16 (1.28 ± 0.46) mg/kg; protein-bound CEL, 1.76–4.90 (2.80 ± 0.95) mg/kg; total AGEs, 2.69–8.15 (5.52 ± 1.84) mg/kg (Table 2). The amount of free CML was 3.3–11.2 (7.2 ± 2.6) times as much as free CEL, but the sum of free AGEs generally accounted for a small portion of the total AGEs (sum of free and protein-bound CML and CEL) in grass carp muscle, ranging from 8.1% to 46.0% ($22.9 \pm 15.2\%$) of the total AGEs. For protein-bound AGEs, there was more CEL than CML in grass carp muscle, and the ratio of CEL to CML ranged from 1.3 to 4.8 with 2.4 on average. There was a relatively large variation in AGEs contents among individual grass carp. Particularly for free CML, the relative standard deviation (RSD, the percentage of the sample standard deviation to the average) reached 89%.

For raw catfish muscle ($n = 21$), the AGEs levels were: free CML, 0.03–0.42 (0.11 ± 0.09) mg/kg; free CEL, 0.18–30.11 (6.50 ± 7.19) mg/kg; protein-bound CML, 0.38–1.46 (0.71 ± 0.27) mg/kg; protein-bound CEL, 0.48–8.63 (3.08 ± 2.70) mg/kg; total AGEs, 1.39–39.74 (10.41 ± 9.82) mg/kg (Table 3). Except for protein-bound CML, the RSD of other three AGEs were above 80%, indicating a very high biological variation in AGEs levels between individual catfish, particularly for free CEL (RSD = 111%).

Similar to grass carp, catfish generally contained higher levels of CEL than CML. CEL accounted for $83.2 \pm 15.4\%$ (40.0–97.5%) of the total AGEs in catfish muscle. However, unlike grass carp muscle which contained very low levels of free CEL (0.16 ± 0.10 mg/kg), catfish muscle was relatively high in free CEL (6.50 ± 7.19 mg/kg), accounting for 9.3–75.8% (average $51.1 \pm 18.1\%$) of the total AGEs in catfish muscle.

The amounts of AGEs in a few raw fish have been reported, but often species and condition of the fish muscle and the forms of AGEs (whether free and protein-bound) are not reported. Chao et al. (2009) reported about 0.12 mg/kg of protein-bound CML in salmon (species not identified) and about 0.20 mg/kg in cod (species not identified). Chen and Smith (2015) found much higher levels of protein-bound CML in fish muscle: 1.92 mg/kg for salmon (species not identified) and 1.07 mg/kg for tilapia (species not identified). Uribarri et al. (2010) reported that the protein-bound CML determined based on an enzyme-linked immunosorbent assay (ELISA) was 517 kU/100 g for previously frozen salmon (species not identified) and 527 kU/100 g for fresh salmon (species not identified).

Our study is the first to show a large biological variation in AGEs among fish muscle of the same species, providing a possible explanation for somewhat inconsistent CML levels in fish muscle reported by different research groups. In addition, most of the reported studies on dietary AGEs focused on CML, but in our study CEL was included. This is significant since CEL levels were generally higher than CML in individual grass carp and catfish muscle, indicating that quantification of

CEL is equally or perhaps even more important than that of CML. What is more, very high levels of free CEL were found in the muscle of some individual catfish tested. This implies a large biological variation in free CEL as well as the necessity of differentiating between free and protein-bound AGEs in foods instead of quantifying free and protein-bound AGEs as a whole which is a common practice with ELISA as quantification method.

3.4. Effects of heat treatments on the amounts of AGEs in fish muscle

Figs. 3 and 4 show the levels of AGEs in raw and heat treated grass carp and in catfish muscle. Heat affected the formation of free AGEs and protein-bound AGEs in fish muscle differently. Heat had little or no effects on the levels of free AGEs in fish muscle (Figs. 3A & B, Figs. 4A & B). Free CML in grass carp muscle did not increase with heating [0.39 ± 0.10 mg/kg (raw), 0.40 ± 0.08 mg/kg (30 min heating)]; there was also little change in the level of free CEL in grass carp muscle [0.08 ± 0.03 mg/kg (raw), 0.10 ± 0.03 mg/kg (30 min heating)]. For catfish muscle, heat treatments significantly affected the levels of free CML [0.08 ± 0.03 mg/kg (raw), 0.09 ± 0.03 mg/kg (5 min), 0.11 ± 0.03 mg/kg (10 min), 0.12 ± 0.03 mg/kg (30 min)], although the amounts of free CML in raw and heat treated catfish muscle were very low (0.08–0.13 mg/kg). The overall impact of heat treatment on the formation of free CML in catfish muscle was minor. No significant difference in free CEL levels was observed between raw and heat treated catfish muscle [3.49 ± 4.25 mg/kg (raw), 3.67

± 4.27 mg/kg (30 min)]. These results were consistent with our previous study indicating that commercial sterilization (121 °C, 10 min) had no significant effect on the levels of free CML and CEL in muscle foods, including pork (hind leg, tenderloin, belly), beef (rump, ribeye, short plate), and chicken (chicken breast, chicken leg) (Sun et al., 2016a).

The amounts of protein-bound CML and CEL in fish muscle increased as the heating (100 °C) time increased (Figs. 3 C&D, Figs. 4 C&D). The average amounts of protein-bound CML in grass carp muscle increased from 0.96 mg/kg (± 0.17 mg/kg, raw) to 5.50 mg/kg (± 1.89 mg/kg, 30 min heating), and protein-bound CEL increased from 2.93 mg/kg (± 1.19 mg/kg, raw) to 6.03 mg/kg (± 2.42 mg/kg, 30 min heating). The averages of protein-bound AGEs in catfish muscle were: CML, 0.75 ± 0.20 mg/kg (raw), 3.71 ± 0.94 mg/kg (30 min); CEL, 2.11 ± 2.24 mg/kg (raw), 3.46 ± 2.71 mg/kg (30 min). Higher amounts of protein-bound AGEs were formed in fish muscle as the severity of the heat treatments increased; similar results were also observed for other foods (Sun et al., 2015; Zhang et al., 2011).

Raw fish muscle generally contained higher levels of protein-bound CEL than CML, but more protein-bound CML than CEL was formed during heating. Following 30 min of heat treatment, the average amounts of protein-bound CML and CEL in fish muscle were similar. A zero-order reaction fitted better than the first- or second-order reaction for both protein-bound CML and CEL in either grass carp or catfish muscle during 30 min of heating. However, the r^2 and the reaction rate for protein-bound

CML or CEL formed in the muscle of each individual fish varied greatly: CML, $r^2 = 0.49\text{--}0.99$, $K = 0.04\text{--}0.26$; CEL, $r^2 = 0.60\text{--}1.00$, $K = 0.01\text{--}0.19$). Although zero-order reaction fitted very well for the formation of CML or CEL in the muscle of some individual fish during heating, it did not work for about half of the other tested individual fish. This further indicated the complexity of the mechanisms of AGEs formation during heating, which was strongly affected by the biological variation of individual fish. The formation of AGEs in more homogenous food or model food systems, such as ground beef (Sun et al., 2015) and sugar-casein model system (Morales and Van Boekel, 1996) were reported as zero-order reactions, implying that the concentration of a tested AGE did not affect its formation rate.

Several different pathways leading to the formation of CML and CEL have been proposed or recognized, but there is no reported study explaining the disparity between the formation of free CML or CEL and protein-bound CML or CEL upon heating. The formation of free CML or CEL requires the glycation of lysine, a free amino acid, while the formation of protein-bound CML or CEL involves the glycation of protein *via* the ϵ -amino group of lysine residues. Since lysine molecule contains α -amino group and α -carboxylic acid group in addition to ϵ -amino group, which is much more reactive than lysine residue in a protein, lysine in fish muscle may rapidly participate in chemical reactions (such as acylation, simple acid-base reaction) other than those leading to the formation of free CML or free CEL upon heating (Sun et al., 2016b); therefore, heating has little or no effect on the formation of free CML or CEL

in fish muscle.

4. Conclusions

There is a large biological variation in AGEs levels among individual fish of the same species. High levels of free and protein-bound CEL were found in the muscle of some catfish but not others, indicating the importance of quantifying CEL in addition to the more commonly analyzed CML to better understand the overall AGEs profiles and the formation of AGEs in muscle foods. Heating had little or no effect on the levels of free CML and CEL in fish muscle. Protein-bound CML and CEL in fish muscle increased substantially and were formed at higher amounts at longer heating time. After 30 min of heating, protein-bound CML in grass carp muscle increased 2.4–10.8 fold, and in catfish muscle increased 2.1–8.5 fold; protein-bound CEL in grass carp increased 27%–242%, and in catfish muscle increased 28% to 224%. Heat exerted different effects on free AGEs and protein-bound AGEs in fish muscle, indicating the importance of analyzing both free and protein-bound AGEs separately, particularly for studying the kinetic of AGEs formation. The formation rate of protein-bound CML or CEL in fish muscle during heating could not be modelled with simple kinetic functions (including 0, 1st, 2nd-order reactions), although zero-order reaction fitted well for the muscle of some individual fish, which further indicated the complexity of AGEs formation in fish muscle during heating and the strong impact of biological variation of individual fish on AGEs formation.

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Figure captions

Fig. 1. Sample location of a 10-cm width of steak cut off from (A) grass carp and (B) catfish (only the white muscle above the lateral line on both sides of the steak was used).

Fig. 2. LC-MS/MS chromatograms of fish muscle spiked with internal standard d₄-N^ε-carboxymethyllysine (d₄-CML) obtained through multiple reaction monitoring in positive-ion mode.

Fig. 3. Effects of heat treatments (100 °C; 5, 10, 30 min) on the amounts of (A) free N^ε-carboxymethyllysine (CML), (B) free N^ε-carboxyethyllysine (CEL), (C) protein-bound CML, and (D) protein-bound CEL in grass carp muscle ($n = 3$). (^{abc} Different letters indicated that the average amounts of protein-bound CML or CEL in raw and heat treated fish muscle were significant different, $p < 0.05$).

Fig. 4. Effects of heat treatments (100 °C; 5, 10, 30 min) on the amounts of (A) free N^ε-carboxymethyllysine (CML), (B) free N^ε-carboxyethyllysine (CEL), (C) protein-bound CML, and (D) protein-bound CEL in catfish muscle ($n = 3$). (^{abc} Different letters indicated that the average amount of free or protein-bound CEL in raw and heat treated fish muscle were significant different, $p < 0.05$).

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Fig. 4. Effects of heat treatments (100 °C; 5, 10, 30 min) on the amounts of (A) free *N*^c-carboxymethyllysine (CML), (B) free *N*^c-carboxyethyllysine (CEL), (C) protein-bound CML, and (D) protein-bound CEL in catfish muscle (*n* = 3). (^{abc} Different letters indicated that the average amounts of free or protein-bound CEL in raw and heat treated fish muscle were significant different, *p* < 0.05).