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Abstract

The aim of the study was to characterize Maillard reactions in meat under different cooking treatments. Considered temperature-time combinations included raw samples (control), 58, 80, 98 and 160° C for 72 min, 118° C for 8 min and 58° C for 17 hours. Furosine, a marker for heat treatment, was detected in all groups with roasting having a 4-fold increase over the control. Sous-vide treatment at 80°C, boiling and autoclaving also contribute to a significant increase in furosine. Nε-carboxymethyllysine, an indicator for advanced glycation end products, showed negligible amount in control, but increased with cooking temperature, with oven samples showing the highest values. A similar increasing trend was observed in lanthionine, covalently bonded protein crosslinks, which arises due to severe thermal regimes. Simultaneously, glycation and deamidation formation were tracked in meat proteins through peptidomics to highlight residue level changes that might affect nutrient value in processed muscle based foods.

Keywords	Furosine; lanthionine; protein crosslinks; carboxymethyllysine; advanced glycation end products; Maillard reaction; cooked meat; residue level modifications
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This paper deals with the effect of an assortment of heat treatments, reflecting those most used for heat processed meat products, on the levels of Maillard reactions markers and on the consequent protein modifications of pork proteins, including formation of furosine, carboxymethyllysine and lanthionine. Protein residue level modification was also addressed through proteomics.

Both cooking time and cooking temperature showed a clear effect on most of these parameters, indicating that the type of heat process for meat products leads to different extent of Maillard reactions and derived protein modifications that might eventually influence the nutritional quality and the protein bio-accessibility. Peptide modifications, such as formation of carboxymethlylysine or pyroglutamic acid or deamidation were also detected at residue level. Interestingly, studied proteins showed a different level of modifications.

The information obtained provides with knowledge about the extent of protein structural modifications under specific cooking conditions, and may be useful in the optimization of heat processing for meat products aiming for optimized protein nutritional features.

HIGHLIGHTS

- Maillard reactions in pork under different heat treatments were studied.
- Early stage Maillard reactions and AGEs increased with higher cooking temperature and time
- Glycation and deamidation were tracked in meat proteins through peptidomics
- Proteins showed a different extent of modification due to Maillard reactions at the residue level

Advanced glycation end products, protein crosslinks and post translational modifications in pork subjected to different heat treatments.

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Abstract

The aim of the study was to characterize Maillard reactions in meat under different cooking treatments. Considered temperature-time combinations included raw samples (control), 58, 80, 98 and 160° C for 72 min, 118° C for 8 min and 58° C for 17 hours. Furosine, a marker for heat treatment, was detected in all groups with roasting having a 4-fold increase over the control. Sous-vide treatment at 80°C, boiling and autoclaving also contribute to a significant increase in furosine. N^{ε}-carboxymethyllysine, an indicator for advanced glycation end products, showed negligible amount in control, but increased with cooking temperature, with oven samples showing the highest values. A similar increasing trend was observed in lanthionine, covalently bonded protein crosslinks, which arises due to severe thermal regimes. Simultaneously, glycation and deamidation formation were tracked in meat proteins through peptidomics to highlight residue level changes that might affect nutrient value in processed muscle based foods.

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1. Introduction

Cooking does not only make meat microbiologically safer, but also enhances its aroma, colour and palatability (Lund & Ray, 2017). However, undesired and unintentional outcomes such as loss of essential amino acids and generation of hazardous compounds occur under the influence of thermal processing, which has a detrimental effect on meat nutritive quality and safety (Trevisan, De Almeida Lima, Sampaio, Soares, & Markowicz Bastos, 2016). With cooking operations intermediate Amadori compounds are formed, following different chemical pathways that produce aroma, flavour and browning, important for consumer acceptance. However, heat treatment of food also results in development of advanced glycation end products (AGEs) that are potentially toxic and decrease the nutritional value of protein (Henle, 2009).

During the early stage of Maillard reactions (MRs), furosine acts as an indicator of the extent of damage in food items (Pompei, Spagnolello, & Alimentari, 1997) and on their slow degradation, partial AGEs are formed (Li, Liu, Meng, & Wang, 2017). Out of many reactions during non-enzymatic browning, when lysine reacts with reducing sugars, fructosylysine (FL) is formed, and further oxidized to N^{ε}-carboxymethyl lysine (CML), a frequently used marker of dietary AGEs (Račkauskiene et al., 2015; Roldan et al., 2015). As much chemical methods determine formation of CML, such glycation modifications could also be tracked via proteomics which provides a well-established analytical platform. The fact that AGE formation was higher in frying and roasting rather than boiling could be confirmed through redox proteomics as mentioned by Hu et al. (2017).

Heat and alkali treatments during food processing also result in the formation of dehydro- and cross-linked amino acids, such as lysinoalanine (LAL), histidinoalanine and lanthionine (LAN) in proteins (Friedman, 1999). With a proton from cystine abstracted and persulfide elimination in the β position, dehydroalanine (DHA) is yielded which then attacks the nucleophilic side of amino acids resulting in the above mentioned cross-links. These DHA-derived compounds and covalent cross-links (LAN and LAL), apart from decreasing protein digestibility and texture also impact tertiary protein structure with an increase in molecular weight (Wada, 2014).

The intake of MRPs from processed foods has a possible correlation with serum AGE levels in humans with the possibility that it might promote oxidative damage, aging, diabetes, obesity and cardiovascular diseases (Trevisan et al., 2016). Some studies suggest that dietary AGEs are potential indicators of oxidative stress and inflammation (Chen & Scott Smith, 2015). Furthermore, no association of dietary AGEs was found with protein bound AGEs in human body, while significantly increasing free CML levels in plasma and urine (Scheijen et al., 2017). However, the risk effects of dietary AGEs on human health are still contentious (Sun et al., 2015).

Typically, the amount of formed AGEs is dependent upon several parameters, like cooking method, temperature, time or protein and fat content (Scheijen et al., 2016). There is however very little information about the extent of MRs and the consequent formation of MRPs, including AGEs, in different types of meat products. While cooked meat has been considered as a main source of AGEs in diets (Goldberg et al., 2004), such information was based on enzymatic assays, which have been demonstrated totally inaccurate for food matrixes (Poulsen et al., 2013).

Therefore, our work aimed to get a deeper knowledge on how thermal treatments of pork, with different combinations of time and temperature that mimic those most commonly used for meat processing, could induce Maillard and cross-link modifications in pork. Early and late stage MRPs were tracked chemically, opening the possibility of use them as markers for thermal treatment in cooked meat products. Assessment of AGEs content through detection of CML in cooked meat samples so that guidelines could be developed for evaluating associated risks with dietary AGE consumption in the future. Furthermore, impact of protein glycation was also investigated on amino acid residues via a peptidomic approach to gain a deeper understanding about residue level modifications.

2. Materials and methods

2.1. Chemicals (Analytical grade)

Reagents used in this experiment are enlisted below: Iodoacetamide (IAA), Phosphate buffered saline (PBS), Perfluoropentanoic Acid (CF_3 $(CF_2)_3COOH$) and DL-lanthionine were brought from Sigma Aldrich, Missouri, USA. Other chemicals like Sodium dodecyl sulphate (SDS) and 1, 4-Dithio-DL-Threit (ol) (DTT) were obtained from AppliChem GmbH, Darmstadt, Germany. Urea (CH_4N_2O), Sodium Acetate and Hydrochloric Acid (HCl) were purchased from Merck KGaA, Darmstadt, Germany. Buffers including Acetonitrile (CH_3CN) and Formic Acid (FA) were outsourced from VWR International, Søborg, Denmark. External and internal standards included N^{ε}-carboxymethyl-L-Lysine (CML), Furosine dihydrochloride and deuterated lysine (L-Lysine-4, 4, 5, 5 -d4 hydrochloride) and they were from Polypeptide Group (Strasbourg, France). Millipore-Milli-Q system (Milli-Q Plus, Bedford, MA) treated water was used for buffer preparation.

2.2. Processing Design

Seven female pigs weighing in the range of 83 - 86 kg were collected from Danish Crown (Supplier No. 77752, Denmark). Animals were CO_2 stunned and killed by exsanguination. pH values ranged from 5.5-5.6 and the proportion of carcass lean ranged 59 – 63 %. Carcasses were stored for 24h at 4°C. *Longissimus lumborum* muscles from both sides of the carcass were selected. Steaks of 2 cm thickness were chopped, labelled and packed in vacuum bags (LogiCon EM-628824 - Vacuumpose 200 x 270 x 0,090 mm^3, Kolding, Denmark) and kept at -80° C. Study design included 7 pigs x 7 cooking methods x 3 steaks, for a total of 147 steaks. Sample replicates were then thawed and cooked.

2.3. Cooking treatments

Pork chops were cooked in various ways, trying to reflect the most common heat treatments and cooking methods for meat. Treatments were RAW (control), OV16072 (roasted in convective oven at 160 °C for 72 min), B9872 (braised vacuum packaged in simmering water at 98 °C for 72 min), SV5872 (*sous vide* treatment at 58 °C for 72 min in a thermostatized water bath), SV5817 (*sous vide* treatment at 58 °C for 17 hours, in the same water bath), SV8072 (*sous vide* treatment at 80

°C for 72 min, also in the same water bath) and AC1188 (autoclave treatment at 118 °C for 8 min, with an F-value = 2.56). Those chops to be cooked under 100° C, were coded and vacuum-packed in Cryovac CN 300 bags (Sealed Air Corporation, North Carolina, USA); those aiming for cooking at 118 °C were put in LogiCon vacuum bags (EM-62890, Kolding, Denmark) while oven samples were roasted unpackaged. A Type T external probe (fitted to a Testo 176 T4 data logger) was attached to a dummy sample in each batch to track the time-temperature profile of the meat core and surface. After cooking, samples were submerged under ice cold water at 4 °C. Replicates were then packed again, coded and stored at -80 °C.

2.4. Protein determination

Protein determination of steak samples was performed after they were measured at a wavelength of 280 nm following a procedure previously described by (Mitra, Lametsch, Akcan, & Ruiz-Carrascal, 2018).

2.5. Tracking Furosine

Furosine content was determined in meat samples as described by Jansson et al. (2014) with major adaptations. 2.5 g of frozen meat was homogenized by Ultra Turrax T25 for 30 sec with 20,500 rpm in 20 mL of PBS (0.01 M, pH 7.4). Acid hydrolysis was made by mixing 1 mL of meat homogenate, 3 mL of 10 M HCl was added in a screw cap glass tube and nitrogen (2 atmosphere pressure) was bubbled through the mixture for 2 min to make it homogenous and prevent it from coming in contact with oxygen. The tubes were sealed and placed in the oven at 110° C for 18 hours. After heating, samples were cooled in a fume hood and cooled mixture was filtered through a filter paper (Syrevasket foldefilter 3FF-15 cm, 20 µm, Frisenette ApS, Knebel, Denmark). 1 mL of the filtrate was pipetted out by a 3-piece single-use syringe (Omnifix-F, B. Braun Melsungen AG, Germany) and was subjected to pass through a 0.45 µm disposable filter (Minisart NML Plus, Sartorius Stedim Biotech GmbH, Goettingen, Germany). The sample was diluted 5 times in 3M HCl and 300 µL was transferred into HPLC Vials (Phenomenex, Danaher Corporation, USA). Detection of furosine was performed on a HPLC System (Agilent 1100 series, USA) with a Diode Array Detector (DAD, Gilson, USA), using a Supelco Supercoil LC-8 Column (Sigma-Aldrich Inc.)

and was triggered onto the autosampler prior analysis. With a flow rate of 1 mL/ min, 0.06 M sodium acetate buffer solution was operated as the mobile phase in an isocratic mode. The injection volume was 10 µL, and the DAD detection (UV) was set at 280 nm. The temperature of the column was kept steady at 25 °C by a chilling-heating system in a column oven (Jones Chromatography 7955, SpectraLab Scientific Inc., Canada). Quantification was performed by using external calibration with furosine dihydrochloride. Furosine concentration was expressed in mg/100 g protein. All the analyses were carried out in triplicates.

2.6. AGEs and protein crosslinks

Acidic hydrolysis of meat samples was performed in order to quantify AGEs and protein cross-links via LC-MS/MS. Modifications and major adaptations were done to the protocol as proposed by Roldan et al. (2015). Briefly, 50 mg of meat were homogenized with 1 mL 6 M HCl and incubated for 24 hours at 110 °C. Thereafter, HCl was evaporated by gentle nitrogen flow. Dry samples were dissolved in 1 mL water, sonicated for 5 minutes at room temperature and centrifuged at 19,000 rpm at 4° C. Supernatants were diluted 1:20 (v/v) in water containing 1 µg/mL deuterated lysine (internal standard for LC-MS/MS quantification). Following a second centrifugation step (19,000 rpm at 4°C), 10 µL of supernatant were injected for LC-MS/MS analysis.

Simultaneous determination of CML and LAN was performed by reverse-phase UHPLC coupled with mass spectrometer (ThermoScientific Q-Exactive Orbitrap, USA), using electrospray ionization in positive mode. Source parameters were optimized by auto-tuning the mass spectrometer via direct injection of standard solutions in the ionization chamber. The method was designed for a two-step separation of analytes of interest: 100% aqueous buffer (5 mM perfluoropentanoic acid in water, buffer A) from 0 to 5 minutes for detection of LAN, followed by a 0 to 50% organic mobile phase gradient (5 mM perfluoropentanoic acid in 100% acetonitrile, buffer B) from 5 to 15 minutes, when detection of CML was achieved. Flow was then as follow: 50 to 100 % B (15 to 17 minutes); 100% B (17 to 22 minutes); 100 to 0% B (22 to 24 minutes) and 100 % A (24 to 27 minutes). Identification of peaks was performed by monitoring the typical m/z ratio for each analyte and two of the most abundant derived fragments, using ThermoScientific Xcalibur software. With a

retention time of 10.4 min and 12.3 min and with m/z ratio of 205.1 and 151.1, CML and deuterated lysine were detected. Two daughter fragments were identified which were 84.1 and 130.1 for CML and 88.1 and 134.1 for deuterated lysine. Simultaneously, LAN was detected with a retention time of 3 min and m/z ratio of 209.1, and the most abundant derived daughter ion was identified as 120. Quantification was performed based on a previously derived external calibration curve in which the peak area ratios of pure analytes compared to the areas of the internal standard detected in each run were calculated. Standard concentrations ranged from 5 to 10,000 ng/mL. The resulting concentrations in meat samples were then referred to the known protein content of each sample and expressed as mg/100 g protein.

2.7. Peptidomic approach to trace modifications

Samples were first hydrolysed by using trypsin following the method previously described by Mitra et al. (2018). After acidification, peptide modification of samples were analysed by a Dionex 3000 RSLC UHPLC system (Thermo Fisher Scientific, Hvidovre, Denmark) coupled with a Q Exactive mass spectrometer (Thermo Fisher Scientific, Hvidovre, Denmark) following the conditions previously described (Mitra et al., 2018). Tracked site specific modifications taken from Deb-Choudhury et al. (2014) are enlisted in Table 1. Carbamidomethylation was added as static modification and rest all modifications uploaded was dynamic. Different amino acid residue targets were set in accordance to the modification type and their mass shift were recorded. In addition to it, a separate database search was also conducted to check susceptibility of lysine *via* participation in AGEs formation in myosin and actin for the salt soluble fraction and in β-enolase and myoglobin for water soluble fraction. Apart from this, one the most important post-translational modifications, referred to as deamidation was also tracked in myoglobin in our experiments as literature suggests that amount of this modification can play a crucial role in protein unfolding and utility (Cañete, Mora, & Toldrá, 2017). Total abundance of selected proteins (myosin, actin, beta-enolase and myoglobin) and calculation of the modification ratio were calculated as explained in Mitra et al. (2018), using equation 1.

Equation 1: Modification Ratio = (peptide abundance (sample) / protein abundance (sample)).

2.8. Statistical Analysis

One-way ANOVA was used with cooking treatment as the independent variable, using the General Linear Model (GLM) procedure (SPSS 22.0, IBM, USA). Descriptive statistics and tukey's test were performed to determine variations among the mean values. When the effect was significant ($P < 0.05$), Tukey's test was used at 5% level to make pair wise comparisons between sample means. All values represented are shown as mean with standard error bar.

3. Results and discussion

3.1. Furosine

Furosine often referred to as (ε-N-(furoylmethyl)-$_L$-lysine), is considered to be an indirect indicator for Amadori products and often times related to early stage Maillard reaction products (Henle, 2009). Fig. 1 details the amount of furosine formation induced by cooking treatments. Such reactions could also be influenced by the availability of reducing sugars like glucose and ribose that react with ε-amino groups of lysine (Roldan et al., 2015). No increase in furosine was observed between RAW and SV5872 groups, probably owing to the fact that the extent of formation of this type of compounds is likely to occur at higher temperatures. But at this same temperature and with longer time (17 h) a significant increase was traced from RAW group, possibly because longer time might positively influence the formation of indirect Amadori compounds. However, there was no increase in furosine content in samples cooked at 58ºC from 72 min to 17 h. With increase in temperature, SV8072, B9872 and AC1188 groups had significantly higher values than RAW and 58ºC *sous vide* groups. This is a very similar trend observed by Pompei and Spagnolello (1997), who showed that the higher formation of furosine in cooked hams was directly linked to increasing heating temperature. To support the above statement, our interpretation is strengthened by the observation that when we compare AC1188 group with SV5817 samples, furosine formation is higher at 118 °C than 58 °C irrespective of the duration of the treatment. Additionally, OV16072 samples had a 4-fold increase in furosine concentration from the RAW ones, suggesting increased reaction between lysine residues and reducing sugars probably due to the same reason as mentioned above. On top of that, oven samples were the only unpackaged ones, which lead to a strong dehydration of the surface during the process, and due to that, probably to a marked decrease in water activity. It is well known that low water activity enhances the rate of MRs, which might also contribute to the much higher levels found in oven cooked samples.

However, for B9872, SV8072 and AC1188 sample groups, there was no difference in furosine content, even though there was a marked elevation in temperature. This might be due to the fact

that, even if furosine production was high, some of these compounds might get degraded as MR progressed, in the process generating intermediate and end products (Trevisan et al., 2016). With such modifications as instigated by MR, lysine loses its biological value with 40 percent of the Amadori product getting transformed into furosine (Rufián-Henares, Delgado-Andrade, Jiménez-Pérez, & Morales, 2007; Schmidt, Boitz, & Mayer, 2017). Therefore, these results point out to an important decrease in nutritional values of meat proteins as cooking temperature increases.

3.2. Tracing CML as an AGE marker

CML, being an important indicator for AGEs, was measured in all the sample groups as illustrated in Fig. 2. In the RAW and SV5872 samples, CML values were quite similar indicating to those from samples cooked at lower temperature CML formation was limited. A further confirmation of CML limitation was also observed in SV5817 group when samples had same values with the previous treatments even though the heat treatment took 17 hours. Samples that were braised at 98° C and autoclaved at 118° C did have a significant difference with raw samples, but were similar to SV8072 group, suggesting that CML formation had quite a similar trend with furosine formation. One of the possible reasons for the autoclaved samples to have no increase in CML values was possibly because at this temperature N^{ε}-carboxyethyl Lysine (CEL) formation is highly favoured and preferred more than CML generation; chemistry behind such observation may be due to the faster reaction rate of methylglyoxal with lysine, rather than the reaction of glyoxal with lysine, at pasteurization temperatures (Yu et al., 2016). Alternately, another possible way of describing limited formation of CML can be presence of carnosine as an anti-glycating agent that inhibits crosslink and protein glycation induced by reducing sugars and reactive aldehydes (Roldan et al., 2015).

Interestingly when AC1188 and SV5872 groups were compared it could be noticed that temperature had a role to play in the formation of CML. As reported by Račkauskiene et al. (2015), CML formation could be based upon two pathways; firstly via oxidative cleavage, the enediol form of N^{ε}-fructosyllysine may give rise to CML formation; secondly via the Namiki pathway where the amino group of lysine can react with GO. In fact, even at a holding time of 8 min, values were

higher in samples cooked at 118ºC than in those cooked at 58 °C for 72 min. In addition to it, OV16072 samples, had a high significant increase in CML values than all the treatment groups which was approximately a 3.5 fold increase from B9872, AC1188 and SV8072 samples, following a similar trend to that reported by Chen & Scott Smith et al. (2015). Such elevated rise in CML formation can be attributed to the fact that carbonyls from lipid oxidation might contribute to the formation of CML via the MR route (Yu et al., 2016). Moreover, upon dehydration caused by roasting, water soluble precursors would be transferred to the meat surface with a higher degree of exposure, and at a lower water activity, hence AGEs formation was accordingly influenced (Chen & Scott Smith, 2015). The CML results reported by Roldan et al. (2015) were in fact not in agreement with the ones reported here in our study, possibly because their core temperature was kept at 73° C, while in our case the internal temperature in oven samples reached 135 °C. Hence, it seems clear that AGEs generated from the core also regulated the total glycation pool other than the surface. At any rate, these results confirm that temperature plays a pivotal role in the formation of AGEs.

3.3. Covalent protein cross-links

Formation of crosslinks like LAN occurs as a consequence of cooking treatments, as depicted in Fig 3. When food proteins are exposed to heat, alkali and oxidative treatments, inter and intramolecular crosslinking occur. LAN is formed from an early dehydration of cysteine residue to form dehydroalanine, which then reacts with thiol groups of cysteine to form a crosslinked derivative (Clerens, Plowman, & Dyer, 2012; Singh, 1991). According to Yu et al., (2016), reports suggest that formation of LAN were much below the level of detection and in some cases after prolonged boiling no traces were found as detected by HPLC. However, with our mass spectrometry method, we were able to detect LAN as a protein covalent crosslink in heated meat products after acidic hydrolysis, possibly due to the higher sensitivity of the mass spectrometry-based determination method. Even though formation of LAN is known to occur under alkaline conditions, the formation of LAL by heating in the acidic and neutral pH regions has also been

reported (Friedman, 1999), and so has been its presence in heated meat (Hasegawa, Mukai, Gotoh, Honjo, & Matoba, 1987).

The presence of low levels of LAN in RAW samples is surprising and difficult to ascribe to any controlled factor. Levels of LAN did not significantly increase for SV5872, SV5817 and SV8072 groups as compared to RAW. Reports have also suggested that significant amount of LAN were also traced in keratin polypeptides that were derived from bovine skin (Friedman, 1999), suggesting that the LAN found in the RAW pork could also be naturally present.

A significantly increasing trend could be observed when meat steaks were subjected at a braising temperature of 98° C and at an autoclaving temperature of 118° C thereby confirming that higher temperature might have a role to play in formation of crosslinks. In spite of having no significant differences in LAN values among B9872, AC1188 and OV16072, when we compare RAW to OV16072, we did see a 3-fold increment in this crosslinking process. Owing to which, cross linking formation might influence protein aggregation, due to which protein digestibility could be impaired with reduced enzymatic proteolysis as stated by Wada et al., (2014). On the other hand, Hendriks et al., (2002) stated that LAN had no significant correlation to amino acid digestibility. Nevertheless, although there are inconsistencies in formation of LAN under different heating regimes, our results might direct us to come to a conclusion that temperature might be an imperative factor in influencing crosslink formation.

3.4. Qualitative Overview of residue level changes

3.4.1. Heat induced and other modifications

Residue level modifications which are induced thermally are documented in Table 2. Heat induced modifications like CML and CEL could be successfully detected in the LCMS analysis. Deamidation, dehydration, di-dehydro and pyroglutamate formation have been tracked and mapped along with their site specific positions. With myosin being the most abundant protein, it was more susceptible towards these types of modifications. Surprisingly, no CML and CEL were tracked in myoglobin and actin. The reason for absence of CML in both actin and myoglobin could be attributed to the fact that they exhibited much more heat stability even after autoclaving meat samples (Yu, Morton, Clerens, & Dyer, 2016). Nevertheless, deamidation was seen to occur quite extensively in all the meat fractions but with limited amount in myoglobin.

The ratio of lysine modification was quantified in myosin and beta-enolase as shown in Fig 4A and 4B respectively. In myosin, some modifications were traced in RAW samples possibly due to sampling, packaging, mincing or postmortem handling prior the freeze thaw process (Yu, Morton, Clerens, & Dyer, 2015a) or via formation of actomyosin bridge formation (Yu et al., 2016). In SV5872, SV8072, AC1188 and SV5817 groups, an increase in mean value of lysine modification ratio was observed suggesting that heating might have exposed these hydrophobic amino acids as protein unfolded (Sun, Zhou, Zhao, Yang, & Cui, 2011). With respect to B9872 and OV16072 groups, mean values were quite high compared to RAW ones and other groups indicating that such strict treatments might have had a faster effect on lysine modification to CML. Such elevated increase in CML could be explained by three theories, firstly, by interaction of carbonyls with lipid peroxidation products (Hu et al., 2017), secondly by a possible faster kinetic reaction between radicals and reducing sugars forming more stable products (Utrera et al., 2014) and thirdly with dehydration, an increase in pro-oxidant concentration that might contribute to advanced MR (Utrera & Estévez, 2013). In beta enolase, a slightly different trend could be seen. While RAW and SV5872 group had negligible CML modification, SV8072 and SV5817 samples had higher mean ratios directing us to hypothesize that higher temperature and longer time did have an influence in

glycation modification reactions. A similar scenario for B9872 group in myosin and beta enolase was noted. For AC1188, mean ratio was a bit lower possibly due to a holding time of 8 min. Surprisingly, for the oven samples, lower mean ratio values can be attributed to the fact that such glycation based compounds might have participated onto further advanced reactions or perhaps have been aggregated (Yu, Morton, Clerens, & Dyer, 2015b) due to which proteomic detection was difficult.

Pyro-glutamic formation could also be seen on almost all the meat proteins except myoglobin. Such formations not only take place at higher temperatures but are also possible at relatively milder conditions. In fact pyro-glutamic acid formation is not caused by glycation or oxidation but it is believed to occur due to non-enzymatic heating that result in cyclization reactions where a free amino group from glutamine or glutamic acid gets converted into a lactam (Deb-Choudhury et al., 2014). According to Yu et al., (2016), the conversion of glutamic acid (Glu, E) to pyro-Glu is at a much slower rate than that of Glutamine (Gln, Q). This is quite in accordance with our results where we identified only 10 modified peptides for E and 16 modified peptides for Q in myosin. Even for actin, the only modified pyro-Glu formation was for Q as represented by **Q**EYDEAGPSIVHR. Although, protein crosslinking was identified chemically in our aforesaid mentioned results, through proteomics, there was no detection of dehydroalanine in any of the proteins, in accordance with previous reported heat modification of meat proteins (Deb-Choudhury et al., 2014). Among other modifications, di-dehydro conversion was also noticed only in actin in a serine residue. The possibility of such a transformation exists as was also confirmed by Yu et al., (2015) where di-dehydro of a threonine residue was spotted. In this case either serine or threonine could participate, as both of them have hydroxyl groups in their amino acid side chains. Dehydration modifications could also be observed quite a lot in serine and threonine residues in myosin rather than the other proteins.

3.4.2. Deamidation

Modification of amino acids play quite an important role in influencing flavour, taste properties and meat chemistry. Deamidations could be tracked in nearly all the proteins, and more specifically, in

the four selected ones (Fig 2). One of the key factors explaining the extent of deamidation is the availability of asparagine, which is deamidated at much higher rates than glutamine, possibly because of the side chain amide group from the peptide nitrogen (Robinson & Robinson, 2001). This holds true for our case as well, in which we see from Table 2 that number of asparagine residues deamidated are more than glutamine residues, be it in myosin, actin, beta-enolase or myoglobin. Additionally, the peptide sequence, HG**N**TVLTALGGILK, derived from myoglobin, with the letter N highlights the possibility of asparagine getting deamidated more than glutamine. Lastly, for deamidation to occur, it is preferred that N and Q residues are always present at the extreme end of the peptide chain rather than being situated in central positions where susceptibility might be low (Cañete et al., 2017).

Fig 4C illustrates the deamidation ratio in myoglobin. From our qualitative data, we do observe conversion of glutamine and asparagine to glutamic acid and aspartic acid respectively indicating possible deamidation. The chemistry behind it could be described as removal of ammonia from the peptide chain via hydrolysis of the amide groups where glutamine or asparagine residue is transformed into its carboxylic form (Cañete et al., 2017). According to Cañete et al., (2017), temperature, pH and salting conditions are the main drivers behind deamidation, and such deamidations could also be responsible for protein unfolding and protein hydrolysis. Deamidation in RAW group was present, possibly due to sample handling, including freeze-thaw process. Values in in SV5872, SV5817 and SV8072 groups were not very different to RAW, indicating that sous vide cooking methods at moderate temperature had not much to contribute to this modification. For high temperature groups like B9872, OV16072 and AC1188, deamidation rates increased up to many fold times, probably due to increased thermal impact. This is in accordance with Izzo et al., (1993), who reported a positive correlation between high temperature and the increase of deamidation ratio.

Deamidation ratio is somehow connected to protein functionality. Thus, Cañete et al., (2017) suggested that a deamidation of 2-6 % might be beneficial for improving nutritional quality. However, excessive deamidation rates might impair protein quality as well, due to higher

possibilities of protein aggregation via interaction by hydrophobic and electrostatic forces (Lei, Zhao, Selomulya, & Xiong, 2015). Hence temperature has possibly a profound effect on deamidation.

4. Conclusion

This experimental study elucidates the progressive stages of MR from formation of furosine to generation of AGEs and protein crosslink indicators in the form of LAN. Oven cooked samples showed the highest levels for all these indicators, followed by meat cooked in the autoclave and braised, pointing out to a clear effect of temperature on the development of MR in meat. On the other hand, low temperature cooking methods seems to generate not much of furosine, AGEs or LAN. Thus, compared to roasting and autoclaving, meat cooked at lower temperature will contribute very little to MRP generation and might be a decisive strategy for reducing dietary AGEs. Determination of post-translational modifications at peptide level like deamidation and pyro-glutamate conversion has provided an insight of the susceptibility of amino acid residues to chemical modifications likely to occur in the most abundant meat proteins. Through proteomics, we also identified lysine modifications and losses that might impair meat eating quality and nutrient uptake. Therefore, such advanced techniques deployed in this study will provide a fundamental platform in order to choose optimum cooking treatments that perhaps will have less effect on the nutritional quality of meat proteins. Concomitantly, emphasis should be given onto how such glycation and other modifications could regulate nutrient utilization and enzymatic hydrolysis which in turn could influence digestibility behaviour and biological value of essential quality nutrients.

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5. References

Cañete, M., Mora, L., & Toldrá, F. (2017). Deamidation post-translational modification in naturally generated peptides in Spanish dry-cured ham. *Food Chemistry*, *229*, 710–715. http://doi.org/https://doi.org/10.1016/j.foodchem.2017.02.134

Chen, G., & Scott Smith, J. (2015). Determination of advanced glycation endproducts in cooked meat products. *Food Chemistry*, *168*, 190–195. http://doi.org/10.1016/j.foodchem.2014.06.081

Clerens, S., Plowman, J. E., & Dyer, J. M. (2012). Food proteomics: mapping modifications. In J. Heazlewood (Ed.), *Proteomic applications in biology* (pp. 3–33). Intech.

Deb-Choudhury, S., Haines, S., Harland, D., Clerens, S., Van Koten, C., & Dyer, J. (2014). Effect of cooking on meat proteins: Mapping hydrothermal protein modification as a potential indicator of bioavailability. *Journal of Agricultural and Food Chemistry*, *62*(32), 8187–8196. http://doi.org/10.1021/jf502668w

Delgado-Andrade, C., Seiquer, I., Haro, A., Castellano, R., & Navarro, M. P. (2010). Development of the Maillard reaction in foods cooked by different techniques. Intake of Maillard-derived compounds. *Food Chemistry*, *122*(1), 145–153. http://doi.org/10.1016/j.foodchem.2010.02.031

Friedman, M. (1999). Chemistry, biochemistry, nutrition, and microbiology of lysinoalanine, lanthionine, and histidinoalanine in food and other proteins. *Journal of Agricultural and Food Chemistry*, *47*(4), 1295–1319. http://doi.org/10.1021/jf981000+

Goldberg, T., Cai, W., Peppa, M., Dardaine, V., Baliga, B. S., Uribarri, J., & Vlassara, H. (2004). Advanced glycoxidation end products in commonly consumed foods. *Journal of the American Dietetic Association*, *104*(8), 1287–1291. http://doi.org/10.1016/j.jada.2004.05.214

Hasegawa, K., Mukai, K., Gotoh, M., Honjo, S., & Matoba, T. (1987). Determination of the lysinoalanine content in commercial foods by gas chromatography-selected ion monitoring. *Agricultural and Biological Chemistry*, *51*(11), 2889–2894. http://doi.org/10.1271/bbb1961.51.2889

Hendriks, W. H., Butts, C. A., Thomas, D. V., James, K. A. C., Morel, P. C. A., & Verstegen, M. W. A. (2002). Nutritional quality and variation of meat and bone meal. *Asian-Australasian Journal of Animal Sciences*, *15*(10), 1507–1516. http://doi.org/10.5713/ajas.2002.1507

Henle, T. (2009). Maillard Reaction of Proteins and Advanced Glycation End Products (AGEs) in Food. *Process-Induced Food Toxicants*, (2), 215–242. http://doi.org/10.1002/9780470430101.ch2g

Hu, L., Ren, S., Shen, Q., Chen, J., Ye, X., & Ling, J. (2017). Proteomic study of the effect of different cooking methods on protein oxidation in fish fillets. *RSC Adv.*, *7*(44), 27496–27505. http://doi.org/10.1039/C7RA03408C

Izzo, H. V., Lincoln, M. D., & Ho, C. T. (1993). Effect of Temperature, Feed Moisture, and pH on Protein Deamidation in an Extruded Wheat Flour. *Journal of Agricultural and Food Chemistry*, *41*(2), 199–202. http://doi.org/10.1021/jf00026a010

Jansson, T., Clausen, M. R., Sundekilde, U. K., Eggers, N., Nyegaard, S., Larsen, L. B., … Bertram, H. C. (2014). Lactose-hydrolyzed milk is more prone to chemical changes during storage than conventional ultra-high-temperature (UHT) milk. *Journal of Agricultural and Food Chemistry*, *62*(31), 7886–7896. http://doi.org/10.1021/jf501671z

Lei, L., Zhao, Q., Selomulya, C., & Xiong, H. (2015). The effect of deamidation on the structural, functional, and rheological properties of glutelin prepared from Akebia trifoliata var. australis seed. *Food Chemistry*, *178*, 96–105. http://doi.org/10.1016/j.foodchem.2015.01.081

Li, Y., Liu, X., Meng, L., & Wang, Y. (2017). Qualitative and quantitative analysis of furosine in fresh and processed ginsengs. *Journal of Ginseng Research*, 0–5. http://doi.org/10.1016/j.jgr.2016.12.004

Lund, M. N., & Ray, C. A. (2017). Control of Maillard Reactions in Foods: Strategies and Chemical Mechanisms. *Journal of Agricultural and Food Chemistry*, *65*(23), 4537–4552. http://doi.org/10.1021/acs.jafc.7b00882

Mitra, B., Lametsch, R., Akcan, T., & Ruiz-Carrascal, J. (2018). Pork proteins oxidative modifications under the influence of varied time-temperature thermal treatments: A chemical

and redox proteomics assessment. *Meat Science*, *140*(December 2017), 134–144. http://doi.org/10.1016/j.meatsci.2018.03.011

Pompei, C., Spagnolello, A., & Alimentari, T. (1997). Influence of Tumbling and Dextrose on Furosine Content in Cooked Ham. *Society*, *8561*(97), 4080–4083. http://doi.org/10.1021/jf970116n

Poulsen, M. W., Hedegaard, R. V., Andersen, J. M., de Courten, B., Bügel, S., Nielsen, J., … Dragsted, L. O. (2013). Advanced glycation endproducts in food and their effects on health. *Food and Chemical Toxicology*, *60*, 10–37. http://doi.org/10.1016/j.fct.2013.06.052

Račkauskiene, I., Pukalskas, A., Venskutonis, P. R., Fiore, A., Troise, A. D., & Fogliano, V. (2015). Effects of beetroot (Beta vulgaris) preparations on the Maillard reaction products in milk and meat-protein model systems. *Food Research International*, *70*, 31–39. http://doi.org/10.1016/j.foodres.2015.01.026

Robinson, N. E., & Robinson, a B. (2001). Deamidation of human proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(22), 12409–12413. http://doi.org/10.1073/pnas.221463198

Roldan, M., Loebner, J., Degen, J., Henle, T., Antequera, T., & Ruiz-Carrascal, J. (2015). Advanced glycation end products, physico-chemical and sensory characteristics of cooked lamb loins affected by cooking method and addition of flavour precursors. *Food Chemistry*, *168*, 487–495. http://doi.org/10.1016/j.foodchem.2014.07.100

Rufián-Henares, J. A., Delgado-Andrade, C., Jiménez-Pérez, S., & Morales, F. J. (2007). Assessing nutritional quality of milk-based sport supplements as determined by furosine. *Food Chemistry*, *101*(2), 573–578. http://doi.org/10.1016/j.foodchem.2006.02.016

Scheijen, J. L. J. M., Clevers, E., Engelen, L., Dagnelie, P. C., Brouns, F., Stehouwer, C. D. A., & Schalkwijk, C. G. (2016). Analysis of advanced glycation endproducts in selected food items by ultra-performance liquid chromatography tandem mass spectrometry: Presentation of a dietary AGE database. *Food Chemistry*, *190*, 1145–1150. http://doi.org/10.1016/j.foodchem.2015.06.049

Scheijen, J. L. J. M., Hanssen, N. M. J., van Greevenbroek, M. M., Van der Kallen, C. J., Feskens, E. J. M., Stehouwer, C. D. A., & Schalkwijk, C. G. (2017). Dietary intake of advanced glycation endproducts is associated with higher levels of advanced glycation endproducts in plasma and urine : The CODAM study. *Clinical Nutrition*, (March), 1–7. http://doi.org/10.1016/j.clnu.2017.03.019

Schmidt, A., Boitz, L. I., & Mayer, H. K. (2017). A new UHPLC method for the quantitation of furosine as heat load indicator in commercial liquid milk. *Journal of Food Composition and Analysis*, *56*, 104–109. http://doi.org/10.1016/j.jfca.2016.12.014

Singh, H. (1991). Modification of food proteins by covalent crosslinking. *Trends in Food Science and Technology*, *2*(C), 196–200. http://doi.org/10.1016/0924-2244(91)90683-A

Sun, W., Zhou, F., Zhao, M., Yang, B., & Cui, C. (2011). Physicochemical changes of myofibrillar proteins during processing of Cantonese sausage in relation to their aggregation behaviour and in vitro digestibility. *Food Chemistry*, *129*(2), 472–478. http://doi.org/10.1016/j.foodchem.2011.04.101

Sun, X., Tang, J., Wang, J., Rasco, B. A., Lai, K., & Huang, Y. (2015). Formation of advanced glycation endproducts in ground beef under pasteurisation conditions. *Food Chemistry*, *172*, 802–807. http://doi.org/10.1016/j.foodchem.2014.09.129

Trevisan, A. J. B., De Almeida Lima, D., Sampaio, G. R., Soares, R. A. M., & Markowicz Bastos, D. H. (2016). Influence of home cooking conditions on Maillard reaction products in beef. *Food Chemistry*, *196*, 161–169. http://doi.org/10.1016/j.foodchem.2015.09.008

Utrera, M., & Estévez, M. (2013). Impact of trolox, quercetin, genistein and gallic acid on the oxidative damage to myofibrillar proteins: The carbonylation pathway. *Food Chemistry*, *141*(4), 400–4009. http://doi.org/10.1016/j.foodchem.2013.06.107

Utrera, M., Parra, V., & Estévez, M. (2014). Protein oxidation during frozen storage and subsequent processing of different beef muscles. *Meat Science*, *96*(1), 812–820. http://doi.org/10.1016/j.meatsci.2013.09.006

Wada, Y. (2014). Effects of Different Industrial Heating Processes of Milk on Site-Speci fi c Protein

Modi fi cations and Their Relationship to in Vitro and in Vivo Digestibility. *Journal of Agricultural and Food Chemistry*, *62*, 4175–85.

Yu, L., Gao, C., Zeng, M., He, Z., Wang, L., Zhang, S., & Chen, J. (2016). Effects of raw meat and process procedure on Nε-carboxymethyllysine and Nε-carboxyethyl-lysine formation in meat products. *Food Science and Biotechnology*, *25*(4), 1163–1168. http://doi.org/10.1007/s10068-016-0185-5

Yu, T., Morton, J. D., Clerens, S., & Dyer, J. M. (2016). Cooking-Induced Protein Modifications in Meat. *Comprehensive Reviews in Food Science and Food Safety*, *0*, 1–19. http://doi.org/10.1111/1541-4337.12243

Yu, T. Y., Morton, J. D., Clerens, S., & Dyer, J. M. (2015a). In-depth characterisation of the lamb meat proteome from longissimus lumborum. *EuPA Open Proteomics*, *6*(1), 28–41. http://doi.org/10.1016/j.euprot.2015.01.001

Yu, T. Y., Morton, J. D., Clerens, S., & Dyer, J. M. (2015b). Proteomic Investigation of Protein Profile Changes and Amino Acid Residue Level Modification in Cooked Lamb Meat: The Effect of Boiling. *Journal of Agricultural and Food Chemistry*, *63*(41), 9112–9123. http://doi.org/10.1021/acs.jafc.5b03324

Yu, T. Y., Morton, J. D., Clerens, S., & Dyer, J. M. (2016). Proteomic investigation of protein profile changes and amino acid residue-level modification in cooked lamb longissimus thoracis et lumborum: The effect of roasting. *Meat Science*, *119*, 80–88. http://doi.org/10.1016/j.meatsci.2016.04.024

Table 1. List of heat induced and other modifications of amino acid residues under the influence of cooking.

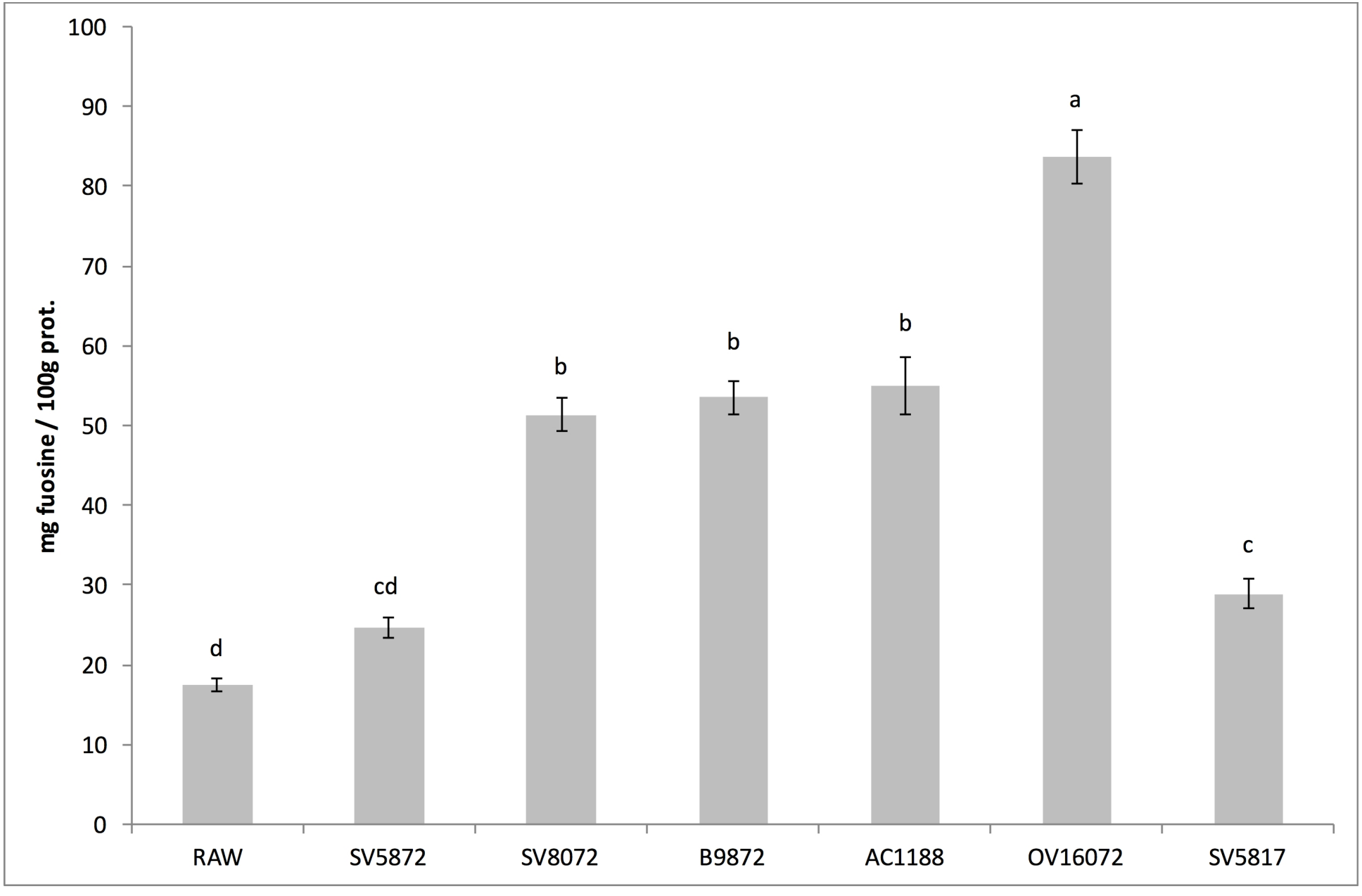
Table 2. Overview of Maillard induced and other modifications in peptide sequences at amino acid residue level with positions.

Figure 1. Effect of processing on the formation of furosine (mg /100 g protein) as an early stage Maillard reaction product. Data shown is represented as mean with standard error bars. Different letters were ascribed for spotting significant differences ($P < 0.05$).

Figure 2. Determination of CML (mg /100 g protein) content as an indicator for AGEs. Data shown is represented as mean with standard error bars. Significant differences ($P < 0.05$) were denoted by different letters.

Figure 3. Effect of cooking treatments on the formation of LAN (mg/100 g protein) as protein cross-link. Data shown is represented as mean with standard error bars. Different letters were ascribed for spotting significant differences ($P < 0.05$).

Figure 4. Ratio highlighting susceptibility to Maillard and other modifications - (A) Lysine modification in Myosin. (B) Lysine modification in beta-enolase. (C) Deamidation ratio in Myoglobin. Only mean values have been shown for a qualitative overview.


mg fuosine / 100g prot.
100
90
80
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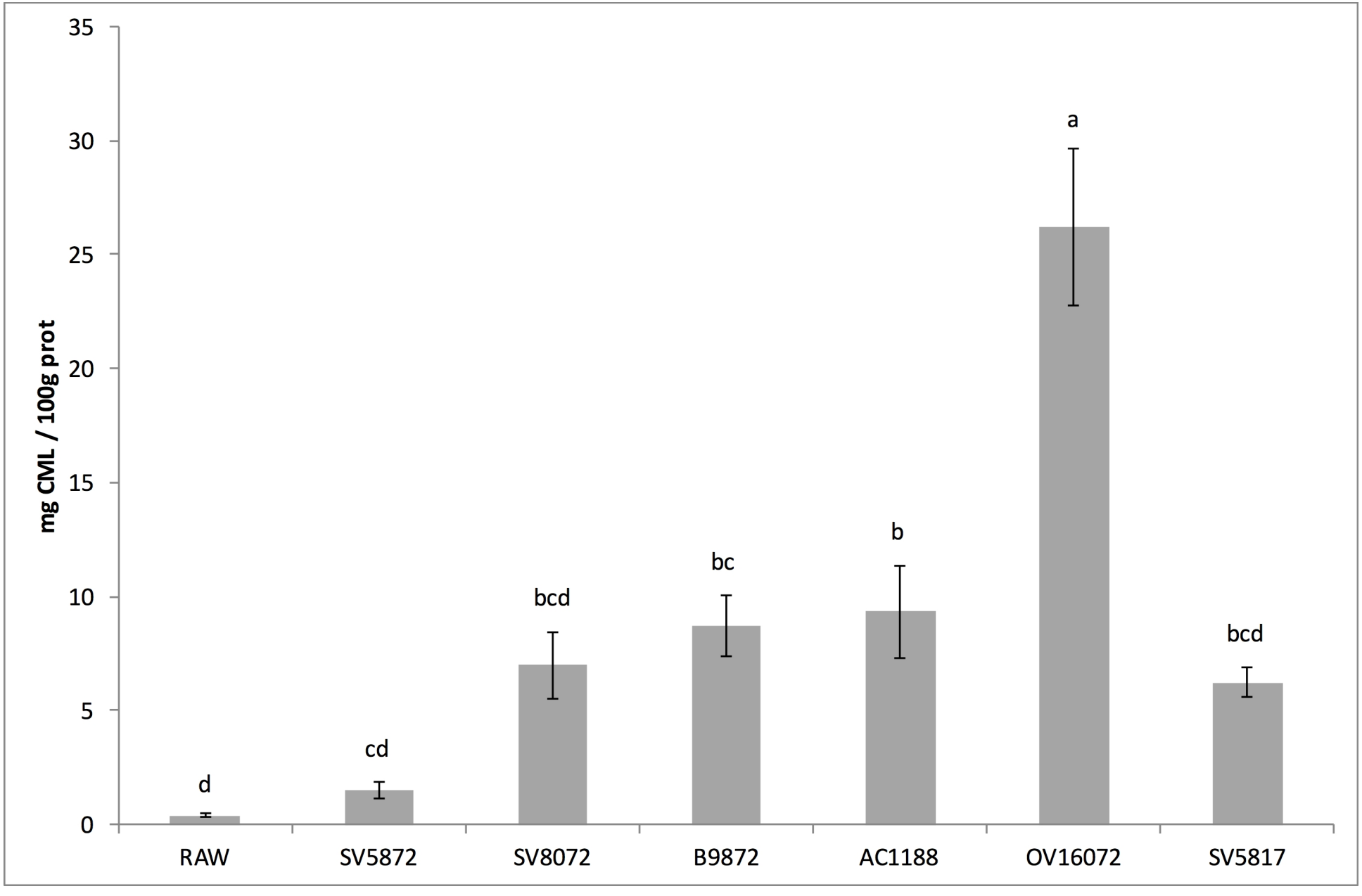
mg CML / 100g prot
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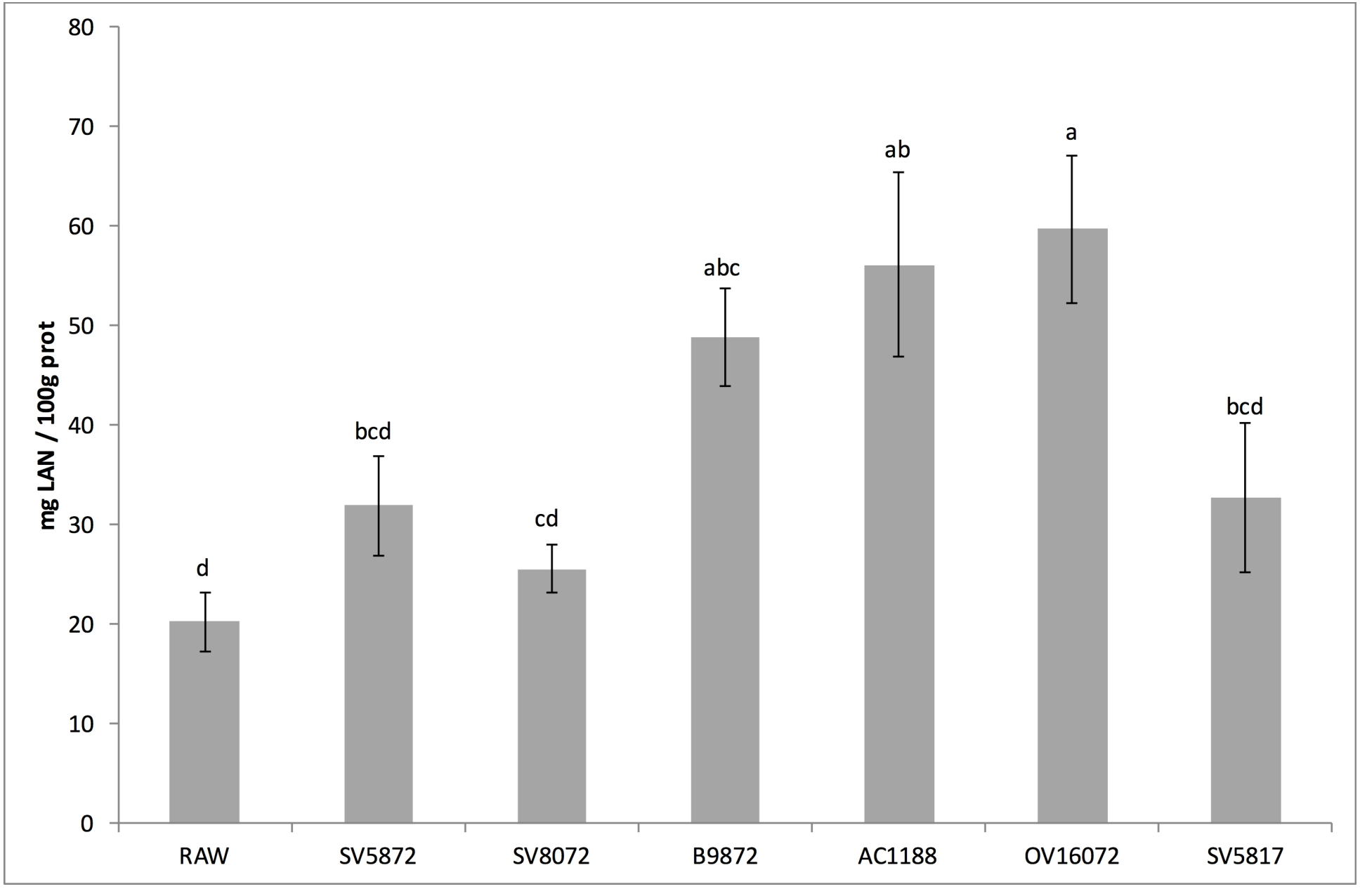
mg LAN / 100g prot
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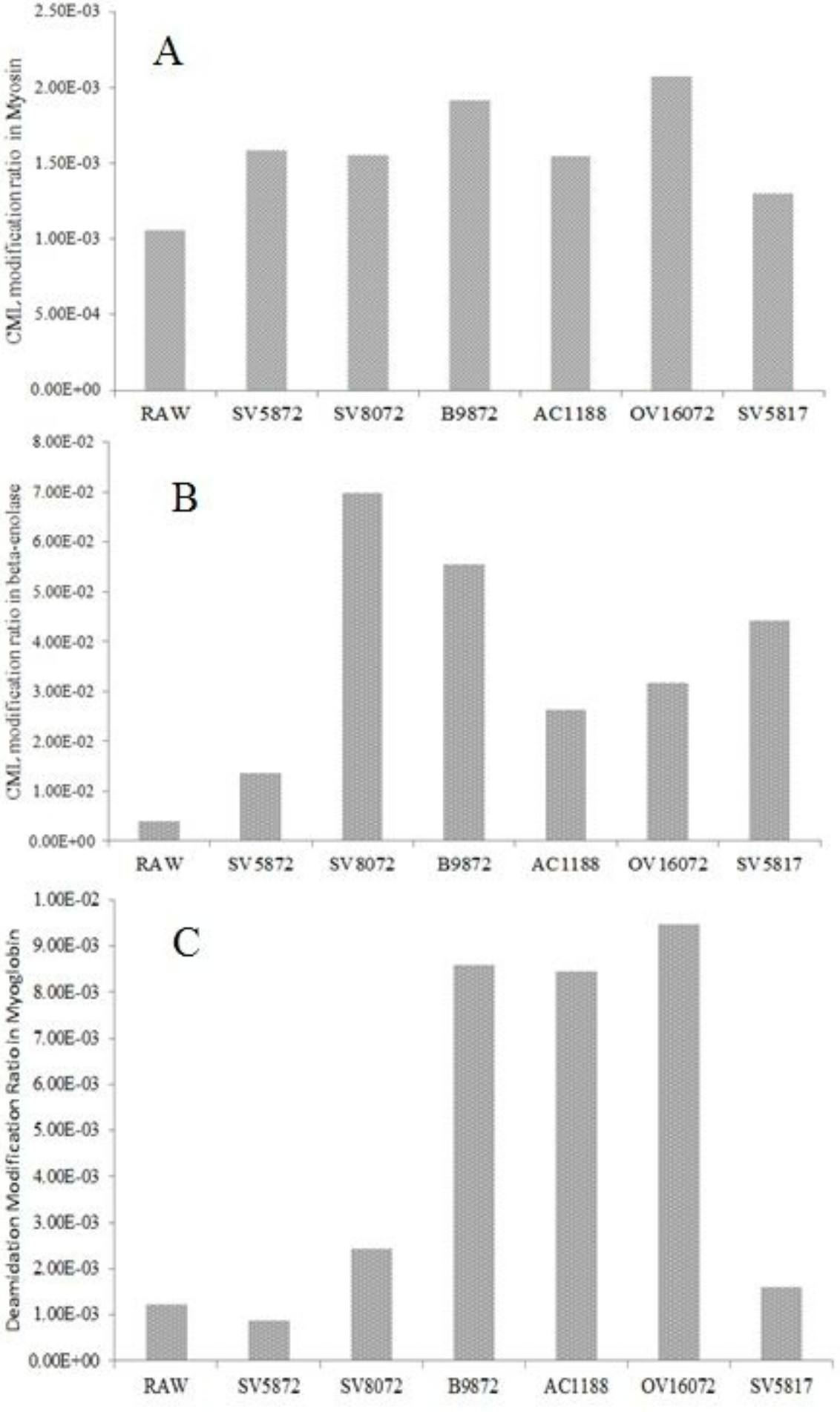
A
CML, modification ratio in Myosin
2.50E-03
2.00E-03
1.50E-03
1.00E-03
5.00E-04
0.00E+00
RAW
SV5872
SV8072
B9872
AC1188
OV16072
SV5817
B
CML, modification ratio in beta-enolase
8.00E-02
7.00E-02
6.00E-02
5.00E-02
4.00E-02
3.00E-02
2.00E-02
1.00E-02
0.00E+00
RAW
SV5872
SV8072
B9872
AC1188
OV16072
SV5817
C
Deamidation Modification Ratio in Myoglobin
1.00E-02
9.00E-03
8.00E-03
7.00E-03
6.00E-03
5.00E-03
4.00E-03
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RAW
SV5872
SV8072
B9872
AC1188
OV16072
SV5817

Table 1. List of heat induced and other modifications of amino acid residues under the influence of cooking.

Slno	Modification	Amino Acid Residue	Position	Formula	Mass Shift (Da)
1	Hex (2)	KR	any	C(12) H(20) O(10)	324.10
2	Carboxymethylation (CML)	KUW	any	C(2) H(2) O(2)	58.01
3	Carboxyethylation (CEL)	HK	any	C(3) H(4) O(2)	72.02
5	Deamidation	NQR	any	H(-1) N(-1) O(1)	00.98
6	Dehydration	DSTY	any	H(-2) O(-1)	-18.01
7	Didehydro	STY	any	H(-2)	-02.01
8	Dehydroalanine (Tyr to Dha)	Y	any	H(-6) C(-6) O(-1)	-94.04
8	Acetyl	HKSTY	any	C(2) H(2) O	42.01
9	Pyro-glu	Q	N-terminal	H(-2) O(-1)	-17.02
10	Pyro-glu	E	N-terminal	H(-3) N(-1)	-18.01

Table 2. Overview of Maillard induced and other modifications in peptide sequences at amino acid residue level with positions.

Protein	Sequence	Amino Acid	Position	Modification
Myosin	EALV**S**QL**S**R	Serine	S5; S8	2xAcetyl
	YAAWMI**YTYS**GLFCV**T**VNP**YK**WLPV**Y**NAEVV**T**A**Y**R	Tyrosine/Threonine/Lysine/Serine	Y/T/K/S (any)	1xAcetyl
	QLDEKD**T**LVSQLSR	Threonine	T7	1xAcetyl
	ALQEA**H**QQALDDLQAEED**K**VNTLTK	Histidine; Lysine	H6; K19	2xCEL
	QLEAE**K**LELQSALEEAEASLEHEEGK	Lysine	K6	1xCEL
	QLEAE**K**LELQSALEEAEASLEHEEGK	Lysine	K6	1xCML
	QLDE**K**EALVSQLSR	Lysine	K5	1xCML
	IAE**K**DEEIDQLK	Lysine	K4	1xCML
	T**K**LEQQVDDLEGSLEQEKK	Lysine	K2	1xCML
	V**K**NAYEESLDQLETLKR	Lysine	K2	1xCML
	T**K**EDLAK	Lysine	K2	1xCML
	T**K**YETDAIQR	Lysine	K2	1xCML
	T**K**LEQQVDDLEGSLEQEK	Lysine	K2	1xCML
	LAQESTMDIENDKQQLDE**K**	Lysine	K13	1xCML
	V**K**NAYEESLDQLETLK	Lysine	K2	1xCML
	LETDIS**Q**I**Q**GEMEDIIQEAR	Glutamine	Q7; Q9	2xDeamidated
	V**Q**LLHT**Q**NTSLINTK	Glutamine	Q2; Q7	2xDeamidated
	KKLETDIS**Q**I**Q**GEMEDII**Q**EAR	Glutamine	Q11; Q (any)	2xDeamidated
	KLETDIS**Q**I**Q**GEMEDII**Q**EA**R**	Glutamine/Arginine	Q/R (any)	2xDeamidated
	QAEEAEEQS**N**V**N**LSK	Asparagine	N10; N12	2xDeamidated
	ELTY**Q**TEEDR	Glutamine	Q5	1xDeamidated
	KL**Q**HELEEAEER	Glutamine	Q3	1xDeamidated
	L**Q**DLVDK	Glutamine	Q2	1xDeamidated
	QLDEKEALVSQLSR	Glutamine	Q1	1xDeamidated
	QLDEKDTLVSQLSR	Glutamine	Q1	1xDeamidated
	KKLETDIS**Q**I**Q**GEMEDIV**Q**EAR	Glutamine	Q	1xDeamidated
	AL**Q**EAH**QQ**TLDDL**Q**AEEDKV**N**TLTK	Glutamine/Asparagine	Q/N (any)	1xDeamidated
	E**N**K**N**L**QQ**EISDLTE**Q**IAEGGK	Glutamine/Asparagine	Q/N (any)	1xDeamidated
	NAYEESLD**Q**LETLK	Glutamine/Asparagine	Q/N (any)	1xDeamidated
	NL**QQ**EISDLTE**Q**IAEGGK**R**	Glutamine/Asparagine/Arginine	Q/N/R (any)	1xDeamidated
	ADIAESQV**N**KLR	Asparagine	N9	1xDeamidated
	AAYLQGL**N**SADLLK	Asparagine	N8	1xDeamidated
	ELE**N**EVENEQK	Asparagine	N4	1xDeamidated
	VK**N**AYEESLDQLETLK	Asparagine	N3	1xDeamidated
	LI**N**ELSAQK	Asparagine	N3	1xDeamidated
	VK**N**AYEESLDQLETLKR	Asparagine	N3	1xDeamidated
	LI**N**DLTAQR	Asparagine	N3	1xDeamidated
	A**N**SEVAQWR	Asparagine	N2	1xDeamidated
	AEAHFSLIHYAGTVDY**N**ITGWLDK	Asparagine	N17	1xDeamidated
	HADSVAELGEQID**N**LQR	Asparagine	N14	1xDeamidated
	NTQGVLK	Asparagine	N1	1xDeamidated
	NAYEESLDQLETLKR	Asparagine	N1	1xDeamidated
	VQLLHTQ**N**TSLI**N**TKK	Asparagine	N (any)	1xDeamidated
	QATEAI**RN**L**R**	Asparagine/Arginine	N/R (any)	1xDeamidated
	SR**N**DAI**R**	Asparagine/Arginine	N/R (any)	1xDeamidated
	NL**RN**T**Q**GVLK	Asparagine/Arginine/Glutamine	N/R/Q (any)	1xDeamidated
	ELE**N**EVE**N**E**Q**KR	Asparagine/Glutamine	N/Q (any)	1xDeamidated
	NL**QQ**EISDLTE**Q**IAEGGK	Asparagine/Glutamine	N/Q (any)	1xDeamidated
	QAEEAEE**Q**S**N**V**N**LSK	Asparagine/Glutamine	N/Q (any)	1xDeamidated
	V**Q**LLHT**QN**TSLI**N**TK	Asparagine/Glutamine	N/Q (any)	1xDeamidated
	ADIAES**Q**V**N**K	Asparagine/Glutamine	N/Q (any)	1xDeamidated
	I**Q**LEL**NQ**VK	Asparagine/Glutamine	N/Q (any)	1xDeamidated
	L**Q**DAEEHVEAV**N**AK	Asparagine/Glutamine	N/Q (any)	1xDeamidated
	V**R**ELE**N**EVE**N**E**Q**K**R**	Asparagine/Glutamine/Arginine	N/Q/R (any)	1xDeamidated
	S**N**AACAALDKK	Asparagine	N2	1xDeamidated
	C**N**GVLEGIR	Asparagine	N2	1xDeamidated
	EQ**Y**EEEQEAK	Tyrosine	Y3	1xDehydrated
	QLEEE**T**K	Threonine	T6	1xDehydrated
	QAF**T**QQIEELKR	Threonine	T4	1xDehydrated
	QA**T**EAIR	Threonine	T3	1xDehydrated
	IHFG**TT**GK	Threonine	T (any)	1xDehydrated
	EL**TY**QTEEDRK	Threonine/Tyrosine	T/Y (any)	1xDehydrated
	DTQLHL**DD**AIR	Threonine/Aspartic Acid	T/D (any)	1xDehydrated
	EQ**DTS**AHLER	Threonine/Aspartic Acid/Serine	T/D/S (any)	1xDehydrated
	ELEGEVE**S**EQKR	Serine	S8	1xDehydrated
	ELEEI**S**ER	Serine	S6	1xDehydrated
	EALV**S**QLSR	Serine	S5	1xDehydrated
	SELQAALEEAEA**S**LEHEEGK	Serine	S13	1xDehydrated
	TKYET**D**AIQR	Aspartic Acid	D6	1xDehydrated
	KLEG**D**LK	Aspartic Acid	D5	1xDehydrated
	L**D**EAEQIALK	Aspartic Acid	D2	1xDehydrated
	E**S**IFCIQYNIR	Serine	S2	1xDehydrated
	H**D**CDLLR	Aspartic Acid	D2	1xDehydrated
	EKS**E**LK	Glutamic Acid	E(any), N-Term	1xGlu->pyro-Glu
	EALVSQLSR	Glutamic Acid	E(any), N-Term	1xGlu->pyro-Glu
	EL**E**G**E**V**E**S**E**QKR	Glutamic Acid	E(any), N-Term	1xGlu->pyro-Glu
	EL**E**N**E**V**E**N**E**QK	Glutamic Acid	E(any), N-Term	1xGlu->pyro-Glu

	EL**E**N**E**V**E**N**E**QKR	Glutamic Acid	E(any), N-Term	1xGlu->pyro-Glu
Myosin (Continued)	**E**LTYQT**EE**DRK	Glutamic Acid	E(any), N-Term	1xGlu->pyro-Glu
	EQDTSAHL**E**R	Glutamic Acid	E(any), N-Term	1xGlu->pyro-Glu
	EQY**EEE**Q**E**AK	Glutamic Acid	E(any), N-Term	1xGlu->pyro-Glu
	EL**EE**IS**E**R	Glutamic Acid	E(any), N-Term	1xGlu->pyro-Glu
	ESIFCIQYNIR	Glutamic Acid	E(any), N-Term	1xGlu->pyro-Glu
	QIE**Q**EKSEL**Q**AALEEAEASLEHEEGK	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QSSEEGGTKK	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QSSEEGGTK	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QAFT**QQ**IEELK	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QAEEAEE**Q**SNVNLSK	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QAEEAEE**Q**SNTNLSK	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QREE**Q**AEPDGTEVADK	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QVE**Q**EKSEI**Q**AALEEAEASLEHEEGK	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QKYEETHAELEAS**Q**K	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QLDEKDTLVS**Q**LSR	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QLEAEKLEL**Q**SALEEAEASLEHEEGK	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QAFT**QQ**IEELKR	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QVE**Q**EK	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QIE**Q**EK	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QAEEAEE**Q**ANTNLSK	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
	QATEAIR	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
Actin	**SY**ELPDGQVI**T**IGNER	Serine/Tyrosine/Threonine	S/Y/T (any)	1xAcetyl
	IWHHTFY**N**ELR	Asparagine	N8	1xDeamidated
	SYELPDGQVITIG**N**ER	Asparagine	N14	1xDeamidated
	SYELP**D**GQVITIGNER	Aspartic Acid	D6	1xDehydrated
	QE**YD**EAGP**S**IVHR	Aspartic Acid/Serine/Tyrosine	D/S/Y (any)	1xDehydrated
	FRCPETLFQPSFIGMESAGIHETTYN**S**IMKCDIDIR	Serine	S27	1xDidehydro
	QEYDEAGPSIVHR	Glutamine	Q(any), N-Term	1xGln->pyro-Glu
Beta-enolase	**H**ITGEK	Histidine	H1	1xAcetyl
	LAQSNG**W**GVMVSHR	Tryptophan	W7	1xCML
	HIADLAGNPDLVLPVPAFNVINGGSHAGN**K**	Lysine	K30	1xCML
	NG**K**YDLDFK	Lysine	K3	1xCML
	VVIGMDVAASEFYRNG**K**YDLDFK	Lysine	K17	1xCML
	HIADLAGNPDLVLPVPAFNVI**N**GGSHAG**N**K	Asparagine	N22; N29	2xDeamidated
	TAI**Q**AAGYPDKVVIGMDVAASEFYR	Glutamine	Q4	1xDeamidated
	NGKYDLDFK	Asparagine	N1	1xDeamidated
	NGKYDLDFKSPDDPSR	Asparagine	N1	1xDeamidated
	HIADLAG**N**PDLVLPVPAF**N**VI**N**GGSHAG**N**K	Asparagine	N (any)	1xDeamidated
	DAT**N**VGDEGGFAP**N**ILE**NN**EALELLK	Asparagine	N (any)	1xDeamidated
	FGA**N**AILGVSLAVCK	Asparagine	N4	1xDeamidated
	NYPVV**S**IE**D**PF**D**Q**DD**WK	Aspartic Acid/Serine	D/S	1xDehydrated
	EILDSR	Glutamic Acid	E(any), N-Term	1xGlu->pyro-Glu
Myoglobin	HG**N**TVLTALGGILK	Asparagine	N3	1xDeamidated