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N^ε-(carboxymethyl)-L-lysine content in cheese, meat and fish products is affected by the presence of copper during elaboration process

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Abstract Formation of dietary advanced glycation end products has been extensively studied, principally with the aim to decrease their intake. In this work, the relationship between copper potentially present during food elaboration and N^ε-(carboxymethyl)-L-lysine (CML) concentrations, has been examined for the first time. For CML determination, a reversed phase liquid chromatography-electrospray ionization-ion trap tandem mass spectrometry procedure, based on acid hydrolysis, ethyl chloroformate derivatization and quantification in MRM mode was set-up, yielding method quantification limit of 98 $\mu g\ kg^{-1}$; copper was determined by ICP-MS. For eleven commercial cheeses, CML and Cu were found in the ranges 3.70–8.58 $\mu g\ g^{-1}$ and 0.08–15.5 $\mu g\ g^{-1}$, respectively, suggesting an inverse relation between these two parameters. For beef, chicken, Mexican pork "carnitas" and salmon, the CML concentration was lower in the item cooked in Cu casserole while element concentration was increased, as compared to this same raw material prepared in Teflon™ (except for "carnitas"). Concentration-dependent effect of Cu, manifest by decreased CML formation, was confirmed evaluating conversion percentage of chemically protected lysine (ZLys) to ZCML in the absence and in the presence of different Cu concentrations (50.0% and 20.4% conversion for Cu:ZLys molar ratio 0:1 and 0.04:1,

respectively). Consistent results obtained in the analysis of three different sample types point to the inhibitory effect of copper during CML formation; however, it should be stressed that Cu is only one parameter within a complex set of factors/conditions involved in glycation process. Although better understanding of the observed effect at molecular level is needed, the results obtained in this work strongly suggest beneficial effect of copper, inhibiting glycation process during food elaboration.

Keywords Glycation · Food · N^ε-(carboxymethyl)-L-lysine · Copper

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Introduction

Formation pathways of advanced glycation end products (AGEs), their structural characterization and quantification of individual compounds are highly demanded in biomedical research, in clinical practice and in studies of glycation reactions occurring during food processing [1, 2]. Within the large and heterogeneous group of precursors, intermediates, propagators and finally generated irreversible AGEs, N^ε-(carboxymethyl)-L-lysine (CML) is one of the commonly measured compounds [3]. Formation of this stable AGE starts by binding the epsilon amine group of lysine to the electrophilic carbonyl moiety of glyoxal (GO) and is followed by a series of reduction and oxidation processes that can be favored by catalytical action of redox-cycling metal ions [1, 4, 5]. CML has been accepted as a versatile biomarker of AGEs, because of a variety of sources of GO in vivo and during thermal treatment of food and because CML can be derived from any stage of glycoxidation or from lipoxidation processes [6–8]. Solid evidence has been accumulated on the involvement of AGEs (CML among others)

in the pathogenesis and progression of chronic diseases [9–11]. It has also been reported that restriction of dietary AGEs is beneficial for human health; however, potential toxicity of these compounds contained in food is still under investigation [1, 12].

The determination of CML in foods has often been reported [3, 13–19]. Its formation during culinary treatments and in the presence of natural or synthetic antiglycation agents has been studied [20–22]; nonetheless, potential effect of redox-cycling metals available during food processing has not been evaluated. Owing to the proposed dual role of copper in a course of Maillard reaction, this topic seems relevant. It is known that free copper promotes oxidation processes [23] whereas formation of its stable complexes with some amino acids, glycation agents and with intermediate compounds (Amadori products) affects synthesis of compounds responsible of sensorial properties and in particular, inhibits food browning upon heating [24, 25]. In regard with CML or other AGEs, pro-oxidative character of copper favors their synthesis in food but opposite effect can be expected as the result of copper complexation with glycation substrates, intermediates or final products. Of note, the involvement of copper in protein glycation in vitro has been demonstrated [26–28] hence this metal ion might have an impact on the concentration of protein-bound CML in food.

Many traditional cuisines utilize copper cookware because this metal is an excellent heat conductor so foods can be cooked evenly without scorching. One of the Mexican gastronomic specialities, "carnitas", is prepared in copper casseroles by braising pork meat, seasoned with lime juice and salt with addition of oil or lard, for approximately 3 h. On the other hand, copper vats are used in the elaboration of Swiss-type cheeses or Protected Designation of Origin (PDO) Italian hard cheeses. Copper ions released from the vessel confer enhanced sensory properties mainly because they help to regulate bacterial communities during cheese elaboration and maturation [29, 30]. In Finland and in USA, stainless steel vessels are utilized and copper sulfate is added directly to milk yielding up to 15 mg kg^{-1} of copper in cheese [30].

The goal of this study was to examine if copper possibly released or intentionally added during food preparation might influence CML content in the final product. For this purpose, Cu and CML were determined in different types of cheese as well as in home-made "carnitas", in beef meat and in fish, all of them prepared in Cu or non-Cu casseroles. Furthermore, effect of copper was also evaluated during chemical synthesis of CML. Set-up of the analytical procedure for CML determination, consisting of ethyl chloroformate derivatization and reversed phase liquid chromatography separation with electrospray ion trap tandem mass spectrometry in multiple reaction monitoring mode (HPLC-ESI-ITMS/MS), is an additional contribution of the present work.

Materials and methods

Reagents and samples

All chemicals were of analytical reagent grade. HPLC grade ethanol, methanol, pyridine, chloroform, acetonitrile (Fisher Scientific) and deionized water (18.2 MΩ cm, Labconco) were used throughout.

Standards of N^ε-(carboxymethyl)-L-lysine (CML) and N^ε-(carboxy[^{2}H$_2$]methyl)-L-lysine (CML-d$_2$) were purchased from PolyPeptide; stock standard solution of copper (1000 mg L^{-1}) was from Sigma and inductively coupled plasma mass spectrometry (ICP-MS) internal standard mix from Agilent Technologies.

The following Sigma-Aldrich reagents were used: ethyl chloroformate, sodium borohydride, sodium cyanoborohydride, boric acid, sodium bicarbonate, sodium hydroxide, ammonium formate, nitric acid, hydrochloric acid, hydrogen peroxide, N^α-(carbobenzyloxy)-L-lysine (ZLys-OH), glyoxylic acid and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES). Amino acids: alanine, glycine, arginine, proline, phenylalanine, histidine, methionine, cysteine, glutamic acid and tyrosine were also from Sigma.

All food products were purchased from regional supermarkets in the city of Guanajuato, Mexico; these included eleven types of cheese [1—edam, 2—Gruyere (PDO), 3—Grana Padano (PDO) 4—emmental (Swiss), 5—emmental (USA), 6—"asadero", 7—ricotta, 8—mascarpone, 9—fresh goat, 10—semi-cured goat; 11—cured goat], fresh salmon, round tip beef steak, pork loin and chicken breast. Frozen cheese samples were shredded on a fine plastic grater and freeze-dried. Three portions (about 100 g each) of raw meat or salmon were cooked in parallel in three different casseroles made of copper, aluminum covered with Teflon™ and Pyrex glass, respectively. Beef, salmon and chicken breast were sliced (about 1.5 cm thick), casseroles were preheated with 30 mL of canola oil (5 min) and the pieces were fried for 8 min (4 min at each side). Internal temperature measured immediately after cooking was in the range 59–62 °C for Pyrex glass, 67–71 °C for Teflon™ and 69–73 °C for copper casserole (IR Thermometer Gun, SPER Scientific). Pork loin was chopped into squares (4 cm), drizzled with a lime juice (5 mL per dish) and kept in the kitchen oven at 170 °C for 60 min. Internal temperature of such prepared "carnitas" was 82–87 °C for each casserole; however, meat browning was observed only in copper and Teflon™ vessels. No salt or condiments were added in the above treatments. After cooking, each item was homogenized in a blender with addition of deionized water. The obtained mixtures were freeze-dried.

CML determination by HPLC-ESI-ITMS/MS

For fat elimination, 2 mL of methanol:chloroform (1:2 v/v) were added to 50 mg aliquots of the freeze-dried material, the mixtures were vortexed and centrifuged (13,000g, 10 min) recovering the pellets. Next, 0.5 mL NaBH$_4$ 2 M (in NaOH 0.1 M) and 2 mL borate buffer 0.2 M, pH 9.2, were added to each pellet and to complete the reduction, the samples were left overnight at 4 °C. Acid hydrolysis was carried out by adding 2.5 mL hydrochloric acid 12 N, the vials were flushed with nitrogen, sealed and heated at 110 °C for 24 h. The hydrolyzates were diluted to 5 mL; 1 mL aliquots were taken and evaporated on a heated plate (60 °C). The obtained residues were reconstituted in deionized water with addition of internal standard (50 µL CML-d$_2$ 800 µg L^{-1}) and sodium hydroxide 1 M yielding the final volume of 400 µL. During calibration, sample matrix was matched by amino acids mix prepared in hydrochloric acid (details given in Table 1S, ESM); in a series of Eppendorf tubes 80 µL of this mix, 50 µL CML-d$_2$ (800 µg L^{-1}) and varying aliquots of CML (2000 µg L^{-1}) were placed and the volume in each tube was brought to 400 µL yielding CML concentrations: 0, 50, 100, 200, 400, 600, 800 µg L^{-1}.

For derivatization, 500 µL of ethanol:pyridine (4:1, v/v) were added to each sample/calibration solution (400 µL), followed by two successive additions of 25 µL of ethyl chloroformate. After each addition, the mixtures were agitated releasing generated carbon dioxide. Finally, 500 µL of sodium bicarbonate 50 mM were added to adjust pH 9.0 and the derivatized compounds were extracted with 500 µL of chloroform; after centrifugation (13,000g 10 min), the organic phase was evaporated at room temperature and reconstituted in 200 µL of the mobile phase (ammonium formate 5 mM:methanol, 60:40 v/v). The samples were filtered (0.22-µm Whatman filters) and analyzed using an UltiMate 3000 liquid chromatograph (Dionex, Thermo Scientific) with electrospray ionization—ion-trap mass spectrometer AmaZon SL (Bruker Daltonics).

The LC–MS system was controlled by Hystar V3.2 where the data were processed by Data Analysis V4.1 SP2 and QuantAnalysis V2.0 SP2 (Bruker Daltonics). The chromatographic column Kinetex C18 (150 × 3 mm, 2.6 µm) was used with a Security Guard ULTRA Cartridge C18, both from Phenomenex. The column was maintained at 35 °C and the injection volume was 5 µL. Two mobile phases, ammonium formate 5 mM, pH 3.5 (A) and methanol (B), were used with the linear gradient: 0 min 40% B, 0–17 min 80% B, 17-20 min 40% B (column re-equilibration), at the total flow rate 0.3 mL/min. Column effluent was introduced to the ESI source operated in a positive mode with the following parameters: spray voltage 4500 V; plate voltage 500 V; nebulizer gas pressure 14 psi (N$_2$); dry gas 6 L min^{-1} (N$_2$) and source temperature 200 °C. Mass spectra were obtained by means of UltraScan mode in the *m/z* range 100–450, with an ion charge control (ICC) target setting 100,000 and the maximum accumulation time 70 ms, two averages per experiment and a rolling averaging of one. The smart parameter settings (SPS) was used for automatic optimization of the trap drive level for *m/z* 405, compound stability 100%, trap drive level 100%.

MS/MS spectra were acquired using helium as the collision gas; the precursor ions were obtained with this same ICC setting as for MS and with single average per experiment. For multiple reaction monitoring mode, fragmentation amplitude 0.4 V, isolation width *m/z* ±2, cut-off 200 and 100 ms dwell time were applied for each ion. Ion transitions used for individual compounds were *m/z* 405 → 359 for CML and 407 → 361 for CML-d$_2$ (*m/z* for qualifier ions were 331 and 333, respectively). For phenylalanine, fragmentation amplitude 0.6 V, cut-off 150 and transition *m/z* 266 → 220 (*m/z* for qualifier ion 192), were adopted. Bruker QuantAnalysis software was utilized, taking peak area of the quantifier ions from respective MRM chromatograms and computing IS-normalized values for CML.

For the recovery experiments, fresh goat cheese (sample 9), salmon and "carnitas" prepared in glass casserole, were used. Prior to acid hydrolysis, 50 and 150 µL of CML standard 2000 µg L^{-1} were added to the aliquots of freeze-dried sample (100 and 300 ng of CML, respectively).

ICP-MS determination of metals

Acid digestion was performed using 50 mg aliquot of the freeze-dried material to which 250 µL of internal standard solution (2 mg L^{-1} each of In, Y, Bi, Rh; 5 mg L^{-1} of Sc; and 10 mg L^{-1} of Li) and 500 µL of concentrated nitric acid were added. The samples were left overnight, then heated to 120 °C in a heating block for 3 h, mixed with 500 µL of hydrogen peroxide and heated again (120 °C, 30 min). The addition of hydrogen peroxide was repeated, and the final volume was brought to 2 mL with deionized water. The samples were centrifuged (13,000g, 10 min), 200 µL portions were diluted 25-fold with deionized water and introduced to the ICP-MS system. An inductively coupled plasma mass spectrometer (Model 7500ce; Agilent Technologies) with a Meinhard nebulizer and Peltier-cooled spray chamber (2 °C) was used with the previously reported instrumental operating conditions [31]. The isotopes ^{65}Cu, ^{63}Cu were monitored and standardized to ^{89}Y and ^{115}In signals. Calibration was performed with Agilent commercial standard at copper concentrations of 0, 0.5, 1.0, 2.0, 5.0, 10, 25 and 50 µg L^{-1}, and with the internal standards, Y and In (10 µg L^{-1} each). The copper instrumental detection limit was 51 ng L^{-1}; method detection limit 57 µg kg^{-1} was evaluated using four times diluted digest of mascarpone cheese [32]. For accuracy checking, two certified reference materials were used;

National Institute of Standards and Technology (NIST) 1643d Trace Elements in Water and NIST 1572 Citrus Leaves. Copper concentrations found in triplicate analysis of these two reference materials were 19.7 ± 0.8 and $16.3 \pm 0.5 \mu g \, g^{-1}$, respectively (certified values 20.5 ± 3.8 and $16.5 \pm 1.0 \mu g \, g^{-1}$).

Synthesis of chemically protected CML in the presence and in the absence of Cu(II)

To examine the effect of Cu(II) on CML formation, 100 µL of ZLys-OH solution (5.0 µmol) were added to a series of Eppendorf tubes and mixed with an aliquot of Cu(II) containing 0, 25, 50, 100, 200 nmol of copper, respectively; then, 150 µL of glyoxylic acid solution (8.0 µmol) were added and in each tube, the volume was brought to 800 µL with HEPES 25 mM, pH 7.4. The mixtures were kept at 80 °C for 4 h and after addition of 200 µL of sodium cyanoborohydride 5 mM, were centrifuged (13000*g*, 10 min) [33]. The supernatant was diluted 1:1 with a mobile phase and 40 µL were injected to the chromatographic system. Liquid chromatograph was an Agilent series 1200 with a diode array spectrophotometric detector (Agilent Technologies, Palo Alto, CA, USA) and chromatographic column was a Luna C8 (250 × 3 mm, 5 µm) from Phenomenex. The separation of ZLys and ZCML was achieved with a mobile phase containing ammonium formate 30 mM, pH 6.4, and acetonitrile (88:12) at the flow rate 0.5 mL min⁻¹ and setting column temperature at 30 °C; spectrophotometric detection was carried out at 259 nm. The identity of Z-CML was confirmed by high resolution mass spectrometry. A column fraction (7.6–8.4 min) was introduced by direct infusion to electrospray ionization source of maXis impact ESI-QTOF-MS spectrometer (Bruker Daltonics). ESI was operated in a positive mode with ion spray voltage 4500 V, end plate offset 500 V, nitrogen dry gas 4 L min⁻¹, drying temperature 180 °C and nebulizing gas pressure 0.4 bar; the lock-mass standard *m/z* 299.2945 (methyl stearate) and the *m/z* range 50–1250 were used. The exact *m/z* value was 339.15510 with the mass error 0.2 ppm with respect to that theoretically calculated (339.15506). Figure 1S in the Electronic Supplementary Material (ESM) shows theoretical and experimental isotopic pattern of protonated molecule.

Results and discussion

Set-up of HPLC-ESI-ITMS/MS procedure for CML determination

Derivatization of CML with ethyl chloroformate converts primary and secondary amine groups to respective ethyl carbamates and at the same time, two carboxylic groups

are esterified by reacting with alcohol present in medium [34, 35]. In addition to the target compound, all amino acids present in food hydrolysates were susceptible to this derivatization hence any interference potentially affecting CML determination by HPLC-ESI-IT-MS/MS had to be observed. The following four aspects were taken into consideration: (1) elimination of potential interferents prior to the on-column injection; (2) ensuring chromatographic resolution of CML, (3) matching food matrix in calibration solutions and (4) attaining high sensitivity in multiple reaction monitoring mode (MRM).

As to the first aspect, food samples were defatted before acid hydrolysis, as described in Materials and methods. To eliminate polar and ionic species present in food hydrolyzates after derivatization, pH 9 was adjusted with sodium bicarbonate 50 mM and non-polar ethyl chloroformate derivatives were extracted to chloroform. It should be stressed that strict pH control was critical in this procedure.

MRM conditions were examined and comparatively evaluated using pyridine mixture with methanol or ethanol during derivatization. In each case, different fragmentation amplitudes and cut-off values were tested yielding about two times higher intensity of CML quantifier ion in the presence of ethanol (*m/z* $405 \rightarrow 359$) then while using methanol (*m/z* $377 \rightarrow 331$, details given in Fig. 2S, ESM). For that reason, ethanol was used in further work.

In the presence of food matrix, baseline separation of derivatized CML in a reasonable short time was challenging. Specifically, using ammonium formate and methanol as typical mobile phases for reverse phase separations coupled to ESI(+)-MS detection, troublesome co-elution with phenylalanine caused serious ionization suppression compromising CML quantification. Suitable adjustment of gradient elution (see "Materials and methods") allowed for separation of the two compounds as shown in Fig. 1; retention times for phenylalanine, CML and CML-d₂ derivatives were 12.69 ± 0.08, 13.24 ± 0.03 and 13.22 ± 0.02 min, respectively, with total chromatographic run of 20 min (17 min separation plus 3 min column re-equilibration). To avoid any other interference due to the high excess of amino acids with respect to CML, it was decided to match the composition of calibration solutions to that of food samples. An aliquot of standards mix containing alanine, glycine, arginine, proline, phenylalanine, histidine, lysine, methionine, cysteine, glutamic acid and tyrosine was added to each calibration solution in the amount equivalent to about 3 mg of digested proteins present in the sample aliquot taken for derivatization (composition of calibration solutions given in Table 1S, ESM). By so doing, the elution profiles and peak intensities were similar in total ion chromatograms obtained for the mix of amino acids and for the real-world samples (Fig. 3S in ESM shows total ion and extracted ion chromatograms for amino acids mix and total ion chromatogram for semi-cured
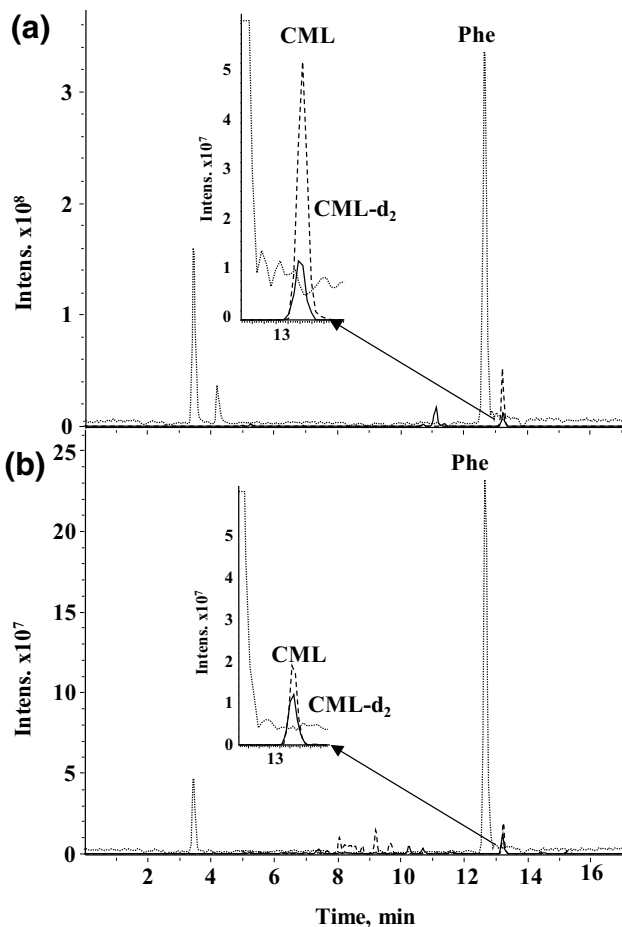
Fig. 1 Chromatograms obtained in MRM mode for: *dotted lines* phenylalanine, m/z 266→220; *dashed line* CML, m/z 405→359; *solid line* CML-d_2, m/z 407 → 361. **a** Matrix-matched CML standard solution (CML 1000 µg L^{-1}, CML-d_2 200 µg L^{-1}). **b** Salmon cooked in glass casserole

Fig. 2 MRM mode chromatograms obtained for CML (m/z 405 → 359) analyzing the sample of salmon cooked in glass casserole: *solid lines* without standard addition; *dashed lines* 100 ng CML added prior to acid hydrolysis, *dotted lines* 300 ng CML added (quantitative results presented in Table 1)

two-point standard addition to salmon cooked in glass casserole, are presented. CML concentrations determined in this sample, in fresh goat cheese and in "carnitas" prepared in glass casserole, are provided in Table 1. Average percentage CML recoveries obtained for the above samples based on three replicates were in the range 86.0–105% indicating acceptable method performance in terms of analytical accuracy.

Analysis of cheese samples presenting varying Cu concentrations

The main goal of this work was to examine if copper present during food elaboration might affect CML concentration in the final product. In the first approach, HPLC-ESI-IT-MS/

goat cheese). In Fig. 1, chromatograms of matrix-matched calibration solution and for salmon cooked in glass casserole are presented, both obtained in MRM mode for CML, CML-d_2 and phenylalanine. As already mentioned before, baseline separation of the analyte from the principal interferent as well as the same peak shape for CML and for internal standard were obtained during calibration and for the real-world sample.

Taking CML-d_2 normalized analyte signals acquired in MRM mode (peak area), linear regression matrix-matched calibration yielded acceptable linearity ($R^2 > 0.999$) with the detection and quantification limits of 0.5 and 1.96 µg L^{-1}, respectively; method detection and quantification limits were 23 and 98 µg kg^{-1} of the freeze dried mass [32].

For accuracy testing of the HPLC-ESI-IT-MS/MS procedure, CML was added to three different freeze-dried food samples prior to acid hydrolysis, as described in Materials and methods. In Fig. 2, MRM chromatograms obtained for
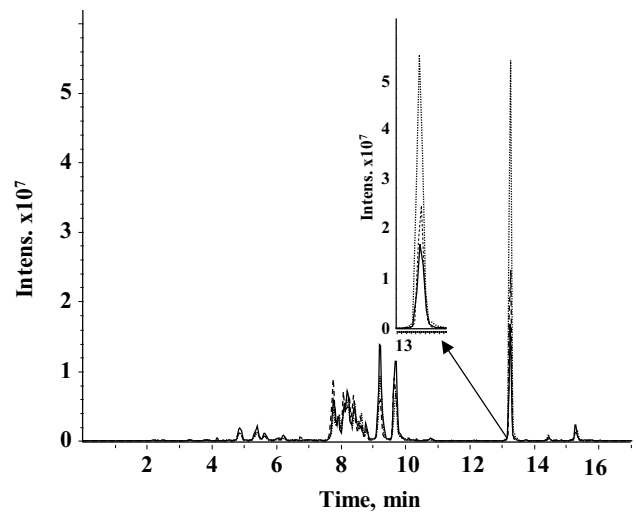
Table 1 Recovery results obtained in two-point standard addition performed for fresh goat cheese (1), salmon cooked in glass casserole (2) and "carnitas" prepared in glass casserole (3)

CML added, ng	CML found, mean ± SD ng per 50 mg of the freeze-dried sample		
	1	2	3
0	210 ± 12	406 ± 15	725 ± 10
100	296 ± 19	499 ± 18	827 ± 13
Recovery, %	86.0	93.0	102
300	509 ± 31	674 ± 20	1041 ± 28
Recovery, %	99.7	89.3	105

CML standard was added to the freeze-dried mass as described in materials and methods, mean value with respective standard deviation were obtained based on three replicates

MS procedure described in the previous section was used for the determination of CML in eleven samples of commercial cheeses. The concentrations found ranged from 3.70 ± 0.03 µg g^{-1} for cured goat cheese to 8.58 µg g^{-1} for ricotta (mean value for all samples 4.75 ± 1.26 µg g^{-1}). Noteworthy, similar concentrations (0.1–5.8 µg g^{-1}) had been previously reported in different types of cheese [15, 17] which further confirms analytical reliability of our data. Results obtained for CML in all samples are presented in Table 2S (ESM) together with concentrations of copper determined in these same samples by ICP-MS. Copper was found in the concentration range from 0.08 ± 0.01 µg g^{-1} (mascarpone) to 15.53 ± 0.61 µg g^{-1} (Gruyere PDO) with average for all samples 3.53 ± 4.96 µg g^{-1}. This relatively wide range and the respect of the regulatory manufacturing standards confirm that copper was released from vats during elaboration of certain cheese types such as Gruyere PDO, Grana Padano PDO, Swiss emmental.

In Fig. 3, CML found in nine samples is plotted against Cu concentrations and the obtained chart suggests an inverse relationship between these two parameters (ricotta and cured goat cheese were excluded as explained below). Figure 3 also shows that the observed relation could be described by a logarithmic trend line with $R^2 = 0.5965$. Of note, in addition to the proposed effect of copper, CML formation in cheese is influenced by many factors such as thermal treatment or ripening conditions. This multifactor effect was clearly observed in the analysis of ricotta; CML concentration found in this cheese (8.58 ± 0.61 µg g^{-1}) was almost two times higher as compared to edam, emmental (USA) or "asadero" (range 4.37–4.91 µg g^{-1}) even though all these products contained similar Cu concentration (range 0.28–0.62 µg g^{-1}). Furthermore, commercial cheeses might become contaminated with copper at different stages of production/packaging/storage [36, 37] so possible contribution of Cu in the glycation processes was not the same in each product. As an example, fresh, semi-cured and cured goat cheeses presented
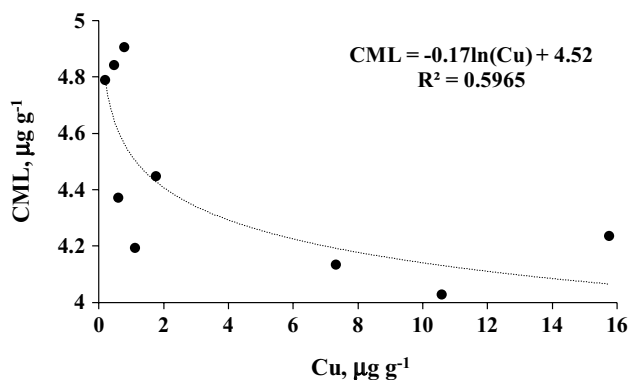
similar Cu concentrations (1.01–1.60 µg g^{-1}) yet CML concentration in cured cheese (3.70 ± 0.03 µg g^{-1}) was lower than in the other two products (4.19–4.45 µg g^{-1}).

Based on the above considerations, the obtained data are insufficient for attempting any mathematical modelling of the relationship between CML and Cu content in cheese; however, these data encouraged us for the evaluation of potential inhibitory effect of copper on CML formation in other food items cooked under controlled conditions and with the known source of Cu.

Analysis of meat and fish products cooked in Cu and non-Cu casseroles

To obtain further evidence on the suggested copper effect, beef, pork, chicken and salmon were cooked in parallel using three different types of casserole and in each food item obtained, CML and Cu were determined. For animal meat prepared in non-Cu casseroles, copper concentration decreased in the following order: beef > pork > chicken, in agreement with already existing data [38, 39]. Relatively lower concentrations were found in salmon, suggesting that the analyzed fish came from non-contaminated environment [40]. When meat or fish was heated in copper casserole, element concentrations increased (range from 7.66 ± 0.03 µg g^{-1} for salmon to 139.3 ± 0.5 µg g^{-1} for "carnitas"), as compared to glass (from 1.01 ± 0.03 µg g^{-1} for salmon to 2.98 ± 0.03 µg g^{-1} in beef) and Teflon™ vessels (from 0.92 ± 0.01 µg g^{-1} in salmon to 2.46 ± 0.06 µg g^{-1} in "carnitas"). Of note, fish, beef and chicken were cooked for 8 min whereas 1 h heating was applied for "carnitas" and raw pork loin was seasoned with a lime juice favoring copper release from casserole. It is not surprising though that Mexican pork dish contained the highest element concentration (139.3 ± 0.5 µg g^{-1}). Detailed results obtained for Cu and CML in all food items are presented in Table 3S (ESM) whereas Fig. 4 shows CML concentrations determined in four products, each of them prepared in three different casserole types. At the first glance, it can be observed that the highest CML content corresponded to "carnitas", and was followed by beef, chicken and salmon, respectively. Similar order can be noted in two food CML databases reported recently [17, 41].

Most importantly, salmon, chicken and beef prepared in copper casserole contained lower CML concentrations with respect to these same items prepared in Teflon™ vessels, as revealed by unpaired t test ($p < 0.05$, except for the beef cooked in copper against Teflon™, Fig. 4). This finding is consistent with the results obtained for commercial cheeses (Figs. 3, 4) and supports our hypothesis of inhibitory Cu effect on CML formation in food. On the other hand, CML contents in the items cooked in Pyrex glass were lower at statistical level as compared Teflon™
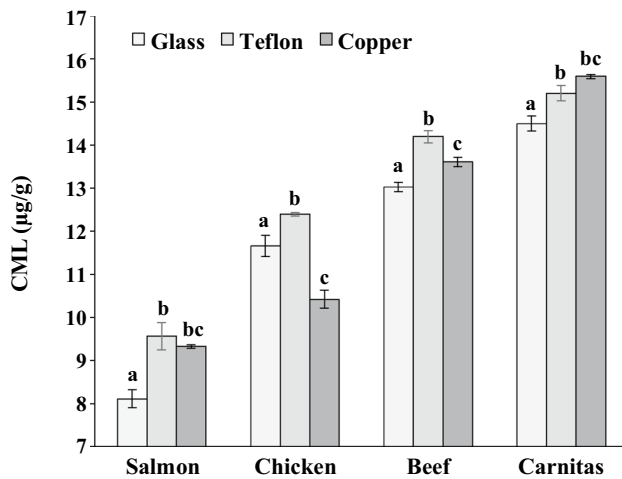

Fig. 3 Relation between copper and CML concentrations found in cheese samples (quantitative results presented in Table 2S, ESM)

$$CML = -0.17\ln(Cu) + 4.52$$
$$R^2 = 0.5965$$

Fig. 4 CML concentrations found in four food items cooked in glass, Teflon™ and copper casserole, respectively (mean value with respective standard deviation is shown in each case based on three replicates, the results are presented in Table 3S, ESM. For comparison of CML levels obtained for this same item prepared in three different casserole types, unpaired *t* test was applied; *different letters* indicate statistically significant difference found at $p < 0.05$)

or copper casseroles (except for chicken), which should be ascribed to lower thermal conductivity of glass with respect to other two materials. Even though this same kitchen stove, the same heating setting and cooking time were used for three casserole types, internal temperature of the meal registered at the end of cooking was different: 59–62 °C for Pyrex glass, 67–71 °C for Teflon™ and 69–73 °C for copper, confirming that cooking conditions is an important factor affecting CML formation [5]. Since Teflon™ and copper provided similar heating capacity, decreased CML concentration observed in Cu casserole could be directly related to metal release to food.

The impact of copper seems to be concentration-dependent and influenced by heating time, because "carnitas" prepared in Cu casserole contained higher CML concentration with respect to the dish heated in non-Cu vessels. The result obtained for this specific sample confirms that multiple mechanisms are involved in glycation process.

To the best of our knowledge, this is the first study evaluating quantitatively the relationship between CML and Cu in foods, yet AGEs formation in the presence of Cu has been studied in other experimental systems [26, 28, 33, 42–46]. Specifically, concentration-dependent effect of copper was observed in a recent proteomic study carried out on human serum albumin (HSA) in vitro glycated with methylglyoxal; whereas physiological and sub-physiological copper concentrations inhibited HSA glycation as compared to the glycation of the Cu-devoid protein, at supra-physiological level, copper clearly favored glycation [28].
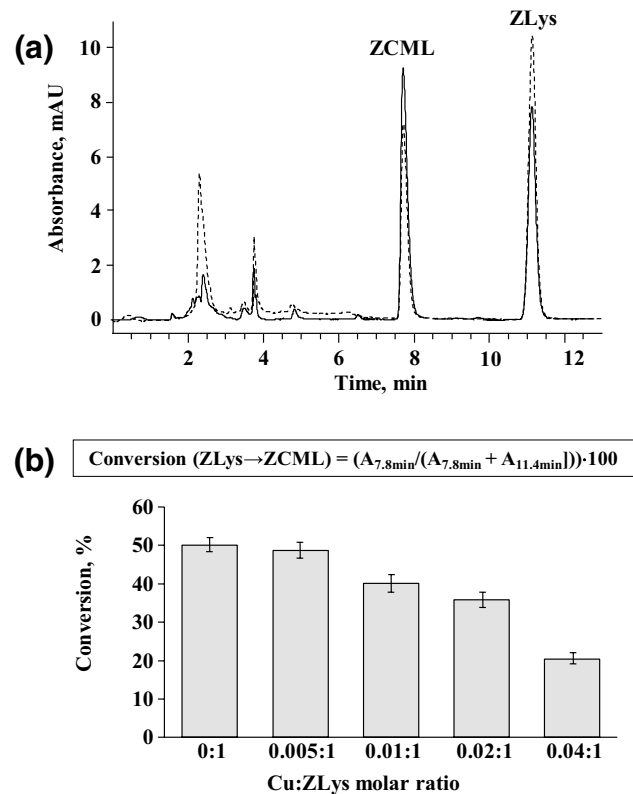

Fig. 5 Effect of Cu(II) added to the reaction mixture on the synthesis of ZCML (synthesis described in materials and methods). **a** *Solid line* HPLC–DAD chromatogram obtained for the reaction mixture without Cu(II) addition and *dotted line* chromatogram obtained for the mixture containing molar ratio Cu(II):ZLys 2:10. **b** Percentage of ZLys conversion to ZCML for different Cu(II): Z-Lys molar ratios

Effect of Cu(II) during synthesis of chemically protected CML

Chemical synthesis of CML was the third experimental system applied in this work to demonstrate inhibitory effect of copper during glycation reaction. ZLys was used to simulate protein structure, in which N-terminal amine group is available for glycation. Glyoxylic acid was utilized as a glycation agent, under conditions described previously [33]. Once the identity of ZCML was confirmed by high resolution mass spectrometry (see Materials and methods and Fig. 1S, ESM), synthesis was carried out in the presence of different Cu(II) concentrations covering the range of Cu:ZLys molar ratio 0.005:1–0.04:1. To evaluate conversion rate ZLys to ZCML, the reaction mixtures were analyzed after 4 h of incubation (80 °C) by high performance liquid chromatography with diode array spectrophotometric detection (detailed protocol given in Materials and methods). In Fig. 5a, typical chromatograms are presented for the mixture not containing Cu(II) and for the mixture with Cu(II):ZLys ratio 0.02:1; ZCML and ZLys eluted with retention times

7.81 ± 0.02 and 11.4 ± 0.03 min, respectively. In can be observed that the intensity of ZCML signal was lower when copper was present during synthesis (Fig. 5a). Since the two compounds presented practically identical absorption spectra (λ_{max} = 259 nm), carboxymethylation of ZLys was evaluated based on the respective peak areas, according to the formula given in Fig. 5. Percentage of ZLys conversion to ZCML obtained in this experiment are presented in Fig. 5b. Under reaction conditions applied in this work, the reaction yield was 50% after 4 h incubation of the mixture not containing copper whereas copper addition resulted in gradual decrease of ZCML formation in a concentration-dependent manner. Even though the composition of reaction mixture and chemical conditions during synthesis were certainly different as compared to those existing during food elaboration, the results obtained in different parts of this work consistently point to the inhibitory effect of copper during CML formation.

Possible mechanism underlying inhibitory effect of copper on CML formation in food

During Maillard reaction, actual effect of copper relies on the compromise between pro-oxidative action of free copper and protection of chemical species participating in browning processes by their complexation [23–25]. In particular, stable complexes of Cu(II) with amino acids as alanine, glycine or lysine have been reported [23, 47, 48]. It was also demonstrated that copper causes enhanced production of Strecker aldehydes via oxidative decarboxylation of its amino acid complexes upon heating; for free amino acids, this process would favor generation of aroma-active volatile compounds [24, 49] whereas for amino acids incorporated in protein structure, interaction with copper might avoid their participation in AGEs formation [50]. Indeed, decreased AGEs formation in the presence of Cu [26, 28] and other metal ions [45, 51] has been reported.

In this work, interaction of Cu(II) with ZLys was confirmed by UV/Vis spectrophotometric measurements. In a series of reaction mixtures that covered Cu:ZLys molar ratios from 1:0.8 to 1:20, a gradual shift of absorption maximum obtained for pure copper sulfate toward lower wavelengths was observed with increasing concentration of ZLys, in agreement with some earlier studies reporting formation of Cu(II)-Lys complex [47, 52] (Fig. 4S, ESM). Since ZLys contains chemical group attached to alpha amine by peptide-like bond, the obtained results suggest reactivity of lysine residues potentially accessible for Cu(II) in tertiary protein structure. In addition to the interaction with lysine, glycation agents and glycation intermediates might be also "inactivated" by copper binding and this aspect is a challenge for future studies.

As already noted, formation of advanced glycation end products in foods is governed by highly complex reaction network which is modulated by the conditions applied in food elaboration (cooking time, temperature, humidity, presence of redox-active food components, salts and spices, pH, etc.) [1, 5]. In this regard, CML content found in different items was clearly affected not only by copper but also by other factors, as discussed in section of cheese analysis. Furthermore, lower temperature achieved in glass casserole was a prevalent factor deciding of lower CML concentration in meat or fish as compared to these same items cooked in Teflon™ or Cu casseroles. Finally, "carnitas" prepared in copper was the sole sample presenting higher CML content with respect to the same dish prepared in Teflon™ vessel, despite relatively high content of Cu. Since this specific dish required longer heating as compared to other items and pork meat was seasoned with a lime juice, the obtained result further confirms that glycation is a multifarious process and that the effect of copper can be overlapped by variety of other factors/conditions.

Based on the above discussion, future studies of the specific Cu effect on CML formation in food should be carried out under strict control of other, potentially participating factors, and under strict control of Cu concentration.

Conclusions

Many foods contain copper as a natural component of raw materials and additionally, copper can be released from vessel in which food was prepared or it can be purposely added to enhance sensorial properties. The role of copper and other redox-cycling metal ions during Maillard reaction has often been discussed in the literature whereas in this work, relation between copper and N^ε-(carboxymethyl)-L-lysine concentrations in different food items has been studied for the first time. Three sample types were analyzed: (1) commercial cheeses, (2) beef, chicken, pork and salmon cooked in Cu and non-Cu casseroles and (3) reaction mixtures after chemical synthesis of ZCML carried out in the absence and in the presence of different copper concentrations. Among multiple factors contributing in the CML formation in foods; the results obtained consistently pointed to the inhibitory, concentration-dependent effect of copper. In line with some earlier studies, the protective role of copper was ascribed to the formation of complexes with lysine or other chemical species participating in glycation.

Providing reliable CML quantification by HPLC-ESI-ITMS/MS was an important aspect of this study. The procedure was refined by setting the experimental conditions of pre-column derivatization with ethylchloroformate; to avoid potential interferences caused by complex chemical matrix of foods and high excess of amino acids present after acid

hydrolysis, samples were defatted, derivatized compounds were extracted from alkalinized solution (pH 9) to chloroform and matrix-matched standard solutions were used for calibration. Accuracy of CML determination was demonstrated by satisfactory percentage recoveries (86.0–105%) obtained in two-point standard addition experiment performed in three different food items.

Further studies are needed for better understanding of Cu effect on AGEs formation in food at molecular level; however, traditional use of copper casseroles seems to have additional beneficial effect in terms of decreased protein glycation.

Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

Human or animal rights All authors declare that this study does not include any experiments with human or animal subjects.

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