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Analytical Methods

Comparative evaluation of three different ELISA assays and HPLC-ESI-ITMS/MS for the analysis of N$^{\varepsilon}$-carboxymethyl lysine in food samples

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ABSTRACT

N$^{\varepsilon}$-carboxymethyl-lysine (CML) is measured in food, but there is a controversy concerning the most convenient yet reliable method(s) for this task. This work compares three different ELISA assays and HPLC-ESI-ITMS/MS for the analysis of CML in several food items. The four methods showed the same decreasing order of CML concentration: beef, bacon > chicken > fish > dairy products > grain products > fruits/vegetables. HPLC-ESI-ITMS/MS results highly correlated with those obtained by ELISA performed with monoclonal CML-antibody ($\beta = 0.98$, $p < 0.0001$) whereas My Bio Source® kit results were not correlated with those provided by Lamider®. Small differences of CML concentrations in food items prepared by different culinary treatment were clearly distinguished by HPLC-ESI-ITMS/MS, but could not always be detected by ELISA. This work demonstrates a reasonable relationship between CM determined by ELISA and HPLC-ESI-ITMS/MS and therefore supports the implementation of ELISA in food CML/AGEs screening.

1. Introduction

The non-enzymatic reaction of sugars with proteins, known as the Maillard or browning reaction, leads to formation of advanced glycation end products (AGEs) (Brownlee, Vlassara, & Cerami, 1984), a heterogeneous group of compounds (Ahmed, Dobler, Dean, & Thornalley, 2005). AGE accumulation in the body has been implicated in the pathogenesis of age-related diseases such as diabetes, neurodegenerative disorders and cardiovascular disease (Baumann et al., 2008; Nin et al., 2011; Poulsen et al., 2013). AGEs are also generated in food and common examples of these compounds are N$^{\varepsilon}$-carboxymethyl lysine (CML), methylglyoxal-lysine dimers (MOLD), pentosidine and pyrraline (Poulsen et al., 2013; Uribarri et al., 2010). Clinical studies have shown that high consumption of dietary AGEs is associated with indicators of oxidative stress and inflammation, which may play an important role in causing chronic disease (Uribarri et al., 2007). In animal models, restriction of dietary AGEs (dAGEs) prevents vascular and kidney dysfunction (Zheng et al., 2002), diabetes type 1 or type 2 (Peppa et al., 2003), and improves insulin sensitivity (Sandu et al., 2005). In humans, high vs. low-CML diets have been shown to increase levels of serum AGEs, inflammation markers, endothelial dysfunction, and to impair flow-mediated dilation and insulin sensitivity (Birlouez-Aragon et al., 2010; Luévano-Contreras, Garay-Sevilla, Wrobel, Malacara, & Wrobel, 2013); however the published results on the toxic effects of dAGEs remain controversial (Ames, 2008; Nguyen, 2006; Poulsen et al., 2013). In order to gain further insight on dAGEs as a risk factor for human health, it is mandatory to have accurate and easily reproducible standard methods for their quantification in both foods and biological samples (Scheijen et al., 2016).

ELISAs have been the most common approach for CML measurement in clinical and basic research with a convenient variety of commercial kits currently available for easy implementation in most

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laboratories. Even though not so straightforward in execution as ELISAs, analytical chemistry methods offer important advantages in terms of their high selectivity and capability for precise assessment of low CML concentrations in chemically complex samples, including food products. Noteworthy, using authentic CML standard, quantitative results are expressed as the analyte mass per sample volume/mass or per its protein content; in such form, the results can be easily compared among different food types, food processing, and different experiments or laboratories. Analytical procedures based on liquid or gas chromatography separation coupled to optical or mass spectrometry detection have been proposed for CML quantification in biological and food matrices (Ames, 2008; Assar, Moloney, Lima, Magee, & Ames, 2009; Charissou, Ait-Ameur, & Birlouez-Aragon, 2007; Chen & Smith, 2015; Scheijen et al., 2016; Wang, Tian, Sun, Lin, & Li, 2015; Zhou et al., 2015). When mass spectrometry is applied, deuterium-labelled analyte can be used as internal standard allowing for enhanced analytical performance (Poulsen et al., 2013; Tareke, Forslund, Lindh, Fahlgren, & Östman, 2013; Uribarri et al., 2015). Fragmentation of suitable precursor ion enables for multiple or selective reaction monitoring mode (MRM or SRM) hence, ensure higher selectivity toward target compound and also lower detection limit because spectral background is efficiently removed (Teerlink, Barto, Brink, & Schalkwijk, 2004; Thornalley et al., 2003). Of note, MRM or SRM are especially well suited for routine analyses because of lower susceptibility to incomplete chromatographic separation and no need for using expensive high resolution mass spectrometry instrumentation.

The present work has been focused on the comparative evaluation of three different ELISA assays and HPLC-ESI-ITMS/MS as applied for the analysis of various food items. These items were selected covering a range of foods typically consumed in Mexico such as grain-related products, meat, fish and fruits/vegetables. Data obtained by individual procedures were compared by means of statistical analysis of correlation; moreover we also evaluated the capability of these procedures to detect changes in CML content when the same raw material was submitted to different culinary treatments. Principal component analysis was also used to compare the performance of the four methods in the analysis of foods. The results provided by all four procedures reinforced the concept from currently published dietary CML databases: the highest content of CML is in high-heat cooked foods of animal origin and the lowest content is in plants-derived foods. On the other part, analytical chemistry procedure was the most suitable for assessing relatively small differences between CML concentrations whereas poor precision and susceptibility to matrix effects were the common ELISA limitations. The obtained results and the discussed limitation would be useful in the field of food analysis where reliable CML determination by ELISA assays is an issue.

2. Materials and methods

2.1. Food items and their preparation

Twenty food items commonly consumed in Mexico were selected. For potatoes, meat and fish, different culinary methods were used in order to: (i) evaluate the effect these treatments might have on CML formation and (ii) examine capabilities of the applied procedures to assess changes in CML content due to different culinary treatment.

The following products were purchased from regional supermarkets in the city of Guanajuato, Mexico: fresh apples, fresh serrano pepper, corn and wheat tortillas, potatoes, plain yogurt, American cheese, salmon, tuna, round tip beef steak, chicken breast, bacon, and “chicharrón” (Mexican style pork rinds). The intent was to analyze fresh foods, excluding deep frozen, dried or canned products. The cooking methods included boiling, poaching, frying and grilling. No salt or condiments were added, except for soy sauce for grilled tuna. Raw potatoes were boiled for 20 min. Meats were transported to the laboratory on ice, and were processed immediately. Slices of one hundred grams (about 1.5 cm thick) were obtained from the same piece of raw meat and cooked by the different procedures. Poached items (chicken and salmon) were placed in 500 mL of water at 90 °C for 10 min. Chicken breast and round tip beef steak (thin cut) were pan-fried with 30 mL of oil at 170 °C for 10 min, and turned over every two minutes. Beef steak was grilled at 150 °C for 4 min for medium steak or 8 min for well-done steak, and each piece was turned over, every two minutes. Salmon and tuna were grilled at 150 °C for 8 min; in addition, tuna was also grilled with soy sauce (15 mL). Smoked bacon was pan-fried at 170 °C with 5 mL of oil. Potato fries and chicken nuggets were purchased from a global fast food establishment.

Each item was prepared in duplicate and kept overnight at 4 °C before further processing. Duplicates were pooled and were homogenized in a blender with addition of phosphate-buffered saline (PBS) and further diluted with PBS to reach 1:10 m/v ratio. The obtained mixtures were freeze-dried. Proteinase K solution (Roche, 15.6 mg/mL) was added to 50 mg aliquot of the freeze-dried homogenates re-suspended in PBS, ensuring enzyme concentration 0.2% (based on protein mass) and the samples were incubated at 37 °C overnight. Protein content in the freeze-dried homogenates was estimated by Bradford spectrophotometric assay using 0.1 M sodium hydroxide for extraction, Biorad-Protein-Assay was used for this purpose. For enzyme deactivation, the mixtures were heated for 10 min at 80 °C. Finally, the samples were centrifuged (10,000*g*, 10 min), diluted as needed and analyzed by ELISA.

2.2. ELISA assays

Two commercial ELISA kits were used in this work, following the manufacturer indications. ELISA-1 was human carboxy-methyl lysine-advanced glycation end product (CML-AGE) ELISA Kit from My Bio Source® (San Diego Cal. USA MBS263691) whereas ELISA-2 was a competitive inhibition assay for total advanced glycation end products in human serum from Lamider® (Lamider Co. Mexico city).

For ELISA-3, the monoclonal CML-specific antibody was kindly provided by the group at the Mount Sinai School of Medicine (Cai et al., 2002). Since this was not a commercial kit, the calibrator was prepared in our laboratory as described elsewhere (Takeuchi et al., 2001), yet with some modifications. Specifically, to 50 mg aliquot of bovine serum albumin from Bio Basic Inc., (product AD 0023 Lot. 1109BI1J22342), 15 mg of glyoxylic acid and 30 mg of sodium cyanoborohydride in 1 mL phosphate buffer 400 mM, pH 8.5, were added and the mixtures were incubated at 37 °C during 24 h. The preparation was then dialyzed against PBS and diluted to reach final protein concentration of 1000 μg/mL. To determine CML content in this calibrator, 20 μL were digested with hydrochloric acid, diluted as suitable, and processed by HPLC-ESI-ITMS/MS (as described in the next section). Based on three technical replicates, the concentration found in the analyzed solution was 15.5 ± 0.47 μg CML/mL, therefore, CML-BSA 1000 μg/mL was equivalent to 309 μg CML/mL.

ELISA-3 was performed as a competitive assay, according to the established method (Mitsuhashi, Vlassara, Founds, & Li, 1977). In brief, 96-well ELISA plate was coated with 100 μl/well of 3 μg/mL CML-BSA (corresponding to 0.929 μg CML/mL) prepared in a coating buffer (0.1 M $NaHCO_3$, pH 9.6, with 0.02% NaN_3, m/v) and kept overnight at 4 °C. Wells were washed three times with 200 μL of washing buffer (PBS with 0.05% Tween-20, m/v and 1 mM NaN_3), then blocked with 100 μL of SuperBlock™ blocking buffer in PBS (Thermo Scientific, #37515), at room temperature for 1 h. After three further rinses with washing buffer, an aliquot of sample or calibration solution (50 μL) was added to the wells, followed by 50 μL of anti-CML antibody solution prepared in a diluting buffer containing PBS, 0.02% Tween-20 andv2% normal goat serum (final dilution 1/1000); antibody had not been added to control wells. ELISA plate was gently agitated at room temperature for 2 h and wells were again rinsed three times with washing buffer. An aliquot (100 μL) of alkaline phosphatase conjugated second antibody (MP

Biomedicals, Goat anti- mouse IgG(H + L)) was added to each well in dilution buffer containing 1% normal goat serum, the plate was incubated at 37 °C for 1 h. Finally, wells were washed with three portions of washing buffer and 100 µL of alkaline phosphatase substrate (pNPP, Sigma #N-9389) were added to each well. Once OD at 405 nm in control wells reached value 1.0–1.5, the optical density was measured in all wells. The calibration solutions were prepared from CML-BSA standard 1000 µg/mL (309 µg CML/mL) by appropriate dilution to obtain the concentration range 1.2–154 ng CML/mL.

2.3. HPLC-ESI-ITMS/MS procedure

An aliquot (20 mg) of the freeze-dried and homogenized food item was reduced with a mixture of 400 µL sodium borohydride 2 M (in 0.1 M NaOH) and 1 mL borate buffer 0.2 M, pH 9.2, overnight at 4 °C. Proteins were precipitated with 200 µL of perchloric acid 2 M (10 min, −20 °C) and lipids were extracted using 1 mL of methanol:chloroform 1:2 (v/v). Acid hydrolysis was carried out by adding 1 mL hydrochloric acid 6 N to the pellet, the vial was flushed with nitrogen, sealed and heated at 110 °C during 20 h. The hydrolyzate was evaporated on a heated plate (60 °C), the residue was reconstituted in deionized water with addition of sodium hydroxide (1 M) to a final volume of 1 mL; 200 µL were taken for derivatization. Calibration was carried out using 200 µL aliquots of standard solutions containing 5.0, 10, 50, 100, 250 y 500 µg/L CML (purchased from PolyPeptide). Once internal standard was introduced (50 µL de CML-d2 600 µg/L, also from PolyPeptide), 500 µL of methanol:pyridine (4:1, v/v) and 50 µL of ethyl chloroformate were added, the latter in two successive portions separated by agitation. Finally, 500 µL of sodium bicarbonate 50 mM were added to adjust pH 9.0 and the derivatized compounds were extracted to chloroform (500 µL) (Petrovic, Futas, Chandoga, & Jakus, 2005), the organic phase was evaporated (hot plate, 60 °C) and reconstituted in 200 µL of the mobile phase A (methanol: ammonium formate 5 mM, 55:45 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 chromatographic column was Kinetex C18 (150 × 3 mm, 2.6 µm) with a Security Guard ULTRA Cartridge C18, both from Phenomenex.

The injection volume was 10 µL, column temperature 30 °C, gradient elution with mobile phase A and methanol (B) as follows: 0-8 min, 55% B; 8–9 min, 95% B; 9–10 min 95% B; 10–12 min 55% B (column re-equilibration) and a total flow rate 0.2 mL/min.

ESI source was operated in a positive mode with the following parameters: spray voltage 4500 V; plate voltage 500 V; nebulizer gas pressure 0.8 bar (N_2); dry gas 6 L/min (N_2); source temperature 200 °C and capillary exit voltage 140 V. Mass spectra were obtained in UltraScan mode in the *m/z* scan range 100–450. For multiple reaction monitoring (MRM), fragmentation amplitude 0.6 V, isolation width *m/z* ± 2 and 70 ms dwell time were applied for each ion. CML fragmentation ions were *m/z* 331 as quantifier and *m/z* 345 as qualifier (precursor ion *m/z* 377); for CML-d2 (precursor *m/z* 379), the quantifier and qualifier ions were *m/z* 333 and *m/z* 347, respectively.

For accuracy checking, 100 ng of CML standard was added to the samples of cooked potato and salmon grilled 8 min prior to acid hydrolysis.

2.4. Statistical analysis

The results presented are means obtained for three independent replicates, standard deviations were calculated using Microsoft Excel 2010.

Statistical analysis of correlation was carried out for the results obtained by each pair of the four methods; one-way ANOVA with post hoc Tukey HSD test was used to compare CML or AGE concentrations found by each method in these same items prepared by different culinary treatments and unpaired *t*-test was used to compare CML or AGEs concentrations in poached and grilled salmon by the four methods. Significance level was established at $p < 0.05$. The software used was Statistica for Windows (Stat Soft Inc., Tulsa, OK).

To observe possible relationships among analytical results obtained using the four different procedures, we performed principal component analysis (PCA) (Unscrambler 7.5, Camo, Norway).

Significance level was established at $p < 0.05$.

3. Results and discussion

3.1. Set-up of the HPLC-ESI-ITMS/MS method

Alkyl chloroformates have been accepted as versatile derivatization agents and are widely used in the analysis of amines, carboxylic acids, phenols, etc. (Husek, 1998). Using gas chromatography-mass spectrometry, efficient conversion of CML to its ethyl amino ester was demonstrated with the advantage of short reaction time, no need for heating and no need for water elimination (Petrovic et al., 2005). In this work, ethyl chloroformate derivatives of CML and CML-d2 were obtained prior to liquid chromatography separation; by so doing, potentially interfering polar sample components could be eliminated during extraction to chloroform and robust non-polar target compounds presented adequate retention on a reversed phase column while using mobile phases compatible with ESI source. In Fig. 1a, typical chromatograms acquired in MRM mode are presented for CML-d2 as IS, and for CML in salmon grilled during 8 min. Linear regression calibration presented acceptable linearity ($r^2 > 0.997$); quantification limit for CML was 3.4 µg/L on column and 0.18 µg/g as referred to the freeze-dried sample mass. The chromatograms obtained for non-spiked salmon grilled 8 min (MRM mode for CML and for internal standard) and those obtained in the recovery experiment for cooked potato (MRM mode for CML) are presented in Fig. 1a and b, respectively. The evaluated percentage recoveries, 108% for potato and 104% for salmon samples,

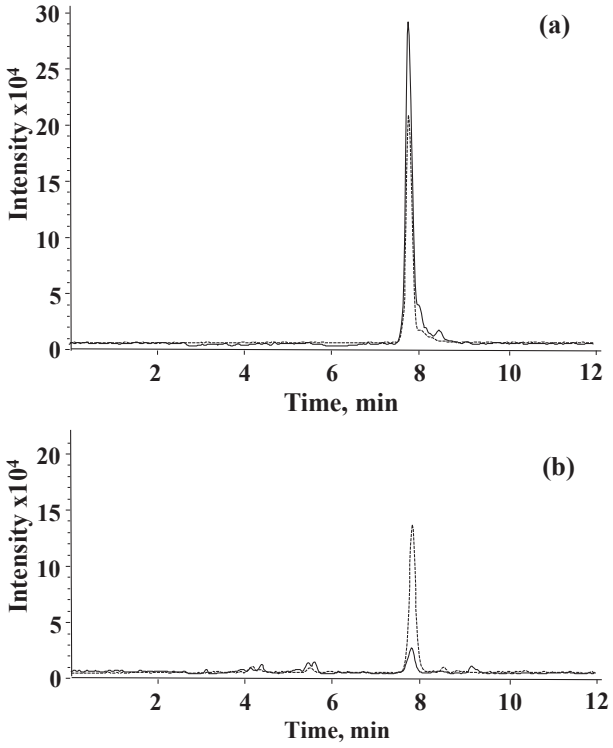

Fig. 1. Typical HPLC-ESI-ITMS/MS chromatograms acquired in MRM mode. a) (—) salmon grill 8 min (CML, *m/z* 377 → 331); (·····) internal standard (CML-d2, 150 µg/l, *m/z* 379 → 333). b) (—) cooked potato and (·····) this same sample after standard addition, 100 µg/l CML (CML, *m/z* 377 → 331).

were indicative of acceptable accuracy.

3.2. ELISA assays used for dAGEs measurement

Our intent was to include assays of different selectivity toward CML and based on different ELISA types. In particular, ELISA-1 is a direct, double antibody sandwich assay designed for human samples; it uses monoclonal CML antibody and polyclonal biotin-labelled secondary antibody thus ensuring high specificity. In the kit's instruction, the calibrator is referred to as "human CML-AGE standard" without further details and the results are expressed as calibrator-equivalent (CEQ) mass per volume of the solution (CEQ ng/mL); the calibration is carried out in the concentration range 1.56-100 CEQ ng/mL.

ELISA-2 was selected as a commercial kit of low selectivity; it employs polyclonal antibody in a competitive assay and is recommended for the assessment of total AGEs in human serum. According to the kit's manual, glycated bovine serum albumin is used for calibration; AGE unit is defined as fluorescence signal obtained for 1 mg/mL calibrator solution ($\lambda_{ex}/\lambda_{em}$ = 370 nm/440 nm) and the results for the real-world samples are expressed as mU/mL; in our experiments the calibration range 2.5–100 mU/mL could be applied. Even though ELISA-2 measures fluorescent AGEs, it was used here to provide further evidence on the utility of CML as a reliable marker of food AGEs. It should also be stressed that none of the two kits (ELISA 1 and 2) have been used previously for the analysis of food products, except for a very recent study in which ELISA-2 was applied for total AGEs measurement in biscuits (Martinez-Saez et al., 2017).

Finally, ELISA-3 employed CML monoclonal antibody which had already been utilized for food analysis (Goldberg et al., 2004). In that case, the calibrator was prepared in our laboratory as described in Materials and methods; consequently, CML content in the real-world samples could be presented directly, as micrograms of CML per gram of food, using the calibration range 1.2–154 ng CML/mL. Noteworthy, in the previous studies using this same antibody (Goldberg et al., 2004; Uribarri et al., 2010), results were provided in AGEs units per 100 g of food (kU/100 g) – referring the analyte content to the equivalent amount of glycated standard, but without quantitative evaluation of protein glycation rate in this standard (Uribarri et al., 2010).

3.3. Results of CML/AGEs determination in food products by ELISA and HPLC-ESI-IT-MS/MS

Quantitative data obtained for all food items are presented in Table 1; using concentration units proper for each procedure, mean values with respective standard deviations are given based on three replicates. In the last column of this Table 1, CML contents available in the most complete so far ELISA-derived database are included (Uribarri et al., 2010). On the other hand, few previous studies employed analytical chemistry procedures for CML determination in foods and these are discussed below emphasizing similarities or discrepancies between the reported values and our HPLC-ESI-ITMS/MS results (Chen & Smith, 2015; Hull, Woodside, Ames, & Cuskelly, 2012; Scheijen et al., 2016; Sun et al., 2015, 2017; Zhou et al., 2015).

Despite evident differences observed in Table 1 for these same products analyzed by four procedures, the following decreasing order of CML/AGE concentrations can be observed: beef and bacon > chicken > fish > dairy products > grain products > fruit/vegetables. Similar order can be noted in the published ELISA database (the last column in Table 1) and this order has also been confirmed in a recently published study performed using liquid chromatography – tandem mass spectrometry procedure; 7.3 μg/g CML in beef steak grilled 5 min, 7.5 μg/g in fried breaded chicken, 4.1 μg/g in fried salmon, 0.4 μg/g in cheese (32% fat), 0.1 μg/g in raspberry yogurt drink, 0.1 μg/g in boiled or fried potatoes (Scheijen et al., 2016). It is known that cooking conditions alter the initial content of AGEs in food and in the cited study, CML in boiled and non-breaded fried chicken was lower than that in breaded fried breast (1.8 μg/g, 3.4 μg/g and 7.5 μg/g, respectively) (Scheijen et al., 2016). As shown in Table 1, CML concentration found in poached chicken was indeed lower with respect to nuggets or pan-fried breast but non-breaded fried breast, which contained less CML than chicken nuggets; this apparent discrepancy most probably resulted from the use of a different raw meat type (we analyzed commercial chicken nuggets, elaborated from ground meat). In agreement with our results, application of liquid chromatography-mass spectrometry yielded higher CML in the beef steak well-done versus medium roasted (14.38 μg/g, 12.30 μg/g) and also in fried as compared to baked chicken breast (44.48 μg/g against 23.54 μg/g) (14.38 μg/g, 12.30 μg/g) (Hull et al., 2012). In another study, CML concentration was found to be about six times lower in the inner part as compared to the surface layer of fried beef (3.13 μg/g versus 20.3 μg/g), chicken (2.99 μg/g versus 17.6 μg/g) and salmon (1.68 μg/g versus 12.2 μg/g) (Chen & Smith, 2015). Even though the entire piece of food was always homogenized prior to measurements in the present work, our HPLC-ESI-ITMS/MS results were comparable with those corresponding to the surface layer of meats (15.7 μg/g in fried beef, 16.2 μg/g in fried chicken and 12.4 μg/g in grilled salmon, Table 1). Despite consistent results described above (Chen & Smith, 2015; Hull et al., 2012; Scheijen et al., 2016), some other studies have provided substantially different CML values; such was the case of a study from China (Zhou et al., 2015), where French fries contained higher CML as compared to our results (12.3–19.5 μg/g versus 1.57 μg/g) while for fried bacon the concentration in the cited work was lower than ours (8.64 μg/g versus 17.2 μg/g). Furthermore, CML content reported in cheddar cheese was 23.2 μg/g (Assar et al., 2009) against 3.4 μg/g provided by our analytical method (Table 1). Differences of CML levels in beef products have often been discussed and the observed variability was associated with the selection of retail beef cut, mincing, initial fat content, the use of preservatives and condiments, water content and the type of heat treatment applied (Chen & Smith, 2015; Sun et al., 2015, 2017). In this study, round tip steak (thin cut) was pan-fried and grilled for 4 or 8 min without salt/species; whereas short grilling caused less CML formation as compared to the frying procedure, opposite effect was observed when grilling time was increased to 8 min. Concentration determined by liquid chromatography – mass spectrometry method in 4 min grilled steak (11.8 μg/g) was consistent with that reported for low-fat ground beef baked in aluminium10 min with no additives (12.37 μg/g) (Sun et al., 2017). As already mentioned before, Hull et al. also reported increase of CML in well-done as compared to medium beef steak (Hull et al., 2012).

Mexican "chicharrón" is elaborated by frying pork skin. Lower CML content was found in this product as compared to the red meat (Table 1).

To the best of our knowledge, CML has not been previously measured in wheat/corn tortilla, products which are Mexican staple food. Of interest, our results (2.09 μg/g, 2.51 μg/g, respectively – second column in Table 1) were lower as compared to the range 7.6–54.2 μg/g reported for cereal-based products (Hull et al., 2012). Furthermore, it has been demonstrated that CML formation in bread crust is favored as compared to the crumb; in whole meal bread Assar et al. informed the values 46.1 μg/g and 4.45 μg/g, respectively (Assar et al., 2009). Apparently, corn grain and wheat processing during tortilla elaboration has lower effect on CML formation in comparison to the production of other foods based on these same cereals.

We did not find any data for grilled tuna based on analytical chemistry methods; nonetheless, in the ELISA-derived database (Uribarri et al., 2010), CML content (5113 kU/100 g) exceeds those for poached and grilled salmon (2292 kU/100 g and 3347 kU/100 g, respectively). Our LC-MS results confirm higher CML concentration in tuna with respect to salmon, in agreement with the existent database and also in agreement with the relatively high lysine and arginine content in tuna as compared to other fishes (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012; Department of Agriculture).

Table 1
Concentrations of CML determined in food items by HPLC-ESI-ITMS/MS and CML/AGEs by ELISA assays. Mean value with respective standard deviation is given for each product based on three independent replicates. (< QL – found below quantification limit of the procedure; ND – not detected; NR - not reported).

Food item	LC-MS (μg/g)[a]	ELISA-1 (CEQng/mL)[b]	ELISA-2 (mU AGEs/mL)[c]	ELISA-3 (μg/g)[a]	ELISA-database (kU/100 g)[d]
Fresh apple	< QL	1.48 ± 0.90	4.63 ± 1.54	1.00 ± 0.11	13
Fresh serrano pepper	1.10 ± 0.18	11.2 ± 0.8	ND	1.22 ± 0.25	NR
Flour tortilla	2.09 ± 0.15	6.74 ± 0.35	5.73 ± 1.95	1.73 ± 0.34	NR
Corn tortilla	2.51 ± 0.67	2.78 ± 0.73	8.88 ± 2.23	1.96 ± 0.45	NR
Fried potatoes	1.57 ± 0.11	5.71 ± 0.66	4.55 ± 0.74	1.61 ± 0.24	1522
Boiled potato	1.15 ± 0.08	2.59 ± 0.25	5.91 ± 2.47	1.29 ± 0.11	17
Natural yogurt	2.92 ± 0.25	2.90 ± 0.41	2.81 ± 0.52	2.4 ± 0.32	3
American cheese	3.40 ± 0.21	3.10 ± 0.68	6.01 ± 0.26	4.1 ± 0.19	4040
Poached salmon	9.81 ± 0.72	5.71 ± 0.75	24.6 ± 3.4	10.2 ± 1.3	2292
Grilled salmon (8 min)	12.4 ± 1.0	7.51 ± 1.21	29.1 ± 2.3	12.0 ± 0.9	3347
Grilled tuna (8 min) with soy sauce	15.2 ± 0.9	8.89 ± 0.43	25.0 ± 3.7	12.1 ± 2.2	5113
Grilled tuna (8 min)	16.9 ± 1.0	9.08 ± 0.46	21.5 ± 7.3	21.2 ± 2.9	NR
Pan-fried beef steak	15.7 ± 1.1	5.37 ± 0.96	25.9 ± 2.9	16.8 ± 1.4	10058
Grilled beef-steak (4 min)	11.8 ± 0.7	4.32 ± 0.63	22.8 ± 2.3	12.7 ± 0.9	7416
Grilled beef-steak (8 min)	19.3 ± 0.9	6.43 ± 0.25	33.4 ± 1.4	19.8 ± 1.2	NR
Chicken nuggets	12.5 ± 0.45	6.85 ± 0.21	25.7 ± 3.8	14.3 ± 1.4	7722
Poached chicken	9.31 ± 0.8	5.82 ± 0.14	12.6 ± 2.5	9.42 ± 1.1	1101
Pan-fried chicken breast	16.2 ± 1.2	8.60 ± 1.04	29.2 ± 3.3	19.4 ± 0.8	4938
Fried bacon	17.2 ± 0.2	9.63 ± 0.92	34.7 ± 5.2	15.7 ± 1.2	9158
"Chicharrón" Mexican style pork rinds	7.19 ± 0.93	6.13 ± 0.64	19.1 ± 4.2	5.73 ± 0.48	NR

[a] Micrograms CML per gram of freeze-dried food item.
[b] Calibrator equivalent nanograms of CML per ml of the solution.
[c] U defined as fluorescence signal obtained for 1 mg/mL calibrator (AGE-BSA) solution.
[d] U unit equivalent to the amount of glycated calibrator (AGE-BSA) (Uribarri et al., 2010).

3.4. Comparative evaluation of ELISA and HPLC-ESI-IT-MS/MS performance in the analysis of dAGEs

In the first approach, correlation analysis was carried out taking mean values obtained for each food item in the four procedures (the results presented in Table 1S, electronic supplementary material, ESM). Statistical significance was found for each pair of assays; the best correlation ($r = 0.9796$, $p < 0.0001$) corresponded to HPLC-ESI-ITMS/MS and ELISA-3 whereas the poorest relationship ($r = 0.4402$, $p = 0.052$) was found for ELISA-1 vs ELISA-2 results. The latter observation is not surprising in the view of different selectivity of these two assays toward AGEs. On the other hand, CML measured by ELISA-1 or ELISA-3 and AGEs assessed by ELISA-2, correlated well with CML determined by HPLC-ESI-ITMS/MS; good correlation was also found for CML determined by ELISA-3 and AGEs measured by ELISA-2 (Table 1S, Fig. 1S ESM). Noteworthy, HPLC-ESI-ITMS/MS measured protein-bound CML while ELISA kits provided total CML (or AGEs) values; such different selectivity had no impact on correlation results in consistency with previous studies informing on low percentage of free CML in raw products and its dramatic decrease during heat treatment (Sun et al., 2017). Overall, the above results support the feasibility of CML as indicator of AGE formation in food.

To enhance the scope of comparative evaluation, CML values from the available database were added, six items not reported in this database were excluded (see Table 1) and correlation analysis was repeated. As shown in Table 2, the direct relationship was conserved for each pair of procedures used in this work. In fact, elimination of six items resulted in better correlation of ELISA-1 with other procedures (Table 1S and Table 2). It should be stressed at this point that ELISA-2 and ELISA-1 kits were designed for clinical samples and not for food analysis; therefore the complex and heterogeneous composition of food items in this study might potentially interfere with their immunoassays. In particular, fresh serrano pepper seemed to be a problematic sample because no AGEs were detected by ELISA-2 and CML seemed highly overestimated using ELISA-1 (Table 1). On the other hand, CML values from ELISA-derived database did not present significant correlation with any of the four procedures (Table 2). This finding, however, may be related to the fact that our study was carried out using identical food items for all four procedures, while results from databases had certainly been built analyzing foods from completely different raw products. It is encouraging however that correlation coefficients between the four procedures and the food-database were relatively high ($r > 0.46$, Table 2).

Table 2
Statistical analysis of correlation evaluating consistency of the results obtained for food items included in ELISA-derived database (Uribarri et al., 2010) using four different procedures (mean values taken for comparison, $p \leq 0.05$; r – correlation coefficient, p – probability).

	ELISA-1	ELISA-2	ELISA-3	ELISA database
LC-MS	r = 0.8419 p = 0.0001	r = 0.9507 p = 0.0001	r = 0.9773 p = 0.0001	r = 0.4614 p = 0.097
ELISA-1	–	r = 0.8261 p = 0.0001	r = 0.7960 p = 0.001	r = 0.5079 p = 0.064
ELISA-2		–	r = 0.9433 p = 0.0001	r = 0.5021 p = 0.067
ELISA-3			–	r = 0.4801 p = 0.082

Although correlation analysis based on mean values obtained in three replicates of each sample suggested suitability of all ELISA assays for CML/AGE determination in foods, repeatability in these assays was worse as compared to HPLC-ESI-ITMS/MS results. Specifically, relative standard deviations were in the range 2.41–60.8% (mean 13.5%) for ELISA-1, 4.20–41.8% (mean 18.5%) for ELISA-2, 4.10–23.0% (mean 10.9%) for ELISA-3 whereas for analytical method the range was 1.20–16.4% (mean 7.88%). To further examine the feasibility of ELISA for measurement of CML as representative AGE in foods, the results obtained for beef steak, chicken and salmon cooked using different recipes, were compared. In Fig. 2, CML/AGE contents in three different beef preparations are presented showing the same decreasing order as assessed in all four procedures: steak grilled 8 min > fried steak > steak grilled 4 min; nonetheless, ANOVA indicated statistical

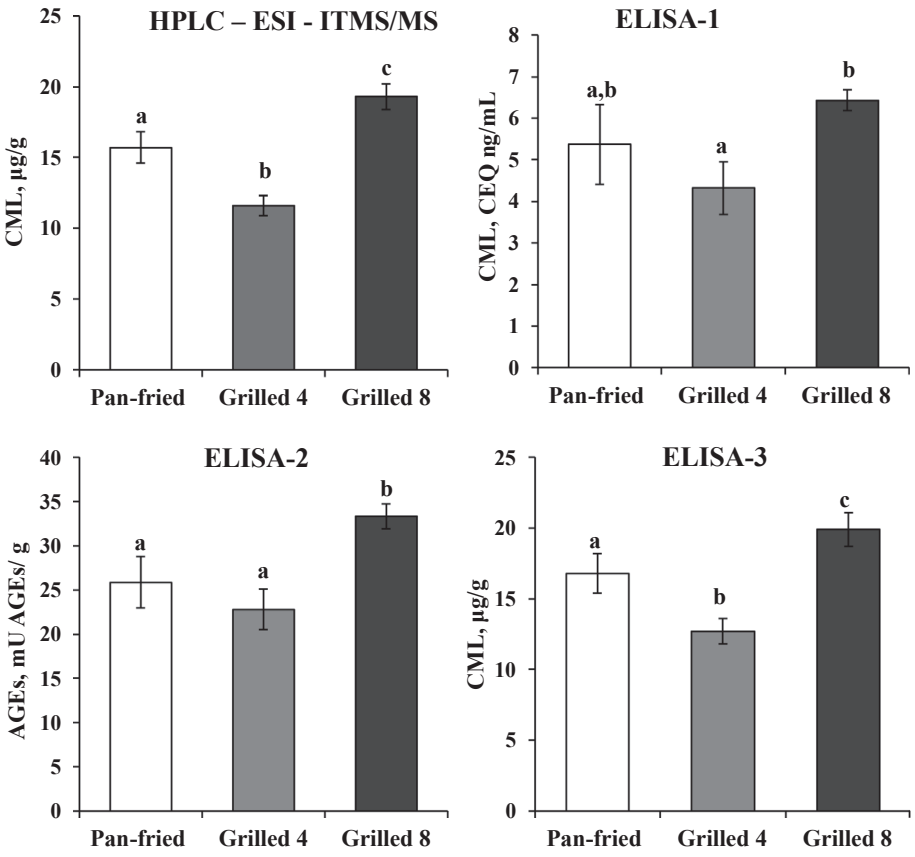

Fig. 2. CML/AGEs concentration found in three different beef steak preparations by each of four methods. (The bars show mean values from three replicates with respective standard deviation; different letters indicate statistically significant difference, one-way ANOVA with post hoc Tukey HSD test, $p < 0.05$).

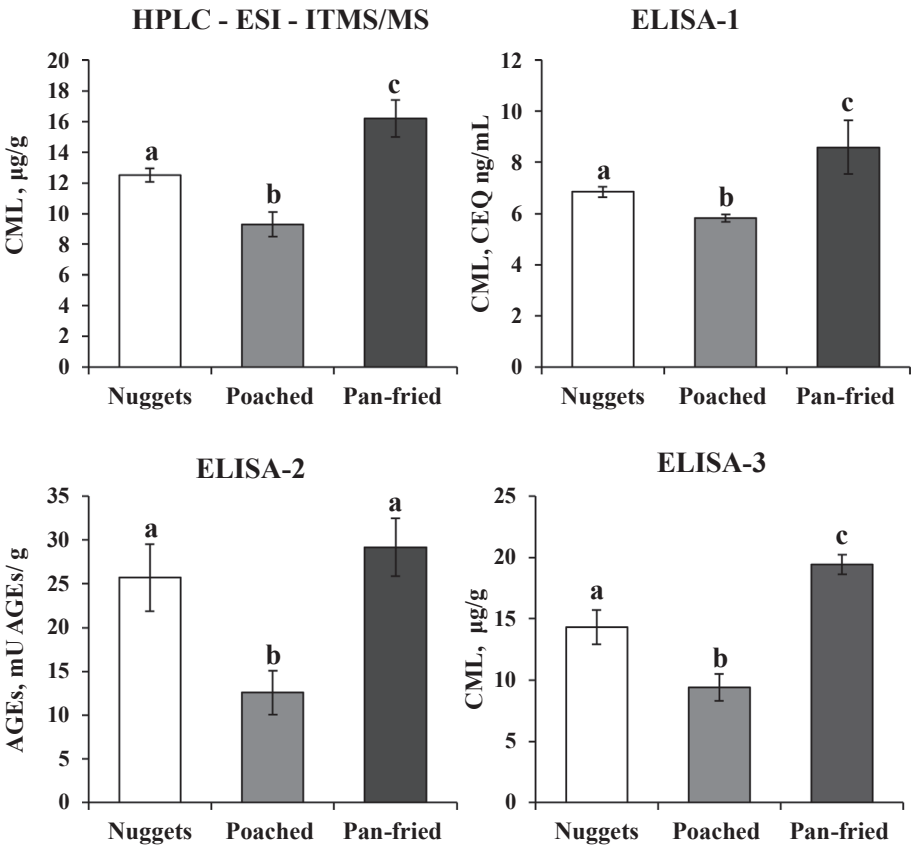

Fig. 3. CML/AGEs concentration found in three different chicken meat preparations by each of four methods. (The bars show mean values from three replicates with respective standard deviation; different letters indicate statistically significant difference, one-way ANOVA with post hoc Tukey HSD test, $p < 0.05$).

significance of these differences only for LC-MS and for ELISA-3. Data obtained for the two commercial kits enabled to distinguish CML/AGE concentrations in steak grilled applying different time periods (4 min and 8 min). The results obtained for chicken products also presented similar tendency for each of the four procedures: fried breast > nuggets > poached breast; in this case however, only concentrations obtained for ELISA-2 in fried breast and chicken nuggets could not be differentiated with statistical significance (Fig. 3). The results obtained for poached and grilled salmon indicated significantly lower CML content in the poached product only for HPLC-ESI-ITMS/MS (Fig. 2S, ESM). Finally, CML/AGE concentrations found in boiled and fried potatoes did not present significant differences in any procedure. CML content in corn tortilla was significantly higher than in wheat tortilla by HPLC-ESI-ITMS/MS and by ELISA-3 ($p < 0.05$). On the contrary, both commercial kits indicated statistically higher CML/AGEs in wheat as compared to corn tortilla, which seems to confirm susceptibility of these kits to matrix interferences.

As a complementary approach for comparative evaluation of the four different procedures used in this work, principal component analysis has been carried out. The purpose was to find possible similarities in the performance of the four methods while analyzing the same food items, independently on the concentration units used in each method. Initially, all individual results for all items had been considered; however, variables (CML or AGE concentrations) corresponding to serrano pepper and grilled tuna (with or without soya sauce) were detected as outliers and were removed in further analysis. As already noted before, inconsistent results were obtained for serrano pepper by the two commercial kits, most probably due to matrix effects. Particularly dark color of tuna, its high arginine and lysine content suggest that other glycation products might be formed during grilling, potentially affecting the use of CML as AGE-representative compound. Indeed, AGEs in tuna (and also in animal meat) measured by ELISA-2 were consistently higher than CML in these same products (Table 1). Furthermore, LC-MS, ELISA-1 and ELISA-3 indicated higher CML concentrations in tuna grilled without soy sauce, whereas ELISA-2 showed lower AGEs content in this item as compared to tuna grilled without sauce (Table 1).

Principal component analysis (PCA) model obtained after exclusion of above three food items is presented in Fig. 4; the first two components (PC1, PC2) accounted for 99% of total data variability indicating statistical significance of the model. On the score plot (Fig.4a), distribution of the food items in a space of reduced dimensionality is accordingly to their CML content. In particular, PC1 enabled to separate meat/fish items with higher CML/AGEs concentrations from those corresponding to plant-derived foods and dairy products with lower CML/AGEs contents. In Fig.4b, the X-loading plot shows locations of the four procedures in these same coordinates PC1 and PC2. At the first glance, close association can be observed between LC-MS and ELISA-3 indicating their similar performance in the analysis of foods. The two commercial kits are clearly away from LC-MS - ELISA-3 cluster, confirming their different (poorer) analytical capability as already inferred form correlation analysis and from ANOVA results. All procedures presented positive PC1 values, which indicates that all of them provided more consistent results for relatively high CML/AGEs in foods; among them, specifically ELISA-1 presented a tendency for over-estimation of low CML concentrations.

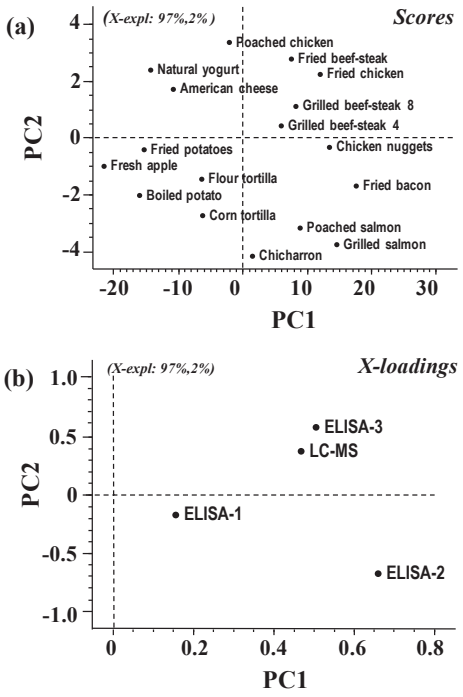

Fig. 4. Principal component analysis (PCA) of data obtained in the analysis of food items by four different procedures: (a) scores plot showing the distribution of food items and (b) X-loadings plot showing distribution of procedures used for CML/AGEs determination, both in the reduced dimensionality of PC1 and PC2.

4. Conclusions

In this work, three ELISA assays and HPLC-ESI-ITMS/MS were comparatively evaluated for the analysis of CML as a representative AGE in twenty food items. In general, CML concentrations were in agreement with those previously reported in the same foods while using both analytical chemistry procedures and immunochemical assays. Furthermore, results for total AGEs measured by ELISA-2 showed acceptable consistency with the three CML-selective procedures. The results obtained support the use of ELISA assays for the measurement of CML in foods and suggest that CML might be adopted as chemical indicator of total AGEs. In particular, ELISAs seem to be especially well suited for the analysis of products with relatively high CML/AGE concentrations; however, these assays presented poorer precision as compared to liquid chromatography-mass spectrometry method and apparently were affected by matrix effects. It should be stressed at this point that the two commercial kits utilized in this work are recommended for the analysis of clinical samples and that no commercial kit is actually offered for food analysis. Of note, the analytical chemistry method enabled to distinguish CML concentrations in these same food items prepared by different culinary treatments, while these often small differences could not always be detected at the statistical level by the ELISA assays. In this regard, the least consistent with liquid chromatography-mass spectrometry data were those obtained with ELISA-1. Since final CML/AGE concentration in food products would always depend on the type of raw material used and the cooking technique, the detailed description of these factors is needed to enable for any comparative evaluation of the results. Finally, the results of this work confirm direct relationship between CML and total AGEs in specific foods and encourage the use of ELISA assays in food CML/AGE screening.

Conflict of interest

The authors declare to have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.09.098.

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