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Simultaneous analysis of lysine, N^{ε}-carboxymethyllysine and lysinoalanine from proteins

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Abstract

Protein quality was assayed by simultaneous measurement of lysine (Lys), carboxymethyllysine (CML) and lysinoalanine (LAL). GC-FID analysis of *N-tert*-butyl dimethylsilyl (tBDMSi) derivatives of these amino acids was undertaken. tBDMSi derivates were separated on a CP-SIL 5CB commercially fused silica capillary column (25 m × 0.25 mm i.d., 0.25 μm film thickness) employing a thermal gradient programmed from 200 to 300 °C. The identity of tBDMSi derivatives of Lys, CML and LAL was established by GC–MS while FID detection was employed for quantification. Analytical parameters such as linearity (lysine 350–4200 μM, LAL 3–81 μM, CML 16–172 μM), precision (1–13% variation coefficients), accuracy (85–108% average recovery) and limits of detection (lysine 0.4 mg/100 g protein, LAL 5.0 mg/100 g protein, CML 3.4 mg/100 g protein) and quantification (lysine 1.4 mg/100 g protein, LAL 15.2 mg/100 g protein, CML 11.2 mg/100 g protein) were determined for validation of the analytical approach. Model systems and real foods have been studied. Kinetic of CML formation from different food proteins (BSA, soy protein, casein and gluten) was performed employing model systems. Carboxymethylation rate depended on the source of protein. Maillard reaction progressed to advanced stages damaging the protein quality of stored infant foods, soy drinks, boiled eggs and dry powdered crepes. CML values ranged from 62 to 440 mg/100 g protein were measured. LAL was also formed during boiling eggs (21–68 mg/100 g protein) indicating additional damage by crosslinking reaction. In agreement, lysine content was affected by both food processing and storage.

Keywords: Gas chromatography; Lysine; Lysinoalanine; N^{ε}-Carboxymethyllysine; Protein

1. Introduction

Lysine bound to proteins, which is one of the most limiting essential amino acid, is greatly degraded during food processing and the subsequent storage by both Maillard and crosslinking reactions [1,2]. Quantitative analysis of lysine and its degradation products formed from these key chemical events have been widely employed as individual chemical marker of protein quality [2].

It is generally accepted that Maillard reaction consists of a series of subsequent and parallel reactions, which are commonly divided into three stages known as early, advanced and final. The early stage products of this reaction also called Amadori rearrangement products may undergo several degradation reactions during severe or prolonged storage or thermal treatment of foods forming "advanced glycation end products" (AGEs). These compounds are also formed *in vivo* and they have been associated to the development of various pathological processes, such as premature ageing, diabetes, atherosclerosis, Alzheimer disease and renal failure [3]. Moreover, a significant correlation has been found between dietary and circulating AGEs in humans, so AGEs intake may constitute a risk factor for tissue injury [4–7]. N^{ε}-

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Carboxymethyllysine (CML) is one of the best characterized AGEs. This compound is formed by oxidative degradation of Amadori compounds or reaction of lysine with products of autoxidation of ascorbic acid. Moreover, other oxidative pathways such as lipid peroxidation and serine oxidation may also be involved [8–10]. CML formation can be estimated in food or protein model system mainly through gas chromatography (GC) [9,11–14], high performance liquid chromatography (HPLC) [15–19], matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) [20,21,22] and enzyme-linked immunosorbent assay (ELISA) [5,23–27].

Lysinoalanine [LAL, N^{ε}-(D,L-2-amino-2-carboxyethyl)-L-lysine] has also been used as a marker of thermal damage in foods. The mechanisms of the formation of this unnatural crosslinking amino acid take place in two steps. The first step consists of the formation of dehydroalanine residue from *O*-phosphorylserine, *O*-glycosylserine or cystine, which is a hydroxide ion-catalyzed reaction, followed by the second reaction that involves the double bond of dehydroalanine with a nucleophilic side chain of another amino acid, such as the ε-amino group of lysine [28,29]. High pH, temperatures, and long exposure times favoured the formation of LAL in protein-containing foods. LAL has been determined in foods using ion-exchange chromatography [30,31], GC [32–36], thin layer chromatography and HPLC [18,37–43].

The aim of this paper was to find out feasible analytical conditions for assessing loss of protein quality due to Maillard and crosslinking reactions in both model systems and real foods by a single injection of the sample. The method here described is based on those previously reported by other authors for GC analysis in separate of the twenty protein forming amino acids, CML and LAL, respectively [9,36,44].

2. Experimental

2.1. Chemicals

N^{ε}-Carboxymethyl-L-lysine was purchased from NeoMPS Groupe SNPE (Strasbourg, France). Lysinoalanine was obtained from Bachem AG (Bubendorf, Switzerland). Glyoxylic acid monohydrate (G4627-10G), bovine serum albumin (BSA, A2153-10G), 2,6-diaminopimelic acid and wheat gluten (G5004-500G) were obtained from Sigma–Aldrich (St. Louis, MO). Sodium cyanoborohydride and MTBSTFA (*N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide) were supplied by Fluka Chemie GmbH (Buchs, Switzerland). Sodium dihydrogen phosphate monohydrate, di-sodium hydrogen phosphate anhydrous, triethylamine and *N*,*N*-dimethylformamide were from Merck KgaA (Darmstadt, Germany). Hydrochloric acid 37% was supplied by Prolabo (Fontenay sous Bois, France). Soy protein was obtained from Manuel Riesgo, S.A. (Madrid, Spain). High purity water was produced in-house in a Milli-Q synthesis A10 system (Millipore, Bellerica, MA, USA) and was used throughout.

Caseins were isolated from bovine skim milk by isoelectric precipitation at pH 4.6 with 1 M HCl and centrifugation at 4500 × *g* and 5 °C for 15 min. The precipitate containing caseins was washed three times with 1 M sodium acetate–acetic acid buffer pH 4.6, dialyzed against water and lyophilized.

Pure fructosyl-lysine (FL) was obtained in-house according to the procedure previously described by Finot and Mauron [45].

2.2. Model systems

A comparative study of the kinetic of CML formation from different food proteins (BSA, soy protein, casein and gluten) was carried out. Carboxymethylation was performed according to the procedure previously described by Berg et al. [46] with slight modifications. Proteins (25 mg) were diluted in 1 mL of 0.2 M phosphate buffer pH 8. Aliquots (2 mL) of the buffered protein solutions were mixed with 145 μL of 0.3 M glyoxylic acid and 145 μL of freshly prepared 0.9 M sodium cyanoborohydride followed by incubation at 37 °C with constant stirring at 1400 rpm. Samples were taken at 0, 5 and 10 min and later at 24 and 48 h followed by storage at −20 °C until analysis. Carboxymethylation was conducted in duplicate.

2.3. Food samples

Three infant foods containing 88% skim milk and 8.8% mixed cereals (wheat, corn, rice, oat, barley, rye, sorghum and millet) added with 0.9% of honey or 1.1% of fruits (banana, orange and apple) were supplied by Hero España SA (Murcia, Spain). Freshly produced samples were stored at 37 °C for 9 months. Samples were analyzed both before and after the storage.

Boiled eggs were produced in-house and the deterioration of their protein quality as a consequence of the processing was evaluated. Grade A hen eggs, medium size (53–63 g), laid by 7–8 days were obtained from a local farm and heated for 30 and 60 min. Boiling point was achieved after 14 min of heating. Shelling and separation of egg albumen from yolk were done carefully and manually. Samples were stored at −20 °C until analysis. Egg albumen was analyzed. Treatments were carried out in duplicate.

Various commercial foodstuffs like sterilized skimmed milk, condensed milk, whole powdered milk, evaporated milk, cocoa-milkshake, dry powdered crepes, wheat bread and two soy drinks were also studied.

2.4. GC analysis

Samples containing 22 mg of protein were mixed with 320 μg of diaminopimelic acid (DAP) as internal standard and digested with 4 mL of 6 M HCl in Pyrex glass bottles at 110 °C for 24 h. Prior to their incubation, samples were degassed using a stream of helium for 2 min. The hydrolyzates were cooled at room temperature and filtered through a Whatman 40 paper filter.

In order to prevent the formation of CML during acid hydrolysis, a reduction step with sodium borohydride prior to hydrolysis of samples has been proposed for converting FL into hexitol-lysine, which is not a precursor of CML [15–18]. With the aim to estimate the level of formation of CML from FL solutions

of 0.5, 1.6, 2.0 and 2.4 mg/mL were hydrolyzed and analyzed following the procedure proposed in this paper.

Pure standards and aliquots of hydrolyzed sample (500 μL) were evaporated to dryness under vacuum at 38–40 °C with a rotary evaporator. *N*,*N*-Dimethylformamide (165 μL) and triethylamine (15 μL) were added to the dry sample and stirred for 1 min. Afterwards MTBSTFA (100 μL) were added and the reaction mixture heated at 65–70 °C for 1 h forming the corresponding *N*-*tert*-butyldimethylsilyl (tBDMSi) derivatives. Derivatization reaction was stopped by cooling at room temperature and the derivatives were injected to the capillary column.

The chromatography was performed by using a Hewlett Packard 6890 (Waldbronn, Germany) gas chromatograph equipped with a flame ionization detector (FID). A CP-SIL 5CB commercially fused silica capillary column (25 m × 0.25 mm i.d., 0.25 μm film thickness) (Chrompack, Middelburg, The Netherlands) was used. The carrier gas (nitrogen) flow rate was 1.2 mL/min. The make-up gas was also nitrogen at a flow rate of 20 mL/min. Injector and detector temperatures were 280 and 300 °C, respectively. Injections were carried out in split-less mode and injection volume was 3 μL. The oven temperature was programmed as follows: 200 °C ramp to 250 °C at 10 °C/min, hold for 42 min at 250 °C, ramp to 300 °C at 50 °C/min, hold for 5 min at 300 °C. Data were acquired by means of HP ChemStation software (Hewlett Packard, Wilmington, DE, USA). All the analyses were carried out in duplicate and the data were expressed as mg/100 g of protein. Quantitative values were calculated by internal standard method.

GC–MS was performed with a Hewlett Packard 6890 GC coupled with Hewlett Packard 5973 quadrupole Mass Spectrometer (Palo Alto, CA, USA). A HP-5MS column (30 m × 0.25 mm i.d., 0.25 μm) (Hewlett Packard) was used. The carrier gas was helium. Injections were carried out in split-less mode and injection volume was 1 μL. Injector and detector temperatures were 280 and 300 °C, respectively. The oven temperature was programmed as follows: 1 min at 80 °C, ramp to 200 °C at 50 °C/min, ramp to 250 °C at 10 °C/min, hold for 42 min at 250 °C, ramp to 300 °C at 50 °C/min, hold for 5 min at 300 °C. Mass spectra were recorded in electron impact mode at 70 eV. Data were acquired and processed by G1701CA ChemStation (Hewlett Packard).

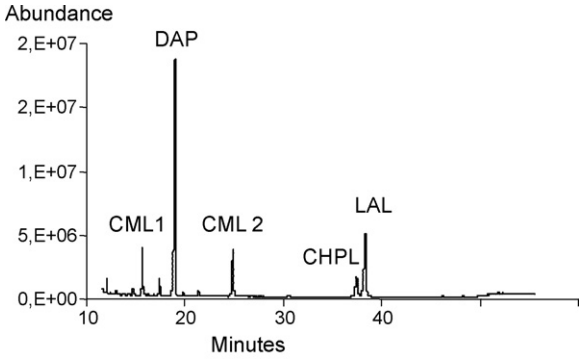
2.5. Statistical analysis

Statistical analysis (Statgraphics plus 3.1 statistical software package) of data was performed by one-way analysis of variance with a level of significance at 95%.

3. Results and discussion

3.1. Identification and quantification of lysine, LAL and CML

Retention time of lysine, LAL and CML peaks were determined using pure standards. CML presented two peaks in the chromatogram, representing the triple and quadruple silyl derivatives, so that both peaks were used for quantification.



CML1= Tri-*tert*-butyl-silyl-CML
CML2= Tetra-*tert*-butyl-silyl-CML
DAP= 2,6-diaminopimelic acid (internal standard)
CHPL= *N*-(1-(carboxy-3-hydroxypropyl)-L-lysine)
LAL=Lysinoalanine

Fig. 1. GC–MS chromatogram corresponding to *N*-*tert*-butyldimethylsilyl derivatives of CML and LAL.

Hasenkopf et al. [9] also obtained two CML derivatives using the same derivatization reagent. CML, lysine and LAL eluted within 45 min. Relative retention times were 0.43 for the lysine derivative, 0.77 and 1.34 for the triple and quadruple silyl CML derivatives, respectively, and 2.10 for the LAL derivative.

Mass spectral data of pure standards were also recorded. The GC–MS profile of a mixture of pure CML, LAL and DAP and the mass spectra of CML and LAL are shown in Figs. 1 and 2, respectively. A peak identified as CHPL (N^{ε}-1-(carboxy-3-hydroxypropyl)-L-lysine) was detected in pure CML.

Typical fragmentation patterns corresponding to tBDMSi derivatives of CML, CHPL and LAL are shown in Table 1. Data agreed with those previously reported by others [9,36]. As can be observed in Table 1, the most common MS fragments found in EI mass were as follows: $[M-15]^+$ $-CH_3$; $[M-57]^+$ $-C(CH_3)_3$; $[M-85]^+$ $-C(CH_3)_3-CO$; $[M-131]^+$ –OtBDMS; $[M-159]^+$ –COOtBDMS; $[M-131-159]^+$ –OtBDMS–COOtBDMS. CML showed two different derivatization products binding three

Table 1
Typical fragmentation pattern obtained for the *N*-*tert*-butyldimethylsilyl derivatives of CML, CHPL and LAL

	m/z	Fragment
CML1 (tri-*tert*-butyl-silyl-derivative)	256	M-131-159
	300	M-R-2H[a]
	489	M-57
	546	M
CML2 (tetra-*tert*-butyl-silyl-derivative)	300	M-R-2H[a]
	370	M-131-159
	501	M-159
	575	M-85
	603	M-57
CHPL	545	M-159
	647	M-57
LAL	387	M-131-159-15
	632	M-57

[a] Fragment corresponding to lysine binding two tBDMS groups with losing of $-C(CH_3)_3$.

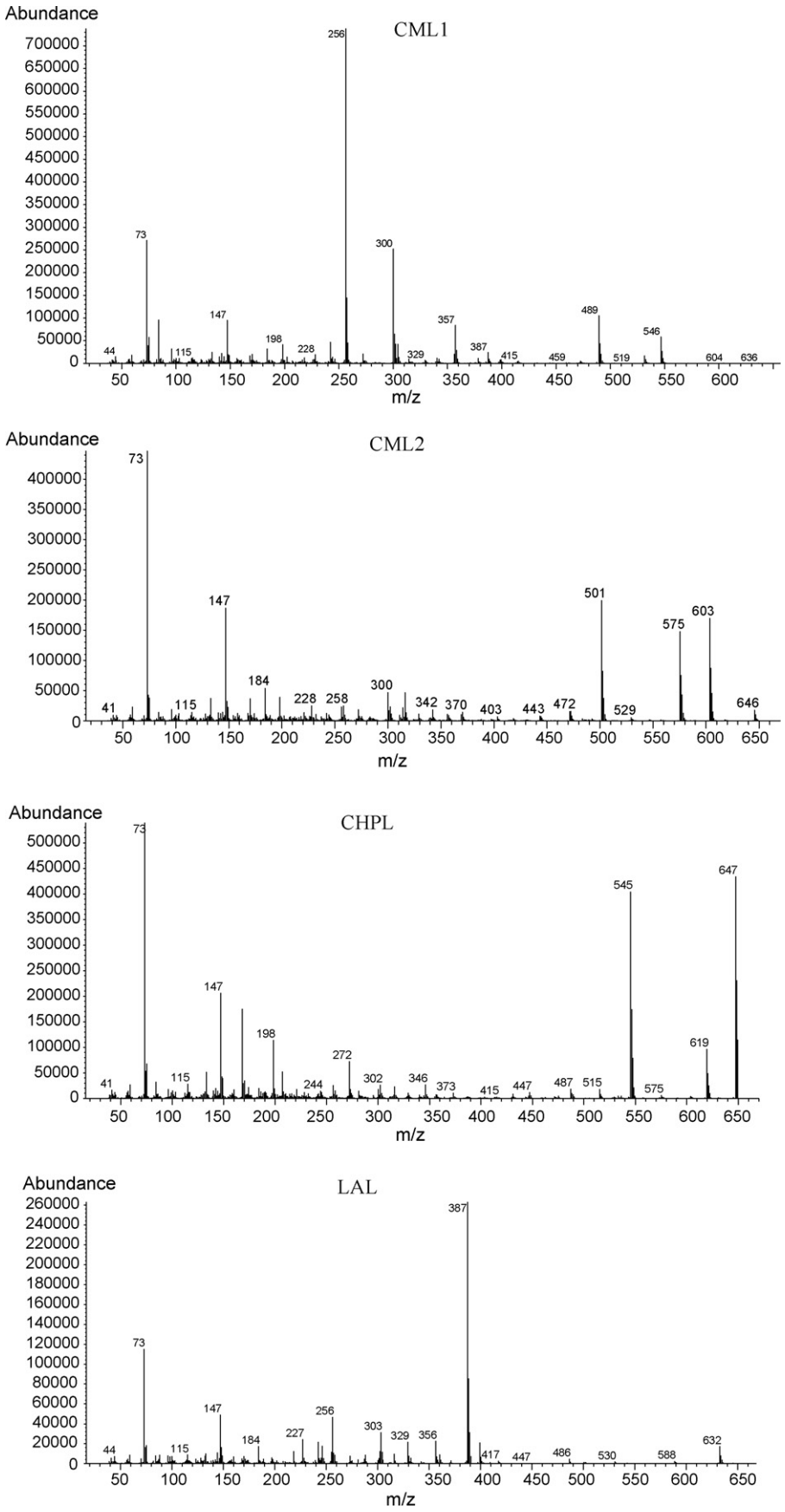


Fig. 2. Mass spectra of *N-tert*-butyldimethylsilyl derivatives of CML, CHPL and LAL.

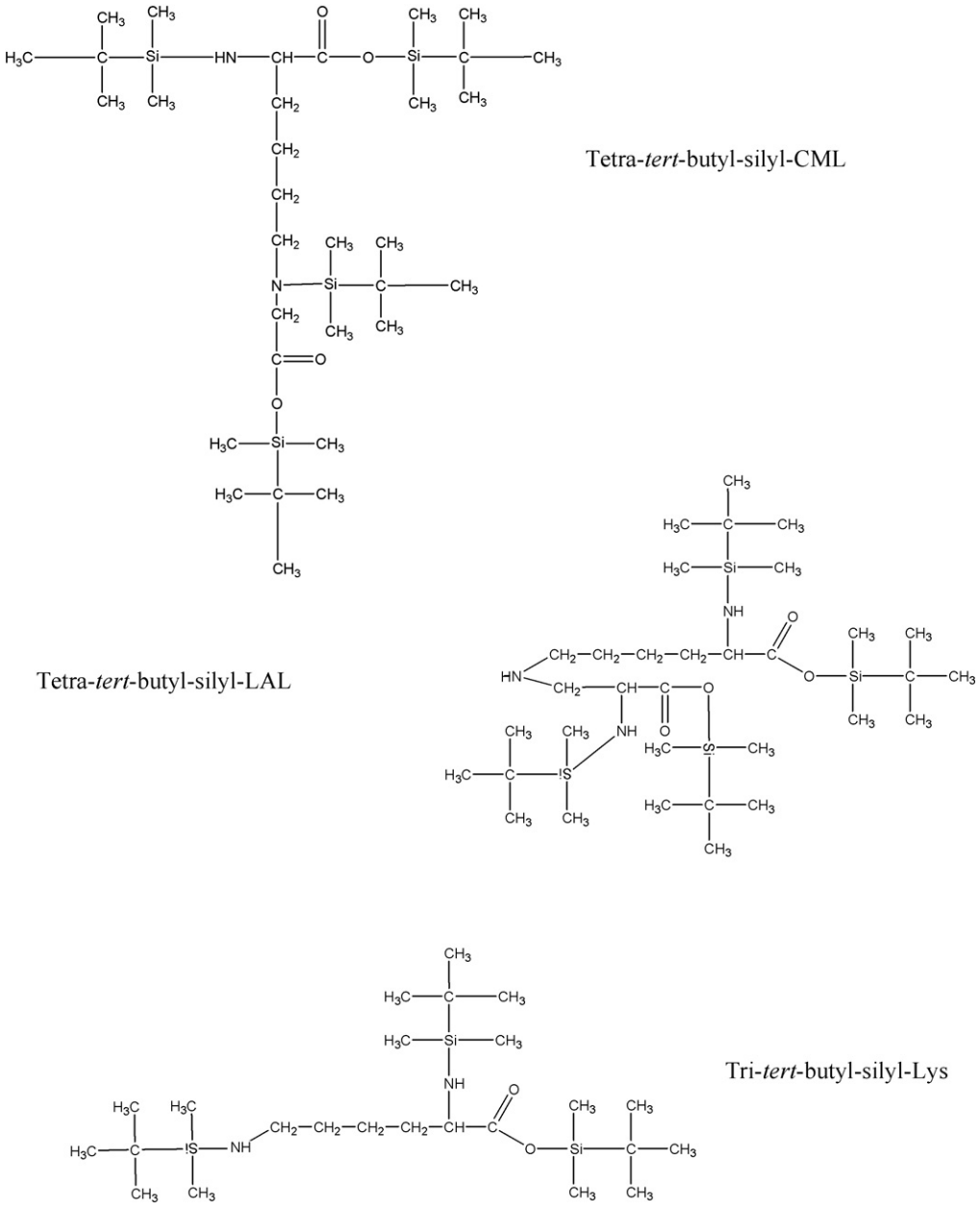


Fig. 3. Structures of tBDMSi CML, tBDMSi LAL and tBDMSi Lys.

and four silyl groups, respectively. CML [M^+] ion mass of CML with three silyl groups was detected while that corresponding to the derivatives with four silyl groups was not obtained. Similar results were observed for CHPL and LAL [M^+] ions that were not identified. Instead $[M-57]^+$ ion corresponding to LAL with four silyl groups and the loss of $C(CH_3)$ was detected. Ion with *m/z* 387 $[M-159-131-15]^+$ due to simultaneous loss of COO–tBDMSi, –O–tBDMSi and CH_3 was also observed.

Quantitative values were calculated from FID peak areas using the internal standard method. Response factors were determined by injecting standards of different concentration containing internal standard several times on different days (three times by duplicate). The range of linearity of FID response was checked by employing calibration curves of lysine, LAL and CML, respectively. Calibration curves of these three pure substances were separately constructed employing concentration of lysine equivalent to 350–4200 μM, LAL 3–81 μM, CML 16–172 μM and a known quantity of DPA (421 μM). Response factors values obtained for CML, LAL and lysine were 1.95 ± 0.18, 3.62 ± 0.33 and 1.22 ± 0.04, respectively. The differences between response factors values can be ascribed to the differences in the structures of tBDMSi Lys, tBDMSi LAL and tBDMSi CML (Fig. 3).

3.2. Validation of the method

CML detection and quantification limits (LOD and LOQ) of 3.35 mg/100 g protein (2.63 mg/L in derivatized)

and 11.16 mg/100 g protein (8.77 mg/L in derivatized) were obtained. LOD was calculated as three times the S/N (signal-to-noise) ratio near to the retention time of CML peaks while LOQ was considered ten times this ratio. A linear relationship ($y = 0.557x - 0.072$, $r = 0.981$) between the response measured as peak area and concentration of CML over the studied range was observed. One-way analysis of variance of the response factor of CML demonstrated significant homogeneity of the variance between the samples over the range of concentration studied since a F-value of 1.010 with $P = 0.412$ ($P > 0.05$) was obtained. The precision of the method determined for the whole procedure including sample work-up ranged from 1.6 to 10.0% that can be considered adequate for quantitative analysis of CML according to the bibliography [17,26,27,47–49]. Accuracy was estimated by carrying out recovery assays. Known amounts of CML standard (27 and 88 μg) were added to hydrolyzates of UHT milk and soy liquid beverage. CML was measured in both unspiked and spiked samples and recoveries were calculated. The average recovery values were 84.45 ± 1.74 and 107.76 ± 10.24% for UHT milk and soy beverage, respectively. Data agreed with those described by others [47–49].

LAL LOD and LOQ were 5.0 mg/100 g protein (3.93 mg/L in derivatized) and 15.2 mg/100 g protein (11.64 mg/L in derivatized), respectively. Recovery of LAL was 91.1%. Linearity for LAL was verified in the previously mentioned range ($y = 3.6624x + 0.0155$; $r = 0.986$). Precision values obtained were similar to those obtained by Montilla et al. [36].

Lysine LOD and LOQ of 0.43 mg/100 g protein (0.34 mg/L in derivatized) and 1.43 mg/100 g protein (1.12 mg/L in derivatized) were obtained. Linearity for lysine was verified in the studied range ($y = 1.221x + 1^{-14}$; $r = 0.999$). The precision ranged from 0.9 to 13.4%.

3.3. Formation of CML from pure Amadori compound

CML formation was not detected in hydrolyzates of FL 0.5 mg/mL. Hydrolyzates from FL solutions of 1.6, 2.0 and 2.4 mg/mL yielded 0.49% of CML. Levels of CML found by us were lower than those described by other authors (3.0–5.5%) employing HCl 7.8N for acid digestion of FL [15,50]. Food samples containing FL concentrations lower than 9090.9 mg/100 g protein do not need a reduction step as part of the sample preparation for CML analysis because formation of CML would not be detected under the assayed conditions. FL values determined in the samples under study (data no shown) were lower than 9090.9 mg/100 g protein. No formation of CML in model system was expected; thus, carboxymethylated proteins were obtained employing glyoxylic acid in the presence of sodium cyanoborohydride. Therefore, reduction was removed from our protocol making CML analysis simpler.

Charissou et al. [14] determined CML in meat and milk products under hydrolysis conditions similar to those used in this paper and also omitted reduction step because it appeared not necessary since no overestimation was observed. These authors have not detected CML in samples with high concentration of FL (furosine, a product from acid hydrolysis of FL, ranged from 100 to 200 mg/100 g protein).

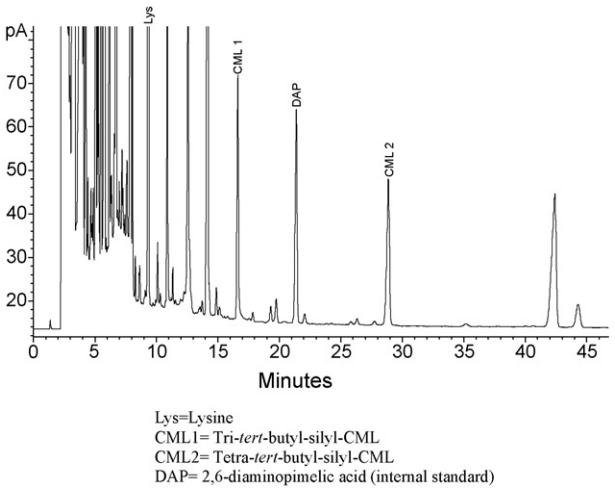


Fig. 4. GC-FID chromatogram corresponding to carboxymethylated BSA by incubation with glyoxylic acid and sodium cyanoborohydride for 24 h.

3.4. Model systems

Model systems consisting in food proteins (BSA, soy, casein and gluten) incubated with glyoxylic acid and sodium cyanoborohydride were studied to know the influence of protein source on the rate of carboxymethylation. Proteins were incubated for different times (0, 5 and 10 min, 24 and 48 h) at 37 °C. GC-FID chromatogram corresponding to carboxymethylated BSA (incubation 24 h with glyoxylic acid and sodium cyanoborohydride) is shown in Fig. 4. As shown in Fig. 5, the CML formation strongly depended on the nature of the involved protein. The BSA was the protein in which higher amounts of CML were formed, followed by soy, casein and gluten. Most part of CML is formed already at time point 0 (except in gluten) that can be ascribed to the high reactivity of the studied proteins with glyoxylic acid and sodium cyanoborohydride. As expected proteins containing higher number of lysine residues showed higher CML values. Gluten is the protein with major number of

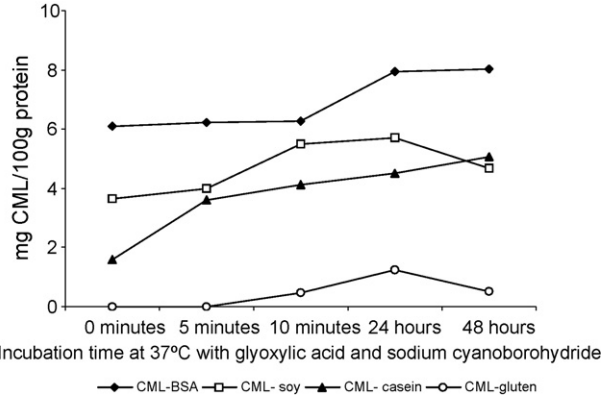


Fig. 5. CML formation in model systems protein–glyoxylic acid–sodium cyanoborohydride.

disulphur bonds, hindering the approach to lysine residues [51]. Therefore, the reaction mixture containing this protein showed the lowest formation of CML.

Rate of CML formation was faster for soy protein compared with BSA and gluten (Fig. 5). As can be observed, kinetic of carboxymethylation depends on the number of available lysine to be carboxymethylated and the structural complexity of proteins. Maximum values of CML were detected in soy model systems at 10 min of incubation; the state stage of CML formation was achieved at 24 h in BSA and gluten model systems while it took longer than 48 h in casein model system. Concentrations of carboxymethylated soy protein and gluten decreased after 48 h of incubation that can be ascribed to a degradation of the compound by giving rise to the final stages of MR compounds.

Carboxymethylation levels obtained for casein and BSA agreed with those described by others under similar conditions [18,46]. Yields of formation of carboxymethylated BSA from 34 to 52% have been previously reported. To the best of our knowledge, no data related to carboxymethylation of gluten and soy protein have been published.

In addition, CHPL was detected in carboxymethylated BSA, soy, casein and gluten, as well as in CML standard. This compound was described for the first time as a specific ascorbylated product by Hasenkopf et al. [9]. These authors did not observe CHPL formation by glycation. In this paper, proteins have been carboxymethylated with sodium cyanoborohydride and glyoxylic acid which is an oxidation product of glucose that might be also employed as precursor of the commercial pure standard CML. Data suggested that CHPL might be considered as by-product of CML synthesis.

3.5. Foods

Protein quality of foods was estimated by determining lysine, CML and LAL.

3.5.1. Lysine

Table 2 shows data on lysine found in the studied foods. Lysine content of infant foods decreased during storage at 37 °C for 9 months from 6760–7620 mg/100 g protein [52] to 4711–5663 mg/100 g protein because of the blockage of lysine mainly due to Maillard reaction.

Table 2
Lysine and CML contents expressed as mg/100 g protein

	Lysine	CML
Infant foods, 9 months, 37 °C	4711 ± 399	69 ± 7
Infant foods with honey (0.9%), 9 months, 37 °C	5511 ± 119	118 ± 12
Infant foods with fruits (1.1%), 9 months, 37 °C	5663 ± 698	102 ± 3
Boiled eggs, 30 min	10,504 ± 1038	227 ± 18
Boiled eggs, 60 min	8694 ± 77	234 ± 12
Soy drink 1	8532 ± 1144	62 ± 6
Soy drink 2	7585 ± 548	132 ± 2
Dry powdered crepes	1119 ± 33	440 ± 10

Values expressed as mean ± standard deviation (two determinations of the same sample).

Lysine content in eggs decreased as a function of the heating treatment time. Therefore, lysine in boiled eggs for 60 min (8694 mg/100 g protein) was minor than in boiled eggs for 30 min (10,504 mg/100 g protein) due to the development of Maillard and crosslinking reactions. Similar levels of lysine values were detected in the studied soy drinks that were also similar to those detected for boiled eggs for 60 min.

Among the samples under study, dry powdered crepes and infants foods were those exhibiting lowest lysine values. Levels of this amino acid depend on its richness in food proteins. Moreover, the content of this essential amino acid is very sensitive to manufacturing and storage conditions as it has been observed for boiled eggs and stored infant foods. In general, lysine values found here agreed with those previously reported [53]. Additionally, information related to the pathway of lysine degradation was extracted by CML and LAL analyses of foods.

3.5.2. Maillard reaction products: CML

CML was not detected in just manufactured milk-cereal based infant foods; however, levels of CML from 69 to 118 mg/100 g protein were measured in milk-cereal based infant foods stored at 37 °C for 9 months (Table 2). Low levels of CML (1.1 mg/100 g protein) have been found in mixed cereals for infants [15]. Birlouez-Aragon et al. [25], Fenaille et al. [19] and Charissou et al. [14] found that CML in infant formulas ranged from 0.1 to 6.3 mg/100 g. In agreement with data reported by others [25], greater CML amount was formed in infant food added with honey compared with only cereal-milk based infant foods and those added with fruits. During infant food manufacturing process, the early stages of the Maillard reaction are mainly responsible for the protein quality deterioration. AGEs like CML required longer storage period and/or higher temperature for their generation. Furosine, a product from acid hydrolysis of FL, has been described as adequate and early quality indicator of the protein quality deterioration by means of Maillard reaction during manufacturing of infant cereals [54].

We did not found CML in sterilized skimmed milk, condensed milk, powdered milk, evaporated milk, bread and cocoa-milkshake. Drusch et al. [17] did not detect CML in pasteurized or UHT milk products that was explained on the basis of intensity of the thermal processes commonly employed for manufacturing of these foods. Quantifiable amounts of CML have been detected in sterilized milk (34.3 mg/100 g protein, $n = 1$), powdered milk (7.1 mg/100 g protein, $n = 1$), evaporated milk (49.9 mg/100 g) and cocoa milk (15.7 mg/100 g) [17]. On the other hand, Büser et al. [12], Hewedy et al. [16] and Fenaille et al. [19] detected CML in UHT milk (1.1–25.9 mg/100 g), Hartkopf et al. [15] in condensed milk (39.0 mg/100 g) and Charissou et al. [14] in cereal products, like cookies (0.5–3.5 mg/100 g), toasted bread (0–1.3 mg/100 g) and corn flakes (0.6–0.8 mg/100 g). However, to date ranges of CML in foods have not been established. This fact may be explained on the basis of the complexity

of the pathway of formation of this compound and the lack of simple methods of analysis for its determination as a routine quality parameter.

3.5.3. Crosslinking products: LAL

LAL was only detected in eggs boiled for 30 and 60 min. Values obtained were similar to those obtained in a previous work (21 and 68 mg/100 g protein for heating 30 and 60 min, respectively) [36] and in the range described by other authors [55,56].

LAL has not been previously detected in milk-cereal based infant foods added with fruits and vitamins [43] or powdered milk [56]. However, this crosslinked amino acid has been found in evaporated milk [56], UHT milk [19,42,56], sterilized milk [42], growing milks [43] and infant formulas [19,42,43].

Formation of CML and LAL is influenced by many facts including food composition, manufacturing and storage conditions. In foods possessing high content of protein and also reducing sugars which are submitted to thermal processes during manufacturing, like milk, furosine has been described as a good quality indicator. Also CML may be employed for drawing a map of applied manufacturing process. Crosslinked amino acids are considered as good quality index of proteins employed as food ingredients and of frauds like adulteration of fresh cheeses with caseinates, etc. [41]. However, few and contradictory data have been published about LAL and CML in foods. There is a lack of information about the level of these compounds in food and dietary intake of them as a consequence of a lack of methods for their routine analysis. Because both reactions take place simultaneously during food manufacturing, we considered the method here proposed may help to obtain a preliminary overview of protein damage and the pathway responsible for this.

4. Conclusions

Although the method described here has a slightly less sensitive result than those previously proposed by others, its application allows an estimation of the main causes of protein damage during processing and storage of foods by simultaneous analysis of three protein quality indicators, lysine, CML and LAL. To improve the detection sensitivity data may be collected by GC–MS in the SIM mode recording *m/z* of the derivatives for a narrow range of time close to their retention time as proposed by Hasenkopf et al. [9]. However, in our opinion, GC-FID method has adequate results for food quality control.

The rate of both Maillard and crosslinking reactions strongly depended on the nature of the involved protein. CML were detected in stored milk cereal infant foods, soy drinks, boiled eggs and dry powdered crepes, but not in freshly produced infant foods, sterilized skim milk, condensed milk, powdered milk, evaporated milk, cocoa-milkshake and bread. LAL was only detected in boiled eggs. Maillard reaction products seem to be the better chemical indicators of protein quality than LAL in the studied foods. However, the simultaneous analysis of them provides useful and complete information related to the protein damage suffered during food manufacturing that is supported by eggs data.

Reduction prior to hydrolysis can be avoided when dealing with carboxymethylation model systems and food products containing FL quantities lower than 9090.9 mg FL/100 g protein. In addition, CPHL seems to be a by-product of CML synthesis.

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