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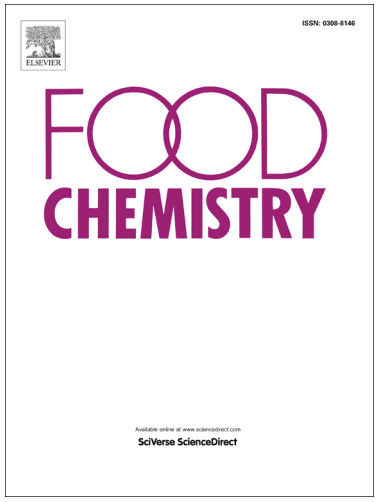

Analytical methods

Quantification of Nε-(2-Furoylmethyl)-L-lysine (furosine), Nε-(Carboxymethyl)-L-lysine (CML), Nε-(Carboxyethyl)-L-lysine (CEL) and Total Lysine through Stable Isotope Dilution Assay and Tandem Mass Spectrometry

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Abstract

The control of Maillard reaction (MR) is a key point to ensure processed foods quality. Due to the presence of a primary amino group on its side chain, lysine is particularly prone to chemical modifications with the formation of Amadori products (AP), Nε-(Carboxymethyl)-L-lysine (CML), Nε-(Carboxyethyl)-L-lysine (CEL). A new analytical strategy was proposed which allowed to simultaneously quantify lysine, CML, CEL and the Nε-(2-Furoylmethyl)-L-lysine (furosine), the indirect marker of AP. The procedure is based on stable isotope dilution assay followed by, liquid chromatography tandem mass spectrometry. It showed high sensitivity and good reproducibility and repeatability in different foods. The limit of detection and the RSD% were lower than 5 ppb and below 8%, respectively. Results obtained with the new procedure not only improved the knowledge about the reliability of thermal treatment markers, but also defined new insights in the relationship between Maillard reaction products and their precursors.

Keywords: Maillard reaction, LC-MS/MS, CML, CEL, lysine, furosine

Abbreviations: Maillard reaction (MR), Maillard reaction end products (MRPs) Nε-(2-Furoylmethyl)-L-lysine (furosine), Nε-(Carboxymethyl)-L-lysine (CML), Nε-(Carboxyethyl)-L-lysine (CEL),

List of compounds: Nε-(2-Furoylmethyl)-L-lysine (furosine, PubChem CID: no items) , Nε-(Carboxymethyl)-L-lysine (CML, PubChem CID: 123800), Nε-(Carboxyethyl)-L-lysine (CEL, PubChem CID: no items), lysine (PubChem CID: 5962).

1. Introduction

The final quality of many industrial food products depends on food formulation and processing design resulting in the formation of a huge variety of molecules as a consequence of thermal treatments and chemical changes (van Boekel, Fogliano, Pellegrini, Stanton, Scholz, Lalljie, et al., 2010). Along with lipid oxidation, the Maillard reaction (MR) occupies a prominent place in the final quality of food being responsible not only for the desired color and aroma compounds but also for the formation of potentially toxic Maillard reaction end products (MRPs). The reaction between reducing sugars and amino groups is the first step in the Maillard cascade: the formation of the stable 1-amino-1-deoxy-2-ketose the Amadori product (AP) and 2-amino-2-deoxyaldose Heyns products represents the starting point of the many chemical pathways of this reaction (Hodge, 1953). The presence of an amino group on the side chain of lysine makes this amino acid particularly sensitive to the carbonyls attachments. The modifications arising from the lysine blockage resulted in the formation of a bewildering array of molecules: Nε-(1-Deoxy-D-fructos-1-yl)-L-lysine (fructosyl-lysine), Nε-(Carboxymethyl)-L-lysine (CML), Nε-(Carboxyethyl)-L-lysine (CEL), pentosidine, pyrraline, lysino-alanine, 5-hydroxymethylfurfural (HMF), α-dicarbonyls and aroma key odorants (Yaylayan & Huyghuesdespointes, 1994). Fructosyl-lysine, CML and CEL represent the most widely studied MRPs, and they are often used as biomarker of food quality (Erbersdobler & Somoza, 2007; Nguyen, van der Fels-Klerx, & van Boekel, 2013). As highlighted in **Figure 1**, the acid hydrolysis adopted to release free amino acids from the polypeptide chain promote the conversion of the 1-deoxy-fructosyl-L-Lysine (AP) through a cyclized Schiff base, into the of Nε-(2-furoylmethyl)-L-lysine (furosine) which is a compound that can be quantified after protein hydrolysis and it has been widely used as marker of thermal treatment particularly in the dairy products (Krause, Knoll, & Henle, 2003).

The formation of CML and CEL from the oxidation of ARP and HRP has been well characterized (Nguyen, van der Fels-Klerx, & van Boekel, 2013). Carbohydrate fragmentation allows the

formation of glyoxal and methylglyoxal that readily react with lysine residues yielding the glycoxidation products CML and CEL, respectively (Ahmed, Thorpe, & Baynes, 1986). Moreover, CML and CEL can be formed via the Namiki-pathway through three subsequent steps: Schiff base production, glycolaldehyde alkylimine synthesis, oxidation and formation of glyoxal or methylglyoxal which react with lysine to yield CML and CEL. Another route of CML and CEL formation is linked to lipid peroxidation as glyoxal and methylglyoxal can derive from polyunsatured fatty acids (Hidalgo & Zamora, 2005). Moreover, the two markers can be also formed from fragmentation and subsequent glycation of ascorbic acid and dehydroascorbic acid (Leclere, Birlouez-Aragon, & Meli, 2002).

From the analytical point of view the identification of these markers of heat treatment can be approached in several ways (Tessier & Birlouez-Aragon, 2012). Furosine is used as indirect marker of quality control of moderately heat-treated dairy samples. The golden standards for furosine detection are ion-exchange chromatography, reverse phase high performance liquid chromatography (RP-HPLC) with UV detection, (Henle, Zehetner, & Klostermeyer, 1995) capillary electrophoresis and ion-pairing HPLC by using sodium-heptanosulphonate (Vallejo-Cordoba, Mazorra-Manzano, & Gonzalez-Cordova, 2004). These procedures had several drawbacks mainly related to the modifications occurring during sample preparation: the acidic hydrolysis does not allow the differentiation between AP and glycosyl-amine; overestimation or underestimation linked to the acidic hydrolysis might occur due to the formation of further intermediates and end-products (Pischetsrieder & Henle, 2012).

As for furosine, CML and CEL analysis implies acidic hydrolysis to hydrolyze peptide bonds followed by their quantification that could be performed by different instrumental methods (Nguyen, van der Fels-Klerx, & van Boekel, 2013). In some papers a pre-column derivatization with o-phthalaldehyde was used to allow the detection by florescence detector (Hartkopf, Pahlke, Ludemann, & Erbersdobler, 1994), while a widely used approach for CML and CEL detection is

gas or liquid chromatography coupled with tandem mass spectrometry. Specifically, multiple reaction monitoring (MRM) mode improves the sensitivity, reduces the coefficient of variability and ruled out the problems of derivatization (Delatour, Hegele, Parisod, Richoz, Maurer, Steven, et al., 2009). A double derivatization is required for GC separation and this bottleneck highlights the advantages of LC-MS/MS detection: no derivatization, highest sensitivity and good reproducibility (Charissou, Ait-Ameur, & Birlouez-Aragon, 2007; Fenaille, Parisod, Visani, Populaire, Tabet, & Guy, 2006). Moreover CML, CEL and lysine detection is possible also by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) that allows relative quantification of protein lactosylation and it is a reliable method to monitor the early Maillard reaction as well as MRPs during milk processing (Meltretter, Becker, & Pischetsrieder, 2008).

The aim of the present paper, was to further improve the existing methodologies for the detection of lysine and MRPs. A new method was designed which included direct hydrolysis along with stable isotope dilution assay coupled with solid phase extraction and ion pairing liquid chromatography tandem mass spectrometry (LC-MS/MS). The developed procedure allowed the simultaneous detection of total lysine, furosine, CML and CEL. The method was tested on several foods: milk, infant formulas, cookies, bread slices. The robustness after several injections and the reliability of the results obtained were evaluated in soybean-based feed products obtained under severe thermal treatment conditions. Data demonstrated satisfactory analytical performances on all tested samples and results were perfectly in line with those previously obtained.

2. Material and methods

2.1 Chemicals and reagents

Acetonitrile, methanol and water for solid phase extraction (SPE) and LC-MS/MS determination were obtained from Merck (Darmstadt, Germany). The ion pairing agent perfluoropentanoic acid, trichloroacetic acid, hydrochloric acid (37%) and the analytical standards L-lysine hydrochloride

and [4,4,5,5-d_4]-L-lysine hydrochloride (d_4-Lys) were purchased from Sigma-Aldrich (St. Louis, MO). Analytical standards Nε-(2-Furoylmethyl)-L-lysine (furosine), Nε-(Carboxymethyl)-L-lysine (CML) and its respective deuterated standard Nε-(Carboxy[$^2H_2$]methyl)-L-Lysine (d_2-CML) were obtained from Polypeptide laboratories (Strasbourg, France), Nε-(Carboxyethyl)-L-lysine and its internal standard Nε-(Carboxy[$^2H_4$]ethyl)-L-lysine (d_4-CEL) were purchased from TRC-Chemicals (North York, Canada).

2.2 Foods samples

Powdered infant formula and milk samples were purchased in a local market, biscuits samples and bread slices were prepared according previous papers published by our group (Fiore, Troise, Mogol, Roullier, Gourdon, Jian, et al., 2012; Vitaglione, Lumaga, Stanzione, Scalfi, & Fogliano, 2009). UHT milk was prepared according to the procedure previously described (Troise, Fiore, Colantuono, Kokkinidou, Peterson, & Fogliano, 2014). Raw milk (protein, 3.5%; fat, 1%) was purchased in a local market.

2.2.1 Soybean samples

One batch of quartered raw soybeans was purchased from Rieder Asamhof GmbH & Co. KG (Kissing, Germany).The raw soybeans were further processed at the hydrothermal cooking plant of Amandus Kahl GmbH & Co. KG (Reinbeck, Germany). First, the beans were short-term conditioned to reach a temperature of 80 °C after 45 seconds. Afterwards, the beans entered a hydrothermic belt cooker at 72 °C and left inside for 3 min at a temperature of 70 °C. Then they were expanded at 117 °C using an annular gap expander (Typ OEE 8, Amandus Kahl GmbH & Co. KG, Reinbeck, Germany). The expanded soybeans were collected in a drying wagon for 10 min. Then they were dried with air at 65 °C for 10 min and cooled for another 10 min to reach a final moisture content of 12%. Afterwards, the expanded soybeans were autoclaved for 0, 5, 10, 15, 20,

25, 30, 35, 40, 45, 50 and 60 min at 110 °C and 1470 mbar using a fully controlled autoclave (Typ HST 6x9x12, Zirbus Technology GmbH, Bad Grund, Germany).

2.3 Samples preparation

Lysine and its derivatives Nε-(2-Furoylmethyl)-L-lysine (furosine), Nε-(Carboxymethyl)-L-lysine (CML), Nε-(Carboxyethyl)-L-lysine (CEL) were analyzed considering previous papers (Delatour, et al., 2009; Fenaille, Parisod, Visani, Populaire, Tabet, & Guy, 2006; Troise, Dathan, Fiore, Roviello, Di Fiore, Caira, et al., 2014) and introducing several modifications. Briefly, 100 mg of each sample was accurately weighed in a screw capped flask with PTFE septa and 4 mL of hydrochloric acid (6 N) was added. The mixture was saturated by nitrogen (15 min at 2 bar) and hydrolyzed in an air forced circulating oven (Memmert, Schwabach, Germany) for 20 h at 110° C. The mixture was filtrated by polyvinylidene fluoride filters (PVDF, 0.22 Millipore, Billerica, MA) and 400 µl was dried under nitrogen flow in order to prevent the oxidation of the constituents. The samples were reconstituted in 370 µl of water and 10 µL of each internal standard d_4-Lys, d_2-CML and d_4-CEL was added in order to obtain a final concentration of 200 ng/mg of samples for both standards. Samples were loaded onto equilibrated Oasis HLB 1 cc cartridges (Waters, Wexford, Ireland) and eluted according to the method previously described, then 5 µl was injected onto the LC/MS/MS system.

2.4 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Separation of furosine, CML, CEL, lysine and their respective internal standards was achieved on a reversed – phase core shell HPLC column (Kinetex C18 2.6 µm, 2.1 mm x 100 mm, Phenomenex, Torrance) using the following mobile phases: A, 5 mM perfluoropentanoic acid and B, acetonitrile 5 mM perfluoropentanoic acid. The compounds were eluted at 200 µL/min through the following gradient of solvent B (t in [min]/[%B]): (0/10), (2/10), (5/70), (7/70), (9/90), (10/90), (12/10), (15/10). Positive electrospray ionization was used for detection and the source parameters were

selected as follows: spray voltage: 5.0 kV; capillary temperature: 350 °C, dwell time 100 ms, cad gas and curtain gas were set to 45 and 5 (arbitrary units). The chromatographic profile was recorded in MRM mode and the characteristic transitions were monitored in order to improve selectivity using an API 3000 triple quadrupole (ABSciex, Carlsbad, CA). All relevant parameters are summarized in **Table 1**.

2.5 Analytical performances

CML, CEL, furosine and total lysine were quantified using a linear calibration curve built with specific solutions of CML spiked with d_2-CML, lysine and furosine spiked with d_4-lysine and CEL spiked with d_4-CEL (final concentration of internal standards: 200 ng/ml) dissolved in water. The limit of detection (LOD) and the limit of quantitation (LOQ) were monitored according to the signal to noise ratio (Armbruster, Tillman, & Hubbs, 1994). The coefficients of determination r^2 for the 4 analytes were tested plotting the ratio between the pure compounds and their respective internal and the concentration of the pure compounds in the linearity range 5-1000 ng/mL. The internal standard ratio was used for the quantification and the relative standard deviation of intraday and interday assay was monitored three times each day and six times in different days. The recovery test was monitored according to the concentration of the internal standards used and to the ratio between labeled compounds and native compounds.

2.6 Statistical analysis

All of the analyses were performed in quadruplicate and the results expressed as mg/100 g of protein. Statistical calculations were performed using Matlab R2009b (Natick, MA) while for mass spectrometry data, Analyst version 1.4.2 (Applied Biosystems, Carlsbad, CA) was used.

3. Results and discussion

3.1 Liquid chromatography set up

Under the above described chromatographic conditions, typical retention time of CML and d_2-CML was 7.11 min, for d_4-Lys and Lys it was 7.23 min, for furosine it was 7.91 min, while for CEL and d_4-CEL it was 7.36 min (**Figure 2**). Previous papers highlighted the problems due to the poor retention of amino acids and their derived molecules on silica bonded and C-18 column (Frolov & Hoffmann, 2008). Preliminary trials performed using C-18 column without the ion pairing agent confirmed this feature: the retention was poor and the analytes co-eluted with the impurities on the front of the chromatographic run with the consequent partial suppression of the signal associated to the markers. Inadequate separation of the analytes was obtained also using polar end-capped column; however a significant improvement was obtained using with this column perfluoropentanoic acid as ion pairing agent. In these experimental conditions, the retention time followed a typical reversed phase profile according to the polarity and to the steric hindrance of each molecule, as previously observed by other papers published earlier (Fenaille, Parisod, Visani, Populaire, Tabet, & Guy, 2006; Troise, Fiore, Roviello, Monti, & Fogliano, 2014). The presence of the ion pairing agent charged the core shell residues increasing the retention and promoting the selectivity of the positively charged CML, CEL, furosine, lysine and their respective internal standards. The presence of a core shell phase increased of the resolution which directly reflects the good performances of the reported method, the shape of the peak was maintained over each batch and the retention time shift was lower than 0.5 min, highlighting the robustness of the analytical performances.

3.2 Mass spectrometry set up

Mass spectrometry conditions were optimized by infusing singularly the seven standards directly in the ion source. Collision energy, declustering potential, tube lens voltage along with spray voltage and interface temperature were monitored in order to favor the formation of the typical fragmentation pattern (Delatour, et al., 2009). The lysine derived compounds underwent the formation of the fragment ion at 130 *m/z* which corresponds to the pipecolic acid generated by the

subsequent cyclization of the side chain of lysine and the loss of ε- amino group, similarly the mass shift for deuterated standards *d4*-CEL and *d4*-Lys was +4 Da as consequence of the fragmentation occurred on the side chain of lysine (**Figure S1** in supplementary material section) (Yalcin & Harrison, 1996). The MRM revealed the loss of formic acid giving the typical fragment at *m/z* 84; the mass shift for the deuterated molecules was +4. The seven standards were also infused inside the chromatographic flow in order to evaluate the interferences due to the ion pairing agent or to the solvent and the results revealed that no enhancement or suppression effect can be ascribed to the parameters monitored.

3.3 Analytical performances

The analytical performances of the method were tested against reproducibility, repeatability, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, carry-over and coefficient of correlation (r^2). Before and after each batch, three solutions of acetonitrile and water (90:10; 50:50 and 10:90) were injected in order to verify the absence of any contaminants with the same signal and the same retention time of the analyzed molecules. The limit of detection and the limit of quantitation were determined according to the procedure previously described. The concentration 0.1 ppb resulted in no signal, while the LOD was 0.5 ppb for CML and lysine while for CEL and furosine it was 1 and 3 ppb, respectively. The slight differences among CML, CEL and furosine can be related to the different stability in the injection conditions. By injecting these concentrations the signal to noise ratio was always higher than 3. The LOQ were 5 ppb for CML, CEL and Lysine while for furosine it was 9 ppb, as highlighted in **Table 2**. These values were perfectly in line with those previously described for CML, CEL and lysine quantification by MS/MS (Delatour, et al., 2009; Tareke, Forslund, Lindh, Fahlgren, & Ostman, 2013) while for furosine the performance of LOD and LOQ were below the values previously reported in milk (Bignardi, Cavazza, & Corradini, 2012). According to the LOD and LOQ, linearity was achieved in the range 5-1000 ppb for CML, CEL and lysine, while for furosine the linearity range was between 9 and 1000 ppb. The carryover

effect was tested injecting after each point of the calibration curves a solution consisting in acetonitrile and water (50:50, v/v) and verifying the absence of the target compounds. The linearity of the calibration curves was evaluated three times in the same day (intraday assay for the reproducibility) and three times for three subsequent days (interday assay for the repeatability) using the ratio between the target compounds and their respective internal standard. The RSD (%) among the three curves was always lower than 8%, demonstrating that external factors had marginal impact on the performance of the method. Each point of the calibration curves was monitored using two specific transitions: the most intensive fragment was used as quantifier, the lowest as qualifier. For CML, CEL, furosine and lysine, the respective transitions of *m/z* 205−84.1, *m/z* 219.1−84.1, *m/z* 255.1−130.2, and *m/z* 147.2−130.2 were used as quantifier, whereas *m/z* 205−130.2, *m/z* 219.1−84.1, *m/z* 255.1−84, and *m/z* 147.2−84.1 were used as qualifier. CML was quantified using *d2*-CML as internal standard (*m/z* 207−144.1 and 207−84 for quantification and confirmation, respectively), CEL was quantified using *d4*-CEL (*m/z* 223−134.1 and 223 − 84 for quantification and confirmation, respectively) whereas for furosine and lysine, *d4*-lysine was used (*m/z* 151.2−134.1 and *m/z* 151.1− 88 for quantification and confirmation, respectively). The use of *d4*-lysine as internal standard for the quantification and recovery of furosine was optimized by monitoring the relative intensity of furosine standard towards *d4*-CEL, *d2*-CML and *d4*-lysine. A mixture of the four standards (10 ppm) was directly infused in the ion source. Results revealed that the intensity of the signal at *m/z* 151.2 and *m/z* 255 were similar and both were 15% higher than the signal of *d2*-CML and *d4*-CEL.

The response of the method in food was tested during each batch evaluating the ratio between the target compounds and the internal standard, these procedures confirmed and deepened the aspects linked to the recovery assay: in each sample the ratio between the area of the analyte and the area of the deuterated compounds was compared towards the calibration curve in order to obtain the final concentration of the analytes in the matrix. The intensity of the internal standard in the samples and

220 in the standard was compared and the RSD (%) between the spiked samples and the spiked
221 standards was always lower than 10%. The recovery test was monitored in all the food matrix
222 according to the intensity of the internal standard, the results were 91.1 ± 8.4, 84.2 ± 7.4, 88.0 ± 6.9
223 for *d2*-CML, *d4*-CEL and *d4*-Lysine.

224 *3.4 CML, CEL, furosine and total Lysine in food*

225 Powdered samples were freeze dried prior analysis in order to remove the interferences due to the
226 humidity. The extraction procedure of MRPs is characterized by three key steps: the reduction with
227 sodium borohydride, the hydrolysis with hydrochloric acid and the stable isotope dilution assay
228 prior ion pairing solid phase extraction. According to the nature of protein and to their concentration
229 each of the above listed can influence the yield and the efficiency of the extraction. The reduction
230 with sodium borohydride promotes the conversion of free fructosyl-lysine into hexitol-lysine in
231 order to avoid the overestimation of CML, CEL (Niquet-Leridon & Tessier, 2011). Moreover, the
232 use of sodium borohydride is recommended when the concentration of free unstable Amadori
233 products is high. Unfortunately, the use of this reducing agent had several drawbacks: protein
234 degradation and free counterpart losses during the reduction, precipitation and purification
235 procedure; moreover, the use of sodium borohydride can interfere with the release of furosine with
236 the above mentioned reduction of fructosyl-lysine into hexitol-lysine. After several preliminary
237 measurements it was decided to avoid the reduction. A good compromise between the detection of
238 furosine and that of CML/CEL was achieved controlling the oxidation under nitrogen. In particular,
239 prior the acidic hydrolysis the screw capped flasks were saturated with nitrogen in order to reduce
240 the effect of autoxidation and control the reaction pathway (Yaylayan & Huyghuesdespointes,
241 1994).

242 The use of hydrochloric acid is a mandatory step for the hydrolysis of peptide bonds and for the
243 release of amino acids, MRPs and for the conversion of fructosyl-lysine into furosine. Different

concentrations of protein per mL of hydrochloric acid can lead to different efficiency of the hydrolysis with the consequent underestimation of lysine content. In the present study, the extraction procedure was optimized in order to promote the dehydration reaction that leads to the formation of furosine and to the release of MRPs (Krause, Knoll, & Henle, 2003; Mossine & Mawhinney, 2007). Further studies will be conducted in order to compare the effect of time and concentration of hydrochloric acid on lysine release, mainly in protein rich samples.

The above described analytical performances were tested in food and feed samples in order to verify the robustness of the method. Several thermally treated foods were tested: powdered infant formula, low lactose milk, lab scale UHT milk, biscuits samples, bread (all prepared according to three different procedures previously described by our group) and powdered soybean-based feed products (prepared at industry scale). All data are summarized in **Table 3**. The concentration of CML in powdered infant formula analyzed ranged from 8.22 ± 0.31 mg/100 g of protein to 14.81 ± 0.92 mg/100 g of protein, while CEL and furosine ranged from 0.71 ± 0.02 mg/100 g of protein to 1.31 ± 0.11 mg/100 g of protein and 471.9± 22.3 mg/100 g of protein to 639.4± 21.1 mg/100 g of protein, respectively. The concentration of total lysine varied from 9.89 ± 0.88 to 13.12 ± 0.78 % of total protein. In low lactose milk the content of lysine was 5.21± 0.30 g/100 g of protein, while the concentration of CEL and furosine was 0.28 mg ± 0.01 mg/100 g of protein and 12.32 ± 0.31 mg/100 g of protein, respectively. CML was 1.28 mg ± 0.11 mg/100 g of protein and this value was perfectly in line with the one previously obtained. Lab scale UHT milk was prepared in order to verify the effect on raw cow milk; while the lysine content was of the same order of magnitude of the low lactose milk (4.71 ± 0.22 mg/100 g of protein), the concentration of the three markers of the MR was 18.41 ± 0.93, 1.12 ± 0.02 and 14.41 ± 1.02 mg/100 g of protein for CML, CEL and furosine respectively. The results obtained were perfectly in line with those previously obtained for the three categories of milk (Fenaille, Parisod, Visani, Populaire, Tabet, & Guy, 2006; Tareke, Forslund, Lindh, Fahlgren, & Ostman, 2013), specifically the CML in low lactose milk was similar

to one previously obtained by our group for LC-MS/MS analysis (Troise, et al., 2014). The concentration of CML and furosine was closed to the range previously obtained: 2.2 – 30.8 and 0.8 – 3.7 mg/100 g of protein for furosine and CML, respectively (de Sereys, Muller, Desic, Troise, Fogliano, Acharid, et al., 2014) .

In bakery products CML content was 43.75 ± 2.02 and 27.15 ± 0.61 mg/100 g of protein for biscuits samples and bread slices, respectively, while CEL and furosine were 46.25 ± 3.01 and 10.01 ± 0.61 and 10.91 ± 0.01 and 98.55 ± 4.61 mg/100 g of protein for biscuits and bread, respectively. The lysine content was almost similar in the two products: 5.01 ± 0.04 and 5.81 ± 0.04 g/100 g of protein, even if the protein content was 6% and 8% for biscuits and bread. The results here reported were of the same order of magnitude as the ones previously reported. Hull et al., analyzed several kinds of bread and other bakery products and the concentration of CML ranged from 2.6 to 45.1 mg/100 g of protein for wheaten bread and potato bread, respectively (Hull, Woodside, Ames, & Cuskelly, 2012). On the other hand He and coworker reported higher values for wholemeal bread: CML ranged from 66.72 to 109.9 mg/100g of protein and CEL ranged from 53.30 to 82.04 mg/100 g protein for bread, while in biscuits samples the concentrations varied from 50.8 to 116.7 and 15.87 to 45.26 mg/100g protein for CML and CEL, respectively (He, Zeng, Zheng, He, & Chen, 2014). Interestingly, the concentration of furosine in bread (after 20 min at 200° C) is similar to the one reported by Capuano and coworker: after 13 min the concentration of furosine increased up to 200 mg/100 g of protein and it quickly decreased up to 20 mg/100 g protein at the end of the thermal treatment (Capuano, Ferrigno, Acampa, Ait-Ameur, & Fogliano, 2008). A similar kinetic profile was observed also by Ramirez-Jimenez and coworker in sliced bread: the concentration of furosine at the end of the process was 79.3 mg/100 g of protein while after 12 min it reached a concentration higher than 200 mg/100 g protein (Ramirez-Jimenez, Garcia-Villanova, & Guerra-Hernandez, 2001). In biscuit samples the kinetic profile revealed similar trends to the ones obtained for bread; as a consequence at the end of the thermal process the concentration of furosine value of

10.01 ± 0.61 mg/100 g of protein was comparable to those of sucrose-containing cookies reported by previous authors (Gökmen, Serpen, Açar, & Morales, 2008).

The above described analytical performances were evaluated in industrially prepared soybean feeds in order to verify the main advantages of the method on industrial sampling. The simultaneous quantification of the four analytes allowed a direct overview of the extent of the MR, where the concentration of lysine and the formation of furosine, CEL and CML can be easily related to the final quality of foods using a single extraction and a single injection. According to the procedure described in material and methods section, soybeans were incubated at 110° C for one hour in an autoclave and the kinetic profile was reported in **Figure 3.** The initial concentration of lysine was 3.45 ± 0.12 g/100 g of protein while CML, CEL and furosine were 9.94 ± 0.74, 0.98 ± 0.04 and 24.24 ± 1.74 mg/100 g of protein respectively. After 30 minutes the concentration of furosine reached the highest values: 108.01 ± 8.97, then it rapidly decreased up to 60.58 ± 3.75 mg/100 g of protein after 55 min. According to the reaction mechanism the degradation of the Amadori products was followed by the increase of CML: at the end of the thermal treatment its concentration was higher than 76 mg/100 g of protein. CEL reached the maximum concentration after 45 minutes (2.41 ± 0.24 mg/100 g of protein), then it decreased probably due to degradation processes or to the blockage of methylglyoxal by other compounds. The degradation of lysine was constant throughout the thermal treatment, after 60 min lysine concentration was 2.60 ± 0.08 g/100 g of protein thus around 23%. Several studies reported the effect of soy proteins in the development of the MR focusing on soy health benefits and on the presence of functional molecules able to control the extent of the MR (Palermo, Fiore, & Fogliano, 2012).

This paper represents the first example of a systematic study on the relationship between thermal treatments, MR and soybean products in feeds and in pet food a topic recently attracting the attention of the scientific community. In fact, it has been observed that the average daily intake (mg/kg body weight$^{0.75}$) of HMF is 122 times higher for dogs and 38 times higher for cats than

319 average intake for adult humans. Possible health risks, such diabetes and renal failure, can be
320 associated to the intake of MRPs not only in human, but also in pets (van Rooijen, Bosch, van der
321 Poel, Wierenga, Alexander, & Hendriks, 2013).

322 **4. Conclusion**

323 The analytical method allowed a comprehensive approach in the analysis of MRPs, simultaneously
324 determining both lysine and its heat-induced derivatives. Up to now the golden standards for MRPs
325 detection were RP-HPLC with UVvis detection for furosine and LC-MS/MS for CML, CEL and
326 lysine, respectively. These results showed that the extraction procedure with nitrogen and
327 hydrochloric acid provided a good compromise for the simultaneous detection of the four analytes.
328 The analytical performances showed high sensitivity and good reproducibility and repeatability in
329 several foods. Quantitative data were fully in line with those previously obtained by other authors
330 on similar foods. The simultaneous detection of the four analytes offered a sensitive tool for the
331 kinetic modeling on neoformed contaminant reaction routes monitoring the precursor lysine, the
332 intermediate furosine via the indirect analysis of the Amadori products and the end-products CEL
333 and CML. The simultaneous monitoring of all compounds allowed to minimize the variability
334 among different samples and to combine the reaction steps starting from lysine blockage, Amadori
335 compounds formation and fragmentation, CML and CEL formation.

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339 **The authors declare no conflict of interests.**

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454 **Figure legend**

455 **Figure 1:** Effect of glucose and dicarbonyls on the formation of protein-bound MRPs. At the
456 bottom the structure of β-Lactoglobulin (Brownlow, Cabral, Cooper, Flower, Yewdall, Polikarpov,
457 et al., 1997).

458 **Figure 2:** Extracted ion chromatogram of the four target molecules and their respective internal
459 standards

460 **Figure 3:** Kinetic profile of the precursor lysine (green), intermediate, furosine (blue) and end-
461 products, CML and CEL (red).

462 **Figure S1:** Fragmentation pathway for lysine and its deuterated internal standard *d4*-lysine. The
463 structures of pipecolic acid and 1,2,3,4-tetrahydropyridin-1-ium ion was reported (Yalcin &
464 Harrison, 1996).

465

466 **Table legend**

467 **Table 1:** Mass spectrometry set up

468 **Table 2:** Analytical performances for the four analytes and their respective internal standards

469 **Table 3:** MRPs concentration after 8 replicates in different samples, the results for CML, CEL and
470 furosine were reported as mg/100 g of protein, except for lysine. The results were compared to the
471 AGE Database (Technische Universität Dresden, 2014).

Tables

Table 1

Compounds	$[M+H]^+$	Fragments	CE (V)	DP (V)
CML	205	84	29	30
		130.2	27	30
***d_2*-CML**	207	84	30	20
		144	21	20
		130	17	20
Furosine	255.1	130	18	21
		84.4	28	21
Lys	147.2	130.2	16	30
		84.1	24	30
***d4* –Lys**	151.3	134.1	15	30
		88.2	26	30
CEL	219.2	130.3	20	30
		84.0	28	30
***d4*-CEL**	223	134.1	18	25
		88.0	30	25

Table 1: Mass spectrometry set up

Table 2

Compound	LOD	LOQ	RSD [%]	Linearity range	r^2	Recovery
CML	0.5 ppb	5 ppb	7	5-1000 ng/ml	> 0.99	91.1 ± 8.4
CEL	1 ppb	5 ppb	5	5-1000 ng/ml	> 0.99	84.2 ± 7.4
Lysine	0.5 ppb	5 ppb	5	5-1000 ng/ml	> 0.99	88 .0± 6.9
Furosine	3 ppb	9 ppb	8	9-1000 ng/ml	> 0.99	88.0 ± 6.9

Table 2: Analytical performances for the four analytes and their respective internal standards

Table 3:

Food	CML	CEL	Furosine	Lysine (g/100 g protein)
Infant formula -1	8.22 ± 0.31	0.71 ± 0.02	471.91 ± 22.31	9.89 ± 0.88
Infant formula -2	10.4 ± 0.52	0.85 ± 0.06	542.53 ± 11.91	12.24 ±0.91
Infant formula -3	10.9 ± 1.03	1.10 ± 0.05	574.5 ± 44.12	13.12 ± 0.78
Infant formula -4	14.81 ± 0.92	1.31 ± 0.11	639.4 ± 21.11	10.28 ± 1.01
Age Database	0.6 – 40.5	/	Up to 1819	/
Low lactose milk	1.28 ± 0.11	0.28 ± 0.01	12.32 ± 0.31	5.21 ± 0.30
Age Database	1.4	/	/	/
Lab scale UHT milk	18.41 ± 0.93	1.12 ± 0.02	14.41 ±1.02	4.71 ± 0.22
Age Database	0.9-8.3	/	12.4 – 220.0	/
Biscuits	43.75 ± 2.02	46.25 ± 3.01	10.01 ± 0.61	5.01 ± 0.04
Bread slices	27.15 ± 0.61	10.91 ± 0.01	98.55 ± 4.61	5.81 ± 0.04
Age Database	2.6 – 45.1	/	/	/

Table 3: MRPs concentration after 8 replicates in different samples, the results for CML, CEL and furosine were reported as mg/100 g of protein, except for lysine. The results were compared to the AGE Database (Technische Universität Dresden, 2014).

Figure-1

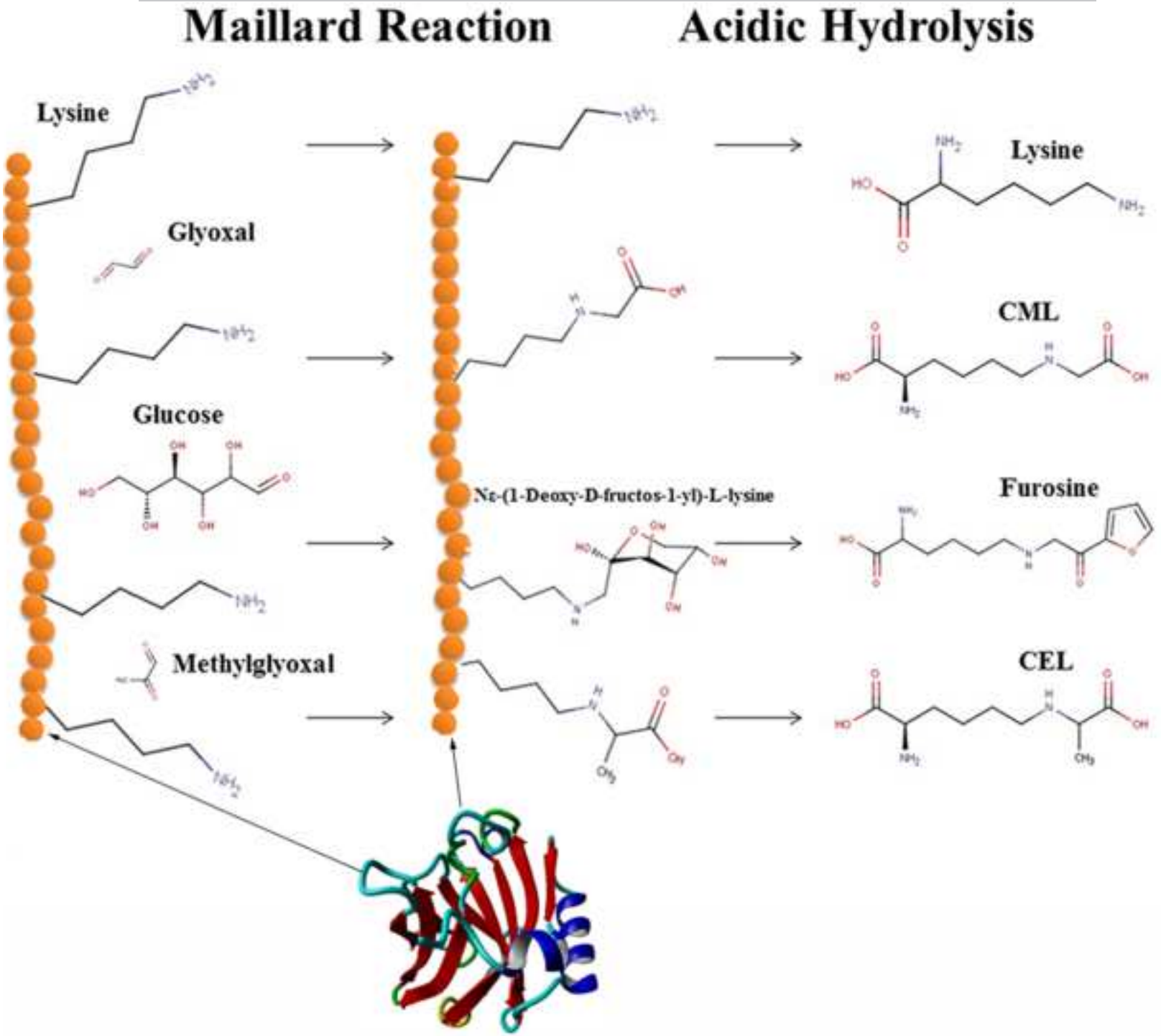


Figure-2

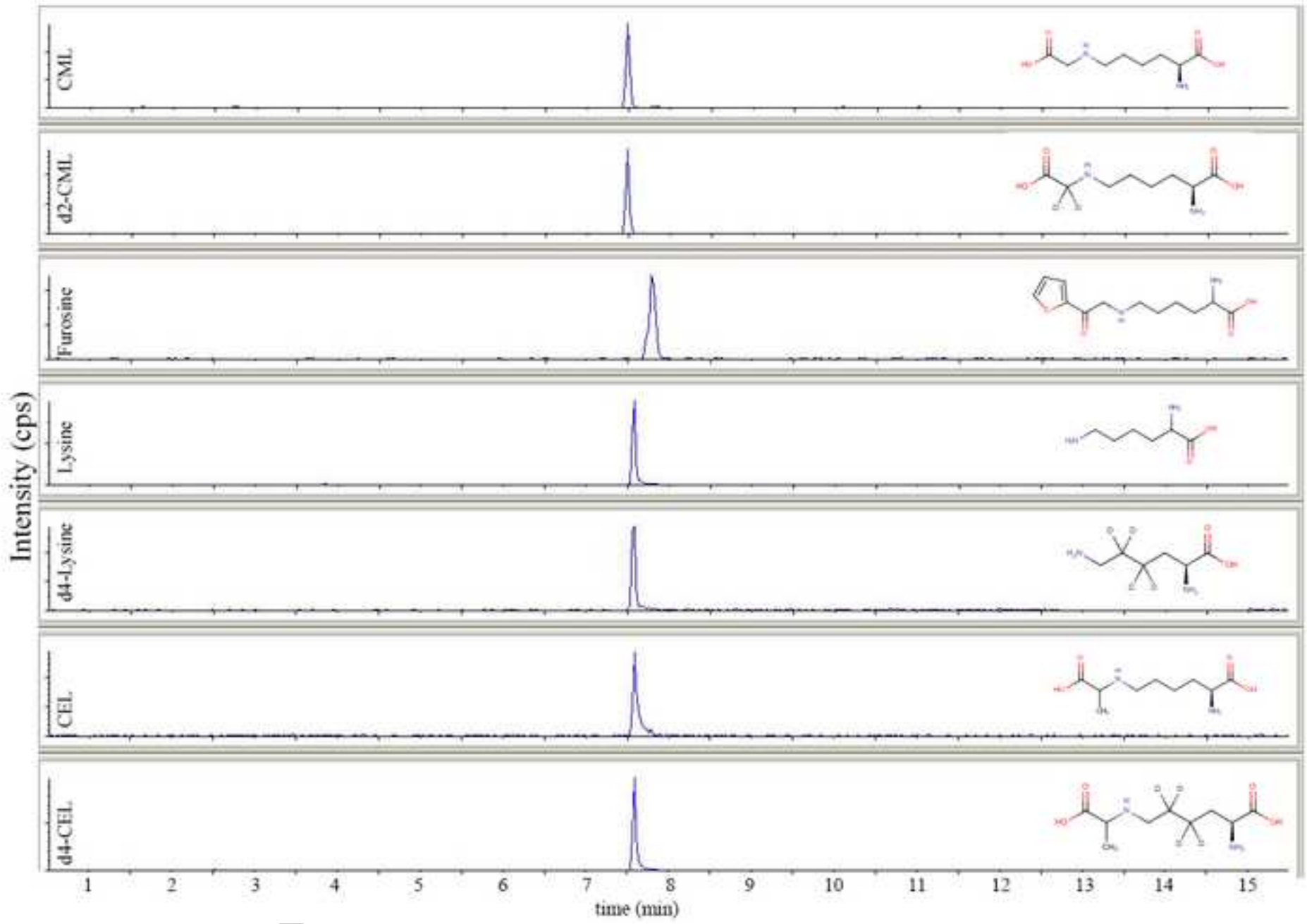
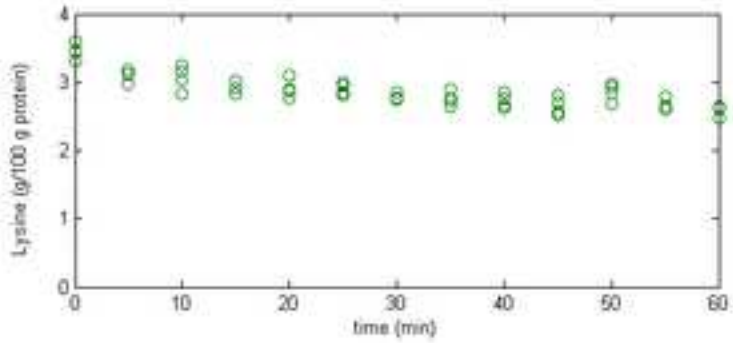
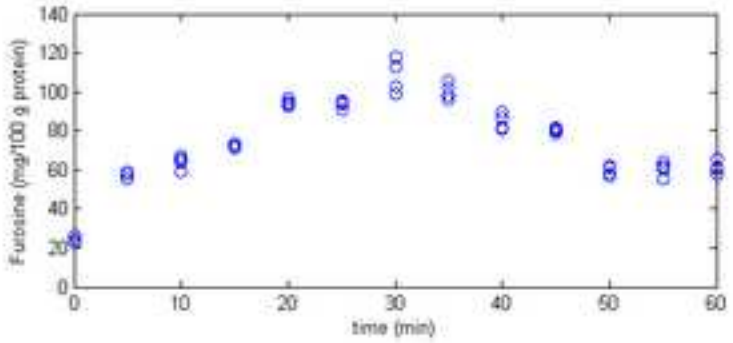
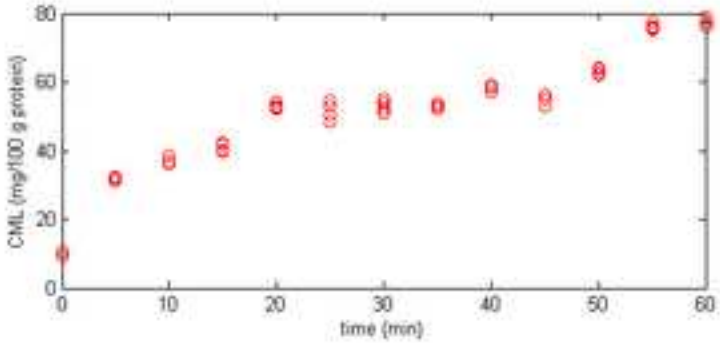
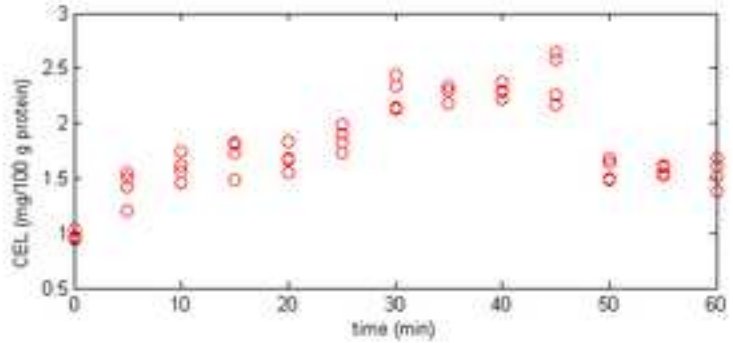


Figure-3









- Tandem mass spectrometry and stable isotope dilution ensured reliable performances.
- The method achieved simultaneous detection of CML, CEL, Lysine and furosine.
- CML, CEL, Lysine and furosine were quantified in several foods.
- The analysis of the four markers paved the way for a better quality control.