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The rehabilitation of raw and brown butters by the measurement of two of the major Maillard products, N^{ε}-carboxymethyl-lysine and 5-hydroxymethylfurfural, with validated chromatographic methods

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ABSTRACT

The fat food group, especially butter, has so far been thought to have a high N^{ε}-carboxymethyl-lysine (CML) content. However recent data have challenged this opinion. The objective of this article was to determine not only CML content but also that of 5-hydroxymethylfurfural (HMF) in raw and cooked butters. The first aim of this study was to verify the accuracy of the LC–MS/MS and LC–UV methods used for the quantification of CML and HMF. The tests on fortified butter samples showed recovery values of 72% for CML and 78% for HMF. The amounts of CML in raw and cooked butters were 0.25 ± 0.03 and 2.22 ± 0.56 μg/g, respectively. The level of HMF in cooked butters was 61 ± 40 μg/g. No CML was detected in clarified butter, and no HMF was detected in raw and clarified butters. The results indicate that the contribution of butter alone to the exposure to CML and HMF is very low.

1. Introduction

Some Maillard reaction products such as Amadori products, pentosidine, and pyrraline have been well characterised during the past decades and are currently used as markers of the reaction during the process of food production as well as in the final products and also during storage (Poulsen et al., 2013). Among these markers, N^{ε}-carboxymethyl-lysine is the one which has been most widely researched (Henle, 2003; Tessier & Birlouez-Aragon, 2012). It has also been used to classify foods according to their level of neoformed compounds, also known as "glycotoxins" (Uribarri et al., 2010). Serum and urinary free CML which originate partly from food sources have aroused the interest of biologists who are currently examining the CML rate of absorption (Alamir et al., 2013) and its biological activity therein (Henle, 2005; Poulsen et al., 2013).

Several studies have suggested that dietary CML increases the *in vivo* pool of CML after intestinal absorption and contributes to the development of degenerative pathologies (Tessier & Birlouez-Aragon, 2012). It is therefore important to determine which food groups are most likely to be the major contributors of CML in the diet, especially when required to make nutritional recommendations to vulnerable populations such as infants and diabetics. Some databases available in scientific articles (Goldberg et al., 2004; Uribarri et al., 2010) and on nutrition websites indicate that the highest CML concentrations are found in food produced with a high fat content. However, we and other analytical chemists came to doubt the accuracy of the immunoassay used to estimate the level of CML in foods and carried out tests using liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods, in order to achieve more accurate results. It has since been widely acknowledged that the best analytical method to quantify CML, both in foods (Tessier, Jacolot, & Niquet-Léridon, 2014) and biological samples (Thornalley & Rabbani, 2014), is indeed the stable isotopic dilution analysis LC–MS/MS.

The fat food group, including oil and butter, has until now been considered as one containing foods high in CML. However recent data using accurate LC–MS/MS methods have challenged this observation (Hull, Woodside, Ames, & Cuskelly, 2012).

The objective of the current study was first to validate the pre-analytic processing of high fat matrices, which is a critical step often underestimated, and then to determine the level of CML in

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raw and cooked butters using an LC–MS/MS method. A second neoformed compound, 5-hydroxymethylfurfural (HMF), was quantified in butter samples to complete the current study. HMF is both a marker of the Maillard reaction between amino acids and sugars and a marker of the sugar degradation reactions commonly known as caramelisation (Capuano & Fogliano, 2011). It is therefore useful to measure HMF as a complementary marker of the chemical modification of food in addition to the CML measurement. A pre-validation of the extraction of HMF from cooked butter was carried out in accordance with that aim.

The second and final objective of this study was to determine the amount of CML in a selection of foods and to compare it with the data obtained previously using an immunoassay.

2. Materials and methods

2.1. Chemicals

Perfluoropentanoic acid (NFPA, 97%) was purchased from VWR International (Fontenay sous bois, France) whereas hydrochloric acid 37%, sodium hydroxide, sodium borohydride, boric acid, lysine, HMF and Carrez solutions (potassium hexacyanoferrate (III) trihydrate and zinc sulfate heptahydrate) were provided by Sigma–Aldrich (Saint Quentin Fallavier, France). Both CML and (D_2)-CML were purchased from PolyPeptide Laboratories France SAS (Strasbourg, France) while ($^{15}N_2$)-lysine was from CortecNet (Voisins-le-Bretonneux, France). Stock solutions of calibrators were prepared in UHQ water except for CML and lysine which were prepared in NFPA (20 mM in UHQ water). Acetonitrile and water (both HPLC grade) were purchased from Sodipro (Echirolles, France).

2.2. Food selection and preparation

Winter and summer butters were obtained from the National Center for Interprofessional Dairy Industries (Paris, France). Each butter was studied in a regular and clarified form (also known as ghee). Portions of regular and clarified butter were cooked at 150 and 180 °C and analysed for CML, lysine and HMF immediately after cooling. The cooking times were determined as the time needed to reach the formation of a brown colour at each temperature in the regular butter. A second cooking time corresponding to twice the first one was also tested. Table 1 summarises the codes used to label each sample prepared in triplicate.

For the second part of this study, a total of 24 foods was selected based on a list of food items already analysed for CML in 2004 by Goldberg et al. They were purchased in duplicate (2 brands/item) from local supermarkets and may thus not have been exactly of the same composition as those purchased by Goldberg et al. approximately ten years ago in North America. The 14 food items which did not need any culinary process after purchasing, were analysed before their expiry date. The other 10 were prepared using recipes typical of Western countries, or according to the instructions on the packaging. Each preparation of cooked food was done in triplicate.

Table 1
Cooking conditions applied to butter samples, and codes used to label each sample prepared in triplicate.

Cooking conditions		Identification code			
Temperature (°C)	Duration (min)	Summer butter	Winter butter	Summer clarified butter	Winter clarified butter
NA	0	SB0	WB0	SCB0	WCB0
150	25	SB25	WB25	SCB25	WCB25
150	50	SB50	WB50	SCB50	WCB50
180	6	SB6	WB6	SCB6	WCB6
180	12	SB12	WB12	SCB12	WCB12

2.3. Sample preparation for recovery studies of CML, lysine and HMF in butter

The effectiveness of the two chromatographic methods for the quantification of CML, lysine and HMF were tested by performing recovery studies.

2.3.1. CML and lysine recovery study

In order to prepare a standard of protein-bound CML, a CML-rich casein sample was prepared according to the method of Faist, Müller, Drusch, and Erbersdobler (2001). Briefly, solutions of casein (18 g/L) and glyoxylic acid (143 g/L) prepared in the same phosphate buffer (0.2 M, pH 7.4) were mixed and incubated at 37 °C with a continuous adjustment of the pH to 7.4. After 2 h of incubation, a solution of sodium cyanoborohydride (56 g/L) prepared in the same phosphate buffer was added in excess. The mixture containing this reducing agent was incubated for 16 additional hours. The synthetised CML-rich casein was then purified using a dialysis membrane with a molecular weight cut-off of 12,000–14,000 Daltons, freeze-dried and stored at −20 °C until analysis and further usage.

The concentration of CML in the CML-rich casein standard was measured using the method developed by Niquet-Léridon and Tessier (2011) and described below. The concentration of lysine in native casein was also measured using the same method.

CML and lysine recovery studies were performed using cooked winter butters heated for 6 (WB6) and 12 min (WB12). Each cooked butter sample was spiked with three known amounts of CML-rich casein (approximately 1.4, 2.8 and 5.6 μg CML/g butter) or three amounts of casein (approximately 0.1, 0.24 and 0.48 mg lysine/g butter). Each spiked sample was prepared in triplicate. Each original or spiked sample was analysed either for CML or lysine

2.3.2. HMF recovery study

A commercial standard of HMF was used to evaluate the extraction efficiency of our method applied to butter. Recovery studies were performed using winter butters cooked for 6 (WB6) and 12 min (WB12). Each cooked butter sample was spiked with three known amounts of HMF (approximately 2, 4 and 8 μg HMF/g butter) and analysed according to the method described below.

2.4. Analysis of N^{ε}-carboxymethyl-lysine and lysine using LC–MS/MS

CML and lysine were analysed three times as described in our previous study (Niquet-Léridon & Tessier, 2011). Briefly, samples equivalent to 10 mg of protein were reduced with 1.5 mL of 0.2 M borate buffer (pH 9.5) and 1 mL of 1 M sodium borohydride (in NaOH 0.1 M) for 4 h at room temperature. Hydrochloric acid was added to a final concentration of 6 M and the samples were hydrolysed at 110 °C for 20 h. Three hundred microlitres of each hydrolysate were dried in a Speed-Vac concentrator (ThermoFisher Scientific, Courtaboeuf, France). Each residue was dissolved in 300 μL of internal standards (D_2)-CML and ($^{15}N_2$)-lysine and filtered through a membrane filter (0.45 μm) before chromatography.

LC–MS/MS was performed on a Surveyor MS pump plus system (ThermoFisher Scientific, Courtaboeuf, France) coupled, by an ESI probe, to a Finnigan LTQ ion trap mass spectrometer (ThermoFisher Scientific, Courtaboeuf, France) working in tandem mode. The chromatographic column was a Hypercarb column

(100 × 2.1 mm, 5 μm; ThermoFisher Scientific) protected with a guard column of the same material. The elution was performed at a flow rate of 0.2 mL/min using a solution of 20 mM NFPA in water as solvent **A** and acetonitrile as solvent **B**. The percentage of solvent **B** increased from 0 to 50% throughout the 20-min separation. The conditions of the ESI positive interface were similar to those described by Niquet-Léridon and Tessier (2011). Measurement of analyte and internal standard were performed using selected reaction monitoring of the specific transitions, m/z 205.0 → m/z 130.0 for CML; m/z 147.0 → m/z 130.0 for lysine; m/z 207.0 → m/z 130.0 for (D_2)-CML and m/z 149.0 → m/z 131.0 for ($^{15}N_2$)-lysine. Quantification of CML and lysine was achieved by measuring the ratio between the area under the peaks for the analyte and its corresponding internal standard, and comparison with the standard curves.

2.5. Analysis of N^{ε}-carboxymethyl-lysine using ELISA

CML was measured in a selection of food samples using the Oxiselect™ CML competitive ELISA kit (Cell Biolabs, San Diego, CA). Only the food items which were in a liquid form at room temperature were tested. In addition, two different brands of powdered hypoallergenic infant formulas were analysed. The immunoassay was used according to the manufacturer's instructions. Briefly, food sample (50 μL) was added in the wells coated with CML-BSA (5 ng per well, overnight at 4 °C) and anti-CML antibody (50 μL diluted in phosphate buffer saline; 1:1000) was added for 1 h at 37 °C. After washing, the horseradish peroxidase-conjugated secondary antibody (1:1000) was added for 1 h at room temperature. The substrate solution was finally added and optical density was read at 450 nm. The relationship between volume and weight was established for each sample and the results were expressed as μg CML/mg of food according to the corresponding standard curve.

2.6. Analysis of free 5-hydroxymethylfurfural

The quantification of free HMF was performed in triplicate according to the method of Garcia-Villanova, Guerra-Hernandez, Martinez-Gomez, and Montilla (1993). Briefly, 0.4 g of sample were diluted in 7 mL of demineralised water. After mixing for 1 min, the sample was centrifuged for 10 min at 5000 rpm. The supernatant was collected and the extraction procedure was repeated twice. All supernatants were pooled with 0.5 mL of each of Carrez solution I and II. After a second centrifugation, the supernatant was transferred into a 25-mL volumetric flask and diluted to the mark with demineralised water. After filtration through a membrane filter (0.45 μm), 25 μL were injected into an HPLC system composed of a Surveyor LC System coupled to a Surveyor PDA (ThermoFisher Scientific, Courtaboeuf, France). HMF was separated from other compounds on a Luna C18 column (250 × 4.6 mm, 4 μm; Phenomenex, Le Pecq, France) equipped with a pre-column of the same phase and eluted isocratically with water:acetonitrile (95:5, v/v) at a flow rate of 1 mL/min. The detection was made at 284 nm. The external standard method was used for quantification of HMF.

2.7. Nitrogen and protein determination

The nitrogen content of the samples was determined in triplicate by combustion method using a LECO FP528 nitrogen analyser (LECO France, Garges les Gonesse, France) according to the Dumas method. One hundred milligrams of the powder were weighed in tin foil and analysed in duplicate using the AOAC method 990.03. The milk protein–nitrogen conversion factor of 6.38 was used for calculation of the protein content expressed in g/100 g.

2.8. Statistical analysis

Statistical analyses were performed to check the possible differences between the two chosen food samples. Data were analysed statistically by Mann–Whitney tests using XLSTAT software, version 2011.4.02 (Addinsoft USA, New York, NY).

3. Results and discussion

3.1. Results of the recovery studies

The validations of the two analytical methods for the analysis of CML & lysine and HMF have been described in previous publications. Most validation characteristics such as the linearity, the precision and the limit of quantification were still valid for the current study (data not shown). However the trueness needed to be confirmed on our food matrix of interest: that is cooked butter. Since no certified reference material is available, the trueness of both methods has been expressed as a percentage of recovery by the assay of butter samples spiked with three known increasing amounts of analyte, with three replicates of each added amount.

CML in dairy products is mainly present as bound to proteins. A CML-rich casein standard was synthesised and characterised in order to spike the butter with the most realistic form of CML. The analysis of the CML-rich casein standard indicated that 36% of lysine was modified into CML, corresponding to a final concentration of 58.0 mg of CML/g of glycated casein. We believe that a recovery study using a pure standard of free CML would have less validity than our study achieved through the use of a protein-bound CML standard. The recovery from the fortified butter samples ranged from 73% to 76% in winter butter heated for 6 min at 180 °C (WB6) and from 62% to 77% in winter butter heated for 12 min at 180 °C (WB12) (Table 2). The results obtained in cooked butters are satisfactory (72% on average) and fall within the range accepted by the European Union (Commission Decision 2002/657/EC 2002).

To demonstrate the accuracy of the analytical procedure for the determination of lysine in butter a recovery study was also performed using in this case a standard of pure casein as a source of lysine added at three concentrations to butter samples. Table 2 shows that the difference between the expected true concentration of lysine and the measured concentration, expressed as a percentage of recovery was from 86% to 121% in winter butter heated for 6 min at 180 °C (WB6); and from 78% to 93% in winter butter heated for 12 min at 180 °C (WB12). An average recovery of 93% for the analysis of lysine in cooked butters was fully satisfactory.

Unlike CML and lysine, HMF is found in food as a free reaction product. It was thus possible to use a pure analyte standard added to butter samples at different concentrations for the recovery study. The test on cooked butter samples shows recoveries between 73% and 82% (Table 2). This is in accordance with the guideline range of the European Union (Commission Decision 2002/657/EC 2002).

It is important that the methods employed in preparing food samples for testing are carefully optimised and validated because of the diversity of matrices found in food. It should not be taken for granted that a method which is reliable for one matrix is also reliable for another. We believe that above all other validation criteria, a recovery test is essential.

3.2. CML and lysine in raw and cooked butters

The nutritional composition of butter is dependent on the quality of milk which itself is dependent on the diet of the cow. This results in the distinction between what it is commonly named

Table 2
Experimental values obtained in the recovery tests for CML, lysine and HMF.

Sample	CML (μg/g butter)				Lysine (mg/g butter)				HMF (μg/g butter)			
	Test sample	Amount added	Spiked sample	Recovery (%)	Test sample	Amount added	Spiked sample	Recovery (%)	Test sample	Amount added	Spiked sample	Recovery (%)
WB6-0	0.82 ± 0.20	0	NA	NA	0.07 ± 0.02	0	NA	NA	0.78 ± 0.06	0	NA	NA
WB6-1	0.82 ± 0.20	1.4	1.66 ± 0.05	75	0.07 ± 0.02	0.1	0.2 ± 0.00	121.5	0.78 ± 0.06	2	2.16 ± 0.65	77.8
WB6-2	0.82 ± 0.20	2.8	2.74 ± 0.22	75.8	0.07 ± 0.02	0.24	0.27 ± 0.02	86.3	0.78 ± 0.06	4	3.48 ± 0.30	72.8
WB6-3	0.82 ± 0.20	5.6	4.71 ± 0.05	73.4	0.07 ± 0.02	0.48	0.52 ± 0.02	96.5	0.78 ± 0.06	8	6.63 ± 0.42	75.5
WB12-0	1.01 ± 0.12	0	NA	NA	0.08 ± 0.00	0	NA	NA	22.3 ± 2.12	0	NA	NA
WB12-1	1.01 ± 0.12	1.4	1.63 ± 0.06	67.7	0.08 ± 0.00	0.1	0.16 ± 0.00	93.3	22.3 ± 2.12	2	20.1 ± 0.98	82.6
WB12-2	1.01 ± 0.12	2.8	2.92 ± 0.15	76.6	0.08 ± 0.00	0.24	0.25 ± 0.00	78	22.3 ± 2.12	4	21.2 ± 0.50	80.4
WB12-3	1.01 ± 0.12	5.6	4.12 ± 0.01	62.4	0.08 ± 0.00	0.48	0.46 ± 0.01	83.1	22.3 ± 2.12	8	24.8 ± 1.05	81.8
Mean recovery values	71.8				93.1				78.5			

winter and summer butters, which come from cows fed with hay and fresh grass, respectively. The major nutritional difference between the two types of butter is the proportion of saturated fats (*versus* unsaturated fats) which in turn affects the physicochemical properties of butter. For instance the winter butter that was used in our study contained 68.8% saturated fat compared to 63.2% in the summer butter. The winter butter also contained fewer total fatty acids than the summer butter (91.6 and 94.2, respectively). In order to address our question in a comprehensive way both types of butters were studied. In addition their respective clarified versions were also studied. Clarified butters were prepared in a food factory by removing water, casein and sugars from the butter.

The amounts of CML found in raw winter and summer butters were low and similar ($p > 0.05$), 0.27 ± 0.01 and 0.21 ± 0.01 μg/g of butter, respectively (Fig. 1). These values are consistent with those reported by Assar, Moloney, Lima, Magee, and Ames (2009), and Hull et al. (2012), who indicated a concentration of 0.37 and 0.30 μg/g butter, respectively. The presence of CML in raw butter is mainly attributable to the presence of CML in milk and unlikely to the formation of CML during butter processing. When expressed in mmol/mol lysine, the concentrations of CML in samples WB0 (0.39 ± 0.02) and SB0 (0.33 ± 0.03) were similar to what has been described in whole milk (Hull et al., 2012).

In raw clarified butters no CML was detected (data not shown). It should be mentioned that the limit of detection for the assay was estimated to be 3 ng CML/g sample. This lack of CML in clarified butter was expected since caseins and other proteins had been removed during the processing. In addition no CML was found in heated clarified butter whatever the temperature and duration of cooking (data not shown).

The effect of heating on the formation of CML in butter is presented in Fig. 1A. An increase of the amount of CML was observed both in winter and summer butters, with a slight but significantly higher amount of CML in heated winter butter (means of the 4 cooking conditions: 2.51 ± 0.62 *versus* 1.93 ± 0.34 μg/g butter, $p < 0.01$). At 150 °C the formation of CML was not dependent on the heating time whereas at 180 °C a significant decrease of the CML concentration was observed when the duration of heating was doubled ($p < 0.01$). This was the first time to our knowledge that a kinetic of degradation of CML was observed in heated food. It is well documented that some Maillard reaction products undergo degradation when exposed to a prolonged heat treatment (Kocadagli, Goncuoglu, Hamzalioglu, & Gokmen, 2012) but the potential instability of CML during heating has never been described and would need to be confirmed with a full kinetic study.

Since the Maillard reaction products are mainly formed from two basic amino acids, which are arginine and lysine, the degradation of the latter was tracked in butter during heating. Again, no lysine was detected in clarified butter. In raw winter and summer butters the amounts of lysine were found to be 470 ± 4.9 and 511 ± 4.1 μg/g butter, respectively (Fig. 1B). An extensive loss of lysine was then observed when butter was heated. The losses varied from 67% to 84% in winter butter and from 69% to 86% in summer butter. The degradation of lysine was not constituently proportional to the extent and temperature of heating. The complex pathways of degradation and release of lysine during the Maillard reaction (Hodge, 1953) may explain this observation. The degradation of lysine (Fig. 1B) was markedly higher than the formation of CML (Fig. 1A). This result confirms that the formation of CML represents only a small fraction of the whole modifications which occur to lysine during the Maillard reaction and other chemical reactions. It is well known, for instance, that the yield of formation of Amadori compounds such as fructoselysine is much higher than the yield of formation of advanced Maillard reaction products (Cerny, 2008).

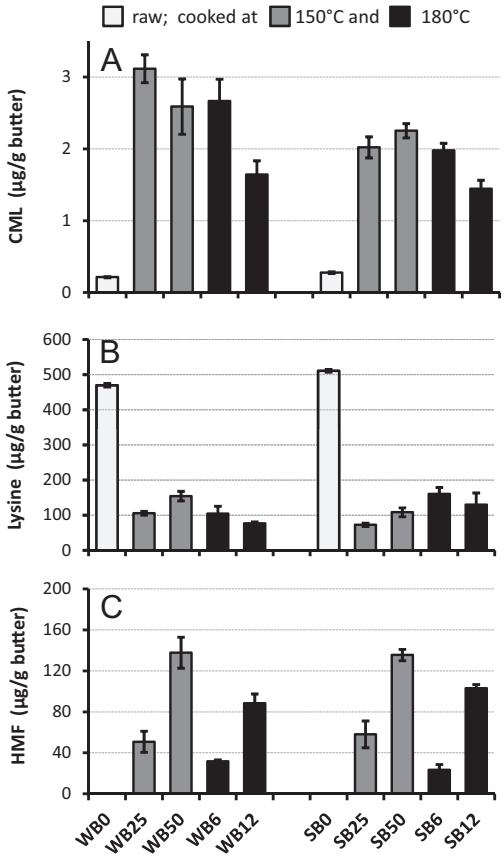

Fig. 1. CML, lysine and HMF in winter (WB0) and summer (SB0) raw butters. CML, lysine and HMF in winter and summer butters cooked at 150 °C for 25 and 50 min, and at 180 °C for 6 and 12 min.

3.3. HMF in raw and cooked butters

Butter has never been listed among the food commodities which contain a significant amount of HMF. In the current study HMF was detected neither in raw butters (Fig. 1C) nor in raw or heated clarified butters (data not shown). It can be concluded that there is no HMF in these butters or at least that its concentration is below the limit of quantification of our analytical method (1.8 μg HMF/g sample). Since the rate of formation of HMF in foods is directly and mainly related to the temperature and the duration of heat treatments it was expected that some HMF would be found in heated butters. The butters cooked at 150 °C for 25 min and 180 °C for 6 min were nearly identical as far as browning was concerned, but the formation of HMF was higher under the former conditions, both in winter (50.8 ± 10.3 *versus* 31.7 ± 1.4 μg/g, respectively, $p < 0.01$) and summer (58.1 ± 13.1 *versus* 23.5 ± 5.2 μg/g, respectively, $p < 0.01$) butters (Fig. 1C). When the different cooking times were doubled the rate of the formation of HMF increased from 2 to 4-fold to reach up to 138 ± 15.1 μg HMF/g butter (WB50, Fig. 1C). This confirms that the concentration of HMF in butter is related to the heat load applied during cooking and that even prolonged treatment does not lead to a major decomposition of HMF in other neoformed compounds (Morales, 2008). The rate of HMF formation, unlike that of CML, seems to remain higher than its rate of degradation during extended cooking. When the cooking times were doubled the difference in the amounts of HMF between winter and summer cooked butters remained almost constant.

3.4. Contribution of butter to the dietary intake of CML and HMF

It is important to know which foods 'as consumed' are most likely to be the major contributors of neoformed compounds in the diet. This is important for epidemiological studies focusing on the potential health effects of CML and HMF, for studies that aim to mitigate the formation of these compounds during domestic or industrial food processing, and for dieticians and physicians who want to apply the precautionary principle to avoid an excessive exposure in patients with diabetes or renal impairment, and other vulnerable populations.

For an adult diet containing a mean amount of CML of 5 mg/day (Tessier & Birlouez-Aragon, 2012), the contribution of a reasonable portion of butter (20 g) to the exposure to CML is negligible (WB0 and SB0: 0.004 and 0.006 mg of CML/portion). After excessive heating the same portions of butter produced amounts of CML varying from 0.028 to 0.062 mg. That would correspond to approximately 1% of the total exposure. For the purpose of the current study the heating of the butter was deliberately increased to attain a butter of a dark brown colour. This worst practice scenario is obviously not recommended for many culinary reasons other than food safety. But it demonstrates that even in this situation almost no CML is formed. The results of the analyses performed by LC–MS/MS on raw and cooked butters cast doubt upon the previous results obtained using an ELISA method, which proves to be inappropriate for this evaluation (Goldberg et al., 2004; Uribarri et al., 2010).

A 20-g portion of raw or clarified butter will produce no HMF whereas the same portion size of brown butter will produce 0.4 to 2.7 mg of HMF. We attempted to assess the contribution of butter to the total exposure to HMF. However it must be made clear that there is no consensus among scientists on a mean daily amount of HMF found in an occidental diet (Capuano & Fogliano, 2011). If the value of 9.7 mg/day is taken as a mean daily intake it can be estimated that a portion of butter heated for 6 min at 180 °C (WB6 and SB6) will produce no more than 0.6 mg of HMF. In this case we can estimate that brown butter contributes to approximately 6% of the total exposure. The highest HMF levels were found in food commodities other than butter or other fat products (Capuano & Fogliano, 2011). Although HMF is usually formed in carbohydrate-rich foodstuffs we proved that heated butter could also contain some amounts of HMF when heated. This is due to the fact that a significant heat load is applied to this fat product during processing. The current data indicate that heated butter contains as much HMF as bread when the values are compared as mg of HMF per kg of food (Ramirez-Jimenez, Garcia-Villanova, & Guerra-Hernandez, 2000). However it is worth pointing out that the daily consumption of butter (Dairy Economy in Figures, 2013) *versus* that of cereal-based products had to be taken into account in the calculation of the relative contribution to the exposure to HMF. Due to its high dietary intake, an average of 170 g/day in Europe (Quilez & Salas-Salvado, 2012), bread is therefore one of the major contributors to the exposure to HMF.

3.5. CML levels in a selection of food categories – comparison between 2 studies

Despite the intensive scientific activity around CML since its discovery (Ahmed, Thorpe, & Baynes, 1986; Büser & Erbersdobler, 1986) there remain many grey areas in relation to its origin in foods and thus in relation to the relative contribution of the different food groups to the dietary exposure to CML.

There are two research teams that have tried to quantify CML in a wide range of food products and published their results in the form of databases (Goldberg et al., 2004; Hull et al., 2012; Uribarri et al., 2010). The most commonly used database of the two has been proposed as the foundation for the so-called 'dietary advanced glycation end-product assessment' in clinical and epidemiological studies (Uribarri et al., 2010). Unfortunately this popular database is based on results obtained from experiments using an immunoassay which has been found by several analytical chemistry experts to be unsuitable for the task (Henle, 2008; Tessier et al., 2014). The most obvious error in the database concerns the content of CML found in butter. The content of lysine, the CML precursor, is known to be very low in butter compared with other foods and yet the calculated content of CML in the database is high. This disputed research finding was the reason for carrying out the current study. The final objective, however, was to determine the amount of CML in a selection of foods and to compare it with the data obtained previously using the immunoassay.

Fig. 2 shows the relative concentration of CML (in arbitrary unit) found by Uribarri et al. (2010) in 24 food items classified into 3 food categories as rich in fats, proteins and carbohydrates, and also, in comparison to those quantities of CML (in μg/g or mL of food) found in the current study. Fig. 2 shows clearly that when CML was measured using an immunoassay (Uribarri et al., 2010) its quantification was overestimated for most fat-rich products and underestimated for most carbohydrate-rich products. The data presented above already indicate that butter is unlikely to be a significant source of CML. In addition the analysis of olive oil and mayonnaise confirms that the fat group contains low amounts of CML. Within this category only roasted almonds prove to contain a high CML value. In contrast to the fat group, the carbohydrate group contains relatively high amounts of CML. Bread and pancakes are among the richest in CML. Our findings are consistent with the data of Assar et al. (2009) and Henle (2003) who also found significant amounts of CML in bakery products. They are in complete contrast with the data obtained using an immunoassay.

Another discrepancy observed between the two studies in Fig. 2 was the relatively low CML values found in milk products when an immunoassay was used compared to the higher values measured by LC–MS/MS. The analysis of milk products using LC–MS/MS method is one of those most studied and validated (Delatour et al., 2009). There is no doubt in this case that some UHT or

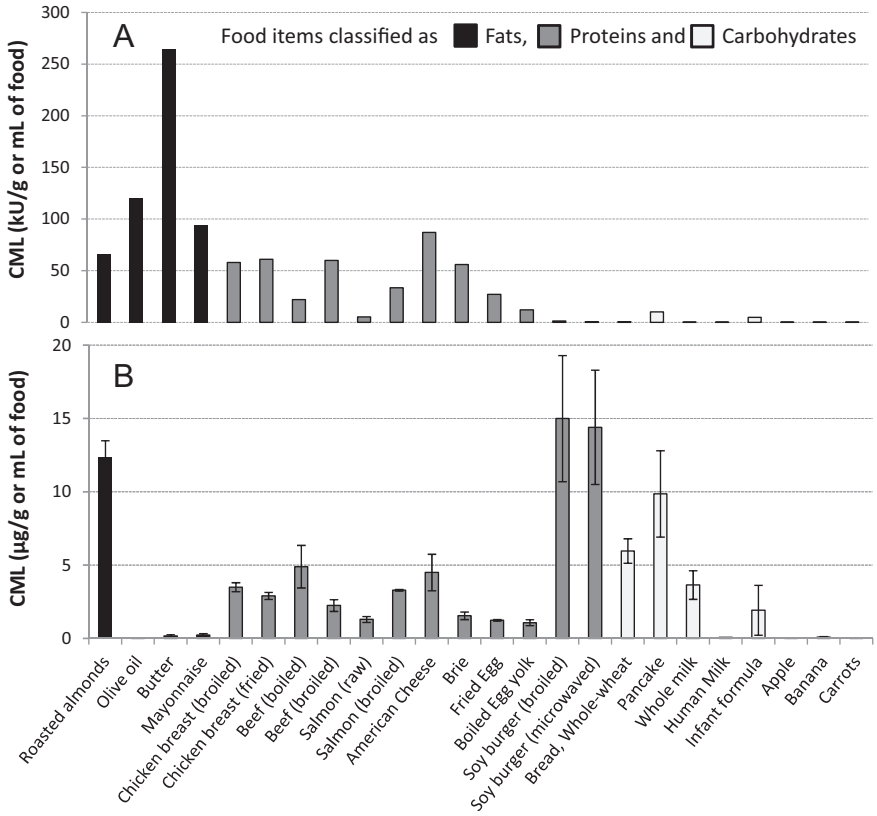

Fig. 2. CML levels in a selection of food categories. Comparison between A – a published database based on results obtained from experiments using a competitive enzyme-linked immunosorbent assay (Goldberg et al., 2004; Uribarri et al., 2010) and B – the current data obtained using stable isotope dilution analysis/liquid chromatography–tandem mass spectrometry.

Table 3
Comparison of CML levels in 6 milk-based foods and 6 fat-rich foods analysed using ELISA and LC–MS/MS.

Sample	ELISA		LC–MS/MS		Difference between values[a]
	CML (µg/g)	RSD (%)	CML (µg/g)	RSD (%)	
Whole milk 1	3.91	±7.2	4.34	±3.4	11%
Whole milk 2	0.75	±5.3	2.96	±1.7	295%
Infant formula 1	3.45	±6.1	4.17	±11.7	21%
Infant formula 2	1.80	±13.9	2.09	±7.6	16%
Hypoallergenic infant formula 1	5.28	±25.7	7.76	±6.9	47%
Hypoallergenic infant formula 2	11.40	±1.0	12.1	±11.1	6%
Butter 1	<LOQ	/	0.24	±8.3	/
Butter2	<LOQ	/	0.12	±8.3	/
Mayonnaise 1	<LOQ	/	0.18	±5.5	/
Mayonnaise 2	<LOQ	/	0.3	±3.3	/
Olive oil 1	<LOQ	/	0.004	±25.0	/
Olive oil 2	<LOQ	/	0.004	±25.0	/

RSD: relative standard deviation.
LOQ: limit of quantification (0.002 µg/g).
[a] Difference calculated as $((CML_{LC-MS/MS}-CML_{ELISA})/CML_{ELISA}) \times 100$.

condensed milks and some infant formulas show significant amounts of CML.

In order to complete the comparison between LC–MS/MS and ELISA methods it would have been interesting to analyse the 24 food items using a commercial immunoassay. Unfortunately only the food samples which are in a liquid form at room temperature were found to be technically suitable for the ELISA test in our laboratories. The analysis of solid food using the ELISA method was not possible and would have required the development and the validation of sample preparation protocols which is not the purpose of the current study. To our knowledge the protocol required in the preparation of the diverse food samples for the quantification of CML has never been described in detail in any publication. Therefore the four liquid samples selected in this study, presented in Table 2 (two samples of whole milk and two samples of infant formulas) were the only ones that it was possible to test with the ELISA kit. In addition two hypoallergenic infant formulas samples were analysed using both LC–MS/MS and ELISA to complete the comparison between the two methods. Table 3 shows a satisfactory correlation between ELISA and LC–MS/MS data ($r = 0.968$) for 6 samples of milk. For these samples the CML levels measured using LC–MS/MS were always higher than the ones measured using ELISA but it is impossible to tell which method gives the more accurate value without the use of a

certified reference material that is not commercially available. A similar trend was observed previously on gruel samples analysed using the two methods (Tareke, Forslund, Lindh, Fahlgren, & Östman, 2013). Charissou et al. reported that the discrepancy between methods was sample dependent (Charissou, Ait-Ameur, & Birlouez-Aragon, 2007). A difference of a factor of ten in CML levels was observed, for instance, when the two methods were compared (ELISA and GC–MS) in liquid infant formulas whereas no difference was observed between the two methods when powdered infant formulas were analysed.

It was not possible to detect CML in butter, mayonnaise or olive oil samples using ELISA (Table 3). Only a very low signal below the limit of quantification was able to be observed. The very low solubility of the antibodies of the ELISA kit that we observed in the three lipophilic matrices tested is the most likely reason for the incompatibility of the immunoassay with foods rich in fat. This latest observation together with the results obtained using LC–MS/MS indicate strongly that the data obtained previously by Uribarri et al. (2010) on butter, mayonnaise and olive oil are inaccurate.

The conclusion of this comparative study is that the use of published data coming from methods based on immunochemistry should not be used to estimate the presence of CML in foods. The use of these methods for the quantification of CML in liquid milk products could be used only if a full validation of the method has been previously carried out. In any case, great care should be taken in interpreting the results. The use of these methods is not recommended for foods rich in fats and any solid foods unless a precise description of the sample preparation protocol, including the use of an internal standard, is presented. Pending significant improvement of the immunochemical assays the stable isotope dilution analysis LC–MS/MS must remain the reference method. It must be made clear that our statement that the LC–MS/MS is the most appropriate tool for the quantification of CML is corroborated by authors using clinical examples (Thornalley & Rabbani, 2014).

4. Conclusion

LC–MS/MS proved to be a valuable method for the analysis of CML in butter samples. Using this validated technique and another method for the quantification of HMF we have provided evidence that the browning of butter during cooking is due to the Maillard reaction between proteins and sugars. The excessive heating model used in this study showed that there is a formation of both CML and HMF as well as a degradation of lysine in brown butter. When clarified butter was heated neither browning nor the markers of the Maillard reaction was detected. Raw butter contained only traces of CML and no detectable HMF.

The present study has clearly demonstrated that the contribution of butter to the exposure to CML and HMF is low. The detailed investigations about the effects of excessive heating on the stability of the fatty acids in butters were carried out in the course of the study but are not presented in this article. In brief, the oxidation of the winter and summer butters during heating was always very low (<3 meq of O_2/kg butter) and no significant increase of the amount of cholesterol oxide was measured. It is the high proportion of saturated fatty acids in the total fat content of butter which is responsible for its stability under heating.

The volatile compounds were not the subject of any experiments in the study. Although some of these provide flavour enhancing benefits other have been described as toxic when inhaled and are of concern to those involved in preparing food. A recent study, however, showed that the total amount of volatile α-dicarbonyl compounds found in butter heated at 100 °C was much less than that of those found in margarine, safflower oil and beef fat heated under the same conditions (Jiang, Hengel, Pan, Seiber, & Shibamoto, 2013). Heated butter should not then be included in the fat category likely to be damaging to health when ingested, or inhaled at higher temperatures.

We may therefore conclude that butter, which contains a high proportion of stable saturated fatty acids and a very low proportion of potentially toxic neoformed compounds, is perfectly suitable for cooking. At a time when the link between saturated fat and the risk of cardiovascular diseases is being called into question (Chowdhury et al., 2014) our study supports the rehabilitation of butter, raw and cooked, in a balanced diet.

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