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ORIGINAL ARTICLE

Determination of N^{ε}-(carboxymethyl)lysine in food systems by ultra performance liquid chromatography-mass spectrometry

Shima H. Assar · Catherine Moloney · Maria Lima · Ronald Magee · Jennifer M. Ames

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Abstract We report the use of ultra pressure liquid chromatography (UPLC), coupled to a tandem mass spectrometer operated in multiple reaction monitoring mode to determine the advanced glycation endproduct, N^{ε}-(carboxymethyl)lysine (CML). The procedure was applied to acid hydrolyzates of protein isolated from a range of foods (milks processed at different temperatures, butter, cheese, infant formulae, bread, raw and cooked minced beef and olive oil). Highest levels of CML were determined in white bread crust (15.2 ± 0.63 mmol/mol Lys), wholemeal bread crust (13.1 ± 0.61 mmol/mol Lys) and evaporated full-fat milk (4.86 ± 0.77 mmol/mol Lys). Lowest levels of CML were measured in raw minced beef beef (0.03 ± 002 mmol/mol Lys), raw full-fat cow's milk (0.08 ± 0.03 mmol/mol Lys) and pasteurized skimmed cow's milk (0.09 ± 0.002 mmol/mol Lys). CML could not be detected in olive oil.

Keywords N^{ε}-(carboxymethyl)lysine · Maillard reaction · Advanced glycation endproducts · Ultra performance liquid chromatography-mass spectrometry · Dietary advanced glycation endproducts

List of abbreviations

AGEs	Advanced glycation endproducts
ALEs	Advanced lipoxidation endproducts
ARP	Amadori rearrangement product
CML	N^{ε}-(carboxymethyl)lysine
FL	Fructoselysine
MR	Maillard reaction
NFPA	Nonafluoropentanoic acid
UPLC-MS	Ultra performance liquid chromatography-mass spectrometry
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid

S. H. Assar · C. Moloney · M. Lima · J. M. Ames (✉)
Human Nutrition and Health Group, School of Biological Sciences, Queen's University Belfast, David Keir Building, Stranmillis Road, Belfast BT9 5AG, UK
e-mail: j.m.ames@qub.ac.uk

R. Magee
School of Chemistry and Chemical Engineering, Queen's University Belfast, Belfast BT9 5AG, UK

Introduction

The Maillard reaction (MR) is a type of non-enzymic browning that comprises of a cascade of chemical reactions between a carbonyl compound, typically a reducing sugar, and an amino compound, for example, the epsilon amino group of lysine residues within protein (Ames 2003). The MR can be divided into three stages, known as the 'early', 'advanced' and 'final' MR (Nursten 2002) First, the MR generates a reversible Schiff's base adduct which spontaneously rearranges to form the more stable early glycation product, called the Amadori rearrangement product (ARP) (Ames 1992). The advanced stage comprises of a complex network of reactions leading to a wide range of products, including compounds termed advanced glycation endproducts (AGEs). The final stage of the MR involves the formation of melanoidins, which are nitrogen-containing macromolecular materials (Ames 2003). AGEs are formed in vivo in all tissues, at a higher rate in diabetes, and amounts accumulate with age in long-lived tissue (Baynes 1991; Vlassara et al. 1991). It has been documented that AGEs, formed both endogenously and exogenously, may contribute to the complications of diabetes and uremia

(Vlassara et al. 2002; Henle 2003). The diet is the major source of exogenously formed AGEs and it is well established that AGEs are formed in foods during heat processing and long-term storage (Förster and Henle 2003). They are generated during all types of cooking, including baking, frying and boiling, and their levels increase with the extent of heat treatment.

The amount of MR products ingested with commonly consumed foods such as heated milk, bakery products and coffee has been estimated to be 500–1,200 mg of ARPs (calculated as FL) and 25–75 mg of AGEs [mainly N^{ε}-(carboxymethyl)lysine (CML) and pyrraline], which is much higher than the total amount of AGEs in plasma and tissues (Henle 2003). CML is a well-characterized and extensively studied AGE. CML may form by different pathways in food including oxidation of FL (Ahmed et al. 1986) and the direct reaction of glyoxal, a product of the oxidation of sugar or the Schiff base (Glomb and Monnier 1995; Thornalley et al. 1999), with the ε-amino group of lysine. Although it was originally classified as an AGE (Thorpe and Baynes 2002), it was subsequently detected as a product of the reaction between lipid peroxidation products and protein (Wells-Knecht et al. 1995; Frye et al. 1998). Thus, it is also an advanced lipoxidation endproduct (ALE) (Baynes 2002). In addition, CML is generated during oxidation of ascorbic acid (Dunn et al. 1990). In contrast to several other AGEs and the ARP, CML is acid stable. This facilitates its analysis and CML has been used as an indicator of the MR in foods and biological systems (Tauer et al. 1999; Erbersdobler and Somoza 2007).

To better understand the impact of ingested dietary AGEs on health, robust methods of analysis are required (Ames 2008). A number of isotope dilution LC-MS/MS methods have been reported for the quantification of CML in biological matrices in recent years (Thornalley et al. 2003; Teerlink et al. 2004; Delatour et al. 2006; Fenaille et al. 2006; Schettgen et al. 2007). Various analytical methods are available for the detection and quantification of CML in food. These include ELISA (Tauer et al. 1999; Dittrich et al. 2006; Goldberg et al. 2004), HPLC (Delgado-Anderade et al. 2007; Drusch et al. 1999), GC-MS (Charissou et al. 2007) and LC-MS/MS (Fenaille et al. 2006). ELISA methods can be useful and accurate providing they are validated for each food matrix to which they are applied. The more selective instrumental methods, especially using LC coupled to MS, are the procedures of choice when the goal is to accurately quantify CML (Ames 2008). Reported levels of CML in foods determined by instrumental methods are summarized in Table 1.

Ultra performance liquid chromatography (UPLC) uses columns packed with solid support particles of 1.7 μm diameter. The UPLC liquid handling system operates such columns at pressures of 15,000 psi (~1,000 bar), compared to HPLC which generally uses pressure of 6,000 psi (400 bar). UPLC offers significant theoretical advantages over HPLC in resolution, speed and sensitivity of analysis, especially when coupled to a mass spectrometer (Churchwell et al. 2005).

In order to obtain accurate data for the level of CML in complex matrices like food, suitable sample preparation is also essential to remove interferences and maximize sensitivity in the final analysis. There are no reports of the effect of sample preparation on the measured amount of CML. Furthermore, most reports concerning the level of CML in foods deal mainly with milks and infant formulae (Table 1).

Therefore, the aim of this paper is to report procedures for the sample preparation of a range of foods (dairy products, infant formulae, bread, meat and olive oil) prior to the determination of CML by UPLC-MS/MS.

Materials and methods

Materials

The following chemicals were obtained from Sigma (Gillingham, UK): boric acid, 99.5%; sodium hydroxide, 98%; sodium borohydride, trifluoroacetic acid (TFA) and nonafluoropentanoic acid (NFPA) 97%. Other chemicals and their suppliers were as follows: methanol free from acetone (analytical reagent) and diethyl ether (analytical grade) (Riedel-de–Haën, Seelze, Germany), hydrochloric acid, 37%, (J.T. Baker, Devender, The Netherlands), acetonitrile, for HPLC (Chromanorm, Leuven, Belgium), chloroform (GPR) (Bios Europe, Skelmersdale, UK), trichloroacetic acid (TCA) (BDH, Poole, UK), CML (NeoMPS, Strasbourg, France), lysine (Sigma, Gillingham, UK), d_4-lysine (Cambridge Isotopes, Andover, MA, USA). d_4-CML was kindly provided by Professor S. R. Thorpe (Department of Chemistry and Biochemistry, University of South Carolina, CO, USA).

Food samples

Samples of pasteurized (full-fat and skimmed) milk, evaporated milk, white bread, wholemeal bread, butter, Cheddar cheese, olive oil, raw minced beef (fat content = 19.8%) and liquid infant formulae were purchased from a local supermarket. Raw bovine milk (Friesian breed) was provided by a local farmer in Northern Ireland. The protein, carbohydrate and fat content of the food samples (with the exception of raw milk) as stated on the packaging is given in Table 2. Values for raw milk were obtained from the Food Standards Agency (2002).

Table 1 Summary of literature reports of N^{ε}-(carboxymethyl)lysine (CML) content of various foods determined by instrumental methods

Sample	CML	Analytical method	References
Raw milk	337 ± 94 nM	LC-MS/MS	Ahmed et al. (2005)
Pasteurised milk	877 ± 47 nM	LC-MS/MS	Ahmed et al. (2005)
Pasteurised milk	16.3 ± 3.3 mg/kg protein	LC-MS/MS	Fenaille et al. (2006)
Pasteurized milk	ND[a]	HPLC	Drusch et al. (1999)
Pasteurized milk	16 mg/kg protein	LC-MS/MS	Fenaille et al. (2006)
Sterilized milk	2,066 ± 497 nM	LC-MS/MS	Ahmed et al. (2005)
Sterilized milk	343 mg/kg protein	HPLC	Drusch et al. (1999)
UHT milk	ND	HPLC	Drusch et al. (1999)
UHT milk	29–46 mg/kg protein	LC-MS/MS	Fenaille et al. (2006)
UHT milk	259 mg/kg protein	GC-MS	Buser et al. (1987)
Norwegian whey cheese	1691 mg/kg protein	HPLC	Drusch et al. (1999)
Evaporated milk	0–1,015 mg/kg protein	HPLC	Drusch et al. (1999)
Condensed milk	390 mg/kg	HPLC	Hartkopt et al. (1994)
Pasteurised sweetened flavoured milk	41–93 mg/kg protein	HPLC	Drusch et al. (1999)
Sterilized flavoured milk	164 mg/kg protein	HPLC	Drusch et al. (1999)
Chocolate milk	0–413 mg/kg protein	HPLC	Drusch et al. (1999)
Coffee cream	0–618 mg/kg protein	HPLC	Drusch et al. (1999)
Ice cream mix	150 mg/kg protein	HPLC	Drusch et al. (1999)
Powdered infant formulae	26–140 mg/kg protein	LC-MS/MS	Fenaille et al. (2006)
Powdered infant formulae	9–12 mg/kg protein	GC-MS	Charissou et al. (2007)
Hypoallergenic powdered infant formulae	135–322 mg/kg protein	LC-MS/MS	Fenaille et al. (2006)
Infant formula	30 mg/kg protein	HPLC	Hartkopt et al. (1994)
Liquid infant formulae	54–72 mg/kg protein	LC-MS/MS	Fenaille et al. (2006)
Liquid infant formulae	5–25 mg/kg protein	GC-MS	Charissou et al. (2007)
Mixed cereal for infants	11 mg/kg	HPLC	Hartkopt et al. (1994)
Cookies	5–35 mg/kg protein	GC-MS	Charissou et al. (2007)
Toast	0–13 mg/kg protein	GC-MS	Charissou et al. (2007)
Cornflakes	6–8 mg/kg protein	GC-MS	Charissou et al. (2007)
Sausage	23 mg/kg	HPLC	Hartkopt et al. (1994)
Grilled, baked or steamed beef	ND	GC-MS	Charissou et al. (2007)
Grilled, baked or steamed salmon	ND	GC-MS	Charissou et al. (2007)
Processed malt	408 mg/kg	HPLC	Hartkopt et al. (1994)
Whole meal (diet A)[b]	1,570 ± 7.1 mg/kg protein	HPLC	Delgado-Anderade et al. (2007)
Whole meal (diet B)[b]	530 ± 5.7 mg/kg protein	HPLC	Delgado-Anderade et al. (2007)

[a] Not detected

[b] Diets A and B included essentially the same foods but components of diet B were processed to minimize the formation of MRPs

Determination of moisture content of bread samples

The moisture content of bread crust and crumb was determined according to the AOAC method by drying to constant weight in an oven at 105°C (Park 1996).

Preparation of cooked minced beef

To prepare fried minced beef, raw minced beef (50 g) was fried in a preheated pan over high heat for 1 min and then at medium heat for 2 min. To prepare boiled minced beef, raw minced beef (50 g) was added to 250 mL boiling water and boiled for 3 min.

Sample preparation

Butter was melted in a water bath (50°C, 3 min) and the fat layer was removed with a Pasteur pipette. The well mixed residual material (200 µL) was delipidated by extracting with diethyl ether (4 × 200 µL). The defatted sample was used for sodium borohydride reduction. Olive oil (200 µL) was extracted with acidified water (3 × 200 µL, pH 3.5).

Table 2 Macronutrient content (%) of analyzed foods

Sample	Fat	Protein	Carbohydrate
Raw milk (Friesian breed)	3.9[a]	3.2[a]	4.6[a,b]
Pasteurised skimmed milk	0.1	3.4	5.0
Pasteurised whole milk	3.5	3.2	4.7
Evaporated whole milk	9.0	8.2	11.7
Infant formula (adapted)	3.6	1.4	7.3
Infant formula (follow-up)	3.3	1.8	9.2
Butter	82.4	1	0.8
Hard cheese	34.4	25	0.1
White bread crust	1.8	9.7	56.6
Wholemeal crust	2.8	14	49.9
White bread crumb	1.5	8.2	47.8
Wholemeal bread crumb	2.2	11	39.1
Minced beef meat	19.8	18.4	0.1

[a] Literature average value for the composition of raw bovine milk (Food Standards Agency 2002)

[b] Lactose content of raw bovine milk is about 3% (Food Standards Agency 2002)

The aqueous extract was used for acid hydrolysis. Cheese (1 g) was homogenized in sodium borate buffer (0.2 M, pH 9.2, 5 mL) prior to sodium borohydride reduction. Bread crust was separated from the crumb with a knife. The separated crust and crumb were frozen in liquid nitrogen and ground in an analytical mill (IKA, Staufen, Germany) prior to sodium borohydride reduction. Raw, boiled and fried minced beef were cut into small pieces, frozen in liquid nitrogen for 5 min, stored at −80°C overnight and freeze-dried. Freeze-dried meat was finely ground using a Minimix Paint Shaker (Merris Engineering, Maidenhead, UK). Other food samples required no preparation prior to sodium borohydride reduction.

Sodium borohydride reduction

A quantity of solid food sample, equivalent to 2 mg protein, was added to sodium borate buffer (0.2 M, pH 9.2, 500 μL). Liquid foods, equivalent to 2 mg protein, were mixed with sodium borate buffer (0.5 M, pH 9.2) to a final buffer concentration of 0.2 M. Sodium borohydride solution (2 M in 0.1 N NaOH) was added to achieve a final concentration of 0.1 M sodium borohydride. Samples (which were emulsions when the food was liquid and suspensions when the food was solid) were reduced overnight at 4°C.

Protein isolation

Three methods were used to isolate protein from sodium borohydride-reduced food samples. The first method involved adding TCA solution (60%) to achieve a final TCA concentration of 20% in the sample. Centrifugation was applied at 5,000×*g* for 10 min to precipitate the protein. The recovered protein was washed with TCA solution (20%, 500 μL). The second procedure used an ultrafiltration device with a 5 kD-molecular-mass cut-off membrane (Amicon, London, UK). The sample (skimmed milk) was centrifuged at 5,000×*g* for 10 min and the retentate was used for acid hydrolysis. The Folch method (Folch et al. 1957) was the third procedure. Chloroform–methanol (2:1, v/v, 1 mL) was added to the reduced protein suspension and protein was recovered by centrifugation at 5,000×*g* for 10 min. Protein isolated by TCA precipitation or the Folch procedure was dissolved in 0.2 M NaOH (1 mL). The volume of the retentate containing protein recovered by ultrafiltration was made up to 1 mL with distilled water for protein determination. Protein isolates were prepared in triplicate for each food.

Protein hydrolysis

Isolated protein (2 mg) was hydrolyzed in 6 M HCl (1 mL) at 110°C for 24 h in 2 mL screw closure polypropylene O-ring sealed vials (Sarstedt, Leicester, UK). Acid was removed under vacuum (Speed Vac, Thermo Electron Cooperation, Milford, MA, USA) and the dried protein hydrolysate was reconstituted in 1% aqueous TFA (1 mL).

Protein hydrolyzate clean-up

Aliquots of reconstituted protein hydrolysate (equivalent to 200 μg protein), containing appropriate amounts of d_4-lysine and d_4-CML (internal standards), were cleaned up on a 3 mL Supelco C_{18} cartridge (Sigma, Gillingham, UK). Conditioning of the C_{18} cartridge was performed with methanol (3 mL) followed by 1% aqueous TFA (3 mL). After applying the protein hydrolysate solution, amino acids were eluted with 1% TFA in methanol:water (20:80, v/v, 3 mL). The elute was dried under vacuum and reconstituted in aqueous 5 mM NFPA, (200 μL) prior to analysis by UPLC-MS.

UPLC-MS

Analyses were performed using a Waters (Manchester, UK) Acquity UPLC module interfaced to a Waters Premier XE triple quadruple mass spectrometer. Separations were performed in an Acquity UPLC™ BEC C_{18} column (2.1 × 50 mm, 1.7 μm particle size; Waters) housed in a column oven at 50°C. The mobile phases were: eluent A, i.e., aqueous 5 mM (0.13%) nonafluoropentanoic acid (NFPA) or aqueous 0.1% TFA, and eluent B, acetonitrile (ACN). Eluent A (90%) was maintained for 0.5 min

Table 3 Multiple reaction monitoring (MRM) conditions of analysis

Compound	Parent mass (Da)	Daughter mass (Da)	Collision energy (eV)
CML	205.3	83.6	19
D_4-CML	209.0	87.7	19
Lysine	146.9	83.8	17
D_4-lysine	150.8	87.7	17

followed by a linear gradient from 90% eluent A to 60% solvent A over 3.3 min. The flowrate was 0.2 mL/min. The injection volume was 7.5 µL, equivalent to 7.5 µg of protein. Separated analytes were detected by the MS operated in electrospray ionization (ESI) positive mode with multiple reaction monitoring (MRM). Significant MS operating parameters were: source temperature, 130°C; desolvation temperature, 400°C; cone voltage, 20 eV; capillary voltage, 3 kV. Table 3 details the MRM conditions of analysis. Analytes were quantified by reference to an external standard calibration curve by plotting MS area ratio against amount ratio (unlabelled compound:deuterated compound).

Statistical analysis

Statistical analysis (ANOVA) was performed to determine differences between two groups of means ($P < 0.05$). The limit of detection (LOD) was defined as the CML concentration equivalent to 3 SD of the hydrochloric acid sample blank. The LOQ was defined as the CML concentration equivalent to 10 S.D. of the hydrochloric acid sample blank.

Results

UPLC-MS conditions of analysis

This work was undertaken to establish an isotope dilution UPLC-MS/MS method of analysis to determine CML and to apply the method to a range of food samples. The choice of mobile phase modifier strongly influences the sensitivity of an MS method of analysis and two modifiers, i.e., TFA and NFPA, were compared. Figure 1 illustrates the much greater signal intensity observed for NFPA (800 and 1,470%, respectively for lysine and CML) compared to TFA. In addition, lysine and CML were completely resolved using NFPA but coeluted with TFA, using the same solvent gradient. Therefore, we selected 5 mM NFPA as the acidifier for our method. The chosen solvent gradient resulted in a short total run time of 3.3 min and an inter-injection time of 8 min.

The most intense fragment ion was selected as the daughter ion for each analyte. These ions corresponded to the same fragment ion for lysine and CML for both the unlabelled and deuterated compounds. Daughter ion masses were 4 mass units higher for the deuterated compounds, compared to the unlabelled compounds, due to the presence of 4 deuterium atoms. The cone voltage and collision energy were selected to optimize the MS response. These analytical conditions resulted in a LOD for CML of

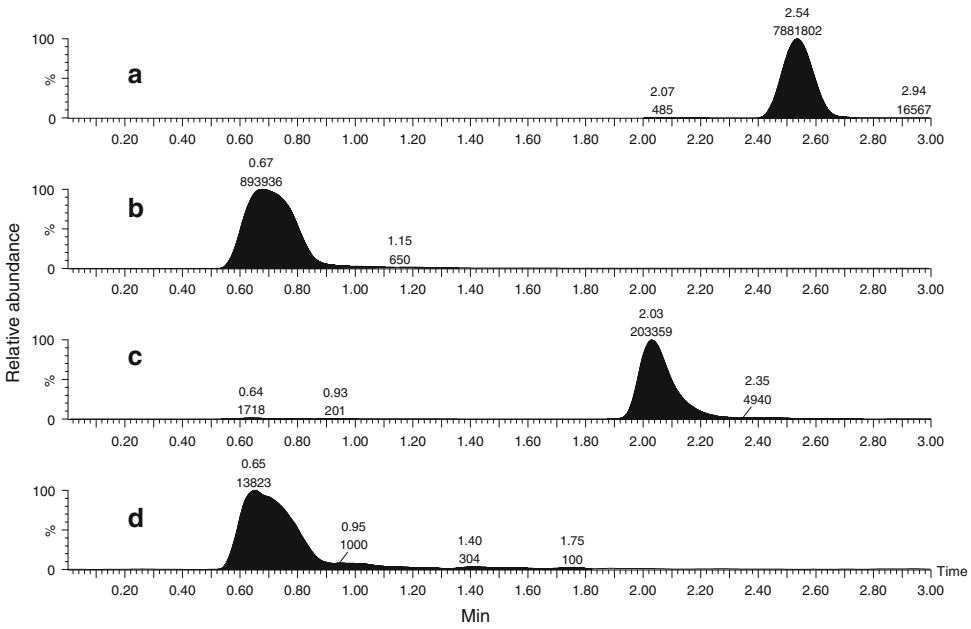

Fig. 1 Comparison of the MS response for lysine and N^{ε}-(carboxymethyl)lysine (CML) when TFA or NFPA is the acidifier in LC solvent A. **a** Lysine, 5 mM NFPA. **b** Lysine, 1% TFA. **c** CML, 5 mM NFPA. **d** CML, 1% TFA

0.009 mmol/mol Lys and a limit of quantification (LOQ) of 0.03 mmol/mol Lys.

Effect of protein isolation method on measured amount of CML

The effect of the method of protein isolation on determined CML was explored using skimmed milk. This food was chosen due to its very low lipid content (0.1%, see Table 1) and because, being a liquid food, it was amenable to protein isolation by ultrafiltration. The data obtained are given in Table 4. The measured level of CML in skimmed milk obtained when TCA precipitation was used to isolate the protein was not significantly different ($P > 0.05$) from the value obtained when protein was isolated using either the Folch extraction or ultrafiltration. However, when protein was isolated by ultrafiltration, the CML level was significantly lower ($P < 0.05$) than when the Folch extraction was applied.

Table 4 Effect of protein isolation method on measured CML level in skimmed milk[a]

Method	CML (mmol/mol Lys)
TCA precipitation	0.06 ± 0.02 (a, b)
Folch	0.08 ± 0.02 (b)
Ultrafiltration	0.04 ± 0.01 (a)

[a] Values represent mean ± SD ($n = 3$). Different letters in brackets indicate significant difference ($P < 0.05$) between different methods of protein isolation within the column

Determination of CML in food samples

Due to its ease of use and low cost, TCA precipitation was selected to isolate protein from most food samples. However, fat is not removed completely by TCA precipitation and thus a protein precipitate containing some fat results which could lead to artefactual CML formation during sample workup. Therefore, the Folch extraction was used to separate protein from foods possessing a higher fat content, i.e., butter and cheese. Ultrafiltration was not generally used because it is of limited value since it neither removes fat nor other high mass components, e.g., starch.

The CML content of the analyzed foods is summarized in Table 5. All hydrolyzates were analyzed in triplicate by UPLC-MS on two different days to estimate the inter-day reproducibility of the entire method with data being expressed as millimole CML/mole lysine. The intra-day reproducibility of the method was examined by analyzing

Table 5 N^{ε}-(carboxymethyl)lysine content of foods determined by UPLC-MS/MS in MRM mode

Food	CML (mmol/mol Lys)	CML mg/kg protein[b]	CML mg/kg food[c]
Raw milk[d]	0.08 ± 0.03	9.3	0.30
Pasteurized skimmed milk	0.09 ± 0.02[b]	10.4	0.35
Pasteurized whole milk	0.14 ± 0.02	16.2	0.52
Evaporated whole milk	4.86 ± 0.77	563	46.2
Infant formula (adapted)	1.54 ± 0.02	[e]	[e]
Infant formula (follow-up)	1.06 ± 0.03	[e]	[e]
Butter	0.32 ± 0.02	37.1	0.37
Cheddar cheese	0.20 ± 0.05	23.2	5.80
White bread crust	15.2 ± 0.63	382	37.1
Wholemeal bread crust	13.1 ± 0.61	329	46.1
White bread crumb	1.25 ± 0.24	31.4	2.58
Wholemeal bread crumb	1.61 ± 0.49	40.5	4.45
Raw minced beef	0.03 ± 0.02	3.9	0.72
Boiled minced beef	0.21 ± 0.06	27.3	5.02
Fried minced beef	0.47 ± 0.12	61.1	11.2

[a] Data are expressed as the mean ± SD ($n = 3$)

[b] Data were calculated using values for the lysine content of the relevant protein reported by Belitz et al. (2004): dairy samples (lysine content of bovine milk protein = 8.3%), beef samples (lysine content of beef muscle protein = 9.3%), bread samples (lysine content of wheat protein = 1.8%)

[c] Data were calculated using the protein content of each food reported in Table 2

[d] Protein was isolated by TCA precipitation. The value is non-significantly higher than the value quoted in Table 4 for pasteurized skimmed milk, due to the use of different batches of milk

[e] The lysine content of the protein component of the infant formulae is unknown and therefore the CML content of these samples in milligram/kilogram protein or milligram/kilogram food is not reported

individual hydrolyzates in triplicate twice on the same day. There was no significant difference ($P > 0.05$) between the data obtained on two different days or on two occasions on the same day. The average coefficient of variation of the entire method was 10%. The CML content is also expressed as milligram/kilogram protein and as milligram/kilogram food. Values for mg CML/kilogram protein were calculated by applying literature (Belitz et al. 2004) values for the lysine content of bovine milk protein (8.3%), beef muscle protein (9.3%) and wheat protein (1.8%) to the three food groups, i.e., dairy, meat, bread. Values for mg CML/kilogram protein were calculated using the protein content of the foods listed in Table 2.

Rate of conversion of lysine to CML (millimole CML/mole lysine)

Among the dairy samples, lowest levels of CML were determined for raw milk (0.08 ± 0.03 mmol/mol Lys) and pasteurized skimmed milk (0.09 ± 0.02 mmol/mol Lys). The CML content of pasteurized whole milk was almost double that for pasteurized skimmed milk and this is attributed to lipid oxidation being a source of the CML precursor, glyoxal. Cheddar cheese and butter manufacture frequently involve use of pasteurized milk (Belitz et al. 2004; Sundaram Gunasekran and Mehmet 2003) and the levels of CML in these foods (0.20 ± 0.05 and 0.32 ± 0.02 mmol/mol Lys, respectively) were of the same order of magnitude as that of pasteurized whole milk. There are few reports of CML in cheese, but, using HPLC, Drusch et al. (1999) could detect CML in only one of 50 cheese samples. The milk with the highest level of CML was evaporated milk (4.86 ± 0.77 mmol/mol Lys), in line with previous reports (Drusch et al. 1999; Charissou et al. 2007).

The CML content of liquid infant formulae was 1.54 ± 0.02 mmol/mol Lys for adapted milk and 1.06 ± 0.03 mmol/mol Lys for the follow-up product.

The level of CML in bread crust (13.1 ± 0.61 mmol/mol Lys for wholemeal bread crust and 15.2 ± 0.63 mmol/mol Lys for white bread crust) was around tenfold higher, compared to bread crumb (1.61 ± 0.49 mmol/mol Lys for wholemeal bread crumb and 1.25 ± 0.24 mmol/mol Lys for white bread crumb). There was little difference between values for wholemeal and white bread.

For this study, raw minced beef was cooked by either boiling or frying. Both the cooking procedures involved the addition of no other ingredients, such as cooking oil. The cooking time (3 min) was the same for both boiling and frying. Raw beef, as well as both cooked samples, was analyzed. CML was present in raw meat (0.03 ± 0.02 mmol/mol Lys), boiled meat (0.21 ± 0.06 mmol/mol Lys) and fried meat (0.47 ± 0.12 mmol/mol Lys).

We could not detect CML in olive oil above the level present in the blank.

CML content of protein (milligram CML/kilogram protein)

Expression of CML as a proportion of the protein isolated from the food takes account of the lysine content of the protein, which is much lower for wheat protein (1.8%) compared to dairy protein (8.2%) or muscle protein (9.3%) (Belitz et al. 2004). The calculated values for mg CML/kg protein given in Table 5 generally compare well with those reported in the literature for similar products (Table 1). This is especially the case for the milks. Highest values were calculated for evaporated milk and bread crust while the lowest value was calculated for raw minced beef. Using the lysine content of bovine milk protein, calculated values for infant formulae (not reported in Table 5) were 179 and 123 mg CML/kg protein, i.e., 2–4-fold higher than those reported by Fenaille et al. (2006) and Charissou et al. (2007). This may be attributed to the lysine content of bovine milk protein not being appropriate for the estimation of the lysine content of the protein of these samples which also contained soy protein and egg in undeclared amounts.

CML content of food (milligram CML/kilogram food)

Expression of CML as milligram/kilogram food takes account of the protein content of the food and facilitates comparison of the CML content of foods 'as consumed'. The values listed in Table 5 reveal that evaporated milk and bread crust contain very similar levels of CML and were the richest sources of CML among the foods analyzed, followed by fried minced beef. Lowest levels were calculated for raw and pasteurized milks, butter and raw minced beef.

Discussion

Trifluoroacetic acid is routinely used as a mobile phase acidifier for LC-MS because it is volatile and gives excellent resolution of sample components. However, TFA is an ionization suppressor and this results in reduced signal intensity and ultimately the sensitivity of the method. Another frequently used mobile phase modifier is formic acid. We did not assess the suitability of this material for the method reported here but while formic acid gives improved MS response compared to TFA, analyte resolution is often poor (Garcia 2005). NFPA has been used to determine CML in plasma by LC-MS (Teerlink et al. 2004). In an MRM analysis, analyte coelution inevitably leads to reduced sensitivity since the MS must

analyze coeluting compounds simultaneously but, due to its alkyl chain, NFPA is able to increase the affinity of sample amino acids for the stationary reverse phase (Garcia 2005). Compared to TFA, NFPA resulted in a vastly increased MS signal coupled with excellent resolution of lysine and CML.

The analysis of different samples (which will exert different matrix effects), coupled with the expression of LOD and LOQ in different units in different studies, makes it difficult to compare the sensitivities of various LC-MS/MS methods with the procedure reported here for the determination of CML. Nevertheless, since we could determine the low levels of CML present in raw milk, our procedure would seem to be of the same order of sensitivity as that reported by Thornalley et al. (2003). Several of the methods in the literature report run times of 20–30 min (Thornalley et al. 2003; Teerlink et al. 2004; Schettgen et al. 2007), while our method takes 3.3 min to complete the solvent programme and the inter-injection time is 8 min. Thus our method has clear advantages when many samples need to be analyzed.

Compared to ultrafiltration, we determined higher values for CML in skimmed milk when TCA precipitation or the Folch extraction were used for protein extraction. One possible reason for this might be the presence of trace impurities, e.g., heavy metals, in the TCA and Folch solvents, which would promote oxidation of trace lipid and sugar, as well as MR intermediates in the milk, leading to the formation of CML during sample workup. Although there was no significant difference between the values for CML in skimmed milk determined following isolation of the protein by TCA precipitation compared to the other protein isolation methods, values for CML increased consistently in the order ultrafiltration $<$ TCA precipitation $<$ Folch extraction, indicating that the method of protein isolation influences the measured amount of CML. Further work with a range of food samples is warranted to establish the dependence of determined CML on protein isolation method prior to developing and recommending an optimized procedure.

Several authors report that ARPs can oxidize to CML during hydrolysis in 6 M HCl at elevated temperatures (Hartkopt et al. 1994; Drusch et al. 1999), although Charissou et al. (2007) could not detect the formation of CML from FL when isolated protein was subjected to acid hydrolysis without prior reduction with sodium borohydride. To be certain that CML was not produced from ARPs in this study, all food samples were reduced with sodium borohydride prior to protein isolation.

As expected, the amount of CML determined within each food group (dairy product, bread and meat) increased with the degree of heat treatment. Raw milk contained one of the lowest measured levels of CML in the current study. The high sensitivity of isotope dilution HPLC-MS/MS resulted in a previous report of CML in raw milk (Ahmed et al. 2005). Pasteurization is a relatively mild heat treatment, typically involving heating the milk at 72°C for 15 s to destroy pathogens (Lewis and Heppell 2000). Drusch et al. (1999) reported that a heat treatment that is more severe than pasteurization is required for CML to be formed at levels that were measurable by their HPLC method. Evaporated milk was the dairy sample containing the highest level of CML expressed as millimole/mole Lysine. It was also the sample with the highest calculated CML protein content and had a similar CML content to breadcrust when expressed as milligram CML/kilogram food (Table 5). This is attributed to the high solids content and the more intense heat treatment applied, compared to pasteurized milk. Briefly, the manufacture of evaporated milk involves standardizing full fat raw milk so that is contains 8% fat and 18% solids-not-fat (Brennan 2006). Phosphate, citrate and bicarbonate salts are added to prevent coagulation of the final product especially during storage, and these salts are able to promote CML formation in a glucose–lysine model system (Ludemann and Erbersdobler 1990). The milk is then pre-heated at 120–122°C for several minutes, concentrated by vacuum evaporation at 50–60°C, and sterilized at 100–120°C for 15–20 min (Brennan 2006).

The carbohydrate:protein and lipid:protein ratios are much higher in infant formulae and this will promote CML formation, resulting in the higher observed levels of CML, compared to pasteurized milk and in agreement with the literature (Fenaille et al. 2006). A combination of factors make liquid infant formulae highly susceptible to the Maillard reaction. These include high levels of lactose and lysine, pasteurization and sterilization to ensure microbiological safety, and length of storage time before consumption. Formulae are enriched with vitamins and minerals and some of these, e.g., vitamin C and iron, will promote CML formation.

The level of CML (millimole/mole Lysine) in bread crust was the highest of all the foods analyzed in this study. During the baking of bread, the dough surface may reach a temperature of 230–250°C, leading to evaporation of water from the surface and sealing of the loaf to form a crust (Wirltz 2003) and a combination of reduced moisture and high temperature favours the MR. In comparison, the centre of the dough reaches a temperature of $\sim$100°C, or slightly higher, and possesses a higher moisture content than the crust (Wirltz 2003), leading to a tenfold reduction in CML level.

Of all the food analyzed in this study, raw minced beef contained one of the lowest levels of CML. The higher level of CML in fried meat, compared to boiled meat, is attributed to the greater extent of lipid oxidation and the higher thermal impact encountered during frying,

compared to boiling. In contrast, Charissou et al. (2007) could not detect CML in beef or salmon following cooking in a steam oven, a dry oven or grilling. The fat content of the beef and salmon were not stated but may have been around 5% for beef and 13% for salmon (Food Standards Agency 2002), compared to 19.8% for the minced beef we used (Table 2). This higher fat content, together with the use of the sensitive UPLC-MS/MS method, accounts for the detection of CML in the current study.

When considering the CML content of foods 'as consumed', it is important to take account of the amount of each food consumed as part of a regular diet. For example, although evaporated milk and breadcrust contain similar levels of CML, probably most individuals consume more breadcrust than evaporated milk on a weekly basis. Similarly, for most consumers, the weekly consumption of cheese and fried meat (with calculated CML contents of 5.8 and 11.2 mg CML/kg food) is likely to be much higher than that of evaporated milk (CML content = 46 mg/kg milk) and thus cheese and fried meat are likely to be more important contributors to dietary CML load than evaporated milk. In the past, epidemiological studies that include the collection of food frequency consumption data have recorded insufficient information about the processing undergone by the food prior to consumption to allow meaningful estimates of intake of CML (or other thermally generated dietary components).

A previous study (Goldberg et al. 2004), using an ELISA based on an anti-CML monoclonal antibody, reported CML to be present in olive oil at a level that was 24-fold higher than cow's milk, 12-fold higher than pancake and 2-fold higher than fried chicken breast. Interestingly, in the same study using the ELISA, CML was determined in butter at a level about twice that in olive oil (Goldberg et al. 2004), while, using UPLC-MS/MS, we measured CML in butter at a level similar to that in pasteurized milk. Since olive oil does not contain any protein we suggest that a matrix effect has led to an apparently high level of CML in olive oil and butter determined using the ELISA.

In conclusion, all steps of the analysis need to be carefully considered when determining AGEs in food. Initial sample treatment should minimize exposure to heat and air. Treatment of the food with sodium borohydride is recommended to reduce ARPs and lipid oxidation products to their alcohol forms. These precautions will prevent artefactual formation of CML. Precipitation of protein with TCA is appropriate for most foods but the Folch extraction is recommended for foods with a higher lipid content. Cleanup of the protein hydrolyzate on an adsorbent cartridge prior to UPLC-MS is essential to preserve the UPLC column and to protect the MS. UPLC-MS/MS in MRM mode is a rapid and sensitive tool to identify and quantify CML in food. Future work should focus on extending the method to other AGEs, applying the method to a wider range of food samples and understanding the components of the diet that are important contributors to AGE load.

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