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Synthesis, tandem MS- and NMR-based characterization, and quantification of the carbon 13-labeled advanced glycation endproduct, 6-*N*-carboxymethyllysine

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Summary. 6-*N*-carboxymethyllysine (CML), generated by the glycation and/or oxidation of lysine residues, has been measured in biological materials and food products using techniques such as ELISA, HPLC with fluorescence detection and mass spectrometry methods. Only limited information has been reported regarding the preparation of standards labeled with either deuterium, ^{13}C or ^{15}N atoms to be used as internal standards. In the present paper, a synthesis of carbon-13 labeled CML is described using 1,2-$^{13}C_2$-glyoxylic acid and 2-*N*-acetyllysine as starting materials. The resulting labeled 2-*N*-acetyl-CML was purified by HPLC-UV as a dibutyl ester. After a deprotection step, the yield was evaluated to be 53% when the reaction was conducted 17 h at 37°C. CML was extensively studied by ^{1}H- and ^{13}C-NMR and the fragments observed in the collision induced dissociation (CID) spectrum were also assigned. Finally, the standards of CML and carbon-13 labeled CML were accurately quantified based on ^{1}H-NMR and tandem MS using lysine as an internal reference.

Keywords: CML – Synthesis – Stable isotope – MS – NMR

Introduction

The term glycation (non-enzymatic glycosylation) is employed to refer to the reaction of reducing sugars with side-chain amino groups in proteins, giving rise to intermediate compounds which further undergo intramolecular rearrangements to ultimately procure the stable so-called advanced glycation endproducts (AGEs) (Monnier and Wu, 2003). So far, several AGEs have been isolated and characterized, and it appears that they belong to two categories. The first one includes adducts, *i.e.* modifications which imply a sugar fragment and a single aminoacid, while the second one is made up of crosslinks. For instance, pentosidine (Sell and Monnier, 1989) and glucosepane (Lederer and Bühler, 1999) have been characterized as lysine-arginine crosslinks whereas it was shown that vesperlysines are crosslinked products from two lysine side-chains (Nakamura et al., 1997). 5-Methyl-5-hydroimidazolone and argpyrimidine (Oya et al., 1999) have been described as arginine adducts while much attention has been devoted to 6-*N*-carboxymethyllysine (CML), initially reported as a degradation product of the Amadori compound fructoselysine (Ahmed et al., 1986). It has been suggested that, *in vivo*, AGEs contribute to pathophysiologies associated with the long term complications of diabetes and aging (Baynes and Thorpe, 2000). Interestingly, some studies evaluated the level of CML in plasma proteins of diabetic rats and humans, and the results demonstrate an increase in the number of adducts when compared to control animals or subjects. In control rats, the CML level was evaluated at 33 ± 4 adducts per 10^6 lysine whereas it doubled to 62 ± 8 CML per 10^6 lysine in the diabetic animals (Thornalley et al., 2003). In healthy humans, CML content in plasma proteins was shown to range from 15 to 42 ng per mg of protein, whereas the range was increased to 56–73 ng per mg of protein in type I diabetic patients (van de Merbel et al., 2004). In human lens proteins, an age-related accumulation of protein-bound CML was reported, and the measurements show that CML levels in 70-year-old subjects are approximately 2-fold higher than in 15-year-old subjects (Ahmed et al., 1997). It is still unclear whether dietary CML (and AGEs in general) significantly contributes to the overall CML protein load *in vivo*, but some preliminary results suggest that, in diabetic patients, an AGE-rich diet may lead to the induction of inflammatory mediators (Vlassara et al., 2002; Goldberg et al., 2004). Therefore, to gain

insight in the metabolism and toxicological significance of AGEs and CML in particular, it appears unavoidable to develop reliable and robust analytical tools for the analysis of CML in both biological samples and food products.

The determination of CML is often based on ELISA tests which procure a good sensitivity (Koschinsky et al., 1997; Wautier et al., 2004), but this technique lacks selectivity and non-specific interactions may lead to either an overestimation of the amount or even false positive responses (Miki Hayashi et al., 2002). It is, therefore, not possible to compare values from samples with different origin or composition. To circumvent this limitation, several groups have developed HPLC methods with fluorescence detection, and such a coupling was successfully applied to determine CML after *ortho*-phthaldialdehyde (OPA)- or 9-fluorenylmethoxycarbonyl (FMOC) chloride-based derivatization (Drusch et al., 1999; van de Merbel et al., 2004). To further increase the sensitivity and certainty of the determination, new methods using single- or triple-stage mass spectrometer were used to measure CML in biological samples and food products. For instance, gas chromatography-mass spectrometry (GC-MS) was employed in the selected ion monitoring mode to determine CML in biological samples as the *N*-trifluoroacetyl methyl ester (Dunn et al., 1999). A screening method, including the analysis of CML, was also described for the analysis of several AGEs by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-MS/MS) (Thornalley et al., 2003), and both CML and 6-*N*-carboxyethyllysine were simultaneously measured in human plasma proteins by LC-MS/MS (Teerlink et al., 2004). For such methods, the use of a stable isotope analogue is mandatory to ensure a reliable quantification, particularly in complex matrices such as biological samples and foodstuffs (Delatour, 2004).

So far, a few groups have reported the preparation of CML but the yields of the synthesis were not always indicated, and the spectroscopic features of the compound were incomplete (Climie and Evans, 1982; Ahmed et al., 1986; Liardon et al., 1987; Knecht et al., 1991; Reddy et al., 1995; Bergmann et al., 2001; Nagai et al., 2003). Therefore, at the present time, it is still difficult to efficiently prepare the CML standard and any isotopomer, especially with a documented and reliable purity suitable for analytical purposes. In this paper, a convenient method is described for the preparation of carbon 13-labeled CML with a 2 Da-upmass shift. The method encompasses the reaction of 1,2-$^{13}C_2$-glyoxylic acid with 2-*N*-acetyllysine followed by a purification using reverse-phase HPLC-UV after the conversion of the carboxylic acid groups into *n*-butyl esters (this step ensures good chromatographic separation of 2-*N*-acetyl-CML from unreacted 2-*N*-acetyllysine). The intermediate 1,2′-*O,O*-di-*n*-butyl-2-*N*-acetyl-6-*N*-carboxymethyllysine is characterized by collision-induced dissociation (CID), and CML further extensively studied by CID, ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR). Due to the non-specific absorption of CML in the UV-visible range, a reliable quantification of the standards based on NMR and mass spectrometry (MS) is also proposed.

Material and methods

Chemicals

2-*N*-acetyllysine, sodium cyanoborohydride ($NaCNBH_3$) and glyoxylic acid monohydrate were obtained from Sigma-Aldrich-Fluka (Buchs, Switzerland), and 1,2-$^{13}C_2$-glyoxylic acid monohydrate (>99% atom ^{13}C) was purchased from Cambridge Isotope Laboratories (Innerberg, Switzerland). Hydrochloric acid (3 N) in *n*-butanol was provided by Regis Technologies (Morton Grove, IL, USA), and Merck (Darmstadt, Germany) supplied both sodium carbonate and HC1 37%. D_2O (99.95% atom D) was from Dr. Glaser (Basel, Switzerland). Lysine monohydrate was obtained from Serva (Heidelberg, Germany).

Synthesis of 2-N-acetyl-CML and its carbon-13 labeled derivative

2-*N*-acetyllysine (30 mg, 159.4 μmol) was dissolved in 5 mL of 100 mM sodium carbonate (pH 10) with 24 mg (260.7 μmol, 1.6 eq.) of glyoxylic acid (or 1,2-$^{13}C_2$-glyoxylic acid) and 100 mg of sodium cyanoborohydride, and the resulting solution was incubated overnight (*ca.* 17 h) at 37°C. Then, the solution was split into five 1 mL-aliquots and 50 μL of HC1 37% was added to each fraction to discard residual sodium cyanoborohydride. Then, the fractions were evaporated to dryness with a rotary concentrator (SVC-100H, Savant, Farmingdale, NY, USA) prior to purification by HPLC. The synthesis was down scaled 10-fold for the preparation of 2-*N*-acetyl-($^{13}C_2$)-CML.

Purification by HPLC-UV

The dry residues were dissolved in 1.5 mL of HC1 (3 N) in *n*-butanol by vigorous mixing. Insoluble salts were eliminated by centrifugation at 14,000 rpm during 3 min, and the resulting supernatant incubated 1 h at 65°C. Then, the solution was evaporated to dryness by rotary concentration with a SVC-100H device (Savant, Farmingdale, NY, USA) prior to resuspension in 200 μL of water for further purification by HPLC. The HPLC system consisted of a HP series 1050 (Hewlett-Packard, Bremen, Germany) equipped with a binary pump. The column was a Symmetry-Prep C18 (7.8 × 150 mm, 7 μm) from Waters (Milford, MA, USA) and the flow rate set at 2 mL/min. The wavelength of the UV-detector was selected at 214 nm. The gradient was: from 0 to 1 min: 95% of solvent A (water with 0.1% TFA); from 1 to 15 min: linear gradient to reach the ratio 20/80 for solvent A/solvent B (water/acetonitrile 80/20, containing 0.1% TFA); from 15 to 17 min: ratio 20/80 for solvent A/solvent B. Under these conditions, typical retention times were 9.2 min for l-*O*-*n*-butyl-2-*N*-acetyllysine and 12.3 min for 1,2′-*O,O*-*n*-butyl-2-*N*-acetyl-CML (Fig. 1). The fractions corresponding to 1,2′-*O,O*-*n*-butyl-2-*N*-acetyl-CML (1,2′-*O,O*-*n*-butyl-2-*N*-acetyl-($^{13}C_2$)-CML) were collected for further release of CML and ($^{13}C_2$)-CML.

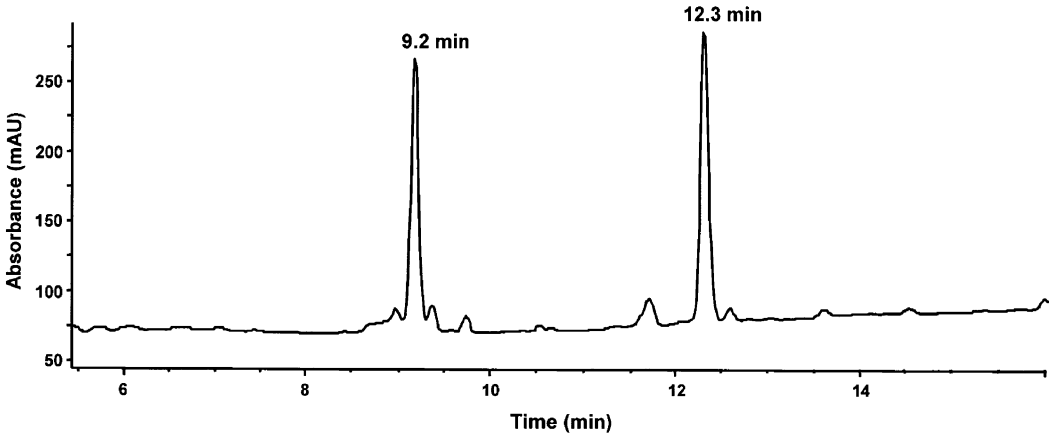

Fig. 1. Chromatographic profile of the butylated reaction mixture containing unreacted 1-*O*-*n*-butyl-2-*N*-acetyllysine (9.2 min) and l,2′-*O*,*O*-di-*n*-butyl-2-*N*-acetyl-CML (12.3 min) recorded at 214 nm

CML and ($^{13}C_2$)-CML release

Fractions collected by HPLC were pooled, and the resulting solution evaporated to dryness prior to redissolution in 6 N HC1 (1.5 mL). The acidic solution was incubated at 110°C for 24 h to eliminate both acetyl and *n*-butyl groups, and subsequently evaporated to dryness by rotary concentration (SVC-100H, Savant, Farmingdale, NY, USA). The dry residue (17.2 mg for CML, yield 53%) was dissolved in appropriate solvents for a comprehensive examination by tandem-MS, ^{1}H- and ^{13}C-NMR.

Mass spectrometry

l,2′-*O*,*O*-di-*n*-butyl-2-*N*-acetyl-CML, CML and ($^{13}C_2$)-CML were analyzed by electrospray in the positive mode using a TSQ Quantum instrument (Thermo, San José, CA, USA). The compounds were first dissolved in a mixture of acetonitrile/water 60/40 containing 0.1% formic acid. Spectra were recorded by infusing the solutions under a continuous 5 μL/min-flow rate. The spray voltage was set at 4.0 kV with a capillary temperature of 250°C. For collision-induced dissociation (CID) experiments, the collision gas (argon) pressure was set at 1.5 mTorr.

NMR spectroscopy

The samples (l–2 mg) subjected to NMR spectroscopic analysis were prepared in Wilmad 520-1A (2.5 mm o.d.) NMR tubes supplied by Sigma-Aldrich (St Louis, MO, USA), and dissolved in 180 μL of D_2O. The spectra were recorded on a Bruker DPX-360 apparatus equipped with a broadband multinuclear z-gradient probehead, at 360.13 MHz for the proton and 90.56 MHz for the carbon-13 nucleus. The probe temperature was 21°C for the recording of the ^{1}H-spectrum and slightly higher for the ^{13}C-spectra due to heteronuclear composite pulse decoupling. The chemical shifts (in parts per million) are expressed with respect to tetramethylsilane (TMS) as a reference. The assignment of the proton and carbon signals was based on the data of ^{1}H- and ^{13}C-1D-spectra, DEPT 135 spectrum and HSQC (two-dimensional inverse detected ^{13}C–^{1}H heteronuclear direct bond shift correlation with gradient selection) and HETCOR (heteronuclear correlation by polarization transfer) as well as HMBC (two-dimensional inverse detected ^{13}C–^{1}H heteronuclear long-range shift correlation with gradient selection) experiments. HSQC experiment was optimized for coupling constants at 140 Hz whilst the delay for the evolution of the long range coupling was set at 0.0625 s ($J = 8$ Hz) for the HMBC experiment.

Results

Synthetic route and optimisation of the procedure

The purpose of the present work was to design a convenient method for the preparation of stable isotope labeled CML to be used as an internal standard for an accurate quantification by isotope dilution liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). So far, a few approaches were reported in the literature for the synthesis of CML, but limited information was provided regarding the characterization of the compound and yields were seldom mentioned. Ahmed et al. (1986) reported the synthesis of CML by incubating 2-*N*-formyllysine in the presence of iodoacetic acid, but the reaction time was long (40 h) and the yield 30%. The compound was characterized by electron impact MS of CML derivatized as the 2,6-*N*,*N*-diacetyl-CML 1,2′-dimethyl ester. Other groups prepared CML by using glyoxylic acid (Liardon et al., 1987; Knecht et al., 1991; Reddy et al., 1995; Nagai et al., 2003) but yields were not always reported and the quality of the final compound was evaluated by electron impact only (*vide supra*). The purification procedure usually involves tedious ion-exchange chromatography to first isolate both CML and lysine from other compounds, and then separate CML from lysine (Ahmed et al., 1986). As an alternative, Teerlink et al. have used reverse-phase HPLC to recover deprotected CML and 6-*N*-carboxyethyllysine (Teerlink et al., 2004); however, based on our experience, the chromatographic separation of CML from unreacted lysine is not very efficient, especially when these compounds are loaded in high amounts onto the column for attempting to obtain observable UV signals. Our approach encompasses the reaction of 1,2-$^{13}C_2$-glyoxylic acid with 2-*N*-acetyllysine prior to

Table 1. Time course formation of 2-*N*-acetyl-CML incubated with glyoxylic acid in the presence of sodium cynaoborohydride (the ratio 2-*N*-acetyl-CML/2-*N*-acetyllysine was evaluated chromatographically on the basis of peak areas)

Incubation time (h)	Ratio 2-*N*-acetyl-CML/2-*N*-acetyllysine
1	0.84
2	0.81
4	1.01
6	1.10
17	1.16
70	1.94

purification by reverse-phase HPLC-UV and final release of ($^{13}C_2$)-CML by incubation in 6 N HC1. CML is a very polar compound poorly retained on an octadecylsilyl silica phase; thus, it was converted into the l,2′-di-*n*-butyl ester prior to purification by HPLC, and the retention time of the derivative was high enough to ensure both an efficient separation from the unreacted 1-*O*-*n*-butyl-2-*N*-acetyllysine (Fig. 1), and a suitable final purity. The hydrochloric acid-based elimination of the acetyl and *n*-butyl groups does not require any material- and time-consuming purification steps since hydrochloric acid, resulting acetic acid and *n*-butanol are simply eliminated by rotary evaporation. With these conditions, the compound of interest is obtained with a good yield (53%), and both MS and NMR experiments demonstrate that a satisfactory purity was achieved for analytical purposes (*vide infra*). The influence of the incubation time on the formation of 2-*N*-acetyl-CML was studied, and our results (Table 1) indicate that the reaction of glyoxylic acid on the amino group of the side chain of lysine is initially fast with a rapid slow down of the process. After a 1–2 h incubation at 37°C, approximately 45% of 2-*N*-acetyllysine is converted into 2-*N*-acetyl-CML whereas 70 h are required to reach a 66% conversion. For reasons of convenience, we have chosen to use an overnight (*ca.* 17 h) incubation; with this incubation time, 53% of 2-*N*-acetyllysine is transformed into 2-*N*-acetyl-CML. Extending the incubation time by 53 hours yielded only an additional 13% conversion. The incubation of 2-*N*-acetyllysine and glyoxylic acid at a higher temperature (50°C) was abandoned since it enhanced the formation of byproducts observed in the chromatographic profile (data not shown).

Fragmentation of 1,2′-O,O-di-n-butyl-2-N-acetyl-CML by CID

The MS analysis of l,2′-*O*,*O*-di-*n*-butyl-2-*N*-acetyl-CML by electrospray in the positive mode gives rise to a prominent ion at m/z 359, corresponding to the *pseudo*-molecular ion $[M+H]^+$ of the compound of interest. When fragmented in the collision cell, this ion leads to the formation of various product ions. The protonation of the nitrogen atom in position 2 of the ion $[M+H]^+$ initiates a rearrangement of the acetyl group with a loss of CH_2CO ($\Delta M = -42$ amu) and the concomitant formation of the ion at m/z 317 (Fig. 2). The proton transfer from the hydrogen N-2 to an oxygen of the carboxylic group in the position 2′ initiates another rearrangement leading to the loss of acetic acid ($\Delta M = -60$ amu) and the formation of the ion m/z 257 with the protonation of the nitrogen N-6. When transferred to the keto group of the –COOH moiety in the position 1 of the ion at m/z 257, the proton is likely to mediate the loss of *n*-butylformate ($\Delta M = -102$ amu) with the subsequent formation of the ion at m/z 155. From the ion at m/z 257, the loss of formic acid can also occur and procure the ion at m/z 211. The loss of 56 amu for the transition observed from the *pseudo*-molecular ion to the ion at m/z 303 can be rationalized in terms of an elimination of *n*-but-1-ene from the –COOH moiety in position 1. Several tautomeric forms of the ion at m/z 303 can be drawn to explain the formation of the ions at m/z 243 and 198 with losses of acetic acid ($\Delta M = -60$ amu) and $(HO)_2CH–NH–CO–CH_3$ ($\Delta M = -105$ amu), respectively.

Fragmentation of CML and ($^{13}C_2$)-CML by CID

The analysis of CML or ($^{13}C_2$)-CML by electrospray MS in the positive mode provides a spectrum with a prominent ion at m/z 205 or 207 respectively, corresponding to the *pseudo*-molecular ion $[M+H]^+$. The fragmentation of the ion $[M+H]^+$ by tandem-MS gives rise to five major product ions at m/z 187, 159, 142, 130 and 84 for CML (Fig. 3). The transition m/z 205 → 187 ($\Delta M = -18$ amu) is rationalized in terms of a loss of water with a subsequent loss of carbon monoxide (product ion at m/z 159), leading to the formation of the carbocation with the charge centered on the carbon C-2. The carbocation at m/z 159 may rearrange to procure the tautomeric form with the amino group protonated in the position 2 which further decomposes by eliminating ammonia to generate the ion at m/z 142. This scheme is supported by the fragmentation pattern of ($^{13}C_2$)-CML which procures product ions at m/z 189, 161 and 144, indicating that the 6-*N*-carboxymethyl group is still present in the structures of these ions. The ion $[M+H]^+$ may also be protonated on the nitrogen atom in the position 6, and decompose by eliminating 2-aminoethanoic acid to form the product ion

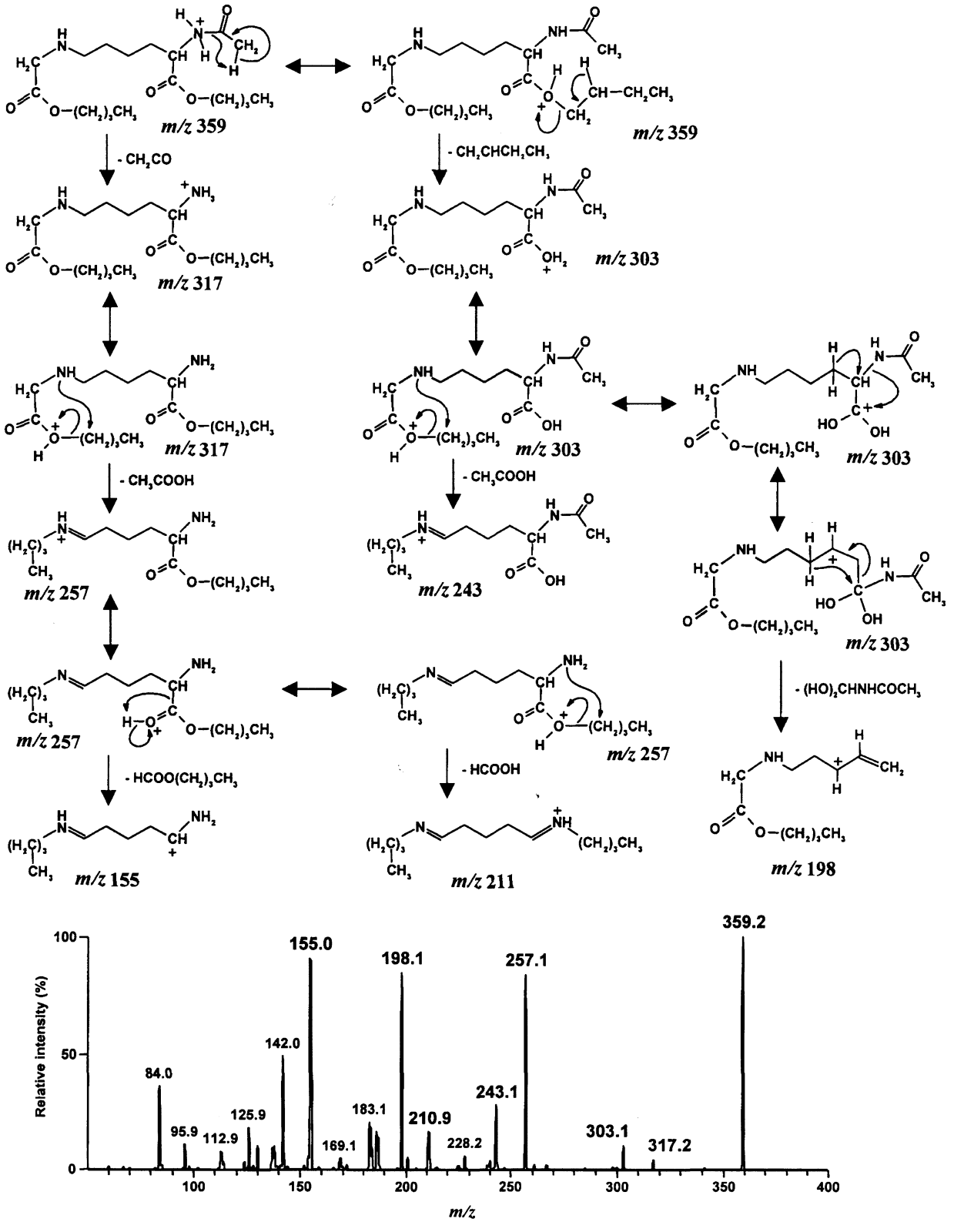

Fig. 2. CID-spectrum of 1,2′-*O,O*-di-*n*-butyl-2-*N*-acetyl-CML recorded at 30 eV (bottom), and postulated fragmentation pathway of the protonated molecular ion at m/z 359 (top)

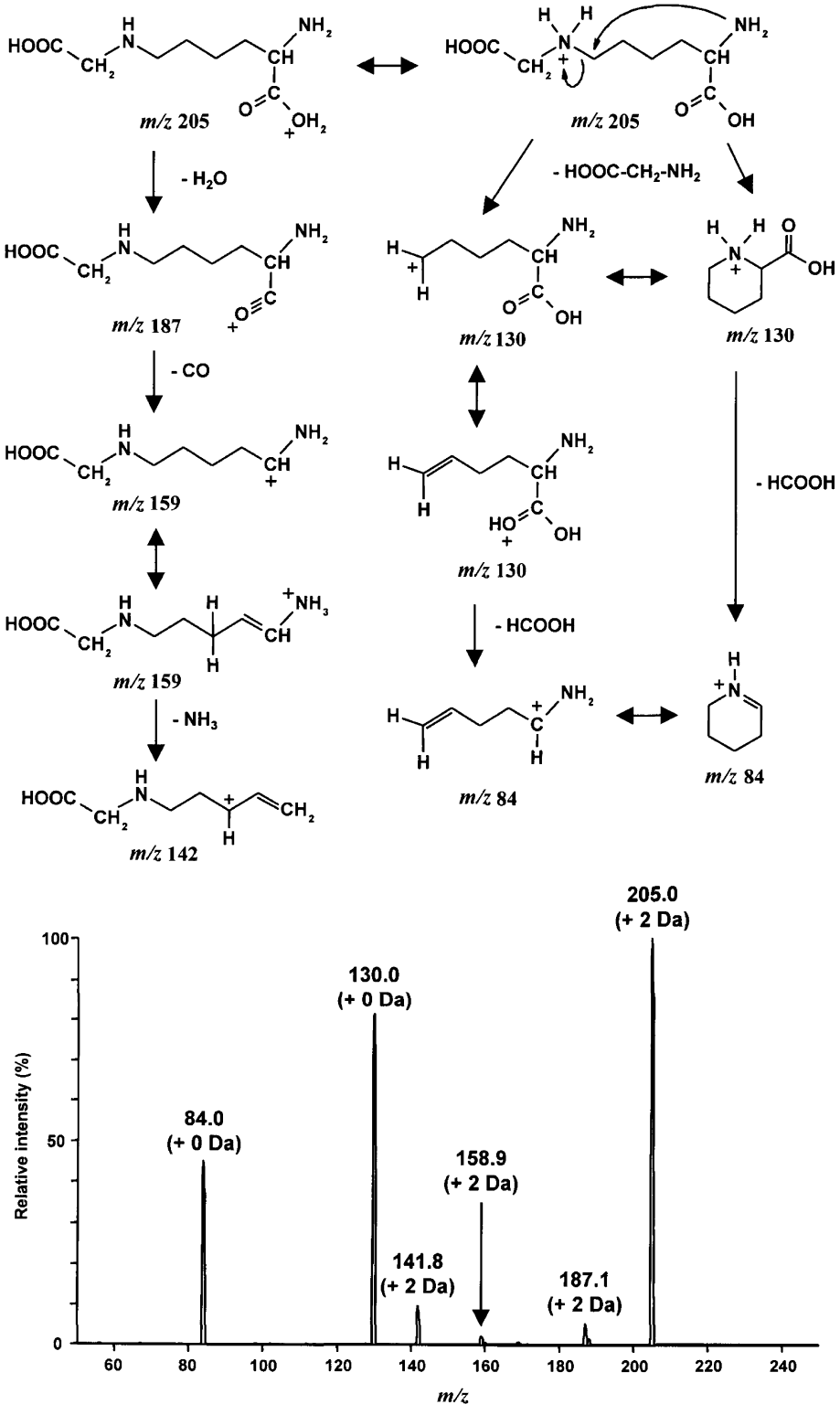

Fig. 3. CID-spectrum of CML recorded at 12 eV (bottom), and postulated fragmentation pathway of the protonated molecular ion at m/z 205 (top). The upmass shift observed in the CID-spectrum of ($^{13}C_2$)-CML is indicated in brackets

at m/z 130. The loss of the two glyoxylic acid-originating carbon atoms is confirmed by the CID-spectrum of ($^{13}C_2$)-CML which does not show any upmass shift of the product ion at m/z 130. The product ion at m/z 130 can further decompose by elimination of formic acid to give rise to the product ion at m/z 84. Alternatively, based on

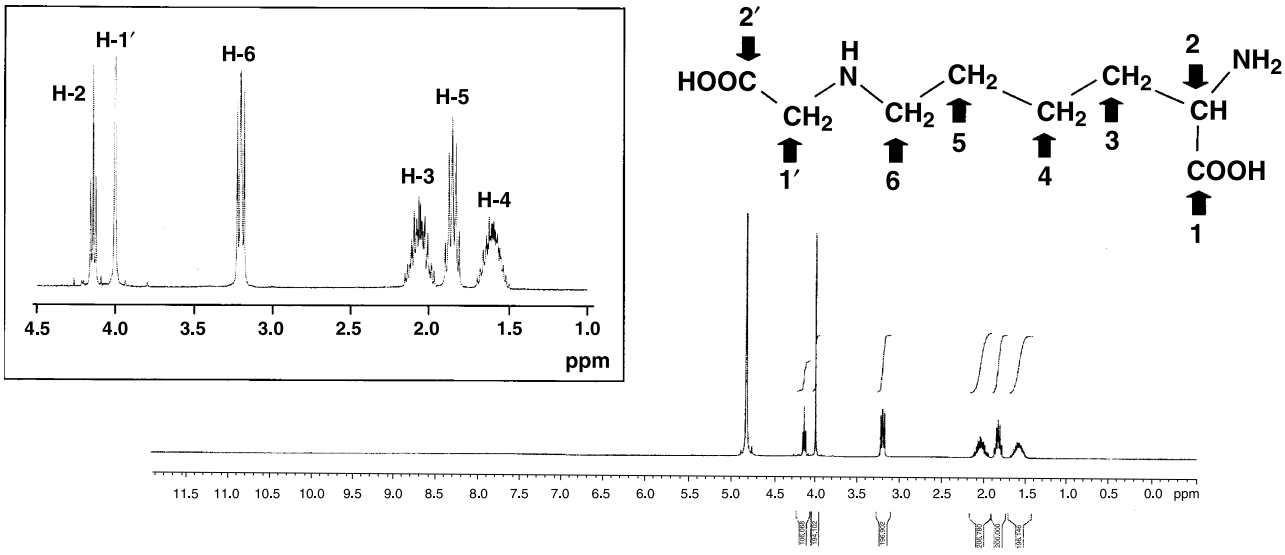

Fig. 4. Proton-NMR 360 MHz spectrum of CML in D_2O recorded at 21°C (chemical shifts are expressed with respect to TMS)

previous observations (Dookeran et al., 1996; Yalcin and Harrison, 1996; Fenaille et al., 2004), it may be suggested that the decomposition of the ion at m/z 205 undergoes a simultaneous cyclization to lead to six-membered ring structures for the ions at m/z 130 and 84.

NMR analysis of CML

The ^{1}H-spectrum of CML, recorded in D_2O, exhibits six resonance signals at $\delta = 1.61$, 1.85, 2.06, 3.21, 4.02 and 4.15 ppm whose relative intensities are 2:2:2:2:2:1, respectively (Fig. 4). This profile unambiguously demonstrates that the signal at $\delta = 4.15$ ppm should be assigned to the proton H-2. Further support for this assignment is obtained on the basis of the multiplicity of the signal which appears as a triplet, corresponding to the ^3J coupling with the two hydrogen atoms in the position 3. The two protons H-l′ do not couple with any other hydrogen atom, and the pertaining signal should appear as a singlet. Thus, the hydrogen atoms at position 1′ are assigned to the signal at $\delta = 4.02$ ppm, which is the only singlet observed in the ^{1}H-spectrum. The assignment of the two protons H-6 is inferred from the multiplicity of the resonance signals within range 3.5–1.0 ppm. The signal of the protons H-6 should appear as a triplet because the ^3J coupling with the two protons H-5 is the only one detectable; consequently, the signal at $\delta = 3.21$ ppm is assigned to the hydrogen atoms H-6. Further assignment of the hydrogen atoms is obtained on the basis of the examination of both HSQC (one-bond ^{13}C–^{1}H scalar correlated 2D NMR experiment) and HMBC (long-range ^{13}C–^{1}H scalar correlated 2D NMR experiment) 2D-maps. Inspection of the HSQC and HETCOR maps show three cross-peaks between the proton signals at $\delta = 4.15$, 4.02 and 3.02 ppm and the carbon signals at $\delta = 53.2$, 47.9 and 47.6 ppm, respectively (Fig. 5). Therefore, the carbon atoms C-2, C-6 and C-l′ are assigned to the resonance signals at $\delta = 53.2$, 47.6 and 47.9 ppm, respectively. The assignment of the carbon C-2 is supported by the DEPT experiment (not shown) which shows only one positive signal at $\delta = 53.2$ ppm. In the HMBC map (Fig. 6), one cross-peak is observed between the proton signal at $\delta = 4.02$ ppm (H-l′) and the carbon signal at $\delta = 169.6$ ppm, whilst another cross-peak is found between the signal at $\delta = 4.15$ ppm (H-2) in the proton scale with the signal at $\delta = 172.6$ ppm in the carbon scale. These correlations demonstrate that the quaternary carbon C-1 is resonating at $\delta = 172.6$ ppm, and the C-2′ at $\delta = 169.9$ ppm. This is supported by the DEPT spectrum which does not exhibit any signal within the 180–160 ppm range. In the HMBC map, a cross-peak is observed between the carbon signal at $\delta = 53.2$ ppm (C-2) and the proton signal at $\delta = 2.06$ ppm, demonstrating that the latter signal is due to the protons H-3. Consequently, the signal at $\delta = 29.8$ ppm is assigned to the carbon in the position 3 (cross-peak between the signal of protons H-3 and the carbon signal at $\delta = 29.8$ ppm in the HSQC). The HMBC map exhibits a low-intensity correlation between the two H-3 protons at $\delta = 2.06$ ppm and the carbon signal at $\delta = 21.9$ ppm. This could be rationalized in terms of the occurrence of a ^3J coupling between the protons H-3 and the carbon C-4. Therefore, the signal at $\delta = 21.9$ ppm is assigned to the carbon C-4. Finally, the carbon signal at $\delta = 25.5$ ppm is assigned to the carbon in

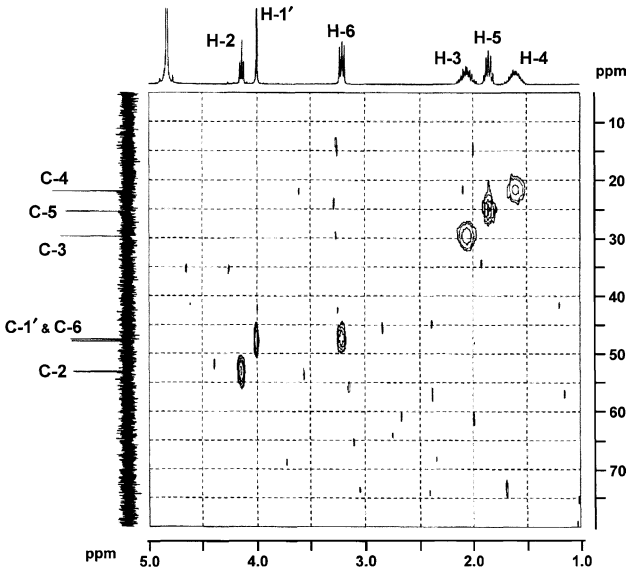

Fig. 5. Two-dimensional inverse detected ^{13}C–^{1}H heteronuclear direct bond shift correlation with gradient selection (HSQC) map of CML in D_2O (21°C)

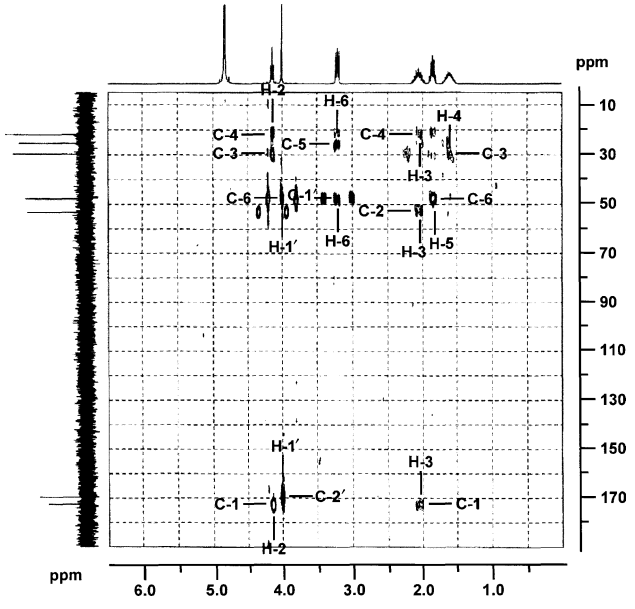

Fig. 6. Two-dimensional inverse detected ^{13}C–^{1}H heteronuclear long-range shift correlation with gradient selection (HMBC) map of CML in D_2O (21°C)

the position 5 and the signal at $\delta = 1.61$ ppm to the hydrogen atoms H-5. This is consistent with the finding that a cross-peak is observed in the HMBC map between the protons H-6 ($\delta = 3.21$ ppm) and the carbon signal at $\delta = 25.5$ ppm, which can be interpreted as a ^{2}J heteronuclear coupling. This is further supported by the occurrence of a low-intensity cross-peak between the signal at $\delta = 2.06$ ppm (protons H-5) and the carbon C-3 at

Table 2. Proton coupling constants (^{2}J in Hz) of CML

Hydrogen atoms position	Constant
2–3	6.8
3–4	n.d.
4–5	8.2
5–6	8.3

n.d. not determined

Table 3. Carbon-13 chemical shifts (δ in ppm, referenced to TMS) of CML in D_2O at room temperature

Position	Signal
1	172.6
2	53.2
3	29.8
4	21.9
5	25.5
6	47.6
1′	47.9
2′	169.7

$\delta = 29.7$ ppm, which can be rationalized in terms of a ^{3}J heteronulcear coupling. The proton coupling constants are given in Table 2 and the assignment of the ^{13}C-signals summarized in Table 3.

Quantification of the standards of CML and ($^{13}C_2$)-CML

The UV-spectrum of CML does not exhibit any characteristic absorption bands, and the molar extinction coefficient at any wavelength above 220 nm is so low that the accurate quantification of the standard is illusive with this method. To circumvent this drawback, we developed a method based on NMR and MS to estimate the amount of material (both CML and ($^{13}C_2$)-CML) obtained by the synthesis. The ^{1}H-NMR spectrum of lysine (recorded in D_2O at 21°C) exhibits two triplets at $\delta = 3.39$ and 3.01 ppm, assigned to the protons H-2 and H-6 respectively. These two resonance signals do not interfer with any other signal observed in the ^{1}H-NMR spectrum of CML (Fig. 4), and may be used as internal references for the quantification of CML. Hence, we prepared a aqueous stock solution of lysine, diluted to a concentration similar to the one estimated in the aqueous solution of CML. Equal volumes of lysine and CML solutions were mixed, dried and the dry residue dissolved in D_2O prior to ^{1}H-NMR spectrum recording. The relaxation time of the NMR experiment was optimised ($5 \times T_1$ with $T_1 = 3.2$ s), and the signals of the resulting spectrum were integrated to

calculate the molar ratio lysine:CML. The quantification of ($^{13}C_2$)-CML was carried out by LC-MS/MS (refer to Teerlink et al., 2004 for the analysis conditions) using transitions $m/z \rightarrow 130$:m/z $207 \rightarrow 130$ (13 eV) and m/z $205 \rightarrow 84$:m/z $207 \rightarrow 84$ (22 eV) to evaluate the ratio CML:($^{13}C_2$)-CML.

Discussion

Much attention has been devoted to the analysis of CML to gain insights into the role of protein glycation in pathologies such as diabetes (Dyer et al., 1993) and aging (Dunn et al., 1991). In this respect, LC-MS/MS which combines sensitivity and selectivity appears to be the technique of choice to quantitatively determine this protein modification in either biological tissues/fluids or foodstuffs. The main limitation of the method are matrix-effects which may strongly affect the response factor of the compound of interest, leading to erroneous measurements (Ikonomou et al., 1990; Choi et al., 2001; Delatour, 2004). The use of an isotope dilution method, requiring the preparation of an isotopomer, is the only way to avoid this. Several quantification methods using synthetic CML standards were described in the literature. In one case, CML was quantified by GC-MS in lens proteins but no internal standard was used to ensure an accurate measurement (Dunn et al., 1989). Interestingly, it was shown that matrix effects may also interfer in GC-MS methods, leading to lower accuracy when no isotopomer is employed as internal standard (Mottier et al., 2000). The measurements performed by Thornalley et al. (2003) for the evaluation of CML residues in both cellular and plasma proteins constitute a reliable set of data since carbon-13 hexalabeled CML was used as internal standard. Similarly, Teerlink et al. (2004) measured CML in human plasma proteins by LC-MS/MS using tetradeuterated CML for the isotope dilution. However, in these two papers, the preparation of CML (and the corresponding isotopomer) was described in scant details and no information was provided regarding both the purity and the quantification of the standard.

In the present paper, we describe the synthesis of carbon-13 labeled CML based on the reaction of 1,2-$^{13}C_2$-glyoxylic acid with 2-*N*-acetyllysine, which avoids the preparation and purification of labeled 2-*N*-acetyllysine. Both temperature and incubation time were optimised to reach a satisfactory yield in short time and it was estimated to be 53% after a 17 h-reaction time at 37°C. In addition, our results suggest that the yield can be improved to 66% with a 70 h-reaction time at the same temperature.

For the purification of the compound of interest we developed an original reverse-phase HPLC strategy which combines an efficient separation of the starting material (2-*N*-acetyllysine) and a final good purity. The retention times of both 2-*N*-acetyllysine and 2-*N*-acetyl-CML as well as the detection at 214 nm were improved by converting the compounds to their *n*-butyl ester derivatives. The intermediate 1,2′-*O,O*-di-*n*-butyl-2-*N*-acetyl-CML was characterized by CID, and CML was extensively studied by CID, ^{1}H- and ^{13}C-NMR. Finally, we proposed a reliable approach for the quantification of the standards of CML and ($^{13}C_2$)-CML based on ^{1}H-NMR and MS using lysine as an internal reference. Last, it must be pointed out that the current methodology can be extended to the synthesis of other labeled modifications of lysine such as 6-*N*-carboxyethyllysine or 6-*N*-carboxymethyl-5-hydroxylysine.

Our method is of particular interest since the formation of CML may originate from various sources, leading to artifactual carboxymethylation of lysine during sample preparation if an unappropriate clean-up procedure is employed. It is assumed that CML is not only a marker of glycoxidative stress but also an indicator of lipoxidative and oxidative stresses (Thorpe and Baynes, 2002), and several pathways have been suggested to be involved in its formation. It is well established that CML is a degradation product of fructoselysine (Ahmed et al., 1986), and it is formed in the presence of glyoxal (Glomb and Monnier, 1995; Wells-Knecht et al., 1995), ascorbate (Dunn et al., 1990), fatty acids (Anderson et al., 1999). It was also observed that salts such as sodium dihydrogenphosphate, sodium diphosphate or ferric chloride may induce the carboxymethylation of lysine to give rise to CML (Drusch et al., 1999). These results suggest than the measurement of CML in complex matrices should be carried out with extreme care to avoid artifactual conversion of lysine or fructoselysine into CML during the process of sample preparation. In this regard, mass spectrometry and stable isotope standards appear as suitable tools to identify the sources of artifacts, and develop alternative procedures to accurately measure endogenous CML.

References

Ahmed MU, Thorpe SR, Baynes JW (1986) Identification of N^{ε}-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. J Biol Chem 261: 4889–4894

Ahmed MU, Brinkmann Frye E, Degenhardt TP, Thorpe SR, Baynes JW (1997) N^{ε}-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. Biochem J 324: 565–570

Anderson MM, Requena JR, Crowley JR, Thorpe SR, Heinecke JW (1999) The myeloperoxidase system of human phagocytes generates

N^{ε}-(carboxymethyl)lysine on proteins: a mechanism for producing advanced glycation end products at sites of inflammation. J Clin Invest 104: 103–113

Baynes JW, Thorpe SR (2000) Glycoxidation and lipoxidation in atherogenesis. Free Rad Biol Med 28: 1708–1716

Bergmann R, Helling R, Heichert C, Scheunemann M, Mäding P, Wittrisch H, Johannsen B, Henle T (2001) Radio fluorination and positron emission tomography (PET) as a new approach to study the *in vivo* distribution and elimination of the advanced glycation end-products N^{ε}-carboxymethyllysine (CML) and N^{ε}-carboxyethyllysine (CEL). Nahrung 45: 182–188

Choi BK, Hercules DM, Gusev AI (2001) Effect of liquid chromatography separation of complex matrices on liquid chromatography-tandem mass spectrometry signal suppression. J Chromatogr A 907: 337–342

Climie IJG, Evans DA (1982) ^{13}C-nuclear magnetic resonance spectroscopy as a probe of enzyme environment – II. Effect of solvent and pH on ^{13}C-chemical shifts in derivatized amino-acid models. Tetrahedron 38: 697–711

Delatour T (2004) Performance of quantitative analyses by liquid chromatography-electrospray ionisation tandem mass spectrometry: from external calibration to isotopomer-based exact matching. Anal Bioanal Chem 380: 515–523

Dookeran NN, Yalcin T, Harrison AG (1996) Fragmentation reactions of protonated α-amino acids. J Mass Spectrom 31: 500–508

Drusch S, Faist V, Erbersdobler HF (1999) Determination of N^{ε}-carboxymethyllysine in milk products by a modified reversed-phase HPLC method. Food Chem 65: 547–553

Dunn JA, Patrick JS, Thorpe SR, Baynes JW (1989) Oxidation of glycated proteins: age-dependent accumulation of N^{ε}-(carboxymethyl)lysine in lens proteins. Biochemistry 28: 9464–9468

Dunn JA, Ahmed MU, Murtiashaw MH, Richardson JM, Walla MD, Thorpe SR, Baynes JW (1990) Reaction of ascorbate with lysine and protein under autoxidizing conditions: formation of N^{ε}-(carboxymethyl)lysine by reaction between lysine and products of autoxidation of ascorbate. Biochemistry 29: 10964–10970

Dunn JA, McCance DR, Thorpe SR, Lyons TJ, Baynes JW (1991) Age-dependent accumulation of N^{ε}-(carboxymethyl)lysine and N^{ε}-(carboxymethyl)hydroxylysine in human collagen. Biochemistry 30: 1205–1210

Dyer DG, Dunn JA, Thorpe SR, Bailie KE, Lyons TJ, McCance DR, Baynes JW (1993) Accumulation of Maillard reaction products in skin collagen in diabetes and aging. J Clin Invest 91: 2463–2469

Fenaille F, Tabet J-C, Guy PA (2004) Study of peptides containing modified lysine residues by tandem mass spectrometry: precursor ion scanning of hexanal-modified peptides. Rapid Commun Mass Spectrom 18: 67–76

Glomb MA, Monnier VM (1995) Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. J Biol Chem 270: 10017–10026

Goldberg T, Cai W, Peppa M, Dardaine V, Suresh Baliga B, Uribarri J, Vlassara H (2004) Advanced glycoxidation end products in commonly consumed foods. J Am Diet Assoc 104: 1287–1291

Ikonomou MG, Blades AT, Kebarle P (1990) Investigations of the electrospray interface for liquid chromatography/mass spectrometry. Anal Chem 62: 957–967

Knecht KJ, Dunn JA, McFarland KF, McCance DR, Lyons TJ, Thorpe SR, Baynes JW (1991) Effect of diabetes and aging on carboxymethyllysine levels in human urine. Diabetes 40: 190–196

Koschinsky T, He C-J, Mitsuhashi T, Bucala R, Liu C, Buenting C, Heitmann K, Vlassara H (1997) Orally absorbed reactive glycation products (glycotoxins): an environmental risk factor in diabetic nephropathy. Proc Natl Acad Sci USA 94: 6474–6479

Lederer MO, Buhler HP (1999) Cross-linking of proteins by Maillard processes – Characterization and detection of a lysine-arginine cross-link derived from D-glucose. Bioorg Med Chem 7: 1081–1088

Liardon R, de Weck-Gaudard D, Philippossian G, Finot P-A (1987) Identification of N^{ε}-carboxymethyllysine: a new Maillard reaction product, in rat urine. J Agric Food Chem 35: 427–431

Miki Hayashi C, Nagai R, Miyazaki K, Hayase F, Araki T, Ono T, Horiuchi S (2002) Conversion of Amadori products of the Maillard reaction to N^{ε}-(carboxymethyl)lysine by short-term heating: possible detection of artifacts by immunohistochemistry. Lab Invest 82: 795–808

Monnier VM, Wu X (2003) Enzymatic deglycation with amadoriase enzymes from *Aspergillus* sp. as a potential strategy against the complications of diabetes and aging. Biochem Soc Trans 31: 1349–1353

Mottier P, Parisod V, Turesky RJ (2000) Quantitative determination of polycyclic aromatic hydrocarbons in barbecued meat sausages by gas chromatography coupled to mass spectrometry. J Agric Food Chem 48: 1160–1166

Nagai R, Araki T, Miki Hayashi C, Hayase F, Horuichi S (2003) Identification of N^{ε}-(carboxyethyl)lysine, one of the methylglyoxal-derived AGE structures, in glucose-modified protein: mechanism for protein modification by reactive aldehydes. J Chromatogr B 788: 75–84

Nakamura K, Nakazawa Y, lenaga K (1997) Acid-stable fluorescent advanced glycation end products: vesperlysines A, B, and C are formed as crosslinked products in the Maillard reaction between lysine or proteins with glucose. Biochem Biophys Res Commun 232: 227–230

Oya T, Hattori N, Mizuno Y, Miyata S, Maeda S, Osawa T, Uchida K (1999) Methylglyoxal modification of protein. Chemical and immunochemical characterization of methylglyoxal-arginine adducts. J Biol Chem 274: 18492–18502

Reddy S, Bichler J, Wells-Knecht KJ, Thorpe SR, Baynes JW (1995) N^{ε}-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. Biochemistry 34: 10872–10878

Sell DR, Monnier VM (1989) Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process. J Biol Chem 264: 21597–21602

Teerlink T, Barto R, ten Brink HJ, Schalwijk CG (2004) Measurement of N^{ε}-(carboxymethyl)lysine and N^{ε}-(carboxyethyl)lysine in human plasma protein by stable-isotope-dilution tandem mass spectrometry. Clin Chem 50: 1222–1228

Thornalley PJ, Battah S, Ahmed N, Karachalias N, Agalou S, Babaei-Jadidi R, Dawnay A (2003) Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. Biochem J 375: 581–592

Thorpe SR, Baynes JW (2002) CML: a brief history. Int Cong Series 1245: 91–99

van de Merbel NC, Mentink CJAL, Hendriks G, Wolffenbuttel BHR (2004) Liquid chromatographic method for the quantitative determination of N^{ε}-carboxymethyllysine. J Chromatogr B 808: 163–168

Vlassara H, Cai W, Crandall J, Goldberg T, Oberstein R, Dardaine V, Peppa M, Rayfield EJ (2002) Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. Proc Natl Acad Sci USA 99: 15596–15601

Wautier M-P, Boulanger E, Guillausseau P-J, Massin P, Wautier J-L (2004) AGEs, macrophage colony stimulating factor and vascular adhesion molecule blood levels are increased in patients with diabetic microangiopathy. Thromb Haemost 91: 879–885

Wells-Knecht KJ, Zyzak DV, Litchfield JE, Thorpe SR, Baynes JW (1995) Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. Biochemistry 34: 3702–3709

Yalcin T, Harrison AG (1996) Ion chemistry of protonated lysine derivatives. J Mass Spectrom 31: 1237–1243

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