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Short Communication

Determination of N^{ϵ}-carboxymethyllysine by a reversed-phase high-performance liquid chromatography method

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Abstract

To determine N^{ϵ}-carboxymethyllysine (CML) in foods a RP-HPLC method after derivatisation with *o*-phthaldialdehyde was developed. To prevent an overestimation of the CML values by the formation of CML from Amadori products during hydrolysis a borohydride reduction precedes the hydrolysis. A comparison of the determination with and without reduction shows that during hydrolysis 2–12 times more CML than originally present can be formed. With the analytical conditions described in this paper it is possible to obtain measurable amounts of this trace substance in spite of the much higher values for other amino acids. The CML contents in selected processed food items varied between 11 mg in a preparation from mixed cereals for infants to 408 mg/kg protein in a processed malt product. CML is suitable as indicator of heat damage in processed or stored foods, being more stable than the Amadori compounds determined, *e.g.* in form of furosine.

1. Introduction

We recently published a comparison of modified HPLC and ion-exchange chromatography methods for the determination of furosine [1], an indicator for Maillard-type lysine damage. Another helpful indicator for heat damage is N^{ϵ}-carboxymethyllysine (CML), an oxidative derivative of the Amadori-compound ϵ-fructoselysine [2,3]. Additionally, CML is formed from the reaction of lysine and ascorbate [4,5] (Fig. 1).

Analysis of CML is complicated by the fact that CML is also formed from the Amadori products during acid hydrolysis normally applied for amino acid determination in foods, resulting in an overestimation of the CML values. However, by reducing the Amadori products it is possible to prevent the formation of CML during hydrolysis [6,7].

Methods for the determination of CML are gas–liquid chromatography of the heptafluorobutyryl isobutyl ester of CML [8,9] or GC–MS methods [4,6,7] and reversed-phase HPLC methods after derivatisation of CML with *o*-phthaldialdehyde (OPA) [10,11]. In this paper we describe a specific clean-up procedure followed by HPLC which is useful to determine specifically the CML concentration, arising from food processing.

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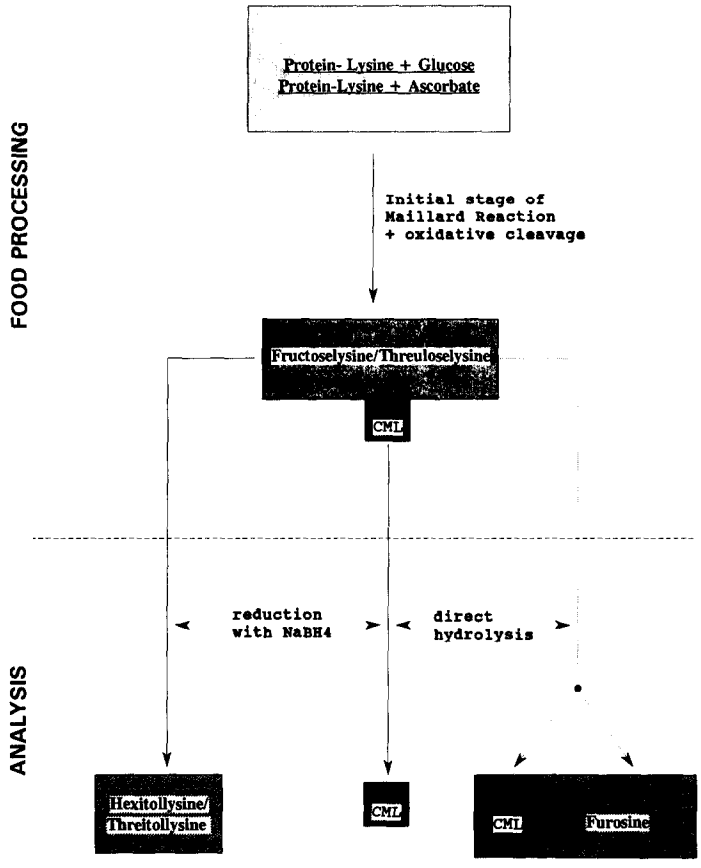

Fig. 1. Formation of CML during food processing and analysis (hydrolysis).

2. Experimental

The CML standard was synthesized by Lüdemann [10] and compared to another standard prepared by Liardon *et al.* [12]. The other reagents (all analytical grade or chromatographic grade) were obtained from Merck (Darmstadt, Germany).

An equimolar mixture of lysine monohydrochloride and glucose in an 88% (w/v) aqueous solution was used as a model sample, which was heated for 3 to 30 h, and then hydrolysed for 20 h with 7.8 *M* hydrochloric acid at 110°C.

The food samples were homogenised or pulverized before analysis. The initial masses of the food samples were chosen in a range between 2 and 5 g or ml, respectively. The sample masses were chosen according to the expected fructoselysine values in order to be sure that also high contents of fructoselysine will be reduced. Thus, all samples could be reduced with the same quantity of sodium borohydride. All foods were analysed either after direct hydrolysis or after borohydride reduction prior to hydrolysis.

The weighed samples were incubated with 20 ml of 0.2 *M* borate buffer (pH 9.1) and 10 ml of 1 *M* sodium borohydride (solved in 0.1 *M* sodium hydroxide). The incubation was continued for 3 h at room temperature. The reduced samples of solid foods were first adjusted with 50 ml 12.5 *M* hydrochloric acid to a molarity of 7.8 and then filled up to 90 ml with 7.8 *M* hydrochloric acid. The reduced samples of liquid foods were adjusted with 58 ml of 12.5 *M* hydrochloric acid and then filled up to 90 ml with 7.8 *M* hydrochloric acid. The samples without reduc-

tion were hydrolysed with 7.8 *M* hydrochloric acid (90 ml final volume). All samples were hydrolysed for 20 h under reflux. Afterwards the hydrolysates were concentrated by rotary evaporation to a volume of 10–20 ml and filled up with water to 50 ml. After filtration (Schleicher & Schüll 602H, Dassel, Germany) 5 ml of each filtrate were concentrated again by rotary evaporation nearly to dryness, resolved in 0.4 *M* borate buffer (pH 9.5) and filled up with borate buffer to make 10 ml, followed by an ultrafiltration (Schleicher & Schüll PH 79). The obtained ultrafiltrates were used for analysis after derivatisation with OPA.

The OPA reagent contained 54 mg OPA dissolved in 1 ml methanol, 50 μl 2-mercaptoethanol, and borate buffer (pH 9.5, 0.4 *M*) to make 10 ml.

CML was analysed using the reversed-phase HPLC-method after derivatisation with OPA. For the precolumn derivatization 100 μl of the standard solution or the hydrolysate were mixed with 500 μl of the OPA reagent in a 1.5-ml Eppendorf vial. This mixture was manually injected in a 20-μl sample loop (Rheodyne 7125, Cotati, USA). After a reaction time of exact 90 s the valve was switched into the inject position and the sample was injected onto the column (Spherisorb 5 C_{18} column; Promochem, Wesel, Germany). Two pumps (ConstaMetric III/IIIG), controlled by a gradient master (1601) from LDC (Gelnhausen, Germany) were used for a gradient elution at a flow-rate of 1.0 ml/min. The CML derivative was eluted with a gradient of the following buffers: (A) sodium acetate buffer (pH 6.70, 0.05 *M*)–methanol (96:4, v/v) and (B) methanol. A gradient was carried out from 15 to 70% B in 35 min, then hold for 4 min at 70% B, reset in 4 min to 15% B, and equilibrate for 12 min. The gradient was not linear but exponential ("exponent 3"). Therefore, the composition of the solvents during the gradient time is given in Table 1. The detection was evaluated with a fluorescence detector (F-1050) from Merck–Hitachi (Darmstadt, Germany), using an excitation wavelength at 340 nm and an emission wavelength at 455 nm. Data acquisition and peak integration were evaluated with an integrator C-R4A Chromatopac from Shimadzu (Duisburg, Germany).

Table 1
Composition of the solvent during the gradient time of the HPLC analysis

Time (min)	A (%)	B (%)
0	85	15
5	85	15
10	84	16
15	80	20
20	75	25
25	65	35
30	50	50
35	30	70

The whole analysis of CML lasted 55 min. Under the conditions described above clear and well separated CML peaks were obtained (Fig. 2).

3. Results and discussion

In our experiments with lysine–glucose model mixtures, treated by the reduction method with sodium borohydride, only 8–20% of the CML contents compared to the not reduced (direct hydrolysed) samples were determined (Fig. 3). This finding is very similar to results reported previously [13] comparing in the heated lysine–glucose models the samples without any hydrolysis with the not reduced, direct hydrolyzed counterparts. From the results a formation of 3.2–5.6% of fructoselysine to CML can be calculated. This is in agreement with reports of Dunn *et al.* [6] and Knecht *et al.* [7] from glycosylated biological material that a fraction of 3–5% fructoselysine undergoes oxidative cleavage to form CML during acid hydrolysis. The rate of formation during hydrolysis seems to be relatively small. However, due to the fact that the ratio of fructoselysine to CML is high a considerable amount of CML is formed.

These results demonstrate that all hydrolyses for CML must be performed after a borohydride reduction in order to transform fructoselysine to

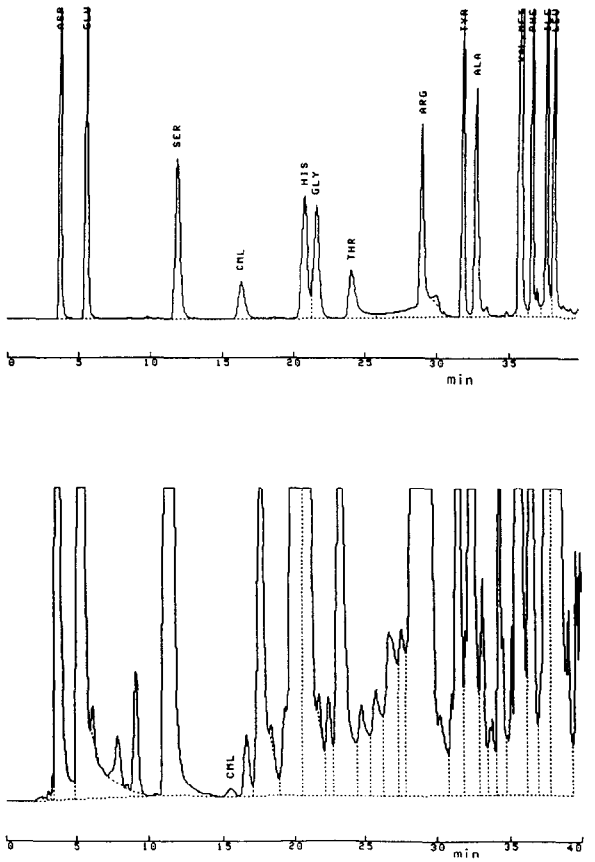

Fig. 2. HPLC chromatogram of a CML and amino acid standard and of a food sample.

hexitollysine, which does not produce CML (Fig. 1). With this procedure the formation of CML during hydrolysis can be prevented. As described elsewhere, the good correlation of $r = 0.996$ between the reduced and hydrolyzed samples and the equivalents without any hydrolysis of the heated lysine–glucose models demonstrated the accuracy of the reduction procedure [13]. Since furosine is also not formed from hexitollysine, efficiency of the reduction can be controlled by checking the absence of furosine.

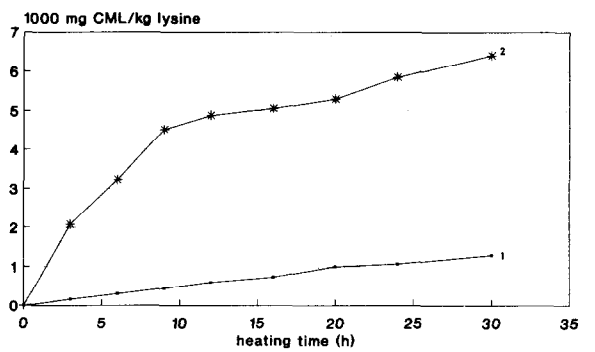

Fig. 3. Comparison of the CML levels (1000 mg CML/kg lysine) in the reduced (1) and not reduced (2) model samples.

To estimate the formation of CML during the hydrolysis in foods a comparison of not reduced (direct hydrolysed) and reduced food samples was carried out. The CML values in the food samples after reduction were only 8–55% of the data obtained in the samples without reduction (Fig. 4). All CML values obtained by the borohydride procedure were significantly lower than CML data in food samples described before. Due to the fact that the ratio of formation of CML during acid hydrolysis is not constant it seems to be not possible to correct mathematically older data from CML analyses of not reduced samples.

The lowest CML contents of the analyzed foods were determined in a preparation from mixed cereals for infants (11 mg) and in a sausage (23 mg CML/kg protein). This indicates a short and slight heat treatment during the processing. On the other hand, the highest CML contents were obtained in a condensed milk (390

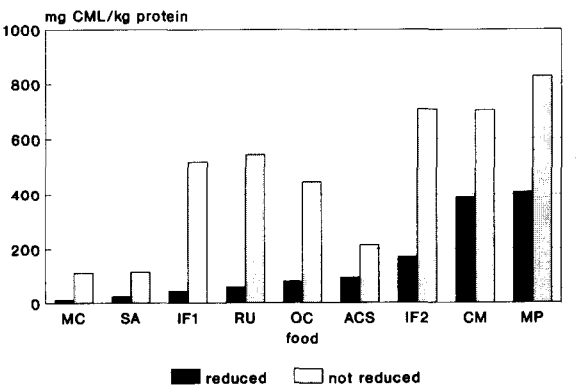

Fig. 4. Comparison of the CML levels (mg CML/kg protein) in the reduced and not reduced food samples. MC = Preparation from mixed cereals for infants; SA = sausage; IF1 = infant formula 1; RU = rusk; OC = oat cereals; ACS = asparagus creme-soup; IF2 = infant formula 2; CM = condensed milk; MP = processed malt product.

mg) and in a processed malt product (408 mg CML/kg protein), foods which were treated severely during processing. This confirms the conclusion that CML is suited as an indicator for heat damage in foods.

References

[1] J. Hartkopf and H.F. Erbersdobler, *J. Chromatogr.*, 635 (1993) 151–154.
[2] M.U. Ahmed, S.R. Thorpe and J.W. Baynes, *Fed. Proc.*, 44 (1985) 1621.
[3] M.U. Ahmed, S.R. Thorpe and J.W. Baynes, *J. Biol. Chem.*, 261 (1986) 4889–4894.
[4] J.A. Dunn, M.U. Ahmed, M.H. Murtiashaw, J.M. Richardson, M.D. Walla, S.R. Thorpe and J.W. Baynes, *Biochemistry*, 29 (1990) 10 964–10 970.
[5] U. Albrecht, J. Hartkopf and H.F. Erbersdobler, *Ernährungsumschau*, 39 (1992) 164.
[6] J.A. Dunn, D.R. McCance, S.R. Thorpe, T.J. Lyons and J.W. Baynes, *Biochemistry*, 30 (1991) 1205–1210.
[7] K.J. Knecht, J.A. Dunn, K.F. McFarland, D.R. McCance, T.J. Lyons, S.R. Thorpe and J.W. Baynes, *Diabetes*, 40 (1991) 190–196.
[8] W. Büser, H.F. Erbersdobler and R. Liardon, *J. Chromatogr.*, 387 (1987) 515–519.
[9] W. Büser and H.F. Erbersdobler, *Milchwisssenschaft*, 41 (1986) 780–785.
[10] G. Lüdemann, *Untersuchungen über Entstehen und Vorkommen von N^{ϵ}-Carboxymethyllysin in Lebensmitteln* (*Agr. Sci. Dissertation Series*, Vol. 6), Institut für Humanernährung und Lebensmittelkunde, University of Kiel, Kiel, 1989.
[11] R. Badoud, F. Hunston, L. Fay and G. Pratz, in P.A. Finot, H.U. Aeschbacher, R.F. Hurrell and R. Liardon (Editors), *The Maillard Reaction in Food Processing, Human Nutrition and Physiology* (*Advances in Life Sciences*), Birkhäuser, Basel, Boston, Berlin, 1990, pp. 79–84.
[12] R. Liardon, D. de Weck-Gaudard, G. Philippossian and P.-A. Finot, *J. Agric. Food Chem.*, 35 (1987) 427–431.
[13] J. Hartkopf and H.F. Erbersdobler, *Z. Lebensm.-Unters.-Forsch.*, 198 (1994) 15–19.