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Gas chromatographic determination of N-carboxymethyl amino acids, the periodate oxidation products of Amadori compounds

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ABSTRACT

Periodate oxidation of Amadori compounds (1-amino-1-deoxy-2-ketoses), formed by the glycosylation of primary amino groups of free amino acids or N-terminal and ε-lysyl amino groups in peptides and proteins with aldoses, leads to the formation of N-carboxymethyl amino acids. The latter are then analysed by gas chromatography as their ethyl ester N-ethoxycarbonyl derivatives, after a straightforward derivatization reaction with ethyl chloroformate in a mixture of ethanol, pyridine and water. The amounts of N-carboxymethylated amino acids directly reflect the extent of glycosylation of the various amino sites. Application of this approach to food products is discussed.

INTRODUCTION

Free amino acids and ε-amino groups of lysine residues in pepides and proteins readily react with reducing sugars, particularly aldoses, to form 1-amino-1-deoxy-2-ketoses upon Amadori rearrangement of an intermediate glycosylamine [1]. This glycosylation reaction occurs during food processing and storage. It represents the first step in a series of complex transformations which eventually lead to the generation of brown pigments and numerous desirable or undesirable flavour compounds. This process is known as the Maillard reaction or non-enzymatic browning [2–5].

In food products, the blockage of the ε-amino group of lysine residues can be initiated under very mild conditions: a milk powder stored at room temperature leads to the progressive loss of this essential amino acid and eventually to the development of a brown colour. Under physiological conditions, enzymes, proteins and DNA can also be modified by glycosylation [3–5]. This process is of special concern in, *e.g.*, diabetic subjects, where physiological and immunological functions may be impaired.

Recently, we proposed a new approach to the measurement of both protein-bound and free Amadori products [6], consisting in determining the N-carboxymethylamino acid derivatives (CM-AAs) arising from the periodate oxidation of the corre-

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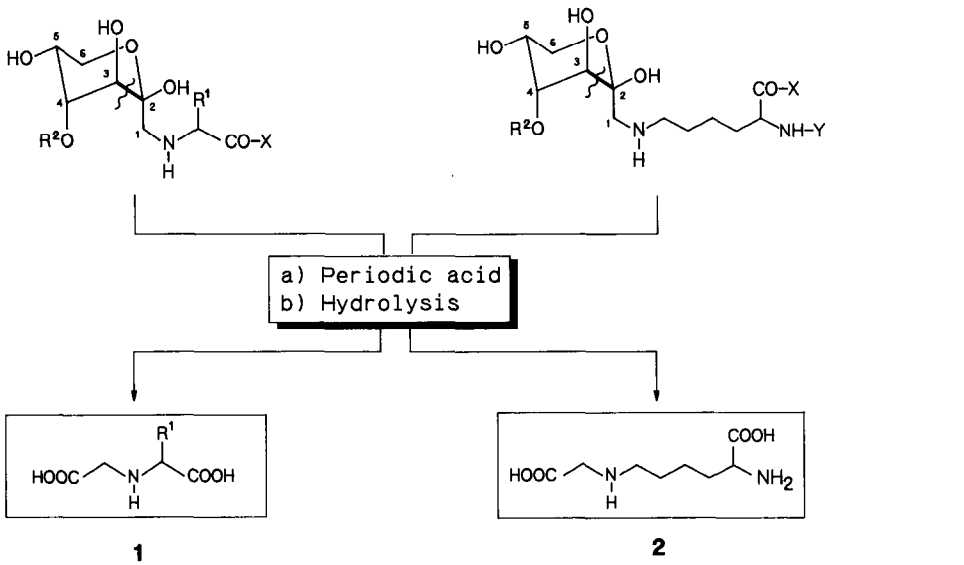

Fig. 1. Reaction pathway for the periodate oxidation of Amadori compounds derived from α-amino acids and ε-lysine residues with glucose (R^2 = H) or lactose (R^2 = galactosyl). Formation of N-carboxymethyl amino acids (CM-AAs, **1**) and N^{ε}-caboxymethyllysine (ε-CM-Lys, **2**).

sponding N-glycosylated species (Fig. 1). In this paper, we report a simple analytical method for the determination of various CM-AAs by gas chromatography (GC) after a straightforward derivatization reaction with ethyl chloroformate.

EXPERIMENTAL

Materials

N-Carboxymethyl derivatives of alanine (CM-Ala), valine (CM-Val), leucine (CM-Leu), isoleucine (CM-Ile), phenylalanine (CM-Phe) and the mono and bis derivatives of lysine (α-CM-Lys, ε-CM-Lys and bis-CM-Lys) were synthesized by reaction of the corresponding amino acids with glyoxylic acid according to Kihlberg *et al.* [7]. All compounds were fully characterized by NMR and mass spectrometry and gave satisfactory elemental analyses. The N-carboxymethyl derivative of glycine (CM-Gly, iminodiacetic acid), O-benzyl-L-serine and periodic acid were purchased from Fluka (Buchs, Switzerland). Ethyl chloroformate was obtained from Aldrich (Steinheim, Germany).

Apparatus

A Hewlett-Packard HP-5890 gas chromatograph with an HP-7673A autosampler and an HP-3392A integrator was used; data were acquired and treated using an HP-3350 laboratory automation system. Hydrolyses were performed using a Thermoreactor TR 105 dry-block heater (E. Merck, Darmstadt, Germany).

Separation conditions

The conditions were as follows: DB-5 fused-silica capillary column (30 m ×

0.32 mm I.D.) (J&W Scientific); carrier gas (helium) pressure, 10 p.s.i.; splitless injector temperature, 250°C; oven temperature programme 50°C (1 min), 30°C/min to 200°C, 5°C/min to 300 (5 min); flame ionization detector temperature, 300°C.

Samples

Dehydrated tomato powder was obtained from a food product development centre (Francereco, Beauvais, France). Model milk powder samples, the water activity of which were adjusted using saturated aqueous magnesium chloride, were heated for various periods of time at 40–50°C to induce glycosylation.

Sample treatment

Approximately 150 mg (exactly weighed) of sample were homogenized in 10 ml of water in a 260 × 40 mm I.D. Pyrex tube with a 29/32 ground joint. A 10-ml volume of a 100 m*M* aqueous periodic acid solution was added and the mixture was left in the dark for 2 h at room temperature with occasional shaking. Excess of periodic acid was decomposed with 2 ml of 2 *M* sodium thiosulphate, then water and concentrated hydrochloric acid were added to make a final volume of 150 ml in 6 *M* HCl. The tube was fitted with a reflux condenser and the mixture was heated at 110°C for 24 h for the milk powders and 2 h for the tomato samples. The hydrolysate was diluted with water and filtered. An aliquot was evaporated to dryness under a stream of nitrogen and reconstituted in 1 ml of 0.1 *M* HCl. O-Benzylserine (internal standard, 25.6 nmol) was added and the mixture was applied to a small column (20 × 4 mm I.D.) of Dowex 50W-X4. After the resin had been rinsed with 10 ml of water, the compounds of interest were eluted with 2 ml of 3 *M* ammonia solution and the solvent was removed under a stream of nitrogen.

Derivatization

The eluate from the cation-exchange resin was dissolved in 100 μl of water–ethanol–pyridine (60:32:8, v/v/v) and 5 μl of ethyl chloroformate were added. After 2 min at room temperature the mixture was diluted with 0.5 ml of saturated aqueous sodium hydrogencarbonate and the derivatives were extracted with 2 ml of dichloromethane. The organic phase was dried (sodium sulphate) and the solvent removed *in vacuo*. The residue was reconstituted in 100 μl of dichloromethane containing hydrocarbons (C_{16}, C_{20}, C_{24}, C_{28}) before injection.

RESULTS

On treatment with periodic acid, Amadori compounds gave rise to the formation of CM-AAs by selective cleavage of the C-2–C-3 bond (Fig. 1). These uncommon amino acids were derivatized with ethyl chloroformate in ethanol–pyridine–water according to the reaction scheme in Fig. 2 and analysed by capillary GC.

Fig. 3 shows the separation of a standard mixture of CM-AAs as their ethyl ester N-ethoxycarbonyl derivatives, including N-carboxymethylglycine (CM-Gly), -alanine (CM-Ala), -valine (CM-val), -leucine (CM-Leu), -isoleucine (CM-Ile), -phenylalanine (CM-Phe) and the mono and bis derivatives of lysine (α-CM-Lys, ε-CM-Lys and bis-CM-Lys). By the use of this type of derivatization it was possible to separate α-CM-Lys and ε-CM-Lys on a apolar stationary phase (SE-54). In con-

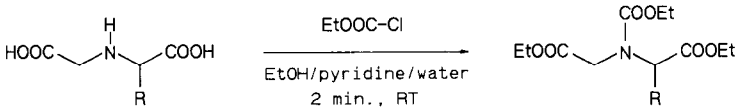

Fig. 2. Formation of ethyl ester N-ethoxycarbonyl derivatives of CM-AAs by reaction with ethyl chloroformate. Et = Ethyl.

trast, using the classical derivatization procedure including esterification with isobutyl alcohol and acylation with pentafluoropropionic acid, these two compounds could only be resolved on a medium-polar phase (OV-1701). The CM-AA derivatives were less volatile than the corresponding amino acids but the elution orders of these two series of compounds were identical. The ethyl ester N-ethoxycarbonyl derivatives were stable for at least 2 weeks at room temperature (variation of response factors below 0.5% for all the CM-AAs studied).

Calibration graphs were linear in the range 2–15 μg/ml (correlation coefficients 0.981–0.991) for all the compounds investigated. This narrow range was chosen because of the low concentrations expected in the actual samples.

The reliability of the determination of the α-, ε- and bis-CM-Lys was tested. Blank samples (non-oxidised milk hydrolysates) were spiked at three different levels, then passed through the cation-exchange resin. The recoveries were better than 80% for all the CM-AAs investigated. The spiked samples were then derivatized and analysed in several replicates. The results are reported in Table I.

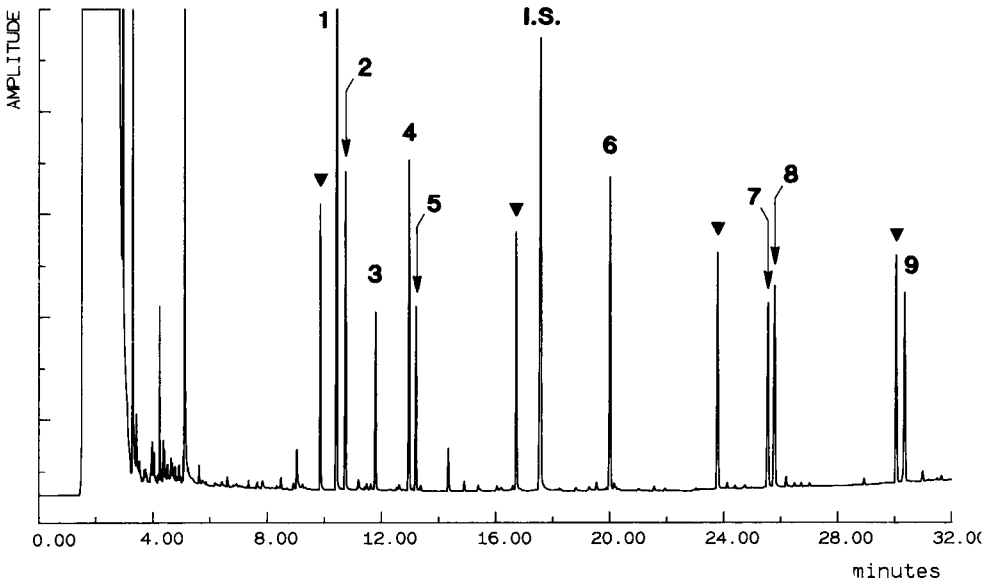

Fig. 3. Separation of a standard mixture of CM-AAs after derivatization with ethyl chloroformate. Peaks: 1 = CM-Gly; 2 = CM-Ala; 3 = CM-Val; 4 = CM-Leu; 5 = CM-Ile; 6 = CM-Phe; 7 = α-CM-Lys; 8 = ε-CM-Lys; 9 = bis-CM-Lys; I.S. = O-benzyl-L-serine; ▼ = added hydrocarbons (C_{16}, C_{20}, C_{24}, C_{28}).

TABLE I

PRECISION AND ACCURACY OF THE DETERMINATION OF THE VARIOUS CM-LYS DERIVATIVES IN SPIKED MILK HYDROLYSATES

The amount of standards were added to 1 ml of hydrolysate containing 0.5 mg of sample.

Derivative	Amount added (μg)	Amount measured (μg)	R.S.D.[a] (%)	n^a
α-CM-Lys	1	0.8	10.5	3
	6	6.4	8.8	6
	10	9.8	8.3	5
ε-CM-Lys	1	0.7	6.7	3
	6	6.6	5.1	4
	10	9.7	10.3	4
Bis-CM-Lys	1	1.3	6.8	2
	6	6.4	4.9	4
	10	10.3	6.9	5

[a] R.S.D. = relative standard deviation; n = number of replicates.

Two food samples were chosen to test the method: a tomato powder for its relatively high content of free amino acids and reducing sugars, and a milk powder for evaluating the extent of blockage of the ε-lysine residues. Both samples were oxidized with periodic acid then hydrolysed. After clean-up through a strong cation-exchange resin, the hydrolysates were derivatized as described above and analysed by GC.

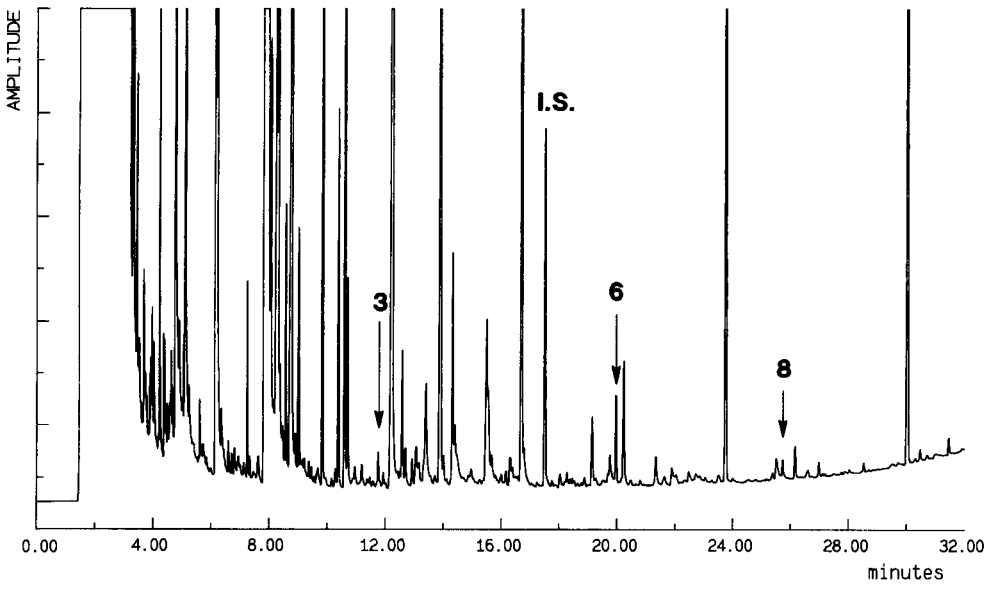

Fig. 4. Chromatogram of a tomato powder after periodate oxidation and ethyl chloroformate derivatization. Peaks as in Fig. 3.

Several CM-AAs were detected in the tomato sample, as shown in Fig. 4. These were CM-Val (2 μmol/g), CM-Phe (18 μmol/g) and ε-CM-Lys (<2 μmol/g), the identities of which were confirmed by mass spectrometry. Other derivatives could not be identified owing to interferences with common protein-hydrolysate amino acids. In a deliberatly heat-damaged milk powder, only ε-CM-Lys was found (80 μmol/g).

DISCUSSION

In the past, free Amadori products have been determined by GC [8] or ion-exchange high-performance liquid chromatography [9] while protein-bound Amadori compounds derived from the glycosylation of ε-lysine groups with glucose or lactose have been determined by measuring the furosine which is released on acid hydrolysis [10]. As mentioned earlier, a new approach has been proposed recently to establish the extent of glycosylation which consists in the determination of the CM-AAs that are released by oxidative degradation of Amadori compounds.

The determination of amino acids by GC is usually performed after esterification of the carboxylic acid group and perfluoroacylation of the remaining functional groups (NH_2, OH, SH). We used this two-step procedure for the determination of CM-AAs in milk samples [6]. However, this derivatization method is time consuming, and high temperatures and anhydrous conditions are needed to obtain high and reproducible yields. In this study, CM-AAs were analysed by GC as their ethyl ester N-ethoxycarbonyl derivatives after reaction with ethyl chloroformate (Fig. 4). This new procedure was developed recently by Husek [11] for the determination of various amino acids and carboxylic acids. Applied to the different CM-AAs, quantitative derivatizations were obtained using very mild reaction conditions (2 min at room temperature).

Not all theoretically expected CM-AAs were investigated because of a lack of reference compounds. Moreover, several derivatives may not be formed at all during the periodate treatment owing to the presence of oxidizable functional groups (*e.g.*, in methionine or tyrosine). In products containing proteins or large peptides, ε-CM-Lys (CML) is expected to be present in much larger amounts than the other CM-AAs as the ε-lysine groups largely predominate. This is verified in milk samples wherein only CML was detected as a result of the oxidative cleavage of the lactose-lysine Amadori compound. On the other hand, other CM-AAs can be formed in systems containing relatively large amounts of free or N-terminal amino acids. In dry tomato powder, the presence of noticeable amounts of Amadori products was first shown by Reutter and Eichner [9]; other CM-AAs were detected as shown in Fig. 2.

It should be mentioned that CML is not only formed by periodate oxidation. It has also been detected in food and in biological systems and was shown to be the consequence of the breakdown by oxygen of fructose-lysine Amadori compound [4]. Up to now, however, no other CM-AAs which could have been generated under similar conditions have been identified.

CONCLUSION

It has been shown that CM-AAs produced by periodate oxidation of Amadori products can be readily derivatized with ethyl chloroformate under very mild condi-

tions. The determination of these uncommon amino acids by GC as their ethyl ester N-ethoxycarbonyl derivatives can be used to evaluate the sites and the extent of glycosylation of the various primary amino groups in amino acids, peptides and proteins. This approach provides a new tool for investigating the glycosylation reaction both in food products and in biological materials.

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