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Note

Identification and determination of N-ε-carboxymethyllysine by gas–liquid chromatography

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We recently published[1] a reliable method for the determination of furosine, an indicator for Maillard-type heat damage. Furosine proved to be a helpful indicator for the lysine sugar complexes fructoselysine, lactuloselysine, maltuloselysine, etc.

The suggestion that the determination of pyridosine, a second indicator of Maillard-type heat damage, was possible simultaneously proved to be wrong. The first electron impact (EI) mass spectra had led us to the assumption that the peak formed was pyridosine, but strict re-examination of these mass spectra together with chemical ionization (CI) mass spectra led to the conclusion that the compound was in fact N-ε-carboxymethyllysine.

Carboxymethyllysine was first identified as a product of heat damage in foods and biological material by Ahmed *et al.*[2,3] and Liardon *et al.*[4]. In 1975 it was found in the urine of hospitalized patients by Wadman *et al.*[5] but they found no connection with food or nutrition. Liardon *et al.*[4] determined the compound as the pentafluoropropionyl isopropyl derivative and synthesized some pure material. A sample of this standard was tested in our laboratories, and confirmed our findings.

Carboxymethyllysine is formed by oxidative cleavage of the sugar derivative erythronic acid from the fructoselysine residue (Fig. 1). It promises to be an interesting indicator of heat damage and to be of biochemical interest itself.

EXPERIMENTAL

The materials and methods employed were similar to those described previously[1]. Carboxymethyllysine and furosine were determined as their heptafluorobutyryl isobutyl esters using thermionic phosphorus–nitrogen detection (PND)[1,6]. The mass spectra of the unknown peak and of the carboxymethyllysine standard were principally identical. Examples of the mass spectra are given in Figs. 2 and 3.

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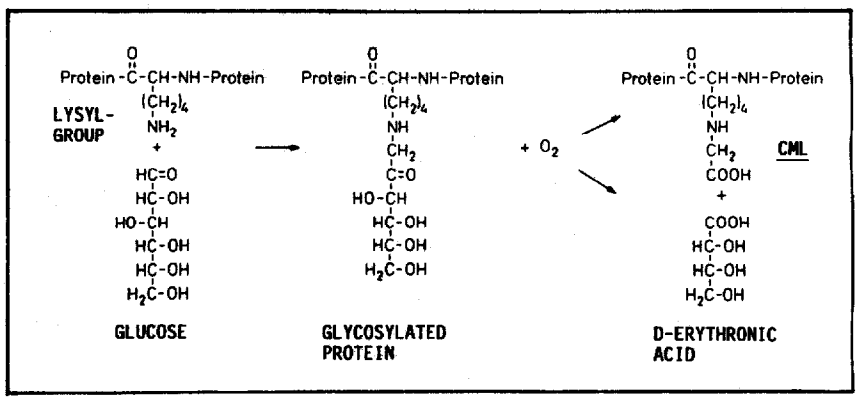

Fig. 1. Reaction scheme for the formation of N-ε-carboxymethyllysine (CML) from a protein, glycosylated at the ε-amino group of lysine.

The compounds to be analysed and all the other amino acids for gas–liquid chromatographic (GLC) analysis were prepared by esterification with isobutanol in 3 *M* hydrochloric acid and acylation with heptafluorobutyric anhydride as described previously[6]. The food samples corresponding to 200 mg of crude protein (Kjeldahl-nitrogen × 6.25, *i.e.* 100 g of protein contains 16 g of nitrogen) were hydrolysed in Pyrex glass bottles with 25 ml of 7.75 *M* hydrochloric acid for 23 h at 110°C in an oven. Compared to the hydrolysis with 6 *M* hydrochloric acid the higher concentration of acid gives a greater yield of furosine[1].

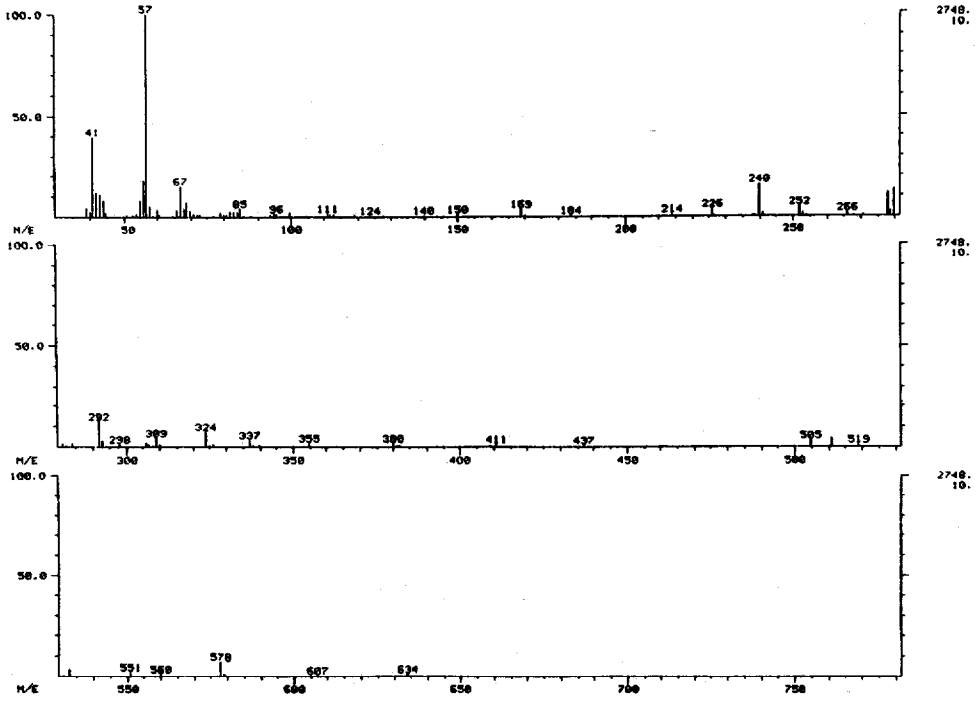

Fig. 2. 70-eV EI GC–MS mass spectrum of the heptafluorobutyryl isobutyl ester of carboxymethyllysine.

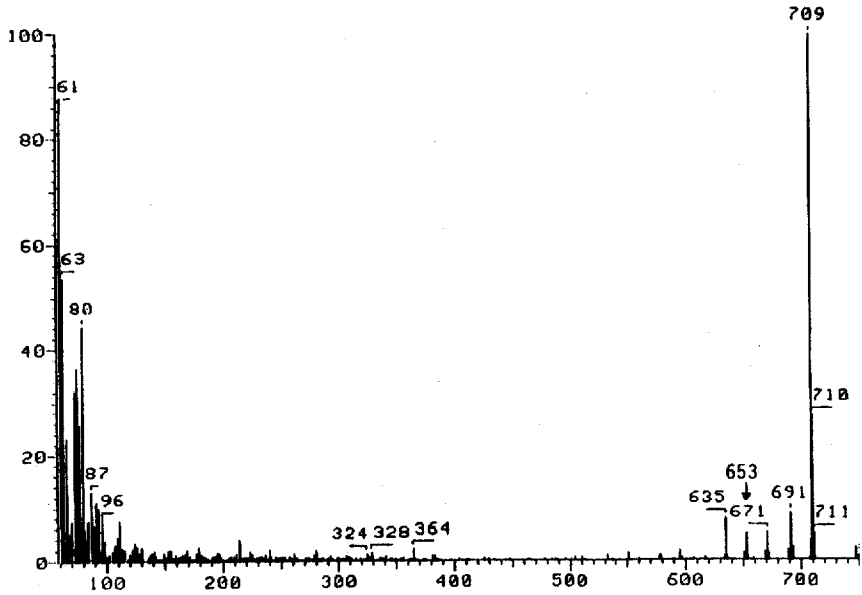

Fig. 3. Isobutane CI GC–MS mass spectrum of the heptafluorobutyryl isobutyl ester of carboxymethyllysine.

The chromatography was performed by using a Sigma 1 B gas chromatograph (Perkin-Elmer, Überlingen, F.R.G.) with a silanized Pyrex glass column as described previously[1,6]. For the capillary gas chromatography a Perkin-Elmer Sigma 8300 chromatograph with a fused-silica column (50 m × 0.32 mm I.D.) coated with 0.3-μm dimethylsilicon, Permaphase and PND were used. The chromatographic conditions were similar to those described elsewhere, with the exception of the flow-rates of carrier gas (helium), 2 ml/min, of hydrogen, 3 ml/min, and of air, 100 ml/min[7]. For analysis, 1.0 μl of the sample was injected with a splitting ratio of 1:30.

EI mass spectra of the heptafluorobutyryl isobutyl ester of carboxymethyllysine were measured with a 1020 MAT quadrupole mass spectrometer (Finnigan, Bremen, F.R.G.) operating at 70 eV after GLC separation on a 30-m SE-30 fused-silica capillary column. The oven was programmed from 60 to 280°C at 20°C/min. Isobutane CI mass spectra were measured with a Finnigan MAT 8230 spectrometer after separation on a 20-m SE-54 glass capillary column. The mass spectra are shown in Figs. 2 and 3.

RESULTS AND DISCUSSION

In Fig. 4 a GLC chromatogram from a sterilized liquid formula diet based on milk is shown. The carboxymethyllysine peak is well separated from those of other compounds. This is a great advantage of the method in comparison to ion-exchange chromatography where carboxymethyllysine is eluted after methionine, and it is not easily detected and identified. In that context, high concentrations of the hydrolysates have to be analysed in order to obtain measurable amounts of such trace substances. This is obviously the reason why carboxymethyllysine remained hidden in food ma-

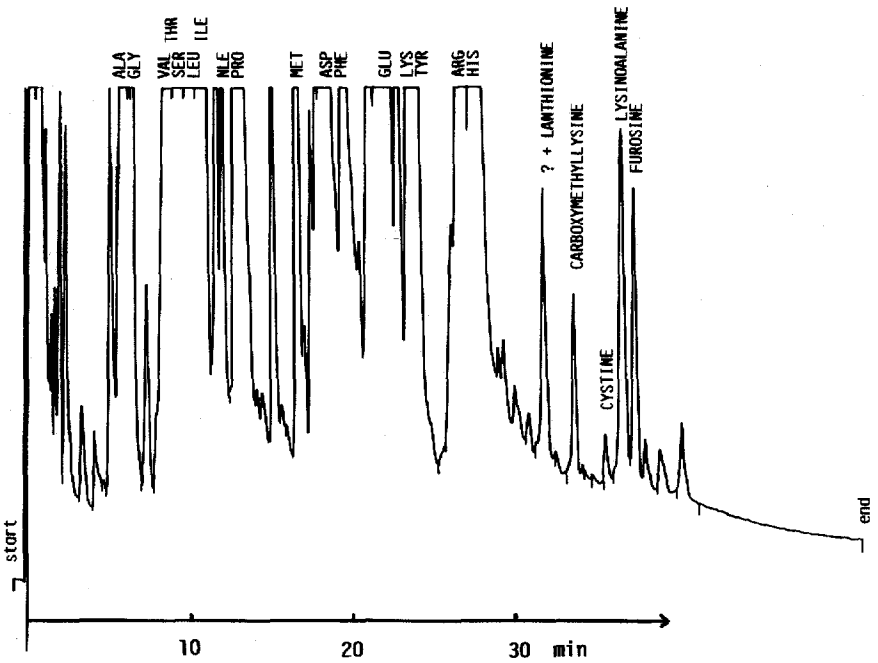

Fig. 4. Chromatogram of an hydrolysate from a sterilized liquid formula diet based on milk, showing the amino acid heptafluorobutyryl isobutyl esters. In order to obtain measurable values for carboxymethyllysine, the concentration of the hydrolysate (and of the most common amino acids) is greatly increased. The carboxymethyllysine peak may contain traces of an impurity as can be seen from Fig. 3 in which the peaks at *m/z* 364 and 671 probably belong to impurities eluted in the same GC peak.

terial for so long. Traces of an impurity, however, may be coeluted with carboxymethyllysine as is shown by the ions at *m/z* 364 and 671 (see Fig. 3), which are not observed in the carboxymethyllysine reference spectrum.

Since carboxymethyllysine is a derivative of fructoselysine, its formation should depend on the amount of fructoselysine in the food and thus on its indicator furosine. As Fig. 5 shows, this seems to be the case at least for material prepared under comparable conditions like the ultra heat treated (UHT) milks. This figure is

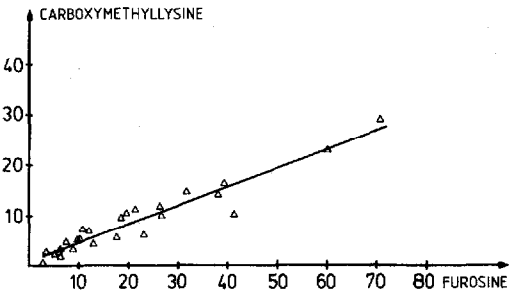

Fig. 5. Correlation between furosine and carboxymethyllysine determined by the present GLC method. The results are given as mg furosine and carboxymethyllysine per litre of UHT-treated milk: $y = 0.38x + 0.73$; $R = 0.96$.

TABLE I

MEAN VALUES FOR FUROSINE AND CARBOXYMETHYLLYSINE (CML) IN SEVERAL FOODS

Given in mg/kg crude protein = in mg/160 g of nitrogen.

Food samples	*n*	*Furosine*	*CML*
UHT milk samples	27	629 ± 503	259 ± 197
Dairy creamer (roller dried)	8	20 751 ± 1291	2126 ± 54
Polony, canned	8	519 ± 198	316 ± 63
Herring, baked with soy sauce, canned	8	842 ± 39	642 ± 185
Chili con carne, canned	8	349 ± 42	336 ± 32

identical with Fig. 5. in our previous publication[1] except that now carboxymethyllysine instead of the incorrectly identified pyridosine is correlated to furosine.

The conditions for the formation of carboxymethyllysine depend on oxidative processes and thus on the presence of oxygen and (possibly) metal ions, alkaline conditions and other factors. It can therefore be assumed that the formation of carboxymethyllysine is not constant under all conditions of food processing. Table I shows the results of preliminary experiments, which demonstrate the validity of this assumption[7]. More detailed results of analyses in foods and model proteins are necessary to determine the exact conditions of the formation of carboxymethyllysine and its dependence on fructoselysine.

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