Journal of Chromatography, 346 (1985) 363–368
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 989

Note

Determination of furosine by gas–liquid chromatography

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(Received July 1st, 1985)

We have recently published[1] a method for the determination of lysinoalanine, a product of heat-damaged proteins, by gas–liquid chromatography (GLC). The question then arose as to whether this method would also be able to determine simultaneously furosine, another indicator of heat damage, lysinoalanine and the main nutritive amino acids. Furosine, ε-N-(2-furoylmethyl)-L-lysine, is formed at a constant rate from fructoselysine or lactuloselysine during hydrolysis with 7.75 *M* hydrochloric acid (Fig. 1). We first detected this helpful indicator of the main products of early Maillard reaction 20 years ago[2]. Heyns *et al.*[3] and Finot *et al.*[4] investigated its structure and called it furosine. Meanwhile, furosine proved to be an ideal indicator

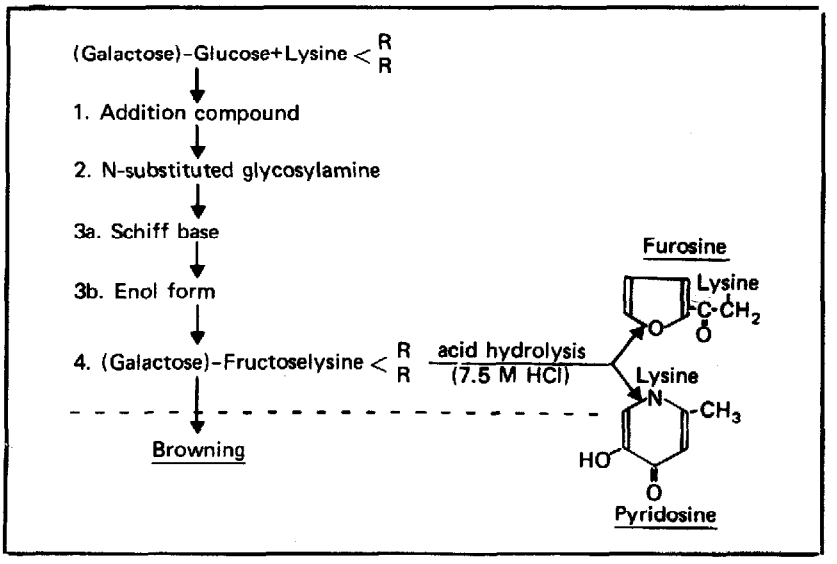

Fig. 1. Initial steps of the Maillard reaction with formation of furosine and pyridosine.

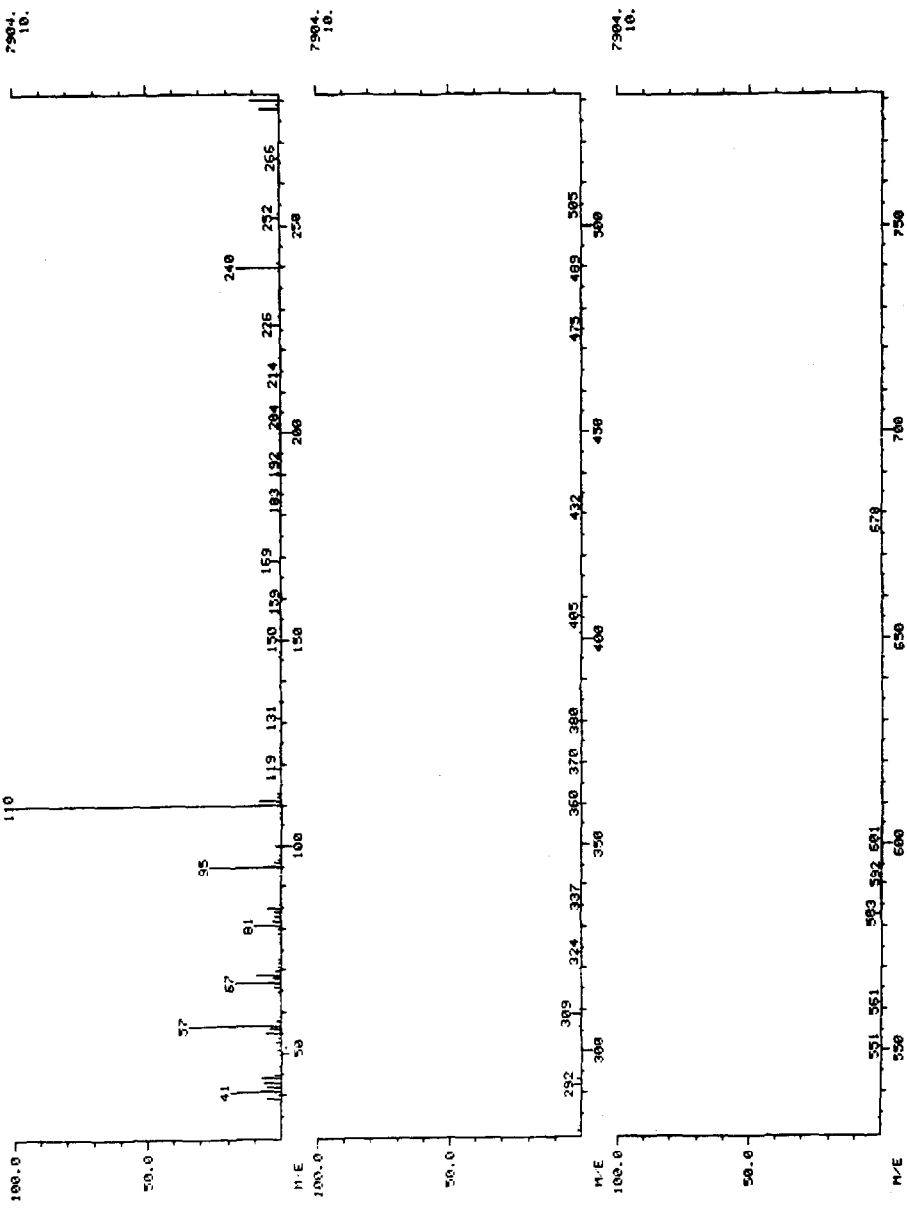

Fig. 2. Mass spectrum of the heptafluorobutyryl isobutyl ester of furosine, measured with a 1020 MAT Finnegan quadrupole mass spectrometer operating at 70 eV, after GLC separation. The observed peaks at *m/e* 110 and 95 correspond to the 2-furoylmethyl moiety of furosine.

of the above mentioned amadori compounds which themselves are very unstable during the hydrolysis of heat-damaged proteins. Furosine determinations are applied in food science and nutrition, protein evaluation and quality control, *e.g.*, ref. 5, as well as in clinical research and in medical biochemistry for diabetic control and measuring the damage caused to proteins by elevated blood sugar concentrations, *e.g.*, ref. 6.

A method for the simultaneous determination of furosine and lysinoalanine by ion-exchange chromatography was published by us in 1979[7]. The determination of pyridosine, a second derivative formed upon acid hydrolysis of the fructoselysine moiety, proposed proved not to be easy in routine analyses of commercial food samples, but seems to be applicable in the GLC method described here. Furthermore, the method presented is suitable for those food laboratories which are not equipped with an amino acid analyser. To our knowledge, this is the first method to determine furosine and pyridosine by GLC.

EXPERIMENTAL

The material and methods were similar to those described previously[1]. Furosine and the other amino acids were determined as their heptafluorobutyryl isobutyl esters using a thermionic phosphorus–nitrogen detector[1] as described by MacKenzie and Tenaschuk[8]. As a pure standard for furosine and pyridosine is not available, we used a hydrolysate of pure ε-fructoselysine (prepared some years ago[9]) in order to detect the furosine and pyridosine peaks on the GLC chromatogram. Moreover, the identification of these peaks was confirmed by GLC–mass spectrometry (MS). The mass spectrum of the heptafluorobutyryl isobutyl ester of furosine was comparable to that of the trifluoroacetyl methyl ester reported by Finot *et al.*[4], as regards the main peaks, including the base peak corresponding to the 2-furoylmethyl moiety which is not derivatized. These peaks could also be found in the mass spectrum of furyl-2-methylketone. An example of the mass spectra is given in Fig. 2.

Furthermore, several hydrolysates from our routine analyses on an amino acid analyser, known to be rich in furosine, were used for comparison. A GLC chromatogram from such a food sample is shown in Fig. 3.

The compounds to be analysed and all the other amino acids for GLC analysis were prepared by esterification with isobutanol-3 *M* hydrochloric acid and acylation with heptafluorobutyric anhydride as described previously[1]. The food samples corresponding to 200 mg of crude protein (N × 6.25) 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 (yield 20%), the higher concentration of acid gives a greater yield of furosine (constant in the range 32–36%)[5,7,10].

The chromatography was performed by using a Sigma 1 B gas chromatograph (Perkin-Elmer, Überlingen, F.R.G.) with a thermionic phosphorus–nitrogen detection (PND) as described previously[1]. The mass spectrum of the heptafluorobutyryl isobutyl ester of furosine was measured with a 1020 MAT quadrupole mass spectrometer (Finnegan, Bremen, F.R.G.) operating at 70 eV, after GLC separation on a 30-m fused-silica capillary column. The oven was programmed from 60 to 280°C at 20°C/min.

The calculation of the furosine content leads only to semiquantitative and

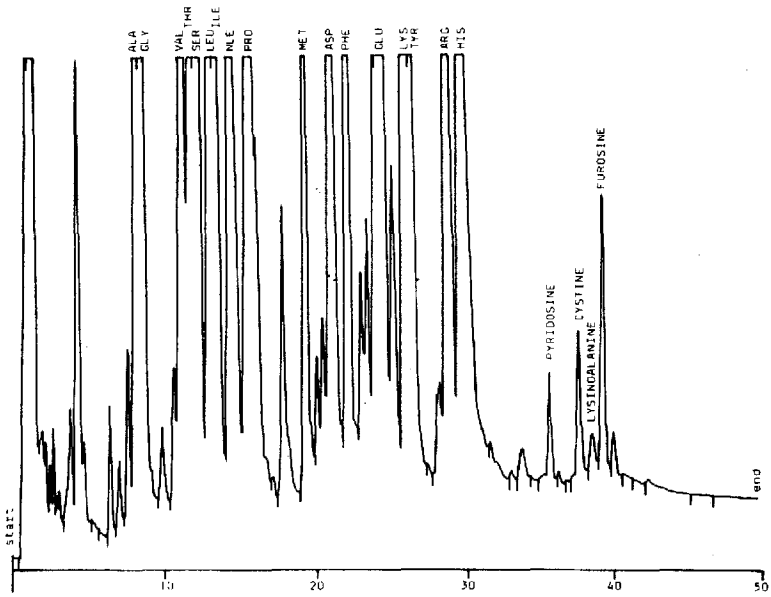

Fig. 3. Chromatogram of a hydrolysate from sterilized coffee cream showing the amino acid heptafluorobutyryl isobutyl esters. In order to obtain measurable values of the interesting substances, the concentration of hydrolysate (and of the most common amino acids) is greatly increased.

relative values since no furosine standard was available. The behaviour of the derivatives during chromatographic separation and the assumption that the response factors of the amino acids, using nitrogen-selective detection, depend on the nitrogen content of the molecule, formed the basis of the approximate calculation. Considering the nitrogen contents of the common amino acids and their response factors, a response factor of about 1.0 for furosine and pyridosine can be assumed. The furosine contents of food samples analysed by the GLC method were in good agreement with the amounts determined by ion-exchange chromatography (IEC) using the ninhydrin reaction for detection and another method of calculation.

RESULTS AND DISCUSSION

As Fig. 3 shows, the peak of furosine is fairly well separated from that of lysinoalanine. Difficulties are encountered only when there are very different amounts of furosine and lysinoalanine in a sample. Pyridosine is easily detectable and present in considerable amounts. Owing to the use of PND, purification of the samples was not necessary even in dark brown food material. One analysis requires approximately 50 min. The sensitivity of the determination is similar to that of normal IEC as is seen from Fig. 4. This figure shows a comparison of the furosine levels in ultra high temperature-(UHT)-treated milk samples analysed by IEC and by GLC. It should be mentioned that the detection of furosine in fresh commercial UHT-treated milks was not possible in earlier investigations[11] and can be performed only with the new and more sensitive generation of amino acid analysers.

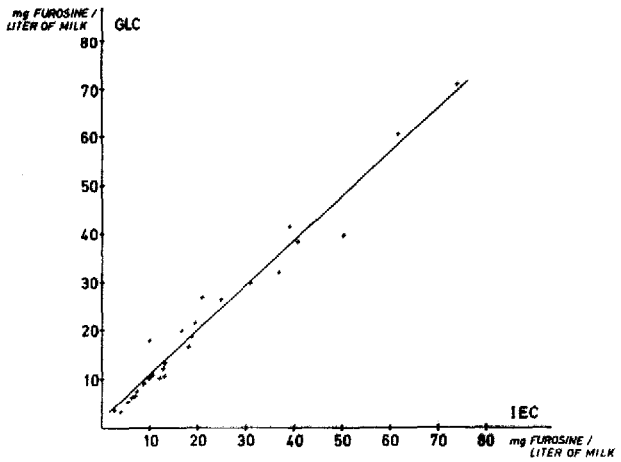

Fig. 4. Comparison of furosine determinations by IEC and the present GLC method. The results shown are given as mg furosine per litre of UHT-treated milk: $y = 0.92x + 1.89$; correlation coefficient, $R = 0.98$.

The advantage of the method presented here is that lysinoalanine, furosine and pyridosine can be analysed simultaneously, which is difficult to achieve by IEC. In particular, pyridosine is not easily detectable by an amino acid analyser equipped with highly resolving resins for rapid analyses. The fact that pyridosine is helpful in the evaluation of heat damage is demonstrated in Fig. 5. The ratio of furosine to pyridosine is not always constant in every kind of food, but there is a significant correlation in milk products.

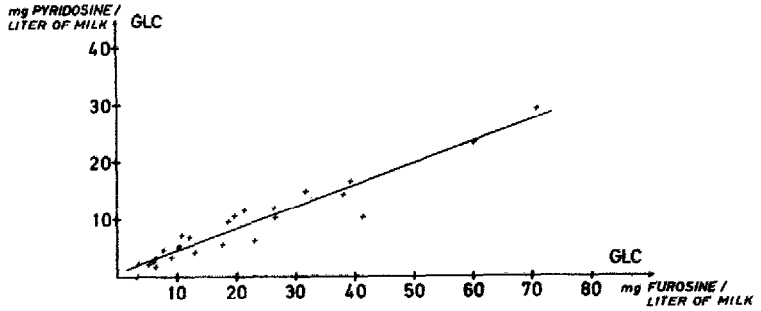

Fig. 5. Correlation between furosine and pyridosine determined by the present GLC method. The results are given as mg furosine and pyridosine per litre UHT-treated milk: $y = 0.38x + 0.73$; $R = 0.96$.

However, with the method presented here it seems to be possible for a laboratory equipped with a gas–liquid chromatograph and a thermionic phosphorus–nitrogen detector to analyse furosine and pyridosine in food samples and biological fluids.

ACKNOWLEDGEMENT

We thank Dr. P.Rösner, Kriminalpolizeiamt Kiel, for obtaining the mass spectra and for helpful comments.

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