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Note

Determination of lysinoalanine as the heptafluorobutyryl isobutyl ester derivative by gas–liquid chromatography

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Lysinoalanine is a cross-linking product, which develops during heat and/or alkali treatment of proteins by the reaction of the ε-amino group of lysine with dehydroalanine. Dehydroalanine itself is formed by β-elimination of O-substituted serine and of cystine[1]. The protein cross-linking formed by these reactions between or within the protein chain reduces the digestibility of the protein. Moreover, amino acid utilization is impaired with a loss of cystine and a decrease in the availability of lysine[2]. The fact that lysinoalanine causes cytomegaly in the epithelial cells of certain regions in the kidneys of rats gave rise to many discussions about the general toxicity of the component[3]. Although toxicity in man seems to be unlikely as the adverse effects are absent in species other than the rat, a worldwide screening for lysinoalanine in many food proteins started some years ago and is still continuing.

The determination of lysinoalanine is carried out mainly by ion-exchange chromatography. As it is commonly found in small amounts (only 100–10,000 ppm) in proteins, it is a minor constituent in food proteins and can be separated only with difficulty from the other common amino acids. These problems and the fact that few food laboratories are equipped with an amino acid analyser are the reasons why lysinoalanine is determined in only few places in the world. The possibility of a sensitive and specific method for its determination by gas–liquid chromatography GLC would therefore be very helpful. The method presented here is suited for this purpose and is superior to earlier GLC methods.

EXPERIMENTAL

The amino acid calibration standard, type hydrolysis, containing 2.5 μmole of each amino acid was obtained from Calbiochem-Behring (Hoechst, La Jolla, U.S.A.) and lysinoalanine from Miles (Elkhard, U.S.A.). Heptafluorobutyric anhydride and other reagents were obtained from Merck (Darmstadt, F.R.G.). Ethyl acetate, isobutanol and acetic anhydride (all analytical-reagent grade) were additionally purified by fractional distillation. Isobutanol–3 *M* HCl was prepared as described else-

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where[4,5]. All reagents were stored at 0°C, but were allowed to equilibrate to room temperature before use. The samples corresponding to 200 mg of crude protein (N × 6.25) were hydrolysed in Pyrex glass bottles with 25 ml of 6 *M* hydrochloric acid for 23 h at 110°C in an oven. After the hydrolysis, norleucine was added as an internal standard in amounts similar to the expected concentrations of amino acids *vs.* lysinoalanine. The samples were centrifuged (20,000 *g*) and filtered through a 0.6-μm micropore filter before derivatization.

From the hydrolysates 25–50 μl were dispensed into a 1.5-ml vial provided with a PTFE fastener and were evaporated at *ca.* 40°C using a stream of dry nitrogen. Isobutanol–3 *M* HCl (100 μl) was then added and the solution was heated in an oven at 120°C for 30 min. After the first 5 min the vials were removed from the oven for a short time and, while hot, agitated for 5–10 sec using an REAX 1R mixer (Heidolph, Kehlheim, F.R.G.). After the 30-min heating time the vials were cooled to room temperature and opened. The excess of the reagent was evaporated at 40°C using a stream of dry nitrogen, then 100 μl of heptafluorobutyric anhydride were added and the vials were heated again as described above at 150°C for 10 min. The vials were then cooled to room temperature and the contents evaporated just to dryness using a stream of dry nitrogen. Thereafter the residue containing the N-(O)-heptafluorobutyryl isobutyl ester derivatives were dissolved in 25 μl of ethyl acetate by agitating as described above. The samples were analysed immediately or stored at −20°C.

The chromatography was performed by using a Perkin-Elmer (Überlingen, F.R.G.) Sigma 1B gas chromatograph with thermionic nitrogen–phosphorus detection (NPD) and a Sigma 115 gas chromatographic integrator system and a silanized Pyrex glass column (2.5 m × 2.2 mm I.D.). The column packing consisted of 3% SE-30 coated on 100–120 mesh Gas-Chrom Q (Serva, Heidelberg, F.R.G.). After a 2-min starting time at 100°C the oven was programmed to increase from 100 to 250°C at 4°C/min. The other chromatographic conditions were injector temperature 250°C, detector temperature 300°C, carrier gas (N_2) flow-rate 20 ml/min, hydrogen flow-rate 3 ml/min, air flow-rate 100 ml/min and NPD bead set to 4.0 A. For analysis 1.0–1.5 μl of the sample were injected together with about 0.5 μl of acetic anhydride as described elsewhere[4,5].

RESULTS AND DISCUSSION

The chromatogram of amino acids including lysinoalanine from a hydrolysate of a heat-damaged casein is shown in Fig. 1, in which lysinoalanine appears at the end of the chromatogram just after cystine. Fig. 2 shows the correlation of lysinoalanine determined by the GLC method compared with the results of determinations on an amino acid analyser[6]. The correlation is very good for samples with both low and higher lysinoalanine concentrations.

Generally we experienced no problems with the separation of lysinoalanine from the other common amino acids. Owing to the use of NPD, purification of the sample was not necessary and no baseline rise was evident during temperature programming. Concentration of the protein in the food samples, in order to concentrate lysinoalanine, was also not necessary. One analytical run needs approximately 50 min. The sensitivity of the determination is similar to that in normal ion-exchange

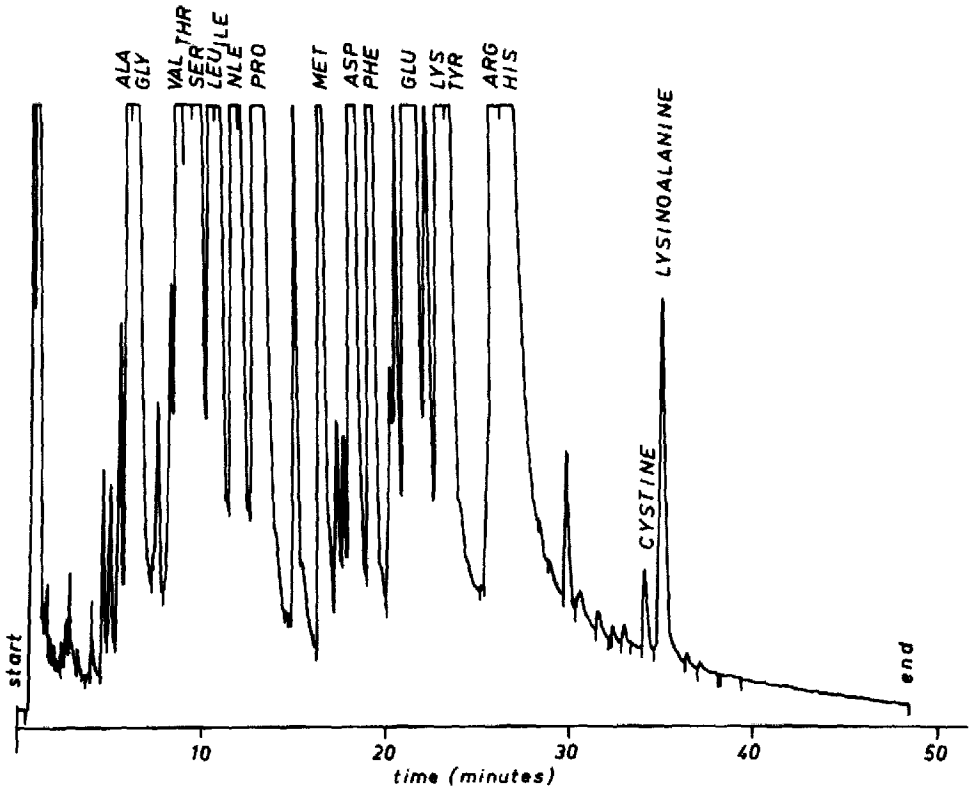

Fig. 1. Chromatogram of a hydrolysate from an alkali-treated caseinate showing the amino acid heptafluorobutyryl isobutyl esters. In order to obtain measurable lysinoalanine values the concentration of the hydrolysate (and of the most common amino acids) is increased (see text).

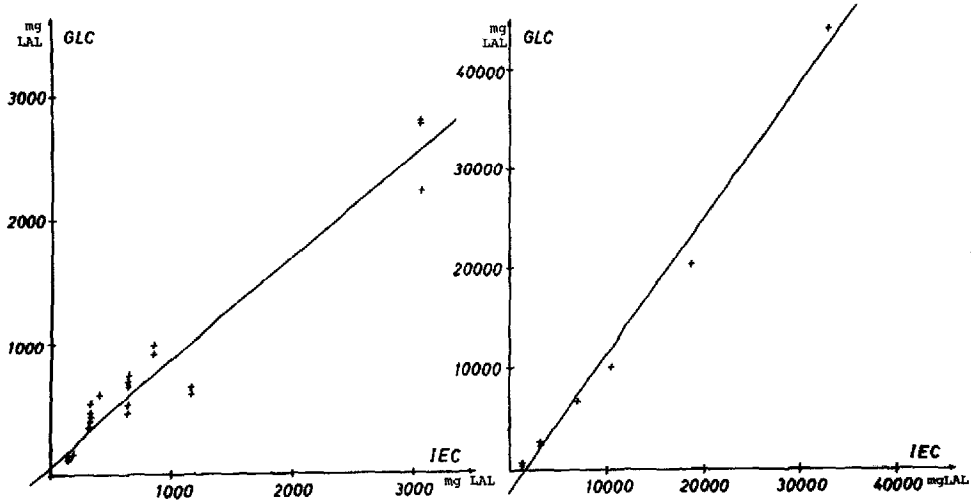

Fig. 2. Comparison of lysinoalanine determinations by ion-exchange chromatography (IEC) and the present GLC method. Owing to the wide range of lysinoalanine concentrations the plot is divided in two parts (100–3000 and 1000–40,000 ppm in crude protein). Results show lysinoalanine (LAL) in mg/kg crude protein. Left graph: $y = 0.83x + 54.63$; correlation coefficient $R = 0.97$. Right graph: $y = 1.32x - 1598.92$; $R = 0.99$.

chromatography with a limit for exact determination of about 100 ppm[6,7]. This is a much better sensitivity than reported elsewhere for other GLC procedures[8]. We experienced some problems, however, with the NPD response which is mainly influenced by the flow-rates of the carrier gas and hydrogen and by the heating current supplied to the rubidium silica bead[9]. In order to eliminate these complications it is recommended that the standard additions procedure is used and that the results are verified with an external standard every five runs.

The advantage of the method is that lysinoalanine can be determined in the same way as most other common amino acids with sufficient sensitivity and reproducibility as the detector does not record most of the interfering compounds. This is especially important for maillard-damaged proteins, which probably could not be analysed without purification by using a flame-ionization detector. In this context small irregularities must be accepted. From preliminary results we suggest that under the same conditions furosine and pyridosine, which are formed from fructoselysine during hydrolysis of maillard-damaged samples[10], can also be determined and separated from lysinoalanine.

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