

Note

Determination of lysinoalanine by densitometry

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Lysinoalanine (LAL), the trivial name of N^ε-(DL-2-amino-2-carboxyethyl)-L-lysine, is an amino acid which can arise in food proteins from lysyl and seryl residues^{1,2}. This compound has recently attracted considerable attention because of its ability to induce renal lesions in rats³⁻⁵. In most studies on LAL, this compound was determined by means of an amino acid analyzer^{1,3,5,6}. Recently, however, Sternberg *et al.*⁷ have described a method consisting of thin-layer chromatography (TLC) and densitometry.

This note describes our experience with this method, in particular the protein hydrolysis, the chromatographic procedure and the colouring with ninhydrin.

MATERIALS AND METHODS

Amino acids

Lysinoalanine, ornithinoalanine [N^δ-(DL-2-amino-2-carboxyethyl)-L-ornithine, OAL] and lysinomethylalanine [N^ε-(DL-1-methyl-2-amino-2-carboxyethyl)-L-lysine, LAL (Me)] were synthesized by the Central Institute for Nutrition and Food Research TNO, according to the method of Okudo and Zahn⁸. S-β-(4-Pyridylethyl)-L-cysteine (PEC) was also synthesized by the same Institute according to the method of Cavins and Friedman⁹. The following amino acids were purchased: AA₅ standard amino acid solution (Calbiochem, Los Angeles, Calif., U.S.A.), containing lysine, histidine, arginine, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine; lantionine, DL- and *meso*-forms (Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.); mixed DL- and DL-*allo*-δ-hydroxylysine hydrochloride (Sigma, St. Louis, Mo., U.S.A.); L-ornithine monohydrochloride (Fluka, Buchs, Switzerland); DL-methioninsulphon (Fluka); DL-norleucine (Fluka); L-1-methylhistidine monohydrate (Calbiochem); L-3-methylhistidine (Calbiochem); DL-2,3-diaminopropionic acid hydrochloride (Fluka); DL-tryptophan (E. Merck, Darmstadt, G.F.R.); DL-cysteic acid (Koch-Light, Colnbrook, Great Britain); DL-γ-aminobutyric acid (Hoffmann-La Roche, Basle, Switzerland); D-galactosamine hydrochloride (J. T. Baker, Phillipsburgh, N.J., U.S.A.), D-glucosamine hydrochloride (Biochem. Res. Corp., Los Angeles, Calif., U.S.A.) and N-ε-methyl-L-lysine hydrochloride (Sigma).

Reagents

Precoated cellulose layers (0.1 mm thickness) were used (Macherey, Nagel & Co., Düren, G.F.R.; Cel 300-10). The plates were densitometrically tested; plates of irregular thickness were not used. The ninhydrin solution was prepared by dissolving 1 g of ninhydrin in a mixture of 97 ml of *n*-butanol and 3 ml of glacial acetic acid. A copper sulphate solution was obtained by dissolving 2 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 10 ml of distilled water and then adding 30 ml of 95% ethanol. Ninhydrin, acetic acid, copper sulphate, ethanol and 37% hydrochloric acid were purchased from E. Merck, *n*-butanol and formic acid from J. T. Baker. All the reagents used were analytical-reagent grade.

The samples investigated, *i.e.*, alkali-treated soy proteins, sodium caseinate and gelatine, were commercial products.

Sample preparation

The sample (200–250 mg dry weight) was hydrolyzed by boiling in 200 ml of 6 *N* hydrochloric acid for 22 h. The hydrolysate was evaporated to dryness in a rotary evaporator at 50°. The residue was redissolved in 20 ml of water and the evaporation was repeated twice. In some cases it was necessary to filter the solution before the last evaporation. The final residue was dissolved in 10 ml of water. This solution is suitable for chromatography. Solutions obtained from samples with a content higher than 800 ppm of LAL (on a dry weight basis) have to be diluted with water.

Chromatography

The samples were applied by repeated spotting using a 5- μl SGE syringe fitted with a PTFE nozzle, with the aid of a stream of warm air. The maximum allowable amount of hydrolysate per spot corresponds to 125 μg dry weight of the sample. The spots should be as small as possible (diameter *ca.* 3 mm). LAL was applied to each plate, in amounts up to 0.10 μg , in separate spots for calibration.

Ascending chromatography was performed⁷ in the solvent system 90% formic acid–*n*-butanol–water (4:23:5) in an unsaturated tank (Desaga, Heidelberg, G.F.R.). The distance travelled by the solvent was 17 cm from the start. The development was performed three times, the elution time for each run being 4 h.

Measurements of spots

After drying the developed plate for 0.5 h at room temperature, it was sprayed with the ninhydrin solution and dried for 10 min at 110°. The layer was sprayed subsequently with the copper sulphate solution and dried for 30 min at room temperature. The spots were scanned with a Vitatron TLD 100 densitometer (Vitatron, Dieren, The Netherlands) connected to a Vitatron UR 402 integrating recorder. The operating conditions were: transmittance mode $\log(-)$; filter, 525 nm; level d; diaphragm, 0.25 mm; stroke distance, 5 mm; scan speed, 3 cm/min; recorder chart speed, 10 cm/min; integrator 7; span variation from 9/25 to 9/90, dependent on the spot intensity and background signal of the sample. Peak areas were calculated by means of the integrator readings and corrected for fluctuations in the background absorption due to variations in layer thickness as indicated by the manufacturer¹⁰. The amount of LAL in each hydrolysate was calculated from the mean of three determinations made on one plate.

RESULTS AND DISCUSSION

Hydrolysis

Protein hydrolysis can be carried out in sealed tubes or under total reflux. We think the sealed-tube technique is less convenient for routine determinations than the open-flask hydrolysis under total reflux. Moreover, disadvantage of the sealed-tube technique is the limited sample size, which may result, in some cases, in samples which are not representative of the material investigated. Both the sealed-tube method, followed by removal of the bulk of HCl in a desiccator over NaOH as used by Sternberg, and the open-flask hydrolysis were applied to three samples, *i.e.*, alkali-treated casein, a commercial product containing a whipping agent and alkali-treated soy protein. Differences in chromatographic separation were not observed. Slump⁶ has already demonstrated that, for most proteins, hydrolysis in 6 *N* hydrochloric acid for 22 h under total reflux is sufficient for complete liberation of LAL.

Chromatography

A prerequisite for densitometry is the complete separation of the given compound from interfering substances. In this respect, both Eastman cellulose sheets (catalogue number 13255) used by Sternberg *et al.* and the plates of Macherey, Nagel & Co. (MN), which we used, were suitable. However, in the case of MN plates, the R_F value of LAL is somewhat larger, and LAL is further removed from other ninhydrin-positive substances, resulting in a better chromatogram. Moreover, we found that MN cellulose plates were in general more homogeneous than Eastman sheets, which results in a lower background noise. An example of a densitogram, showing the results of LAL determinations on MN plates and on Eastman sheets, is provided in Fig. 1.

Not all types of cellulose plates are suitable for separation: it was found, that under the same chromatographic conditions, LAL interferes with other ninhydrin-positive substances, *i.e.*, lanthionine and cysteic acid, when Eastman cellulose sheets containing a fluorescence indicator (catalogue number 13254) were used. Precoated cellulose layers of Merck (catalogue number 5716) are also unsuitable. The Gelman chamber applied by Sternberg is not essential for LAL separation.

The method was also tested on ornithinoalanine (OAL), a compound which, like LAL, may arise during alkali treatment of proteins¹¹. It was observed that, on the Eastman sheet as well as on the MN plate, the spots of both compounds coincide. However, in alkali-treated products (*i.e.*, soy proteins, caseinates and foaming agents), investigated on an amino acid analyzer, OAL was found to be absent or present only in negligible amounts¹². If the presence of both OAL and LAL has still to be demonstrated, the ratio of the solvent components should be changed to 4:23:4. This system was not investigated further.

LAL (Me), an amino acid which theoretically can be formed from threonyl and lysyl residues⁶, shows a slightly higher R_F value than LAL. Furosine and pyridosine, compounds arising in acid hydrolysates of heated, proteinaceous products rich in reducing sugars, were also tested for their possible interference with the LAL determination. A hydrolysate of a lactose-hydrolyzed skimmed milk powder, in which the presence of both furosine and pyridosine was demonstrated by an amino acid

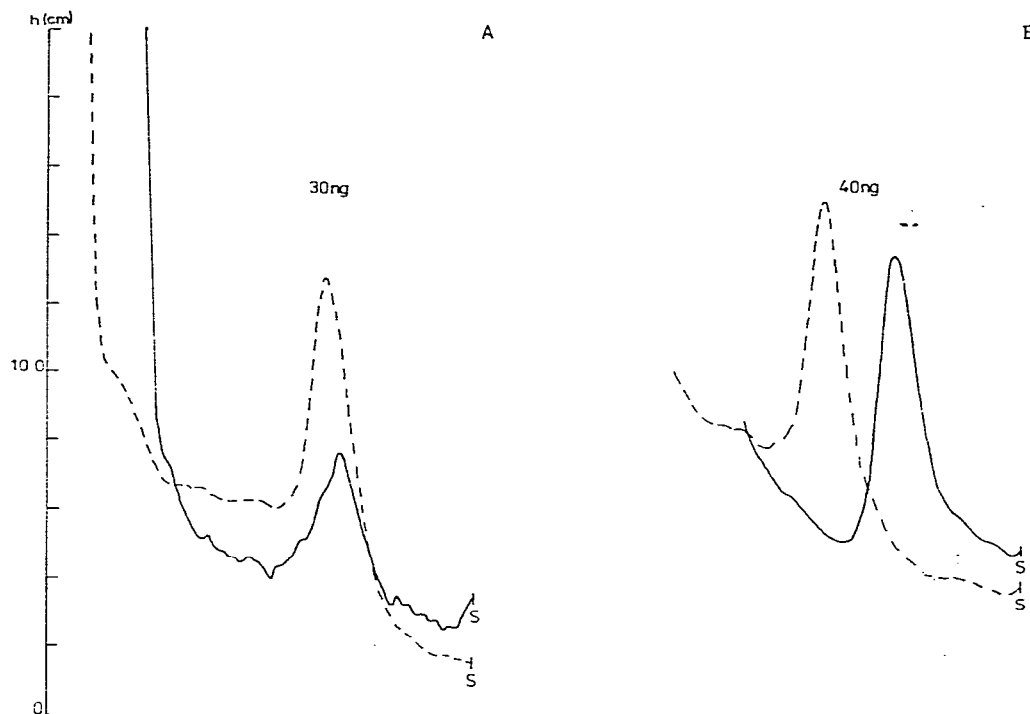


Fig. 1. Densitograms, showing LAL determinations on MN cellulose plates (---) and on Eastman cellulose sheets (—). A = Calcium caseinate (270 ppm LAL); B = sodium caseinate (340 ppm LAL); S = start.

analyzer, was submitted to chromatography. No ninhydrin-positive compound was found with a R_F value equal to that of the LAL spot.

A complete chromatogram showing the tested amino acids and some samples is shown in Fig. 2. The R_F value of LAL in a sample was slightly lower than that of reference LAL.

Precoated silica gel plates (E. Merck, silica gel 60 F₂₅₄; catalogue number 5715; thickness 0.25 mm) were also investigated because of their better homogeneity as compared to cellulose plates. The eluent was *n*-butanol-isopropanol-acetic acid-water (4:4:4:5). After prewashing the plates with the eluent, ascending chromatography was performed for 16 h. Ninhydrin colouring was carried out as described above (absorbance maximum, 497 nm). LAL was separated from most other amino acids usually present in protein hydrolysates, with the exception of OAL, LAL (Me), 3-methylhistidine and N- ϵ -methyllysine. However, colour development is much poorer compared to that on the cellulose layer, resulting in a detection limit four times that observed on cellulose. The colour is also less stable. On other silica gel plates (Woelm silica gel F_{254/366}; Macherey, Nagel & Co. silica gel N-HR/UV₂₅₄; Bakerflex IB-F and Merck silica gel 60 without fluorescence indicator) LAL was not separated from one of the amino acids of the standard amino acid mixture, under the same chromatographic conditions.

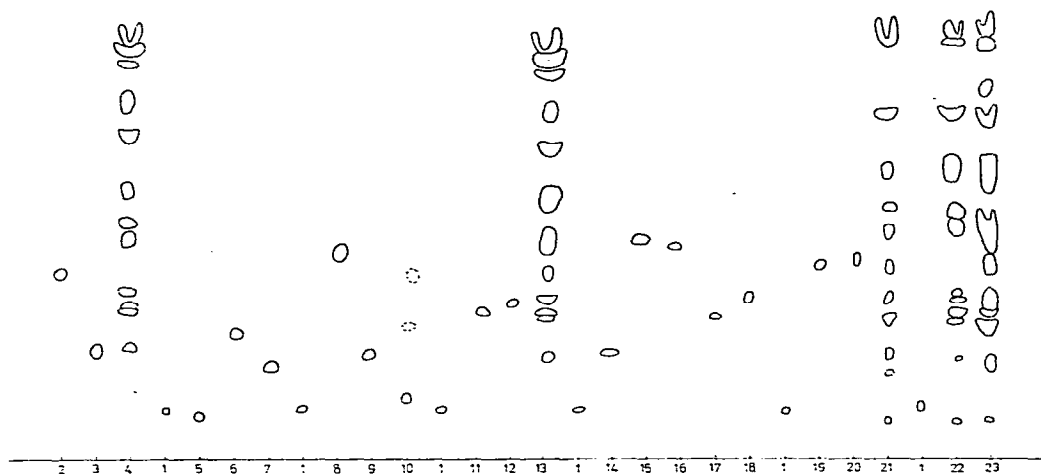


Fig. 2. Thin-layer chromatogram of amino acids on MN cellulose plates. Spots: 1 = LAL; 2 = PEC; 3 = hydroxylysine; 4 = standard amino acid mixture; 5 = OAL; 6 = ornithine; 7 = lanthionine; 8 = γ -aminobutyric acid; 9 = 2,3-diaminopropionic acid; 10 = LAL(Me); 11 = galactosamine; 12 = glucosamine; 13 = lactose-hydrolyzed skimmed milk powder; 14 = cysteic acid; 15 = methioninsulphon; 16 = tryptophan; 17 = 1-methylhistidine; 18 = 3-methylhistidine; 19 = N- ϵ -methyl-L-lysine; 20 = norleucine; 21 = alkali-treated soy protein (2800 ppm LAL); 22 = sodium caseinate (550 ppm LAL); 23 = alkali-treated soy protein (40 ppm LAL).

Conditions for ninhydrin spraying

A comparison of different ninhydrin reagents demonstrated that the optimal colour intensity and stability was attained by spraying with 1% ninhydrin solution followed by spraying with copper sulphate solution, as described in Materials and methods. Moreover, the standard deviation is lower than that obtained by spraying with ninhydrin alone. The copper sulphate spray shifts the colour maximum from 550 to 525 nm. Spraying with a mixture of ninhydrin and copper sulphate results in a very poor colour formation. For the LAL concentrations investigated, spraying a few days after elution resulted in no colour formation. When this is the case, a good colour can still be obtained by spraying with the eluent, drying in a hood for 0.5 h and treating with ninhydrin and copper sulphate as already described.

Some other spray reagents were investigated with the intention of improving the sensitivity of the determination. Spraying with *o*-phthalaldehyde (Fluka), recently described by Lindberg¹³ as a very sensitive reagent for amino acids on thin-layer plates, does not lead to a lower detection limit for LAL as compared to spraying with ninhydrin. This is also true when spraying with fluorescamine (Hoffmann-La Roche). Applying the procedure described by Mendez and Lai¹⁴ to LAL, the minimum detectable amount was 0.02 μg .

Linearity and sensitivity

A linear relationship exists between the amount of LAL spotted up to 0.10 μg and the corresponding peak areas, the limit of detection being 0.005 μg or 40 ppm in the sample (dry weight basis). Amounts of LAL exceeding 0.10 μg per spot were not tested.

Reproducibility and comparison with the amino acid analyzer

The reproducibility of the procedure was tested with four different samples, each being hydrolyzed six times (Table I). For the same hydrolysates, LAL was also

TABLE I

COMPARISON OF RESULTS OBTAINED BY TLC DENSITOMETRY AND BY THE AMINO ACID ANALYZER (AAA)

Sample	Method	Amount of LAL (ppm)	Range (ppm)	Standard deviation	Coefficient of variation (%)
Alkali-treated soy protein	TLC	40	30- 55	10 ($n = 6$)	25
	AAA	140	125- 165	15 ($n = 6$)	11
Alkali-treated soy protein	TLC	2800	2550-3000	220 ($n = 6$)	8
	AAA	3000	2600-3400	320 ($n = 6$)	11
Sodium caseinate	TLC	550	400- 650	87 ($n = 6$)	16
	AAA	680	610- 800	81 ($n = 6$)	12
Gelatine	TLC	250	218- 308	39 ($n = 6$)	16
	AAA	165	148- 170	12 ($n = 6$)	7

measured by means of an amino acid analyzer according to Slump's method A⁶. For both methods the standard deviation is calculated from

$$S = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n - 1}}$$

and the coefficient of variation from

$$v = \frac{S}{\bar{x}} \cdot 100\%$$

The comparatively large standard deviation of the densitometric method, in particular in the low concentration range, is partly due to the tailing of a ninhydrin-positive compound present in a very high concentration in comparison with LAL, or to incomplete separation of the LAL spot from an unknown ninhydrin-positive compound. This, in some cases, makes it rather difficult to determine the area enclosed by the LAL peak. Except for alkali-treated soy protein (2800 ppm), the results of both methods are, with respect to the products investigated, statistically different (t -test; $P = 95\%$). Possibly, in both methods, other unknown ninhydrin-positive compounds, dependent on the sample, are co-determined. A more extensive comparison of both methods in different foods and food products will be published elsewhere¹⁵.

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