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Note

Fluorophotometric assay of amino acids by using automated ligand-exchange chromatography and pyridoxal–zinc(II) reagent

A method for the quantitative separation of amino acids and related compounds in protein hydrolyzates and in physiological fluids into individual components by ion-exchange chromatography, with colour development by the ninhydrin reaction for each component, was established by SPACKMAN *et al.*[1]. This method has been rapidly developed in about the last 10 years. The limit of sensitivity for amino acids when special equipment is used approaches 10^{-9} mole of sample. Although the sensitivity of the ninhydrin reaction is sufficient for many purposes, situations often occur in biochemical studies in which more sensitive assays for amino acids are needed. As fluorimetry is known to increase the sensitivity of assays by several orders of magnitude, some investigators tried to develop a fluorimetric assay for amino acids[2–5]. Recently, SAMEJIMA and co-workers[6,7] reported a new fluorescence reaction of primary amines, amino acids and peptides with phenylacetaldehyde and ninhydrin, and applied it to the determination of amino acids separated by the procedure of SPACKMAN *et al.*[1].

In previous papers[8,9], a new fluorophotometric method for the determination of amino sugars and amino acids was described. This paper concerns the application of the selective fluorescent reaction with pyridoxal and zinc(II) to an automatic amino acid analyzer.

Experimental

Materials. Amino acids were obtained from Ajinomoto Inc., Tokyo. Pyridoxal hydrochloride was the product of Sigma Chemical Co., U.S.A. Other chemicals were of reagent grade.

Reagent solution. Pyridoxal–zinc(II) reagent solution was freshly prepared before use by dissolving 0.100 g of pyridoxal hydrochloride and 1.000 g of zinc acetate in 1000 ml of 2.0% pyridine–methanol. Buffers were prepared in accordance with Table I.

Procedure. Amino acid analyses were performed by ligand-exchange chromatography as described by ARIKAWA[10] and GANNO AND ARIKAWA[11]. A Hitachi Model 034 liquid chromatograph and a Hitachi Model 204 fluorescence spectrophotometer equipped with a mercury lamp were used.

A complete amino acid analysis was performed in two steps. The acidic and neutral amino acids were eluted from a 95 × 6 mm pre-column and a 450 × 6 mm column of sulphonated polystyrene resin (Hitachi Custom Ion Exchange Resin 2613) equilibrated at 55° with buffer I (pH 4.10). The basic amino acids were eluted from a 200 × 6 mm column packed with a similar resin and equilibrated at 55° with buffer II (pH 5.10). The flow-rate through the column was 30 ml/h. The effluent

TABLE I

COMPOSITION OF BUFFERS USED FOR THE SEPARATION OF AMINO ACIDS

Constituent	*Amount added per 10 l of buffer*	
	I[a]	*II*[b]
Sodium acetate	41.0 g	738.0 g
Glacial acetic acid	118.0 ml	185.0 ml
0.5 *M* zinc acetate	7.0 ml	20.0 ml
Ethyl alcohol	800.0 ml	—
Benzyl alcohol	—	110.0 ml
25% Brij-35	40.0 ml	40.0 ml

[a] Buffer used for separation of acidic and neutral amino acids (pH 4.10).
[b] Buffer used for separation of basic amino acids (pH 5.10).

stream was mixed with pyridoxal–zinc(II) reagent solution pumped at the rate of 120 ml/h (4 parts of reagent solution per 1 part of effluent) and allowed to react at 65–75° for 10 min (30 m × 1.0 mm reaction coil of PTFE capillar tubing). After the reaction, the mixture was passed through the flow cell in the fluorophotometer, which was equipped with a Toshiba Y-42 primary filter and set at 365 nm with excitation and at 485 nm with emission. The flow diagram is shown in Fig. 1.

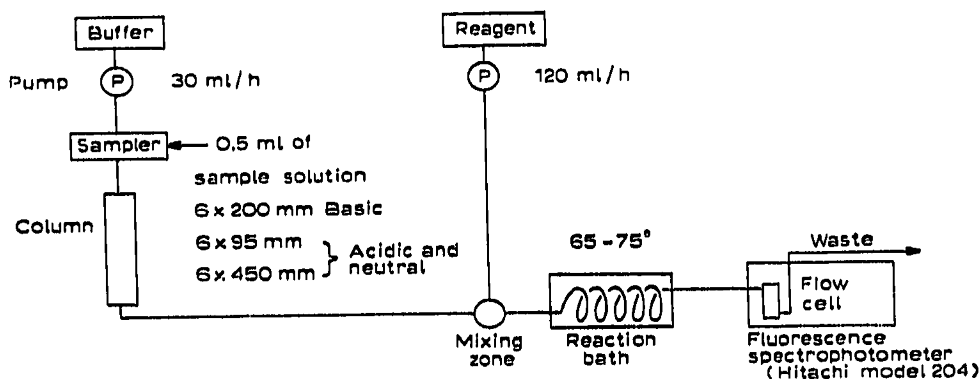

Fig. 1. Flow diagram of amino acid analyzer using the fluorophotometric method with pyridoxal–Zn(II) reagent.

Results and discussion

The following parameters were examined in order to determine the optimal conditions for the separation and determination of amino acids.

Type of eluent. The fluorescent reaction used in this study is based on the formation of chelates between N-pyridoxylidene amino acids and Zn(II). The citrate buffers used by SPACKMAN *et al.*[1] could not be used in the present system because citrate is a strong chelating agent and interferes to produce a fluorophor, N-pyridoxylidene amino acid–Zn(II) chelate, in this fluorescent reaction. ARIKAWA[10] and GANNO AND ARIKAWA[11] described ligand-exchange chromatography based on the ability of amino acids to form complexes with Zn(II). The buffer used in that system

did not contain citrate and any other chelating agents, as shown in Table I. Ligand-exchange chromatography was therefore chosen for use in our experiments.

Effect of reaction temperature. The experiments were carried out at various temperatures of the reaction bath in order to determine the optimal reaction temperature. An aliquot of amino acid solution was injected into the mixing zone with a microsyringe. The results are shown in Fig. 2. The fluorescence intensity increased rapidly with increase in temperature and reached a maximum. Neutral and acidic amino acids exhibited maximum intensities at 70–80°, while the basic amino acid lysine exhibited the maximum at 65°. The temperature of the reaction bath was therefore set at 65–75°, and the waste was subjected to a back-pressure of about 1 atm so as to prevent the methanol from boiling.

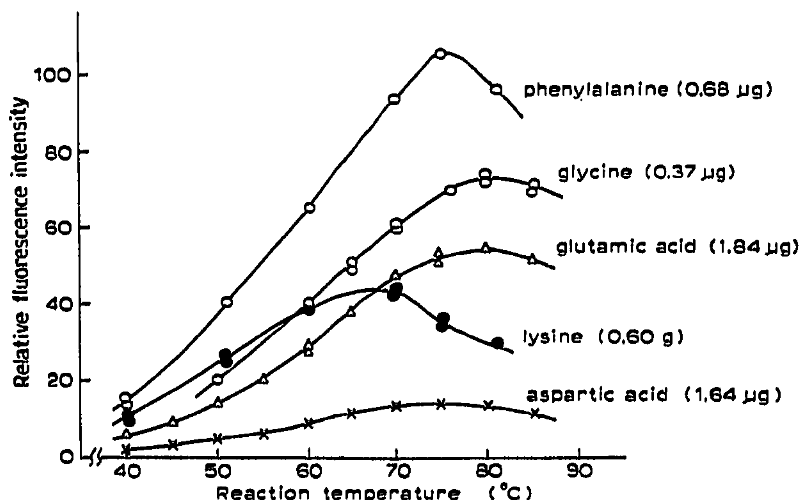

Fig. 2. Effect of the reaction bath temperature on fluorescence intensity.

Effect of ratio of reagent to eluent. The mixing ratio of reagent to eluent was varied by changing the flow-rates of both. As shown in Fig. 3, the fluorescence intensity increased rapidly when the mixing ratio of reagent to eluent was varied from 3:1 to 6:1. However, a decrease in the flow-rate of eluent increased the elution times of amino acids. The flow-rate of eluent was therefore set at 30 ml/h, at which rate a good resolution of each amino acid was obtained, and the flow-rate of reagent at 120 ml/h.

Typical chromatograms obtained for the separation of a standard mixture of amino acids under the above conditions are presented in Figs. 4 and 5. Each peak represents 0.005 µmole of basic amino acid. Under the best conditions, 0.001 µmole of each amino acid was determined by the automated fluorophotometric procedure. It seems that one twentieth to one fiftieth of the usual amount of sample can be determined with this instrument. The calibration curve was achieved by use of the peak heights. The relationship between the concentration of amino acids and the peak height or peak area was linear. The stability of the reagent was tested by checking the same amount of a standard solution of glycine every day. Reagent solution kept

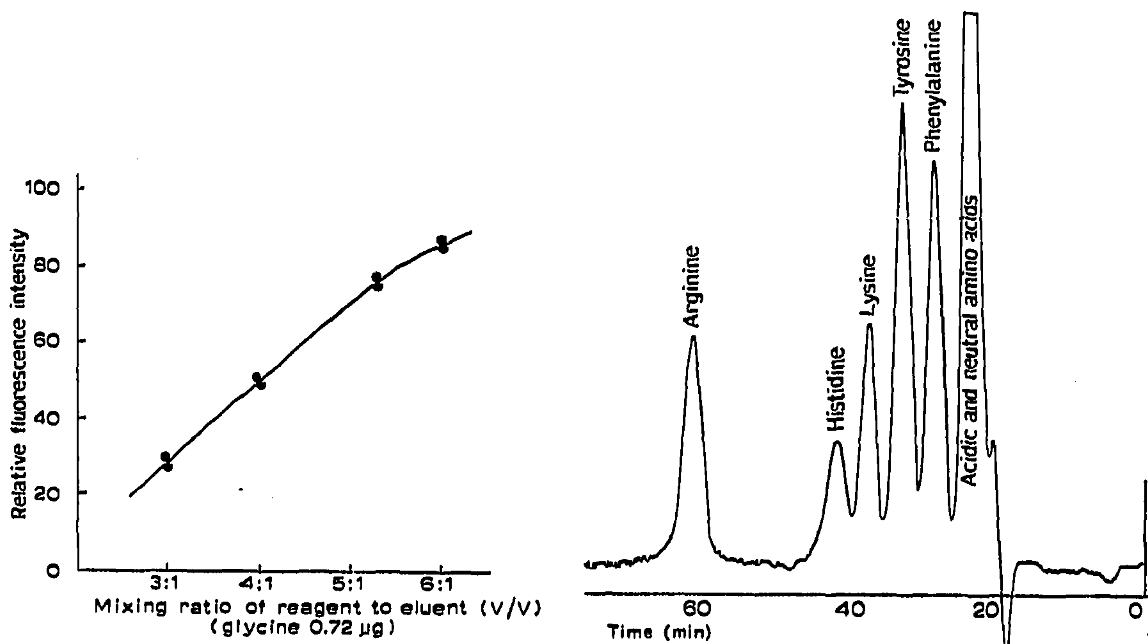

Fig. 3. Effect of the mixing ratio of reagent to eluent on fluorescence intensity.

Fig. 4. Chromatogram monitored by automated fluorimetric assay using the flow cell of a Hitachi Model 204 fluorescence spectrophotometer. The sensitivity was set arbitrarily to minimize the noise level. Each peak represents 0.005 µmole of basic amino acid.

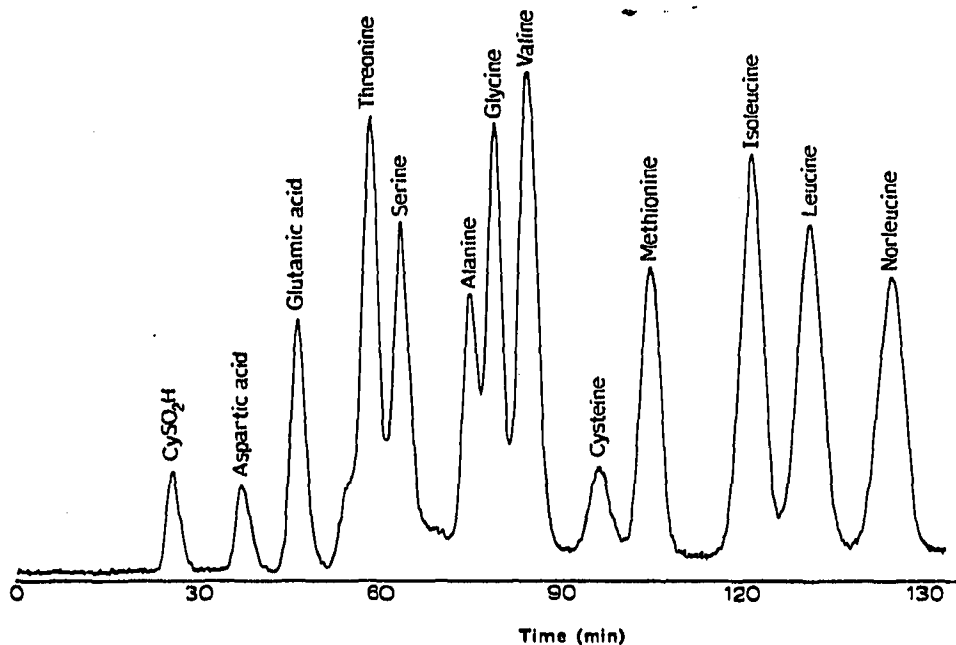

Fig. 5. Chromatogram of acidic and neutral amino acids. Each peak represents 0.01 µmole of sample.

in a refrigerator at −10° for 1 week could be used in the assay just as well as a freshly prepared reagent solution.

Amino sugars, such as glucosamine and galactosamine, also yield fluorescence and can be determined by this procedure. Although proline and hydroxyproline, which have no primary amino groups, gave no fluorescence, and tryptophan and ammonia exhibited no fluorescence, this procedure is for most amino acids one to two orders of magnitude more sensitive than the usual ninhydrin method.

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