SCI. REP. KYOTO PREF. UNIV. (NAT. SCI. & LIV. SCI.), NO. 34. Ser. B, p.15~21 (NOV. 1983)

Studies on the Pre-treatment of Green-Tea Extract with SEP-PAK or Sephadex for Amino Acid Analysis

KOZO OHTSUKI, MAKOTO KAWABATA, KUNIKO TAGUCHI and HIROSHI KOKURA*

(Received July 20, 1983)

Rapid chromatographic methods to clean up green-tea extracts for amino acid analyses were studied. The column packings of the clean-up chromatography were SEP-PAK C_{18} cartridge (Waters) and Sephadex (Pharmacia) G-10, G-25, and G-50. Among these column packings, it was shown that Sephadex G-50 was the most suitable for the separation of the free amino acids in green-tea extracts from tannins and the other contaminants, because of the good recovery of amino acids, the low dilution of the samples, and the good removal of contaminants. This method can also be applied to remove the brown pigment in the 6 N hydrochloric acid hydrolysate of the other samples.

Introduction

The amino acid analyses of green tea by using an amino acid analyser or a high performance liquid chromatograph have been reported[1-3]. In these analyses, it is the most important problem to clean up, without loss of any amino acids, green-tea extracts or green-tea hydrolysates with 6 N hydrochloric acid, because some tannins, pigments and the other contaminants of the tea samples liable to make the quality of the column packings of the analyser worse.

It is not enough only to filter various samples, especially colored samples, with use of Membrane filter or Millipore filter before an injection, to keep the analytical column clean, because some samples may precipitate on the column after the filtration. To prevent these effects, some pre-treatments of samples with the same kind of resin as the analytical column are often carried out. An example of this pre-treatment is to use a SEP-PAK cartridge C_{18}[4].

It is recommended to use Millipore filter and SEP-PAK to clean up various samples before a sample applying to high performance liquid chromatograph to guard an analytical column[4].

For amino acid analyses, the pre-treatment of a sample with a small column of cation ion-exchange resin is often carried out, but the adsorbed amino acids must be eluted with 2N ammonium hydroxide, which is not suitable eluent some time because susceptible amino acids will be changed.

Laboratory of Food Chemistry, Department of Food Science, Kyoto Prefectural University
* Kyoto Prefectural Tea Research Institute

Several methods to clean up green-tea extracts or green-tea hydrolysates were studied in this report.

Materials and Methods

Tryptophan and the other standard amino acids were purchased from Ajinomoto Co., Ltd. Ethyl gallate (Tokyo Kasei Kogyo Co., Ltd.) and the other chemicals (Guaranteed grade) were obtained from Nakarai Chemical Co., Ltd. Green tea (Sen-cha) was purchased from a super market.

Green tea (1g) was extracted with 10ml of hot water (80℃) for 10 minutes and the extract was filtered with a stainless-steel strainer and Membrane Filter TM-2P, 0.45μm, (25mmφ, Toyo Roshi Co., Ltd.).

The eluate from the column was fraction-collected with a mini-collector, SJ-1410SR (ATTO Co., Ltd.). Tryptophan content of the column eluates was measured at 280nm with a Hitachi spectrophotometer, model 100-10. Amino acids were determined by reaction of the column eluates with ninhydrin-titanus chloride solution and measuring the absorbance at 570nm.[5)-7)]. Total tannins were determined by reaction of the eluates (0.5ml) with 0.5ml of 0.1% ferrous chloride-0.5% sodium potassium tartrate and 1.5ml of 0.1M sodium potassium phosphate buffer, pH 7.5, and by measuring the absorbance at 540nm. Ethyl gallate (0.025%) was used as standard, and is defined to be equivalent to $\frac{2}{3}$ times of the tannins in green tea.

Amino acid analyses were carried out on a column of Hitachi Resin # 2618 (0.5×40cm) at 55℃ with buffer-flow rate of 40ml/hr and ninhydrin solution-flow rate of 20ml/hr. The first buffer solution was 0.2 N Na^+ citrate, pH 3.25, the second buffer solution was 0.2 N Na^+ citrate, pH 4.25, and the third buffer solution was 0.35 N Na^+ citrate-0.10 N Na^+ borate, pH 9.8[8)]. The ninhydrin solution was the same as that stated above.

Results and Discussion

When samples are prepared for amino acid analysis or high performance liquid chromatography, the sample preparations are always filtered with Membrane filter or Millipore filter before injection to protect the analytical column of the apparatuses. But the filtration is not always effective for the guard of the columns, because the sample will precipitate or change their nature under some conditions after the filtration, and will stick to the top of the column. The next method often used to protect the analytical column is the use of a guard column.

This is packed with the same type of column packing as the analytical column in the flow-line of liquid chromatograph, just in front of the analytical column. This method is actually effective to protect the analytical column from the sample contaminants, but when the samples are too many, the guard column must be replaced too often. The third method to guard the analytical column is utilizing a SEP-PAK (Waters) or a equivalent device which is not equipped in the flow-line of the liquid chromatograph, but is manipulated to filter a sample with a syringe. In this report SEP-PAK C_{18} (ODS)

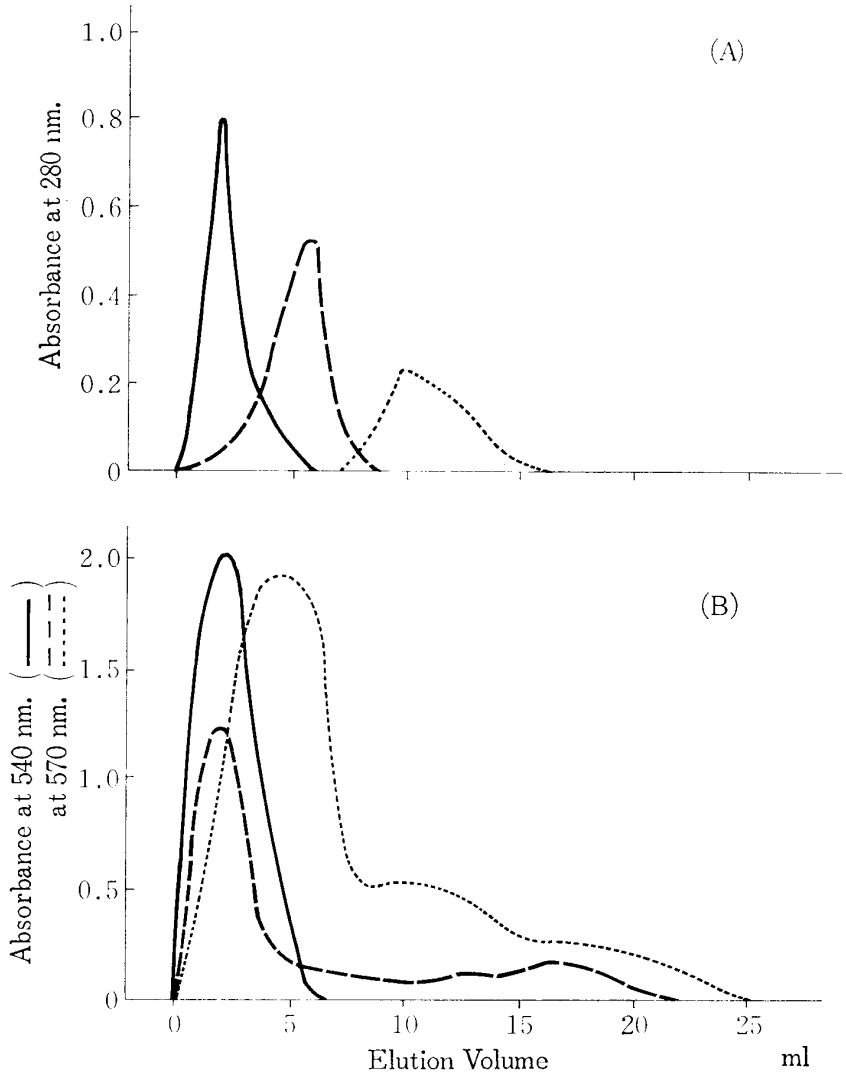

Fig. 1. Chromatograms of Tryptophan and Green-Tea Extract on SEP-PAK (ODS).
(A) : One ml of 0.25 mM tryptophan was eluted with 0.1 N Na^+ citrate buffer, pH 2.2, (——), 7% ethanol (- - -), or distilled water (·····).
(B) : One ml of green-tea extract was eluted with distilled water (——and - - -) or 7% ethanol (·····). The eluates were reacted with ferric chloride (——) for the determination of tannins and with ninhydrin (- - -and·····) for the determination of amino acids and peptides.

and Sephadex G-10, G-25, and G-50 were used to separate free amino acids from the other contaminant and tannins in green-tea extracts.

Chromatograms of tryptophan, as one of free amino acid, and green-tea extract on SEP-PAK were given in Fig. 1. Tryptophan was eluted slowly, Fig. 1 (A), in distilled water as eluting solvent compared with that in citrate buffer or in 7% ethanol. The elution of the other free amino acids were not so affected as that of tryptophan and almost corresponded to the elution position of tryptophan in citrate buffer. It is essential to minimize the dilution of the sample in the pre-treatment of green-tea extract, in order to obtain the acurate and high-sensitive data of amino acid analyses, we must use citrate buffer as an eluting solution to clean up the green-tea extracts.

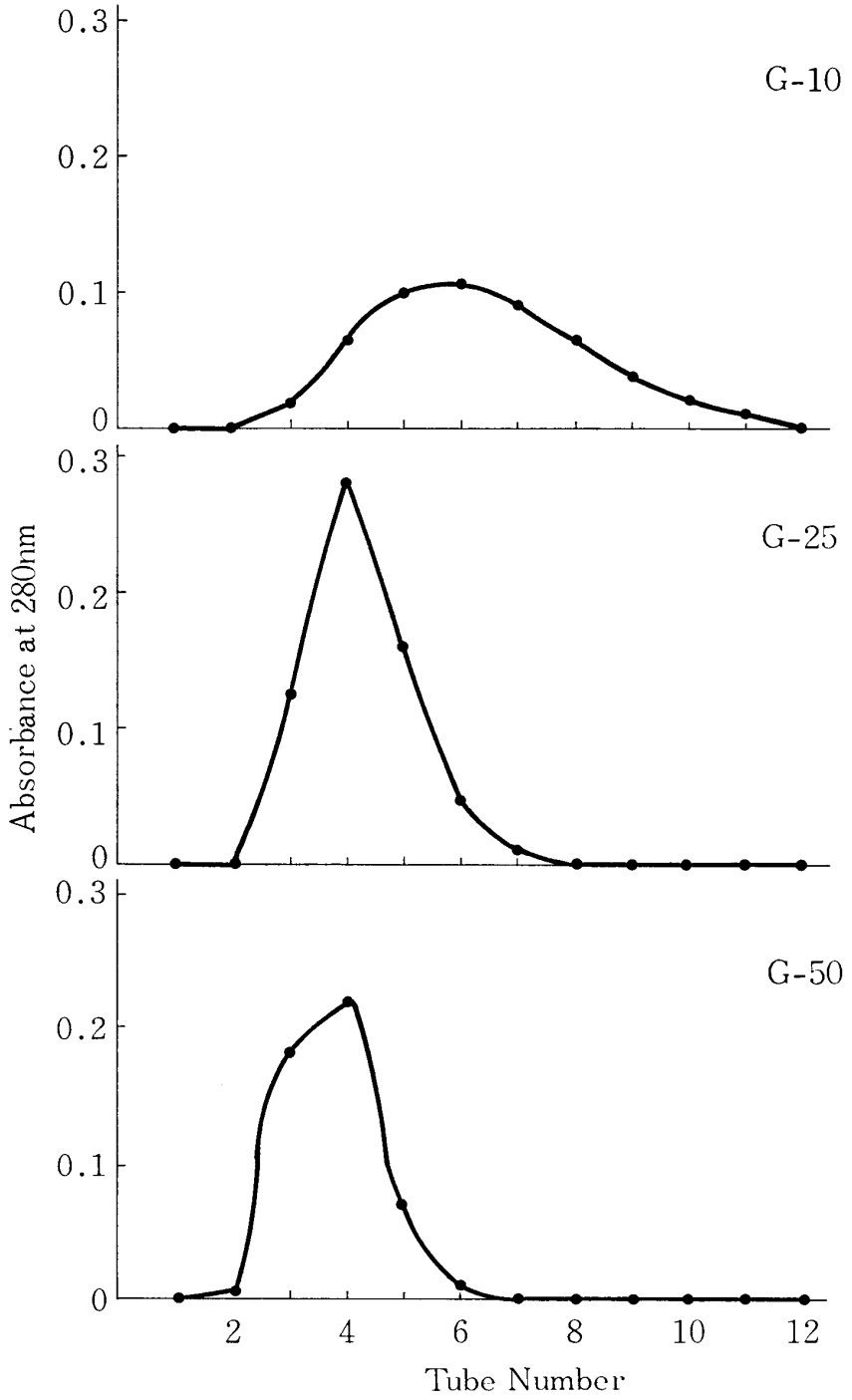

Fig. 2. Elution Patterns of Tryptophan. Column (1.4×3 cm) : Sephadex G-10. G-25, and G-50. One ml of 0.25 mM tryptophan was eluted with 0.1 N Na^+ citrate buffer, pH 2.2, and the eluate was collected every 1.9 ml.

Fig. 1. (B) shows that tannins and free amino acids in green-tea extract co-eluted by eluting with distilled water. It was also observed that tannins and free amino acids co-eluted from the column equilibrated with 0.1N Na^+ citrate buffer (the data is not shown in figures.). These results indicate that SEP-PAK C_{18} is not proper to use for clean-up the green-tea extracts, because we wanted to remove the tannins, which is liable to be oxidized and change to contaminants on the analytical column, without losses of amino acids.

Sephadex G-10, G-25, and G-50 were the next column packings to check the cleaning ability for the green-tea extracts. Acidic amino acid such as aspartic acid, neutral amino acid, alanine, and basic amino acid, arginine, were all eluted from fraction number 2 to 4 of Sephadex chromatngraphy in Fig. 2, as checked by color development with ninhydrin. The elution patterns of tryptophan from Sephadex G-10, G-25, and G-50 were characteristic as shown in Fig. 2. Since the dilution of samples or tryptophan, was the least, and the recovery of standard amino acid mixture was over 97±3% after passing through the column of Sephadex G-50, it is better to clean up the green-tea extract on Sephadex G-50. The low dilution of the sample make it easy to attain a high-sensitive amino acid analysis.

When a green-tea extract was applied on a Sephadex G-50 column as given in Fig. 3, all the ninhydrin-positive substances were eluted from 4ml to 10ml, on the other hand, the tannins were eluted over wide range of the elution. By this chromatography,

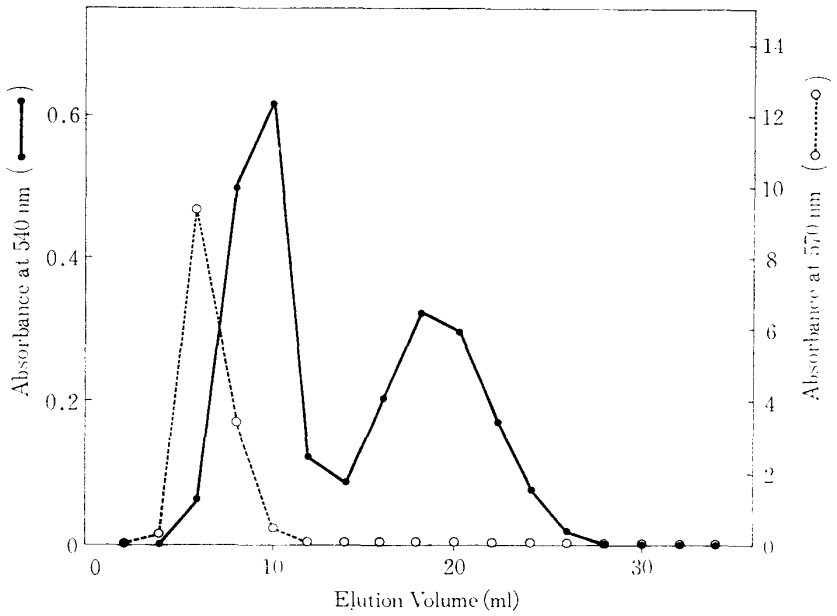

Fig. 3. Separation of Free Amino Acids from Tannins in Green-Tea Extract. Column: Sephadex G-50 (0.8×10 cm). Green-tea extract (0.5 ml) was eluted with 0.005 N HCl. The eluates were reacted with ferric chloride (——) and ninhydrin (……).

about 77% of the tannins in the tea extract were removed from the amino acid fractions. In case of a high sensitive amino acid analysis is not needed, twice or the longer column of Sephadex G-50 column must be prepared to get the higher separation of the tannins from the amino acid in green-tea extracts.

A typical chromatogram of the amino acid analysis by sodium-citrate buffer system were shown in Fig. 4. It is known that theanine, one of specific amino acids to green tea, asparagine, and glutamine are included together with the other ordinary amino acid. Since the amino acid analytical system shown in Fig. 4, was a rapid analytical one, the resolution of the amino acids around threonine, serine, and theanine was not so attained as that by lithium-citrate buffer system. Theanine can be

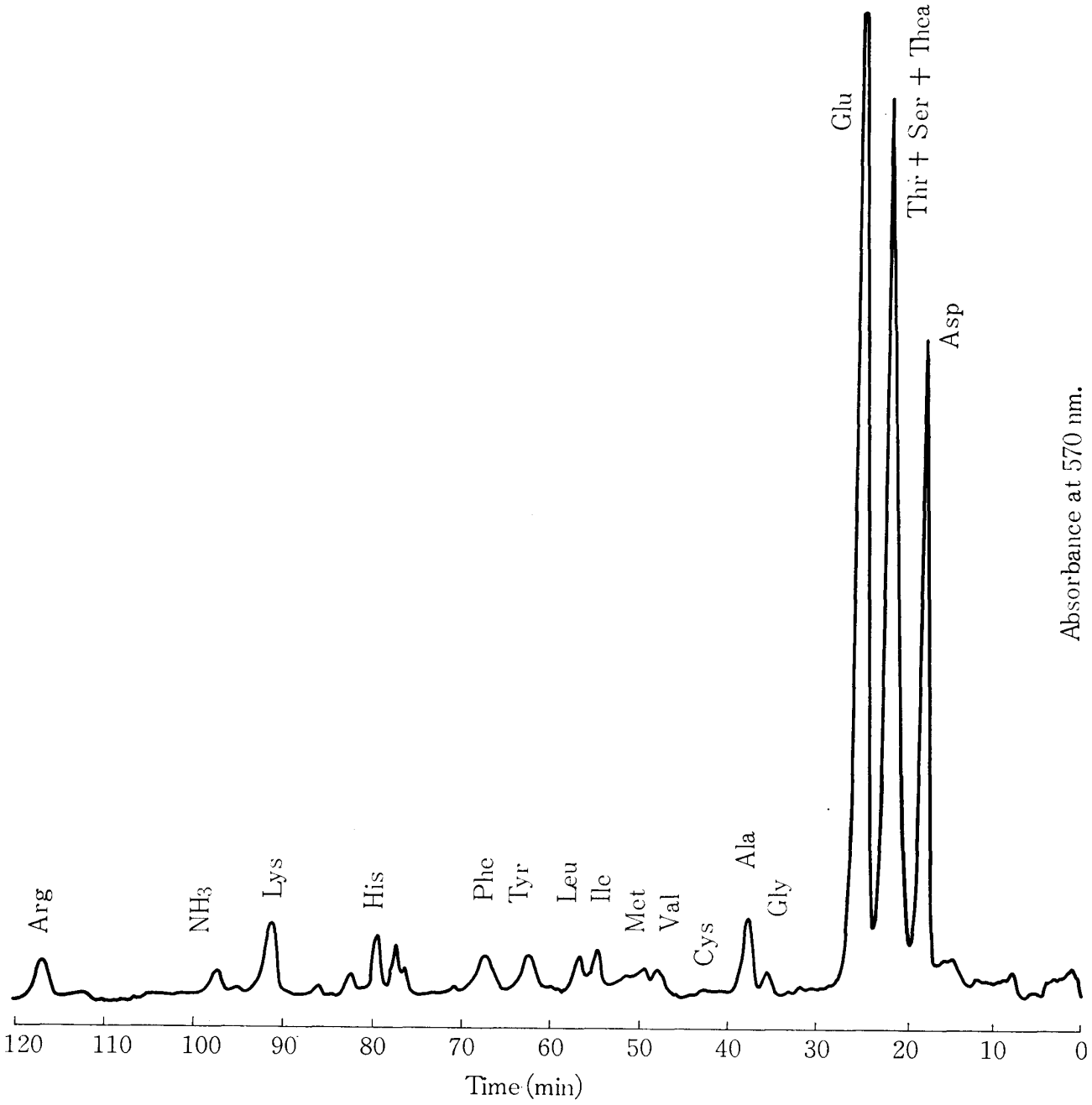

Fig. 4. Amino Acid Analysis of Green-Tea Extract. Column (0.5×40 cm) : Hitachi Resin # 2618 at 55℃. Buffer 1 : 0.2 N Na^+ citrate, pH 3.25. Buffer 2 : 0.2N Na^+ citrate, pH 4.25. Buffer 3:0.45 N Na^+ citrate-borate, pH 9.8.

determined after the tea extract is hydrolyzed with 6N hydrochloric acid and by subtracting the glutamic acid value in Fig. 4. from that in the hydrolysate.

After a green-tea sample was hydrolyzed with 6 N hydrochloric acid at 110° C for 24 hours to 72 hours, the hydrolysate was always stained to brown. In such a case, the brown pigments together with tannins and the other contaminants could be removed from the hydrolysate without loss of amino acids by the chromatography on Sephadex G-50 as mentioned above.

Acknowledgments

We thank Misses S. Fukutome, T. Hisazumi, and H. Konishi for help in performing a part of the experiment.

References

1) M, Nakagawa and N, Ishima: The relation of chemical content of green tea liquors with their their taste. J. Food Sci. Tech. (Japan) **20**. 119 (1973)
2) S. Hirose and S. Tamada: A quantitative determination of amino acids in tea by high pressure liquid chromatography. Chagyo Kenkyu Hokoku **49,** 61 (1979)
3) K. Ohtsuki, M. Kawabata, K. Taguchi, and H, Kokura: High performance liquid chromatography of free amino acids in green tea extract, Studies on prelabeling with dansyl chloride and the separation of the dansyl amino acids. Sci. Rep. Kyoto Pref. Univ. (Nat. Sci. and Liv. Sci.) **30,** B13 (1979)
4) SEP-PAK cartridges instruction sheet: Sample preparation made easy with SEP-PAK cartridges. Waters (Millipore Co., Ltd.)
5) L. B. James: Amino acid analysis, the reduction of ninhydrin reagent with titanous chloride. J. Chromatogr. **59,** 178 (1971)
6) L. B. James: Amino acid analysis, II. Ninhydrin reduction with titanous chloride. J. Chromatogr. **152,** 298 (1978)
7) S. Rokushika, F. Murakami, and H. Hatano: Sigle-wavelength detection for amino acid analysis. J. Chromatogr. **130,** 324 (1977)
8) K-T. D. Lin: Use of a low-salt, alkaline buffer in the elution of basic amino acids from a single-column amino acid analyzer. J. Chromatogr. **132,** 160 (1977)

Laboratory of Food Chemistry,

Department of Food Science,

Kyoto Prefectural University

Shimogamo Kyoto, 606, Japan