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A modified gradient elution procedure for single column amino acid analysis*

Amino acid analysis of connective tissue proteins poses problems not encountered with most protein hydrolysates. The difficulty arises in the separation of hydroxyproline from aspartic acid, and separation of hydroxylysine, lysinonorleucine and the desmosines from other basic amino acids. Of the current procedures in the literature[1-4], only the method of MILLER AND PIEZ[1] resolves these amino acids in a length of time suitable for routine analysis. However, the ninhydrin reagent commonly used with standard amino acid analysis is not compatible with their buffer system. Amino acid color yields are only 25% of maximum at the start of the run, with quite a significant increase in the base line toward the end of the run.

Recently a rapid procedure of programmed analysis has been described which gives good separation of the amino acids in collagen in less than 4 h[5]. This procedure is not practical for most of the standard analyzers, as it requires four buffer changes and three temperature changes.

This paper describes a rapid method that resolves all the amino acids arising from connective tissue proteins on a single column, with no temperature change, and utilizing the same buffers and reagents used in the standard two-column method[6,7].

Methods

A Beckman Model 116 amino acid analyzer with the starting buffer line connected to a nine chambered Varigrad gradient device was used in these experiments. The long column, 0.9 × 69 cm, was packed to a column height of 50 cm with UR-30 resin. Flow rates were 70 ml/h for the buffer and 30 ml/h for the ninhydrin reagent. Water jacket temperature was maintained at 56° throughout the run.

The ninhydrin reagent, the 0.20 *N* sodium citrate buffer, pH 3.25 and the 0.35 *N* sodium citrate buffer, pH 5.25, were prepared as described in the Beckman manual. The pH 2.91 buffer was prepared the same as the pH 3.25 buffer except two percent of the water was replaced with *n*-propanol and the pH adjusted with concentrated HCl. Forty-three grams of sodium chloride were added per liter of pH 5.25 buffer to bring the final sodium concentration to 1.08 *N*.

The gradient was prepared as shown in Table I.

The column was equilibrated with the pH 2.91 buffer prior to the start of the analysis. After application of the sample, the starting buffer line was connected to the Varigrad and the run started. A change to the pH 5.25 buffer was made after 130 min.

Results and discussion

Fig. 1 shows a chromatogram of a synthetic mixture of amino acids. Peak retention times for amino acids not shown in the chromatogram are: cysteic acid, 28 min; 3-hydroxyproline, 43 min; methionine sulfoxides, 51 min; isodesmosine, 175 min; lysinonorleucine, 190 min; and ornithine, 201 min.

If the buffers were not free from contaminating ammonia, a shoulder remained after the ammonia peak, and resulted in an increased baseline through histidine

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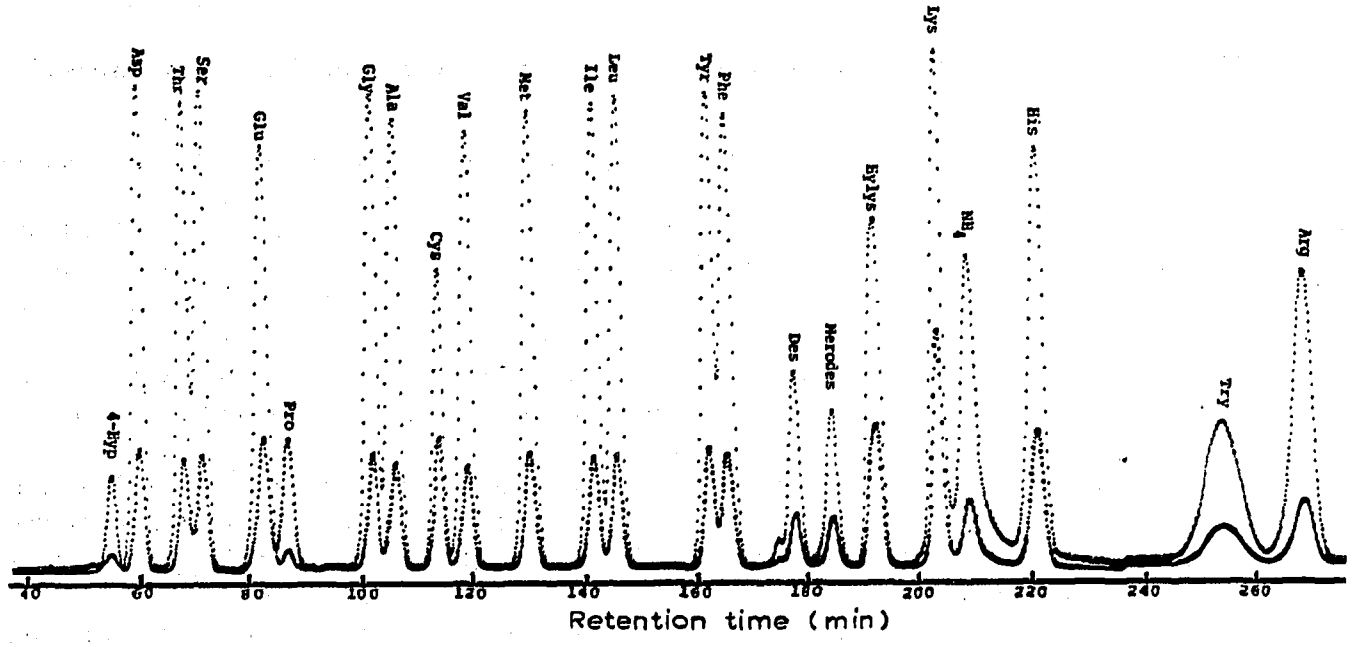

Fig. 1. Chromatogram of a synthetic mixture of amino acids using the procedure described in the text.

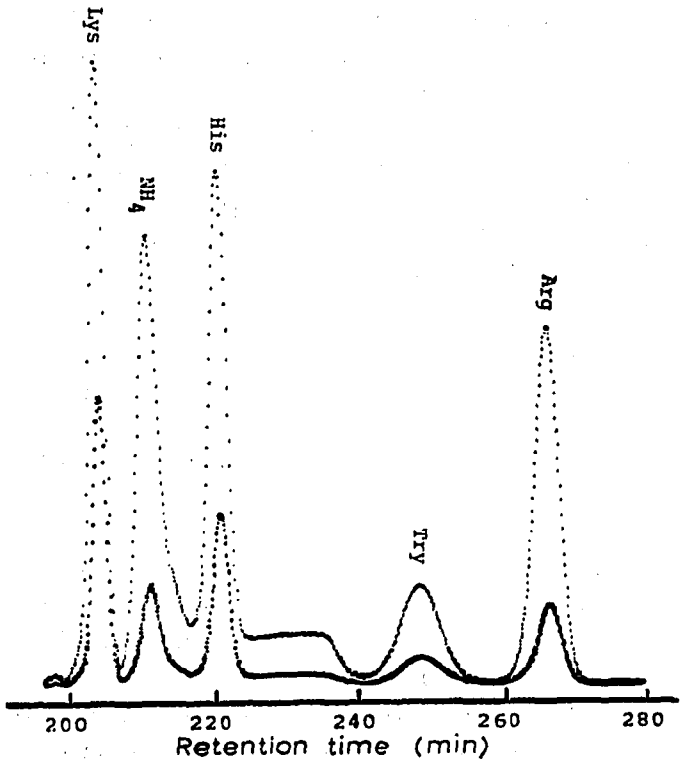

Fig. 2. Latter part of a gradient elution chromatogram, illustrating the ammonia plateau that appears when the pH 2.91 buffer contains ammonia.

TABLE I

PREPARATION OF THE VARIGRAD GRADIENT

Chamber	*pH 2.91 (ml)*	*pH 3.25 (ml)*	*0.4 N Na-citrate (ml)*
1	36	—	—
2	36	—	—
3	—	36	—
4	—	30	6
5	—	26	10
6	—	18	18
7	—	6	30
8	—	—	36
9	—	—	36

(Fig. 2). Unless the ammonia was present in large amounts, however, this did not affect the resolution or accuracy of the analysis. This shoulder could be eliminated by redistilling over sulfuric acid the water that was used to make the pH 2.91 buffer.

The low pH of the equilibrating buffer was required for the separation of hydroxyproline from aspartic acid. This separation could also be accomplished by lowering the temperature, but inadequate resolution and broadening of peaks resulted. Increasing the normality of the pH 5.25 buffer as little as 0.03 *N* in sodium resulted in tryptophan and arginine being eluted together.

Washing the column with NaOH after each analysis is not necessary with most protein hydrolysates. The buffer line is cleared with the pH 2.91 buffer and the column equilibrated. The entire process of making the gradient and equilibrating the column to start a new run requires about 20 min.

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1 E. J. MILLER AND K. A. PIEZ, *Anal. Biochem.*, 16 (1966) 320.
2 T. DEVENYI, *Acta Biochem. Biophys. Acad. Sci. (Hung.)*, 3 (1968) 429.
3 R. D. DURBIN, G. F. PEGG AND M. STRMECKI, *J. Chromatog.*, 28 (1967) 429.
4 D. E. PETERSON AND R. W. BERNLOHR, *Anal. Biochem.*, 33 (1970) 238.
5 W. BANDLOW AND A. NORDWIG, *J. Chromatog.*, 39 (1969) 326.
6 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
7 R. W. HUBBARD, *Biochem. Biophys. Res. Commun.*, 19 (1965) 679.

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