Journal of Chromatography, 115 (1975) 236–239

CHROM. 8576

Note

An improved buffer system for use in single-column gradient-elution ion-exchange chromatography of amino acids

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(First received April 14th, 1975; revised manuscript received July 1st, 1975)

Amino acid analysis using ion-exchange chromatography has improved over the years with faster rates of analysis, increased sensitivity and greater reliability of instrumentation[1–3]. The majority of analysers use the stepwise technique developed by Spackman *et al.*[4], but some methods of gradient elution chromatography have overcome some of the disadvantages associated with stepwise systems.

The gradient elution technique of Piez and Morris[5], although representing a considerable advance in ion-exchange chromatography, could not readily be automated for repetitive fast analyses. This was due both to an inherent lack of flexibility, particularly with regard to abrupt pH changes, and to the associated inconvenience of buffer preparation. Subsequent work by Thomas *et al.*[6–8] produced a system which overcame these problems and which did make possible fast, automated amino acid analysis on a routine bases.

Thomas *et al.* achieved separation of the amino acids by producing on the ion-exchange column a pH gradient derived from the accurate mixing of only two buffers, of high and low pH with equimolar sodium concentration. The technique as first reported relied on a citrate–phosphate buffer system[9] and it would now appear from work done in this laboratory that the latter gives somewhat limited control of the pH gradient during elution of the basic amino acids.

This paper describes an alternative, citrate–borate buffer system which gives better control of the pH gradient in the basic region with a resulting improvement in the separation of the appropriate amino acids.

EXPERIMENTAL

Apparatus

A Rank Hilger Chromaspek[10] ion-exchange chromatograph was used to produce the pH gradients and subsequent chromatograms. The instrument was fitted with a 350 × 2.6 mm column filled with Rank Hilger 7μ resin and this, when maintained at 60°, required that the high pressure pump overcame a back pressure of 300–400 p.s.i. to maintain the optimum buffer flow-rate of 0.16 ml/min.

The pH was monitored at the sample resorvoir using a Model 26 pH meter, micro electrode G2221C and reference electrode K1301 (Radiometer, Copenhagen, Denmark).

Reagents

Buffer solutions were prepared as follows: acid buffer (pH 2.2, 0.2 *M* Na^+). 10.5 g citric acid, 11.7 g sodium chloride. 3.5 ml Brij 35 (10% w/v), 2.5 ml thiodiglycol dissolved in water and diluted to one litre: citrate–phosphate basic buffer (pH 11.5. 0.2 *M* Na^+), 14.7 g trisodium citrate·2 H_2O, 6.46 g trisodium orthosphosphate·12 H_2O, 12.5 ml 4% EDTA, 3.5 ml Brij 35 (10% w/v), dissolved in water and diluted to one litre; borate basic buffer (pH 11.5, 0.2 *M* Na^-). 38.13 g disodium tetraborate·10 H_2O dissolved in water and diluted to one litre, the pH was adjusted to pH 11.5 with 0.2 *M* sodium hydroxide and 13.0 ml 4% EDTA and 4.0 ml Brij 35 (10% w/v) were added to the final solution before use.

The amino acid standard was prepared as follows: 1 ml AA5 Standard (Calbiochem, Los Angeles, Calif., U.S.A.), 1 ml *n*-leucine (2.5 μmoles/ml), 1 ml tryptophan (2.5 μmoles/ml) diluted to 50 ml with 0.025 *M* HCl. This gave a solution containing 50 nmoles/ml of each amino acid. The sample loading was 160 μl.

Procedure

The pH gradients were produced by running Chromaspek with a programme drum profile which gave linear stepwise increments in the volume ratio of basic to acid buffer. The stepwidths were such as to allow stabilisation of pH between each ratio change and the resulting pH readings were plotted against percentage of basic buffer for both the citrate–phosphate and citrate–borate systems. Separate chromatograms were then produced using suitable profiles for each buffer system to effect resolution of the amino acids.

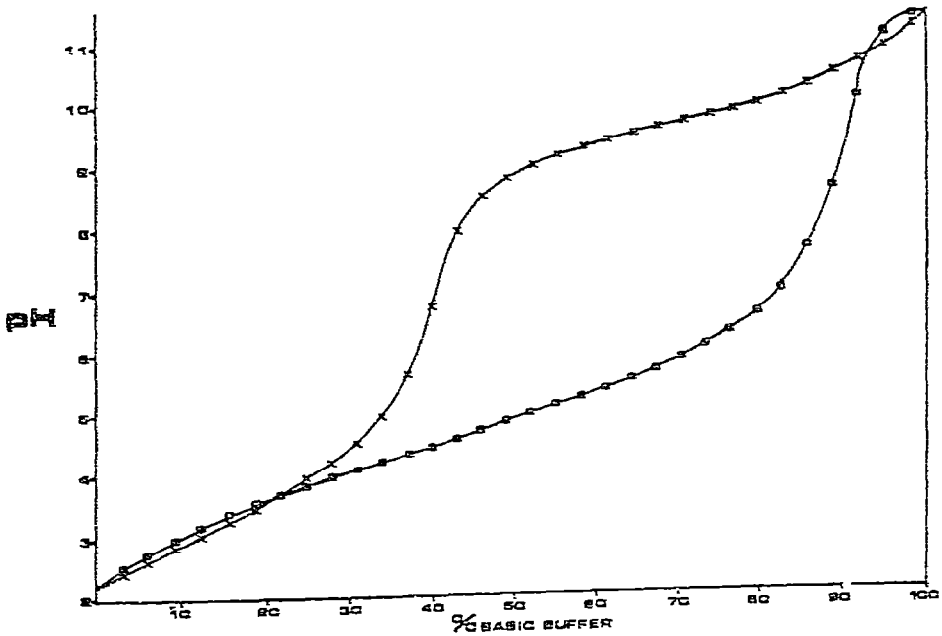

Fig. 1. Relationship between pH and percentage of basic buffer using the citrate–phosphate buffer system (□—□) and the citrate–borate buffer system (▽—▽).

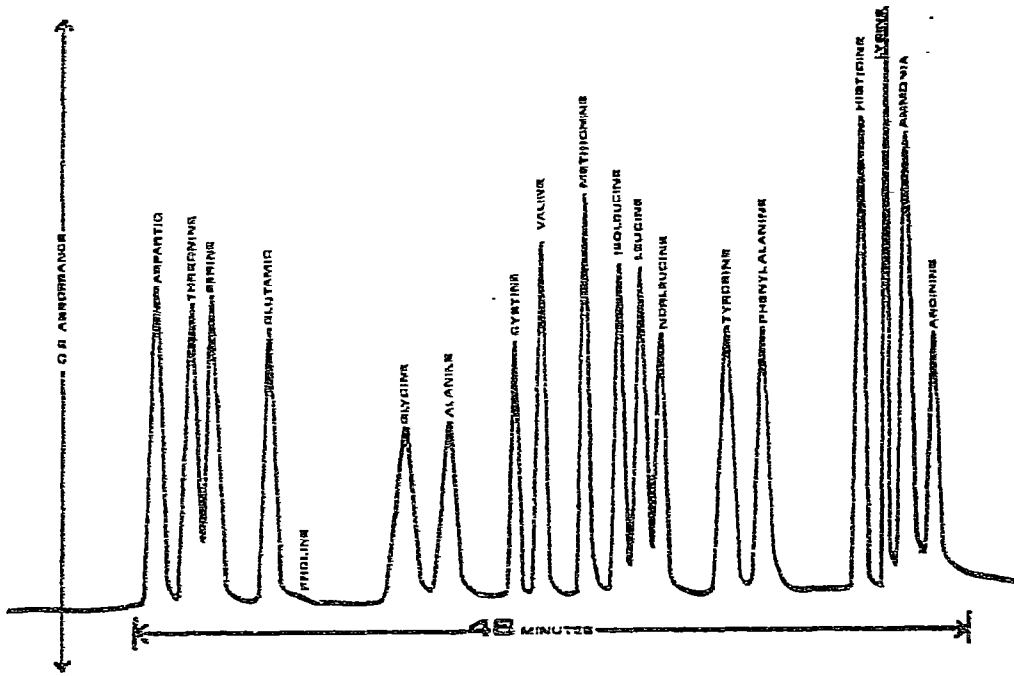

Fig. 2 Chromatogram obtained using the citrate–phosphate buffer system.

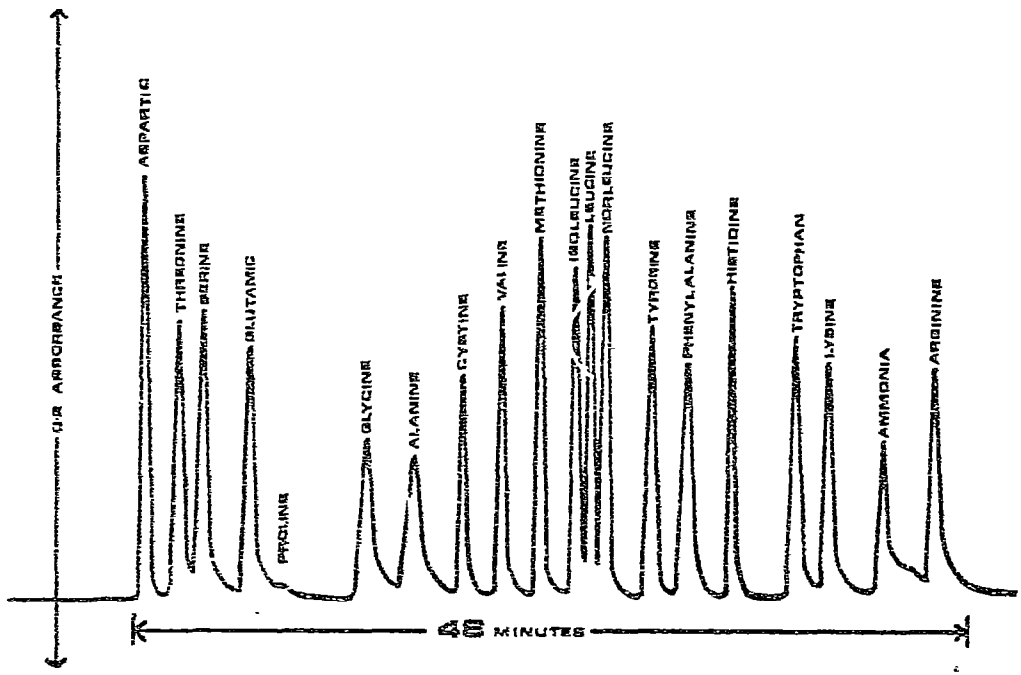

Fig. 3. Chromatogram obtained using the citrate–borate buffer system showing improved separation of the basic amino acids.

RESULTS AND DISCUSSION

When using low molarity buffers for fast elution of the basic amino acids a controliable pH gradient between pH 7.5 and pH 11.0 is required to give adequate separation. Comparison of the graphs of pH *vs.* percentage of basic buffer for the citrate–phosphate and citrate–borate systems (Fig. 1) shows that the rate of rise of pH through this region is significantly lower for citrate–borate buffering, although as a consequence the pH gradient is higher between pH 4 and pH 7.5.

Chromatograms obtained using the two different buffer combinations are shown in Figs. 2 and 3. It can be seen that an improved separation of the basic amino acids has been achieved using the citrate–borate system. At the same time there has been no significant deterioration in the separation of the other acids and no increase has been required in the overall cycle time.

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