Journal of Chromatography, 209 (1981) 41–47
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,610

USE OF MIXED LITHIUM–SODIUM BUFFERS AND POTASSIUM BUFFERS FOR SEPARATION OF THE COMMON FREE AMINO ACIDS IN PLANT MATERIAL ON A TWO-COLUMN SYSTEM

J. V. MORTENSEN
Department of Agricultural Chemistry, State Laboratory for Soil and Crop Research, Lottenborgvej 24, 2800 Lyngby (Denmark)
(First received July 7th, 1980; revised manuscript received December 23rd, 1980)

SUMMARY

A method for the determination of amino acids in plant extracts using a two-column system is described. The acidic and neutral amino acids are separated on a column of Beckman AA-15 resin, with three buffers of mixed lithium citrate–sodium citrate. With the mixed buffers the back pressure on the column was lower than with a pure lithium buffer, but glutamine and asparagine were still well separated from the other amino acids. The basic amino acids and some related compounds are separated on a column of Beckman PA-35 resin, with two buffers of potassium citrate.

INTRODUCTION

The use of lithium citrate buffers has made it possible to separate the amides asparagine and glutamine from each other and from the other amino acids in ion-exchange chromatography[1]. Many modifications of this method have been described[2–7]. These amides have also been separated by use of sodium buffers and temperature programming[8], but at the expense of the separation of aspartic acid and serine. The use of lithium buffers results in a higher column pressure and hence a higher risk of leakage at the connections and valves in the analyzer than experienced with sodium buffers.

Owing to the increased destruction of glutamine at elevated column temperatures[9], it is necessary to keep the column temperature as low as possible, but this results in a higher column pressure. In most of the analytical systems previously described a column temperature of 35–40°C is used until glutamine is eluted from the column.

This paper describes a method for the separation of acidic and neutral amino acids on a long column using mixed lithium citrate–sodium citrate buffers to overcome the pressure problem while maintaining a satisfactory resolution. The use of mixed buffers of this type has been described by Oulevey and Heitefuss[7], but their aim was to obtain a better separation of basic amino acids in a one-column system than could be achieved by use of a pure lithium buffer[10].

0021-9673/81/0000–0000/$02.50

In the two-column system, sodium buffers are generally still used for the elution of basic amino acids from the short column, as no better separation is achieved by lithium buffers. The use of potassium buffers has been described for elution of amines[11], where they were found to shorten the elution time compared with sodium buffers.

We also describe a method using potassium buffers for the elution of basic amino acids and related compounds from the short column.

MATERIAL AND METHODS

Equipment

A Beckman (Palo Alto, CA, U.S.A.) Model 121 amino acid analyzer equipped with an Autolab computing integrator was used. Two columns were employed, one 69 × 0.9 cm with 55 cm Beckman AA-15 resin for the acidic and neutral amino acids, and the other 28 × 0.9 cm with 16 cm Beckman PA-35 resin for the basic amino acids. A Hetofrig cooler was used for cooling the water in the column jackets.

Sample preparation

A synthetic mixture was prepared from amino acids obtained from Sigma (St. Louis, MO, U.S.A.), Merck (Darmstadt, G.F.R.), BDH (Poole, Great Britain) or Hoffmann-La Roche (Basle, Switzerland). The amino acids were dissolved in sodium citrate buffer. The amides glutamine and asparagine were renewed regularly. The plant amino acids were extracted according to Cook and Bieleski[12].

Reagents

The ninhydrin reagent was prepared from 100 g of ninhydrin, 4 l methyl Cellusolve, 1.5 l of 4 *M* sodium acetate buffer, pH 5.5, and 15 ml of 15% titanous chloride for reduction of the ninhydrin[13].

Three buffers were used on the long column and two on the short column for elution of the amino acids. Their compositions are listed in Tables I and II.

TABLE I
COMPOSITION OF BUFFERS FOR ELUTION OF ACIDIC AND NEUTRAL AMINO ACIDS FROM THE LONG COLUMN
The final adjustment of pH was made with HCl or LiOH–NaOH (3 mole/2 mole).

	Buffer		
	A	*B*	*C*
pH	3.00	3.30	4.15
Lithium concentration (*M*)	0.12	0.12	0.12
Sodium concentration (*M*)	0.08	0.08	0.08
Lithium citrate tetrahydrate (g/l)	11.28	11.28	11.28
Sodium citrate dihydrate (g/l)	7.84	7.84	7.84
Thiodiglycol (ml/l)	2.0	2.0	2.0
Caprylic acid (ml/l)	0.1	0.1	0.1
Concentrated HCl (ml/l)	14.0	12.5	9.5

TABLE II
COMPOSITION OF BUFFERS FOR ELUTION OF BASIC AMINO ACIDS FROM THE SHORT COLUMN

	Buffer	
	D	*E*
pH*	3.70	4.15
Potassium concentration (*M*)	0.30	0.40
Potassium citrate monohydrate (g/l)	21.63	21.63
Potassium chloride (g/l)	7.50	15.00
Caprylic acid (ml/l)	0.1	0.1
Concentrated HCl (ml/l)	11.0	9.5
Benzyl alcohol (ml/l)**	10.0	5.0

* Final adjustment made with HCl or KOH.
** Added after adjustment of pH.

For regeneration of the resins, 0.2 *M* lithium hydroxide–sodium hydroxide (Li^+–Na^+ = 3:2) and 0.2 *M* KOH were used for the long and short columns respectively.

Procedure

The flow-rate for the buffers was 70 ml/h and for the ninhydrin reagent 35 ml/h.

After sample injection, the amino acids on the long column were eluted with buffers A, B and C for 57, 90 and 98 min respectively. The two buffer changes were into effect between glutamine and glutamic acid and between cystine and methionine. The resin was regenerated with base for 18 min and equilibrated with buffer A for 44 min.

On the short column the amino acids were eluted with buffers D and E for 122 and 90 min with the buffer change between lysine and tryptophan. The resin was regenerated with base for 10 min and equilibrated with buffer D for 36 min.

The column temperature was lowered to 30°C by a 6-min fast cool step just before the injection of the sample on the long column. It was then maintained for 99 min before being raised to 45°C for the rest of the analysis.

RESULTS

The elution pattern of the first amino acids from the long column with the selected lithium–sodium ratio (0.12:0.08) is shown in Fig. 1, together with the effect of variation in this ratio at the chosen buffer pH and column temperature. This ratio gave a complete separation of the amides from each other and from the other amino acids. Glutamic acid was eluted after the amides and not between them as with the pure lithium buffer, which also gave a complete separation of the amino acids. When sodium formed a large part or all of the cations, poor or no separation of aspartic acid from threonine and of the amides was obtained.

The broad space between glutamic acid and glutamine achieved with the mixed buffer was utilized by placing the first buffer shift there.

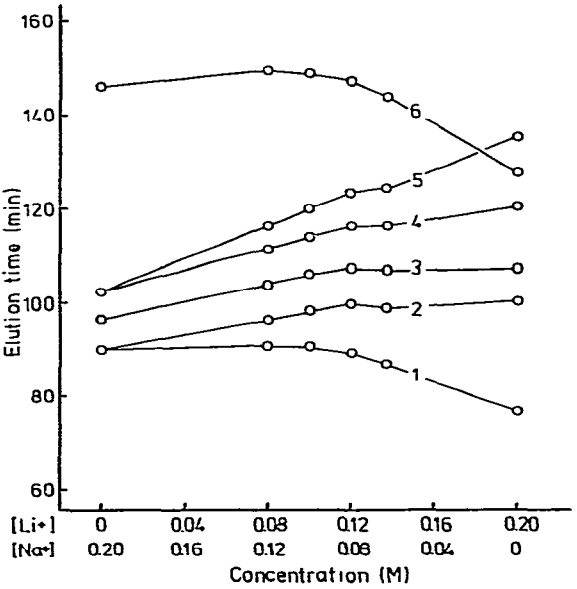

Fig. 1. Effect of the lithium–sodium ratio on the elution behaviour of the first common amino acids from the long column at pH 3.0; column temperature 30°C. Amino acids: 1 = Asp; 2 = Thr; 3 = Ser; 4 = Asn; 5 = Gln; 6 = Glu.

The column back pressure rose from 300 p.s.i. for a pure sodium buffer to 350 p.s.i. at the selection ratio and 450 p.s.i. for a pure lithium buffer.

A pH of 3.00 was found to be optimum for buffer A. Lowering the pH to 2.78 resulted in a much increased elution time and a partial overlapping of aspartic acid and threonine, while raising it to 3.24 resulted in coelution of the amides.

With the mixed buffers B and C the elution order of the common protein amino acids was as obtained for pure sodium or lithium buffers[2]. Unfortunately, α-aminoadipic acid and citrulline were not separated from the glycine–alanine couple and hydroxyproline was eluted together with aspartic acid, but these amino acids are normally present only in negligible amounts compared to the common protein amino acids.

The separation of basic amino acids and related compounds with the potassium buffer (D) is shown in Fig. 2, together with the effects of variation of the pH (a) and of the concentrations of potassium (b) and benzyl alcohol (c). A good separation of the named compounds was achieved except for tyrosine from phenylalanine, which, however, were separated on the long column. Tryptophan and arginine, which were eluted with buffer E, do not appear in the figure.

The optimum values for the parameters of buffer D were a pH of 3.70, a potassium concentration of 0.30 *M* and a benzyl alcohol concentration of 10 ml/l, with a reasonable flow-rate.

While most of the compounds are very sensitive to changes of pH in the tested range, ammonia and ethanolamine are unaffected. A decrease of pH to 3.6 gave a better separation of most of the compounds, but β-alanine came closer to ethanolamine. At pH 4.0 some amino acids are eluted together.

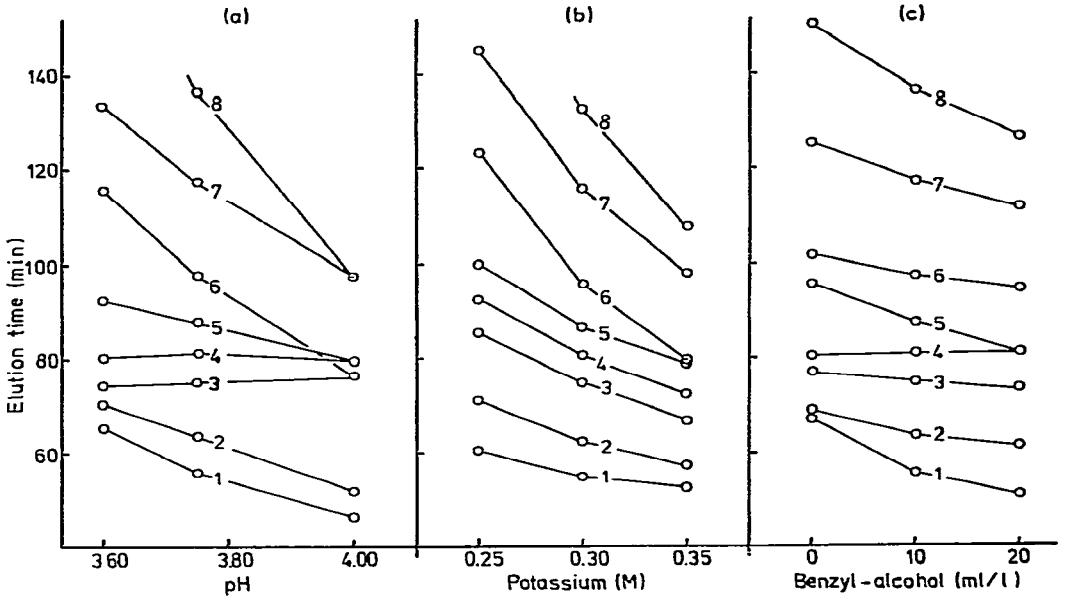

Fig. 2. Effect of the pH (a) and concentrations of potassium (b) and benzyl alcohol (c) on the elution behaviour of various amino compounds from the short column. Temperature 45°C; pH 3.75; potassium concentration 0.30 *M*; benzyl alcohol concentration 10 ml/l. Amino compounds: 1 = Tyr + Phe; 2 = β-Ala; 3 = ethanolamine; 4 = NH_3; 5 = γ-aminobutyric acid; 6 = Orn; 7 = His; 8 = Lys.

The potassium concentration had only a small effect on the elution pattern. With 0.25 *M* K^+ a better separation was achieved than with 0.30 *M* K^+, but at the expense of increased elution time and peak broadening. With 0.35 *M* K^+, ornithine and γ-aminobutyric acid were eluted together.

Addition of benzyl alcohol favoured the separation of β-alanine from tyrosine + phenylalanine because of a specific decrease in the elution time of the aromatic amino acid, especially of tryptophan, which appears late in the chromatogram. Also γ-aminobutyric acid was particularly affected and was eluted together with ammonia at 20 ml of benzyl alcohol per litre of buffer.

Changes in the column temperature had only small specific effects on the elution pattern.

A comparison of the sodium and potassium buffers was undertaken at the values of the buffer parameters found to give the best results with the potassium buffer (Table III). Most of the compounds are eluted faster with potassium than with sodium, except for the aromatic amino acids, tyrosine + phenylalanine and tryptophan.

Running of plant extracts prepared from grass and clover gave clear chromatograms with a good separation of the amino acids; some unidentified peaks appeared, especially before aspartic acid.

TABLE III
EFFECT OF TWO CATION SPECIES ON THE ELUTION TIMES OF VARIOUS AMINO COMPOUNDS FROM THE SHORT COLUMN
Buffer parameters: 0.2 *M* Na^+ or K^+; pH 3.75; 10 ml/l benzyl alcohol. Column temperature: 45°C.

Compounds	*Elution times (min)*	
	With K^+	*With* Na^+
Tyrosine + phenylalanine	55.5	51.5
β-Alanine	63.5	67.9
Ethanolamine	75.0	100.7
Ammonia	81.1	110.7
γ-Aminobutyric acid	87.4	100.7
Ornithine	97.6	137.0
Histidine	117.4	174.6
Lysine	136.2	195.1
Tryptophan	181.6	156.5

DISCUSSION

The mixed lithium–sodium buffer made it possible to separate the amides from each other and from the other amino acids and a lower pressure was obtained than with a pure lithium buffer. A higher proportion of lithium would have improved the separation of the first amino acids, but the column back pressure would have increased and there would have been less space between glutamine and glutamic acid for the buffer shift.

The specific effect of the cation species on the dicarboxylic amino acids is remarkable in that they, contrary to the other amino acids, are eluted faster with lithium than with sodium. Also α-aminoadipic acid is eluted sooner in relation to the other amino acids with lithium buffers[2]. A disadvantage of the mixed buffers is the more complicated preparation.

The effect of pH on the elution time of the amino compounds from the short column is dependent on whether their p*K* values lie within the applied range of pH. However, at the resin surface the pH is 1–2 units lower than in the buffer[14], which explains the stronger effect of pH on ornithine, lysine and histidine with p*K* values of 1.9, 2.2 and 1.8 respectively for their carboxylic group[15] than on β-alanine and γ-aminobutyric acid with p*K* values of 3.6 and 4.0 respectively. The small effect of pH on the elution of tyrosine and phenylalanine, which have p*K* values of about 2.2, is possibly due to a greater binding by non-electrostatic forces. Ammonia and ethanolamine have p*K* values too far outside the actual range of pH to be measurably influenced.

Benzyl alcohol probably acts as a competitor with respect to the adsorption by non-electrostatic forces and therefore exerts its strongest influence on the aromatic amino acids, which made it possible to separate β-alanine from tyrosine + phenylalanine. No effect was observed on ammonia and ethanolamine, which are mostly bound by electrostatic forces.

The faster elution of most of the compounds with potassium than with sodium is undoubtedly due to the smaller radius of the hydrated potassium ion. However, the aromatic amino acids exhibit opposite behaviour, with the advantage that the broad

peak of tryptophan is placed in the great space after lysine when potassium is used and not among the closely spaced amino compounds as with sodium. Tryptophan is anomalous in another respect in that it shows an increased elution time with increasing sodium concentration[16,17].

REFERENCES

1 J. V. Benson, M. J. Gordon and J. A. Patterson, *Anal. Biochem.*, 18 (1967) 228.
2 J. V. Benson, *Anal. Biochem.*, 50 (1972) 477.
3 G. E. Atkin and W. Ferdinand, *Anal. Biochem.*, 38 (1970) 313.
4 C.-P. Kedenburg, *Anal. Biochem.*, 40 (1971) 35.
5 S. B. Melançon and J. Tayco, *J. Chromatogr.*, 63 (1971) 404.
6 J. L. Young and M. Yamamoto, *J. Chromatogr.*, 78 (1973) 221.
7 J. Oulevey and R. Heitefuss, *J. Chromatogr.*, 94 (1974) 283.
8 I. Oreskes, F. Cantor and S. Kupfer, *Anal. Chem.*, 37 (1965) 1720.
9 I. Oreskes and S. Kupfer, *Anal. Chem.*, 39 (1967) 397.
10 H. Lorenz, *Phytochemistry*, 10 (1971) 63.
11 H. Tabor, C. W. Tabor and F. Irreverre, *Anal. Biochem.*, 55 (1973) 457.
12 A. R. Cook and R. L. Bieleski, *Anal. Biochem.*, 28 (1969) 428.
13 L. B. James, *J. Chromatogr.*, 59 (1971) 178.
14 C. W. Davies, *Biochem. J.*, 45 (1949) 38.
15 D. D. Perrin (Editor), *Dissociation Constants of Organic Bases in Aqueous Solution*, IUPAC, Butterworths, London, 1965, p. 473.
16 P. B. Hamilton, *Anal. Chem.*, 35 (1963) 2055.
17 K.-T. D. Lin, *J. Chromatogr.*, 132 (1977) 160.