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AMINO ACID ANALYSIS OF PROTEIN HYDROLYSATES BY LITHIUM BUFFER SYSTEM

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ABSTRACT

A chromatographic procedure for the analysis of amino acid hydrolysates using lithium buffers and a dedicated amino acid analyzer is described. When used with an Hitachi amino acid analyzer model 835-50, the modified system permitted separation to near baseline of 18 common amino acids, plus another 6 amino compounds commonly encountered in such assays. Elution time from injection to arginine was 90 minutes. A major advantage of this system when only one amino acid analyzer is available is the greatly reduced work load compared to the classical switching over between sodium-based and lithium-based systems.

INTRODUCTION

Automated analysis of amino acid mixtures by ion exchange chromatography technique has been available since 1958.[1]The eluting buffers used in such analyses may be categorized by their counterion components, either sodium or lithium. The main advantage of lithium buffers is the ability to resolve the amides asparagine and glutamine present in physiological samples, from glutamic acid, without sacrificing resolution of other amino acids.[2] The main advantage of sodium buffers is their lower cost. For these and other considerations, buffer systems for the analysis of protein hydrolysates invariably use sodium as the counterion, while buffers for physiological fluid analysis use lithium.

These two types of analysis need not only different instrument software and hardware settings, but also different sets of buffers. Changeover procedures that take only a short time include the time dependent programming of buffers and column temperatures, the changeover of analytical columns, and the loading of integrator parameters. Other procedures, however, such as the preparation of a new set of 4 to 5 buffers, and the complete flushing of 5 to 6 buffer lines and valves, consume about two working days. More time and reagents are usually required for the fine tuning of buffer pH to get relatively consistent separation of peaks, before calibration and sample analysis can start.

Such changeovers are a major inconvenience in service situations where only one amino acid analyzer is available for widely varying sample types, and where quick results are required. Another disadvantage of sodium systems is that the separation pattern is often crowded even for the common 18 amino acids. Thus, to give quicker analysis service with less work load and increased resolution a versatile lithium protein hydrolysate buffer system was developed.

MATERIALS, INSTRUMENT AND CONDITIONS

Materials : Where available, chemicals of amino acid analysis grade were used. Lithium chloride, lithium hydroxide dihydrate, anhydrous citric acid and thiodiglycol of Sequanal and pHix® buffer grades were from Pierce Chemical Company. Ethyl and benzyl alcohols of reagent grade are from E. Merck Darmstadt. Brij-35 and caprylic acid were from Wako Pure Chemical Industries Ltd. and Koch-Light Limited respectively. Freshly deionized double distilled water was used for all buffers and ninhydrin reagent preparations. All buffers were finally filtered through 0.45 μm pore size membranes, after pH adjustment with analytical grade hydrochloric acid.

Pierce amino acid standard H hydrolysate mixture was diluted to a final concentration (all compounds) of 0.1 μmole/ml using pH 2.2 lithium loading buffer. Ninhydrin reagent was prepared as described in the instrument manual.[3] Other amino compounds added to the standard H mixture when required included cysteic acid, taurine, S-carboxymethyl-l-cysteine, methionine sulfone, norvaline, norleucine, tryptophan and L-α-amino-β-quanidinopropionic acid (AGPA). Pierce standard physiological acid plus neutral (AN) and basic (B) mixture was diluted in the same manner as the standard H mixture. Compounds added in this case included glutamine, ethanolamine and AGPA.

Instrument : A Hitachi model 835-50 amino acid analyzer was used throughout. The relevant configurations were a single microbore stainless steel column (2.6 mm inside diameter by 15 cm in length) with a maximum of 5 programmable eluting buffers and one regenerant. Control of column temperature was by circulating water heating bath. An ammonia filter set (part no. 835-0306) was connected between the pump and autosampler.

Conditions : The column material was Hitachi Custom Cation-Exchange # 2619F resin. Buffers and ninhydrin flow rates were 0.25 and 0.30 ml/min respectively. A cooling bath (Eyela model CA 101) was used to quickly lower the T4 temperature (68°C) of water in the heating bath back to the T1 temperature (34°C) before each new analysis cycle.

RESULTS AND DISCUSSION

The separation of all amino acids in the hydrolysate standard H mixture plus four additional compounds by the modified 5 lithium buffer system is shown in Fig. 1. Chromatography conditions were based on the following principles :-

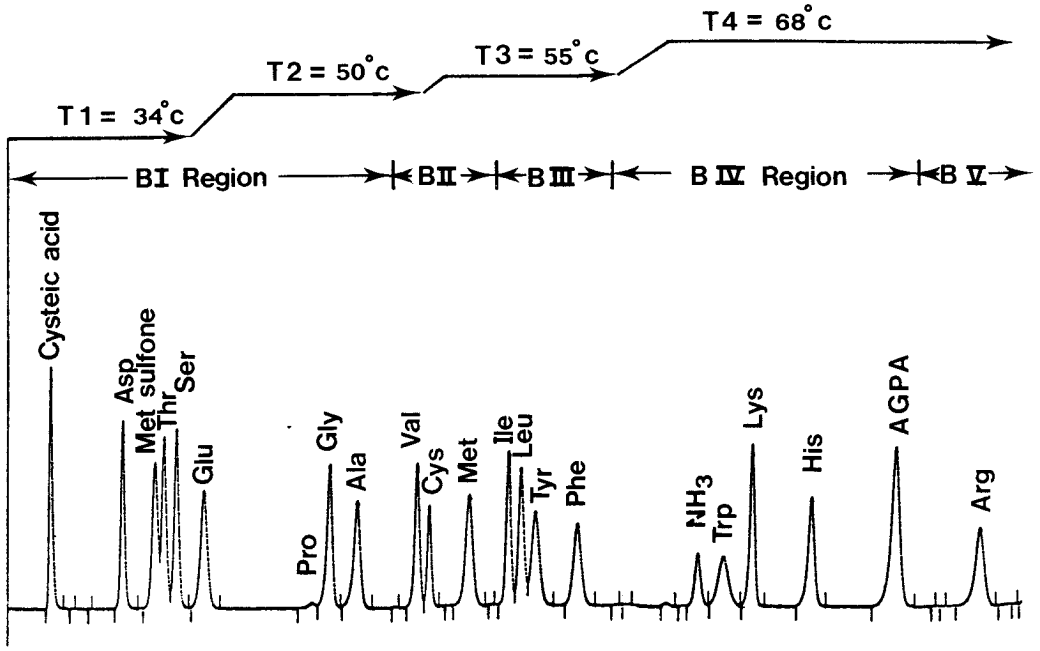

Fig. 1 The separation pattern of amino acid standard H mixture plus cysteic acid, methionine sulfone, trp and AGPA by the modified five buffer, lithium citrate hydrolysate system. Buffer and temperature profiles are also shown.

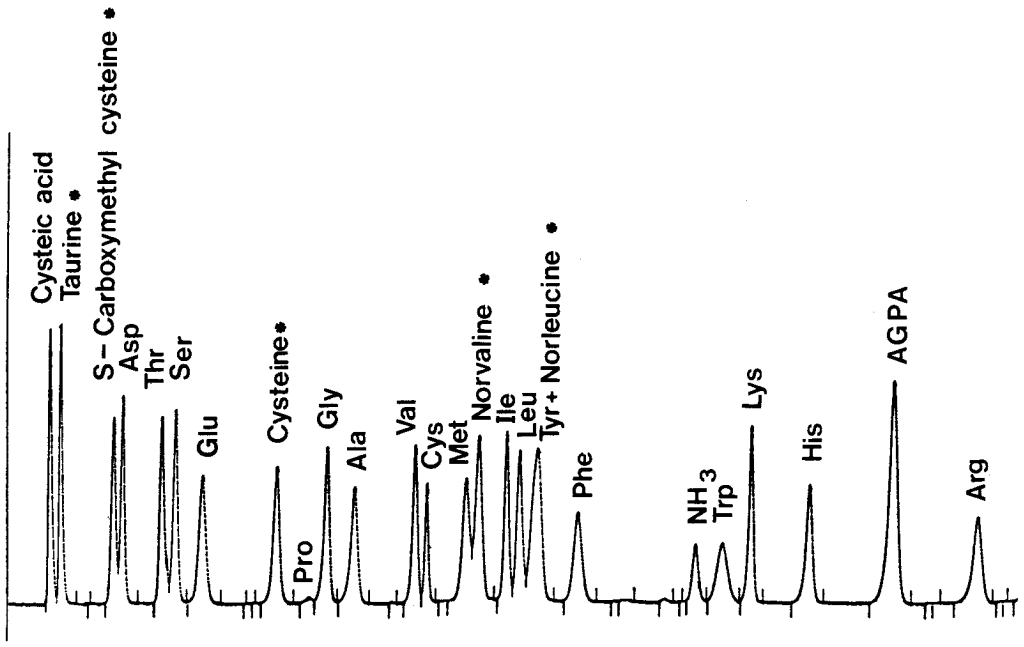

Fig. 2 The separation of 5 additional amino compounds (marked with an asterisk*) including taurine, S-carboxymethyl-cysteine, cysteine, norvaline and norleucine by the modified five buffer, lithium citrate hydrolysate system.

a) pH of buffer I was determined by good separations within the methionine sulfone-threonine-serine and proline-glycine groups. A Tl temperature as low as 34°C was necessary for good separation of methionine sulfone and threonine. After the elution of serine, a higher T2 temperature was used to speed up the elution of other peaks. Buffer I was terminated such that alanine was the last compound eluted in this buffer.

b) pH of buffer II was adjusted so that cystine just fused with valine. The last compound eluted in buffer II was methionine. The T3 temperature began after the valine peak and lasted until just after the elution of phenylalanine.

c) Buffer III region included the compounds isoleucine to phenylalanine. Correct pH of buffer III was indicated by the separation of tryptophan from ammonia in the buffer IV region.

d) Buffer IV terminated after the complete elution of α-amino-β-quanidinopropionic acid (AGPA) which was used as internal standard. The pH of buffer IV was controlled by elution times of ammonia, lysine, histidine and AGPA. The T4 temperature started at the same time as buffer IV and lasted until after arginine was eluted.

e) Buffer V was used only to speed up the elution of arginine.

Analysis time from injection until arginine was 90 min. Total run time including regeneration and equilibration was 114 min. Cysteic acid and methionine sulfone converted from cystine plus cysteine, or methionine, respectively, by performic acid oxidation, were well resolved, as were the other 18 common amino acids. In effect, all compounds were resolved essentially to baseline.

The compositions and pH of the modified 5 lithium hydrolysate system are given in Table 1. Formulae except pH were intentionally kept the same as for the normal physiological analysis system where pH and formulae had previously been optimized compared with the values orginally given in the instrument manual.[3] Differences in pH between the modified hydrolysate and the optimized physiological systems are also shown in Table 1. The addition of saturated lithium hydroxide solution with resultant minor changes in lithium and citrate concentrations to hydrolysate buffer II to convert it to physiological buffer II, or physiological buffers IV and V to convert them to hydrolysate buffers IV and V, respectively, had no noticeable effect on the various chromatogram patterns. Hence all buffers used in the one system could be used in the other analytical system after appropriate pH readjustments. Thus, the main aim of reducing the time consuming procedures involved in switching from physiological to hydrolyzate assays, and vice-versa, was achieved.

Another benefit of the modified lithium chromatographic conditions was the ability to resolve other commonly encountered amino compounds, in particular types of samples as shown in Figs. 2 and 3. Taurine, which is found in fresh water and marine amimals, was almost completely resolved from cysteic acid. S-carboxymethyl-l-cysteine, another converted form for cystine plus cysteine, eluted earlier than, although marginally fused with, aspartic acid. Cysteine eluted as a discrete peak, between

TABLE 1 The compositions and pH of buffers in the modified lithium hydrolysate buffer system. The pH of previously optimized lithium citrate buffers for use in full phusiological assays are included for comparison.

Components	Loading buffer	Buffer I	Buffer II	Buffer III	Buffer IV	Buffer V	Regenerant
$[Li^+]$ [N]	0.155	0.155	0.255	0.805	1.00	1.20	0.35
[Citrate] (M)	0.197	0.197	0.092	0.092	0.051	0.167	-
pH	2.20	3.04	3.37	3.30	4.70	7.80	-
Total volume (ml)	1000	1000	1000	1000	1000	1000	1000
LiCl (g)	4.18	1.00	6.40	29.68	36.83	28.65	-
$LiOH.H_2O$ (g)	2.38	5.52	4.38	4.40	5.50	22.00	14.72
Citric Acid (g)	37.85	37.85	17.68	17.68	9.80	32.08	-
Caprylic Acid (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Brij-35* Soln (ml)	-	4.0	4.0	4.0	4.0	4.0	4.0
Thiodiglycol (ml)	5.0	5.0	5.0	-	-	-	-
Ethyl alc. (ml)	-	40.0	30.0	-	-	-	-
Benzyl alc. (ml)	-	-	-	5.0	-	-	-
Physiological buffer pH	2.20	3.00	3.62	3.33	4.52	7.60	-

*Brij-35 solution was prepared by 25 g of Brij-35 per 100 ml of H_2O

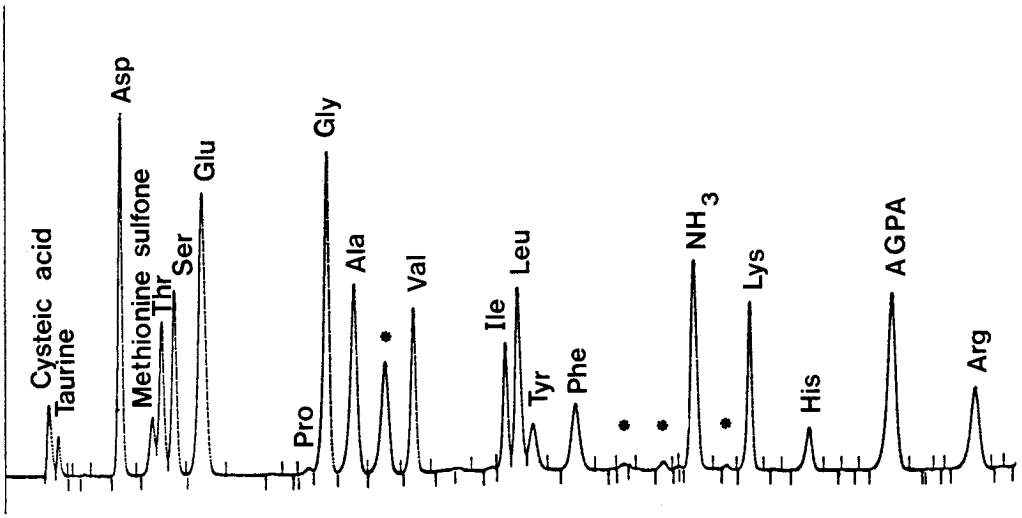

Fig. 3 The amino acid analysis pattern of a prawn head sample oxidized with performic acid prior to acid hydrolysis.[4] Four extra ninhydrin-positive compounds (marked with an asterisk*) were routinely seen.

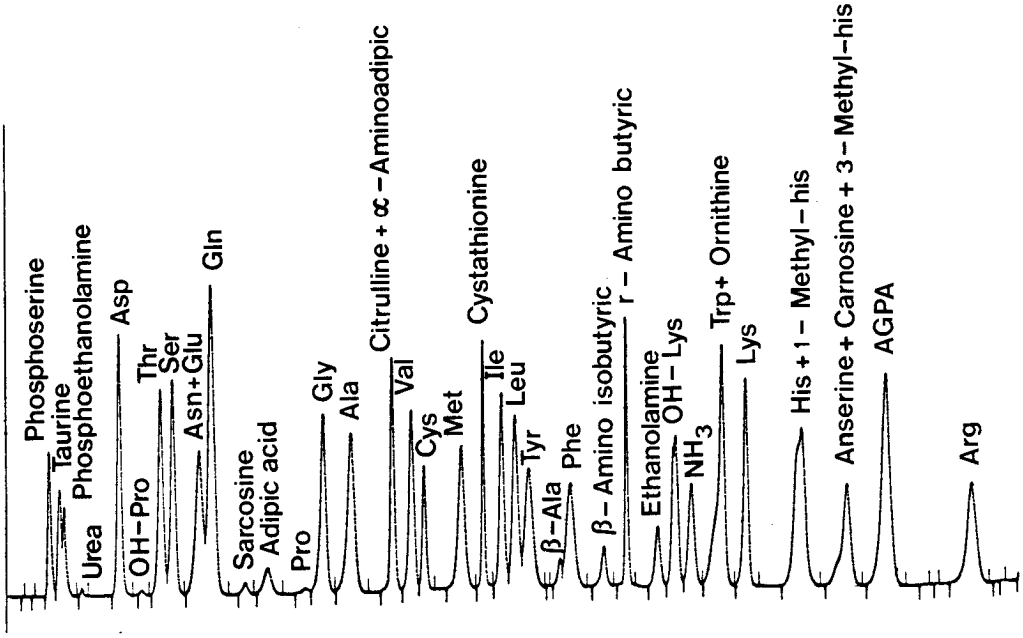

Fig. 4 The separation capability of the modified five buffer lithium hydrolysate program for the analysis of physiological standard acid, neutral and basic mixture plus gln, ethanolamine and AGPA. A total of 28 of the 41 compounds were resolved.

glutamate and proline. AGPA was chosen as internal standard since norvaline fused with methionine, and norleucine was coeluted with tyrosine. The large ninhydrin positive peak shown in Fig. 3 which was routinely found in high amounts in mushrooms and mollusks was well positioned such that the accuracy of peak area integration for other compounds was not affected. Other smaller peaks between phenylalanine and lysine were also well separated from the commonly encountered amino acids. The ability of the modified lithium citrate buffer system to separate other amino acids found in physiological mixtures is shown in Fig. 4. A total of 28 of 41 compounds could be resolved. The shortened lithium chromatographic system may nonetheless be suitable for the assay of free amino compounds in physiological samples having fewer compounds than the standard physiological mixture.

ACKNOWLEDGEMENTS

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บทคัดย่อ

การศึกษานี้ได้นำเสนอวิธีการทางโครมาโตกราฟฟีอีกระบบหนึ่ง สำหรับการวิเคราะห์กรดอะมิโนที่เป็นองค์ประกอบของโปรตีนด้วยเครื่องวิเคราะห์กรดอะมิโนอัตโนมัติและสารละลายบัฟเฟอร์ซึ่งเป็นเกลือลิเธียม ระบบที่ดัดแปลงใหม่นี้ซึ่งทดลองใช้กับเครื่องวิเคราะห์กรดอะมิโนยี่ห้อฮิตาชิ รุ่น 835-50 สามารถแยกกรดอะมิโน 18 ชนิดของโปรตีนและสารประกอบอะมิโนชนิดอื่นที่จะพบได้บ่อยอีก 6 สาร ได้เป็นอย่างดีภายในเวลา 90 นาทีนับจากการเริ่มฉีดตัวอย่างจนถึงพีคของอาร์จินีน ประโยชน์หลักของระบบนี้ จะให้ความคล่องตัวและลดงานเตรียมการวิเคราะห์ลงมากเมื่อมีเครื่องวิเคราะห์กรดอะมิโนใช้เพียงเครื่องเดียว ถ้าเทียบกับวิธีการที่ใช้กันอยู่ซึ่งต้องสลับกันระหว่างระบบที่ใช้เกลือโซเดียมกับเกลือลิเธียม