Aug. 22, 1972 J. R. BENSON 3,686,118

CHROMATOGRAPHIC METHOD

Filed Jan. 11, 1971 2 Sheets-Sheet 1

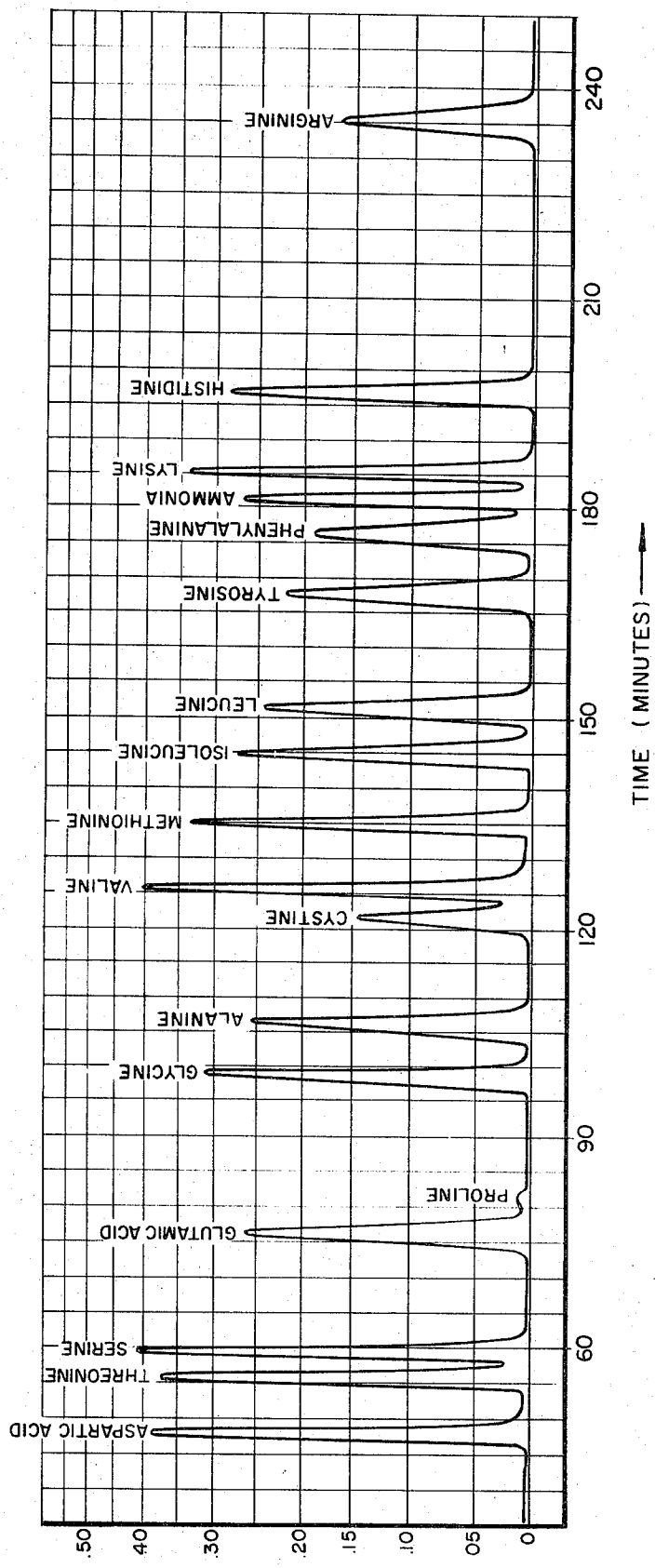


Fig. 1

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CHROMATOGRAPHIC METHOD

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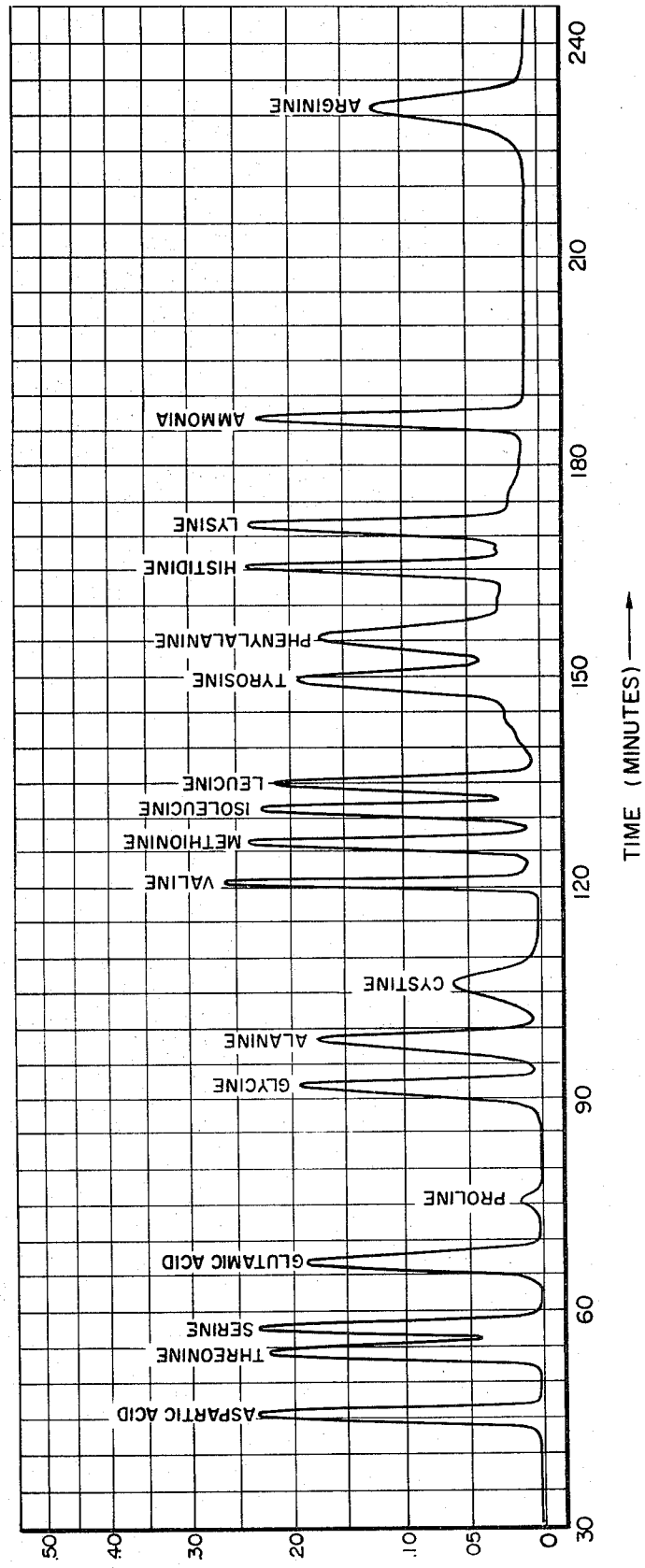


Fig. 2

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CHROMATOGRAPHIC METHOD

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ABSTRACT OF THE DISCLOSURE

An improved chromatographic method for separating the components of a mixed amino acid sample using a cation exchange resin by sequential elution. The buffer solution is an alkali metal salt of an acid which has a pK value of less than 5. The buffer solution is further characterized by a pH value of less than 4.4 and a pH variance of less than 30% during the elution cycle. Suitable buffer anions are citrate, acetate, formate or phosphate. The elution driving force for the more firmly retained amino acids is provided by increasing the cation concentration while maintaining the pH at the above relatively constant level. Separation by means of the above buffer system substantially eliminates baseline shift during subsequent analysis.

BACKGROUND OF THE INVENTION

Mixed amino acid samples of the protein hydrolyzate type (e.g. the 20 alpha amino acids) or physiological fluid type (e.g. urine), have been chromatographically separated by various techniques for the subsequent quantitative determination of the individual components. In a particularly effective technique, a mixed amino acid sample is fed to a column of ion exchange resin composed of a fixed matrix of divinyl benzene cross-linked polystyrene to which is attached a negative functional group, commonly sulfonic acid. The sulfonic group attracts the positively charged amino acids, each of which has a characteristic affinity on the same. Thereafter the individual amino acid components are sequentially eluted and thereby separated in inverse order to the degree of resin affinity for each component from the column under the force of a pressurized feed of an alkali metal citrate buffer solution. A common technique for quantitation is to react the amino acids with ninhydrin reagent to form a compound which absorbs light in the 570 mu wavelength region and to direct the mixture through a flowcell past a colorimeter detector (e.g. a photocell) which is quantitatively responsive to that wavelength. Certain "imino" acids (e.g. proline) form a compound with ninhydrin that absorbs in the 440 mu wavelength region and this may be detected by a second monitoring system. These detectors are connected to a recorder which plots the response as on a chart. Quantitation is performed by time integrating each peak of the chart, each peak having a characteristic position and configuration dependent upon the amino acid type.

When the above chromatographic techniques were developed, a constant composition sodium citrate buffer solution was fed to the top of a comparatively long (e.g. 150 cm.) column to elute the mixed amino acid sample. This technique required excessive elution times; on the order of 48 hours for protein hydrolyzates and 90 hours for physiological fluids.

In an attempt to decrease the time of chromatographic separations, a two column system was developed employing a first long column (150 cm.) and a second short column (15 cm.), as fully described in the article by Spackman, D. C., Stein, W. H., and Moore, S., "Automatic Recording Apparatus for Use in the Chromatography of Amino Acids," 30, Anal. Chem., 1190 (July 1970). According to this system (hereinafter the Stein-Moore technique) the sample is split into two aliquot portions, each of which is supplied to one of the columns. Two discrete buffer solutions at pH values of 3.2 and 4.5 are sequentially supplied to the long column to elute the more readily removable amino acids of one aliquot portion. A third buffer solution at a pH value of 7 is employed to elute the more firmly held amino acids from the second shorter column. By utilizing this two-column system with three discrete buffers, the separation time is significantly less than the aforementioned single column. For example, Spackman, Stein and Moore were able to analyze protein hydrolyzates in about 22 hours and physiological fluids in about 44 hours. Improvements in technology and development of higher resolution resins have enabled many analyzer users today to reduce these analysis times to 4 hours for hydrolyzates and 11 hours for physiological fluids.

Rapid separation in the two column system is accomplished by a balance of one known driving force, the pH of the buffer solution, with the desired degree of resolution. The analysis time is decreased as the buffer pH is increased but there is a consequent decrease in resolution. The more readily removable amino acids are eluted with the lower pH buffer solutions in the long column since a comparatively small elutive driving force is necessary. The more rigidly held amino acids may be eluted by utilizing a higher pH solution in the short column.

In a present day application of the Stein-Moore technique, a three aliquot portion buffer solution of sodium citrate is employed in which the first portion has a pH of about 3.25 with 0.2 N sodium citrate, the second portion has a pH of about 4.25 and the same normality, and the third portion (for the short column) has a pH of 5.35 and a 0.35 normality.

The above two-column technique has a number of serious disadvantages. Errors are readily introduced if the original sample is not divided into two exactly equal portions. Also, a larger amount of any extremely valuable sample is necessary since an additional aliquot is required for the second column. In addition, operation of a two-column system is complicated and the expense of adding automatic sample loading equipment is increased.

One approach to lessen some of the above disadvantages is to employ a single column with a variable buffer system similar to the one employed in the two column system. One such system is described in Hamilton, P. B., "Ion Exchange Chromatography of Amino Acids," 35, Anal. Chem., 2056 (December 1963). Even with this system, excessive separation times are required (e.g. about 24 hours).

A serious problem with either the single or two column system of the above type is a shifting of the baseline during quantitation as the run proceeds. A consequent introduction of substantial error is possible and is a particularly serious problem when small samples are employed (e.g. less than 100 nanomoles) since the distortions become extreme. The cause of the baseline shift was at first hypothesized to be ammonia contaminants in the buffers. This ammonia was supposed to elute from the column in increasing quantities as the pH of the solution increased. Ammonia contamination as the sole cause of baseline shift was questioned on p. 2060 of the above Hamilton article but no substitute theory was offered. That theory, in fact, is disproved since baseline shifts resulting from using buffer solutions from which ammonia contaminants have been previously filtered are still observed, especially in cases where small samples (e.g. less than 50 nanomoles) are analyzed.

SUMMARY OF THE INVENTION AND OBJECTS

It is an object of the present invention to provide a process for chromatographically separating a mixed amino acid sample using an ion-exchange resin column

which overcomes the aforementioned disadvantages of prior techniques.

It is a further object of the invention to provide a chromatographic technique of the above type capable of performing the separation in a short period of time on a single ion-exchange column.

It is a particular object of the invention to provide a technique of the above type making use of a buffer system which substantially eliminates baseline shift in the subsequent analysis.

Further objects of the invention will be apparent from the following description taken in conjunction with the accompanying drawings.

In accordance with the above objects, an improved chromatographic method has been provided for separating the components of a mixed amino acid sample using a single column of a cation-exchange resin. First the sample is supplied to the column so that the amino acid components are retained by the resin. Thereafter, a sufficient quantity of an aqueous buffer solution is fed through the column to sequentially elute and thereby separate the components. In order to elute a typical sampling of amino acid components, such as of the protein hydrolyzate type, and within a reasonably short period of time (e.g. less than 5 hours) it has been found desirable to use a buffer system having a low initial "driving force" and increasing the same as the sequence of the eluted amino acids proceeds from the ones having a lesser to a greater degree of resin affinity. Driving force is defined as the rate at which particular amino acids are eluted from the column by a buffer system. The present invention is predicated on the discovery that conventional means for increasing the driving force to elute the firmly held amino acids, i.e. by increasing the pH value of the buffer solution to a maximum value of substantially greater than 4.0 and by also increasing the total concentration of buffer, causes a baseline shift. It has been discovered that this baseline shift may be substantially eliminated by maintaining the pH value at less than about 4.0 and within relatively small variances. The increase in driving force is supplied by increasing the cation concentration. Suitable buffers include alkali metal salts of citrate, acetate, formate, phosphate, or other anions which possess the properties described hereinafter.

For a protein hydrolyzate sample, it is generally preferred to maintain the buffer solution at a pH of from about 3 to 4.0 during the final stage of elution although it is possible, under certain circumstances, to raise the pH as high as 4.2. One exception to this rule is phosphate which preferably has a pH of between 2.0 and 3.0 during elution in order to be closer to the 2.1 pK value of the acid as explained hereinafter. The total pH variance (defined hereinafter) during the process is no greater than 30% and preferably no greater than 20%. The alkali metal ion concentration is preferred to be at least 500% greater in the final than in the initial stage of elution to provide an adequate increase in driving force for the more difficult to remove amino acids.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a chart illustrating the lack of baseline shift when using the buffer system according to the present invention.

FIG. 2 is a chart illustrating the baseline shift caused by the use of a conventional buffer system.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the present invention, a mixed amino acid sample of the protein hydrolyzate or physiological fluid (e.g. urine) type are separated on a column of a cation exchange resin. For clarity, the following discussion will specifically relate to a protein hydrolyzate sample including 18 amino acid components, as illustrated in FIGS. 1 and 2.

The amino acid sample is supplied to the top of a cationic exchange resin (e.g. of the sulfonic type) so that the amino acid components are distributed in and retained by the resin. Thereafter, an aqueous buffer solution is fed under pressure through the top of the column to separate the amino acid components by sequential elution of the same from the outlet end of the column.

As used herein, the term "pH variation" is defined by the following equation:

$$\text{pH variation} = [(pH_2 - pH_1)/(pH_2 + pH_1)/2)] \times 100\% \quad (1)$$

wherein,

pH_2=the buffer pH value when the last amino acid is eluted

pH_1=the buffer pH value when the first amino acid is eluted.

The cation exchange resin is typically comprised of relatively small particles (e.g. 10–20 microns in diameter) formed of polystyrene cross-linked with a minor portion (e.g. 8%) of divinyl benzene to which are appended a number of negatively charged groups (e.g. sulfonic acid). For use with a single column, a resin depth of 50 to 60 cm. is suitably employed.

An amino acid sample of the protein hydrolyzate type is commonly prepared from an unknown protein sample by mixing the same with an aqueous solution of a strong acid and heating this at elevated temperatures to cause hydrolysis of the protein into separate amino acid components. Some of this sample is then supplied to the top of the ion exchange column wherein the individual amino acid groups are distributed and retained by electrostatic bonding with the functional groups of the resin. The resin affinity for each component is a distinctive characteristic and is employed as the basis for the chromatographic separation. The alkali metal of the buffer solution sequentially displaces each amino acid component from the resin in inverse order to the resin affinity for that component. Referring to the charts of FIGS. 1 and 2, it is seen that in that particular protein hydrolyzate group, aspartic acid is the most weakly held amino acid while arginine is the most strongly held.

A common technique for quantitating the elutions from the column is to react the amino acid components with ninhydrin reagent to form a compound which absorbs light in the 570 mu wavelength region to direct the mixture through a flow cell past a colorimeter detector which is quantitatively responsive to that wavelength. As aforementioned, a second monitoring system responsive to 440 mu may be employed for imino acids. These detectors are connected to a recorder which plots the response on a chart. Quantitation is performed by time integrating each peak which has a characteristic position and configuration on the chart.

In accordance with the present invention, the buffer system employed for the chromatographic separation has particular unique characteristics which enable the separation to be carried out at a rapid rate from only a single column and without any substantial shift of the baseline. This system may also be used for a multiple column system and is effective to prevent baseline shift even where relatively small sample quantities (e.g. 50 nanomoles or less) are employed.

The buffer solution can be supplied to the column in discrete portions totaling say 3 or 4 with the composition of each portion varied. Alternatively, a so-called "gradient" buffer solution may be used in which a continuous feed of buffer is employed with a steady change of composition. For either type of buffer solution system, the variation is predetermined to increase the driving force of the solution as the elution cycle progresses to enable the more firmly held amino acids to be eluted in a reasonable amount of time. If the driving force is too great initially, the requisite degree of resolution among the components may be lost. On the other hand, the rela-

tively low driving force necessary for resolution of the preliminary amino acids might require an excessive elution time for the more strongly held amino acids. In a compromise, a relatively small driving force is employed at the beginning of the cycle and a relatively large driving force at the end.

The control of the pH level during the elution cycle is an important feature in the avoidance of a baseline shift. It has been found that the baseline shift starts to increase substantially at pH values greater than 4.0. This effect is accelerated as the sensitivity of the analysis increases such as by decreasing the sample quantity. Variation of the pH over wide ranges (e.g. greater than 20 or 25%) during the process also causes a baseline shift which increases with the absolute pH value. The optimum condition would be to employ essentially constant pH buffers throughout. Small variations in buffer pH may be tolerated, however, that amount of variation being dependent upon the absolute sensitivity of the particular analysis. Higher sensitivities (i.e. lower sample amounts) require minimizing the pH variance used during the analysis.

The buffer solutions employed in the above invention do not substantially interfere with color development when the amino acid reacts with the ninhydrin reagent. In addition, the acids forming the buffer anions of the present invention have a pK value ($-\log_{10}$ of the dissociation constant) of less than 5. The pK value is preferably not substantially different from the pH of the buffer during elution so that the buffer maintains a reasonable capacity. For example, the pH value preferably varies by no more than plus or minus 30% from the pK of the acid. There are a large number of acids which may fulfill this latter requirement and so which may be used according to the invention since the pH is maintained within a relatively small variance (30%) in contrast to prior art methods. Suitable acids and their corresponding pK values are acetic acid (4.75); formic acid (3.25); phosphoric acid (2.1); and citric acid (3.1).

Suitable buffer solutions of the above include salts of alkali materials such as sodium, potassium and lithium. The buffer anions include citrate, acetate, formate, phosphate, and the like.

A particularly desirable pH range for the buffer solution, except for phosphate buffer, during the first part of the elution cycle is from 3.1 to 3.3 or 3.4 to 3.7 for protein hydrolyzate sample. Using the buffer solution the range of 3.3 to 3.4 can result in difficulties of resolution caused by movement of the mobile cystine component into the area of the glycine and alanine components to cause an overlapping. In a pH range of about 3.1 to 3.3 the cystine is eluted in a free area prior to glycine-alanine and in the range of 3.4 to 3.7 it is eluted in a free area subsequent to the same.

It has been found that a phosphate buffer is effective at a preliminary pH of 2.6 which is closer to the phosphoric acid pK value (2.1). Presumably, other buffer solutions with pK values below 3.0 may be employed with a corresponding lower pH value. The driving force may be applied by using a relatively high initial cation concentration (e.g. 0.5 N or more).

In the aforementioned Stein-Moore technique, the third buffer solution has a pH of 5.35, substantially in excess of the maximum value, 4.0, according to the present invention. A substantial baseline shift occurs with pH values greater than 4.0 especially evident when relatively small samples (e.g. 50 nanomoles or less) are employed. Heretofore, this high pH was deemed necessary to supply the driving force necessary to elute the more firmly held amino acid components in a reasonable period of time.

The requisite driving force for a single column chromatographic system in four hours or less may be accomplished with an increase in alkali metal ion concentration in the citrate buffer while leaving the pH and anion level at a relatively constant value. Thus the variation of the driving force to elute the more firmly held amino acids may be accomplished by adding an inorganic alkali metal salt of a halide, phosphate, sulfate, or the like. The baseline is apparently not sensitive to large increases in the alkali metal ion concentration. Thus, during the initial stages of the elution cycle the alkali metal ion content may be varied from an initial value of about 0.10 to 0.35 mole per liter to a final value from about 0.25 to 3.0 or more moles per liter without effecting a shift in the baseline.

In accordance with the above principles, a particularly effective three stage sodium citrate buffer system for a protein hydrolyzate includes an initial stage in which the sodium concentration ranges from 0.1 to 0.35 and the pH varies from about 3.1 to 3.3 or 3.4 to 3.7. The second and third buffers have a pH of 3 to 4.4, and a sodium ion concentration of 0.2 to 3 moles per liter.

The theoretical explanation for the aforementioned baseline sensitivity to the pH value is not completely understood. It appears that the baseline shift is caused by certain extraneous colored interferents which are eluted from the column of resin and which provide a competing color to the ninhydrin reaction product. Although not intended as a limiting theory, it is hypothesized that the interferents are components of the resin itself which are eluted from the resin in varying amounts depending upon the pH value. This is partially borne out by filling a column of resin with an aqueous solution and varying the pH. It is found that at the higher pH values that a certain characteristic color is imparted to the solution but at the lower pH value this phenomenon does not occur. It could well be that this component is sensed by the detector resulting in a baseline shift. According to this theory, as the pH increases to values of 4.0 or more, the interference is erratically and intermittently emitted from the resin to cause massive distortions of the baseline. This theory also explains why large pH variations even below a pH of 4.0 cause distortion. The baseline would shift since the pH of the buffers correspondingly effects the amount of the interferent emitted.

In order to more clearly disclose the nature of the present invention, a specific example of the practice thereof is here and given. It should be understood, however, that this is done by way of example and is intended neither to delineate the scope nor limit the appended claims.

EXAMPLE 1

A sample comprising a standard calibration mixture containing about 50 nanomoles of the 18 amino acids illustrated in FIG. 1, comparable to a protein hydrolyzate, was placed on top of a 0.9 x 58 cm. packed bed of sulfonic cationic exchange resin having a polystyrene substrate cross-linked with about 8% divinyl benzene and a 15–20μ diameter sold under the designation DC–1A by Durrum Chemical Corporation, Palo Alto, Calif.

Three discrete aqueous sodium citrate buffers (designated A, B and C) were supplied sequentially to the column under a pumping pressure of 280 p.s.i.g. and a constant flow rate of 70 ml./hr. to force the sample through the column. The compositions of the buffers were as follows:

Buffer	pH	Citrate ion (moles/l.)	Sodium (moles/l.)	Chloride (moles/l.)
A	3.25	0.067	0.2	
B	3.50	0.067	0.7	0.5
C	3.65	0.067	1.6	1.4

Prior to the run, buffers A and B were filtered to remove ammonia contaminants.

The buffer sequence for the run was as follows: Buffer A—85 minutes, buffer B—35 minutes, and buffer C—120 minutes for a total analysis time of 240 minutes. The temperature of the column was 45° C. for the first 90 minutes and 65° C. thereafter as controlled by a jacket

of circulating heat transfer fluid (warm water) about the column.

Upon elution, the fully separated amino acids were mixed in a mixing manifold with ninhydrin reagent pumped into the same under sufficient pressure to maintain a flow rate of 35 ml./hour. This mixture was directed to a reaction coil maintained at about 100° C. and the formed reaction products characteristically absorbed light either in the 570μ wavelength (amino acids) or 440μ wavelength (imino acids) as explained above. These colored products were directed into a flow cell through which light at 570μ was passed. A silicon diode sensing photometer monitored the light and provided a voltage to a recorder. Absorbence of light by the colored product caused a loss in voltage to the detector which was registered by the recorder. The chart of FIG. 1 plots the absorbence against time. It is apparent that about 45 minutes elapse before the first amino acid is eluted. The recorded results demonstrate a substantially steady baseline throughout the run.

EXAMPLE 2

In Example 2, a sample of the same type analyzed in Example 1 was used except that the amino acid concentrations were doubled to 100 nanomoles/liter. The same analysis scheme as in Example 1 was used except for the use of a conventional buffer system as follows:

Buffer	pH	Citrate ion (moles/l.)	Sodium ion (moles/l.)	Chloride ion (moles/l.)
A	3.25	0.067	0.2	
B	4.40	0.067	0.1	
C	6.35	0.20	1.6	1.0

The results of Example 2, plotted in the chart of FIG. 2, indicated a marked baseline shift, especially between 100 and 200 minutes of the elution cycle, in comparison to Example 1. This is true even though Example 1 was performed at twice the sensitivity (half the sample concentration) of Example 2. The only apparent reason for the lack of baseline shift in Example 1 is the use of a buffer system according to the invention rather than a conventional one.

What is claimed is:

1. In an improved chromatographic method for separating the components of a mixed amino acid sample such as are present in a protein hydrolyzate or in a physiological fluid using a column of a cation exchange resin the steps of supplying the sample to the column so that the amino acid components are distributed in and retained by the resin; feeding a sufficient quantity of an aqueous buffer solution through the column to sequentially elute and thereby separate the components, said buffer solution comprising an alkali metal salt of an acid with a pK value less than 5 and being characterized by the properties of non-detrimental reactivity to resin and to the sample, said buffer solution further having a pH value less than about 4.0 during the elution cycle and a pH variance of less than 30% the concentration of the alkali metal ion of said acid salt during the beginning stage of elution being from about 0.10 to 0.35 mole per liter and being increased substantially to from about 0.25 to 3.0 moles per liter during the final stage of elution.

2. A method as in claim **1** in which the alkali metal of said alkali metal salt is selected from the group consisting of sodium, lithium and potassium.

3. A method as in claim **2** in which the buffer solution is characterized by a pH of from about 3.1 to 3.3 during the beginning stage of elution and by a pH of from about 3.0 to 4.0 during the final stage of elution.

4. A method as in claim **2** in which the anion of the buffer solution is selected from the group consisting of citrate, acetate, formate and phosphate.

5. A method as in claim **4** in which the anion is citrate.

6. A method as in claim **2** in which the aqueous buffer solution is fed to the column in separate, discrete portions.

7. A method as in claim **2** in which the aqueous buffer solution is fed to the column in a stream having a continuously increasing alkali metal ion content.

8. A method as in claim **2** in which the buffer solution is characterized by a pH of from about 3.4 to 3.7 during the beginning stage of elution and by a pH of from about 3.0 to 4.0 during the final stage of elution.

9. A method as in claim **1** in which the alkali metal ion concentration of said acid salt has a concentration at least 500% greater in the final stage than in the beginning stage of elution.

10. A method as in claim **1** in which the cation exchange resin is of a polystyrene type.

11. A method as in claim **10** in which the polystyrene is cross-linked with a minor portion of divinyl benzene.

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JOHN ADEE, Primary Examiner