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THE COMPLETE GAS–LIQUID CHROMATOGRAPHIC SEPARATION OF THE TWENTY PROTEIN AMINO ACIDS*

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SUMMARY

The complete gas–liquid chromatographic separation of the twenty protein amino acids is presented. In our previous publications, we reported on an effective chromatographic separation of seventeen N-trifluoroacetyl *n*-butyl esters of the amino acids on a packing composed of stabilized grade EGA and acid-washed Chromosorb W (heated at 140° for 12 h). A new column packing has been found to replace the OV-17; it is composed of a mixed phase of OV-17 and OV-210. This is a superior packing and shows quantitative elution, and highly efficient and complete separation of histidine, internal standard tranexamic acid, lysine, arginine, tryptophan, *n*-butyl stearate (I.S.), and cystine. No longer is it necessary to make a separate "subtraction-calculation" for histidine. With these two packings, EGA and mixed siloxane phases, one can now simultaneously analyze and separate the 20 protein amino acids in 30 min or less with automatic electronic integration of all peaks.

A second internal standard (tranexamic acid) is introduced which contains both $-NH_2$ and -COOH functional groups. It can be used to follow the performance of ion-exchange cleanup, thereby increasing the reliability of analysis of complex biological materials.

The use of two columns for separation of the twenty amino acids has important advantages over a single column system with respect to resolution, cross confirmation, and identification of the eluted compounds.

INTRODUCTION

The increasingly wide interest in and importance of amino acids, peptides, and proteins in the medical and nutritional sciences has brought with it the need for ac-

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curate, sensitive, and rapid amino acid analyses. Extensive research investigations conducted in our laboratories have resulted in the development of gas–liquid chromatographic (GLC) techniques for quantitatively analyzing the twenty protein and at least forty non-protein amino acids as their N-trifluoroacetyl (N-TFA) *n*-butyl ester derivatives. The organic reaction conditions for preparing the amino acid N-TFA *n*-butyl esters of the twenty protein amino acids have been established[1–5]. GEHRKE *et al.*[6] in 1968 published a monograph in which details are presented on derivatization, chromatography, instrumental parameters, reagents, and sample ion-exchange cleanup for analysis of amino acids at the macro, semi-micro, and micro levels. However, their complete GLC separation on a single column has been difficult to achieve. GEHRKE AND SHAHROKHI[5], STEFANOVIC AND WALKER[7], and MCBRIDE AND KLINGMAN[8] reported various single columns for the analyses, but quantitative and reproducible elution of arginine, histidine, and cystine was not achieved. Also, DARBRE AND BLAU[9] and DARBRE AND ISLAM[10], using mixed siloxane liquid phase columns, studied the derivatization and GLC separation of the amino acid N-TFA methyl esters, and likewise, they were not able to achieve a quantitative elution of histidine.

In 1968, GEHRKE *et al.*[11] reported a dual column chromatographic system, from which nineteen of the amino acid N-TFA *n*-butyl esters were quantitatively eluted and separated; and the twentieth amino acid, histidine, was obtained after injection of *n*-butanol which converted the diacyl histidine derivative to the monoacyl derivative which now could be resolved. Admittedly this was not an ideal method but it was workable and did provide much useful information. This method employed two columns, one containing 0.325 w/w% of stabilized grade ethylene glycol adipate (EGA) as the liquid phase was used for separation of sixteen amino acid derivatives, and a 1.5 w/w% OV-17 (liquid phase) column was used for the separation and analysis of arginine, tryptophan, histidine, and cystine. This dual column approach was employed as these four amino acid derivatives are not eluted from columns containing polyester liquid phases. However, in the above report, the preferred diacyl derivative of histidine was co-eluted with phenylalanine and aspartic acid using the OV-17 liquid phase columns. Then in 1969, ROACH *et al.*[12] reported an improved GLC method for the quantitative determination of histidine. For the N-TFA *n*-butyl ester of histidine, both of these reported methods required on-column conversion of either diacyl histidine (His) to the monoacyl derivative by injection of *n*-butanol; or conversion of monoacyl to diacyl His by injection of trifluoroacetic anhydride (TFAA). Other limitations in the conversion of the histidine derivative were that these methods were especially dependent on instrumental conditions and chromatographic interactions on the column. Therefore, in these two methods, a subtraction-calculation method for the determination of histidine with the OV-17 column was finally considered the best alternative and more reliable, whereby the aspartic acid (Asp) and phenylalanine (Phe) values obtained previously from the ethylene glycol adipate (EGA) column were subtracted from the single peak containing Asp, Phe, and diacyl His on the OV-17 column.

The major aim of this study was to develop a chromatographic column which would quantitatively elute and resolve His, Arg, Trp, and Cys as single peaks from the other N-TFA *n*-butyl ester amino acids, and thus greatly facilitate routine analysis of amino acids by GLC. An important consideration in this research was that the developed column must not only exhibit the necessary separation characteristics,

but also must yield the separations under the same thermal conditions as for the EGA column. This would then allow simultaneous operation of both columns in a dual column chromatograph, and would give a complete analysis of the twenty protein amino acids in a single programmed temperature cycle of 15 to 30 min duration.

EXPERIMENTAL

Apparatus

A Bendix 2500 Series gas chromatograph with a four-column oven bath, two hydrogen flame detectors, two differential electrometers, a linear temperature programmer, and equipped with a Varian Model A-20 dual pen recorder was used. An Infotronics Corporation CRS-104 digital integrator was used for determining peak areas. Also, the following instruments were used: a Varian Aerograph 2100 and a Packard Instrument Co. 7300 series dual column gas chromatograph with FID detectors.

Solvents were removed from the samples with a CaLab rotary evaporator, "cold finger" condenser filled with liquid nitrogen, and a Welch Duo-Seal vacuum pump.

Pyrex 16 × 75 mm glass screw top culture tubes with teflon lined caps (Corning No. 9826) were used as the reaction vessel for the acylation reactions.

Filters containing shell type charcoal and indicating molecular sieve Type 13X were placed in the lines for removal of trace amounts of hydrocarbons and water in the nitrogen, hydrogen, and air to the chromatograph.

Reagents and supplies

All amino acids used in this study were obtained from Mann Research Laboratories, Inc., New York, N.Y. and were chromatographically pure.

The mixed phase column packing used for separation of His, Arg, Trp, and Cys can be obtained from Analytical Bio-Chemistry Laboratories, Inc., P.O. Box 1097, Columbia, Mo. 65201, and Regis Chemical Company, Chicago, Ill. as Tabsorb-HAc, 100/120 mesh.

n-Butanol was a "Baker Analyzed" reagent. TFAA was obtained from Distillation Products Industries, Rochester, N.Y., and was an "Eastman Grade" chemical. Acetonitrile, a "Baker Analyzed" reagent of high purity, was stored over Drierite in a bottle with a ground glass stopper.

Reference standards of amino acids, TAB(TM), were obtained from Regis Chemical Co., Chicago, Ill. They also provide a complete list of other chemicals and supplies used in GLC of amino acids.

Anhydrous HCl gas was generated by the slow addition of 250 ml of reagent grade HCl into 500 ml of concentrated H_2SO_4. The HCl gas was passed through two drying towers containing concentrated H_2SO_4 and then bubbled into the *n*-butanol.

The *n*-butanol and methylene chloride were redistilled from an all glass system and stored in a glass inverted top bottle to protect it from atmospheric moisture. The methylene chloride and *n*-butanol were refluxed over calcium chloride before distillation.

The OV-17 siloxane liquid phase and 100/120 mesh Supelcoport support material were purchased from Supelco, Inc., Bellefonte, Penn. The OV-210, also a siloxane

liquid phase, was purchased from Applied Science Laboratories, State College, Penn.

For preparation of the mixed phase column packing, the following solutions were prepared: (a) 20.0 mg/ml of OV-17 in acetone, and, (b) 10.0 mg/ml of OV-210 in acetone.

Stearic acid was obtained from Applied Science Laboratories, Inc., State College, Penn. and was 99.8% minimum purity.

Tranexamic acid, trans-4-(aminomethyl)cyclohexanecarboxylic acid, was obtained from KABI Co., Sweden, and is also available from Aldrich Chemical Co., Inc., Milwaukee, Wisc.

Chromatographic columns

EGA column (stabilized). The EGA column packing was prepared and conditioned as described by ROACH AND GEHRKE[13], and contained 0.65 w/w% of stabilized grade EGA on 80/100 mesh Chromosorb W (heated at 140° for 12 h). The packing was then placed in 1.5 m × 4 mm I.D. glass columns.

Properly prepared columns should last about two months and give the desired separation for seventeen amino acid derivatives, depending on the individual column and the types of samples injected. Signs of column degradation are: loss of the valine–glycine separation; loss of resolution in the methionine–hydroxyproline–phenylalanine region and loss of separation for the *n*-butyl stearate–ornithine pair. Also, the TFA peak will be eluted later in the chromatogram as the column deteriorates.

Mixed phase packing. The mixed phase column packing (2 w/w% OV-17, 1 w/w% OV-210) was prepared by first placing 29.1 g of Supelcoport in a 500 ml ridged round bottom flask, and "Nanograde" acetone was added until the liquid level was *ca.* 1/8 in. above the support material. Then, 30.0 ml of solution (a) containing 0.6 g of OV-17, and 30.0 ml of solution (b) containing 0.3 g of OV-210 in acetone were pipetted into the flask. The solvent was slowly removed with the rotary evaporator under partial vacuum over a period of *ca.* 45 min. The dry freely-flowing column packing was then placed in clean, dry 1.5 m × 4 mm I.D. glass columns with gentle tapping. Silanized glass wool plugs were then placed in each end of the column to hold the packing in place. Prior to analytical use, the mixed phase columns were placed in the gas chromatograph and conditioned at 250° for 24 h with a carrier flow of about 50 ml/min of pure N_2.

The OV-17, OV-210 mixed phase columns should last three to six months, depending on the types of samples analyzed. The first sign of column deterioration is usually a loss of quantitative elution of arginine and cystine indicated by a reduced $R.W.R._{\text{a.a./I.S.}}$.

When not in use, the EGA and mixed phase columns should be kept at 200° in the chromatograph with a carrier gas flow of 20–50 ml/min. If the columns must be removed from the instrument, the ends should be tightly closed during storage to exclude atmospheric moisture. The EGA columns must not be subject at any one time to temperatures in excess of 225° for longer than 1 to 2 h.

Preparation of the amino acid N-TFA n-butyl esters

The "direct esterification-acylation" procedure described by ROACH AND GEHRKE[14] was used for the conversion of amino acids to their N-TFA *n*-butyl esters. As the elution pattern of the amino acid derivatives using the mixed phase column

was unknown, "spiking" with standards was used to identify the chromatographic peaks.

Response linearity study

A standard solution containing 0.50 mg/ml of histidine, arginine, tryptophan, and cystine as the N-TFA *n*-butyl esters and 0.50 mg/ml of *n*-butyl stearate in methylene chloride was prepared. Aliquots of this solution were diluted with a methylene chloride solution containing 0.50 mg/ml of *n*-butyl stearate. Solutions containing 0.25, 0.125, 0.0625, and 0.03125 mg/ml of each amino acid derivative were prepared. Four microliters of each solution were injected in duplicate and all the chromatographic peaks were electronically integrated. The response ratio of amino acid to *n*-butyl stearate ($R_{a.a./I.S.}$) plotted against the micrograms of amino acids injected should result in a straight line if no loss of the amino acid derivatives occurs due to a derivative–packing interaction during chromatographic analysis.

RESULTS AND DISCUSSION

A study was made of various silicone liquid phases for the separation of the

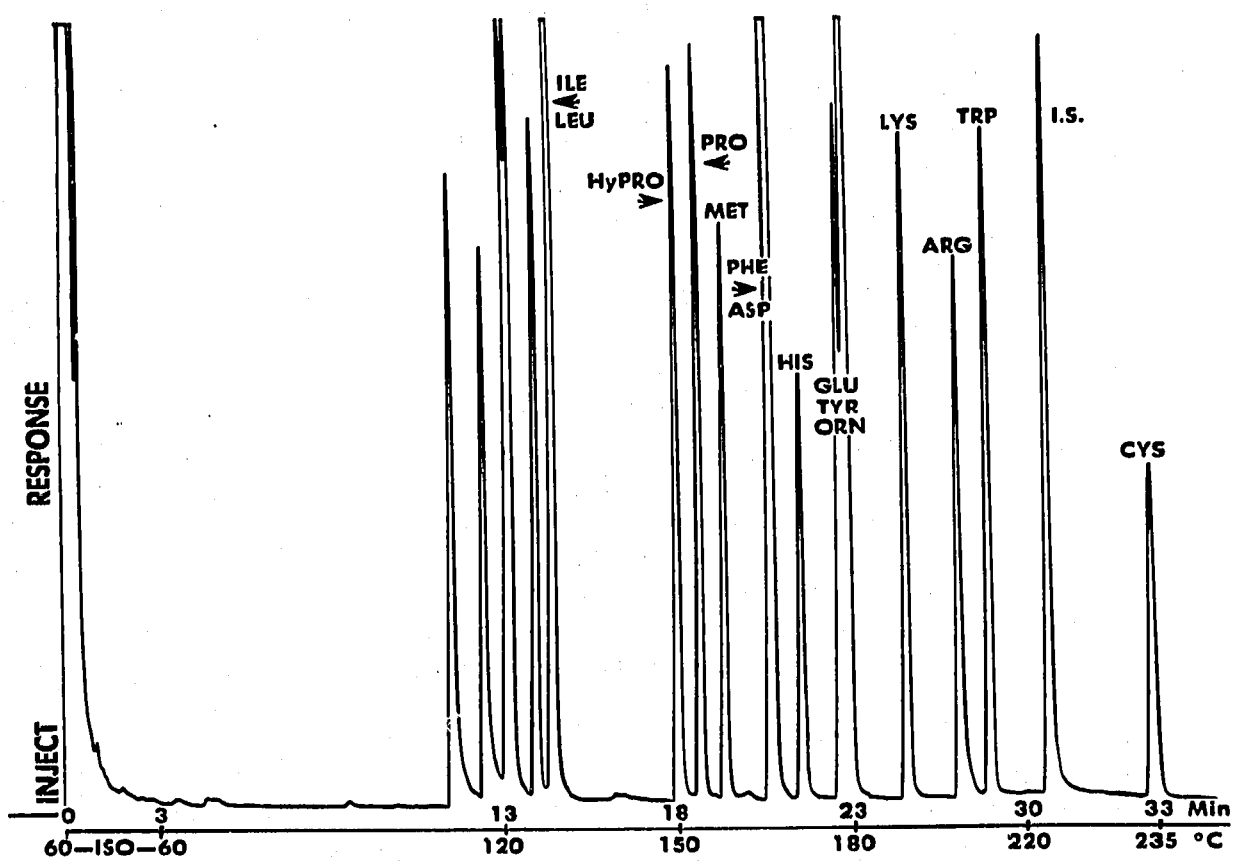


Fig. 1. GLC separation of N-TFA *n*-butyl esters of diacyl histidine, arginine, tryptophan, and cystine. Injected: 0.5 μg of each amino acid. Column: 2.0 w/w% OV-17, 1.0 w/w% OV-210 on 100/120 mesh Supelcoport, 1.5 m × 4 mm I.D. glass. *n*-Butyl stearate as I.S. Conditions: initial temperature, 60°; initial hold, 3 min; program rate, 6°/min; final temperature, 235°; attenuation, 1×10^{-10} a.f.s.

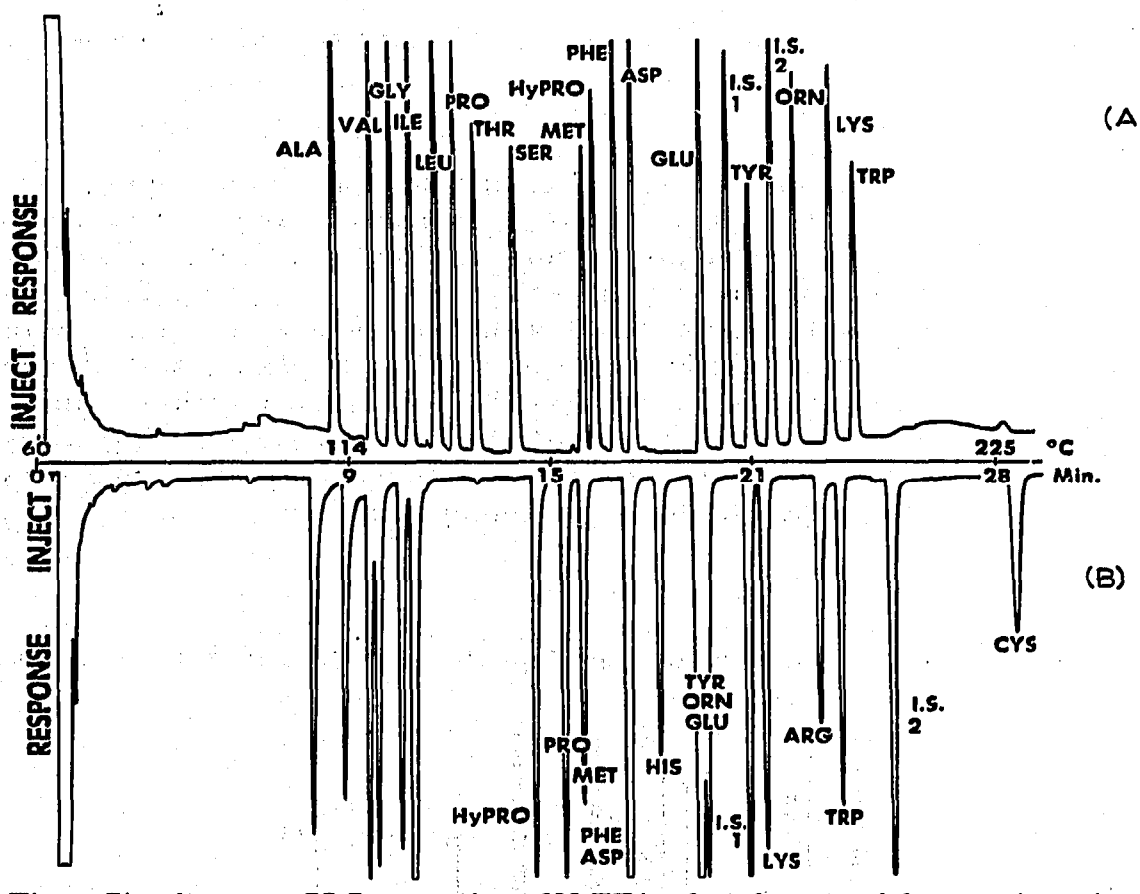
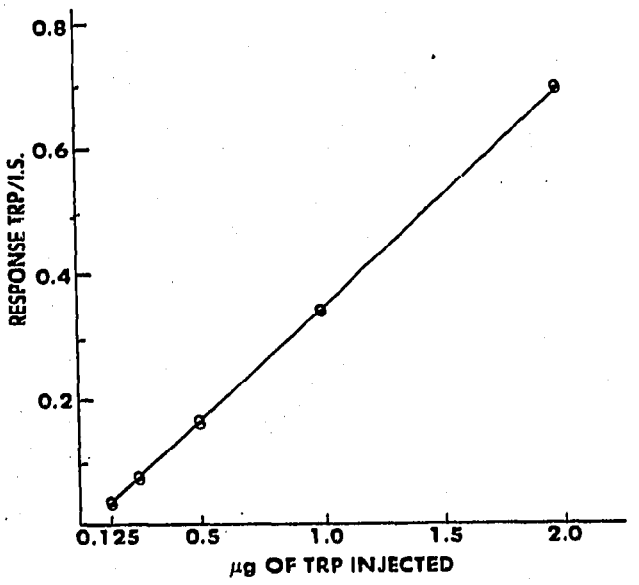


Fig. 2. Simultaneous GLC separation of N-TFA *n*-butyl esters of the protein amino acids. Injected: *ca.* 0.6 μg of each. Column A: 0.65 w/w% stabilized grade EGA on 80/100 mesh AW Chromosorb W, 1.5 m × 4 mm I.D. glass. Column B: 2.0 w/w% OV-17, 1.0 w/w% OV-210 on 100/120 mesh Supelcoport, 1.5 m × 4 mm I.D. glass. Conditions: attenuation, 1 × 10^{-10} a.f.s.; initial temperature, 60°; program rate, 6°/min; final temperature, 225°; I.S. 1 = tranexamic acid; I.S. 2 = *n*-butyl stearate.

N-TFA *n*-butyl esters of histidine, arginine, tryptophan, and cystine, from the other protein amino acids. It was observed that the diacyl histidine derivative was co-eluted with aspartic acid, phenylalanine, or tyrosine derivatives on OV-17, OV-25, and OV-210 columns, respectively. On OV-225, an individual diacyl histidine peak was obtained, however, the response was greatly reduced, which indicated a strong substrate–derivative interaction. Based on previous observations, a mixed phase of OV-17 and OV-210 was then evaluated. In this experiment, a complete separation of diacyl histidine, arginine, tryptophan, and cystine derivatives was achieved as shown in Fig. 1 with a mixed phase of 2.0 w/w% OV-17 and 1.0 w/w% OV-210 on 100/120 mesh Supelcoport, 1.5 m × 4 mm I.D. glass column. Fig. 2 presents the simultaneous chromatography on the mixed phase and EGA columns with a dual column instrument, resulting in a complete separation of the twenty protein amino acids.

In our previous publications, we reported on an effective chromatographic separation of 17 N-TFA *n*-butyl esters of the amino acids on a packing composed of stabilized grade EGA and acid-washed Chromosorb W (heated at 140° for 12 h). This new column packing, composed of a mixed phase of OV-17 and OV-210, is a superior packing and shows quantitative elution, and highly efficient and complete separation



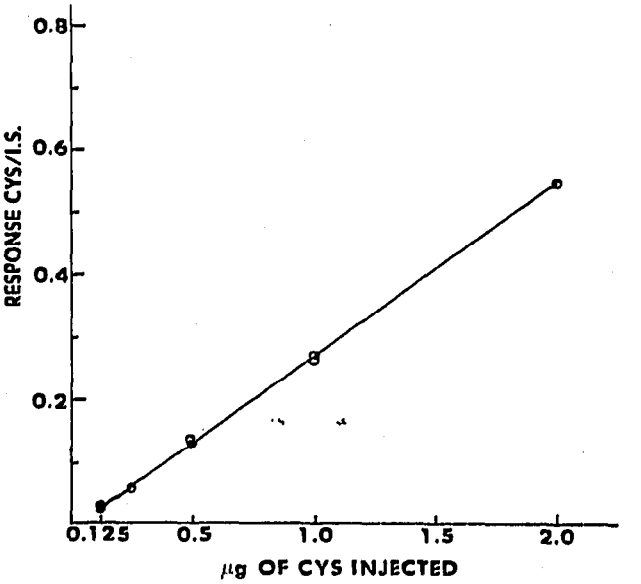
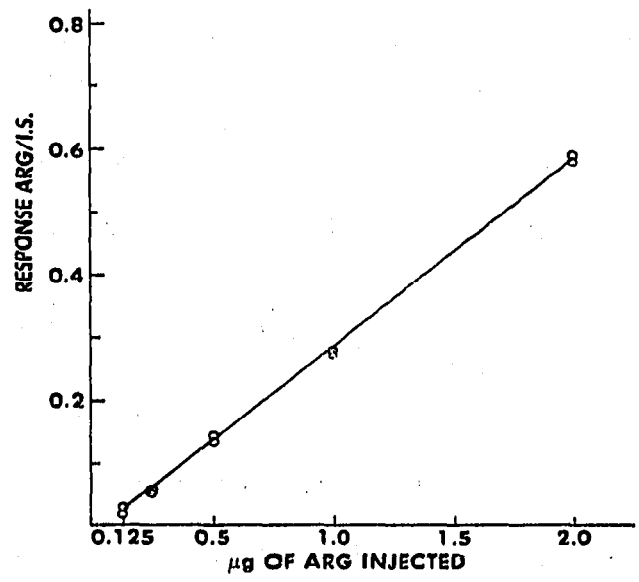


Fig. 3. Chromatographic response of tryptophan N-TFA *n*-butyl ester. Column: 2.0 w/w% OV-17, 1.0 w/w% OV-210 on 100/120 mesh Supelcoport, 1.5 m × 4 mm I.D. glass. Internal standard, *n*-butyl stearate; constant amount injected, 2.0 μg.

Fig. 4. Chromatographic response of cystine N-TFA *n*-butyl ester. Column: 2.0 w/w% OV-17, 1.0 w/w% OV-210 on 100/120 mesh Supelcoport, 1.5 m × 4 mm I.D. glass. Internal standard, *n*-butyl stearate; constant amount injected, 2.0 μg.



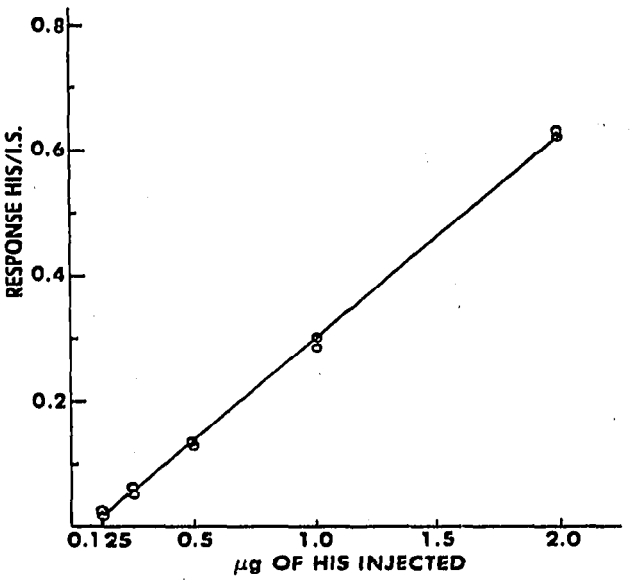


Fig. 5. Chromatographic response of arginine N-TFA *n*-butyl ester. Column: 2.0 w/w% OV-17, 1.0 w/w% OV-210 on 80/100 mesh Supelcoport, 1.5 m × 4 mm I.D. glass. Internal standard, *n*-butyl stearate; constant amount injected, 2.0 μg.

Fig. 6. Chromatographic response of histidine N-TFA *n*-butyl ester. Column: 2.0 w/w% OV-17, 1.0 w/w% OV-210 on 80/100 mesh Supelcoport, 1.5 m × 4 mm I.D. glass. Internal standard, *n*-butyl stearate; constant amount injected, 2.0 μg.

of histidine, internal standard tranexamic acid, lysine, arginine, tryptophan, *n*-butyl stearate (I.S.) and cystine. No longer is it necessary to make a separate "subtraction-calculation" for histidine. With these two packings, EGA and mixed siloxane phases, one can now simultaneously analyze and separate the twenty protein amino acids in 30 min or less with automatic electronic integration of all peaks.

We were also able to demonstrate the quantitative elution of histidine, arginine, tryptophan and cystine over a wide range of amounts of derivatives injected (0.125–2 μg) by response linearity plots which are illustrated in Figs. 3, 4, 5, and 6. In these studies, the amount of internal standard (*n*-butyl stearate) injected was held constant (2 μg).

Tranexamic acid and *n*-butyl stearate were both found to be well suited as internal standards in this dual chromatographic column combination. As tranexamic acid possesses both $-NH_2$ and –COOH functionality, it can easily be used to monitor the performance of the ion-exchange column cleanup for more complex biological samples, thereby increasing the reliability of quantitative analysis of amino acids in complex substances.

CONCLUSIONS

The analysis of amino acids by GLC has been significantly advanced by the quantitative elution and complete separation of all twenty protein amino acids as the N-TFA *n*-butyl esters on an EGA and mixed phase siloxane columns. No longer does one need to make a separate "subtraction-calculation" for histidine, it is separated and measured directly as are all the others.

Histidine, arginine, tryptophan, and cystine are eluted and completely separated with a 1.5 m × 4 mm I.D. glass column containing a mixed phase of 2.0 w/w% OV-17 and 1.0 w/w% OV-210 on 100/120 mesh Supelcoport, and the other sixteen protein amino acids are separated on a column of similar length containing 0.65 w/w% of stabilized grade EGA on 80/100 mesh Chromosorb W. Simultaneous operation of the mixed phase and EGA columns with a dual column instrument, results in a complete analysis of the protein amino acids in 30 min or less.

A second internal standard (tranexamic acid) is introduced which contains both $-NH_2$ and –COOH functional groups. It can be used to follow the performance of ion-exchange cleanup, thereby increasing the reliability of analysis of complex biological materials.

This dual column system enables rapid, accurate, and sensitive amino acid analyses to be performed in which single chromatographic peaks are obtained for each of the twenty protein amino acids. This chromatographic system, combined with the recently reported method for derivatization and GLC analysis of nanogram amounts of amino acids[15], should find a broad range of applications, extending from nutritional research to analyses for environmental pollutants and studies at the cellular level.

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