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GAS–LIQUID CHROMATOGRAPHIC STUDIES ON THE TWENTY PROTEIN AMINO ACIDS: A SINGLE-COLUMN SEPARATION*

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SUMMARY

The results of earlier investigations by GEHRKE *et al.* toward a single column separation of the twenty protein amino acids showed that polar liquid phases decompose the N-trifluoroacetyl (N-TFA) *n*-butyl ester derivatives of arginine, histidine, and cystine. Therefore, this study was to evaluate a number of non-polar liquid phases with regard to their ability to separate and reproducibly elute the N-TFA *n*-butyl ester derivatives.

In 1971, a chromatographic system was reported by GEHRKE *et al.* which employed dual columns; one of 0.65 % ethylene glycol adipate (stabilized grade EGA) as liquid phase on 80–100 mesh acid-washed Chromosorb W for separation of seventeen amino acid derivatives, and a mixed phase column containing 1.0 % (w/w) OV-210 and 2.0 % (w/w) OV-17 on Gas-Chrom Q was used for the separation of histidine, arginine, and cystine. Both columns of the system were operated under the same thermal conditions, thus allowing simultaneous operation.

The complete separation of the twenty amino acids on a single column is ultimately desirable and would aid considerable with respect to speed and cost; yet a dual-column combination is of significant merit in that additional separations are achieved and valuable cross confirmation is obtained on the amino acids in the sample.

A careful study of the separation characteristics of the OV series of siloxane liquid phases demonstrated that none of these phases, singly or in combinations would yield complete and reproducible separation of the protein amino acids on a single column. Studies were then made to determine the optimum initial column temperature and hold time for separation of Ala, Thr, Gly, and Ser, and Phe, Lys, and Tyr by measuring the rates of migration of these derivatives under isothermal conditions on non-polar liquid phases.

The optimum separation of all twenty amino acid derivatives was achieved with a 2.5 m × 2 mm I.D. glass column of 10 % (w/w) Apiezon M on 80–100 mesh HP Chromosorb W with an initial column temperature of 90° for 6–9 min, then a 6°/min program rate to 260°. Analyses of dipeptides containing histidine

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resulted in no apparent on-column conversion of diacyl histidine to the monoacyl derivative. Apiezon M is a useful liquid phase for the single column separation of the protein amino acid N-TFA *n*-butyl esters.

INTRODUCTION

Analysis of amino acids by gas–liquid chromatography (GLC) has been extensively studied due to both the importance of the amino acids, and the speed, sensitivity, and resolving power of GLC.

As the low volatility of the amino acids has prevented their direct analysis by GLC, the formation of suitable derivatives of increased volatility has been the obvious approach toward their analysis. Several different types of derivatives have been studied by a number of investigators[1], however, continued experiments have shown that the N-trifluoroacetyl (N-TFA) *n*-butyl esters are among the most useful derivatives. The organic reaction conditions for derivatizing the twenty protein amino acids to their N-TFA *n*-butyl esters have been established as the result of earlier investigations by ZOMZELY *et al.*[2] and GEHRKE and co-workers[3–5]. Later reports presented improved methods for esterification[6], GLC separation[7], and the detection of extremely small amounts of the protein amino acids[8].

The complete GLC separation of the twenty protein amino acid derivatives on a single column has been an intractable problem, much more difficult than the quantitative synthesis of the derivatives.

In 1962, ZOMZELY *et al.*[2] reported on the use of columns composed of neopentyl glycol succinate polyester on Gas-Chrom A for the separation of amino acid N-TFA *n*-butyl esters. Since then, many investigations aimed at achieving complete GLC separation of the protein amino acids on a single column have been made, however, little success has been achieved. Earlier, ten liquid phases were evaluated by GEHRKE AND SHAHROKHI[9], resulting in the report of a mixed phase of DEGS and EGSS-X on acid-washed Chromosorb W for the separation of the twenty protein amino acid N-TFA *n*-butyl esters; however, quantitative and reproducible elution of arginine, histidine, and cystine was not achieved.

DARBRE AND ISLAM[10] reported on the use of a mixed phase of XE-60, QF-1 and MS-200 on activated Diatoport S for the separation of the N-TFA methyl ester derivatives. XE-60 and QF-1 are 25% cyanoethyl methyl siloxane and 50% trifluoropropyl methyl siloxane, respectively. They successfully separated twenty-two amino acid derivatives, however, loss of histidine was observed during chromatography. Likewise, the separation of the twenty protein amino acids as N-trimethylsilyl *n*-butyl esters was successfully carried out by HARDY AND KERRIN[11] with columns of 0.2% OV-7 on 100–120 mesh textured glass beads. Glycine, arginine, and lysine gave multiple derivative formation with this method.

Ethylene glycol adipate (EGA-stabilized grade) has been studied by STEFANOVIC AND WALKER[12] and ROACH AND GEHRKE[13], and was found to be an excellent liquid phase for the separation of all the protein amino acid N-TFA *n*-butyl esters with the exception of arginine, histidine, and cystine.

In 1968, GEHRKE *et al.*[14] and a year later ROACH *et al.*[15] reported on dual-column chromatographic systems consisting of one column containing EGA, and a

second column with OV-17 as liquid phase from which nineteen of the amino acid N-TFA *n*-butyl esters were quantitatively eluted and separated, however, the twentieth amino acid, histidine, could not be readily analyzed on either of these systems. Using a technique similar to that of ROACH *et al.*[16] for the on-column conversion of monoacyl histidine to diacyl histidine, MOSS *et al.*[16] separated the twenty protein amino acids as their N-heptafluorobutyryl (N-HFB) *n*-propyl ester derivatives on a single column containing 3% OV-1. With this method it was necessary to evaporate the excess heptafluorobutyric anhydride (HFBA) from the sample prior to analysis as the tailing of the HFBA peak prevented the quantitative analysis of alanine and glycine.

In 1971, a chromatographic system was reported by GEHRKE *et al.*[7], which employed dual columns—one of 0.65% ethylene glycol adipate (stabilized grade EGA) as liquid phase on 80–100 mesh acid-washed Chromosorb W was used for the separation of seventeen amino acid derivatives, and a mixed-phase column containing 1.0% (w/w) OV-210 and 2.0% (w/w) OV-17 was used for the separation of histidine, arginine, and cystine. Both columns of the system were operated under the same thermal conditions, thus allowing simultaneous operation. This dual-column system has been successfully used for the analysis of a number of biochemical materials[17].

The complete separation of the twenty protein amino acids on a single column is ultimately desirable and would aid considerably with respect to speed and cost; yet a dual-column combination is of significant merit in that additional separations are achieved and valuable cross confirmation is obtained on the amino acids in the sample.

From the results of investigations toward a single-column separation, it is known that polar liquid phases decompose the N-TFA *n*-butyl ester derivatives of arginine, histidine, and cystine. Therefore, non-polar liquid phases were investigated for the reproducible quantitative elution of the twenty protein amino acids.

The aim of this study was to evaluate a number of liquid phases with regard to their ability to separate, and reproducibly elute, the N-TFA *n*-butyl esters of the twenty protein amino acids. This information would be of value in the development of a single-column system for the analysis of the amino acids by GLC.

EXPERIMENTAL

Apparatus

A Bendix 2500 Series gas chromatograph with a four-column oven bath, four hydrogen flame detectors, two differential electrometers, a linear temperature programmer, and equipped with a Varian Model A-20 dual-pen recorder was used. Peak areas were determined with an Infotronics Corp. CRS-104 digital integrator. Also, a Packard Instrument Company 7300 Series dual-column gas chromatograph with hydrogen flame detectors was used.

Solvents were removed from the samples with a Calab rotary evaporator, "cold finger" condenser filled with dry ice in Methyl Cellosolve, and a Welch Duo-Seal vacuum pump.

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

Filters containing shell-type charcoal and indicating molecular sieve (Type

13X) were placed in the lines for removal of trace hydrocarbons and water in the nitrogen, hydrogen, and air to the chromatograph. For removal of particulate matter from the gas streams, "F" series inline 7-μ sintered stainless-steel filters were obtained from Nupro Company, Cleveland, Ohio, and fitted into the lines.

Reagents and materials

All amino acids used in this study were obtained from Mann Research Laboratories, Inc., New York, N.Y., and were chromatographically pure. Two enzymes, lysozyme from egg white and trypsinogen from bovine pancreas, were obtained from Sigma Chemical Company, St. Louis, Mo. Two dipeptides, glycyl-L-histidine HCl hydrate and L-histidyl-L-leucine hemihydrate, were purchased from Cyclo Chemical, Division Travenol Laboratories, Inc., Los Angeles, Calif.

n-Butanol was a "Baker Analyzed" reagent. The trifluoroacetic anhydride was obtained from Distillation Products Industries, Rochester, N.Y., and was an "Eastman Grade" chemical. Methylene chloride was obtained from Mallinckrodt Chemical Works, St. Louis, Mo., and was Analytical Reagent Grade. Dimethyldichlorosilane (DMCS) for silylating the glass column walls was obtained from Eastman Organic Chemicals, Rochester, N.Y. 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, then bubbled into *n*-butanol until 3 *M* in HCl.

The OV-3, OV-7, OV-25, OV-61, OV-210, OV-225, DC-430, and SE-54 liquid phases and HP Chromosorb W were purchased from Applied Science Laboratories, State College, Pa. The liquid phase SP-2401 and Supelcoport support material were purchased from Supelco. Inc., Bellefonte, Pa. Apiezons L and M were obtained from James G. Biddle Co., Plymouth Meeting, Pa. The Apiezons were purified as follows: 10 g of Apiezon were dissolved in 50 ml of methylene chloride, transferred to a separatory funnel, and allowed to stand overnight for separation of the insoluble material. The clear methylene chloride solution was placed in a 125-ml flat-bottom flask, and evaporated to dryness under vacuum at 80° on a rotary evaporator. A stock solution containing 50.0 mg/ml of purified Apiezon in methylene chloride was then prepared.

Preparation of the 10 % (w/w) Apiezon M column

The support material, 80–100 mesh HP Chromosorb W (9.00 g), was placed in a 250-ml ridged round-bottom flask and just covered with methylene chloride. Then, 20 ml of purified Apiezon M solution containing 50.0 mg/ml in methylene chloride were added to the flask. The solvent was slowly removed using a rotary evaporator at room temperature until just damp. The flask was then immersed in a 60° water-bath under full vacuum for 10 min. The dry packing was then placed in glass columns (2.5 m × 2 mm I.D.) which had been silylated by the procedure recommended by Applied Science Laboratories, Inc.[18]. Silylation of the glass columns consisted of filling the columns with a solution of 10 % DMCS in toluene and allowing to stand overnight, then rinsing with toluene, methanol, and acetone followed by drying at 200°. A vacuum was used to aid in the packing of longer columns, and 1/4-in. plugs of silylated glass wool were placed in the open ends of the column to hold the packing in place. The columns were then placed in a column conditioning

oven and flushed for 30 min with nitrogen carrier gas, then maintained at 240° for 14 h with a nitrogen flow of 15 ml/min.

When not in use, the Apiezon M columns are best kept below 200° with a carrier gas flow maintained at 10–15 ml/min. If the column must be removed from the instrument, the ends should be tightly closed during storage to exclude atmospheric moisture.

Preparation of the other chromatographic columns studied followed the same procedure as described above for the 10 % Apiezon M columns.

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

The "direct esterification-acylation" procedure described by ROACH AND GEHRKE[9] was used for conversion of the amino acids to their N-TFA *n*-butyl esters. For identification of the chromatographic peaks, mixtures of the N-TFA *n*-butyl ester derivatives were "spiked" with single amino acid derivatives.

Hydrolysis of enzymes and dipeptides

Lysozyme and trypsinogen were hydrolyzed by the procedure described by MATSUBARA AND SASAKI[10]. The hydrolysates were prepared at 110° for 21 h with 6 *N* HCl containing 5 % thioglycollic acid (TGA) to prevent destruction of tryptophan, and interferences were removed from the hydrolysates by cation exchange. Prior to hydrolysis, the sample in acid solution was evacuated, swept with nitrogen gas, and this procedure repeated five times.

Calculations

The relative weight response (*RWR*) of each amino acid relative to proline, $RWR_{\text{a.a./pro.}}$, was calculated as follows:

$$RWR_{\text{a.a./pro.}} = \frac{A_{\text{a.a.}}/g_{\text{a.a.}}}{A_{\text{pro.}}/g_{\text{pro.}}} = \frac{A_{\text{a.a.}}}{A_{\text{pro.}}};$$

when equal weights (*ca.* 0.2 mg) of each were used. $A_{\text{a.a.}}$ = area in counts of the amino acid peak and $g_{\text{a.a.}}$ = grams of amino acid in the sample.

Internal standards

Ornithine, tranexamic acid, and stearic acid were used as internal standards as they were well resolved from the other amino acid peaks with a 2.5 m × 2 mm I.D. glass column of 10 % (w/w) Apiezon M on 80–100 mesh HP Chromosorb W (Fig. 3).

RESULTS AND DISCUSSION

Earlier studies have shown that the most difficult problems with regard to the development of a single-column GLC system for amino acid analysis are the following:

(1) Interaction and loss of the derivatives of arginine, histidine, and cystine on the chromatographic column.

(2) Difficulty in separation of phenylalanine, aspartic acid, and histidine derivatives.

(3) Separation of the derivatives of leucine and isoleucine.

The mixed liquid phase column of OV-17 and OV-210 reported by GEHRKE *et al.*[7] has solved the problem of the separation of histidine from aspartic acid and phenylalanine, but aspartic acid and phenylalanine are not resolved with this column, however, these two amino acids are separated on the EGA column.

The separation of leucine and isoleucine can be achieved by use of polar liquid phase columns, but polar phases result in losses of arginine, histidine, and cystine derivatives.

In this study, a number of siloxane liquid phases of differing polarities were studied, and their separation characteristics were observed. The different columns used, supports, conditioning of the column, and the composition of the liquid phases are given in Table I. Figs. 1 and 2 present the elution sequences of the amino acid

TABLE I

COMPOSITION AND CONDITIONING OF CHROMATOGRAPHIC COLUMNS

Column No.	*Column*[a]	*Support*	*Conditioning*	*Siloxane liquid phases of composition*
1	0.3% OV-225	100–120 mesh Supelcoport	250°/3 days	25% cyanopropyl, 25% phenyl, 50% methyl
2	3% OV-25	80–100 mesh HP Chromosorb W	290°/2 days	75% phenyl, 25% methyl
3	3% OV-61	80–100 mesh HP Chromosorb W	290°/2 days	35% phenyl, 65% methyl
4	3% OV-7	80–100 mesh HP Chromosorb W	300°/2 days	20% phenyl, 80% methyl
5	3% OV-3	80–100 mesh HP Chromosorb W	300°/2 days	10% phenyl, 90% methyl
6	3% SP-2401	100–120 mesh Supelcoport	250°/2 days	50% trifluoropropyl, 50% methyl (Supelco Co.)
7	3% OV-210	80–100 mesh HP Chromosorb W	250°/2 days	50% trifluoropropyl, 50% methyl (Applied Science Co.)
8	3% SE-54	100–120 mesh Supelcoport	280°/2 days	1% vinyl, 5% phenyl, 94% methyl
9	3% DC-430	100–120 mesh Supelcoport	290°/2 days	1% vinyl, 99% methyl
10	9% Apiezon L	80–100 mesh HP Chromosorb W	240°/14 h	Made from specially selected hydrocarbon fractions

[a] Polymeric structure of siloxane phases: $(CH_3)_3\text{–Si–O–}\left[\begin{matrix}R & R\\ | & |\\ \text{Si–O–Si–O–}\\ | & |\\ R & R\end{matrix}\right]_x\text{Si–}(CH_3)_3$.

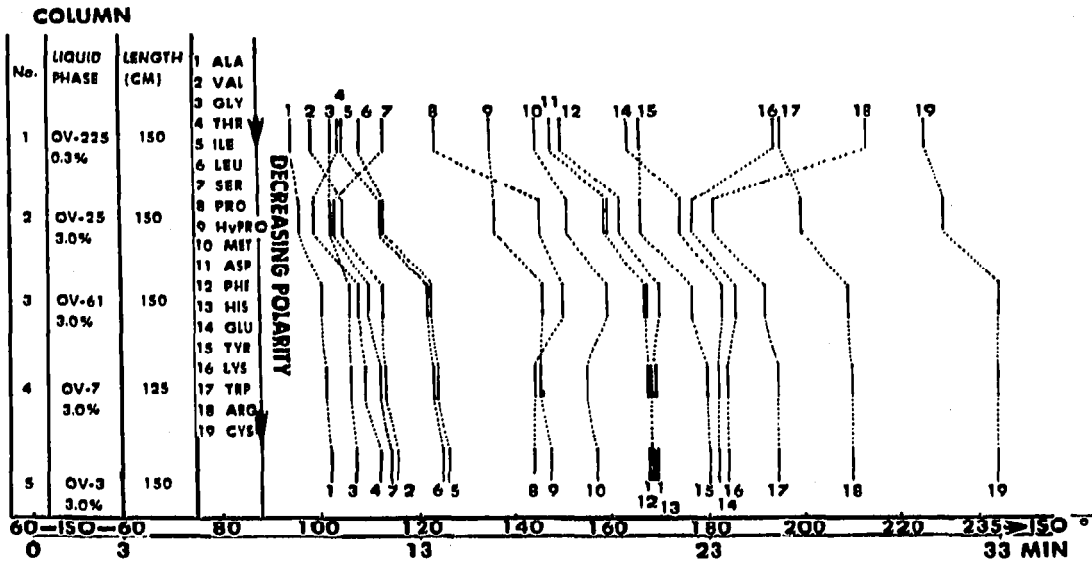


Fig. 1. Effect of liquid phase polarity on the separation of the N-TFA *n*-butyl esters of amino acids. Instrumental conditions: initial temperature 60°, delay 3 min, 6°/min, and final temperature 235°. Nitrogen flow-rate: 50 ml/min.

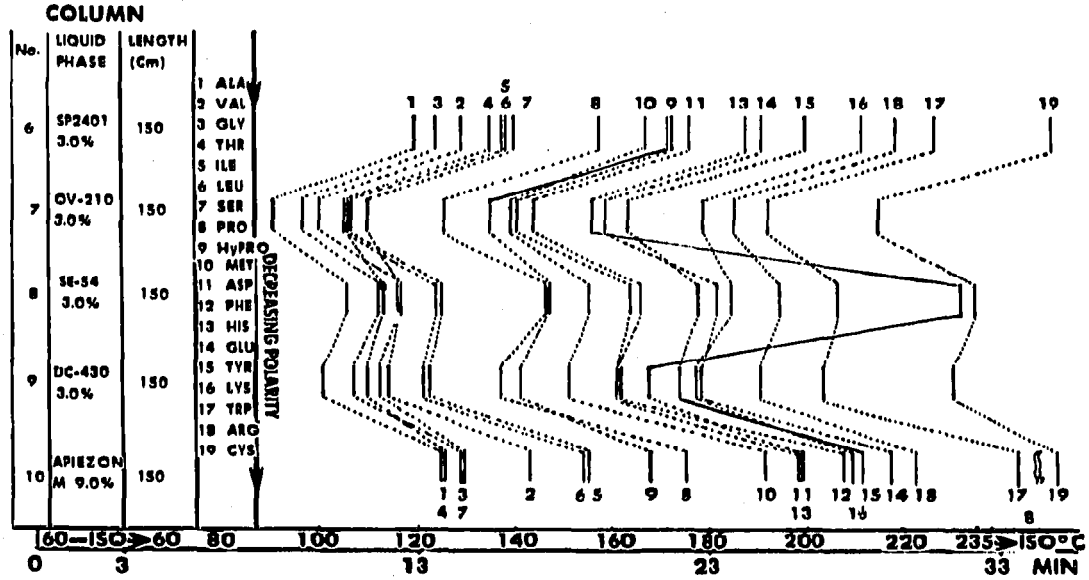


Fig. 2. Effect of liquid phase polarity on the separation of the N-TFA *n*-butyl esters of amino acids. Instrumental conditions as in Fig. 1. Nitrogen flow-rate: 50 ml/min.

N-TFA *n*-butyl esters from columns prepared with the various liquid phases. Each vertical dark line represents the elution time of the amino acid derivative peak on that particular column. These studies show that no single substrate or combination of these substrates would yield the complete separation of the twenty protein amino acid N-TFA *n*-butyl esters on a single column. As will be noted in Fig. 1, decreasing

polarities of liquid phases (OV-25, OV-61, OV-7, OV-3, and OV-1) gave somewhat increased separation of leucine and isoleucine. On polar liquid phases isoleucine is eluted prior to leucine; as the polarity decreases this elution order is reversed and leucine elutes first. This observation suggested the use of liquid phases of less polarity than OV-1 might yield a separation of leucine and isoleucine without the on-column loss of any of the twenty protein amino acid derivatives. According to the characterization of liquid phases by McREYNOLDS[20], and considering the high degree of thermal stability, Apiezon (high-molecular-weight hydrocarbons) was evaluated as a liquid phase (Fig. 3). Initially, the main difficulty was the separation of alanine, threonine,

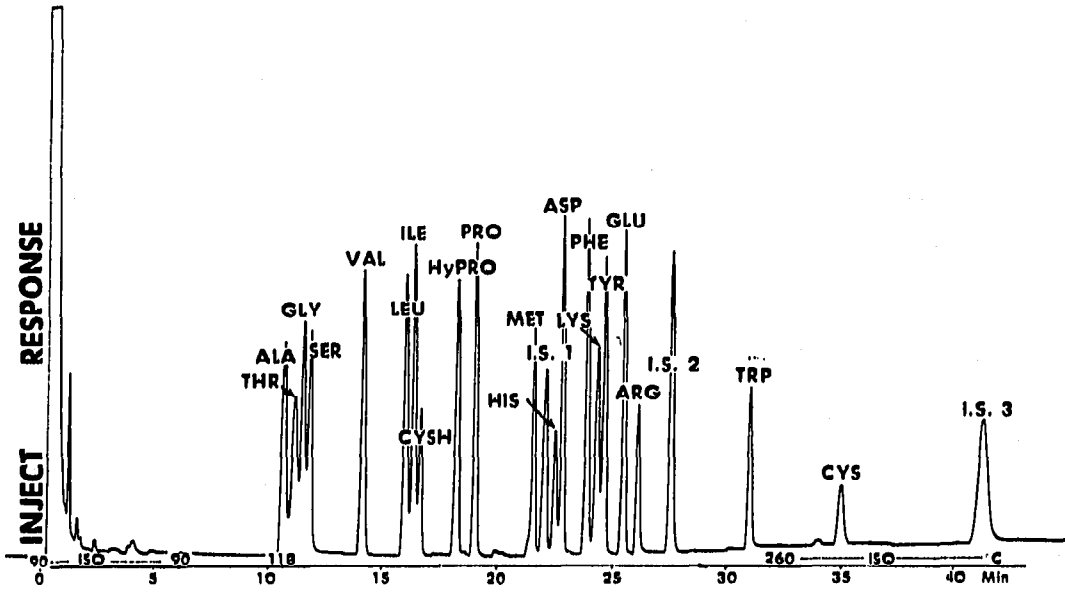
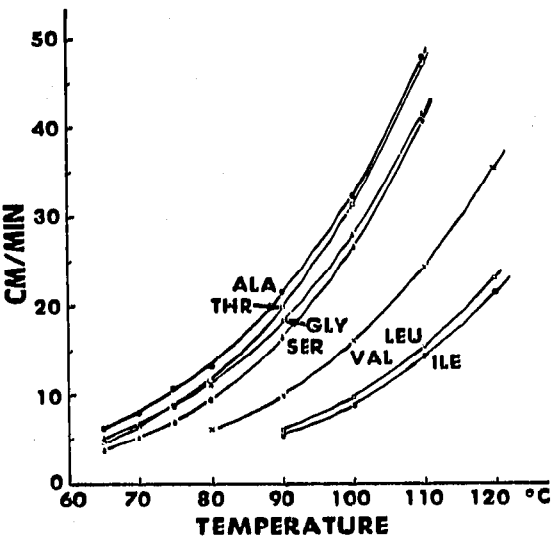


Fig. 3. Single-column GLC separation of the N-TFA *n*-butyl esters of the protein amino acids. Column: 10% purified Apiezon M on 80–100 mesh HP Chromosorb W, 2.5 m × 2 mm I.D. glass. Sample: *ca.* 2.4 μg of each. Attenuation: 1×10^{-10} a.f.s. Instrumental conditions: initial temperature 90°, delay 6 min, 6°/min, and final temperature 260°. Internal standards: (1) ornithine, (2) tranexamic acid, and (3) *n*-butyl stearate.

glycine, and serine with the 10% Apiezon M column. Therefore, a study of the migration rates of these derivatives under isothermal conditions was conducted to find the most effective initial temperature and hold time. At initial temperatures above 90°, separations of the alanine–threonine and glycine–serine pairs were poor. At initial temperatures below 90°, threonine and glycine were not well resolved. With an increased initial delay of 10–20 min at 90°, the separation improved, but the peaks became unacceptably broad. Fig. 4 suggests that a several-minute delay (6–9 min) at 90° gives the greatest separation of alanine, threonine, glycine, and serine.

As the retention times of phenylalanine, lysine, and tyrosine were very similar, a study of their separation was also conducted. Migration rates of phenylalanine, lysine, and tyrosine were measured on the 10% Apiezon M column under isothermal conditions (Fig. 5). The separation of phenylalanine and lysine decreases with increasing temperature, and a program rate of 6°/min was found to be optimal.



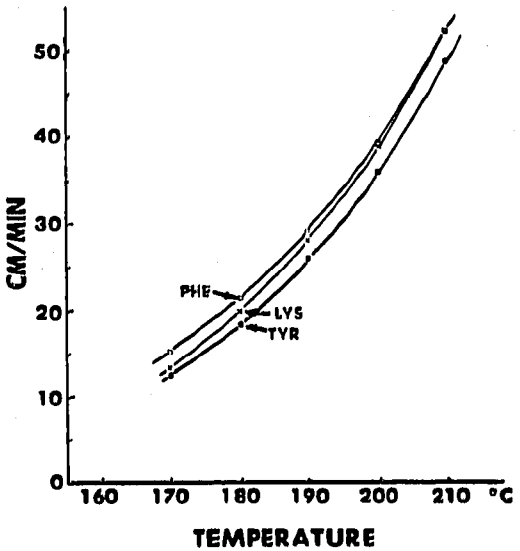


Fig. 4. Migration rates of the N-TFA *n*-butyl esters of amino acids. Column: 10% (w/w) Apiezon M on 80–100 mesh HP Chromosorb W, 77 cm × 2 mm I.D. glass. Temperatures: oven, iso; inlet, 120°; and detector, 150°. Nitrogen flow-rate: 15 ml/min.

Fig. 5. Migration rates of the N-TFA *n*-butyl esters of amino acids. Column: 10% (w/w) Apiezon M on 80–100 mesh HP Chromosorb W, 2.5 m × 2 mm I.D. glass. Temperatures: oven, iso; inlet, 210°; and detector, 260°. Nitrogen flow-rate: 15 ml/min.

The relative weight responses, $RWR_{\text{n.a./pro.}}$, were measured after chromatography on a 2.5 m × 2 mm I.D. column of 10% Apiezon M on 80–100 mesh HP Chromosorb W and are given in Table II. For measurements of these *RWR* values, standard solutions of amino acids were derivatized, analyzed, and the peak areas obtained by electronic integration.

Table III presents a comparison of the relative weight responses of six amino acids obtained from columns of 10% Apiezon M, a mixed phase of 2% OV-17 and 1.0% OV-210, and 0.65% EGA. Excellent precision of results was shown. Tryptophan gave a higher *RWR* (+ 25%) on Apiezon M as compared to the mixed-phase column, and methionine (−14%), histidine (−37%), arginine (−7%), and cystine (−54%) gave lower *RWR* values on the Apiezon M column as compared to the mixed-phase column. No peaks were observed for histidine, arginine, and cystine on the EGA column. This confirms our earlier reports. In general, the FID response for methionine, histidine, arginine, and cystine is considerably lower on most substrates as compared to the response for the other amino acids. This is mainly a result of substrate–derivative interaction.

The 10% Apiezon M column can be used over a period of one to two months without loss of separation and reduction in response of the amino acid derivatives.

In experiments on the elution of histidine, a standard solution eluted quantitatively as the diacyl histidine derivative when histidine was injected in the range of 100 to 600 ng, however, with hydrolysates of lysozyme with standard histidine added at the 2 and 5% level and containing hydroxyproline as the internal standard; the recoveries of the diacyl histidine derivative were 80.0 and 88.9%, respectively.

TABLE II

RELATIVE WEIGHT RESPONSES OF AMINO ACID N-TFA *n*-BUTYL ESTERS ON 10% APIEZON M

A 2.5 m × 2 mm I.D. glass column containing 10% (w/w) Apiezon M on 80–100 mesh HP Chromosorb W was used.

Amino acid	*RWR*					
	0.5 μg[a]		*1.0 μg*[a]		*Av.*	*RSD* (%)
Alanine	0.944	0.946	0.946	0.945	0.945	0.11
Threonine	0.915	0.916	0.909	0.911	0.913	0.36
Glycine	0.836	0.848	0.878	0.872	0.859	2.31
Serine	0.961	0.939	0.938	0.943	0.945	1.34
Valine	1.018	1.019	1.014	1.014	1.016	0.26
Leucine	0.985	0.983	0.977	0.981	0.982	0.36
Isoleucine	1.017	1.019	1.023	1.019	1.019	0.26
Hydroxyproline	0.948	0.949	0.950	0.952	0.950	0.18
Proline[b]	1.000	1.000	1.000	1.000	1.000	—
Methionine	0.713	0.711	0.723	0.720	0.717	0.78
Ornithine	0.680	0.687	0.718	0.714	0.700	2.71
Histidine	0.329	0.335	0.358	0.348	0.342	3.83
Aspartic acid	1.140	1.122	1.110	1.106	1.120	1.37
Phenylalanine	1.102	1.106	1.101	1.103	1.103	0.20
Lysine	0.798	0.812	0.859	0.854	0.831	3.66
Tyrosine	0.960	0.947	0.950	0.954	0.953	0.59
Glutamic acid	1.065	1.060	1.101	1.102	1.082	2.09
Arginine	0.410	0.431	0.450	0.431	0.431	3.78
Tranexamic acid	1.062	1.056	1.089	1.100	1.077	1.96
Tryptophan	0.625	0.634	0.625	0.619	0.626	0.99
Cystine	0.261	0.243	0.238	0.260	0.251	4.70

[a] Amount of each amino acid derivative injected.
[b] Proline as internal standard.

TABLE III

RELATIVE WEIGHT RESPONSES OF N-TFA *n*-BUTYL ESTERS OF AMINO ACIDS ON DIFFERENT SUBSTRATES

Amino acids	*RWR*[a]								
	10% Apiezon M[b]			*Mixed phase*[c]			*0.65% EGA*[d]		
	1	*2*	*Av.*	*1*	*2*	*Av.*	*1*	*2*	*Av.*
Proline[e]	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Methionine	0.675	0.674	0.675	0.786	0.785	0.786	0.707	0.707	0.707
Histidine	0.232	0.210	0.221	0.343	0.360	0.352		No peak	
Lysine	0.847	0.857	0.852	0.855	0.865	0.860	0.804	0.815	0.810
Arginine	0.455	0.437	0.446	0.480	0.475	0.478		No peak	
Tryptophan	0.774	0.782	0.778	0.619	0.642	0.631	0.563	0.612	0.588
Cystine	0.245	0.228	0.236	0.498	0.529	0.513		No peak	

[a] Analyses of a standard solution of amino acid N-TFA *n*-butyl esters.
[b] 2.5 m × 2 mm I.D. glass column packed with 10% (w/w) Apiezon M on 80–100 mesh HP Chromosorb W.
[c] 1.5 m × 2 mm I.D. glass column containing 2.0% (w/w) OV-17 and 1.0% (w/w) OV-210 on 100–120 mesh Gas-Chrom Q.
[d] 1.5 m × 4 mm I.D. glass column packed with 0.65% (w/w) stabilized-grade EGA on 80–100 mesh acid-washed Chromosorb W.
[e] Proline as internal standard.

A chromatogram showing GLC analysis of hydrolyzed lysozyme is given in Fig. 6. This protein contains *ca.* 1 % histidine. In samples containing low concentrations of histidine, we observed that histidine was eluted mainly as the monoacyl derivative.

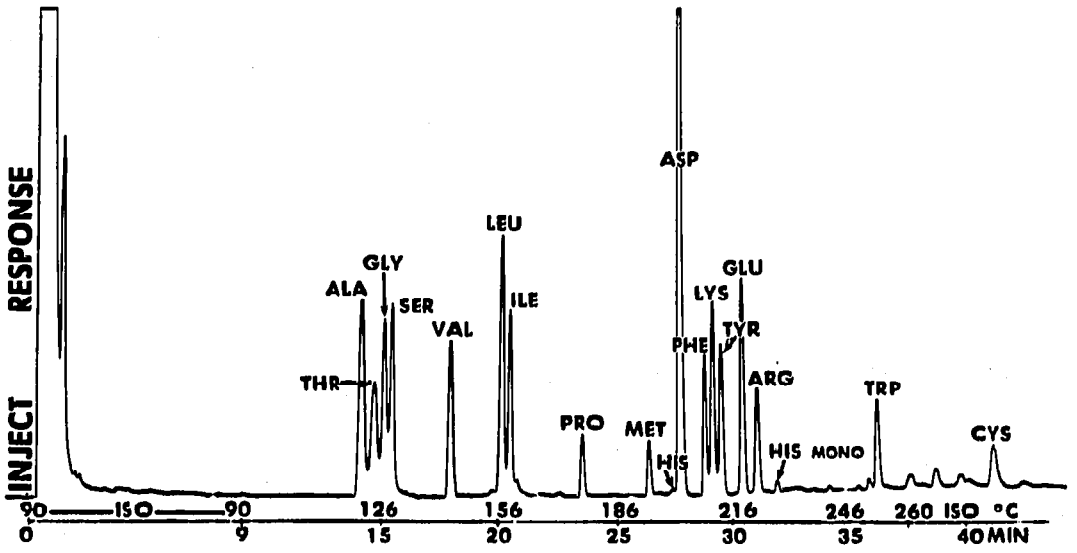


Fig. 6. Single-column GLC analysis of the N-TFA *n*-butyl esters of lysozyme — with cleanup. Column: 10% (w/w) Apiezon M on 80–100 mesh HP Chromosorb W, 2.5 m × 2 mm I.D. glass. Sample: 6.0 mg lysozyme. Hydrolysis: 5 ml 6 *N* HCl with 5% TGA, 21 h, 110°. Instrumental conditions: initial temperature 60°, delay 9 min, 6°/min, and final temperature 260°.

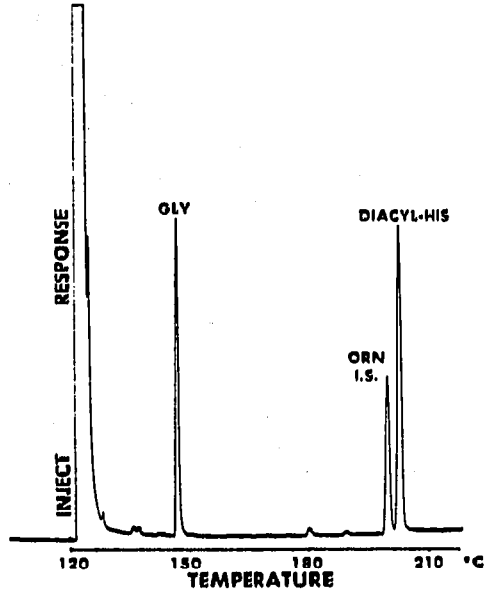


Fig. 7. GLC analysis of the N-TFA *n*-butyl esters of hydrolyzed glycyl-L-histidine. Column: as in Fig. 6. Sample: *ca.* 1.5 mg total. Attenuation: 5 × 10^{-9} a.f.s. Instrumental conditions: initial temperature 120°, 6°/min. Internal standard: ornithine.

However, as shown in Fig. 7, GLC analysis of hydrolyzed glycyl-L-histidine HCl hydrate gave the diacyl histidine derivative without decomposition. The monoacyl derivative was not observed. Table IV gives the molar ratios of dipeptides containing histidine by GLC. Note the good agreement of the experimental data with theory.

TABLE IV

MOLAR RATIOS OF DIPEPTIDES BY GLC ANALYSIS

A 2.5 m × 2 mm I.D. glass column packed with 10% Apiezon M on 80–100 mesh HP Chromosorb W was used.

Amino acid	*Molar ratio*			
	Theory	*GLC analysis*		*Av.*
Histidine/glycine[a]	1	1.09	1.09	1.09
Histidine/leucine[b]	1	0.98	1.00	0.99

[a] 0.5 mg of glycyl-L-histidine HCl hydrate was hydrolyzed in 1 ml of 6 *N* HCl at 110° for 21 h.
[b] 0.15 mg of L-histidyl-L-leucine hemihydrate was hydrolyzed in 1 ml of 6 *N* HCl at 110° for 21 h.

CONCLUSIONS

The three major difficulties with regard to the single-column separation of the twenty protein amino acid derivatives on a single GLC column are: (1) the on-column loss of arginine, histidine, and cystine, (2) separation of aspartic acid, phenylalanine, and histidine, and (3) separation of leucine and isoleucine. A number of siloxane liquid phases were chosen for evaluation as the use of more polar polyester phases generally results in losses of the three amino acids mentioned above. A careful study of the separation characteristics of the OV series of siloxane liquid phases demonstrated that none of these phases, singly or in combinations, would yield complete and reproducible separation of the protein amino acids on a single column. It was noted, however, that liquid phases of decreasing polarity gave somewhat increased separation of leucine and isoleucine. The non-polar hydrocarbon Apiezon was thus selected for study, and was found to be a promising substrate for the separation of the amino acid derivatives.

Studies were conducted to determine the optimum initial column temperature and hold time for the separation of alanine, threonine, glycine, and serine and of phenylalanine, lysine, and tyrosine by measuring the rates of migration of these derivatives under isothermal conditions.

The optimum separation of all twenty amino acid derivatives was achieved with a 2.5 m × 2 mm I.D. glass column of 10% (w/w) Apiezon M on 80–100 mesh HP Chromosorb W with an initial column temperature of 90° for 6–9 min, then a 6°/min program rate to 260°.

Tryptophan gave a higher *RWR* (+ 25%) on Apiezon M as compared to the mixed-phase OV-17/OV-210 column, and methionine (−14%), histidine (−37%), arginine (−7%), and cystine (−54%) gave lower *RWR* values on the Apiezon M column as compared to the mixed-phase column.

Analyses of dipeptides containing histidine resulted in no apparent on-column conversion of diacyl histidine to the monoacyl derivative, however, on analysis of lysozyme and trypsinogen hydrolysates, which contain relatively small amounts of histidine (*ca.* 1 %), we have encountered some difficulties in that diacyl histidine is in part decomposed to the monoacyl histidine on the Apiezon M GLC column. When calculations of the combined peak areas of diacyl histidine and monoacyl histidine were made, the results agreed with the values obtained from classical ion-exchange analyses of the enzymes.

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