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GAS–LIQUID CHROMATOGRAPHY OF PROTEIN AMINO ACIDS

SEPARATION FACTORS*

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SUMMARY

This research reports on chromatographic and instrumental studies which will permit one to accurately, rapidly, and routinely analyze the twenty natural protein amino acids in biological substances. A number of factors influencing the performance of the chromatographic system were evaluated, and the separation characteristics of various polyesters of neopentyl glycol were evaluated. Also, an evaluation was made of support materials which had undergone various heat treatments.

It was shown that the optimum chromatographic performance for neopentyl glycol polyesters was observed at a carbon chain length of ten (neopentyl glycol sebacate). The separation ability of ethylene glycol adipate as a liquid phase was found to be superior to neopentyl glycol sebacate, consistent, and reproducible with respect to time and temperature. Arginine, histidine, and cystine were not reproducibly eluted from this column. This is a result of interaction between the substrate phase and the amino acid derivative. However, seventeen of the amino acid derivatives were well separated and quantitatively eluted in 33 min from columns containing 0.325 w/w % ethylene glycol adipate coated on 80/100 mesh acid-washed heat-treated Chromosorb G.

In general, the retention temperatures for the amino acids were lower and a significant improvement in resolution was noted when columns were prepared with Chromosorb G which had been heated at 450° to 600° for 15 h.

For the analysis of arginine, histidine and cystine, columns containing 1.5 w/w % OV-17 coated on high performance 80/100 mesh Chromosorb G were used. Di-acyl histidine was converted to the mono-acyl derivative by injection of *n*-butanol immediately after injection of the sample. The di-acyl derivative of histidine does not interfere with any of the amino acids on the EGA column.

A dual column chromatographic system of ethylene glycol adipate and OV-17 as the liquid phases is described which quantitatively separates the protein amino acids in 55 min. The quantitative gas–liquid chromatographic analysis of the amino acids in ribonuclease is reported.

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INTRODUCTION

Gas–liquid chromatographic (GLC) methods have been particularly useful for the rapid, accurate, and sensitive analysis of biological materials for their amino acid content. However, this technique has presented certain limitations concerning the routine determination of all twenty of the protein amino acids. The reaction conditions for quantitatively preparing the derivatives of the twenty protein amino acids and at least thirty non-protein amino acids have been determined[1–5]. The major remaining problem has been the inability to elute quantitatively and separate all of the derivatives from a single chromatographic column.

A general review by GEHRKE AND STALLING in 1967[1] discussed the preparation of suitable derivatives of amino acids and their analysis by GLC. LAMKIN AND GEHRKE[2] reported in 1965 that the most suitable derivative for the gas–liquid chromatography of the natural protein amino acids is the N-trifluoroacetyl (N-TFA) *n*-butyl ester. In the same year they reported a procedure for quantitatively preparing the N-TFA *n*-butyl esters of eighteen of the amino acids for GLC analysis. Single chromatographic peaks were obtained for all the common protein amino acids except tryptophan and arginine. Reproducibility of response was found to be good for derivatives carried through the entire chemical and chromatographic procedures.

Studies on the quantitative gas chromatography of amino acids were reported by GEHRKE *et al.*[3] in 1965. The retention temperatures and relative molar responses of the amino acids were determined using a 1.0 m column packed with 1.0 w/w % neopentyl glycol succinate on 60/80 mesh Gas-Chrom A. Acylation studies on arginine were published in 1966 by STALLING AND GEHRKE[4]. The quantitative conversion of arginine to a single derivative, the tris(trifluoroacetyl) derivative, was achieved by conducting the acylation of arginine *n*-butyl ester·HCl at 150° for 5 min in a *closed* tube. Also, tryptophan was converted to the di-acyl derivative producing only one peak, and identical relative peak areas were obtained for each of the other eighteen protein amino acids using either the closed tube acylation method at 150° for 5 min or acylation at room temperature for 2 h.

An investigation of chromatographic factors affecting the separation of the amino acid N-TFA *n*-butyl esters was reported by GEHRKE AND SHAHROKHI[5] in 1966. The aim of that study was to find suitable column packings which would completely separate and allow the quantitative elution of the twenty volatile derivatives on a single column. An evaluation was made of a number of the commercially available polar stationary phases. Combinations of two mixed stationary phases were investigated as well as columns packed with two liquid phases in series. This paper also reported on different column lengths and concentrations of the two stationary phases and the use of temperature programming. Complete resolution of a 20-component mixture of the N-TFA *n*-butyl ester derivatives was achieved with a *mixed* stationary phase column of 0.75/0.25 w/w % of DEGS/EGSS-X. However, reproducible elution of arginine, histidine, and cystine was not obtained using this column. Also, this mixture of stationary phases was found inadequate for routine analysis of the amino acids in proteins as isoleucine and glycine were unresolvable after prolonged use of the column. STEFANOVIC AND WALKER[6] reported that the liquid phase ethylene glycol adipate (EGA) would separate the twenty N-TFA *n*-butyl ester derivatives. These workers reported the optimum loading to be 0.65 w/w % EGA on Chromosorb

W. However, they observed very small peaks for arginine, histidine and cystine, and suggested that these derivatives were decomposed on the column or reacted chemically with the polar liquid phase.

McBRIDE AND KLINGMAN[7] investigated polyesters of increased thermal stability in an effort to find a single column which would separate nanomolar quantities of the N-TFA *n*-butyl esters of amino acids isolated from biological substances. Using a 1.2 w/w % phenyldiethanolamine succinate polyester coated on Gas-Chrom A, they reported good separation for seventeen amino acids but gave no data for arginine, histidine and cystine. DARBRE, BLAU and ISLAM[8,9] have investigated the derivatization and GLC separation of the amino acids. They made extensive use of mixed siloxane liquid phases for the separation of the N-trifluoroacetyl methyl esters. However, histidine was not quantitatively eluted from these columns.

The present paper reports research on chromatographic and instrumental studies which will permit one to analyze the protein amino acids accurately, rapidly, and routinely in biological substances. In this work, a number of factors influencing the performance of the chromatographic system were evaluated, the results being a dual column system which completely resolves and quantitatively elutes in a routine manner the twenty N-TFA *n*-butyl ester derivatives.

Further, in an effort to find a stationary phase which would resolve all of the N-TFA *n*-butyl esters of the protein amino acids, the separation characteristics of various polyesters of neopentyl glycol were evaluated. The effect of increasing carbon chain length of the dicarboxylic acids in the polyesters on the retention volumes of the amino acid derivatives was studied.

Also, an evaluation was made of support materials which had undergone various heat treatments to determine the effect of removal of moisture from the support phase prior to coating with the substrate phase.

Investigations were made on a dual column system containing 0.325 w/w % EGA polyester liquid phase for the separation of seventeen of the amino acid derivatives and 1.5 w/w % OV-17 siloxane liquid phase for the elution of arginine, histidine and cystine. To improve the quantitation of histidine it was converted from the di-acyl to the mono-acyl derivative.

EXPERIMENTAL

Apparatus

A dual hydrogen flame detector gas chromatograph (Microtek Model 220), with strip chart recorders (Texas Instruments, Inc., Model FS1N6A), and a digital readout integrator (Infotronics, Model CRS-11AB/HS/42) were used. A Varian Aerograph Model 1520 dual column unit with two flame ionization detectors and matrix temperature programming was also utilized. Also, a Varian Aerograph Model 2100 instrument with a four-column oven bath, four flame ionization detectors, two dual differential electrometers, and linear temperature programming was used. Both Varian units were equipped with Leeds and Northrup Speedomax W recorders with Model 224 Disc Chart Integrators.

Solvents were removed from samples and reaction mixtures by means of a CaLab rotary evaporator, "Cold finger" condenser, and a Welch Duo-Seal vacuum pump. An electric furnace (Arthur H. Thomas, Co.), vacuum oven and vacuum

desiccator (Precision Scientific Co.) were used to remove moisture from the support material.

Reagents

All of the amino acids used in this study were obtained from Mann Research Laboratories, Inc. or Nutritional Biochemicals Corp., and were chromatographically pure.

Methanol and butanol were "Baker Analyzed" reagents. Chloroform was a "Fisher Certified Reagent". The trifluoroacetic anhydride was obtained from Distillation Products Industries, and was an "Eastman Grade" chemical. Acetonitrile was a "Baker Analyzed" reagent of "Nanograde" purity. Anhydrous HCl, 99.0% minimum purity, was obtained from Matheson Company. *p*-Toluenesulfonic acid was purchased from K and K Laboratories.

The methanol and methylene chloride were redistilled from an all glass system protected from atmospheric moisture. The methanol was first refluxed over magnesium turnings, and the methylene chloride over calcium chloride before distillation. The anhydrous HCl gas was passed through a H_2SO_4 drying tower before bubbling through the butanol or methanol.

Column packings

The following materials were purchased from Analabs, Inc.: neopentyl glycol succinate (NPGS), neopentyl glycol adipate (NPGA), neopentyl glycol sebacate (NPGSb), and ethylene glycol adipate (EGA). The OV-17 substrate was obtained from Applied Science Laboratories, Inc., and DC-550 was purchased from Dow Corning Corp. Neopentyl glycol and brassylic acid were obtained from K and K Laboratories, Inc.

Neopentyl glycol brassylate (NPGB) was prepared as described by JAMES[10], using *p*-toluenesulfonic acid as esterification catalyst. The product was a viscous liquid which solidified at room temperature.

The support material, 80/100 mesh acid-washed Chromosorb G, was obtained from Johns-Manville. 30.000 g of this material were weighed into each of five quartz evaporating dishes, and heated as described prior to coating with the liquid phase. Five different heat treatments were made: (a) 15 h at 100° in a vacuum oven, and (b, c, d and e) all for 15 h in a muffle furnace at 300°, 450°, 600° and 850°, respectively.

Then the dried support phases were allowed to cool in a vacuum desiccator over P_2O_5, and weighed to determine weight loss.

Columns were then prepared using each of the heated supports to observe any change in column performance as a function of the heat treatment. The liquid phases used were 0.5 w/w % NPGSb and 0.325 % w/w EGA.

Column preparation

A known amount of support was added to a 500 ml ridged round bottom flask and covered with the solvent used to dissolve the liquid phase. The stationary phase was weighed into a small Erlenmeyer flask, dissolved in the appropriate solvent, and transferred to the flask containing the support. The flask containing the support and stationary phase was then placed in a 60° water bath, and the solvent was slowly removed with a rotary evaporator and vacuum pump. Glass columns 1.5 m × 4 mm

I.D. were packed by slow addition of the coated support with gentle tapping of the column. Dry glass wool plugs ($^1/_4$ in.) were then placed in the ends of the column.

Four columns containing 0.5 w/w % NPGS, NPGA, NPGSb, and NPGB were placed in the gas chromatograph and conditioned simultaneously. The support material in each case was 80/100 mesh acid-washed Chromosorb G. These columns were conditioned for 15 h at 220° at a carrier gas flow rate of 10 ml/min N_2. Also a 0.325 w/w % EGA column was prepared and packed in a 1.5 × 4 mm I.D. glass column.

Instrumental settings

Column temperature	initial, 65°; final, 220°
Detector temperature	260°
Program rate	2°/min
N_2 carrier flow	64 ml/min
Hydrogen	35 ml/min
Air	400 ml/min
Chart speed	0.25 in./min

Preparation of derivatives

To evaluate the separation characteristics of the liquid phases NPGS, NPGA, NPGSb, and NPGB, a mixture of eight amino acids was converted to their N-TFA *n*-butyl derivatives as described by GEHRKE *et al.*[2]. This mixture included alanine, valine, glycine, isoleucine, leucine, threonine, proline, and serine. These amino acid derivatives are the eight of greatest volatility. The same procedure was used to prepare derivatives for the study of EGA as a liquid phase, and for the evaluation of the heated support materials.

RESULTS AND DISCUSSION

Substrate phase studies

Earlier experiments by GEHRKE AND SHAHROKHI[5] with the previously mentioned mixed liquid phases showed that they were unsuited for routine analysis as the separation and elution characteristics of the column varied with time and temperature. Thus, neopentyl glycol polyesters of increasing carbon chain length were evaluated to determine their ability to resolve the N-TFA *n*-butyl esters of the protein amino acids. The retention temperature of the eight amino acid derivatives on each neopentyl glycol polyester column was noted under similar chromatographic conditions. Table I gives the relative retention volumes for these amino acid derivatives. Alanine was assigned an arbitrary value of 1.00. Fig. 1 graphically shows the relative retention volume *versus* the carbon number of the dicarboxylic acid. From this graph it is clear that maximum separation was achieved at carbon number 10, or neopentyl glycol sebacate. From a structural standpoint there are ten atoms from the trifluoromethyl group to the terminal methyl group of the butyl ester of the amino acids. Also, in the case of leucine and isoleucine there are ten atoms including the terminal aliphatic methyl group to the terminal carbon atom in the *n*-butyl group. Maximum separation could result from the preferential orientation of the aliphatic portion of the amino acid with the CH_2 groups of the polyester and of the polar trifluoroacetyl group with the ester groups in the polyester.

TABLE I

SEPARATION OF AMINO ACIDS WITH POLYESTERS OF NEOPENTYL GLYCOL

Amino acid	*Relative retention volume*			
	Succinate	*Adipate*	*Sebacate*	*Brassylate*
Alanine (1.00)	1.00 (26.8)[a]	1.00 (27.7)	1.00 (25.6)	1.00 (21.1)
Valine	1.11 (29.8)	1.10 (30.4)	1.20 (30.8)	1.19 (25.2)
Isoleucine	1.29 (34.7)	1.29 (35.8)	1.46 (37.4)	1.25 (26.4)
Glycine	1.29 (34.7)	1.29 (35.8)	1.54 (39.5)	1.42 (29.9)
Leucine	1.48 (38.2)	1.40 (38.9)	1.66 (42.5)	1.56 (33.0)
Threonine	1.52 (40.7)	1.40 (38.9)	1.80 (46.5)	1.56 (33.0)
Proline	1.56 (42.0)	1.53 (42.5)	1.99 (51.0)	1.64 (34.3)
Serine	1.75 (46.8)	1.67 (46.2)	2.16 (55.3)	1.66 (35.0)

[a] Retention volume per mg liquid phase, ml/mg.

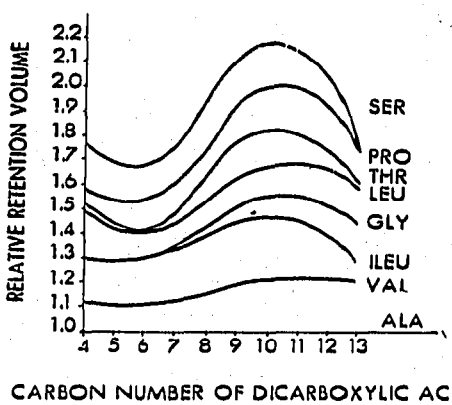

Fig. 1. Relative retention volumes of N-TFA *n*-butyl esters of amino acids with polyesters of neopentyl glycol.

The chromatographic resolution seen in Fig. 2 was achieved on further study with the NPGSb stationary phase. This liquid phase exhibited very good separation ability for the twenty protein amino acids under the appropriate instrumental conditions. However, continued investigation showed that this liquid phase was not satisfactory for the analysis of arginine, histidine and cystine as these derivatives

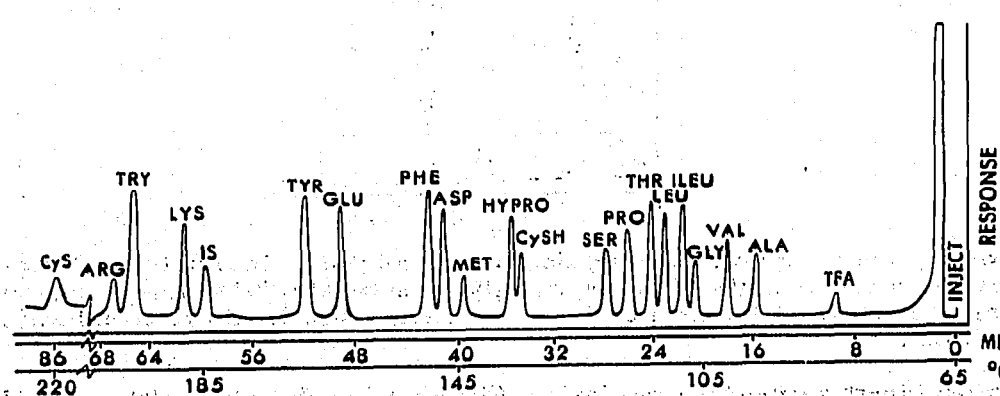

Fig. 2. Separation of amino acids with 0.5 w/w % neopentyl glycol sebacate (NPGSb) on 80/100 mesh acid-washed H.T. Chromosorb G. Column: 1.5 m × 4 mm I.D., glass. N_2 flow: 65 ml/min. Program rate: 2°/min. Internal standard: *n*-butyl stearate. Concentration: 12.5 n*M* each.

were not quantitatively eluted. In many instances only diminished peaks were observed for these amino acid derivatives. However, NPGSb was found to be a good stationary phase for the analysis of seventeen of the protein amino acids.

Support phase studies

Since the N-TFA *n*-butyl ester derivatives of the amino acids are especially susceptible to hydrolysis, it was considered that any water which could be removed from the support material should improve the efficiency of a particular column and result in less support–derivative interaction. The loss of weight on heating the 80/100 mesh acid-washed Chromosorb G to various temperatures was observed. The thermal treatments used and the corresponding weight losses are given in Table II.

TABLE II

THERMAL TREATMENTS AND WEIGHT LOSSES OF CHROMOSORB G

Experiment	*Heat treatment*	*Weight loss* (%)
A	15 h, 100°, vacuum oven	0.08
B	15 h, 300°, muffle furnace	0.52
C	15 h, 450°, muffle furnace	0.54
D	15 h, 600°, muffle furnace	0.63
E	15 h, 850°, muffle furnace	0.64

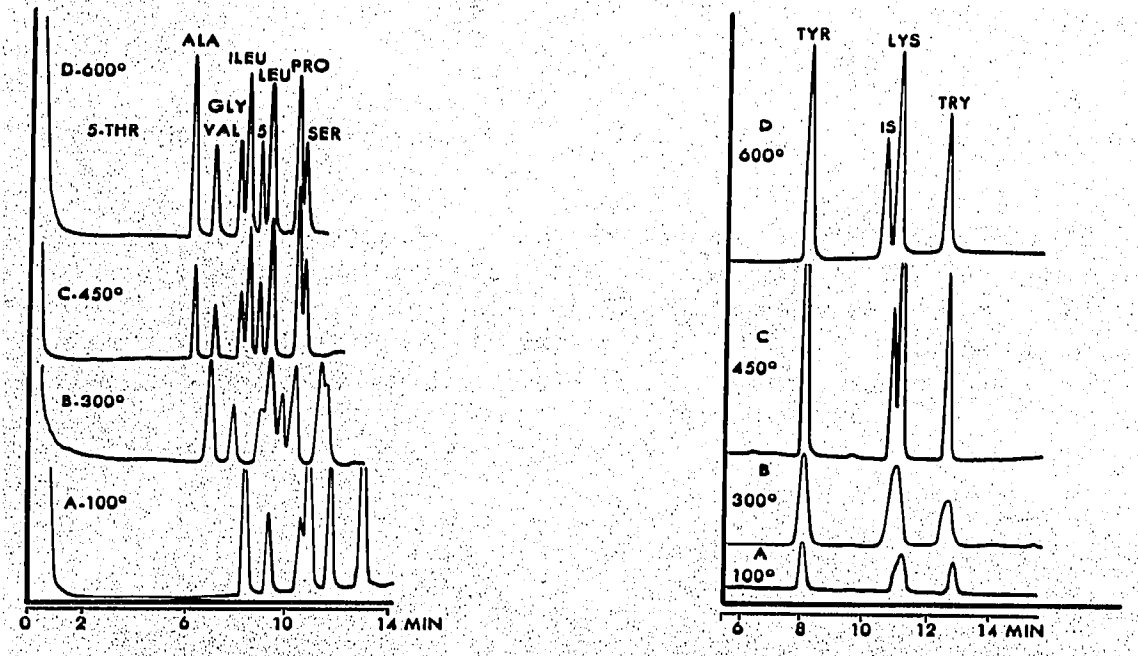

Fig. 3. Effect of heating of the inert support on retention and resolution. Chromatography of N-TFA *n*-butyl esters of amino acids. 0.5 w/w % neopentyl glycol sebacate (NPGSb) on 80/100 mesh acid-washed H.T. Chromosorb G. Column: 1.0 m × 4 mm I.D., glass. N_2 flow: 70 ml/min. Temperature: 80° initial, 5°/min.

Fig. 4. Effect of heating of the inert support on resolution. Chromatography of N-TFA *n*-butyl esters of amino acids. 0.5 w/w % neopentyl glycol sebacate (NPGSb) on 80/100 mesh acid-washed H.T. Chromosorb G. Column: 1.0 m × 4 mm I.D., glass. N_2 flow: 70 ml/min. Temperature: 150° initial, 5°/min.

The performance of these heat-treated (H.T.) support materials was observed after coating each with 0.5 w/w % of NPGSb. These columns were conditioned simultaneously under the same conditions and all of the instrumental settings were the same for each analysis. Fig. 3 shows a comparison of chromatograms obtained on the analysis of a mixture of the eight most volatile amino acid derivatives on support phases heated to different temperatures. An increase in separation was observed for the columns whose support had been heated in the range of 450–600°. Fig. 4 shows a similar comparison of heated supports for the derivatives of tyrosine, lysine, and tryptophan, with *n*-butyl stearate as the internal standard. Increased separation was again achieved with supports heated at 450–600°. A comparison of these heated support materials was then made using a mixture of seventeen amino acid derivatives (Fig. 5). In this experiment EGA was used as the liquid phase. The peak for tyrosine

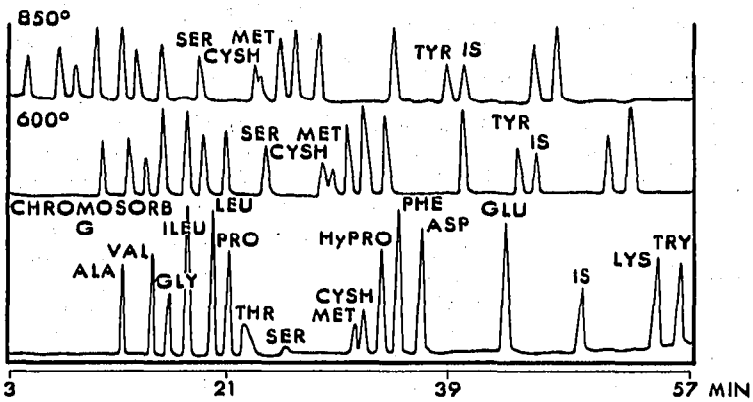

Fig. 5. Effect of heating of the inert support on retention and resolution. Chromatography of N-TFA *n*-butyl esters of amino acids. 0.325 w/w % ethylene glycol adipate (EGA) on 80/100 mesh acid-washed H.T. Chromsorb G. Column: 1.5 m × 4 mm I.D., glass. N_2 flow: 65 ml/min. Temperature: 65° initial, 2°/min. Final temperature 225°.

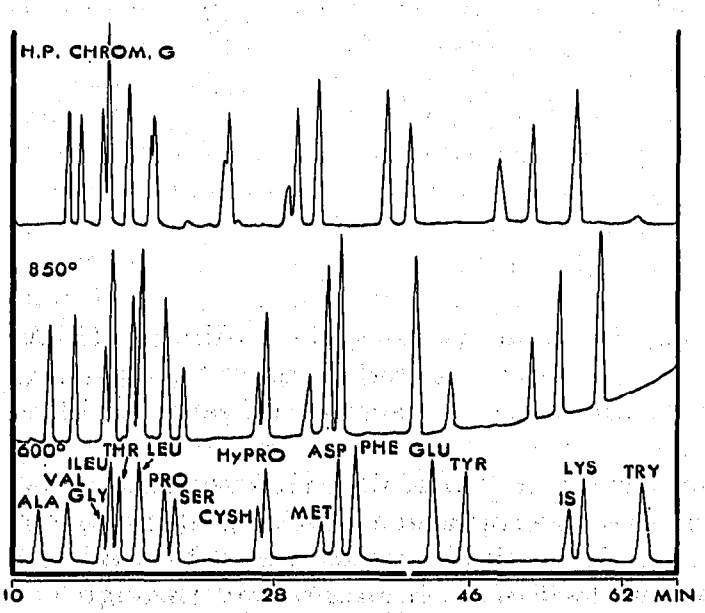

Fig. 6. Effect of heating of the inert support on retention and resolution. Chromatography of N-TFA *n*-butyl esters of amino acids. 0.5 w/w % neopentyl glycol sebacate (NPGSb) on 80/100 mesh acid-washed H.T. Chromosorb G. Column: 1.5 m × 4 mm I.D., glass. N_2 flow: 65 ml/min. Temperature: 65° initial, 2°/min. Final temperature 240°.

was absent, and small for serine on regular Chromosorb G. For the non-heated support, tyrosine was partially converted to the free hydroxy derivative, and eluted with lysine. Fig. 6 shows a similar study with NPGSb as the liquid phase.

Some studies were also made with high performance Chromosorb G, a product silanized by the manufacturer. The retention temperature for each of the amino acid derivatives was noted for each column and is given in Tables III and IV. In general, a decrease in the retention temperature of the amino acids was noted for columns containing the heat-treated support.

From these studies, EGA was found to be the best liquid phase, and Chromosorb G heated at 600° for 15 h the best support phase, for the analysis of seventeen of the protein amino acids.

TABLE III

RETENTION TEMPERATURES OF N-TFA *n*-BUTYL AMINO ACID ESTERS ON CHROMOSORB G

Amino acid	*EGA 0.325 w/w % and Chromosorb G*			
	Acid-washed Chromosorb G	*Acid-washed Chromosorb G 500°*	*Acid-washed Chromosorb G 850°*	*H.P. Chromosorb G DMCS*[a]
Alanine	96	92	87	75
Valine	102	97	92	77
Glycine	105	101	94	81
Isoleucine	107	104	97	83
Leucine	112	108	102	84
Proline	115	111	104	93
Threonine	118	115	107	105
Serine	125	121	114	109
Cysteine	137	131	124	119
Methionine	139	133	125	121
Hydroxyproline	142	136	127	125
Phenylalanine	144	140	130	139
Aspartic acid	149	143	134	145
Glutamic acid	163	157	146	174
Tyrosine	175	165	155	176
Lysine	186	179	165	200
Tryptophan	190	183	168	219

[a] DMCS = dimethylchlorosilane.

The dual column chromatographic system

A chromatographic column of 0.325 w/w % ethylene glycol adipate (EGA) coated on 80/100 mesh, a.w., Chromosorb G previously heated to 600° for 15 h was selected for the separation of all the protein amino acids except arginine, histidine and cystine.

A 1.5 m × 4 mm I.D. U-shaped glass column containing this substrate and support phase was placed into the Model 2100 Aerograph gas chromatograph. A standard reference solution of the amino acid derivatives was prepared by taking a 10 ml aliquot of a solution containing 2.5 mmoles/l of each amino acid through the derivatization method. The closed tube acylation method was used. Five microliters of the derivatized solution were injected into the gas chromatograph.

For the determination of arginine, histidine, and cystine, columns of DC-550

TABLE IV

RETENTION TEMPERATURES OF N-TFA-*n*-BUTYL AMINO ACID ESTERS ON CHROMOSORB G

Amino acid	*NPGSb 0.5% w/w and Chromosorb G*			
	Acid-washed Chromosorb G	*Acid-washed Chromosorb G 500°*	*Acid-washed Chromosorb G 850°*	*H.P. Chromosorb G DMCS*
Alanine	107	97	99	99
Valine	109	103	104	102
Glycine	114	110	110	106
Isoleucine	115	112	112	107
Leucine	116	114	116	107
Threonine	118	118	119	112
Proline	123	122	123	116
Serine	123	125	126	117
Cysteine	137	140	138	130
Hydroxyproline	139	142	139	131
Methionine	148	151	147	142
Aspartic acid	151	154	150	143
Phenylalanine	153	158	153	147
Glutamic acid	167	170	167	158
Tyrosine	173	175	174	163
Butyl stearate	188	190	185	178
Lysine	192	193	190	183
Tryptophan	200	206	198	192
Arginine	—	209	—	—
Cystine	—	220	234	204

and OV-17 coated on 80–100 mesh, high performance (H.P.) Chromosorb G were prepared. These columns were placed in the Model 2100 Aerograph gas chromatograph and evaluated. This experiment resulted in the discovery of two peaks for histidine, *viz.* the mono-acyl and di-acyl derivatives. Then, mixtures of glutamic acid, arginine, histidine, and cystine were derivatized and chromatographed on columns of DC-550 and OV-17. Using extreme care to maintain the anhydrous condition of the sample it was possible to obtain only the di-acyl peak for histidine. This was the moderately volatile component with a retention temperature slightly lower than glutamic acid (top chromatogram, Fig. 7). The di-acyl derivative could be obtained from samples acylated in a closed tube or at room temperature. However, injection of samples that had been opened to the atmosphere a few times resulted in the two peaks for histidine (middle chromatogram, Fig. 7).

Chromatography of the samples on polyester substrate columns under more or less anhydrous conditions resulted in only the less volatile mono-acyl component and at a reduced level of response. It was assumed that the component of higher volatility was the N,N'-bis(trifluoroacetyl) derivative, and that an interaction with the polyester column material resulted in the conversion of the di-acyl histidine derivative to the mono-acyl product.

In addition to the extreme sensitivity of the histidine di-acyl derivative to hydrolysis, it was found that the separation of the di-acyl derivative from aspartic acid was impossible on the siloxane polymer substrate columns. Thus, for the analysis of histidine experiments were made to quantitatively convert the di-acyl derivative

of histidine to its mono-acyl derivative. This was accomplished by injecting 1-butanol into the gas chromatograph immediately after injection of the sample containing histidine (bottom chromatogram, Fig. 7).

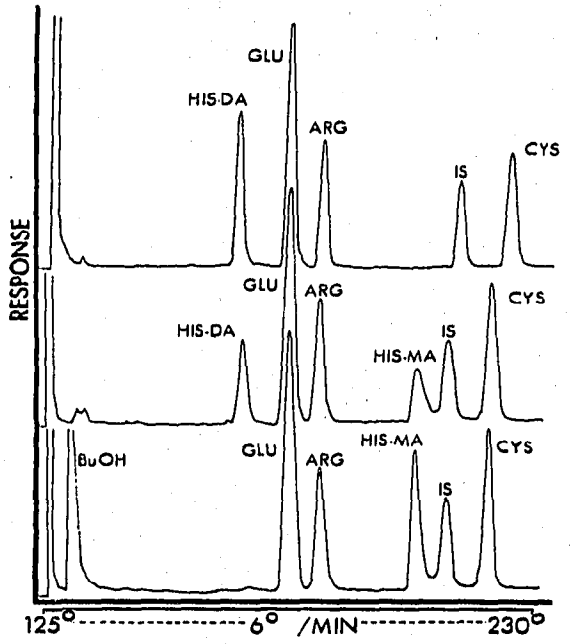

Fig. 7. Chromatograms of a solution containing histidine. Top: Anhydrous condition of sample maintained. Middle: Sample allowed contact with atm. H_2O. Bottom: BuOH injected on column after sample injection. Column: 1 m × 4 mm I.D., 1.5 w/w % OV-17 on 80/100 mesh H.P. Chromosorb G. MA = Mono-acyl, DA = di-acyl.

RESULTS AND DISCUSSION (DUAL COLUMN SYSTEM)

Excellent separation of seventeen protein amino acids was obtained with the 0.325 w/w % EGA column. The instrumental operating and chromatographic conditions were as follows:

Column temperature	initial, 80°; final, 215°
Detector temperature	250°
Program rate	4°/min
N_2 carrier flow	60 ml/min
Hydrogen	50 ml/min
Air	450 ml/min
Chart speed	0.25 in./min

The evaluation of DC-550 and OV-17 as possible substrates for the analysis of arginine, histidine and cystine showed that either of these liquid phases coated on H.P. Chromosorb G would provide good separation. However, considerable substrate bleed was encountered with the DC-550 liquid phase at the elevated temperatures necessary to elute these amino acids of low volatility. Therefore, the OV-17 substrate on 80/100 mesh H.P. Chromosorb G was selected as the second column for the dual column system. A loading level of 1.5 w/w % of the OV-17 produced the most reproducible response factors for these amino acids while maintaining excellent separation.

At a higher loading level of 2.5 w/w %, cystine was not completely eluted from the column.

With a 1 m column of 1.5 w/w % OV-17 on 80/100 mesh H.P. Chromosorb G in the Aerograph Model 1520 chromatograph it was found that the injection of 1 μl of 1-butanol immediately following the injection of the sample would convert all of the histidine to its mono-acyl derivative. One microliter of butanol was selected since it gave reproducible response factors for arginine and cystine as well as histidine when mixtures of the amino acids were analyzed. In order to achieve this conversion of histidine it was necessary to inject 7 μl 1-butanol onto the column when an Aerograph 2100 instrument was employed. The relative molar responses of arginine, histidine and cystine with respect to glutamic acid are presented in Table V. The instrumental conditions were as follows:

Column temperature	initial, 125°; final, 235°
Detector temperature	250°
Program rate	6°/min
Injection port temperature	200°
N_2 carrier flow	70 ml/min
Hydrogen	50 ml/min
Air	450 ml/min
Chart speed	0.5 in./min

TABLE V

RELATIVE MOLAR RESPONSE OF ARGININE, HISTIDINE AND CYSTINE ON DIFFERENT COLUMNS AND INSTRUMENTS

Amino acid	*Column and instrument*	$RMR_{a.a./glu.}$			*Average*
		1[a]	*2*	*3*	
Arginine	Old column[b], Model 1520[c]	0.61	0.62	0.64	0.62
	New column[d], Model 1520	0.70	0.69	0.68	0.69
	New column, Model 2100[e]	0.76	0.74	0.74	0.75
Histidine	Old column, Model 1520	0.37	0.37	0.35	0.36
	New column, Model 1520	0.47	0.47	0.47	0.47
	New column, Model 2100	0.49	0.46	0.47	0.47
Cystine	Old column, Model 1520	0.94	0.95	0.88	0.92
	New column, Model 1520	0.94	0.93	0.93	0.93
	New column, Model 2100	0.62	0.63	0.60	0.62

[a] 1, 2, and 3 are independent samples.
[b] Extensively used column.
[c] Model 1520 Aerograph Gas Chromatograph. Samples injected in this instrument were followed by a 1 μl butanol injection.
[d] Well conditioned new column.
[e] Model 2100 Aerograph Gas Chromatograph. Samples injected in this instrument were followed by a 7 μl butanol injection.

A comparison of the relative molar responses obtained from different columns of the same material showed that it was essential that the response factors of the amino acid derivatives be determined periodically for that column (Table V). Columns of different ages and prior use will give different response factors. However, a column

can be used for at least 50 to 100 analyses. The relative molar response (RMR) of glutamic acid was arbitrarily assigned a value of unity. The relative molar response of each amino acid relative to glutamic acid, $RMR_{a.a./glu.}$, was calculated as follows:

$$RMR_{a.a./glu.} = \frac{A_{a.a.}}{\text{moles}_{a.a}} \Big/ \frac{A_{glu.}}{\text{moles}_{glu.}}$$

where $A_{a.a.}$ = area in counts of amino acid peak.

A chromatogram of the complete separation of the twenty common protein amino acids with a dual column system is shown in Fig. 8. A complete gas chromatographic analysis of a protein hydrolysate can be made as follows:

Place a 1.5 m column of 0.325 w/w % EGA on 80/100 mesh acid-washed H.T. Chromosorb G in an instrument designed to accommodate at least two columns. Also place in the instrument a 1.0 m column of 1.5 w/w % of OV-17 on 80/100 mesh acid-washed H.P. Chromosorb G. By analyzing a derivatized reference standard solution on both columns, the relative molar response of each of the amino acids to

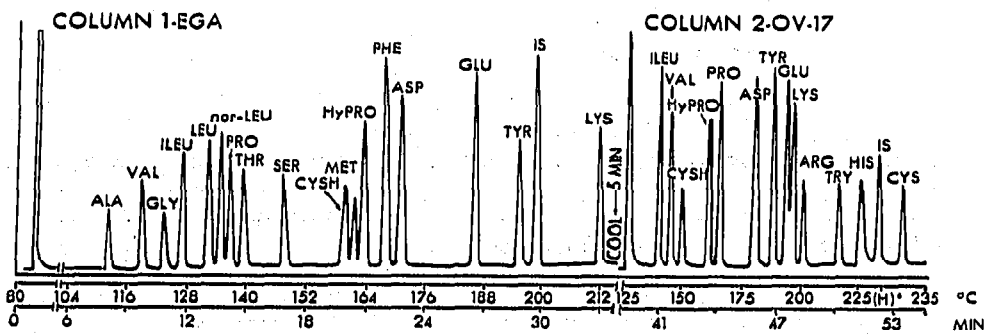

Fig. 8. Separation of all twenty protein amino acids with a dual column system. Column 1: 0.325 w/w % ethylene glycol adipate on 80/100 mesh acid-washed H.T. Chromosorb G, 1.5 m × 4 mm I.D., glass. Initial temperature: 80°, 4°/min. N_2 flow: 60 ml/min. Column 2: 1.5 w/w % OV-17 on 80/100 mesh H.P. Chromosorb G, 1.0 m × 4 mm I.D., glass. Initial temperature: 125°, 6°/min. N_2 flow: 70 ml/min. The injected mixture contained *ca.* 0.02 μmole of each amino acid (~ 2 μg). *Hold at 235°.

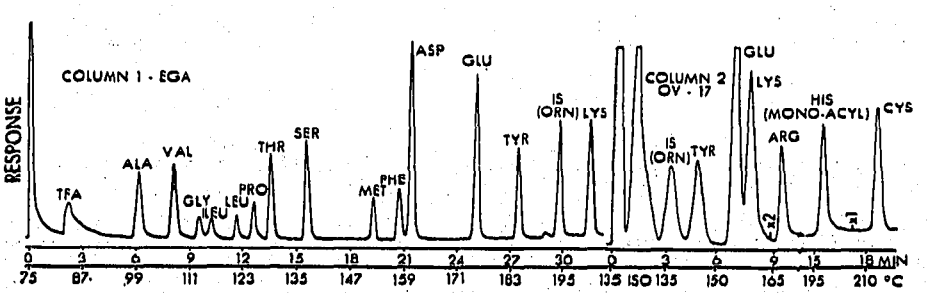

Fig. 9. Gas–liquid chromatogram of ribonuclease N-TFA *n*-butyl esters. Sample: 15 mg in 5.0 ml; 15 μg total amino acids injected (5 μl). Varian Aerograph 2100. EGA column: 0.325 w/w % on 80/100 mesh acid-washed H.T. Chromosorb G, 1.5 m × 4 mm I.D., glass. Initial temperature: 75°, 4°/min. OV-17 column: 1.5 w/w % on 80/100 mesh H.P. Chromosorb G, 1.0 m × 4 mm I.D., glass. Initial temperature: 135°, 5°/min in Microtek MT 220. Three microliters (9 μg total) of sample injected followed by 5 μl *n*-butanol.

the internal standard can be calculated for the particular instrument and column employed. With these RMR factors determined, the protein sample hydrolysates after derivatization can be analyzed on both columns and the w/w % of each amino acid calculated.

The applicability of this method is demonstrated by the GLC analysis of ribonuclease. The complete chromatogram is shown in Fig. 9. Table VI presents the data obtained from both GLC and ion-exchange analyses of ribonuclease, and excellent agreement of the two was obtained.

TABLE VI

AMINO ACID ANALYSIS OF RIBONUCLEASE[a]

Amino acid	*w/w %*			
	Gas–liquid chromatography		*Average*	*Ion exchange chromatography*[b]
Alanine	7.27	7.47	7.37	7.60
Valine	6.81	7.08	6.95	7.00
Glycine	1.75	1.62	1.69	1.83
Isoleucine	1.67	1.61	1.64	2.06
Leucine	2.14	1.92	2.02	2.22
Proline	3.15	2.90	2.98	3.11
Threonine	7.78	7.94	7.86	7.80
Serine	9.66	9.81	9.74	9.41
Methionine	2.55	2.71	2.63	3.38
Phenylalanine	3.26	3.24	3.25	3.62
Aspartic acid	13.78	13.68	13.73	14.07
Glutamic acid	11.93	12.19	12.06	12.08
Tyrosine	6.43	6.29	6.36	6.80
Lysine	10.25	10.17	10.21	10.58
Arginine	5.04	4.83	4.93	4.65
Histidine	3.21	3.58	3.40	3.46
Cystine[c]	6.10	6.36	6.23	6.20
			103.05	105.87

[a] All determinations made on the same hydrolysate which was prepared by hydrolyzing the protein for 18 h at 105° in a closed tube with constant-boiling HCl. Ornithine used as the internal standard.

[b] Average of two independent samples, norleucine as internal standard.

[c] Analyzed as half-cystine by the ion-exchange method.

CONCLUSIONS

These studies have shown that by varying the length of the carbon chain in a polyester substrate phase, the separation characteristics of a chromatographic column can be markedly influenced. The optimum chromatographic performance for neopentyl glycol ployesters was observed at a carbon chain length of ten (neopentyl glycol sebacate).

Although NPGSb exhibited good separation ability of the N-TFA *n*-butyl esters of the protein amino acids, this polyester was not suited for the analysis of arginine, histidine and cystine, as these derivatives were not quantitatively eluted from the column. The separation ability of ethylene glycol adipate as a liquid phase

was found to be superior to NPGSb, consistent, and reproducible with respect to time and temperature. Again, arginine, histidine and cystine were not reproducibly eluted from this column.

The effects of heating the support material prior to coating with the stationary phase were pronounced. In general, the retention temperatures were lower and a significant improvement in resolution was noted when columns were prepared with Chromosorb G which had been heated at 450° to 600° for 15 h. Also, losses in weight (*ca.* 0.5 %) were observed on heating the support material, and the resultant loss of water improved the reproducible elution and separation of the amino acid derivatives when using polyester stationary phases. With unheated Chromosorb G, however, the peak for tyrosine was gone and only a small peak was observed for serine. This was corrected with the heat-treated Chromosorb G.

In all of our experiments it was not possible to elute quantitatively arginine, histidine and cystine from a polyester liquid phase. This is a result of interaction between the substrate phase and the amino acid derivative. However, seventeen of the protein amino acid derivatives were well separated and quantitatively eluted in 33 min from columns containing 0.325 w/w % EGA coated on 80/100 mesh acid-washed heat-treated Chromosorb G.

For the analysis of arginine, histidine and cystine, columns containing 1.5 w/w % OV-17 coated on high performance 80/100 mesh Chromosorb G gave excellent results. Di-acyl histidine was converted to the mono-acyl derivative by injection of *n*-butanol immediately after injection of the sample. This technique enabled mono-acyl histidine to be eluted at a position on the chromatogram which did not coincide with any other amino acid derivative. The amount of butanol necessary for the quantitative conversion of di-acyl histidine to the mono-acyl derivative must be determined for each instrument.

A dual column chromatographic system of EGA and OV-17 as the stationary phases was prepared from which all twenty of the protein amino acids were quantitatively eluted and separated in 55 min. The quantitative GLC analysis of the amino acids in ribonuclease is reported. This chromatographic system was very stable, and recent studies with these columns have shown that excellent results can be obtained for samples containing only 1 μg of each amino acid with 50 ng of each being injected. Further studies have shown that this system also permits a semiquantitative analysis of samples containing 100 ng of each amino acid, with 5 to 10 ng being injected.

It is concluded that amino acids can be quantitatively determined in proteins, peptides, and biological substances by gas–liquid chromatography. The subject of a paper in preparation covers the applications and details of macro, semimicro and micro methods.

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REFERENCES

1 C. W. GEHRKE AND D. L. STALLING, *Separation Sci.*, 2 (1967) 101.
2 W. M. LAMKIN AND C. W. GEHRKE, *Anal. Chem.*, 37 (1965) 383.
3 C. W. GEHRKE, W. M. LAMKIN, D. L. STALLING AND F. SHAHROKHI, *Biochem. Biophys. Res. Commun.*, 13 (1965) 328.
4 D. L. STALLING AND C. W. GEHRKE, *Biochem. Biophys. Res. Commun.*, 22 (1966) 329.
5 C. W. GEHRKE AND F. SHAHROKHI, *Anal. Biochem.*, 15 (1966) 97.
6 M. STEFANOVIC AND B. L. WALKER, *Anal. Chem.*, 39 (1967) 710.
7 W. J. MCBRIDE, JR. AND J. D. KLINGMAN, *Anal. Biochem.*, submitted for publication.
8 A. DARBRE AND K. BLAU, *J. Chromatog.*, 29 (1967) 49.
9 A. DARBRE AND A. ISLAM, *Biochem. J.*, 106 (1968) 923.
10 A. T. JAMES, *J. Chromatog.*, 2 (1959) 552.