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QUANTITATIVE GAS–LIQUID CHROMATOGRAPHY OF HISTIDINE*

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SUMMARY

The quantitative gas–liquid chromatographic analysis of histidine was easily accomplished using two different methods and the mono and diacyl N-TFA *n*-butyl esters of histidine as the derivatives. To obtain quantitation, histidine must be present entirely as either the monoacyl or as the diacyl derivative. There are certain advantages in using the diacyl derivative, but this derivative was not separated from the N-TFA *n*-butyl ester of aspartic acid on any of the siloxane columns evaluated (OV-1 to OV-25).

A quantitative, reproducible method has been developed and is presented by which histidine can be analyzed as its diacyl N-TFA *n*-butyl ester. In this method the diacyl derivative formed during acylation was converted to the monoacyl derivative by evaporation of the excess acetylating reagent, TFAA. Thus, histidine is present as the monoacyl derivative when the sample is injected 'on column'. Then, the monoacyl derivative was converted to the diacyl derivative by direct 'on column' injection of 4 μl of TFAA. When the TFAA was injected immediately after the methionine peak, the diacyl derivative synthesized on the column was precisely eluted between tyrosine and glutamic acid. No interference was observed with any of the other amino acid derivatives. The separation of the diacyl derivative of histidine from the aspartic acid derivative was made possible because the retention temperature of the monoacyl derivative is different from that of the diacyl histidine derivative or the derivative for aspartic acid.

The quantitative elution of all twenty of the protein amino acids as their N-TFA *n*-butyl esters has been achieved on a dual column system of 0.325 w/w % of stabilized EGA on heat-treated Chromosorb G and 1.5 w/w % OV-17 on high performance Chromosorb G as the stationary phases. *Alternatively*, for sixteen amino acids, a 1.5 m glass column with packing of 0.65 w/w % of stabilized EGA on 80/100 mesh acid-washed Chromosorb W dried at 140° for 12 h can be used.

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In another approach, sixteen of the protein amino acids can be determined as their N-TFA *n*-butyl esters on a 1.5 m × 4 mm I.D. glass column consisting of 0.325 w/w % of stabilized EGA on heat-treated Chromosorb G (or 0.65 w/w % of stabilized EGA on 80/100 mesh acid-washed Chromosorb W dried at 140° for 12 h) while arginine, histidine, tryptophan, and cystine are chromatographed as their trimethylsilyl (TMS) derivatives on a 1.75 m × 4 mm I.D. glass column containing a *mixed* phase of 3.0 w/w % OV-7 and 1.5 w/w % OV-22.

The TMS derivative offers a unique advantage in that the derivatization reaction is completed at 135° for 4 h in a closed tube with no transfers or removal of reagents. Silylation of amino acids to their TMS derivatives holds considerable promise not only for the quantitative analysis of arginine, histidine, tryptophan, and cystine, but for all of the protein amino acids. The analysis of these amino acids in known mixtures and ribonuclease as the TMS derivatives is reported. An average recovery of 102.5% was obtained. The reaction conditions and chromatography of the TMS derivatives of the twenty protein amino acids and some non protein amino acids are the subject of a separate manuscript.

A simplified determination of histidine can be made by a calculation of the $AREA_{HIS}$ on the OV-22 column from the $AREA_{HIS+ASP}$ on the same column.

INTRODUCTION

Due to their speed and sensitivity, gas–liquid chromatographic (GLC) methods have proved to be most useful for the analysis of amino acids in biological materials. However, the low volatility of the amino acids has prevented their direct analysis by GLC; therefore, volatile derivatives of the amino acids must be prepared. LAMKIN AND GEHRKE[1] reported in 1965 that the most suitable derivative for the gas–liquid chromatographic analysis of the natural protein amino acids is the N-trifluoroacetyl (N-TFA) *n*-butyl ester. A general review by GEHRKE AND STALLING[2] in 1967 discussed the preparation of the N-TFA *n*-butyl esters of the amino acids and their analysis by GLC.

In 1966, GEHRKE AND SHAHROKHI[3] reported complete resolution of a mixture of the N-TFA *n*-butyl esters of the protein amino acids using a mixed stationary phase column of 0.75/0.25 w/w % of diethylene glycol succinate/ethylene glycol succinate–siloxane copolymer. However, reproducible elution of arginine, histidine, and cystine was not achieved using this column. STEFANOVIC AND WALKER[4] reported separation of all twenty N-TFA *n*-butyl ester amino acid derivatives with the liquid phase ethylene glycol adipate (EGA). The fact that these workers observed small peaks for arginine, histidine, and cystine using 0.65 w/w % EGA on Chromosorb W suggested that these derivatives were either decomposed on or interacted with the polar EGA column.

GEHRKE *et al.*[5] reported on an EGA and OV-17 dual column system for the separation of all twenty N-TFA *n*-butyl ester derivatives of the protein amino acids. A 1.5 m column containing 0.325 w/w % EGA polyester as the liquid phase was used for separation of sixteen of the amino acid derivatives while a 1 m column containing 1.5 w/w % OV-17 siloxane as the liquid phase was used for the quantitation of arginine, tryptophan, histidine, and cystine. Diacyl histidine was eluted

with aspartic acid when OV-17 was used as the liquid phase, thus diacyl histidine was converted to monoacyl histidine by direct 'on column' injection of *n*-butanol immediately after injection of the sample. The monoacyl histidine derivative was eluted at a position which did not coincide with any other amino acid derivative. To this time no other reports have been made on the quantitation and GLC analysis of histidine, arginine, tryptophan, and cystine.

However, our reported method of converting the diacyl histidine N-TFA *n*-butyl ester to the monoacyl derivative by injection of *n*-butanol immediately after injection of the sample was satisfactory but dependent on a number of chromatographic and instrumental parameters as follows: A known correct amount of *n*-butanol must be injected to obtain good results, and the amount of *n*-butanol injected is dependent on the particular instrument used, the quantity of sample injected, injector temperature, carrier flow rate, and perhaps other parameters. Further, the quantitation of histidine by our previous method was also severely dependent on the condition of the chromatographic column. The difficulties observed with this technique have generally limited its use to research samples.

This paper reports on three different GLC methods for the separation and quantitative GLC analysis of histidine. In two of the methods, the N-TFA *n*-butyl ester derivatives are used, and in the third method, histidine is converted to the trimethylsilyl (TMS) derivative using bis(trimethylsilyl)trifluoroacetamide (BSTFA) as reported by GEHRKE *et al.*[6].

In the *first method*, histidine was analyzed as the monoacyl derivative. The diacyl N-TFA *n*-butyl ester of histidine was converted to the monoacyl derivative by removal of the excess acylating reagent, trifluoroacetic anhydride (TFAA). In the *second method*, the excess TFAA was removed to convert the diacyl derivative to the monoacyl form. Then the sample from the reaction mixture was injected and the monoacyl derivative was reconverted on the chromatographic column to the diacyl derivative by injection of 4 μl of TFAA. Histidine was then analyzed as the diacyl derivative which was now eluted from the OV-22 column between tyrosine and glutamic acid since histidine entered the column as the monoacyl derivative, and thus is eluted at a later position on the chromatogram. In both of these methods, 0.325 w/w % EGA was used for analysis of sixteen of the amino acids while a 1.5 w/w % OV-22 column was used for the separation of arginine, histidine, tryptophan, and cystine.

In the *third method*[7], histidine was converted to its trimethylsilyl (TMS) derivative (HIS_3), and chromatographed on a column containing a *mixed* liquid phase of 3.0 w/w % OV-7 and 1.5 w/w % of OV-22. Arginine, tryptophan, and cystine were also determined as their TMS derivatives (ARG_4, TRY_3, CYS_4) under the same derivatization and chromatographic conditions. The subscript denotes the number of TMS groups on the amino acid. The other sixteen amino acids are determined as their N-TFA *n*-butyl esters on a 0.325 w/w % EGA column as reported earlier[5].

EXPERIMENTAL

Apparatus

A Microtek Model 220 dual hydrogen flame detector gas chromatograph equipped with a Varian Model 30 recorder, and a Varian Aerograph Model 2100 gas

chromatograph with a four-column oven bath, four flame ionization detectors, two dual differential electrometers, and equipped with a Varian Model 20 recorder were used. A Packard Instruments Co. Model 7300 dual column gas chromatograph with hydrogen flame detectors and equipped with a Honeywell Electronik 16 strip chart recorder was also used. A digital readout integrator (Infotronics, Model CRS 104) was used for determining peak areas.

Solvents were removed from the samples with a CaLab rotary evaporator, 'cold finger' condenser, and a Welch Duo-Seal vacuum pump, or the samples were taken just to dryness with a stream of dry nitrogen gas.

Filters containing activated charcoal and $CaSO_4$ were used for purification of the N_2 gas.

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

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. The trifluoroacetic anhydride was obtained from Distillation Products Industries, and was an 'Eastman Grade' chemical. Acetonitrile, a 'Baker Analyzed' reagent of 'Nanograde' purity, was stored over drierite in a bottle with a ground glass stopper. Anhydrous HCl, 99.0% minimum purity, was obtained from the Matheson Company.

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

Bis(trimethylsilyl)trifluoroacetamide, the trimethylsilylating reagent, was obtained from Regis Chemical Co., Chicago, Ill.

Columns

Stabilized grade ethylene glycol adipate was obtained from Analabs, Inc., and coated on 80/100 mesh Chromosorb G which had been heat-treated as described by GEHRKE *et al.*[5]. The EGA column material was packed into 1.5 m × 4 mm I.D. glass columns.

The OV-7 and OV-22 siloxane substrates were purchased from Supelco, Inc. The support material for the OV-22 columns was 80/100 mesh high performance (H.P.) Chromosorb G, and the support material for the *mixed* phase columns was 100/120 mesh H.P. Chromosorb G. The glass columns were 1.0 m × 4 mm I.D. for the OV-22, and 1.75 m × 4 mm I.D. for the *mixed* phase columns.

The column packing was prepared by first adding a known amount of support material to a 500 ml ribbed round bottom flask then adding the solvent used to dissolve the stationary phase until the liquid level was about 1/4 in. above the support material. The stationary phase(s) was weighed into a small erlenmeyer flask, dissolved in the appropriate solvent, and then transferred to the flask containing the

support. The flask containing the support and stationary phase was placed in a 60° water bath, and the solvent was removed with the rotary evaporator under partial vacuum.

Derivatization

N-TFA n-butyl esters. The derivatization technique of GEHRKE *et al.*[2] was used for the conversion of the amino acids to their N-TFA *n*-butyl esters. A complete summary of this technique is included in a recent monograph[8]. Two different mixtures of amino acids were converted to their N-TFA *n*-butyl esters in order to study the quantitation of histidine. One of these mixtures contained only histidine, ornithine, glutamic acid, arginine, tryptophan, and cystine; while the other mixture contained all of the protein amino acids with the exception of cysteine.

Trimethylsilyl derivatives. To establish the quantitation of silylation to the TMS derivatives and chromatographic procedures, a solution containing known amounts of glutamic acid, arginine, histidine, tryptophan, and cystine in 0.1 *N* HCl was prepared. Aliquots of this solution containing *ca.* 4 mg of total amino acids were placed in 16 × 75 mm Pyrex glass silylation tubes, and taken just to dryness by placing the tubes in a dry bath heated at *ca.* 100° ± 4°, and flushing with a stream of dry nitrogen. To ensure complete removal of moisture, *ca.* 2 ml of methylene chloride was then added, and the sample again taken just to dryness in the above manner.

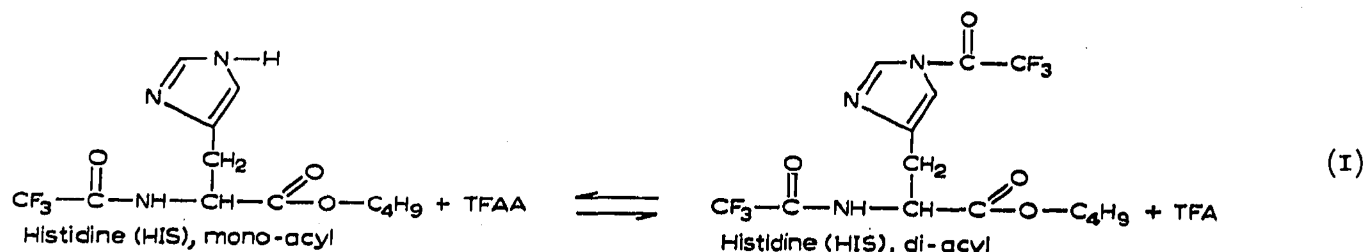
Conversion of the amino acids to their TMS derivatives was accomplished by adding acetonitrile as the silylation solvent, and an equal amount of BSTFA. This corresponds to a 30 molar excess of BSTFA/amino acids. It was convenient to add phenanthrene, the internal standard, from a stock solution containing a known amount of phenanthrene dissolved in acetonitrile.

The tube was then placed in an oil bath and heated at 135° for 4 h, then removed and cooled under the tap. The sample was injected into the previously mentioned mixed phase column, and the peak areas for each of the amino acids were measured electronically. The % recovery of the amino acids was calculated using relative molar response values, $RMR_{a.a./phen.}$, which were obtained from at least three independent analyses of standard stock solutions of the amino acids.

RESULTS AND DISCUSSION

Method I: direct analysis of monoacyl histidine

The excess TFAA in the sample was removed to convert the diacyl N-TFA *n*-butyl ester of histidine to the monoacyl derivative prior to injection on the column. In the presence of a large, greater than 50 molar, excess of TFAA, histidine was converted quantitatively to the diacyl derivative. However, when this large excess of TFAA was removed, the diacyl derivative was essentially quantitatively converted to the monoacyl form by a shift in the equilibrium represented by reaction 1. The TFAA can be effectively removed by evaporating the sample to dryness using dry nitrogen, redissolving the sample in 2 ml of methylene chloride, drying again, and redissolving in methylene chloride. The sample prepared in this manner is ready for injection.



Thus, histidine was chromatographed as the monoacyl derivative formed in this manner. Repeated analysis of an equimolar solution of the protein amino acids using this technique resulted in a range for the relative molar response (RMR) values of 0.36 to 0.45 for the monoacyl derivative of histidine. The RMR of glutamic acid was arbitrarily assigned a value of unity. The relative molar response of any amino acid relative to glutamic acid, $RMR_{a.a./glu.}$ can be calculated as follows:

$$RMR_{a.a./glu.} = \frac{A_{a.a.}}{moles_{a.a.}} \Big/ \frac{A_{glu.}}{moles_{glu.}}$$

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

This method of determining histidine as its monoacyl N-TFA *n*-butyl ester is of limited use due to the difficulty in obtaining precise RMR values for histidine. Reduced RMR values for arginine, tryptophan, and cystine were also obtained when this method was used.

The difficulties experienced with the determination of histidine as its monoacyl N-TFA *n*-butyl ester suggested that the diacyl derivative might be the derivative of choice. For this reason, several commercially available liquid phases were evaluated for chromatographic separation and substrate–derivative interaction, including OV-1, OV-3, OV-7, OV-11, OV-17, OV-22, OV-25, and XE-60. The peak observed for the diacyl histidine derivative coincided with the aspartic acid derivative with all of the OV columns; whereas the diacyl derivative underwent considerable decomposition when XE-60 (cyanopropylsiloxane) was used as the liquid phase.

Method II: analysis as diacyl histidine

The diacyl derivative of histidine was not separated from aspartic acid on any of the columns evaluated. Thus, another approach to the problem was needed. Experiments were conducted on the conversion of the histidine diacyl derivative to the monoacyl derivative as previously described, then injection of the monoacyl histidine derivative onto the OV-22 column in methylene chloride as solvent. The monoacyl derivative was then converted to the diacyl derivative by direct 'on column' injection of TFAA. In this way the diacyl histidine derivative could be eluted at a position on the chromatogram which does not coincide with the aspartic acid derivative, since prior to injection of TFAA histidine exists as the monoacyl derivative, and thus proceeds through the column at a slower rate.

An appropriate aliquot of a standard amino acid mixture was derivatized as described by GEHRKE *et al.*[2] using TFAA–CH_2Cl_2 (1:1, v/v) as the acylation medium. A 1.0 ml aliquot of the derivatized sample was placed in an acylation tube and evaporated just to dryness by passing a stream of dry nitrogen gas via a glass capillary into the neck of the tube. The sample was then redissolved in 2.0 ml of methy-

lene chloride and again dried with a stream of nitrogen as previously described. The sample was then dissolved in 1.0 ml of methylene chloride and mixed in an ultrasonic bath for 5 sec. The sample was then ready for injection onto the column. At this point, all of the histidine was present as the monoacyl derivative when the sample was injected, but after injection of 4.0 μl of TFAA the monoacyl histidine was converted in the column to the diacyl derivative. It was found that maximum separation was achieved when the TFAA was injected immediately after elution of the methionine peak.

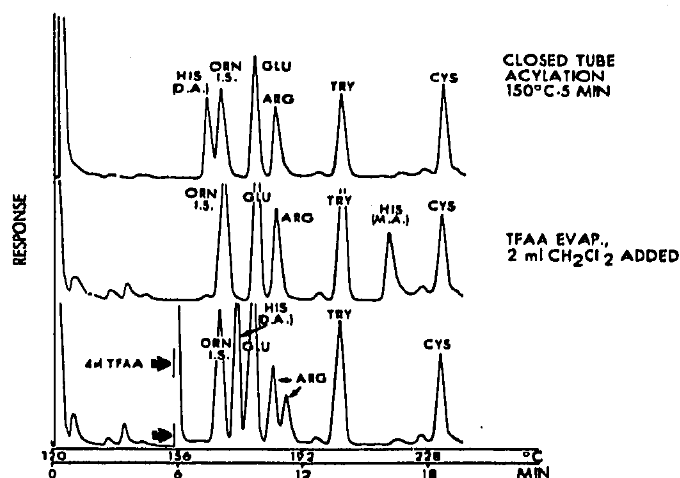


Fig. 1. GLC of mono- and diacyl histidine. Amino acid N-TFA *n*-butyl esters. Sample: 5 mg in 4.0 ml, 5 μg total amino acids injected (4 μl). TFAA–CH_2Cl_2 (1:1, v/v). Column: 1.5 w/w % OV-22 on 80–100 mesh H.P. Chromosorb G; 1 m × 4 mm I.D. glass. Initial temperature 120°, 6°/min.

A known solution of six amino acids was interesterified and then acylated in a TFAA–CH_2Cl_2 (1:1, v/v) reaction medium. The chromatogram is shown at the top of Fig. 1. Note that the diacyl derivative of histidine is eluted just prior to ornithine. This is the same retention time as for the aspartic acid derivative. The monoacyl derivative of histidine was eluted just prior to cystine as shown in the second chromatogram in Fig. 1. The monoacyl derivative was prepared by removing the excess TFAA as described previously. The third chromatogram gives the position at which the diacyl derivative of histidine was eluted after 'on column' injection of 4 μl of TFAA 6 min after injection of a sample in which the histidine was present as the monoacyl derivative. The monoacyl histidine derivative was quantitatively converted to the diacyl derivative and is now eluted just before glutamic acid. Under these experimental conditions, two peaks were observed for arginine which resulted from excessive drying of the sample, causing a partial conversion of the triacyl derivative of arginine to the diacyl derivative. Diacyl arginine was then converted to the triacyl derivative in the column after injection of TFAA. In quantitative analysis the two triacyl arginine peaks are summed.

The importance of this method is demonstrated by the two sets of RMR data presented in Table I. One set of data was obtained from analysis of derivatized samples which had not been dried under nitrogen. The second set of data was obtained from derivatized samples which had been treated as described above. The

TABLE I

RMR OF N-TFA *n*-BUTYL ESTERS OF AMINO ACIDS WITH AND WITHOUT EXCESS TFAA
RMR of glutamic acid assigned a value of 1.00.

Amino acid	*RMR*[a,b], *TFAA present*					*R.S.D. (%)*	*RMR*[a-d], *TFAA removed*					*R.S.D. (%)*
	1	*2*	*3*	*4*	*Av.*		*1*	*2*	*3*	*4*	*Av.*	
Histidine, diacyl	0.62	0.65	0.63	0.64	0.64	1.3	0.65	0.66	0.64	0.67	0.66	1.4
Ornithine	0.75	0.76	0.77	0.77	0.76	1.4	0.75	0.76	0.77	0.77	0.76	1.0
Glutamic acid	1.00	1.00	1.00	1.00	1.00	—	1.00	1.00	1.00	1.00	1.00	—
Arginine	0.73	0.74	0.75	0.75	0.75	1.0	0.68	0.70	0.71	0.69	0.70	2.2
Tryptophan	0.72	0.72	0.72	0.71	0.72	0.6	0.72	0.74	0.71	0.71	0.72	1.4
Cystine	0.83	0.86	0.86	0.85	0.85	1.4	0.78	0.79	0.81	0.81	0.80	1.5

[a] Each value is a single determination on 1.5 w/w % OV-22.
[b] Acylated at 150° for 5 min in TFAA–CH_2Cl_2 (1:1, v/v).
[c] TFAA evaporated; 2 ml of CH_2Cl_2 added, then dried with N_2 gas (2×); redissolved in 2 ml CH_2Cl_2.
[d] After 18 min, 4 µl TFAA injected immediately after methionine peak.

RMR values for histidine are for the diacyl derivative, with the two sets of data showing excellent agreement.

A study was then made to determine the stability as a function of time of the N-TFA *n*-butyl esters of the amino acids in the absence of an excess of TFAA. A comparison of the data presented in Table II indicates that the stability of the derivatives while standing at room temperature in closed tubes is not dependent on the presence of an excess of TFAA. This is due to the fact that 'on column' injection of TFAA results in the quantitative acylation of arginine, histidine, tryptophan, and cystine. These four amino acids are analyzed in this way since they are not eluted quantitatively from columns containing EGA as the liquid phase. Apparently some

TABLE II

STABILITY OF THE N-TFA *n*-BUTYL ESTERS OF AMINO ACIDS AS A FUNCTION OF TIME
Chromatographed on 1.5 w/w % OV-22, 1 m × 4 mm I.D. column.

Amino acid	*RMR, sample 1*[a,d]			*RMR, sample 2*[a-d]		
	Initial	*24 h*	*48 h*	*Initial*	*24 h*	*48 h*
Histidine, diacyl[e]	—	—	—	0.60	0.61	0.67
Tyrosine	1.11	1.12	1.21	1.02	1.01	1.07
Glutamic acid	1.00	1.00	1.00	1.00	1.00	1.00
Lysine	0.73	0.72	0.78	0.69	0.70	0.76
Arginine	0.68	0.66	0.63	0.64	0.64	0.68
Tryptophan	0.67	0.67	0.57	0.70	0.67	0.67
Cystine	0.78	0.74	0.87	0.79	0.74	0.65

[a] A mixture of twenty amino acids acylated at 150° for 5 min in TFAA–CH_2Cl_2 (1:1, v/v).
[b] TFAA evaporated; 2 ml of CH_2Cl_2 added, then dried with N_2 gas (2×); redissolved in 2 ml CH_2Cl_2.
[c] After *ca.* 18 min, 4 µl TFAA injected immediately after methionine peak.
[d] Samples 1 and 2 were aliquots from the same derivatization volume. RMR of glutamic acid arbitrarily assigned a value of 1.00.
[e] Cannot be separated from aspartic acid.

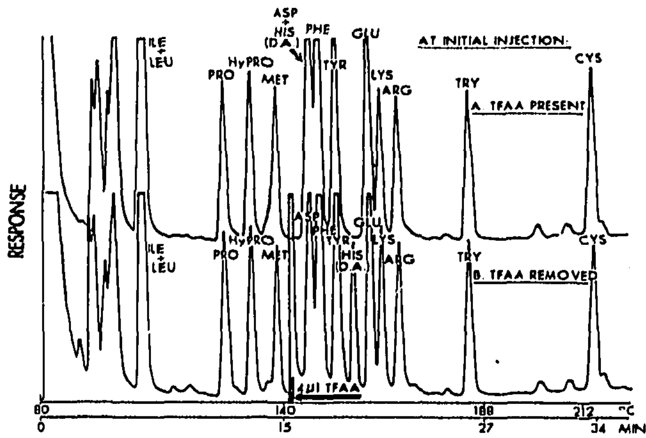


Fig. 2. GLC of diacyl histidine. Amino acid N-TFA *n*-butyl esters. Sample: 6 mg in 6.0 ml, 5 µg total amino acids injected (5 µl). Closed tube acylation 150°, 5 min. TFAA–CH_2Cl_2 (1:1, v/v). Column: 1.5 w/w % OV-22 on 80–100 mesh H.P. Chromosorb G; 1 m × 4 mm I.D. glass. Initial temperature 80°, 4°/min.

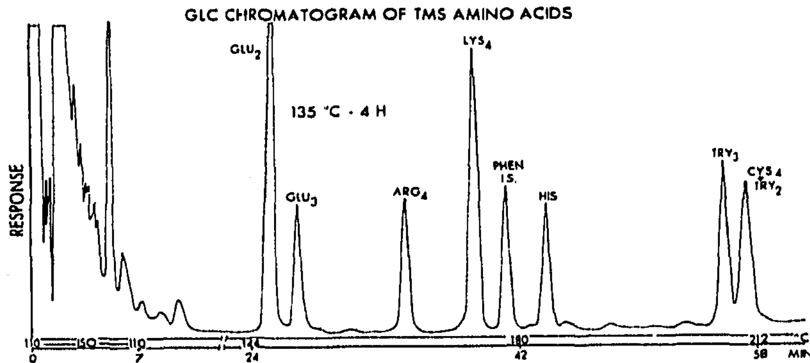


Fig. 3. GLC of TMS amino acids. Sample: 6 mg in 3.0 ml, 10 µg total amino acids injected (5 µl). BSTFA–CH_3CN (1:1, v/v). Column: mixed liquid phase of 3.0 w/w % OV-7 and 1.5 w/w % OV-22 on 100–120 mesh H.P. Chromosorb G; 1.75 × 4 mm I.D. glass. Initial temperature 110°, 7 min hold, then 2°/min.

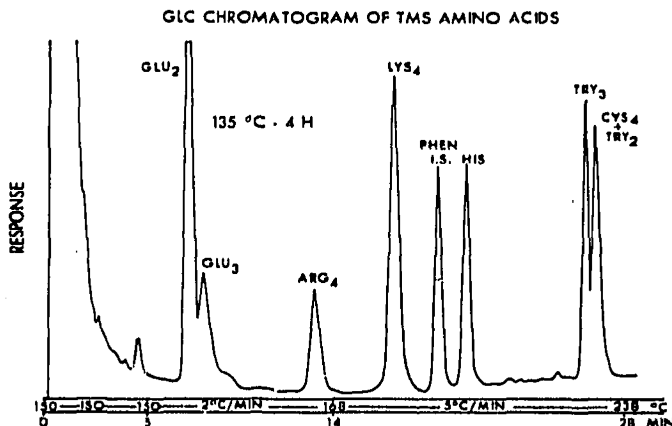


Fig. 4. GLC of TMS amino acids. Sample: 6 mg in 3.0 ml ,10 µg total amino acids injected (5 µl). BSTFA–CH_3CN (1:1, v/v). Column: mixed liquid phase of 3.0 w/w % OV-7 and 1.5 w/w % OV-22 100–120 mesh H.P. Chromosorb G; 1.75 × 4 mm I.D. glass. Initial temperature 150°, 5 min hold, then 2° for 9 min, then 5°/min.

TABLE III

GAS-LIQUID CHROMATOGRAPHIC ANALYSIS OF TMS AMINO ACIDS IN A MIXTURE

Amino acid	*Amino acid (mg)*					
	Added	*Recovered by GLC*[a,b]			*Av.*	*Recovery (%)*
$GLU_2 + GLU_3$	1.152	1.186	1.169	1.169	1.174	101.9
ARG_4	0.902	0.965	0.870	0.918	0.917	101.6
LYS_4	0.820	0.838	0.773	0.783	0.798	97.3
HIS_3	0.984	1.078	1.041	1.003	1.042	105.9
$TRY_2 + TRY_3$	1.012	0.989	0.909	0.943	0.947	93.6
CYS_4	0.968	1.075	0.992	0.968	1.011	104.4

[a] Phenanthrene internal standard.
[b] Each value represents an independent analysis.

kind of substrate-derivative interaction occurs. Chromatograms of a mixture of nineteen amino acids are presented in Fig. 2. The TFAA injection was made immediately after the methionine peak and resulted in the elution of the diacyl histidine derivative between tyrosine and glutamic acid.

Calculation of $AREA_{HIS}$ *from* $A_{HIS+ASP}$ *on OV-22*. A simplified determination of histidine can be made in yet another way. After acylation of the sample, it is injected on an OV-17 or OV-22 column. Histidine is present as the diacyl derivative and elutes with the aspartic acid derivative. The combined area for the non-separated histidine and aspartic acid derivatives on the OV-22 column is determined by integration. Also, a calibration mixture which does not contain histidine is used for the determination of the RMR_{ASP} on OV-22.

Then, the area for histidine is calculated with the following eqn. (1).

$$A_{HIS} \text{ on OV-22} = A_{HIS+ASP(OV\text{-}22)} - A_{ASP(EGA)} \times \frac{RMR_{ASP(OV\text{-}22)}}{RMR_{ASP(EGA)}} \times \frac{A_{I.S.(OV\text{-}22)}}{A_{I.S.(EGA)}} \quad (1)$$

The calculated area, A_{HIS} on OV-22, can then be used to calculate the w/w % of histidine in the sample. Eqn. (1) for A_{HIS} on OV-22 can be reduced to eqn. (2).

$$A_{HIS} \text{ on OV-22} = A_{HIS+ASP(OV\text{-}22)} - A_{ASP(EGA)} \times \frac{A_{I.S.(OV\text{-}22)}}{A_{I.S.(EGA)}} \quad (2)$$

This is based on experimental evidence showing that the RMR ratio is nearly one (1.00). If a substrate-derivative interaction takes place, the RMR ratio will not be equal to one (1.00). This ratio should be checked periodically and if a significant deviation occurs then eqn. (1) must be used.

Method III: silylation of histidine

A third method, mentioned in the INTRODUCTION, for the analysis of histidine makes use of another derivative, the trimethylsilyl derivative (TMS) of histidine (HIS_3). With this approach, sixteen of the amino acids may be determined as their N-TFA *n*-butyl esters on an EGA column. The remaining amino acids, arginine,

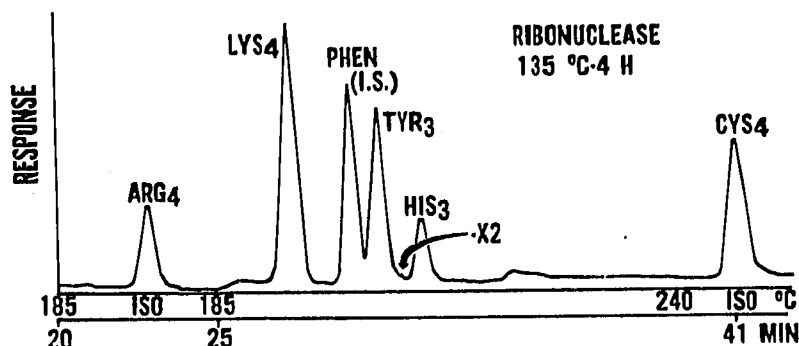


Fig. 5. GLC of ribonuclease TMS acids. Sample: 4 mg in 2.0 ml, 8 μg total amino acids injected (4 μl). Column: 3.0 w/w % OV-7 and 1.5 w/w % OV-22 on 100–120 mesh H.P. Chromosorb G.

histidine, cystine, and tryptophan, are then determined as their TMS derivatives on a *mixed* phase of 3.0 w/w % of OV-7 and 1.5 w/w % of OV-22 coated on H.P. Chromosorb G.

The quantitation of trimethylsilylation and chromatographic analysis of glutamic acid, arginine, lysine, histidine, tryptophan, and cystine as their TMS derivatives was established from analysis of a solution containing known amounts of these amino acids. The $RMR_{a.a./phen.}$ values used in the calculations for the recovery of these amino acids were obtained from repeated analyses of an equimolar amino acid stock solution. Typical chromatograms of these amino acids are shown in Figs. 3 and 4. Fig. 3 is a 58 min analysis, while Fig. 4 was completed in 28 min. The % recovery of each of the amino acids is given in Table III. The recovery using the TMS derivatives was found to be quite good, ranging from 105.9 to 93.6% with an average recovery of 102.5%.

The GLC analysis of histidine, arginine, and cystine in ribonuclease demonstrates the applicability of the trimethylsilylation method for analysis of amino acids in biological substances. A typical chromatogram obtained from analysis of these amino acids in ribonuclease is presented in Fig. 5. Comparison of results by silylation with classical ion-exchange data showed very good agreement, with values of histidine of 3.75% and 3.69%, respectively, for the two methods.

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