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DIRECT ESTERIFICATION OF THE PROTEIN AMINO ACIDS

GAS–LIQUID CHROMATOGRAPHY OF N-TFA *n*-BUTYL ESTERS*

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SUMMARY

The reaction conditions necessary for the "direct esterification" of the protein amino acids to their *n*-butyl esters are described. All of the amino acids were quantitatively esterified in *n*-butanol 3 *N* in hydrochloric acid at 100° with the exception of isoleucine. This "direct esterification" method with *n*-butanol permits a rapid derivatization and analysis by gas–liquid chromatography of the protein amino acids, thus, one of the major disadvantages of the earlier reported method has been removed.

The amino acids were observed to dissolve very slowly in *n*-butanol 6 *N* in hydrochloric acid even when the samples were subjected to ultrasonic mixing. Fairly rapid dissolution occurred in 1.5 *N* hydrochloric acid but a longer esterification time was noted. The optimum concentration of hydrochloric acid was found to be 3 *N* because the amino acids dissolved quickly in this solution with ultrasonic mixing and short esterification times were obtained. The more insoluble amino acids were broken up by ultrasonic mixing, thus increased rates of solution and esterification to the *n*-butyl esters were achieved. The effect of temperature over the range of 90 to 120°, on the rate of esterification was investigated but little effect on the relative molar response values was observed. However, the time of esterification was quite significant for two amino acids, tryptophan and isoleucine. Nineteen of the amino acids were quantitatively esterified with *n*-butanol 3 *N* in hydrochloric acid in 15 min at 100°, but 35 min were required for the esterification of isoleucine. However, with the longer esterification time, tryptophan underwent some decomposition (*ca.* 15%).

An esterification time of 35 min is recommended for samples which are to be analyzed for all of the amino acids including isoleucine and tryptophan; however, only 15 min are required if a slight error in the absolute value for isoleucine is permissible.

In quantitative work, a reference calibration mixture is analyzed under exactly the same experimental conditions as are the samples thus correction is made for any

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breakdown of tryptophan and the response values of all of the other amino acids are placed on the same basis.

Using the "direct esterification" method, recoveries of amino acids from a mixture averaged 99.7% with an average relative standard deviation of 0.92%. This "direct esterification" method allows a rapid, precise, and accurate derivatization of the protein amino acids to their N-TFA *n*-butyl esters with a minimum of transfers and sample handling. A complete gas–liquid chromatographic analysis of the protein amino acids, including derivatization and chromatography, can be completed in less than 1 h.

INTRODUCTION

Gas–liquid chromatographic (GLC) methods have proved to be most useful for the analysis of amino acids in biological substances because of their speed and sensitivity. Since the low volatilities of the amino acids has prevented their direct analysis by GLC, suitable volatile derivatives of the amino acids must be prepared. In 1962, ZOMZELY *et al.*[1] investigated the N-trifluoroacetyl (N-TFA) *n*-butyl esters as possible derivatives. LAMKIN AND GEHRKE[2] reported in 1965 that the most suitable derivative with respect to volatility and chromatography for the GLC analysis of the natural protein amino acids is the N-TFA *n*-butyl ester. The experimental conditions for quantitative derivatization and chromatographic requirements for separation were detailed by GEHRKE AND STALLING[3] in 1967. This was followed by a recent monograph by GEHRKE *et al.*[4] which presents macro, semimicro, and micro methods, reagents, sample preparation, instrumental and chromatographic requirements, and sample ion-exchange cleanup for the quantitative GLC analysis of the protein amino acids as their N-TFA *n*-butyl esters.

The chromatographic properties of several derivatives of the protein amino acids, in addition to the N-TFA *n*-butyl esters, have also been evaluated. Among the derivatives are the trimethylsilyl derivative introduced by RÜHLMANN AND GIESECKE[5] in 1961 and studied more recently by GEHRKE *et al.*[6,7]; the N-acetyl *n*-amyl esters by JOHNSON *et al.*[8]; and the N-trifluoroacetyl methyl esters by SAROFF AND KARMEN[9], by HAGEN AND BLACK[10], by CRUICKSHANK AND SHEEHAN[11], and by DARBRE AND ISLAM[12]. Each of these derivatives has proved to be useful for GLC separations, but none has received the intensive study regarding quantitative analysis as has the N-TFA *n*-butyl ester. An excellent review was written by BLAU[13] in 1968 on the analysis of amino acids by GLC covering the areas of derivatives, methods, and applications.

The N-TFA *n*-butyl esters of the amino acids are less volatile than the N-TFA methyl esters. The N-TFA methyl esters are so volatile that losses can easily occur during the derivatization procedure especially if the samples are concentrated after derivatization. DARBRE AND ISLAM[12] report that there is danger of loss of the more volatile N-TFA methyl esters when the samples are dried for longer than 4 min under vacuum at the temperature of an ice bath. Recent investigations[14] resulted in losses of *ca.* 10% for the more volatile N-TFA methyl esters when the samples were dried under a stream of nitrogen at room temperature; whereas insignificant losses were obtained for the N-TFA *n*-butyl esters dried under the same conditions. On

ca. a 10% loss occurred for the more volatile N-TFA *n*-butyl esters when evaporated at 100°.

During the past eight years, GEHRKE and co-workers[2-4,15] have developed a quantitative single general method for the determination of the twenty protein amino acids and at least forty other non-protein amino acids as their N-TFA *n*-butyl esters.

MUSSINI AND MARCUCCI[16] reported the esterification of the amino acids using diazobutane, but the most common method for preparing esters of the amino acids with the higher alcohols is the reaction of the amino acids with the alcohol in the presence of anhydrous hydrogen chloride. Direct formation of the higher alkyl esters of the amino acids has been a problem because of the lack of solubility of cystine and some of the other amino acids in the higher alcohols. BLAU AND DARBRE[17] suggested dissolving the amino acids in a small amount of anhydrous trifluoroacetic acid before adding the *n*-BuOH·HCl to obviate the solubility problems associated with cystine and some of the others. STALLING *et al.*[18] reduced the solubility problems by first making the methyl esters by direct esterification with methanolic HCl, then interesterification with *n*-butanolic HCl. These workers found that all of the amino acid methyl ester hydrochlorides were soluble in this reagent.

The quantitative GLC methods developed by GEHRKE and co-workers at the University of Missouri have proved to be most satisfactory except that the total derivatization time (methyl ester formation and interesterification) requires about 3.5 h for completion. Thus, in view of the number of laboratories which use this chromatographic method, it was highly desirable to have a faster procedure. From studies directed toward this goal, this research reports a simple, rapid, direct esterification method for preparing the N-TFA *n*-butyl esters of the protein amino acids with *n*-butanol 3 *N* in HCl which can be completed in 15 min. Also, the solubility problems associated with cystine and some of the other amino acids were removed.

EXPERIMENTAL

Apparatus

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, and a Packard Instruments Co. Model 7300 dual column gas chromatograph with hydrogen flame detectors and equipped with a Honeywell Electronik 16 strip chart recorder were 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 filtered nitrogen gas. The filters for purification of the N_2 gas contained activated charcoal and $CaSO_4$.

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

Reagents

All of the amino acids used in this study were obtained from Mann Research Laboratories, Inc., New York, N.Y. 10006, or Nutritional Biochemicals Corp., Cleveland, Ohio, and were chromatographically pure.

Methanol and butanol were "Baker Analyzed" reagents. The trifluoroacetic anhydride was obtained from Distillation Products Industries, Rochester, N.Y. 14603, 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, Joliet, Ill., 60434.

The methanol, *n*-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 *n*-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.

Columns

Stabilized grade ethylene glycol adipate was obtained from Analabs, Inc., Hamden, Conn. 06518 and coated on 80–100 mesh acid-washed (A.W.) Chromosorb W which had been dried at 140° for 12 h as described by ROACH AND GEHRKE[19]. The EGA column material was packed in 1.5 m × 4 mm I.D. glass columns.

The OV-17 and OV-22 siloxane substrates were purchased from Supelco, Inc., Bellefonte, Pa. 16823. The support material for these columns was 80–100 mesh high-performance (HP) Chromosorb G. The OV-22 glass columns were 1.0 m × 4 mm I.D. The OV-22 columns gave essentially the same results as the OV-17 columns reported earlier. Thus, OV-17 and OV-22 columns can be used interchangeably.

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 solvent used to dissolve EGA was dry acetonitrile, and the OV-17 or OV-22 substrates were dissolved in dry methylene chloride. The stationary phase 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 *slowly* removed with a rotary evaporator over a time period of 45 min under partial vacuum. This ensured a uniform coating of the stationary phase on the support material.

ANALYTICAL DERIVATIZATION METHOD

Macro method (1–20 mg total)

Pipet an appropriate aliquot of a protein hydrolysate, amino acid mixture, or ion-exchange cleaned physiological fluid[4] into a 125 ml flat-bottom flask. Add an *exact* amount (0.2–4 mg) of *n*-butyl stearate, ornithine or other internal standard (I.S.) solution to each flask[4].

Dry under a partial vacuum on an all-teflon rotary evaporator placed in a 60° water bath.

Add 1.5 ml of *n*-BuOH 3 *N* in HCl per 1.0 mg of total amino acids, mix on an ultrasonic mixer for at least 15 sec at room temperature, esterify at 100° for 15 min in an oil or sand bath.

Evaporate to dryness under a partial vacuum at 60° on a rotary evaporator

Add 3 ml of anhydrous methylene chloride and 1 ml of trifluoroacetic anhydride (TFAA) for each 10 mg of amino acids and thoroughly mix.

Transfer an appropriate aliquot to a Corning No. 9826 culture tube with teflon-lined screw cap. The amount need not be exact since an I.S. is included.

Acylate at 150° for 5 min in the closed tube placed behind a safety shield.

Semimicro (~ 2 mg total) and micro method (1–200 μg total)

Pipet an appropriate aliquot of sample into a Corning No. 9826 culture tube or micro reaction tube. Add an appropriate *exact* amount of ornithine or other internal standard[4].

Dry by passing a stream of filtered dry N_2 gas over the sample at 100°.

Add 150 μl of *n*-BuOH 3 *N* in HCl per 100 μg of total amino acids. Mix on an ultrasonic mixer for at least 15 sec at room temperature. Esterify at 100° for 15 min.

Evaporate to dryness at 100° using a stream of dry, filtered N_2 gas or by simply placing on a sand bath and allow to evaporate.

Azeotrope water with 150 μl of CH_2Cl_2.

Add 60 μl of CH_2Cl_2 and 20 μl of TFAA for each 100 μg of amino acids (minimum final volume of 80 μl).

Acylate at 150° for 5 min in a closed tube with teflon-lined screw cap placed behind a safety shield. The sample is ready for chromatography.

If the amount of starting sample is only 25–250 ng of each amino acid, concentrate the acylated sample to 50 μl by opening the vial and allow to evaporate by gentle warming. In this case use an acylation solution of 0.2 μl TFAA/40 μl CH_2Cl_2. Inject 4–8 μl of the concentrated sample.

RESULTS AND DISCUSSION

In 1968, GEHRKE *et al.*[15] presented an EGA and OV-17 dual column system for the separation of the N-TFA *n*-butyl esters of all twenty protein amino acids. Recently, ROACH AND GEHRKE[19] reported the use of stabilized EGA coated on dried (140° for 12 h) 80–100 mesh A.W. Chromosorb W for the rapid quantitative analysis of all the protein amino acids except arginine, histidine, and cystine. Also, ROACH *et al.*[20] reported the use of either OV-22 or OV-17 coated on 80–100 mesh, HP Chromosorb G for the rapid quantitative determination of histidine, arginine, tryptophan, and cystine. The gas–liquid chromatographic separations are now so rapid that only 15 to 25 min are required for a complete analysis of the twenty protein amino acids.

The major drawback to the method which was reported earlier for the GLC analysis was the considerable time required for interesterification. Formation of the methyl esters of the amino acids required 30 min and the interesterification required 2.5 h. Because of the considerable time involved in methyl ester formation, then interesterification to the *n*-butyl esters, a concerted effort was made to develop a "direct esterification" procedure.

STALLING *et al.*[18] reported that cystine and some of the other amino acids were difficult to dissolve in *n*-butanol which was 1.25 *N* in HCl. These authors noted that a fine white precipitate formed on the bottom of the container when *n*-BuOH·HCl was added to a dried sample of the amino acids. The present authors also noted this precipitate and considered it important to investigate the effect of HCl concentration

and ultrasonic mixing on the solubility of the amino acids. Ultrasonic mixing at room temperature for about 15 sec resulted in complete dissolution of the white precipitate. The ultrasonic mixing was of primary importance to the esterification reaction since the amino acid precipitates were readily broken up, thus the rates of solution and reaction were significantly enhanced in *n*-butanol 3 *N* in HCl. A study was then made to determine the effects of other reaction conditions: the HCl concentration, the reaction time, and the temperature.

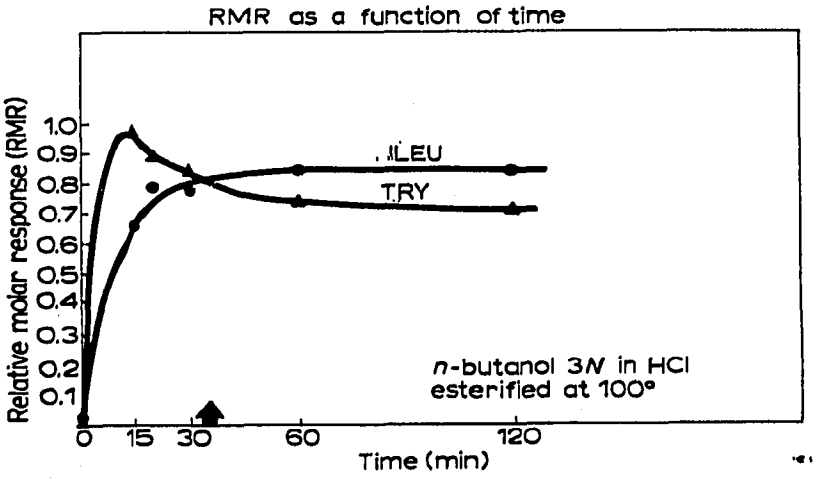

Fig. 1. *RMR* values for isoleucine and tryptophan as a function of esterification time.

TABLE I

THE RELATIVE MOLAR RESPONSE OF AMINO ACIDS AS A FUNCTION OF ESTERIFICATION TIME[a]

Amino acid	*Relative molar response*[b] *(a.a./glu.)*				
	15 min	*20 min*	*30 min*	*60 min*	*150 min*
Alanine	0.54	0.51	0.51	0.53	0.54
Valine	0.71	0.70	0.72	0.71	0.72
Glycine	0.43	0.42	0.42	0.42	0.43
Isoleucine	0.70	0.78	0.80	0.84	0.84
Leucine	0.83	0.81	0.81	0.82	0.82
Proline	0.73	0.71	0.71	0.70	0.71
Threonine	0.64	0.61	0.63	0.63	0.63
Serine	0.55	0.53	0.52	0.53	0.52
Methionine	0.54	0.50	0.52	0.51	0.52
Hydroxyproline	0.76	0.74	0.74	0.74	0.75
Phenylalanine	1.15	1.13	1.13	1.11	1.12
Aspartic acid	0.92	0.91	0.91	0.90	0.91
Glutamic acid	1.00	1.00	1.00	1.00	1.00
Tyrosine	0.95	0.96	0.97	0.95	0.87
Lysine	0.86	0.84	0.85	0.84	0.84
Histidine	0.63	0.63	0.64	0.63	0.61
Arginine	0.62	0.64	0.63	0.62	0.59
Tryptophan	0.97	0.88	0.77	0.74	0.63
Cystine	0.88	0.92	0.92	0.91	0.90

[a] Direct esterification in *n*-butanol 3 *N* in HCl at 100°. *RMR* of glutamic acid assigned a value of 1.

[b] Each value represents an average of two determinations.

Isoleucine was found to be the most difficult amino acid to esterify and tryptophan was partially decomposed in *n*-BuOH·HCl, but tryptophan undergoes decomposition in any acidic media. Since isoleucine was the most difficult amino acid to esterify and some losses were observed for tryptophan, these two amino acids were selected as models for further experiments. The samples were derivatized as described in the Section ANALYTICAL DERIVATIZATION METHOD.

The effect of the concentration of HCl on the esterification reaction was investigated first. Solutions of 1.5, 3.0, and 6.0 *N* HCl in *n*-butanol were studied. In all experiments, 1.5 ml of *n*-BuOH·HCl was added for each 1.0 mg of total amino acids. The amino acids dissolved very slowly in 6 *N* HCl, whereas 1.5 *N* HCl dissolved the samples, but longer times were required.

TABLE II

THE RELATIVE MOLAR RESPONSE OF AMINO ACIDS AS A FUNCTION OF ESTERIFICATION TEMPERATURE[a]

Amino acid	*Relative molar response*[b] *(a.a./glu.)*			
	90°	*100°*	*110°*	*120°*
Alanine	0.53	0.53	0.51	0.52
Valine	0.72	0.72	0.70	0.73
Glycine	0.44	0.44	0.42	0.43
Isoleucine	0.80	0.82	0.82	0.83
Leucine	0.80	0.82	0.81	0.84
Proline	0.71	0.72	0.71	0.74
Threonine	0.63	0.64	0.64	0.65
Serine	0.54	0.55	0.55	0.56
Methionine	0.56	0.55	0.54	0.55
Hydroxyproline	0.76	0.75	0.74	0.76
Phenylalanine	1.13	1.14	1.14	1.16
Aspartic acid	0.89	0.90	0.89	0.91
Glutamic acid	1.00	1.00	1.00	1.00
Tyrosine	0.95	0.96	0.96	0.97
Lysine	0.86	0.85	0.84	0.88
Histidine	0.61	0.62	0.64	0.67
Arginine	0.62	0.61	0.62	0.64
Tryptophan	0.90	0.88	0.81	0.77
Cystine	0.85	0.89	0.90	0.92

[a] Direct esterification in *n*-butanol 3 *N* in HCl for 35 min. *RMR* of glutamic acid assigned a value of 1.

[b] Each value represents a single determination.

A plot of the relative molar response (*RMR*) values for isoleucine and tryptophan as a function of time of esterification is given in Fig. 1. The *RMR* for glutamic acid was arbitrarily assigned a value of unity, and the response values of each amino acid relative to glutamic acid, $RMR_{a.a./glu.}$, were calculated as follows:

$$RMR_{a.a./glu.} = \frac{A_{a.a.}/g_{a.a.}/MW_{a.a.}}{A_{glu.}/g_{glu.}/MW_{glu.}}$$

where $A_{a.a.}$ = area in counts of amino acid peak, $g_{a.a.}$ = grams of amino acid in sample, and $MW_{a.a.}$ = molecular weight of amino acid.

A direct esterification time of 35 min was selected to give maximum *RMR* values for isoleucine and all of the other amino acids with the exception of tryptophan.

The *RMR* values for the amino acids given in Table I were determined at five different esterification times (15 to 150 min). These data show that excellent results can be achieved with an esterification time of 15 min for all of the amino acids except isoleucine. Note that the maximum *RMR* value for tryptophan was obtained for a 15-minute "direct esterification". The maximum *RMR* for isoleucine was obtained after esterification for *ca.* 1 h but little change in *RMR* values resulted after 45 min. Direct esterification of isoleucine at 100° for 15 min yielded an average *RMR* value of about 85% of the maximum value. Since the w/w% recovery depends not only upon the *RMR* of the experimental sample, but also upon the *RMR* for the calibration mixture taken through the complete method, a recovery of 100% can be achieved when both the sample and the calibration mixture are esterified for 15 min under *exactly* the same reaction conditions.

TABLE III

RECOVERY OF AMINO ACIDS IN A MIXTURE BY DIRECT ESTERIFICATION

Amino acid	*Milligrams*[a]		*Recovery* (%)[b]
	Added	*Found*	
Alanine	0.4456	0.435	97.6
Valine	0.5856	0.566	96.7
Glycine	0.3756	0.367	97.7
Isoleucine	0.6560	0.644	98.2
Leucine	0.6560	0.650	99.1
Proline	0.5756	0.564	97.9
Threonine	0.5956	0.591	99.2
Serine	0.5356	0.525	98.0
Cysteine	0.6060	0.591	97.5
Methionine	0.7460	0.746	100.0
Hydroxyproline	0.6556	0.652	99.4
Phenylalanine	0.8260	0.819	99.2
Aspartic acid	0.6656	0.687	103.2
Glutamic acid	0.7356	0.747	101.5
Tyrosine	0.9060	0.912	100.7
Lysine	0.7310	0.748	102.3
Histidine	0.7760	0.771	99.4
Arginine	0.8710	0.908	104.2
Tryptophan	1.0210	1.158	113.4[c]
Cystine	1.2016	1.233	102.6

[a] Each value represents an average of three independent determinations. *n*-Butyl stearate as internal standard. Esterified for 35 min at 100° in *n*-butanol 3 *N* in HCl.

[b] Recovery based on amino acid calibration mixture taken through complete method with interesterification.

[c] High value due to loss of tryptophan in calibration mixture during interesterification for 2.5 h at 100°.

The maximum *RMR* value for tryptophan was obtained on direct esterification for 15 min at 100°. After reaching a maximum, the *RMR* value decreased with time due to the decomposition of tryptophan in the acidic *n*-butanol solution. An average of about 85% of the maximum *RMR* for tryptophan was obtained with a 35-minute esterification. An average recovery of more than 95% was obtained, however, when the sample and calibration mixture were reacted under the same experimental con-

ditions (100° for 35 min). However, samples should be esterified directly at 100° for 15 min to obtain the most accurate tryptophan values. It is well known that tryptophan undergoes decomposition during acidic hydrolysis, thus analysis for tryptophan in acidic hydrolysates is useless. For samples which contain no tryptophan, direct esterification for 35 min at 100° is recommended to assure quantitative esterification of isoleucine.

The effect of esterification temperature on the *RMR* values was investigated in the range of 90 to 120°. The data in Table II demonstrate that the temperature at which the esterification was conducted influenced the *RMR* values only slightly. A temperature of 100° ±5° was selected as the esterification temperature. Using isoleucine and tryptophan as model compounds, the conditions for direct esterification were determined. The next step in the study was to apply these reaction conditions to a mixture containing the twenty protein amino acids. Aliquots containing known

TABLE IV

RELATIVE MOLAR RESPONSE OF N-TFA *n*-BUTYL ESTERS OF AMINO ACIDS[a]

Amino acid	*Relative molar response*[b]				
	1	*2*	*3*	*Av.*	*RSD* (%)
Alanine	0.523	0.518	0.513	0.518	0.97
Valine	0.725	0.731	0.735	0.730	0.69
Glycine	0.427	0.438	0.434	0.433	1.29
Isoleucine	0.829	0.823	0.835	0.829	0.72
Leucine	0.838	0.831	0.827	0.832	0.67
Proline	0.732	0.717	0.724	0.724	1.04
Threonine	0.641	0.636	0.629	0.635	0.95
Serine	0.532	0.540	0.538	0.537	0.78
Cysteine	0.458	0.454	0.446	0.453	1.35
Methionine	0.562	0.551	0.557	0.557	0.99
Hydroxyproline	0.763	0.757	0.745	0.755	1.21
Phenylalanine	1.139	1.140	1.142	1.140	0.14
Aspartic acid	0.917	0.909	0.914	0.913	0.44
Glutamic acid	1.000	1.000	1.000	1.000	—
Tyrosine	0.957	0.946	0.952	0.952	0.58
Lysine	0.863	0.859	0.846	0.856	1.04
Histidine	0.623	0.619	0.630	0.624	0.89
Arginine	0.643	0.637	0.653	0.644	1.26
Tryptophan	0.851	0.840	0.832	0.841	1.13
Cystine	0.932	0.958	0.941	0.944	1.40

[a] Direct esterification in *n*-butanol 3 *N* in HCl at 100°. *RMR* of glutamic acid assigned a value of 1.

[b] Each value represents a single determination.

amounts of the amino acids (~ 5 mg) were dried, an excess of *n*-butanol 3 *N* in HCl (1.5 ml per 1.0 mg total amino acids) was added, the samples were esterified for 35 min, and then acylated with TFAA in a closed tube at 150° for 5 min as described by GEHRKE *et al.*[4]. The calibration mixtures were prepared by first forming the methyl esters of the amino acid and then interesterifying and acylating as described by GEHRKE *et al.*[4]. The recovery data obtained from this experiment are given in Table III. Excellent recovery was obtained for all of the amino acids indicating that the direct esterification of the amino acids was essentially complete. The high value for

tryptophan in the sample resulted from the low *RMR* for tryptophan in the calibration mixture. This resulted from heating tryptophan in an acidic *n*-butanolic solution for a total of 3 h with the corresponding losses.

The *RMR* values for the amino acid derivatives which were obtained using the direct esterification procedure are presented in Table IV. The precision of the data is excellent as evidenced by the relative standard deviations, which in most cases are *ca.* 1% or less.

CONCLUSIONS

The reaction conditions for the "direct quantitative esterification" of the protein amino acids have been established. These experiments conclusively demonstrate that GLC analyses of the amino acids can be made precisely, accurately, and rapidly when the N-TFA *n*-butyl esters of the amino acids are formed directly in *n*-butanol 3 *N* in HCl. A significant advantage of the N-TFA *n*-butyl ester derivative over the N-TFA methyl ester derivative is that no losses occur on concentration at room temperature, whereas serious losses of the methyl esters would occur. Further, the losses of the butyl ester derivative would be minimal if any leaks occurred during derivatization. A complete GLC analysis of the protein amino acids, including derivatization and chromatography, can be completed in less than 1 h. This GLC method should fill an important need of scientists who are faced with the problem of amino acid analyses of biologically important substances.

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