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THE EFFECT OF SALTS ON THE DERIVATIZATION AND CHROMATOGRAPHY OF AMINO ACIDS*

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SUMMARY

This study was made to determine the effects of inorganic salts on the derivatization and chromatography of the N-TFA *n*-butyl esters of the amino acids. In general, the presence of an equal weight of inorganic salts to total weight of amino acids (or $W_{salt}/W_{a.a.}$ of 20 for each amino acid) on the derivatization and chromatography is not serious for qualitative work, but in certain cases can be significant in quantitative work. The following ions cause problems: oxalate, manganese(II), cobalt(II), nickel, zinc, tin(II), lead(II), chromium(III), and iron(III). When a 1:1 ratio of salts to total amino acid is present in the sample, it is suggested that they be removed by the use of ion-exchange chromatography. However, the following ions at a $W_{salt}/W_{a.a.}$ ratio of about 20 are not considered of significance: sodium, potassium, copper(I), silver, magnesium, calcium, barium, mercury(II), aluminum, chloride, bromide, acetate, nitrate, sulfate, and phosphate. The repeated injection of a sample containing salt, such as sodium chloride, results in reduced response for all long retention time amino acids and for the internal standard, butyl stearate. However, the column is not harmed by the repeated injections, for when the injection port end of the column is cleaned, good quantitative results are again obtained.

INTRODUCTION

Gas–liquid chromatographic (GLC) methods have proved to be useful for the analysis of amino acids in biological substances because of their speed and sensitivity. Since the low volatility of the amino acids has prevented their direct analysis by GLC, suitable derivatives of the amino acids must be prepared. ZOMZELY *et al.*[1] investigated the N-trifluoroacetyl (N-TFA) *n*-butyl esters as a possible derivative. LAMKIN AND GEHRKE[2] reported that the most suitable derivative with respect to volatility and

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chromatography for the GLC analysis of the natural protein amino acids is the N-TFA *n*-butyl ester. GEHRKE AND STALLING[3] reported detailed experimental conditions for quantitative derivatization and chromatographic separation, and in 1968 GEHRKE *et al.*[4] wrote a monograph covering macro, semimicro, and micro methods, reagents, sample preparation, instrumental and chromatographic requirements and sample ion-exchange cleanup for the quantitative GLC analysis of the twenty protein amino acids in biological substances. ROACH AND GEHRKE[5] have reported on the use of acid-washed Chromosorb W in place of the heat-treated HP Chromosorb G used in the earlier work. Also, ROACH AND GEHRKE[6] reported an esterification procedure with *n*-butanol · 3 *N* HCl with heating at 100° (15–30 min), which allows one to form the butyl ester derivatives by "direct esterification" rather than by interesterification from the methyl ester. These reports considerably simplified the chromatography and derivatization of the amino acids.

In the analysis of amino acids in sea water, soil, and some biological samples by GLC, one is aware of the presence of cations and anions without knowledge of their effect on the analysis. This study was undertaken to investigate the effects of cations and anions on the derivatization and chromatography of seventeen protein amino acids as the N-TFA *n*-butyl esters.

EXPERIMENTAL

Apparatus

A Micro-Tek MT 220 gas chromatograph with a four-column oven bath, four flame ionization detectors, two dual differential electrometers, and equipped with a Varian Model 30 dual-pen recorder, was used for this study. A digital readout integrator (Hewlett-Packard Model 3370A) was used for determining peak areas.

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 reaction. A 1/32-in. hole was drilled in the center of the caps. This hole was covered with a silicone septum and a teflon liner for entering with a syringe without opening and exposure of the sample to the moisture and air.

A CaLab rotary evaporator, "cold finger" condenser, and a Welch Duo-Seal vacuum pump were used for the removal of solvents in the preparation of column packing.

Reagents

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

n-Butanol was "Baker Analyzed" reagent. 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 high purity was stored over drierite in a bottle with a ground glass stopper. Anhydrous HCl was generated by the slow addition of 250 ml of reagent grade HCl to 500 ml of concentrated H_2SO_4. The HCl gas was passed through two H_2SO_4 drying towers and then bubbled into the *n*-butanol.

The *n*-butanol and methylene chloride were redistilled from an all-glass system and stored in an all-glass inverted top bottle to protect from atmospheric moisture.

The methylene chloride and *n*-butanol were refluxed over calcium chloride before distillation.

Chromatographic column

Stabilized grade ethylene glycol adipate (EGA) was obtained from Analabs, Inc., Hamden, Conn. 06518, and coated on 80/100 mesh acid-washed (AW) Chromosorb W which had not been heated at 140° for 12 h. The EGA column material was packed in a 1.5 m × 4 mm I.D. glass column.

The column packing was prepared by first adding 30.00 g of 80/100 mesh AW Chromosorb W to a 500-ml ribbed round-bottom flask. Acetonitrile was added until the liquid level was about 1/4 in. above the support material. 0.20 g of EGA were weighed into a small erlenmeyer flask, dissolved in 20 ml acetonitrile, and transferred to the flask containing the support. The flask containing the support and substrate was placed in a 60° water bath, and the solvent slowly removed with a rotary evaporator over a period of 1 h under a partial vacuum.

Derivatization

Two milliliters of a stock solution of the seventeen amino acids containing 0.1 mg/ml of each amino acid in 0.1 *N* hydrochloric acid were pipetted into a 16 × 75 mm culture tube. The water was evaporated under a stream of filtered dry nitrogen at 70°. Two milliliters of a solution of 0.1 mg/ml of stearic acid (internal standard, I.S.) in *n*-butanol 3 *M* in HCl were added to the tube. Five milliliters of butanol 3 *M* in HCl were added for each 1.0 mg of total amino acids. The solution was heated at 100° for 45 min to esterify the amino acids, then the butanol was removed with a stream of filtered nitrogen at 70°. One milliliter of a 1:1 solution of chloroform to trifluoroacetic anhydride (TFAA) solution was added, then acylated at 150° for 5 min.

In derivatization of samples containing added salt, the salt was weighed into the reaction tube before the addition of the amino acid solution and the above procedure followed.

Chromatography

For the chromatography and quantitative analysis of the samples, 5.0 μl of the derivatized sample were injected. The first injection on each day was a standard followed by a salt-containing sample, then the standard was reinjected. If the response values for the second standard failed to agree with those for the first standard, the glass wool and top $^1/_2$ in. of column packing were replaced and the above injections repeated. It was necessary to precede and follow each salt-containing sample with chromatography of standards to prove that the chromatographic column would still give the required separation and quantitation.

The same concentration of each amino acid and internal standard was used in all experiments, and the same known volume was injected each time into the chromatography column. Thus, for the standard, the peak area was obtained in such a manner that the same area should be obtained for each amino acid derivative in the sample containing salt. By doing the experiments in this way, one was able to note whether the experimental areas for the amino acids, or internal standard was enhanced or reduced by the added salt.

TABLE I

THE EFFECT OF SALTS ON THE N-TFA *n*-BUTYL ESTER AMINO ACID DERIVATIVES—NON-INTERFERING[a]

Experiments were performed with 4 mg of salt and 4 mg of total amino acid mixture; $W_{salt}/W_{a.a.} = 20$.

Amino acid	$RWR_{a.a./stearic\ acid}$[b]											
	Std.[c]	*NaCl*	*KCl*	*CuCl*	$AgNO_3$	$MgSO_4$	$CaCl_2$	$BaCl_2$[d]	$HgBr_2$	$AlCl_3$	Na_3PO_4	*N*
Alanine	1.14	1.14	1.07	1.09	1.16	1.16	1.21	1.05	1.08	1.15	1.16	1.
Valine	1.14	1.23	1.07	1.07	1.21	1.15	1.16	1.02	1.08	1.15	1.16	1.
Glycine	1.04	1.09	1.08	1.05	1.06	1.06	1.02	1.01	1.03	1.06	1.02	1.
Isoleucine	1.17	1.14	1.08	1.09	1.16	1.18	1.19	1.06	1.09	1.17	1.16	1.
Leucine	1.11	1.17	1.13	1.12	1.13	1.13	1.14	1.11	1.05	1.13	1.11	1.
Proline	1.20	1.19	1.15	1.19	1.19	1.28	1.19	1.14	1.17	1.12	1.20	1.
Threonine	0.93	0.96	0.96	0.94	0.95	0.90	0.94	0.92	0.92	0.95	0.96	0.
Serine	0.99	0.98	0.96	0.99	0.97	1.05	1.04	0.93	0.98	1.00	0.97	0.
Cysteine	0.57	0.58	0.59	0.47	0.55	0.60	0.54	0.56	0.53	0.56	0.59	0.
Methionine	0.73	0.76	0.73	0.74	0.73	0.75	0.82	0.70	0.75	0.67	0.71	0.
Hydroxyproline	0.97	0.99	0.96	0.97	0.99	0.94	0.93	0.96	0.98	0.99	0.96	0.
Phenylalanine	1.23	1.21	1.17	1.24	1.22	1.31	1.29	1.16	1.19	1.19	1.21	1.
Aspartic acid	1.14	1.21	1.18	1.16	1.15	1.13	1.10	1.14	1.16	1.16	1.14	1.
Glutamic acid	1.22	1.18	1.16	1.22	1.20	1.15	1.13	1.14	1.18	1.24	1.22	1.
Tyrosine	0.93	0.94	0.91	0.92	0.92	0.94	0.93	0.83	0.93	0.89	0.93	0.
Lysine	1.04	1.07	1.03	1.05	1.05	1.00	1.01	0.99	1.05	1.11	1.04	1.
Tryptophan	0.45	0.44	0.43	0.43	0.45	0.47	0.47	0.38	0.45	0.40	0.45	0.

[a] A salt was classified as non-interfering if the RWR's varied by less than ±10% from the star values.

[b] $RWR_{a.a./stearic\ acid\ (I.S.)} = \frac{A_{a.a.}/g_{a.a.}}{A_{I.S.}/g_{I.S.}}$.

[c] Average of three independent analyses.

[d] Two waters of hydration.

RESULTS AND DISCUSSION

The data for salts which are classified as non-interfering are listed in Table I. A salt was classified as non-interfering if the relative weight response, $RWR_{a.a./I.S.}$, did not deviate from the value of the standards by more than ±10%. The following ions were found to be non-interfering: potassium, copper(I), silver, magnesium, calcium, barium, sodium, mercury(II), and aluminum as cations; and chlorides, bromides, nitrates, sulfates, and phosphates as anions.

A salt was listed as interfering if the relative weight response, $RWR_{a.a./I.S.}$, deviated from the value of the standards by more than ±10%. The ions classified as interfering are listed in Table II and were manganese(II), cobalt(II), nickel, zinc, tin(II), lead(II), chromium(III), and iron(III), as cations, and oxalate as an anion.

Three explanations can be offered for the observed results given in Table II. These are: (1) reduced volatilization of the internal standard, (2) reduced volatilization of the amino acids, and (3) reduced response due to chelation of the amino acid by the cation.

A reduced volatilization of the internal standard results in a reduced response for the internal standard and thus an apparent increase in the $RWR_{a.a./I.S.}$ for the amino acids. It should be noted that no enhancement of response for any amino acids was observed as the result of the presence of a salt. All relative weight response values

TABLE II

THE EFFECT OF SALTS ON THE N-TFA *n*-BUTYL ESTER AMINO ACID DERIVATIVES—INTERFERING[a]

Experiments were performed with 4 mg of salt and 4 mg of total amino acid mixture; $W_{salt}/W_{a.a.} = 20$.

Amino acid	$RWR_{a.a./stearic\ acid}$[b]									
	Std.[c]	$K_2C_2O_4$	$MnCl_2$	$CoCl_2$	$NiCl_2$	$ZnCl_2$	$SnCl_2$	$Pb(C_2H_3O_2)_2$	$CrCl_3$[d]	$FeCl_3$
Alanine	1.14	1.06	3.14	1.61	2.18	1.40	1.33	1.28	1.71	1.54
Valine	1.14	—[e]	3.02	1.68	2.23	1.43	1.52	1.29	1.74	1.61
Glycine	1.04	—[e]	1.96	1.53	1.76	1.34	1.24	1.25	1.87	1.40
Isoleucine	1.17	—[e]	2.91	1.65	2.19	1.71	1.59	1.37	1.77	1.63
Leucine	1.11	—[e]	2.32	1.67	2.14	1.42	1.27	1.25	1.74	1.43
Proline	1.20	1.26	3.01	1.65	2.31	1.45	1.40	1.59	1.86	1.60
Threonine	0.93	0.94	1.67	1.14	1.76	1.64	1.24	1.18	1.31	1.32
Serine	0.99	0.98	2.46	1.38	1.10	1.20	1.37	1.19	0.91	1.33
Cysteine	0.57	0.58	0.24	0.28	0.36	0.37	0.59	0.60	0.45	0.47
Methionine	0.73	0.74	1.64	0.98	1.20	0.36	0.64	0.91	1.08	0.54
Hydroxyproline	0.97	0.97	1.68	1.64	1.96	1.44	0.76	1.18	1.35	1.36
Phenylalanine	1.23	1.24	2.90	1.68	2.15	1.50	1.47	1.63	1.88	1.63
Aspartic acid	1.14	1.15	1.19	1.43	2.31	1.74	0.91	1.47	1.15	1.52
Glutamic acid	1.22	1.30	0.98	1.17	1.18	0.92	0.93	1.58	1.25	1.11
Tyrosine	0.93	0.92	1.57	0.85	0.02	2.61	0.58	0.93	0.03	1.15
Lysine	1.04	1.03	0.28	0.86	0.36	0.92	1.02	1.09	0.56	0.78
Tryptophan	0.45	0.46	0.39	0.11	0.11	0.88	0.11	0.50	0.09	0.36

[a] A salt was classified as interfering if the RWR's varied by more than ± 10% from the standard values.

[b] $RWR_{a.a./stearic\ acid\ (I.S.)} = \dfrac{A_{a.a.}/g_{a.a.}}{A_{I.S.}/g_{I.S.}}$

[c] Average of three independent analyses.

[d] Six waters of hydration.

[e] Interference due to di-*n*-butyl oxalate.

TABLE III

THE EFFECT OF NaCl ON THE N-TFA *n*-BUTYL ESTER AMINO ACID DERIVATIVES

Experiments were performed with 20 mg NaCl and 10 mg of total amino acid mixture; $W_{salt}/W_{a.a.} = 20$.

Amino acid	$RWR_{a.a./stearic\ acid}$ *and injection number*[a]					
	1	*4*	*6*	*7*	*10*	*11*[b]
Alanine	1.131	1.130	1.149	1.224	1.362	1.139
Glycine	1.044	1.043	1.059	1.137	1.271	1.046
Leucine	1.110	1.124	1.176	1.258	1.301	1.109
Threonine	0.941	0.946	0.957	1.036	1.147	0.946
Cysteine	0.562	0.566	0.573	0.611	0.742	0.568
Hydroxyproline	0.981	0.987	0.994	1.107	1.112	0.991
Aspartic acid	1.138	1.142	1.127	1.068	0.987	1.144
Tyrosine	0.941	0.947	0.910	0.763	0.646	0.946
Lysine	1.002	1.001	0.968	0.041	0.403	1.005
Tryptophan	0.457	0.452	0.396	0.246	0.210	0.459

[a] $RWR_{a.a./stearic\ acid\ (I.S.)} = \dfrac{A_{a.a.}/g_{a.a.}}{A_{I.S.}/g_{I.S.}}$.

[b] The glass wool and the top 1/2 in. of column packing were replaced, then the column was inserted into the same injection port.

larger than those for the standards occurred as the result of a reduced response for the internal standard, not as a result of an enhancement of the response for an amino acid. The second explanation results in a reduced response of the amino acid. This would be expected to have a greater effect on the less volatile amino acids and indeed this trend was observed. The third explanation, again, would result in a reduced response for the amino acids.

A reasonable explanation of all the experimental observations and data in the tables is that the changed RWR values are a result of reduced volatilization of internal standard, amino acid, and perhaps chelation of the amino acids.

In this study, it was often noted that a decrease in the response of the long retention time amino acids occurred and little change was observed in the response for the short retention time amino acids. In many cases this problem was obviated on replacement of the glass wool plug and top $^1/_2$ in. of column packing. This observation resulted in a study of the effect of sodium chloride at a 20:1 (w/w) level to each amino acid in a sample upon repeated injection into a chromatographic column. The results of this experiment are given in Table III. As a build-up occurred of sodium chloride deposits in the injection port the volatilization of the less volatile amino acids decreased. When the glass wool plug was replaced and the first $^1/_2$ in. of column packing was removed, response values were again obtained comparable to the original values. This proves that there has not been a destruction of the column packing, but an interference in the volatilization of the internal standard and amino acids due to deposits of sodium chloride.

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