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Note

Effect of ammonium hydroxide concentration on the recoveries of amino acids during preparation for gas–liquid chromatography

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In preparation for analysis by gas–liquid chromatography (GLC), amino acids on biological samples, or hydrolysates, are separated from potential interfering compounds using a strong cation-exchange resin (sulfonated polystyrene). Ammonium hydroxide at a concentration of 2 N[1,2], 3 N[3], or 7 N[4–6] is used to elute amino acids from the cation-exchange resin. During the initial stages of adapting a cation-exchange procedure for isolation of amino acids from biological substances, it was found that upon subsequent GLC measurement, recoveries of lysine and arginine were less than 50% and highly variable when ammonium hydroxide at concentrations greater than 3 N was used as the eluant. Recovery of arginine was always higher than that of lysine, since NH_4^+ displaces lysine from a resin of sulfonated polystyrene more readily than it displaces arginine[7,8], the recovery of lysine rather than arginine was expected to be higher. This note reports the effects of high concentrations of ammonium hydroxide upon the GLC measurement of lysine and arginine in a solution of amino acids of known concentration, and an improved recovery of amino acids in the presence of dilute ammonium hydroxide solutions.

EXPERIMENTAL

Amino acid, and internal and external standards were dissolved in aqueous 0.1 N hydrochloric acid. An amino acid (amino acids purchased as kit LAA-21 from Sigma, St. Louis, MO, U.S.A.) solution containing 2.5 mM L-alanine, glycine, L-valine, L-threonine, L-serine, L-leucine, L-isoleucine, L-proline, L-methionine, L-aspartic acid, L-phenylalanine, L-glutamic acid, L-lysine–HCl, L-tyrosine, L-arginine–HCl, and L-histidine–HCl and 1.25 mM L-cystine was prepared. Cycloleucine (Aldrich, Milwaukee, WI, U.S.A.) was used (2.5 mM) as an internal standard. Pipecolic acid–HCl (Sigma) was used (2.5 mM) as an external standard.

The solvents under study included distilled deionized water and 1, 2, 3, 4 and 5 N ammonium hydroxide. To triplicate 3-ml samples of each solvent in 13 × 100 mm

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TABLE I

MEAN RELATIVE MOLAR RATIOS AND COEFFICIENTS OF VARIATION FOR AMINO ACIDS PREPARED FOR GAS-LIQUID CHROMATOGRAPHY FROM SOLUTIONS OF VARIOUS CONCENTRATIONS OF AMMONIUM HYDROXIDE

$$RMR = \frac{\text{(peak area amino acid/molar concentration amino acid)}}{\text{(peak area internal standard/molar concentration internal standard)}}$$

C.V. = (Standard deviation/mean) × 100.

Amino acid	*Treatment*											
	Water		*Ammonium hydroxide (N)*									
			1		*2*		*3*		*4*		*5*	
	RMR	*C.V.*	*RMR*	*C.V.*	*RMR*	*C.V.*	*RMR*	*C.V.*	*RMR*	*C.V.*	*RMR*	*C.V.*
Alanine	0.69	3.9	0.71	1.4	0.67	4.0	0.69	4.7	0.64	15.9	0.50	25.8
Glycine	0.64	0.9	0.63	1.6	0.56	9.3	0.59	4.4	0.60	5.8	0.55	8.5
Valine	0.95	3.3	0.94	2.1	0.97	4.1	0.96	3.6	0.90	10.7	0.78	19.7
Threonine	0.96	3.3	0.95	1.0	0.88	8.9	0.91	4.0	0.97	4.1	0.91	6.4
Serine	0.86	4.7	0.86	1.2	0.79	8.6	0.80	3.8	0.89	3.9	0.85	10.0
Leucine	1.06	0.9	1.09	0.9	1.12	3.6	1.10	2.7	1.11	2.5	1.05	1.9
Isoleucine	0.98	1.0	0.95	3.8	1.03	6.5	0.96	3.6	0.93	17.4	0.86	16.5
Proline	0.86	0.0	0.90	0.7	0.89	3.6	0.89	1.9	0.96	5.7	0.94	9.8
Methionine	0.89	1.1	0.90	1.7	0.88	10.8	0.87	2.3	0.79	15.2	0.75	14.4
Aspartic acid	1.14	0.0	1.12	1.0	1.19	0.9	1.13	1.2	1.28	11.6	1.26	15.3
Phenylalanine	1.34	1.1	1.35	2.3	1.59	22.3	1.40	3.9	1.47	8.0	1.45	12.4
Glutamic acid	1.16	0.5	1.14	0.5	1.14	5.1	1.14	1.8	1.32	16.2	1.32	16.7
Lysine	1.04	6.7	1.02	5.4	0.78	41.8	0.91	7.3	1.16	26.4	1.21	19.8
Tyrosine	1.36	0.8	1.37	2.8	1.37	2.9	1.40	2.1	1.61	13.6	1.54	17.7
Arginine	1.01	4.7	0.96	4.6	0.53	74.9	0.86	10.8	0.78	20.6	0.87	7.6
Cystine	0.89	2.6	0.85	3.6	0.62	47.5	0.83	11.3	1.69	53.1	1.31	—

culture tubes, were added 100 μl of the amino acid solution and 100 μl of the internal standard. Culture tubes were kept in an ice bath and all solutions were stored at 4°C before use. The culture tubes were capped with PTFE-lined screw-caps and the solutions were thoroughly mixed, frozen immediately at −30°C, and lyophilized.

The lyophilized amino acid residues were prepared as the isobutyl-N(O)-heptafluorobutyryl esters[9,10]. A co-injection of heptafluorobutyric anhydride (HFBA) with each preparation of amino acid esters (dissolved in methylene chloride) was necessary to obtain repeatable relative molar ratios (RMR) for the esters of serine, arginine and cystine. With a co-injection of HFBA, the histidine ester appeared between the esters of phenylalanine and glutamic acid with a low recovery relative to the internal standard. Results for the histidine ester are, therefore, not reported. RMR, as defined by Gehrke and Stalling[11], and noted in the footnote of Table I, were calculated ($n = 3$) with cycloleucine as the internal standard. Coefficients of variation (C.V.) for the mean RMR of each amino acid were calculated according to Snedecor and Cochran[12].

The absolute, rather than relative, recoveries of individual amino acids from a cation-exchange resin (AG 50W-X8, H^+, 200–400 mesh; Bio-Rad Labs. Richmond, CA, U.S.A.) were determined in triplicate with 1 *N* ammonium hydroxide as the eluent, using the procedure of Boila and Milligan[9]. A 100-μl volume of the solution of amino acids plus 100 μl of internal standard solution were each added to a column (155 × 10 mm I.D.) of cation-exchange resin (bed-volume of 1 ml) and eluted with 1 *N* ammonium hydroxide. Recoveries of amino acids and cycloleucine from the column of cation-exchange resin were calculated using pipecolic acid as the external standard (100 μl) added to the combined fractions eluted with 10 ml 1 *N* ammonium hydroxide plus a 5-ml water wash. The combined eluate was frozen to −30°C, lyophilized and prepared fro chromatography.

RESULTS AND DISCUSSION

At a concentration of 2 *N* ammonium hydroxide or higher, mean RMR of several amino acids were different than for the treatments entailing distilled deionized water or 1 *N* ammonium hydroxide (note phenylalanine, lysine, tyrosine, arginine and cystine, Table I). A reduced RMR indicates a lower recovery of an amino acid relative to the internal standard, while an increased RMR indicates a higher recovery of an amino acid relative to the internal standard. In addition to changes in RMR, C.V. of RMR for each amino acid listed in Table I were increased which indicates that there was a greater variability in the recovery of amino acids at higher concentrations of ammonium hydroxide. This increased variability for RMR, at 2 *N* ammonium hydroxide or higher, precludes precise quantitative measurement of the concentration of amino acids in biological samples.

The amounts of the amino acids recovered from the ion-exchange chromatography using 1 *N* ammonium hydroxide as the eluant were 90%, or more, of the amounts added with the exception of lysine and arginine. Recoveries of lysine and arginine were approximately 80% with C.V. of 2.5% and 3.2%.

Clearly, the variability for RMR of individual amino acids, among triplicate analyses, can be reduced by using 1 *N* ammonium hydroxide to elute the amino acids from a sulfonated polystyrene cation-exchange resin in preparation for GLC.

At this low concentration of ammonium hydroxide the recovery of amino acids and internal standard from the resin are maximized and a repeatable RMR is obtained.

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