Journal of Chromatography, 357 (1986) 330–334
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 531

Note

Analysis of amino acids as *tert.*-butyldimethylsilyl derivatives by gas chromatography

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(First received November 25th, 1985; revised manuscript received February 4th, 1986)

N-Methyl-N-*tert.*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) is a relatively new silylation reagent which leads to the *tert.*-butyldimethylsilyl (TBDMS) derivative. Active hydrogen atoms on oxygen, nitrogen and sulfur are quickly replaced[1,2] and yield derivatives that are as much as 10 000 times more stable to hydrolysis than the corresponding trimethylsilyl derivatives[3]. TBDMS derivatives are stable enough so that the derivatives may be concentrated if necessary for improved sensitivity or they may be injected directly with the reaction solvent and reagent[4]. Separation of derivatized amino acids by gas chromatography is much quicker than with conventional amino acid analyzers and involves inexpensive, relatively simple equipment. Therefore, gas chromatographic analysis lends itself to routine analysis of many samples.

In this study MTBSTFA was used to prepare the TBDMS derivatives of the twenty amino acids commonly found in proteins as well as several sulfur-containing amino acids. The derivatized amino acids were separated on a widebore, bonded silicone phase, glass capillary column. The method was tested on two protein hydrolyzated.

EXPERIMENTAL

MTBSTFA, with 1% *tert.*-butyldimethylchlorosilane (TBDMCS) as a catalyst to facilitate derivatization of alcohols and amines, was obtained from Regis (Morton Grove, IL, U.S.A.). About 1–2 mg of dry amino acid(s) were placed in a vial and 100 μl of acetonitrile and 100 μl of MTBSTFA solution were added. Alternatively, aqueous mixtures of amino acids were added to a vial and dried in a vacuum oven at 40°C for 30 min prior to derivatization. Retention times were identified for derivatives of each individual amino acid. Quantitative reaction of most amino acids required heating at 95°C for 1 h. A mixture containing all of the amino acids was differentially derivatized by heating at increasing temperatures for 1 h at each temperature. The vial was first heated at 25°C for 1 h, a sample taken for the gas chromatograph, reheated for 1 h at 60°C, sampled once again, and so on at 70, 86, 102 112 and 125°C. Samples were thoroughly stirred with a vortex device every 15 min to facilitate their solution and, therefore, reaction. By this method, an ideal reaction temperature could be determined to allow quantitative derivatization without deg-

0021-9673/86/$03.50

radation of the derivatives. Several reaction solvents were also investigated: acetonitrile, tetrahydrofuran, carbon disulfide, and no solvent.

Derivatized amino acids were analyzed with a 60 m × 0.75 mm I.D., SPB-5 bonded silicone phase, 1 μm thick, on a wide-bore capillary glass column (Supelco, Bellefonte, PA, U.S.A.) using hydrogen as the carrier gas at 64 cm/s. On-column injection was used as recommended by some authors when analyzing amino acids on glass capillary columns[5]. Since temperature programming was used and hydrogen was used as the carrier gas, the carrier gas was regulated by flow control instead of pressure regulation to avoid a continuously falling baseline[6]. Temperature programming was used as follows (Tracor Model 560 gas chromatograph, Houston, TX, U.S.A.): 165°C for 5 min; 4°C/min to 300°C; 300°C for 5 min. Nitrogen at 40 ml/min was used as the makeup gas, hydrogen for the flame ionization detector was used at 15 ml/min (in addition to the carrier gas) and air at 300 ml/min.

To determine the precision and accuracy of the method, solutions containing approximately 2, 5, 12.5, 25, 50 or 100 μg of each amino acid and 50 μg of S-methylcysteine (internal standard) were evaporated in triplicate, derivatized at 95°C for 1 h and injected. Since the injection volume was typically 1 μl, 10–500 ng of each amino acid were actually injected into the chromatograph. By applying linear regression with the molar amount of each amino acid as the independent variable against the area of each amino acid divided by the area of S-methylcysteine (the internal standard), the calculated slope represents the molar relative detector response for a particular amino acid.

Two sample proteins, hen ovalbumin (99%, Sigma, St. Louis, MO, U.S.A.) and bovine serum albumin (96–99%, Sigma), were hydrolyzed in 6 N hydrochloric acid for 24 h in tubes first purged with nitrogen and then evacuated, then analyzed for amino acid composition as described above.

RESULTS AND DISCUSSION

Of the solvents tested, acetonitrile was by far superior in terms of amount of derivatization for a given temperature and time, as well as for the least amount of degradation as indicated by extraneous peaks. Derivatization without solvent was not as rapid as using acetonitrile and also led to more extraneous peaks.

The separation of the twenty amino acids is shown in Fig. 1 with their retention times. Norleucine and S-methylcysteine were added as internal standards. All of the twenty amino acids commonly found in proteins, except for glutamate and arginine, were resolved. Arginine was not derivatized until the reaction temperature exceeded 95°C, and when it was derivatized it gave two peaks, the larger of which co-eluted with glutamate. It is possible that they may be separable using a fused-silica column or mass spectrometer to quantitate both of these components.

As shown in Fig. 2, the relative amounts of amino acids present after reaction at each temperature of the stepwise derivatization scheme reveal that there is a trade off between quantitative derivatization and decomposition of some of the derivatized amino acids. The curve for threonine is typical for serine, lysine, and histidine; the curve for glutamine is typical for norleucine and asparagine; and the curve for valine is typical for the remaining of the twenty amino acids. Of the protein amino acids, only glutamine, and to a smaller degree, asparagine, break down under the conditions of derivatization.

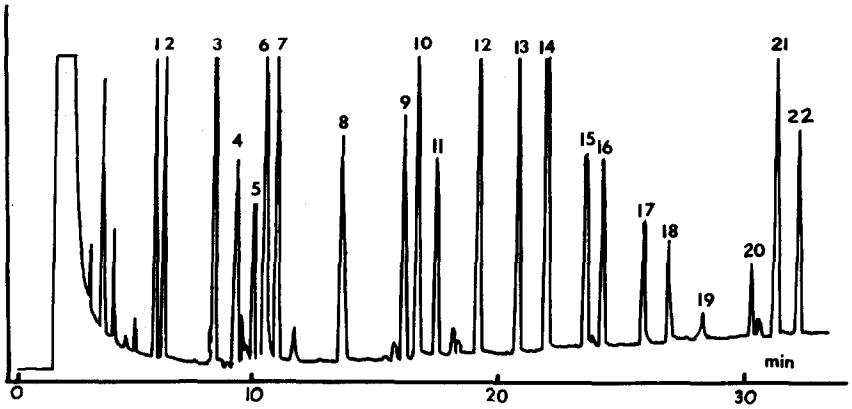

Fig. 1. Separation of the twenty common amino acids, norleucine, and S-methylcysteine. Retention times (min) are given in parentheses; some amino acids not present (NP) are also given. Amounts: 25–100 ng of each amino acid. Peaks: 1 = alanine (5.68); 2 = glycine (6.04); homocysteine thiolactone (7.09 NP); 3 = valine (8.08); 4 = leucine (9.00); partially underivatized serine (9.41 NP); 5 = isoleucine (9.77); 6 = norleucine (10.19); 7 = proline (10.72); hypotaurine (12.74 NP); 8 = S-methylcysteine (13.31); 9 = methionine (15.78); 10 = serine (16.33); 11 = threonine (17.09); ethionine (17.45 NP); 12 = phenylalanine (18.78); 13 = aspartate (20.38); 14 = cysteine (21.56); 15 = glutamate/arginine (23.11); methionine sulfone (23.62 NP); 16 = asparagine (23.83); homocysteine (24.17 NP); cysteine sulfinic acid (24.32 NP); 17 = lysine (25.47); 18 = glutamine (26.46); 19 = arginine-2 (27.75); 20 = histidine (29.92); 21 = tyrosine (31.74); 22 = tryptophan (31.74); methionine DL-sulfoxide (27.00 and 24.23 NP).

The precision and accuracy of this method as a means of quantitating free amino acids is shown in Table I. Linear regression was applied to the triplicate samples at concentrations of 10, 25, 62.5, 125, 250 or 500 ng of amino acid per μl injected after derivatization. Values for glutamate and alanine were corrected for small peaks which were present in a reagent blank. Excellent linear fits of the data were obtained for each amino acid. If any of the three values at a single concentration for a particular amino acid were not within 20% of the mean, those three values were then not included in the regression analysis. This eliminated the 10 ng/μl concentration for cysteine, glutamate, asparagine, lysine, glutamine, histidine and tryptophan, in-

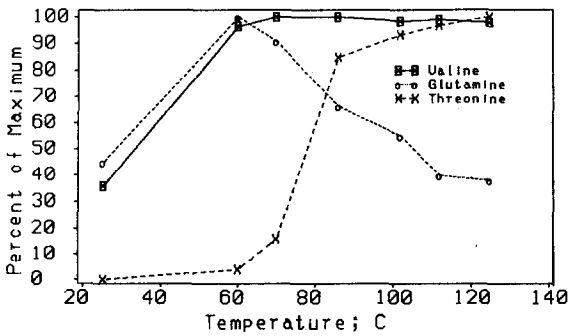

Fig. 2. Stepwise derivatization of some representative amino acids. The amino acids were held at each temperature for 1 h, sampled and heated at the next highest temperature for 1 h.

TABLE I

LINEAR REGRESSION ANALYSIS OF RELATIVE MOLAR RESPONSE AGAINST AMINO ACID CONCENTRATIONS

Relative molar response was calculated as the peak area of the amino acid divided by the peak area of the internal standard, S-methylcysteine.

Amino acid	*No. of observations*	*Slope**	*Intercept*	*Coefficient of determination*
Alanine	18	1.007 (±0.8)	0.004	0.999
Glycine	18	0.893 (±1.2)	0.006	0.999
Valine	18	1.106 (±1.4)	0.016	0.999
Leucine	18	1.135 (±1.4)	0.021	0.999
Isoleucine	18	1.171 (±1.4)	0.031	0.999
Proline	18	1.095 (±2.0)	−0.047	0.999
Methionine	18	1.020 (±1.5)	0.015	0.999
Serine	18	1.293 (±0.8)	−0.004	0.999
Threonine	18	0.876 (±5.4)	0.003	0.993
Phenylalanine	18	1.371 (±0.8)	0.015	0.999
Aspartate	18	1.412 (±1.1)	0.010	0.999
Cysteine	12	1.236 (±1.3)	−0.029	0.999
Glutamate	12	1.209 (±4.9)	0.056	0.997
Asparagine	15	1.023 (±2.2)	−0.049	0.999
Lysine	15	1.325 (±2.7)	−0.056	0.999
Glutamine	15	0.885 (±5.3)	−0.096	0.994
Histidine	12	1.118 (±5.2)	−0.187	0.996
Tyrosine	18	1.723 (±4.2)	−0.007	0.996
Tryptophan	15	1.160 (±4.2)	−0.054	0.996

* Numbers in parentheses indicate the 95% confidence interval of the slope as % of slope.

TABLE II

AMINO ACID ANALYSIS OF BOVINE SERUM ALBUMIN AND HEN OVALBUMIN

Amino acid	*Composition of bovine serum albumin (%)*		*Composition of hen ovalbumin (%)*	
	Experimental	*Literature**	*Experimental*	*Literature***
Alanine	4.44	4.99	4.89	5.36
Glycine	1.46	1.38	2.39	2.32
Valine	4.46	5.01	5.07	5.96
Leucine	8.58	10.59	6.48	7.94
Isoleucine	1.96	2.25	4.10	6.04
Proline	3.90	4.01	2.62	3.04
Methionine	0.68	0.71	3.40	1.57
Serine	3.01	3.50	6.11	6.75
Threonine	3.75	4.95	3.00	3.42
Phenylalanine	4.88	5.87	5.36	6.86
Aspartate	7.44	9.43	7.78	8.04
Glutamate	10.97	14.48	10.93	14.48
Lysine	12.00	11.25	6.67	5.52
Histidine	2.54	3.54	1.61	2.08
Tyrosine	3.84	4.56	3.41	3.33

* From refs. 7 and 8.

** From ref. 9.

dicating that the methods may not be sensitive at this level for these particular amino acids. The 25 ng/μl values for histidine and glutamate were also excluded. Cysteine concentration at 500 ng/μl was not within the linear range; however, all other amino acids tested exhibited a linear response through a maximum concentration of 500 ng/μl.

The excellent resolution and linear response makes this method suitable for analysis of biological samples. The method has been applied to the determination of the amino acid composition of bovine serum albumin and hen ovalbumin (Table II). There is good agreement between the literature values and those obtained by this method. S-Methylcysteine made a good internal standard as it was stable to hydrolysis and its derivatization product is also stable; furthermore, it is usually not found in biological samples in appreciable quantities.

Besides increased stability over trimethylsilyl derivatives, TBDMS derivatives of amino acids offer several advantages over other derivatives used for gas chromatography. It is a simple (one-step) and rapid derivatization process. This derivative has interesting application to gas chromatography–mass spectrometric analysis due to the frequently prominent ion at 57 units less than the molecular ion[10]. Amino acid analysis has not been studied extensively by capillary gas chromatography of the TBDMS derivatives. The method described here should provide for a straightforward approach to be taken in amino analysis of a wide array of biological media.

ACKNOWLEDGEMENTS

This work was supported by USPHS Grants AM 21712 (J.A.M.), AM 32521 (R.D.S.), USDA Grant 82CRCR-1-1153 (R.D.S.) and by the College of Agricultural and Life Sciences of the University of Wisconsin-Madison.

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