Journal of Chromatography, 130 (1977) 151–160

CHROM. 9347

GAS–LIQUID CHROMATOGRAPHY OF THE N-HEPTAFLUOROBUTYRYL ISOBUTYL ESTERS OF FIFTY BIOLOGICALLY INTERESTING AMINO ACIDS

ROLAND J. SIEZEN* and TIMOTHY H. MAGUE**

Institute of Marine Resources, University of California at San Diego, La Jolla, Calif. 92093 (U.S.A.)

(First received March 1st, 1976; revised manuscript received May 10th, 1976)

SUMMARY

Fifty amino acids are analyzed as their N-heptafluorobutyryl isobutyl esters by gas chromatography on a column of 3% OV-101 on Gas-Chrom Q.

The identification and quantification of unusual amino acids, specifically those occurring in the marine environment, in the presence of common protein amino acids is reported.

Regular relationships between elution temperatures and amino acid structures are found, thus allowing the estimation of elution temperatures of other amino acids.

INTRODUCTION

Hušek and Macek[1] have recently reviewed the present knowledge of the gas–liquid chromatography of amino acids. Good single-column separations of protein amino acid derivatives include the analysis of the N-heptafluorobutyryl (HFB) *n*-propyl esters, as described by Moss and co-workers[2,3], and the analysis of the N-HFB isoamyl esters, as introduced by Zanetta and Vincendon[4] and later modified and supplemented with mass spectral data by Felker and Bandurski[5]. However, biological materials with a relative abundance of aspartic and glutamic acids cannot be analysed satisfactorily using these derivatives, because aspartic and glutamic acids do not separate well enough from methionine, phenylalanine and tyrosine.

A superior method, using the N-HFB isobutyl esters, has since been developed by MacKenzie and Tenaschuk[6–8].

We are currently applying this method in order to identify and quantify free and bound amino acids in marine phytoplankton cultures, in marine sediments and in dissolved and suspended organic matter in sea water[9]. The suspended material with a diameter of about 0.5–200 μm is customarily referred to as "particulate

* Present address: Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands.

** Present address: Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine 04575, U.S.A.

matter", and it consists primarily of detritus (dead organisms and their fragments) and living phytoplankton and bacteria. Particulate organic matter is an extremely important part of the marine food chain, and knowledge of its chemical composition is required in order to study the food balance in the sea.

Many non-protein amino acids occur in the marine environment, such as ornithine[10,11], β-alanine[11], hydroxylysine[11], 3,4-dihydroxyphenylalanine[11], citrulline[12], argininosuccinic acid[12], 2-aminoadipic acid[13], 2-aminobutyric acid[13], 2-aminoisobutyric acid[13], 2,5-diaminoadipic acid[13], 4-aminobutyric acid[13], 2,6-diaminopimelic acid[14], 3,4-dihydroxyproline[15], 6-N-trimethylhydroxylysine[16] and iodotyrosines[17]. The detection of minute concentrations of these components prompted this study of the gas chromatography of rarely occurring, biologically interesting amino acids. The results are of general interest for the rapid determination of trace amounts of these amino acids in plant and animal tissues, sera and urines.

EXPERIMENTAL

Reagents and glassware

Single amino acids were obtained from Sigma (St. Louis, Mo., U.S.A.), except 3,4-dihydroxyproline and 6-N-trimethyl-5-hydroxylysine, which were a gift from Dr. B. E. Volcani. Solutions containing 2.5 μmole/ml of each individual amino acid were prepared in 0.1 *N* HCl. Amino acid standard mixture (2.5 μmole/ml in 0.1 *N* HCl), heptafluorobutyric anhydride (HFBA) and acetonitrile (silylation grade) were from Pierce (Rockford, Ill., U.S.A.).

Isobutanol, dichloromethane and ethyl acetate (all Mallinckrodt, St. Louis, Mo., U.S.A.) were redistilled from an all-glass apparatus after refluxing for 2–6 h over anhydrous calcium chloride or magnesium turnings. Isobutanolic HCl was prepared by bubbling anhydrous hydrogen chloride through a trap with concentrated sulfuric acid and then into isobutanol at 0° until the solution was 3 *N* in HCl as determined by weighing. Acetonitrile and isobutanol–3 *N* HCl were stored in a nitrogen atmosphere at −20°. Aliquots were taken by inserting a syringe needle through a rubber septum. To prevent deterioration of HFBA, 200-μl portions were dispensed in ampoules, which were sealed after flushing with dry nitrogen and stored at −20°.

All glassware was heated at 450–500° for 2–6 h before use so as to remove any contaminating organic material.

Preparation of amino acid derivatives

Derivatization was performed in 1-ml Reactivials capped with Teflon Mininert valves (Pierce). The Reactivials were heated during derivatization and evaporation in a Reacti-therm heating module (Pierce). A small amount of silicone oil was added to each vial compartment in order to speed heat transfer to the vial. Samples containing 25–250 nmoles of each amino acid were dried at 60° by inserting a needle through the valve and blowing dry nitrogen through the vial. Subsequently, the last traces of water were removed azeotropically with 100 μl of dichloromethane.

For esterification of the amino acids, 100 μl of isobutanol–3 *N* HCl was added to the vial, which was then flushed with nitrogen before closing the valve. The vial was placed in an ultrasonic bath (40°) for 10 min so as to dissolve the amino acids, and then heated at 110° for 30 min. Only the lower portion of the vial was submerged

in the silicone oil, thus allowing the sample to reflux. The sample was dried at 60° with dry nitrogen and re-dried after addition of 100 μl of dichloromethane.

For acylation, 40 μl of acetonitrile and 20 μl of HFBA were added and the vial was flushed with nitrogen before closing the valve. The vial was then heated at 110° for 20 min. After cooling, the sample was taken just to dryness with a stream of nitrogen at room temperature, and redissolved in an appropriate volume of anhydrous ethyl acetate.

Gas chromatography

A Varian Aerograph 1400-10 gas chromatograph equipped with a flame-ionization detector and a linear temperature programmer was used. Samples were analysed on a 6 m × 2 mm I.D. coiled glass column packed with 3% OV-101 on Gas-Chrom Q, 80–100 mesh (Applied Science Labs., State College, Pa., U.S.A.). We coated the support material with OV-101 with the aid of a fluidizer[18].

The chromatographic conditions were: injector temperature, 225°; detector temperature, 285°; temperature program, 80°, isothermal for 5 min, then 80–250° at 6°/min and finally 250° isothermal; carrier gas (nitrogen) flow-rate, 18 ml/min.

Injection of 1–10 nmoles of each amino acid, dissolved in 1–2 μl of ethyl acetate, gave excellent results. If histidine was present in the sample, acetic anhydride (Mallinckrodt) was injected directly after the sample, or added to the sample before injection. In both instances the volume ratio of sample to acetic anhydride was approximately 2:1 (see ref. 6).

Quantification of peak areas was performed by using a Varian Aerograph 480 electronic digital integrator and a Hewlett-Packard 3373B integrator simultaneously. The molar response of each derivative relative to the norleucine internal standard (RMR_{NLE}) was calculated.

RESULTS AND DISCUSSION

Fig. 1A shows the separation of the N-HFB isobutyl esters of the protein amino acids on 3% OV-101/Gas-Chrom Q. Each peak represents about 2 nmoles of amino acid. A sharp peak for histidine is found only if the sample is co-injected with acetic anhydride, which causes an on-column conversion of the monoacyl into the diacyl derivative[1,6,19]. MacKenzie and Tenaschuk[6] obtained almost identical results on a 3% SE-30/Gas-Chrom Q column.

Table I summarizes the retention times, retention temperatures and relative molar responses of 50 amino acid derivatives. Each relative molar response is an average of at least three determinations. For the protein amino acids the standard deviations were less than 5% (5–8 determinations), except those for cysteine, cystine, arginine and histidine, which were about 20–30%. A higher acylation temperature should give more constant molar responses[1,5,8], but for the sake of convenience both esterification and acylation were carried out at 110° in this study.

The separation of eighteen biologically interesting non-protein amino acids is illustrated in Fig. 1B. Tyrosine occurs as a contaminant of 3-iodotyrosine (MIT). The two peaks for hydroxylysine (HYL) represent the DL isomers and DL-*allo*-isomers[3,7], but we have no explanation for the homoserine (HSER) double peak. Obviously, some of these amino acids will interfere with the common protein amino

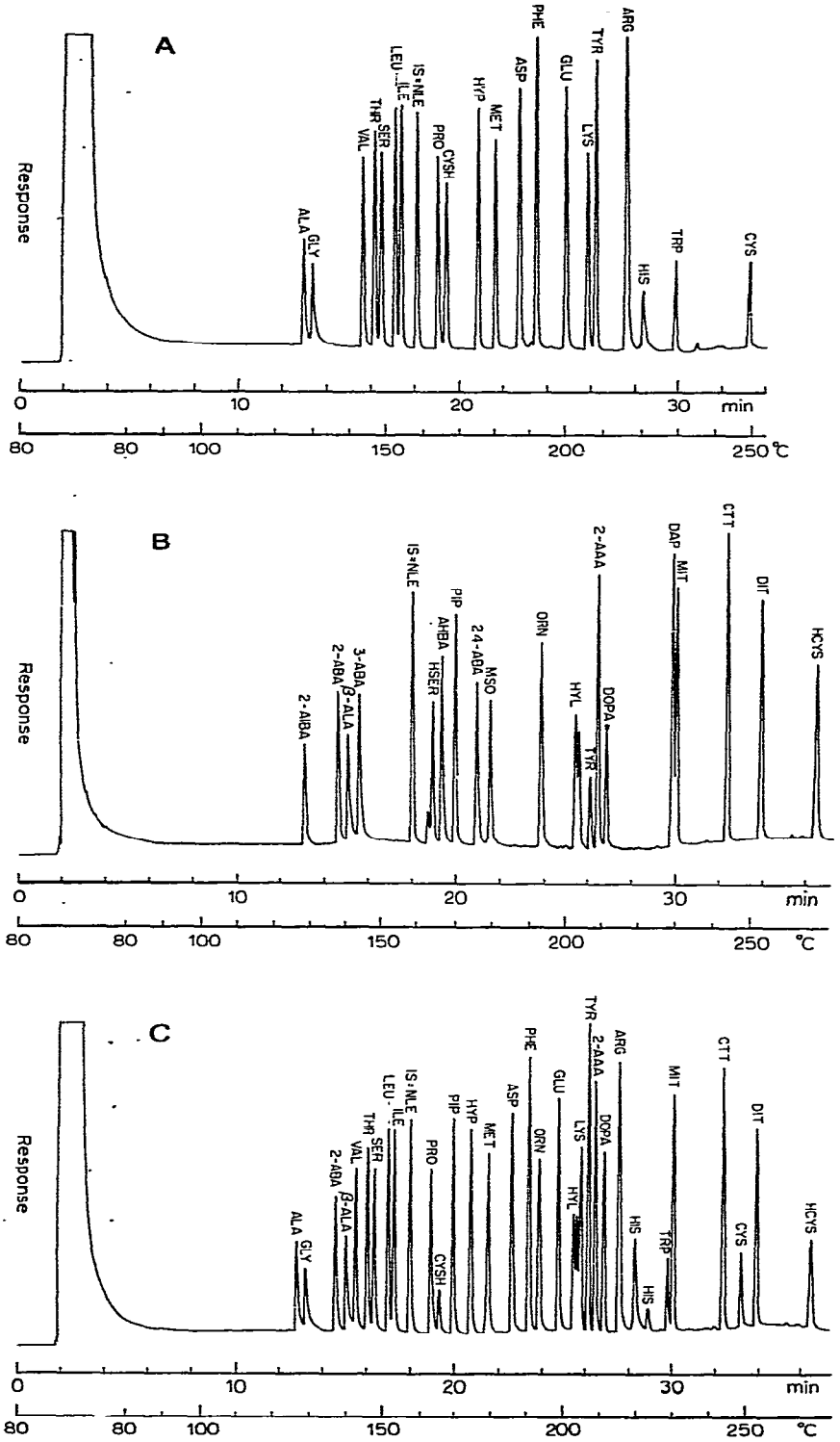

Fig. 1. Gas chromatographic separations of the N-HFB isobutyl esters of amino acids (for abbrevia tions see Table I) on a 6 m × 2 mm I.D. glass column packed with 3% OV-101 on Gas-Chrom Q. Temperature program: 5 min isothermal at 80°, followed by 6°/min to 250°, and then isothermal at 250°. Other column conditions are given in the text. A mixture containing 100 nmoles of each amino acid was derivatized. Each peak represents about 2 nmoles of amino acid derivative. The sample was injected in 1 μl of ethyl acetate, followed by injection of 0.5 μl of acetic anhydride in (A) and (C).

TABLE I

RETENTION TIME, RETENTION TEMPERATURE AND RELATIVE MOLAR RESPONSE (RMR_{NLE}) OF N-HFB ISOBUTYL ESTERS OF AMINO ACIDS

All are L-amino acids unless otherwise specified. Each non-protein amino acid was derivatized separately and in mixtures of amino acids.

Amino acid	*Abbreviation*	*Time (min)*	*Temperature (°C)*	*RMR_{NLE} value*
Cysteic acid		12.2	123	0.14*
Alanine	ALA	12.8	127	0.55
2-Aminoisobutyric acid	2-AIBA	13.0	128	0.48
Glycine	GLY	13.2	129	0.50
2-Aminobutyric acid	2-ABA	14.6	137.5	0.75
Sarcosine		14.7	138	0.56
β-Alanine	β-ALA	15.1	140.5	0.63
Valine	VAL	15.5	143	0.83
DL-3-Aminobutyric acid	3-ABA	15.6	143.5	0.72
DL-3-Aminoisobutyric acid		15.6	143.5	0.75
Threonine	THR	16.1	146.5	0.93
Norvaline	NVA	16.2	147	0.83
Serine	SER	16.4	148.5	0.84
Leucine	LEU	17.0	152	1.00
Isoleucine	ILE	17.3	154	0.98
Norleucine (internal standard)	NLE	18.0	158	1
4-Aminobutyric acid	4-ABA	18.0	158	0.8**
Homoserine	HSER	18.8	163	0.12
		19.0	164	0.61***
Proline	PRO	19.0	164	0.85
DL-4-Amino-3-hydroxyb utyric acid	AHBA	19.4	166.5	0.90
Cysteine	CYSH	19.4	166.5	0.5§
S-Methylcysteine	SMC	19.5	167	0.76
3,4-Dihydroxyproline		19.7	168	1.5§§
DL-Pipecolic acid	PIP	20.0	170	0.94
4-Hydroxyproline	HYP	20.8	175	0.95
2,4-Diaminobutyric acid	2,4-ABA	21.0	176	0.80
Methionine	MET	21.6	179.5	0.86
Methionine sulfoxide	MSO	21.6	179.5	0.56
Asparagine/aspartic acid	ASP	22.7	186	1.08
Phenylalanine	PHE	23.5	191	1.28
Ornithine	ORN	23.9	193.5	0.94
Citrulline		23.9	193.5	0.48§§§
Glutamine/glutamic acid	GLU	24.8	199	1.10
5-Hydroxylysine (DL and DL-*allo*)	HYL	25.5	203	1.10
		25.6	203.5	
Lysine	LYS	25.8	205	0.97
Tyrosine	TYR	26.2	207	1.28
DL-2-Aminoadipic acid	2-AAA	26.5	209	1.20
3,4-Dihydroxyphenylalanine	DOPA	26.9	211.5	1.0†
Arginine	ARG	27.6	215.5	2.2§
S-Carboxymethylcysteine		28.0	218	1.08
Histidine	HIS	28.3	220	0.6§,††
Tryptophan	TRP	29.9	229.5	0.45†††
DL-2,6-Diaminopimelic acid	DAP	29.9	229.5	1.20
3-Iodotyrosine	MIT	30.0	230.5	1.03

(Continued on p. 156)

TABLE I (*continued*)

Amino acid	*Abbreviation*	*Time (min)*	*Temperature (°C)*	*RMR_{NLE} value*
Cystathionine	CTT	32.4	244.5	1.22
Cystine	CYS	33.2	249	0.7§
3,5-Diiodotyrosine	DIT	34.0	250 (isothermal)	1.00
DL-Homocystiine	HCYS	36.5	250 (isothermal)	0.90

* The unexpectedly early elution and low RMR_{NLE} value for cysteic acid were continuously found.

** As 4-aminobutyric acid elutes at the same position as norleucine, its RMR_{NLE} value was determined indirectly by comparing its molar response with those of amino acids other than the internal standard.

*** Homoserine elutes as a double peak for unknown reasons.

§ Recovery of cysteine, cystine, arginine and histidine is variable (see text).

§§ The RMR_{NLE} value for 3,4-dihydroxyproline is very approximate, because the exact concentration of this amino acid in solution was not known.

§§§ Citrulline breaks down during derivatization, yielding ornithine. The lower RMR_{NLE} value for citrulline suggests that this conversion is incomplete.

† The RMR_{NLE} value for 3,4-dihydroxyphenylalanine is approximate, as the derivative is unstable, decaying at least 50% within 24 h.

†† A sharp peak for histidine is found only when the sample is co-injected with acetic anhydride. This is the diacyl derivative, and the monoacyl derivative sometimes gives a low recovery at 28.9 min (223.5°), as shown in Fig. 1C.

††† The low RMR_{NLE} value for tryptophan is due to partial degradation during acidic esterification.

acids. However, we found that the following seventeen amino acids can be identified and quantified in the presence of protein amino acids: cysteic acid, 2-aminobutyric acid, sarcosine, β-alanine, norleucine, 4-aminobutyric acid, 3,4-dihydroxyproline, pipecolic acid, 2,4-diaminobutyric acid, ornithine/citrulline, 2-aminoadipic acid, 3,4-dihydroxyphenylalanine, S-carboxymethylcysteine, 3-iodotyrosine, cystathionine, 3,5-diiodotyrosine and homocystine. Fig. 1C shows an example of the separation of equimolar amounts of 32 protein and non-protein amino acids. In this chromatogram two peaks can be seen for histidine, indicating an incomplete conversion of the monoacyl into the diacyl derivative. Norvaline, 2-aminoisobutyric acid, 3-aminoisobutyric acid and 3-aminobutyric acid can be detected, but they separate poorly from the protein amino acids. Of all the amino acids studied here, only three co-elute with a protein amino acid: homoserine with proline, 4-amino-3-hydroxybutyric acid with cysteine, and 2,6-diaminopimelic acid with tryptophan. Methionine sulfoxide and S-methylcysteine co-elute with the amino acids from which they are derived. Citrulline breaks down to ornithine during derivatization. No detectable peaks are found for taurine, 6-N-trimethyl-5-hydroxylysine and argininosuccinic acid, either because the derivatives are unstable or are not eluted from the column.

Some of these non-protein amino acids have not been studied before by gas chromatography, whereas others have been analysed as their N-trifluoroacetyl (TFA) *n*-butyl esters[20–23]. Both methods are complementary, in other words certain amino acids can be separated as their N-HFB isobutyl esters and not as their N-TFA *n*-butyl esters, and *vice versa*.

In Table II, modifications in amino acid structure are correlated with changes in elution temperature of the N-HFB isobutyl esters. Some general rules are evident:

all the structural modifications listed bring about an increase in the elution temperature, with the exception of chain branching, which gives a 5–10° lower elution temperature. The largest increase (40–50°) is found with the introduction of an additional amino group or carboxyl group, due to the accompanying introduction of another acyl or ester group in the derivative. Chain elongation by one methylene group usually gives an upward shift of 10–15°. There are a few exceptions, such as the chain elongations glycine to alanine (—2°), serine to threonine (—2°) and cysteine to S-methylcysteine (+0.5°).

By extrapolating in Table II we can make reasonable estimates of the elution positions of other amino acids: a few predictions are given in italics, most of which are based on the 10° increase for chain elongation by one carbon atom. The estimation of molar responses is also possible, as linearity of response with carbon number has been shown for the N-TFA *n*-butyl esters[22] and the N-TFA methyl esters[24] of amino acids possessing the same reactive groups. However, conditions should be selected so that maximum yields of amino acid derivatives can be obtained, and this was beyond the scope of the work presented here.

The identification of unknown peaks in a gas chromatogram is more complicated. Although Table II can be used to narrow down the possibilities, mass spectrometry must be applied in order to elucidate the structure.

The results of an application of this gas chromatographic method of analysis to a protein hydrolysate are shown in Fig. 2 and Table III. We determined the amino acid composition of organic particulate matter filtered from surface water at Scripps pier and at a station 3 miles offshore from the pier. Glycine and glutamic acid occur

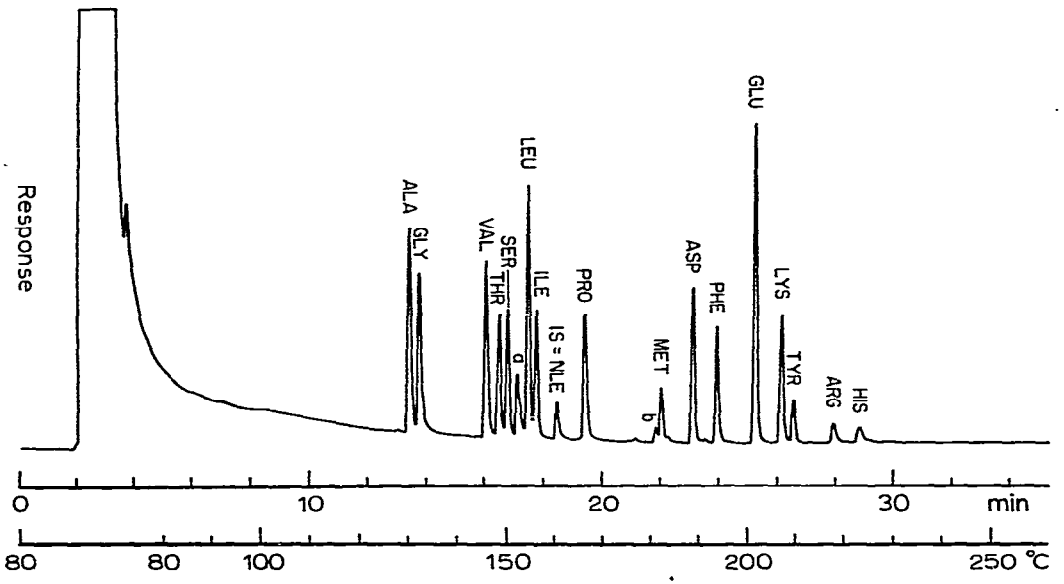

Fig. 2. Gas chromatographic separation of the N-HFB isobutyl esters of the amino acids from particulate matter filtered from Scripps pier surface water (see Table III for isolation procedure). Column conditions were as in Fig. 1. In this instance 1 μl of ethyl acetate containing 0.5% of the sample was injected, followed by 1 μl of acetic anhydride. The internal standard peak represents about 0.6 nmole of norleucine.

TABLE II

ELUTION TEMPERATURES (°C) OF N-HFB ISOBUTYL DERIVATIVES IN RELATION TO AMINO ACID STRUCTURE

The temperatures in italics are estimates. Abbreviations (see also Table I): 2-AHA = 2-aminoheptanoic acid, 2-AOA = 2-aminooctanoic acid, HILE = homoisoleucine, 2-APA = 2-aminopimelic acid, SEC = S-ethylcysteine, SEHC = S-ethylhomocysteine, HCYSH = homocysteine, ACA = azetidine-2-carboxylic acid, HPIP = hydroxypipecolic acid, DAA = 2,5-diaminoadipic acid, LAN = lanthionine, 2-ASA = 2-aminosuberic acid.

Modification	*Chain elongations*						
None (basic structures)	GLY (129)	ALA (127)	2-ABA (137.5)	NVA (147)	NLE (158)	2-AHA (*168*)	2-AOA (*178*)
Carbon chain branching			2-AIBA (128)	VAL (143)	LEU (152) ILE (154)	HILE (*162*)	
$-NH_2$ shift			3-ABA (143.5) 4-ABA (158)				
		β-ALA (140.5)					
$-NH_2$ addition			2,4-ABA (176)	ORN (193.5)	LYS (205)		
-OH addition		SER (148.5)	THR (146.5) HSER (164)				
$-CH_3 \rightarrow -COOH$			ASP (186)	GLU (199)	2-AAA (209)	2-APA (*219*)	2-ASA (*229*)
$-CH_2- \rightarrow -S-$			CYSH (166.5)	SMC (167) *HCYSH (179)*	SEC (*177*) MET (179.5)	SEHC (*190*)	
Cyclization			ACA (*158*)	PRO (164)	PIP (170)		
$-NH_2$ shift, -OH addition			AHBA (166.5)				
$-NH_2$ and -OH addition					HYL (203)		
Cyclization, -OH addition				HYP (175)	HPIP (*181*)		
$-CH_3 \rightarrow -COOH$, $-NH_2$ addition					DAA (*219*)	DAP (229.5)	
$-CH_3 \rightarrow -COOH$, $-NH_2$ addition $-CH_2- \rightarrow -S-$						LAN (*235*)	CTT (244.5) CYS (249)

TABLE III

AMINO ACID COMPOSITION OF PARTICULATE MATTER

Particulate matter (0.5–200 μm) was filtered from 2 l of sea water and hydrolysed with 6 *N* HCl for 20 h at 110° after addition of internal standard (norleucine). The amino acids were subsequently obtained by AG 50W-X8 cation-exchange chromatography and derivatized as described in the text (see Fig. 2 for gas chromatogram). As several protein amino acids are known to decompose partly during hydrolysis, the relative molar responses were determined for a standard mixture of amino acids after hydrolysis, ion-exchange chromatography and derivatization. These molar responses (not shown) differed slightly from those given in Table I and were used in calculating amino acid compositions of hydrolysates. Because the recoveries of cysteine, cystine and arginine were variable and often negligible, these amino acids are not included.

Amino acid	*Scripps pier surface water (Feb. 4, 1975)*		*3 miles offshore Scripps pier surface water (Feb. 11, 1975)*	
	(nmole/l)	*Residues per 100 residues*	*(nmole/l)*	*Residues per 100 residues*
Ala	482	10.6	96	9.0
Gly	518	11.3	150	14.0
Val	418	9.2	63	5.9
Thr	257	5.6	55	5.1
Ser	290	6.4	92	8.6
a*	133	2.9	2	0.2
Leu	373	8.2	77	7.2
Ile	288	6.3	45	4.2
Pro	229	5.0	43	4.0
Hyp	4	0.1	2	0.2
b*	22	0.5	0	0
Met	107	2.3	27	2.5
Asp	313	6.9	112	10.4
Phe	171	3.7	42	3.9
Glu	544	11.9	135	12.6
Lys	243	5.3	72	6.7
Tyr	64	1.4	37	3.4
Arg	nd**	nd**	nd**	nd**
His	108	2.4	23	2.1
Trp	nd**	nd**	nd**	nd**
Cys	nd**	nd**	nd**	nd**
Total	4564	100.0	1073	100.0

* a, b = unknown compounds; concentrations calculated assuming $RMR_{NLE} = 1.0$.
** nd = not determined.

in the highest concentrations, followed by alanine, leucine, aspartic acid and valine. These amino acid compositions resemble those obtained for various species of marine phytoplankton in culture[11,13]. The pier water was taken during a "red tide" bloom of the dinoflagellate *Gonyaulax polyedra*, which probably accounts for the more than 4-fold higher total concentration of amino acids. The amino acid composition of this particulate matter possibly reflects the composition of this organism. This sample contains 3% of component (a), which elutes between serine and leucine at 150°, and a smaller amount of component (b), eluting just before methionine at 179°. Both can be uncommon amino acids specific for *Gonyaulax*. In this particular case, mass

spectrometry must be included in order to identify these extra components, as they do not correspond to any of the 50 amino acids investigated here.

ACKNOWLEDGEMENTS

We are greatly indebted to Dr. Osmund Holm-Hansen for allowing us to perform this work in his laboratory. Dr. B. E. Volcani kindly gave us 3,4-dihydroxyproline and 6-N-trimethylhydroxylysine. We thank Dr. H. J. Hoenders and Mr. H. A. Kramps for critically reading the manuscript.

This work was supported in part by the U.S. Atomic Energy Commission Contract No. AT(11-1) GEN, P.A. 20, and by NATO-fellowship N84-117 awarded to R.J.S. by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

REFERENCES

1 P. Hušek and K. Macek, *J. Chromatogr.*, 113 (1975) 139.
2 C. W. Moss, M. A. Lambert and F. J. Diaz, *J. Chromatogr.*, 60 (1971) 134.
3 C. W. Moss and M. A. Lambert, *Anal. Biochem.*, 59 (1974) 259.
4 J. P. Zanetta and G. Vincendon, *J. Chromatogr.*, 76 (1973) 91.
5 P. Felker and R. S. Bandurski, *Anal. Biochem.*, 67 (1975) 245.
6 S. L. MacKenzie and D. Tenaschuk, *J. Chromatogr.*, 97 (1974) 19.
7 S. L. MacKenzie and D. Tenaschuk, *J. Chromatogr.*, 104 (1975) 176.
8 S. L. MacKenzie and D. Tenaschuk, *J. Chromatogr.*, 111 (1975) 413.
9 R. J. Siezen and T. H. Mague, submitted for publication.
10 O. Holm-Hansen and R. A. Lewin, *Physiol. Plant.*, 18 (1965) 418.
11 E. T. Degens, in D. W. Hood (Editor), *Organic Matter in Natural Waters*, Institute of Marine Science, College, Alaska, 1970, p. 77.
12 L. Fowden, in R. A. Lewin (Editor), *Physiology and Biochemistry of Algae*, Academic Press, New York, London, 1962, p. 189.
13 Y. K. Chau, L. Chuecas and J. P. Riley, *J. Mar. Biol. Assoc. U.K.*, 47 (1967) 543.
14 O. Holm-Hansen, R. Prasad and R. A. Lewin, *Phycologia*, 5 (1965) 1.
15 T. Nakajima and B. E. Volcani, *Science*, 164 (1969) 1400.
16 T. Nakajima and B. E. Volcani, *Biochem. Biophys. Res. Commun.*, 39 (1970) 28.
17 R. Scott, *Nature (London)*, 173 (1954) 1098.
18 R. J. Leibrand and L. L. Dunham, *Res. Develop.*, (Sept. 1973) 32.
19 D. Roach, C. W. Gehrke and R. W. Zumwalt, *J. Chromatogr.*, 43 (1969) 311.
20 D. J. Casagrande, *J. Chromatogr.*, 49 (1970) 537.
21 F. Raulin, P. Shapshak and B. N. Khare, *J. Chromatogr.*, 73 (1972) 35.
22 M. Sakamoto, K.-I. Kajiyama and H. Tonami, *J. Chromatogr.*, 94 (1974) 189.
23 V. Amico, G. Oriente and C. Tringali, *J. Chromatogr.*, 116 (1976) 439.
24 A. Islam and A. Darbre, *J. Chromatogr.*, 71 (1972) 223.