Journal of the Oceanographical Society of Japan
Vol. 27, No. 1, pp. 27 to 33, February 1971

Gas-liquid Chromatographic Determination of Amino Acids and Vertical Distribution of Proteinaceous Substances in Sea Water*

Hosaku KAWAHARA** and Yoshiaki MAITA**

Abstract: Gas-liquid chromatographic method by GEHRKE *et al.* was applied to the determination of the dissolved proteinaceous substace. Sea-water samples were evaporated in vacuum to the volume of one twentieth of the initial volume and separated from deposited inorganic salt crystals. Thirteen kinds of amino acids except histidine, arginine and cystine were quantitatively determined for artificial sea water containing sixteen standard amino acids with accuracy of about five percent and with yield of 80 to 103 percent. This method has advantage comparing with paper or thin layer chromatography on the precision and comparing with ion-exchange method on sensitivity.

The difference of amino acid concentration between the sea-water samples of different depth and different region was observed. The total amounts of dissolved amino acids were found to be between 33 and 92 μg per liter in sea water of Pacific Ocean taken at the surface to 1,500 m in depth.

1. Introduction

It was assumed that proteinaceous substances are present in sea water, since albuminoid nitrogen was detected in surface water by NATTELER (1892). Despite of the importance on the nitrogen cycle of ocean water there is little information about the distribution of proteinaceous substances. The difficulty on the determination of the dissolved amino compounds present in sea water is caused by removing a large amount of inorganic salts from the minor organic constituents. Recently, sensitive methods were developed and various organic substances dissolved in natural water were identified.

GEHRKE and Co-workers (1965~1967) have established the quantitative determination of amino acids by gas-liquid chromatography. We applied their method to the amino acid analysis of sea water. The application of this method to the amino acids in sea water has never been published. As pointed out by several authors the determination of amino acids by paper and thin layer chromatography always accompanies the error of about ten percent order. The automatic analysis of amino acids is the most accurate method. In this method 0.1 to 4.0 micromoles of each amino acids are usually used for each analysis so that large amounts are required for very dilute samples as sea water (SPACKMAN *et al.*, 1958).

TATSUMOTO *et al.* (1961) have semiquantitatively determined thirteen protein amino acids in the surface sea water of Gulf of Mexico and other areas by the coprecipitation of organic matter with ferric hydroxide. PARK *et al.* (1962), using the same procedure, detected approximately seventeen different amino acids in sea water taken from depths of up to 3,500 m at the central Gulf of Mexico. PALMORK (1963) has applied to sea water the dinitrophenyl method for the identification of N-terminal amino acid of protein developed by SANGER (1945). CHAU *et al.* (1966) have made a comparison between the several methods of concentrating the amino acids from sea water. They have described that very poor recoveries were obtained by coprecipitation with ferric hydroxide, while high recoveries were obtained by the procedure of PALMORK (1963). SIEGEL *et al.* (1966) determined the concentration of dissolved amino acids from saline waters by ligand exchange. Recently, STARIKOVA *et al.* (1969) examined the amino acids in the Black Sea by paper chromatography.

* Received December 21, 1970
Contribution No. 41, from The Research Institute of North Pacific Fisheries, Hokkaido University
** Faculty of Fisheries, Hokkaido University

2. Experimental methods

Evaporation of sea water: Two to five liters of filtered sea-water sample were directly evaporated until one twentieth of the initial volume by a rotary vacuum evaporator at a temperature lower than 60°C, removing the precipitated salts by filtration. Residues were washed with the mixture of ethanol, water, and concentrated hydrochloric acid, 80:20:1 by volume. Filtrates and washings were reduced in volume and were completely dried. In the case of the higher evaporation ratio than twenty the obtained viscous solution prevents the formation of coarse crystals that can be separated easily, and drying up of the mother liquor containing amino acids.

Hydrolysis of proteinaceous substances: The concentrates of sea water were subjected to hydrolysis in 6N-hydrochloric acid for 24 hours at 100°C. After hydrolysis the acid was removed as completely as possible under vacuum. Residues were dissolved by small amount of distilled water and were adjusted to the pH 2 with 2N-sodium hydroxide. These solutions were passed through an ion exchange resin of Dowex 50×8, hydrogen type, in a column 2.5 cm in diameter and 50 cm in length. The loaded column was washed with 0.01 N-hydrochloric acid and was eluted with two liters of 2N-ammonia solution. Eluates were completely evaporated under vacuum at a temperature lower than 60°C.

Esterification of amino acids: The concentrates were esterified in 10 m*l* of methanol containing 1.2±0.1 milliequivalent of anhydrous hydrogen chloride per m*l* for 30 minutes at room temperature. The methanol was removed by vacuum distillation at 60°C. The methylesters were converted to the n-butyl ester by 10 m*l* of n-butanol containing 1.2±0.1 milliequivalent of anhydrous hydrogen chloride per m*l* for 180 minutes at 90±3°C. The butanol was removed by vacuum distillation at 60°C. The n-butylester hydrochlorides were then trifluoroacetylated by adding 2.5 m*l* of dimethylchloride and 0.5 m*l* of anhydrous trifluoroacetic acid. The temperature of the oil bath was maintained at 150±5°C. Sealed glass tube containing the butylester of samples and the reagents described above was immersed in oil bath during five to fifteen minutes. The solvent and excess acid were removed by vacuum distillation at room temperature.

Reagents used in this experiment are guaranteed grade which obtained from Wako Pure Chemicals Co. Ltd. Methanol and butanol were dried on calcium oxide and magnesium turning prior to use. The other organic solvents were also purified using the fractionating column of one meter in length filled with fillers "Heli Pack".

Gaschromatography: Instrument used is the programmed-temperature gas chromatograph with the flame ionization detector of Hitachi Seisakusho Model K-23. Esterified samples were dissolved in 0.5 m*l* of chloroform and the known amount of internal standard was added prior to analysis.

3. Results

(A) Determination of recovery of amino acids from artificial sea water: For the pur-

Table 1. Recoveries of protein amino acids from artificial sea water.

Amino acid	Recovery (%)
Alanine (1)	100.9±4.1
Valine (1)	97.3±2.7
Isoleucine (1)	100.9±3.7
Glycine (1)	103.1±6.3
Leucine (1)	103.1±2.8
Threonine (2)	103.7±5.4
Proline (1)	101.8±2.9
Serine (1)	90.4±4.2
Hydroxyproline (2)	80.8±0.3
Methionine (2)	84.7±2.4
Phenylalanine (1)	96.1±2.6
Aspartic acid (1)	95.4±2.0
Glutamic acid (1)	99.2±1.8
Tyrosine (2)	93.6±5.6
Lysine (1)	101.2±3.2
Cystine (2)	100.0±20.4

(1) 2 m.×3 mm. (i.d.) glass column packed with 60/80 mesh acid washed chromosorb W and mixed liquid phases of 0.75/0.25 w./w.% of DEGS/EGSS-X.

(2) 2 m.×3 mm. (i.d.) glass column packed with 80/100 mesh acid washed chromsorb W and liquid phase of 1.5 w./w.% of silicone OV-17.

pose of examining the loss of amino acids in these procedure, artificial sea water (LYMAN and FLEMING, 1940) was prepared and the sixteen kinds of standard amino acids were added in one liter of the artificial sea water with the range of one to twenty micromoles.

The artificial sea water containing amino acids was evaporated and was ion exchanged by the same procedure described above. As shown in Table 1 the recoveries of all amino acids except arginine and histidine were the ranges between 80 and 103 percent.

(B) The recovery of proteinaceous substances by acid hydrolysis: The conventional method for recovering of all amino acids from proteinaceous substances in sea water is the hydrolysis with 6N-hydrochloric acid. However, it is said a part of amino acids are decomposed during acid hydrolysis. Amino acids releasing from the protein in the various periods of hydrolysis were examined by using the hard protein prepared from inner ligament (resilium) of a kind of molluscan shell (*Spisula sachalinensis*). According to this results the appropriate period of hydrolysis was 24 hours, but a few percents of amino acids were lost by the decomposition under this condition.

(C) Calibration by using internal standard for the determination of amino acids: The quantity of the amino acids dissolved in sea water were calculated by comparing the areas of their peak and the internal standard (n-butyl palmitate). Relative molar response of the amino acids to the internal standard are shown in Table 2, and the calibration of internal standard is given in Fig. 1. The least amount of detection under this gas chromatographic condition is about 0.5 μg per sample injected to gas chromatography. The limitation of the identification of amino acids is about 2.5×10^{-9} grams per sample injected, because the sensitivity of gas chromatograph is possible to raise moreover 200 times than the condition described above.

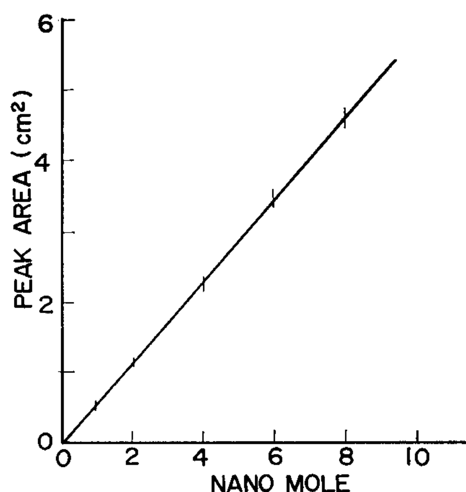

Fig. 1. Calibration by internal standard (butyl ester of palmitic acid) for the determination of amino acids. The internal standard was injected to gas chromatograph in the volumes between 1 to 8 microliter of 0.02 millimoles per 10 m*l* of chloroform solution.

Table 2. Relative molar response of each amino acid to the internal standard.

Amino acid	Relative molar response	Standard deviation
Alanine	0.809	±0.017
Valine	1.018	±0.047
Isoleucine	1.151	±0.086
Glycine	0.591	±0.052
Leucine	1.008	±0.068
Threonine	1.043	±0.041
Proline	0.897	±0.081
Hydroxyproline	1.132	±0.052
Methionine	1.085	±0.054
Phenylalanine	1.260	±0.018
Aspartic acid	1.083	±0.052
Glutamic acid	1.073	±0.000
Tyrosine	0.885	±0.022
Lysine	0.779	±0.041
Cystine	0.410	±0.064

The figures indicating in Table 2 are the mean values of three individual gas chromatograms of the authentic amino acid esters.

(D) Impurities in the reagent used in this experiment: The reagents; hydrochloric acid, ammonium hydroxide, ethanol, methanol, butanol, dimethylchloride, chloroform, trifluoroacetic acid, redistilled water, and ion exchange resin, were carefully purified. Blank test was made by the same procedure and the same volumes as the analyses of sea water samples. The gas chromatogram was shown in Fig. 2. A peak was detected at the position between internal

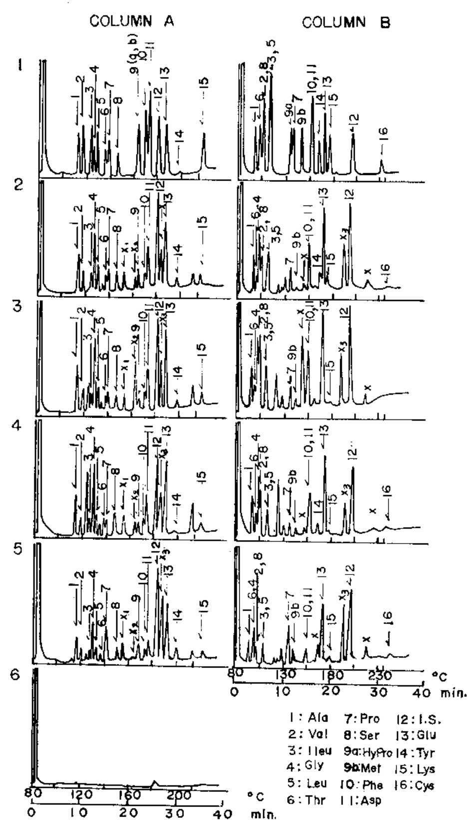

Fig 2. Gas chromatograms of standard amino acids and representative sea-water samples which were analyzed by two different species of packing materials. Conditions used gas chromatographic measurements were as follows;

Columns: Column A; 2 m×3.1 mm, i. d. glass column packed with 60/80 mesh acid washed Chromosorb W and mixed liquid phase of 0.75/0.25 w./w.% of DEGS/EGSS-X.

Column B; Same column in diameter as described above packed with 80/100 mesh acid washed Chromosorb W and liquid phase of 1.5 w./w.% of Silicone OV-17.

Column temperature: Initial 80°C (Columns A and B), final 215°C (Column A) and 235°C (Column B)

Program rate: 4°C/min. (Column A) and 5°C/min (Column B)

Carrier flow, N_2 gas: 60 ml/min. at 80°C

Air: 650 ml/min. Hydrogen: 37 ml/min. Chart speed: 1 cm/min.

1: Standard amino acid, 2: Surface sample, 3:20 meter 4:50 meter, 5:100 meter, 6: Reagent blank

standard amino acid peaks. This impurity does not correspond to the peak of amino acid. Absolute value of this peak is thought to be less than 0.2 μg per liter of sample.

(E) Dissolved proteinaceous substances in sea water: Water samplings were performed by using Van Dorn samplers from the surface to 1,500 m depths at one station in the southeastern Japan (22°00′N, 142°00′E), in January 1968 and three station of near shore (southern Hokkaido) and off shore (Kuroshio region).

Each ten liters of samples was filtered through glass fiber filter (Whatman, GF/C) immediately after sampling and was frozen to −20°C in deep freezer. Fourteen kinds of amino acids were confirmed. The identified amino acids were as follows; alanine, valine, isoleucine, glycine, leucine, serine, phenylalanine, aspartic acid, glutamic acid, tyrosine, lysine, threonine, proline, and methionine. The gas chromatograms of the standard amino acids and the representative water samples which were analyzed by two different kinds of columns are shown in Fig. 2. These results are summerized in Table 3.

4. Discussion

Accuracy: In gas-liquid chromatography of amino acids except in the case of cystine, we obtained the accuracy of about five percent as shown in Table 1. GEHRKE *et al.* described that the accuracy of gas-liquid chromatography was about three percent for macro amount of amino acids. The accuracy of about ten percent for paper and thin layer chromatography, three percent for automatic amino acid analyzer by ion exchange chromatography has been reported.

Sensitivity: Gas-liquid chromatography has high sensitivity. The limitatation of the identification of 2.5×10^{-9} grams of amino acids was recognized by author's gas chromatographic experiments and 10^{-10} grams by TOMITA (1967), while the limitation of detection has been generally said about 10^{-6} grams for paper and thin layer chromatography and about 10^{-5} grams for ion exchange method.

Time required for analysis: Gas chromatographic method needs about 60 hours: 5 hours

Table 3. The amino acid composition in sea water taken from Pacific Ocean at the position of 22°00′N and 142°00′E in January 1968. Amino acids are expressed as micrograms of amino acid and amino nitrogen contained in one liter of samples.

Amino acid \ Depth (m)		0	20	50	100	198	1009	1500
Alanine	μg/l	5.6	6.6	6.3	8.9	3.2	1.0	6.8
	N-μg/l	0.88	1.04	0.99	1.40	0.50	0.16	1.07
Valine		2.5	2.9	2.1	3.3	1.3	0.6	2.9
		0.30	0.35	0.25	0.39	0.16	0.07	0.35
Isoleucine		1.5	1.4	1.7	2.1	0.9	0.5	2.1
		0.16	0.15	0.18	0.22	0.10	0.05	0.22
Glycine		11.5	13.6	16.7	25.6	7.1	4.1	2.4
		2.15	2.54	3.12	4.78	1.32	0.77	0.45
Leucine		3.2	2.7	2.6	3.7	1.5	0.9	2.9
		0.34	0.29	0.28	0.40	0.16	0.10	0.31
Serine		Tr.	7.3	13.5	7.2	2.9	2.7	6.4
		—	0.97	1.79	0.96	0.38	0.36	0.85
Phenylalanine		1.8	2.4	2.3	2.7	1.3	1.2	2.5
		0.15	0.20	0.20	0.23	0.11	0.10	0.21
Aspartic acid		6.1	6.4	5.9	7.3	2.2	1.5	4.4
		0.64	0.67	0.62	0.77	0.23	0.16	0.46
Glutamic acid		9.0	11.7	15.7	16.2	5.6	2.5	21.9
		0.86	1.11	1.49	1.54	0.53	0.24	2.08
Tyrosine		6.9	6.1	3.4	4.3	8.6	4.2	6.7
		0.53	0.47	0.26	0.33	0.66	0.32	0.52
Lysine		2.0	2.8	2.1	4.5	1.0	1.3	1.9
		0.38	0.54	0.40	0.86	0.19	0.25	0.36
Threonine		2.3	2.9	2.2	—	0.4	0.6	0.8
		0.27	0.34	0.26	—	0.05	0.07	0.09
Proline		0.3	3.5	3.8	5.3	7.1	11.5	7.9
		0.04	0.43	0.46	0.65	0.86	1.40	0.96
Methionine		Tr.	0.6	0.4	0.6	0.5	0.4	0.7
		—	0.06	0.04	0.06	0.05	0.04	0.07
Total	μg/l	52.7	70.9	78.7	91.7	43.6	33.0	70.3
	N-μg/l	6.70	9.12	10.34	12.59	5.30	4.09	8.00

for vacuum evaporation, 24 hours for acid hydrolysis, 24 hours for removing inorganic salts by ion exchange, 4 hours for esterification, and an hour for gas chromatography. On the other hand, ion exchange or paper and thin layer chromatographic method would be need more time than gas chromatographic procedure. Gas chromatography offers advantages over other chromatographic methods, because of its speed, accuracy and sensitivity.

Materials of gas chromatographic columns: In earlier experiments, we used the stainless columns packed with silicious earth as solid phase and 10 wt.% of poly-neopentyl glycol sebate and 10 wt.% of silicone OV-17 as liquid phase. Hydroxy-amino acids such as serine and threonine decomposed in a column and in a metallic injection part by metal catalytic action and were detected scarcely.

Golay column which castor wax was coated, 150 feet in length, 0.01 inches in inside diameter, was tested with the constant oven temperature. Satisfactory results were obtained in that respect of the separation of each components. Avoiding the metal catalytic action we adopted the on-column system by glass column. Good separation was obtained for the fourteen kinds of amino acids using the substrate phases of 0.75/0.25 w./w.% of DEGS/EGSS-X and 1.5 w./w.% of silicone OV-17. Histidine and

Table 4. Free amino acids composition of the surface sea water.

Station / Amino acid	I (1) μg/l	II (2) μg/l	III (3) μg/l
Alanine	37.0	8.1	0.8
Valine	4.2	6.2	0.6
Isoleucine	11.6	6.7	0.7
Glycine	9.8	18.4	3.0
Leucine	3.2	16.5	2.3
Serine	tr.	tr.	tr.
Phenylalanine	6.4	14.2	4.9
Aspartic acid	26.8	7.4	0.8
Glutamic acid	44.4	4.2	14.7
Tyrosine	1.0	tr.	—
Lysine	11.1	13.4	4.3
Proline	12.9	tr.	1.8
Methionine	tr.	tr.	tr.
Histidine	(12.5)	3.1	(4.1)
Cystine	10.1	tr.	—
Total	191.0	98.2	38.0

(1) One kilometer off Yoshioka, Tsugaru Strait, Hokkaido. Oct. 10, 1968.
(2) 41°08′N, 142°41′E, Southwestern of Cape Erimo. May 21, 1964
(3) 25°01′N, 141°47′E, Southern Japan. Jan. 21, 1968.

arginine have been recovered with less satisfactory results. This reason is probably due to the uncomplete reaction of acylation or the decomposition in gas chromatographic column after sample was injected. Further detailed experiments are necessary relating to this point.

Distribution of proteinaceous substances in sea water: The most abundant amino acids found in sea water were glycine and glutamic acid (Table 3). Total amounts of amino acids in sea water showed a considerable vertical change. A large quantity of glycine was detected at the depths between surface and 100 m. Glutamic acid was also changed vertically. Table 4 shows the horizontal distribution of the free amino acids present in the surface sea water. Regional variation was found with respect to the quantity and the composition as shown in Table 4. The sea water of point I, near coast of Yoshioka, Hokkaido, seems to be effected by the land run off.

Unknown peaks were found on gas chromatograms, but these peaks were not identified.

Table 5. Vertical distribution of the nitrogen compounds found in Pacific Ocean (22°00′N, 142°00′E).

N-compound / Depth	NO_2 (μg atom/l)	NO_3 (μg atom/l)	NH_3 (μg atom/l)	Amino-N (μg atom/l)	ΣN (μg atom/l)	Amino-N/ΣN %
0	0.04	2.7	0.3	0.48	3.52	13.7
20	0.00	0.4	0.8	0.65	1.85	35.1
50	0.00	0.3	0.5	0.74	1.54	48.1
100	0.10	1.7	0.0	0.90	2.70	33.3
198	0.08	18.0	0.0	0.38	18.46	2.1
1009	0.03	37.3	0.8	0.29	38.42	0.8
1500	0.05	38.1	1.8	0.57	40.52	1.4

Further experiments are needed to confirm wether these peaks are the naturally occuring components or the decomposable materials by acid hydrolysis.

The amounts of dissolved amino nitrogen and inorganic nitrogen compound in the station of southeastern Japan were given in Table 5. Amino nitrogen accounts for 0.8 to 48.1 percent of the inorganic nitrogen. The maximum peak of amino nitrogen appears near 50 to 100 m depth.

References

CHAU, Y. K. and J. P. RILEY (1966): The determination of amino acids in sea water. Deep-Sea Res., **13**, 1115-1124.

GEHRKE, C. W., W. M. LAMKIN, D. L. STALLING and F. SHAHROKHI (1965): Quantitative gas chromatography of amino acids. Biochem. and Biophys. Res. Comm., **19**, 328-334.

GEHRKE, C. W. and F. SHAHROKHI (1966): Chromatographic separation of n-butyl N trifluoroacetyl esters of amino acids. Anal. Biochem., **15**, 97-108.

LAMKIN, W. M. and C. W. GEHRKE (1965): Quantitative gas chromatography of amino acids. Preparation of n-butyl N-trifluoroacetyl esters. Anal. Chem., **37**, 383-389.

LYMAN, J. and R. H. FLEMING (1940): Composition of sea water. Jour. Mar. Res., **3**, 134-146.

NATTELER, K. (1892-1894): Denkschr. Akad. Wiss. Wien, **59**, 1e Reihe, 83; **60**, 2e Reihe, 49; **61**, 3e Reihe, 23.

DUURSMA (1965): The dissolved organic constituents of sea water. Chem. Oceanogr. Vol. 1, 435. Edited by RILEY, J. P. and G. SKIRROW, Academic Press, London.

PALMORK, K. H. (1963): The use of 2, 4-dinitro-1-fluoro-benzene in the separation of amino acids from sea water. Acta. Chem. Scand., **17**, 1456-57.

PARK, K., W. T. WILLIAMS, J. M. PRESCOTT and D. H. HOOD (1962): Amino acids in deep-sea water. Science, **138**, 531-532.

SANGER, F. (1945): The free amino groups of insulin. Biochem. J., **39**, 507-515.

SIEGEL, A. and E. DEGENS (1966): Concentration of dissolved amino acids from saline waters by ligand exchange. Science, **151**, 1098-1101.

SPACKMAN, D. H., W. H. STEIN and S. MOORE (1958): Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem., **30**, 1190-1206.

STALTING, D. L. and C. W. GEHRKE (1967): Quantitative gas chromatography of amino acids. Interesterification studies. Anal. Biochem., **18**, 118-125.

STARIKOVA, N. D. and R. I. KORZHIKOVA (1969): Amino acids in the Black Sea. Okeanologiya, **9**, 625-636.

TATSUMOTO, M., W. T. WILLIAMS, J. M. PRESCOTT and D. W. HOOD (1961): Amino acids in samples of surface sea water. Jour. Mar. Res., **19**, 89.

TOMITA, I. (1967): Gaschromatographic separation of amino acids and peptides. Tanpakushitsu Kakusan Koso (Protein, Nucleic acid and Enzyme), Kyoritsu Shuppan Co., Tokyo, **12**, 1069-1080.

アミノ酸のガスクロマトグラフィーによる定量法と海水中の溶存蛋白様物質の垂直分布について

川原鳳策　米田義昭

要旨　海水中の溶存蛋白様物質の定量にガスクロマトグラフ法を応用した．蛋白構成アミノ酸の14種類が2種類の異なるカラム充てん剤を用いたガスクロマトグラフによって分離定量された．アルギニンおよびヒスチジンはガスクロマトグラフィーによって再現性のある結果を与えず，今後更に検討する必要がある．

人工海水中に加えた16種のアミノ酸中アルギニン，ヒスチジン，およびシスチンを除く13種のアミノ酸は80～103％の範囲で，±5％の精確さでもって分離定量された．

海水中の溶存蛋白様物質の垂直分布，および二，三の表面海水の水平的な変化をしらべた．外洋水（22°00′N, 142°00′E）の表面から1,500 m までの7層について全蛋白様物質は 33～92 $\mu g/l$ の値であった．3地点で採取した表面海水は 38, 98, および 191 $\mu g/l$ の値が得られた．アミノ酸の種類ではグリシンとグルタミン酸が多量に含まれている傾向を示した．窒素化合物の総量（NO_2+NO_3+NH_3+Amino-N）に対してアミノ態窒素の占める比率は 0.8～48.1％ であり，表面より 100 m 層間のアミノ態窒素の占める比率は深層と比較して高い値を示した．