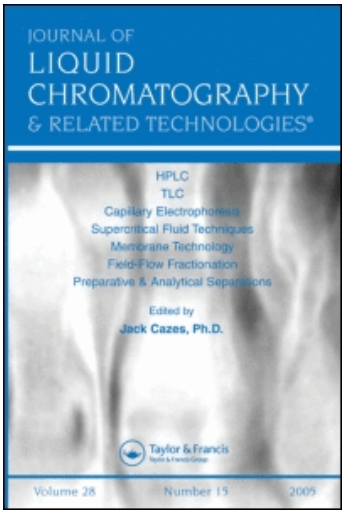

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ABSTRACT

The amino acid compositions were determined in four different algal species: the temperate *Thalassiosira rotula* and *Phaeocystis globosa* and the polar *Thalassiosira antarctica* and *Phaeocystis antarctica.*

An extraction procedure was developed to obtain amino acids from phytoplankton species. The amino acids were analysed after precolumn derivatization with 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl); the procedure was optimized and the samples were analysed quantitatively on a Superspher RP8 C_8 column. The good stability of the FMOC derivatives was utilized to develop a new semipreparative chromatographic method for the separation and collection of the most important amino acids.

INTRODUCTION

The importance of nutrient supply for phytoplankton production in aquatic systems and the roles of these processes in the sequestration of atmospheric carbon dioxide and its transport into deep water via the biological pump have long been established.[1] Dugdale and Goering[2] introduced an outstanding model and an innovative application of ^{15}N methodology, which contributed considerably to our current understanding of the nitrogen cycle. These authors distinguish "new" and "regenerated" primary phytoplankton production. The former is associated with allochthonous nutrients, mainly nitrate and dinitrogen, whereas the latter is fuelled by compounds regenerated *in situ*, such as ammonium and dissolved organic nitrogen (DON). Despite the contention of Dugdale and Goering that "the regenerated fraction of available nitrogen is not entirely measured by the ammonium uptake and that organic species are potentially important...", DON has remained conspicuous by its absence in most nutrient uptake studies.

Few recent studies shed new light on the role of DON in phytoplankton nutrition and ecology.[3,4] One highly important feature is the rapid exudation of DON by natural phytoplankton assemblages, a process which might to some extent be responsible for the earlier-mentioned mystery of the imbalance ^{15}N.[5] The DON released by planktonic organisms is thought to be a nitrogen source for primary products.[4] This DON pool presumably consists of easily degradable molecules, such as amino acids and small peptides.

To clarify the biosynthetic routes of nitrogen-containing nutrients, we combined the ^{15}N labeling technique with chromatographic separation and subsequent fluorometric detection of the amino acids. The purpose of the investigation was to elucidate the variations in amino acid composition of different phytoplankton species and to develop a semipreparative reversed-phase high performance liquid chromatographic (RP-HPLC) method suitable for collection of the most important amino acids. Since spectroscopic measurements of the ^{15}N abundance require contamination-free preparation of the samples, the eluent system should not involve traditional solvents such as acetonitrile or nitrogen-containing buffers. Moreover, the critical minimum for

reliable spectroscopic measurements of the $^{15}N/^{14}N$ ratio amounts to a few μg of nitrogen, which demonstrates the need for the application of both analytical and semipreparative chromatography.

The most useful RP-HPLC methods for amino acid analysis include precolumn derivatization, the amino acids being converted into compounds that absorb or fluoresce in the UV-visible wavelength range. Several reagents have been developed for this purpose. Phenyl isothiocyanate[6,7] reacts with both primary and secondary amino acids to yield stable phenylthiocarbamoyl derivatives, which can be detected via their UV absorption. The main drawback of this ligand is its low sensitivity. o-Phthalaldehyde (OPA)[8-11] is itself nonfluorescent, but it reacts rapidly with primary amino acids at room temperature to form highly fluorescent isoindoles. The disadvantages of this method are the poor reactivity of OPA with secondary amino acids and the low stability of the reaction product. 1-Dimethylaminonaphthalene-5-sulfonyl (dansyl) chloride[12-14] forms fluorescent adducts with amino acids but lacks selectivity (it reacts with both -OH and $-NH_2$ groups) and requires rather long reaction times and high reaction temperatures.

In contrast 9-fluorenyl-methoxycarbonyl chloride (FMOC-Cl)[15-31] reacts rapidly with amino acids to form highly fluorescent and stable adducts. The major disadvantage of this method is the need for excess reagent, which must be removed prior to chromatography by extraction or by addition of a second reagent (1-aminoadamantane, ADAM). The expected low amino acid content of phytoplankton and the need for semipreparative chromatography made us opt for the utilization of FMOC-Cl in combination with fluorometric detection.

EXPERIMENTAL

Algal Cultures

Experiments were carried out with four different algal species: the temperate *Thalassiosira rotula* and *Phaeocystis globosa*, and the polar *Thalassiosira antarctica* and *Phaeocystis antarctica*. The temperate species were isolated from the North Sea, and the polar ones from the Weddell Sea. Stock cultures were kept at a temperature of 15 $\pm$ 0.5°C and 4 $\pm$ 0.5°C, and at a light intensity of 93 μmol m^{-2} s^{-1} and 40 μmol m^{-2} s^{-1}, respectively. These conditions correspond to the natural condition in the North Sea and Weddell Sea. Light was provided from above by fluorescent tubes (Philips, TL 54); a light/dark cycle of 16/8 h was applied for the temperate algae and permanent light for the polar ones. The culture medium was natural sea water from the Weddel Sea, filtered on 0.2 μm cellulose acetate filters (Sartorius) and enriched

with nutrients according to Von Stosch and Drebes,[32] with the exception of iron and silicate, which were added at 1/10 of the recommended concentrations. Stock cultures were diluted regularly to maintain the cells in the exponential growth phase.

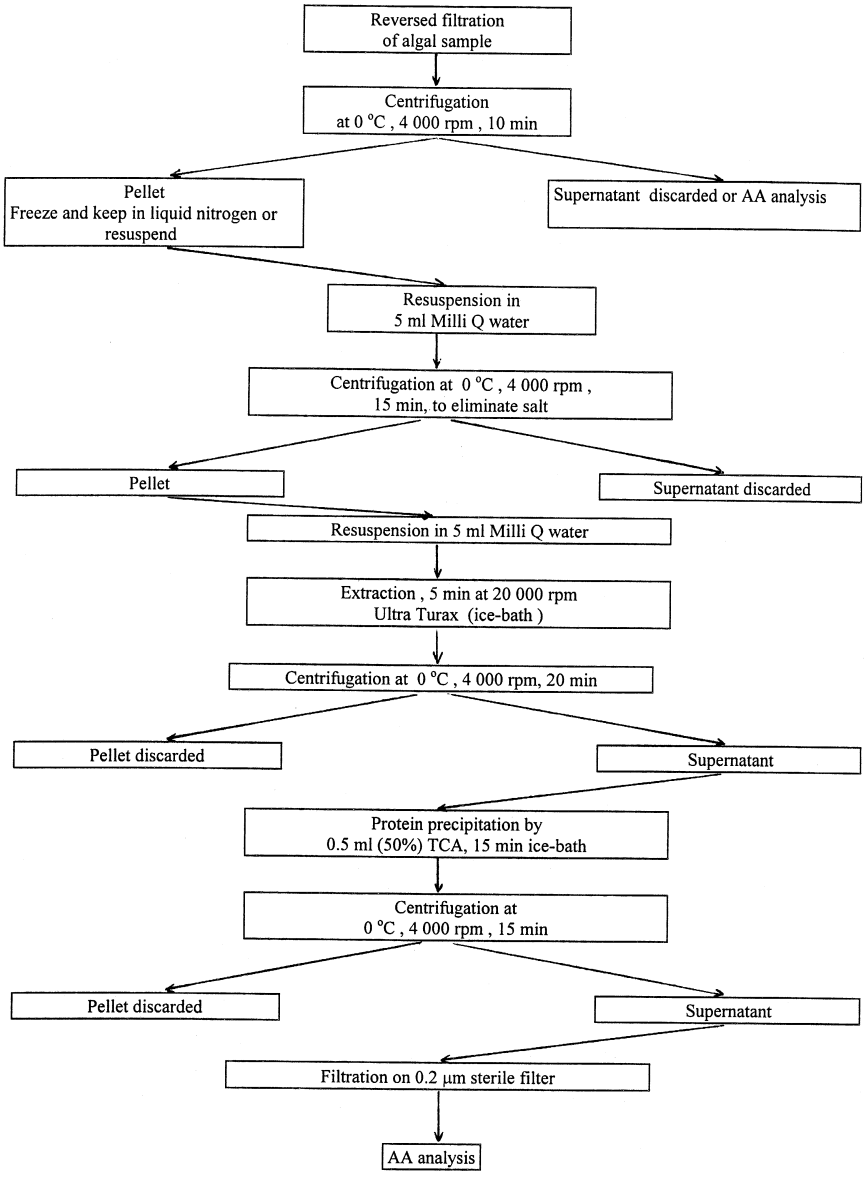
Sample Preparation

The procedure elaborated is outlined in Scheme 1. In a preliminary step, the algal culture (1-2 l) was preconcentrated by reversed filtration through a plankton gauze with a mesh size of 10 μm, resulting in maximal removal of sea-water. The residue was centrifuged at 0°C and 4 000 rpm for 10 min, with the supernatant being discarded or used for amino acid analysis. The pellet was frozen in liquid nitrogen and stored for later analysis, when it was resuspended in 5 mL milliQ water and centrifuged at 0°C and 4 000 rpm for 15 min. This washing step proved very important to maintain the chromatographic column in good condition and to increase its lifetime. The supernatant was discarded and the pellet was resuspended a second time in 5 mL milliQ water. In this extraction step, the phytoplankton cells were disrupted with an Ultra-Turax T25 high-speed disperser (Janke & Kunkel Gmbh, Staufen, Germany) for 5 min at 20 000 rpm in an ice-bath. Completeness of cell disruption was controlled microscopically. After centrifugation for 20 min (4 000 rpm and 0°C), the proteins were precipitated for 15 min in an ice-bath after the addition of 0.5 mL trichloroacetic acid (TCA) (50%). The proteins were separated by centrifugation (4 000 rpm at 0°C for 15 min) from the liquid phase containing the amino acids.

Originally, this solution was used for amino acid analysis. In some experiments, an increase in amino acid content was observed with time; this was attributed to dissociation of the remaining proteins which could not be removed during the final centrifugation step. In an additional filtration step, therefore, the supernatant was filtered on a 0.2 μm sterile Hollow Fiber Syringe Filter (Dyna Gard, Microgon Inc., Laguna Hills, CA, USA) a syringe being used. After filtration, the amino acids were determined without delay.

Chemicals

Acetonitrile, methanol, tetrahydrofuran (THF), and acetone, all of HPLC grade, and boric acid, sodium hydroxide, and acetic acid of the highest purity available (Suprapur) were purchased from Merck. Hydrochloric acid and sodium acetate were of p.a. quality (Merck). FMOC-Cl and ADAM were from Fluka (Buchs, Switzerland), ADAM.HCl was from Sigma (St. Louis, MO, USA) and amino acid standards were from Pierce (Rockford, IL, USA) or from Fluka.



Scheme 1. Extraction procedure.

Chromatographic Equipment

The chromatographic system consisted of an L-6200A Intelligent Pump, an L-3000 Multi Channel Photo Detector, a T-6300 Column Thermostat, a D-6000 HPLC-Manager with Interface (Merck-Hitachi, Darmstadt, Germany), and a fluorescence detector Model 420 (Waters Chromatography, Milford, MA, USA), with a 254 nm excitation filter and a 313 nm emission filter. The samples were introduced with a Rheodyne Model 7125 valve (Cotati, USA) equipped with a 20 or 500 μL loop. Analytical separations were carried out on a Superspher 60RP8 C_8, 250x4 mm I.D., 4 μm particle size column (Merck, Darmstadt, Germany) and on a Vydac 218TP54 C_{18} (250x4.6 mm I.D.), 5 μm particle size column (The Separation Group, Hesperia, CA, USA). Semipreparative separations were performed on a Vydac 218TP1510 C_{18} (250x10 mm I.D.), 15 μm particle size column.

Chromatographic Conditions for Separation

The analytical separation of amino acids on the Superspher RP8 C_8 column was carried out with a modified gradient system developed by Merck.[33] Component A of the mobile phase consisted of 0.1 M sodium acetate (pH 4.4): THF = 95: 5 (w/w) and component B was acetonitrile. The flow rate was kept constant at 1.25 mL/min; the gradient profile was: 0 min 15% B, 16 min 18% B, 17.5 min 40% B, 20 min 40% B, 35 min 50% B, 36 min 100% B, 41 min 100% B, and 42 min 15% B. The temperature of the column was kept at 45°C. A typical chromatogram is shown in Figure 1. In spite of careful sample preparation and derivatization procedures an increase in the retention of Arg relative to the other amino acids was observed for aged columns. This could not be compensated for with the mobile phase used in routine analysis.

Semipreparative separations were carried out on a Vydac column with 10 mm I.D., but the procedure was optimized for the analytical column with 4.6 mm I.D. The results obtained for the analytical separations were readily transferred to semi-preparative chromatography by increasing the flow rate to 4 mL/min. The acetonitrile-free eluent consisted of sodium acetate buffer, methanol and THF. In preliminary experiments, 0.05 M sodium acetate buffer (pH 4.4) was used as the hydrophilic component of the eluent, and methanol as the organic modifier. The first peaks appearing in the chromatogram were those of Asn and Gln. Asp, Glu, Ser, and Arg appeared in a single peak, and neither modification of the starting composition of the eluent nor variation of the slope of the gradient improved the separation of these compounds. An increase of the pH of the sodium acetate buffer to 5.5 resulted in separation of the peaks of Asp and Glu at the beginning of the chromatogram. The coelution of Ser and Arg persisted, however.

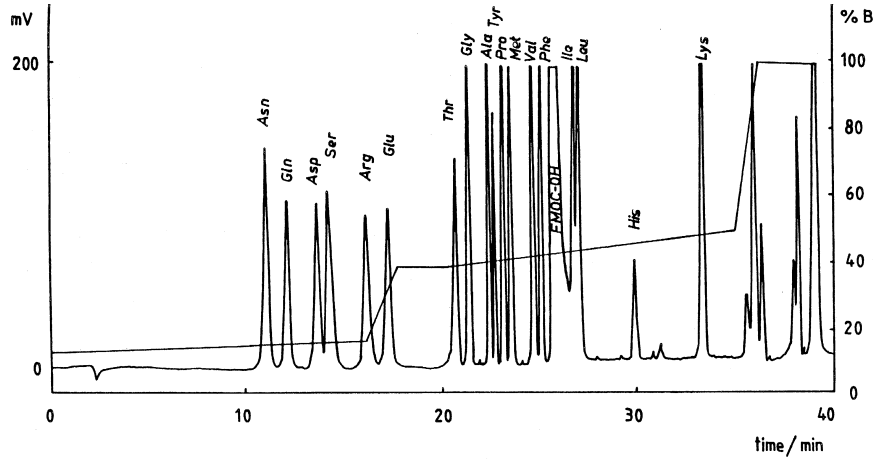


Figure 1. Separation of FMOC-amino acid standard. Operating conditions: Column: Superspher RP8 C_8. Flow rate: 1.25 mL/min. Solvent A: 0.1 M sodium acetate (pH 4.4):THF=95:5 (w/w). Solvent B: acetonitrile. Gradient profile: see Materials and Methods. Excitation at 254 nm, emission filter at 310 nm. Column temperature: 45°C.

Any further increase in pH deteriorated the resolution of these peaks. The separation of Ser and Arg was made possible by the addition of THF as a third component to the mobile phase. The final composition of the eluent was: component A 0.05 M sodium acetate buffer (pH 5.5), component B pure THF in a constant concentration of 3% (v/v) while component C was methanol. In the gradient profile, only the volume ratio A/C was varied: 0 min 97/0% A/C, 4 min 65/32% A/C, 17 min 60/37% A/C, 27.5 min 55/42% A/C, 30 min 35/62% A/C, 40 min 35/62% A/C, 41 min 0/97% A/C, 45 min 0/97% A/C, and 46 min 97/0% A/C. The flow rate was 1.25 mL/min and the temperature of the column was kept at 45°C. A typical chromatogram is shown in Figure 2. Decrease of the THF content from 3% (v/v) to 2% (v/v) improved the separation of the peaks of Met and Val, and of Ile and Leu+Phe at the end of the chromatogram, but at the same time the resolution of Ser and Arg decreased. The best way to increase the separation of Met and Val was to eliminate the THF from the mobile phase after 30 min. Leu and Phe were not separable in the methanol-containing mobile phase system on the Vydac column.

Preparation of FMOC Derivatives

To 100 μL of amino acid standard (1-50 μM) or to 100 μL of algal sample 100 μL of borate buffer (0.5 M, pH 8.0) was added. Before the addition of

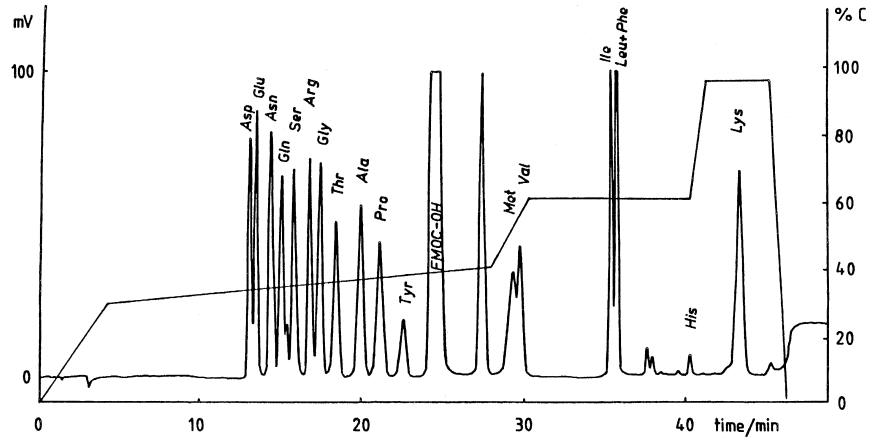


Figure 2. Separation of FMOC-amino acid standard. Operating conditions: Column: Vydac 218TP54 C_{18}. Flow rate: 1.25 mL/min. Solvent A: 0.05 M sodium acetate (pH 5.5). Solvent B: THF. Solvent C: methanol. Gradient profile: see Materials and Methods. Excitation at 254 nm, emission filter 310 nm. Column temperature: 45°C.

borate buffer, the pH of the sample was adjusted to about pH 7-8 by the addition of 2 M NaOH. After shaking, 100 μL of FMOC-Cl dissolved in acetone was added. The concentration of reagent was generally 3 mM. After 5 min, the reaction was stopped by the addition of 100 μL of ADAM (12 mM) and after a further 1-min waiting period, the reaction mixture was injected.

RESULTS AND DISCUSSION

Optimization of Derivatization Procedure

A survey of the literature dealing with the derivatization of amino acids reveals that different authors have proposed different optimal conditions. Following the procedure of Betner and Földi,[19,22,23] we observed that the yield for some amino acids was consistently too low at FMOC-Cl and ADAM concentrations of 1 and 12 mM, respectively and a reaction time of 45 s. The derivatization procedure was therefore reinvestigated. In a first series of experiments with standard mixtures of amino acids (20 μM each), the concentrations of FMOC-Cl and ADAM were kept constant, whereas the reaction time was varied.

Table 1

Relative Yields (%) of FMOC Derivatives of Amino Acids Depending on Time of Reaction

Amino Acids (AA)	Relative Yields (%) Time of Reaction(s) 45	150	300	600
Asn	100	105	110	110
Gln	100	105	108	108
Asp	100	140	161	198
Ser	100	119	128	123
Arg	100	104	105	104
Glu	100	136	145	161
Thr	100	122	126	134
Gly	100	103	103	103
Ala	100	113	118	118
Tyr	100	96	87	78
Pro	100	100	100	99
Met	100	99	99	100
Val	100	106	104	104
Phe	100	102	101	101
Ile	100	108	80	44
Leu	100	109	96	67
His	100	112	107	106
Lys	100	90	88	86

Initial concentrations: [amino acids] = 20 μM each; [FMOC-Cl] = 3.0 mM; [ADAM] = 12.0 mM. Molar ratio of FMOC-CL/MAA = 8.3

The results in Table 1 are normalized: the peak areas for reaction times of 150, 300, and 600 s were related to those obtained for a reaction time of 45 s (taken as 100%). Clearly, the yields of some amino acids increased with increasing reaction time (especially for Asp, Glu, Ser, Thr, and Ala) and reached a maximum value after 10 min for initial amino acid and FMOC-Cl concentrations of 20 μM and 3.0 mM, respectively. The decreased yield of Tyr may be explained by the measurement of the monosubstituted derivative. The decreases in the peak areas of Ile and Leu are due to the incorrect integration of the peaks on the tailing of FMOC-OH and can be compensated by increasing the concentration of ADAM. Einarsson[17] also observed a loss of the doubly derivatized Lys, which was probably due to adsorption on the wall of the glass vial.

Table 2

Relative Yields (%) of FMOC Derivatives of Amino Acids Depending on Initial Concentration of FMOC-Cl

Amino Acids (AA)	Relative Yields (%) Concentration of FMOC-Cl (mM) 1.0	3.0	6.0	12.0
Asn	100	111	113	110
Gln	100	108	114	110
Asp	100	161	219	241
Ser	100	120	138	135
Arg	100	105	108	107
Glu	100	161	171	170
Thr	100	135	145	144
Gly	100	117	136	132
Ala	100	133	144	140
Tyr	100	92	85	78
Pro	100	109	104	107
Met	100	99	98	94
Val	100	103	103	101
Phe	100	101	100	99
Ile	100	101	89	---
Leu	100	108	50	---
His	100	58	46	34
Lys	100	94	36	23

Initial concentrations : [amino acids] = 10 μM (each); {ADAM} = 12.0 mM. Molar ratio of FMOC-Cl/ΣAA = 5.5 - 66.6. Reaction time: 5 min.

It was observed that the reaction time could be reduced to 3 min without any decrease in the maximal efficiency for an amino acid concentration of 2 μM and FMOC-Cl and ADAM concentrations of 3 mM and 12 mM, respectively. In another series of experiments, the amino acid concentration, the ADAM concentration, and the reaction time were kept constant at 10 μM, 12 mM, and 5 min, respectively, and the concentration of FMOC-Cl was varied from 1 to 12 mM.

As can be seen in Table 2, the yield was significantly affected by the FMOC-Cl concentration. The values are normalized to the values obtained at 1 mM FMOC-Cl. Especially Asp, Ser, Glu, Thr, Gly, and Ala furnished better yields with increasing FMOC-Cl concentration.

Table 3

Response of Peak Area Relative to That of Ala for FMOC Derivatives Detected by Fluorescence Method

Amino Acids (AA)	Relative Responses	Literature[25]
Asn	1.01	---
Gln	1.00	---
Asp	0.75	0.31
Ser	1.02	0.88
Arg	0.97	1.08
Glu	1.06	0.50
Thr	0.85	0.80
Gly	1.07	1.44
Ala	1.00	1.00
Tyr	0.47	0.89
Pro	1.00	1.43
Met	0.79	0.93
Val	1.00	1.27
Phe	0.95	1.39
Ile	1.62	1.36
Leu	1.11	1.22
His	0.28	0.19
Lys	1.68	2.15

Initial concentrations: [amino acids] = 20.0 μM each, [FMOC-Cl] = 3.0 mM. Molar ratio of FMOC-Cl/ΣAA = 8.3. Reaction time: 5 min.

The concentration of ADAM influenced the peak shape and intensity of FMOC-OH; 12 mM was found to be optimal. Higher concentrations of ADAM decreased the peak intensity of FMOC-OH, but elution of the excess ADAM and the FMOC-ADAM adduct required a longer time. In conclusion, we suggest a reaction time of 5 min, a FMOC-Cl concentration of 3 mM and an ADAM concentration of 12 mM.

Table 3 shows the fluorescence yields for different amino acids relative to Ala. The relative response for most of the amino acids was near to 1.0, and significantly increased compared to the low responses reported in the literature for Asn, Gln, Asp, Glu and His. The yield of Tyr was lowered below 0.5. The precision of the procedure was examined by analysing ten replicate standard amino acid mixtures and comparing the peak areas for the respective mixtures.

Table 4

Free Amino Acid Composition of Algal Samples

Free Amino Acids	*Thalassiosira Antarctica*	*Thalaiosira Rotula* Mol %	*Phaeocystis Antartica*	*Phaeocystis Globosa*
Asn	1.1	1.2	6.2	1.3
Gln	14.5	7.7	4.1	13.1
Asp	3.9	8.5	4.1	1.3
Ser	2.3	3.6	5.0	14.4
Arg	3.6	0.8	2.1	1.3
Glu	14.6	44.5	0.1	6.6
Thr	1.6	2.4	0.1	4.0
Gly	2.5	3.9	51.7	30.2
Ala	13.8	14.2	7.5	4.0
Tyr	0.2	1.2	0.1	8.0
Pro	28.0	2.4	7.5	8.0
Met	0.6	1.6	0.1	1.3
Val	5.6	1.6	6.2	2.6
Phe	0.4	3.2	4.2	2.6
Ile	1.5	0.1	0.1	0.1
Leu	0.9	0.1	0.1	0.1
His	0.1	1.0	0.1	0.1
Lys	4.0	1.8	0.1	1.3
Total Conc. (μM)	176.8	49.2	47.8	7.6

The relative standard deviation for the peak areas ranged from 6% for Tyr to 3% for the other amino acids, at 10 μM. The detection limit was 300 fmol, at a signal-to-noise ratio of 3:1, which depended rather on the impurities in the solvents and on the sample matrix. The correlation coefficients for concentration versus response were $r>0.997$ for all derivatives except serine ($r=0.992$).

Analysis of Algal Samples

Four phytoplankton species were analysed for their free amino acid contents after preconcentration as described in Scheme 1. The results are summarized in Table 4. The samples exhibited considerable interspecies

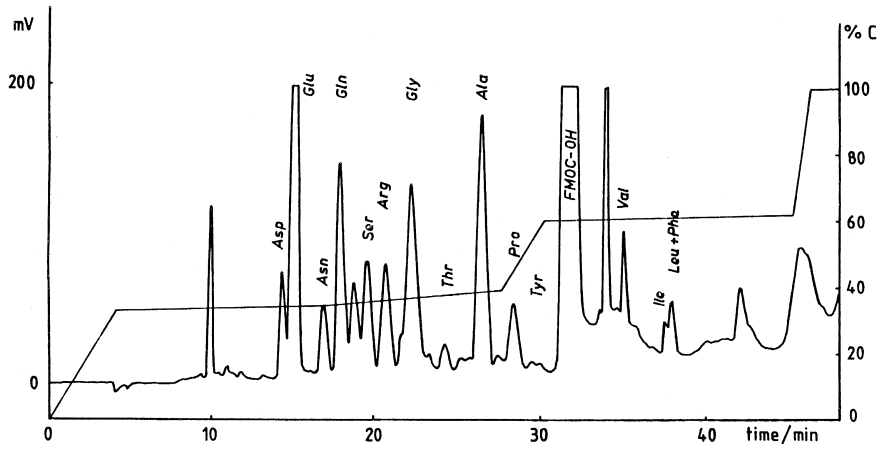


Figure 3. Semipreparative separation of FMOC-amino acids of *Thalassiosira rotula*. Total concentrations of amino acids: 375 μM. Operating conditions: Column: Vydac 218TP1510 C_{18}. Flow rate: 1.25 mL/min. Solvent A: 0.05 M sodium acetate (pH 5.5). Solvent B: THF. Solvent C: methanol. Gradient profile: see Materials and Methods. Excitation at 254 nm, emission filter 310 nm. Column temperature: 45°C. Injected volume: 250 μL.

differences, with concentrations varying between 176.8 and 7.6 μM. Hence, the results of HPLC analysis were normalized by calculating the ratio of each concentration to the total amino acid concentration. The five predominant amino acids (concentrations >5 mol%) in *Thalassiosira antarctica* were Pro, Glu, Gln, Ala, and Val, while those in *Thalassiosira rotula* were Glu, Ala, Asp, Gln, and Gly, which is in agreement with the results of Martin-Jézéquel,[34] who found similar abundances in *Thalassiosira rotula*. For *Phaeocystis antarctica*, Gly, Ala, Pro, Asn, and Val were predominant, whereas for *Phaeocystis globosa*, Gly, Ser, Gln, Pro, and Tyr were present in highest concentrations. The five most frequent amino acids accounted for 73.7-79.1% of the free amino acid pool.

Semipreparative Chromatography of Algal Samples

In order to test the suitability of the semipreparative amino acid separation, sample preparation and derivatization were carried out on a very dense culture of *Thallasiosira rotula* (Figure 3 and Table 5). The chromatogram (Figure 3) shows the separation of an original sample of 250 μL with a total amino acid concentration of 375 μM. Increases of the injected volume mostly decreased the peak resolution.

Table 5

Purities of Separated Amino Acid Fractions of Sample of *Thalassiosira Rotula*

Amino Acid (AA)	Original Conc. (μM)	Purity of Fraction(%)	Main Impurity Amino Acid	(%)
Asp	18.8	52	Glu	48
Glu	72.1	>99	Asp	>1
Asn	14.2	50	Glu	10
			Gln	17
Gln	70.5	>97	Glu	>3
Ser	25.6	>95	Gln	2
			Gly	3
Arg	8.8	85	Ser	4
			Gly	3
Gly	41.9	>97	Ala	2
Thr	20.6	75	Gly	25
Ala	57.8	>99	---	---
Pro	19.3	98	Ala	2
Tyr	3.2	not collected	---	---
Met	>0.1	not collected	---	---
Val	5.1	88	Ala	4
Ile	4.4	80	Leu+Phe	20
Leu+Phe	7.3	80	Ile	20
Hys	>0.1	not collected	---	---
Lys	5.2	not collected	---	---

For the separation of individual amino acids, the start and end-point of fraction collection were chosen at the minimum level between the two neighboring peaks. The collected fractions were re-chromatographed and the purities of fractions were determined. As can be seen in Table 5, Gln, Glu, Ser, Gly, Ala, and Pro fractions could be obtained with a purity of >95%. The purity of a fraction strongly depends on the start and end-point of collection. Purer fractions can be obtained by decreasing the time of collection, but at the cost of the maximal amount of amino acid.

Finally, we tested the feasibility of labeling the amino acids with ^{15}N in order to clarify the intra- and interspecies differences in N incorporation by phytoplankton. The incubation experiment was primarily designed to assay the significance of ^{15}N enrichment in the different amino acids. The purpose was not to estimate the precision and accuracy levels of observed ^{15}N abundances.

Therefore, a dense phytoplankton culture was prepared under similar conditions as described in the Methods section, but with labeled (99% ^{15}N) rather than natural (0.37% ^{15}N) nitrate. Free amino acids were preconcentrated and separated by semipreparative chromatography, as described above. The different collected fractions were submitted to emission spectrometric determination of their ^{15}N abundances after conversion of the bound nitrogen into N_2 gas.[35] A majority of the amino acids exhibited a significant enrichment, with abundances ranging from 0.37% to > 1.0%.

These preliminary results were not translated into incorporation rates, since the phytoplankton was grown under extremely nutrient-rich conditions. Nevertheless, the traceable ^{15}N enrichment of several amino acids opens up perspectives for future nitrogen uptake studies.

Further experiments are needed to validate the procedure for measuring the rates of incorporation. The utility of the procedure described here, however, considered to have been demonstrated.

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