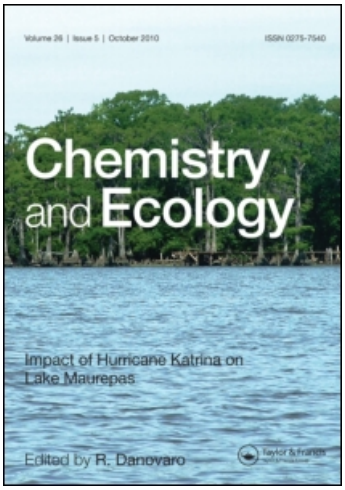


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# A High Performance Liquid Chromatographic Determination of Free Amino Acids in Natural Waters in the Picomolar (M X $10^{-12}$) Range Suitable for Shipboard Use

Roger Evens[a]; James Braven[a]; Leslie Brown[a]; Ian Butler[b]
[a] Department of Environmental Sciences, Plymouth Polytechnic, Plymouth, Devon, U.K. [b] Marine Biological Association of the U.K., Citadel Hill, Plymouth

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# A High Performance Liquid Chromatographic Determination of Free Amino Acids in Natural Waters in the Picomolar ($M \times 10^{-12}$) Range Suitable for Shipboard Use

ROGER EVENS, JAMES BRAVEN and LESLIE BROWN

*Department of Environmental Sciences, Plymouth Polytechnic, Plymouth, Devon, U.K.*

*and*

IAN BUTLER

*Marine Biological Association of the U.K., Citadel Hill, Plymouth*

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A reliable method for the determination of amino acids, suitable for routine shipboard use, is presented. Using reverse-phase high-performance liquid chromatography with computer-controlled gradient elution optimization, the amino acids commonly found in sea-water were clearly resolved and determined at the picomole level. A procedure was also developed to enable repeat injections of sea water samples to be made without deterioration of column material or performance. Thus the analysis was made more operator-independent and consequently more suitable for use at sea.

## INTRODUCTION

Productivity studies in the English Channel have shown that the phytoplankton growth that occurs in the Spring strips from the water the inorganic nitrate which has built up during the winter months. The nitrate concentration in the euphotic zone of the water column then remains low throughout the summer months, the nitrogen available for phytoplankton growth during this period being in the form of ammonia, urea, and other nitrogenous products recycled by the biota (Butler *et al.*, 1979). There is now considerable evidence that some species of phytoplankton preferentially utilise specific forms of dissolved nitrogen (Harvey *et al.*, 1976;

Schell *et al.*, 1974; Sepers, 1977). The nature of the dissolved nitrogen available for phytoplankton growth is therefore one of the factors which decides which particular competing phytoplankton species will have optimum conditions of growth.

In the English Channel studies, about half the dissolved nitrogen constituents in the Summer have been identified and measured individually. It is known that the amino acids make up a significant part of the unidentified fraction but until recently no simple reliable method was available for their estimation. The main difficulty was that amino acids in sea water are present in μg quantities dissolved in a 3.5% saline solution and the high salt content gave rise to analytical difficulties. The development of HPLC presented an opportunity to overcome these difficulties and Lindroth and Mopper (1979) demonstrated the HPLC separation of amino acids in sea water following direct injection using pre-column fluorescence derivatisation with o-phthaldialdehyde. Initially we attempted to use this method but were unable to obtain consistent results for quantitation of individual amino acids. Column deterioration was also very rapid.

The procedure described in this paper has been developed from Lindroth and Mopper's method and gives consistent results both in fresh and saline waters.



## MATERIALS AND METHODS

### Reagents

All solvents used in the analyses were Fisons HPLC grade. Distilled water was double-distilled from glass. All other compounds were obtained from BDH and were of the purest grade commercially obtainable. Standard solutions of amino acids (1m mol/l) were stored at 4°C.

Phosphate buffer (pH 7.50) was prepared by titrating a 0.1 M solution of disodium phosphate with 0.1 M monosodium phosphate to pH 7.50 and borate buffer (pH 9.50) was prepared by titrating 0.4 M boric acid with sodium hydroxide to pH 9.50.

Ortho-phthaldialdehyde (OPA) reagent was prepared by dissolving 135 mg OPA in 5 ml HPLC grade methanol, adding mercaptoethanol (100 μl) and making up to 25 ml with borate buffer (pH 9.5). The reagent was stored unrefrigerated in glass and under these conditions was stable for 7 days if a further 20 μl of mercaptoethanol was added after 3 days.

The eluting solvents and buffer were degassed by filtration through a 0.45 μm Millipore filter.

### Apparatus

The solvent delivery system consisted of the following: Two Waters Associates 6000A solvent delivery pumps with a Waters 720 System

Controller and a Waters U6K injector. Detection was achieved using either a Perkin Elmer MPF 3 fluorescence spectrophotometer fitted with a flow cell ($\lambda$ ex = 345 nm, $\lambda$ em = 440 nm) or a Waters 420 filter fluorometer (excitation filter 335 nm, emission filter 450 nm). A Phillips PM 8252 dual-channel recorder was used. A Hewlett Packard 3390 A recorder with an integrator was connected in parallel with the Phillips recorder and the integrator was programmed to convert peak areas to mole fractions of the appropriate amino acid. Sample injection was done with Hamilton syringes; Oxford variable volume automatic pipettes were used to dispense reagents.

A Shandon Southern column (100 × 5 mm), packed with Hypersil 5–7 $\mu$m octadecylsilane (ODS), and fitted with a Shandon guard column packed with Whatman pellicular (25–37 $\mu$m) ODS was used in the analyses.

A set of acid washed glassware (10% HCl) was kept specifically for use in these analyses.



### Procedure

*Collection and handling* Samples of sea water were collected and immediately filtered through a 0.45 $\mu$m filter under low vacuum. The analyses were carried out as soon as possible after collection and usually within 48 h. When this time was exceeded the samples were deep frozen. Although storage by this method appeared to be satisfactory in most cases it occasionally gave rise to anomalous results. It is recommended that the storage capacity of the samples should be checked as this may vary not only seasonally but from one location to another.

Great care must be taken in the handling of glassware and apparatus to avoid contamination, and as finger marks are one source of amino acids (Hamilton, 1965) it is recommended that suitable protective gloves should always be worn.

Syringes were repeatedly flushed with water before and after each injection and filled with water between injections. Cleanliness was checked by monitoring a distilled water injection between analysis.

## RESULTS

### Analysis

The filtered water sample (500 $\mu$l) was mixed with 100 $\mu$l of OPA reagent in a glass stoppered flask and, after 2 minutes, 300 $\mu$l of 0.4 M boric acid added to quench the reaction and lower the pH to 7.50. A 100 $\mu$l sample of this mixture was then injected onto the column with the following programme:

| Time minutes | Flow rate | $CH_3CN$ (%) | Phosphate Buffer (%) | Curve |
|---|---|---|---|---|
| 0 | 4 | 3 | 97 | Linear |
| 6 | 4 | 7 | 93 | Linear |
| 14 | 4 | 10 | 90 | Linear |
| 24 | 4 | 20 | 80 | Isocratic |
| 30 | 4 | 20 | 80 | Linear |
| 36 | 4 | 33 | 67 | Linear |

Retention times of various compounds under these conditions is given in Table I and a typical recorder trace obtained from a sea water sample analysed at sea is shown in Figure 1.



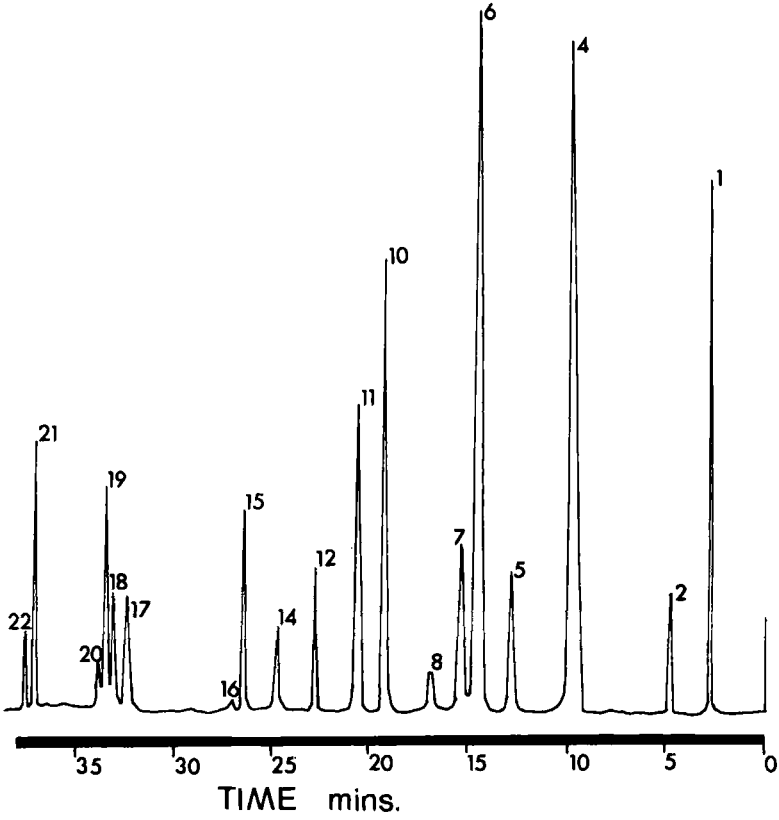


FIGURE 1 Chromatogram obtained from the analysis of a 150 μl sample of sea water. For peak identification see Figure 2.

TABLE I

Retention times of standard compounds

| Amino acid | Time in minutes |
|---|---|
| Aspartic acid | 3.0 |
| Cystine | 4.0 |
| Glutamic acid | 5.1 |
| Asparagine | 9.7 |
| Serine | 10.1 |
| Histidine | 12.9 |
| Glycine | 14.9 |
| Threonine | 16.0 |
| Arginine | 16.4 |
| β-Alanine | 18.9 |
| Alanine | 19.7 |
| Tyrosine | 21.1 |
| α-amino butyric acid | 22.9 |
| Ethanolamine | 23.8 |
| γ-amino butyric acid | 24.7 |
| Valine | 27.2 |
| Methionine | 27.9 |
| Tryptophan | 33.0 |
| Phenylalanine | 33.2 |
| Isoleucine | 33.6 |
| Leucine | 34.0 |
| Ornithine | 37.5 |
| Lysine | 38.1 |



After 50 injections of sea water the guard column was refilled. It was not necessary to refill the chromatography column until at least 200 sea water injections had been made.

### Safety

Great care was taken when using acetonitrile because of its poisonous properties. In our work the effluent from the instrument was discharged directly into a flask containing 5% sodium hypochlorite. This solution was renewed at the start of each day's work.

### Calibration

A calibration mixture containing $44 \times 10^{-12}$ moles of each standard was analysed as already described to yield a calibration chromatogram. The calibration was checked after every 5 injections and was very reproducible. Although amino acid standards produced different fluorescence intensities,

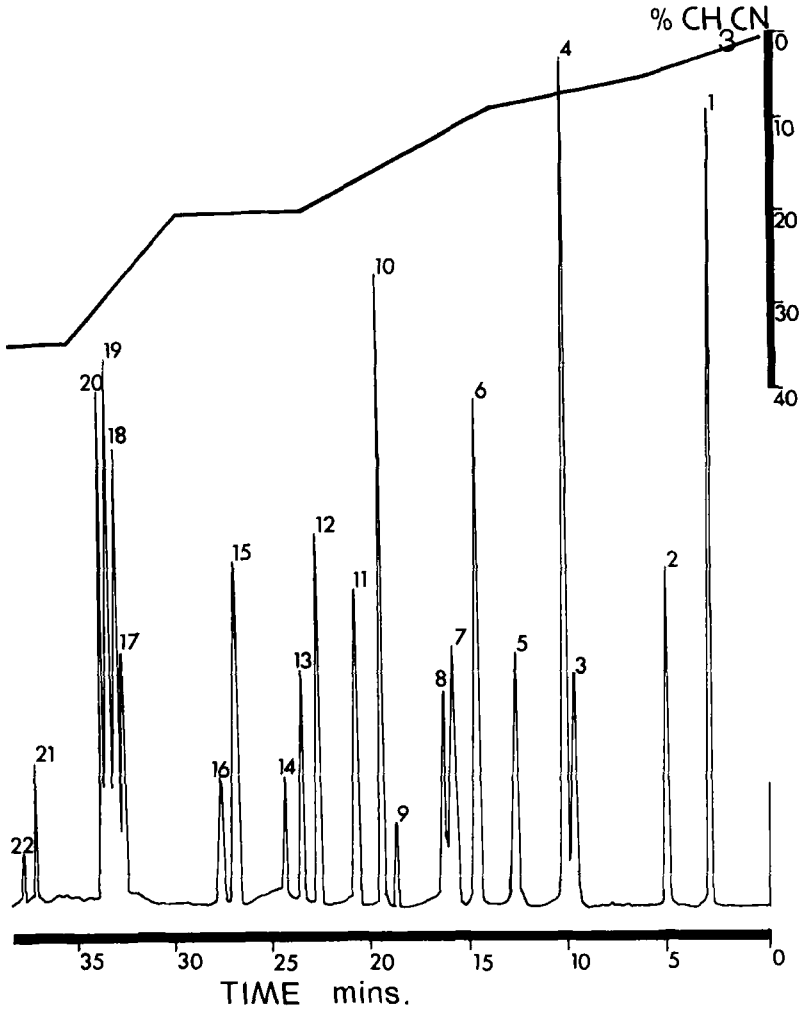


FIGURE 2 Chromatogram obtained from the analysis of an equimolar (44 picomoles) amino acid mixture. Identification of the compounds is as follows: 1. Aspartic acid; 2. Glutamic acid; 3. Asparagine; 4. Serine; 5. Histidine; 6. Glycine; 7. Threonine; 8. Arginine; 9. β-Alanine; 10. Alanine; 11. Tyrosine; 12. α-amino butyric acid; 13. Ethanolamine; 14. γ-Amino butyric acid; 15. Valine; 16. Methionine; 17. Tryptophan; 18. Phenylalanine; 19. Isoleucine; 20. Leucine; 21. Ornithine; 22. Lysine. The $CH_3CN$ elution gradient is also shown.

the fluorescence intensity for each amino acid was the same in distilled, estuarine or sea water. A chromatogram of a mixture of 22 standard compounds together with the gradient profile is shown in Figure 2.

Standard additions of known amounts of amino acids to sea water gave linear responses for each amino acid over the range 1–400 picomoles.

### Reproducibility

Under the conditions specified in this paper the reproducibility of results varied for each amino acid. Table II gives the coefficient of variation for 20 amino acids found in sea water.

### Column Regeneration

After each determination the column was re-equilibrated to the initial conditions for 5 minutes before the next determination. Using this procedure twelve sea water samples (including calibration and blanks) could be analysed in twelve hours.



TABLE II

Reproducibility of the results obtained for the analysis of 33 picomoles of 20 amino acids found in sea water

| Amino acid | Coefficient of variation (based on 6 repeat analyses) |
|---|---|
| Aspartic acid | ± 9% |
| Glutamic acid | ±11% |
| Asparagine | ±12% |
| Serine | ± 7% |
| Histidine | ±13% |
| Glycine | ±28% |
| Threonine | ±12% |
| Arginine | ±13% |
| Alanine | ±11% |
| Tyrosine | ± 8% |
| α-amino butyric acid | ± 9% |
| γ-amino butyric acid | ±10% |
| Valine | ±11% |
| Methionine | ±11% |
| Tryptophan | ±12% |
| Phenylalanine | ±11% |
| Isoleucine | ±10% |
| Leucine | ±10% |
| Ornithine | ±10% |
| Lysine | ±12% |

The coefficient of variation is the standard deviation expressed as a percentage of the mean.

### Shut-down procedure

At the end of each working day the system was washed with 40 ml of water followed by 100 ml of methanol.

## DISCUSSION

The elution system of phosphate buffer and acetonitrile was selected after much preliminary investigation as it gave the clearest separation, base-line stability and reproducibility.

The column deterioration observed by Lindroth and Mopper (1979) and ourselves was overcome by adding 0.4 M boric acid to the reaction solution after 2 minutes reaction time thereby reducing the pH from 9.5 to 7.5. The silica base of the column packing is not soluble at this pH and the column showed no deterioration in performance even after 200 injections. The normal procedure for chromatography of sea water samples of replenishing the guard-column after 50 injections was observed however.

This method has been developed for use both in the laboratory and at sea. It has already been tested at sea during a cruise on the NERC Research Vessel Frederick Russell when it performed well under difficult weather conditions including a Force 8 gale. Computer control of gradient profiles and programmable conversion of peak areas to mole fractions of amino acids not only greatly speeds analysis but also renders the method more useful for shipboard use by making it more operator-independent. The method is at present being used to study the seasonal variation of the amino acids in English Channel waters and the results from these studies will be published elsewhere.



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