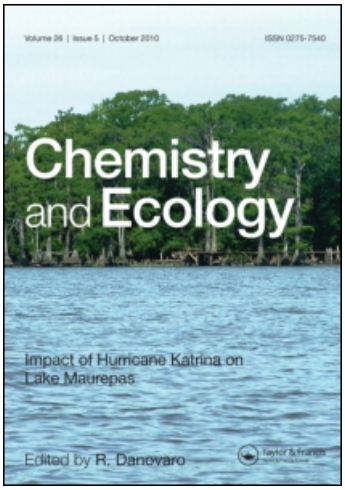

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Some Practical Aspects of Measurements of Dissolved Free Amino Acids in Natural Waters and Within Microalgae by the Use of HPLC

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Some Practical Aspects of Measurements of Dissolved Free Amino Acids in Natural Waters and Within Microalgae by the Use of HPLC

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A detailed method for the analysis of dissolved free amino acids (DFAA) and ammonium by reverse phase high performance chromatography of fluorescent derivatives using commercial columns and guard columns is described in detail for the newcomer. The method is flexible enough to allow for minor differences between different HPLC pump and gradient systems. For DFAA, the method is sensitive and reproducible down to high femptomole/low picomole levels, suitable for analysis of DFAA in natural waters down to the low nanomolar range. Aspects of reproducibility, sensitivity, sample preparation, cell extraction and sample storage are considered.

INTRODUCTION

Dissolved organic nitrogen (DON) forms a large component of total N in mature marine ecosystems, such as mid ocean waters (Williams and Druffel, 1987) and summer temperate waters (Butler *et al.*, 1979). An important component of DON is dissolved free amino acids (DFAA) but initially research was restricted because of problems in quantification (Dawson and Mopper, 1978). At the

beginning of this decade a major advance was made with the development of HPLC based methods for analysis of DFAA at natural concentrations (Lindroth and Mopper, 1979; reviewed by Mopper and Dawson, 1986). Measurements of DFAA have now been made in sea water (Mopper and Lindroth, 1982; Braven *et al.*, 1984; Laanbroek *et al.*, 1985; Williams and Poulet, 1986), fresh water (Jørgensen, 1987), sediment pore water (Jørgensen *et al.*, 1980) and in rain (Mopper and Zika, 1987). These studies have shown the importance of DFAA in the N-cycle. More specific studies have shown the role of DFAA in the nutrition of marine bacteria (Amano *et al.*, 1982; Ferguson and Sunda, 1984) and of phytoplankton (reviewed by Flynn and Butler, 1986). Studies of the internal pool of DFAA in algae suggest that they may be used as biomarkers of N-status (Admiraal *et al.*, 1986; Haberstroh and Ahmed, 1986; Flynn and Al-Amoudi, 1988). Other studies have shown the role of phytoplankton (Hammer and Brockman, 1983) and microflagellates (Andersson *et al.*, 1985) in DFAA release, and the possible use of DFAA released by phytoplankton as a chemosensory stimulus to copepods (Gill and Poulet, 1988).

In order to facilitate the more frequent use of HPLC in such studies it is important that methods are described in detail. In almost all reports of such methods, and of their use, various important procedural steps, perhaps obvious to the expert but vital to the newcomer, are poorly explained. The aim of this communication is to describe methods for the analysis of low picomole amounts of α amino acids using columns and guard columns obtained from international suppliers. In particular, methods have been described (a) in sufficient detail for the inexperienced user and (b) which are flexible enough to cater for different HPLC systems. Methods and problems of FAA collection, extraction from algae, and storage are explored.

Three methods have been tried. The methods of Lindroth and Mopper (1979) and Evens *et al.* (1982) use phosphate buffer as the inorganic solvent, although the former uses methanol (MeOH) and the latter acetonitrile as the organic solvent. Both of these methods also make use of columns and, perhaps of lesser importance, guard columns, packed by the workers. This complicates attempts at method duplication, especially if facilities for packing are not available. The method of Jones and Gilligan (1983) uses acetate

buffer with the addition of tetrahydrofuran (THF) as the inorganic solvent and MeOH as the organic solvent and, unlike the other two methods, as originally described used a commercial analytical column. The guard column described by Jones and Gilligan (1983), and used by others (O'dell and Stephens, 1986), was a 45 × 2.1 mm column which they packed with Whatman ODS Co:Pell or similar. Although tap-packing is not difficult, and is economical, it may not always be convenient and the desire was to develop a system which was easy to maintain. A cartridge guard column was considered preferable. After testing the other methods and experiencing considerable difficulty in obtaining satisfactory results, the Jones and Gilligan (1983) method was that considered the best starting point.

MATERIALS AND METHODS

Instrumentation

The system used consisted of two Waters 510 pumps, Waters 680 gradient controller, Waters U6K injector fitted with a trigger switch, Waters 740 AC fluorimeter (excitation filter 335 nm, emission filter 450 nm), Waters 740 data module (integrator), and a chart recorder for use in monitoring pressure and documenting gradient shape during method development. The use of a variable volume injector, rather than a fixed loop injector, can have its advantages. Lengths of tubing between injector, guard column, column and detector (0.009" i.d.) were kept as short as possible.

Liquid handling

HPLC grade water (>14 megΩ) supplied by a MilliQ (Millipore) water purification system was used at all times, all solvents and chemicals were of the highest quality obtainable (HPLC grade where possible).

Where possible all-glass equipment (filter apparatus, beakers, bottles etc.), or disposable plastic containers (obtained from microbiological suppliers) were used. A set of glassware was kept specifically for the analysis, soaked in 10% HCl and rinsed in

double distilled and finally HPLC grade water. At all times gloves were worn to minimize contamination by amino acids on hands (Hamilton, 1965).

Columns

A Beckman 150 × 4.6 mm Ultrasphere (5 μm spherical particle, octadecylsilane (ODS) C_{18}, endcapped) analytical column was used, preceeded by a Waters Guard-Pak guard column fitted with μBondapak (10 μm irregular particle ODS C_{18}, endcapped) inserts.

Columns and guard columns were preconditioned and stored as detailed by the manufacturers. Inserts were replaced every 40–50 injections when the chromatography (peak shape or resolution) deteriorated; this was usually due to irreversible accumulation of organics rather than blocked frits (increasing back pressure) in the insert. Regeneration of the analytical column was performed by the start up programme; further regeneration and column maintenance was performed as detailed by the column manufacturer.

Mobile phases

Solvent A was 90:10 acetate buffer:MeOH; 0.05 M sodium acetate trihydrate was titrated to pH 6.8 with acetic acid. The solvent could be stored for up to 48 h at 4°C. Solvent B was MeOH. 500 ml of each solvent was just sufficient for 10 injections including start up and shut down. Solvents, and 500 ml water, were degassed by vacuum filtration through 0.2 μm Durapore (Millipore) filters on the day of use. Tetrahydrofuran (THF), typically 0.25%, was added to solvent A after degassing. Filters were rinsed first with 20 ml water or MeOH, as appropriate, in order to remove extractables; some makes of filters appeared to have high levels of extractables (e.g. Gelman Vericel) which bind to the column leading to premature guard column failure.

Standards and internal standard

1 mM solutions of chromatographically pure L-amino acids were made in HPLC grade water. It was convenient to make these in groups (i.e. acidic, amidic, aliphatic hydroxy, aliphatic neutral,

aromatic, basic and sulphur), especially when developing the gradient. Using these, 20 μM solutions were made and stored at −20°C as detailed below: Storage of samples. On the day of use a calibration standard was made at the required concentration (50, 100 or 200 nM; warning—see Results: storage of samples). The 20 μM standards were replaced every month although no deterioration was apparent after 6 months. Stock internal standards (2-amino *n*-butyric acid was usually used) were made and stored as for other standards, and made up to 2.5, 5 or 10 μM on the day of use.

Derivatization

The derivatization reagent was made by dissolving 0.135 g o-phthalaldehyde (OPA) in 5 ml MeOH and making up to 25 ml with borate buffer (1.9 g sodium tetraborate in 50 ml water). 2 ml volumes were filter sterilized (Gelman 0.2 μm Acrodisc) into sterile 7 ml plastic bijous (see below: storage of samples) and showed no signs of deterioration over 2 months. On the day of use, 10 μl 2-mercaptoethanol was added to 2 ml OPA stock solution. Boric acid (1.24 g in 50 ml water) was stored for a maximum of 2 weeks at room temperature.

Derivatization was performed in disposable 1.5 ml microtubes (Eppendorf type). A simple tool was used to open the microtube without risk of touching the inside of the lid; the lip on the lid of some makes of microtube is very small. 20 μl internal standard was added to 480 μl sample. Care was taken to ensure that any sample which had been stored frozen had warmed to room temperature. The reaction was started by the addition of 100 μl derivatization reagent and the mixture shaken. After 2 min the reaction was halted by the addition of 300 μl boric acid and shaken. This addition also lowers the pH to below 7.5; the silica base of the columns breaks down at pH < 2 and > 7.5. A volume, typically 200 μl, was loaded into the injector and injected onto the column exactly 3 min after the start of the derivatization.

Gradients

For all gradients, flow rates (and hence pressure) were altered slowly to minimize damage to the column. Aqueous solvents (other

than 50:50 MeOH:water) were never allowed to stand in the system; a minimum flow rate of 0.1 ml min^{-1} was used. Column and guard column temperatures were maintained at 27–28°C by the use of a simple thermistor controlled heater (a coil of wire wound on a glass tube as the element) attached to the column and both analytical and guard columns surrounded with expanded polystyrene for insulation.

Start up. The column and guard column were stored with 50:50 MeOH:water and attached to the instrument; pumps were stored containing 100% MeOH. Pump A was primed on water and pump B on MeOH. The priming valve was shut and programme 1 (Table I) run. This washed the column in 100% MeOH, removing any fluorescent material, replaced the MeOH with water and then slowed to 0.1 ml min^{-1}. The pumps were stopped and the solvent line for pump A was placed in solvent A (degassed and with typically 0.25% v/v THF added—see Results: method development and Figures 1 & 2), and 20 ml solvent drawn through the priming valve. The injector was flushed as per the manufacturer's instructions. Programme 2 (Table I) was run for 2 min followed by programme 3 (the main gradient programme) to equilibrate the column. 34 min later the first injection was made.

Chromatography. The main gradient programme 3 (Table I) was a multi-step progression from 0% to 80% MeOH followed by an increase to 100% MeOH to bring off any other organics, and a decrease and re-equilibration to 0% MeOH (Figure 2). This increase from 80% to 100% MeOH may be omitted, but a more rapid deterioration of column efficiency may be noted. Time between injections was 34–36 min. It was found convenient to use a timed event programme to indicate when to start the next derivatization so that injections could be made within a minimum time.

Shut down. If it was necessary to stop the analysis, for example to replace the guard column, programme 4 (Table 1) was used to decrease pressure gently. Programme 2, followed by 3, was used to start it again.

After the last gradient was complete, programme 4 was run and

TABLE I
Chromatography programmes. All steps in gradients are linear

Programme 1 (Start up)

Time (min)	Flow (ml/min)	%A (water)	%B (MeOH)
initial	0.1	0	100
2.0	2.0	0	100
15.0	2.0	0	100
20.0	2.0	100	0
30.0	2.0	100	0
32.0	0.1	100	0

Programme 2 (Speed up); Programme 4 (Slow down) has flow rates reversed.

Time (min)	Flow (ml/min)	%A (Solv. A)	%B (MeOH)
initial	0.1 (2.0)	100	0
2	2.0 (0.1)	100	0

Programme 3 (Chromatography)

Time (min)	Flow (ml/min)	%A (Solv. A)	%B (MeOH—Solv. B)
initial	2.0	100	0
0.5	2.0	80	20
7.0	2.0	80	20
9.0	2.0	58	42
11.0	2.0	58	42
12.0	2.0	48	52
14.0	2.0	48	52
14.5	2.0	30	70
19.0	2.0	20	80
22.0	2.0	0	100
26.0	2.0	100	0
32.0	column equilibrated, prepare for next injection.		

Programme 5 (Shut down)

Time (min)	Flow (ml/min)	%A (water)	%B (MeOH)
initial	0.1	100	0
2.0	2.0	100	0
16.0	2.0	100	0
22.0	2.0	50	50
28.0	2.0	50	50
30.0	0.1	50	50

pumps stopped. Pump A was flushed (primed) with 50 ml water and programme 5 (Table I) run to wash out the buffer (solvent A) and finish with the column in 50:50 MeOH:water. The injector was flushed during this procedure. Pump A was then primed with MeOH, the priming valve shut and pumps turned off.

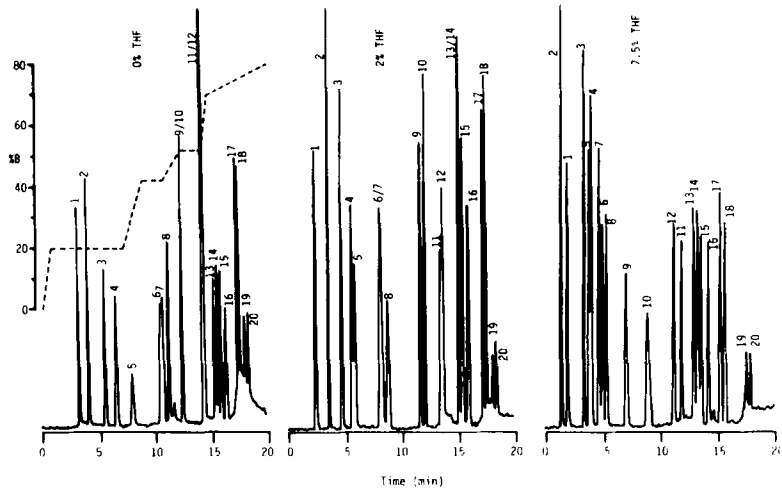

FIGURE 1 Effect of addition of different amounts of THF to solvent A. Peaks were not identified but presumptive movements of peaks are indicated by numbers. Note the difference in retention times of some components and the way in which some components change order. The amount of THF typically added was 0.25%; see Figure 2. Proportion of solvent B is indicated (– – –).

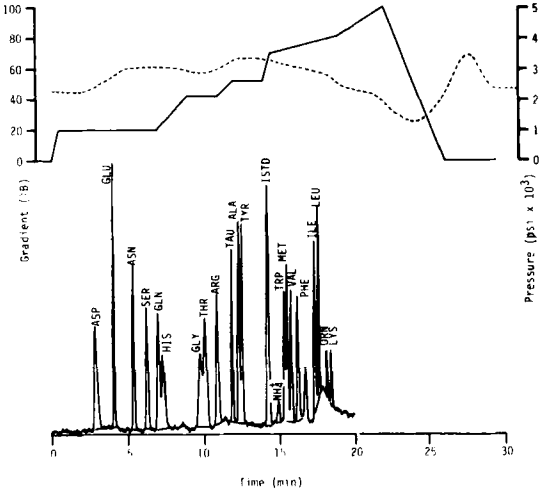

FIGURE 2 Chromatogram of 20 amino acids, NH_4^+ and 2-amino *n*-butyric acid (as internal standard—ISTD). Sample amino acids at 5 pmole each, NH_4^+ at 50 pmoles, on the column. Percentage solvent B and changes in pressure during the chromatography (as described in programme 3, Table I) are also shown. 0.25% THF was added to solvent A. Proportion of solvent B (——) and typical column pressure (– – –) are indicated.

Filtration of samples

A simple apparatus was used (Figure 3) in order to filter particulates, to sterilize the sample and store it in chemically clean conditions. The plunger of a sterile 10 ml plastic syringe was withdrawn down to the 5 ml mark under aseptic conditions (either in a sterile cabinet or working in the hot air rising from a bunsen flame, with care being taken not to touch any of the internal surfaces of the equipment even with gloved hands). A sterile 0.2 μm filter (Millipore "Millex" or Gelman "Acrodisc" was attached to this syringe and the barrel of a 5 ml syringe attached to the other end of the fiter using a short (1 cm) length of PVC tubing. The sample (5 ml) was poured into the upper barrel and the plunger of the lower pulled down 0.5 ml to the 5.5 ml mark. By maintaining the air gap in the lower syringe at 5.5 ml the vacuum applied to the

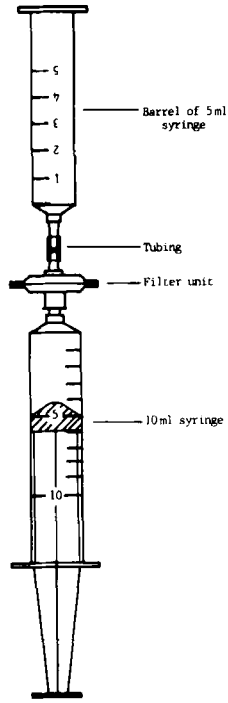

FIGURE 3 Filtration apparatus. Construction and use are described in the text.

filter never exceeded, say, 7.5 cm Hg. If there was an air-lock in the filter, slight pressure was applied on the plunger to expel the air. Using this simple apparatus, small volumes (<5 ml) could be gently filter-sterilized into a syringe and later, if required, transferred asceptically into sterile plastic bijous for storage (see below).

Cell extraction

In order to measure the intracellular pool of FAA in algae, the FAA must be extracted into a solvent compatible with the derivatization method. Cells were collected on to a suitable filter (Whatman GF/F were used) under gentle vacuum (<10 cm Hg) using acid washed filtration apparatus. No cell rupture was detected by analysis of the media after filtration. Filters were not washed as the risk of rupturing the cells (Fuhrman and Bell, 1985; Goldman and Dennett, 1985) was considered to outweigh errors associated with any relatively small amounts of DFAA from the media remaining in the filter matrix. Filters were placed in acid washed glass centrifuge tubes and extractant added. Tricarboxylic acid (TCA) extraction was compared to hot water extraction. For the TCA extraction, the method was similar to that of Haberstroh and Ahmed (1986); the cells were incubated with 4 ml 0.3 M TCA at 4°C for 15 or 30 min and neutralized with the addition of 0.4 ml 3.0 M NaOH. Hot water extraction was performed by adding 2 ml HPLC grade water and incubating at 80°C for periods of 15 or 30 min, centrifuging, removing the supernatant, and repeating with another 2 ml water. The first 2 ml was added back to the test tube (which now contains 4 ml) and recentrifuged; the top 2–3 ml of supernatant was recovered.

An alternative (preferred) method for hot-water extraction was to load cells (10^6 maximum) on to a filter using the apparatus in Figure 3. After the medium had been drawn out of the filter unit, a 2 ml syringe containing 2 ml HPLC grade water at 70°C was attached in place of the 10 ml (or larger) syringe, and another 2 ml syringe with its plunger at 0 attached to the other end. The cells were then resuspended in water by drawing water from one syringe to the other; the shear forces help to disrupt the cells. The apparatus was incubated at the desired temperature (60–70°C—check that this is suitable for the syringes and filter used) with occasional flushing of

the water from one side of the filter to the other. After the incubation (30–60 min), the water was forced through to the lower syringe leaving the cell debris on the other side of the membrane. Centrifugation was not required. An additional freeze-thaw step may be included with the filter + cells, with 2 empty 2 ml syringes attached, frozen to −20°C and the cells thawed and extracted by the addition of 2 ml water at 70°C as described. This method is convenient when taking many samples and has been used successfully to extract FAA from microalgae, including cyanobacteria, and from yeast.

Storage of samples

It is unusual for samples for chemical analysis to be sterilized (Butler, personal communication) but for samples containing compounds which may be substrates for microbes, sterilization may be considered desirable. With the advent of disposable ready-to-use filters, filter sterilization has become very simple. Samples of growth medium or sea water were filter sterilized using the apparatus shown in Figure 3.

Storage of solutions containing low concentrations of amino acids requires that two conditions are met; the container must be chemically clean (if reusable it may be acid-washed and rinsed in HPLC grade water) and biologically clean (sterile). As an extra precaution, freezing of the sample may be considered. Two types of container were tested; acid washed glass vials (such as those used in scintillation counting) and sterile polystyrene 7 ml bijous (used in microbiological applications). For convenience plastic bijous were used for the storage of amino acid standards and internal standards; 3–4 ml were filter sterilized (0.2 μm Gelman "Acrodisc") into each bijou and stored at −20°C (more than 4 ml sometimes cracked the bijou on freezing).

RESULTS AND DISCUSSION

Method development

Initial experiments were conducted using the Shandon column and guard column described by Evens *et al.* (1982) (gradient shown in

Figure 1, similar to that described in Table 1 with 0.5% THF added to solvent A) even though the size of the column and of the packing is different to that described in the method of Jones and Gilligan (1983). The HPLC system used is a high pressure mixing system with a static mixer, but the method of Jones and Gilligan (1983) has also been used with success using a single-head pumps and dynamic mixing (e.g. Beckman/Altex system) (Jones and Gilligan, 1983; Lu and Stephens, 1984; O'dell and Stephens, 1986). The basic method appears to be robust both against differences in equipment and minor differences in columns.

Optimum separation using the column and guard column described in materials & methods was achieved by running a series of solvents with different amounts of THF (0, 0.25, 0.5, 1, 2.5, 5 & 7.5%) in solvent A using two variants of the gradient (alterations in composition of solvents and periods of isocratic elution—for examples, see Jones and Gilligan, 1983); the aim was to separate the mixture into the known number of individual peaks. Some results are shown in Figure 1, using a gradient similar to that in Table I. A separation was obtained by the addition of 0.25% or 7.5% THF although elution order was different. It was found to be essential that THF was added to the solvent *after* degassing. The gradient was then altered further to optimize separation.

Slight alterations of gradient sometimes had significant effects, often much later in the elution than the change, and improvements in separation of some peaks led to poorer separation of others. The gradient also had to be altered slightly to prevent co-elution of peaks of interest with any buffer peaks (these were identified by running the gradient without an injection). Quantification of leucine was adversely affected when the top of the buffer peak coincided with its peaks in the latter part of the gradient. Another factor in development was that slight alterations in gradient sometimes affected peak area, especially of ornithine and lysine. During earlier experiments, attempting to decrease the time for separation using the method of Evens *et al.* (1982), similar effects were noticed, together with a more rapid loss in column efficiency associated with shorter gradients. Although it is possible that some of this deterioration was due to an increased number of injections during a day (regeneration by flushing with 100% solvent B for >15 min was normally only performed once a day), it appears that a compromise

must be reached between the speed of analysis and the life of the guard and analytical columns.

Having achieved a separation of all the compounds, an internal standard (ISTD) was chosen such that the ISTD peak was not merged with any other; 2-amino-butyric acid was used for the final chromatography.

Linearity was verified over the range of 2–24 pmoles each amino acid applied to the column ($r^2 > 0.99$). Care was taken not to exceed the linear response of the detector by using too high a gain setting (a gain exceeding 32 also increased baseline noise). It was also noted that quantification was improved when calibration standards of comparable concentration to the unknowns were injected; the injection of standards of 25–50 pmoles each FAA when the unknown injections had <5 pmoles each amino acid could result in errors in quantification of, especially, merged peaks. Likewise, the concentration of the internal standard was kept within the same range. There was no advantage in injecting large amounts of sample on to the column; this only resulted in a shortened guard column life. The method described was typically calibrated for analysis of sea water samples with 5.3 pmoles each FAA (200 μl injection from a solution of 50 nM each FAA—Figure 2) with good reproducibility (Table II).

Quantification was found to depend on the repeatability of all stages of derivatization and liquid handling. The use of microtubes for derivatization prevented any risk of carry-over or of sample dilution by water used for rinsing. The microsyringe used for injection was rinsed thoroughly and kept filled with water between injections. Timing of derivatization and of injection is critical. Even though the addition of boric acid stops the reaction it does not prevent degradation of the products (Lindroth and Mopper, 1979), so that it was just as important to inject at a constant time as it was to halt the derivatization reaction.

Reproduction was sensitive to temperature, column equilibration and to the condition of the guard column. Deterioration of the guard column had a more marked effect on the separation and quantification of some amino acids than others. In particular, ornithine and lysine were affected. However, the degradation was gradual and corrections made by the routine injection of standards. The integrator was calibrated at the start of the day and standards

TABLE II

Repeatability (95% confidence limits expressed as % of mean – $n = 6$) and retention times (RT) at 26°C with 0.25% THF in Solvent A of amino acids spiked into sea water. Average amount of FAA on column was 7.5 pmole each amino acid (equivalent to about 70 nM each in sea water). Internal standard was 2-amino *n*-butyric acid (RT = 14.3 min)

Amino acid	RT (min)	confidence limits (±% of mean)
ASP	3.0	1.6
GLU	4.1	2.0
ASN	5.5	3.8
SER	6.4	4.8
GLN	7.1	2.6
HIS	7.4	3.6
GLY	9.8	8.8
THR	10.1	1.4
ARG	11.0	2.8
TAU	12.0	2.2
ALA	12.4	3.8
TYR	12.6	2.1
NH_4^+	15.0	3.1
TRP	15.4	1.3
MET	15.6	1.6
VAL	15.8	1.6
PHE	16.3	1.7
ISO	17.4	3.5
LEU	17.7	2.7
ORN	18.2	5.3
LYS	18.5	4.8

were injected at intervals (every 4–5 unknowns) but not recalibrated; it was essential that the final injection was of a standard. A correction was made for any deterioration of column efficiency by plotting the injection number for the standards against the reported concentration, the % error for each injection calculated and the data for the unknowns corrected.

Sampling of DFAA from media and the use of artifical media

The needs to gently filter-sterilize small volumes of liquid into a chemically clean container were met by the use of the apparatus described (Figure 3). The pre-packed sterile filters had no detectable effect on the DFAA composition. This arrangement of syringe

and filter was used successfully from a small boat in a moderate swell. The same apparatus was used for filtration of cultured algae with no indication of cell rupture even using high cell densities. Filtration must be gentle and the volume low as cell rupture can occur due to shear stress worsened by the accumulation of many cells on the filter (Fuhrman and Bell, 1985; Goldman and Dennett, 1985). No rupturing of algal cells was detected using this method even when filtering 5 ml of 5×10^6 cells ml^{-1} of the diatom *Phaeodactylum tricornutum.*

In laboratory cultures organic buffers are frequently used. It is important that such buffers, especially Tris, which has an amino group which reacts with OPA, are not used. The presence of small amounts of Tris even after harvesting and washing organisms in Tris-free media, interfered with analysis of glycine and threonine. For marine work, carbonate buffer is preferable because it is the natural buffer. Suitable media were F2 (Guillard and Ryther 1962), essentially enriched filtered aged sea water (aged sea water has very low levels of DFAA), or the artificial sea salt Instant Ocean (Aquarium Systems), with the addition of F2 vitamins and trace elements. Bicine or Hepes were found to be suitable if an organic buffer was essential. It was also found that the presence of more than 20 μM NH_4^+, added as a N-source for algae, interfered with attempts to detect release of tryptophan, methionine or valine and especially of phenylalanine and lysine (these elute after NH_4^+).

Care was taken at all times to exclude accidental addition of FAA, and to remove any organic matter on glassware which may have broken down to release FAA on autoclaving. The addition of FAA to test for uptake by organisms was after autoclaving by 0.2 μm filter (Gelman Acrodisc or similar) with flasks containing no organisms also set up as controls against abiotic changes in FAA concentrations (see Results: storage of samples).

Extraction of FAA from intracellular pools

TCA extraction was compared to hot water extraction (Figure 4). The TCA extraction has the advantage that it is used as part of a method of cell fractionation (Roberts *et al.* 1955; Haberstroh and Ahmed 1986). For extraction of FAA it has the disadvantage that

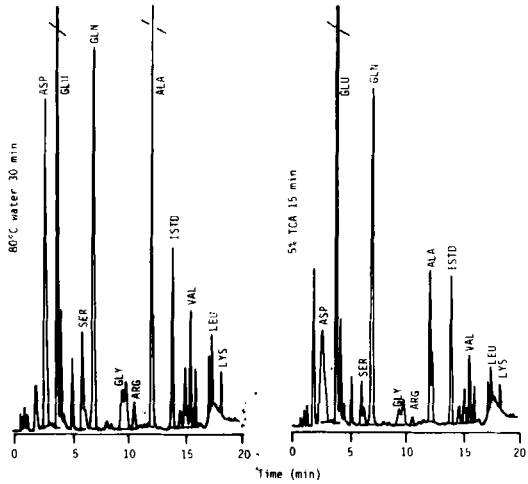

FIGURE 4 Differences between extraction of cells of the diatom *Phaeodactylum tricornutum* by use of 80°C water or 5% trichloroacetic acid (TCA). Note the poor recovery of GLY, THR, ARG and ALA; although the shape of the ASP peak in the TCA sample is bad (suggesting adverse effects of TCA on separation) the area is similar.

the extract requires neutralization with NaOH (Haberstroh and Ahmed 1986) as the pH is unsuitable for derivatization. Even when this was done, TCA appeared to adversely affect the chromatography using the method described, resulting in misshapen peaks (e.g. of ASP—Figure 4). More importantly, TCA extraction of FAA from *Phaeodactylum tricornutum* was incomplete compared to that with hot water. Amounts of some components were seriously underestimated (e.g. SER, GLY, THR, ARG & ALA—Figure 4). Hot water extraction appeared more suitable as recovery is better and the extract is already at a suitable pH. Thoresen *et al.* (1982) recommend heat and osmotic shock for extraction of inorganic N from algae.

Both the TCA and hot water methods were shown to be linear with the quantity of cells. The period of extraction should be determined for the organisms in question and kept at a minimum. For *Phaeodactylum tricornutum,* the injection of FAAs from $<10^4$ cells (0.0015 μl packed cell volume) was adequate to allow estimation of concentrations.

Storage of samples

Several experiments were conducted to examine effects of storage for different periods and under different conditions. Some results are shown in Table III.

In two experiments aged sea water (which contains almost no detectable DFAA) was spiked with 50 nM each FAA, fresh unfiltered samples were compared to those which were filter-sterilized using the method described earlier, dispensed either into acid washed glass vials or into sterile plastic bijous and stored frozen (−20°C) or, for some bijous, at 4°C. Triplicate samples were

TABLE III

Storage of samples. a) Fresh sea water spiked with approx. 50 nM each FAA, filtered, and analysed immediately or after storage in plastic at 4°C or −20°C for 72 h. b) Aged sea water spiked with approx. 100 nM each FAA and 4 μM NH_4^+, analysed before and after filtration and after 24 h storage at −20°C in plastic or glass. Results for 72 h storage similar. For both experiments triplicate samples were analysed. Results are nM

	(a) *filtered spiked sea water* stored in plastic			(b) *spiked aged sea water* stored at −20°C			
	fresh	4°C	−20°C	fresh	filtered	24 h plastic	24 h glass
ASP	68	86	88	98	103	102	100
GLU	59	55	56	97	94	106	105
ASN	52	51	52	94	94	101	102
SER	200	58	64	101	101	104	109
GLN	50	51	52	97	96	100	98
HIS	114	55	52	96	98	90	98
GLY	131	60	62	101	102	110	105
THR	66	51	51	94	96	100	105
ARG	58	50	51	94	93	99	102
TAU	49	50	50	97	92	101	97
ALA	108	53	57	97	99	105	104
TYR	57	51	53	96	97	102	100
NH_4^+	—	—	—	4260	4112	4276	4359
TRP	47	47	47	93	90	97	98
MET	46	49	49	93	94	103	104
VAL	72	49	49	93	93	101	102
PHE	53	51	50	90	93	96	98
ISO	51	60	62	94	96	97	101
LEU	56	51	52	95	94	99	103
ORN	49	47	50	81	85	76	85
LYS	43	43	44	75	78	74	78

taken over a period of up to 72 h. No statistical differences could be detected; filtration by the method described did not alter the levels of DFAA and plastic bijous were just as suitable (and more convenient) than acid washed glass vials (Table IIIb). Temperature (4 or −20°C) did not affect storage but freezing is preferred as an extra safeguard against any biotic degradation.

In the other two experiments, fresh sea water was used, either spiked with a further 50 nM each FAA or not spiked. In both cases some amino acids, notably SER, GLY and ALA, appeared to be lost (over 50% loss for some) from solution within 24 h of sampling with or without filtration, in plastic or in glass. Other amino acids, however, showed no loss suggesting that bacterial contamination was not to blame (Table IIIa).

The cause of this deterioration of the sample on storage is unknown. The aged sea water did not come from the same location as the fresh samples and ageing will result in changes in the water so it is possible that chemical complexing which removes FAA occurs in some circumstances. Ferguson and Sunda (1984) warn of the importance of excluding metal contamination in experiments using DFAA, and Riley and Segar (1970) suggest metals in sea water may complex with DFAA. Tatsumoto *et al.* (1961) used iron to complex FAA from sea water as a preparative step in analysis. Jørgensen and Søndergaard (1984) question whether FAA are actually free, especially in the presence of clays (clay particles may pass through 0.2 μm filters used in sterilization). In the light of this problem, in experiments using cultured organisms it is essential that control flasks, with no organisms, are set up so that any abiotic effects can be monitored. Many culture media, especially after autoclaving, have precipitates which may increase the likelihood of an abiotic loss of DFAA.

Ideally samples should be analysed fresh but this may conflict with the need to replicate analysis of each sample. However, errors in analysis may be less than that incurred by storage. Given that storage will be inevitable, filtration and dispensing into sterile bijous as described has several advantages over other methods. The method is quick and simple, the filter holder and storage vessel are new, removing the risk of cross contamination, and the sterile sample in the syringe does not appear to undergo further deterioration over a 24 h period at 15–20°C (sufficient to transport the sample back to a laboratory).

CONCLUSIONS

Sources of error

It is important to remember that the analysis of DFAA in, for example, sea water requires analysis at the limits of sensitivity and yet the volumes of samples injected in such analysis (200–500 μl) exceed that typically injected in analytical HPLC by an order of magnitude. Injections of samples such as cell extracts may contain organics which will irrevocably bind to the column packing. It is essential that a guard column is used but it is also important to use high grade solvents. If high grade water is not available, extra protection may be afforded by the installation of a SepPak C_{18} (Waters, Millipore) cartridge (first rinsed with 10 ml MeOH and 10 ml water by syringe) followed by a 25 mm 0.4 μm Durapore (Millipore) filter in the solvent feed line to pump A. Because of the extra resistance to flow, flow rates should not exceed, say, 2.5 ml min^{-1}; the normal solvent filter should be removed or cavitation of solvent may occur. One SepPak will clean 1 l of solvent (about 20 injections worth).

During the course of this work, various sources of error became apparent and some are summarized in Table IV. Errors in analysis may be divided into three areas, although errors in one area may affect another. These areas are chromatographic (associated with pumps, columns, guard columns and solvents), sampling (filtration, extraction, storage, pipetting and derivatization), and quantitative (bad baseline fitting, inappropriate sensitivity, calibration errors).

Chromatographic errors

The most obvious indication of a fault in the chromatography is a degradation in the appearance of a chromatogram of a standard, such as peak splitting, poor resolution (especially separation of merged peaks such as glycine and threonine), changes in retention times and in areas or heights of standard peaks. Plate counting may be performed on the internal standard peak.

Problems associated with solvents include:– inadequate degassing (do not degas before the solvent has reached room temperature and check the pumps have been primed correctly); incorrect amount of THF added (THF must be added *after* degassing and beware using

TABLE IV
Some errors and their possible causes using the method described (Table I, Figure 2; 0.25% THF added)

1. Bad peak shape	bad sample (TCA, pH) or shoulder peak bad tube connections or blocked injector column/guard deterioration temperature of guard ≠ analytical column
2. Split single peak	column void or bad tube connections bad column/guard deterioration 2 peaks, 1 unknown!
3. Noisy baseline	solvents not degassed or pump faults detector gain too high sample or solvent with high organic load inappropriate integrator sensitivity
4. Peak shape good but bad separation	temperature too low THF degraded or amount wrong
5. Bad replication of RT	temperature changes
6. Variation in ISTD area	pipette or injection error bad derivatization co-elution with unknown
7. Poor separation of GLY/THR	amount of THF incorrect, too much & HIS/GLN merge column/guard deterioration
8. Loss of LYS/ORN	guard column deterioration
9. Sudden loss of one or more FAA	biotic effect (bacteria) abiotic effect
10. Bad quantitation but chromatogram good & ISTD area good	bad derivatization timing (LYS, ORN, GLY, NH_4^+) integration error (baseline, merged peaks) peak identification error degradation of sample (bacteria) guard column deterioration
11. Bad linearity	detector gain too high replication of derivatization pipetting & time

old THF (>6 months) because HPLC grades are not stabilized and so degrade with time).

Problems associated with the columns include:- degeneration of the packing (do not use a guard column beyond 50 injections); blocked frits (increase back pressure—samples which have suspended material in them should not be injected); inadequate equilibration (a minimum of 25 ml of solvent A should be pumped through before another injection is made and if a large excess of fluorescent material is injected the column may need to be regenerated before further use as per the manufacturer's instructions); column temperature is inappropriate or unstable (maintain temperature within the range 26–28°C, and ensure that both

analytical and guard columns are at the same temperature—many commercial column heaters do not heat a guard column).

Other problems include a partly blocked injector (the sample is not loaded on to the column evenly) and badly fitting connections which no longer have zero-dead volumes (in particular those between the guard column and the analytical column which are released when changing the Guard–Pak insert).

Sample errors

Sample errors may be divided into those associated with collection and storage of the sample or of standards, and errors during derivatization. Routine calibration, by pipetting small volumes of water into a beaker on an accurate balance, will show both operative and instrument errors in pipetting. Pipettes should have tip ejectors. A Channey adapter is a useful accessory on the (100 or 200 μl) microsyringe used for injection.

Errors associated with collection of samples include:– the rupture of cells thus liberating their internal pool of amino acids (filter small volumes under low vacuum—ideally the method should be checked using a suspension of representative organisms); inadequate extraction of amino acid pools from organisms (the method must be checked for linearity with numbers of cells) or extraction which results in the breakdown of peptides or proteins (a minimum period and concentration or temperature should be used).

Errors associated with storage are mainly due to chemical or biological contamination from the container; the use of disposable sterile plastic containers is an advantage. Routine filter-sterilization of samples also appears to be a sensible precaution, but samples of filters from different manufacturers should be checked to ensure that they do not contain extractables which interfere with the sample. Abiotic changes to samples, such as sorbtion on to the walls of the container or in/on to microparticles which are not removed by filtration are more of a problem. The typical error between repeated injections on to the HPLC may be less than that likely to be caused by storage; it is better to have a slightly erroneous measurement of fact than an accurate measurement of an artefact. Unfortunately, sample deterioration may be evident only when it is too late. It should also be borne in mind that there can be

significant differences in FAA concentrations even in duplicate samples collected from the same geographical location, although the general trends may be similar. It is important that errors associated with analysis are not mistaken for natural variability, or vice versa. As for all aspects of chromatography, methods of sample collection and storage (period, temperature and container) should be standardized.

Equally important is the short term storage of standards and internal standards. In particular, caution must be used when using standards made up to low (<50 nM each FAA) concentrations because bacterial uptake may alter the levels significantly over the course of the day suggesting changes in column efficiency when this may not be so. Such standards should be made up fresh from the concentrates before each injection.

Errors associated with derivatization are due to pipetting faults and timing of the reaction quenching and injection. In theory this step could be automated but other problems due to degradation of the samples in the rack are likely to compensate for gains in derivatization accuracy. Changes in the area of the internal standard (ISTD) peak may not only be caused by changes in efficiency of the separation but also by errors in the derivatization and injection. As with sample storage, the use of disposable plastic containers for the derivatization minimizes the risk of contamination of the sample. Some workers use a 1 min derivatization period (e.g. Lindroth and Mopper, 1979); the author did not find significant differences between 1 and 2 min derivatizations. However, it is essential that the reaction time *and* the time of injection into the system be kept constant because of the instability of, in particular, the NH_4^+, glycine and lysine derivatives (Lindroth and Mopper, 1979).

Quantitative errors

These errors may be unique to the detection of the signal and its analysis, or may be caused by either, or both, of the chromatographic and sample errors. It is important to look critically at the location of the baseline and at identification of merged peaks (especially where one appears as a shoulder on another) as plotted by the integrator rather than to believe implicitly in the final numbers. It is desirable to use an integrator with a raw data

reprocessing capability. All modern integrators have a reprocessing capability and computer based systems are very comprehensive. However, reprocessing the data is no compensation for bad chromatography.

An internal standard must be used both as a check for derivatization and of chromatography, and for quantification. The choice of standard depends on individual requirements (ethanolamine, 2-amino n-butyric acid, and α-aminoadipic acid have been used) but the peak should ideally be near the centre of the elution profile, or near peaks of special interest, not merging with other peaks, and not naturally present at detectable levels in the sample. When working on a new application a sample must first be analysed without the addition of an internal standard in order to check for possible co-elution.

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