Vol. 89: 73–79, 1992 | MARINE ECOLOGY PROGRESS SERIES Mar. Ecol. Prog. Ser. | Published November 19

Non-protein free amines in microalgae: consequences for the measurement of intracellular amino acids and of the glutamine/glutamate ratio

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ABSTRACT: An unidentified *o*-phthaldialdehyde positive substance is often present at significant concentrations in extracts of intracellular amino acids from diatoms, dinoflagellates and prymnesiophytes. This compound elutes in reverse phase HPLC protocols similar to that of Lindroth & Mopper (1979; Analyt. Chem. 51: 1667–1674), near or with glutamine or histidine and may thus result in errors of quantification of those amino acids. As a consequence, errors in the estimation of the Gln/Glu ratio, an index of C-N status in algae, are possible. In addition there are other compounds, some present at significant levels (notably a compound in prymnesiophytes), which elute near or with other protein amino acids. A modification to the HPLC protocol is described which not only results in the elution of the major compounds away from any of the other common 20 amino acids, but which also gives a better separation of glycine and threonine which formally were often resolved poorly.

INTRODUCTION

Quantification of the intracellular amino acid (InAA) pool of algae has been found to be useful in studies of algal physiology (e.g. Admiraal et al. 1986, Flynn & Al-Amoudi 1988, Martin-Jézéquel et al. 1988, Al-Amoudi & Flynn 1989, Rijstenbil et al. 1989, Anderson et al. 1990, Martin-Jézéquel et al. 1992). In particular the ratio of intracellular Gln/Glu has been shown to be valuable as an index of the C-N status (Flynn 1990b, 1991). The method of quantification used in such studies usually relies on the precolumn derivatization of alpha amino acids with *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol to give highly fluorescent derivatives which can be separated by reverse phase gradient HPLC. Various versions of the protocol have been developed (e.g. Lindroth & Mopper 1979, Evens et al. 1982, Jones & Gilligan 1983, Flynn 1988), the major differences being in the composition of the mobile phases (usually phosphate or acetate buffers with methanol or acetonitrile as the organic phase).

While examining extracts from dinoflagellates an amino acid, formally identified as histidine in the protocol of Flynn (1988), consistently had a retention time slightly earlier than that for histidine in the standard injection. Using the fact that small additions of tetrahydrofuran (THF) to the HPLC solvents affect the chromatography of different amino acids in different ways (thus effectively providing a convenient route to using different solvent systems – see Flynn 1988), we have examined extracts of InAA and established that there is indeed at least 1 major unidentified substance which may have interfered with peak identification in previous studies.

MATERIALS AND METHODS

The HPLC system comprised a high pressure mixing gradient pump system (Waters Millipore 510 pumps, 680 controller and U6K injector), Linear Instruments LC304 scanning fluorometer (excitation 332 nm, emission 456 nm, slit 20 nm), with an AutoChrom Apex integration system. The analytical column (150 mm × 4.6 mm, 5 µm end-capped ODS C_{18} at 31°C) was either a Beckman Ultrasphere or an HPLC Technology Ultratechsphere, protected by a Waters GuardPak with µBondaPak inserts (replaced every 50 injections). The mobile phases were 9:1 50 mM Na-acetate buffer

(pH 6.8) : methanol (Solvent A), and 100 % methanol (Solvent B). If required, unstabilised tetrahydrofuran (THF) was added to the solvent(s) after the bulk of the solvent(s) had been filter degassed (0.2 µm Millipore Durapore filter). The derivatizing reagents and process was as described by Flynn (1988).

The gradient system when using the Ultrasphere column was as described by Flynn (1988). Various gradient systems were tested with the Ultratechsphere column; the system finally chosen contained 3.2 % THF in Solvent A and 3 % THF in Solvent B with a gradient of 100 % A at 0 min, 80 % A at 7 min, 48 % A at 14 min, 20 % A at 19 min, 0 % A at 22 min, returning to 100 % A by 26 min with the next injection at 30 min. Gradient steps were linear and the flow rate was 2 ml min^{-1}.

In all the chromatograms presented, the internal standard (ISTD) was DL-2-amino-*n*-butyric acid. There was no evidence at any time of selectivity for D- and L-isomers of protein amino acids.

Collection and extraction of algae was either as described in Flynn (1988), or cells were collected under low (<50 mm Hg) vacuum onto 13 mm diameter preashed glassfibre filters (Gelman A/E). The filter, in a 1.5 ml microtube (Eppendorf pattern), was stored at −20 °C. Extraction was by addition of 1 ml of HPLC grade water at 70 °C (dispensed using a BCL8000 repeating pipette with 60 ml syringe; Boehringer Mannheim) and incubation at 70 °C for 30 min. After centrifugation, ca 750 µl of the supernatant was withdrawn and stored at −20 °C until analysis. For added precision (to account for different amounts of extractant added or for water in the filter matrix – this is especially important if using 25 mm filters when collecting from field populations) the microtubes were weighed before centrifugation and again after removal of the supernatant and subsequent drying of the microtube + filter, with the extract volume calculated assuming 1 ml = 1 g.

RESULTS

Fig. 1 shows sections of chromatograms obtained with the Ultrasphere column (used as described by Flynn 1988) for a standard (Fig. 1a), an extract from the dinoflagellate *Aureodinium pigmentosum* (Fig. 1b), and a standard co-chromatographed with extract (Fig. 1c). In the standard, the retention times of glutamine and histidine differ by 0.3 min. In the extract, the retention times of glutamine and the next peak differ by 0.25 min. Fig. 1c shows clearly that the peak after glutamine in the extract elutes between glutamine and histidine. This unknown is hereafter termed 'X'.

Fig. 2 shows separations of amino acid standards using the Ultratechsphere column with the solvents (but with 0 % THF) and gradient of Flynn (1988) and using the new solvents and gradient as described in 'Materials and Methods'. The chromatogram in Fig. 2a is very similar to that obtained using the Ultrasphere column (Flynn 1988). Note the different elution order of some amino acids in Figs. 2a & b; the addition of THF affects the retention times of different OPA-amino acid complexes in different ways. Overlaid on these are chromatograms obtained with a standard co-chromatographed with an extract from *Aureodinium pigmentosum* using the appropriate solvent and gradient conditions. In the original solvent/gradient system with the Ultratechsphere column (Fig. 2a), X co-elutes with Gln (compare Figs. 1c & 2a). With the new gradient/solvent system (Fig. 2b), X elutes as a separate peak away from any others. In addition, an additional peak (TT2) is present between taurine and tyrosine (Fig. 2b).

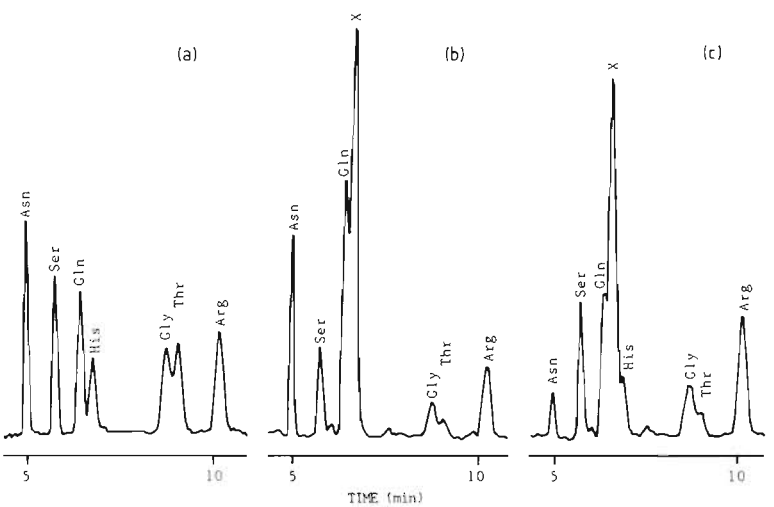

Fig. 1 Detail from chromatograms made using the method of Flynn (1988) with a Beckman Ultrasphere column showing (a) a standard, (b) extract from the dinoflagellate *Aureodinium pigmentosum*, and (c) standard co-chromatographed with extract

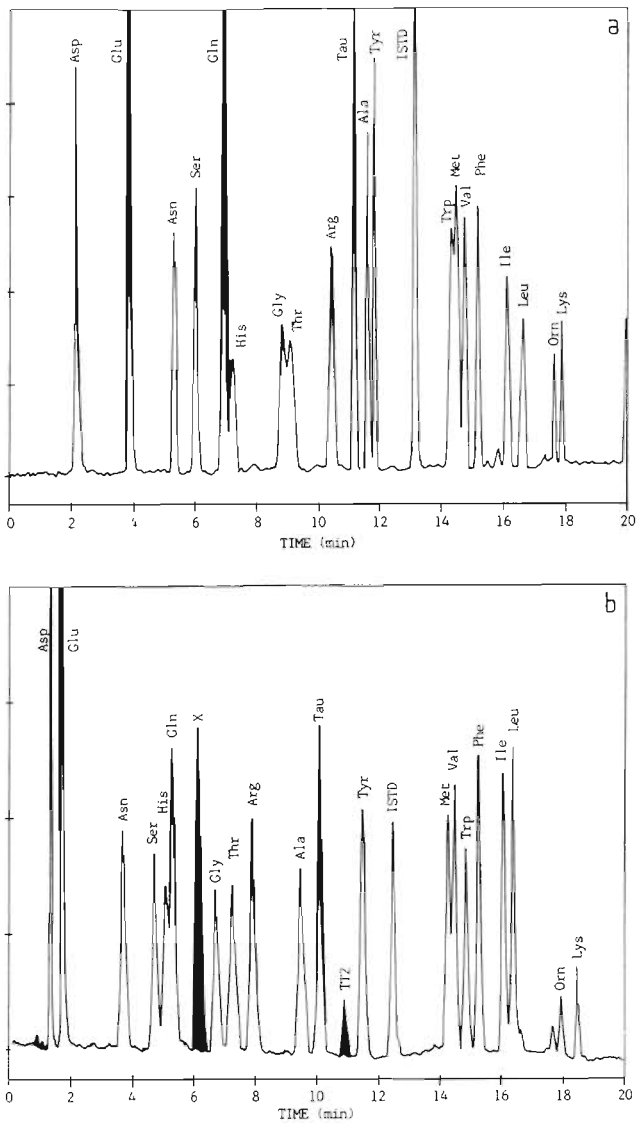

Fig. 2. Chromatograms using (a) the HPLC Technology Ultratechsphere column and the gradient and solvents (0 % THF) of Flynn (1988), or (b) the gradient and solvents described in 'Materials and Methods'. Open peaks represent 200 nM concentrations of standard amino acids. Closed peaks are from a standard co-chromatographed with an extract from the dinoflagellate *Aureodinium pigmentosum*

The intracellular amino acid composition for other dinoflagellates (which usually contain X) will be presented elsewhere; dinoflagellates also contain the nonprotein amino acid taurine. Examples of chromatograms of extracts from other algae are presented in Figs. 3 & 4.

The diatoms *Phaeodactylum tricornutum* and *Cyclotella cryptica* both have significant amounts of X (Fig. 3a, b) as do the prymnesiophytes *Chrysochromulina polylepis* and *Emiliania huxleyi* (Fig. 3c, d) and *Isochrysis galbana* (not shown). Prymnesiophytes also have another significant peak, TT1, which, at least in *Isochrysis*, can on occasion be the largest peak in the extract and accumulates in the growth medium. TT1 coelutes with alanine in the system of Flynn (1988). *Chrysochromulina* had a large unidentified peak at 3.4 min (Fig. 3c). Neither the diatoms nor the prymnesiophytes tested contain taurine. The prasinophytes Omega 48-23 (isolated from the Atlantic) and *Tetraselmis subcordiformis* (Fig. 3e, f) do not have significant amounts of X although, as with all others, there are various smaller unidentified peaks present and the nonprotein amino acid taurine is present at high concentration.

The chlorophytes *Chlorella stigmatophora* (Chlorococcales), *Stichococcus minor* (Ulotrichales) and *Dunaliella primolecta* (Volvocales) do not have significant amounts of X, although again there are other compounds, notably Y which elutes at 14.2 min (Fig. 4a, b, c) and GX1 eluting just after glutamine (Fig. 4a, b). The eustigmatophytes *Ellipsoidion* sp. (Fig. 4d) and *Nannochloropsis oculata* (not shown) also lack significant amounts of X. *Porphyridium purpureum* (Rhodophyta) lacks X but has an unknown (GT) which elutes between glycine and threonine (Fig. 4e), and the cyanobacterium *Synechococcus* sp. also lacks X (Fig. 4f).

DISCUSSION

Most examinations of InAA in algae have been performed using the same or similar phases for separation, namely reverse phase end-capped ODS columns with (usually) methanol as the organic eluent. For complex chromatography such as for amino acids, where more than 20 peaks may elute in as many minutes, the consequence is that an unknown compound could co-elute with a known amino acid.

Both the system of Flynn (1988) using the Ultrasphere column, and the similar system using the Ultratechsphere column (Fig. 2a) give an adequate separation of all the protein amino acids in the standard mixture. However, with the Ultrasphere column the unknown compound X elutes in such a position that the unwary could misidentify it as histidine (Fig. 1b), while with the Ultratechsphere column X coelutes with glutamine (Fig. 2c). Glutamine is an amino acid of fundamental importance in the assimilation of intracellular ammonium; changes in concentrations of glutamine occur with changes in C-N status. For calculation of the Gln/Glu ratio (Flynn 1990b, 1991) it is of paramount importance that both glutamine and glutamate are estimated accurately. Flynn & Al-Amoudi (1988) and Flynn (1990a) misidentified X (where present) as histidine. The work by Flynn and coworkers

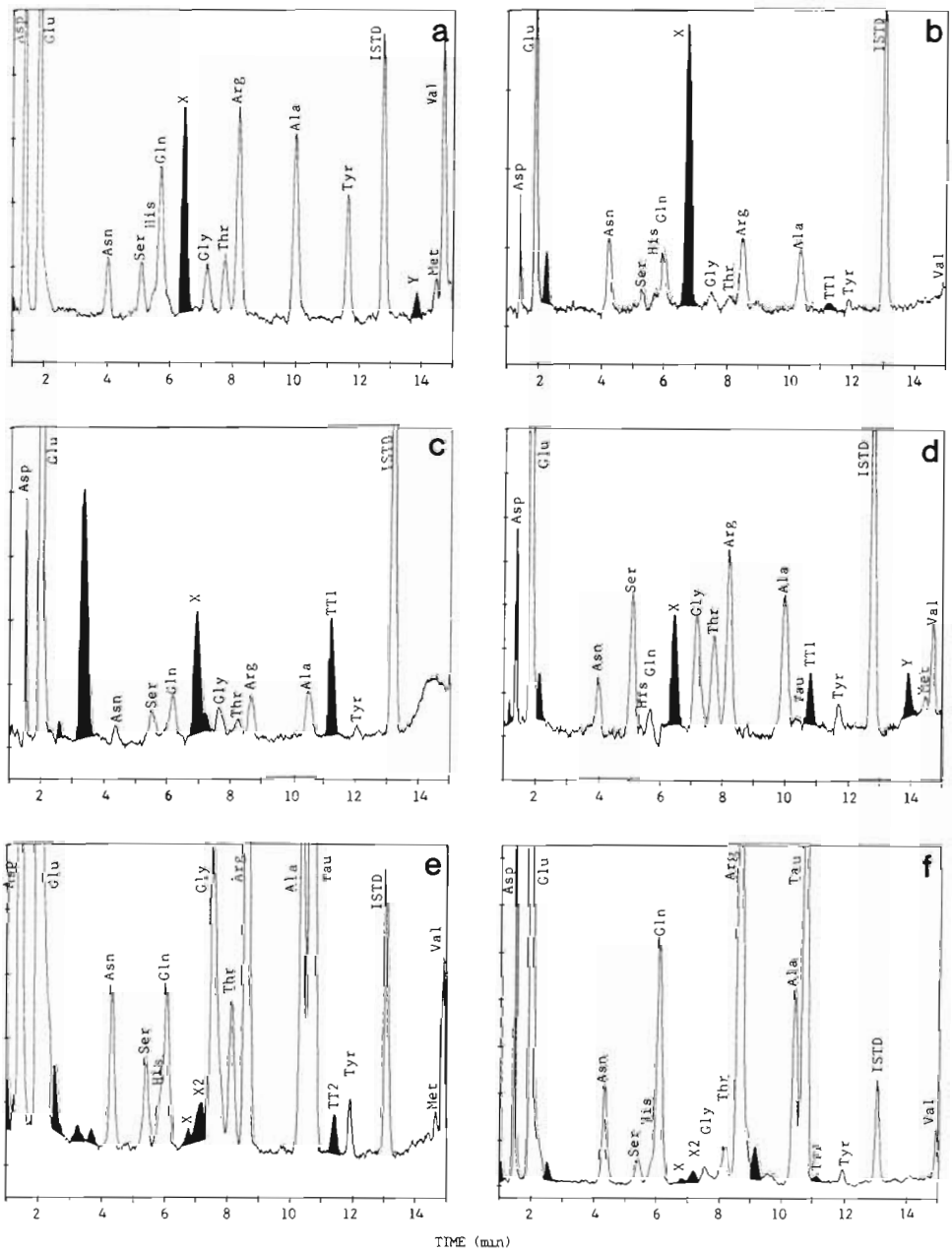

Fig. 3. Chromatograms of extracts made using the methods described in 'Materials and Methods' with the new HPLC protocol. Closed peaks are of unidentified non-protein amines. (a) *Phaeodactylum tricornutum*; (b) *Cyclotella cryptica*; (c) *Chrysochromulina polylepis*; (d) *Emiliania huxleyi*; (e) unidentified prasinophyte Omega 48-23; and (f) *Tetraselmis subcordiformis*

concerning Gln/Glu (summarized up until 1990 in Flynn 1990b) is unaffected as X did not co-elute with glutamine.

Few works mention the presence of unidentified peaks at all yet we often observe them, although most are only of minor quantitative importance. If one assumes that the OPA derivative of X fluoresces in a similar quantitative fashion to most of the others, and that it contains 1 N per molecule, then it is often present at concentrations similar to the those of serine or glycine and sometimes very much higher. One would expect that a peak of that magnitude would have been reported if observed by other workers and thus one concludes that in analyses of InAA from diatoms, dinoflagellates and prymnesiophytes, X has not formerly been resolved from protein amino acids. The same can be said about the presence of TT1 in prymnesiophytes.

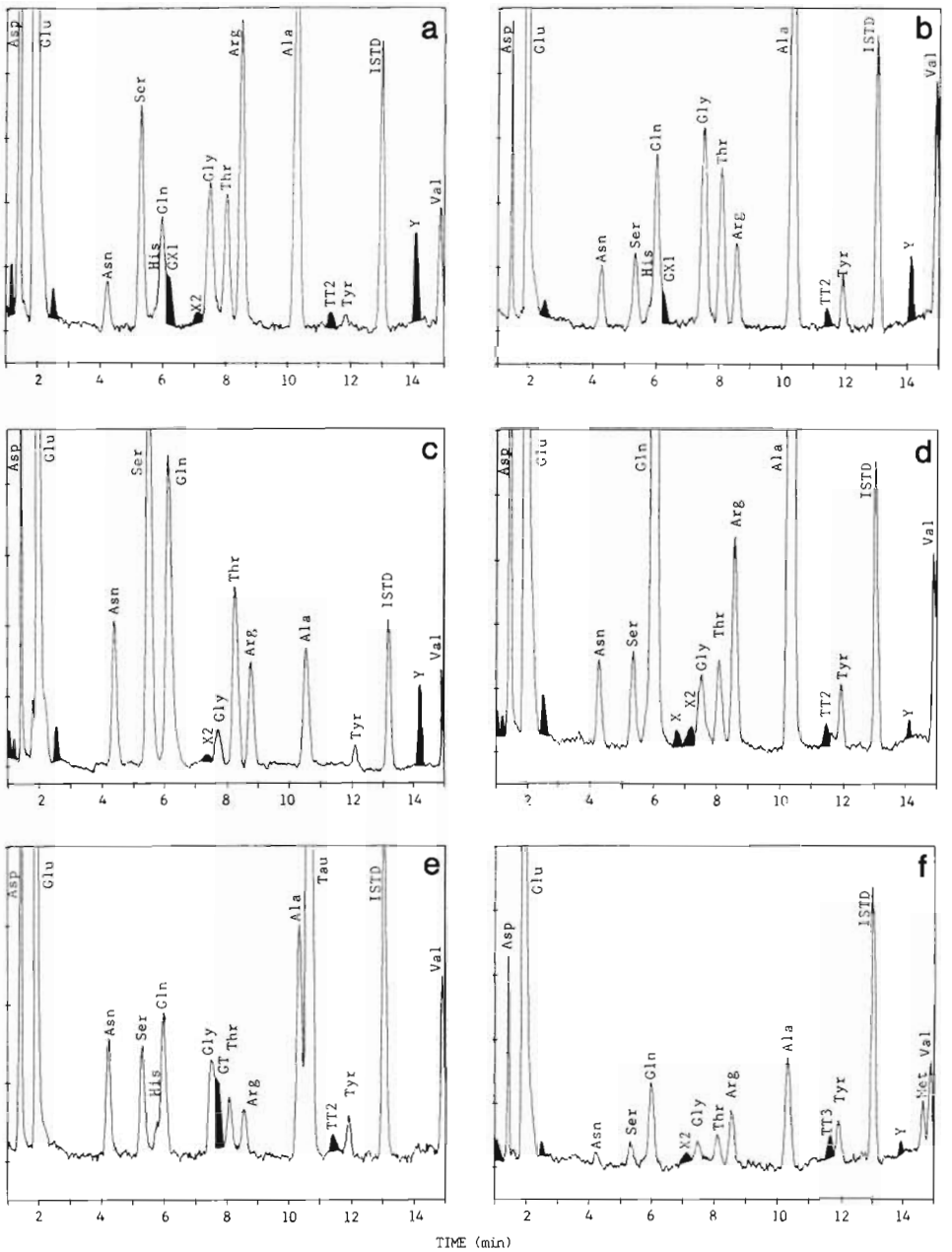

Fig. 4. As in Fig. 3, but (a) *Chlorella stigmatophora*; (b) *Stichococcus minor*; (c) *Dunaliella primolecta*; (d) *Ellipsoidion* sp.; (e) *Porphyridium purpureum*; and (f) *Synechococcus* sp.

Lu & Stephens (1984), Admiraal et al. (1986) and Rijstenbil et al. (1989) all report InAA for diatoms. In each instance histidine is reported as a minor peak. Conversely, concentrations of glutamine appear very high. The work of Lu & Stephens (1984) is for the diatom *Phaeodactylum tricornutum*; they report little histidine while Flynn & Al-Amoudi (1988) report a significant amount of that amino acid (now known to be X). The works of Admiraal et al. (1986) and Rijstenbil et al. (1989), using similar HPLC systems to that of Lindroth & Mopper (1979), include data for cultures during N-stressed growth which still have high concentrations of glutamine and/or high ratios of Gln/Glu. Haberstroh & Ahmed (1986), studying N-refeeding in the diatom *Skeletonema costatum*, used a tertiary (not binary) HPLC gradient but the mobile phases were still similar to those used by others except that the first solvent contained 1 % THF. Again no mention is made of

unidentified peaks but in this data set (contrary to that of Rijstenbil et al. 1989), histidine is reported to be a significant amino acid.

Martin-Jézéquel et al. (1988) examined a wide range of algae. In some of these algae, including *Isochrysis galbana*, several diatoms and the dinoflagellate *Gymnodinium aureolum*, glutamine was identified as a major amino acid (even in stationary phase cultures) with histidine low or absent, while in the dinoflagellates *Gymnodinium simplex* and *Scrippsiella trochoidea*, histidine was important. In our studies (authors' unpubl. data), using both the system of Flynn (1988) and that described here, *Isochrysis*, a natural population of *Gymnodinium* and several cultured dinoflagellates were found to contain little histidine, relatively low glutamine and significant amounts of X. Turpin & Harrison (1978), who used a dedicated amino acid analyzer, make no comment about the presence of unidentifiable amino acids in the dinoflagellate *Gymnodinium simplex*, and unlike Martin-Jézéquel et al. (1988) they did not detect significant histidine in this species.

We suggest that in previously reported examinations of InAA from diatoms, dinoflagellates and prymnesiophytes using OPA HPLC methods similar to that of Lindroth & Mopper (1979), a reported high concentration of histidine is probably actually X and that (in the absence of any evidence to the contrary) in all other instances X is co-eluted with glutamine and thus any calculations of Gln/Glu are most likely high (although trends of high values correlating with a good N-status are probably still observed).

It is clearly important for checks to be made when examining intracellular amino acids in algae by analysis of the sample extracts under different chromatographic conditions by alteration of phase composition; the use of THF enables selectivity to be altered whilst retaining other phase conditions. The HPLC system described can separate the compound from the usual amino acids. In addition, it also provides a good separation of glycine from threonine, amino acids which often do not separate well (if at all) and are often reported as Gly-Thr. As disadvantages, ammonium does not elute as a sharp peak and leucine elutes near a reagent peak.

The identity of these compounds is under investigation. However, over 300 non-protein amino acids have been described for plants (Runeckles & Conn 1974, Conn 1981, Rosenthal 1982). Many have no known function although they have been used for taxonomic purposes with certain compounds being unique for specific groups, as would appear to be the case in microalgae. For the algae which we have examined: dinoflagellates usually contain X, taurine and TT2; diatoms contain X; prymnesiophytes contain X and TT1; prasinophytes contain taurine; chlorophytes contain neither X, taurine nor TT1; the rhodophyte tested contained taurine and TT2.

The OPA derivatizing reagent does not react solely with alpha amino acids. Glutathione (a peptide of glycine-cystine-glutamate) reacts with OPA giving a peak eluting at 1 min. The mole-sensitivity of OPA to glutathione (containing 3 N molecule) is less than 20 % of that for glutamate (which contains 1 N molecule) in the HPLC system described. Clearly if one includes short peptides as potential unknowns then the range of possible compounds increases further, while care must also be taken not to assume that small peaks represent insignificant concentrations of such compounds.

Acknowledgements. This work was funded by the Natural Environment Research Council. The extract from *Chrysochromulina polylepis* was taken while K.J.F. was working with Professor Paasche funded by the British Council and NAVF.

LITERATURE CITED

Admiraal, W., Peletier, H., Laane, R. W. P. M. (1986). Nitrogen metabolism of marine planktonic diatoms; excretion, assimilation and cellular pools of free amino acids in seven species with different cell size. J. exp. mar. Biol. Ecol. 98: 241–263

Al-Amoudi, O. A., Flynn, K. J. (1989). Effect of nitrate-N incorporation on the composition of the intracellular amino acid pool of N-deprived *Tetraselmis marina*. Br. Phycol. J. 24: 53–61

Anderson, D. M., Kulis, D. M., Sullivan, J. J., Hall, S., Lee, C. (1990). Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Mar. Biol. 104: 511–524

Conn, E. E. (ed.) (1981). Secondary plant products. The biochemistry of plants 7. Academic Press, London

Evens, R., Braven, J., Brown, L., Butler, I. (1982). 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. Chem. Ecol. 1: 97–106

Flynn, K. J. (1988). Some practical aspects of measurement of dissolved free amino acids in natural waters and within microalgae by the use of HPLC. Chem. Ecol. 3: 269–293

Flynn, K. J. (1990a). Composition of intracellular and extracellular pools of amino acids, and amino acid utilization of microalgae of different sizes. J. exp. mar. Biol. Ecol. 139: 151–166

Flynn, K. J (1990b). The determination of nitrogen status in microalgae. Mar. Ecol. Prog. Ser. 61: 297–307

Flynn, K. J (1991). Algal carbon-nitrogen metabolism: a biochemical basis for modelling the interactions between nitrate and ammonium uptake. J. Plankton Res. 13: 373–387

Flynn, K. J., Al-Amoudi, O. A. (1988). Effects of N-deprivation and darkness on composition of free amino acid pool and on amino acid release from diatom *Phaeodactylum tricornutum* Bohlin. J. exp. mar. Biol. Ecol. 119: 131–143

Haberstroh, P. R., Ahmed, S. I. (1986). Resolution by high pressure liquid chromatography of intracellular and extra-

cellular free amino acids of a nitrogen deficient marine diatom, *Skeletonema costatum* (Grev.) Cleve, pulsed with nitrate or ammonium. J. exp. mar. Biol. Ecol. 101: 101–117

Jones, B. N., Gilligan, J. P. (1983). *o*-Phthaldialdehyde precolumn derivatization and reversed-phase high-performance liquid chromatography of polypeptide hydrolysates and physiological fluids. J. Chromatogr. 266: 471–482

Lindroth, P., Mopper, K. (1979). High performance liquid chromatographic determination of subpicomolar amounts of amino acids by pre-column fluorescence derivatization with o-phthaldialdehyde. Analyt. Chem. 51: 1667–1674

Lu, M., Stephens, G. C. (1984). Demonstration of net influx of free amino acids in *Phaeodactylum tricornutum* using high performance liquid chromatography. J. Phycol. 20: 584–589

Martin-Jézéquel, V., Poulet, S. A., Harris, R. P, Moal, J., Samain, J. F. (1988). Interspecific and intraspecific composition and variation of free amino acids in marine phytoplankton. Mar. Ecol. Prog. Ser. 44: 303–313

Martin-Jézéquel, V., Sournia, A., Birrien, J.-L. (1992). A daily study of the diatom spring bloom at Roscoff (France) in 1985. III. Free amino acids composition studied by HPLC analysis. J. Plankton Res. 14: 409–421

Rijstenbil, J. W., Wijnholds, J. A., Sinke, J. J (1989). Implications of salinity fluctuations for growth and nitrogen metabolism of the marine diatom *Ditylum brightwellii* in comparison with *Skeletonema costatum*. Mar. Biol. 101 131–141

Rosenthal, G. A. (1982). Plant nonprotein amino and imino acids. Biological, biochemical and toxicological properties. Academic Press, London

Runeckles, V. C., Conn, E. E. (eds.) (1974). Metabolism and regulation of secondary plant products. Recent advances in phytochemistry 8. Academic Press, London

Turpin, D. H., Harrison, P. J. (1978). Fluctuations in free amino acid pools of *Gymnodinium simplex* (Dinophyceae) in response to ammonia perturbation: evidence for glutamine synthetase pathway. J. Phycol. 14: 461–464

This article was presented by M. J. Dring, Belfast, Northern Ireland

Manuscript first received: June 2, 1992
Revised version accepted: October 1, 1992