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High-Performance Liquid Chromatographic Method To Determine Ammonium Ion and Primary Amines in Seawater

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INTRODUCTION

The close interactions of ammonium ion and amino acids with various autotrophic and heterotrophic organisms in aquatic foodwebs (*1–10*) make it desirable to simultaneously measure these two types of dissolved nitrogen in seawater or lake water. Ammonium ion in seawater is now most commonly measured colorimetrically (*11*), and dissolved free amino acids (DFAA) are either measured as individual compounds by high-performance liquid chromatography (HPLC) (*12*) or as groups of primary amines after reaction with Fluorescamine (*13*) or *o*-phthalaldehyde (OPA) (*14–17*). The advantages and disadvantages of measuring individual amino acids vs measuring primary amines as a group (i.e., the "global" approach) in aquatic ecosystems have recently been discussed in detail (*14*).

o-Phthalaldehyde, in combination with 2-mercaptoethanol, forms strongly fluorescing derivatives with most amino acids in aqueous solutions (*18*) and has been widely used for measuring amino acids (and ammonium ion) both as a postcolumn (*19*) and precolumn (*12*) reagent. It is also a useful reagent for measuring primary amines as a group, but if the primary amines are not first isolated from ammonium ion (e.g., refs 16 and 17), the results must be corrected for ammonium ion interference (*14*). Although the fluorescence response is generally much lower for ammonium ion than for most organic primary amines (*17*), this correction is important because concentrations of ammonium ion are commonly higher than those of primary amines in natural waters. For example, in sediment pore waters, ammonium ion concentrations are usually several times higher than those of total free amino acids (*20*).

Ammonium ion can also be measured as a fluorescent derivative(s) of OPA (*21–24*), but likewise, if it is not first separated from amino acids, measurements are subject to interferences from primary amines. This potential interference has recently been substantially reduced, in a flow injection technique, by using sulfite rather than 2-mercaptoethanol in the OPA reagent (*24*). Ammonium ion can also be separated from primary amines by increasing the pH of the water and allowing the ammonia to diffuse into receiving solutions through porous membranes in continuous-flow systems (*22, 25*). Alternatively, ammonium ion can be separated from primary amines by ion-exchange chromatography and then reacted with OPA and measured fluorometrically (*21, 26*). This approach not only removes potential interferences but also can provide measurements of both ammonium ion and primary amines in the same sample. Like other OPA techniques, it has the advantages of small sample size and multiday stability of buffers and reagents. The inconveniences of the chromatographic method, as originally described for ammonium ion analysis (*21*), are low fluorometric response for ammonium ion relative to that for primary amines, decreased column efficiency with increasing salinity, and the need to assemble a specialized analytical system driven by gas pressure to make the measurements.

In this paper, we describe an improved HPLC method to analyze small water samples for both ammonium ion and primary amines. A high-efficiency cation-exchange column, combined with modified buffer and reagent solutions, a pulse-damped HPLC pump, and a peristaltic pump-driven postcolumn OPA detection system, provides rapid and complete resolution and sensitive analysis of ammonium ion and primary amines in seawater.

EXPERIMENTAL SECTION

The HPLC system (Figure 1) was assembled from an isocratic HPLC pump (Altex 100a or Anspec 909), a pulse damper (Scientific Systems Inc., Model LP-2), a sample injection valve (Rheodyne 7125) equipped with a 50-μL sample injection loop, a machined 28-mm × 2.4-mm-i.d. Delrin column containing a high-performance strong cation-exchange resin (5-μm beads of sodium-form sulfonic acid cation exchanger with 12% cross-linked polystyrene/divinylbenzene polymeric matrix; St. John Assoc.), a postcolumn OPA reaction system held at 47 °C, and a fluorometric detector (Gilson Model 121). Light filters in the fluorometer were a Corning 7-60 (maximum transmission at 356 nm) for excitation and a Corning 3-71 (sharp cutoff at 482 nm) for emission.

The mobile-phase buffer was prepared by adding 8.0 g of NaCl and 3.0 g of boric acid to about 490 mL of distilled, deionized water (DDW), adjusting the pH to 10.10 with NaOH solution (final volume 500 mL) and passing the solution through a 0.22-μm pore size nylon filter that had been rinsed with DDW. The OPA reagent was prepared as previously described (*21*), except that the pH was adjusted to 7.0 instead of 10.5 to optimize the fluorometric response of OPA–ammonium ion (*23*) relative to that for OPA–amino acid derivatives. Care was exercised to prevent human exposure to caustic NaOH during buffer preparation or to KOH, OPA, or 2-mercaptoethanol during reagent preparation. The 2-mercaptoethanol was handled under a hood to prevent vapor inhalation.

Mobile-phase buffer was pumped through the column at a flow rate of 0.25 mL min^{-1}, and reagent solution was pumped into the postcolumn mixing coil at a rate of ca 0.10 mL min^{-1}. For sample injection, at least 0.4 mL of water sample was passed through a 0.22-μm pore size filter directly into the injector sample loop (50 μL). The filter membrane was held in a low-dead-volume filter holder (*27*), modified to include a 22 gauge needle with a blunt end for the Rheodyne injection valve. Four-tenths milliliter of sample was adequate to rinse the filter and sample loop and leave a clean 50-μL sample in the loop for injection and analysis. A solution of EDTA (ethylenediaminetetraacetic acid; ca 1%) in NaOH (ca 2%) was sometimes pumped through the column between sample runs to remove impurities and maintain column efficiency.

A 1 mM NH_4Cl solution in DDW and a standard amino acid mixture (AA-S-18, Sigma Chemical Co.), containing 2.5 nmol μL^{-1} NH_4Cl and 17 amino acids, were stock solutions for the ammonium ion and primary amine standards. To calculate the primary amine concentrations, 1 μL of the standard mixture was considered to contain 37.5 nmol of primary amines less basic than ammonium ion that eluted in the major primary amine peak and 2.5 nmol each of ammonium ion and arginine that eluted as separate peaks.

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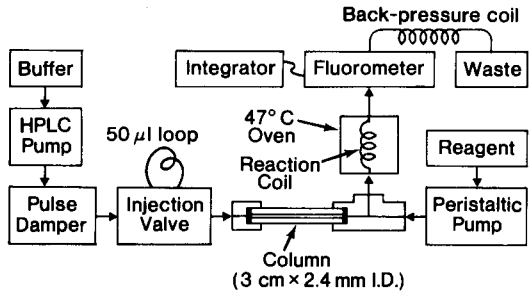

Figure 1. Schematic diagram of HPLC system for ammonium ion–primary amine analysis.

Proline was not considered in the calculations because it is not a primary amine and does not produce a fluorescing reaction product with OPA (*18*). The fluorometric response per unit primary amine is slightly less for this mixture than it would be for most single amino acids, such as glycine, because OPA reaction products of cystine and lysine fluoresce less strongly than those of the other primary amines (*28*). Working standard solutions were prepared in seawater directly before they were injected. For standard curve calculations, peak heights from the unspiked samples were first subtracted from those of the spiked samples to force the calibration curves to intersect the origin.

The method was evaluated by using Gulf of Mexico seawater that had been stored in the laboratory at room temperature for several weeks; it was depleted of measurable ammonium ion and labile primary amines.

RESULTS AND DISCUSSION

Fluorometric ammonium ion–primary amine analysis can be accomplished either by precolumn OPA derivitization followed by reversed-phase separation and fluorometric detection, as is often done for reversed-phase amino acid analysis (*12*), or by initial cation-exchange separation of the dissolved compounds followed by postcolumn OPA derivitization and fluorometric detection, as is done in traditional cation-exchange amino acid analysis (e.g., refs 19 and 29). We preferred the latter approach because high-performance cation-exchange columns can effectively separate ammonium ion from primary amines, postcolumn reaction conditions are precisely controlled and reproducible, derivative formation is easily automated, the potential formation of multiple derivatives of ammonium ion or amino acids does not complicate analytical interpretation, and finally, the cation-exchange resins are more stable and resistant to column deterioration over extended periods of use under aqueous conditions than are silica-based reversed-phase columns. The cation-exchange columns can be used for thousands of analyses without loss of separation efficiency and can be easily cleaned by flushing with a solution containing NaOH and EDTA.

Chromatograms from seawater with added amino acids and ammonium ion yielded near-baseline resolution of primary amine groups and ammonium ion (Figure 2). The high-performance cation-exchange resin provided improved resolution of ammonium ion from primary amines in seawater relative to that obtained with 7- or 8-μm mean bead diameter resins with 8% cross-linkage (data not shown). Resins with 12% cross-linkage have greater separation factors than those with 8% cross-linkage but tend to be less efficient at high flow rates because diffusion through the resin is slower than for 8% cross-linked resin (*30*). Pumping the buffer at a relatively slow flow rate (0.25 mL min^{-1}) through a short column containing the refined small bead 12% cross-linked resin provided rapid analysis of ammonium ion and primary amines with excellent resolution of peaks (e.g., Figure 2).

As previously noted (*17*, *21*), the fluorometric response of OPA derivatives is linear for both ammonium ion and primary amines over a wide range of concentrations. In the present study, the relationship between compound(s) concentration

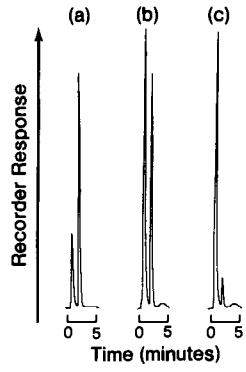

Figure 2. Chromatograms from stored Gulf of Mexico seawater fortified with different levels of added primary amines and ammonium ion. (a) 0.75 μM primary amines, 10 μM ammonium ion, and 0.05 μM arginine (peak below detection limit) added; (b) 3.0 μM primary amines, 10.1 μM ammonium ion, and 0.2 μM arginine added; (c) 3.0 μM primary amines, 1.2 μM ammonium ion, and 0.2 μM arginine added.

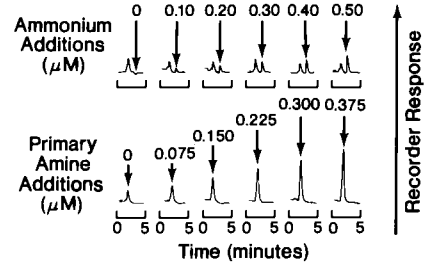

Figure 3. Chromatograms of stored seawater fortified with zero and low levels of ammonium ion or primary amines.

and fluorometric response was linear ($r > 0.999$) for ammonium ion concentrations ranging from 0.1 to 40 μM and for primary amine concentrations ranging from 0.1 to 9 μM in seawater. The practical detection limits observed for ammonium ion and primary amines (ca 0.1 μM or 5 pmol $injection^{-1}$; Figure 3) depended on having an electronically stable detector and a relatively pulseless flow [such as offered by a gas-driven pumping system (*21*) or by using a pulse damper with an HPLC pump, as in this study]. Although detection limits lower than 0.1 μM are potentially possible with this system, they would not be meaningful for most seawater studies unless extreme precautions are taken to prevent contamination of samples and mobile-phase buffer with ammonium ion or amino acids. A small peak for primary amines (equivalent to about 0.1 μM primary amines in our seawater) was observed even when time was allowed for bacteria to deplete the labile amino acids. This peak was not caused by naturally fluorescing compounds in the seawater as it was not observed when the reagent was replaced by distilled water. It is apparently caused by compounds that form fluorescent derivatives with OPA but are not completely removed by bacterial degradation. We have consistently observed a similar, but larger, stable primary amine peak in lake water (*31*).

Postcolumn OPA reaction systems optimized for amino acid or primary amine analysis usually employ OPA reagents adjusted to pH 9.5–10.0 (*28*). When a pH near this range is used for ammonium ion analysis (e.g., ref 21), and measurements are made with fluorometric light filters also optimized for OPA amino acid analysis (excitation at 340 nm; emission at 455 nm), the response factor is more than 10-fold higher for most amino acids than for ammonium ion (*17*). This difference in response lowers the sensitivity and precision of ammonium ion analysis if more than moderate amounts of primary amines are present, particularly if resolution of the components is not complete. When the pH of the reagent was adjusted to 7 (*23*) and an emission light filter suited to the optimum emission wave-

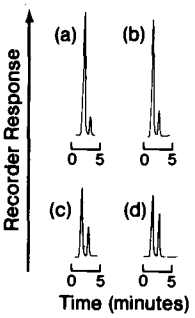

Figure 4. Comparative chromatograms for primary amines (0.75 μM) and ammonium ion (2 μM) examined at two reagent pH's and with two emission filters. (a) Reagent pH 9.5, maximum light transmission at 450 nm; (b) reagent pH 7, maximum light transmission at 450 nm; (c) reagent pH at 9.5, sharp cutoff for light filter at 482 nm; (d) reagent pH at 7.0, sharp cutoff for light filter at 482 nm.

length for ammonium ion (486 nm; 22) was used, the difference in fluorometric response for primary amines relative to that for ammonium ion was reduced to a factor of about 4 (Figure 4).

The efficiency of cation-exchange separation of ammonium ion from primary amines is influenced by sample salinity (*21*). Successful resolution of the components in seawater previously required relatively large injection volumes (0.2-mL sample loop) and comparatively long retention times (about 6 min for the ammonium ion peak; *21*). We have now achieved rapid (ammonium ion retention time of 2.7 min) and complete separation of ammonium ion from the two groups of primary amines in 50 μL of seawater by using the improved high-efficiency HPLC cation-exchange resin and a pH 10.10 buffer containing 8 g of NaCl and 3 g of boric acid. Complete separation (near baseline resolution) was obtained even when largely different concentrations of the components are present in a sample. If primary amines more basic than ammonium ion (e.g., arginine) are not detectable in the samples, as is usually the case in natural waters, the analysis is complete in 4 min. If arginine is present, about 6 min is required per sample (Figure 2).

The described HPLC method is particularly useful for measuring ammonium ion and primary amines in natural or experimental systems where rapid analysis of small samples is desired. We have recently used it on board a ship in the Gulf of Mexico to measure ammonium ion excretion rates of a variety of benthic invertebrates ranging in size from less than 100 μg to several grams dry weight (unpublished data). Figure 5a shows chromatograms for initial and final measurements of ammonium ion concentrations in seawater (4 mL) surrounding a single marine polychaete over an incubation interval of 2.1 h. Pore water samples can also be analyzed on board a ship immediately after samples are extruded and centrifuged. Because of the high sensitivity of the method, small samples (e.g., 10 μL of pore water) can easily be diluted (e.g., to 1 mL), filtered, and analyzed rapidly for both ammonium ion and primary amines (*33*). Likewise the method provides convenient and rapid analysis of field samples where volumes are limited (e.g., samples from benthic flux-measurement chambers; Figure 5b) or of samples from experimental microcosms where conversions of amino acids to ammonium ion, or vice versa, can be examined (*2*, *3*). The features of dual measurement of ammonium ion and primary amines, small sample size, immediate filtration, direct injection, short analysis time, and wide concentration range make this HPLC fluorometric technique ideal for field and experimental examination of nitrogen dynamics in both marine waters and freshwaters.

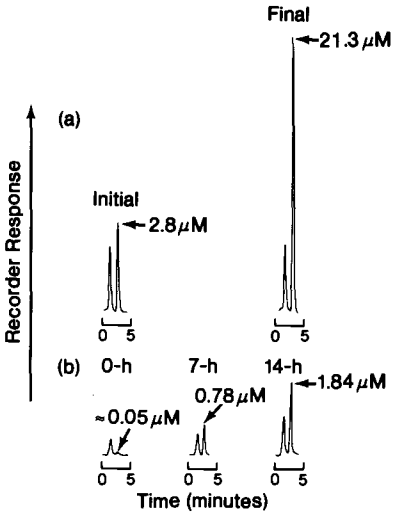

Figure 5. Examples of field application of the described method to examine ammonium ion fluxes in the Gulf of Mexico. (a) Chromatograms from seawater surrounding a marine polychaete (*Magelonidae* sp.; incubation volume = 4.0 mL) for samples collected at the beginning and end of a 2.1-h incubation interval. The polychaete was placed in the incubation bottle several minutes before the sample was taken for the initial measurement. Note, a more sensitive fluorometer setting (range = 0.02) was used for the initial measurement than for the final one (range = 0.05). (b) Chromatograms for seawater samples collected from a benthic flux-measurement chamber (*32*) about 0, 7, and 14 h after the chamber was remotely placed above Gulf of Mexico sediments. Indicated concentrations are for ammonium ion; primary amine concentrations were not quantified in these samples.

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