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## Improved chromatographic analysis of $^{15}\text{N}:^{14}\text{N}$ ratios in ammonium or nitrate for isotope addition experiments

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### Abstract

Estimating nitrogen transformation rates in aquatic ecosystems by isotope dilution techniques is simplified by directly measuring nitrogen isotopic ratios for  $\text{NH}_4^+$  in the water using high performance cation exchange liquid chromatography (HPLC). Modifications of HPLC conditions and implementation of a median-area method for retention time determination improved and linearized a previously reported sigmoid relationship between the retention time shift ( $\text{RT}_{\text{shift}}$ ) of the  $\text{NH}_4^+$  peak and the ratio of  $^{15}\text{NH}_4^+ : [\text{Total NH}_4^+]$  in seawater fortified with  $^{15}\text{NH}_4^+$ . Increasing the temperature of the HPLC column from 47 to 85 °C increased mobile phase buffer flow rate relative to column back pressure, decreased the retention time for  $\text{NH}_4^+$ , and allowed the buffer pH to be optimized relative to the  $\text{pK}$  of  $\text{NH}_4^+$ . The use of median-area rather than maximum-height to define the retention time of  $\text{NH}_4^+$  further improved the linearity ( $r > 0.995$ ) of the relationship between the ratio  $^{15}\text{NH}_4^+ : [\text{Total NH}_4^+]$  and  $\text{RT}_{\text{shift}}$  over the range of isotope ratios. Reduction of  $\text{NO}_3^-$  to  $\text{NH}_4^+$  by adding zinc dust to acidified (pH 2) seawater or lakewater samples, followed by pH neutralization, and subsequent analysis of  $\text{NH}_4^+$  isotope ratios by HPLC, extended application of the method to isotope dilution experiments with  $\text{NO}_3^-$ . Advantages of this direct-injection method over mass-measurement approaches traditionally used for isotope dilution experiments include small sample size and minimal sample preparation.

### 1. Introduction

Nutrient transformation rates must be measured to understand the ecology and biogeochemistry of aquatic ecosystems. The dynamics of C and P are often studied by using the radioactive isotopes  $^{14}\text{C}$  and  $^{32}\text{P}$  as tracers (e.g. Steeman Nielsen, 1951; Schlinder et al., 1972; Harrison, 1983a; Raj and Jacobsen, 1990; Bentzen and Taylor, 1991). Tracer studies with nitrogen are usually done with the stable isotope  $^{15}\text{N}$  that is measured by mass or emission spectrometry. Isotope dilution or enrichment experiments with  $^{15}\text{NH}_4^+$  (e.g. Harrison, 1978,

1983b; Blackburn, 1979; Caperon et al., 1979; Glibert et al., 1982),  $^{15}\text{NO}_3^-$  (Dugdale and Goering, 1967; Goering et al., 1970) and  $^{15}\text{N}$ -labeled organic nitrogen compounds (Kirchman et al., 1989; Bronk and Glibert, 1991; Gardner et al., 1993) have provided useful information about the cycling of nitrogen in benthic and pelagic freshwater and marine ecosystems.

Relatively large samples of water are required for isotope dilution or enrichment experiments with  $^{15}\text{N}$  compounds because mass detection methods used for stable isotopes are not nearly as sensitive as those used for radioactive isotopes.

Nitrogen in particles can be simply concentrated and prepared for mass analysis by filtration and Dumas combustion, but dissolved forms of nitrogen ( $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and dissolved organic nitrogen) must first be removed from the water, concentrated, and dried before they can be converted to  $\text{N}_2$  for mass analysis (Harrison, 1983b). Direct measurement of nitrogen ion isotope ratios in the aqueous phase would prevent the need for this multistep procedure that requires large volumes of sample water.

As an alternative approach to mass analysis, nitrogen isotope ratios for  $\text{NH}_4^+$  in isotope dilution experiments can be determined directly in small water samples by high performance cation exchange liquid chromatography (HPLC; Gardner et al., 1991, 1993). The HPLC technique is not efficient enough to separate the two isotopic forms of  $\text{NH}_4^+$  into separate chromatographic peaks, but the ratios can be determined from a shift in  $\text{NH}_4^+$  retention time ( $\text{RT}_{\text{shift}}$ ), caused by  $^{15}\text{NH}_4^+$ , relative to that of an internal standard of natural-abundance  $\text{NH}_4^+$  in mobile phase buffer that is injected at a measured time interval before the sample. The  $\text{RT}_{\text{shift}}$  occurs because the ratio of  $^{15}\text{NH}_4^+ : ^{15}\text{NH}_3$  is slightly larger than the ratio of  $^{14}\text{NH}_4^+ : ^{14}\text{NH}_3$  at pH's near the  $\text{p}K$  for  $\text{NH}_4^+$  (about pH 9; Gardner et al., 1991). The shape of the calibration curve is sigmoid in shape, a factor that makes the method less sensitive at low and high isotope ratios than in the mid portion of the curve where concentrations of  $^{15}\text{NH}_4^+$  and  $^{14}\text{NH}_4^+$  are similar (Gardner et al., 1991, 1993). It would be desirable to equalize the response factor over the whole range of the curve to improve the relative sensitivity for low (and high) ratios of  $^{15}\text{NH}_4^+ : [\text{Total } \text{NH}_4^+]$  and to linearize the shape of the calibration curve.

The value of using HPLC to quantify isotope ratios would be further increased if its use could be extended to doing isotope dilution experiments with dissolved  $\text{NO}_3^-$  in natural waters. This goal could potentially be accomplished by reducing  $\text{NO}_3^-$  to  $\text{NH}_4^+$  in sample water (e.g. Stainton et al., 1977) followed by HPLC analysis of the isotope ratios of the resulting  $\text{NH}_4^+$  ions.

In this paper, we linearize the calibration relationship between  $\text{RT}_{\text{shift}}$ 's and  $^{15}\text{NH}_4^+ :$

Total  $\text{NH}_4^+$  ratios in aqueous samples and describe a  $\text{NO}_3^-$  reduction step that extends the capability of the method to isotope dilution experiments with  $^{15}\text{NO}_3^-$ . Application of the method to isotope dilution and enrichment experiments in the Gulf of Mexico and Saginaw Bay, Lake Huron, will be reported separately.

## 2. Methods

### 2.1. High performance liquid chromatographic system

High performance liquid chromatographic conditions were similar to those recently described (Gardner et al., 1991, 1993) except that column temperature and method of  $\text{RT}_{\text{shift}}$  determination were modified to linearize the relationship between isotope ratio and  $\text{RT}_{\text{shift}}$  (see details below). Briefly, the HPLC system consisted of an ISCO 260D syringe pump operated in the constant pressure mode, an Alcott Model 728 Autosampler equipped with a Valco Model EC6W fast electronically-activated injection valve with a 50  $\mu\text{l}$  sample loop, a heated (Standard CROCO-CIL HPLC column heater) 30 cm  $\times$  4mm i.d. stainless steel column containing a strong cation exchange resin (5  $\mu\text{m}$  beads of the sodium form of sulfonic acid cation exchanger with 12% cross-linked polystyrene-divinylbenzene polymeric matrix; St. John Associates), an assembled post-column reaction system, and a Gilson 121 Fluorometric detector equipped with a Corning 7-60 excitation light filter (maximum transmission at 356 nm) and a Corning 3-71 emission filter (sharp cutoff at 482 nm). Sample signal from the detector was recorded either with a Shimadzu Integrator (Model C-R3A) or with a computerized Galactic software system that determined retention times both by a maximum peak height algorithm (Galactic Center X) or by a post-run program (Galactic program COL-RT.ABP) designed to determine median-area retention time, i.e. the position in the peak where the area of the peak before the retention time is the same as the area after the retention time.

The mobile phase buffer [12 g boric acid + 12 g NaCl + 0.8 g disodium ethylenediaminetetraacetic

acid in 1 l water, adjusted to the desired pH with NaOH, fortified with 0.5 ml of Brij 35, and filtered (0.2  $\mu\text{m}$  pore size nylon; Gardner et al., 1993)] was passed through the column at flow rates ranging between 0.14 and 0.17  $\text{ml min}^{-1}$ , depending on HPLC conditions. The *o*-phthalaldehyde (OPA) reagent, modified from Hare (1975) and Gardner and St. John (1991), was an aqueous solution of boric acid (30  $\text{g l}^{-1}$ ) adjusted to pH 7.0 with KOH, and then mixed with a solution of 0.5 g OPA dissolved in 10 ml MeOH and 0.5 ml 2-mercaptoethanol. Four ml of Brij 35 was added for pump-seal lubrication and the reagent was filtered (distilled water-rinsed 0.45  $\mu\text{m}$  pore size Millipore). In the post-column reaction system, OPA reagent was pumped at a flow rate of 0.1  $\text{ml min}^{-1}$  using an Anspec 909 (currently available as an Alcott 760) HPLC pump equipped with a micro-bore head. After the OPA reagent was mixed with the column eluate via a *T*-fitting, the mixture was passed sequentially through a heated (ca. 40°C) 1 m teflon reaction tube, the fluorometric detector, and a 100 psi back-pressure regulator (Upchurch U446; to prevent post-column degassing).

## 2.2. Sample analysis

The fluorometer and oven heater were turned on and the syringe pump and reagent reservoir were loaded with mobile phase buffer and reagent, respectively. Pump flows were started before the sample trays of the autoinjector were loaded to allow the chromatographic system to equilibrate. The syringe pump was operated at constant pressure (up to 3600 psi) selected to give the desired mobile phase flow rate. Odd-numbered injection vials in the Autosampler were each loaded with a 4  $\mu\text{M}$  standard solution of natural abundance  $\text{NH}_4^+$  (i.e. 99.63%  $^{14}\text{NH}_4^+$ ) prepared in mobile phase buffer. Samples, or calibration curve standards, to be analyzed were placed in even-numbered vials. Isotope mixture standards were prepared in water having the same salinity as the samples to be analyzed. Triplicate sequential sets of standard  $\text{NH}_4^+$  and sample  $\text{NH}_4^+$  vials were prepared for each sample so that values from three replicate chromatograms could be averaged to yield the  $\text{RT}_{\text{shift}}$  measurement. The autosampler

was programmed so that the water in the even-numbered vials would be injected at a precise time interval (5.0 or 7.0 min) after the internal standard solutions in the odd-numbered vials were injected. Sufficient time (33 to 48 min, depending on chromatographic conditions) was allowed for the sample  $\text{NH}_4^+$  to elute before the next internal standard  $\text{NH}_4^+$  solution was injected. Under these conditions, the syringe pump (266 ml capacity) contained sufficient buffer to analyze all standards and samples from a filled autoinjector tray. The tray capacity of 64 vials allowed injection of 10 triplicate pairs of samples and standards (60 vials). Vials in the remaining final open slots were loaded with distilled water that was injected to rinse any deposited salts from the sample injector valve. After standards and samples were loaded, auto-sampler injections were begun and all the data were recorded, on the Shimadzu integrator and/or the Galactic computerized data system, as one chromatographic run.

## 2.3. Analysis of $^{15}\text{NO}_3^-$ : [Total $\text{NO}_3^-$ ] ratios

Nitrate was reduced to  $\text{NH}_4^+$  by reacting the sample with zinc under acidified conditions (Stainton et al., 1977). However, instead of using a packed zinc column, we mixed approximately 150 mg of zinc dust directly into 15 ml of sample filtrate that had been acidified with 140  $\mu\text{l}$  of 2 N Ultrex  $\text{H}_2\text{SO}_4$  (Baker). This approach was convenient and avoided the potential problem of  $\text{NH}_4^+$  carry-over that could occur in a zinc column when small sample volumes are used. Nitrate reduction efficiency was 60–100%, depending on sample matrix. After 15–20 min, the sample pH was adjusted to ca. 8.0 by addition of 3 ml boric acid buffer (the same as the mobile phase buffer). The resulting flocculent zinc hydroxide precipitate along with remaining zinc powder was removed by passing the sample through a 0.2  $\mu\text{m}$  pore size nylon filter, and the  $^{15}\text{NH}_4^+$  : [Total  $\text{NH}_4^+$ ] ratio was determined by HPLC as described above. Standard  $\text{NO}_3^-$  solutions containing different  $^{15}\text{N}$  enrichments were treated in exactly the same way as the samples to establish calibration curves for  $^{15}\text{NO}_3^-$  : [Total  $\text{NO}_3^-$ ] ratios.

To accurately determine the isotopic ratios of the

reduced  $\text{NO}_3^-$ , it was necessary to make corrections for the presence of any  $\text{NH}_4^+$  that was in the seawater sample before the zinc reduction step. The correct ratio ( $R'$ ) was determined as:

$$R' = R - [\text{NH}_4^+] (R - R_{\text{NH}_4^+}) / [\text{NO}_3^-]$$

where  $R'$  is the actual  $^{15}\text{NO}_3^- : [\text{Total NO}_3^-]$  ratio,  $R$  is the  $^{15}\text{NH}_4^+ : [\text{Total NH}_4^+]$  ratio determined from the  $\text{RT}_{\text{shift}}$  after  $\text{NO}_3^-$  reduction,  $[\text{NH}_4^+]$  is the  $\text{NH}_4^+$  concentration before sample reduction,  $R_{\text{NH}_4^+}$  is the  $^{15}\text{NH}_4^+ : [\text{Total NH}_4^+]$  ratio for the sample before reduction, and  $[\text{NO}_3^-]$  is the concentration of  $\text{NO}_3^-$  reduced to  $\text{NH}_4^+$ .

If the  $\text{NH}_4^+$  present before reduction is at natural abundance (i.e. 99.63%  $^{14}\text{NH}_4^+$ ), and assumed to be 100%  $^{14}\text{NH}_4^+$  for the purpose of experimental calculations, the above equation is simplified to:

$$R' = R([\text{NO}_3^-] + [\text{NH}_4^+]) / [\text{NO}_3^-]$$

#### 2.4. Quality assurance

The HPLC method can effectively measure  $^{15}\text{NH}_4^+ : [\text{Total NH}_4^+]$  ratios for isotope dilution or enrichment experiments over the range of salinities observed in freshwater, coastal marine, and oceanic system if standards are prepared in water having approximately the same salinity as the samples. The accuracy and precision of isotope ratio data obtained by the  $\text{RT}_{\text{shift}}$  method depends on precise control of HPLC conditions. In particular, column temperature must be carefully regulated and the flow rates of both mobile phase buffer and reagent must be precise. Column temperature is accurately controlled by a column heater/controller. The syringe pump, operated at constant pressure, provides a precise mobile phase buffer flow rate. The pump delivers more precise flows in the constant pressure mode than in the constant flow mode at low flow rates because under constant pressure the column flow is not affected by slight leakage around the syringe pump seal that may vary with the position of the plunger in the syringe cylinder. In the constant-flow mode, any differential leakage around the syringe pump seal during an analytical run can affect the actual flow rate of mobile phase buffer

through the column and affect  $\text{RT}_{\text{shift}}$  values (Gardner et al., 1993). The reagent pump is equipped with a microbore head to assure precise flow control and a flow of He is constantly passed over the degassed reagent to prevent imprecision caused by dissolved gasses in the reagent solution. Averaging results from three sequential chromatograms for each sample reduces random variability in  $\text{RT}_{\text{shift}}$ 's. The mean precision of replicate measurements also provides an index of the quality of the data being collected. Seals for both pumps are changed when the baseline becomes more noisy than usual or when standard errors of the mean for sets of replicate injections reach an overall average value of more than about 0.02 min per set over the course of an analytical run.

### 3. Results and discussion

#### 3.1. Criteria for use of $\text{RT}_{\text{shift}}$ for isotope ratio determination

Determination of relative concentrations of two components in a single unresolved chromatographic peak can be quantified by measuring the  $\text{RT}_{\text{shift}}$  and comparing it to  $\text{RT}_{\text{shift}}$ 's from calibrated standards if the following criteria are met:

- (1) the components are isolated from other interfering compounds,
- (2) they have slightly different retention times from each other, and
- (3) they have otherwise consistent (preferably identical) chromatographic behaviors and detector responses.

These criteria are met by cation exchange fractionation of  $\text{NH}_4^+$  isotopes at pH's near the pK of  $\text{NH}_4^+$ . The first criterion is achieved because high performance cation exchange chromatography combined with post-column OPA reaction and fluorescent detection is relatively selective for amino acids (primary amines) and  $\text{NH}_4^+$  (Hare, 1975; Gardner and St. John, 1991). Adjusting the mobile phase pH to values near the pK of  $\text{NH}_4^+$  allows  $\text{NH}_4^+$  to be isolated from most amino acids, but arginine (arg) has a retention time similar to that of  $\text{NH}_4^+$ . The two compounds co-elute at

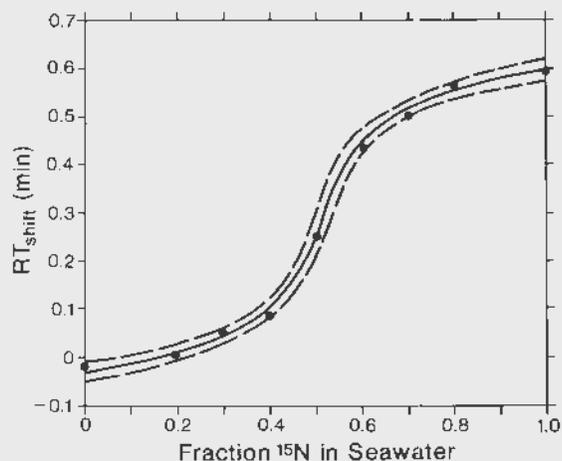


Fig. 1. Relationship between the  $[\text{}^{15}\text{NH}_4^+]/[\text{Total NH}_4^+]$  ratio (= Fraction  $^{15}\text{N}$ ) and  $\text{RT}_{\text{shift}}$  in seawater (salinity = 22 ppt). Column temperature = 35°C; Mobile phase buffer pH = 10.25; Flow rate = 0.14 ml min $^{-1}$ . Error bands are 95% confidence intervals for the calibration curve.

47°C (Gardner et al., 1991) but can be separated by manipulating column temperature (Long and Geiger, 1969). The second criterion is achieved because the two isotopic forms of  $\text{NH}_4^+$  have slightly different retention times on cation exchange resins due to the difference between the two isotopes in the equilibrium reaction between non-ionized  $\text{NH}_3$  and  $\text{NH}_4^+$  in water. A slightly higher portion of  $^{15}\text{NH}_4^+$  than of  $^{14}\text{NH}_4^+$  exists in the cationic form at equilibrium at pH's near the  $\text{pK}$  for  $\text{NH}_4^+$  (Urey et al., 1937; Ishimori, 1960). The third criterion is met because the chemical reactivity and chromatographic behavior of the two isotopic forms of  $\text{NH}_4^+$  are virtually identical.

### 3.2. Modification of chromatographic conditions and retention time algorithm to optimize and linearize the calibration curve

#### Chromatographic conditions

The previously described calibration curve  $\text{RT}_{\text{shift}}$  vs. the ratio of  $[\text{}^{15}\text{NH}_4^+]/[\text{Total NH}_4^+]$  is best described by a sigmoid relationship (Gardner et al., 1993). Factors controlling the shape of the sigmoid relationship for  $\text{RT}_{\text{shift}}$  vs. isotope ratio have not been thoroughly examined, probably because the  $\text{RT}_{\text{shift}}$  concept is not normally used

to quantify peak component ratios in chromatography. To investigate factors affecting the shape of the calibration curve, we modified column temperature and buffer pH and changed to a median-area method for calculating retention time.

Lowering the column temperature from 47 to 38°C, to achieve separation of  $\text{NH}_4^+$  from arg, changed the shape of the sigmoid curve by lengthening the tails and increasing the center slope of the sigmoid pattern (Gardner et al., 1993). Thus, column temperature can significantly affect the response factors in different regions of the curve (e.g. Fig. 1).

In an attempt to equalize the calibration curve response, but still separate  $\text{NH}_4^+$  and arg, we examined the effects of increasing the temperature of the cation exchange column. A column temperature of 65°C caused arg to elute before rather than after  $\text{NH}_4^+$  and decreased column back-pressure, presumably due to the decreased viscosity of water with an increase in temperature. Ammonium retention time decreased with increasing temperature even when mobile phase buffer flow rates were held approximately constant. For example, at 65°C the retention time was 28 min as compared to 36 min at 47°C and 49 min at 35°C for buffer flow rates of 0.12–0.14 ml min $^{-1}$ . Net pressures (= inlet pump pressure minus HPLC system eluent back pressure regulated at 100 psi) required to maintain these flow rates ranged from 3600 psi (the approximate recommended upper limit for the column resin beads) at 35°C down to 2000 psi at 65°C. At a temperature of 65°C, a buffer flow rate of ca. 0.13 ml min $^{-1}$ , and a buffer pH of 10.26, the shape of the calibration curve (Fig. 2) was much more linear than had been observed at lower temperatures (e.g. Fig. 1). However, the total change in  $\text{RT}_{\text{shift}}$ , over the range of isotope ratios (0–1.0), was reduced to about 0.2 min (Fig. 2) as compared to  $\text{RT}_{\text{shift}}$ 's of up to 0.7 min that were observed at lower column temperatures and correspondingly longer  $\text{NH}_4^+$  retention times. This reduction in the  $\text{RT}_{\text{shift}}$  range decreased the signal-to-noise ratio of the  $\text{RT}_{\text{shift}}$  response factor relative to isotope ratio changes and resulted in relatively large confidence bands in the  $\text{RT}_{\text{shift}}$  vs.  $[\text{}^{15}\text{NH}_4^+]/[\text{Total NH}_4^+]$  ratio calibration curve (Fig. 2). Lowering the pH of the buffer to 9.75 increased the  $\text{NH}_4^+$  retention time

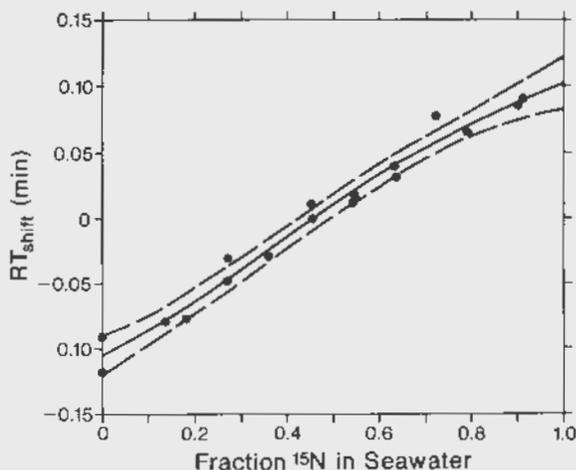


Fig. 2. Relationship between the  $[\text{}^{15}\text{NH}_4^+]/[\text{Total NH}_4^+]$  ratio and  $\text{RT}_{\text{shift}}$  in seawater (salinity = 22 ppt). Column temperature =  $65^\circ\text{C}$ ; Mobile phase buffer pH = 10.25; Flow rate =  $0.12 \text{ ml min}^{-1}$ .

and the  $\text{RT}_{\text{shift}}$  range but again resulted in a sigmoid curve with relatively flat tails (data not shown).

The above results indicate that the linearity of the calibration curve improves, but that the magnitude of the  $\text{RT}_{\text{shift}}$  range (in minutes) decreases, as a function of column temperature. To optimize the linearity of response but still increase the  $\text{RT}_{\text{shift}}$  range, we increased the column temperature to

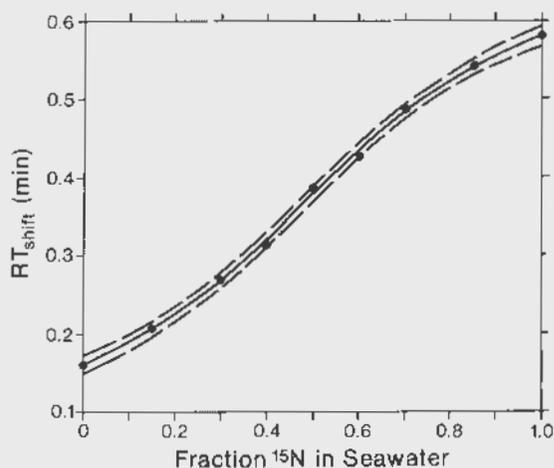


Fig. 3. Relationship between the  $[\text{}^{15}\text{NH}_4^+]/[\text{Total NH}_4^+]$  ratio and  $\text{RT}_{\text{shift}}$ , determined from maximum-height retention times, in seawater (salinity = 22 ppt). Column temperature =  $85^\circ\text{C}$ . Mobile phase buffer pH = 9.36; Flow rate =  $0.17 \text{ ml min}^{-1}$ .

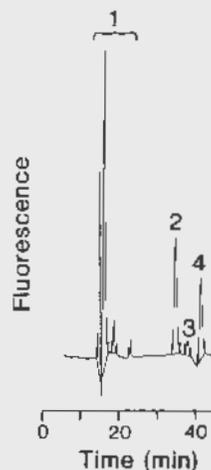


Fig. 4. Chromatogram showing the separation of  $\text{NH}_4^+$  from arg and other amino acids in lake water. 1 – amino acid peaks; 2 – internal standard of  $\text{NH}_4^+$  in mobile phase buffer; 3 – arg in lake water; 4 =  $\text{NH}_4^+$  in lake water. Chromatographic conditions as specified in Fig. 3.

$85^\circ\text{C}$  but lowered the pH of the mobile phase buffer to 9.36, a value nearer the  $\text{pK}$  for  $\text{NH}_4^+$ . At this temperature, column back pressure was relatively low so the pH of the buffer could be optimized relative to the  $\text{pK}$  for  $\text{NH}_4^+$  without extending the  $\text{NH}_4^+$  retention time beyond practical limits. A net column pressure of 2500 psi resulted in a flow rate of about  $0.17 \text{ ml min}^{-1}$  and an  $\text{NH}_4^+$  retention time of c. 34 min. The calibration curve obtained under these conditions was still moderately sigmoid in shape, but the relative response factors were more uniform over the span of the calibration curve (Fig. 3) than had been obtained at lower temperatures and at buffer pH's of  $>10$  (Gardner et al., 1993; Fig. 1). To prevent overlap of the arg peak (that eluted 3.7 min before  $\text{NH}_4^+$ ) with the internal standard  $\text{NH}_4^+$  peak, the time interval between internal standard and sample injection was extended from 5.0 to 7.0 min. Under these conditions, arg in the sample was chromatographically resolved from both the sample  $\text{NH}_4^+$  and the internal standard  $\text{NH}_4^+$  peaks (Fig. 4).

#### Retention time algorithm

The integrator algorithm for calculating retention time is a major factor affecting the

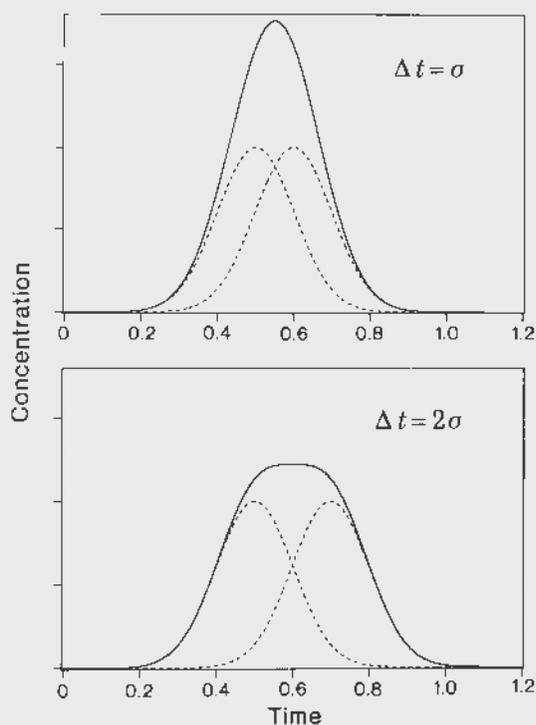


Fig. 5. Illustration of two unresolved Gaussian peaks (---) and the profiles resulting from their summation (—). Top: separation of unresolved peaks by one standard deviation ( $\sigma$ ). Bottom: separation of unresolved peaks by  $2\sigma$ .

linearity of the response of  $RT_{\text{shift}}$  to the isotope ratio. Retention times are most commonly calculated by defining the time corresponding to the maximum response of the peaks of interest. This approach is satisfactory in the accurate determination of retention times for ideally behaved single-component peaks, but is less desirable for determining  $RT_{\text{shift}}$ 's caused by the presence of two components within a single unresolved peak (as in the analysis of  $^{15}\text{NH}_4^+$  in an isotopic mixture of  $^{15}\text{NH}_4^+$  and  $^{14}\text{NH}_4^+$ ). As illustrated in Fig. 5, two ideal Gaussian profiles are not resolved for separation times less than  $2\sigma$ , where  $\sigma$  (an indicator of peak width) defines the standard deviation of the peak profile (Snyder and Kirkland, 1979). If the difference in retention times of the individual components within the profile is small (e.g.  $1\sigma$ , Fig. 5), the resulting  $RT_{\text{shift}}$  curve is only slightly sigmoid and may be approximated as a linear relationship with only slight error (Fig. 6). This curve resembles

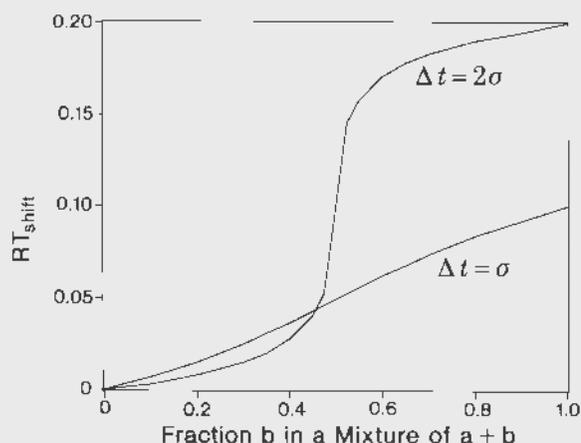


Fig. 6. Theoretical maximum-height  $RT_{\text{shift}}$  response as a function of composition for two unresolved components separated by  $1\sigma$  and  $2\sigma$ .

experimental data shown in Fig. 2. As the hypothetical time difference in the component separation increases to  $2\sigma$  (Fig. 5, bottom), the sigmoid nature of the calibration curve becomes much more pronounced (Fig. 6) and resembles experimental results for the chromatograms run at  $35^\circ\text{C}$  (Fig. 1). It is clear from Fig. 6 that this change in response pattern inherently limits the optimization of the maximum-height algorithm for defining the  $RT_{\text{shift}}$ . That is, a near-linear response is obtained over the range of the  $RT_{\text{shift}}$  curve only when component separation is small relative to peak width. As the component separation increases, the sensitivity increases but only at the cost of an increase in the sigmoid nature of the calibration curve. This result is consistent with the qualitative picture that a small change in the ratio of components will have a significant effect in the maximum-height retention time for mixtures having nearly equal composition (0.4–0.6 of one component) but will have a much more modest contribution when the composition differs widely. While this variation in the sensitivity can be accommodated by using a detailed calibration curve (Gardner et al., 1993), it causes the method to be insensitive at low and high isotope ratios and requires that a relatively large number of standards be analyzed for accurate calibration.

These difficulties can be overcome by calculating the  $RT_{\text{shift}}$  based on the median area of the

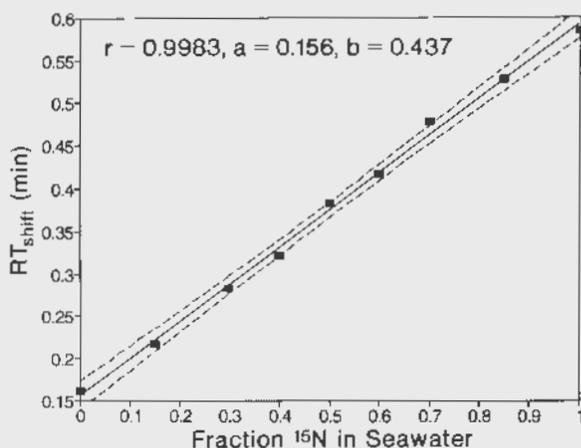


Fig. 7. Relationship between the  $[\text{}^{15}\text{NH}_4^+]:[\text{Total NH}_4^+]$  ratio and  $\text{RT}_{\text{shift}}$ , determined from median-area retention times, in seawater. Data were collected from the same chromatograms as those shown in Fig. 3.

unresolved peak. The median-area method describes the retention time as the vertical line that divides the peak into equal portions and is an accurate measure of the center of mass or centroid of the peak. Based on the technique of statistical moments, this first moment is the most accurate means of characterizing the retention time even for a single, fully resolved component (Bidlingmeyer and Warren, 1984). For two unresolved components, this method is an accurate measure of the  $\text{RT}_{\text{shift}}$  caused by the effective weighting of each component within the overall unresolved peak. As a result of this direct correspondence between median-area retention time and component composition, a linear calibration curve is expected over the entire composition range regardless of the degree of separation of the two components comprising the peaks.

Assessment of the utility of this approach to the isotope application is accomplished using the median-area retention time determination. This algorithm calculates the centroid of the peak by first dividing the peak into intervals ( $dt$ ) equally distributed across the profile and then summing the product of intensity [ $I(t)$ ] and time ( $t$ ) for each interval. This summation is then normalized to the peak area yielding the centroid retention

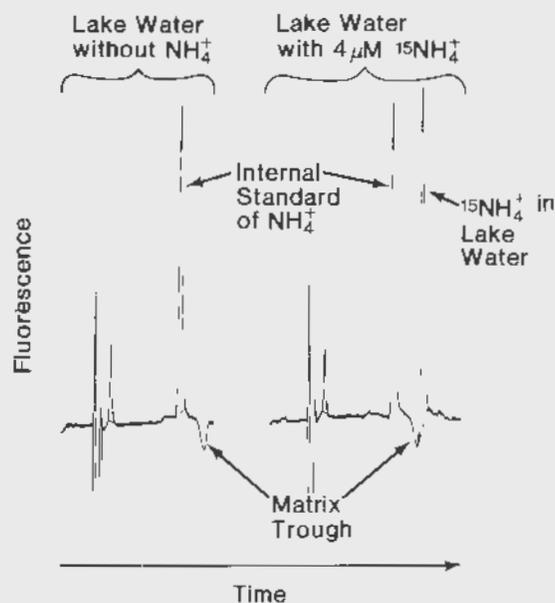


Fig. 8. Chromatograms of lake water and of lake water with added  $\text{NH}_4^+$  to show the position of the  $\text{NH}_4^+$  peak in relation to the matrix-trough caused by the injection of lake water.

time ( $ct_R$ ), i.e.

$$ct_R = \frac{\sum tI(t)dt}{\text{area}}$$

This approach was evaluated by calculating the  $\text{RT}_{\text{shift}}$  for measurements of isotope composition using both methods. The calibration curve resulting from the maximum response method is clearly sigmoid in shape (Fig. 3). In contrast, the same chromatograms evaluated using the median-area retention method resulted in a linear calibration curve with a correlation coefficient of 0.998 (Fig. 7). Thus, the median-area centroid determination provides a simple and direct means for obtaining uniform calibration curves for the  $\text{RT}_{\text{shift}}$  in this isotopic method.

As previously noted (Gardner et al., 1991), maximum-height retention time shifts are slightly biased at low  $\text{NH}_4^+$  concentrations because the  $\text{NH}_4^+$  elutes at the edge of a matrix-trough caused by the direct injection of seawater or lake water (Fig. 8). The trough is not observed for the internal standard of  $\text{NH}_4^+$  because the standard is prepared in mobile phase buffer. An advantage of using the median-area method for retention time

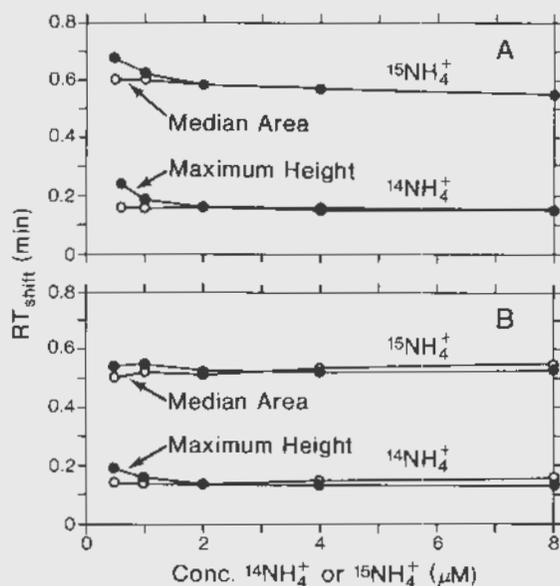


Fig. 9. Comparison of matrix-trough bias on  $RT_{\text{shift}}$  at low  $\text{NH}_4^+$  concentrations with the maximum-height and median-area methods of retention time determination. (A) Scawater (22 ppt salinity). (B) Saginaw Bay water (0 ppt salinity).

determination was that it prevented the  $RT_{\text{shift}}$  matrix bias at low  $\text{NH}_4^+$  concentrations that was observed with the maximum-height algorithm (Fig. 9). Calculation of median area was apparently not measurably affected by the sloping baseline caused by the trough.

### 3.3. Measurements of $[\text{NO}_3^-] : [\text{Total NO}_3]$ ratios

Reduction of  $\text{NO}_3^-$  in seawater or lake water, followed by the measurement of  $RT_{\text{shift}}$  values for  $\text{NH}_4^+$ , resulted in a linear relationship between median-area  $RT_{\text{shift}}$  and the  $[\text{NO}_3^-] : [\text{Total NO}_3]$  ratios that was nearly identical to the relationship for  $\text{NH}_4^+$  standards (Fig. 11). The  $RT_{\text{shift}}$  axis intercept was lower for the reduced-nitrate curve (near the origin) than for the standard-ammonium curve (about 0.15 min). This difference can be attributed to the matrix changes caused by the nitrate-reduction and pH-adjustment procedures. Differences in the pH and chemical composition of the sample relative to that of the mobile phase buffer determine the position of the  $RT_{\text{shift}}$  intercept. However, these differences do not affect isotope ratio determinations so long

as the samples and calibration curve standards have the same pH's and chemical matrices. Regression coefficients for  $\text{NO}_3^-$  calibration relationships are similar to those obtained for  $\text{NH}_4^+$  ( $r > 0.995$ ).

Previously published methods to isolate  $\text{NO}_3^-$  for mass or emission spectrometry analysis include the formation of an azo dye followed by solvent extraction (McCarthy et al., 1984; Lipschultz et al., 1986) and reduction to  $\text{NH}_4^+$  followed by steam distillation (Horrigan et al., 1990). Although the method described here is not sensitive enough to measure natural levels of  $^{15}\text{NO}_3^-$  in water, it has several advantages over previous methods for isotope dilution experiments, including small sample volume requirement (15 ml), low susceptibility to contamination, and minimal sample preparation. Although not yet tried, this method could also potentially be extended to determining the  $^{15}\text{N}$  fraction in dissolved organic nitrogen, after photo-oxidation of the DON to  $\text{NO}_3^-$  (Stainton et al., 1977), in experiments where added  $^{15}\text{N}$  is expected to be converted to  $^{15}\text{N}$ -DON (Bronk and Glibert, 1991).

### 3.4. Practical considerations for isotope addition experiments

The sample preparation procedure of freezing, thawing, and loading small volumes of filtrate onto the HPLC autosampler for  $^{15}\text{NH}_4^+$  isotope ratio analysis is time-efficient relative to the more extensive sample preparation steps required for mass measurement techniques. Sample preparation time for  $^{15}\text{NO}_3^-$  isotope analysis is of course increased by the need to convert the nitrate to ammonium before HPLC analysis. Daily preparation of  $^{15}\text{NH}_4^+$  samples and standards, and loading the autoinjector requires approximately 1 h per each sample set (triplicate injections of 6–7 water samples and 3 isotope standard solutions) that can be analyzed over a 24 h period. Mobile-phase buffer and reagent are prepared in 21 batches once per 6 days of HPLC run time.

Comparison of isotope ratio results from refrozen and thawed filtrates with those that had been initially thawed and analyzed several months earlier indicated that the results were either not

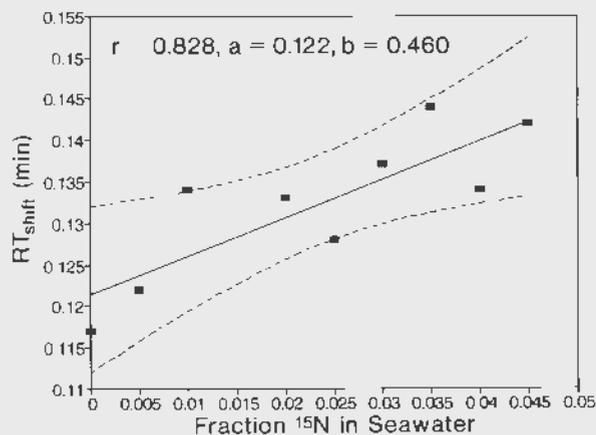


Fig. 10. Relationship between the  $^{15}\text{NH}_4^+$  : [Total  $\text{NH}_4^+$ ] ratio and  $\text{RT}_{\text{shift}}$  at low isotope ratios.

significantly different from each other or that samples from the second analyses produced slightly lower ratios than the first ones (Table 1). The latter observation, observed for *T-1* observations in the light where total ammonium concentrations were low, were apparently caused by slight contamination of the filtrates with atmospheric ammonium during the refreezing, storage, and thawing of the samples. These results indicate that it is advantageous to analyze frozen samples as soon as feasible and to manipulate samples as little as possible before analysis. In practice, the HPLC method is quite robust and it is seldom necessary to analyze refrozen samples. For example, in the course of analyzing 308 samples for isotope ratios over a period of 3 months, all initial measurements were successful so that none of the refrozen samples needed to be analyzed again.

To evaluate the possible utility of the improved method for measuring low ratios of  $^{15}\text{NH}_4^+$  : [Total  $\text{NH}_4^+$ ], we ran a standard calibration curve for ratios ranging from 0 to 0.045 in a total concentration of added ammonium of  $4 \mu\text{M}$  (Fig. 10). Although the scatter is quite high in the data, the linear relationship is significant ( $r = 0.83$ ). These data suggest that the method could potentially be used for relatively low isotope ratio comparisons if replication is sufficient and precautions are taken to prevent contamination. However, the HPLC technique is not optimally suited for this application because the precision of  $\text{RT}_{\text{shift}}$  determinations is

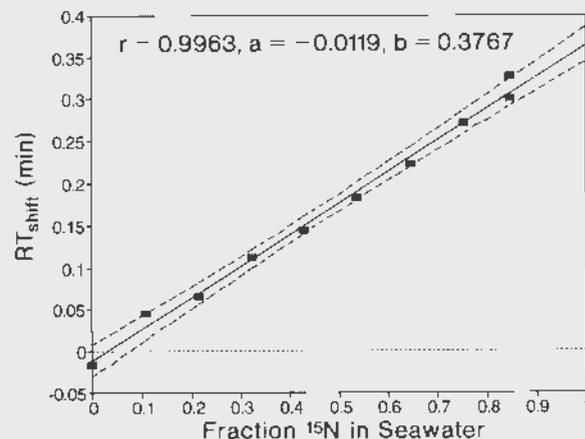


Fig. 11. Calibration curve for the relationship of  $\text{RT}_{\text{shift}}$  vs.  $[\text{NO}_3^-] : [\text{Total NO}_3^-]$  for seawater samples (salinity = 34 ppt). Before HPLC analysis, samples were treated with acidified zinc powder to reduce the  $\text{NO}_3^-$  to  $\text{NH}_4^+$  and adjusted to pH 8 with boric acid buffer.

approximately constant over the whole range of isotope ratios (e.g. see Figs. 7 and 9). Thus the ratio of measurement error to signal is increased at low isotope ratios. For this reason, more meaningful results are obtained for ratio changes observed over a relatively large portion of the curve, as can occur for high-level additions of isotopes in relatively dynamic systems, than for small changes expected with tracer-level additions.

The HPLC technique is thus most suitable for experiments where comparatively high-concentration isotope additions and ratios of  $^{15}\text{NH}_4^+$  : [Total  $\text{NH}_4^+$ ] do not greatly affect experimental interpretations. Ideal environments are coastal and other environments where turnover rates of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  are high or in experiments designed to examine the potential fate of N in organic nitrogen substrate addition experiments (e.g. Gardner et al., 1993). The HPLC method provides an ideal approach for examining nutrient turnover in water flowing over intact sediment cores where nutrient fluxes are high but water volumes are relatively small (unpubl. data). The relative merits of using high vs tracer additions of  $^{15}\text{N}$  for isotope addition experiments in natural waters have been previously discussed by Harrison (1983b).

Table 1

Comparison of isotope ratio results observed for refrozen and thawed samples analyzed in July 1994 to results from the same samples that had been initially thawed and analyzed in May 1994

Sample	T-0 (0 h)			T-1 (2.35 h)			T-2 (9.25 h)		
	NH <sub>4</sub> <sup>+</sup> Conc. ( $\mu$ M)	Ratio		NH <sub>4</sub> <sup>+</sup> Conc. ( $\mu$ M)	Ratio		NH <sub>4</sub> <sup>+</sup> Conc. ( $\mu$ M)	Ratio	
		May	July		May	July		May	July
<b>Dark</b>									
A	4.2	0.98	0.93	2.3	0.81	0.75	7.2	0.48	0.45
B	3.9	0.95	0.93	2.0	0.75	0.75	7.5	0.38	0.38
C	4.2	0.90	0.91	2.1	0.78	0.75	7.2	0.42	0.38
Mean	4.1	0.94	0.92	2.1	0.78	0.75	7.3	0.43	0.40
SE	0.1	0.02	0.01	0.1	0.02	0	0.1	0.03	0.02
<b>Light</b>									
D	4.1	0.93	0.90	0.4	0.60	0.47	0.3	nd	nd
E	4.2	0.89	0.94	0.4	0.63	0.41	0.3	nd	nd
F	4.1	0.95	0.95	0.3	0.60	0.40	0.2	nd	nd
Mean	4.1	0.92	0.93	0.4	0.61	0.43	0.3	nd	nd
SE	0.03	0.02	0.02	0.03	0.01	0.02	0.03	nd	nd

Isotope ratios were measured in waters from an isotope dilution experiment conducted on the Mississippi River plume surface water (salinity = 15 ppt) in the Gulf of Mexico in July 1993. Portions of a common water sample were fortified with  $4 \mu\text{M } ^{15}\text{NH}_4^+$  and incubated either in the dark (A, B and C) or under natural light (D, E and F) for intervals of 0 h, 2.35 h, or 9.25 h. Ammonium was analyzed onboard ship by the method of Gardner and St. John (1991) shortly after samples were taken. Note, the dark and light replicate results were each obtained from three separate incubation bottles and thus were treatment replicates rather than analytical replicates on the same treatment waters.

nd = not detected.

#### 4. Conclusions

The above results provide insights about factors controlling the relationship between ammonium isotope ratio and  $\text{RT}_{\text{shift}}$  in chromatographic analysis of isotope ratios. Column temperature, mobile phase buffer pH, and the algorithm for determining  $\text{RT}_{\text{shift}}$  are important variables affecting the shape and magnitude of  $\text{RT}_{\text{shift}}$  vs isotope ratio curves. At a column temperature of  $85^\circ\text{C}$ , a buffer pH of c. 9.4, and with the use of median-area method for determining retention time, we were able to produce a linear relationship over the whole range of the calibration curve. Development of a linear relationship describing these variables simplifies the use of  $\text{RT}_{\text{shift}}$  for isotope ratio determinations and makes the technique more applicable to measuring low and high ratios of  $[\text{}^{15}\text{NH}_4^+] : [\text{Total NH}_4^+]$  than was the case with the previously described sigmoid relationship. Incorporation of a  $\text{NO}_3^-$  reduction

step extends the potential use of the HPLC method to isotope dilution or enrichment experiments with nitrate. The described modifications, incorporated into isotope dilution and enrichment experiments, makes feasible the convenient measurement of nitrogen transformations in a variety of coastal and other nutrient-rich aquatic ecosystems.

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