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TECHNICAL NOTE

Simplifying and improving the extraction of nitrate from freshwater for stable isotope analyses†

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Determining the isotopic composition of nitrate (NO_3^-) in water can prove useful to identify NO_3^- sources and to understand its dynamics in aquatic systems. Among the procedures available, the 'ion-exchange resin method' involves extracting NO_3^- from freshwater and converting it into solid silver nitrate ($AgNO_3$), which is then analysed for $^{15}N/^{14}N$ and $^{18}O/^{16}O$ ratios. This study describes a simplified methodology where water was not pre-treated to remove dissolved organic carbon (DOC) or barium cations (added to precipitate O-bearing contaminants), which suited samples with high NO_3^- (≥400 μM or 25 mg L^{-1} NO_3^-) and low DOC (typically <417 μM of C or 5 mg L^{-1} C) levels. % N analysis revealed that a few $AgNO_3$ samples were of low purity (compared with expected % N of 8.2), highlighting the necessity to introduce quality control/quality assurance procedures for silver nitrate prepared from field water samples. Recommendations are then made to monitor % N together with % O (expected at 28.6, *i.e.* 3.5 fold % N) in $AgNO_3$ in order to better assess the type and gravity of the contamination as well as to identify potentially unreliable data.

1. Introduction

The common occurrence of elevated concentrations of nitrate (NO_3^-) in freshwater has long been a cause of concern for human/animal health (ingestion of contaminated water linked to a number of diseases[1]) and the environment (nitrate occurrence can be associated with eutrophic conditions[2]). In response to these problems, environmental policies have been implemented in many countries to limit NO_3^- levels in water systems. However, such a task remains challenging, partly because nitrate can come from multiple sources (*e.g.* artificial and organic fertiliser applications, human/animal waste effluents, soil nitrogen, atmospheric deposits), which makes identifying and controlling the main contamination difficult. To identify NO_3^- sources, a number of methods have been previously used, among which is dual stable isotope analyses. This technique, which combines the measurements of $^{15}N/^{14}N$ and $^{18}O/^{16}O$ ratios in dissolved NO_3^-

Environmental impact

This study ('Improving and simplifying the extraction of nitrate from freshwater for stable isotope analyses' by E. Minet, R. Goodhue, C. E. Coxon, R. M. Kalin and W. Meier-Augenstein) is aimed at researchers who use stable isotope analyses to study nitrate sources and dynamics in inland waters. In this manuscript, we described a simplified 'ion-exchange resin' methodology of nitrate extraction suited for freshwater with high nitrate (≥400 μM or 25 mg L^{-1} NO_3^-) and low dissolved organic carbon levels (typically <417 μM of C or 5 mg L^{-1} C) typical of highly productive sand and gravel aquifers. Simplifications to the original method were brought to reduce sample preparation and the cost of consumables. Furthermore, we introduced a straightforward quality control and quality assurance procedure that can help detect potentially unreliable samples and strengthen the worth of much needed stable isotope data.

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(referred to as δ^{15}N–NO_3^- and δ^{18}O–NO_3^-, expressed in units of permil‰ relative to AIR and V-SMOW primary standards respectively), has drawn substantial attention for two main reasons. Firstly, some of the main N sources generate nitrate with characteristic isotopic composition.[3] Secondly, biochemical reactions affecting NO_3^- can cause distinctive isotopic fractionation (*e.g.* denitrification, mineralisation–immobilisation turnover[3,4]), which gives some insights on nitrate dynamics.

Nowadays, δ^{15}N–NO_3^- and δ^{18}O–NO_3^- in nitrate can be measured according to three analytical methods:[5] two 'denitrifier methods' requiring Isotope Ratio Mass Spectrometry (IRMS) analysis of N_2O gas generated by bacterial or chemical means, and a procedure known as the 'ion-exchange resin method' whereby NO_3^- is extracted from freshwater and converted into solid silver nitrate ($AgNO_3$) that is analysed by IRMS.[6] The latter methodology, which isolates nitrate from other N- and O-bearing species, has been successfully used by many, but it is labour-intensive. Minor modifications have been adopted by some to ease the sample preparation,[7] one of the most significant changes being the precipitation of O-bearing contaminants (mainly sulfate and phosphate) with barium chloride ($BaCl_2$) prior to passing water through an anion-exchange resin so that $AgNO_3$ is ready for both δ^{15}N and δ^{18}O analyses. The primary aim of this research was to further simplify the methodology for freshwater samples. A secondary aim was to explore the variability of $AgNO_3$ purity (based mainly on % N content) prepared from field water samples. Ultimately, this led to a quality control/assurance procedure that discriminates potentially unreliable data.

2. Materials and methods

2.1. Sample preparation

Nitrate was extracted from 305 water samples[8] (253 mainly from groundwater in a sand/gravel aquifer and 52 from soil-water). A preliminary step consisted of filtering water with 0.45 µm nylon filters and measuring concentrations of nitrate and other major ions within 24 hours of collection.[8] Thereafter, an aliquot of each sample containing 100 µmol of nitrate (*i.e.* 6.2 mg NO_3^-) was portioned off. As nitrate levels were between 32 and 2455 µM (≥400 µM or 25 mg L^{-1} NO_3^- in 85% of the samples), volumes used ranged between 0.041 and 3.1 L (≤0.250 L in 85% of the samples). Each water sample underwent five steps (first two steps completed in the laboratory within 48 hours of collection):

Step 1. Removal of O-bearing contaminants: addition of 4 mL of 1 M $BaCl_2$ (large excess) to precipitate sulfate (up to 4000 µmol) and phosphate to a lesser extent (levels typically low in sub-surface water), storage at 4 °C overnight, filtration through a 0.2 µm nylon membrane.

Step 2. Extraction of nitrate: sample passed through the anion-exchange resin column (2 mL Bio-Rad AG1-X8, 200–400 mesh in Cl^- form, capacity of 1200 µeq mL^{-1}, pre-conditioned by dripping through 4 mL of 1 M HCl and rinsed) at a flow rate no faster than 1 L h^{-1}, resin rinsed and column filled with deionised water, storage at 4 °C until later elution. The resin was kept wet at all times.

Step 3. Elution of nitrate: five 3 mL increments of 3 M HCl passed through the column (elutant kept chilled to minimise volatilisation of HNO_3), each increment kept in the column for 30 seconds before slowly blowing the column dry, rinsing (whole step carried out in the dark).

Step 4. Neutralisation: elutant immediately neutralised by slow addition of about 6 g of silver oxide (three different batches of high-grade Ag_2O heavily contaminated with nitrate were previously washed[6]) until pH reached 5–6 (checked with pH-paper), filtration through a 0.2 µm nylon membrane to remove excess Ag_2O and silver chloride (AgCl), rinsing (whole step carried out in the dark).

Step 5. Freeze-drying: $AgNO_3$ solutions (about 40 mL) frozen at −75 °C, then freeze-dried in the dark (and stored in amber vials in a desiccator).

Unlike the simplified method,[7] water samples were not pre-treated with a cation-exchange resin, used by others to remove barium cations (Ba^{2+})[7] or DOC[9] before the anion-exchange resin. To process samples, apparatus similar to that described by Silva *et al.*[6] or the USGS[9] were used.

2.2. IRMS measurements

δ^{15}N and δ^{18}O analyses of silver nitrate were carried out by Continuous Flow-Isotope Ratio Mass Spectrometry (CF-IRMS). δ^{15}N analyses (about 0.8 mg per $AgNO_3$ sample) were made in duplicate with an CE Instruments Series 1112 flash elemental analyser coupled to a ThermoFinnigan Delta$^{\text{Plus}}$ isotope ratio mass spectrometer *via* a Conflow III Interface, which allows the introduction of reference gases N_2 and CO_2 (the former is used to calibrate the instrument for δ^{15}N and % N analyses, the latter to calibrate for % C analyses). The oxidation tube was set to 900 °C, the reduction tube to 650 °C and the gas chromatography (GC) 2 m-column (standard CE P/N:26008205 type) to 40 °C to separate N_2 and CO_2 peaks (peak-to-peak separation was approximately 120 s, peak start slope set at 0.2 mV s^{-1} and peak end slope set at 0.8 mV s^{-1}). δ^{18}O analyses were also made in duplicate (about 0.2 mg per $AgNO_3$ sample) with a high temperature conversion elemental analyser (TC/EA) coupled to a Delta$^{\text{Plus}}$ XP isotope ratio mass spectrometer *via* a Conflow III Interface, which allows the introduction of reference gas CO (used to calibrate the instrument for δ^{18}O analyses). Samples were dropped with a Costech Zero-Blank solid auto-sampler into the reactor, whose temperature was set at 1350 °C while the post-reactor GC 0.60 m-column (standard MolSieve 5A type) was maintained at 95 °C to separate the undesirable N_2 peak (and potentially formed NO) from the CO peak that follows (peak-to-peak separation higher than 80 s, peak start and end slopes set at 0.8 mV s^{-1}). A laboratory standard L-alanine (δ^{15}N = −1.7‰) was run in blocks before, during, and after δ^{15}N analyses in order to correct δ^{15}N–NO_3^- results to the primary standard $\delta^{15}N_{\text{AIR}}$. Similarly, reference material IAEA-NO-3 (δ^{18}O = 25.6 ± 0.2‰) was run in order to correct δ^{18}O–NO_3^- data to $\delta^{18}O_{\text{V-SMOW}}$. Silver boats (4 × 6 mm) used to encapsulate samples and reference materials were also inserted empty at the beginning of each batch for blank correction. Each chromatograph was examined visually before processing with Isodat NT software. In particular, it was checked that (i) the integration of the gas reference peaks was reproducible, (ii) the samples' peaks (N_2 and CO_2 during δ^{15}N analyses, N_2 and CO peaks during δ^{18}O analyses) were correctly separated and integrated (although N_2 had no utility during δ^{18}O analyses), (iii) the peaks' shape was as expected and (iv) the baseline was stable before, between and after the peaks. Standard deviations (SDs) for duplicated δ^{15}N analyses of $AgNO_3$ samples were in line with the analytical precision of the instrument (≤0.1‰, measured from L-alanine): SDs were below 0.1‰ in 90% of the samples (273/305) and always better than 0.3‰. For δ^{18}O analyses, SDs were also in line with the analytical precision (≤1.2‰, measured from IAEA-NO-3): SDs were below 0.5‰ in 79% of the samples (240/305) and always better than 2.1‰.

2.3. Elemental analyses

During δ^{15}N analyses, L-alanine standard (15.7% N and 40.4% C) was also used for the calibration of % N and % C, which were measured in duplicate in $AgNO_3$ ($n = 284$ for % N, $n = 206$ for % C). SDs of % N compared well with the analytical precision (≤0.8): SDs were better than 0.5 and below 0.1% N in 93% of tested samples (265/284). During % C analyses, SDs were below 0.2 (analytical precision ≤ 2.2) in the few samples whose % C was above the detection limit (0.1% C).

3. Results

3.1. Testing sulfate removal, DOC accumulation and isotopic fractionation

Levels of sulfate (SO_4^{2-}) ($n = 273$) ranged between 91.6 and 716 μM, which based on volumes portioned off, equated to 11.4 to 845 μmol of SO_4^{2-} to be removed. Removal (step 1), which is crucial because sulfate has a higher affinity for the resin than nitrate (according to the resin manufacturer), was previously tested on 1 L solutions of 1041 μM sulfate that had been treated with 4 mL of 1 M $BaCl_2$ and 0.2 μm nylon-filtered after three hours at 4 °C. This proved highly efficient as no SO_4^{2-} was detected in the filtrate. Phosphate (in $H_2PO_4^-$ and HPO_4^{2-} form, as pH ranged between 6.7 and 7.6[8]) was not analysed, but its low affinity for the resin and its typically low concentration in sub-surface water meant that it should be quantitatively precipitated along with sulfate. Bicarbonate (HCO_3^-) was not measured either, but its equally low affinity for the resin and its likely conversion to CO_2 by acidification in step 3 ruled it out as a source of O contamination. Besides SO_4^{2-}, unwanted O (and N) may also be introduced by DOC, which was present at low level in the sand/gravel aquifer[10] (121 ± 34 μM of C). It was decided not to treat samples for DOC, but instead to rely on the filtration capacity of the resin and to monitor % C in $AgNO_3$ (the top part of the resin was heavily discoloured from light yellow to dark brown for some samples, which could have been indicative of DOC accumulation in the resin): % C in $AgNO_3$ was systematically low (≤2%) and below the detection limit in most samples.

To ensure that no significant isotopic fractionation occurred during the preparation of $AgNO_3$, the methodology was tested according to the following plan: three potassium nitrate salts (KNO_3) were converted into silver nitrate after 125 mL solutions of 800 μM of nitrate underwent the whole extraction process. Resulting δ^{15}N–NO_3^- and δ^{18}O–NO_3^- values were then compared with values measured in KNO_3 salts (see Table S1 in the ESI†). Overall, results did not show any systematic trend: the apparent isotopic enrichment factor $\Delta_{AgNO_3-KNO_3}$ for δ^{15}N varied between −0.7‰ and 0.1‰, whereas the range for δ^{18}O was wider between −0.9‰ and 1.6‰.

It should be noted that most of Cl^- anions (a minor competitor to NO_3^- adsorption—at equal concentrations—on the anion exchange resin sites) present in water at the end of step 1, *i.e.* prior to passing samples through the anion exchange resin, were accounted for by $BaCl_2$ addition rather than "naturally" occurring Cl^-. In fact, more than 95% of total Cl^- content at the end of step 1 (*i.e.* "natural" Cl^- + Cl^- from $BaCl_2$) originated from $BaCl_2$ alone in 98.2% of tested samples (277/282), and this exogenic Cl^- fraction was never lower than 72%. Overall, between 8012 and 11 049 μeq of chloride were passed through the column (compared with 100 μeq of nitrate).

3.2. Elemental composition of $AgNO_3$ samples

% N measured in $AgNO_3$ prepared from field water samples (see Fig. S1A in the ESI†) ranged from 1.7 to 8.3 (median of 7.8), with 90% of these samples (257/284) showing % N between 7.0 and 8.3. % C (see Fig. S1B in the ESI†) was measured above the detection limit (0.1) and below 2 in only 7.3% of tested $AgNO_3$ samples (15/206). In two $AgNO_3$ test samples (δ^{15}N–NO_3^- [1] = −20.3 ± 0.2‰ and δ^{18}O–NO_3^- [1] = 14.8 ± 1.3‰, δ^{15}N–NO_3^- [2] = −20.5 ± 0.1‰ and δ^{18}O–NO_3^- [2] = 14 ± 0.2‰) prepared from one of the three KNO_3 salts (δ^{15}N–NO_3^- = −20.4 ± 0.2‰ and δ^{18}O–NO_3^- = 13.4 ± 0.9‰), % N was measured at 7.6 ± 0.4 and 7.8 ± 0.1 respectively.

Plotting % N in $AgNO_3$ (x coordinate) against the volume of water passed through the resin (y coordinate) (see Fig. S2 in the ESI†) resulted in a small but highly significant and negative correlation (Spearman rank coefficient $r_s = -0.24$, slope = −0.26, $p \leq 0.0001$). Importantly, all volumes smaller than 0.250 L (*i.e.* nitrate level ≥ 400 μM) resulted in silver nitrate with high % N (≥6.7). Plotting % N against total chloride content at the end of stage 1 (*i.e.* "natural" Cl^- + Cl^- from $BaCl_2$) also disclosed a negative correlation, which was small but nonetheless significant at the 0.05 level ($r_s = -0.12$, slope = −6.2, $p = 0.048$). On the other hand, the plotting of % C against % N or against the volume of water was not significant ($p \gg 0.05$).

4. Discussion

The modification brought to the simplified 'ion-exchange resin method' (*i.e.* no pre-treatment of water other than sulfate precipitation with $BaCl_2$ before passing samples through the anion-exchange resin) left Ba^{2+} cations in solution before the nitrate extraction step. However, experiments carried out on test solutions of three pure nitrate salts showed that this causes little isotopic fractionation of the extracted nitrate for δ^{15}N (absolute value of $\Delta_{AgNO_3-KNO_3} \leq 0.7$‰). The absolute value of $\Delta_{AgNO_3-KNO_3}$ for δ^{18}O was larger (≤1.6‰) but nonetheless in line with the analytical precision. Lesser precision can happen during δ^{18}O analyses with the TC/EA if the N_2 peak and/or formed NO (*m*/*z* 30) tail into the CO peak to impair the integration, which was not seen on most chromatograms. Ratio trace 30/28 (see Fig. S3 in the ESI†), which shows where the peaks were detected and integrated during δ^{18}O analyses, was generally flat (back at baseline) between N_2 and CO. As small quantities analysed (about 0.2 mg) inevitably result in higher background noise, sample heterogeneity is likely to add up to the poorer precision. Nevertheless, different GC settings and a larger peak-to-peak separation time might have brought some improvement to batches with less precision. The method modification also left DOC in solution, yet the vast majority of $AgNO_3$ samples was free of measurable C contamination. This confirmed that low DOC levels were not a cause for concern for subsurface freshwater, even for nitrate levels as low as 32 μM (and volumes as large as 3.1 L). The lack of relationship between % C in $AgNO_3$ and the volume of water passed through the resin suggested that even higher volumes (*i.e.* >3.1 L) could be processed without problem of DOC contamination. Sulfate was another unlikely source of O contamination for $AgNO_3$. In fact, tests showed that 4 mL of 1 M $BaCl_2$ was more than adequate to safely precipitate up to (and very likely more than) 1000 μmol of sulfate.

90% of silver nitrate samples prepared from field water showed % N between 7.0 and 8.3, which confirmed that $AgNO_3$ preparation was consistent. In the absence of DOC contamination, it was then assumed that all N accounted for in $AgNO_3$ was in nitrate form and bound to silver. If this assumption is correct, it meant that $AgNO_3$ samples were on average 95% pure (median % N of 7.8 divided by expected % N for pure $AgNO_3$ of 8.2). However, contamination levels would be better estimated with both % N (expected at 8.2) and % O (expected at 28.6): deviation of the % O to % N ratio from the 3.5 expected value would characterise the presence of non-nitrate N and/or O in $AgNO_3$ (contaminants with the same % N and % O as silver nitrate would be unnoticed). Unfortunately, % O data (values can usually be obtained with the TC/EA by integrating the CO peak) were not available. In the remaining 10% silver nitrate samples prepared from field water, however, % N ranged between 1.7 and 6.9. In the absence of % O, the nature of the impurities can only be speculated. Nevertheless, % N recorded in two test samples (*i.e.* KNO_3 converted into $AgNO_3$) gave some evidence that any % N lower than 8.2 does not necessarily affect $\delta^{15}N–NO_3^-$ and $\delta^{18}O–NO_3^-$ by a large margin: the absolute value of $\Delta_{AgNO_3–KNO_3}$ for these two samples ($\leq$0.2‰ for $\delta^{15}N$ and $\leq$1.4‰ for $\delta^{18}O$) was close to the analytical precision of the mass spectrometers. Importantly, these differences should not impact nitrate source identification, which is based on a comparatively much wider range of values[3] (*e.g.* $\delta^{15}N$ in nitrate derived from synthetic fertilisers often expected between −4 and 4‰ while nitrate derived from organic human/animal wastes expected between 10 and 22‰; $\delta^{18}O$ in synthetic nitrate measured between 18 and 22‰ while nitrate derived from natural nitrification often estimated between 0 and 5‰[11]). It should be noted that besides the amount of N- and O-bearing contaminants present in $AgNO_3$, $\delta^{15}N$ and $\delta^{18}O$ is also affected by the (N and O) isotopic composition of these contaminants: impurities present in large quantity (*i.e.* in "low N" samples) with δ values very different from that of NO_3^- will compromise isotopic data the most. In addition to % O measurement, further analyses (*e.g.* % Ag, % S, % P, *etc.*) may then be necessary in a more thorough quality control/assurance procedure to precisely identify the contaminations, to define an acceptable contamination threshold and to ultimately decide what samples should be excluded.

The highly significant and negative correlation ($p \leq 0.0001$) observed between % N in $AgNO_3$ and the volume of water passed through the resin showed that the resin was losing efficiency at low nitrate levels, especially below 400 μM of nitrate (*i.e.* volumes > 0.250 L). This trend was expected since larger volumes translate into more contaminants disturbing NO_3^- adsorption on the resin exchange sites. A similarly negative relationship between % N and total chloride content suggested that Cl^- interference played a role. Yet this role appeared to be minor ($p < 0.05$) considering that the column's capacity (2400 μeq) was widely exceeded by chloride loading (≥8012 μeq). On the other hand, DOC was not responsible for any decrease in % N (no relationship between % N and % C in $AgNO_3$).

5. Conclusions

Nitrate extraction from water according to a modified 'ion-exchange resin method' (*i.e.* no pre-treatment of water other than sulfate precipitation with $BaCl_2$ before passing samples through the anion-exchange resin) proved highly manageable and reliable for samples with high NO_3^- (≥400 μM or 25 mg L^{-1} NO_3^-) and low DOC (typically <417 μM of C or 5 mg L^{-1} C) levels. In addition to reducing sample preparation, the simplification also reduced the cost of consumables (*e.g.* no use of a cation-exchange resin to remove DOC or Ba^{2+}).

Monitoring % N in $AgNO_3$ prepared from field water samples identified a few samples of low purity. But the gravity of the contamination was unclear without % O data (% O to % N ratio expected at 3.5 in pure silver nitrate). In a more thorough quality/control procedure, the measurement of other parameters (*e.g.* % Ag, % S, % P) would have been required to precisely determine the nature of the contaminants, to define a contamination threshold and to ultimately decide what samples should be excluded.

Low % N in $AgNO_3$, recorded with large volumes of water passed through the resin, showed that the methodology loses some reliability with low nitrate water (<400 μM) and high volumes (>0.250 L). Determining the limit of this method towards lower NO_3^- levels could prove useful for future studies. Since larger volumes require more sulfate (and phosphate) to be removed, attention could also be given to the impact of more $BaCl_2$ being added on the nitrate extraction yield. In that case, the introduction of O-bearing contaminants by precipitates of $BaSO_4$ (and $Ba_3(PO_4)_2$), which has a very low but finite solubility, should be investigated. Finally, the overloading of the resin with chloride (total chloride content ≫ column's capacity) had a marginal detrimental effect on nitrate adsorption.

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