Procedure for hypobromite oxidation of ammonium to nitrite, followed by azide conversion to nitrous oxide

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Reference: Zhang et al. (2007) “Sensitive Measurement of NH4+ 15N/14N (δ15NH4+) at Natural Abundance Levels in Fresh and Saltwaters.” *Analytical Chemistry* **79,** 5297–5303. [doi:10.1021/ac070106d](https://doi.org/10.1021/ac070106d)

**Before starting**

* Muffle (450°C, 5 h) 20 mL crimp-cap vials, other glassware, and aluminum foil. In the course of the procedure, ~1-2 large (1 L), 1 medium (200 mL), and 2 small (10-50 mL) beakers, 1 small screwcap bottle, and 1 25 mL graduated cylinder will be required. Use muffled glassware for all reagents and liquid transfers. Work on muffled aluminum foil.
* For lowest blanks, use boiled ultrapure water for dilution of samples. Boil on a hot plate and let cool to ambient temperature before use (can speed up cooling by submersion in ice. The boiled water can be kept in a screwcap bottle and used over a few days.
* At all stages, work as cleanly as possible.

**Day 1**

1. Dilution of samples

The acceptable range for the method is ~10-40 nmol ammonium (~2-8 µM), which will generate 20-80 nmol N2O. For samples above 8 µM, dilution is required. For lowest blank values, use boiled ultrapure water for dilution. Pick the biggest target sample size possible from this range. Calculate the amount of sample required to reach the target, typically in 5 mL of water.[[1]](#footnote-1) A template for calculation of dilutions is attached. If <500 µL of sample will be used, dilute into 5 mL MQ water. If >500 µL, adjust water amount down accordingly.

If nitrite removal is not required the next step can be skipped, but adding HCl seems to improve yields, so consider including it even if nitrite is not found in the samples.

1. Removal of nitrite

Reagent: sulfamic acid (1 mM), HCl (6M)

Remove nitrite by adding 1mM sulfamic acid (SFA) at 1.5x the measured nitrite concentration. Use the attached template to calculate the appropriate SFA addition, given ammonium and nitrite concentrations. If nitrite concentrations are unknown, but suspected to be low, samples can be treated with a standard addition of 50 µL 1mM sulfamic acid per 5mL prepared volume. For samples with no nitrite, adding the HCl alone seems to help yields later, and so can be done at this stage.

Into a new 15 mL Falcon tube, add (1) water, (2) HCl (46 µL, 6M) and SFA (calculated for each sample), and then (3) sample and shake well. Let sit overnight (or all day) at room temperature. Also prepare a ‘SFA blank’ that contains water, HCl, and the maximum amount of SFA used in the run. If samples with no nitrite are included, add a ‘HCl blank’ with water and HCl.

**Day 2**

1. Sample transfer

Transfer all samples to 20 mL glass vials with a 5 mL pipet, or by careful pouring. Cover each vial with rubber stoppers, using clean technique, as soon as it is exposed to the room and whenever it is not in use. Pipet tips can be reused between samples but should be rinsed 5x with MQ water between each sample.

Also prepare blanks and standards as indicated in the runsheet template, with boiled ultrapure water. Choose two of the standards from the set IAEA-N1, IAEA-N2, USGS25, and USGS26, as appropriate for primary standardization,[[2]](#footnote-2) plus 1 or more check standard (the preceding list plus IAEA-305A are possibilities; N1 and N2 should always be run). Primary standards should be prepared at 3 concentrations bracketing the target, as indicated on the runsheet template. As BrO– method blanks, have 2 vials, each with 5 mL ultrapure water.

1. Preparing hypobromite reagent

All bromine reagents, as well as arsenite, are highly toxic and should be handled with care. Use gloves and careful glove technique to work with them. Clean up spills immediately, dispose of waste in the appropriate container, and wash hands thoroughly and frequently.

Stock solutions: Bromate/bromide stock solution, 0.6 g sodium bromate mixed with 5.0 g sodium bromide in 250 mL milliQ water (good for ≥5 months). Sodium arsenite, 0.39 M, 5.1 g sodium arsenite in 100 mL water. 6M HCl.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Number of samples | Water (mL) | Stock solution (mL) | HCl (mL) | NaOH (mL) |
| 40 | 10 | 0.2 | 0.6 | 10 |
| 60 | 15 | 0.3 | 0.9 | 15 |
| 80 | 20 | 0.4 | 1.2 | 20 |
| 100 | 25 | 0.5 | 1.5 | 25 |

The following procedure is written for ~40 mL of reagent, good for ~80 samples. Scale up or down accordingly (see table). Prepare the reagent in the fume hood.

1. Make a hypobromite (BrO-)working solution: **in the dark**, add 0.4 mL of the stock solution to 20 mL deionized water, followed by 1.2 mL 6M HCl, to produce Br2. Stir vigorously on a magnetic stirplate. Let react 5 minutes in the dark.
2. Add 20 mL of 10M NaOH with vigorous stirring. BrO- is produced.
3. Reagent degrades rapidly; use within an hour.
4. Hypobromite oxidation

Continue working in the fume hood.[[3]](#footnote-3)

1. Add 0.5 mL BrO- to each 5 mL sample. Immediately after each BrO- addition, mix each vial vigorously while also holding down the cap to prevent spillage.
2. Let the sample react for 30 minutes at room temperature (pH 14).
3. Add 0.1 mL arsenite solution to stop reaction and remove excess BrO-.[[4]](#footnote-4) Shake each vial vigorously, again, while holding down the cap. Let sit ≥30 minutes.

*Samples are stable and can be stored overnight or even for several days at this stage if necessary, but care should be taken to keep protect from contamination.*

1. Pre-azide neutralization

* Neutralize all samples, which were brought to pH 14 by the hypobromite procedure. For standards and most samples, add ~400 μL 6M HCl to each sample to accomplish this. This approach is reliable and needs no further check for highly-diluted wastewater samples. But for other types of samples, especially strongly-buffered materials, check the pH with a drop of sample onto pH for a subset of samples. Aim for pH of samples to be ≤ 7 (and as low as 1 to 2 is okay).

1. Azide conversion of nitrite to N2O

Sodium azide is toxic and can also be explosive when mixed with excess HCl or in contact with metals. Before using sodium azide (NaN3) review hazards associated with it. Work carefully, wear gloves, clean spills immediately, wipe large spills with pH 12 water (0.01 M NaOH), dispose of all waste in the proper vessel (typically the ‘azide sharps’ container in the fume hood.

Perform all of the following steps in the fume hood.

Prepare azide reagent: for 20 mL of azide (good for up to ~60 samples), use a 10 mL syringe to take 13 mL of 2M NaN3 from the working stock[[5]](#footnote-5) and place it in crimptop vial or bottle. To this, add the same volume of 20% (v/v) acetic acid to make 20 mL of a 1:1 azide-acetic acid mixture.

1. Purge the azide reagent with N2 for ~ 2 hours before use.[[6]](#footnote-6) For purging use two long needles and one short exit needle. Remove exit needle before removing the second purging needle, and then turn N2 gas flow off.
2. Prepare nitrite standards N7373 and N10219 at the same concentration target as the samples, in 5 mL MQ water. At the same time, prepare 2 x ‘azide blanks,’ ultrapure water that the azide reagants will be added to.
3. Using a 1 mL luer-lock syringe and needle, add 300 μL of the azide-acetic acid mixture to each sample. Mix by shaking, and then place upside-down in the vial rack. Let react for ≥30 minutes. Dispose of the azide-contaminated syringe and needle in the “Azide Sharps” waste.
4. After ≥30 minutes add 200 μL of 10M NaOH, using a 1 mL luer-lock syringe and needle, to stop the reaction. Make sure the solution is well-mixed and store the vials upside-down. Dispose of the needle in “Azide Sharps” waste. Samples are now ready for analysis.
5. Disposal of measured samples

After measurement, carefully place the measured vials in the large barrel that is typically kept in the isotope lab. When full, this barrel is suitably labelled and placed in the waste collection room, and a new one is started.

**Trouble-shooting guide**

*What do I do when…*

*…no or uniformly small N2O peaks appear?*

The azide step probably failed? Check azide reagents and repeat. Or it is an IRMS problem and this is the wrong troubleshooting guide for you!

*…only nitrite standards give N2O peaks?*

Likely a problem with the BrO– working solution. Repeat the procedure. If NaBr/NaBrO2 stock solution is older or has not been used successfully recently, replace it, but this is not the first thing to do.

*…peaks are smaller than expected, but well above background.*

The procedure worked but had a poor yield. Take care with quality control, but the data could still be acceptable.

*…only standards have good N2O yields?*

The pH of your samples probably did not get into a good range. Try again, checking pH at the time of HCl addition more carefully.

*…only samples have good N2O yields?*

This is a rare but annoying problem, probably also attributable to pH problems or a low-quality working solution.

*…excessively high backgrounds are seen?*

Any steps that were not performed with maximal cleanliness, try to improve. But if you are following good practices in this way, consider whether any reagents (Br-stock, arsenite, HCl, NaOH) may have become contaminated.

*…samples show larger than expected peaks?*

Likely, additional N atoms were converted to a labile form, perhaps during storage. Do not trust data. Consider remeasuring by an alternative technique (diffusion method?).

1. Higher volumes of up to ~8 mL can be tried for low-concentration samples. Up-scale all reagents (SFA, HCl, BrO–, arsenite, HCl, azide, and NaOH) accordingly when going above 5 mL target size. This is necessary because of the pH-modifying properties of each of these reagents. [↑](#footnote-ref-1)
2. Standards should bracket the expected range in δ15N seen in the samples.

   |  |  |  |  |  |  |
   | --- | --- | --- | --- | --- | --- |
   | **Standard** | IAEA-N1  (± 1 s.d.) | IAEA-N2  (± 1 s.d.) | USGS25  (± 1 s.d.) | USGS26  (± 1 s.d.) | IAEA-305A (95% CI) |
   | **δ15N (‰ vs air)** | 0.43 ± 0.07 | 20.41 ± 0.12 | -30.41 ± 0.27 | 53.75 ± 0.24 | 39.8 ± 0.5 |

   [↑](#footnote-ref-2)
3. Often these steps are often performed in the dark, but this may not be necessary. A little more light is certainly okay. [↑](#footnote-ref-3)
4. These steps can be overlapped, i.e., arsenite addition can start 30-35 minutes after BrO- was added to the first sample. [↑](#footnote-ref-4)
5. For instructions on preparing this working stock, refer to the azide method protocol [↑](#footnote-ref-5)
6. Start azide purging before arsenite additions for optimal timing [↑](#footnote-ref-6)