**Determination of ammonium cation concentrations in resin bag extracts**

**Reaction details**

The following protocol details a modified phenol-hypochlorite reaction for quantifying ammonium cation concentrations, a well-established method for ammonium (NH4-N) concentrations in resin bag and soil extracts. The reaction moves forward by allowing ammonia to react with hypochlorite to form chloramine. Chloramine then couples with two phenols: sodium nitroprusside and sodium salicylate. Sodium citrate and sodium tartrate are included in the reaction to avoid precipitation of calcium, magnesium, and other hydroxide compounds. The reaction forms a green-blue pigment after a brief incubation period, where the intensity of blue is proportional to the ammonium concentration in each extract. The reaction turns a yellow pigment if there is too much ammonium present.

**Reagent Preparation**

1. **Sodium salicylate solution:**

In a 100 mL volumetric flask, bring to volume with ultrapure water (MilliQ):

* + - 6.8 g sodium salicylate
    - 5.0 g sodium citrate
    - 5.0 g sodium tartrate
    - 0.025 g sodium nitroprusside (in refrigerator)

1. **Sodium hydroxide solution:**

In a 100 mL volumetric flask, bring to volume with ultrapure water:

* + - 6.0 g sodium hydroxide

1. **Bleach solution (make daily prior to curves):**

In a 15 mL centrifuge tube:

* + - 0.2 mL bleach
    - 9.8 mL sodium hydroxide solution (reagent 2)

1. **Sample matrix (2.0 M NaCl/0.1 M HCl):**

In a 1 L volumetric flask, bring to volume with ultrapure water:

* + - 116.88 g sodium chloride
    - 8.4 mL concentrated hydrochloric acid

1. **100 ppm NH4+ stock solution (make weekly):**

In a 500 mL volumetric flask, bring to volume **with the sample matrix**

* + - 235.8 mg ammonium sulfate

**Standard Curve**

1. Dilute the 100 ppm NH4+ stock solution to 10 ppm by adding 150 μL stock solution to 1350 μL sample matrix in a centrifuge tube
2. Create a 7-point standard curve by diluting the 10 ppm NH4+ solution in sample matrix using the dilutions in Table 1

**Table 1**: Dilutions for the 7-point standard curve

|  |  |  |
| --- | --- | --- |
| [NH4-N] | 10 ppm NH4+ (μL) | Matrix (μL) |
| 0 | 0 | 1000 |
| 0.3 | 30 | 970 |
| 0.5 | 50 | 950 |
| 1.0 | 100 | 900 |
| 2.5 | 250 | 750 |
| 5.0 | 500 | 500 |
| 10.0 | 1000 | 0 |

**Procedure**

1. Add 40 μL of each standard in triplicate in columns 1-3 of the well plate (Figure 1)
2. Add 40 μL of random standards in wells H1-H8 as duplicates. These will serve as a positive control and will be calculated as unknowns to test for the accuracy of the standard curve (Figure 1)
3. Add 40 μL of the sample matrix in wells H9-H11. These will serve as both the negative control and the blanks in the assay
4. Add 40 μL of unknown samples into any empty wells. Be sure to add unknowns as duplicates (i.e. in two separate wells). Sample replicates will be averaged to account for any unaccounted sample heterogeneity
5. Add 80 μL of the salicylate solution to each well using a multichannel pipette and solution reservoir
6. Add 80 μL of the bleach solution to each well using a multichannel pipette and solution reservoir
7. Place a clean lid on the 96-well plate, shake for 60 seconds, and incubate for 50 mins at room temperature
8. Turn on the microplate reader and open the computer software. Select a project, or create a new one, and select the “ammonium\_resin” protocol. This protocol will shake the well plate once a plate is loaded and measure each well at 650nm given the plate design in Figure 1

**A few notes**

* Vortex each standard and unknown before pipetting into wells to homogenize sample
* Work quickly and efficiently to reduce evaporation error. Use a multichannel pipette with a solution reservoir to speed up salicylate and bleach addition process
* The standard curve may need to be altered to avoid extrapolating values from the curve. If a plate has any unknown concentration (not absorbance) values that are >10% larger than the largest concentration value in a standard curve, the standard curve should be expanded and plate should be assayed again
  + To avoid unnecessary labor, run a sample standard curve with a single replicate and problematic unknowns to see if any additional modifications to the curve are needed before re-assaying entire plate
* Samples will turn a yellow color if the concentration is too high. If this occurs, problematic unknowns need to be re-assayed along with an additional standard curve using a diluted sample : salicylate : bleach ratio
  + Steve Allison’s protocol recommends that “high” ammonium concentrations be diluted such that 20 μL of sample is assayed against 90 μL salicylate solution and 90 μL bleach solution
  + The exact sample : salicylate : bleach ratio may need to be further diluted if a particular sample has especially high ammonium concentrations
* While the microplate reader software creates a standard curve, linear regression for the standard curve, and determines concentrations of all unknown samples, this is not necessarily in a reproducible format, so it is recommended that the user export the plate data with the well plate id and absorbance values to do calculations on their own (see “Microplate analysis” below). There is an R script titled “NH4\_plate\_analysis.R” in the “lab\_protocols” GitHub repository that should help streamline these calculations

**Figure 1**

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Description automatically generated

**Figure 1** Layout of 96-well plate with standards, positive controls, blanks, and sample loadings

**Microplate analysis**

* Calculate the mean of the blanks (wells H9-H11) and subtract all absorbance measurements by this value
* Construct a linear regression with known standard concentration as the independent variable and blank-corrected absorbance value as the dependent variable and check the R2 value. A passable R2 value is anything greater than 0.98. Individual wells can be removed to achieve a better fit
* Calculate the concentration of each well by subtracting the blank-corrected absorbance by the intercept of the linear regression equation and dividing this value by the slope of the linear regression equation:
* Calculate the coefficient of variation for each duplicate positive control. The coefficient of variation is calculated as the standard deviation divided by the mean. Acceptable controls are <10%
* Calculate ammonium concentrations of each unknown sample by quantifying the mean of each duplicate
* The unknown samples are expressed in ppm, which is equivalent to mg L-1. If measuring with soil extracts, units can be converted to mg NH4-N kg-1 by multiplying the amount of extractant used in L (40 μL = 4.0×10-5 L), then dividing by the dry mass of the soil extracted (kg)

**Further protocol and reaction details**

Protocol modified from protocols supplied from Aimée Classen ([https://classenlab.com/)](https://classenlab.com/) and Steve Allison (<https://allisonlab.bio.uci.edu/>). Protocol is based on a modified phenol-hypochlorite reaction originally described in Weatherburn (1967) and further described in Forster (1995) and Rhine *et al.* (1998).

**References**

Forster, J. C. (1995). Soil sampling, handling, storage and analysis. In *Methods in Applied Soil Microbiology and Biochemistry* (pp. 49–121). Academic Press. https://doi.org/10.1016/B978-012513840-6/50018-5

Rhine, E. D., Mulvaney, R. L., Pratt, E. J., & Sims, G. K. (1998). Improving the Berthelot reaction for determining ammonium in soil extracts and water. *Soil Science Society of America Journal*, *62*(2), 473. https://doi.org/10.2136/sssaj1998.03615995006200020026x

Weatherburn, M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry*, *39*(8), 971–974.