7

Nitrate and Nitrite

Key Words: Nitrate; nitrite; methemoglobin; blood pressure; asphyxia; spinach; spongy cadmium column; zinc metal; sodium nitrate; sodium nitrite; ammonia buffer solution; Jones reductor.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

Occasionally forages accumulate nitrates in quantities that are toxic to some farm animals. Under certain environmental conditions, some plants may accumulate high concentrations of nitrates. The factors that can influence the accumulation of nitrates in the plants are drought, shade, use of herbicides, and application of nitrogenous fertilizers. Nitrates are not very toxic, but they are readily converted by bacteria in the alimentary tract into nitrites, which are much more toxic. In cattle and sheep this conversion takes place in the rumen and in horses in the cecum. Nitrites pass easily from the gastrointestinal tract into the blood, where they combine with hemoglobin in the red blood cells to form methemoglobin, a compound that is incapable of taking up and transporting oxygen **(Fig. 1)**. Consequently the clinical signs of nitrite poisoning are those associated with oxygen deficiency and include general weakness and a fall in blood pressure. Death may follow from asphyxia. Young animals and babies are particularly at risk because the small volume of blood that they contain requires only a small amount of nitrite to convert all the hemoglobin to methemoglobin. Pregnant animals that are affected, but do not die, may abort later.

From: *Methods in Molecular Biology, vol. 393: Plant Secondary Metabolites*
By: H.P.S. Makkar, P. Siddhuraju and K. Becker © Humana Press Inc., Totowa, NJ

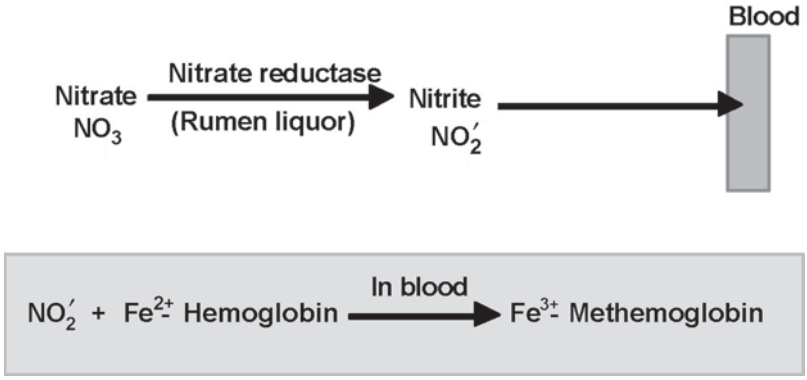

Fig. 1. Mechanism of action of nitrate.

1.2. Present in

Spinach, raps, sorghum, grasses.

1.3. Principle of Assay

The sample is extracted with distilled water and the aqueous extract is clarified with zinc hydroxide. Nitrate is reduced to nitrite on a spongy cadmium column (the nitrite originally present is unaltered); sulfanilic acid is diazotized by the nitrite and coupled with *N*-(1-naphthyl)-ethylenediamine dihydrochloride to form a pink azo dye, the absorbance of which is measured at 550 nm using a spectrophotometer. For those samples containing both nitrite and nitrate, the nitrite content is first determined from the unreduced sample filtrate, and then the total nitrite content (existing nitrite and nitrite formed from nitrate) from the column eluate. The nitrate content is then calculated by difference ***(1–4)***.

2. Materials

1. *Ammonia buffer solution, pH 9.6–9.7.* Dilute 20 mL of concentrated HCl (37%) to 500 mL with distilled water, mix, and add 50 mL concentrated NH_4OH (25%). Dilute this solution to 1 L with distilled water and mix thoroughly. Check the pH of the solution and adjust it to 9.6 to 9.7 using HCl or NH_4OH as necessary.
2. *Zinc sulfate solutions (0.42 M).* Dissolve 12 g $ZnSO_4.7H_2O$ in approximately 70 mL distilled water and then make the volume up to 100 mL with distilled water.
3. *Sodium hydroxide solution (2%).* Dissolve 2 g sodium hydroxide in 100 mL distilled water.
4. *Cadmium sulfate solutions (0.14 M).* Dissolve 3.7 g $CdSO_4.8H_2O$ in approximately 70 mL distilled water and make the volume up to 100 mL with distilled water.

5. *Zinc metal (about 10 cm sticks)*—Fisher Scientific (Scwerte, Germany), Catalogue No. Z-13, or equivalent.
6. *Sulfanilic acid solution, 1% in 30% acetic acid.* Dissolve 10 g of sulfanilic acid in 700 mL of distilled water, then add 300 mL of acetic acid (100%) and mix thoroughly (do not add acetic acid until the sulfanilic acid has dissolved completely). Store at room temperature.
7. *N-(1-naphthyl)-ethylenediamine dihydrochloride (Marshall`s reagent), 0.1% in 60% acetic acid.* Weigh 100 mg *N*-(1-naphthyl)-ethylenediamine dihydrochloride and dissolve in 100 mL of 60% acetic acid. Store in a refrigerator. It is stable for 1 week.
8. *Color reagent.* Mix equal volumes of the sulfanilic acid and Marshall's reagent solutions just before use.
9. *Sodium nitrate standard solutions. I. Stock solution, 1 mg/mL*: Dissolve 500 mg of $NaNO_3$ in approximately 300 mL distilled water in a 500-mL volumetric flask. Add to it 50 mL of the NH_4Cl buffer and make the volume up to 500 mL with distilled water. The solution is stable at 4°C for 1 to 2 weeks. *II. Working solution, 10 µg/mL*: Transfer 1 mL of stock solution to a 100-mL volumetric flask, make the volume to 100 mL with distilled water and mix thoroughly. Prepare the solution every day.
10. *Sodium nitrite standard solutions. I. Stock solution, 500 µg/mL*: Dissolve 250 mg $NaNO_2$ in approximately 300 mL distilled water in a 500-mL volumetric flask. Add 100 mL of the ammonia buffer and make the volume up to 500 mL with distilled water. Mix thoroughly and store it at 4°C (stable for 1 week). *II. Working solution, 5 µg/mL*: Transfer 1 mL stock solution to a 100-mL volumetric flask, make the volume up to 100 mL with distilled water, and mix the solution thoroughly. The solution should be prepared fresh every day.

3. Method

3.1. Preparation of Standard Curve for Sodium Nitrite

1. Add 0.0, 1.0, 2.0, 4.0, 6.0, and 10 mL of $NaNO_2$ working solution to separate 50-mL volumetric flasks. Add 9.0 mL of the ammonia buffer and 5 mL of 60% acetic acid solution to each flask and immediately proceed to step 2 (*see* **Note 1**).
2. Add 10 mL of the color reagent, make the volume up to 50 mL with distilled water, mix thoroughly, and let it stand for 25 min in the dark.
3. Set the spectrophotometer at 550 nm and measure absorbance of the above solutions against the blank (0 standard solution).
4. Prepare a calibration curve by plotting absorbance against micrograms of $NaNO_2$ (range: 5 to 50 µg).
5. The absorbance range should extend from 0 to 0.6 approximately.

3.2. Preparation of Modified Jones Reductor

1. Place three to five zinc (Zn) sticks in each of the two 800-mL beakers containing 500 mL $CdSO_4$ solution.

2. Remove Zn sticks every 2 to 3 h [or as soon as a thick layer of cadmium (Cd) forms] and scrape off the spongy metallic Cd by rubbing sticks against each other (important: Cd must be kept covered with aqueous solution at all times).
3. After 6 to 8 h, wash deposits with two 500-mL portions of distilled water.
4. Transfer Cd with distilled water to a high-speed blender and blend for 2 to 3 s.
5. Retain 8 to 40 mesh particles and repeat blending to increase yield of particles.
6. Wash particles with 0.1 N HCl, stir occasionally with a glass rod, and leave it overnight in acid.
7. Stir once to degas and decant. Then wash with two 500-mL portions of distilled water.
8. Fill the modified Jones reductor in a chromatographic column (glass tube of approximately 300 mm in length and 10 mm in internal diameter) plugged with a glass wool and fixed with a stopcock.
9. Add Cd to a depth of 8 to 10 cm and drain occasionally during filling but do not allow liquid level to fall below top of Cd bed. During filling, Cd bed should always be dipped in distilled water.
10. Eliminate bubbles in Cd bed by tapping sides of column and wash Cd column with 25 mL of the ammonia buffer and drain to top of Cd bed (*see* **Note 2**).

3.3. Testing Efficiency of Cadmium Column

1. Mix 6 mL $NaNO_3$ working solution (10 µg/mL) and 5 mL of the ammonia buffer and pour on the Cd column.
2. Adjust flow rate to 3 to 5 mL/min. After column is emptied, wash with 15 mL of distilled water.
3. Collect the eluate in a 50-mL volumetric flask and wash the column with additional approximately 10 mL of distilled water and again collect the eluate in the same 50-mL volumetric flask. Add to the flask 5.0 mL of 60% acetic acid.
4. Add 10 mL of the color reagent, make the volume up to 50 mL with distilled water, and mix well. Keep the solution in the dark for 25 min.
5. Prepare a blank in the same manner by taking 6 mL of distilled water through steps 1 and 4 (*see* **Note 3**).
6. Read the absorbance in the spectrophotometer at 550 nm. The $NaNO_2$ concentration, as determined from the standard curve, should be about 48.7 µg/mL if 100% conversion occurs.
7. If there is less than 90% conversion, recondition the column by passing 25 mL of 0.1 N HCl followed by two 25-mL portions of distilled water and 25 mL of the ammonia buffer and repeat steps 1–6. Check efficiency of each column once a week.

3.4. Preparation of Extract

1. Weigh a 10-g sample (ground if dry, and cut into small pieces if fresh) and blend with 70 mL of distilled water and 12 mL of 2% NaOH solution in a blender until smooth slurry is formed (generally takes about 5 min) (*see* **Note 4**).

2. Transfer the slurry into a 200-mL volumetric flask, and rinse blender with 30 to 50 mL of distilled water. Then mix the suspension well by swirling. Take out one or two drops of suspension from the flask and check the pH with pH paper. If the pH is between 8 and 10, heat the contents in a water bath (50–60°C) until the temperature of the suspension reaches close to 50°C. If the pH is less than 8, add additional amounts of 2% NaOH solution until the pH rises to 8 to 10, and then heat as above.
3. Occasionally swirl the contents while heating. Maintain the temperature at about 50°C for an additional 10 min, mixing occasionally.
4. Add 10 mL of $ZnSO_4$ solution and mix by swirling. If no white precipitate of $Zn(OH)_2$ becomes visible after the addition of $ZnSO_4$ solution, add 2 to 5 mL of 2% NaOH solution and keep mixing to avoid excessive addition of 2% NaOH solution.
5. Cool to room temperature in a water bath and dilute to volume 200 mL with distilled water and mix thoroughly.
6. Filter the content through filter paper (Whatman No. 1), discarding the first 20 mL filtrate, into a 250-mL glass-stoppered flask. Re-filter if the extract is not clear.

3.5. Determination of Nitrite

1. Transfer a 10-mL aliquot of the above filtrate to a 50-mL volumetric flask. Add 9.0 mL of the ammonia buffer and 5.0 mL of 60% acetic acid.
2. Add 10 mL of the color reagent and make the volume up to 50 mL with distilled water.
3. Mix thoroughly and place in the dark for 25 min.

3.6. Determination of Nitrate Plus Nitrite

1. Mix a second 10-mL aliquot of filtrate with 5 mL of the ammonium buffer and pass through the modified Jones Reductor (*see* **Note 5**).
2. Wash the column with about 15 mL of distilled water.
3. Collect the eluate in a 50-mL volumetric flask and wash the column with additional approximately 10 mL of distilled water and again collect the eluate in the same 50-mL volumetric flask. Add to the flask 5.0 mL of 60% acetic acid.
4. Add 10 mL of the color reagent and make the volume up to 50 mL with distilled water. Then mix well and leave it in the dark for 25 min.

3.7. Preparation of Reagent Blank

Carry 70 mL of distilled water through steps 2 to 7 described in Section 3.4.

3.8. Calculations

1. After color development, read the absorbance of unreduced blank and unreduced sample filtrates against the blank (0 standard solution; step 3 in **Section 3.1.**). This corresponds to the presence of nitrite in the sample.

2. Read the absorbance of reduced blank and reduced sample eluates against the same blank (0 standard solution; step 3 in **Section 3.1.**). This corresponds to the presence of nitrate plus nitrite in the sample.
3. Subtract the blank values from the corresponding sample values.
4. Determine the concentration of nitrite in each case from the standard curve.
5. Calculate the concentration of nitrite in the unreduced and reduced sample filtrates.
6. The difference between the two values is a measure of the nitrate concentration, equivalent to nitrite.
7. Finally, calculate the concentrations of nitrite and nitrate in the original sample.

Example

Absorbances:	Reagent blank, unreduced	NO_2	a
	Reagent blank, reduced	NO_2/NO_3	b
	Sample, unreduced	NO_2	c
	Sample, reduced	NO_2/NO_3	d

I. $NaNO_2$ determinations:

Absorbance (due to $NaNO_2$) = A (sample NO_2) − A (blank NO_2)
$= c - a$
$= x$

From standard curve, absorbance $x = m\,\mu g$

Since this is from a 10-mL aliquot from a total of 200 mL, nitrite in 200 mL = (200/10) $m = 20m\,\mu g$.

Since the original sample wt. was 10 g, the $NaNO_2$ concentration is $20m\,\mu g/10\,g = 2m\,\mu g/g = 2m$ ppm.

II. $NaNO_3$ determination:

Absorbance (due to $NaNO_3/NaNO_2$) = A (sample NO_3/NO_2) − A (blank NO_3/NO_2)
$= d - b$
$= y$

From standard curve, absorbance $y = n\,\mu g$

Since this is from a 10-mL aliquot, from a total of 200 mL, total $NaNO_2$ (from $NaNO_3$ plus $NaNO_2$ present in the sample) = (200/10) × $n = 20n\,\mu g$.

Since the original sample was 10 g, total $NaNO_2$ is $20n\,\mu g/10\,g = 2n\,\mu g/g$ or $2n$ ppm.

$NaNO_2$ from $NaNO_3$ in the sample = $2n - 2m = 2\,(n - m)$ ppm.

The conversion factor from $NaNO_2$ [molecular weight (MW) 69] to $NaNO_3$ (MW 85) is 1.23.

Therefore, $NaNO_3$ level in the sample is 2.46 $(n - m)$ ppm.

4. Notes

1. Since nitrite is unstable in acidic solutions the color reagent should be added immediately after adding the 60% acetic acid solution.
2. When the column is drained, the liquid level should be just at top of the Cd bed (±2 mm).
3. If high blank is obtained for NO_3 on Cd column, follow the following washing schedule to remove absorbed residual NO_3 from Cd column:

   Pass 50 mL of 1 N NaOH through Cd column. Wash column with distilled water until the pH of the effluent is close to neutral pH (pH 7–8). Then pass 25 mL of 0.1 N HCl and again wash column with distilled water. Finally, wash column with 25 mL of the ammonia buffer. This should be done each day and the columns be kept ready for later use. Omit the above steps if columns are new, i.e., being used for the first time.

4. If the results are beyond the range of the standard curve, then dilutions must be made to the sample extract. Do not dilute the final reaction mixture after color development has occurred. Appropriate dilutions must also be made to the sample blanks. Conversely, if the absorbance is low on using 10 mL of the sample extract, higher volumes can be taken for the color development, and absorbance should be recorded against appropriate blanks.
5. Recondition the column after each analysis and store the Cd under the ammonia buffer between analyses.

References

1. Sen, N. P., and Donaldson, B. J. (1978) Improved colorimetric method for determining nitrate and nitrite in foods. *J. Assoc. Offic. Anal. Chem.* **61**, 1389–1394.
2. Sen, N. P., Lee, Y. C., and McPherson, M. (1979) Comparison of two extraction procedures for recovering nitrite from cured meat products. *J. Assoc. Offic. Anal. Chem.* **62**, 1186–1188.
3. Sen, N. P., and McPherson, M. (1978) Interference by ascorbic acid in the determination of nitrite by the separate color reagent addition technique. *J. Food Safety*, **1**, 247–255.
4. Hamilton, J. E. (1976) Collaborative study of the colorimetric determination of nitrate and nitrite in cheese. *J. Assoc. Offic. Anal. Chem.* **59,** 284–288.