1. Introduction

Assays of nitrite and nitrate have become increasingly important in recent years for health care and economic reasons. While nitrate is an essential plant nutrient, it constitutes a risk to human health because it plays a part in the formation of methemoglobin and nitrosamines. Negligible amounts of nitrite occur in plants and drinking water. However, under unfavorable conditions nitrite may enter the food chain via microbial reduction of nitrate thus endangering human health.

2. Application

This semimicro method for determination of nitrite and/or nitrate can be used to investigate waste water, water, environmental samples, plant material, foodstuffs, drugs, cosmetics and biological samples.

3. Principle

Nitrate is reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of the enzyme nitrate reductase (NR) (1).

\[
(1) \text{Nitrate} + \text{NADPH} + \text{H}^+ \rightarrow \text{Nitrite} + \text{NADP}^+ + \text{H}_2\text{O}
\]

The nitrite formed reacts with sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride to give a red-violet diazo dye (2).

\[
(2) \text{Nitrite} + \text{sulfanilamide} + \text{N-(1-naphthyl)-ethylenediamine} \rightarrow \text{diazo dye}
\]

The diazo dye is measured on the basis of its absorbance in the visible range at 540 nm (Hg 546 nm).

4. The test combination contains

1. **Bottle 1** with 22 ml solution, consisting of potassium phosphate buffer, pH 7.5 and stabilizers.
2. **Bottle 2** with 7 tablets, each tablet contains 0.5 mg NADPH, 0.01 mg FAD and stabilizers.
3. **Two bottles 3** each containing 4 ml nitrate reductase, lyophilized.
4. **Two bottles 4** each containing 8 ml color reagent I, consisting of sulfanilamide and stabilizers.
5. **Two bottles 5** each containing 8 ml color reagent II, consisting of N-(1-naphthyl)-ethylenediamine dihydrochloride and stabilizers.

4.1. Preparation of the solutions

1. Use the contents of bottle 1 undiluted.
2. Place one tablet from bottle 2 in a beaker and dissolve in 3 ml of the solution from bottle 1. (Remove the tablet from bottle 2 using the tweezers provided.) The resulting solution is reaction mixture 2 and is sufficient for 12 nitrate determinations.
3. Dissolve the contents of one bottle 3 in 0.7 ml of redistilled water to give solution 3.
4. Use the contents of one bottle 4 undiluted.
5. Use the contents of one bottle 5 undiluted.

4.2. Stability of the solutions

Solution 1 is stable for 1 year at +2 to +8° C. Bring solution 1 to +15 to +25° C before use. The contents of bottle 2 and 3 are stable for 1 year at +2 to +8° C. Prepare reaction mixture 2 immediately before use. Bring reaction mixture 2 to +15 to +25° C before use. Solution 3 is stable for 2 weeks at +2 to +8° C. Solution 4 is stable for 3 months at +2 to +8° C. Solution 5 is stable for 3 months at +2 to +8° C.

4.3. Reagents

4.3.1. **Sodium nitrite-standard solutions**

Accurately weigh 75.0 mg (± 0.1 mg) sodium nitrite (e.g. Merck 6569) into a 100 ml volumetric flask, dissolve in redistilled water and make up to the mark with the same solvent (stock solution = 500 mg nitrite/l). Prepare a nitrite calibration curve by diluting the stock solution with redistilled water to give concentrations in the range from 5.00 mg and 0.05 mg nitrite/l.

4.3.2. **Potassium nitrate - standard solution**

Accurately weigh 81.5 mg (± 0.1 mg) Potassium nitrate (e.g. Merck 5065, Suprapur) into a 100 ml volumetric flask, dissolve in redist. water and make up to the mark with the same solvent (stock solution = 500 mg nitrate/l). Prepare a nitrate calibration curve by diluting the stock solution with redistilled water to give concentrations in the range from 5.00 mg and 0.05 mg nitrate/l.

5. Determination

| Wavelength | 540 nm (Hg 546 nm) |
| Glass cuvette | 1.00 cm, semimicro |
| Temperature | +20 to +25° C |
| Volume | 1.27 ml |
| Measurement against blank | Sample solution: 0.05 mg - 5.00 mg nitrite or nitrate/l |

1 Carry out the measurement at 540 nm if using a spectrophotometer with a mercury vapor lamp.
2 Disposable cuvettes may be used instead of glass cuvettes.

6. Measurement

### Pipette into cuvettes

| Blank nitrate | Sample nitrate | Blank nitrate + nitrate | Sample nitrate + nitrate |
| Sample | 0.500 ml | - | 0.500 ml |
| Redist. water | 0.770 ml | 0.270 ml | 0.500 ml | - |
| Reaction mixture 2 | - | - | 0.250 ml | 0.250 ml |
| Solution 3 | - | - | 0.020 ml | 0.020 ml |
| Mix, incubate for 30 min at +15 to +25° C, read off A1 and add | - | - | - | - |
| Color reagent I | 0.250 ml | 0.250 ml | 0.250 ml | 0.250 ml |
| Color reagent II | 0.250 ml | 0.250 ml | 0.250 ml | 0.250 ml |
| Mix, allow to stand in dark at +15 to +25° C for 10 to 15 min, read off A2 | - | - | - | - |

1 Before adding the sample solution rinse the pipette supplied with the test or the tip of the piston pipette with the sample.
2 e.g. using a spatula or by swirling after sealing, e.g. with Parafilm.
7. Calculation

Graphical evaluation

The result is calculated from the calibration curves constructed using the standard solutions. Plot the change in absorbance obtained for the sodium nitrite and potassium nitrate standard solutions on the y-axis against the corresponding nitrite or nitrate concentrations in mg/l on the x-axis.

\[
\Delta A_{\text{nitrite}} = (A_2 - A_1)_{\text{nitrite}} - (A_2 - A_1)_{\text{Blank nitrite}}
\]

\[
\Delta A_{\text{nitrite + nitrate}} = (A_2 - A_1)_{\text{nitrite + nitrate}} - (A_2 - A_1)_{\text{Blank nitrite + nitrate}}
\]

\[
\Delta A_{\text{nitrate}} = \Delta A_{\text{nitrite + nitrate}} - \Delta A_{\text{nitrite}}
\]

- Determine the concentrations of nitrite and nitrate in the sample from the calibration curves using the change in absorbance measured.
- If the sample has been diluted during preparation, the result must be multiplied by the dilution factor F.
- When analyzing solid or semisolid samples that have to be weighed out, calculate the result with respect to the mass of sample.

Content nitrite = \(\frac{C_{\text{nitrite}} \times 1000}{\text{Mass}_{\text{sample in g/l sample solution}}}\) (mg nitrite/kg sample)

Content nitrate = \(\frac{C_{\text{nitrate}} \times 1000}{\text{Mass}_{\text{sample in g/l sample solution}}}\) (mg nitrate/kg sample)

The results are determined as sodium nitrite and potassium nitrate.

The conversion factor from NaNO₂ to nitrite (NO–2) is 46.006 : 68.995 = 0.667 and from KNO₃ to nitrate (NO₃⁻) 62.005 : 101.11 = 0.613.

8. Notes on performing the test

8.1. Calibration curves

The calibration curves do not have to be plotted every time a determination is made. It is sufficient to check the calibration curves from time to time and to include a sodium nitrite or potassium nitrate standard solution as a control.

8.2. Determination of nitrite

If only nitrite is to be determined, follow the procedure shown in columns 1 and 2 of the pipetting scheme.

8.3. Concentration of the sample solution

The sample solution should be diluted to give a nitrite or nitrate concentration between 0.05 mg/l and 5.00 mg/l.

9. Specificity

Under the given conditions, nitrate reductase reacts specifically with nitrate ions.

10. Limit of detection

The limit of detection of the method is 0.02 mg/l for nitrite and nitrate.

A high degree of scatter is to be reckoned with in this trace range because of the small measurement signal.

11. Linearity

The method is linear in the range from 0.02 mg nitrite or nitrate/l sample solution to 5.00 mg nitrite or nitrate/l sample solution.

12. Interference

Manganese ions in the sample (\(> 5 \mu g/cuvette\)) considerably delay the reaction of nitrate. Chloride ions only have a distinct effect at concentrations \(> 4.3 mg/cuvette\). Cyanide ions (\(> 1.6 \mu g/cuvette\)) and sulfide ions (\(> 100 \mu g/cuvette\)) completely deactivate nitrate reductase.

13. Notes on sample preparation

Use clear, colorless or faintly colored solutions directly or dilute to a concentration of < 5.00 mg nitrite or nitrate/l before using in the test. Filter turbid solutions or clarify using Carrez solutions and dilute such that the nitrite or nitrate concentration is below 5.00 mg/l.

The diluted solution can be used in the test without further treatment.

13.1. Examples

13.1.1. Determination of nitrite and nitrate in water, waste water, drinking and mineral water

If necessary, dilute clear, colorless water samples to a nitrite or nitrate concentration of < 5.00 mg/l and use in the test. Filter turbid water samples before use. Filter samples containing carbon dioxide or swirl for 1 min to remove the gas, neutralize if necessary and use in the test.

13.1.2. Determination of nitrite and nitrate in fermentation samples and cell culture media

Centrifuge the samples if necessary and stop enzymatic processes. Place in a water-bath at 60°C for about 15 min, centrifuge and if necessary dilute the supernatant such that the nitrite or nitrate concentration is less than 5.00 mg/l and use in the test. Alternatively, the samples may be deproteinized with concentrated Carrez solutions (see 13.1.5 Meat and meat products).

13.1.3. Determination of nitrite and nitrate in juice

Accurately weigh about 5 g of well homogenized sample into a 100 ml volumetric flask, add about 20 ml redist. water and mix. Add 5 ml of diluted Carrez I solution (3.60 g potassium hexacyanoferrate(II) \(K_4[Fe(CN)_{6}] \times 3H_2O/100 ml\), mix well, add 5 ml of Carrez II solution (2.20 g zinc sulfate, ZnSO₄ × 7 H₂O/100 ml) and mix well once again. Adjust to pH 8 with sodium hydroxide solution (1 M). Fill the flask up to the mark with redist. water, mix, filter and if necessary centrifuge. Use the clear filtrate for the test.

13.1.4. Determination of nitrite and nitrate in fruit and vegetables

Accurately weigh about 3 g of well homogenized sample into a 100 ml beaker and add 60 ml of hot (approx. 60–70°C) redist. water. Shake and allow to stand for about 15 min on a water-bath at 60–70°C. Treat samples that are intensely colored or that have a high starch content with Carrez solutions (see 13.1.2 Meats). Allow the contents of the beaker to cool to +15 to +25°C, transfer quantitatively to a 100 ml volumetric flask, make up to the mark with redist. water, mix, filter and if necessary centrifuge. Discard the first few ml and use the clear filtrate for the test.

13.1.5. Determination of nitrite and nitrate in meat and meat products

Accurately weigh about 6 g of well homogenized sample into a 100 ml beaker, add about 50 ml of boiling redist. water, mix and boil on a water-bath for about 15 min. Allow to cool to +15 to +25°C and successively add 3 ml each of concentrated Carrez I solution (15.0 g potassium hexacyanoferrate(II) \(K_4[Fe(CN)_{6}] \times 3H_2O/100 ml\)) and concentrated Carrez II solution (30.0 g zinc sulfate, ZnSO₄ × 7 H₂O/100 ml) and mix well. Acidify with 1 M hydroxide solution and mix well. Stir for about 15 min, centrifuge and if necessary dilute the supernatant such that the nitrite or nitrate concentration is less than 5.00 mg/l and use in the test. Filter turbid solutions or clarify using Carrez solutions and dilute such that the nitrite or nitrate concentration is below 5.00 mg/l. The diluted solution can be used in the test without further treatment.

13.1.6. Use of clear, colorless or faintly colored solutions directly or dilute to a concentration of < 5.00 mg nitrite or nitrate/l before using in the test. Filter turbid solutions or clarify using Carrez solutions and dilute such that the nitrite or nitrate concentration is below 5.00 mg/l. The diluted solution can be used in the test without further treatment.
**13.1.6. Determination of nitrite and nitrate in dairy products**

Accurately weigh about 2 g of well homogenized sample into a 100 ml conical flask, add about 50 ml of boiling redist. water and mix. Boil on a water-bath for about 15 min, allow to cool to +15 to +25°C and successively add 3 ml each of concentrated Carrez I and Carrez II solutions (see 13.1.5 'Meat and meat products'), mixing well after each addition. Adjust to pH 8 with sodium hydroxide solution (1 M) and mix again. Transfer the contents of the conical flask quantitatively to a 100 ml volumetric flask and make up to the mark with redist. water. Transfer an aliquot to a centrifuge tube, centrifuge at 3,000 x g (6,000 rpm, r = 7 cm) for 15 min and filter the supernatant (folded filter paper or membrane filter). Discard the first few ml and use the clear filtrate for the test.

**13.1.7. Determination of nitrite and nitrate in cheese and processed cheese**

Accurately weigh about 3 g of well homogenized sample into a 100 ml volumetric flask, add about 20 ml of redist. water and heat in a microwave oven until the cheese is melted (e.g. at 1000 W for about 10 s). Stir with a spatula and microwave again until the mixture boils gently. Add about 50 ml of boiling redist. water and boil on a water-bath for about 15 min, allow to cool to +15 to +25°C and successively add 5 ml each of concentrated Carrez I and Carrez II solutions (see 13.1.5 'Meat and meat products'), mixing well after each addition. Adjust to pH 8 with sodium hydroxide solution (1 M) and mix again. Transfer the contents of the conical flask quantitatively to a 100 ml volumetric flask and make up to the mark with redist. water. Transfer an aliquot to a centrifuge tube, centrifuge at 3,000 x g (6,000 rpm, r = 7 cm) for 15 min and filter the supernatant through a membrane filter (e.g. Millex HV13 from Millipore). Discard the first few ml and use the clear filtrate for the test.

**13.1.8. Determination of nitrite and nitrate in baby food**

Accurately weigh about 1.5 g of well homogenized sample into a 100 ml conical flask, add about 50 ml of boiling redist. water and mix. Boil on a water-bath for about 15 min, allow to cool to +15 to +25°C and successively add 3 ml each of concentrated Carrez I and Carrez II solutions (see 13.1.5 'Meat and meat products'), mixing well after each addition. Adjust to pH 8 with sodium hydroxide solution (1 M) and mix again. Transfer the contents of the conical flask quantitatively to a 100 ml volumetric flask and make up to the mark with redist. water. Transfer an aliquot to a centrifuge tube, centrifuge at 3,000 x g (6,000 rpm, r = 7 cm) for 15 min and filter the supernatant through a membrane filter (e.g. Millex HV13 from Millipore). Discard the first few ml and use the clear filtrate for the test.

**14. Technical notes**

**14.1. Checking of reagents and equipment**

The following points are important because of the high sensitivity of the nitrite/nitrate colorimetric test.

a) The filter papers or membrane filters must be free from nitrite and nitrate. In case of doubt it is recommended to test the filter material by washing with redist. water and using the washings in the test.

b) The solutions used to prepare the samples must also be free from nitrite and nitrate.

**14.2. Clarification with Carrez solutions**

After adding Carrez solutions I and II it is important that the pH is adjusted to 8.0 ± 0.2.

**References**


**Ordering Information**

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**Changes to previous version**

Editorial Changes

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