

# Nitrate (NO<sub>3</sub><sup>-</sup>)

## UV method

for the determination of nitrate (NO<sub>3</sub><sup>-</sup>) in foodstuffs and other materials

**Cat. No. 10 905 658 035**

Test-Combination for 3 × 13 determinations

**BOEHRINGER MANNHEIM / R-BIOPHARM**  
Enzymatic BioAnalysis / Food Analysis

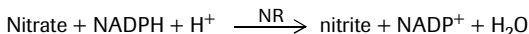
For use in *in vitro* only

Store at 2-8°C

For recommendations for methods and standardized procedures see references (2)

### Principle (Ref. 1)

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



The amount of NADPH oxidized during the reaction is stoichiometric to the amount of nitrate. The decrease in NADPH is measured by means of its light absorbance at 334, 340 or 365 nm.

### The Test-Combination contains

1. Bottle 1 with approx. 40 ml solution, consisting of: imidazole buffer, pH approx. 7.8
2. Bottle 2 with approx. 40 tablets, each tablet contains: NADPH, approx. 0.5 mg, FAD, approx. 0.01 mg
3. Three bottles 3 with approx. 4 U nitrate reductase each (as lyophilizate)

### Preparation of solutions

1. Use contents of bottle 1 undiluted.
2. Dissolve **one** tablet of bottle 2 with **one** ml solution of bottle 1 in a beaker or test-tube dependent on the number of determinations for each assay (blank and sample). This results in reaction mixture 2\*. Use the enclosed forceps to take the tablet out from bottle 2.
3. Dissolve contents of one bottle 3 with 0.8 ml redist. water. This results in solution 3.

### Stability of reagents

Solution 1 is stable at 2-8°C (see pack label).

Bring solution 1 to 20-25°C before use.

The contents of bottles 2 and 3 are stable at 2-8°C (see pack label).

Reaction mixture 2 has to be prepared freshly before use\*.

Bring reaction mixture 2 to 20-25°C before use.

Solution 3 is stable for 2 weeks at 2-8°C.

### Procedure

Wavelength<sup>1</sup>: 340 nm, Hg 365 nm or Hg 334 nm

Glass cuvette<sup>2</sup>: 1.00 cm light path

Temperature: 20-25°C

Final volume: 3.050 ml

Read against air (without a cuvette in the light path) against water or against blank<sup>3</sup>

Sample solution: 0.3-30 µg nitrate/assay<sup>4</sup> (in 0.100-2.000 ml sample volume)

Pipette into cuvettes	Blank	Sample
reaction mixture 2*	1.000 ml	1.000 ml
sample solution**	-	0.100 ml
redist. water	2.000 ml	1.900 ml

Mix\*\*\*, read absorbances of the solutions (A<sub>1</sub>) after approx. 3 min and start reaction by addition of:

solution 3	0.050 ml	0.050 ml
------------	----------	----------

Mix\*\*\*, read absorbances of the solutions (A<sub>2</sub>) **exactly 40 min after measuring of A<sub>1</sub>** directly one after the other. **After exactly another 20 min** read again absorbances of the solutions (A<sub>3</sub>) one after the other.

\* For simplification of the assay performance it is also possible to pipette directly 1.000 ml of solution 1 into the cuvette and add 1 tablet from bottle 2. After dissolution of the tablet with the aid of a spatula continue working as described in the procedure. The difference in volume of ca. 1 % (increase of volume by 1 tablet per 3.050 ml assay volume) has to be taken into account in the calculation by multiplication of the result with 1.01.

\*\* Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.

\*\*\* For example, with a plastic spatula or by gentle swirling after closing the cuvette, e. g. with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

1 The absorption maximum of NADPH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; if spectralline photometers equipped with a mercury vapor lamp are used, measurements are taken at a wavelength of 365 nm or 334 nm.

2 If desired, disposable cuvettes may be used instead of glass cuvettes.

3 For example, when using a double-beam spectrophotometer

4 See instructions for performance of the assay

Determine the absorbance differences (A<sub>1</sub>-A<sub>2</sub>) and (A<sub>2</sub>-A<sub>3</sub>) for both, blank and sample.

Absorbance difference of the blank = (A<sub>1</sub>-A<sub>2</sub>)<sub>blank</sub> - 2 × (A<sub>2</sub>-A<sub>3</sub>)<sub>blank</sub>

Absorbance difference of the sample = (A<sub>1</sub>-A<sub>2</sub>)<sub>sample</sub> - 2 × (A<sub>2</sub>-A<sub>3</sub>)<sub>sample</sub>

ΔA<sub>nitrate</sub> = absorbance difference<sub>sample</sub> - absorbance difference<sub>blank</sub>

The measured absorbance difference ΔA<sub>nitrate</sub> should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions for performance of assay" and "Sensitivity and detection limit", pt.4).

If the absorbance difference of the sample (ΔA<sub>sample</sub>) is larger than 1.000 (measured at 340 nm or Hg 334 nm) or 0.500 (measured at Hg 365 nm), the concentration of nitrate is too high. The sample solution must therefore be diluted according to the dilution table.

### Calculation

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\epsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

V = final volume [ml]

v = sample volume [ml]

MW = molecular weight of the substance to be assayed [g/mol]

d = light path [cm]

ε = extinction coefficient of NADPH at:

$$340 \text{ nm} = 6.3 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]}$$

$$\text{Hg } 365 \text{ nm} = 3.5 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]}$$

$$\text{Hg } 334 \text{ nm} = 6.18 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]}$$

It follows for nitrate (NO<sub>3</sub><sup>-</sup>):

$$c = \frac{3.050 \times 62.0}{\epsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A = \frac{1.891}{\epsilon} \times \Delta A \text{ [g nitrate/l sample solution]}$$

If the sample has been diluted on preparation, the result must be multiplied by the dilution factor F.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$\text{Content}_{\text{nitrate}} = \frac{c_{\text{nitrate}} \text{ [g/l sample solution]}}{\text{weight}_{\text{sample}} \text{ in g/l sample solution}} \times 100 \text{ [g/100 g]}$$

### 1. Instructions for performance of assay

The amount of nitrate present in the assay has to be between 0.3 µg and 30 µg. In order to get a sufficient absorbance difference, the sample solution is diluted to yield an nitrate concentration between 0.03 and 0.3 g/l.

#### Dilution table

Estimated amount of nitrate per liter	Dilution with water	Dilution factor F
< 0.3 g	-	1
0.3-3.0 g	1 + 9	10
3.0-30 g	1 + 99	100
> 30 g	1 + 999	1000

If the measured absorbance difference (ΔA) is too low (e.g. <0.100), the sample solution should be prepared again (weigh out more sample or dilute less strongly) or the sample volume to be pipetted into the cuvette can be increased up to 2.000 ml. The volume of water added must then be reduced to obtain the same final volume in the assays for sample and blank. The new sample volume v must be taken into account in the calculation.

## 2. Technical information

- 2.1 The "diluted Carrez solutions" are sufficient in the determination of nitrate in fruit and vegetable juices. The "concentrated Carrez solutions", however, have to be used in the analysis of meat and meat products for complete removal of enzymes (from muscle or from starter cultures). It is necessary in both cases that the pH of the solution is slightly alkaline (pH 8) after the addition of sodium hydroxide.
- 2.2 **When determining nitrate in trace-level compound analysis, some points have to be taken into consideration in order to get satisfying results:**
- Paper or membrane filters have to be free from nitrate.
  - Adsorbents recommended for sample preparation (polyvinylpyrrolidone PVPP, bentonite, ect.) have also to be free from nitrate.
  - The Carrez solutions must be free from nitrate, too.

### To check for the absence of nitrate:

- Wash paper or membrane filter with redist. water and use 2.000 ml of the washing water for the assay (blank with redist. water).
- Add water to the adsorbents in the volume/quantity ratio given in the instructions, mix and filter; use 2.000 ml of the filtrate for the assay (blank with redist. water).
- Mix redist. water, Carrez-I-solution, Carrez-II-solution and sodium hydroxide solution in the ratio given in the instructions, mix and filter; use 2.000 ml of the filtrate for the assay (blank with redist. water).

Also in the case of small amounts of nitrate in the assay, follow the instructions: read absorbances  $A_2$  exactly 40 min after measuring  $A_1$  and read  $A_3$  after another exactly 20 min.

## 3. Specificity (Ref. 1.1)

Nitrate reductase is to a large extent specific for nitrate ions. The nitrite ions which are formed during the nitrate reduction are converted only with neglectable reaction rate. Chlorate ions react with relatively high reaction rate, but not perchlorate and iodate ions.

In the analysis of commercial potassium nitrate, results of approx. 100 % have to be expected.

## 4. Sensitivity and detection limit (Ref. 1.1)

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume  $v = 2.000$  ml and measurement at 340 of a nitrate concentration of 0.1 mg/l sample solution (if  $v = 0.100$  ml, this corresponds to 2 mg/l sample solution).

The detection limit of 0.15 mg/l is derived from the absorbance difference of 0.010 (as measured at 340 nm) and a maximum sample volume  $v = 2.000$  ml.

## 5. Linearity

Linearity of the determination exists from 0.3  $\mu\text{g}$  nitrate/assay (0.15 mg nitrate/l sample solution; sample volume  $v = 2.000$  ml) to 30  $\mu\text{g}$  nitrate/assay (0.3 g nitrate/l sample solution; sample volume  $v = 0.100$  ml).

## 6. Precision

In a double determination using one sample solution, a difference of 0.005 to 0.015 absorbance units may occur. With a sample volume of  $v = 0.100$  ml and measurement at 340 nm, this corresponds to a nitrate concentration of approx. 1.5-5 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.015-0.05 g/100 g can be expected.)

The following data have been published in the literature:

5.8 $\mu\text{g}$ /assay	n = 10	CV = 2.7 %	
17 $\mu\text{g}$ /assay	n = 10	CV = 2.8 %	
28 $\mu\text{g}$ /assay	n = 10	CV = 1.3 %	(Ref. 1.1)

Radish juice:  $r = 77.3$  mg/l  $s_{(r)} = \pm 27.3$  mg/l  
 $R = 112.2$  mg/l  $s_{(R)} = \pm 39.7$  mg/l

Carrot juice:  $r = 12.2$  mg/l  $s_{(r)} = \pm 4.3$  mg/l  
 $R = 17.7$  mg/l  $s_{(R)} = \pm 6.3$  mg/l

Beetroot juice:  $r = 42.8$  mg/l  $s_{(r)} = \pm 15.1$  mg/l  
 $R = 64.1$  mg/l  $s_{(R)} = \pm 22.7$  mg/l

Spinach baby food:  
 $x = 64$  mg/kg  $r = 15.5$  mg/kg  $s_{(r)} = \pm 5.5$  mg/kg  
 $R = 24.5$  mg/kg  $s_{(R)} = \pm 8.7$  mg/kg (Ref. 2.1)

## 7. Interference/sources of error

Nitrite ions of the sample inhibit the main reaction at concentrations higher than 70  $\mu\text{g}$ /assay. Manganese ions ( $>5$   $\mu\text{g}$ /assay) retard the nitrate conversion. Chloride ions inhibit only at a concentration higher than 4.3 mg/assay. Cyanide ions ( $>1.6$   $\mu\text{g}$ /assay) and sulfite ions ( $>100$   $\mu\text{g}$ /assay) inactivate the nitrate reductase completely.

## 8. Recognizing interference during the assay procedure

- 8.1 If the conversion of nitrate has been completed according to the time given under "Procedure", it can be concluded in general that no interference has occurred.
- 8.2 Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml): the measured differences in absorbance should be proportional to the sample volumes used.
- When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.
- 8.3 Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample and assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.
- 8.4 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

## 9. Reagent hazard

The reagents used in the determination of nitrate are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulation 67/548/EEC and subsequent alteration, supplementation and adaptation guidelines. However, the general safety measures that apply to all chemical substances should be adhered to.

After use, the reagents can be disposed of with laboratory waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

## 10. General information on sample preparation

In carrying out the assay:

Use **clear, colorless and practically neutral liquid samples** directly, or after dilution according to the dilution table, and of a volume up to 2.000 ml; Filter **turbid solutions**;

Degas **samples containing carbon dioxide** (e.g. by filtration);

Adjust **acid samples** to pH 8 by adding sodium or potassium hydroxide solution;

Adjust **acid and weakly colored samples** to pH 8 by adding sodium or potassium hydroxide solution and incubate for approx. 15 min;

Treat **"strongly colored" samples** that are used undiluted or with a higher sample volume with polyvinylpyrrolidone (PVPP) - (e.g. 1 g/100 ml);

Crush or homogenize **solid or semi-solid samples**, extract with water or dissolve in water and filter if necessary; resp. remove turbidities or dyestuffs by Carrez clarification;

Deproteinize **samples containing protein** with Carrez reagents;

Extract **samples containing fat** with hot water (extraction temperature should be above the melting point of the fat involved). Cool to allow the fat to separate, make up to the mark, place the volumetric flask in an ice bath for 15 min and filter; alternatively clarify with Carrez-solutions after the extraction with hot water.

**Note:** With some sample solutions (e.g. from coffee, colored juice concentrates) a creep reaction is observed occasionally, even before the measuring of  $A_1$ . In such cases it is recommended to add polyvinylpyrrolidone, PVPP (1 g/100 ml) or bentonite (1 g/100 ml) to the sample, to stir for 2 min and subsequently to filter the sample material.

### Carrez clarification:

Pipette the liquid sample into a 10 ml volumetric flask which contains approx. 60 ml redist. water, or weigh sufficient quantity of the sample into a 100 ml volumetric flask and add approx. 60 ml redist. water. Subsequently, carefully add 5 ml Carrez-I-solution (potassium hexacyanoferrate(II) (ferrocyanide), 85 mM = 3.60 g  $K_4[Fe(CN)_6] \times 3 H_2O/100$  ml) and 5 ml Carrez-II-solution (zinc sulfate, 250 mM = 7.20 g  $ZnSO_4 \times 7 H_2O/100$  ml). Adjust to pH 7.5-8.5 with sodium hydroxide (0.1 M; e.g. 10 ml). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

The "concentrated" Carrez solutions have to be used for the preparation of meat and dairy samples (see pt. 11: application examples).

## 11. Application examples

### Determination of nitrate in water, drinking-water and mineral water

Clear and colorless water samples are used undiluted for the assay or after dilution according to the dilution table. Turbid water samples are filtered and used for the assay.

CO<sub>2</sub>-containing samples are filtered, neutralized if necessary, and used for the assay.

### Determination of nitrate in beer

Beer samples are filtered or stirred for 1 min to remove the CO<sub>2</sub>. Add 0.1 g bentonite to 10 ml CO<sub>2</sub>-free beer sample and stir with a glass rod for approx. 2 min. Filter and use 1.000 ml for the assay.

### Determination of nitrate in fruit juice and vegetable juice

Exactly weigh approx. 15 g of homogenized sample into a 100 ml volumetric flask, add approx. 20 ml redist. water and mix. For clarification, add one after the other and mix after each addition: 5 ml diluted Carrez-I solution (3.60 g potassium hexacyanoferrate(II),  $K_4[Fe(CN)_6] \times 3 H_2O/100$  ml) and 5 ml diluted Carrez-II solution (7.20 g zinc sulfate,  $ZnSO_4 \times 7 H_2O/100$  ml). Adjust with sodium hydroxide (1 M) to pH 8. Fill up to the mark with redist. water, mix and filter. Use the clear filtrate ( $v = 0.100-0.500$  ml) for the assay.

### Determination of nitrate in wine

Neutralize the sample if necessary. When analyzing red wine add additionally 1 g polyvinylpyrrolidone (PVPP) per 20 ml wine and stir for approx. 5 min. Filter and use the filtrate for the assay.

### Determination of nitrate in fruits and vegetables

Mince the sample and homogenize. Exactly weigh approx. 5 g sample material into a 100 ml beaker and add approx. 60 ml hot redist. water (approx. 70°C). Shake and incubate for approx. 15 min at 60–70°C. Strongly colored products (e.g. beetroot) or samples with a high content of starch (e.g. potatoes) treat additionally with Carrez reagents (see under fruit juice and vegetable juice). Allow to cool to 20–25°C and transfer quantitatively into a 100 ml volumetric flask. Fill up to the mark with redist. water and filter. Take 0.100-1.000 ml of the filtrate for the assay.

### Determination of nitrate in meat and meat products

Mince the sample and homogenize. Exactly weigh approx. 5 g sample material into a 100 ml beaker and add approx. 50 ml boiling redist. water. Keep boiling for 15 min and allow to cool to 20–25°C. Add one after the other 3 ml concentrated Carrez-II solution (15.0 g potassium hexacyanoferrate(II),  $K_4[Fe(CN)_6] \times 3 H_2O/100$  ml) and 3 ml concentrated Carrez-I solution (30.0 g zinc sulfate,  $ZnSO_4 \times 7 H_2O/100$  ml) each and mix after each addition. Adjust to pH 8 with sodium hydroxide (1 M). Transfer the contents of the beaker quantitatively into a 100 ml volumetric flask, fill up to the mark with redist. water and mix. For fat separation place the flask for 20 min in a refrigerator and filter. Discard the first few ml of the filtrate. Use the clear filtrate for the assay.

If the filtrate is not clear, the sample solution has to be prepared freshly by using 4 ml (maximum 10 ml) concentrated Carrez-I-solution and concentrated Carrez-II-solution, each.

### Determination of nitrate in dairy products and cheese

Mince the sample and homogenize. Exactly weigh approx. 5 g sample material into a 100 ml beaker and continue working as described under "meat and meat products".

### Determination of nitrate in baby food

Mince the sample and homogenize. Exactly weigh approx. 5 g sample material into a 100 ml beaker and add approx. 50 ml boiling redist. water. Keep boiling for 15 min and allow to cool to 20–25°C. Add one after the other diluted Carrez-I solution and diluted Carrez-II solution and mix after each addition (for preparation see under fruit juice and vegetable juice. Continue working as described under "meat and meat products".

## 12. Further applications

The method may also be used in the examination of pharmaceuticals, cosmetics, and in environmental analysis and in research when analyzing biological samples.

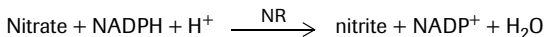
## References

- 1.1 Beutler, H.-O., Wurst, B. & Fischer, S. (1986) Eine neue Methode zur enzymatischen Bestimmung von Nitrat in Lebensmitteln, Deutsche Lebensmittel-Rundschau **82**, 283-289
- 2.1 Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG; Untersuchung von Lebensmitteln: Bestimmung von Nitrat in Gemüsesäften, 26.26-2 (Mai 1988); Bestimmung von Nitrat in Gemüsebrei für Säuglinge und Kleinkinder, 48.03.05-1 (Mai 1988); Bestimmung des Nitrit- und Nitratgehaltes in Wurstwaren nach enzymatischer Reduktion, 08.00-14 (Dezember 1990)
- 2.2 Brautechnische Analysenmethoden, Band II, 2. Auflage, S. 139-141 (1988), Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission (MEBAK), herausgegeben von F. Drawert im Selbstverlag der MEBAK, Freising
- 2.3 Deutsche Norm DIN EN 12014-5 (August 1997) Bestimmung des Nitrat und/oder Nitritgehaltes, Teil 5: Enzymatische Bestimmung des Nitratgehaltes in gemüsehaltiger Säuglings- und Kleinkindernahrung
- 2.4 European Standard EN 12014-5 (August 1997) Determination of nitrate and/or nitrite content, Part 5: Enzymatic determination of nitrate content of vegetable containing food for babies and infants
- 3.1 Rohm, H. & Winkler-Macheiner, U. (1987) Eine enzymatische Methode zur Nitratbestimmung in Anwendung auf Molkenpulver und Käse, ERNÄHRUNG/NUTRITION **11**, 9-13
- 3.2 Beutler, H.-O., Wurst, B. & Henniger, G. (1986) Enzymatic Determination of Nitrate, Poster-Präsentation bei Association of Official Analytical Chemists Annual International Meeting, Scottsdale, AZ, USA
- 3.3 Kretzschmar, R. & Kretzschmar, T. (1988) Enzymatische Nitratbestimmung in kommunalem Abwasser, Vom Wasser **70**, 119-128
- 3.4 Brauner-Glaesner, G. & Beutler, H.-O. (1989) Ringversuchsergebnisse zur enzymatischen Nitratbestimmung, Lebensmittelchem. Gerichl.Chem. **43**, 123-126

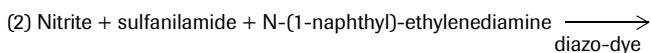
# 13. Colorimetric method for the determination of nitrate and nitrite in sausages/meat products acc. to Arneht (s. Ref. 1.1)

## Principle (Ref. 1.1)

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



Nitrite reacts with sulfanilamide and N-(1-naphthyl)-ethylenediammonium dichloride with the formation of a red diazo-dyestuff (2).



The amount of the diazo-dyestuff formed during the reaction is stoichiometric to the amount of nitrite. The dyestuff is measured by means of its light absorbance at 540 nm (Hg 546 nm) in the visible range.

## Reagents

- I. Dissolve 0.2 g bromothymol blue in 100 ml methylated ethanol (96 %; w/w).
- II. Carrez-I-solution: 150 g  $\text{K}_4[\text{Fe}(\text{CN})_6] \times 3 \text{ H}_2\text{O}$ /l redist. water.
- III. Carrez-II-solution: 230 g  $\text{Zn}(\text{CH}_3\text{COO})_2 \times 2 \text{ H}_2\text{O}$ /l redist. water.
- IV. Use the contents of bottle 1 (imidazol buffer, pH approx. 7.8) of the Test-Combination Nitrate undiluted.  
The solution is sufficient for approx. 36 assays.  
The solution is stable at 2–8°C (see pack label).
- V. Use the tablets from bottle 2 (NADPH, FAD) of the Test-Combination Nitrate unchanged.  
The tablets are sufficient for approx. 36 assays.  
The tablets are stable at 2–8°C (see pack label).
- VI. Use the contents of bottle 3 (nitrate reductase) of the Test-Combination Nitrate unchanged.  
Each bottle contains sufficient enzyme for approx. 12 assays.  
The contents of bottle 3 are stable at 2–8°C (see pack label).
- VII. Buffer/enzyme solution  
Dissolve the contents of one bottle 3 of the Test-Combination Nitrate with 12 ml imidazole buffer solution (IV) from bottle 1 of the Test-Combination Nitrate, add 50 mg Titriplex III (di-sodium-ethylen-dinitrilo-tetraacetate-dihydrate) and mix.  
Prepare the solution freshly before use.
- VIII. Color reagent  
Solution A: Dissolve 8 g sulfanilamide in 500 ml redist. water in a warm water bath, cool to room temperature and filter, if necessary. While stirring add 330 ml concentrated hydrochloric acid (free from nitrate and nitrite), fill up to 1000 ml with redist. water.  
The solution is stable at 20°C for several weeks.  
Solution B: Dissolve 330 mg N-(1-naphthyl)-ethylene-diammonium dichloride in 250 ml redist. water.  
The solution is stable in a brown bottle at 20°C for 1 week.  
Working solution: Mix **daily** for each assay 1.5 ml solution A and 1.5 ml solution B.
- IX. Sodium nitrite working standard solutions  
Dissolve 200 mg sodium nitrite to the nearest 0.1 mg with 500 ml redist. water in a volumetric flask. Dilute 25 ml of this stock solution in a 500 ml volumetric flask with redist. water (= 20 mg sodium nitrite/l). Pipette 10, 20, 30 and 40 ml of the diluted stock solution into 200 ml volumetric flasks. Add 0.2 ml bromothymol blue solution (I), titrate with NaOH (1 M) until the color changes to blue. Add 2 ml Carrez-I-solution (II) and 2 ml Carrez-II-solution (III), mix after each addition, fill the volumetric flasks to the mark with redist. water, mix and filter. Discard the first parts of the filtrate.  
The working standard solutions (with 1 mg, 2 mg, 3 mg and 4 mg sodium nitrite/l) have to be prepared daily.

## X. Nitrate standard solutions

Dissolve 300 mg potassium nitrate (to the nearest 0.1 mg; e.g. Merck Darmstadt, Cat. no. 5065, suprapur) with 500 ml redist. water in a volumetric flask. Dilute 25 ml of this stock solution in a 500 ml volumetric flask with redist. water (= 30 mg potassium nitrate/l). Pipette 10, 20, 30 and 40 ml of the diluted stock solution into 200 ml volumetric flasks. Add 0.2 ml bromothymol blue solution (I), titrate with NaOH (1 M) until the color changes to blue. Add 2 ml Carrez-I-solution (II) and 2 ml Carrez-II-solution (III), mix after each addition, fill the volumetric flasks to the mark with redist. water, mix and filter. Discard the first parts of the filtrate.

The standard solutions (with 1.5 mg, 3 mg, 4.5 mg and 6 mg potassium nitrate/l) have to be prepared daily.

## Procedure

Wavelength <sup>1</sup> :	540 nm (Hg 546 nm)
Glass cuvette <sup>2</sup> :	1.00 cm
Incubation volume:	6.000 ml
Measuring volume:	approx. 3 ml
Read against water:	
Sample solution:	3–12 µg potassium nitrate, resp. 2–8 µg sodium nitrite/assay (in 2.000 ml sample solution) <sup>3</sup>

## a) Determination of nitrite

Pipette into test tubes:	Working standard NaNO <sub>2</sub> 1 mg/l	Working standard NaNO <sub>2</sub> 2 mg/l	Working standard NaNO <sub>2</sub> 3 mg/l	Working standard NaNO <sub>2</sub> 4 mg/l	Sample
sample solution	–	–	–	–	2.000 ml
working standards (IX)	2.000 ml	2.000 ml	2.000 ml	2.000 ml	–
redist. water	1.000 ml	1.000 ml	1.000 ml	1.000 ml	1.000 ml
color reagent (VIII)	3.000 ml	3.000 ml	3.000 ml	3.000 ml	3.000 ml
Mix, incubate for 30 min at room temperature in the dark. Read the absorbances of the solutions.					

## b) Determination of nitrate and nitrite

Pipette into test tubes:	Standard KNO <sub>3</sub> 1.5 mg/l	Standard KNO <sub>3</sub> 3 mg/l	Standard KNO <sub>3</sub> 4.5 mg/l	Standard KNO <sub>3</sub> 6 mg/l	Sample
tablet (V)	1 tablet	1 tablet	1 tablet	1 tablet	1 tablet
sample solution	–	–	–	–	2.000 ml
standard solutions (X)	2.000 ml	2.000 ml	2.000 ml	2.000 ml	–
buffer/enzyme solution (VII)	1.000 ml	1.000 ml	1.000 ml	1.000 ml	1.000 ml
Incubate at room temperature for 60 min. Add					
color reagent (VIII)	3.000 ml	3.000 ml	3.000 ml	3.000 ml	3.000 ml
Mix, incubate for 30 min at room temperature in the dark. Read the absorbances of the solutions.					

**Note:** It is not necessary to determine the calibration curve in each series of analyses. It is sufficient to determine the calibration curves from time to time, and to run 1 working standard sodium nitrite and 1 standard potassium nitrate for assay control in each series of analyses.

1 On spectrophotometers, measurements are taken at 540 nm; if spectralline photometers equipped with a mercury vapor lamp are used, measurements are taken at 546 nm.  
2 If desired disposable cuvettes may be used instead of glass cuvettes.  
3 See instructions

## Calculation

### Graphical evaluation

The calculation of results is done with calibration curves prepared with standard solutions. Plot the absorbance values for the sodium nitrite working standard solutions and the potassium nitrate standard solutions (ordinate) against the individual sodium nitrite and potassium nitrate concentrations (abszissa) in a coordinate system. A linear relationship should result.

One gets for the sample solution  $A_{\text{nitrate} + \text{nitrite}}$  and  $A_{\text{nitrite}}$

the difference  $A_{\text{nitrate} + \text{nitrite}} - A_{\text{nitrite}}$  gives  $A_{\text{nitrate}}$ .

Determine the sodium nitrite and potassium nitrate concentration from the resp. absorbance values by means of the calibration curves.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$\text{Content}_{\text{nitrite}} = \frac{c_{\text{nitrite}} \times 1000}{\text{weight}_{\text{sample}} \text{ in g/l sample solution}} \quad [\text{mg nitrite/kg sample}]$$

$$\text{Content}_{\text{nitrate}} = \frac{c_{\text{nitrate}} \times 1000}{\text{weight}_{\text{sample}} \text{ in g/l sample solution}} \quad [\text{mg nitrate/kg sample}]$$

The results are calculated as sodium nitrite and potassium nitrate.

The calculation of nitrate ( $\text{NO}_3^-$ ) from  $\text{KNO}_3$  is done with the factor  $62.005/101.11 = 0.613$ , the calculation of  $\text{NO}_2^-$  from  $\text{NaNO}_2$  with the factor  $46.006/68.995 = 0.666$

### 13.1 Instructions for Performance of assay

The amount of nitrite, of nitrate resp., in the assay should range between 2–8  $\mu\text{g}$  of nitrite, 3–12  $\mu\text{g}$  of nitrate, resp.. The sample solution is diluted sufficiently to yield a nitrite concentration between 1–4 mg/l, a nitrate concentration between 1.5–6 mg/l.

### 13.2 Instructions for sample preparation

Weigh approx. 10 g sample material to the nearest 1 mg into a wide mouth Erlenmeyer-flask, add 50 ml redist. water and homogenize for 30 to 60 s with an Ultra-Turrax. Rinse the shaft of the homogenizer with 50 ml hot redist. water. Add 0.2 ml bromothymol blue solution (I), titrate with NaOH (1 M) until the color changes (if necessary check with a pH-meter:  $< 8.5$ ; there may be no clear filtrates after Carrez clarification if the pH is higher), and incubate for 15 min in a bath with boiling water.

Cool the flask to 20–25°C and transfer the contents quantitatively into a 200 ml volumetric flask. Add one after the other 2 ml Carrez-I-solution (II) and 2 ml Carrez-II-solution (III) (4 ml each in the analysis of samples rich in connective tissue), and mix after each addition. Fill the volumetric flask to the mark with redist. water, mix and filter with a fluted filter paper. Discard the first parts of the filtrate. Use the clear filtrate for the assay.

### 13.3 Precision

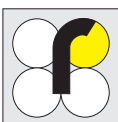
The following data for the determination of nitrite have been published in the literature:

Boiled sausage:

$$\begin{array}{lll} x = 37 \text{ mg/kg} & r = 6 \text{ mg NO}_2/\text{kg} & s_{(r)} = \pm 2 \text{ mg NO}_2/\text{kg} \\ & R = 8 \text{ mg NO}_2/\text{kg} & s_{(R)} = \pm 3 \text{ mg NO}_2/\text{kg} \end{array} \quad (\text{Ref. 2.1})$$

### References

- 1.1 Armeth, W. & Herold, B. (1988) Nitrat/Nitrit-Bestimmung in Wurstwaren nach enzymatischer Reduktion, *Fleischwirtschaft* **68**, 761–764
- 2.1 Amtliche Sammlung von Untersuchungsverfahren nach §35 LMBG: Untersuchung von Lebensmitteln: Bestimmung des Nitrit- und Nitrat-Gehaltes in Wurstwaren nach enzymatischer Reduktion, L 08.00-14 (Dezember 1990)
- 2.2 Schweizerisches Lebensmittelbuch, Kapitel 22 (Diätetische Lebensmittel und Speziallebensmittel)/12.3 (1990): Provisorische Methode der Subkommission 5



R-BIOPHARM AG  
Landwehrstr. 54  
D-64293 Darmstadt  
Telefon + 49 61 51 / 81 02-0  
Fax + 49 61 51 / 81 02-20  
www.r-biopharm.com

