Enzymatic UV assay for the determination of nitrate (NO_3^-) in foodstuffs and other sample materials
Test combination for 50 determinations

For *in vitro* use only
Store between 2 - 8 °C

This test was conducted using the following matrices: meat and fish products, vegetable purées, juices, and powders (kale, spinach, lettuce, arugula, carrots), as well as water, wine, beer, and milk.

For detailed results and further information on validation data, please refer to the validation report.

Other foodstuffs or sample materials can be tested and must be validated by the user.

Enzytec™ *Liquid* Nitrate E8370 can be used for the colorimetric determination of nitrate and nitrite in meat and meat products (*Arneth* method). A separate application is available for this purpose (see Chapter 7. Supporting Documents).

1. Test principle

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

$$\text{Nitrate} + \text{NADPH} + H^+ \xrightarrow{\text{NR}} \text{nitrite} + \text{NADP}^+ + H_2O$$

The amount of NADPH oxidized in this reaction is stoichiometric to the amount of nitrate. NADPH is measured based on its specific absorption at a wavelength of 340 nm. The result is expressed as mg/L or mg/kg nitrate.

2. Reagents

2.1. Content & composition

The test is suitable for manual and automated processing. With manual processing, the reagents are sufficient for 50 determinations. The number of determinations for automated processing is increased by a multiple. However, this depends on the used device.

- Reagent 1: 2 x 50 mL with buffer, NADPH
- Reagent 2: 2 x 12.5 mL with buffer, nitrate reductase

2.2. Reagent preparation

The reagents are ready-to-use and be allowed to reach room temperature (20 - 25 °C) before use. Do not interchange components between kits of different batches.

2.3. Storage & stability

The reagents are stable until the indicated shelf life (see labeling) even after opening at 2 - 8 °C if handled properly. Do not freeze reagents.

2.4. Safety & disposal

This product/test is only suitable for use within the scope of its intended purpose. The instruction for use must be strictly followed.

General safety rules for working in chemical laboratories should be applied. Do not swallow! Avoid contact with skin and mucous membranes.

This kit may contain hazardous substances. For hazard notes on the contained substances, please refer to the appropriate safety data sheets (SDS) for this product. After use, the reagents can be disposed of with the laboratory waste. Packaging materials may be recycled.

3. Sample preparation

- Sample preparation for manual and automated testing is identical.
- The samples should be brought to room temperature before measurement.
- Use liquid, clear and almost neutral sample solutions directly or after dilution with dist. water to a concentration within the measuring range (see performance data).
- Filter or centrifuge turbid solutions.
- Degas samples containing carbonic acid.
- Store samples in a cold and dry room protected from light.
- **Important:** the enzymatic system is highly sensitive for nitrate. Ensure that reagents used for extraction are free from nitrate e.g. water and chemicals.
- Use separate tips for each sample extract (and control solutions) to avoid cross-contamination, pre-flush the tip before pipetting.
- Weigh samples with a high fat content into a volumetric flask and extract with hot water; allow sample solution to cool down for fat separation (e.g. 15 min in an ice bath); fill volumetric flask up to the mark with water, filter aqueous solution before testing.
- If necessary, decolorize strongly colored samples with PVPP:
  - 1 g PVPP + at least 10 mL sample solution
  - Incubate for 10 min at RT and invert on a roller mixer, centrifuge at 4000 rpm for 5 min
  - Remove supernatant and filter through a pleated filter
- For clarification of protein-containing samples, Carrez-clarification is recommended.
- Carrez-clarified samples with **low** nitrate concentrations require a Carrez reagent blank (CRB). This has to be prepared by using 15 mL of Carrez-clarified water instead of 15 g of sample.
  Important: perform the pH adjustment with 1 M NaOH. Otherwise, the Carrez-clarified water appears cloudy and the measurement is impaired. This solution will be used **instead** of the water reagent blank (RB) mentioned in section *4. Assay procedure*. Please also note the calculation in section *5. Calculation of results*.
- **Diluted** Carrez solutions:
  - Carrez-I: 36 g/L potassium hexacyanoferrate (II) trihydrate
  - Carrez-II: 72 g/L zinc sulfate heptahydrate
- **Concentrated** Carrez solutions:
  - Carrez-I: 150 g/L potassium hexacyanoferrate (II) trihydrate
  - Carrez-II: 300 g/L zinc sulfate heptahydrate

3.1. Potable and Mineral water

- Filter, vortex, or briefly treat in an ultrasonic water bath if the water contains carbon dioxide.
- Use undiluted. Sample volume: 500 µL to 1000 µL

3.2. Beer

- Filter, vortex, or briefly treat in an ultrasonic water bath if the beer contains carbon dioxide.
- Add 0.1 g of bentonite to 10 mL of CO_2-free beer, then vortex or shake.
- Filter through a syringe filter.
- Sample volume: at least 200 µL

3.3. Wine

- Neutralize the wine with 1 M NaOH..
- Red wine: add 1 g PVPP to 20 mL wine, shake automatically for 5 minutes, and filter through a syringe filter.
- White wine: use directly or filter before use if cloudy.
- Sample volume: at least 200 µL

3.4. Fruit and vegetable juices

- Weigh in approx. 15 g of juice sample **exactly** into a beaker or a 50 mL centrifuge tube.
- Add 20 mL distilled water and mix.
- Add 5 mL each of **diluted** Carrez I and Carrez II solution in succession and mix.
- Adjust to pH 8.0 ± 0.1 with 1 M NaOH.
- Transfer to a 100 mL volumetric flask, fill to the mark with distilled water and mix.
- Filter the supernatant through a pleated filter or, if necessary, a syringe filter.
- Recommended sample volume: 100 µL for vegetable juice and 500 µL for fruit juice

3.5. Fruit and vegetables

- Carefully homogenize the samples and weigh in approx. 2.5 g **exactly** 50 mL beaker.
- Add 30 mL of distilled water heated to 70 °C and mix.
- Incubate for 15 min in a water bath heated to 60 - 70 °C.
- Add 2.5 mL of **diluted** Carrez I and Carrez II solution in succession and mix. Then allow to cool to room temperature.
- Adjust to pH 8.0 ± 0.1 with 1 M NaOH.
- Transfer to a 50 mL volumetric flask, fill to the mark with distilled water and mix.
- Filter the supernatant through a pleated filter or, if necessary, a syringe filter.
- Recommended sample volume: 100 µL for vegetables and 500 µL for fruits

3.6. Infant formula

- Weigh in approx. 2.5 g **exactly** into a 50 mL beaker.
- Add 25 mL of boiling distilled water and mix.
- Incubate in a boiling water bath for 15 min, then allow to cool to room temperature.
- Add 2.5 mL each of **concentrated** Carrez I and Carrez II solution in succession and mix.
- Adjust to pH 8.0 ± 0.1 with 1 M NaOH.
- Transfer to a 50 mL volumetric flask, fill to the mark with distilled water and mix.
- To separate the fat, place the flask in a refrigerator at 2 to 8 °C for 20 min.
- Filter the supernatant through a pleated filter or, if necessary, a syringe filter.

3.7. Meat, fish, and dairy products (protein-containing samples, based on the processing of meat and sausage products for the determination of nitrate/nitrite according to Arneth)

- Carefully homogenize the samples and weigh in approx. 5 g **exactly** into a 100 mL beaker.
- Add 20 mL distilled water and suspend the sample.
- Add 30 mL boiling distilled water and mix.
- Incubate in a boiling water bath for 15 min, then allow to cool to room temperature.
- Add 3 mL each of **concentrated** Carrez I and Carrez II solution in succession and mix.
- Adjust to pH 8.0 ± 0.1 with 1 M NaOH.
- Transfer to a 100 mL volumetric flask, fill to the mark with distilled water and mix.
- To separate the fat, place the flask in a refrigerator at 2 to 8 °C for 20 min.
- Filter the supernatant through a pleated filter or, if necessary, a syringe filter.

4. Assay procedure

Wavelength:	340 nm
Temperature:	20 - 25 °C or 25 - 37 °C
Photometer alignment:	against air (without cuvette)
Measuring range:	30 - 300 mg/L

	RB / CRB	**Samples / controls**
Reagent 1 **Sample / control** **Dist. water**	2000 µL - 100 µL	2000 µL 100 µL -
Mix, incubate for 3 min at 20 - 25 °C or 25 - 37 °C. Read absorbance **A_1** at 340 nm, then add:		
Reagent 2	500 µL	500 µL
Mix, incubate at 20 - 25 °C for **exactly 40 min** or at 25 - 37 °C for **exactly 20 min**, then measure the absorbance **A_2**.		
Incubate **another exactly 20 min** at 20 - 25 °C or **another exactly 10 min** at 25 - 37 °C and **read** the absorbance **A_3**.		

4.1. Instructions for performing the test

- A multistepper pipette for adding reagent 1 and reagent 2 is recommended. Use a single tip for each component.
- The (Carrez) reagent blank value must be determined **once for each run** and subtracted from **each** sample result.
- Due to the creep reaction, after the second measurement, a third absorption measurement must be carried out **after exactly 20 min** (20 - 25 °C) or **after exactly 10 min** (25 - 37 °C). The OD difference will be used to correct the creep reaction.

5. Calculation of results

5.1. Calculation of sample solutions

5.1.1. Concentration of nitrate

$$\Delta A_{\text{RB or CRB}} = (A_1 \times df - A_2) - 2 \times (A_2 - A_3)$$
$$\Delta A_{\text{Sample or control}} = (A_1 \times df - A_2) - 2 \times (A_2 - A_3)$$
$$\Delta A_{\text{Nitrate}} = \Delta A_{\text{sample or control}} - \Delta A_{\text{RB or CRB}}$$

df: Dilution factor
RB: Reagent blank
CRB: Carrez reagent blank

$$df_{100\mu L} = \frac{\text{sample volume + volume R1}}{\text{total test volume}} = \mathbf{0.808}$$

Stated df of 0.808 **applies for a basic application of 100 µL**. Increasing the sample volume is possibly applicable (max. 1000 µL; refer to validation report). **With constant reagent volumes** conversion of dilution factor (df) is necessary.
If the sample volume is increased, the test system may be affected. In general, this must be checked depending on the matrix. **It is necessary to adjust the (Carrez) reagent blank to the increased sample volume.**

$$\mathbf{C}_{\text{Nitrate}}\ \mathbf{[g/L]} = \frac{(V \times MW \times \Delta A)}{(\varepsilon \times d \times v \times 1000)} = \mathbf{0.2559 \times \Delta A} \times F$$

If the sample extract was diluted before measurement, this result has to be multiplied with the pre-dilution factor F.

V:	Test volume (basic application) [mL]	= 2.600
MW:	Molecular weight [g/mol]	= 62.0
d:	Optical path [cm]	= 1.00
v:	Sample volume (basic application) [mL]	= 0.100
ε:	Extinction coefficient NADHP [L/mmol x cm]	= 6.3 (at 340 nm)

5.2. Calculation of solid samples

$$\text{Content}_{\text{Nitrate}}\ [\text{g}/100\ \text{g}] = \frac{C_{\text{Nitrate}}\ [\text{g/L sample solution}]}{\text{weight}_{\text{Sample}}\ \text{in g/L sample solution}} \times 100$$

5.3. Controls & acceptance criteria

Controls or reference samples should be carried along for quality control during each run. Recovery of aqueous standard solutions should be within 100 ± 5 %. For this purpose, we recommend the use of reference materials or standard solutions. For example:

- NIST SRM 3185 Aqueous solution
- NIST 1546a Meat homogenate
- LGC 7114 Kale powder
- FAPAS Quality Control Material:
  - Nitrate in Lettuce Puree; T15163QC
  - Nitrate in Spinach Puree; T15166QCsale
  - Nitrate in Meat; T15167QC

6. Performance data

6.1. Specificity & side activities

The nitrate reductase is specific for nitrate (NO_3^-). No side activities were identified for any of the substances examined during the validation of this test (please also refer to the validation report).

6.2. Interferences

Sulfite and sodium chloride do not interfere at or below 7.5 g/L. Neither high citric acid concentration of 10 g/L nor 3 g/L of ascorbic acid interfere in this test.

A known interferant for the nitrate reductase is manganese ion (II). Manganese concentrations in food are at maximum 10 mg/kg in oysters and blue mussels. It is certain, that theses concentrations not interfere the nitrate measurement due to the dilution factor after extraction in any way.

6.3. Linearity, measuring range & sensitivity

Linearity is given up to 300 mg/L nitrate, with the recommended measuring range between 30 and 300 mg/L.

The limit of detection (LoD) was determined according to method DIN 32645:2008-11 in buffered aqueous solution. This results in an LoD of 7 mg/L and 0.8 mg/L nitrate for a sample volume of 100 µL and 500 µL, respectively. The limit of quantification (LoQ) was determined by precision profile and confirms a concentration of 10 mg/L and 1.5 mg/L for 100 µL and 500 µL sample volume, respectively.

To determine concentrations < 30 mg/L (especially in matrix samples), the sample volume should be increased to 200, 500, or 1000 µL.

7. Supporting documents

On request, we offer the following documents:

- Recommendation: Colorimetric determination of nitrate and nitrite in meat and meat products according to Arneth with the use of Enzytec™ Liquid Nitrate (E8370)
- Enzytec™ *Liquid* Nitrate Validation report
- Enzytec™ *Liquid* Sample preparation guide
- Enzytec™ *Liquid* Nitrate Excel templates for results calculation
- Enzytec™ *Liquid* Troubleshooting guide

Safety data sheets (SDS) und certificates of analysis (CoA) are available in digital under the following link
https://eifu.r-biopharm.com/

8. Limits of this method

Test results may vary depending on the sample matrix, the individual test procedure and the laboratory environment. Detection and quantification limits depend on the respective sample matrix and the extraction method. For detailed results and further information, please refer to the current validation report.

For the present enzymatic test, only stated, exemplary matrices could be validated due to the large number of foodstuffs and other sample materials.

When analyzing a non-validated matrix, it is recommended to verify the results obtained by means of spike experiments. If necessary, a suitable sample preparation validation for the sample matrix of interest will need to be performed and validated.

9. Services & technical support

On request, we offer the following services:

- Customized troubleshooting
- Data & results analysis
- Customer workshops & webinars
- Automation: application support and technical service

10. Disclaimer

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