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 (36 – 46 °F)

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

Detailed results and information regarding associated validation data are found in the Validation Report.

The test may be used with other foods or samples material, provided that these are subjected to individual validation by the user.

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

1. Test principle

Nitrate is reduced by reduced nicotinamide-adenine dinucleotide phosphate (NADPH) to nitrite 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 it depends on the device.

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

2.2. Reagent preparation

The reagents are ready-to-use and be allowed to reach room temperature (20 – 25 °C / 68 – 77 °F) before use. Do not interchange components between kits of different batches.

2.3. Storage & stability

If stored as directed and between 2 – 8 °C (36 – 46 °F), reagents remain stable until the printed expiration date, even after opening. Reagents must not be frozen.

2.4. Safety & disposal

The test is intended solely for the intended use as described. The provided Instructions for Use must be strictly followed.

Follow standard chemical safety procedures when handling this product. Do not swallow. Avoid contact with skin or mucous membranes.

Detail safety information for individual components is available in the corresponding Safety Data Sheets (SDS).

Dispose of used reagents as laboratory waste in compliance with all relevant regulations. Packaging materials are to be recycled according to local regulations.

3. Sample preparation

3.1. General

- Sample preparation for manual and automated testing is the same.
- Samples solutions should be brought to room temperature before measurement.
- Use liquid, clear, colorless and almost neutral sample solutions directly or dilute sufficiently to yield a nitrate concentration within the stated measuring range (refer to performance data).
- For turbid test samples: Filter by using fluted paper filter or syringe filter or centrifuge the test solution in a reaction tube (recommended 3000 rpm for at least 5 minutes) until a clear filtrate or supernatant is obtained.
- Degas samples containing carbonic acid, e.g. by stirring in a beaker, applying a brief ultrasonic burst, filtration or centrifugation.
- If necessary, decolorize **strongly** colored samples with polyvinyl-polypyrrolidone (PVPP, e.g., 1 g/100 mL sample). Incubate for 10 minutes at room temperature and invert the solution on a roller mixer. Centrifuge at 4000 rpm for at least 5 minutes and filter in addition, until a clear supernatant is obtained.
- Crush and homogenize solid and semi-solid samples. Weigh a sufficient quantity of sample in a volumetric flask (considering the measuring range), extract with water; fill up to the mark and filter if necessary (by using fluted paper or syringe filters) or centrifuge in reaction tubes. Use Carrez clarification if necessary.
- 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 minutes in an ice bath); fill volumetric flask up to the mark with water, filter aqueous solution before testing.
- For clarification of protein containing samples, Carrez clarification is recommended as described in the following subsections.
  The following chemicals, among others, are required for this:
  a. **Diluted** Carrez solutions:
     - Carrez I: 36 g/L potassium hexacyanoferrate (II) trihydrate
     - Carrez II: 72 g/L zinc sulfate heptahydrate
  b. **Concentrated** Carrez solutions:
     - Carrez I: 150 g/L potassium hexacyanoferrate (II) trihydrate
     - Carrez II: 300 g/L zinc sulfate heptahydrate
- **Important:** the creep reaction to be determined during the analysis is influenced by Carrez reagents. Therefore, Carrez-clarified samples with **low** nitrate concentrations require a Carrez reagent blank (CRB). This must be prepared using, for example, 15 ml of Carrez-clarified water instead of 15 g of sample.
  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. Manual test procedure*. Note the calculation in section *5. Calculation of results*.
- **Important:** the enzymatic system is highly sensitive for nitrate. Ensure that reagents used for extraction are free from nitrate e.g. water and chemicals.
  Store samples in a cold and dry environment protected from light and prevent cross-contamination.

3.2. 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

3.3. 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

3.4. 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

3.5. 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

3.6. 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 (158 °F) and mix.
- Incubate for 15 minutes in a water bath heated to 60 – 70 °C (140 – 158 °F).
- 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.

3.7. 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 minutes, 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 – 8 °C (36 – 46 °F) for 20 minutes.
- Filter the supernatant through a pleated filter or, if necessary, a syringe filter.

3.8. 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 minutes, 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 – 8 °C (36 – 46 °F) for 20 minutes.
- Filter the supernatant through a pleated filter or, if necessary, a syringe filter.

4. Manual test procedure

Wavelength:	340 nm
Temperature (measurement):	20 – 37 °C (68 – 99 °F)
Photometer alignment:	against air (without cuvette)
Measuring range:	30 – 300 mg/L

	Reagent blank / Carrez reagent blank	**Sample / control**
Reagent 1 **Sample / control** **Dist. water**	2000 µL - 100 µL	2000 µL 100 µL -
Mix, incubate for **3 minutes at 20 – 37 °C (68 – 99 °F)**. **Read** absorbance $\mathbf{A_1}$, then add:		
Reagent 2	500 µL	500 µL
Mix, incubate at 20 – 25 °C (68 – 77 °F) for **exactly 40 minutes** or at 25 – 37 °C (77 – 99 °F) for **exactly 20 minutes**, then **read** absorbance $\mathbf{A_2}$.		
Incubate **another exactly 20 minutes** at 20 – 25 °C (68 – 77 °F) or **another exactly 10 minutes** at 25 – 37 °C (77 – 99 °F) and **read** absorbance $\mathbf{A_3}$.		

Important notes for assay procedure

- The (Carrez) reagent blank value must be determined **in each series of measurement** and subtracted from **each** sample result.
- Specified incubation times were validated at 37 °C (99 °F). The test may generally perform within a range between **20 – 37 °C (68 – 99 °F)**.
- Use separate tips for each sample extract and the control solutions to avoid cross-contamination; rinse the tip before pipetting.
- A multistepper pipette is recommended for adding reagents. Use a separate tip for each component.
- Stirring spatulas are recommended for mixing each individual cuvette. Remove these from the cuvette immediately before measuring the absorbance
- Always wait for the reaction to end or for the absorbance to stabilize.
- Due to the creep reaction, a **third absorbance measurement A_3** must be taken after the second one, **after exactly 20 minutes** (20 – 25 °C / 68 – 77 °F) or **after exactly 10 minutes** (25 – 37 °C / 77 – 99 °F). The OD difference is used to correct the creep reaction (extrapolation).
- If the measured absorbance difference of the samples is too small (< 0.020), the sample solution must be prepared again with a higher weight or a lower dilution. Increase the sample volume if concentrations close to LoQ are expected.
- If the absorbance difference of the samples is very large (e.g., > 1.500), the sample solution must be diluted if necessary.

5. Calculation of results

5.1. Concentration of nitrate in sample solutions

The extinction difference ΔA must be calculated for each sample:

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

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

$$\Delta A_{\text{Nitrate}} = \Delta A_{\text{sample or control}} - \Delta A_{\text{RB or CRB}}$$

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

$$df = \frac{\text{sample volume [mL]} + \text{volume R1 [mL]}}{\text{test volume [mL]}} = 0.808$$

The specified df value of **0.808 applies to a base application of 100 µL**. An increase in sample volume is possible (max. 1000 µL; refer to validation report). **While keeping reagent volumes unchanged**, this requires **conversion of the reagent dilution factor (df) accordingly**.

Increasing the sample volume may influence test performance. This must generally be checked depending on the matrix. **The (Carrez-)reagent blank value must be adjusted to the changed sample volume.**

The concentration of nitrate in sample solutions is calculated using Lambert-Beer's law:

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

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

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

5.2. Calculation of solid samples

When analyzing solid and semi-solid samples that have to be weighed in for the extraction of the sample, the content is related to the sample weight:

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

5.3. Controls & acceptance criteria

Control or reference samples should be included in each run for quality control purposes. 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

The recovery of aqueous control solutions should be 100 ± 5 %, for extracted food samples, it should be within the range of 90 – 110 %.

6. Performance data

6.1. Specificity & side activities

Nitrate reductase is specific for nitrate (NO_3^-). No side activities were identified for any of the substances tested during validation (N-nitrosodimethylamine, N-nitrosodipropylamine, nitrite, sulfite, and ascorbic acid).

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 (sample volume of 100 µL) with a recommended measuring range of 3 – 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* Nitrate Excel templates for results calculation
- Enzytec™ *Liquid* Nitrate Technical Information
- Enzytec™ *Liquid* Sample preparation guide
- Enzytec™ *Liquid* Troubleshooting guide

Safety data sheets (SDS) and certificates of analysis (CoA) are available in digital form, quoting the batch number, via the following link:

https://eifu.r-biopharm.com/

8. Limits of this method

Test results may vary depending on the sample matrix, specific test implementation, and laboratory environmental conditions. Detection and quantification limits are dependent on respective sample matrices extraction procedures. Refer toto the current Validation Report for details.

For this test, only the matrices explicitly listed in the documentation were validated, due to the wide variety of food products and other potential sample materials.

When analysing non-validated matrices results should be verified by performing spiking (fortification) experiments. If appropriate or necessary, a suitable sample preparation procedure for the respective matrix must be developed and validated.

The responsibility for validating non-validated matrices and for ensuring the suitability of the assay for its intended use lies solely with the user.

9. Services & technical support

Upon request, we offer the following services, among others:

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

10. Disclaimer

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d. Failure to otherwise use, and when necessary validate or verify, suitable controls, samples, matrices, or processing procedures;
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