

VALIDATION OF AN ENZYMATIC METHOD FOR NITRATES DETERMINATION FROM VEGETABLE PRODUCTS

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Abstract: *Nitrates and nitrites are natural components of soil, derived from mineralization of nitrogenous organic substances of vegetable and animal origin. Plants absorb nitrogen from soil, mainly as nitrates or ammonium forms.*

This paper presents the results of researches carried out to validate an enzymatic method for nitrates determination in vegetable products. Within this method, the nitrate is reduced by the reduced nicotinamide adenine dinucleotide phosphate (NADPH) to nitrite, in the presence of nitrate-reductase (NR).

In the case of the enzymatic method for nitrates determination in vegetable products, the calibration curve achieved with 9 standard levels of nitrate ions (with three rehearsals for each one), in the range of concentrations 6 mg/L – 30 mg/L, beginning from origin and the linearity coefficient (r^2), is 0.9999. For 10 vegetable samples grown in open field (egg plants, bell peppers, green peppers and onion) the average nitrate concentration of vegetable species varied in the range 27.91 mg NO_3^-/kg – 82.85 mg NO_3^-/kg , and repeatability was in the range 1.33 mg NO_3^-/kg – 1.50 mg NO_3^-/kg .

The determination of nitrates in a cabbage sample, by three analysts, led to an average nitrate concentration of 107.42 mg NO_3^-/kg , relative standard deviation RSD(R), and the determined concentration is 0.81 %, and reproducibility is 2.45 mg NO_3^-/kg .

The detection limit is 0.15 mg/L and quantification limit is 0.30 mg/L.

In the case of onion sample grown in open field, the nitrates concentration was 55.5 mg NO_3^-/kg , and uncertainty was 0.68 mg NO_3^-/kg .

Key words: *linearity, repeatability, reproducibility, limit of detection, limit of quantification*

Introduction

In growing and development of horticulture-wine-growing plants, the improvement of soil fertilization state and achievement of high and constant crops, organic or mineral fertilizers are applied in the soil [1].

Nitrates and nitrites are natural components of the soil from nitrogen mineralization of organic substances of plant and animal origin. Nitrogen

mineralization takes place primarily through the existing micro-organisms in soil. In areas with temperate climate, this process is carried out with maximum intensity in hot season [2].

Plants absorb nitrogen from soil, mainly as nitrates or ammonium forms.

Vegetables and fruits can accumulate also nitrogen, as nitrate and nitrite forms. The mineral nitrogen amount in tissues of vegetable and fruit is much more in the case of species which the nitrates'

reduction happens in leaves and when light intensity and temperature are lower [3].

High concentration of nitrates in plants (especially in vegetables) means a hazard for human and animal body, for two reasons: the possibility of methemoglobin appearance at children and nitrates conversion to nitrites in saliva and formation of cancerigen nitrozamines in the intestinal tract [2].

In a normal diet, 54% of nitrates content comes through vegetables consumption. In the daily ingestion of vegetable products, especially in the case of vegans, the percentage can be much more, 75 – 80% [2].

Taking into consideration the cancerigen potential of nitrosamines, the high content of nitrates in vegetable products, presents a potential hazard.

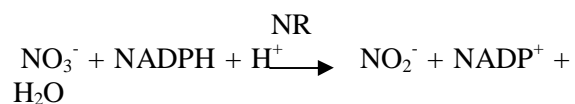
In order to determine nitrates within vegetable products, the following methods can be used:

- Spectrophotometer methods
- High performance liquid chromatography method, using UV detector
- High performance ion chromatography, using electrical conductivity detector
- Potentiometer method with selectively electrode
- Enzymatic method

This paper presents the results of the performed researches carried out to validate an enzymatic method for nitrates determination in vegetable products. This enzymatic method presents no risks for the analyst, eliminating the danger of using metallic cadmium and α – naphthyl amine (cancerous substance, which is one of the components of colour reagent) represent within classical spectrophotometer method.

Experimental

This paper presents the results of the performed researches carried out to validate an enzymatic method for nitrates determination in vegetable products. Within this method, the nitrate is reduced by the reduced nicotinamide adenine dinucleotide phosphate (NADPH) to nitrite, in the presence of nitrate-reductase (NR) [4].



The amount of oxidized NADPH is stoichiometrically equal to the nitrate one. The decrease of NADPH amount is measured through absorbance at $\lambda = 340$ nm.



Figure 1. Kit for nitrates determination through enzymatic method

The objective of validation is to demonstrate that a defined analytic system leads to the obtaining of some precious and reproducible results, for a given property. In order to validate the method for nitrates determination in vegetable products, the following performance criteria were established:

- linearity
- accuracy
- precision (repeatability and reproducibility)

- sensitivity (limit of detection, limit of quantification)

- measurement uncertainty

In order to validate the enzymatic method for nitrates determination in vegetable products, we used as matrices the following vegetable species, cultivated in open field: egg plants, bell peppers, green peppers, cabbage and onion.

Results and Discussion

Linearity is defined as the ability of method to provide results proportional with level of measuring.

Calibration curve has to be straight and to start from origin. Any deviations that appear must be known. In order to accomplish the calibration curve, minimum five concentrations are necessary, with three rehearsals for each of them.

Linearity coefficient (r^2) has to be of minimum 0.999, lower values may be accepted if there is a justification for this.

In the case of the enzymatic method for nitrates determination in vegetable products, we achieved a calibration curve with 9 levels of nitrate ions standard (with three rehearsals for each one), in the concentrations range 6 mg/L – 30 mg/L, as following:

- ✓ level 1 – 6 mg/L
- ✓ level 2 – 9 mg/L
- ✓ level 3 – 12 mg/L
- ✓ level 4 – 15 mg/L
- ✓ level 5 – 18 mg/L
- ✓ level 6 – 21 mg/L
- ✓ level 7 – 24 mg/L
- ✓ level 8 – 27 mg/L
- ✓ level 9 – 30 mg/L

The achieved calibration curve starts from origin and linearity coefficient (r^2) is 0.9999.

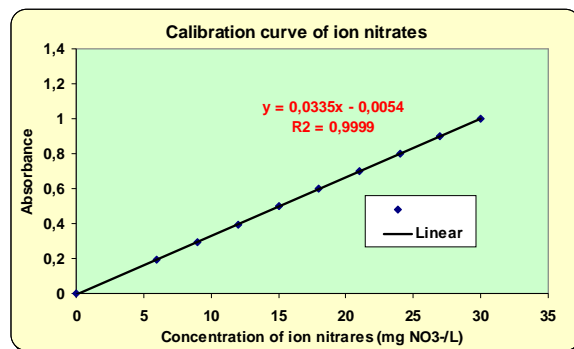


Figure 2. Calibration curve of ion nitrates

The equation of calibration curve is the following:

$$y = 0.0335 x - 0.0054 \quad (1)$$

of which:

y = absorbance measured at $\lambda = 340 \text{ nm}$
x = concentration of nitrate ions, in mg NO_3^-/L

By equation (1), knowing absorbance value, we can directly calculate the concentration of nitrate ions in a test sample, using the following formula:

$$x = \frac{y + 0.0054}{0.0335} \quad (2)$$

Accuracy means the ability to make an analysis with low difference between real value and founded value.

In the case of enzymatic method for determination of nitrate ions in vegetable products, accuracy is expressed through *recovery*.

Recovery is calculated by the equation (3):

$$\text{Recovery (\%)} = (c_d/c_t) \times 100 \quad (3)$$

of which:

c_t – theoretical concentration of nitrate ion, in mg/L

c_d – determined concentration of nitrate ion, in mg/L

In order to determine recovery, in the case of enzymatic method for determination of nitrate ion, we used 9 prepared solutions as in the case of the achievement of calibration curve.

The concentration of nitrate ions of those 9 nitrate solutions was determined in two ways:

- calculated by equation (2)
- using the enzymatic method

The concentrations of nitrate ions of those 9 solutions, using the enzymatic method, were calculated by formula (4):

$$C_{\text{nitrate}} = (1.01 \times V \times MW / \varepsilon \times d \times V) \times \Delta A \text{ in } [\text{mg NO}_3^-/\text{L sample solution}] \quad (4)$$

of which:

1.01 – correction factor of final volume of sample

V – final volume of sample (3.05 mL)

MW – molecular weight of nitrate ion (62 g/mol)

ε – extinction coefficient of NADPH (at $\lambda = 340 \text{ nm}$, $\varepsilon = 6.3 \text{ l} \times \text{mol}^{-1} \times \text{cm}^{-1}$)

d – length of optical way (1 cm)

V – sample volume, in mL

ΔA – difference between variance of sample absorbance and blank absorbance

Difference of absorbance for sample = $(A_1 - A_2)_{\text{sample}} - 2 \times (A_2 - A_3)_{\text{sample}}$

Difference of absorbance for blank = $(A_1 - A_2)_{\text{blank}} - 2 \times (A_2 - A_3)_{\text{blank}}$

$$\Delta A_{\text{nitrate}} = \Delta A_{\text{sample}} - \Delta A_{\text{blank}} \quad (5)$$

Changing these elements, we have obtained:

$$x = \frac{30.316 \times \Delta A_{\text{nitrate}}}{V} \quad (6)$$

The determination of concentrations of nitrate ions of 9 solutions of kalium nitrate was made by the formula (2) and *recovery* was in the range: 98.67 % - 100.23 %.

The determination of concentrations of nitrate ions of those 9 solutions of kalium nitrate was made by the formula (6), using the protocol of the enzymatic method, *recovery* was in the range: 97.00 % - 102.67 %.

Precision is reverberated by the concordance degree between independent

analytical results obtained under specified conditions.

Precision is a measure of results dispersion and is determined in conditions of repeatability and reproducibility.

Repeatability (r) – value below which the absolute difference between results of the two separated tests, achieved on the same test object, the same apparatus, the same executant and in the same short time spell, is below the specified probability limits (as a rule 95%). The limit of repeatability is calculated by the equation (7):

$$r = 2.8 \times S_D(r) \quad (7)$$

of which:

r – limit of repeatability;

$S_D(r)$ – standard deviation, calculated in the case of results obtained in repeatability conditions.

Relative standard deviation, RSD(r), obtained from the achieved results in *repeatability* conditions is calculated by equation (8):

$$RSD(r) = (S_D(r) / \bar{X}) \times 100 \quad (8)$$

of which:

$S_D(r)$ – standard deviation, calculated in case of the obtained results in repeatability conditions;

\bar{X} – arithmetic average of determined concentrations in repeatability conditions.

Reproducibility (R) – value below which the absolute difference between results of a single test, obtained for an identical material, in two or more laboratories, by different operators, on different apparatus, is below the specified probability limits (as a rule 95 %).

Relative standard deviation, RSD(R), obtained from the achieved results in *reproducibility* conditions is calculated by the equation (9):

$$RSD(R) = (S_D(R) / \bar{X}) \times 100 \quad (9)$$

of which:

$S_D(R)$ – standard deviation, calculated from the obtained results in reproducibility conditions;

\bar{X} – arithmetic average of the obtained results in reproducibility conditions.

Standard deviation – measure dispersal tendency of data and is calculated by equation (10):

$$S_D = \sqrt{\frac{\sum (X_i - \bar{X})^2}{n - 1}} \quad (10)$$

of which:

S_D – standard deviation;

X_i – result of measurement;

\bar{X} – arithmetic average of measurement results;

n – number of tests.

In order to determine repeatability in the case of enzymatic method for determination of nitrates in vegetable products, we analysed 10 parallel samples of vegetables cultivated in field (egg plants, bell peppers, green peppers and onion) and we calculated for each vegetable species the following:

- average concentration of nitrates
- value of relative standard deviation RSD(r), in repeatability conditions
- limit of repeatability

In the case of the 10 parallel samples of egg plants analysed to determine nitrates content, the average concentration of nitrates is 65.98 mg NO_3^-/kg , relative standard deviation RSD(r), for determined concentration is 0.77%, and the limit of repeatability is 1.43 mg NO_3^-/kg .

In the case of the 10 parallel samples of bell peppers analysed to determine nitrates content, the average concentration of nitrates is 27.91 mg NO_3^-/kg , relative standard deviation RSD(r), for determined concentration is 1.90%, and limit of repeatability is 1.49 mg NO_3^-/kg .

In the case of 10 parallel samples of green peppers analysed to determine nitrates

content, the average concentration of nitrates is 30.85 mg NO_3^-/kg , relative standard deviation RSD(r), for determined concentration is 1.54%, and limit of repeatability is 1.33 mg NO_3^-/kg .

In the case of 10 parallel samples of onion analysed to determine nitrates content, the average concentration of nitrates is 82.85 mg NO_3^-/kg , relative standard deviation RSD(r), for determined concentration is 0.65%, and limit of repeatability is 1.50 mg NO_3^-/kg .

In order to determine intralaboratory reproducibility in the case of the enzymatic method used in the determination of nitrates in vegetable products, we analysed by three analysts (analyst A – 3 parallel samples, analyst B – 3 parallel samples, analyst C – 3 parallel samples) one cabbage sample, carrots and onion and we calculated:

- average concentration of nitrates
- value of relative standard deviation RSD(R), in reproducibility conditions
- limit of reproducibility

In the case of the analysed cabbage sample by analysts for determination of nitrates content (each analyst performed 3 parallel samples), the average nitrates concentration is 107.42 mg NO_3^-/kg , and relative standard deviation RSD(R), for determined concentration is 0.81% and limit of reproducibility is 2.45 mg NO_3^-/kg .

In the case of the analysed carrots sample by analysts for determination of nitrates content (each analyst performed 3 parallel samples), the average nitrates concentration is 92.27 mg NO_3^-/kg , relative standard deviation RSD(R), for determined concentration is 1.24%, and limit of reproducibility is 3.20 mg NO_3^-/kg .

In the case of the analysed onion sample by analysts for determination of nitrates content (each analyst performed 3 parallel samples), the average nitrates concentration is 71.88 mg NO_3^-/kg , relative standard deviation RSD(R), for determined

concentration is 1.22%, and limit of reproducibility is 2.45 mg NO₃⁻/kg.

Limit of detection of the method is 0.15 mg/L and corresponds to a difference of absorbance between sample and blank ($\Delta A_{\text{nitrate}}$) of 0.010 (absorbances being measured at $\lambda = 340$ nm) and a sample maximum volume $V = 2$ mL.

Limit of quantification of the method is 0.30 mg/L and corresponds to a difference of absorbance between sample and blank ($\Delta A_{\text{nitrate}}$) of 0.020 (absorbances being measured at $\lambda = 340$ nm) and a sample maximum volume $V = 2$ mL.

Uncertainty is a parameter associated with the result of measurement that characterises the dispersion of the values that could reasonably be attributed to the measurand.

In order to estimate uncertainty the following steps should be taken:

- specification of measurand
- identification of the significant sources of uncertainty
- estimation of combined standard uncertainty
- estimation of extended standard uncertainty

Combined uncertainty is calculated with equation (11):

$$u_c = \sqrt{u_1^2 + u_2^2 + u_3^2 + u_4^2 + \dots + u_n^2} \quad (11)$$

of which:

u_c – combined uncertainty

$u_1, u_2, u_3, u_4, \dots, u_n$ – component uncertainties of the budget of uncertainties.

Extended uncertainty is calculated by equation (12):

$$U = k \times u_c \quad (12)$$

of which:

U – extended uncertainty

u_c – combined uncertainty

k – covering factor, for a confidence level of 95%, $k = 2$

Measurand = concentration of nitrates in vegetable products, expressed in mg NO₃⁻/kg.

In the case of determination of nitrates in vegetable products, through enzymatic method, significant sources of uncertainty are:

- ✓ measurement of volume of aqueous extract of sample
- ✓ weighting of sample
- ✓ measurement of absorbance of aqueous extract of sample

Relative standard uncertainty, within enzymatic method of nitrates determination, is calculated by equation (13) [5]:

$$\frac{u(x)}{x} = \sqrt{\left(\frac{u_{c1}}{2}\right)^2 + \left(\frac{u_m}{m}\right)^2 + \left(\frac{u_{abs.}}{\Delta A}\right)^2} \quad (13)$$

of which:

u_{c1} – combined standard uncertainty at measurement of the aqueous extract of sample

u_m – combined standard uncertainty at weighting of sample

$u_{abs.}$ – combined standard uncertainty at measurement of the aqueous extract of sample

m – weight of analysed sample, in g

ΔA – difference between variance of absorbance of sample and blank

x – nitrates concentration of sample, in mg/kg

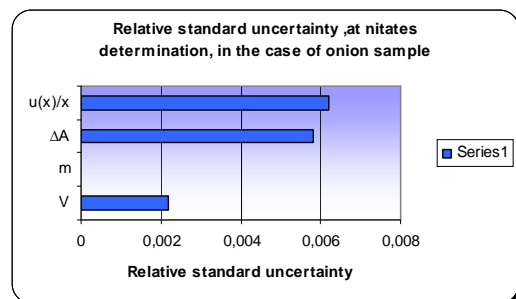


Figure 3. Relative standard uncertainty, at nitrates determination, in the case of onion sample

In the case of the analysed onion sample, by changing the suitable data, we obtained:

$$\frac{u(x)}{x} = 0.0062$$
$$x = 55.5 \text{ mg NO}_3^-/\text{kg sample}$$

$$u(x) = 0.34 \text{ mg NO}_3^-/\text{kg sample}$$

$$U(x) = k \times u(x)$$

of which:

$U(x)$ – extended uncertainty

k – covering factor for a confidence level of 95%, $k = 2$

$$U(x) = 0.68 \text{ mg NO}_3^-/\text{kg sample}$$

Therefore, result can be expressed in this way: $x = 55.5 \pm 0.68 \text{ mg NO}_3^-/\text{kg sample}$

Conclusion

1. This paper presents the results of the performed researches carried out to validate an enzymatic method for nitrates determination in vegetable products.

2. In the case of the enzymatic method for nitrates determination in vegetable products, the calibration curve achieved with 9 standard levels of nitrate ions (with three rehearsals for each one), in the range of concentrations 6 mg/L – 30 mg/L, starts from origin, and the linearity coefficient (r^2) is 0.9999.

3. For 10 vegetable samples grown in open field (egg plants, bell peppers, green peppers and onion) the average nitrate concentration of vegetable species varied in the range 27.91 mg NO_3^-/kg – 82.85 mg NO_3^-/kg , and repeatability was in the range 1.33 mg NO_3^-/kg – 1.50 mg NO_3^-/kg .

4. The determination of nitrates in a cabbage sample, by three analysts, led to an average nitrate concentration of 107.42 mg NO_3^-/kg , relative standard deviation RSD(R), for determined concentration is 0.81%, and reproducibility is 2.45 mg NO_3^-/kg .

5. In the case of carrots sample analysed by analysts to determine nitrates content (each

analyst performed 3 parallel samples), average nitrates concentration is 92.27 mg NO_3^-/kg , relative standard deviation RSD(R), for determined concentration is 1.24%, and limit of reproducibility is 3.20 mg NO_3^-/kg .

6. The detection limit of method is 0.15 mg/L and corresponds to a difference of absorbance between sample and blank ($\Delta A_{\text{nitrate}}$) of 0.010 (absorbances being measured at $\lambda = 340 \text{ nm}$) and a maximum volume of sample $V = 2 \text{ mL}$.

7. The quantification limit of method is 0.30 mg/L and corresponds to a difference of absorbance between sample and blank ($\Delta A_{\text{nitrate}}$) of 0.020 (absorbances being measured at $\lambda = 340 \text{ nm}$) and a maximum volume of sample $V = 2 \text{ mL}$.

8. In the case of onion sample grown in open field, nitrates concentration was 55.5 mg NO_3^-/kg , and uncertainty was 0.68 mg NO_3^-/kg .

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