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# ACCREDITATION OF THE ANALYTICAL METHOD USED FOR NITRATE DETERMINATION IN VEGETABLES

Veronika Kmecl[1] and Dragan Žnidarčič[2,*]

[1]*Agricultural Institute of Slovenia,* Hacquetova 17, 1000 Ljubljana, Slovenia
[2]*Department of Agronomy, Biotechnical Faculty, University of Ljubljana,* Jamnikarjeva 101, 1000 Ljubljana, Slovenia

[*]Corresponding author: dragan.znidarcic@bf.uni-lj.si

*Abstract* - The standard method of EN 12014-7 was used for the determination of nitrate content in vegetables in the Central Laboratory of the Agricultural Institute of Slovenia. Trueness of the method was verified by the analysis of samples in the interlaboratory proficiency testing scheme Bipea (*Bureau Interprofessionnel d¢Etudes Analytiques*). Linearity was confirmed by multiple linear regression; the correlation coefficient ($R^2$) was 0.9993. The limit of detection (LOD) was calculated as 0.8 mg $NO_3^-$/kg and limit of quantification (LOQ) 3.0 mg $NO_3^-$/kg. Relative standard deviation (RSD) of repeatability was 4.6% and RSD of reproducibility 8.9%. The relative uncertainty of repeatability and reproducibility were 10.5% and 20.2%, respectively. The method has been accredited by the French Accreditation Committee *COFRAC* in 2007 and by the national *Slovene Accreditation* in 2012.

*Key words*: nitrates; validation; accreditation; continuous flow method

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## INTRODUCTION

Nitrate is a naturally occurring compound that is a part of the nitrogen cycle (Handley and Raven, 1992), as well as an approved food additive (Walker, 1990). It plays a significant role in the nutrition and function of plants. It is an important component of vegetables due to its potential for accumulation. Vegetables are the largest source of dietary nitrate, contributing more than 90% of nitrate intake (Tamme et al., 2006). In terms of plant anatomy, the nitrate content of vegetable organs can be listed in descending order (most to least) as petiole > leaf > stem > root > inflorescence > tuber bulb > fruit > seed (Hord et al., 2009). Nitrate content in vegetables depends on different factors such the biological properties of plant culture (Blom-Zandstra, 1989), light conditions (Weightman et al., 2006), type of soil, temperature, humidity, frequency of plants in the field, plant maturity, vegetation period, harvesting time or type of fertilization (Gent, 2002; Guadagnin et al., 2005; Santamaria, 2006).

Crops and fruits contain much less nitrate; it is typical for the latter to have a very low capacity to accumulate $NO_3$- (Ysart et al., 1999). Nitrate is relatively non-toxic (Gangolli et al., 1994), but its metabolites and reaction products e.g., nitrite, nitric oxide and N-nitroso compounds, have raised concern because of implications for adverse health effects (Olszówka

and Perucka, 2011) such as methemoglobinemia or "blue baby syndrome" (Knobelock et al., 2000). This is a condition characterized by increased quantities of hemoglobin in which the iron of heme is oxidized to the ferric ($Fe^{3+}$) form. Methemoglobin is useless as an oxygen carrier and thus causes varying degrees of cyanosis (McKnight et al., 1999). In addition, several researches have suggested that the nitrite as such, and nitrate when reduced to nitrite, may react with amines or amides to form carcinogenic N-nitroso compounds (Bruning-Fann and Kaneene, 1993; Hsu et al., 2009). Epidemiological studies have not provided any evidence that there is an increased risk of cancer related to high nitrate intake from sources other than vegetables (Gangolli et al., 1994). On the other hand, there are some suggestions that the protective effect of certain vegetables on the cardiovascular system is related to their high content of inorganic nitrate, which in concert with symbiotic bacteria in the oral cavity is converted into nitrite, nitric oxide and secondary reaction products with vasodilating and tissue-protective properties (Lundberg et al., 2006).

To protect human health, most European countries regulate nitrate content in food (Hmeljak Gorenjak and Cencič, 2013). Commission Regulation No 1881/20063 and Commission Regulation No 1258/20114 (EC, 2011) set maximum levels for nitrates in foodstuffs which were adopted by Slovene regulation. The regulation prescribes maximum levels of specific foodstuff such as fresh spinach, fresh lettuce, rocket, processed cereal-based foods and baby foods for infants and young children.

There are several analytical methods for the determination of nitrates in different samples; automated colorimetric methods using reduction with cadmium, direct spectrophotometric methods, ion chromatographic method, polarimetric methods, fast UV screening methods, tests with nitrate-selective electrodes or quick tests with Reflectoquant applicable in the field (Raikos et al., 1988). The most suitable method for nitrate determination in vegetable samples is considered to be the automated colorimetric method with reduction with cadmium. In our laboratory, the automated colorimetric method (CFA, continuous flow analysis technique) was used for the determination of mineral nitrogen in soil samples and gave accurate and reliable results (Kmecl et al., 2005).

Further, we wanted to introduce the analytical method for nitrate determination in plant samples. The Agricultural Institute of Slovenia led the study for the monitoring and assessment of pollution by nitrates of agricultural products in collaboration with the Ministry of Agriculture in Slovenia. For this purpose, we validated and accredited the method for determination of nitrate in vegetables according to EN 12014-7 (1998).

## MATERIALS AND METHODS

### *Method development*

Analyses of mineral nitrogen (N-min) were conducted on filtered samples using a Skalar San++ multichannel continuous flow autoanalyzer, with channels set up for simultaneous determination of nitrate-N plus nitrite-N analyses, using automated procedures based on the colorimetric techniques of Henriksen and Selmer-Olsen (1970).

### *Preparation of reagents and standard solutions:*

First grade reagents and deionized Milli-Q water were used. A standard solution of $NO_3$-N (10.000 mg $NO_3$-N $L^{-1}$): 6.068 g $NaNO_3$ was dissolved in 100 ml deionized Milli-Q water. In the preparation of analysis, adequate aliquots of standard solution were taken and diluted with deionized Milli-Q water. Standard solution $NO_2$-N (10 000 mg $NO_3$-N $L^{-1}$): 4.9486 g $NaNO_2$ was dissolved in 100 mL deionized Milli-Q water. In the preparation of analysis, adequate aliquots of standard solution were taken and diluted with deionized Milli-Q water.

### *Solutions*

Thirty-three g of potassium sodium tartrate ($C_4H_4O_6KNa.4H_2O$) was dissolved in ±800 ml water; 24 g of trisodium citrate ($C_6H_5O_7Na_3.2H_2O$) was added and diluted to 1 L with water; 3 ml of Brij-35 was

added. Six g of potassium hydroxide (KOH) was dissolved in 1 L water; 3 ml Brij-35 was added. Twelve g of copper (II) sulphate ($CuSO_4.5H_2O$) was dissolved in 1 L deionized water Milli-Q. 1.7 g hydrazine sulphate ($N_2H_6SO_4$) was dissolved in ±800 ml deionized Milli-Q water; 1.5 ml copper sulphate solution was added and diluted to 1 L with Milli-Q water.

*Sulfanilamide reagent*

100 ml 85 % orthophosphorous acid ($H_3PO_4$), 10 g sulfanilamide ($C_6H_8N_2O_2S$) and 0.5 g N-naphthylethylene diamine dihydrochloride ($C_{12}H_6Cl_2N$) were added to a 1 L flask. Deionized Milli-Q water was used to dilute the solution to 1 L. Brij-35 was 30%)

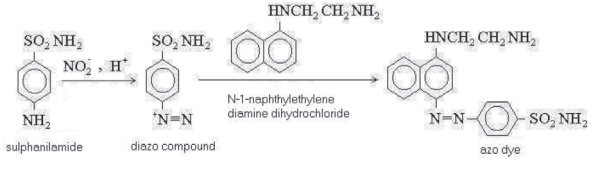
*Sample preparation*

Frozen vegetable samples of lettuce were homogenized. Before extraction, the samples were kept at -18°C. The extraction was conducted with water. Forty g of the frozen, homogenized sample was mixed with 360 mL of water and homogenized for at least 1 min and filtered. The extract was analyzed using a continuous flow analyzer (CFA) on the same day.

*Principle of nitrate determination in vegetables by CFA*

Nitrate is reduced to nitrite with hydrazine sulphate; the nitrite ion reacts with sulfanilamide under acidic conditions to form a diazo compound. This compound reacts with N-1-naphthylethylene diamine dihydrochloride to form a reddish-purple azo dye, measured photometrically at 540 nm. The result is the sum of nitrate and nitrite. To express only the nitrate, the preliminary determined nitrite has to be subtracted.

Reduction of nitrate into nitrite: $NO_3^- + 2e^- \rightarrow NO_2^-$




Transformation of $NO_2^-$ to the reddish-purple azo-dye:

Method validation

Method validation was performed on the frozen sample of lettuce. We verified the accuracy, linearity, limit of detection (LOD), limit of quantification (LOQ), precision (repeatability and reproducibility) and the uncertainty (Miller and Miller, 1988; Eurachem, 1998; ISO 13528, 2005).

*Accreditation*

In 2007, the Central Laboratory of the Agricultural Institute of Slovenia submitted an application to the French Accreditation Committee (COFRAC) to accredit a method for nitrate determination ($NO_3^-$) in vegetables according to EN 12014-7.

RESULTS AND DISCUSSION

*Method development*

The flow diagram performed with CFA is presented in Fig. 1.

Due to the complexity and sensitivity of the process, a recovery of reaction (reduction of nitrates to nitrites) was determined. For this purpose, standard solutions $KNO_3$ and $NaNO_2$ of the concentration of 2.0 mg $NO_3^-$-N/L were prepared. The recoveries ranged between 90 and 105%; we concluded that the reduction of nitrate to nitrite was quantitative.

*Method validation*

Trueness was verified by the analysis of samples from the proficiency-testing scheme organized by Bipea (Bureau Interprofessionnel d'Etudes Analytiques, France). The statistical treatment in Bipea is carried out in accordance with ISO 13528 standard "Statistical methods for use in proficiency testing by inter-laboratory comparisons". The minimum and the maximum values (mean ± 2x standard deviation) define the tolerance interval inside which the result can

**Table 1**. $NO_3$ content in vegetable samples.

| Name of scheme | Sample | Unit | Criteria (Min. - Max.) | Laboratory results | Z -value |
|---|---|---|---|---|---|
| Bipea, No. 19 ($NO_3^-$) | 1 | mg/kg | 1001 - 1669 | 1279 | -0.34 |
| | 2 | mg/kg | 852 - 1420 | 1089 | -0.33 |
| | 3 | mg/kg | 2022 - 3370 | 2903 | 0.61 |
| | 4 | mg/kg | 1710 - 2850 | 2619 | 1.19 |
| | 5 | mg/kg | 578 - 964 | 746 | -0.26 |
| | 6 | mg/kg | 1935 - 3225 | 2722 | 0.44 |
| | 7 | mg/kg | 1333 - 2221 | 1550 | -1.02 |
| | 8 | mg/kg | 1552 - 2588 | 2105 | 0.14 |
| | 9 | mg/kg | 16 - 38 | 20 | -1.27 |
| | 10 | mg/kg | 462 - 770 | 716 | 1.30 |
| | 11 | mg/kg | 156 - 260 | 256 | 1.85 |
| | 12 | mg/kg | 168 - 280 | 264 | 1.43 |
| | 13 | mg/kg | 970 - 1616 | 1371 | 0.48 |
| | 14 | mg/kg | 633 - 1055 | 956 | 1.06 |
| | 15 | mg/kg | 646 - 1076 | 875 | 0.13 |
| | 16 | mg/kg | 331 - 551 | 469 | 0.51 |
| | 17 | mg/kg | 128 - 212 | 182 | 0.57 |

**Table 2**. Results of multiple linear regression analysis.

| Concentration level (mg $NO_3^-$-N /L) | 0.40 | 0.80 | 1.20 | 1.60 | 2.00 |
|---|---|---|---|---|---|
| Instrument response (mg $NO_3^-$-N /L) | 0.39 | 0.78 | 1.20 | 1.63 | 1.99 |
| | 0.40 | 0.81 | 1.19 | 1.60 | 2.00 |
| | 0.39 | 0.79 | 1.20 | 1.63 | 1.99 |
| | 0.44 | 0.78 | 1.20 | 1.60 | 2.00 |
| | 0.38 | 0.81 | 1.20 | 1.60 | 2.00 |

| Relation : y = b·x + a | | Slope (b) | Intercept (a) |
|---|---|---|---|
| Factors of the curve | | 1.00 | -0.003 |
| S.D. of the factors | | 0.01 | 0.01 |
| Lower limit | 99% | 0.99 | -0.02 |
| Upper limit | 99% | 1.02 | 0.02 |

| | |
|---|---|
| S.D. of the residues | 0.02 |
| Limit of detection | 0.02 |
| Limit of quantification | 0.07 |

| Linearity test | | | | | | |
|---|---|---|---|---|---|---|
| Sources of variation | Square differences | Degree of freedom | Estimated variance | F | Critical value | 1% |
| Regression | 8 | 1 | 8.04 | 36545.68 | 8.10 | *The linear model is fit* |
| Error of the model | 0 | 3 | 0.0003 | | | |
| Experimental error | 0 | 20 | 0.0002 | 1.44 | 4.94 | *The range is correct* |
| Sum | 8 | 24 | | | | |

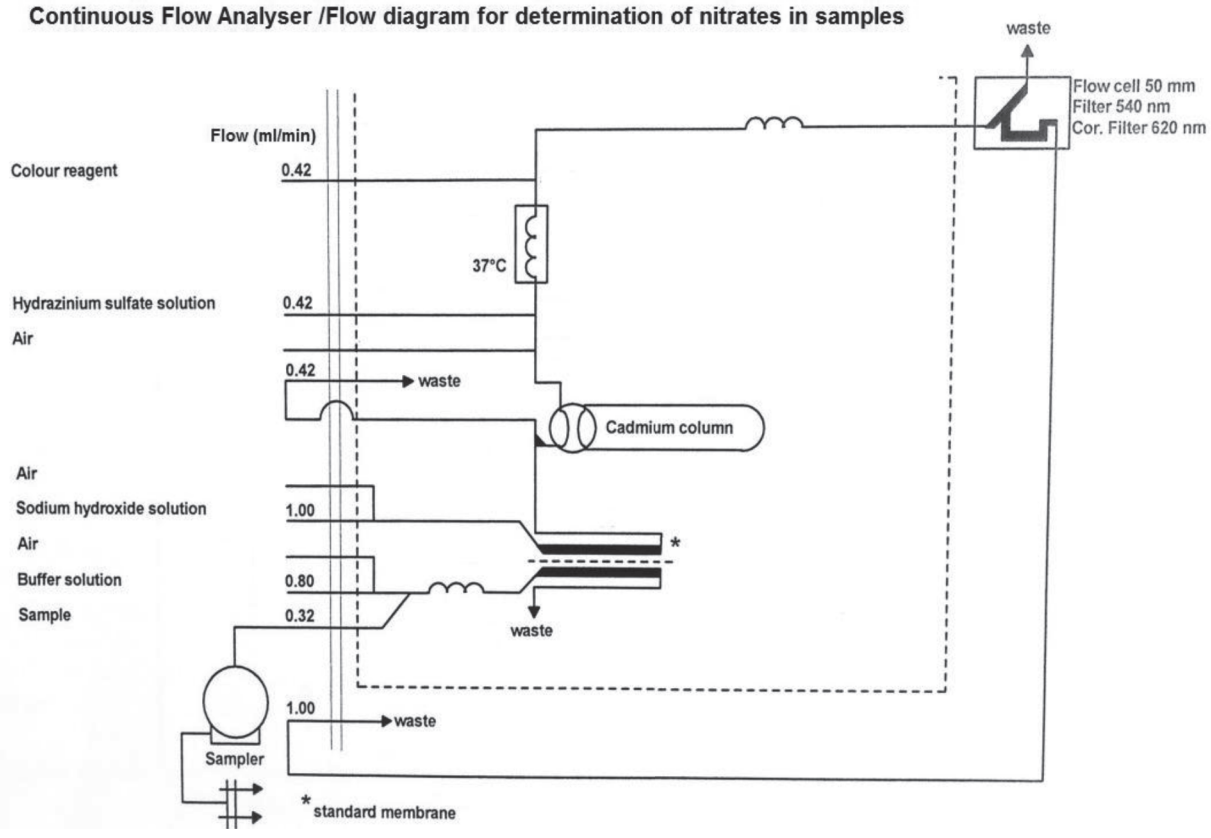



**Fig. 1.** Flow diagram for determination of $NO_3^-$ in vegetable samples/Continuous Flow Analyser (Skalar, San++).

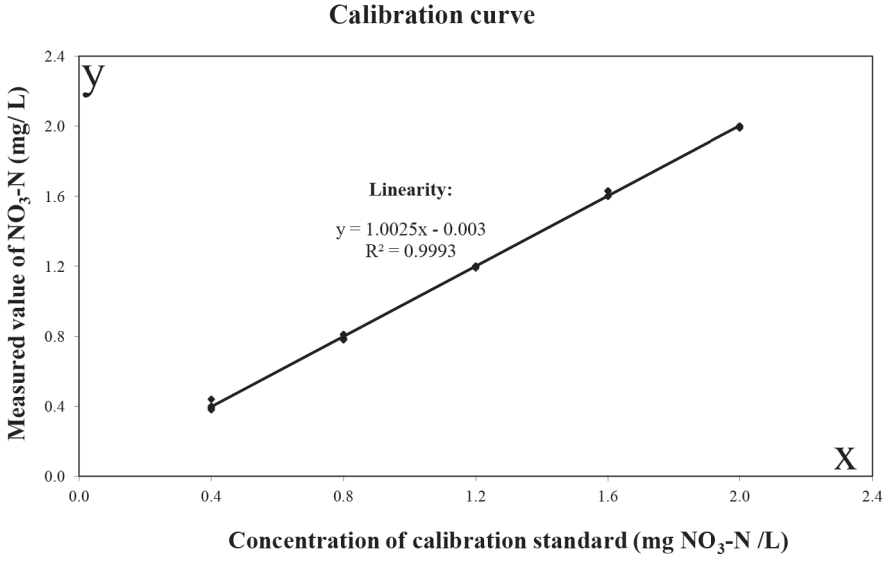



**Fig. 2.** Linearity, expressed by equation and correlation coefficient ($R^2$) of the calibration curve (five calibration standards with concentrations 0.4, 0.8, 1.2, 1.6 and 2.0 mg /L as $NO_3^-$-N): linear equation y = 1.0025 x -0.003; correlation coefficient $R^2$= 0.9993.

**Table 3**. Repeatability and reproducibility of the obtained data

| Sample of lettuce | $NO_3^-$ |
|---|---|
| Means of the levels (mg/kg) | 82.1 |
| Standard deviation (SD) of repeatability of the level (mg/kg) | 3.81 |
| Relative standard deviation (RSD) of repeatability of the level (%) | 4.6 |
| Stand. deviation (SD) of reproducibility of the level (mg/kg) | 7.36 |
| Relative standard deviation (RSD) of reproducibility of the level (%) | 9.0 |

**Table 4**. Uncertainty of the obtained data

| Sample of lettuce | $NO_3^-$ |
|---|---|
| Means of the levels (mg/kg) | 82.1 |
| Uncertainty of repeatability (mg/kg) | 8.61 |
| Relative uncertainty of repeatability (%) | 10.5 |
| Uncertainty of within laboratory reproducibility (mg/kg) | 16.6 |
| Relative uncertainty of within laboratory reproducibility (%) | 20.2 |

be accepted as correct. The tolerance interval, Z-value, minimal and maximal value and results obtained in our laboratory are presented in Table 1.

The linearity of method was confirmed by the analysis of five calibration standards with concentrations of 0.4 mg/L, 0.8 mg/L, 1.2 mg/L, 1.6 mg/L, 2.0 mg/L (as $NO_3^-$ N) in five repetitions. The data were calculated by multiple linear regression, which gave us the correlation coefficient of calibration curve (Table 2). The correlation coefficient ($R^2$) was 0.9993 (Fig. 2).

LOD and LOQ were calculated using a linearity test. The correlation coefficient 0.9993 confirms a high degree of linearity and the straight line could be used as a calibration curve at determine LOD and LOQ. Regarding this test, the value of LOD was calculated as 0.02 mg $NO_3$-N/L and LOQ was 0.1 mg $NO_3$-N/L. Taking into account the sample preparation, LOD was 0.8 mg $NO_3^-$/kg and LOQ was 3.0 mg $NO_3^-$/kg (for real vegetable sample).

For the determination of precision, we analyzed samples of lettuce in a concentration range of 82.1 mg/kg $NO_3^-$ during 10 days, with 2 repetitions each day. ANOVA was used in the analysis of experimental data. Dispersions of results were checked with the Cochran test and outliers with the Grubbs test. We calculated the standard deviation of repeatability (measurements within one day) and the standard deviation of reproducibility (measured several days consequently). The results are shown in Table 3.

The repeatability and reproducibility of results are presented with standard deviation (SD) and relative standard deviation (RSD). The SD of repeatability was two times lower than that of reproducibility. In the concentration range of 82.1 mg $NO_3^-$/kg, the RSD of repeatability of the level was 4.6% and of reproducibility 9.0%. The results obtained in one day were more reproducible as compared to results obtained between days.

We calculated the uncertainty of repeatability and uncertainty of within laboratory reproducibility by multiplying standard deviation of repeatability and reproducibility by Student's t factor for 9 degrees of freedom and a 95% confidence level ($t_{95,9}$= 2.262). Uncertainty for measurement of the lettuce sample in the concentration range of 82.1 mg $NO_3^-$/kg is presented in Table 4.

Uncertainty of reproducibility (16.6 mg/kg) was almost two times higher than uncertainty of repeat-

ability (8.61 mg/kg). Relative uncertainty of repeatability was 10.5% and relative uncertainty of reproducibility 20.2 %. The results indicated a significant uncertainty of the measurements. The reason is insufficient homogeneity of the fresh lettuce samples. The results of validation are within the values, specified by the standard EN 12014-7.

**Accreditation**

The method for determination of nitrates in vegetables developed in our lab was confirmed by the French Accreditation Committee *COFRAC* and accredited in 2007, the national accreditation body (*Slovenska akreditacija)* in 2012.

## CONCLUSION

The Central Laboratory of the Agricultural Institute of Slovenia has been involved in the monitoring program and assessment of contamination of agricultural products with nitrates. For this purpose, we validated and accredited the method for determination of $NO_3^-$ in vegetable samples according to EN 12014-7. Frozen vegetable samples of lettuce were extracted with water and the measurement of $NO_3^-$ was performed with a continuous flow analyser (CFA). Validation of the method revealed that the results are accurate and precise; trueness of measurements was verified by the successful collaboration of our laboratory in the proficiency testing scheme Bipea for many years; precision was proven from repeatability and reproducibility data. RSD of repeatability of the level was 4.6% and RSD of reproducibility 8.9%. We determined the uncertainty of repeatability (8.61 mg $NO_3^-$/kg) and uncertainty of reproducibility (16.6 mg $NO_3^-$/kg) in the concentration range of 82.1 mg $NO_3^-$/kg lettuce. The linearity was verified by multiple linear regression. The correlation coefficient of calibration curve was 0.9993 and confirmed a high degree of linearity in the concentration range 0.4 to 2.0 mg $NO_3$-N /L. Limit of detection and limit of quantification were calculated using a linearity test. LOD was 0.8 mg $NO_3^-$/kg and LOQ 3.0 mg $NO_3^-$/kg.

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