Evaluation of the nitrate and nitrite content of vegetables commonly grown in Slovenia

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Abstract

Nitrate (NO_3^-) and nitrite (NO_2^-) levels of a total 1195 samples of nine different vegetables (lettuce, potato, cabbage, carrot, string beans, tomato, cucumber, cauliflower and pepper) collected at several locations of an intensive agricultural area in Slovenia were analysed during a period of 13 years. The content of NO_2^- and NO_3^- ions in commercial mature samples was determined using a segmented flow analyser. The average NO_3^- content was the highest in lettuce (962 mg/kg), cabbage (795 mg/kg), string beans (298 mg/kg), carrot (264 mg/kg), cauliflower (231 mg/kg), potato (169 mg/kg) and was moderately high in cucumber (93 mg/kg) and pepper (69 mg/kg). A low NO_3^- content was found in tomato (<10 mg/kg). The average values of NO_2^- did not exceed 0.5 mg/kg, with the exception of potato (1.08 mg/kg). Six samples of lettuce exceeded the maximum permissible level of NO_3^- according to current European Union (EU) legislation. Based on the results of our investigation, we assessed the approximate daily intake (DI) of NO_3^- and NO_2^- to human body. The results indicated that with the consumption of potato, the daily intake per inhabitant is close to the acceptable DI permitted in EU.

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Introduction

Nitrates are naturally occurring compounds of nitrogen and the formation of nitrates is an integral part of the nitrogen cycle in the environment. They appear from fertilisers, decaying plants, manure and other organic residues (Prasad and Chetty, 2008). Nitrates are found in the air, soil, water and food. They are also used as a food additive, mainly as a preservative and antimicrobial agent (Walker, 1990; Gangolli *et al.*, 1994; Spejiers, 1996; Speijers and van der Brandt, 2003).

Until recently, concern over the amounts of nitrate and nitrite in our diet has been due to the relationship between nitrate and nitrite and infant methemoglobinemia. The role of nitrites in the formation of carcinogenic nitrosamines has led to some public apprehension about the nitrite content of our food (Knobelock *et al.*, 2000). The nitrate (NO_3^-) nitrogen (N) form has a low level of acute toxicity but it can be transformed into the nitrite N (NO_2^-) form, which has much higher acute toxicity (Santamaria, 2006). It has been estimated that about 4-8% of the nitrate from the diet may be reduced to nitrite by the micro flora in the oral cavity (Ashby, 2008; Lundberg and Weitzberg, 2009). Some studies have shown that nitrate exposure is correlated with gastric cancer risk due to the endogenous formation of N- nitroso compounds (Jakszyn and González, 2006).

The intake of nitrite is normally low compared with toxic level doses, but nitrite in food is considered to be a health problem primarily because its presence in both food and the human body may lead to the formation of nitrosamines. Such effects have been shown in animal experiments but their relevance to humans is still uncertain (Nie *et al.*, 2009). There is thus increasing concern about contamination in food, especially vegetables, with nitrate and nitrite (Petersen and Stoltze, 1999; Vaessen and Schothorst, 1999; Ysart *et al.*, 1999). According to Knight *et al.* (1987) vegetables are generally considered the main source of dietary nitrate in the human diet. Some vegetables, such as Swiss chard (*Beta vulgaris* L.) (Parks *et al.*, 2008), rape (*Brassica campestris* L.), Chinese cabbage (*Brassica chinensis* L.), spinach (*Spinacia oleracea* L.) (Chen *et al.*, 2004), lettuce (*Lactuca sativa* L.) (Huett and White, 1992) and salad rocket (*Eruca sativa* Mill.) (Cavaiuolo and Ferrante, 2014), contain nitrate at significant levels. These vegetables often contain nitrate concentrations above 2500 mg kg^{-1}, especially when they are cultivated in greenhouses (Santamaria, 2006). On the other hand, the nitrate content of vegetables can also be affected by the geographical region, day (light) intensity and duration, soil texture, soil temperature, humidity, density of plants in the field, vegetation period, season of harvest and processing time (Guadagnin *et al.*, 2005; Tamme *et al.*, 2006; Weigtman *et al.*, 2006; Parks *et al.*, 2012).

To protect human health, most European countries have regulated the nitrate content in food (Santamaria, 2006). Commission Regulation EU No 1258/2011 (European Commission, 2011) amending Regulation EC No 1881/2006 regulates maximum levels for nitrates in vegetables, and these have also been adopted by Slovene regulations (Table 1).

The Joint Expert Committee of Food and Agriculture (JECFA) and the European Commission's Scientific Committee on Food (SCF) have set admissible nitrate and nitrite intake values for the human body. The acceptable daily intake (ADI) for NO_3^- is 0-3.7 mg kg^{-1} body weight per day and for NO_2^- 0-0.07 mg kg^{-1} body weight per day. These values are equivalent to 222 mg of NO_3^- or 4.2 mg of NO_2^- per day for a 60 kg adult (Hmeljak and Cencič, 2013). The objective of the research was to determine nitrogen forms and nitrogen rates of nitrate accumulation in various vegetable samples commonly grown in Slovenia and to compare them with relevant legislation. The defined approximate daily intake (ADI) of nitrates and nitrites to the human body were compared with the ADI acceptable in EU. The study was partly based on a previous investigation (Sušin *et al.*, 2006) and was supplemented with new data of nitrate and nitrite daily intake estimates in the Slovene population.

Materials and methods

Plant material collection

Over a period of 13 years, nine species of vegetable (lettuce, potato, cabbage, carrot, string beans, tomato, cucumber, cauliflower and pepper) were included in monitoring, and a total of 859 samples were taken (Table 2). Samples were collected from eight agricultural production areas (Celje, Koper, Kranj, Ljubljana, Maribor, Murska Sobota, Nova Gorica, and Novo Mesto) evenly all over Slovenia (Figure 1). All production areas in this study were managed in accordance with guidelines of the Chamber of Agriculture and Forestry of Slovenia.

Figure 1. Sampling sites in Slovenia (1-Nova Gorica, 2-Koper, 3-Kranj, 4-Ljubljana, 5-Novo Mesto, 6-Celje, 7-Maribor, and 8-Murska Sobota).

Table 1. Regulated values of nitrates in foodstuff (European Commission, 2011).

Foodstuff	Harvest period	Maximum nitrate levels (mg kg^{-1})
Fresh spinach (*Spinacia oleracea*)	-	3500
Preserved, deep frozen or frozen spinach	-	2000
Fresh lettuce (*Lactuca sativa*) (greenhouse and open air grown lettuce) excluding Iceberg type lettuce (greenhouse grown lettuce)	1st October to 31st March 1st October to 31st March (lettuce grown in the open air) 1st April to 30th September (lettuce grown under cover) 1st April to 30th September (lettuce grown in the open air)	5000 4000 4000 3000
Iceberg type lettuce	1st October to 31st March (lettuce grown under cover) 1st April to 30th September (lettuce grown in the open air)	2500 2000
Rucola (*Eruca sativa, Diplotaxis sp., Brassica tenuifolia, Sisymbrium tenuifolium*)	1st October to 31st March 1st April to 30th September	7000 6000
Processed cereal-based foods and baby foods for infants and young children	-	200

Table 2. Information of plant samples analysed during thirteen consecutive seasons (2002-2014).

Vegetable	Harvest time	Part of plant	Analysed samples (n)
Lettuce	May-October	Leaves	319
Cabbage	September-October	Vegetative buds	67
String beans	June-September	Pods	92
Carrot	July-September	Roots	65
Cauliflower	September-October	Flowers	106
Potato	July-September	Tuber	267
Cucumber	July-September	Fruits	80
Pepper	August-September	Fruits	95
Tomato	July-September	Fruits	104

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Samples were taken from producers directly at the production sites at the time of commercial maturity of products. Only healthy und undamaged samples, fulfilling market demands, were collected. Immediately after sampling, the samples were taken to the laboratory in polyethylene bags using a portable refrigerator.

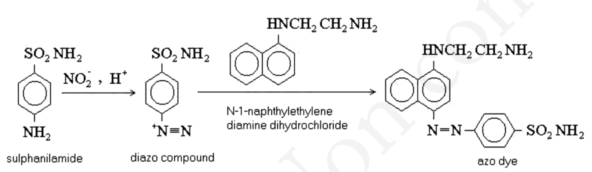
Samples preparation

Among the vegetables that were taken to the laboratory, the leafy part of the sample was analysed in lettuce, the vegetative buds of cabbage, the pods of string beans, the roots of carrot, the flowers of cauliflower, the tubers of potato and the fruits of cucumber, pepper and tomato. Prior to analysis, the fresh plant samples were minced and homogenised and 20 g of the sample was weighed into a 200 mL flask, 150 mL hot deionised water milliQ was added and the flask heated on a water bath for 20 minutes at a temperature of 95°C. Two mL of Carezzo solution (300 g/L $ZnSO_4$ x $7H_2O$) and 10 mL borax solution (50 g/L $Na_2B_4O_7$ x $10H_2O$) were added. The flask was filled up to the mark with deionised water milliQ. The extract was filtered through filter paper (Schleicher: Schuell, No. 5891) and the filtrate was analysed according to Naumann and Bassler (1988).

Determination of nitrate and nitrite

Nitrate and nitrite in vegetables were determined with a Segmented Flow Analyzer (AA II, Bran+Luebbe). The first step was the reduction of nitrate to nitrite on a copper coated cadmium column ($NO_3^- + 2e^-$ ® NO_2). The second step was the reaction of nitrites with appropriate reagents to form a colored compound: nitrites react with sulphanilamide under acidic conditions to form a diazo compound. It couples with N-(1-Naphthyl)ethylenediamine dihydrochloride (NEDD) to form a purple azo-dye.

Scheme of transformation of NO_2^- into redish-purple azo-dye:



The intensity of colored compound was measured photometrically by a Segmented Flow Analyzer at a wavelength of 540 nm. The result is the sum of nitrate and nitrite. To express only the nitrate, the preliminarily determined nitrite must be subtracted.

A flow diagram for determination of NO_3^- in vegetable samples by continuous flow analysis (CFA) (San^{++}) is also presented in Figure 2.

Confirmation of quality assessment data

The method for determination of nitrates in vegetables is accredited in our Agrochemical Laboratory of the Agricultural Institute of Slovenia (Kmecl and Žnidarčič, 2015). The accuracy of measurements was verified in collaboration with the Dutch international inter-laboratory comparative scheme WEPAL (Wageningen Evaluating Programme for Analytical Laboratories) and the French comparative scheme BIPEA (Bureau Interprofessionnel d'Etudes Analytiques). The values found were within the 95% confidence interval for the test materials.

Data analysis and calculation of daily intake of nitrate and nitrite

Data were statistically analysed for each vegetable separately using the statistical software package R (R Development Core Team, 2010). Classical descriptive statistics was done. We determined the median content of NO_3^- and/or NO_2^-, the coefficient of variation within an individual vegetable, the class in which the vegetable can be placed according to the median content and the number of individual samples analysed.

From the obtained data we calculated the daily intake (DI) of nitrates and nitrites into the human body. DI was calculated on estimations based on annual vegetable consumption per inhabitant.

Results and discussion

Content of the nitrate form in vegetables

The nitrate content of selected vegetables in Slovenia is presented in Figure 3. The results obtained show a considerable variation in the nitrate content within the same vegetable species. As

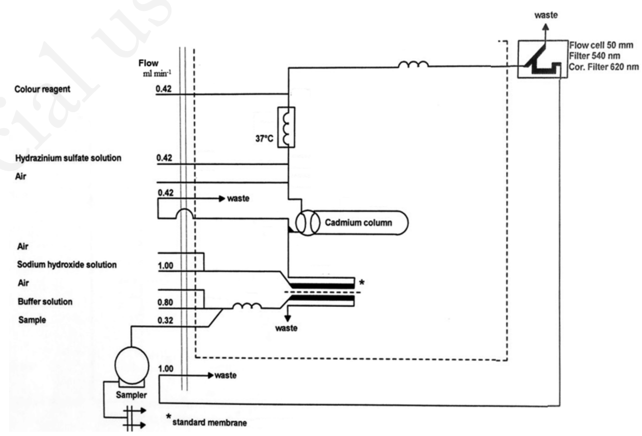


Figure 2. Flow diagram for determination of nitrate in vegetable samples by continuous flow analysis (San++).

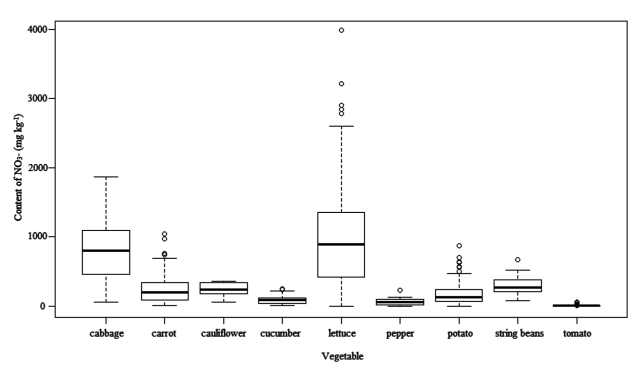


Figure 3. Box plots of nitrate contents (mg kg^{-1}) in nine vegetables across all locations. Boxes encompass the upper and lower quartiles, while the line indicates the median. Circles are outliers.

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previously described, the nitrate content in vegetables depends on many factors, such as soil properties, fertiliser usage, cultivation and weather conditions, which are unknown and whose effects are impossible to account for in this study. Considering all these different factors, wide ranges and large standard deviation may occur (Pennington, 1998). On the other hand, the differentiation in nitrate concentration can be explained by the variable intensity of metabolic processes in the different organs of plants (Kovacik, 1994). This may also explain the high variation among the findings presented in this study. Leafy vegetables (lettuce and cabbage) accumulated the highest nitrate content compared to other vegetables. A high amount of NO_3^- was found in lettuce but the median concentration of it was 962 mg kg^{-1}. The range of NO_3^- in cabbage was from 56 to 1964 mg kg^{-1}, and it was under maximum levels for leafy vegetables. The obtained results appear to be about 200% higher than the values reported by Yordanov *et al.* (2001) and Czech *et al.* (2012) in their studies on cabbage harvested in Bulgaria and Poland. It seems that lettuce contributes to the highest dietary nitrate intake from vegetables in Slovenia. DeMartin and Restani (2003) in Italy, Tamme *et al.* (2006) in Estonia, Merino *et al.* (2006) in Sweden and Menard *et al.* (2008) in France also found a very high content of nitrate in lettuce. On the other hand, some findings did not agree with these reports. For example, Fytianos and Zarogiannis (1999) in Greece reported that the nitrate content in lettuce was 282 mg/kg^{-1}. Some researchers have suggested (Hsu *et al.*, 2009) that this dissimilarity may be due to horticultural practices, such as the use of nitrate-based fertilisers.The median nitrate contents for vegetables such as string beans, carrot, cauliflower and potato (100 to 300 mg kg^{-1} of NO_3^-) were similar and comparable to the results obtained for vegetables in other European countries, particularly those reported in Italy (DeMartin and Restani, 2003) and Poland (Jaworska, 2005). String beans accumulated the most nitrates (298 mg kg^{-1}), followed by cauliflower (231 mg kg^{-1}), carrot (264 mg kg^{-1}) and potato (169 mg kg^{-1}). Moderate concentrations of NO_3^- were found in cucumber (93 mg kg^{-1}) and pepper (69 mg kg^{-1}). The least nitrates were found in tomato samples (on average 3 mg kg^{-1}), although the coefficient of variation was high. Present legislation in the European Union regulates the maximum values of nitrates in spinach, lettuce, rucola and processed cereal-based foods (Table 1). When the analysed values of nitrates in lettuce were compared to the prescribed limits, six samples out of a total of 319 exceeded the maximum admissible value.

Content of the nitrite form in vegetables

The results for nitrite in vegetables are shown in Figure 4. The nitrite content in vegetables was not as high as that of nitrate. This observation is consistent with the results of Petersen and Stoltze (1999) and Correia *et al.* (2010), who found only minor quantities of nitrites in several vegetables.

The highest nitrites were found in potato, with a median concentration of 1.08 mg kg^{-1}. The range of individual samples was between LOQ and 7.64 mg kg^{-1} of NO_2^-. Due to the very high coefficient of variation (100%), some samples deviated markedly from the average value. In other vegetable samples, the average nitrite concentration was under 0.3 mg kg^{-1}. A high variability of nitrite measurements was observed in lettuce, tomato and cucumber samples (CV= 60-100%), in which the range of NO_3^- was between LOQ and 1.58. Although vegetables contain a minor amount of nitrite, this can be increased significantly by microbiological reduction of nitrate if they are stored incorrectly. It was suggested by Zhong *et al.* (2002) that this is especially a problem for leafy vegetables, because soil adhering to them may be difficult to remove completely.

Nitrate and nitrite forms intake by human body

According to Hmeljak and Cencič (2013), the primary variables for nitrate intake are the type of vegetables consumed, the levels of nitrate in the vegetables and the amount of vegetables consumed. For example, Van Velzen *et al.* (2008) reported that nitrate from leafy vegetables is absorbed very effectively, resulting in an absolute nitrate bioavailability of around 100%.

Based on the data obtained, we calculated the daily intake (DI) of nitrates and nitrites and compared it with the acceptable daily intake (ADI) valid in he European Union. The DI was calculated on estimations based on annual vegetable consumption per inhabitant over a period of 13 years. The results indicated that potato is the most consumed agricultural product in Slovenia (the average consumption per inhabitant is as high as 74.8 kg potato per year) (Table 3).

The average nitrate content of 267 potato samples was 169 mg kg^{-1} (Table 4). In terms of potato consumption, the nitrate DI is 34.6 mg per inhabitant. Considering the maximum measured nitrate content of potato (871 mg kg^{-1}), the DI increase to 179 mg per inhabitant. This value is close to the ADI 222 mg of NO_3^- that is permitted in the EU. The nitrate daily intake of lettuce and cabbage samples is 79.9 mg per inhabitant (lettuce) and 67.8 mg per inhabitant (cabbage) and 33.7 mg per inhabitant (carrot) considering the maximum measured content of nitrates in both cultures. The DI for other vegetables (string beans, tomato, cucumber, cauliflower and pepper) does not reach even 10 mg of NO_3^- per inhabitant (Table 4). If the potential intakes of nitrate from vegetables in

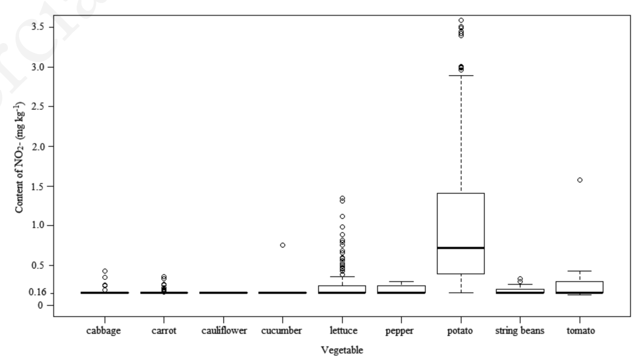

Figure 4. Box plots of nitrite contents (mg kg^{-1}) in nine vegetables across all locations. Boxes encompass the upper and lower quartiles, while the line indicates the median. Circles are outliers. Limit of quantification is at 0.16 mg kg^{-1}.

Table 3. Consumption of vegetables (kg) per inhabitant per year in Slovenia.

Vegetable	Average (2002-2014) Consumption (kg/inh.)/year
Lettuce	6.4
Cabbage	12.6
String beans	4.4
Tomato	18.6
Cucumber	3.4
Cauliflower	1.6
Carrot	11.8
Pepper	6.2
Potato	74.8

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Table 4. Nitrate intake (Slovenian inhabitant per day).

Vegetable	Content of NO_3^- (mg kg^{-1}) (average value)	Content of NO_3^- [(mg/inh.)/day] (average value)	Content of NO_3^- (mg kg^{-1}) (max value)	Content of NO_3^-, [(mg/inh.)/day] (max value)	Coefficient of variation (%)
Lettuce	962	16.9	3986	79.9	70
Cabbage	795	27.4	1964	67.8	60
String beans	298	3.6	675	8.1	50
Tomato	6.4	0.3	60	3.1	150
Cucumber	93	1.0	245	2.3	70
Cauliflower	231	1.0	360	1.6	50
Pepper	69	1.2	225	3.8	90
Potato	169	34.6	871	179	80
Carrot	264	8.5	1042	33.7	90

Acceptable daily intake (NO_3^-): 3.7 mg kg^{-1} body weight day^{-1} (=222 mg for 60 kg person).

Table 5. Nitrite intake (Slovenian inhabitant per day).

Vegetable	Content of NO_2^- (mg kg^{-1}) (average value)	Content of NO_2^- [(mg/inh.)/day] (average value)	Content of NO_2^- (mg kg^{-1}) (max value)	Content of NO_2^-, [(mg/inh.)/day] (max value)	Coefficient of variation (%)
Lettuce	0.28	0.01	1.35	0.02	70
Cabbage	0.17	0.01	0.43	0.01	30
String beans	0.18	0.002	0.33	0.004	20
Tomato	0.25	0.01	1.58	0.08	80
Cucumber	0.18	0.002	0.76	0.01	60
Cauliflower	<0.16	/	<0.16	/	/
Pepper	0.20	0.003	0.30	0.01	30
Potato	1.08	0.22	7.64	1.57	100
Carrot	0.17	0.005	0.36	0.01	20

Acceptable daily intake (NO_2^-): 0.07 mg kg^{-1} body weight day^{-1} (=4.2 mg for 60 kg person).

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Slovenia is compared with those in other European countries, it appears that the nitrate intake estimated in this study is substantially higher than the intake estimates reported from the UK (Knight *et al.*, 1987), Denmark (Petersen and Stoltze, 1999), Italy (De Martin and Restani, 2003), Estonia (Tamme *et al.*, 2006) and France (Menard *et al.*, 2008). The most likely explanation for this difference is the higher consumption of vegetables in Slovenia. The situation was similar in the assessment of dietary intake of nitrites (Table 5). Only potato samples contained higher concentrations of NO_2^- (maximum content was 7.64 mg kg^{-1}) and we estimated a nitrite intake of 1.57 mg of NO_2^- per inhabitant per day. This result is half of the value for the acceptable daily intake for nitrite (ADI: 4.2 mg of NO_2^-; for 60 kg person). Our investigation indicates that none of the other crops (lettuce, cabbage, string beans, tomato, cucumber, cauliflower and pepper) are a matter of concern for nitrite intake.

Conclusions

Growing concern over nitrate toxicity has produced a number of studies on the nitrate and nitrite contents of fresh vegetable samples. Nitrite and nitrate levels of nine vegetable samples from an intensive horticultural area in Slovenia were analysed over a period of 13 years and their levels compared to those reported in recent literature. The results are in the range of others reported in different European countries. The most nitrates were found in leafy vegetables, *i.e.* lettuce and cabbage. We determined average values of NO_3^- between 700 and 1000 mg kg^{-1} in these crops. Six of a total of 319 lettuce samples (2%) exceeded the maximum admissible value recommended by the EU. According to present knowledge, ingestion of the studied vegetables is considered to be beneficial for the population despite their nitrate and nitrite contents.

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