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NITRATE IN LEAFY GREEN VEGETABLES AND ESTIMATED INTAKE

Danijel Brkić[1], Jasna Bošnir[1], Martina Bevardi[1], Andrea Gross Bošković[2], Sanja Miloš[2] Dario Lasić [1], Adela Krivohlavek[1], Aleksandar Racz[3], Ana Mojsović – Ćuić[3], Natalija Uršulin Trstenjak[4]

[1]Institute of Public Health „ Dr. Andrija Štampar", Mirogojska cesta 16, Zagreb, Croatia
[2]Croatian Food Agency, I. Gundulića 36 b, Osijek, Croatia., [3] Zagreb University of Applied Health Sciences, Mlinarska 38, Zagreb, Croatia., [4]University North, University Centre Varaždin, 104. brigade 3, Varaždin, Croatia

Corresponding Author's E-mail: aleksandar.racz@zvu.hr

Abstract

Background: Vegetarian diets are rich in vegetables. Green leafy vegetables are foods that contain considerable amounts of nitrate, which can have both positive and negative effects on the human body. Their potential carcinogenicity and toxicity have been proven, particularly after the reduction of nitrate to nitrite itself or just serving as a reactant with amines and/or amides in the formation of N-nitroso compounds -N-nitrosamines and other nitrogen compounds which may have high levels of nitrate. The aim of this study was to determine whether there is a significant difference, considering the location and seasonal sampling period, in the level of nitrate in certain types of green vegetables, all in order to be able to assess their intake, and possible impact on human health, especially knowing that exposure to nitrate can be potentially higher for vegetarian population group.
Materials and Methods: For this purpose, the sampling of 200 different leafy green vegetables was conducted, all of which could be found in free sale in the Republic of Croatia. The sampling was conducted during two seasonal periods - the spring and autumn period. In the springtime, lettuce (sem), spinach (pinacho), kale (kale), chard (mangel) and cabbage (brassica) were sampled, and in autumn lettuce, spinach, kale, chard and arugula. Samples were analyzed using high performance liquid chromatography (HPLC) with UV detection.
Results: The results from the spring sampling phase were in the range of 603 mg/kg for cabbage - 972 mg/kg for chard, and for autumn phase of 1.024 mg/kg for chard to 4.354 mg/kg for the arugula. The results showed that there were significant differences ($p < 0.05$) for most of the samples analyzed, considering the sampling locations and time period.
Conclusion: The results indicate that the analyzed vegetables contain significant amounts of nitrate in their composition, which represents relatively significant, but still acceptable intake into the human body.

Key words: leafy green vegetables, nitrate, HPLC, season period, daily intake

Introduction

Nitrates are, besides being used as food additives, found in nature as part of the nitrogen cycle, and play an important role during nutrition, growth and development of plants. Because of their cumulative properties, they are an important part of vegetables (Lucarini et al., 2012; Boink, 2001; EFSA, 2008). Besides leafy vegetables that may contain a substantial proportion of nitrate, studies have shown that other types of vegetables such as oilseeds, grains, tubers and nuts also contain nitrate (Gundimeda,1993). Absorption of nitrate occurs most often from natural sources, but vegetables accumulate a significant portion of nitrate from nitrogen-based fertilizers, which are used for fertilizing plants for faster and bigger growth (Shahid Umar, 2007). Leafy vegetables, such as lettuce or spinach, contain the highest concentrations of nitrate (Iammarino, 2014). Approximately 80% of dietary nitrates are derived from vegetable consumption. Sources of nitrites include vegetables, fruit, and processed meats, which means that human exposure to nitrate is usually associated with intake through vegetables, and to a lesser extent, with other foods and water (Temme, 2011). Dietary nitrates are essentially inert and acquire biological activity only with nitrate reduction to nitrite and then serving as a reactant with amines and/or amides in the formation of N-nitroso compounds. The main sites of reduction are in the saliva (nitrate to nitrite), and the stomach and blood vessels (nitrite to NO). As such, nitrate serves as a source, via successive reduction, for the production of nitrite and nitric oxide as well as other metabolic products. Nitrites are also produced endogenously through the oxidation of nitric oxide and through a reduction of nitrate by commensal bacteria in the mouth and gastrointestinal tract (Norman, 2009).

Determination of nitrate content of vegetables is important because in the academic community there are still human safety controversies surrounding nitrate and nitrite in the diet (Sindelaar,2012), such as "dietary nitrate – good or bad?" (Gilchrist, 2010) or "noxious or nutritious"? (Weightman, 2013).

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The starting point is the fact that toxic properties of nitrate are not expressed to a greater extent, but the possibility of creating metabolites such as nitrites, N-nitrosamines and various other nitrogen compounds that have very harmful toxicological effects on human health, and this gives nitrate special attention.

It should be noted that their harmful effects are associated with the occurrence of methemoglobinemia, and some carcinogen effects have been attributed to them as well (Temme, 2011; Keszai, 2013; Della Valle, 2013; Walker, 1990; Knobeloch,, 2000; Santamaria, 2006). However, recent epidemiological evidence suggests that increased consumption of vegetables reduces the risk of cardiovascular disease (Joshipura, 2011) and some types of cancer (Terry, 2001). These benefits cannot be explained just by the intake of the antioxidant factors in vegetables, but many non-antioxidant factors should be considered, such as inorganic nitrate. Furthermore, the strength of the evidence linking the consumption of nitrate and nitrite-containing plant foods to beneficial health effects supports the consideration of these compounds as nutrients. (Maacha, 2012). This opinion is supported by a large body of evidence (results and conclusions of a number of experimental and clinical studies) showing that dietary nitrate supplementation at doses that are commonly found in vegetable-rich diets exerts beneficial effects especially on the cardiovascular system (Naseem, 2005), blood pressure lowering (Larsen, 2005), vaso-protective (Webb, 2008; Bahra, 2012), cardiovascular risk benefit (Tang, 2011), including a just published paper summarizing the therapeutic potential of nitrate and nitrite for conditions such as myocardial infarction, stroke, systemic and pulmonary hypertension, and gastric ulceration (Bondonono,2016). Norman (2009) has explained the physiologic context for potential health benefits suggesting that dietary nitrate, derived primarily from vegetables, could contribute to cardiovascular health „via effects on nitric oxide (NO) status beacause NO plays an essential role in cardiovascular health. It is produced via the classical L-arginine–NO-synthase pathway and the recently discovered enterosalivary nitrate–nitrite–NO pathway. The discovery of this alternate pathway has highlighted dietary nitrate as a candidate for the cardioprotective effect of a diet rich in fruit and vegetables.“

Approximately 11-41% of daily intake of nitrate enters the organism. Acceptable daily intake (ADI) for nitrate was determined by the Scientific Committee on Food (SCF) in 2002 and amounts from 0 to 3.7 mg/kg body weight/day, which is equivalent to the intake of 222 mg nitrate/day for an adult weighing 60 kg. Studies show that the average adult daily consumes approximately 400 g of various vegetables, from which it can be concluded that the average intake of nitrate is 157 mg/day (FAO/WHO, 2013).

If we take into account the consumption of fruits, which contain less nitrate than vegetables, total intake of nitrate ranges from 81 to 106 mg/day for the majority of the European population, which is within the ADI value. It has been shown that potential problems can represent the portion of the population that consumes only leafy vegetables in large quantities, and in whom it can result in exceeding the ADI value (FAO/WHO, 2013; Mitek, 2013).

Literature data mention significant amounts of nitrate present in green vegetables, although the legislation determines the maximum permitted level only in certain types of lettuce, spinach and arugula. The aim of this study was to determine the level of nitrate in leafy green vegetables for which there are permitted maximum levels (MAQ), as well as those for which these values are not defined, and it is expected that these products will be consumed, and will contribute to the intake of nitrate in the human body. In addition, the aim was to determine whether seasonal periods, associated with cultivation of green vegetables, have an impact on nitrate levels. Sampling was planned in four markets in the largest Croatian cities and their shopping centers during two seasonal periods: spring and autumn. After sampling and conducted analyses, the objective was to determine whether there are significant differences in the amounts of nitrate depending on the location and the sampling period, and whether the determined values represent a significant intake of nitrate in the human body.

Material and Methods

This paper analyzes a total of 100 samples of green leafy vegetables. Vegetables were collected during the spring and autumn of 2012. Sampling was conducted randomly at markets and retail chains in four cities in the Republic of Croatia: Zagreb, Rijeka, Split and Osijek, and was based on sampling domestic local products. Sampling locations were selected in such a way as to take into consideration the largest offer of various green vegetables, as well as their consumption. The weight of the sample was at least 1 kg. All samples were put into paper bags, and after sampling were stored in coolers and promptly delivered to the laboratory for analysis. In accordance with the prescribed procedure, the samples were homogenized within 24 hours from sampling. The majority of samples was prepared immediately after homogenization, while others were stored at low temperatures in the freezer (EC, 2006a).

In the springtime, a total of 100 samples of green leafy vegetables were sampled, 25 samples at each location (5 samples of lettuce (*Lactuca sativa L*), 5 samples of spinach *Spinacia oleracea L.)*, 5 samples of kale *(Brassica oleracea var. sabellica)*, 5 samples of chard (*Beta vulgaris subsp. Vulgaris*) and 5 samples of cabbage) (*Brassica oleracea var. Capitata*).

In the autumn period, a total of 100 samples of green leafy vegetables were sampled, 25 samples at each location (5 samples of lettuce (*Lactuca sativa L*), 5 samples of spinach *Spinacia oleracea L.)*, 5 samples of kale *(Brassica oleracea var. sabellica)*, 5 samples of chard (*Beta vulgaris subsp. Vulgaris*) and 5 samples of arugula (*Eruca sativa*), which was sampled instead of cabbage from the spring period).

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The sampling procedure, transport of samples to the laboratory, and preparation of samples for analysis, was conducted in accordance with the requirements of the Directive 1882/2006 on the methods of sampling and analysis for the official control of the levels of nitrate in food. (EC, 2006a)

For identification and quantification of nitrate, high performance liquid chromatography (HPLC) technique was used, with UV detection as a recommended technique by the European Commission (Directive) (EC, 2006a). After homogenization of the sample, 10 g ± 0.05 g was weighed in a volumetric flask of 500 ml, 400 ml of water at >80°C was added, and homogenized with the magnetic stirrer. The sample was cooled and then filtered through a membrane filter, after which the determination of the quantity of nitrate was carried out by HPLC. Sample recovery percentages of the method were between 60 and 120% for samples in which the quantity of nitrate amounted to 500 mg/kg, while for specimens in which the quantity of nitrate was 500 mg/kg or more, sample recovery percentages ranged in an acceptable range of 90 to 120%. Fresh samples were prepared for analysis within 24 hours from sampling. When this was not possible, samples were frozen and stored in a freezer until the beginning of the analysis, but not more than 6 weeks.

The amount of nitrate in the sample was determined by the method of external standard using the calibration curve. To prepare the calibration curve, it is necessary to determine the peak area for ≥4 different concentration levels (area of the method) with at least three repeats for each concentration level. The sample prepared for chromatographic analysis is placed in the HPLC autosampler. Chromatography was carried out under the following conditions: a mobile phase used was a solution of potassium hydrogen phosphate (10 g/L of KH_2PO_4; pH 3.0), mobile phase flow rate was 1 ml/min, temperature - ambient, detector - UV 214 nm, the volume of analyzed sample - constant 20 µL. The result was expressed in mg/kg according to the formula: mg/kg=a(mg/kg) x b (ml)/weighing(g), where a=concentration read from the chromatogram, b = dilution (EC,2006a; Chou,2003; Di Mateo,1997).

The data is presented in tables and graphs. An analysis of normality of distribution was performed using Kolmogorov-Smirnov test, and in accordance with the results obtained, in the further processing parametric tests and data presentation were used. Nitrate levels are shown using arithmetic means and the corresponding standard deviations, and standard errors in tables and in figures. Arithmetic means instead of the range of nitrate content for each group has been presented in tables to enable comparison of the nitrate values found in the vegetable samples in our study with the same types of vegetables in other studies where only means values have been presented, and to compare the results with the maximum permitted levels in the European and national legislative. Complete data are available on demand from authors.

The differences between the levels of nitrate in the spring and the autumn were analyzed using the independent t-test. All P values of less than 0.05 were considered statistically significant. SPSS for Windows, version 19.0.0.1 software package was used for statistical analyses.

Results

The research results are presented in two groups. The first group refers to seasonal differences in the level of nitrate in various kinds of vegetables sampled at specific locations, and are shown in Tables 1 to 5. Second group of results are those related to seasonal differences in the level of nitrate in certain locations for each type of vegetables, and are shown in Tables 6 to 11. Table 12 provides an overview of all the analyzed samples collected at four locations over two years.

Monitoring nitrate in green vegetables is prescribed by the Directive on contaminants, according to which the obligation of each state is to conduct regular monitoring of the presence of nitrate in lettuce, spinach, and arugula (EC,2006b; EC,2006c).

Besides lettuce, spinach and arugula, this research also included other green vegetables for which MAQ is not defined, such as chard, kale and cabbage. This research was conducted during the spring and autumn periods, in order to gain insight into the amount of nitrate during the spring and autumn periods of vegetable growth, and whether determined quantities can adversely affect health. For this purpose, a total of 200 samples of green vegetables were analyzed. From Table 1, which gives an overview of nitrate in green vegetables sampled during 2012-2013 at four locations, and during two periods in the Republic of Croatia. It is evident that the lowest levels of nitrate were detected in kale (603.0 mg/kg) sampled in the spring period, followed by a lettuce with detected levels of nitrate of 703.7 mg/kg in the same period, spinach with 706.2 mg/kg, cabbage with 718.8 mg/kg, and the largest amount of 972.2 mg/kg was found in chard. During the autumn period, contrary to the spring, the lowest levels of nitrate were found in chard and amounted to 1024.7 mg/kg. Somewhat higher amounts were found in kale 1181.4 mg/kg, followed by a lettuce in which the amount of nitrate was 1264.8 mg/kg, and spinach with an amount of 2013.1 mg/kg. With the entry of the Directive into force, according to which required nitrate control is extended to arugula, sampling of the above mentioned vegetable was conducted, and the determined amount was 4354.9 mg/kg. Looking at seasonal differences in the level of nitrate in the analyzed types of vegetables at the level of all the locations together, it is evident that significant differences were found for lettuce, spinach and kale. The exception is chard, in which there were no significant differences, considering the location where it was sampled. The impact of seasonal period on the level of nitrate could not be determined in the cabbage and arugula, being collected during only one of the periods (Table 2).
Taking into account the seasonal period when the samples were sampled, and the locations where they were sampled, the results indicate significant differences in the concentration of nitrate in Zagreb, and they were recorded for all

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vegetables except cabbage (Table 3). Similarly, in samples of vegetables sampled in the Split area, there were significant differences for all vegetables except cabbage (Table 4), while at the location of Rijeka, seasonal differences in the levels of nitrate were not recorded (Table 5).

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Table 1: Nitrate quantities in sampled green vegetables during 2012, at four locations and in two seasonal periods, in the republic of Croatia.

Nr.	**Sampling location**	**Sample type**	**Nitrate (mg/kg) spring period**	**Nitrate (mg/kg) autumn period**	**Sample type**	**Nitrate (mg/kg) spring period**	**Nitrate (mg/kg) autumn period**	**Sample type**	**Nitrate (mg/kg) spring period**	**Nitrate (mg/kg) autumn period**	**Sample type**	**Nitrate (mg/kg) spring period**	**Nitrate (mg/kg) autumn period**	**Nitrate in cabbage (mg/kg) spring period**	**Nitrate in arugula (mg/kg) autumn period**
1.	Rijeka	Lettuce	638	1173	Spinach	1213	1888	Kale	277	942	Chard	809	600	718	4679
2.	Rijeka	Lettuce	1037	890	Spinach	1054	2351	Kale	45.6	855	Chard	442	830	212	4786
3.	Rijeka	Lettuce	354	585	Spinach	1281	887	Kale	267	595	Chard	2166	773	856	4191
4.	Rijeka	Lettuce	722	657	Spinach	1267	1200	Kale	322	114	Chard	2209	1295	473	4185
5.	Rijeka	Lettuce	578	477	Spinach	1264	1960	Kale	858	693	Chard	2234	884	508	3880
6.	Zagreb	Lettuce	696	1822	Spinach	126	1887	Kale	450	403	Chard	580	1470	444	4311
7.	Zagreb	Lettuce	1076	2784	Spinach	171	1525	Kale	493	1270	Chard	844	2761	492	4628
8.	Zagreb	Lettuce	874	2940	Spinach	364	845	Kale	354	36.3	Chard	1701	1923	230	4946
9.	Zagreb	Lettuce	668	1440	Spinach	475	876	Kale	510	787	Chard	1427	2327	525	5370
10.	Zagreb	Lettuce	1073	2005	Spinach	395	1909	Kale	809	710	Chard	695	2820	277	5246
11.	Osijek	Lettuce	397	341	Spinach	133	3096	Kale	291	1425	Chard	754	1833	614	4874
12.	Osijek	Lettuce	253	1054	Spinach	863	3244	Kale	116	1950	Chard	312	436	215	4682
13.	Osijek	Lettuce	173	1015	Spinach	1010	3412	Kale	297	2128	Chard	515	475	315	4865
14.	Osijek	Lettuce	449	690	Spinach	1322	2087	Kale	664	1202	Chard	794	358	531	4531
15	Osijek	Lettuce	1135	546	Spinach	36.6	2493	Kale	264	978	Chard	1779	468	286	3089
16	Split	Lettuce	2358	1364	Spinach	688	2247	Kale	915	1619	Chard	19.6	81.8	1320	3228
17	Split	Lettuce	239	1610	Spinach	666	2337	Kale	1451	2165	Chard	653	65.5	1620	3437
18	Split	Lettuce	714	1423	Spinach	670	2645	Kale	920	1461	Chard	872	708	1469	4810
19	Split	Lettuce	69.7	1068	Spinach	495	876	Kale	1020	1692	Chard	41.2	266	1588	3391
20	Split	Lettuce	571	1413	Spinach	632	2498	Kale	1737	2604	Chard	598	121	1684	3969
Mean value			**703.7**	**1264.8**		**706.2**	**2013.1**		**603.0**	**1181.4**		**972.2**	**1024.7**	**718.8**	**4354.9**

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Table 2: Seasonal Differences in Nitrate Levels Regarding Vegetable Type at All Locations Together.

Sample type: all locations together		**N**	**Arithmetic mean**	**SD**	**Standard error**	**t**	**P**
Lettuce	Nitrate content in mg/kg in spring	20	703.74	500.59	111.94	-3.32	0.004
	Nitrate content in mg/kg in autumn	20	1264.85	711.80	159.16		
Spinach	Nitrate content in mg/kg in spring	20	706.28	432.29	96.66	-6.4	<0.001
	Nitrate content in mg/kg in autumn	20	2013.15	793.12	177.35		
Kale	Nitrate content in mg/kg in spring	20	603.03	444.03	99.29	-4.36	<0.001
	Nitrate content in mg/kg in autumn	20	1181.47	699.55	156.42		
Chard	Nitrate content in mg/kg in spring	20	972.24	697.41	155.94	-0.24	0.814
	Nitrate content in mg/kg in autumn	20	1024.77	878.75	196.49		

Table 3: Seasonal Differences in Nitrate Levels Regarding Vegetable Type in Zagreb

SAMPLE TYPE: ZAGREB		**N**	**ARITHMETIC MEAN**	**SD**	**STANDARD ERROR**	**T**	**P**
Lettuce	NITRATE CONTENT IN MG/KG IN SPRING	5	877.40	196.51	87.88	-5.4	0.006
	NITRATE CONTENT IN MG/KG IN AUTUMN	5	2198.20	641.71	286.98		
Spinach	NITRATE CONTENT IN MG/KG IN SPRING	5	306.20	150.39	67.26	-3.96	0.017
	NITRATE CONTENT IN MG/KG IN AUTUMN	5	1408.40	523.00	233.89		
Kale	NITRATE CONTENT IN MG/KG IN SPRING	5	523.20	170.85	76.41	-0.62	0.568
	NITRATE CONTENT IN MG/KG IN AUTUMN	5	641.26	459.35	205.43		
Chard	NITRATE CONTENT IN MG/KG IN SPRING	5	1049.40	488.69	218.55	-3.41	0.027
	NITRATE CONTENT IN MG/KG IN AUTUMN	5	2260.20	571.57	255.61		

Table 4: Seasonal differences in nitrate levels regarding vegetable type in split

Sample type: SPLIT		**N**	**Arithmetic mean**	**SD**	**Standard error**	**t**	**P**
Lettuce	Nitrate content in mg/kg in spring	5	790.34	913.07	408.34	-1.43	0.227
	Nitrate content in mg/kg in autumn	5	1375.60	195.79	87.56		
Spinach	Nitrate content in mg/kg in spring	5	630.20	78.24	34.99	-5.19	0.007
	Nitrate content in mg/kg in autumn	5	2120.60	712.26	318.53		
Kale	Nitrate content in mg/kg in spring	5	1208.60	368.45	164.78	-13.44	<0.001
	Nitrate content in mg/kg in autumn	5	1908.20	469.16	209.82		
Chard	Nitrate content in mg/kg in spring	5	436.76	384.93	172.15	1.22	0.290
	Nitrate content in mg/kg in autumn	5	248.46	268.78	120.20		

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Table 5: Seasonal differences in nitrate levels regarding vegetable type in Rijeka.

Sample type: RIJEKA		**N**	**Arithmetic mean**	**SD**	**Standard error**	**t**	**P**
Lettuce	Nitrate content in mg/kg in spring	5	665.80	248.32	111.05	-0.7	0.522
	Nitrate content in mg/kg in autumn	5	756.40	277.82	124.24		
Spinach	Nitrate content in mg/kg in spring	5	1215.80	94.05	42.06	-1.47	0.216
	Nitrate content in mg/kg in autumn	5	1657.20	597.61	267.26		
Kale	Nitrate content in mg/kg in spring	5	353.92	301.52	134.84	-1.37	0.242
	Nitrate content in mg/kg in autumn	5	639.80	323.63	144.73		
Chard	Nitrate content in mg/kg in spring	5	1572.00	874.06	390.89	2.019	0.114
	Nitrate content in mg/kg in autumn	5	876.40	257.16	115.01		

Table 6 shows the seasonal differences in the level of nitrate according to the type of vegetables sampled in Osijek. It was found that there are significant differences in the amounts of nitrate in spinach and kale during seasonal sampling. In all significant differences, autumn nitrate concentrations were higher compared to the results obtained in the spring.

Table 6: Seasonal differences in nitrate levels regarding vegetable type in osijek

Sample type: OSIJEK		**N**	**Arithmetic mean**	**SD**	**Standard error**	**t**	**P**
Lettuce	Nitrate content in mg/kg in spring	5	481.40	381.66	170.68	-0.92	0.410
	Nitrate content in mg/kg in autumn	5	729.20	305.36	136.56		
Spinach	Nitrate content in mg/kg in spring	5	672.92	562.91	251.74	-5.88	0.004
	Nitrate content in mg/kg in autumn	5	2866.40	556.75	248.98		
Kale	Nitrate content in mg/kg in spring	5	326.40	202.64	90.62	-4.45	0.011
	Nitrate content in mg/kg in autumn	5	1536.60	489.16	218.76		
Chard	Nitrate content in mg/kg in spring	5	830.80	564.63	252.51	-0.3	0.779
	Nitrate content in mg/kg in autumn	5	714.00	627.26	280.52		

Looking at the results obtained and seasonal differences in the level of nitrate in certain locations, for each type of vegetable analyzed, we can conclude that the nitrate values obtained are significant in all sampling locations. Seasonal differences in the level of nitrate in lettuce were only significant in Zagreb, while at other locations, although present, the differences were not statistically significant. The obtained values of nitrate in analyzed spinach samples were not significant only in Rijeka, while at all other locations autumn values were significantly higher. Seasonal differences in the level of nitrate in kale were not significant in Zagreb and Rijeka, while in Split and Osijek values in autumn were significantly higher. With chard, we can determine that seasonal differences in the level of nitrate are significant in Zagreb, and in spring period value is almost twice the value recorded in the autumn period (Tables 7-11).

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Table 7: Seasonal differences in nitrate levels at particular locations

Sample type: all vegetables		**N**	**Arithmetic mean**	**SD**	**Standard error**	**t**	**P**
Zagreb	Nitrate content in mg/kg in spring	25	629.96	378.21	75.64	-5.12	<0.001
	Nitrate content in mg/kg in autumn	25	2281.65	1544.40	308.88		
Split	Nitrate content in mg/kg in spring	25	920.42	597.37	119.47	-4.78	<0.001
	Nitrate content in mg/kg in autumn	25	1883.97	1253.33	250.67		
Rijeka	Nitrate content in mg/kg in spring	25	872.18	614.02	122.80	-2.35	0.028
	Nitrate content in mg/kg in autumn	25	1654.80	1463.06	292.61		
Osijek	Nitrate content in mg/kg in spring	25	540.74	421.92	84.38	-4.54	<0.001
	Nitrate content in mg/kg in autumn	25	2050.88	1534.37	306.87		

Table 8: Seasonal differences in nitrate levels at particular locations for lettuce

Sample type: lettuce		**N**	**Arithmetic mean**	**SD**	**Standard error**	**t**	**P**
Zagreb	Nitrate content in mg/kg in spring	5	877.40	196.51	87.88	-5.4	0.006
	Nitrate content in mg/kg in autumn	5	2198.20	641.71	286.98		
Split	Nitrate content in mg/kg in spring	5	790.34	913.07	408.34	-1.42	0.227
	Nitrate content in mg/kg in autumn	5	1375.60	195.79	87.56		
Rijeka	Nitrate content in mg/kg in spring	5	665.80	248.32	111.05	-0.7	0.522
	Nitrate content in mg/kg in autumn	5	756.40	277.82	124.24		
Osijek	Nitrate content in mg/kg in spring	5	481.40	381.66	170.68	-0.92	0.410
	Nitrate content in mg/kg in autumn	5	729.20	305.36	136.56		

Table 9: Seasonal differences in nitrate levels at particular locations for spinach

Sample type: spinach		**N**	**Arithmetic mean**	**SD**	**Standard error**	**t**	**P**
Zagreb	Nitrate content in mg/kg in spring	5	306.20	150.39	67.26	-3.96	0.017
	Nitrate content in mg/kg in autumn	5	1408.40	523.00	233.89		
Split	Nitrate content in mg/kg in spring	5	630.20	78.24	34.99	-5.19	0.007
	Nitrate content in mg/kg in autumn	5	2120.60	712.26	318.53		
Rijeka	Nitrate content in mg/kg in spring	5	1215.80	94.05	42.06	-1.46	0.216
	Nitrate content in mg/kg in autumn	5	1657.20	597.61	267.26		
Osijek	Nitrate content in mg/kg in spring	5	672.92	562.91	251.74	-5.88	0.004
	Nitrate content in mg/kg in autumn	5	2866.40	556.75	248.98		

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Table 10: Seasonal differences in nitrate levels at particular locations for kale

Sample type: kale		**N**	**Arithmetic mean**	**SD**	**Standard error**	**t**	**P**
Zagreb	Nitrate content in mg/kg in spring	5	523.20	170.85	76.41	-0.621	0.568
	Nitrate content in mg/kg in autumn	5	641.26	459.35	205.43		
Split	Nitrate content in mg/kg in spring	5	1208.60	368.45	164.78	-13.443	<0.001
	Nitrate content in mg/kg in autumn	5	1908.20	469.16	209.82		
Rijeka	Nitrate content in mg/kg in spring	5	353.92	301.52	134.84	-1.37	0.242
	Nitrate content in mg/kg in autumn	5	639.80	323.63	144.73		
Osijek	Nitrate content in mg/kg in spring	5	326.40	202.64	90.62	-4.45	0.011
	Nitrate content in mg/kg in autumn	5	1536.60	489.16	218.76		

Table 11: Seasonal differences in nitrate levels at particular locations for chard

Sample type: chard		**N**	**Arithmetic mean**	**SD**	**Standard error**	**t**	**P**
Zagreb	Nitrate content in mg/kg in spring	5	1049.40	488.69	218.55	-3.42	0.027
	Nitrate content in mg/kg in autumn	5	2260.20	571.57	255.61		
Split	Nitrate content in mg/kg in spring	5	436.76	384.93	172.15	1.22	0.290
	Nitrate content in mg/kg in autumn	5	248.46	268.78	120.20		
Rijeka	Nitrate content in mg/kg in spring	5	1572.00	874.06	390.89	2.01	0.114
	Nitrate content in mg/kg in autumn	5	876.40	257.16	115.01		
Osijek	Nitrate content in mg/kg in spring	5	830.80	564.63	252.51	0.3	0.779
	Nitrate content in mg/kg in autumn	5	714.00	627.26	280.52		

Table 12: Nitrate amounts in mg/150 g of green vegetables with average daily consumption per capita

Sample type	Lettuce		Spinach		Kale		Chard		Cabbage	Arugula
Seasonal period	spring	autumn	spring	autumn	spring	autumn	spring	autumn	spring	autumn
Mean nitrate values in mg/kg	703.7	1264.8	706.2	2013.1	603.0	1181.4	972.2	1024.7	718.8	4354.9 /44 g
Mean nitrate values in mg/150 g	105.5	189.7	105.9	301.9	90.5	177.2	145.8	153.7	107.8	191.6 /44 g
% of daily nitrate intake regarding total intake (222.0 mg/day)	47.5	85.4	47.7	135.9	40.7	79.8	65.7	60.8	48.5	86.3

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Discussion

The findings have shown that following levels of nitrate were detected: kale (603.0 mg/kg), lettuce (703.7 mg/kg) spinach (706.2 mg/kg) cabbage (718.8 mg/kg), and chard (972.2 mg/kg) in the spring period and chard (1024.7 mg/kg), kale (1181.4 mg/kg), lettuce (1264.8 mg/kg) and spinach (2013.1 mg/kg) and arugula (4354.9 mg/kg) in the autumn period. The results showed that there were significant differences ($p < 0.05$) for most of the samples analyzed, considering the sampling locations and the time period. The results are important since the consumption of vegetables is constantly increasing, especially in the developed countries. In the Republic of Croatia, there is currently no reliable data on the consumption of fresh vegetables per capita, therefore the data of the European Food Safety Authority (EFSA) is used, and according to which the consumption of fruits and vegetables is 400 g/capita. It should be noted that people whose diet is based on the input of large quantities of vegetables are more likely to have an excessive intake of nitrate than the average population.

The European Food Safety Authority, in their scientific studies, gave recommendations about the acceptable daily intake (ADI) of nitrate in the human body and for an adult it amounts to 3.7 mg/kg body weight/day, i.e. for a person with body weight of 60 kg, it is 222.0 mg/day. Intake is based on the total nutrient load from all sources, especially food, water and air, but also from cigarettes (EFSA, 2008; Selenka,1975). Regarding the food, the largest amount of nitrate is consumed with fresh vegetables, and it is anticipated that an average person can consume daily about 400 g of fresh fruit and vegetables, while for the arugula a maximum is set to 44 g per day, because the amount of 47 g/day may contribute to higher intake of nitrate into the organism (Bundesinstitute Für Risikobewertung, 2003). The consumption of 150 mg of vegetables per day is also considered safe, i.e. it is estimated that with the abovementioned quantity of vegetables, more than the recommended amount of nitrate will not be taken in. Taking into account the recommended intake of green vegetables during the day, and nitrate values obtained in the analyzed vegetables, it is evident (Table 12) that the intake of nitrate is lower in the spring, compared to the autumn period. Literature data shows a similar study conducted in Iran, where the results obtained during the autumn period are also higher than in the spring period (Nowruz, 2012). It can also be seen that by consumption of spinach (135.9%) and lettuce (85.4%) in the autumn period, the largest percentage of the daily intake of nitrate in the body is taken in. Given the high value of nitrate in the samples of arugula, as well as in similar studies, it was found that consuming 44 mg arugula/day will not exceed the acceptable daily intake of nitrate. It should be noted that the intake of nitrate in children could be increased due to the frequent consumption of spinach, if it is combined with commercial baby food that contains nitrate, which can lead to the methemoglobinemia (MetHb) (Dusdieker,1994; Greer,2005).

Studies on daily dietary intake of nitrate in the human body have been conducted in different European Union countries like Slovenia (Susin, 2006), Estonia (Tamme, 2006), Denmark (Petersen, 1999), Spain (Pardo-Marín, 2010; Quijano, 2016), Poland (Mitek, 2013; Szymczak, 1999), Italy (Lucarini, 2012), Belgium (Temme, 2011), Finland (Penttilä, 1990), and France (Menard,2008), as well as in some other countries in the world (Santamaria, 1999), like Korea (Chung, 2003), China (Zhong, 2002), Honk Kong (Chung, 2011), the USA (Nuñez de González, 2015)
In all these studies, daily intakes of nitrate in the body were determined based on the results of research into the presence of nitrate in various types of food.

In the Slovenian study the average nitrate contents were the highest in lettuce (1074 mg/ kg), cabbage (881 mg/ kg), string beans (298 mg/ kg) and carrot (264 mg/ kg), and they were moderately high in potato (158 mg/ kg), silage maize (122 mg/ kg), strawberries (94 mg/ kg), cucumbers (93 mg/kg) and cereals (49 mg/kg) (Susin, 2006).
In Estonia the highest mean values of nitrates were detected in dill (2936mg/kg), spinach (2508mg/ kg), lettuce (2167 mg/kg) and beetroot (1446 mg/kg) (Tamme, 2006).

The results of the Belgian research suggest that half of the daily intake of nitrate into the body is through vegetables, especially lettuce, and 20% is through water and water-based drinks (Temme, 2011).
Significant nitrate intake by eating lettuce was confirmed by the study conducted in Denmark, where samples of vegetables sampled in shopping centres were analyzed, and lettuce from the winter period contained more nitrate than lettuce from the summer period. High amounts of nitrate were found in dill (2.936 mg/kg), spinach (2.508 mg/kg), lettuce (2.167 mg/kg) and red beet (1.446 mg/kg) (Petersen,1999).

In the USA (Nuñez de González,2015) nitrate mean values contents were conventional broccoli (394 mg/kg), cabbage (418 mg/kg), celery (1496 mg/kg), lettuce (851 mg/kg) and spinach (2797 mg/kg). In China (Zhong,2002) the mean nitrates level was higher in A. tuberosum Roth (5150 mg/ kg) and spinach (4259 mg/kg), intermediate in radish (1878 mg/kg) and Chinese cabbage (1740 mg/kg). Most of the above mentioned data are in accordance with the study in the Republic of Croatia, with spinach, lettuce and arugula being vegetables that contribute most to the intake of nitrate in the human body. The findings of this study are also in accordance with the findings in few rare studies conducted in Croatia such as a rather old study from the Independence War period when the low levels of nitrate were achieved in lettuce (1820–2237 mg/kg) and less than 1000 mg/kg in radicchio grown outdoors (Custic, 1994). Another study was conducted in 1999 when the nitrate levels found in potato samples (196 mg/kg) and cabbage samples (911 mg/kg) were lower than the contents reported for most other countries (Sebecic, 1999). Finally in the study from 2002 nitrate content in fresh spinach ranged from 95.79 to 415.08 mg/kg (Markovic, 2002).

The fact that for the first time a systematic sampling was performed in 4 different Croatian main areas, and according to a seasonal sampling scheme, is the most important strength of the study. As every study, this one also has certain limitations among which it is necesary to emphasize that the study did not take into consideration the

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production practices thus it was not possible to differ conventional from bio or organic production which is another reason for the use of mean average values of nitrate. The geographic differences that were compared might be influenced by transportation and reselling of vegetables in different parts of the country, that is why the authors cannot be 100 percent be sure that the vegetables sampled from different regions were actually grown in that same region. The potato was not included in the study regardless of the fact that potato is the most common vegetable in the Croatian diet and responsible for the important percentage of daily intake. Finally, further examination of nitrate concentrations in vegetables in Croatia, as well as investigation and examination of the influence of war-time operations on accumulation of these toxic contaminants are necessary.

Conclusion

The obtained research results indicate that, in addition to vegetables for which MAQ value is determined, other green vegetables also contain nitrate. It is important to emphasize that arugula contains the highest amounts of nitrate and consequently significantly influences nitrate intake. However, given the recommended daily intake of arugula, the results are within the acceptable ranges because they do not exceed the maximum permissible intake of nitrate in the human body. The research revealed that there are significant differences when it comes to seasonal period of growing vegetables, because it has been proven that the values of nitrate in vegetables that were analyzed in the autumn period are much higher than those analyzed in the springtime. We can also conclude that the intake of nitrate in the human body through vegetables does not differ from the amounts that are established in other countries, therefore, Croatian population is not exposed to amounts of nitrate that could endanger health. It is important to emphasize that due care should be taken with spinach consumption in childhood, but also in people who consume vegetables in larger quantities than recommended, because in this way, they take in larger amounts of nitrate, which, if continuing for a longer period of time, could adversely affect health. For a complete assessment of nutrient loads to the body, it is necessary to carry out the analysis of other vegetables and fruits, meat and meat products, and drinking water and water-based drinks as well.

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