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A STATISTICAL STUDY ON NITRATE CONTENTS IN A SET OF SOME HORTICULTURAL PLANTS USED IN MEDITERRANEAN DIET

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ABSTRACT. In this paper we consider a set of fresh horticultural plants, used in Mediterranean diet, and we carry out a statistical analysis. In particular, we illustrate the nitrate contents in these vegetables, obtained in laboratory, as a sample variable and we study its frequency distribution, we compute its average, variance and standard variation and we work out some graphic representations. The derived results are more deepened with respect to those ones obtained in a previous paper, where a classification in ordered classes of nitrate contents was taken into consideration. From the results it is seen that the average value of nitrate contents, ingested by human organism by the consumption of the analyzed fruits and vegetables, does not constitute a risk for human health, when the assumption of these vegetables is limited, in agreement with the values recommended by medical standards to prevent diseases, For this reason Mediterranean diet is recommended, because prescribes to eat many portions of fruits and vegetables to be in good health.

1. Introduction

In a previous contribution (see Matarese Palmieri and Scinelli 2014) a statistical examination was given on nitrate contents in a set of 26 horticultural plants, following a classification in 5 ordered classes of this content (see Bianco and Pimpini 1990 and also Zangheri 1976; Salisbury and Ross 1984; Traverso 1990; Venturelli 1995) obtaining results very handy and practical to be applied in studying different vegetables. In this paper the carried out results are more deepened and the statistical analysis conducted on the same set of plants is quantitative. The contents of nitrates (obtained in laboratory) are examined as a variable and correspond to the average of three measures estimated on each species of vegetable. Nitrates are salts of nitric acid and are an essential vegetal nutrient absorbed by the roots of plants from the soil. They originate from living matter as a result of degradation processes, mainly due to microorganisms, leading to the formation of simple compounds. Once absorbed, the nitrates are used for the synthesis of complex substances essential to the structures and functions of plants. The accumulation of nitrates in the foods can be influenced by several factors, including their level of concentration in the ground (closely related to the level of fertilization adopted, to its permeability and composition) and the light

radiation, which, by acting on specific enzymes present in vegetables, can lead to a greater degradation rate of nitrates. Nitrates of potassium and sodium are used as food additives to promote the conservation of certain foods. Indeed, in certain circumstances, nitrates may be transformed into nitrites. The excess of nitrates may constitute a risk for both environment and human health, but the biological cultivations reduce the content of nitrates in plants. Nitrate contents decrease in the following order depending on the following different parts of a plant: petiole, leaf, stem, root, inflorescence, tuber, bulb, fruit, seed. Furthermore, there are many factors that have as effect accumulation of NO_3 in vegetable tissues coming from: genetic factor, environment (atmospheric humidity, temperature, irradiance, photoperiod), agricultural practices (use of herbicides, synthetic nitrogen fertilizers, nitrogen and other chemical nutrients). European Member states amended EC Regulations fixing limits for nitrate in vegetables, trying to harmonize the different national limits, that vary according the seasons and for open-grown vegetables and under glass-grown vegetables (see De Martin and Restani 2003; Menard *et al.* 2008; Iammarino *et al.* 2013; Quijano *et al.* 2017). In Section **3** we define the sample variable X_1, describing 26 fresh horticultural plants. All samples were analysed in triplicate. In Section **4** we introduce the sample variable X_2, illustrating the nitrate contents in the considered horticultural plants. Then, we worked out the frequency distribution, the average, variance and standard deviation of X_2, and some statistical graphic representations, discussing the results. In particular, we conclude discussing the recommendations, given by international and national organizations for nutrition, as OMS (World Health Organization), FAO (Food and Agriculture Organization), to avoid high content of nitrates to reduce the risk of several diseases. All samples were analysed in triplicate.

2. Materials and methods

2.1. Samples. Twenty-six horticultural plants were collected in triplicate, marketing them in the same sicilian market. Coming from the same territory, where the manufacturers have utilized similar coltivation conditions. All chemicals were of HPLC grade and all reagents, eluent, standard and sample solutions used for the determination were prepared by ultrapure water with a specific conductivity less than 18 mS cm^{-1} % purchased from Romil (Milan, Italy). The analytes, nitrite and nitrate ions were purchased from Dionex (Sunnyvale, CA, USA). In this study 3% acetic acid solution was used, prepared from ultrapure acetic acid (99.9%) purchased from J.T. Baker (Deventer, the Netherlands). Mobile phase (9mM Na_2CO_3) was prepared from 0.5 M sodium carbonate obtained from Dionex.

2.2. Standard solution and sample preparation. Standard mixture concentrations ranged from 30 to 150 mg l^{-1} in ultrapure water (99.9%). Standard solutions were prepared daily by serial dilution of the standard mixture prior to use. After homogenisation and agitation with a vortex, 10 gr of every sample were transferred into a 20 ml volumetric flask, put in ultrasonic bath for 30 min and after they were centrifuged at 5000 rpm per 5 min, spiked with 2ml of 3% acetic acid and brought to volume by ultrapure water. A total of 1ml of this solution was diluted with ultrapure water again up to 50 ml.

2.3. Equipment. Analysis were performed by an ICS 1000 ion chromatography system (Dionex) equipped with an isocratic pump, a conductivity detector, a guard column (Dionex

Ion Pac AG9-HC, 4 x 50 mm) to prevent potential fouling of the analytical column, a highcapacity anion exchange analytical column (Dionex Ion Pack AS9-HC, 4 x 250 mm, 9 μm), 25 μl sample loop, and an anion self-regenerating suppressor (ASRS 300, 4 mm). Data acquisition and instrument control were performed using Chromeleon software.

2.4. Ion exchange chromatography analysis. All experiments were performed at room temperature, with flow rate of 1.0 ml min^{-1} and 35^0 C flow cell temperature. Suppressor current was fixed at 45 mA. The isocratic elution was carried out using a 9 mM sodium carbonate solution. The standard and sample solutions were filtered through 0.22 μm glass-microfibre GMF Whatman chromatographic filter before entering the IEC system. Data collection was performed in triplicate. Ultrapure water was injected before the unknown samples.

2.5. Anions determination. For anions determination we follow the method used by Di Bella *et al.* (2012). The data showed that Na_2CO^3 concentration of 9 mM permitted a fast separation of the two anions (about 15 min). The chromatographic peaks were well resolved and consequently the quantifications steps were easy. Identification of analytes was carried out by comparing the retention times in the sample with those of the standard mixture. For quantification a calibration curve was obtained for each analyte by plotting peak areas versus their concentrations.

2.6. Validation of IEC analysis. The relative standard deviations (RSD %) on retention times and on peak area were determined by considering a mixture of standard anions at the concentration level of 2.5 (nitrite and nitrate). The measurements were performed, in the conditions reported above, within the same day (n=3). The highest RSD values were 1.4% and 2.2% for T_R and 2.4% and 3.7% for areas. The linearity of the used method was assessed by analysing seven standard solutions obtained from the standard mixture. Three replicate analyses were performed at each concentration level. Good linearity was observed in each concentration range, with linear correlation coefficients $(R_2) > 0.9873$. As per European Pharmacopoeia (2005), the limits of detection (LODs) and of quantification (LOQs) were experimentally calculated as a signal-to-noise ratio of 3 and 10, respectively. The accuracy of the method described for the determination of anions in the samples was evaluated at three spiking levels, with three replicates for each evel. For the recovery test, a sample was previously analysed and then fortified with a known amount of standard anions. For the anions, the recoveries were around 88%. All RSDs$<$ 2.8%, which shows the good precision of the method used for the determination of anions in horticultural plants.

3. Statistical analysis: introduction of the sample variable X_1

In this section we consider a sample of horticultural plants as a variable X_1, defined as follows:

$$X_1 : S \longrightarrow T_1, \tag{1}$$

where the elements of the set S are the analyzed plants and the range T_1 contains the corresponding numbers, that order in increasing way the considered plants: $T_1 = \{1, 2, ...26\}$ (see Table 1).

Table 1. Sample variable X_1: 26 fresh horticultural plants

S	T_1	
FAMILY	GENERA AND SPECIES	
Asteracae	*Cichorium endivia* L. var. *latifolium* Hegi	1
	Cichorium intybus L.	2
	Lactuca sativa L.	3
	Scorzonera hispanica L.	4
Liliaceae	*Allium cepa* L.	5
	Allium porrum L.	6
	Asparagus officinalis L.	7
Brassicaceae	*Brassica oleracea* var. *italica* Plenck	8
	Brassica oleracea L. var. *Sabauda* L.	9
	Brassica rapa L.	10
	Brassica rapa L. var. *rapa*	11
	Raphanus sativus	12
Cucurbitaceae	*Cucumis melo* L.	13
	Cucurbita moschata	14
	Cucumis sativus L.	15
Convolvulaceae	*Ipomoea batatas* L.	16
Graminaceae	*Zea mays* L. var. *saccharata* Körn	17
Leguminosae	*Phaseolus vulgaris* L.	18
	Pisum sativum L.	19
Polygonaceae	*Rhéum rhaponticum* L.	20
Solanaceae	*Capsicum annuum* L.	21
	Lycopersicon esculentum Mill.	22
	Solanum melongena L.	23
	Solanum tuberosum L.	24
Umbelliferae	*Apium graveolens* L. var. *dulce*	25
	Petroselinum crispum	26

4. Variable X_2 describing the nitrate contents in horticultural plants

In this Section we give a statistical analysis of nitrate contents (expressed in mg/kg of fresh weight of edible plant) present in 26 analyzed horticultural plants, on the basis of obtained data in laboratory.

Then, we define the variable X_2 describing these nitrate contents as follows:

$$X_2 : S \longrightarrow T_2, \tag{2}$$

where the elements of set S are the horticultural plants under consideration $S = \{1, 2, ..., 26\}$ and the range T_2 contains the corresponding content of nitrates, obtained in laboratory, expressed in mg/kg of fresh weight of edible plant of the considered plants with own calculated standard deviation (see Table 2_a). Such values correspond to the average of three

measures estimated on each species of vegetable, purchased in the same sicilian market. Following some authors (see Bianco and Pimpini 1990) there exist a classification of the content of nitrates in 5 classes, i. e.: class 1 (indicating nitrates *very low* content (< 200 mg/kg)); class 2 (indicating nitrates *low* content (200 - 500 mg/kg); class 3 (indicating nitrates *medium* content (500 - 1000 mg/kg); class 4 (indicating nitrates *high* content (1000 - 2500 mg/kg) and class 5 (indicating nitrates *very high* content (> 2500 mg/kg). In Matarese Palmieri and Scinelli (2014) the authors studied statistically the nitrates content in the examined plants following this classification.

Therefore, we define the frequency distribution $F(X_2)$ of the variable X_2, as follows:

$$F(X_2) : T_2 \longrightarrow W_2, \tag{3}$$

where T_2 contains the content of nitrates (expressed in mg/kg of fresh weight of edible plant) of the considered plants, and the elements of W_2 are the relative frequencies corresponding to the values of nitrates content (see Table 2_b). From the obtained data, presented in Table 2, we construct the graphic representations of X_2 and its frequency distribution $F(X_2)$ (see Figures 1 and 2).

Finally, we compute the average $M(X_2)$ of X_2:

$$M(X_2) = \frac{\sum_{i=1}^{19} x_i f_i}{\sum_{i=1}^{19} f_i} = \frac{19045,0}{26} = 732,5\ mg/kg, \tag{4}$$

where x_i (i = 1, 2,...,19) are the values of X_2 and f_i (i = 1, 2,...,19) the relative frequencies, with $\sum_{i=1}^{19} f_i = 1$, the variance V and the standard deviation σ given by:

$$V = \frac{\sum_{i=1}^{19}(x_i - M)^2 f_i}{\sum_{i=1}^{19} f_i} = 21.307.610,5/26 = 819.523,48\ \ (mg/kg)^2, \tag{5}$$

$$\sigma = \sqrt{V} = 905,27\ \ mg/kg. \tag{6}$$

From these results it is seen that the average value $732,5\ \ mg/kg$ of nitrates content in 26 analyzed horticultural plants indicates that eating fruits and vegetables of the considered sample does not constitute risk for the human health, when the consumption of these vegetables is limited. In fact, scientific committee for food (SCF) established an acceptable daily intake (ADI) of nitrate content of 3,65 mg per kg of body weight/day. Furthermore, the negative effects of nitrate contents are contrasted by the consumption of nutraceutical and antioxidant substances (carotenoides, vitamins C, E and other ones, selenium, crude fibre, glucosinolates, isothiocyanates, flavonoids, phenols) contained in fruits and vegetables (see also Matarese Palmieri and Scinelli 2015a, Matarese Palmieri and Scinelli 2015b). The ADI of nitrate content (in mg) per 60 kg of body weight/day is $219,0\ mg$ (ADI60). The ADI of fresh edible plant per 60 kg of body weight, expressed in Kg ($ADI60_{fp}$) is given in the set T_3 of Table 3 (calculated by the expression: $ADI60_{fp} = \frac{219.0\ \text{mg}}{\text{fresh plant mg}}\ Kg$) and represented in Fig. 3. Finally, we calculate the covered DIA (CDIA), expressed in %, using the formula: $X_{CDIA} = \frac{0,000219\ kg}{ADI60_{fp}\ \text{Kg}} \times 100\ \%$ (see the set T_4 in Table 4).

5. Tables and figures

Table 2. a) Variable X_2 (Content of nitrates mg/kg of fresh weight of edible plant) and b) its frequency distribution $F(X_2)$

S	T_2
Cichorium endivia L. var. *latifolium* Hegi	1200 ± 0.34
Cichorium intybus L.	150 ± 0.15
Lactuca sativa L.	3000 ± 0.24
Scorzonera hispanica L.	250 ± 0.18
Allium cepa L.	250 ± 0.25
Allium porrum L.	1500 ± 0.12
Asparagus officinalis L.	190 ± 0.28
Brassica oleracea var. *italica* Plenck	400 ± 0.31
Brassica oleracea L. var. *Sabauda* L.	600 ± 0.27
Brassica rapa L.	600 ± 0.19
Brassica rapa L. var. *rapa*	350 ± 0.21
Raphanus sativus	2600 ± 0.26
Cucumis melo L.	93 ± 0.18
Cucurbita moschata	590 ± 0.28
Cucumis sativus L.	250 ± 0.15
Ipomoea batatas L.	150 ± 0.25
Zea mays L. var. *saccharata* Körn	30 ± 0.12
Phaseolus vulgaris L.	53 ± 0.22
Pisum sativum L.	53 ± 0.26
Rhéum rhaponticum L.	2400 ± 0.19
Capsicum annuum L.	25 ± 0.22
Lycopersicon esculentum Mill.	16 ± 0.16
Solanum melongena L.	460 ± 0.29
Solanum tuberosum L.	35 ± 0.31
Apium graveolens L. var. *dulce*	2600 ± 0.24
Petroselinum crispum	1200 ± 0.19

(a)

T_2	W_2
16	1/26
25	1/26
30	1/26
35	1/26
53	2/26
93	1/26
150	2/26
190	1/26
250	3/26
350	1/26
400	1/26
460	1/26
590	1/26
600	2/26
1200	2/26
1500	1/26
2400	1/26
2600	2/26
3000	1/26

(b)

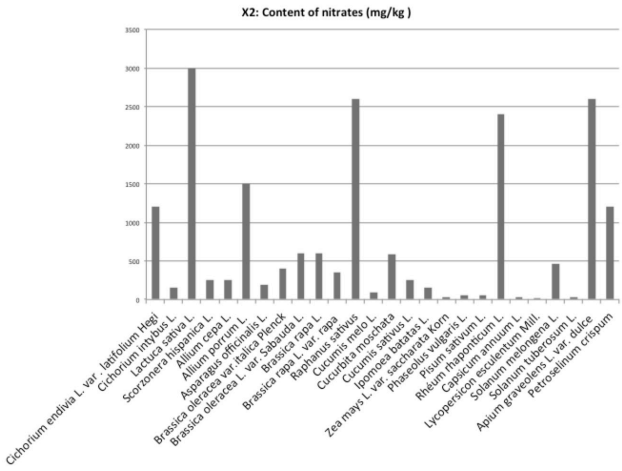

Figure 1. Diagram of the variable X_2.

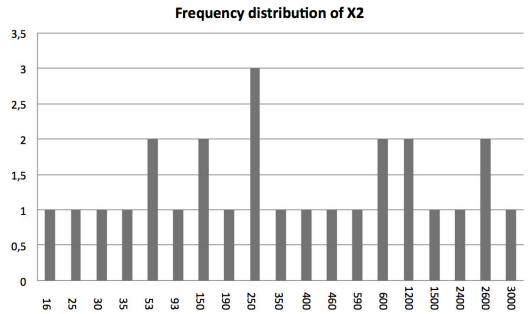

Figure 2. Diagram of the frequency distribution of X_2.

Table 3. ADI (acceptable daily intake) of fresh edible plant per 60 Kg of body weight ($ADI60_{fp}$) (in Kg)

S	T_3
Cichorium endivia L. var. *latifolium* Hegi	0,182
Cichorium intybus L.	1,46
Lactuca sativa L.	0,073
Scorzonera hispanica L.	0,876
Allium cepa L.	0,876
Allium porrum L.	0,146
Asparagus officinalis L.	1,152
Brassica oleracea var. *italica* Plenck	0,547
Brassica oleracea L. var . *Sabauda* L.	0,365
Brassica rapa L.	0,365
Brassica rapa L. var. *rapa*	0,625
Raphanus sativus	0,084
Cucumis melo L.	2,354
Cucurbita moschata	0,371
Cucumis sativus L.	0,876
Ipomoea batatas L.	1,46
Zea mays L. var. *saccharata* Körn	7,3
Phaseolus vulgaris L.	4,132
Pisum sativum L.	4,132
Rhéum rhaponticum L.	0,091
Capsicum annuum L.	8,76
Lycopersicon esculentum Mill.	13,687
Solanum melongena L.	0,476
Solanum tuberosum L.	6,257
Apium graveolens L. var. *dulce*	0,084
Petroselinum crispum	0,182

Table 4. Covered DIA (CDIA) in %

S	T_4
Cichorium endivia L. var. *latifolium* Hegi	0,120%
Cichorium intybus L.	0,015%
Lactuca sativa L.	0,300%
Scorzonera hispanica L.	0,025%
Allium cepa L.	0,025%
Allium porrum L.	0,150%
Asparagus officinalis L.	0,019%
Brassica oleracea var. *italica* Plenck	0,040%
Brassica oleracea L. var . *Sabauda* L.	0,060%
Brassica rapa L.	0,060%
Brassica rapa L. var. *rapa*	0,035%
Raphanus sativus	0,260%
Cucumis melo L.	0,009%
Cucurbita moschata	0,059%
Cucumis sativus L.	0,025%
Ipomoea batatas L.	0,015%
Zea mays L. var. *saccharata* Körn	0,003%
Phaseolus vulgaris L.	0,005%
Pisum sativum L.	0,005%
Rhéum rhaponticum L.	0,240%
Capsicum annuum L.	0,003%
Lycopersicon esculentum Mill.	0,002%
Solanum melongena L.	0,046%
Solanum tuberosum L.	0,004%
Apium graveolens L. var. *dulce*	0,260%
Petroselinum crispum	0,120%

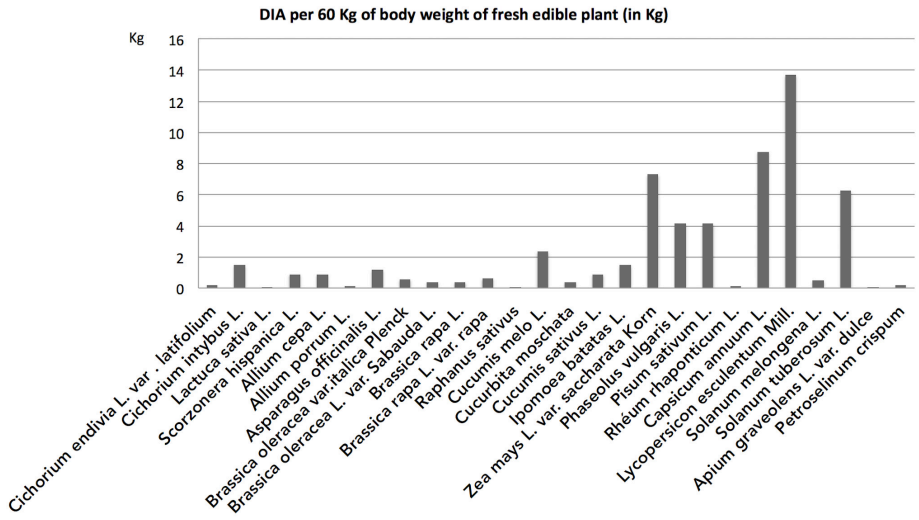

Figure 3. Diagram of ADI (acceptable daly intake) of fresh edible plant per 60 Kg of body weight ($ADI60_{fp}$) (in Kg).

6. Conclusions

It is well known that high concentrations of nitrates are considered toxic. The conversion of nitrates to nitrites can occur in food during its preparation or within human body. In fact, when nitrates are combined with amines, present in foods or derived from protein degradation processes, occurring in stomach, then they can produce nitrosamines, recognized as carcinogens. There are many directives and recommendations from international and national organizations for nutrition, as WHO (World Health Organization), FAO (Food and Agriculture Organization), EFSA (European Food Safety Authority), regarding the risk limits of nitrates content. Thus, the reduction of dietary nitrate is a advisable preventive measure. Nitrate levels in plants lower than nitrate threshold values indicate good practices in agriculture.

In this paper, a statistical population of 26 horticultural plants was analyzed. The contents of nitrates (obtained in laboratory) were examined as a variable and correspond to the average of three measures estimated on each species of vegetable. It was seen that the average value of nitrate contents, intaken by human organisms by the consumption of the considered fruits and vegetables, does not constitute a risk when the assumption of vegetables is limited. Furthermore, the negative effects of nitrate contents in the Mediterranean diet (see Keys and Keys 1975; Metro *et al.* 2018) are contrasted promoving a consumption of agrifood containing nutraceutical and antioxidant substances (carotenoides, vitamins C, E and other ones, selenium, crude fibre, glucosinolates, isothiocyanates, flavonoids, phenols). Vitamin C is capable of inhibiting conversion of nitrites to nitrosamines, transforming them into nitric oxide (see Traverso 1990; Venturelli 1995; Matarese Palmieri and Scinelli 2015b; Cicero *et al.* 2019).

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