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ORIGINAL ARTICLE

Evaluation of Nitrate and Nitrite Levels in Leafy Vegetables to Assess the Potential Health Risks in Tehran, Iran

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KEYWORDS

Nitrate and nitrite level;
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ABSTRACT: The nitrate and nitrite content of four leafy vegetables (parsley, garden cress, coriander, and dill) from different regions of Tehran province, Iran (north, south, east, and west) were determined by spectrophotometer method at 538 nm. The results indicated that the dill had the highest nitrate concentration (402.944 mg kg^{-1}), while the coriander had the highest nitrite concentration (0.672 mg kg^{-1}), and these values were significantly higher than other vegetables ($P<0.05$). Garden cress had the lowest nitrate and nitrite concentrations, 288.556 and 0.378 mg kg^{-1}, respectively. The estimated daily intake (EDI) and target hazard quotient (THQ) of both nitrate and nitrite were below the standard threshold. The EDI values for nitrate were 0.2216, 0.1980, 0.1746, and 0.1587 (mg kg^{-1} body weight/day) for dill, coriander, parsley, and garden cress, respectively, indicating that the health risk associated with consuming these vegetables was lower than the standard limit (3.7 mg kg^{-1} body weight /day). The THQ results for nitrate and nitrite samples revealed that they were all lower than 1, implying that the risk level is acceptable for chronic risk health exposure to leafy vegetables. In conclusion, our results showed that the studied vegetables were safe to consumption based on nitrate and nitrite concentrations.

INTRODUCTION

Leafy vegetables have various health benefits. They are low in calories and fat but high in dietary fiber, vitamins, minerals, antioxidants, etc. WHO (World Health Organization) recommends a daily intake of at least 400 g of fruit and vegetables, including different leafy vegetables. Leafy vegetables are an inseparable part of the human diet as they may confer protection against chronic diseases such as cardiovascular disease, stroke, diabetes, and some cancers due to the presence of various micronutrients and fibers [1-2]. However, despite the nutritional benefits of vegetables, they may also contain high levels of nitrate and nitrite, which may have detrimental health consequences [3].

Nitrate and nitrite are naturally occurring compounds in soil, water, and plants. The concentration of these compounds in leafy vegetables depends on various factors, including fertilization, irrigation, storage condition, processing methods, etc. [4]. Although these compounds are vital nutrients for plant growth and metabolism, they may accumulate in leafy vegetables and causes some health risks to humans when consumed in excess [5]. More precisely, high amounts of nitrate and nitrite in food can lead to some health problems. In fact, nitrate compounds in vegetables are essentially

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inactive. Still, they become physiologically active when nitrate is reduced to nitrite and then interacts with hemoglobin, affecting oxygen transfer and causing Methemoglobinemia. Nitrite can also react with secondary amines to form nitrosamines or N- nitroso compounds, which are known to be carcinogenic, teratogenic, and mutagenic [3, 6 and 7].

Nitrate concentrations in vegetables vary among families, species, and cultivars. Previous studies have demonstrated that different leafy vegetables have different capacities for nitrate accumulation, related to different locations of nitrate reductase activity and different degrees of nitrate absorption and transfer in the plan [3]. Moreover, it has been reported that nitrate accumulation in various parts of a plant is different, i.e., petiole, leaf, and stem organs have the highest nitrate content [9]. Indeed, since different leafy vegetables tend to have higher nitrate concentrations than other types of vegetables, and they are almost an integral part of the human diet in the Iranian cousin; it is crucial to assess the nitrate and nitrite levels to evaluate their potential health hazard [9]. A joint expert committee of Food and Agriculture (JECFA) and the European Commission's Scientific Committee on Food (SCF) recommended acceptable daily intake (ADI) for adults of 3.7 and 0.07 mg kg^{-1} body weight for nitrate and nitrite, respectively [6].

Given the significance of nitrate and nitrite concentrations for human health, and the substantial contribution of leafy vegetables to the human diet, this study quantified the nitrate and nitrite concentrations in four commonly consumed leafy vegetables - parsley, dill, coriander, and garden cress - in the Tehran province of Iran. These vegetables, typically consumed raw, form a considerable portion of the daily diet in Iran.

MATERIALS AND METHODS

Materials

All chemicals used in this study were of analytical grade. Sodium nitrate, sulphanilamide, hydrochloric acid, N-(1-naphthyl) ethylene diamine dihydrochloride, zinc acetate, potassium (II) hexacyanoferrate and saturated disodium Tetraborate. H_2O was purchased from the Merck Company (KgaA, Darmstadt, Germany).

Methods

Sampling

The study was conducted in accordance with the International Standard (ISO) 6635-1984 [10], utilizing the Griess diazotization reaction as the primary methodological approach. Data was collected from multiple prominent fruit and vegetable markets located in the North, South, East, and West regions of Tehran, Iran (as labled in Figure 1). The study focused on four vegetables that are commonly consumed in Iran: Garden Cress (*Lepidium sativum*), Coriander (*Coriandrum sativum L.*), Parsley (*Petroselinum crispum*), and Dill (*Anethum graveolens*). These were selected for their significance in the local food basket and their prevalence in Iranian dietary habits. The aim was to determine the concentration of nitrate and nitrite within these vegetables. Totally, 48 samples were collected randomly, with each vegetable type represented by 12 samples. The collection period spanned from April to June 2023. Each sample, weighing a minimum of 1 kg, was stored in LDPE zippered plastic bags, placed in a cold box, and promptly transported to the laboratory for analysis. It should be noted that the samples were fresh vegetables, chosen through sensory examination to ensure they were free from any damage that could be attributed to prolonged storage or decay.

Figure 1. The study markets areas: A. Iran, B. Distribution of Fruit and vegetable markets in Tehran in this study are as follow: Ekbatan market, Sheikh Bahaei fruit and vegetable market, Qasr fruit and vegetable market, and Municipal fruit and vegetable market. The market locations are marked with red lables.

Extraction

In the lab, each sample was thoroughly cleaned and washed with distilled water to remove any undesirable pollution. Then, samples were well chopped and ground using a ceramic pestle and mortar to obtain a homogeneous mixture. Approximately 10 g of the prepared sample were carefully weighed using a balance and transferred into a 200 mL beaker with 5 mL of Borax solution ($Na_2B_4O_7.10H_2O$). Then 100 mL of distilled water at 70-80 ˚C was added. Next, the beaker was heated in a boiling water bath for 15 minutes while being agitated occasionally. Then, 2 mL of Potassium (II) hexacyanoferrate ($K_4Fe(CN)_6.3H_2O$) solution and 2 mL of zinc acetate solution ($Zn(CH_3COOH)_2.2H_2O$) were added successively and stirred after each addition. After cooling, the contents were transferred into a 200 mL volumetric flask. The resulting solution was stirred and filtered with an ashless filter paper (Whatman No.42). If the filtered solution was turbid, it was passed through the same filter paper several times to make it completely clear [10].

Nitrite measurement

A volume of 15 mL of the filtered solution was transferred into a 50 mL volumetric flask. This was followed by the addition of 5 mL of Griess reagent I (sulphanilamide), 3 mL of hydrochloric acid solution, and approximately 30 mL of distilled water. The mixture was then thoroughly combined and stored in a dark environment at ambient temperature. Subsequently, 1 mL of Griess reagent II (N- (1- naphthyl) ethylene diamine dihydrochloride) was introduced to the solution, mixed well, and returned to the dark environment for a duration of 5 minutes at room temperature. After a total of 15 minutes, a red complex was formed. The absorbance of this complex was measured at a wavelength of 538 nm using a Thermo Scientific GENESYS 10 UV-Visible spectrophotometer. The absorbance of a blank solution was subtracted from the absorbance of the sample solution. The concentration of nitrite in the sample was then determined from the standard curve, as per reference [10].

Standard curve

Accurately pipetted 0.00 mL, 0.50 mL, 0.1 mL, 0.20 mL, 0.40 mL, 0.50 mL and 0.60 mL of 10.0 μg mL^{-1} sodium nitrite (NaNO2) standard solution into the colorimetric tubes and proceeded according to the above procedure. Using blank solution as a reference, the absorbance was measured at 538 nm, the concentration of sodium nitrite was plotted as the horizontal axis, the measured absorbance value was plotted as the vertical axis, the standard curve was drawn, and the regression equation was calculated (Figure 2) [11].

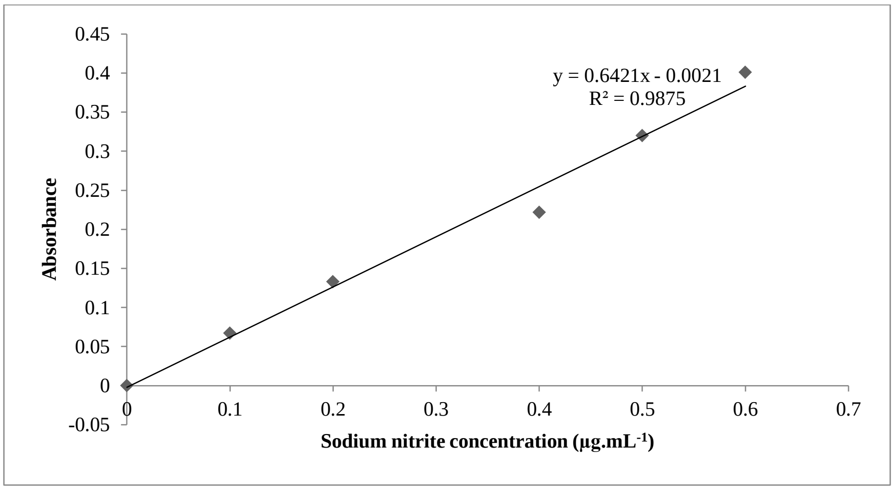

Figure 2. Calibration graph of the standard sodium nitrite solution.

Nitrite calculations

The amount of nitrite (milligrams in one kilogram of the product) is estimated using the following relationships:

$$N_1 = M_1 \times (200/(V_1 \times m_0)) \qquad (1)$$

Where N_1 (mg kg^{-1}) represents the nitrite concentration; m_1 (g) is the mass of the sample; V_1 (mL) is the consumption volume of the filtered solution; m_o is the nitrite mass calculated according to the linear equation [10].

Nitrate evaluation

The nitrate measurement involved extracting the sample with hot water and precipitating the proteins by increasing the solution of potassium (II) hexacyanoferrate and zinc acetate, filtering the resulting solution, reducing the nitrates to nitrites with metallic cadmium, adding Griess reagent I and Griess reagent II to the filtered solution sequentially. Finally, the red complex formed by nitrite was measured at 538 nm [10].

Nitrite measurement

A volume of 10 mL of the filtered solution, as described in section 2.2.1.2, containing nitrate ions (NO_3^-) in the range of 30 to 120 µg, was pipetted into a 25 mL Erlenmeyer flask. This flask contained approximately 2 g of cadmium and 5 mL of an NH_4Cl buffer solution with a pH of 9.4. The flask was sealed and subjected to magnetic stirring for a duration of 5 minutes. Following this, the solution was filtered using filter paper, and the resultant clear solution was collected in a 50 mL volumetric flask. The Erlenmeyer flask and the filter were rinsed multiple times with small volumes of water, with the rinse water being collected in the same volumetric flask. This was then diluted with distilled water. The total nitrite content was then measured according to the established nitrite measurement procedure, utilizing 10 mL of the final filtered solution. A blank solution, prepared by substituting the sample solution with 10 mL of distilled water and repeating the previous steps, was used to correct the absorbance readings. Then to determine the total nitrite content from the nitrite standard curve, the corrected absorbance was used as a reference [10].

Nitrate calculation

The amount of nitrate (milligrams in one kilogram of the product) is estimated by the following equation:

$$N_2 = 1.348 \times (\frac{m2 \times 10000}{V3V2V0} - \frac{m1 \times 200}{V1 \times V3}) \qquad (2)$$

Where N_2 (mg kg^{-1}) represents the nitrate concentration; m_2 (g) is the nitrite mass calculated according to the linear equation. V_2 (mL) volume of sample solution used for spectrophotometer measurement. V_3 (mL) is the consumption volume of the filtered solution; m_0, m_1, V_0 and V_1 have the same meanings as mentioned in equation 1., and 1.348 is the ratio between the molecular weight of nitrate (NO_3^-) and nitrite ions (NO_2^-) [10].

Health risk assessment

Health risk assessment was performed using a non-carcinogenic hazard quotient (HQ) based on vegetable consumption containing nitrate and nitrite. HQ<1 indicated no obvious hazard for consumers. The estimated daily intake (EDI) of nitrate and nitrite via leafy vegetable consumption was calculated using Equation 3 [12].

$$EDI = \frac{C \times IR \times ED \times EF}{BW \times AT} \qquad (3)$$

Where C (mg kg^{-1}) presents nitrate or nitrite concentrations, IR (kg/person/day) is ingestion rate of leafy vegetables, ED means 30 years' exposure duration for adults, EF is 350 days per year exposure frequency (every day of a week), BW is adults body weight (70 kg), AT is 10950 days (ED×365 days) non carcinogenic adults average life time [12].

The non-carcinogenic risk was calculated using Equation 4.

$$HQ = \frac{EDI}{RFD} \qquad (4)$$

Where RFD is oral reference dose and are 7.0 and 0.33 mg kg^{-1} body weight/day for nitrate and nitrite, respectively [12].

To calculate total hazard quotient (THQ) of nitrate and nitrite, the equation 5 was used:

$$THQ = \sum(HQ\ NO_3^- + HQ\ NO_2^-) \qquad (5)$$

Statistical analysis

SPSS v.16 (SPSS Inc, USA) was used to analyze the data. All experiments were done in 12 replicates (n=12), and a completely randomized design (CRD) was performed. One-way analysis of variance (ANOVA) was done for all samples, and comparison among means was carried out using Duncan's test at the significant level of 0.05.

RESULTS AND DISCUSSION

Nitrate and nitrite content in leafy vegetables

Our study revealed significant nitrate concentration variations across all examined vegetables. Table 1 shows that dill had the highest nitrate concentration (402.944 mg kg^{-1}), significantly higher coriander, parsley, and garden cress, which had concentrations of 360.002, 317.526, and 288.556 mg kg^{-1}, respectively. As per Table 1, the standard nitrate limit is 2000 mg kg^{-1} for both parsley and dill, indicating their safe consumption given our results. Nitrate levels in leafy vegetables are influenced by factors like irrigation water type, fertilizer use, and harvest time [10]. Nitrate transport via the xylem and accumulation in mesophyll cell vacuoles explain the higher nitrate levels in leafy vegetables compared to fruit-bearing or hypogeal vegetables [12].

Botanically, nitrate concentrations in leafy vegetables vary among families like Amaranthaceae, Apiaceae, Asteraceae, and Brassicaceae, which are known for nitrate-accumulating species [9-13]. Our study found that garden cress from Brassicaceae family had a significantly lower nitrate concentration (288.556 mg kg^{-1}) than the dill, coriander, and parsley that all of which belong to the apiaceous family (P<0.05). These results confirmed that nitrate concentration varies among different families [13]. Ali et al. [10], reported similar results in a study. Another study on the impact of nitrogen fertilizers on nitrate concentrations in different vegetables, confirmed the variation of nitrate concentration among families [14]. A study conducted in 2002-2003 on the vegetables grown in Turkey also reported similar nitrate range for garden cress, aligning with our results [15]. In another study by Jalali et al. [16], nitrate concentrations for parsley, dill, coriander, and cress were determined to be similar to our results.

Nitrate accumulation in leafy vegetables can also vary among intraspecific cultivars, often due to morphological differences. More precisely, it has been reported that morphological differences among cultivars may be responsible for nitrate accumulation [13]. In our study, the nitrate differences in dill, parsley, and coriander, all from the same family, can be attributed to their morphological variations. Cantliffe reported significant nitrate accumulation differences between smooth and savoury spinach leaves [17 and 18]. This can be explained by nitrate's role as an osmoticum, for retaining turgor and causes leaf expansion under conditions where photosynthetic capacity is too low to produce organic solutes [19]. Another study also confirmed that vegetables with higher nitrate accumulation tend to have higher water content and produce less dry matter [20]. Studies confirm a correlation between nitrate accumulation and water content in leafy vegetables [21 and 22]. This explains the nitrate concentration differences among vegetables from the same family, such as the higher nitrate concentration in dill due to its higher water content (88-87%) [23], compared to parsley (82-83.15%) [24 and 25] and coriander (84%) [26]. So, the higher content of nitrate can be explained by the higher water content of dill, since its leaves need nitrate to keep them fresh and expand. Various studies, including those by Nowrouz et al. [27] and Kyriacou et al. [28] reported higher nitrate concentrations in dill compared to parsley and coriander, aligning with our findings.

Nitrite concentrations in vegetables can also vary with age, with younger or earlier-harvested leaves having lower nitrate levels [13]. Optimal harvest times are 66-70 days [29] for dill, 47-70 days [30 and 31] for parsley, and 45-70 days [32] for coriander. Dill's longer harvest time could contribute to its higher nitrate accumulation. This is consistent with findings from Santamaria et al [33], Santamaria et al [34], Konstantopoulou et al. [35], and Koukounaras et al. [36]. Table 1 reveals the nitrite content of the vegetables studied. Coriander had the highest nitrite content (0.672 mg kg^{-1}), while dill, parsley, and garden cress had significantly lower levels (0.623, 0.556, and 0.378 mg kg^{-1}, respectively). Nitrite levels exceeding 100 mg kg^{-1}can pose health risks [37]. However, all studied vegetables had nitrite content below 1 mg kg^{-1}, indicating they are safe for consumption. Nitrite accumulation in leafy vegetables is generally low due to post-harvest low rate of nitrate-to-nitrite conversion [38].

Table 1. Nitrate and nitrite content in leafy vegetables and comparison of Nitrate concentration in our study with WHO limit [39].

Leafy vegetable	NO_3 (mg kg^{-1})	NO_2 (mg kg^{-1})	NO_3 WHO standard limit (mg kg^{-1})
Parsley	317.526 ± 14.255^{c}	0.556 ± 0.011^{c}	2000
Garden cress	288.556 ± 2.651^{d}	0.378 ± 0.006^{d}	-
Coriander	360.002 ± 13.435^{b}	0.672 ± 0.005^{a}	-
Dill	402.944 ± 10.169^{a}	0.623 ± 0.005^{b}	2000

Lowercase letters in each column indicate significant differences between treatments. Results were shown as Mean ± SD and the number of amples were n=12.

Acceptable daily intake (ADI) and estimated daily intake (EDI)

According to WHO and FAO, the recommended daily consumption of fruits and vegetables for an adult is 400 g, leading to an average nitrate intake of 157 mg kg^{-1} accounting for 11-14% of daily nitrate absorption [1]. The Joint FAO/WHO Expert Committee on Food Additives reaffirmed a nitrate ADI of 0-3.7 mg kg^{-1} in 2002 [40]. Our results, presented in Tables 2 show that the nitrate EDI for all studied vegetables was below the JECFA's ADI standard limit, indicating a low health risk. Among studied vegetables, The EDI of dill (0.2216) was higher than that of coriander (0.1980), parsley (0.1746), and garden cress (0.1587), further confirming the low health risk associated with these vegetables.

The Joint FAO/WHO Expert Committee on Food Additives reaffirmed a nitrite ADI of 0-0.07 mg kg^{-1} [40]. All vegetables studied had an EDI below this limit, with coriander having the highest at 0.0036 mg kg^{-1}. The lower nitrate and nitrite EDIs may be due to reduced nitrate contamination, avoidance of sewage irrigation, lesser use of nitrate fertilizers, and consumption below the recommended daily intake of 400 g per person [7 and 41].

Table 2. Acceptable daily intake and total hazard quotients of nitrite from consumption of various vegetables (Mean ± SD)

	NO_2 concentration				**NO_3 concentration**			
Leafy vegetable	**NO_2 (mg kg^{-1})**	**EDI**	**HQ**	**THQ**	**NO_3 (mg kg^{-1})**	**EDI**	**HQ**	**THQ**
Parsley	0.556 ± 0.011^c	0.0031 ± 0.0002^c	0.0093 ± 0.0006^c	0.0342 ± 0.0006^c	317.526 ± 14.255^c	0.1746 ± 0.0078^c	0.0249 ± 0.0011^c	0.0342 ± 0.0006^c
Garden cress	0.378 ± 0.006^d	0.0021 ± 0.0001^d	0.0063 ± 0.0004^d	0.0290 ± 0.0005^d	288.556 ± 2.651^d	0.1587 ± 0.0014^d	0.0227 ± 0.0003^d	0.0290 ± 0.0005^d
Coriander	0.672 ± 0.005^a	0.0036 ± 0.0001^a	0.0112 ± 0.0003^a	0.0395 ± 0.0011^b	360.002 ± 13.435^b	$0.1980 \pm .0074^b$	0.0283 ± 0.0011^b	0.0395 ± 0.0011^b
Dill	0.623 ± 0.005^b	0.0034 ± 0.0001^b	0.0104 ± 0.0003^b	0.0420 ± 0.001^a	402.944 ± 10.169^a	$0.2216 \pm .0056^a$	0.0317 ± 0.0008^a	0.0420 ± 0.001^a

Lowercase letters in each column indicate significant differences between treatments. Results were shown as Mean±SD, and the number of samples were n=12.

Non-carcinogenic hazard risk

Nitrate toxicity directly correlates with daily intake, and its health risks depend on consumption patterns, consumer body weight, and inherent nitrate risks [7 and 10]. A Target Hazard Quotient (THQ) below 1 indicates acceptable chronic health risk from leafy vegetable consumption [42]. As per Tables 2, the Hazard Quotient (HQ) and THQ of all samples are below 1, indicating that that consuming them is safe in the Tehran province. Specifically, the adult THQ for parsley, garden cress, coriander, and dill were 0.0342, 0.0290, 0.0395, and 0.0420, respectively.

In a study by Jalali *et al*. [16], nitrate accumulation health risks in cress, dill, parsley, and coriander was reported as 0.0001, 0.005, 0.02, and 0.0006, respectively, aligning with our findings. In another study by Seilsepour [43] on pollutant concentration in edible leafy vegetables, risk assessment and hazard quotient index, the Hazard Quotient (HQ) values for nitrate was reported 0.22 for dill, 0.29 for coriander, 0.16 for parsley, and 0.28 for cress. Their results were higher than what we achieved, which means that the hazard risk of our studied vegetables was lower than what they observed in their study, i.e., the vegetables cultivated in Tehran province were safer.

CONCLUSIONS

This study aimed to evaluate the concentrations of nitrate and nitrite in four commonly consumed vegetables - dill, coriander, garden cress, and parsley - in the Tehran province of Iran. The findings confirmed that the nitrate and nitrite concentrations in all the vegetables studied were within the acceptable range as stipulated by the World Health Organization (WHO), thereby indicating their non-toxicity. Furthermore, the study unequivocally established that the Estimated Daily Intake (EDI) of nitrate and nitrite for all the vegetables studied was lower than the Acceptable Daily Intake (ADI) standard limit set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), suggesting a low health risk associated with their consumption. Our results affirmed the safety of these vegetables, in terms of nitrate and nitrite content, for consumption in the studied region during the specified period. The results of this study could be useful for further meta-analyses on nitrate and nitrite levels in vegetables.

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Conflict of interests

The authors declare no conflict of interest.

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