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Development of a Cost-Effective Method for Nitrate and Nitrite Determination in Leafy Plants and Nitrate and Nitrite Contents of Some Green Leafy Vegetables Grown in the Aegean Region of Turkey

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An accurate, fast, easily applicable, and cost-effective method for the determination of nitrate and nitrite was developed. This method was much more reliable than the cadmium column reduction method, which is a tedious and time-consuming procedure and not easily applicable. The principle of the method was reduction of nitrate to nitrite with cadmium acetate solution and zinc powder and then treatment with Griess reagent. Recovery of the method changed from 92.9 to 102.8%, and detection limit was found as 31.4 mg/kg. Coefficient of variation was 3.16% for intraday precision. Nitrate and nitrite contents of 10 types of leafy vegetables native to the Aegean region of Turkey were determined. Wild radish, chicory, fennel, blessed thistle, blue mallow, and chard were analyzed for the first time. Nitrate contents were found between 354.8 mg/kg for iceberg lettuce and 4653 mg/kg for wild radish. Tested vegetables contained <26.33 mg/kg nitrite.

KEYWORDS: Nitrate; nitrite; nitrate and nitrite determination; leafy vegetables

INTRODUCTION

Recently, concentrations of harmful nitrogen-containing compounds, such as nitrate and nitrite, have increased in foods and drinks (*1*−*3*). Nitrate is a final product of biochemical oxidation of organic nitrogen (*4*−*6*). Although nitrate ion is not directly toxic, it can readily be converted to harmful nitrite ion by microbial reduction in food products and in the human body, especially in the oral cavity and in the stomach (*7*−*9*). Nitrite has several adverse effects upon human health (*10*). Nitrite can interact with hemoglobin to form methemoglobin by oxidation of ferrous iron (Fe^{2+}) to the ferric state (Fe^{3+}), thus preventing or reducing the ability of blood to transport oxygen, a condition described as methemoglobinemia that is dangerous, especially in infants (the so-called blue-baby syndrome) (*6*). In the stomach, the reaction between nitrite and secondary amines leads to the formation of nitrosamines (*N*-nitroso compounds), some of which are known as carcinogenic, teratogenic, and mutagenic and increase risk of cancer of the stomach and esophagus (*4*, *9*, *11*, *12*). Gastric cancer is the third leading cause of death in men after lung and prostate cancer and is the fourth leading cause of death in women throughout the world (*9*).

Vegetables play an important role in human nutrition because they are an outstanding source of vitamins, minerals, and biologically active compounds (*12*). On the other hand, the presence of nitrate in foods is mainly due to the plants, taking nitrogen from the soil in this ionic form. The use of nitrogen-containing fertilizers increases nitrate concentration in the soil and, therefore, the nitrate content of plants grown therein is above the normal level. In addition, a small amount of nitrite is added to meat products to protect them against *Clostridium botulinum* and to enhance flavor (*13*). Although useful as a curing agent, residual nitrite in meat products represents a health risk to humans (*14*). It was estimated that plants contribute about 85 and 16−43% of the dietary intakes of nitrate and nitrite, respectively, in a number of societies (*4*). Nitrate concentrations in vegetables vary enormously, ranging from around 1 to 10000 mg/kg of fresh weight (*15*). Especially the leafy vegetables are the major sources for the dietary intake of nitrate. Beetroot, celery, lettuce, spinach, and radish are the greatest sources of nitrate, with the nitrate content often exceeding 1000 mg/kg, whereas broad bean, peas, cauliflower, potato, onion, and sweet corn generally contain < 200 mg/kg (*16*). There are many factors affecting the nitrate and nitrite contents of vegetables. The geographical region, the particular season of harvest, the choice of cultivar, the use of nitrogen-containing fertilizers, growing conditions, light conditions, humidity, plant diseases, insect damage or contact with herbicides, deficiency of some minerals in soil, wastewater, processing conditions, storage time, and conditions affect the nitrate and nitrite contents (*15*).

According to the related national regulations set by the Turkish Food Codex (*17*), the maximum allowable level of nitrate in fresh spinach (*Spinacia oleracea*) is 2500−3000 mg/kg. The maximum allowable levels for fresh lettuce (*Latuca sativa*) are 3500−4500 mg/kg (grown in a greenhouse) and 2500−4000 mg/kg (grown in the open field). The levels for iceberg lettuce are 2500 mg/kg (grown in a greenhouse) and 2000 mg/kg (grown in the open field). The acceptable daily intake (ADI) for nitrate was set at 3.7 mg/kg of body weight by the European Union Scientific Committee for Food, and the ADI value for nitrite was set at 0.06 mg/kg of body weight (*18*).

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Table 1. Green Leafy Vegetables[a]

botanical name	English name	Turkish name	family	cultured or wild
Raphanus raphanistrum	wild radish	turp otu	Cruciferae	wild
Cichorium intybus	chicory	radika	Chicory	cultured/wild
Foenuculum vulgare	fennel	arapsaçi	Umbelliferae	wild
Cnicus benedictus	blessed thistle	şevketi bostan	Asteraceae	wild
Portulaca oleracea	purslane	semiz otu	Portulacaceae	cultured/wild
Spinacia oleracea	spinach	ispanak	Amaranthaceae	cultured
Malva sylvestris	blue mallow	ebegümeci	Malvaceae	wild
Beta vulgaris	chard	pazi	Amaranthaceae	cultured
Lactuca sativa	lettuce	marul	Asteraceae	cultured
Lactuca sativa	iceberg lettuce	iceberg marul	Asteraceae	cultured

[a] Classified by Davis (*27*).

The simultaneous determination of nitrite and nitrate concentrations is of rapidly increasing interest for the food industry (*19*). The majority of the methods involve spectrophotometric procedures (*8*, *11*, *20*, *21*). Among these, the cadmium column reduction method is the most widely used (Cd column−Griess method). The principle of the method is the reduction of nitrate to nitrite with the cadmium column and then treatment with Griess reagent. Nitrite undergoes diazotization and is determined by UV−vis spectrophotometry. However, this method is tedious, time-consuming, and not easily applicable. Monitoring the reducing capacity of the cadmium column is necessary because it changes with time. When reducing capacity decreases below 80%, the column is regenerated. In recent years, several analytical procedures including ion-exchange chromatography, ion-pair reversed phase HPLC, and capillary electrophoresis have been developed for the determination of these ions (*9*, *22*, *23*). Electrochemical sensors can also be used for the determination of nitrate and nitrite ions (*24*).

Edible wild and cultured plants are found in countries with different climates. Plant foods will be increasingly important to ensure a long and healthy life. In the Aegean region of Turkey, a plateful of raw or cooked vegetables (cultured or wild) is commonly served with the main meal. Various green leafy vegetables are the most abundant in the Mediterranean region and are available to purchase from the open markets or to pick from nature. They are picked in spring before the flowers appear. Furthermore, most of the green leafy vegetables have been used traditionally for their health benefits including prevention of cancer, antiedema effects, diuretic effects, as treatment of intense coughing, and as tranquilizers, tonics, and soporific drugs (*25*). The main contrast for the nutritional benefits of these plants is the presence of certain antinutritional and toxic substances such as nitrate, oxalate, and saponin (*26*). Therefore, the objective of this study was to develop an accurate, simple, fast, and cost-effective method for quantifying nitrate and nitrite contents of vegetables and to determine, for the first time, nitrate and nitrite contents of some green leafy vegetables grown in the Aegean region of Turkey.

MATERIALS AND METHODS

Materials. *Samples*. Ten types of green leafy vegetables native to the Aegean region of Turkey were examined (**Table 1**). All samples were grown in the open field and purchased from local markets in İzmir (the biggest city in the Aegean region), from May to November 2007. For each type, three different samples supplied at different times were tested, each of them in triplicate. One kilogram of each vegetable packed in a polyethylene bag was purchased. Edible parts of vegetables were assayed, and disease-infected plants or leaves were discarded. All vegetables were kept at refrigeration temperature and analyzed within 24 h. The classification of green leafy vegetables tested was based on the taxonomy of Davis (*27*) as presented in **Table 1**. Of the 10 leafy vegetables tested, production numbers were available only for spinach, lettuce, and iceberg lettuce as 25400, 15700, and 180 t/year in İzmir, respectively (*28*).

Reagents. Analytical grade reagents were used. Sodium nitrite, sodium nitrate, cadmium acetate dihydrate, sulfanilic acid, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, glacial acetic acid, ammonia, potassium ferrocyanide trihidrate, dipotassium hydrogen phosphate, orthophosphoric acid, and disodium tetraborate decahydrate were purchased from Merck (Darmstadt, Germany). Zinc sulfate heptahydrate and zinc acetate dihydrate were purchased from Riedel-de Haën (Seeize, Germany), acetonitrile (HPLC grade) from Lab-Scan (Dublin, Ireland), and zinc powder from BDH (Poole, U.K.). Distilled water used throughout the procedure was supplied from the distillation apparatus of Hamilton Laboratory Glass Limited (Margate Kent, U.K.).

Griess Reagent. Griess reagent was prepared by modifying the official method of AOAC International (*29*). A 2.10 g amount of sulfanilic acid was dissolved in 250 mL of 15% (v/v) acetic acid solution (HOAc) by heating on a steam bath. *N*-(1-naphthyl)ethylenediamine·2HCl solution was prepared by dissolving 0.750 g of the reagent in 30 mL of distilled water by heating on the steam bath and then pouring while still hot into 250 mL of 15% (v/v) HOAc. This solution was mixed with sulfanilic acid solution and filtered when necessary. Griess reagent was prepared weekly and stored in a brown glass bottle in a refrigerator.

Nitrate Stock Solution (1000 mg/L). A 137.1 mg amount of primary standard sodium nitrate, previously dried for 24 h at 105 °C, was dissolved in sufficient water and then diluted to 100 mL. For preparing standard nitrate solutions stock nitrate solution was diluted with distilled water.

Nitrite Stock Solution (1000 mg/L). A 150.0 mg amount of primary standard sodium nitrite, previously dried for 1 h at 110 °C, was dissolved in sufficient water and diluted to 100 mL. For preparing standard nitrite solutions stock nitrite solution was diluted with distilled water.

Cadmium Acetate Solution (5%, w/v). A 5.78 g amount of cadmium acetate dihydrate was dissolved in sufficient water with the addition of 1 mL of glacial acetic acid and diluted to 100 mL with distilled water.

Methods. *Sample Preparation*. The official procedure TS 6183 from the Institute of Turkish Standards (*30*) was followed with modification. A 25 g amount of sample was homogenized with 80 mL of distilled water in a high-speed blender for 5 min and transferred into a 400 mL beaker; 100 mL of hot water (70−80 °C) and 10 mL of 5% (w/v) $Na_2B_4O_7 \cdot 10H_2O$ solution were added and then mixed and heated for 15 min. The mixture was clarified by adding 5 mL of 23% (w/v) $ZnSO_4 \cdot 7H_2O$ solution and shaking for 15 s, then adding 5 mL of 15% (w/v) $K_4Fe(CN)_6 \cdot 3H_2O$ solution and shaking for 15 s. After cooling, sample was diluted to 250 mL with distilled water and filtered through a Whatman (no. 40) filter paper, and the extract was analyzed immediately.

Nitrate Analysis by Spectrophotometric Method. Nitrate analysis was achieved according to the official procedure TS 4080 from the Institute of Turkish Standards (*31*) with modification. The principle of the method was reduction of nitrate to nitrite with cadmium acetate solution and zinc powder and then colorimetric determination of nitrite with Griess reagent. A 1 mL aliquot (0.5 mL of extract was used for wild radish and fennel, which contained > 2500 mg/kg nitrate) of the sample extract was transferred into a 50 mL volumetric flask, and 14 mL of distilled water and 2 mL

of 25% NH_3 solution were added. After the solution had been mixed, 500 mg of zinc powder was added. The suspension was mixed, and 1 mL of 5% (w/v) cadmium acetate solution was added by blowing into the sample with a pipet. The sample was allowed to stand for 5 min, diluted to 50 mL with distilled water, and, after vigorous shaking, filtered through a Whatman (no. 40) filter paper. To an 8 mL portion of the solution in a glass tube was added 1 mL of glacial acetic acid and 1 mL of Griess reagent. After standing for 30 min, absorbance of the red-violet azo compound was read at 538 nm against a reagent blank using a spectrophotometer. This optical density was a measure of both nitrate and nitrite in the sample due to contribution of the nitrite content, which might occur in the food sample being analyzed. Therefore, nitrate concentration of the sample was determined after subtraction of the corresponding optical density of nitrite from the above-mentioned optical density. Nitrate concentration was calculated by using a nitrate calibration curve. To evaluate the recovery rate of nitrate determination, 4 mL of standard nitrate solution (20 mg of nitrate/L) and 10 mL of distilled water were added to a 1 mL (0.5 mL for wild radish and fennel) aliquot of sample extract in a 50 mL volumetric flask, and the analysis was completed with the same procedure by reducing nitrate to nitrite and then treatment with Griess reagent.

Calibration Curve for Nitrate. Certain volumes of 100 mg/L standard nitrate solution (0.8, 1.4, 2.0, 3.0, 5.0, 7.5, 10.0, and 12.5 mL) were transferred into 50 mL volumetric flasks separately and then diluted to 15 mL with distilled water. The same reduction procedure was applied before treatment with Griess reagent. A calibration graph was constructed by plotting absorbance values against nitrogen concentrations as milligrams per liter nitrate in the solution treated with Griess reagent. Although the solution treated with Griess reagent contains nitrite ions, to simplify the calculations corresponding milligrams per liter nitrate concentrations (instead of nitrite concentrations) were used in abscissa for the construction of the calibration curve. Thus, the concentration of nitrate in vegetables in milligrams per kilogram was found by multiplying the value obtained for vegetable from the abscissa by 500 (by 1000 for wild radish and fennel).

Nitrite Analysis and Calibration Curve. To determine nitrite contents of samples, the official method of AOAC International (*29*) was used with slight modification. A 2 mL aliquot of the sample extract was diluted to 50 mL with only distilled water. To an 8 mL portion of the solution was added 1 mL of glacial acetic acid and 1 mL of Griess reagent. After standing for 30 min, absorbance of the sample was read at 538 nm (half of this absorbance value was used in the subtraction procedure in the preceding nitrate determination, and one-fourth of this optical density was used in a subtraction procedure in wild radish and fennel analyses). Nitrite concentration was calculated by using a nitrite calibration curve. To find the recovery rate of nitrite determination, 2 mL of standard nitrite solution (8 mg of nitrite/L) was added into 2 mL of sample extract, and the solution was diluted to 50 mL with distilled water. The analysis was completed by treatment with Griess reagent. A nitrite calibration curve was prepared by treating 8 mL portions of standard nitrite solutions, with nitrite concentrations ranging from 0.01 to 0.40 mg/L, with 1 mL of glacial acetic acid and 1 mL of Griess reagent and plotting absorbance values against nitrite concentrations.

HPLC Determination of Nitrate and Nitrite. Simultaneous determination of the nitrate and nitrite in the sample extract was achieved according to ref *22*.

Apparatus. HPLC determinations were performed by using an Agilent 1100 liquid chromatograph (Agilent, Santa Clara, CA) equipped with an Agilent 1100 diode array detector (DAD) set at 205 nm, an Agilent 1100 quaternary pump, and an Agilent 1100 autosampler. The chromatographic column was μ-Bondapak NH_2 (10 μm particle size, 300 mm × 3.9 mm i.d., Waters Corp., Milford, MA).

Absorbance of the red-violet azo compound was measured with a Cary 50 UV−vis spectrophotometer (Varian, U.K.).

Statistical Analysis. All of the statistical analyses were performed with the SPSS 10.0 statistics package program. Statistical analysis of data was achieved by using one-way analysis of variance (ANOVA), Duncan post-test, and Pearson correlation test. Comparison of the proposed spectrophotometric method with HPLC method was performed by the *t* test. In all data analyses, a value of $P < 0.05$ was considered to be statistically significant. Interday precision of the nitrate analysis was estimated by calculating pooled (within-sample) standard deviation (*32*).

Table 2. Percentage of Recovery for Nitrate and Nitrite Analyses

	nitrate		nitrite	
vegetable	mean[a]	SD[b]	mean	SD
wild radish	100.1	10.1	100.2	4.1
chicory	100.4	9.5	97.7	1.8
fennel	101.2	4.8	98.2	1.1
blessed thistle	102.1	5.8	96.8	8.6
purslane	95.4	5.6	98.7	3.3
spinach	92.9	1.2	98.4	2.7
blue mallow	102.8	9.2	95.7	4.3
chard	100.1	8.1	96.9	1.8
lettuce	97.6	6.7	97.4	2.1
iceberg lettuce	94.5	4.9	98.1	3.6

[a] Mean of nine replicates. [b] SD, standard deviation.

Table 3. Mean Values for Nitrate Contents of Green Leafy Vegetables (Milligrams per Kilogram)[a]

	sample			
vegetable	1	2	3	overall mean
wild radish	4652	5764	3544	4653 a
chicory	990.3	386.3	548.4	641.7 d
fennel	2187	4022	3575	3261 b
blessed thistle	403.3	590.2	961.7	651.7 d
purslane	1735	1069	2290	1698 c
spinach	338.8	156.9	653.4	383.0 d
blue mallow	2123	2433	2522	2359 bc
chard	2401	3142	1706	2416 bc
lettuce	439.9	297.1	857.4	531.5 d
iceberg lettuce	256.4	381.5	426.5	354.8 d

[a] Overall mean values with different letters are significantly different from each other ($P < 0.05$).

RESULTS AND DISCUSSION

Performance Characteristics of the Method for Nitrate and Nitrite Determinations. In our study, a more reliable method was developed by modifying the official procedure TS 4080 from the Institute of Turkish Standards (*31*), which had been used for the analysis of fizzy soft drinks with very simple food matrices. The developed method was applied to a range of fresh and processed vegetables using a different composition of Griess reagent. Performance characteristics of the method were estimated, and validation against the HPLC method was realized. The nitrate calibration curve was linear from 0 to 12500 mg of nitrate/kg of food. The regression equation was $y = 0.1385x + 0.0128$ with a correlation coefficient of 0.9991. The limit of detection was calculated to give a signal-to-noise ratio of 3 and found as 31.4 mg of nitrate/kg of food. To find the nitrate content of vegetables containing < 31.4 mg/kg nitrate, it is possible to experiment with > 1 mL of sample extract. Recovery rates of nitrate determinations were calculated for each sample type. As seen from **Table 2**, nitrate recovery was satisfactory and varied from 92.9 to 102.8%.

The interday precision of the nitrate analysis was estimated by calculating the pooled standard deviation and found as 124.1 mg of nitrate/kg of food. This value indicates the precision of nitrate analyses of 10 leafy vegetables including 90 replicates (**Table 3**). As can be calculated from **Table 3**, the mean nitrate content of all these leafy vegetables was 1695 mg/kg. Then, the coefficient of variation (CV) of interday precision was calculated as 7.32%. The intraday precision was estimated from the nitrate determinations

Table 4. Mean Values for Nitrite Contents of Green Leafy Vegetables (Milligrams per Kilogram)[a]

	sample			
vegetable	1	2	3	overall mean
wild radish	0.40	nd	nd	0.13 b
chicory	nd	nd	15.99	5.33 b
fennel	8.30	nd	nd	2.77 b
blessed thistle	nd	nd	nd	nd
purslane	28.63	12.79	37.58	26.33 a
spinach	nd	nd	nd	nd
blue mallow	nd	55.33	3.81	19.71 ab
chard	nd	nd	15.55	5.18 b
lettuce	nd	nd	nd	nd
iceberg lettuce	nd	nd	nd	nd

[a] Overall mean values with the different letters are significantly different from each other ($P < 0.05$). nd, not detected.

Table 5. Validation of the Developed Spectrophotometric Method (SpM) against the HPLC Method[a]

blue mallow				wild radish				chicory			
nitrate		nitrite		nitrate		nitrite		nitrate		nitrite	
SpM	HPLC	SpM	HPLC	SpM	HPLC	SpM	HPLC	SpM	HPLC	SpM	HPLC
1491	1498	nd	nd	2252	2187	nd	nd	nd	26.1	nd	nd
1505	1505	nd	nd	2123	2179	nd	nd	nd	27.9	nd	nd
1478	1478	nd	nd	2150	2186	nd	nd	nd	28.9	nd	nd
1445	1495	nd	nd	2128	2185	nd	nd	nd	29.9	nd	nd
1467	1482	nd	nd	2095	2195	nd	nd	nd	27.9	nd	nd
1477[b]	1492	nd	nd	2150	2186	nd	nd	nd	28.1	nd	nd

[a] Nitrate and nitrite contents are given in mg/kg, and detection limit of SpM for nitrate is 31.4 mg/kg. nd, not detected. [b] Mean value.

of a blue mallow sample as 10 replicates. Nitrate contents of this sample were found as 1514, 1455, 1464, 1430, 1440, 1425, 1413, 1372, 1401, and 1513 mg/kg. On the basis of this set of data, mean value and standard deviation were 1443 and 45.6 mg/kg, respectively. The CV for intraday precision was calculated as 3.16%.

In nitrite determination (**Table 4**) the calibration curve was linear from 0 to 100 mg of nitrite/kg of food. The regression equation was $y = 0.2299x - 0.0028$ with a correlation coefficient of 0.9979. Recovery rates of nitrite determinations were calculated for each sample type. As seen from **Table 2**, the nitrite recovery was between 95.7 and 100.2%.

Validation of the Developed Spectrophotometric Method (SpM) against the HPLC Method. Validation of the developed SpM was performed against the HPLC method. As seen from **Table 5**, nitrate and nitrite contents of three selected vegetables (blue mallow, wild radish, and chicory) were determined according to both SpM and HPLC methods. Mean nitrate values of blue mallow were 1477 and 1492 mg/kg for SpM and HPLC methods, respectively. There was no difference between these mean values ($P < 0.05$). Mean nitrate values for wild radish were 2150 and 2186 mg/kg for SpM and HPLC methods, and these mean values were also the same ($P < 0.05$). The mean nitrate value for chicory was 28.1 mg/kg for the HPLC method. The nitrate concentration of this vegetable was below the detection limit (31.4 mg/kg) according to the SpM method. Nitrite concentrations of blue mallow, wild radish, and chicory were below the detection limits for both methods. Consequently, SpM and HPLC methods gave the same results for nitrate and nitrite concentrations of green leafy vegetables.

Nitrate and Nitrite Contents of Green Leafy Vegetables. Nitrate and nitrite contents of 10 green leafy vegetables are given in **Tables 3** and **4**. As seen from the **Table 3**, the lowest average nitrate values were obtained for iceberg lettuce (from 256.4 to 426.5 mg/kg with the mean value of 354.8 mg/kg), and the highest average nitrate values were obtained for wild radish (from 3544 to 5764 mg/kg with the mean value of 4653 mg/kg). Thus, it can be concluded that wild radish contributes a lot to dietary nitrate intake from green leafy vegetables during the winter and spring months in the Aegean region of Turkey. As is seen from **Table 4**, the lowest average nitrite values were obtained for blessed thistle, spinach, lettuce, and iceberg lettuce (not detected). The highest average nitrite values were obtained for purslane, from 12.79 to 37.58 mg/kg. Nitrite levels in vegetables may increase during postharvest storage by the action of indigenous bacteria and/or the presence of nitrate reductase, especially when they are left at room temperature or higher (*9*). This may explain the presence of nitrite in purslane. Whereas nitrate contents of tested vegetables were high in most cases and varied from 156.9 to 5764 mg/kg, nitrite contents were < 55.33 mg/kg. An average nitrate concentration of 1695 mg/kg was detected for the green leafy vegetables tested, whereas the average nitrite concentration was 5.94 mg/kg. These results are in accordance with the related literature. As found by Ximenes et al. (*33*), nitrite levels in fresh leafy vegetables were usually < 2 mg/kg. Likewise, it was demonstrated that there was no detectable nitrite in 94% of edible fresh retail vegetables (*34*). When the overall mean values for nitrate and nitrite were compared (**Tables 3** and **4**), there was not a correlation between nitrate and nitrite values of the green leafy vegetables tested. Likewise, Amr and Hadidi (*4*) did not find any correlations between nitrate and nitrite contents of vegetables grown in the open field.

Examination of Differences in Nitrate and Nitrite Contents between Different Types of Vegetables. When the overall mean nitrate levels in leafy vegetables were compared, there were statistically significant differences between different types (**Table 3**). Among the leafy vegetables tested, wild radish had the highest nitrate content, whereas chicory, blessed thistle, spinach, lettuce, and iceberg lettuce had the lowest ($P < 0.05$). Statistically significant differences were also detected between the samples belonging to the same type, as the harvest time, growing conditions, farming practises, climate, soil quality, level and type of fertilization, and other factors affect the nitrate and nitrite contents of vegetables. According to results of ANOVA, three wild radish samples were statistically different from each other ($P < 0.05$) (**Table 3**). The same differences were also valid for the other leafy vegetables tested except blue mallow, as blue mallow samples 2 and 3 were the same. These findings correlated well with the literature. On the basis of Jaworska (*3*), plants harvested in July showed the smallest contents of nitrites and the greatest contents of nitrates, and those in September the smallest contents of nitrates, but the greatest contents of nitrites. When the nitrite levels were compared, there were also statistically significant differences between different types (**Table 4**). It is obvious from the data presented in **Table 4** that there were significant differences between purslane and the other types including wild radish, chicory, fennel, blessed thistle, spinach, chard, lettuce, and iceberg lettuce ($P < 0.05$).

Comparison of the Nitrate and Nitrite Contents of Tested Vegetables with Those in the Literature. Nitrate and nitrite contents of green leafy vegetables tested have not been studied before except for spinach, purslane, lettuce, and iceberg lettuce. Erkmen et al. (*35*) reported the nitrate and nitrite contents of 31 types of fresh vegetables, including lettuce, spinach, and purslane, available at retail markets in İstanbul, Turkey. Nitrite contents ranged from 0.10 to 2.96 mg/kg and nitrate contents from 2.92 to 2055.5 mg/kg. Nitrate values for spinach are greater, and nitrite values of purslane are smaller than our results, whereas the other values are in accordance with our data. Öztekin et al. (*23*) determined nitrite and nitrate in spinach, parsley, dill, and leek.

Nitrite was not detected in these vegetables, but nitrate contents varied from 130 to 2820 mg/kg. The nitrate content of spinach is considerably higher than our result, but the nitrite value of spinach is in accordance with our result. Amr and Hadidi (*4*) studied the effects of cultivar and harvest date on the nitrate and nitrite contents of the edible parts of spinach, lettuce, cabbage, squash, parsley, cauliflower, tomato, and cucumber. Nitrate levels in these vegetables were generally low (the lowest average of 0.13 mg/100 g, and the highest of 4.77 mg/100 g). Nitrite levels, on the other hand, ranged from nondetectable to a maximum level of 0.29 mg/100 g. Nitrite contents of lettuce and spinach are in accordance with our results, but nitrate values are much smaller than our results. Prasad and Chetty (*12*) studied the nitrate contents of four commonly consumed fresh leafy vegetables (Chinese cabbage, celery, lettuce, and English cabbage) supplied from markets in Fiji. The results of the study showed that nitrate-N contents in fresh leafy vegetables ranged from 1297 to 5658 mg/kg. The value for lettuce is much greater than we found. Hsu et al. (*9*) quantified nitrate and nitrite contents of a range of vegetables. It was demonstrated that nitrate contents ranged from 48.0 to 4849.6 mg/kg and nitrite contents varied from nondetectable to 19.6 mg/kg. The nitrite content of iceberg lettuce is the same as our result, but the nitrate content is lower than ours.

Importance of the Current Study to the Field. An accurate, simple, fast, and cost-effective spectrophotometric method was developed for quantifying nitrate and nitrite contents in vegetables. The principle of the method is the reduction of nitrate to nitrite with cadmium acetate solution and zinc powder and then spectrophotometric determination of nitrite with Griess reagent. This method is much more reliable than the cadmium column reduction method. The proposed method is easily applicable, and it is not necessary to monitor the reducing capacity of the cadmium acetate solution−zinc powder system. In addition, preparation of a cadmium column, which is a difficult and time-consuming procedure, is not necessary. Also, the method does not need any sophisticated instruments like HPLC. Recoveries of nitrate and nitrite determinations are greater than 92.9 and 95.7%, respectively. The nitrate calibration curve is linear up to 12500 mg of nitrate/kg of food, with a correlation coefficient of 0.9991. The nitrite calibration curve is linear up to 100 mg of nitrite/kg of food, with a correlation coefficient of 0.9979. The limit of detection is 31.4 mg of nitrate/kg of food, and the coefficient of variation was calculated as 3.16% for intraday precision of nitrate determination. Validation of the developed spectrophotometric method was achieved against the HPLC method. Both methods gave the same results for nitrate and nitrite contents of vegetables ($P < 0.05$).

Nitrate and nitrite contents of 10 types of green leafy vegetables native to the Aegean region of Turkey were determined. Wild radish, chicory, fennel, blessed thistle, blue mallow, and chard were analyzed for the first time. Nitrate contents were found between 354.8 mg/kg for iceberg lettuce and 4653 mg/kg for wild radish. According to the related national regulations (*17*), nitrate contents of all green leafy vegetables except wild radish were found to be under the toxic levels. With regard to the nitrite values, the tested vegetables contained < 26.33 mg of nitrite/kg of food. The current study demonstrated that green leafy vegetables commonly consumed in the Aegean region of Turkey and possibly in the Mediterranean basin may contain considerable amounts of nitrate. Daily nitrate intake depends on the type and quantity of the vegetables consumed, and it may be concluded that wild radish contributes to the highest dietary nitrate intake from green leafy vegetables. Unfortunately, production numbers about wild radish are not available as it is not a cultured plant (*28*). As high dietary nitrate and nitrite intake may increase the risk of gastrointestinal cancers due to the formation of carcinogenic nitrosamines, it is vital to make dietary changes for low consumption of vegetables with high nitrate content.

LITERATURE CITED

(1) Johnson, C. J.; Kross, B. C. Continuing importance of nitrate contamination of groundwater and wells in rural areas. *Am. J. Ind. Med.* **1990**, *18* (4), 449–456.

(2) Van Leeuwen, F. X. R. Safe drinking water: the toxicologist's approach. *Food Chem. Toxicol.* **2000**, *38*, S51–S58.

(3) Jaworska, G. Content of nitrates, nitrites, and oxalates in New Zealand spinach. *Food Chem.* **2005**, *89*, 235–242.

(4) Amr, A.; Hadidi, N. Effect of cultivar and harvest date on nitrate (NO_3^-) and nitrite (NO_2^-) content of selected vegetables grown under open field and greenhouse conditions in Jordan. *J. Food Compos. Anal.* **2001**, *14*, 59–67.

(5) Mikuška, P.; Vecera, Z. Simultaneous determination of nitrite and nitrate in water by chemiluminescent flow-injection analysis. *Anal. Chim. Acta* **2003**, *495*, 225–232.

(6) Cemek, M.; Akkaya, L.; Birdane, Y. O.; Seyrek, K.; Bulut, S.; Konuk, M. Nitrate and nitrite levels in fruity and natural mineral waters marketed in western Turkey. *J. Food Compos. Anal.* **2007**, *20*, 236–240.

(7) Bories, P. N.; Bories, C. Nitrate determination in biological fluids by an enzymatic one-step assay with nitrate reductase. *Clin. Chem.* **1995**, *41*, 904–907.

(8) Siu, D. C.; Henshall, A. Ion chromatographic determination of nitrate and nitrite in meat products. *J. Chromatogr., A* **1998**, *804*, 157–160.

(9) Hsu, J.; Arcot, J.; Lee, N. A. Nitrate and nitrite quantification from cured meat and vegetables and their estimated dietary intake in Australians. *Food Chem.* **2009**, *115*, 334–339.

(10) Connolly, D.; Paul, B. Rapid determination of nitrate and nitrite in drinking water samples using ion-interaction liquid chromatography. *Anal. Chim. Acta* **2001**, *441*, 53–62.

(11) Kazemzadeh, A.; Ensafi, A. A. Sequential flow injection spectrophotometric determination of nitrite and nitrate in various samples. *Anal. Chim. Acta* **2001**, *442*, 319–326.

(12) Prasad, S.; Chetty, A. A. Nitrate-N determination in leafy vegetables: study of the effects of cooking and freezing. *Food Chem.* **2008**, *106*, 772–780.

(13) Stalikas, C. D.; Konidari, C. N.; Nanos, C. G. Ion chromatographic method for the simultaneous determination of nitrite and nitrate by post-column indirect fluorescence detection. *J. Chromatogr., A* **2003**, *1002*, 237–241.

(14) Sîríken, B.; Özdemir, M.; Yavuz, H.; Pamuk, Ş. The microbiological quality and residual nitrate/nitrite levels in Turkish sausage (*soudjouck*) produced in Afyon Province, Turkey. *Food Control* **2006**, *17*, 923–928.

(15) Walters, C. L. Nitrate and nitrite in foods. In *Nitrates and Nitrites in Food and Water*; Hill, M. J., Ed.; Ellis Horwood: London, U.K., 1991; pp 93−112.

(16) MAFF. Nitrate, nitrite and *N*-nitroso compounds in foods. 20th Report of the Steering Group on Food Surveillance, Food Surveillance Paper 20; HMSO: London, U.K., **1987**.

(17) Turkish Food Codex, Appendix 16, Ankara, 2008.

(18) EU Scientific Committee for Food Opinion on nitrate and nitrite. European Commission DG III Brussels *Expressed on 22 September 1995*-Annex 4 to document III/56/95, CS/ CNTM/NO3/20-FINAL.

(19) Butt, S. B.; Riaz, M.; Iqbal, M. Z. Simultaneous determination of nitrite and nitrate by normal phase ion-pair liquid chromatography. *Talanta* **2001**, *55*, 789–797.

(20) Elliott, R. J.; Porter, A. G. A rapid cadmium reduction method for the determination of nitrate in bacon and curing brines. *Analyst* **1971**, *96*, 522–527.

(21) Sanderson, J. E.; Ross Consaul, J.; Lee, K. Nitrate analysis in meats; comparison of two methods. *J. Food Sci.* **1991**, *56* (4), 1123–1124.

(22) Foodstuffs − Determination of nitrate and/or nitrite content − Part 2: HPLC/IC method for the determination of nitrate content of

vegetables and vegetable products, EN 12014-2; European Committee for Standardization: Brussels, Belgium, 1997.
(23) Öztekin, N.; Nutku, M. S.; Erim, F. B. Simultaneous determination of nitrite and nitrate in meat products and vegetables by capillary electrophoresis. *Food Chem*. **2002**, *76*, 103–106.
(24) Badea, M.; Amine, A.; Palleschi, G.; Moscone, D.; Volpe, G.; Curulli, A. New electrochemical sensors for detection of nitrites and nitrates. *J. Electroanal. Chem*. **2001**, *509*, 66–72.
(25) El, S. N.; Karakaya, S. Radical scavenging and iron-chelating activities of some greens used as traditional dishes in Mediterranean diet. *Int. J. Food Sci. Nutr*. **2004**, *55* (1), 67–74.
(26) Özcan, M. M.; Akbulut, M. Estimation of minerals, nitrate and nitrite contents of medicinal and aromatic plants used as spices, condiments and herbal tea. *Food Chem*. **2007**, *106*, 852–858.
(27) Davis, P. H. *Flora of Turkey and the East Aegean Islands*; Edinburg University Press: Edinburgh, Scotland, 1965−1985; Vol. 1−9.
(28) Republic of Turkey, Ministry of Agriculture and Rural Affairs, Data of İzmir Province Agriculture Directorate, **2009**.
(29) *Official Methods of Analysis of AOAC International*, 15th ed.; AOAC International: Arlington, VA, 1990; pp 843−844.
(30) *Fruits, Vegetables and Derived Products, Determination of Nitrite and Nitrate Content, TS 6183*; Institute of Turkish Standards: Ankara, Turkey, 1988.
(31) *Fizzy Soft Drinks, TS 4080*; Institute of Turkish Standards: Ankara, Turkey, 1992.
(32) Skoog, D. A.; West, D. M.; Holler, F. J. *Fundamentals of Analytical Chemistry*; Saunders College Publishing: Philadelphia, PA, 1996; pp 30.
(33) Ximenes, M. I. N.; Rath, S.; Reyes, F. G. R. Polarographic determination of nitrate in vegetables. *Talanta* **2000**, *51*, 49–56.
(34) Hunt, J.; Turner, M. K. A survey of nitrite concentrations in retail fresh vegetables. *Food Addit. Contam*. **1994**, *11*, 327–332.
(35) Erkmen, G.; Orak, H.; Şatıroğlu, S. Nitrate and nitrite content of fresh vegetables of Turkish origin. *Turk. J. Chem*. **1990**, *14*, 196–200.

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