The Effect of Home Cooking Method and Refrigeration Processes on the Level of Nitrate and Nitrite In Spinach

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Introduction

Nitrites and nitrates are the compounds naturally found in the environment which are often ingested by human via vegetables, meats and drinking water. Nitrate can convert into nitrite, which is less abundant in the environment by bacterial activity.¹ These two compounds have been known as unwanted compounds in the diet because of their carcinogenic effect,¹² causing some diseases such as methemoglobinemia in infants¹³ and congenital malformations in children whose mother had drunk water with nitrate concentrations greater than 5ppm during pregnancy.³ Furthermore, increase in the nitrate level leads to oxalate accumulation in leafy vegetables such as spinach which can cause nutrient deficiency and increase the risk of kidney stones.⁴ Nevertheless, some other studies have indicated that nitrite accumulation in the blood and tissues is a biological source of NO which can reduce the blood pressure. It can enhance gastroprotection, has good effects on cardiac function,
and plays a role in mitochondrial respiration.\textsuperscript{7,8}

Nitrites are found in large amounts in vegetables, which are responsible for 70-90\% of dietary nitrate intake.\textsuperscript{9,11} Among vegetables, green leafy ones contain higher levels of nitrate.\textsuperscript{11} Spinach is one of these vegetables which has been shown in several studies to contain high levels of nitrate, compared with other vegetables. This amount can be up to 5 grams nitrate per kg of fresh spinach.\textsuperscript{5,12-14} Furthermore, this vegetable is affected by various processes, including cooking and refrigeration before consuming, which can affect its nitrate and nitrite levels. As many studies indicate that boiling reduces the nitrate content of spinach or other vegetables,\textsuperscript{15-17} however, it cannot affect their nitrite level.\textsuperscript{15,17}

Although the effects of freezing on the nitrate and nitrite in vegetables have been examined in many studies,\textsuperscript{15-19} few investigations have been conducted on the effect of refrigeration on the nitrate and nitrite content. At present, there are conflicting results. As Chung and colleagues showed, storing vegetables at 5±1 °C, in spite of keeping them in ambient temperature, has no effect on nitrate and nitrite levels over 7 days.\textsuperscript{20} But Chew and colleagues indicated that refrigeration at 4 °C, after blanching at 100 °C water for 1 minute, leads to reduced nitrate and increased nitrite levels in two types of leafy vegetables called \textit{Amaranthus gangeticus} and \textit{Amaranthus paniculatus}.\textsuperscript{21}

The Acceptable Daily Intake (ADI) for nitrate is 3.7 mg/kg/day, so the nitrate intake of a 60 kg adult should not be more than 222 mg nitrate per day\textsuperscript{3,11,22} and the ADI for nitrite is 0-0.07 mg/kg/day.\textsuperscript{23} Therefore, nitrate and nitrite intake, especially for those who consume lots of vegetables in their diet, must be reduced to a minimum level.\textsuperscript{22}

Most studies that examined the effect of cooking on nitrate and nitrite level of vegetables have investigated on the boiling method. The present study aimed to investigate the effect of a cooking method on spinach, as one of the high nitrate vegetables. This method, which is common in Iran, has not been investigated in other studies before. The effect of refrigeration on nitrate and nitrite level of the cooked spinach, which has been investigated in a few studies, was studied, as well.

**Materials and Methods**

**Instruments**

Samples were analyzed using Waters HPLC (High Performance Liquid Chromatography) system, which was equipped with a UV Waters 2487 Dual λ Absorbance Detector, Waters 1525 Binary HPLC Pump and C18 Waters Spherisorb ® 5 μm ODs2 (250×4.6) column. For degassing the solutions, YL9100 HPLC System Vacuum Degasser was utilized. pH measurements were determined using a Metrohm827 pH meter. Sigma Laboratory Centrifuges (3k30) was utilized to centrifuge the samples before injection, and Elmasonic S60H ultrasonic system was used for preparation of samples.

**Chemicals:** All reagents were purchased from Merck Company.

**Preparation of Spinach**

Spinach was bought from green groceries in Shiraz, Fars, Iran in 2014. After removing their visible soil and non-edible parts, they were cut into 4-7 cm pieces. Then, they were washed with deionized water for 3 times and drained. A part of raw spinach was set aside to measure the nitrate and nitrite levels and the other parts were heated in a closed container until almost all water of the spinach was evaporated (about 40 min without adding water). A part of the cooked spinach was used to measure the nitrate and nitrite levels and the others were kept in 4 °C refrigerator for 5 days.

**Sample Preparation for Analysis**

After the initial preparation, spinach samples were prepared for HPLC injection based on the Chou and colleagues’ study.\textsuperscript{24} All glassware were soaked in NaOH solution and then washed with deionized water for several times.

**Preparation of Mobile Phase and Standard Solutions**

Two aqueous methanol concentrations (40, 30\% v/v) and different pH values (5.5-7.5) were tested. Different amounts of Octylamine (0.010, 0.0125, 0.015, and 0.020 M) were added to the solutions. Finally, the solutions were passed from 0.22μm filter. Eventually, the optimal condition of the mobile phase (30\% methanol, pH 6.5 with 0.015 M Octylamine) was used in the experiment.

Some standard solutions containing 3.12, 6.25, 12.5, 25 and 50 μg/mL nitrite and nitrate were prepared on the day of experiment and injected to HPLC system.

**HPLC Analysis**

Several flow rates were tested (0.5, 0.8, 1 mL/min) and the optimal one was 0.8 mL/min. The detected UV wavelength and the injection volume were 213 nm and 5 μL, respectively. HPLC column was refreshed by passing a mixture solution of water and methanol (50:50 v/v) at the end of the analysis.

**Statistical Analysis**

The collected data were analyzed using SPSS,
version 18. Descriptive statistics were used to report the mean and standard deviation. The levels of nitrite and nitrate in spinach were compared before and after the cooking process and also before and after refrigeration storage, using Wilcoxon test following the test of the normality of the parameters. The significance level was \( P<0.05 \).

**Results**

After examining various conditions to set up the HPLC system, the optimum mobile phase for detection and separation of two peaks of nitrate and nitrite was 30% (v/v) aqueous methanol with addition of 0.015 M Octylamine which was adjusted in 6.5pH value. The flow rate was 0.8 mL/min.

The contents of nitrate in raw and cooked spinach are given in Table 1. As shown in the table, fresh spinach had the lowest contents of nitrate that significantly increased as much as 410.16 ppm (121%) after cooking (\( P=0.012 \)), but a significant decrease (246.20 ppm (33%)) was observed after refrigeration storage of the cooked spinach (\( P=0.012 \)).

Table 2 shows the contents of nitrite in the raw and cooked spinach. Cooking reduces the nitrite level of spinach by 13% (3.5 ppm), but this change was not significant. No significant change was observed in the nitrite content after refrigeration, as well.

**Discussion**

The aim of this study was to evaluate the effect of the cooking and refrigeration processes on the level of nitrite and nitrate in spinach. As shown in the Tables, concentration of nitrate and nitrite in spinach samples varied widely. This can occur because of the plant variety, rainfall, light, amount of fertilizer, and other growth conditions.\(^{13,25} \) The mean nitrate concentrations in raw spinach was 336.54±182.2 mg/kg, comparable to the content given by Gajda and colleagues\(^{26} \) and Petersen and colleagues;\(^{27} \) they found that the level of nitrates in spinach varied from 29 to 6757 mg/kg and 48 to 5630 mg/kg, respectively.

The mean nitrite concentration in raw spinach was 26.49±10.07 mg/kg. Based on the usual content of nitrite reported in vegetables, the raw spinach was characterized by a high content of nitrite, because the nitrite level of vegetables is low, usually below 2 mg/kg.\(^{28,29} \) However, depending on the type of vegetables, light, the amount and type of the fertilizer used and other conditions, this amount can be increased. As Chou and colleagues found out, the nitrite concentration of vegetables is more than the amount reported, which was 122±8.5 mg/kg in spinach.\(^{24} \) The results are in agreement with those reported by Petersen and colleagues too, indicating that the nitrite level of spinach varied from 0 to 162 mg/kg with a mean amount of 11±30 mg/kg.\(^{27} \) Nitrite concentration of fresh spinach is reported 9.5-197.5 mg/kg by Iammarino and colleagues.\(^{30} \) Nevertheless, some studies determined the nitrite concentration of spinach about 0 mg/kg by HPLC method\(^{14} \) and about 1 mg/kg by spectrophotometry method.\(^{15} \)

The other reason for the high nitrite concentration is poor storage conditions of vegetables.\(^{31} \) In fact, improper storage conditions may increase the activity of the nitrate-reducing bacteria which results in nitrate reduction and nitrite enhancement.\(^{27,28} \) Studies that reported a higher nitrite level of spinach than its usual concentrations in vegetables; due to improper storage conditions Phillips, Heisler and colleagues and Aworth and colleagues determined a nitrite concentration of 10-22 mg/kg, 140 mg/kg and 4-24 mg/kg in spinach, respectively.\(^{31} \) Though in this study, spinach samples were kept in a good condition after purchase, but chopping spinach in the preparatory phase could lead to this condition. Moreover, since spinach used in this study was purchased from the green groceries in the city, it is possible that storage conditions were poor during harvesting and transporting them from farms.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Nitrate (ppm)</th>
<th>Mean</th>
<th>S.D</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw spinach (1st day)</td>
<td>8</td>
<td></td>
<td>336.54*</td>
<td>182.20</td>
<td>74.71-587.82</td>
</tr>
<tr>
<td>Cooked spinach (1st day)</td>
<td>8</td>
<td></td>
<td>746.70*</td>
<td>594.54</td>
<td>112.92-1706.02</td>
</tr>
<tr>
<td>Cooked spinach after refrigeration (5th day)</td>
<td>8</td>
<td></td>
<td>482.50*</td>
<td>409.43</td>
<td>94.96-1178.13</td>
</tr>
</tbody>
</table>

Different letters in the same column indicate statistically significant differences (\( P<0.05 \))

**Table 2:** Contents of nitrite in raw and cooked spinach

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Nitrate (ppm)</th>
<th>Mean</th>
<th>S.D</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw spinach (1st day)</td>
<td>8</td>
<td></td>
<td>26.49*</td>
<td>10.07</td>
<td>14.27-41.91</td>
</tr>
<tr>
<td>Cooked spinach (1st day)</td>
<td>8</td>
<td></td>
<td>22.99*</td>
<td>10.49</td>
<td>14.99-39.07</td>
</tr>
<tr>
<td>Cooked spinach after refrigeration (5th day)</td>
<td>8</td>
<td></td>
<td>22.08*</td>
<td>10.44</td>
<td>14.30-36.76</td>
</tr>
</tbody>
</table>

Different letters in the same column indicate statistically significant differences (\( P<0.05 \))
to vegetable field and then to green groceries in the
city, which usually takes about 1 day. According to
Chung and colleagues, storing vegetables at ambient
temperature (22±1 °C) can reduce nitrate and increase
nitrite after 2-3 days.20 Tamme and colleagues also
concluded that storing vegetable juices at ambient
temperature causes a reduction in nitrates and increase
in the nitrite during the 2 days.29

The cooking process mentioned before, (heating
for 40-45 min without adding any water, until almost
all water of the spinach was evaporated) increased
the level of nitrate of spinach as much as 410.16 ppm.
However, Jaworska showed that boiling reduces the
nitrate level in spinach and New Zealand spinach.13
Prasad and colleagues in their effort to study the
effects of cooking and freezing on nitrate level of
leafy vegetables also concluded that boiling for
10 minutes reduced the nitrate content by 47-56% in
leafy vegetables.14 Moreover, Leszcynska and
colleagues observed that boiling for 10-15 minutes
led to a reduction in the nitrate level in vegetables,
too.32 Although other studies indicated that cooking
can reduce the nitrate level of vegetables, it should
be pointed out that cooking method of those studies
is different from that used in this study; in others,
some water had been added to vegetables first, and
after boiling, they were drained and the water was
discarded.15,16,32 But no water was added to vegetables
in this study and cooking process continued until the
tissue of spinach became soft and almost all its water
was evaporated. As a result, no water was discarded.
So, it is likely that nitrate, which is a water-soluble
compound, remained in spinach and was condensed
during cooking. Other studies have also confirmed
that, depending on the method of boiling and duration
of cooking and the variety of vegetables, changes in
nitrite and nitrate levels are different.32

A slight reduction (3.5 ppm) was observed in the
nitrite level after the cooking process, but the change
was not statistically significant. Although cooking
method of this study was different from previous
studies, the result is similar to that of Jaworska15 and
Huarte-Mendicoa and colleagues,17 investigating the
effect of cooking on nitrate and nitrite of spinach, New
Zealand spinach,15 and broccoli.17 On the other hand,
Leszcynska and colleagues could not observe a clear
effect of boiling on nitrite in cruciferous vegetables;
they observed that boiling had no significant effect on
the nitrite level of some types of cruciferous vegetables
(curly kale, broccoli and green cauliflower), but it
caus a significant decrease in white cauliflower and
significant increase in Brussels sprouts. Cieslik
observed a reduction in the nitrite level after boiling
of potato, too. These results also show that, depending
on the duration of cooking and the variety of vegetables,
changes in nitrite and nitrate are different.12

Refrigeration at 4°C for 5 days reduced the
nitrate level of spinach about 264.2 ppm, but it had
no significant effect on the nitrite concentration.
The results of previous research in this field are
inconsistent. Bosch and colleagues determined the
concentration of nitrite of frozen spinach after boiling
and refrigerating at 2-5 °C. They observed that the
concentration of nitrite and nitrate became higher and
lower, respectively, as the spinach was stored longer in
the refrigerator.33 Chew and colleagues also observed
reduction in nitrate and enhancement in nitrite after
blanching Amaranthus gangeticus and Amaranthus
paniculatus, two types of leafy vegetables, in boiling
water (100 °C) for 1 minute and refrigerating them at 4
°C.31 However, Chung showed that storing vegetables
at 5±1 °C, unlike storing them at ambient temperature,
had no effect on the nitrate and nitrite content during
7 days. It is worth mentioning that the measurement
of nitrite and nitrate levels in Chung’s study was
performed on raw vegetables,30 however, Bosch
and Chew’s studies were performed on processed
vegetables. This point and the difference in processing
could be the reason of these contradictions. In the
current study, spinach was cooked through different
methods before refrigeration, so the reduction in nitrate
concentration was similar to Bosch and colleagues33
and Chew and colleagues’ studies,31 but the result
obtained about nitrite was different. This can be due
to differences in the previous process of refrigeration,
which may have a greater role in conversion of nitrite
to other nitrogen derivatives. Because, in addition
to enzymatic mechanisms, whose probability is low
here, nitrite can convert into NO by non-enzymatic
mechanisms, e.g. via chemical reduction.34 Indeed,
nitrate and nitrite are parts of nitrogen cycle; in
addition to converting into each other, they can also
convert into other nitrogenous derivatives. Spoelstra,
in determination of nitrite level in silage, revealed that
nitrate converts to ammonia and nitrous oxide by the
action of bacteria eventually, and nitrite and nitric acid
are intermediate products of these transformations.35

One of the strong points of this study was
investigation of the effect of a common method of
cooking of spinach in Iran which has not been studied
before. This study showed that this type of cooking
is not a proper method to reduce nitrate and nitrite
intake from spinach. Cooked spinach is usually kept
in refrigerator for some days for other uses; this study
revealed how nitrate and nitrite levels of spinach
change after refrigeration for 5 days. However, as
previously mentioned, it seems that differences in
the previous process of refrigeration are effective
in the obtained result. In this study, it was found,
though it wasn’t a part of the objectives of the study,
that the amount of nitrite in raw spinach was high
comparable to the usual content of nitrite reported
in vegetables. So investigation of the causes of this

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issue in agricultural conditions in Iran, including management of agricultural water and fertilizers and other effective conditions, is necessary.

**Conclusion**

Based on the usual content of nitrate and nitrite reported in spinach, the raw spinach was characterized by a low content of nitrate but a high content of nitrite. This could be due to weather conditions, amount of the fertilizers used, and other growth conditions. Moreover, it can indicate damage to the spinach tissue and activation of nitrate reductase during harvesting, transporting, or preparing the spinach.

It seems that cooking spinach without adding water for 40-45 minutes, which was used in this study, is not a proper method to reduce nitrate and nitrite intake. For this purpose, it is better to use other cooking methods such as boiling followed by removing the boiled water. Furthermore, this study showed that refrigerating spinach, which was cooked via the mentioned method, at 4 °C can significantly reduce the nitrites’ concentration.

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**Conflict of Interest:** None declared.

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