Determination of Nitrate Content in Organic and Conventionally grown Vegetable Leaves in Sri Lanka using Spectrophotometry

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Abstract

Introduction and objective: Nitrate is essential for the healthy growth of plants. However, consuming a diet with high levels of nitrate has been linked to an increased risk of fatal conditions, such as methaemoglobinaemia, and gastric cancer. The main aim was to determine the nitrate content. The extraction is a necessary analytical step. Five types of organic and conventionally grown leafy vegetables were taken. Organic vegetables are grown without the use of pesticides, synthetic fertilizers, sewage sludge, genetically modified organisms, or ionizing radiation. Five locally available leafy vegetables (Spade leaf, Spinach, Drumstick leaves, Dwarf copperleaf, Beetroot leaves) were purchased from an organic farm in Arugambay and conventional in the Wellimada, Sri Lanka. Extraction of nitrate was achieved through spectrophotometry technique.

Method: Absorbance of each sample was obtained in three replicates and the calibration graph of nitrate standards was used to obtain the nitrate concentration of each sample.

Results: Results showed that the nitrate content in both organic and conventional samples of spinach (13.5 ± 0.14 μg/ g, 26.2 ± 0.27 μg/ g), drumstick leaves (16.2 ± 0.14 μg/ g, 73.4 ± 1.2 μg/ g) and dwarf copperleaf (16.2 ± 0.14 μg/ g, 77.3 ± 0.41 μg/ g) leaves is lower compared to Spade leaf (18.3 ± 0.09 μg/ g, 108.8 ± 0.11 μg/ g) and Beetroot leaves (48.4 ± 7.1 μg/ g, 56.9 ± 0.34 μg/ g).

Conclusion: The conventional samples of these leafy vegetables contain higher nitrate content in comparison to the organic samples and those leafy vegetables can be recommended for the nutritional purposes because of the low nitrate content.

Keywords: European Commission’s Scientific Committee for Food (ECSCF), Joint Expert Committee of the Food and Agriculture (JECFA), Acceptable Daily Intake (ADI), Nitrate Content, and Methaemoglobinaemia.
1. Introduction

Pollution is one of the high prevalent issue all over the world after the industrial revolution. Nitrate is one of the hazardous chemical compounds present and raised with the increased environmental pollution. (González et al., 2010). This pollution has caused severe conflict to environment, growth of plants, and animals and human health. Vegetables are part of the essential diet with most rapid and lowest cost of the human life style. These vegetable are the main route for the nitrate to enter and accumulate within the human body compared to all the other route of entries for it’s consumed by humans every single day (Khoshtinat et al., 2009). Plants depend on nitrate for nutrition, to enhance their biological function (Akon et al., 2009; Ozdestan and Uren, 2012). However, when plants especially crops tend to absorb nitrate more than required it could develop negative functions when consuming (Ahamed, 2009; Cárdenas-Navarro et al., 1999).

The nitrate content differs in concentration of part to part from the same plant. Out of all leaves of the vegetables tend to have higher nitrate content and could become harmful. Nitrate is transported through the xylem to the rest of the parts continuously by which the storage vegetables, such as carrot, beans and potato showcase less concentration when studied (Ekart et al., 2013). Many diseases occur due to nutritional deficiencies and they are treated with healthy diet plan (Gadomska et al., 2009).

By nature nitrate is a non-toxic compound to humans when consumed below acceptable daily intake (Uhegbu et al., 2011). Nitrate can be endogenously transformed to nitrite by bacterial and mammalian metabolic pathways. Thus, becomes highly toxic when nitrite accumulate in a high level (Santamaria, 2006). The metabolite nitrite may produce a number of serious health effects in humans and animals (Prasad and Chetty, 2008). This endogenously produced nitrite was, shown to react with amines (e.g., nitrosatable compounds) to produce N-nitroso compounds in the human gut. Many of these compounds have been found to be carcinogenic in all vitro tests, although readily formed compounds which is N-nitrosoproline was not carcinogenic in human (Bryk et al., 2003; Zhou, 2000).

High level of nitrate is also found in blood, urine, and cancer tissue causing bladder cancer, Non-Hodgkin’s lymphoma and stomach cancer (Basker, 1992). In addition, high level of nitrate intake is also linked with miscarriage (Afzali and Elahi, 2014). Nitrate may cause a condition called methemoglobinemia (also known as “blue baby disease”) mainly in infants under three month of age. Methemoglobinemia is a condition characterized by increased quantities of hemoglobin in which the iron of heme is oxidized to the ferric (Fe3+) form. Nitrite ion in contact with RBCs oxidizes ferrous iron in Hgb to the ferric state, forming stable methemoglobin incapable of oxygen transport) and resultant anoxia (Chan, 2011; Kapil et al., 2010).

The Scientific Committee on Food (SCF) established in 1974, was the main committee providing the European Commission with scientific advice on food safety. Its responsibilities have been transferred to the European Food Safety Authority (EFSA), The Joint FAO/WHO Expert Committee on Food Additives (JECFA), and the United Nations’ World Health Organization (WHO) have set the same Acceptable Daily Intake (ADI) of nitrate, in 1995, at 3.65 mg/kg body
weight (Anjana et al., 2007; Umar et al., 2007; Ziarati, 2012). For a real assessment, however, average daily intake form all other nitrate sources should be taken into account.

In Sri Lanka, leaf vegetables are cultivated under both conventional and organic farming systems. Organic farming means vegetables are grown without the use of pesticides, synthetic fertilizers, sewage sludge, genetically modified organisms, or ionizing radiation. The extreme use of the fertilizers and pesticides in farming with the threat of these chemicals in plant has become one of the most important awareness issues in this country. However, there are no sufficient data available regarding the nitrate content of many leafy vegetables.

Spade leaf (*Centella asiatica*) is a very popular medicinal herb used widely in Sri Lanka. It is used to treat several diseases like, ulcers, diarrhoea, fever, hair and skin diseases. It is also good in increasing memory power (Jana and Moktan, 2013). Spinach (*Spinacia oleracea*) is very nutrient-dense food, which is used as antioxidant, and anti-inflammatory properties and many studies have shown that consumption of spinach may keep human brain as young and agile (Ahmadi et al., 2010; Iammarino et al., 2014). Out of all green leafy vegetables, drumstick leaves (*Moringa oleifera*) contains the highest β-carotene and carotene content and can be a suitable diet for nutrient deficiency especially to treat vitamin A deficiency (Croitoru et al., 2015). Epidemiological studies have shown that high consumption of carotenoids present in leafy vegetables may reduce the risk of several chronic diseases, including age-related macular degeneration and cardiovascular disease (Lundberg et al., 2011).

The dwarf copperleaf (*Alternanthera sessilis*) is nutrients than other leafy vegetables (Lyons et al., 2006). It is used to treat cough, wounds, bronchitis, flatulence, and diabetes. The sessile is also used as a home remedy throughout Asia and Africa to treat gastrointestinal problems and even hepatitis (Chung, 2003). Beetroot leaves (*Beta vulgaris*), sometime called Beet Greens, are a great source of nutrients and fibers (Grzebelus and Baranski, 2001). They are one of the ideal curry along with rice in Sri Lanka and they used to treat cancer to build immunity.

The aim of this work was to determine the nitrate content in some leafy vegetables using different solvent and compare the nitrate content among them.

2. Material and Methods

2.1 Material

2.1.1. Chemicals

Most of the chemicals in this study were of analytical grade. Sodium carbonate, sulphuric acid, silver sulphate, sodium hydroxide and, toluene were purchased from Sigma Aldrich Corporation (Sri Lanka) and phenol was purchased from Glorchem Enterprise (Sri Lanka). Silver sulphate was purchased from Mas chemical (Sri Lanka) and were used according to the manufacturer’s instructions.
2.2 Methods

2.2.1. Sample collection

Five conventional leafy vegetables were purchased from conventional farms in Wellimada, Sri Lanka and the five organic leafy vegetables were purchased from ICEI Ecowave organic Outlet, Arugambay, Sri Lanka.

2.2.2. Nitrate standard solutions

A stock solution of 100 mg ml\(^{-1}\) of nitrate was prepared. Absorbance of 08 standard solutions (0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 20.0, 25.0 μgml\(^{-1}\)) was measured in triplicates in a spectrophotometer at 407 nm. Then a calibration graph (absorbance versus concentration of nitrate) was plotted.

2.2.3. Extraction of nitrate in leafy vegetables

All purchased leafy vegetables were cleaned and dried in sunlight for 3 days. Then dried vegetables were ground until homogeneous powder granulometry was evaluated using a home blender. Then it was transfer into the named container and stored at room temperature. The procedures were carried out for all the vegetables samples. 10g of the sample was taken into a 250 ml beaker. Then 70 ml of distilled water and 2.5ml of 4 % sodium hydroxide was added to it. Then the content was mixed well and then it was warmed at 80° C for 25min with occasional shaking using water bath. The resulting solution was filtered through a fluted filter paper (Whatman 1) into a 100 ml volumetric flask.

Then the filtered solution was diluted by adding distilled water up to the mark of the volumetric flask. An aliquot of 04 ml was taken into a labelled falcon tube then it was cooled into the refrigerator for 5 minutes. Then 01 ml of 5 % silver sulphate solution was added followed by subsequent addition of 7 ml of 98% sulphuric acid and 0.1 ml of 5 % phenol solution. Then the solution was allowed to stand for 20min while shaking occasionally.

The resulting mixture was extracted by adding 5 ml of toluene for 10 minutes with occasional shaking. Then the lower aqueous layer was discarded. The organic phase was washed twice with 10 ml of distilled water by shaking for 2 min and each time discarding the aqueous phase. Then the organic phase was extracted again by shaking for 1 min with 10 ml of 10 % sodium carbonate solution.

2.2.4. Spectrophotometer determination

Distilled water was used to adjust zero Absorbance and the spectrophotometer was set to the wavelength at 407 nm. Then the extracted resulting mixture was pipetted into a cuvette, then the sides of the cuvette were wiped and it was placed in the spectrophotometer chamber. Then dilution was carried out by taking 3 ml of extracted sample with 1.5 ml distilled water.
Then the diluted resulting mixture was pipetted out into a wiped cuvette. Then it was placed in the spectrophotometer chamber and the measurements were made. The same procedure was repeated thrice to confirm repeatability. Then the original observation and the nitrate content were calculated by using below given formula.

\[
\text{Nitrate content in vegetables} = \frac{C \times 100}{W_s \times 4} \quad (W_s \text{ – Weight of the sample used})
\]

\(C\) – Concentration of nitrate in the sample as from calibration graph (μg / ml)

### 2.2.5. Determine the concentration of the sulphuric acid required for effective nitrate extraction

Sulphuric acid standards solutions (7 ml of 40 %, 60 % and 80 %) were prepared. Then 4 ml of diluted solution was pipetted out and transferred equally into the 04 labelled falcon tube (0, 40, 60 and 80 %) respectively and it was cooled in refrigerator for 5 minutes. Then 1 ml of 5 % silver sulphate and 0.1 ml of phenol was added into the all labelled falcon tube. Then 7 ml of 40 %, 60 % and 80 % of sulphuric acid was added to the respective labelled falcon tubes. Then the same extraction procedures were carried out to the each falcon tube as described above. Then the absorbance was measured in triplicates in spectrophotometer. Then a graph of absorbance against percentage (%) acid was plotted.

### 2.2.6. Determine the concentration of phenol required for effective nitrate extraction

Phenol standards solutions (0.1 ml of 3 %, 5 %, 8 %, 10 % and 15 %) were prepared. Then 4 ml of diluted solution was pipetted out and transferred equally into the 05 labelled falcon tube respectively and it was cooled in refrigerator for 5 minutes. Then 1 ml of 5 % silver sulphate and 7 ml of 98 % sulphuric acid was added into the all labelled falcon tubes. Then 0.1 ml of 3, 5 %, 8 %, 10 % and 15 % phenol was added to the labelled falcon tubes. Then the same extraction procedures were carried out to the each falcon tube as described above. Then the absorbance were made in triplicates in spectrophotometer. Then a graph of absorbance against percentage (%) phenol was plotted.

### 2.2.7. Determine the concentration of sodium carbonate required for effective nitrate extraction

Sodium carbonate standards solutions (10 ml of 3 %, 5 %, 8 %, 10 % and 15 %) were prepared. Then 4 ml of diluted solution was taken into a 05 labelled falcon tube then it was cooled into the refrigerator for 5 minutes. Then 01 ml of 5% silver sulphate solution was added to each falcon tubes followed by subsequent addition of 7 ml of 98 % v/v sulphuric acid solutions respectively. Then 0.1 ml of 5 % phenol solution was added to each falcon tubes. Then the solution was allowed to stand for 20 min while shaking occasionally. The resulting mixture was extracted by adding 5 ml of toluene to each falcon tube for 10 minutes with occasional shaking. Then the lower aqueous layer was discarded. The organic phase was washed twice with 10 ml of distilled water by shaking for 2 min and each time discarding the aqueous phase.

Then the organic phase extracted again by shaking for 1 min with 10 ml of 3, 5, 8, 10 and 15 % sodium carbonate solution. Then the absorbance were made in triplicates in spectrophotometer. Then a graph of absorbance against percentage (%) acid was plotted.
3. Results

3.1 Calibration graph of the standard nitrate solution.

![Graph of absorbance (A.U) versus concentration (µg/ml⁻¹) of nitrate.]

*Figure 1:* Graph of absorbance (A.U) versus concentration (µg/ml⁻¹) of nitrate.

3.2 Comparison of nitrate between the organic and conventional leafy vegetables.

![Determined nitrate amount in the organic and conventional leafy vegetables.]

*Figure 2:* Determined nitrate amount in the organic and conventional leafy vegetables.
3.3 Effects of sulphuric acid, phenol and sodium carbonate in nitrate determination

![Figure 3: Effect of sulphuric acid concentration on the nitrate.](image)

![Figure 5: Effect of sodium carbonate concentration on the nitrate determination.](image)
Figure 4: Effect of phenol concentration on the nitrate determination.

4. Discussion

Figure 1 shows an excellent linearity of the calibration graph $y = 0.0368x - 0.0039$ shows the gradient of 0.0368, intercept $-0.0039$ and with $R^2 = 0.9999$ which is very close to +1. Figure 2, shows that nitrate content was found in detectable amount in all the leafy vegetables investigated. It was found that spinach, drumstick leaves, dwarf copperleaves contain the lowest amount of nitrate ($13.5 \pm 0.14 \mu g/ g, 16.2 \pm 0.14 \mu g/ g, 16.2 \pm 0.14 \mu g/ g$) in comparison with the nitrate contents of samples like spade leaves, and beetroot leaves which contains the higher amounts ($18.3 \pm 0.086 \mu g/ g, 48.4 \pm 7.087 \mu g/ g$).

Vegetables such as spinach, drumstick leaves, dwarf copperleaves and beetroot leaves contain the lowest amount of nitrate ($26.2 \pm 0.274 \mu g/ g, 73.4 \pm 1.201 \mu g/ g, 77.3 \pm 0.413 \mu g/ g, 56.9 \pm 0.339 \mu g/ g$) in comparison with nitrate content of sample like spade leaves which contains the highest amount ($108.8 \pm 0.112 \mu g/ g$).

The nitrate content in this study was found to be lower in organic leafy vegetables compared to conventional leafy vegetables as shown in figure 2. In organic spinach, the nitrate content was found to be the lowest ($13.5 \pm 0.138 \mu g/ g$) whereas to the conventional spade leaves that contains the highest nitrate content ($108.8 \pm 0.112 \mu g/ g$) among all the leafy vegetables. In conventional spinach, the nitrate content was doubled ($26.2 \pm 0.274 \mu g/ g$) than the organic one ($13.5 \pm 0.138 \mu g/ g$). In organic drumstick leaves and dwarf copperleaf, nitrate content was significantly equal ($13.5 \pm 0.138 \mu g/ g$) compared to conventional that was unequal; (dwarf copperleaf contains $77.3 \pm 0.413 \mu g/ g$ amount of nitrate and the drumstick leaves contains $73.4 \pm 1.201 \mu g/ g$ amount of nitrate although among them dwarf copperleaf was higher.

Difference of nitrate content between organic and inorganic is maximum in spade leaves ($90 \mu g/ g$), minimum in beetroot leaves ($12.95 \mu g/ g$). The possible reasons for maximum and minimum differences between organic and inorganic nitrate content due to the ecological factors changes. A study performed in Sri Lanka found maximum
difference between the organic and inorganic nitrate content and they concluded that nitrate contents are changed based on the significant ecological damage. (Gunatilake and Iwao, 2010; Silva and Lathiff, 2013).

Nitrate content of various parts of a plant differs (Tamme et al., 2010). Vegetables that are consumed with their roots, stems and leaves have a high nitrate, whereas those with only fruits and melons as consumable parts have a low nitrate accumulation (Hogstad et al., 1997; Majd et al., 2010). In the organic spade leaves belongs to the vegetable plants, which accumulate less nitrates than other leafy vegetables. In contrast, in the conventional spade leaves belongs to the vegetable plants, which accumulate high nitrates than other leafy vegetables.

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Nitrogen-rich organic fertilizers can also generate lower nitrate contents, but when mineralization conditions are very favorable they can also lead to high nitrate accumulations. (Nemade, and Attarde, 2014). The use of organic fertilization with slowly or moderately available nitrogen (especially composts) is key to explaining the generally observed lower nitrate accumulation in organic vegetables (Custic et al., 2003; Narayana and Sunil, 2009).

The effect of climate on nitrate accumulation has been studied and it was found that nitrate content was lower in years that had a high rainfall. In warm and wet climate, increased accumulation of nitrate is possible, regardless of whether the nitrogen originates from organic or mineral sources (Zhou et al., 2000). A comparable study performed in Austria on 17 vegetables found lower nitrate contents (40 % to 86 %) in organic vegetables (Basker, 1992). In Germany, a comparison on carrots showed 61 % less nitrates in organic ones with spinach being an exception (Rauter and Wolkerstorfer, 1982). In contrast, two other studies performed on tomato in Israel and carrot in Norway did not show noticeable differences (Pommer and Lepschy, 1985).

Nitrate content of vegetables depends on a number of external and internal factors. From external factors supply of substrate with nitrate, light, time of day, temperature, season, supply with water, relative humidity, carbon dioxide concentration in the air, supply with biogenic elements, the influence of the accompanying cations, heavy metals, herbicides, chemical properties of the soil, location, time of sowing, time and method of harvest, storage conditions, etc (Anjana and Iqbal, 2007; Uwah et al., 2009).

Among the internal factors, the most important is the genetic specificity in the accumulation of nitrate (differences between species and differences within genotypes), the distribution of nitrate in certain parts of the plant and the age of the plants (Burns et al., 2011; Burns et al., 2012). Among that, light intensity and nitrogen fertilization have been identified as the major factors that influence the nitrate content in vegetables.

Taking spade leaf is one the favourable dishes at diet of Sri Lankans and most of these vegetable leaves highly consumed. According to our knowledge, studies have been carried out to determine nitrate content of beetroot taproots and not of beetroot leaves (Grzebelus and Baranski, 2001; Zhong et al., 2002).
Acceptable Daily Intake (ADI) for nitrate ion as recommended by the European Commission’s Scientific Committee that the acceptable nitrate amount is enough to say that ADI is < 3.65 mg/kg body weight (Anjana et al., 2007). The table 1 illustrates the amount of nitrate in 100 g of the both organic and convention leafy vegetable samples based on the bodyweight. This values indicate all of the leafy vegetable samples were not exceeded the acceptable daily intake. However, a study performed in India on spinach found exceeded the nitrate amount (34%) than the recommended acceptable daily intake (Anjana et al., 2007).

**Table 1:** Nitrate content of samples and the amount (mg) of nitrate ingested per day when consuming 100 g of the vegetables.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Nitrate amount in organic (mg/day)</th>
<th>Nitrate amount in inorganic (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spade leaf</td>
<td>1.83</td>
<td>10.88</td>
</tr>
<tr>
<td>Spinach</td>
<td>1.35</td>
<td>2.61</td>
</tr>
<tr>
<td>Drumstick leaves</td>
<td>1.62</td>
<td>7.34</td>
</tr>
<tr>
<td>Dwarf copperleaf</td>
<td>1.62</td>
<td>7.73</td>
</tr>
<tr>
<td>Beetroot leaves</td>
<td>4.84</td>
<td>5.69</td>
</tr>
</tbody>
</table>

Sulphuric acid has a marked effect on determination of nitrate. The figure 3 indicated sharp rise in the amount of nitrate detected as the concentration of the acid is increased. Nitrate was not detected when sulphuric acid concentration was below 40 %. The use of 98 % sulphuric acid was therefore recommended for the procedure. The figure 4 indicated there was no significant difference in the amount of nitrate determined with sodium carbonate concentration of 3, 5, 8, 10 and 15 %. Therefore, the variation of the concentration of sodium carbonate solution in the procedure does not affect the nitration reaction. However, observation showed that at concentrations higher than 15% the solution became saturated at room temperature. For this reason, 10 % sodium carbonate was recommended.

The Figure 5 indicated high amount of nitrated detected in 5% phenol used therefore it recommended for this procedure. In this study, the nitrate determining method is based on the measurement of the absorbance of yellow sodium nitrophenoxide formed via the reaction of phenol with the vegetable-based nitrate in presence of sulphuric acid and the interfering chlorides were precipitated by the addition of 1ml of 5% silver sulphate solution (Gaya and Alimi, 2006).
The locally available vegetables are valuable and natural sources of nitrate. The results in this study show that both organic and conventional leafy vegetable leaves are source of nitrate; among them conventional leafy vegetable leaves have high nitrate content. However, all obtained nitrate values fall within the acceptable daily intake of the European Commission’s Scientific Committee for Food and those leafy vegetables are recommended for the nutritional purpose. Apart from determining the nitrate content, appropriate concentration of chemicals used during extraction was also analysed. Rational application of organic manure instead of inorganic nutrients, use of physiologically active substances, proper spray of nitrification inhibitors and molybdenum fertilizers, and growing plants under controlled environmental conditions may all be factors that materially reduce nitrate accumulation in leafy vegetables (Shahbazzadegan et al., 2010; Tamme et al., 2006). This study presents a spectrophotometric method usable for simultaneously determining nitrate in leafy vegetables with high sensitivity, and precision. Absorbance measurements were repeated thrice to confirm precision and reliability of the study.

Further work

- Determining the effect of seasons on the level of nitrate in leafy vegetable would be improving this research.
- Determining the effect of genotypes on the level of nitrate in leafy vegetables would be improving this research.
- Determining of nitrite would further add value to this research
- Analysis the possible reasons for the differences between organic and inorganic nitrate content in spade and beetroot respectively.
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My sincere gratitude is to supervisor Miss. Bhagya Jeerasinge who gave me the freedom in exploring the research area and also extending her never ending support and guidance. I am overwhelmingly thankful to Dr. Sajani Dias, Dr. Mathi Kandiah, and Dr. Ranmalee Amarasekara for meeting all the requirements needed at different instances throughout the project. This wouldn’t have been possible without the support of the encouragement and unending support given by my fellow colleagues and laboratory technician. I extend my sincere thanks to them. Lastly, thanking my parents for the immense support and understanding.

This study will be focused on the nitrate contents in some vegetable leaves, and will not use commercially sensitive data or data covered by the UK official Secrets Act, and there will be no outdoor environment field work. Hence, the above need not be taken into consideration. Therefore, this project can be placed in the green ethical considerations category. The research will be undertaken in a research lab in School of Science building. In this project, the vegetable leaves samples used are covered by bioCOSHH and all chemicals used are covered by COSHH regulations. The project supervisor has successfully completed the relevant ethical consideration submission for this research project.
References


