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FOOD SECURITY – NITRATE INTERFERENCE

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Food security in an avid sense refers to the continuous supply of food that is free from disease causative chemicals and microorganisms that cause tribulations. Vegetables are an important source of vitamins, minerals, and bioactive compounds. The complexity with regards to nutritional exploitation of vegetables is the presence of analytes which are antinutritional and toxic in nature. Nitrates are an inherent part of the environment and are therefore present in air, water, soil, food, and particularly present in fruits and vegetables. Fresh vegetables contribute approximately 84% of nitrates in the diet. A decline in dietary nitrate is an advantageous anticipatory measure as the endogenous conversion of nitrate to nitrite has been implicated in the incidence of Methemoglobinemia and Gastric cancer. Thus nitrates are considered a serious threat to public health, if ingested in increasing amount. The present study is devoted to establishing a flow injection analysis (FIA) technique to investigate the nitrate content of ten commonly consumed fresh fruits and vegetables from market in Fiji. Activated carbon extraction was preferred over alkaline extraction and applied. The effects of deep-freezing as well as short-term refrigeration on nitrate content were also determined. The results of the investigation of the nitrate content of the selected Fresh Fruits and vegetables ranges from 25-3785 mg kg^{-1}. Nitrate was determined in the linear range from 1.0 to 20.0 mg L^{-1} with a method detection limit of 0.042 mg L^{-1}. The results from the conducted study will play a significant role in future epidemiological predictions in relation to Methemoglobinemia as well as gastric cancer in Fiji.

Keywords: Nitrate, Fiji vegetables, Flow Injection Analysis, Methemoglobinemia, Gastric Cancer

1.0 Introduction

The general public outlook has a growing anxiety for food security issues. The concept of food security refers to the continuous supply of edible food produce that is free from disease causing microbes and chemicals. The 1996 World Food Summit affirmed that "food security exists when all people at all times have both physical and economic access to sufficient food to meet their dietary needs for a productive and healthy life" (FAO, 1996). Chemistry is the key science holding in concert the concept of food and nutrition. The modern age food and nutrition needs can only be evaluated through the understanding of the chemical elements present in foods. Since the 18^{th} century many of the beneficial components of foods such as vitamins and minerals have been highlighted and defined (Fanzo *et al.*, 2011).

Plant food (vegetables and fruits) play a predominant role as the main source of nutrition in much of the poorer world countries since they are an outstanding resource for vitamins, minerals and biologically active compounds (Kmiecik *et al.*, 2004). Consequently vegetables are high-value crops and provide a consistent income for vegetable farmers in Fiji. The complexity with regards to nutritional exploitation of vegetables is the presence of analytes which are antinutritional and toxic in nature. One such analyte is the chemical compound acknowledged as "nitrate".

Nitrate is an inorganic compound comprising of one atom of nitrogen (N) and three atoms of oxygen (O). It has a molecular weight of 62 g mol^{-1} and its chemical symbol is NO_3^-. Nitrates are an inherent part of the environment and are therefore present in air, water, soil and food (particularly vegetables and fruits). They are also produced endogenously in the human intestine (Tannenbaum *et al.*, 1978). Human nitrate intake is mainly from vegetables, water supplies and from additives/preservatives used in cheese and meat (Wolff & Wasserman, 1972). About 87% of the total nitrate concentration in a normal diet is believed to be a direct result of vegetable intake (Huarte-Mendicoa *et al.*, 1997).

Nitrate content of vegetables may range from 1 to 10000 mg kg^{-1} (Ximenes et al., 2000). The various reason for this wide range are excessive use of fertilizer, crop variety, types of N-fertilizers, light and temperature conditions, lack of water, etc. (Santamaria et al., 2001; Wolff & Wasserman, 1972;). A combination of these factors account for different nitrate values reported in different countries.

The main concern for the public health is the link between nitrates and gastro-intestinal cancers. It is due to the fact that nitrates/nitrites help in the formation of carcinogenic nitrosamines. The elevation of gastric pH >5.5 leads to bacterial growth followed by rapid conversion of nitrate to nitrite. Nitrite is a precursor in the formation of nitrosamines (Tannenbaum & Correa, 1985). Approximately 5% of all dietary nitrates are reduced to nitrites in saliva and the gastrointestinal tract (Santamaria, 2006). Greater nitrite content thus could increase the likelihood of endogenous nitrosation reactions leading to the formation of carcinogenic nitrosamines, which in turn may lead to a greater risk of cancer causation.

Children are the most vulnerable to the toxic effects of these compounds as they have low body weight, immature immune system, and lower gastric pH. Young babies who have low stomach acidity can suffer from infantile methemoblobinamea (White, 1975) as a direct result of nitrates in their diet. This is a condition where nitrite is substituted for oxygen in haemoglobin (Fan *et al.*, 1987) and death may occur as a result of oxygen deprivation i.e. nitrate linked to infantile methemoglobinamea. It has been reported that many babies/infants foods contain nitrate/nitrite (Nitrates and Homemade Baby Food). Therefore, determination of the levels of the nitrite/nitrate in biological, environmental and food samples are extremely important to protect the human health and especially childrens' health (Merusi *et al.*, 2010). Thus it is imperative to determine the nitrate content of common fruit vegetables largely consumed in the Pacific Islands.

In continuation of vegetables nitrate analysis in our laboratory, in the present study an attempt is made to analyze the nitrate contents of nine different fresh fruits and vegetables sold in the local municipal market using Flow Injection Analysis (FIA). The effects of deep freezing as well as short term refrigeration on nitrate content have also been studied.

2.0 Materials and Methods

2.1 Location of the Study Site

Suva is the capital city of the Republic of the Fiji Islands (often referred to simply as Fiji), an independent island nation in the southern Pacific Ocean. It is located on the south-eastern side of the island of Viti Levu.

2.2 Seasonal and Climatic factors

The climate in Fiji is of tropical oceanic in nature and is prevalent throughout the year. Annual temperature remains stable at approximately 25°C. The main islands have a 'wet' side to the south and east and a 'dry' side to the north and west. Rainfall in Fiji is highly unpredictable.

2.3 Experimental Procedure

A flow injection analysis method used for brackish and waste water samples was standardized and applied for the quantification of nitrate in specific vegetable and fruit matrices.

2.3.1 Reagents and Standards

Unless specified otherwise all reagents used in this study were of analytical-reagent grade. The term "water" implies distilled de-ionized water (DDW, 18 M Ω cm^{-1}) and was used for all sample extraction and vegetable sample preparation. All glassware used was soaked in 10% HCl for 24 h, and rinsed many times with DDW prior to use. The standard nitrate solutions were prepared by dissolving 0.25 g of KNO_3 (Biolab, Australia) in 250 mL of water. This gave rise to a 0.004 g ml^{-1} of stock solution. Several serial dilutions were made to obtain the standard concentrations; 1.0, 4.0, 8.0, 12.0, 16.0 and 20.0 mg L^{-1} nitrate.

2.3.1.1 Sulfanilamide Color Reagent

Sulfanilamide (SA) color reagent was prepared in a 1 L volumetric flask, containing 600 mL of water, 25 mL of 85% phosphoric acid and dissolving 10.0 g of Sulfanilamide (Ajax Finechem, Australia). 0.25 g of N-(1-naphthyl)ethylenediamine dihydrochloride (NED) (BDH, England) was added to the solution. The mixture was stirred to dissolve for 30 min and diluted to 1 L. Resulting solution was stored in a dark amber color bottle and discarded when it became pink.

2.3.1.2 Ammonium Chloride Buffer

Ammonium chloride buffer was prepared in a 1 L volumetric flask by dissolving 21.25 g of ammonium chloride (Asia Pacific Specialty Chemicals Ltd, Australia) and 1.0 g of disodium ethylenediaminetetraacetic acid dihydate (EDTA, Sigma-Aldrich) in 800 ml of water. The mixture was diluted to 1 L while the pH was adjusted to 8.5 with 10% NaOH (w/v).

2.3.2 Extraction and Analysis

Two extraction techniques were tested on four types of leafy vegetables (Chinese cabbage, celery, lettuce and tomato). The most efficient technique was then employed through out for the extraction of nitrate in this study.

2.3.2.1 Extraction via activated carbon

The extraction of nitrate using activated carbon was performed using Hunt and Seymore (1985) method with minor modifications. 0.1-0.2 g of freeze-dried vegetable sample was added to 50 mL of water in a screw-capped bottle (120 mL capacity). 100-250 mg of activated carbon was added and the bottle was tightly screw-capped. The mixture was then shaken for 30 min on a Stuart Scientific shaker SF1 as shown in Plate 10. A portion of the extract was filtered through a 0.45 µm Whatman No. 541 (12 cm) membrane filter into a 50 mL polypropylene vial ready for analysis. The first 20 mL of the extract was discarded to prevent any residual contamination from the filter paper. If clear extracts were not obtained, the samples were centrifuged at 3000 rpm for 10 min and filtered again through a 0.45 µm Whatman No. 541 (12 cm) membrane filter.

2.3.2.2 Alkaline Extraction

Sen & Donaldson (1978) method with minor modifications was used for the extraction of nitrate-N in the present study and for its comparison with the activated carbon extraction. 10 g of the homogenized sample was accurately weighed and blended for 5 min with 70 mL of water. Then 12 mL of 2% NaOH was added while pH *ca.* 8 was adjusted with 2% NaOH (avoiding excess NaOH).

The slurry was transferred to a 200 mL volumetric flask and heated on water bath (50-60 ^{0}C) with occasional swirling until the temperature of the suspension reached about 50 ^{0}C. 10 mL of $ZnSO_4$ was added and temperature of the suspension maintained at about 50 ^{0}C for further 10 min. If a white precipitate of $Zn(OH)_2$ did not appear, 2-5 mL of 2% NaOH was added (avoiding excess NaOH).

Contents were cooled to room temperature by immersing flask in cool water bath. The solution was diluted to a fixed volume with water and mixed thoroughly. Then the solution was filtered through a 0.45 µm membrane filter and collected in an Erlenmeyer flask. The solution was kept stoppered until analysis.

2.3.3 Flow Injection System and Apparatus

The extracted samples were analyzed using a Lachat QuickChem 8000 flow injection analyzer. A schematic diagram of the flow injection system employed for the quantification of nitrate is shown in Figure 1. The manifold is equipped with a peristaltic pump, P, (RP-100 Series) having a flow rate of 2.6 mL min^{-1}, an injection valve, V, a copperised cadmium reduction column, C, (Lachat part no. 50327) and a two state switching valve, SV. It is also equipped with a 60-position rack, an auto-sampler with sample volume of 200 µL, a 10 mm path length flow cell and a colorimetric detector, D, having UV filter of 520 nm. The teflon tubes R_1, R_2 and R_3 of 1.07 mm i.d. were used for the carrier water, buffer and reagent flow respectively while teflon tubes used for the manifold connections, the delay coil, DC (70 cm), and the reaction coil, RC (70 cm) were of 0.8 mm i.d.

All pH measurements were made using Hanna instruments microprocessor 8521 pH meter. The pH meter was standardized by the use of standard buffers (Aldrich). A Stuart Scientific shaker model SF1 was used for shaking all samples while extraction of nitrate was carried out. All vegetable samples were freeze-dried in FD3 Freeze Dryer (Analytical Equipment Company, Australia) and extracted samples were centrifuged in a MSE Centaur 2 centrifuge.

2.3.4 Analytical Procedure

The six calibration standards and extracted sample solutions were placed in sample tubes on the 60-position rack. The auto-sampler filled the solutions into injection valve (V) that is then injected into the carrier flow stream (R_1), which merged with the buffer (EDTA and NH_4Cl, pH 8.5) stream, R_2. The sample along with buffer passes through the delay coil, DC, and reaches the two state column switching valve, SV. The switching valve when turned on allows the stream of sample/buffer to progress through the copperised cadmium (Cd/Cu) reduction column, C, reducing the nitrates to nitrites. The stream having nitrites then merges with the color-developing reagent solution, R_3. At this junction a diazotization/coupling reaction proceeds in reaction coil, RC, and forms a purplish-pink dye, giving peaks. The peaks were recorded in a PC connected to the FIA. Finally the solution goes to waste tank. The flow rate of the carrier/buffer and samples was 2.6 mL min^{-1}. The concentration of nitrate-N was determined by measuring the peak area, of the dye formed, at 520 nm using the colorimetric detector. In case of non- injection of standard and samples, the carrier and buffer passed through a parallel

channel, B, and not through C as shown in Figure 8. The FIA was operated at room temperature of 25 °C.

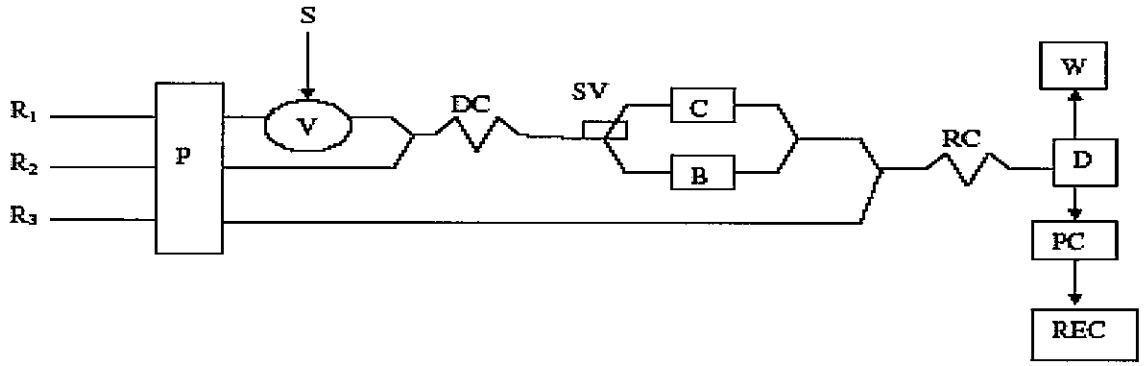

Figure 1 Schematic diagram of the flow injection manifold used for determination of nitrate: R_1, Carrier stream (distilled deionized water); R_2, Buffer stream (NH_4Cl/EDTA); R_3, Color reagent stream (SA/NED); P, Peristaltic pump; V, Injection valve; S, Sample; DC, Delay coil; SV, Two state switching valve; C, Copperised cadmium column; RC, Reaction coil; D, Detector; W, Waste tank; PC, Personal computer; REC, Recorder.

3.0 Results and Discussion

Nitrogen is an important nutrient for the existence of plants and animals alike as it is used to fabricate many crucial constituents, such as proteins, DNA, RNA, vitamins, hormones and enzymes. Plants absorb nitrogen from the environment in the form of simple nitrogenous compounds such as nitrates and ammonia whereas higher animals such as humans get the bulk of their nitrogen (in the form of nitrates) from foods (such as meat and vegetables) and drinking water. Most plants prefer nitrates over ammonia.

Concern about the high content of nitrates in certain foodstuffs such as baby foods and drinking water has long been recognized (Usher & Telling, 1975). Nitrate is seen as an undesirable component of meats, vegetables and drinking water because of its association with infantile methaemoglobinaemia and cancer. Approximately 5% of all dietary nitrates are reduced to nitrites in saliva and the gastrointestinal tract. Greater nitrite content thus could increase the

likelihood of endogenous nitrosation reactions, which in turn may lead to a greater risk of cancer causation.

Suva is the capital city of Fiji. It has the highest urban population in the country. The Suva market is located next to the bus stand, which makes it an ideal and convenient place for consumers to purchase fresh vegetable produce. Thus ten most commonly consumed vegetables and fruits have been assayed in the present study. The methodology employed to quantify nitrate is based on the Greiss protocol (Ivanov, 2004).

3.1 Nitrate content in Fruits and Vegetables

The results of the investigation of the nitrate contents (mean values) of the selected fresh leafy vegetables as well as their range and variability (RSD) are reported in Table 1. The mean nitrate content of the 10 vegetables and fruits ranges from 25 to 3875 mg kg^{-1}. Bele has the highest nitrate content within the studied vegetables whereas oranges have the lowest. It is evident from the results shown in Tables 1 that there is considerable variation of nitrate content between different samples of the same vegetable.

The high variability shown is satisfactory since vegetable nitrate content is known to vary extensively as reported in literature (van der Schee & Speek, 2000). Being partially a photosynthetic process in most plants, nitrate accumulation is understood to be independently reliant on the climate (Cardenas- Navarro *et al.*, 1999). There is a general accord amid researchers that vegetables grown in low irradiance high temperature zones accumulate more nitrates (Cardenas-Navarro *et al.*, 1999).

Fiji on average had both low and high irradiance with relatively high temperatures. Thus high nitrate values in part may be attributed to Fiji's tropical oceanic climate. Each vegetable sample was collected at random from six different vendors early in the morning. The amount of N-fertilizer and the delay time after harvesting could not be ascertained. Variability between the samples may also be accredited to these two factors.

Table 1 Nitrate contents in selected fresh vegetables and fruits using activated carbon extraction technique.

Vegetable	State	Samples Analyzed (n)[a]	Nitrate content (mg kg^{-1})				RSD[c] (%)	R^2
			Min	Max	Mean	SD[b]		
Bele	Fresh	6	2500.00	5522.36	3785.33	1019.83	26.94	0.9986
Chauraiya	Fresh	6	1300.00	2695.00	2022.33	512.69	26.82	0.9997
Dalo leaves	Fresh	6	1200.00	2500.00	1699.67	481.83	28.35	0.9935
Otta	Fresh	6	789.00	1300.00	1066.50	180.42	16.62	0.9970
Garlic	Fresh	6	22.00	45.00	32.50	8.92	27.43	0.9899
Onion	Fresh	6	32.00	52.00	42.00	7.95	18.93	0.9986
Oranges	Fresh	6	15.00	36.00	25.67	7.66	29.84	0.9865
Apples	Fresh	6	25.00	40.00	30.17	5.74	19.03	0.9995
Watermelon	Fresh	6	22.00	45.00	33.83	9.24	27.31	0.9978
Pear	Fresh	6	22.00	38.00	29.67	6.59	22.22	0.9926

[a]Each sample was analyzed in duplicate.

[b]SD: Standard deviation.

[c]RSD: Relative standard deviation.

3.2 Effect of Deep Freezing and Refrigeration

In this study the effects of deep freezing and refrigeration on the nitrate level of the two selected vegetables (Bele and Dalo leaves) was studied over a period of four days. Four samples of each type of vegetable being studied were purchased fresh from the Suva municipal market early in the morning (≈ 0830 hrs). Nitrate was extracted via the activated carbon technique and analyzed on the day of purchase to ascertain the initial (day = 0) nitrate content of each vegetable. Representative four (n = 4) sub-samples from the samples of the two vegetables were placed in (acid washed) labeled freezer bags in a deep freezer at -20°C and in the refrigerator at 4^0C. Each of the sub-samples of the four vegetables was then analyzed for nitrate levels on 1^{st} to the 4^{th} day.

Table 2 show the effects of deep freezing on Bele and Dalo leaves. There has been only a minimal change (<2%) of nitrate content over the 4 day period for both the vegetables. Table 3 shows the effects of refrigeration on the two studied vegetables. As is evident from the results there has been a considerable decrease (reduction of nitrate) in nitrate content over the four day period. In the case of Bele nitrate has decreased from 5 to 15% from day 1 to day 4 and in the case of Dalo leaves a 3 to 12% decrease of nitrate content is evident.

Table 2 shows the effects of deep freezing on the two studied vegetables

	No of days				
Vegetable[a]	0	1	2	3	4
Bele	3200.00[b] ± 465.74[c]	3215.00 ± 675.98	3195.00 ± 540.98	3155.00 ± 786.09	3150.00 ± 675.09
Dalo leaves	1456.00 ± 409.98	1444.00 ± 439.00	1442.00 ± 345.87	1444.00 ± 478.00	1430.00 ± 399.99

[a]Each sample was analyzed in duplicate
[b]Mean values of the nitrate contents in the four different samples of the same vegetable
[c]± value indicate the variability in the different samples of the same vegetable

Table 3 shows the effects of refrigeration on the two studied vegetables

	No of days				
Vegetable[a]	0	1	2	3	4
Bele	3200.00[b] ± 463.52[c]	3215.00 ± 586.25	3195.00 ± 455.22	3155.00 ± 689.23	3150.00 ± 669.51
Dalo leaves	1456.00 ± 199.20	1412.00 ± 250.02	1383.00 ± 299.32	1340.00 ± 248.89	1281.00 ± 302.56

[a]Each sample was analyzed in duplicate
[b]Mean values of the nitrate contents in the four different samples of the same vegetable
[c]± value indicate the variability in the different samples of the same vegetable

4.0 Conclusion

Nitrate is inherent in the environment because of its role in the nitrogen cycle and its versatility. Vegetables get contaminated with nitrate when their nitrate uptake exceeds the amount that they require for normal growth. Farmers apply fertilizers to the soil to increase plant growth. There is a tendency to apply extensive amounts of nitrogenous fertilizers such as NPK and urea as a means of guarantee for a good yield in a minimum amount of time.

The nitrate levels in leafy vegetable (Bele, Dalo leaves, Chauraiya, Otta) can always be expected to be above 1000 mg kg^{-1}. On the other the levels of nitrate found in vegetables such as garlic, onions as well as the studied fruits are unlikely to poses any instantaneous health risk to the general populace. However the health risk to infants from the consumption of leafy vegetables increases exponentially due mainly to their low body weight as well as their immature immune system.

There is a general consensus between dieticians, doctors and food scientists that a balanced diet with at least two servings of fruit and three servings of vegetables everyday is needed for healthy living. Current epidemiological data regarding the potential long term health risks of nitrate levels present in our diet provides conflicting evidence. Vegetables can provide approximately 84% of the nitrates in our diet. Despite the conflicting reports, it is widely acknowledged that reduction of dietary nitrate should be a preventative measure. Good agricultural practice can substantially reduce plant nitrate content while still maintaining healthy plant growth. The use of organic fertilizers is subjected to microbial action and thus will provide a constant supply of nitrate in contrast to mineral N-fertilizers such as NPK. Farmers should be

made aware of the potential health risks of excessive fertilization with regards to cancer and infant methaemoglobinaemia.

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