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Analytical Methods

Flow injection analysis of nitrate and nitrite in commercial baby foods

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ABSTRACT

Commercial baby foods are an easy alternative to home-made meals especially for working parents in a nuclear family therefore it is imperative to determine the nitrate and nitrite content in commercially available baby foods varieties marketed in Fiji. A total of 108 baby food samples were analyzed for nitrate and nitrite using our standardized flow injection analysis (FIA) technique with colorimetric detection technique employing sulfanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride as color reagents where the samples throughput was 38 h^{-1}. The commercial baby food varieties chosen comprised of vegetables, cereals, fruits and milk. The study shows that the nitrate content of the baby foods studied ranges from 2.10 to 220.67 mg kg^{-1} whereas the nitrite content ranges from 0.44 to 3.67 mg kg^{-1}. Typical recoveries of spiked nitrate residues ranged from 92% to 106%. The study shows that the average nitrate content of commercially available baby foods in Fiji descends below the maximum level proposed by the European Union Legislation.

1. Introduction

The "Nitrogen cycle" is reputed to be one of the key nutrient cycles for the sustainability of terrestrial ecosystems (Martinez-Espinoza, Cole, Richardson, & Watmough, 2011). The reduced form of nitrogen is needed by organisms to form crucial parts of proteins and nucleic acids (Galloway, Leach, Bleeker, & Erisman, 2013; Prasad & Chetty, 2008). The nitrate ion forms an intricate link within the "Nitrogen cycle" as 90% of nitrogen uptake in plants is estimated to be in the form of nitrates (Cieslik & Sikora, 1998; Hill, 1996). Vegetables are an exceptional reservoir of vitamins, minerals and biologically active compounds and play an important role in human nutrition (Kmiecik, Lisiewska, & Słupski, 2004; Prasad & Chetty, 2008). However nitrogen introduced into the global nitrogen cycle through human activities has resulted in nitrate contamination in vegetables as high as 10,000 mg kg^{-1} (Ximenes, Rath, & Reyes, 2000). In a normal adult diet 87% of total nitrate intake is believed to come from vegetables (Huarte-Mendicoa, Astiasaran, & Bello, 1997). Vegetables are also included in babies' diets for healthy growth, and as babies have an elevated ingestion of food and fluid on a per kg body weight basis, so their diet needs to be closely monitored by parents and guardians.

The endogenous intake of the nitrate ions through food and water has long been viewed with scepticism. The potential advantageous or unfavorable human health effects arising from the dietary exposure to the impending nitrate metabolites has long been shrouded in controversy (Castanheira et al., 2004). This has resulted in nitrate/nitrite research that has spanned for almost four decades through the quantitative assessment of fresh and processed foods and water supplies (Bryan, Alexander, Coughlin, Milkowski, & Boffetta, 2012). There are two main reasons that have increased public scrutiny and labeled nitrates and nitrites as detrimental to health. Firstly, nitrates have been linked to stomach cancer since nitrates help in the formation of carcinogenic nitrosamines (Butler, 2015; Chetty & Prasad, 2009; Prasad & Chetty, 2008; Tannenbaum & Correa, 1985). Nitrates are chemically unreactive but through microbial reduction to the unstable nitrite ions, spur endogenous nitrosation reactions (Fewtrell, 2004). Thus the International Agency for Research on cancer has labeled nitrates and nitrites taken through food and water as plausible human carcinogens (Catsburg et al., 2014; Hord, Tang, & Bryan, 2009). Stomach cancer has been reported as the second largest cause of cancer mortality worldwide (Cross et al., 2011). Hence detection and determination of nitrate and nitrite in different foods is extremely essential.

The second public health concern is infantile methemoglobinemia (MHb) i.e., 'blue baby syndrome' (Cassidy & Duggan, 2015). It is also referred as 'Acquired' or 'Clinical MHb'. Young babies with low stomach acidity are vulnerable to an onset of MHb due to excessive nitrates in their diet, where nitrite is substituted for oxygen in hemoglobin resulting in asphyxia and eventually death

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(Sanchez-Echaniz, Benito-Fernández, & Mintegui-Raso, 2001; Swann, 1975). The infantile methemoglobinaemia, was first reported in the United States in 1945. The onset of methemoglobinaemia was directly related to the local well water which was used to make reconstituted baby food (Comly, 1945). Thereafter, a number of studies have reported the cases of infantile MHb linked to nitrate/nitrite contaminated well water (used for the preparation of infant formula) as well as through ingestion of vegetable based home-made puree where well water was used having nitrate/nitrite (Bryk, Zalzstein, & Lifshitz, 2003; Faivre, Faivre, Klepping, & Roche, 1976; Herman, Chyka, Butler, & Rieger, 1999; Johnson & Kross, 1990; Keating, Lell, Strauss, Zarkowsky, & Smith, 1973; Knobeloch & Proctor, 2001; Knobeloch, Salna, Hogan, Postle, & Anderson, 2000; Martinez et al., 2013; Sanchez-Echaniz et al., 2001; Savino et al., 2006). However, cases of infantile MHb induced through commercial baby food intake have not been detailed in literature. There is a lack of incidence data in Fiji and as such arises an imminent need to investigate the nitrate and nitrite levels in processed baby foods available in supermarkets. Hill (1996) has reported that the possibility of nitrite formation during processing of baby foods should not be ruled out and be considered (Hill, 1996).

Recently, we have reported the optimization of a FIA method with colorimetric detection and applied it for the determination of the nitrate contents in commonly consumed fresh, cooked and frozen leafy, root and fruit vegetables marketed in Fiji (Chetty & Prasad, 2009; Prasad & Chetty, 2008; Prasad & Chetty, 2011). Therefore, in continuation of our study on Fiji's vegetable foods (Chetty & Prasad, 2009; Prasad & Chetty, 2008; Prasad & Chetty, 2011) and in serious consideration of report by Hill (1996), the present study was initiated after taking into consideration the potential risk of infantile MHb to infants through the intake of commercially available baby foods. Thus the primary aim of the study was to use our standardized flow injection analysis (FIA) method (Prasad & Chetty, 2008) to determine the nitrate and nitrite content of baby foods and to assess the potential risk in the light of the European Commission Regulation (EC) No. 1881/2006 (EC, 2006).

2. Materials and methods

2.1. Reagents and standards

Only analytical reagent grade reagents were utilized in the resultant study. All sample extraction and sample preparations were carried out via the use of distilled de-ionized water (18 MΩ cm^{-1}). The pH of the reaction mixture was maintained through the use of an ammonium chloride (NH_4Cl) buffer. 21.25 g of NH_4Cl (Asia Pacific Speciality Chemicals Ltd., Auburn, NSW, Australia) and 1.0 g of disodium ethylenediaminetetraacetic acid dehydrate (EDTA) (Sigma–Aldrich, U.S.A) were dissolved and diluted to 1 L. The pH was adjusted to 8.5 using NaOH (10% w/v) solution.

The color-developing reagent (Griess Reagent) used for the analytical procedure comprised of sulfanilamide (SA) and N-(1-naphthyl)ethylenediamine dihydrochloride (NED). 10.0 g of SA (Ajax FineChem, NSW, Australia) and 0.25 g NED (BDH, Poole, England) were dissolved in 1L water in a volumetric flask. 100 mL of phosphoric acid (25% v/v) solution was subsequently added and diluted to mark and stored in a dark amber bottle.

The stock solutions (1000 μg mL^{-1}) were prepared from potassium nitrate and potassium nitrite (Biolab, Australia) in 250 mL volumetric flasks. Subsequent serial dilutions were made to acquire standards to construct calibration curves for nitrate and nitrite. The nitrate calibration curve ranged from 1.0 to 20.0 μg mL^{-1}) while nitrite calibration curve ranged from 0.025 to 2.0 μg mL^{-1}).

2.2. Samples

The study design involved ten baby food matrices marketed in Fiji. A blank without added nitrate was also included. Eighteen dissimilar fruit, vegetable, milk and cereal based baby foods were scrutinized. Samples were mainly from four internationally well recognized brands identified as A, B, C and D. All samples were bought from the supermarkets in Suva, the capital city of Fiji. The brand codes and the main ingredients of the baby foods are reflected in Table 1.

2.3. Sample extraction

Approximately 25 g of the baby food sample was weighed on an analytical balance and was homogenized with 200 mL of water (≈100 °C) in a blender and filtered through a Whatman 541 quantitative filter paper. 80 mL of the resultant mixture was transferred to a screw-capped bottle (120 mL capacity). Activated carbon (100–250 mg) with 2 mL each of Carrez reagents I and II were added and the bottle was tightly screw-capped. The mixture was shaken on a Stuart Scientific Shaker SF1 for approximately

Table 1
Nitrate and nitrite contents in various baby foods marketed in Fiji.

Types and contents of baby foods		Nitrate (mg kg^{-1})			Nitrite (mg kg^{-1})		
		Min	Max	Mean ± SD	Min	Max	Mean ± SD
Baby cereal	Wheat based[A]	8.00	12.00	9.48 ± 1.40	nd	nd	nd
	Multigrain banana[B]	10.00	12.50	11.73 ± 0.88	nd	nd	nd
	Wheat, banana and milk[C]	11.10	12.90	12.16 ± 0.70	nd	nd	nd
Fruit and cereal based puree	Apple and banana cereal[B]	20.00	21.8	20.77 ± 0.66	nd	nd	nd
	Apple and oatmeal[B]	16.10	17.45	16.81 ± 0.47	nd	nd	nd
Vegetable based puree	Carrots, broccoli and sweetcorn[D]	180.87	220.67	199.77 ± 14.32	0.56	3.67	2.30 ± 1.34
	Pumpkin and sweetcorn[D]	172.88	184.33	178.96 ± 4.49	0.61	1.23	1.00 ± 0.23
Fruit juice	Apple and prune[B]	10.67	12.88	11.39 ± 0.87	nd	nd	nd
	Apple and blackcurrent[B]	8.90	12.34	9.49 ± 0.54	nd	nd	nd
Fruit puree	Fruit salad[D]	10.78	12.76	11.84 ± 0.78	nd	nd	nd
Milk based puree	Chocolate custard[B]	2.10	3.89	2.90 ± 0.58	nd	nd	nd
Fruit, milk, yogurt and cereal based puree	Fruit salad yogurt[B]	9.99	12.00	11.20 ± 0.92	nd	nd	nd
	Custard with banana[B]	19.87	20.87	20.54 ± 0.48	nd	nd	nd
	Creamy banana porridge[B]	17.00	21.02	19.48 ± 1.53	nd	nd	nd
Meat and vegetable based puree	Sweetcorn and chicken[B]	38.98	45.24	42.32 ± 2.82	nd	nd	nd
	Lamb and vegetables[B]	123.4	141.20	136.93 ± 6.71	0.44	0.76	0.55 ± 0.12
Noodles and vegetable based puree	Chicken noodles and vegetables[B]	45.00	50.34	46.91 ± 2.71	nd	nd	nd
Vegetables and rice based puree	Carrots and rice[B]	87.00	89.56	88.58 ± 0.97	nd	nd	nd

nd: not detected; SD: standard deviation; A,B,C,D: samples' brand codes.

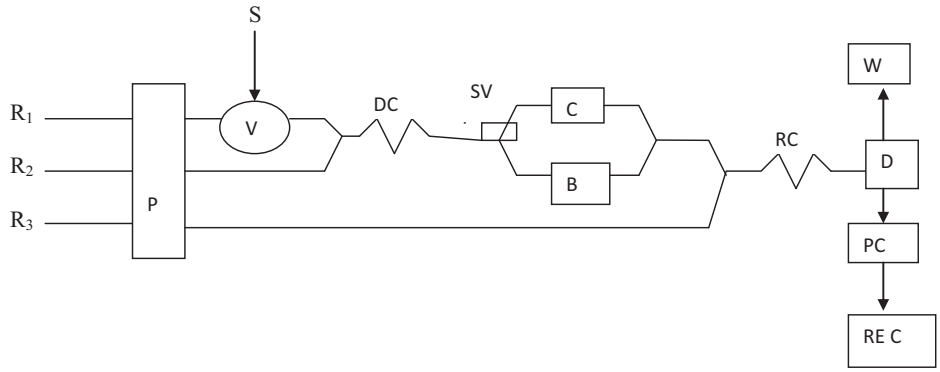

Fig. 1. The flow injection manifold used for the determination of nitrate: R_1, carrier stream (distilled de-ionized water); R_2, buffer stream (NH_4Cl/EDTA); R_3, color reagents stream (SA/NED); P, peristaltic pump; V, injection valve; S, sample; DC, delay coil; SV, two state switching valve; C, copperised cadmium column; B, alternate channel for only nitrite detection; RC, reaction coil; D, detector; W, waste tank; PC, personal computer; REC, recorder.

30 min. The mixture was filtered through HPLC syringe filters (0.45 μm) into 50 mL polypropylene vials ready for analysis.

2.4. Analytical procedure and equipment

Nitrate and nitrite analysis was carried out using a Lachat QuickChem 8000 flow injection analyzer (FIA). The flow injection analyzer used for the investigation of nitrate and nitrite in baby foods is shown in Fig. 1. The manifold was equipped with a peristaltic pump, P, an injection valve, V, a two state switching valve, SV, a copperised cadmium reduction column (C) for the reduction of nitrate to nitrite, and an alternate channel (B) for only nitrite detection. The FIA is equipped with a 60-position rack, an autosampler 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. The details on the instrument and methodology used are outlined in our previous communication (Prasad & Chetty, 2008).

The general principle employed by the FIA methodology is the reduction of nitrate to nitrite using a copperised cadmium (Cu-Cd) reduction column. The resultant stream of nitrite which is stabilized by a buffer at a pH of 8.5 is colorimetrically determined with Griess reagent (mixture of SA and NED). This furnishes the total nitrate and nitrite content of the sample. To quantify only nitrite, the sample is not allowed to pass through the Cu-Cd reduction column. Thus the exclusion of the nitrite values from the total resulted in superlative nitrate quantification in a sample.

The quantification parameters and the figure of merits of the analytical method are listed in Table 2. All pH measurements were carried out using Hanna Instruments Microprocessor 8521 pH meter. For analyte extraction a Stuart Scientific shaker model SF1 was utilized. The calibration graphs for nitrate as well as nitrite were acquired for each run by injecting (in duplicate) six different concentrations of nitrate and nitrite in the range of 1.0–20.0 μg mL^{-1} and 0.025–2.0 μg mL^{-1} respectively.

Table 2
Quantification parameters of flow injection analysis of nitrate and nitrite.

Facet	Parameter
FIA instrument	Lachat QuickChem 8000 FIA
Reduction column used	Copperised cadmium column
Path length	10 mm
Detector	Colorimetric (UV–vis)
UV filter	520 nm
Sample volume	200 μL
Nitrate limit of detection	0.040 μg mL^{-1}
Dynamic range of detection	Nitrate – 1.0–20.0 μg mL^{-1} Nitrite – 0.025–2.0 μg mL^{-1}
Coefficient of determination (R^2)	Nitrate – 0.9974 Nitrite – 0.9970
Sample throughput	38 h^{-1}

2.5. Statistical analysis

The statistical analysis were achieved using Microsoft excel 2007. Research data was subjected to one way analysis of variance (ANOVA) and Pearson Product-Moment Correlation. The standard deviations were calculated for each sample and reported along with the mean value.

3. Results and discussion

3.1. Calibration and analytical quality assurance

All calibration standards were prepared from 1000 μg mL^{-1} stock solutions of nitrate and nitrite and the linear calibration curves were ascertained with six points in each. The calibration equations of the measured peak areas (V s) *versus* analyte concentrations (μg mL^{-1}) were utilized to determine the nitrate and nitrite content of the baby food matrices in μg mL^{-1} which is ultimately stated as mg kg^{-1}. Fig. 2 shows typical FIA signals, i.e., peak profiles for the nitrate calibration standards in the range of 0.0–20.0 μg mL^{-1} (Prasad & Chetty, 2008).

The figures of merit for the quantification of nitrate/nitrite in the baby food samples are presented in Table 2. The precision in terms of relative standard deviation (RSD) for peak areas were below 1% for both the nitrate and nitrite calibration curves. The samples that surpassed the focused calibration range were diluted and re-evaluated. The calibration standards were run before each batch of samples with blanks run before and after sample analysis.

A known concentration of potassium nitrate lying between 0 μg mL^{-1} (blank) and the smallest calibration standard was used to determine the limit of detection (LOD) for nitrate. The standard deviation of seven replicates of this standard was calculated and multiplied by the student's t (=3.14) value at the 99% confidence level to dispense the experimental LOD. The nitrate LOD for the method used is 0.04 μg mL^{-1}.

The accuracy was gauged through the results of the spiked recovery. Only the samples with vegetable based baby food matrix were used for the spiked recovery analysis. Known concentrations of the nitrate solutions (5, 10 and 20 μg mL^{-1}) were added as an internal standard to the extracted samples. The results of the

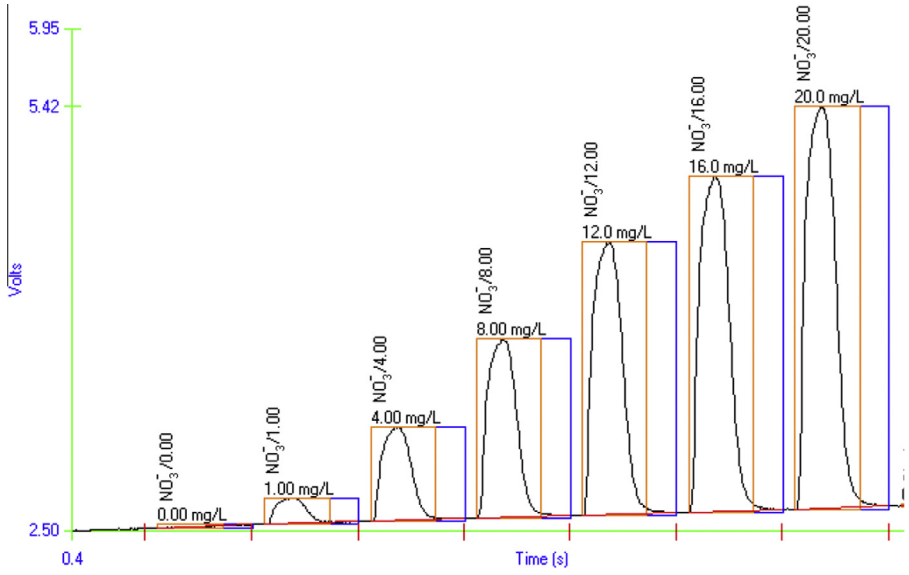

Fig. 2. Typical FIA peak profiles for nitrate standards (0–20 μg mL^{-1}).

Table 3
Determination of nitrate (μg mL^{-1}) extracted from the original and corresponding spiked samples.

Types of baby foods	Samples description	Nitrate μg mL^{-1}		Recovery (%)
		Spiked	Content ± SD	
Vegetable based puree	Pumpkin and sweetcorn[D]	–	9.03 ± 0.01	–
		5.0	14.33 ± 0.00	106.0
		10.0	19.33 ± 0.01	103.0
		20.0	28.63 ± 0.01	98.0
Vegetable based puree	Carrots, broccoli and sweetcorn[D]	–	10.18 ± 0.54	–
		5.0	14.97 ± 0.27	92.1
		10.0	20.28 ± 0.54	101.0
		20.0	29.98 ± 0.54	99.0
Meat and vegetable based puree	Lamb and vegetables[B]	–	6.90 ± 0.16	–
		5.0	12.14 ± 0.08	106.0
		10.0	17.00 ± 0.16	101.0
		20.0	25.50 ± 0.16	93.0
Average				99.89 ± 5.01

SD: standard deviation.

recovery analysis have been presented in Table 3. Typical recoveries of spiked nitrate residues ranged from 92% to 106%. The average recovery was calculated to be 99.89 ± 5.01%.

3.2. Baby food analysis

Baby foods marketed in Fiji are mainly from four brands identified as brands A, B, C and D for the purpose in this study. These are internationally recognized brands. Majority of the samples belong to brand B which was widely available at the time of the present study. Table 1 summarizes the nitrate and nitrite content of the eighteen different baby food samples ($n = 6$) analyzed by FIA. All nitrate and nitrite values are expressed in mg kg^{-1} (w/w).

The nitrate contents in the baby food samples studied ranged from 8.00 to 220.67 mg kg^{-1}. The nitrite was detected only in vegetable based baby food matrices and ranged from 0.44 to 3.67 mg kg^{-1}. In majority of the samples studied, the nitrite was below the detection limit. The average nitrate and nitrite content in the measured samples were 48.66 mg kg^{-1}and 1.28 mg kg^{-1} respectively. The four brands (A, B, C and D) had average nitrate content of 9.48, 33.77, 12.16 and 171.89 mg kg^{-1} respectively. The vegetable based baby foods (Brand D) showed relatively higher nitrate content in comparison to the other baby food matrices. Two samples from Brand D contained nitrate values higher than the current EU legislation (EC, 2006). The main ingredients of these samples were carrots, broccoli and sweet corn. Similar studies carried out in Portugal (Vasco & Alvito, 2011), Spain (Hardisson, Padrón, Frı, & Reguera, 1996) and Estonia (Tamme et al., 2006) have reported nitrate values higher than 200 mg kg^{-1} for baby foods containing broccoli and/or carrots. The nitrate content increases and followed trend as: milk based < cereal based < fruit based < vegetables based. The mean nitrate data (±SD) for the 10 chosen baby food matrices have been compared in Fig. 3. The above mentioned trend is undoubtedly discernible in this graphical representation of the nitrate data.

The nitrite was only detected in sample matrix that had vegetables as a core ingredient. Pearson product-moment correlation (PPMC) test was used to evaluate the existence of a linear relationship between the nitrate and nitrite values in these samples. The PPMC coefficient, $r > 0$ suggests that a positive linear association exists between the nitrate and the nitrite content of the samples that contain vegetables as core ingredients. This data could not be used to identify a cause and effect relationship between nitrate and nitrite as this may not hold true for all such samples. Although,

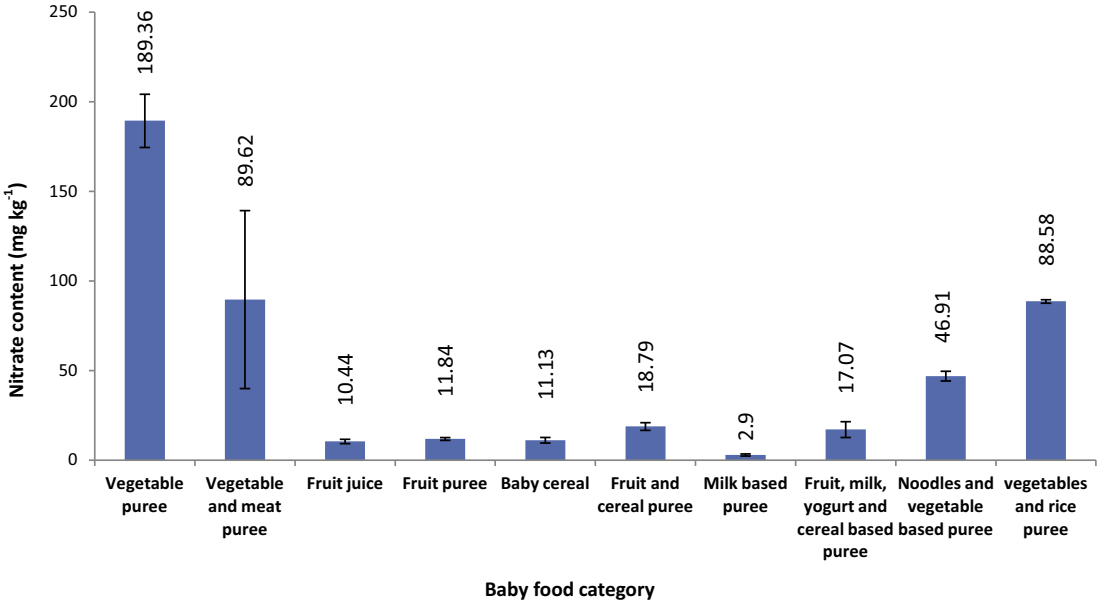

Fig. 3. Comparison of mean nitrate content ($n = 6$) between different commercial baby food categories.

Table 4
Comparison of the nitrate content (mg kg^{-1}) in different vegetable-based food categories in different countries.

Types of baby foods	Fiji	Estonia	Spain	Portugal	Germany
Vegetable puree	173–221	19–208	ndr	90–230	ndr
Vegetable and meat puree	39–141	32–148	20-204	ndr	ndr
Vegetable and cereal puree	ndr	24–162	382	ndr	ndr
Carrots and carrot juice	ndr	76–251	104	ndr	ndr
Average	49	88	92	102	81

Note: Estonia (Tamme et al., 2006); Spain (Hardisson et al., 1996).
Germany (Tamme et al., 2006); Portugal (Vasco & Alvito, 2011).
ndr: no data reported.

it is logical to infer that nitrate can get reduced to nitrite during the processing phase of the baby food manufacturing and as such a positive relationship becomes apparent.

The cereal based baby foods which are from brands A, B and C were subjected to a one-way ANOVA (analysis of variance) test. Thus according to the results ($P > 0.05$), there are no statistically significant differences between the nitrate contents of the cereal based baby food brands. The mean values of nitrate of all the ten baby food matrices were also subjected to a one-way ANOVA. The results clearly identified statistically significant differences ($P < 0.05$) between the mean nitrate values of the various baby food matrices studied. Table 4 shows a comparison of nitrate content in different vegetable based baby food categories in different countries. The average nitrate values of vegetable based baby foods in Fiji are relatively lower than other such studies carried out in Estonia, Spain, Portugal and Germany.

4. Conclusion

The FIA methodology illustrated allows a rapid, accurate and specific measurement of nitrate and nitrite in baby food matrices. The study presents primary data on the nitrate and the nitrite contents of eighteen commercially available baby foods marketed in Fiji. The evaluated baby food samples were sourced from ten common baby food matrices. The nitrate values ranged from 8.00 to 220.67 mg kg^{-1} while nitrite was detected only in vegetable based baby food samples. The average nitrate values fall below the allowable nitrate content for commercial baby foods as stated in the European Union legislation. As shown in Table 4, the average nitrate values in the studied baby food samples are lower in comparison to similar studies undertaken in other countries. Thus it is imperative for manufacturing companies to ensure that the nitrate and the nitrite values are kept to as low as reasonable possible. However, further research is needed to gather food consumption data to evaluate nitrate/nitrite risk assessment to infants and toddlers in Fiji.

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