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Investigation of the Nitrate and Nitrite Contents in Milk and Milk Powder in Taiwan

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ABSTRACT

Cow milk is one of the most important nutrition sources besides breast milk for infants and babies; therefore the detection of nitrate and nitrite in milk is of great importance to the health of infants and babies. Traditional colorimetric method for the determination of the nitrate and nitrite concentrations in food is very tedious. In the present study, 100 samples of milk and milk powder were analyzed by ion chromatography to detect nitrate and nitrite simultaneously without tedious preparation steps. The limit of detection (LOD) for nitrate is 0.33 ppm and the LOD for nitrite 0.07 ppm. The measured nitrate concentration in milk ranged from 0.3 - 417.7 ppm with an average concentration of 92.7 ppm. The nitrite concentration in all milk samples were below the detection limit. The survey information was as the following: (1) the average nitrate concentration of colostrums fortified milk ranged from 14.1 to 136 ppm. (2) the average nitrate concentration of whey fortified milk ranged from 42.6 to 242.8 ppm. (3) the average nitrate concentration of ordinary milk ranged from 57.9 to 157.6 ppm. The dietary intake of nitrate from milk and milk products in Taiwanese adults and children were estimated. All the nitrate exposure levels calculated with the measured results for the different age groups were less than the acceptable daily intake (ADI) of 3.7 mg/ kg body weight per day suggested by WHO. Finally the health effect of nitrate and nitrite in foods was discussed.

Key words: nitrate, nitrite, milk, ion chromatography

INTRODUCTION

Nitrate and nitrite occurred naturally in soil, water and food. Besides the natural sources, nitrate was utilized as fertilizer in agriculture for the production of fruits and vegetables. In meat and cheese preservation, nitrate and nitrite were commonly employed as food additives. Therefore food such as vegetables, drinking water and meat would have more nitrate and nitrite contents as the above consequences of the human activities.

Previous studies showed the health risk associated with nitrate and nitrite in foods for human. For example, infants consuming too much nitrite would have the possibility of methaemoglobinaemia(1). The nitrate in food was reduced to nitrite from bacteria in the saliva, and under the gastric acid conditions would react with secondary amine to form the carcinogenous nitrosoamine(2). Many countries have regulations regarding the maximum levels of nitrate and nitrite in foods. Both the US FDA and EU have established maximum levels regulation for nitrate and nitrite in processed meat products. The Commission Regulation (EC) No. 1881/2006(3) have established maximum levels of nitrate contamination in different foods as the following: (1) The maximum level of nitrate contamination allowed for spinach ranged 2000 - 3500 ppm. (2) The maximum level of nitrate contamination allowed for lettuce was 3000 - 5000 ppm. (3) The maximum level of nitrate contamination allowed for rucola ranged 6000 - 7000 ppm. (4) The maximum level of nitrate contamination allowed for processed cereal-based foods and baby foods was 200 ppm.

The carcinogenicity of nitrate and nitrite in food was still in dispute at different studies. The study by WHO(4) revealed that nitrate was not carcinogenic. However, International Agency for Research on Cancer (IARC)(5) concluded in their study: "ingested nitrate or nitrite under conditions that result in endogenous nitrosation is probably carcinogenic to humans (group 2A)." Recently, the epidemiological study by European Food Safety Authority (EFSA)(6) stated that nitrate and nitrite were not correlated with cancer.

Recent studies have revealed that nitrate and nitrite are healthful dietary components. Nitrate in food could convert into nitrite in our mouth and then in stomach nitrite could convert into NO which has many therapeutic effects to promote human health(7,8). The dietary consumption of

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nitrates and nitrites from vegetables and fruit, according to Hord *et al.*[7], may contribute to the blood pressure lowering from Dietary Approaches to Stop Hypertension (DASH) diet. But the nitrate contents in DASH diet could vary from 174 to 1,222 mg, and 1,222 mg exceeds the World Health Organization's Acceptable Daily Intake (ADI) for nitrate by 550% for a 60-kg adult. Therefore Hord *et al*. questioned the rationale for regulatory limit of nitrate and nitrite consumption from plant foods. Adequate intake of nitrate containing food could help control blood pressure and lower blood pressure[9-11]. Studies have shown nitrate in food could reduce oxidative stress, prevent heart and kidney injuries[12], and reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice[13]. Tang *et al.* have revealed that Chinese have used nitrate to alleviate pain resulting from angina as early as the 8th century[14]. Tang *et al.* demonstrated that the activity of nitrite reductase in many of the Traditional Chinese Medicine (TCM) extracts was greater by 1,000 times than that of biological tissues. In this respect, the NO generated from TCM accomplished therapeutic effect toward cardiovascular disease. Zand *et al.* have proved that NO dietary supplements made of beetroot and Hawthorn berry could restore NO homeostasis in human and reduce triglycerides[15].

Although there were potential health risk for nitrate and nitrite in previous studies, more recent studies have demonstrated the potential health benefit from nitrate and nitrite. Therefore investigation of nitrate and nitrite contents in different foods would be of great importance for both food safety and nutrition intake recommendation. The National Food Residue Database (NFRD) of Ireland revealed that the nitrate levels were in the ranges < 5 - 120 ppm in skim milk powder, < 5 - 88 ppm in acid casein and < 5 - 56 ppm in rennet casein[16]. The nitrite content were in the ranges 0.5 - 3.8 ppm and 0.5 - 5.9 ppm for acid and rennet caseins, respectively. According to the NFRD investigation, contamination of dairy products with nitrates might occur from water and/or gas used in processing and from the use of nitric acid as a cleaning agent in processing plants. The NFRD investigation indicated that relatively high nitrate and nitrite levels might occur in dairy powder products, particularly nitrate in acid casein powder and nitrite in acid and rennet casein powder. The recent death incidents of three children by nitrite tainted milk in China had raised the concern about nitrite content in milk internationally[17]. There were no investigation related to nitrate and nitrite content in milk and milk products in Taiwan. Milk is one of the most important food nutrition sources besides breast feeding for infants and little babies, therefore the detection of nitrate and nitrite in milk and milk products is of great importance to the health of infants and babies.

The traditional AOAC Official Method (993.03) spectrophotometric method could not detect nitrate and nitrite simultaneously[18]. The determination of nitrate would require reduction of nitrate to nitrite using toxic cadmium metal followed by derivatization with Greiss reagents. Through all the tedious steps, the interfering compounds still remained in the sample matrix and the detection limit was thus restricted. McMullen *et al*. have developed ion chromatographic method to detect both nitrate and nitrite in vegetable and fruit baby foods simultaneously[19]. The ion chromatographic results were comparable with the spectrophotometric AOAC Official Method (993.03) and avoided the use of toxic Cd metal. No nitrite was detected and the nitrate contents of baby foods ranged from 96.2 to 1084.2 ppm in the study by McMullen *et al*. This work would adopt the ion chromatographic method[20] to detect nitrate and nitrite in milk and milk powder in Taiwan. Comparison with previous literature survey data and the possible sources contributing nitrate content will also be discussed.

MATERIALS AND METHODS

I. *Reagents and Chemicals*

The reagents and solutions in the present work are all of reagent grade.

Ultrapure water: deionized water with specific resistance greater than 18.0 MΩ. Nitrate standard solution (traceable to SRM from NIST $NaNO_3$ in H_2O 1000 mg/L NO_3 CertiPUR®) and nitrite standard solution (traceable to SRM from NIST $NaNO_2$ in H_2O 1000 mg/L NO_2 CertiPUR®) were from Merck. Glacial acetic acid (ACS reagent grade) was from Echo. Sodium carbonate and sodium bicarbonate (ACS reagent grade) was from Sigma-Aldrich. Milk and milk powder samples were purchased from local supermarkets and convenient stores.

II. *Instrumentations*

The ICS-1500 Ion Chromatography System (Dionex, USA) equipped with suppressed conductivity detector with ASRS 300 as suppressor was employed for ion chromatography analysis. The Chromeleon 6.8 Chromatography Data System (Dionex, USA) was used for instrument control and data analysis. The shaker SA31 was from Yamato Scientific Co.(Tokyo, Japan). The centrifuge was a high-speed refrigerated centrifuge CR21G III from Hitachi (Tokyo, Japan). The ultrasonic bath was provided by DC400H from Delta (Taipei, Taiwan).

III. *Solvents Preparation*

Three percent acetic acid: 3 mL glacial acetic acid was diluted volumetrically to 100 mL with ultrapure water.

Mobile phase solution (8 mM Na_2CO_3 and 1 mM $NaHCO_3$) : 8.48 g Na_2CO_3 and 0.84 g $NaHCO_3$ were dissolved and diluted volumetrically to 100 mL with ultrapure water. The solution was stored at 4°C in the refrigirator and warmed up to room temperature before use. Ten milliliter was measured precisely and diluted volumetrically to 1 L stock. The solution was filtered before use.

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IV. *Sample Preparation*

One gram of sample was weighed into 50 mL volumetric flask. About 30 mL ultrapure water was added on a shaker, and the mixture was put in an ultrasonic bath for 10 min. One milliliter of 3% acetic acid was added to precipitate the proteins in milk. The volume is brought to 50 mL with ultrapure water. The sample was let sit for 20 min and then centrifuged at 7,000 rpm for 10 min. The supernatant was filtered with a 0.22 μm syringe filter. The first 1.5 mL of sample was discarded and the remaining was collected for ion chromatographic analysis.

V. *Ion Chromatography Analysis*

Conditions for ion chromatography were listed in Table 1.

Nitrate and nitrite standard solution preparation : 10 mL nitrate stock standard and 4 mL nitrite stock standard were volumetrically diluted to 20 mL respectively.

Calibration solutions were prepared by taking 10, 20, 40, 100, 200 μL of standard solution and dilution to 10 mL respectively.

Ion chromatogram for 2 ppm standard solution was shown in Figure 1(A). The peak appearing at 4.957 min was nitrite ion and the peak at 6.62 min was nitrate ion. Ion chromatogram for one milk sample was shown in Figure 1(B). The peak at 4.9 min was nitrite ion and the peak at 6.52 min was nitrate ion.

RESULTS AND DISCUSSION

I. *Linearity of Calibration Curve and LOD*

The linearity and LOD of this method were listed in Table 2. A good linearity was achieved in the concentration

Table 1. Condition for Ion Chromatography

Column	IonPac®AS14A Analytical 4 × 250 mm
Column Heater	35.0°C
Mobile phase	Mobile phase solution
Velocity mL/min	1
Injection volumn μL	25
Suppressor	ASRS 300 4-mm Self-Regenerating Suppressor
Detector	Conductivity cell
Data Rate	5.0 Hz
Supp. Current	43 mA
Cell Temp	35.0°C

Table 2. Method linearity (n = 5) and LOD

	Retention time (min)	Linear range (ppm)	Calibration curve	correlation coefficient	LOD (ppm)
nitrite	4.873	0.2 - 4	y = 0.1085x + 0.0099	0.9989	0.07
nitrate	6.483	0.5 - 10	y = 0.0924x + 0.0024	0.9994	0.33

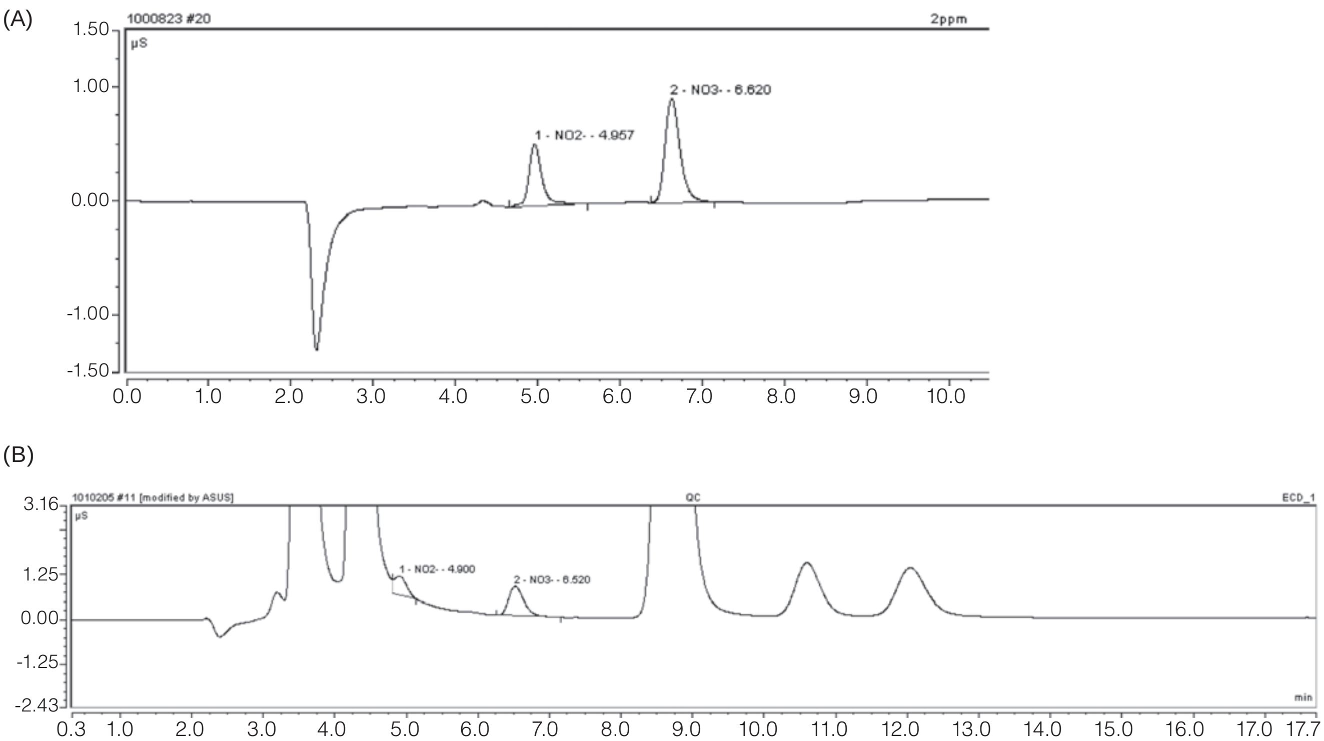

Figure 1. (A) Ion chromatogram for 2 ppm standard solution (B) Ion chromatogram for milk sample.

of 0.2, 0.4, 0.8, 2, 4 ppm for nitrite and in the concentration of 0.5, 1, 2, 5, 10 ppm for nitrate. The correlation coefficients were 0.9989 and 0.9994 for nitrite and nitrate, respectively. The LOD was calculated from the standard deviation of signals and the slope of the calibration curve according to Konieczka *et al.*[21].

II. *Precision and Accuracy*

(I) *Recovery and Precision*

Recovery was evaluated by spiking 0.8 ppm nitrite and 2 ppm nitrate into blank matrix of milk samples with 7 repetitions for three days. The recovery rate was shown in Table 3. For both nitrite and nitrate, the recovery were better than 85%. According to the Dionex Application Note 279[20], the recovery rate of nitrate in milk was 111% and 113%. The high recovery rate for nitrate could be due to the nitrate present in the blank milk samples for recovery study.

Precision of nitrite and nitrate analysis was evaluated by spiking 0.8 ppm nitrite and 2.0 ppm nitrate, respectively, into blank matrix of milk samples with 7 repetitions for three days. The result was shown in Table 3. The RSD's for nitrite and nitrate were all less than 10%.

III. *Survey Results of Nitrate Contents in Milk and Milk Powder*

The survey results for nitrate contents in 100 samples of milk and milk powder were listed in Table 4. The milk samples was from the following categories : colostrums fortified milk powder, whey fortified milk powder, ordinary milk powder, and fresh milk and milk in aseptic package.

As shown in Table 4, milk powders for adult on the average contained the highest nitrate concentration. Adult milks are often fortified with many nutraceuticals, therefore the nitrate concentration are higher than those of infant and growing up milk. The nitrate concentration in milks with whey added for adult consumption is on the average 200 ppm higher than that in the infant formula. The nitrate concentration in milks with colostrum added for adult consumption is on the average 100 ppm higher than the infant formula. The nitrate concentration in milks for growing up consumption is on the average 50 - 108 ppm higher than that in the infant formula. Because there are not too many fortified nutrients and in liquid form with greater amount of water content compared to milk powder, fresh milk and milk in aseptic package have the lowest concentration of nitrate. Different milk products had varied nutrients fortification so that the nitrate contents also varied.

There were many previous studies on nitrate and nitrite concentrations in milks and baby foods. Usually nitrate contents in the milk products and baby foods were less than 200 ppm[16,22-23,26]. In order to increase the nutrition contents, the milk products and baby foods were usually fortified by other ingredients[19,24-28]. The highest nitrite concentration 48.23 ppm was found in whey hydrolysate[26]. Milk products and baby foods might have nitrate concentration as high as 1,760 ppm[27]. The highest nitrate concentration in the present study was 417.7 ppm and nitrite concentration was below the limit of detection. In milk products, the ratio of whey to casein would sometimes be adjusted to approximate human milk, therefore the nitrate content would be different from raw milk. Vegetable ingredients would also change the nitrate contents.

According to Encyclopedia of Dairy Sciences, the nitrate contents in milk powder and whey protein powder were 1 - 102 and 1.9 - 50 ppm[23], respectively. The highest nitrite

Table 3. Precision of nitrite and nitrate

Nitrite	Blank value (ppm)	Recovery (%)	RSD (%)
Day 1	N.D.	99.07	2.7
Day 2	N.D.	85.26	1.4
Day 3	N.D.	88.93	1.7
Nitrate	Blank value (ppm)	Recovery (%)	RSD (%)
Day 1	N.D.*	110.61	4.2
Day 2	N.D.	113.68	9.3
Day 3	N.D.	112.46	2.7

*Not detected

Table 4. Survey results for nitrate contents

Sample type		Nitrate contents (ppm)	Mean contents (ppm)
Colostrums fortified milk powder	Infant formula for less 1 year old (n = 8)	0 - 34.9	14.1
	Growing up for over 1 year old (n = 14)	44.9 - 312.6	122.2
	Adult (n = 2)	15.5 - 256.5	136
Whey fortified milk powder	Infant formula for less 1 year (n = 11)	2 - 91	42.6
	Growing up for over 1 year old (n = 21)	21.4 - 245.5	116.5
	Adult (n = 7)	60.7 - 405	242.8
Ordinary milk powder	Infant formula for less 1 year (n = 5)	0 - 83.2	57.9
	Growing up for over 1 year old (n = 11)	49.3 - 171.5	108.4
	Adult (n = 10)	49.3 - 417.7	157.6
Fresh milk and milk in aseptic package (n = 20)		0.3 - 42.3	14.3

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content in raw milk and milk powder was 1.0 and 8.6 ppm, respectively. Hardisson had surveyed 27 types of infant foods with UV spectrometer. The highest nitrate concentration of 382 ppm was detected in infant food comprised of carrot and rice[(24)]. The nitrite levels were N.D. to 0.05 ppm in Hardisson's study. Vasco *et al.* checked 80 baby foods in Portugal for nitrate contents and the range was from 5 to 230 ppm[(25)] and no measurement about nitrite in his work. According to the study by Vasco *et al.*, the estimated nitrate intake with the highest nitrate content for 3-month-old infant did not exceed the FAO/WHO ADI values. Gapper *et al.* measured nitrate and nitrite contents of 80 dairy products including skim milk, whole milk, buttermilk, infant formulas and milk protein hydrolysate powders in New Zealand. The nitrate contents range in dairy products were 6.4 - 44.18 ppm[(26)]. The highest nitrate content of 44.18 ppm was found in whey hydrolysate. Whey hydrolysate also contaminated with 48.23 ppm nitrite and casein hydrolysate contaminated with 27.24 ppm nitrite.

Whey or whey protein, sucrose and dietary fiber are often added to milk to fortify nutrients. From previous literature survey, these fortified nutrients could have high nitrate contents. Sen *et al.* have studied the nitrate and nitrite concentrations of 15 whey powders[(27)]. The highest nitrate content in whey powder was found to be 1,760 ppm. No detectable nitrite content was measured in Sen's work. Oliveira *et al.* measured nitrate and nitrite contents in 231 whey containing products[(28)]. The contents of nitrate ranged 4.9 - 1250 ppm, and the nitrate in whey-containing milk powder ranged 7.2 - 102 ppm. Sixty percent of the products had nitrate contents in the range of 1.1 - 4.6 ppm. Oliveira *et al.* pointed out that 67% nitrate and nitrite still remained in whey during the cheese manufacturing process. Therefore they concluded whey protein had higher risk of nitrate and nitrite contamination than cheese. The nitrite content ranged N.D.-4.6 ppm in Oliveira's work.

Not only could fortified whey increase the nitrate content in milk, but other nutrients such as sucrose, dietary fiber and lactose could also increase the nitrate level in milk. According to the studies by C. Merusi *et al.*, the nitrate content in sucrose is 4.9 - 6.6 ppm, dietary fiber like fructooligosaccharides and inulin could have nitrate content ranging 10 - 72 ppm[(29)]. Because lactose are manufactured from whey, lactose could also have high nitrate content. Some of the products in the present study exceeded the EU regulation of 200 ppm in infant milk. As seen in literature data and the nutrition labels in the products, the high nitrate contents could come from the contribution of whey, lactose, sucrose and dietary fiber.

IV. *Nitrate Exposure Assessment and Health Effects*

To provide different exposure scenarios for infants less than 1 year old, infant body weight data from Dietary Reference Intakes Tables of Taiwan[(30)] and the average milk consumption information from the DONALD (Dortmund Nutritional and Anthropometrical Longitudinally Designed) study by Kersting *et al.*[(31)] were adopted to calculate the nitrate exposure shown in Table 5. The average milk consumption for 3, 6, 9 and 12 months old infants were 67, 195, 234 and 208 g/day, respectively.

Because milks are the main staple of infants for nutrients, the lower the body weight, the more the nitrate intake. Even with the highest nitrate content of 91 ppm, the calculated nitrate exposure for 3, 6, 9, 12 month infant was 1.1, 2.4, 2.5 and 2.0 mk/kg body weight /day, respectively. The nitrate exposure is still less than the WHO ADI value 3.7 mg/kg body weight /day.

To estimate different exposure scenarios for kids from 1 to 3 years old, the average milk consumption 53 g/day from the DONALD study by Alexy *et al.*[(32)] and the body weight data from Dietary Reference Intakes Tables of Taiwan[(30)] were adopted to calculate the exposure in Table 5. Although growing up milk for 1 - 3 years old kids had more nitrate contents, with reduced milk consumption and more heavy body weight, the calculated highest nitrate exposures 1.274 mg/kg body weight /day are still less than the WHO ADI value of 3.7 mg/kg body weight /day.

To calculate different exposure scenarios for people from 4 to 65 years old, the body weight data from Dietary

Table 5. Nitrate exposure (mk/kg body weight /day) for infants less than 1 year old and 1 - 3 years old kids

Consumption scenarios			Nitrate intake (mg /kg/ bw/ day)					
Age (month)	Weight[a] (kg)	Average milk consumption[b] (g/day)	14.1 ppm[c]	42.6 ppm[d]	57.9 ppm[e]	34.9 ppm[f]	91 ppm[g]	83.2 ppm[h]
3	5.8	67	0.2	0.5	0.7	0.4	1.1	1.0
6	7.5	195	0.4	1.1	1.5	0.9	2.4	2.2
9	8.6	234	0.4	1.2	1.6	0.9	2.5	2.3
12	9.4	208	0.3	0.9	1.3	0.8	2.0	1.8
Age (year)	Weight[a] (kg)	Average milk consumption[i] (g/day)	122.2 ppm[c]	116.5 ppm[d]	108.4 ppm[e]	312.6 ppm[f]	245.5 ppm[g]	171.5 ppm[h]
1 - 3	13	53.0	0.498	0.475	0.643	1.274	1.001	0.699

[a] Reference 31. [b] Kersting *et al.*[(32)]. [c] Mean nitrate content of colostrums fortified milk. [d] Mean nitrate content of whey fortified milk. [e] Mean nitrate content of ordinary milk.[f] Highest nitrate content of colostrums fortified milk. [g] Highest nitrate content of whey fortified milk. [h] Highest nitrate content of ordinary milk. [i] Alexy *et al.*[(33)]

Reference Intakes Tables of Taiwan(30) and average milk consumption information from Nutrition and Health Survey in Taiwan(33) were adopted to calculate the nitrate exposure. Although the milk for the 4 to 65 age group contain the highest nitrate content, the reduced milk consumption and heavier body weight combination make the nitrate intake exposure well below the WHO ADI value. With the highest nitrate content 417.7 ppm, the calculated nitrate exposure for 4 - 6 years old kids and 65 years old adult was only 0.184 ppm and 0.038 ppm, which was 5% and 1% of the WHO ADI value, respectively. Therefore the products surveyed in the present study will not cause food safety risk.

CONCLUSIONS

In the present study, 100 samples of milk and milk powder were analyzed by ion chromatography to detect nitrate and nitrite simultaneously without tedious preparation steps and hazardous Cd metal waste. The LOD's for nitrate and nitrite were 0.33 and 0.07 ppm, respectively. The nitrite concentration in all of the milk samples were below the detection limit. The average nitrate concentration of colostrums fortified milk ranged from 14.1 to 136 ppm. The average nitrate concentration of whey fortified milk ranged from 42.6 to 242.8 ppm. The average nitrate concentration of ordinary milk ranged from 57.9 to 157.6 ppm.

Although some products in this survey contain nitrate concentrations higher than 200 ppm by EU regulation for baby foods, the calculated nitrate exposure value is still lower than the ADI suggested by WHO/FAO. Therefore the products surveyed in the present study will not cause food safety risk. Because the major dietary exposure source of nitrate was from vegetables, the EU nitrate regulation didn't include milk for young and adult. The Codex Alimentarius Commission, US, Canada, Australia and New Zealand also didn't impose regulation of nitrate content in milk products and vegetables. Because sometimes relatively high nitrate and nitrite levels might occur in dairy powder products, investigation of nitrate and nitrite contents in food should be done for food safety precaution.

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