Simultaneous determination of nitrite and nitrate in milk samples by ion chromatography method and estimation of dietary intake

Sajad Chamandust [a], Mohammad Reza Mehrasebi [b], Kourosh Kamali [c], Reza Solgi [d], Jafar Taran[b], Firoozeh Nazari [e], Mir-Jamal Hosseini [f,g]✉

[a] Department of health and safety Food, School of public health, Zanjan University of Medical Sciences, Zanjan

[b] Department of Environmental Health, Faculty of Health, Zanjan University of Medical Sciences, Zanjan, Iran

[C] Department of Public Health, School of Health, Zanjan University of Medical Sciences

[d] Metabolic Disorders Research Center, Golestan University of Medical Sciences, Gorgan, Iran

[e] Food and Drug Administration-Iran University of Medical Sciences, Tehran, Iran

[f] Zanjan Applied Pharmacology Research Center, Zanjan University of Medical sciences, Zanjan, Iran

[g] Department of Pharmacology and Toxicology, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran

Corresponding authors:

Mir-Jamal Hosseini (✉), Assistant Professor of Toxicology

Zanjan Applied Pharmacology Research Center, Zanjan university of Medical sciences, Zanjan, Iran; Department of Pharmacology and Toxicology, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran P. O. Box: 45139-56184.

E-mail:jamal_hossini@yahoo.com;Phone: +98 912 5030167; Fax: +98 2433473639

Running title: Nitrate & nitrite in milk by Ion chromatography

Abstract

The presence of nitrate and nitrite in foods may be considered hazardous after ingestion in the gastrointestinal tract due to their reaction with naturally occurred secondary amines to form potentially carcinogenic nitrosamines. Due to this fact, a new method was developed in this study for the simultaneous determination of nitrite and nitrate in milk samples. After dilution and filtering of milk samples, they were analyzed by ion chromatography. Proposed mobile phase composed of sodium hydrogen carbonate and sodium carbonate (1.0 and 3.2 mmol/L) with a flow rate of 0.7 ml/min. The average recoveries for nitrate and nitrite were higher than 86 and 88%, respectively (with coefficient of variation lower than 10%). The limit of detection for nitrate and nitrite were 0.24 and 0.09 mg/L, respectively). This method was performed on 102 milk samples. The results showed that nitrate was found in all of the samples (100%) with a mean of 34 ± 11 mg/L, while Nitrite was found in none of the samples. The mean intake of nitrate in all age groups was lower than WHO guideline. The present assessment concludes that the maximum contaminant level was equal to 82.8 mg/L nitrate. This method was fast, sensitive and accurate and is capable of being an alternative method in food control laboratories for investigation of nitrite and nitrate content. This is the first study of the determination of nitrite and nitrate and exposure assessment of the Iranian population to these compounds. In addition, this was the first survey of the nitrite and nitrate level in milk, which was widely used in infants and adolescents as one of the basic food components.

Keywords: Nitrates, Nitrites, Milk, Ion chromatography, Exposure assessment

Introduction

The increasing concentration of nitrite and nitrate level in food products such as meat, cheese and fish is commonly used as curing and preservatives, has posed serious hazards to animal and human health [1-3]. However, the therapeutic potential effect of nitrite in the treatment of a number of cardiovascular including ischemia/reperfusion (I/R) injured; hypercholesterolemia induced microvascular inflammation as well as ischemia-induced angiogenesis are established [4]. Previous reports indicate that these compounds may damage the nervous system, liver, kidneys and spleen of small fish [5]. In addition, some study suggests that infant formula has too much nitrite would have the possibility of methaemoglobinaemia [6]. The widespread use of fertilizers, domestic, agricultural and industrial wastes have increased the chances of nitrite and nitrate into manufactured dairy products [7].

Based on many documents, lethal oral doses of nitrite and nitrate for human are established in 80‑800 and 33‑250 mg/kg body weight, respectively. It is proven that nitrite is formed during reduction of nitrate of bacteria in the saliva and under gastric acid condition in reaction with secondary amine to form the carcinogenic nitrosamine [8]. Consequently, it suggested nitrite is 10 times more toxic than nitrate and its potential as a human carcinogen (Group 2A) by the International Agency for Research on Cancer (IARC) [1,9]. The presence of nitrates and nitrites in food product is associated with an increased risk of gastrointestinal cancer and methaemoglobinaemia in infants [10]. Previously, it was noted that physiological roles for nitrate and nitrite in vascular and immune function [11]. Dietary nitrates are derived from vegetables, fruit, processed meats and dairy milk. Nitrites are produced endogenously through the oxidation

and through a reduction of nitrate by commercially bacteria in the gastrointestinal tract [12]. Milk is often regarded as being nature's most complete food consumed by humans [13]. Although, a variety of naturally bioactive peptides have been found in milk which exert a number of health beneficial activities such as boosting natural immune system and reducing the risk of chronic diseases via the a great impact on major body systems including digestive, nervous, endocrine, cardiovascular and diabetes [14,15], but, it is easily exposed with large number of physical pollutants (foreign bodies, radionuclides), chemicals (pesticides, heavy metals, antibiotics, nitrates, and so) and caused undesirable health effects [16,17].

Previous studies confirm that nitrates and nitrites are frequent constituents of many foods including vegetables, fresh and cured meats, dairy products, fruits and grains [12]. Existence of nitrites and nitrates in food such as milk could be considered as hazardous compounds when oxidation and reduction status in the gastrointestinal tract [18]. These compounds have harmful impact on human health due to its reaction with naturally present secondary amines to form potent carcinogenic N-nitrosoamines. Since N-nitroso compounds are easily formed by the interaction of a secondary amino compound with nitrite and nitrate in milks [19]. Also, there is an explosion of interest in the toxicology of the nitroso compounds in the induction of cancer in many tissues such as lung, kidney, liver, bladder, pancreas, esophagus and tongue, brain, colon and bone, depending on the species [19].

Milk could be considered as an important type of food especially for infants because of their nutritional requirements, so quality control in milk and milk products related to trace elements determination has been recommended [16,19]. Based on documents, regulatory authorities (US FDA and EU) have been established maximum residues level for nitrate and nitrite in different

nutrients such as spinach (rang 2000 -3500 mg/L), lettuce (3000-5000 mg/L) and processed cereal-based food and baby food(200mg/L) [21]. In USA and British, maximum permissible values of nitrate intake which enters the human body weekly from different ways is 400 to 450 mg containing 210 and 110 mg from vegetable and meat consumption, respectively, and 85 mg/ liter from drinking water [10].

More recent methods for determination of nitrate and nitrite level have been comprehensively reviewed [10, 22-24]. Using of many methods such as spectrophotometry, fluorimetry and chemiluminescence requiring to tedious extraction step, interfering with matrix and restriction of high detection limit. These make them unsuitable for the routine analysis of large numbers of samples [22,23]. Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography with fluorescence detection methods have higher sensitivity, reliability and selectivity in the isolation of these compounds compared with the other methods available for the exact determination of trace levels of nitrite and nitrate in food samples. But, these methods are expensive, time consuming, and require high amount of solvents which are corrosive or toxic in sample preparation steps [10,24,25].

Due to these disadvantages, interest in analytical techniques which could replace the classical methodology has been increasing. Ion chromatography is a versatile and powerful technique for cationic and anionic compound analysis due to very fast separations and high-resolution, without requiring organic solvent and low amounts of reagents and samples, resulting in a lower residue generation [21,26,27].Given milk is one of the most important sources for infant and babies and adults, it is necessary to determine rapid and reliable analytical methods for determination of

nitrite and nitrate level in food products particularly in milk due to its harmful or health effects on human health.

It is necessary to control the level of nitrite and nitrate in milk since their excess may cause serious health problem for consumers. Unfortunately, there is no investigation related to nitrite and nitrate content in milk or milk product in Iran. Therefore, this study aims to develop an ion chromatography method for analysis of nitrite and nitrate level in milk and exposure assessment of nitrate and nitrite through milk consumption in Iran population for the first time in Iran.

Materials and methods

Materials

Chemicals: All solutions were prepared using analytical grade reagents and deionized water. Sodium nitrite, sodium nitrate and other chemicals or solvents were purchased from Merck (Darmstadt, Germany). Precautions have always been taken to minimize sample contamination. All sample containers and glassware were thoroughly cleaned with 0.1M HCl solution and then finally with deionized water. The blank chromatograms with the double deionized water have shown no nitrite or nitrate peak.

Samples: A total of 102 commercial sterilized cow's milk samples were collected randomly in April -July 2014 from local supermarkets and convenient stores in different provinces of Iran. All the samples were taken and then stored at 4^{o}c until analysis.

Apparatus: The 881 Compact Ion chromatography system (Metrom, Switzerland) equipped with Suppressed conductivity detector with Metrohm suppressor Module (MSM, 50 mmol/L H_2SO_4). The MagIC net (version 2.3) software was used for monitoring system and data analysis. The shaker SA31 was from Yamato Scientific CO.(Tokyo-Japan). The centrifuge was a high-speed refrigerated centrifuge 3-30K from Sigma CO.(Germany). The ultrasonic bath was provided by Powersonic 405 from Daihan Lab Tech CO (Nanyangiu, Korea).

Standard, sample and solvent preparation: Nitrite and nitrate stock standard solutions (200 and 1000 µg/ml) in deionized water were prepared weekly and then these standards were used to prepare mixed working standard for analysis. Working standard solutions were prepared daily by diluting the mix standard with deionized water. A total of 1ml milk sample was diluted with deionized water to obtain a final volume of 50 ml. Then, samples were centrifuged at 10000 rpm for 5 min. The first, 1 ml was discarded and remaining sample was filtered through a 0.22 µm glass-microfiber GMF Whatman chromatographic filter and 25µl injected into the anion chromatography system with conductivity detection after chemical suppression. Mobile phase was made up of sodium hydrogen carbonate (1.0 mmol/L) and sodium carbonate (3.2 mmol/L) with a flow rate of 0.7 ml/min.

Validation Procedure

The validation process consisted of assessing the following parameters: linearity, Limits of detection (LOD), limit of quantification (LOQ) precision, repeatability and accuracy.

System suitability: It was tested by considering the relative standard deviation (RSD) ≤3% of the means obtained from 5 consecutive injections of standard samples in different days.

Linearity: Linearity was established from the standard calibration curves, employing five concentration levels prepared in the range of 0.25 to 5 mg/L and 1.25 to 25 for nitrite and nitrate, respectively. Three replicate injections randomly were performed at each level. 25µl of milk prepared samples or standard solutions were directly injected into the ion chromatography system. The high value of the regression coefficient (R^2>0.998) obtained indicated a good linearity of the analytical response in milk samples.

Precision and accuracy: Precision was determined by three consecutive injections of nitrate and nitrite at five desired concentration levels in intra-day and inter-day. Recovery was determined through the mean obtained for the three independent replicates of a sample spiked at three levels in the range used for the calibration curves.

Limits of detection (LOD) and quantification (LOQ): The LOD and LOQ were estimated by signal-to-noise ratio, 3:1 and 9:1, respectively.

Stability: To investigate the presence of possible degradation products, a stability study of standard solutions in deionized water and spiked samples was conducted. Our results confirmed stability of nitrite and nitrate in spike samples in refrigerator temperature (4°C) after 8 days.

Statistical Analysis

Results were presented as means ± SD. All statistical analyses were performed using the SPSS software, version 17. Assays were performed in triplicate and the mean was used for the statistical analysis.

Results

Our result showed that there is no interference at the retention time of nitrite and nitrate in blank and spiked milk samples. The chromatogram of mixed standard solution (1 mg/L for nitrite and 5 mg/L for nitrate) was shown in Figure 1(A). Our results showed retention time of nitrite and nitrate in the spiked samples were at 9.6 and 12.8 min, respectively. The chromatograms of standard, naturally milk (blank) and spiked sample (1 mg/L for nitrite and 5 mg/L for nitrate) are shown in Figure 1(A), 1(B) and 1(C). The calibration curves were linear over the range of 1.25 to 25 mg/L and 0.25 to 5 for nitrate and nitrite, respectively. Relative standard deviation values (RSD) (<9.5%) of intra-day and inter-day variation also confirmed this method for analyzing nitrite and nitrate in spiked milk samples.

The LOD of nitrite and nitrate using a 25µl loop were 0.09 and 0.24 mg/L, respectively, and the LOQ values were 0.25 and 1.25 mg/L for nitrite and nitrate, respectively. The stability of the column was also evaluated by calculating the retention time of a standard solution of nitrate and nitrite every 10-20 sample injections and the mean of the observed retention time, which was determined to be 12.8 min ± 0.2 SD for nitrate and 9.6 min ± 0.2 SD for nitrite.

Mean recovery values for nitrite and nitrate concentration in the spiked samples at different levels were found to be between 84–93% and 71–97% with RSD % of 2.6-9.5% and 0.2-7.9%, as described as accuracy and precision in Table.1. RSD values for repeatability and reproducibility was varied between 0.3–9.1 and 0.4 –9.8% for nitrite and between 0.1–7.3 and 0.2–8.1% for nitrate, respectively (Table.2).The results of nitrate contents in 102 milk samples are showed in Table 3. The nitrite level was below the LOD in all milk samples and the nitrate concentrations were found in the range of 23.1 to 82.8 mg/L with a mean of 34 ±11mg/L. The lowest and highest level of nitrate was observed in the samples collected from Isfahan and Hamedan, respectively (Table 3).

Exposure to nitrate for each type of food depends on its concentration in food and the amount of food consumed. Nitrate exposure (mg Nitrate/kg body weight/day) is calculated as follows:(daily milk intake (ml)×mean concentration of Nitrate in food (µg/ ml) /(body weight (kg)*1000). The estimated daily intake was calculated for all age groups. There was no data regarding the milk intake in different age groups in Iran, in this study, data on milk daily consumption was extracted from the GEMS/Food regional diets (Middle Eastern consumption of milk and milk products: 116.9 ml/person/day)[30].We used 116.9 ml/person/day as the milk intake by the adults. With regard to "school milk project" in Iran, each student receives 200 ml milk in a day. Therefore, we used at least 200 ml /person/day as the milk intake for the age group 7-19 years old. For children younger than 7 years old, we calculated the nitrate intake according to 4 hypothetical milk consumption figures of 100, 200, 400 and 600 ml. Finally, evaluation of nitrate exposure in milk samples collected from 10 province of Iran shows that nitrate exposure level was lower than WHO ADI value (3.7 mg/kg body weight/day) (Table.4).

Discussion

This is the first survey of nitrite and nitrate level in milk in Iran. In our study the nitrite concentration in all milk samples was below the 0.07 mg/L (LOD). Our results are in agreement with the result of the survey in Italy by Licata et al (2013) and show a low risk of nitrite contamination in milk. In our survey, nitrate was detected in all samples with an average of 34 mg/L and maximum level of 82.5 mg/L. In Taiwan the measured nitrate concentration in 20 milk samples ranged from 0.3 – 42.3 mg/L with an average concentration of 14.3 mg/L[21].

In Romania, an investigation on levels of nitrate in 95 fresh milk samples has been carried out during the years 2007, 2008, 2009 and 2010. The mean level of nitrate was2.66 mg/L ,2.39 mg/L, 3.08 mg/L and2.67 mg/L in 2007, 2008, 2009, 2010, respectively. The level of nitrate also was determined in 40 pasteurized milk samples during the years 2009 and 2010. The average level of nitrate was 2.48 mg/L and2.75 mg/L in 2009 and 2010, respectively [28]. In Turkey, the mean level of nitrate in milk samples from cows, goats and sheep (N= 3) were 22.43, 23.45 and 22.63 mg/kg, respectively. The concentration of nitrite in all milk samples were 0.12 mg/kg [29]. In Italy, Nitrates in 47 goat and ovine milk samples were found to be lower at 146mg/L.

As mentioned above, in all studies, the nitrite and nitrate content in milk samples are less than the regulatory limits on the permissible concentration of nitrate in milk (200mg of nitrate/L in the European Union) [30].The Joint Food and Agricultural Organization/World Health Organization (WHO) in 2006 have set TDI of nitrite and nitrate to be 0.06 and 3.7 mg/kg of body weight [10]. In our study, the estimated daily nitrate intake from milk for all age groups are less than ADI of 3.7 mg/ kg body weight per day suggested by WHO (Table 4). Our results are in

agreement with the report of Yeh et al. 2013 in Taiwan [21]. Since, Ysart et al, 1999 estimated that 2.9% of nitrate intake in the UK was from the consumption of milk, further data on nitrate and nitrite content in other food is needed for an accurate daily intake [32].

Conclusion

This is the first survey on nitrate and nitrite content in milk as well as exposure assessment of the Iranian population to nitrate**.** The results of our study indicate that the nitrate level in all milk samples was less than 200 mg/L by EU regulation in milk and no nitrite level was observed in the samples. Therefore, investigation of nitrate and nitrite contents in different foods would be of great importance for both food safety and nutrition intake recommendation. Although in this study, the mean intake of nitrate was lower than the ADI suggested by WHO/FAO more surveys of nitrate and nitrite contents in different foods such as vegetables, baby food, water, etc. are recommended.

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Figure 1. Chromatograms of (A) 1 ppm of nitrite and 5 ppm of nitrate mix standard in water, (B) Blank samples(naturally contaminant of milk sample with nitrate (C) Spiked of 0.5 mg/L of nitrite and 2.5 mg/L nitrate in milk samples

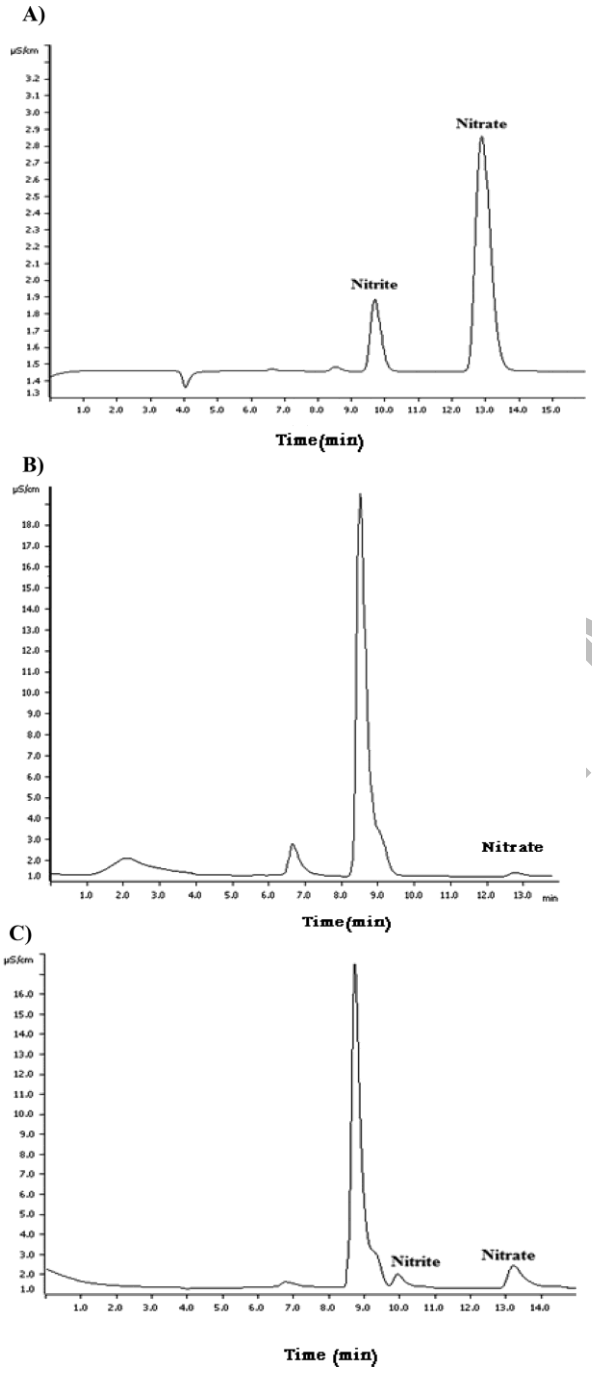

Table.1. Recovery of nitrite and nitrate in Sterilized milk (n=3)

	Spiked level (mg/L)	Mean found (mg/L) ± SD	Recovery %	RSD%
Nitrite	0.25	0.21±0.02	84	9.5
	0.5	0.51±0.03	102.07	5.8
	1	0.87±0.03	87.1	3.44
	2	1.6±0.07	80.36	4.37
	5	4.64±0.12	92.7	2.58
Nitrate	1.25	0.89±0.07	70.8	7.9
	2.5	2.23±0.08	89.31	3.58
	5	4.28±0.07	83.95	1.63

	10	9.13±0.02	91.32	0.21
	25	24.23±0.56	96.89	2.31

Table.2. Inter-day and intra-day precision results for Nitrite and Nitrate

	Spiked level (mg/L)	Intra-day precision %RSD	Inter-day precision %RSD
Nitrite	0.25	9.1	9.8
	0.5	1.82	2.04
	1	1.44	1.49
	2	0.29	0.92
	5	0.27	0.43
Nitrate	1.25	7.3	8.1
	2.5	1.51	0.76
	5	0.89	0.54
	10	0.28	0.18

25	0.14	0.16

Table.3.Levelof nitrate concentration in real milk samples

Province	**No. of sample**	**Positive samples**	**Range (mg/L)**	**Mean (mg/L)**	**Median (mg/L)**
Isfahan	10	100%	23.1-25.4	24.3	24.6
Shiraz	10	100%	23.9-26.1	24.6	24.6
Mashhad	10	100%	24.6-29.1	26.7	26.9
Kerman	10	100%	25.4-29.1	27.6	28.3
Uromia	10	100%	27.6-32.8	29.9	29.9
Tabriz	10	100%	28.4-32.8	30.6	31.3
Zanjan	12	100%	23.1-38.8	32.3	31.7
Lorestan	10	100%	32-39.6	35.1	34.3
Tehran	10	100%	28.4-48.5	40.2	37.7

Hamedan	10	100%	42.5-82.8	63.3	62.7

Table. 4. Estimated mean daily intake of nitrate exposure through milk in Iran

Age (Year)	Mean Weight (Kg)	Average daily milk Consumption (ml)	Mean of Daily intake of nitrate (mg/kg bw/day)[a]									
			Zanjan mg/L	Hamedan mg/L	Lorestan mg/L	Tabriz mg/L	Uremia mg/L	Tehran mg/L	Shiraz mg/L	Mashhad mg/L	Isfehan mg/L	Kerman mg/L
1-3	13.4	100[b]	0.24	0.47	0.26	0.231	0.22	0.29	0.21	0.20	0.18	0.21
1-3	13.4	200[C]	0.49	0.94	0.52	0.463	0.45	0.5	0.4	0.40	0.36	0.42
1-3	13.4	400	0.95	1.88	1.04	0.93	0.8	1.07	0.84	0.81	0.72	0.84
1-3	13.4	600	1.43	2.82	1.57	1.39	1.35	1.62	1.251	1.21	1.07	1.25

4-6	20.8	100[b]	0.15	0.30	0.17	0.15	0.14	0.17	0.14	0.13	0.12	0.14
4-6	20.8	200[C]	0.30	0.61	0.34	0.30	0.29	0.35	0.27	0.26	0.23	0.27
4-6	20.8	400	0.61	1.21	0.67	0.60	0.57	0.69	0.53	0.52	0.46	0.54
4-6	20.8	600	0.92	1.82	1.01	0.90	0.86	1.04	0.81	0.78	0.69	0.81
7-9	28.1	200	0.23	0.448	0.25	0.22	0.21	0.26	0.20	0.19	0.17	0.20
10-12	37.5	200	0.17	0.336	0.19	0.17	0.16	0.19	0.149	0.14	0.13	0.15
13-15	50.0	200	0.13	0.25	0.14	0.12	0.12	0.14	0.11	0.11	0.10	0.11
16-	58.6	200	0.11	0.22	0.12	0.1	0.10	0.12	0.0	0.09	0.08	0.10

19						0			9			
Adult	60.0	116.9	0.06	0.12	0.07	0.06	0.06	0.07	0.06	0.05	0.05	0.06

[a]: Mean of all samples, **Mean of Nitrate exposure (mg/kg body weight/day) =** (Average daily milk intake (mg)* Mean of Nitrate concentration in milk samples)/ (Weight (Kg)*1000)