

Estimation of the Nitrite Concentration in the Raw Milk and Locally Produced Soft Cheese in Different Area of Baghdad, Iraq

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Abstract

Samples for both raw milk and locally produced soft cheese totally (150), 75 for each were collected from different regions of Baghdad from October 2020 to July 2021 to estimate nitrite concentration. Ion exchange chromatography was used to detect the nitrite levels in the samples. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were 0.5 mg/L and 1.7 mg/L respectively. The levels of nitrite in the samples of raw milk were (0.5-1.5 mg/L) with average (0.9 ± 0.4 mg/L), while the concentrations were (0.8-2.3 g/kg) in the soft cheese samples with average (1.4 ± 0.4 g/kg). The nitrite level in the soft cheese was higher than in the raw milk according to the months significantly ($P < 0.05$). The presence of nitrite in milk and cheese indicates a poorness of hygiene practices during milking and post-milking operations, as well as during cheese production.

Keywords: Raw milk, Cheese, Nitrite, Ion Chromatography

تقدير مستويات النتريت في الحليب الخام والجبن الطري المنتج محليا في مناطق مختلفة من بغداد, العراق
الخلاصة

جمعت 150 عينة من الحليب الخام والجبن الطري المحلي (75 كل منهما) من مناطق مختلفة من بغداد امتدت من الشهر العاشر 2020 الى شهر السابع 2021 لتقدير مستوى النتريت. استخدام كروماتوغرافيا التبادل الأيوني للكشف عن مستويات النتريت في العينات. كان حد الكشف وحد القياس الكمي (0.5 و 1.7) ملغم / لتر على التوالي. كانت مستويات النتريت في الحليب الخام ما بين (0.5-1.5) ملغم/ لتر بمعدل (0.9 ± 0.4) ملغم/ لتر. بينما كانت مستويات النتريت في الجبن الطري المحلي (0.8-2.3) غم/ كغم بمعدل (1.4 ± 0.4) غم/ كغم. مستوى النتريت في الجبن الطري المحلي كان اعلى من الحليب الخام وبشكل معنوي وعلى مستوى ($P < 0.05$). يعد وجود النتريت في الحليب والجبن دليل على سوء النظافة والصرف الصحي أثناء عمليات الحلب وما بعد الحلب ، وكذلك أثناء عملية إنتاج الجبن.

Introduction

A number of studies worldwide investigated the presence of nitrate and nitrite residues in the foods (1). Nitrite is considered a chemical preservative agent permitted in versatile

applications such as fertilizers and food preservatives in many countries to stop the growth of bacteria that produce gas such as *coliforms* that contaminate food (2). The acceptable daily intake of nitrite is 0.07 mg/kg body weight (3). In spite of the fact that the amount of nitrite in the ground water or water

surface is usually insignificant in comparison to nitrate (4). However, nitrate can be transformed to nitrite in anaerobic conditions due to microbial contamination (5). Some plants, such as *Rhizobium*, have the nitrogenase enzyme that combines nitrogen and hydrogen to create ammonia (6). In addition, bacteria convert ammonia into nitrite ions (7). In ruminants, microorganisms in the digestive system contents convert dietary nitrate to nitrite, which is then converted to ammonia for use in microbial protein synthesis (8). The initial content of nitrate, as well as the microbial flora and the animal's diet, can all interfere with the conversion of the nitrate to the nitrite, resulting in nitrite accumulation (8). Nitrite causes Methemoglobinemia (Anoxia) particularly in the children after oxidize the ferrous ion in hemoglobin (9). Bacteria can reduce nitrate to nitrite at the acidity pH in the stomach of adults, nitrite can react with the acid, amines and amides in the stomach to produce nitrosamine (10). Nitrosamine is probably carcinogenic to human (11). Contamination of the feed of ruminants by nitrates and nitrites cannot reach their milk because the ruminal microorganisms are degraded by the nitrates and nitrites and both are used for the formation of nitric substances (12). The contamination of milk and dairy products by these substances can occur through water used in the technological process in both harvesting and fabrication, atmospheric air polluted with nitric oxide, and industrial centers (12). The nitrite ion can be found in both water and soil and is released into the atmospheric and aquatic environment through human-made materials such as fertilizers (13). Many methods are used for the detection of nitrite, such as liquid chromatography, molecular absorption spectrometric technique, and the ion exchange chromatography method (14). This study was conducted to estimate the concentrations of nitrite in the raw milk and locally produced soft cheese by ion exchange chromatography in different regions of Baghdad

due to can accurately detect low concentration ions.

Materials and methods

Collection of samples

A total of 150 samples (75 for each of raw milk and locally produced soft cheese) were collected from different regions of Baghdad, three regions of Resafa (Al-Saadar city, Fadhiliya and Al-Sadria) and three regions of Karch (Abu Ghraib, Radwaniyah and Al-Shiela), from local stores, markets, and homemade. The collection and transportation of samples to the laboratory is done by sterilized bottles and poly-ethylene bags in the ice box for chemical analysis. All the solutions were produced with sterile deionized water (DW) and analytical grade reagents. The nitrite standard solution was prepared using NaNO_2 (purity 99.9%) powder purchased from Sigma-Aldrich (USA). Precautions were taken to avoid contamination of the samples (15).

Preparation of samples

The samples of cheese were prepared by weighing, dissolving in warm water, fat and protein precipitation, and finishing with filtration. Four grams of soft cheese sample were weighted into a 200 ml volumetric flask containing about 150 ml of sterile distilled water and 40 ml of raw milk into the flask containing 110 ml sterile distilled water. The samples were kept in the equilibrium water bath at 50 °C for 10 minutes, and then 10 ml of ZnSO_4 solution was added to precipitate the milk fat and protein. Then, 10 ml of NaOH solution was added to each flask to neutralize the pH of the sample, and the material was thoroughly mixed. The samples were cooled at room temperature, poured for subsequent filtration by Whatman number 43 filter paper and an appropriate volume of filtrate was obtained in a 125ml flask. The samples were centrifuged for 10 minutes at 3000 rpm, then filtered again using filter paper (Whatman number 42), pH papers were used to examine the samples to ensure that

the pH was between 7 and 8 (16).

Preparation of standard nitrite solution

1000 mg/L nitrite stock standard solution was prepared in the sterile deionized water by dissolved (1 g) of standard sodium nitrite NaNO_2 (purity 99.9%) powder purchased from Sigma-Aldrich (Germany) in a (1000 mL) flask with sterile deionized (DI) water with concentration in the laboratories of Ministry of Science and Technology.

Apparatus and chemical

Detection the nitrite level was conducted by 881 ion chromatography (Metrohm, Switzerland) with suppressed conductivity detector and Metrohm Suppressor Module (MSM; 50 mM H_2SO_4) was used. Mobile phase solutions were composed of (8 mm/L sodium carbonate Na_2CO_3 and 1 mm/L sodium bicarbonate NaHCO_3). 8.48g of Na_2CO_3 and 0.84g of NaHCO_3 were dissolved and diluted volumetrically in the 100 mL, the flow rate 0.500 ml/ min (16). The injection volume was 20 μL of sample and standard nitrite solution at recording time of 20 minutes, the reaction occur at (24 °C) and pressure 10.47 MPa, the separation was carried out at the same temperature, and sample is injected into a stream of carbonate-bicarbonate eluent (mobile phase) and passed through a series of ion exchangers. Metrosep A SUPP -250 (250 \times 4 mm, 9 μm particle size) with an anion-exchange polymer of polyvinyl alcohol with quaternary ammonium groups used as the carrier material. The column that is used is the Metrohm Manual (8.107.8040EN/2020-1-5) for Metrosep A Supp 5. Separated anions are transformed to highly conductive acid forms in the suppressor, whereas the carbonate-bicarbonate eluent is converted to weakly conductive carbonic acid. Conductivity is used to measure the separated anions in their acid forms. The separation occurs on the basis of retention time in comparison to standards. Quantitation is by measurement of peak area or peak height (15).

Linearity and recovery

Linearity, limit of detection and limit of quantification can evaluate by ion exchange chromatography (17). To determine linearity, seven different aqueous standard solutions were analyzed with concentrations ranging from 0.1-10 mg/L. These seven concentrations (0.1, 0.5, 1, 3, 5, 8, and 10 mg/L) were used to measure the calibration curve for nitrite. At each level of concentration, three replicate injections ($n = 3$) of each sample (raw milk, soft cheese) were performed. Calibration graphs for nitrite (0.1, 0.5, 1, 3, 5, 8, and 10 mg/L) were prepared by completing these concentration to 100 mL volumetric flask with deionized water. 20 μL was injected of each sample in the ion chromatography. The percent relative standard deviation (RSD %) used to calculate the precision. The examination of three replicates of each calibration standard in both raw milk and cheese samples were undertaken on the same day for repeatability evaluation (18). The recovery evaluation was used to determine the trueness. The percentage of recovery was then estimated as average value of three independent replicates. All these solutions are prepared and stored in dark polyethylene bottles at room temperature and protected from light. The LOD and LOQ estimated by the following equations:

$$Y = ax + b$$

$a = \text{slop}$ $b = \text{intercept}$ $N = \text{number of test}$

$\text{SD of intercept} = \text{Standard error of intercept} \times \sqrt{N}$

$\text{LOD} = 3.3 \times (\text{Standard deviation of intercept/ slope})$

$\text{LOQ} = 10 \times (\text{Standard deviation of intercept / slope})$ (18)

Statistical analysis

The data was statistically analyzed

using SAS (Statistical Analysis System) (version 9.1). For more than two groups, a two-way ANOVA and a post-hoc Least Significant Differences (LSD) test were utilized, whereas for two groups, an independent *t* test was used (19).

Results and Discussion

The chromatograms of the standard nitrite solution, raw milk, and cheese samples were shown in figures 1, 2, and 3 respectively. The blank chromatogram with the sterile DW showed no nitrite peak. The retention time for standard nitrite was 13.895 minutes with a height of 223.123 and the concentration of nitrite in the raw milk and soft cheese samples was determined by comparing it with the standard curve. Calibration is the relationship between analyte concentrations and instrument response, in order to estimate the analyte concentration in unknown samples (18). That was accomplished by measuring the instrument response for a set of known-concentration samples (referred to as standards).

The calibration curve was produced by charting the area vs. concentrations. The calibration curve was linear over the range of 0.1 to 10 mg/L for nitrite (Figure 4). Recovery tests on three replicates of raw milk and soft cheese were used to check the method's linearity. The correlation coefficient of seven standard concentrations was ($R^2 > 0.9984$), indicating excellent linearity of analytical response in milk and cheese samples with a range of 0.1-10 mg/L. The mean relative standard deviation (RSD) was less than 5%, and the RSD% (5%) was deemed acceptable and used to confirm the nitrite analysis in the milk and cheese samples. (20). Limit of detection (LOD) is the minimal concentration of analyte that can be detected in the test sample with repeatability precision. Limit of quantification (LOQ) is the lowest concentration of analyte that can be quantified in the test sample with accepted recovery (21). LOD and LOQ of nitrite using 20 μ L loop for nitrite were 0.5 and 1.7

mg/L respectively.

The stability of the column was assessed by calculating the retention time of a standard solution of nitrite and the mean of the observed retention times, which was determined to be $13.895 \pm \text{SD}$ min. The mean values of nitrite recovery for different levels of nitrite concentration in the samples were reported to be between 60 and 200% with average (83%), and an RSD% between (0.1-1.8 %). The recovery investigation was carried out using samples of raw milk and soft cheese fortified with seven different concentrations of standard nitrite, according to the Association of Official Agricultural Chemists (22).

Table 1: Recovery of nitrite (n=3 for each sample)

| N O | Standar d of nitrite (mg/L) | Mean (mg/L) \pm S D | Recover y % | RSD % |
|--------|--------------------------------------|-----------------------------|----------------|----------|
| 1 | 0.1 | 0.2 \pm 0.1 | 200 | 0.1 |
| 2 | 0.5 | 0.3 \pm 0.2 | 60 | 0.2 |
| 3 | 1 | 0.6 \pm 0.2 | 60 | 0.2 |
| 4 | 3 | 1.9 \pm 0.6 | 63.3 | 0.7 |
| 5 | 5 | 3.2 \pm 0.3 | 64 | 0.4 |
| 6 | 8 | 5.3 \pm 1.1 | 66.2 | 1.3 |
| 7 | 10 | 6.8 \pm 1.5 | 68 | 1.8 |

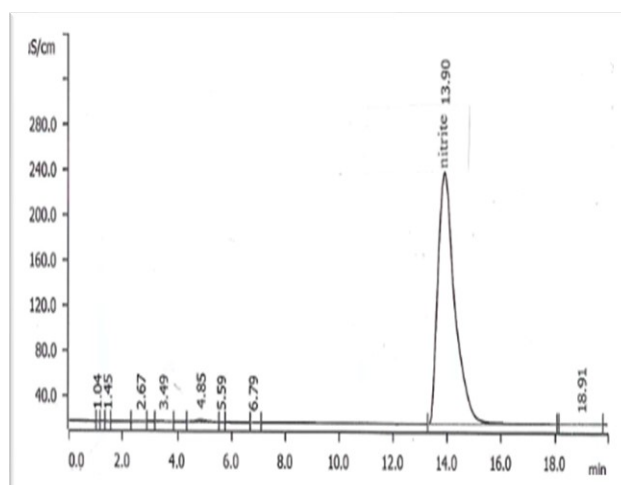


Figure 1: chromatograms of 0.1mg/L of standard nitrite solution

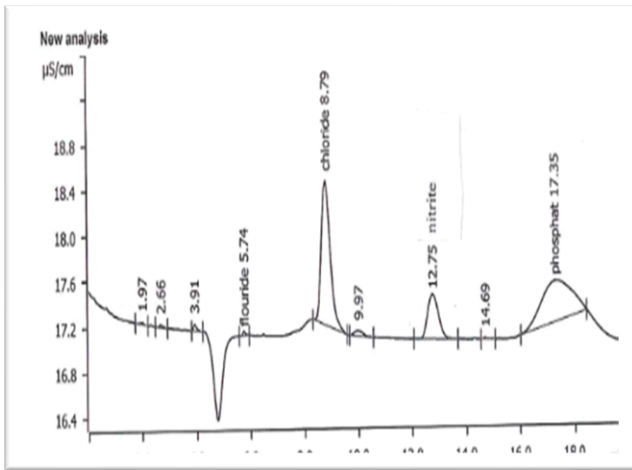


Figure 2: Chromatogram of spiked 0.1 mg/L of nitrite in raw milk sample

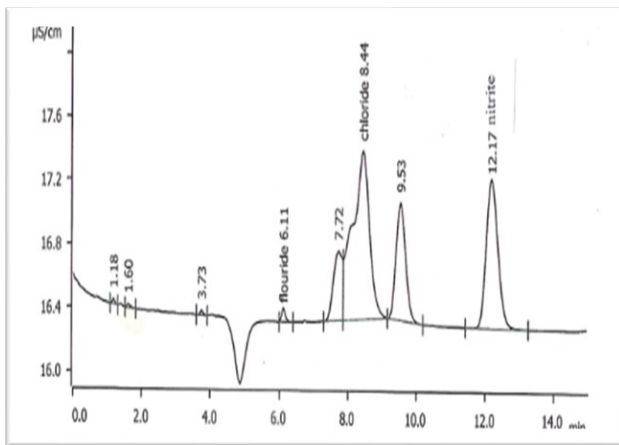


Figure 3: Chromatogram of spiked 0.1 g/kg of nitrite in soft cheese sample

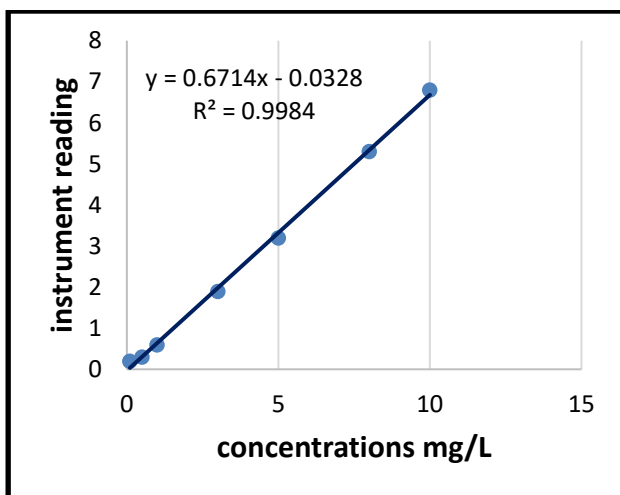


Figure 4: Calibration curve for seven standard nitrite solutions

Table (2) showed the levels of nitrite in raw milk and soft cheese samples according to the months in different regions of Baghdad. The low levels of nitrite in the raw milk samples were observed in October, December and January with a range of (0.5 ± 0.2) mg/L, (0.7 ± 0.4) mg/L and (0.6 ± 0.2) mg/L respectively. While the highest level of nitrite in raw milk was reported in July with a range of (1.5 ± 0.7) mg/L. The levels of nitrite in all raw milk samples were between $(0.5-1.5)$ mg/L, with average (0.9 ± 0.4) mg/L, whereas the limit of detection (0.5) . The high level of nitrite in the soft cheese sample was in July (2.3 ± 0.6) mg/kg with average (1.4 ± 0.4) g/kg. The levels of nitrite in raw milk and soft cheese samples differed significantly ($P < 0.05$) according to the months.

Table 2: Nitrite levels in raw milk and soft cheese samples according to the months.

| Samples Months | Year | NO of Sample | Raw milk mg/L (Mean ± SE) | NO of Sample | Soft cheese mg/kg (Mean ± SE) |
|----------------|------|--------------|---------------------------|--------------|-------------------------------|
| October | 2020 | 8 | 0.5 ± 0.2 g | 8 | 1.7 ± 0.3 b |
| November | | 8 | 1.0 ± 0.4 bc | 7 | 1.5 ± 0.3 ac |
| December | | 7 | 0.7 ± 0.4 f | 7 | 0.9 ± 0.4 e |
| January | 2021 | 8 | 0.6 ± 0.2 fg | 8 | 0.9 ± 0.4 e |
| February | | 7 | 0.9 ± 0.4 e | 8 | 0.8 ± 0.4 e |
| March | | 7 | 1.3 ± 0.5 c | 7 | 1.5 ± 0.7 ac |
| April | | 7 | 1.1 ± 0.4 d | 7 | 2.2 ± 0.5 a |
| May | | 7 | 1.0 ± 0.6 de | 7 | 1.1 ± 0.4 d |
| June | | 8 | 1.3 ± 0.6 b | 8 | 1.1 ± 0.7 d |
| July | | 8 | 1.5 ± 0.7 a | 8 | 2.3 ± 0.6 a |
| Average | | | 0.9 ± 0.4 e | | 1.4 ± 0.4 c |
| LSD | | | | 0.20 | |

Means in the same column with different small letters differ significantly ($P < 0.05$)

Means in the same raw with different capital letters differ significantly ($P < 0.05$)

All raw milk and soft cheese samples were contaminated with nitrite according to the months; this may be related to both external and internal sources, including agricultural drinking water, fertilizer contamination, and forage (2). Furthermore, the concentration of nitrites in the milk and milk products varies widely depending on the degree of contamination, seasonal conditions, whether pasture or grazing was utilized, primary treatment and processing procedures, analytical methods that were used and milk processing technologies (23). The range value of nitrite concentrations of all soft cheese and raw milk samples were between (0.8 to 2.3 g/kg) and (0.5 to 1.5) respectively and according to the (24), the accepted nitrite maximum limits in the cheese were ranged from 0.8 to 2.2 mg/kg, while in the fluid milk were 0.1-1.1mg/L, therefore the results in the current study were within the acceptable limits. The high levels of nitrite in the soft cheese compared to raw milk could be attributed to contamination by nitrate and nitrite in the absence of a good production environment under improper hygienic conditions and through the technological process (25). In addition, cheese contamination can contribute to the nitrate to nitrite conversion during the process of ripening or due to the action of a xanthine oxidase enzyme that is found in the milk (26). Thermophilic (denitrifying bacteria) have varying microbial activities based on the operating conditions of individual processing stages in cheese manufacturing (pH value, temperature, dry substance content), and they can convert nitrate to nitrite, resulting in an increase in nitrite levels in cheese samples (27). An increase in the levels of nitrite during hot months in the raw milk and soft cheese may occur due to microbial action in the summer because of high ambient temperature and less refrigeration conditions that encourage the growth of bacteria (28). *Staphylococci* and *E. coli* bacteria can enhance the conversion of nitrate to nitrite (29). The nitrite levels of raw milk samples varied significantly between cold and hot months, these

finding were in agreement with (30) who found that a significant monthly variation in the nitrite level of raw milk samples as well as the nitrite content was highest in the spring/summer season and lowest in the autumn/winter season. The high levels of pathogenic bacteria and metals identified in the raw milk indicate poor sanitary conditions during milking and post-milking activities, as well as high levels of chemical substance contamination in the environment, which will impair the milk and milk products by these substances (31).

Conclusion

The results in the current study indicated that most of the examined samples of raw milk and locally produced soft cheese load with nitrites ranged from 0.5-1.5 mg/L in the raw milk and 0.8-2.3 mg/kg in the soft cheese, and these concentrations were within the allowed concentrations mentioned by the health organization. Studying the efficacy of another technological method for reducing and/or removing nitrite from milk and local dairy products such as Nanomaterials (Bacteriocin) that produced by lactic acid bacteria and antioxidant agent as ascorbate, ascorbic acid as reducing agent.

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