Reliable HPLC determination of nitrate and nitrite in Egyptian dairy products

Magda Abd El Aziz[1] and Ahmed Salem Sebaei[2*]

[1]Dairy Chem. Department, Animal Production Research Institute, Agriculture Research Centre, Giza, Egypt.
[2]Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Food, Agricultural Research Center, Ministry of Agriculture, Giza, 12311, Egypt.
abdelazizmagda61@yahoo.com, Ahsebaei@gmail.com,

Abstract: Presence of excessive nitrate and nitrite in foodstuff has toxic and carcinogenic effects on human. The present study was to develop HPLC method to determine the two salts in different dairy products in the local Egyptian markets. The optimal conditions of HPLC method were applied by using methanol: water (1:4), N-O ctayl amine at pH 3-4 for the mobile phase at flow rate 0.8 ml/min. Recoveries of nitrate and nitrite were between95-104% and 96-105%, respectively, indicating that the method is quite accurate. So, this method was applied for determination the salts levels in 44 collected samples including UHT milk, fermented milk and cheese from the Egyptian market. The results revealed that the nitrate contents in the samples varied in the range 0.00 - 6.30 mg/kg. High levels of nitrate were observed in the fortified dairy products with fruits or vegetables and processed cheese. However, the nitrite was not detected in all samples. The proposed method is a reliable and applicable method for the determination of nitrate and nitrite levels in dairy products. The mean values for nitrate in investigated samples were lower than ADI levels of WHO. This means that the dairy products which were investigated are safe for consumption.

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1. Introduction

Nitrates and nitrites widely found in human and animal foodstuffs. The National Academy of Sciences indicated that vegetables can normally provide 87% of nitrate in a diet, while the other products including fresh, cured meat and dairy products provide the rest **(Kyriakidis *et al*., 2007).** The content of nitrate and nitrite in diet is not only from natural source but also from food additive and contamination.

Nitrate level in milk related to milking animals which are subjected to nitrate pollution via drinking water and feeding stuffs **(Kyriakidis *et al*, 1997).** Moreover, the 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 (**NFRDI, 2001).** Sodium or potassium nitrate and nitrite are used as food additive in cured meat products and certain types of cheese to prevent growth and toxin production of *Clostridium botulinum* **(Davidson *et al.*, 2002).** Recently, (**EFSA, 2017)** confirmed that nitrites and nitrates as food additives are safety for consumer within safe levels (ADI). The current Acceptable Daily Intake (ADI) for nitrate ion is 0-3.7 mg/kg of body weight per day and establishing an ADI for nitrite ion is 0-0.07 mg/kg of body weight per day, however, it was noted that these ADIs do not apply to infants below the age of 3 months **(WHO, 2003)**.

Excessive of nitrate and nitrite in food have potential health risk for human, the risk of nitrate attributed mainly to its reduction to nitrite during processing and storage as well as in saliva and the gastrointestinal tract by bacterial reduction. Nitrite has a harmful impact on human health due to its reaction with secondary amines to form carcinogenic N-nitrosoamines **(Krul *et al.*, 2004; Winter *et al*., 2007; FCEC, 2016).** Previously, it was found the positive correlation between the level of nitrate added to cheese milk and the levels of N-nitroso-dimethylamine in the cheese (**Gloria *et al.*, 1997**). International Agency for Research on Cancer **(IARC, 2010)** concluded in their study: ingested nitrate or nitrite under conditions that result in endogenous nitrosation is probably carcinogenic to human**.** Furthermore, nitrite ion can combine with hemoglobin to form a methaemoglobin complex and depriving living tissues from oxygen**.** Infants consuming too much nitrite are more susceptible to methaemoglobinaemia (Blue Baby Syndrome) **(WHO, 2011).**

On the other hand, several researches demonstrated the potential health benefit from nitrate and nitrite. The study of **Bryan *et al.*, (2012)** reported that nitrate and nitrite were not associated with cancer. Nitrate in food reduce into nitrite in mouth and then in stomach, nitrite convert into nitrogen oxide (NO) which has therapeutic effects for human health **(Bryan**

and Loscalzo, 2011). Adequate amount intake of nitrate in food helps control blood pressure and reduces high blood pressure (**Sobko *et al.,* 2010; Gilchrist *et al.,* 2011).**

Generally, traditional spectrophotometric methods are used to determine nitrate and nitrite in food. Three alternative spectrophotometric methods for the analysis of nitrate and nitrites in milk and milk products were described in **ISO methods (2004 a, b, c)**. Unfortunately, the spectrophotometric methods cannot detect nitrate and nitrite simultaneously**;** the determination of nitrate requires reduction of nitrate to nitrite using toxic cadmium metal followed by derivatization with Greiss reagents **(Sjöberg and Alanko, 1994)**. A high sensitivity for the detection of trace levels of the analytes is lacked through the tedious steps. For these reasons a variety of analytical methods were developed and applied to determine nitrate and nitrite in water, plants, food, and dairy products, including developed spectrophotometric methods (**Lima *et al.,* 2006**; **Fakhre and Qader, 2013),** polarographic method **(Ximenes *et al.,* 2000)** and capillary electrophoresis (CE) **(Merusi *et al.,* 2010)**. Moreover, a number of IC (**Gapper *et al.,* 2004; McMullen *et al.*, 2005**) and HPLC **(Chou *et al.,* 2003**; **Croitoru, 2012)** methods were developed, which are generally characterized by faster, more accurate and higher sensitivity than the spectrophotometric methods**.**

The investigations of nitrate and nitrite contents in different foods are very importance for both risk analysis and nutrition intake recommendation. In Egypt, there were limitation in evaluation of nitrate and nitrite contents in milk and milk products. Therefore, the objective of this study was to develop the most optimal conditions for the HPLC method, which can be applied to determine nitrate and nitrite levels in some dairy products which the most commonly consumed in Egypt to ensure their safety.

2. Materials and Methods

Materials

Sampling

The present study investigates 44 samples of different Egyptian dairy products including: UHT Milk (n=12) which were different in types between plain milk and fortified with fruit and chocolate, fermented milk (n=12) which were also different in types between plain fermented milk and fortified with fruit, cheese (n=20) categories as soft cheese (as Domiatte and Feta cheese) and hard cheese (as Ras cheese) and processed cheesewere collected from different supermarkets in Cairo and Giza governorates in Egypt during eight months from April2017 to November 2017.

Chemicals

All chemicals and reagents were analytical and HPLC grade. De-ionized Water generated by Milli-Q A10 gradient. Methanol was purchased from Lab-scan with assay 99% and N-Octyl amine98%from MERCK-Schuchardt. Phosphoricacid85%was obtained from PROLABO. Potassium nitrate and sodium nitrite from ACROS Organic products with assay 99.9% wereused in preparation ofthe standard solutions.

Apparatus

Centrifuge from Thermo scientific (USA), model Heraeus Megafuge40 and for extraction the Geno-grinder, SPEX® sample preparation device from UK. Liquid chromatography equipment: High performance liquid chromatography HP 1100 series equipped with Quaternary pump (G1311A), vacuum degasser (G1322), auto-sampler (G1313) and switching valve (G1316-60000) to switch between nitrate-nitrite analysis and another routine application, variable wavelength detector (G1314A) and the analytical column is Eclipse plus C8 150× 4.6 mm × 5µm.

Materials

Preparation of standard solutions:

Stock solutions of nitrate ion (1000 µg/ml) and nitrite ion (1000µg/ml) were prepared by dissolving 0.1647 g of potassium nitrate in 100 ml de-ionized water and0.1513 g of sodium nitrite in 100 ml de-ionized water respectively. For working solution **(**100µg/ml)10 ml of the stock standard solution was diluted in 100 ml de-ionized water. The Calibration solutions, ranging from0.05, 0.1, 0.25, 0.5 and 1.0µg/mlwere prepared by suitable diluting in de-ionized and subjected to HPLC analysis.

Sample preparation

Ten grams of the homogenized dairy sample was weighed into 50 ml plastic tube and 20 ml of methanol: water 1:1 was added. Shake gently and occasionally for five minute at 700 rpm using Geno/Grinder device. Centrifuge the treated sample at 4000 rpm for 5 min., filter the sample using 0.45 µm membrane filter. Inject the finally filtered solution directly into the LC-System. For spiking tests use samples that previously analyzed and proved to be nitrate-free. If clean sample is not available, use contaminated one, at level lower than the LOQ level (0.1mg/kg), Fortify the control samples by adding appropriate volume of the standard solution on the sample (e.g. 0.05 ml of 100 µg/ml standard solution on 10 g sample to obtain 0.5 mg/kg spiking level).

HPLC conditions:

HPLC pump flow rate is 0.8 ml/min. Mobile phase composition is isocratic mixture and each liter contains 800 ml water, 200 ml Methanol, 1.63 ml N-Octyl amine and the pH of the solution was adjusted to be in the range 3-4 using 10%phosphoric acid solution. The separation column is Eclipse plus C8

150× 4.6 mm × 5µm. The injection volume is 10 µl. The Software is Chemstation for LC 3D Rev. B.04.03 [16]. Detector parameter is UV wavelength at 220nm. The concentration of nitrite and nitrate were calculated according to the standard curve.

3. Results and Discussions

HPLC Assay

The present study modified HPLC method which derived from the procedures of **Cheng, and Tsang, (1998)** to determine the nitrate and nitrite in milk and dairy products. The most optimal conditions for the determination were obtained by using methanol and N-Octyl amine with adjusted pH value to 3-4 as the mobile phase and a flow rate of 0.8 ml/min in the modified HPLC method. For one sample analysis, the total analytical time of the method was within 15 min. (**Fig. 1)** represented the retention times of nitrite and nitrate which were 7.6 ± 0.15 min and 10.0 ± 0.10 min, respectively.

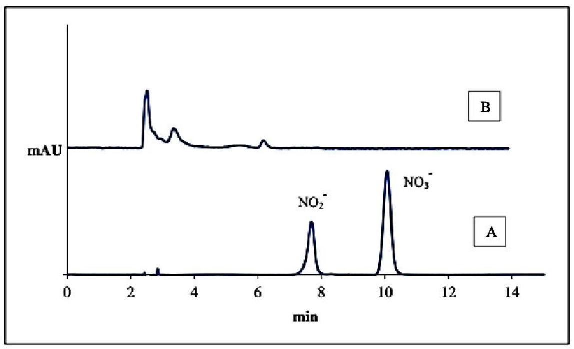

Figure 1: Nitrite and Nitrate HPLC chromatograms for 1.0 mg/kg standard (A) and cheese blank sample (B).

Standard linearity and performance parameters

Nitrite and nitrate were determined based on comparison with a standard curve. The calibration curve was constructed by plotting the area versus the concentration. The calibration curves cover the dynamic range from 0.05 mg/L to 1.0mg/L for nitrite and nitrate in the final solution respectively **(Fig.2)**. Nitrate (NO_3^-) and nitrite (NO_2^-) levels in samples were reported as mg/kg.

The correlation coefficients for the two compounds were both greater than 0.9999, which showed a very good linearity within the range of 0.05-1.00mg/kg **(Table 1)**. To verify the HPLC peaks resolution, column efficiency, and repeatability of a chromatographic system to ensure its adequacy for a routine analysis. These performance characteristics were compared with the predefined acceptance criteria of the guideline provided by FDA's Center for Drug Evaluation and Research **(CDER, 1994)**. The resolution factor and the number of theoretical plates results in **Table (1)** shows that the peaks well-resolved from other peaks and the void the undesirable peak matrices and the peak shape is ideally symmetric as indicated from the tailing factor.

Method Validation

The parameters of method validation were performed according to **EURACHEM (1998, 2002)** guidelines and FDA recommendations **(FDA, 2012)**.

LOQ and LOD:

Limit of quantification (LOQ) is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision. The accuracy analyte peak (response) was identifiable, discrete, and reproducible with a precision 1% for the two peaks. Limit of detection (LOD) is the minimum concentration of analyte that can be detected with acceptable certainty, though not quantifiable with acceptable precision and statistically determined as a triple of the standard deviation of sample blanks spiked at lowest acceptable concentration measured **(Table 2)**. The LOQ practically detected at 0.25 mg/kg for nitrate and nitrite. The method provides a sensitive detection limit 0.03mg/kg for nitrite and 0.02 mg/kg for nitrate which are better than that reported by **Rezaei *et al.* (2014)** and **Chou *et al.*, (2003)** that using the same detection technique of HPLC and comparable with **Dinçkaya *et al.* (2010)** that using amperometric biosensor for detection.

Table 1: Performance parameters for nitrite and nitrate peaks separated by HPLC.

Performance parameter	Nitrite	Nitrate	CDER guideline
Repeatability of peak response (n=5)	0.3%	0.5%	≤1%
Column efficiency (plate count)	6268	8262	>2000
Resolution factor	3.91	5.73	>2.00
Linearity correlation factor	0.99993	0.99991	>0.999
Tailing factor	1.06	1.00	≤2.00

Method Linearity and Test Recovery.

Figure 3 provides the method linearity of nitrite and nitrate. Method linearity was checked by making recovery tests at three different levels of 0.25, 1.0 and 10.0 mg/kg in traditional Domiatte cheese sample. Linearity's were obtained from the tested concentrations range of 0.25-10 mg/ kg for each nitrate and nitrite with a strong correlation coefficient

0.99998 and 0.99992 respectively. The check for method linearity performed with test recoveries for six replicates at the three different levels in cheese samples. As shown in **Table 2**, the recoveries of nitrate and nitrite spiked with three concentrations (0.25, 1.00 and 10.00 mg/kg) into cheese samples were in the range of 95-104% and 96 -105%, respectively, indicating that the method is quite accurate.

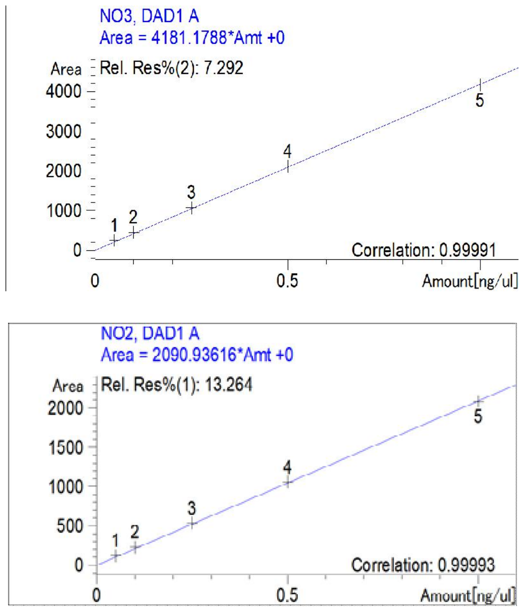

Figure 2: HPLC linear calibration curves for nitrite and nitrate.

Precision and Trueness.

Precision is degree of closeness of replicate measurements under specified conditions. The precision is described by statistical methods such as a standard deviation or confidence limit and less precision is reflected by a larger standard deviation and was classified as repeatability and reproducibility whichwas less than 15% in agreement with FDA guideline **(2012).** Reproducibility of the proposed method was evaluated by the spread (RSD %) of 10 replicates milk and cheese samples at spiking level 5.0 mg/kg for nitrite and nitrate salts over of 10 days (**Table 2**). All repeated analysis obtained RSD values less than 15%, pointing out reasonable high degree of reproducibility. Repeatability of nitrite and nitrate analysis was evaluated by spiking 0.250 mg/kg for nitrite and nitrate into cheese samples with 5 repetitions. The result was shown in **Table (2)**. The RSD's for nitrite and nitrate were all less than 10%.

Trueness is the degree of closeness of the mean value from a series of measurements with the true value or accepted reference value related to systematic error (bias). The method trueness was normally tested by certified reference materialor proficiency tests. Because of unavailability of CRMs or PT samples for nitrate and nitrite salts in dairy products the trueness was calculated by spiked samples at different levels on cheese samples and bias expressed as absolute relative difference percent (RD %) must not exceed 10% **(Table 2)**.

Table (2): Summary of validation parameters results for nitrate and nitrite in dairy products.

Product	Salt	Spiking level (mg/kg)	Recovery (%) ± SD	Bias RD % *	LOD (mg/kg)	LOQ (mg/kg)	Repeatability (RSD %)	Reproducibility (RSD %)
Milk	NO_3	0.25	104 ± 0.7	4.0	0.02	0.25	3	11
		1.00	101 ± 10	1.0				
		10.00	95 ± 7.7	5.0				
	NO_2	0.25	98 ± 1.3	2.0	0.03	0.25	5	12
		1.00	105 ± 4.8	5.0				
		10.00	96 ± 4.2	4.0				
Cheese	NO_3	0.25	95 ± 7.5	5.0	0.02	0.25	4	12
		1.00	95 ± 6.6	5.0				
		10.00	99 ± 5.7	1.0				
	NO_2	0.25	95 ± 6.1	5.0	0.03	0.25	8	14
		1.00	96 ± 4.9	4.0				
		10.00	98 ± 4.1	2.0				
Fermented Milk	NO_3	0.25	95 ± 5.5	5.0	0.03	0.25	4	11
		1.00	96 ± 5.1	4.0				
		10.00	96 ± 4.7	4.0				
	NO_2	0.25	95 ± 6.8	5.0	0.03	0.25	7	13
		1.00	94 ± 5.7	6.0				
		10.00	94 ± 5.3	6.0				

* Relative difference.

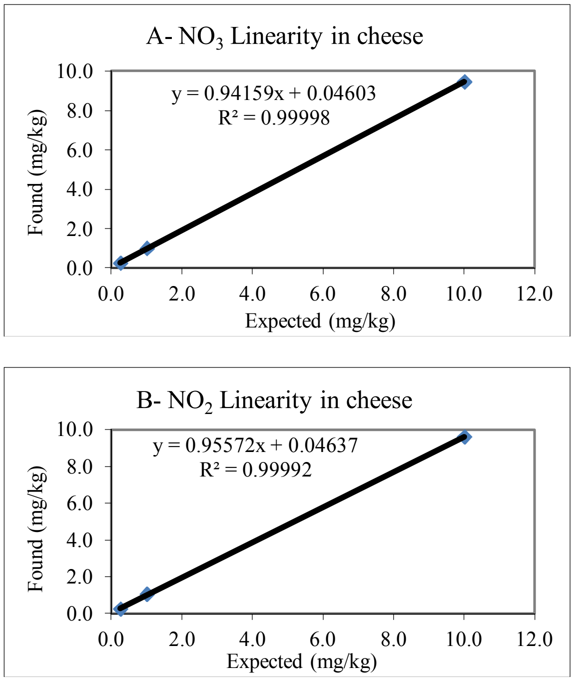

Figure 3: Method of linearity of nitrate and nitrite

Occurrence of nitrate and nitrite in UHT milk, fermented milk and cheese

Nitrate and nitrite contents in 44 dairy samples including UHT milk, fermented milk and cheese samples were inspected for analysis. All samples are free from nitrite salt and the nitrate contents were reported in **Table 3**. From the table, nitrate in plain UHT milk samples were not detected. While, fortified UHT milk with strawberry and banana had the average nitrate concentrations 0.90 and 2.50mg/kg respectively. Also, the data showed the nitrate concentrations in fortified UHT milk with chocolate were higher than that in UHT with strawberry; and with banana.

With regard to fermented milk samples nitrate concentrations appeared in fortified fermented milk with fruits in the range 0.90 – 2.00mg/kg, while disappeared in plain fermented milk. The highest value was found in fortified fermented milk with blueberry.

The analysis of soffit cheese samples revealed that nitrate content in Domiaettecheese was not detected. But it was found in Feta cheese as seen in **Table 3** and the higher value was recorded in fortified Feta cheeses with olive. These results in the line of study **Yeh *et al.*, (2013)** who reported that vegetable ingredients would also change the nitrate contents.

According to the report of **Merusi *et al.* (2010)**, other nutrients such as sucrose, dietary fiber and lactose also increase the nitrate level in milk. Natural foods that are rich in nitrates provide a variety of health benefits, but some nitrate in human body is reduced to nitrite which form nitrosamines and led to risk effects as reported by **EFSA (2008)**. Fresh fruits and vegetables high in nitrates are far few problems compared to meats artificially high in nitrates due to natural antioxidant and other properties which may inhibit the conversion to nitrosamines. So, ascorbic acid may be used as inhibitor to the nitrosamine formation **(FCEC 2016).** In USA, the addition of sodium ascorbate (550 ppm) should be used together with ingoing amounts of sodium nitrite (100 ppm) or potassium nitrite (123 ppm) to pumped bacon to minimize consumer exposure to preformed nitrosamines in bacon **(USDA 2013).**

Table (3): Nitrate content in UHT milk, fermented milk and cheese.

Dairy product	Sample type	Range Nitrate content (mg/kg)	Mean ± SD
UHT milk	Plain UHT milk	ND	-
	UHT milk with strawberry	0.70 - 1.20	0.90 ± 0.20
	UHT milk with banana	2.30 - 2.60	2.50 ± 0.20
	UHT milk with chocolate	4.20 - 4.60	4.30 ± 0.20
Fermented milk	Plain fermented milk	ND	-
	Fermented milk with peach	0.90 - 1.20	1.70 ± 0.30
	Fermented milk with mango	1.20 - 1.30	1.20 ± 0.10
	Fermented milk with blueberry	1.70 - 2.0	1.90 ± 0.20
Cheese	Damietta cheese	ND	-
	Feta cheese	0.00 - 2.90	1.70 ± 1.10
	Feta with olive	3.10 - 6.30	4.10 ± 2.20
	processed cheese	0.50 - 3.80	2.60 ± 2.00
	Ras cheese	0.00 - 4.90	1.90 ± 1.30

In this study the nitrate content in hard cheese (Ras cheese) is ranged from 0- 4.90 with the mean value 1.90mg/kg. Concerning of processed cheese samples the average of nitrate content was

2.60mg/kgwith minimum value 0.50 mg/kg and maximum value 3.80mg/kg. The obtained results also revealed that examined samples of cheese were the lowest permissible limits (50 mg nitrate / kg cheese) according to Egyptian Standards **(1995)** and **Codex Standards (2003)** which considered as safe and permits the use of sodium nitrate singly or in combination with potassium nitrate in different varieties of cheese in amount up to 50 mg/ kg, expresses as $NaNO_3$. Nitrate in the manufacture of certain types of cheese is a common method to control the bacteriological problems of late blowing gassy defect in cheese **(Tompkin, 2005).** The dangerous in most cheese varieties that undergo a long ripening period is the anaerobic spore forming *Clostridi*a. These microorganisms are not destroyed by pasteurization and consequently produce considerable butyric acid fermentation with production of gasses resulting in late blowing of cheese that make it unfit for consumption **(Glórıa *et al.,* 1997)**.

Higher results were *obtained* by **Ali *et al.*, (2006)** who found that nitrate contents were ranged from 0.47 to 23.68 mg/kg in different types of Turkish cheeses. Also, higher concentrations of nitrates in Egyptian cheese samples were recorded by **Ismail and Al-Ashmawy (2008)** who found nitrate content in range between 4.9 and 60.3 mg / kg; 4.11 and 22.47 mg / kg and 10.57 and 37.49 mg / kg in soft, hard, and processed cheese respectively. These values were higher than the obtained data in present study.

With respect to nitrite content in dairy samples the data showed the nitrite concentration in all of the dairy samples was below the detection limit. Theseresults were agreement with **Yeh *et al.* (2013).** Previously, the lower values of nitrites reported by **Renner (1999**) who revealed that the interaction of lipids with nitrite is considered the cause of considerable reduction in the nitrite content of cheese. In addition, **Codex Standard (2003)** stated that negative or small amount of nitrites in cheese can be related to the unstable nature of nitrites.

From the obtained results, it was obvious that the investigated samples of the Egyptian dairy products in the present study are food safety products.

Conclusions

In the present study the optimal HPLC method was successfully applied to determine the nitrate and nitrite in dairy products simultaneously and rapidly. Variety of validation parameters including LOQ, LOD, linearity, precision, trueness, test recoveries, and uncertainty measurement indicate that the method was precise and accurate for determination of nitrate and nitrite in dairy products and it is recommended for food safety monitoring programs and routine analysis. In 44 dairy products samples the level of nitrate was in the range 0.00-6.30 mg/kg. All samples were nitrite free and plain milk, plain fermented milk and Damietta cheese are free from nitrate and nitrite. The highest level of nitrate was found in fortified dairy products with fruits or vegetables. From all investigated samples, processed cheese was the highest detected with nitrate. Generally, the average values of nitrates in the analyzed products were lesser than the maximum level for nitrates recommended by the World Health Organization (WHO) and FAO. This means that the investigated Egyptian dairy products are safe for consumption.

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Conflict of Interest: There is no conflict of interests to reveal the study results.

Ethical Approval: All materials, samples and results are obtained legally.

Informed Consent: The study is performed in accordance to informed consent and all of raw data are real and available. Availability of the raw data: All of raw data con be shared with readers via email of the corresponding author.

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