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Research Article
Influence of Melamine Adulteration and Proteolytic Enzymes on Total Protein Content of Imported Milk Powder

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Abstract

Background and Objective: Milk protein which is the key nutritional component of powdered milk can be negatively affected by the presence of proteolytic enzymes as well as the recent adulteration by melamine to cover the low-income consumers, demand from dairy proteins in developing countries therefore, this study assessed the total protein percentage of imported milk powder as well as the factors which could alter its content. **Materials and Methods:** Total protein and melamine content of 25 full cream imported milk powder were investigated using the formol titration method and ELISA, respectively in addition to measuring the proteolytic enzyme activity by the TNBS method. **Results:** The mean total protein (%) in the tested samples was 27.3±0.048 with 93.33% of the examined samples were following the Egyptian Standard (ES: 1780/2014). Additionally, melamine was detected in all examined milk powder with a mean value of 111.074±4.404 ng L^{-1}, however, they were following the Codex Alimentarius and the European Commission. There was a medium positive and significant correlation between the total protein and melamine content ($p>0.05$). Furthermore, proteolytic microorganisms were detected in 90% of the tested samples with a mean count of $31.19\times10^3\pm16.7\times10^3$ CFU g^{-1}. Hydrolysis Degree% (HD) in the examined samples ranged from 0.05.25 with a mean value of 0.3518±0.06632, whereas the mean amino nitrogen content was 0.1001±0.01872 g/100 g. A strong positive and significant correlation between HD% and the amino nitrogen content ($p>0.05$) was showed. **Conclusion:** Nearly all examined samples were in agreement with the international standards, however, strict periodical monitoring for melamine content in milk and dairy products should be conducted.

Key words: Milk protein, proteolytic enzymes, proteolytic microorganisms, melamine, Hydrolysis degree, amino acid content, milk powder, TNBS, ELISA

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Milk protein is one of the best nutritious proteins that contain amino acids proportion analogous to that synthesized by the human body. Furthermore, all essential amino acids (lysine, tryptophan, phenylalanine, methionine, threonine, isoleucine, leucine, valine and histidine) are needed by the human body and cannot be synthesized on their own were present in the milk proteins. Milk casein contains 45.1 g/100 g, whereas, whey protein has 50.9 g/100 g essential amino acids[1]. Additionally, protein content represents one of the main factors that influence the texture and flavour of milk products and consequently, it is considered as a quality index by many industries[2,3].

Increasing consumer awareness of these health benefits and the importance of dietary protein has increased the global demand for milk protein ingredients as a constant supply of good quality protein. milk powder which is not only used for reconstitution but also as an ingredient in many value-added foods (other dairy products, bakery, confectionery and meat products) owing to its functional properties is a very popular nutrient-rich food characterized by its significant dietary energy and protein content which composed of casein (about 80%), whey proteins (about 20%) and non-protein nitrogenous compounds[4-7].

Milk adulteration with melamine has been widely reported following the Chinese milk and infant formula scandal that reported by the WHO in 2008 whereas, 51,900 infants and young children were hospitalized for urinary problems, possible renal tube blockages and kidney stones in addition to 6 confirmed deaths among infants owing to ingesting melamine-contaminated infant formula[8-11]. In 2007, a pig pet food recall was reported in the United States and Canada because of adulteration with melamine[12]. Furthermore, adulteration of food of animal origin with melamine such as meat and meat products, eggs, fish and non-animal sources such as wheat, rice and beverages have been informed[13-15].

Melamine (2, 4, 6-triamino-1, 3, 5-triazine, $C_3H_6N_6$) is an organic chemical base mostly present in white crystals form rich in nitrogen[16]. It is produced primarily for usage in melamine-formaldehyde resins synthesis, plastics, laminates, commercial filters, coatings, glues and dish and kitchenware's[10,17].

Industrial cheap melamine contains 66.6% nitrogen by weight so it is considered one of the most infamous exogenous adulterants recently added to milk and milk-based products by the unscrupulous producers or their intermediaries to elevate falsely the content of protein and subsequently increasing its apparent value for financial gains and rising sales, in addition to satisfying the growing demand of the low-income consumers for dairy proteins in the developing markets, including many African nations where the border controls are poor and the national food control systems are fragmented[18-20].

Food products adulterated with melamine can easily pass the quality checks and cannot be easily recognized in the routine analysis by the standard Kjeldahl method. Because this method depends mainly on determining the total nitrogen content including the organic protein and non-protein nitrogenous substances such as melamine[11,14].

Recently, food and dairy products contaminated with melamine have created a widespread food safety concern because of their toxicity. Melamine combines with cyanuric and uric acid and forms an insoluble crystal, which in turn block and damage the renal cells causing eventual renal failure and ultimately death[21].

Many countries established a Maximum Residue Limit (MRL) for melamine in different products to enhance food safety which in turn protect the public health[11], the European Union (EU) set 2.5 mg kg^{-1} melamine in high-protein foods and milk products, whereas 0.25 mg kg^{-1} in milk and milk products by the US-FDA[11]. On the other hand, the Ministry of Health in China published new dairy safety standards on April 22, 2010, mentioned that food should not be free from melamine. Otherwise, the intentional adding of melamine to dairy foods is considered banned, even if it was much lower than the MRL[22].

Milk proteins may be negatively affected by the existence of proteolytic enzymes either the native milk alkaline proteinases (plasmin) or the heat-resistant extracellular microbial proteinases that are produced by the proteolytic microorganisms. Some of these microbial enzymes are heat resistant, remain active and can continue to decompose the milk ingredients after the enzyme-producing microbes have been destroyed by sterilization and even pasteurization[23].

The nutrient richness of raw milk makes it easily contaminated by exogenous microorganisms, resulting in a waste of milk resources. Along with the development of low-temperature storage and cold chain transportation technology, the growth of most bacteria in raw milk is inhibited[24]. Nevertheless, psychrotrophic bacteria that can grow under low-temperature conditions have not been inhibited[25]. Among the variety of psychrotrophic bacteria that are present in raw milk, Pseudomonas, Salmonella, Acinetobacter and Alcaligenes. Sterilization of dairy products can effectively inactivate the psychrotrophic bacteria. However, the enzymes secreted by them often have heat

resistance. Accordingly, Abdel-Salam and Soliman[26] reported that psychrotrophic bacteria are capable of producing active proteinases at 149°C (300°F) for 10 sec in 70-90% of raw milk samples.

Thermally resistant proteases can affect the milk powder quality along with the foods to which milk powder is added such as Ultra High Temperature milk (UHT) and the other milk products in various ways, largely by producing bitter peptides, gelation, technological problems, sensory, rheological and functional defects in the produced dairy products that limit their shelf-life[23,27]. Milk proteolysis can be studied by two approaches, proteolytic microorganisms' quantification and determination of the proteases enzyme activity.

Because of all mentioned before, the frequent usage of milk powder as reconstituted and as a major ingredient during the manufacturing of the other dairy products in addition to the great importance of the milk proteins.

This study was conducted to evaluate the protein content of the imported milk powder retailed in the Egyptian markets with investigating the factors which could alter its content such as the presence of heat-stable proteolytic enzymes using the TNBS method as well as the adulteration of milk powder with melamine using ELISA method that constitutes recently a major safety concern worldwide.

MATERIAL AND METHODS

Study area: The study was conducted in the Department of Food hygiene and control, Faculty of veterinary medicine and Department of Food Technology, National Research Center, Egypt from February-September, 2020.

Sample collection: Twenty-five samples of full cream milk powder (Miro, Nestle, Nido, Milky, Top value, Avanti, Mora and Rainbow), imported from Ireland, New Zealand and the European Union were randomly collected from the Egyptian markets and sent to the laboratory without delay in an insulated box for the further examinations.

Determination of total protein content according to the method explained by Moore *et al.*[28] using the formol titration method: The free amino acids, protein-bound amino acids and the peptides react with the formaldehyde, producing methylene amino acid derivatives that change the pKa of these amino groups.

Determination of melamine content was applied according to Garber[29]: Melamine concentration was determined by the indirect competitive ELISA, which is indicated as follows: 96-well polystyrene microtiter plates were coated with 100 µL/well of 0.25 ng mL^{-1} of melamine-BSA solution dissolved in coating buffer overnight at 4°C. The plate was then washed twice with 300 µL/well of washing buffer and blocked with 200 µL/well of blocking buffer by incubation for 2 hrs at 37°C. After that, the blocking buffer was removed and the plate was washed again. Several diluted solutions (standard melamine solution or melamine containing milk samples, 50 µL/well) were added, then melamine MAbs solution (50 µL) was added, followed by 30 min of incubation. The plate was washed again and further incubated with 100 µL/well of goat anti-mouse HRP at 37°C for 30 min. Substrate solutions were added and incubated at 37°C for another 30 min then, a stopping solution was added and the absorbance was determined at 450 nm for each well. Negative control was used from the serum taken before immunization.

Standard curve: The Melamine-BSA (0.20 ng mL^{-1}) was used as a coating antigen and indirect ELISA was carried out as described above. The melamine solution, 0, 5, 10, 20, 40, 60, 80, 100 and 150 ng L^{-1} (PBS, pH 6.6), was analyzed by the immunoassay developed. The standard curve was drawn using the previous standard melamine concentrations with their absorbance at 450 nm (Fig. 1).

Total proteolytic count was conducted according to the method assessed by Abdel-Salam and Soliman[26]: Duplicate plates of standard caseinate agar were inoculated with 0.1 mL from previously prepared serial dilutions, inoculated plates were incubated at 32±1°C for 48-72 hrs. Colonies surrounded by a white or off-white zone of Para casein precipitate are proteolytic microorganisms.

Degree of milk protein hydrolysis was estimated using the trinitrobenzene sulfonic acid (TNBS) method[30]: Half gram of milk powder was weighed exactly into 100 mL volumetric flask, dissolved and adjusted to the mark with SDS 1%, the flask was heated for 15 min in a water bath at 50°C, shake and let to cool to room temperature, the solution was centrifuged for 20 min at 3000 rpm.

Make 2 dilutions: The following volumes of sample solution (supernatant) illustrated in Table 1 were pipetted into 10 mL volumetric flasks, made up to the mark with SDS 1%, the solution was filtered through a low protein-binding filter (0.45 mm).

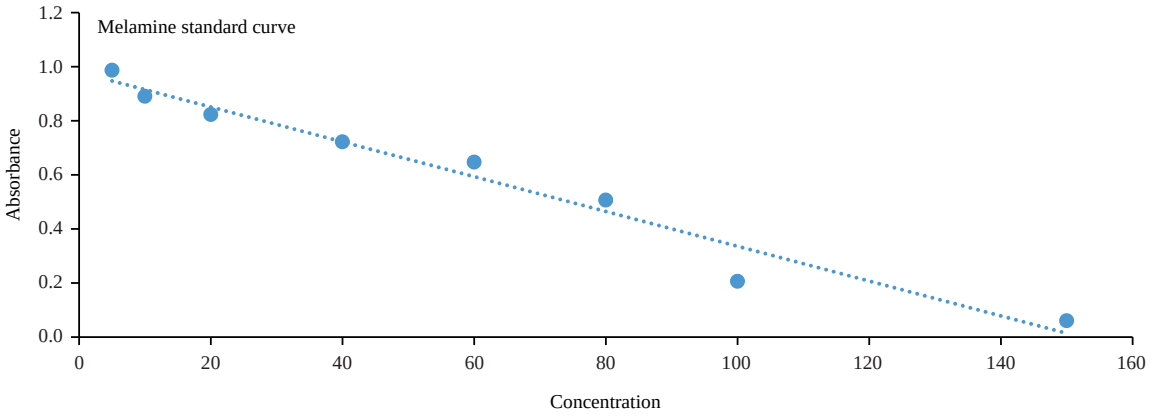

Fig. 1: Standard curve of melamine (ng L^{-1})

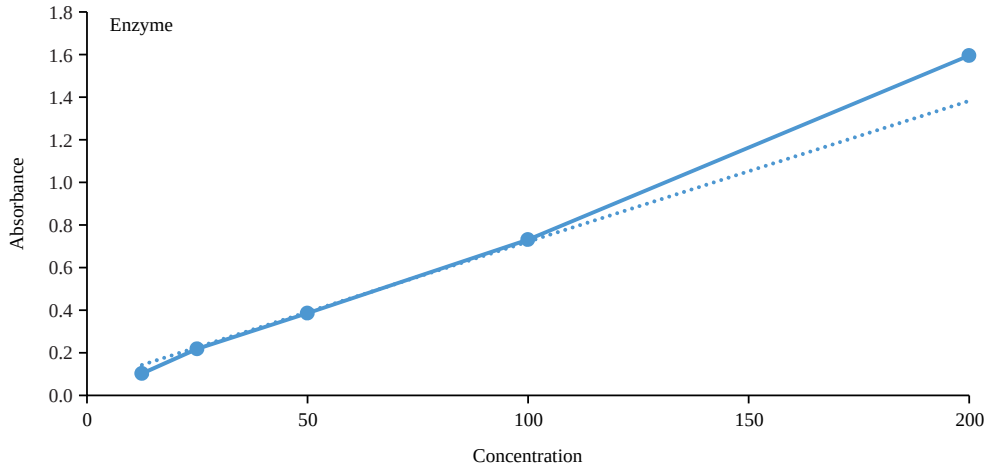

Fig. 2: Standard curve of the milk protein hydrolysis (HD% and amino nitrogen content g/100 g)

Table 1: Sample volumes with their dilution factors according to the degree of the hydrolysis

	Dilution 1 (mL)	Dilution factor (d)	Dilution 2 (mL)	Dilution factor (d)
Partially hydrolysed	As it is	1	5	2
Extensive hydrolysed	5	2	3	3.33
Protein hydrolysate	3	3.33		5

Reaction and spectrophotometric measurement: A total of 250 µL SDS 1% for the blank, 250 µL sample dilutions and 250 µL of each calibration standard were introduced in the respective test tubes. Two mL of sodium phosphate buffer and 2 mL of TNBS solution 0.1% were added subsequently to each tube, closed, mixed and placed in a water bath (covered with aluminium foil) at 50°C for 60 min. The reaction was stopped after 60 min by adding to each tube 4 mL HCl 0.1 M, mixed and let the tubes cool to room temperature for 30 min. The absorbance was measured at 420 nm against air.

Standard curve: Leucine standard solution, 10 mM (65.6 mg L^{-1} Leucine was weighed into 50 volumetric flasks, dissolved and adjusted to the mark with SDS1%). Leucine standard solution, 1 mM (5 mL of the Leucine standard solution10 mM was pipetted into 50 volumetric flasks and adjusted to the mark with SDS1%). Leucine standard solution for calibration curve, the following concentrations of standard leucine solution (750, 500, 250, 125, 100 and 50 nmol/250 µL) were prepared using 10 mM and 1 mM solutions.

The obtained absorbances values were plotted against the concentration of the leucine (nmol/250 µL) and the calibration line was drawn, the correlation coefficient was calculated (which should be >0.995) (Fig. 2).

The amino nitrogen, ($N\text{-}NH_2$) was expressed in g/100 g product, corresponds to:

X_1 : 14.007
V_1 : d/m
V_2 : 10000

$$\text{Hydrolysis Degree (DH)} = \frac{\text{Amino nitrogen (g/100 g)}}{\text{TN}} \times 100$$

X_1 = nmol leucine read on the curve
m = Weight of the test portion (g)
V_1 = Volume used for sample preparation (100 mL)
V_2 = Volume used for the reaction (250 ml)
d = Dilution factor
14.007 = Atomic weight of nitrogen
TN = Total nitrogen (g/100 g)

TN was determined using the Kjeldahl method according to Ramesh *et al.*[31].

Statistical analysis: Results were calculated in the form of Mean+SEM using the program Statistical Package for Social Science (SPSS), version 20. Pearson's correlation coefficient (r) was used to evaluate the correlation between the tested parameters. Significance was considered at p>0.05.

RESULTS

Data illustrated in (Table 2) revealed that the total protein percentage in the examined milk powder samples ranged from 22.6-31.3 with a mean value of 27.31±0.478. Additionally, melamine was found in all examined samples of milk powder with concentrations ranged from 87.77-146.38 and a mean value of 111.074±4.404 ng L^{-1}.

Proteolytic microorganisms count in the tested samples ranged from 9×10^2-42.8×10^4 with a mean value of $31.19\times10^3\pm16.7\times10^3$ CFU g^{-1}. On the other hand, the Hydrolysis Degree % (HD) of the examined samples using the TNBS method ranged from 0.05 to 1.25 with a mean value of 0.3518±0.06632, while the amino nitrogen content ranged from 0.01-0.39 with a mean value of 0.1001±0.01872 g/100 g (Table 3).

Results illustrated in Fig. 3a-c, revealed that there was significant strong positive correlation between HD% and amino nitrogen content (g/100 g) (r = 0.983, p>0.05) (Fig. 3a). Significant medium positive correlation between total protein (%) and melamine (ng L^{-1}) (r = 0.412, p>0.05) (Fig. 3b). While there was negative weak non- significant correlation between melamine (ng L^{-1}) and HD% (r = -0.06, *(*p<0.05) (Fig. 3c).

DISCUSSION

About 93.33% of the examined samples were agreed with the Egyptian standard (ES: 1780/2014)[32]. Milk proteins have excellent nutritional value and functional properties, which are widely exploited in the food industry[33].

Milk proteins are the key nutritional component of the powdered milk, they are composed of casein and whey proteins as major types of protein in addition to numerous minor proteins including the non-protein nitrogenous substances[4,34]. A reliable method is essential for protein estimation for the manufacturers and the international trade. Estimation of protein content in food is mostly done by the Standard Kjeldahl method through multiplying the nitrogen content in a conversion factor, summing constituent amino acids could be an alternative measurement[35].

Our findings of total protein were nearly in agreement with Kajal *et al.*[36], who found that protein content ranged from 25.22-27.01 g/100 g and Sobia *et al.*[37], who found the mean protein content in whole powdered milk was 26.85±0.86%, while lower mean total protein (%) was recorded by Ibrahim *et al.*[32] who found the mean total protein (%) in the examined whole powdered milk was 25.53.

Table 2: Statistical analytical results of total protein (%) and melamine content (ng L^{-1}) in the examined milk powder (n = 30)

Parameters	Minimum	Maximum	Mean±SEM
Total protein (%)	22.6	31.3	27.31±0.478
Melamine (ng L^{-1})	87.77	146.38	111.074±4.404

Permissible total daily intake per day for melamine is 500 ppb kg/b.wt./day (Filazi *et al.*[10])

Table 3: Statistical analytical results of Hydrolysis degree% and the proteolytic microorganisms count in the examined milk powder (n = 30)

Parameters	Minimum	Maximum	Mean±SEM
Amino nitrogen content (g/100 g)	0.01	0.39	0.1001±0.0187
Hydrolysis Degree (DH) (%)	0.05	1.25	0.3518±0.6600
Total proteolytic count (CFU g^{-1}) (n = 27 positive samples)	9×10^2	42.8×10^4	$31.19\times10^3\pm16.7\times10^3$

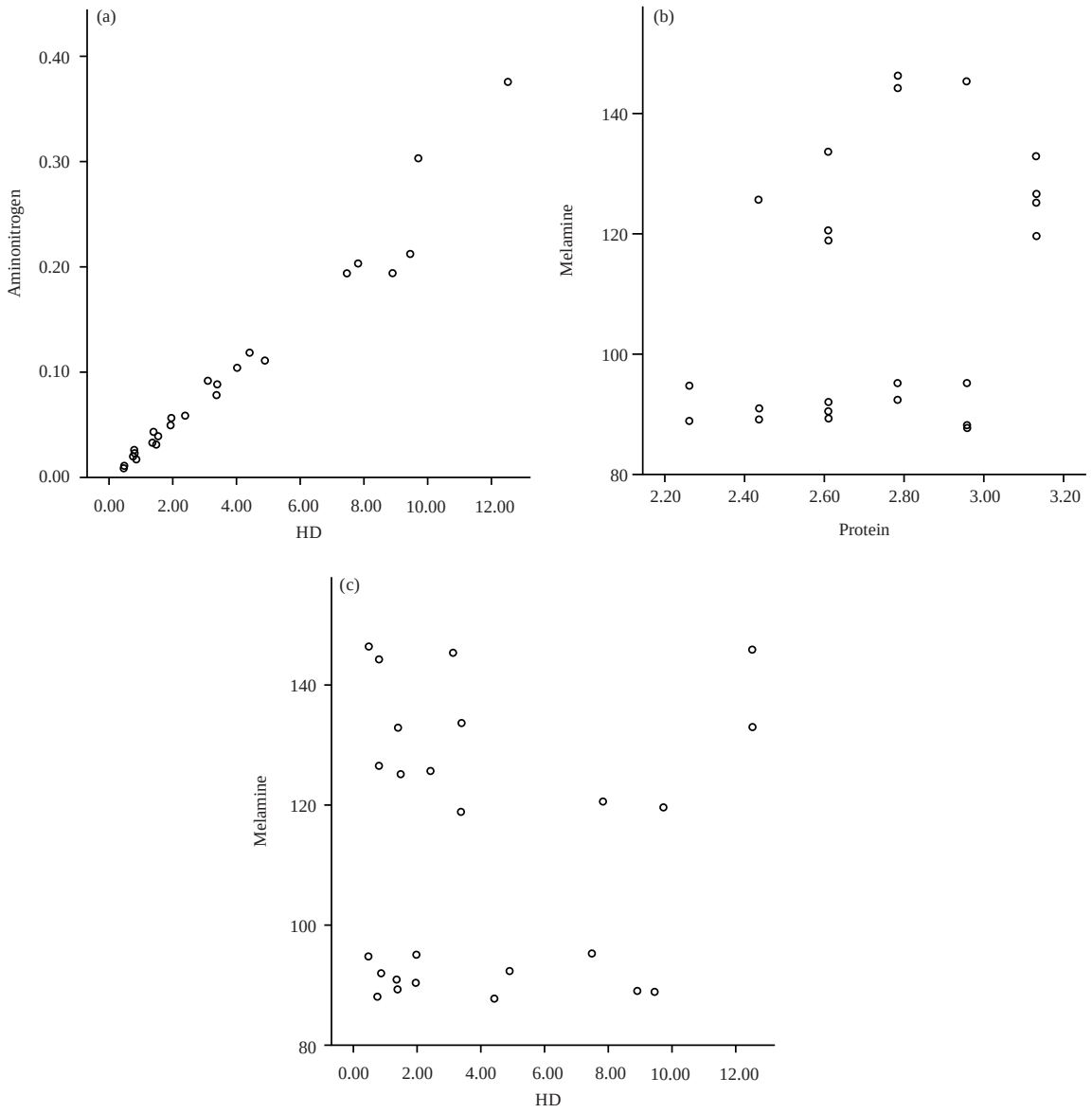

Fig. 3(a-c): Correlation between the total protein (%) and the different examined parameters

The addition of one gram of melamine to 1 L of milk falsely increases the protein content by 0.4%. Moreover, 3.1 g of melamine can be dissolved in water without forming precipitate and the protein content will falsely increase by 1.2% at room temperature. The greater solubility of milk powder in warm water made the added melamine to be greater[15]. Milk powder is consumed as reconstituted milk and the main ingredient in many other dairy products including ice cream, cheese, UHT and even for the preparation of infant food in some instances. Therefore, contamination of milk powder with melamine means contamination of all of these products and represents a major safety concern.

Melamine was found in all examined samples of milk powder. Moreover, the results illustrated revealed that there was a medium positive and significant correlation between the total protein and melamine content ($p>0.05$) (Fig. 3), which means that by adding melamine, there was a false increase in the protein % in the measured samples.

The presence of melamine in all examined milk powder samples could be explained by its presence in the environment, contaminated animal feeds that have been treated with products containing melamine, such as fertilizers or pesticide or the intentional addition of melamine to milk powder to increase the protein count falsely at less cost by the

unethical manufacturers[13,15]. Moreover, milk contamination can result from the packaging materials[10,38]. The excessive intake of melamine above the safety limit (2.5 ppm in the USA and European Union) may result in the formation of insoluble melamine cyanurate crystals in the kidney, which finally causes renal failure and even death[39,40].

The obtained results of melamine were the following that determined by Deabes and El-Habib[41] and Poorjafari *et al.*[42] who evaluated the melamine content in full cream milk powder and found that all examined samples were contaminated. On the other hand, other studies assessed lower percentages of milk powder contamination (6, 60, 8 and 48%)[10,43,44], respectively, whereas higher concentrations of melamine adulterant were determined by Wen *et al.*[45], who determined melamine as high as 2.563 mg kg^{-1} in some of the examined samples, García *et al.*[46] and Schoder and McCulloch[21].

Maximum Residue Limit (MRL) has been established for melamine by many countries to prevent further contamination and fraud, the European Union (EU) set 2.5 mg kg^{-1} melamine in dairy products and high-protein foods, whereas the US-FDA set 0.25 mg kg^{-1} melamine in milk and dairy product. While the presence of melamine contaminant in all examined milk powder samples, none of them exceeded the Codex Alimentarius Commission[46] and the European Commission[10]. Although the detected limit of melamine in all examined samples was low and agree with these standards. The frequent usage of milk powder by the consumers put this low limit at risk, especially that, milk powder enter in the manufacture of many other dairy products and food preparations, this means that these products are also contaminated with melamine and together with the daily intake of milk powder may exceed the safe threshold limit of melamine sit by these organizations which put Tolerable Daily Intake (TDI) of 0. 63 mg kg^{-1} body weight per day by the Food and Drug Administration (FDA) for food and food ingredients other than infant formula[47] and 0.5 mg kg^{-1} body weight by Maleki *et al.*,[48].

Because of the public health risk of the contaminated melamine and to protect human health, intensive melamine controls should be conducted by producers, national safety authorities and importers worldwide. Strict periodical monitoring of melamine content in milk and dairy products should be done together with applying serious controlling programs to prevent the entry of melamine into milk and dairy products. Good Manufacturing Practice (GMP), Good Agriculture Practices and good quality control programs are very effective manner for melamine control in milk. Additionally, further novel studies and techniques should be implemented for melamine control and chelation in milk and milk products.

Proteolysis in milk can be studied by two approaches, quantification of the proteolytic microorganisms and determination of the proteolytic enzyme activity.

Proteolytic bacteria comprise a very large number of species that grow both aerobically and anaerobically. proteolytic bacteria are found among the species of micrococcus, pseudomonas, achromobacter, proteus, alcaligenes, flavobacterium, serratia, all of them are non-spore-forming bacteria. In addition to the spore-forming Bacillus and clostridium species[26,49].

The contaminated organisms in the examined samples may come from air, dust, soil, water, packing materials and the poor hygienic practice applied during the handling, processing and repacking process of the imported powder[50,51]. Additionally, contamination of raw milk with high numbers of microorganisms resulted in high numbers in the produced milk powder especially the spore formers that resist the extreme heat and produce proteolytic enzymes[7].

The ability of the dietary protein source to meet the human nutritional requirements for the necessary amino acids are defined as "protein quality" which is critical to support normal development and growth[52]. There are many different approaches to assessing the protein quality of food[53]. Total or partial hydrolysis of protein and the separation and quantification of the released amino acids or peptides are now widely used[4]. Additionally, Proteolysis in milk powder has been measured by monitoring the changes in nitrogen levels such as the decrease in casein nitrogen or increase in Non Protein Nitrogen (NPN). These changes have been linked to changes in its functionality[54].

The activity of the hydrolytic enzymes produced by psychrotrophic organisms in raw milk is 100% during cold storage. However, several of these enzymes can keep their activity between 60-70% after pasteurization and 30-40% after sterilization of milk. For these reasons, the bacterial enzymes have been the most studied and best described[55]. Moreover, the activity of proteolytic enzymes of native or bacterial origin can lead to undesirable changes not only in flavour (bitterness particularly) but also in texture[26].

There was a strong positive and significant correlation between HD% and the amino nitrogen content ($p>0.05$).

Pseudomonas bacteria have been associated with the spoilage of raw milk and dairy products because of the

production of thermostable proteolytic enzymes[49]. Furthermore, *Bacillus* spp. are widely distributed in the environment and can be introduced into milk powder during production, handling and processing. They can sporulate during milk powder processing, producing extremely heat resistant spores[7,54,56]. Among the bacteria belonging to the genus *Bacillus*, *B. stearothermophilus*, *B. licheniformis*, *B. coagulans*, *B. cereus*, *B. subtilis* and *B. circulans* are the most common species that have a greater capacity to produce a broad spectrum of thermostable extracellular and intracellular hydrolytic enzymes[57,58]. Proteolytic changes caused by *Bacillus* spp. are a significant increase in the concentration of free tyrosine, which can increase in milk up to 2.13 mg mL^{-1} in comparison to their initial values of approximately 0.65 mg mL^{-1} [59]. Proteolysis could be avoided by using good quality raw milk for manufacturing dairy products.

CONCLUSION

Milk protein which is the key nutritional component of powdered milk can be negatively affected by the presence of the protein splitting enzymes either native or microbial in addition to the adulteration with melamine which constitutes a public safety concern since 2008. The study revealed that 93.33% of the examined milk powder samples were following the Egyptian standard (1648/2005). Melamine was detected in all examined samples with the mean value of 111.074±4.404 ng L^{-1}, however, none of them exceeded the Codex Alimentarius Commission and the European Commission. proteolytic microorganisms were detected in 90% of the evaluated samples. Therefore, Good quality raw milk should be used for manufacturing dairy products in addition to conducting a strict periodical monitoring for melamine with applying serious controlling programs that prevent its entry into milk and dairy products such as Good Manufacturing Practice (GMP). Further novel studies and techniques should be implemented for melamine chelation in milk and milk products to avoid its public health risk.

SIGNIFICANCE STATEMENT

This study noticed that 93.33% of the examined milk powder samples were following the total protein % set by the Egyptian standard (1648/2005). Melamine was detected in all examined samples, however, none of them exceeded the Codex Alimentarius and the European Commission. The high contamination level of some examined samples with proteolytic microorganisms was noticed, with the presence of a significant strong positive correlation between HD% and the amino nitrogen. Strict periodical monitoring for melamine content in milk and dairy products should be conducted.

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