



## Original Article



# Melamine a Potent Carcinogen: Detection in Packed and Unpacked Milk Samples Collected from Different Regions of Lahore, Pakistan by ELISA Method

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## ABSTRACT

Melamine is a nitrogen-rich compound often added to milk to enhance its protein contents. The European Commission has established a permissible limit of 2.5mg/kg for melamine, as excessive amounts can lead to kidney stones and other health risks for both infants and adults.

**Objective:** To assess melamine concentrations in both packed and unpacked milk samples from various regions in Lahore, Pakistan. **Methods:** An experimental study was conducted using the Enzyme-Linked Immunosorbent Assay (ELISA) technique to detect melamine levels in milk samples. Measurements were obtained at a wavelength of 450 nm, following standard ELISA protocols. **Results:** The analysis revealed that one unpacked milk sample contained melamine levels exceeding the permissible limit at 6.3±0.1mg/kg, while the other samples were deemed safe for consumption. Additionally, while melamine was detected in packed milk samples, all were within safe consumption levels. **Conclusions:** In conclusion, melamine was not found in the packaged milk samples, adhering to the acceptable limits set by the European Commission in 2009; however, one unpacked milk sample showed a concerning level of melamine.

## INTRODUCTION

Milk is very nutritious for people and serves as an energy source, supplying lactose, fats, proteins, calcium, vitamins, and minerals [1]. Adulteration refers to the deliberate addition, replacement with lower-quality stuff, or elimination of precious elements from food for personal gain, whereas food fraud encompasses the purposeful inclusion of harmful ingredients [2]. This study presented a colorimetric method for detecting melamine in milk using gold nanoparticle aggregation, offering a rapid and visual detection approach [3]. In most regions of Pakistan, low-

quality milk is available, and individuals struggle to find nutritious foods. Several adulterants find their way into milk, such as detergents, water, cane sugar, rice flour, starch, formalin, hydrogen peroxide, sodium chloride, melamine, urea, cyanuric acid, vegetable oil, ammonium sulfate, caustic soda, boric acid, glucose, sorbitol, hypochlorite, salicylic acid and arrowroot [4]. Melamine is an organic industrial substance rich in nitrogen. The compound is NC-NH<sub>2</sub>, a trimer of cyanamide consisting of 67% nitrogen. C<sub>3</sub>H<sub>6</sub>N<sub>6</sub> is the chemical composition of the



compound. This systematic review and meta-analysis evaluates the levels, detection methods, and health risks of melamine and cyanuric acid in milk [5, 6]. Araujo et al., 2012 state that hydrolyzed melamine decomposes into ammelide, ammeline, and cyanuric acid [7]. It is commonly utilized in the manufacture of melamine resins through the reaction with formaldehyde [8]. Additionally, melamine is utilized in the production of plastics, decorative laminates, tableware, molding materials, coatings, flame retardant agents, and adhesives [9]. Besides its various industrial applications, melamine has seen growing utilization in food and dairy items as a synthetic protein source [10]. Due to its elevated nitrogen levels (66%), it is unlawfully included in baby formulas, animal feed, nitrogen fertilizers and milk products as an enhancer of protein [11]. The addition of melamine to packaged milk has become a widespread practice, as businesses employ it to boost market prices, enhance nitrogen levels in milk, and lower product expenses [12]. While melamine is safe in minimal amounts, a TDI (tolerable daily intake) threshold of 0.2 mg/kg has been established using dose-response evaluations from sub-chronic rat research. Additionally, safety thresholds of 1mg/kg melamine in baby formulas and 2.5mg/kg in other products have been set. Many countries have established Maximum Residue Limits (MRL) for melamine across various products to protect public health and ensure food safety. For example, the USFDA established the MRL of melamine in dairy products, milk foods and milk at 0.25 mg/kg, stressing that infant formula available to consumers of United States must be completely melamine-free, whereas the European Union (EU) set the MRL of melamine in dairy items and high-protein foods at 2.5 mg/kg [13, 14]. The present research can aid this effort in Pakistan by concentrating on the easy determination of melamine levels in milk products, marking an initial step toward regulating melamine in food items from local markets. Thus, the primary objectives of this investigation aimed to measure melamine levels and its associations between the protein concentrations of different packaging samples of milk and milk baby formulae with a foundation of local marketplaces in Pakistan.

To accomplish these goals, ELISA based approach was employed to evaluate the presence of melamine in different milk items.

## METHODS

This experimental study employed the AgraQuant@ Melamine ELISA kit was used for the current experimental study. It contained melamine standard solutions (0, 20, 100 and 1000ppb), melamine enzyme conjugate, substrate solution, stop solution and wash buffer. ELISA microplate reader (Model: EZ Read 2000, biochrom), centrifuge machine, digital pipettes and microtubes, distilled water

were also required for the test. Various samples of packed and unpacked milk (n=10 for packed and n=10 for unpacked) were collected randomly from different locations of Lahore. The unpacked milk samples were obtained from local market and milkmen providing milk at domestic levels in different regions of Lahore. The packed milk samples were collected from some highly recommended and commonly known supermarkets and stores from various locations in Lahore. The samples were kept in refrigerator after collection at -20°C. 5mL of milk samples were pipetted out in tubes for centrifugation for 10 minutes at 1500rpm. The fat layer was removed and the clear milk serum was collected for analysis. 150µL of the melamine standards and samples were poured into antibody coated wells. 50µL of the conjugate was also added and mixed well by carefully pipetting up and down 3 times. These wells were incubated for 30 minutes at room temperature. The contents of the microwells were emptied and washed with diluted wash buffer solution for a total of 4 washes each. The wells were dried on multilayer absorbent paper towel. 100µL of the substrate solution was added to the wells and then kept for 20 minutes at room temperature. 100µL of the stop solution was added and noted the absorbance within 10 minutes at 450 nm by using an ELISA microplate reader [15]. A standard curve using the absorbance values of the melamine standards was constructed and calculated the melamine concentration in the milk samples based on the standard curve.

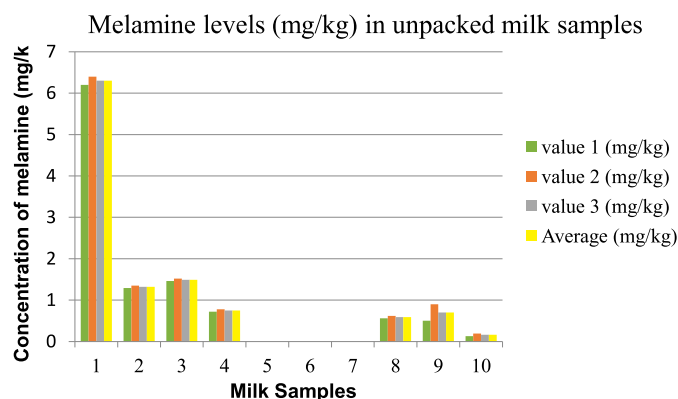
## RESULTS

LOD and LOQ of the ELISA kit method was 8.0µg/kg and 0.1-0.5mg/kg as prescribed on Kit brochure. Melamine levels were noted in mg/kg (ppm) with a set of 3 readings for each sample and made an average value out of it for each sample. Table 1 shows different readings of melamine observed under ELISA reader and their average value in mg/kg in unpacked milk samples.

**Table 1:** Melamine Levels (mg/kg) in unpacked Milk Samples

Samples	Value 1 (mg/kg)	Value 2 (mg/kg)	Value 3 (mg/kg)	Average (mg/kg) Mean ± SD
1	6.2	6.4	6.3	6.30 ± 0.10
2	1.29	1.35	1.32	1.32 ± 0.03
3	1.46	1.52	1.49	1.49 ± 0.03
4	0.72	0.78	0.75	0.75 ± 0.03
5	NOT DETECTED			
6	NOT DETECTED			
7	NOT DETECTED			
8	0.56	0.62	0.59	0.59 ± 0.03
9	0.50	0.90	0.70	0.70 ± 0.20
10	0.13	0.19	0.16	0.16 ± 0.03

The results in figure 1 showed that sample 1 is unfit for human consumption as it has melamine beyond permissible levels of 2.5mg/kg.



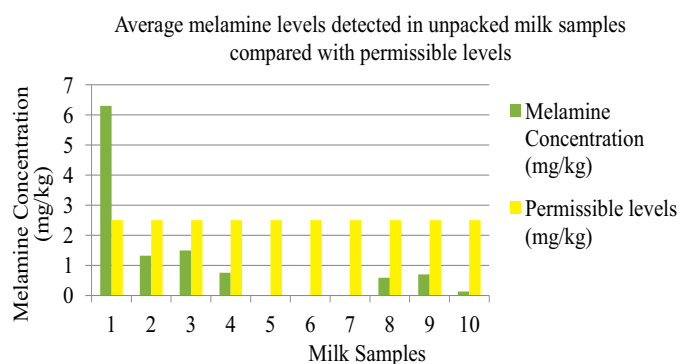
**Figure 1:** Comparison of melamine levels(mg/kg)in unpacked milk samples

Melamine concentrations detected in unpacked milk samples collected from different regions of Lahore (Table 2).

**Table 2:** Results of unpacked Milk Samples

Samples	Melamine in unpacked samples (mg/kg)	Results
1	6.30	Not fit for human consumption
2	1.32	Fit for human consumption
3	1.49	Fit for human consumption
4	0.75	Fit for human consumption
5	0	Fit for human consumption
6	0	Fit for human consumption
7	0	Fit for human consumption
8	0.59	Fit for human consumption
9	0.70	Fit for human consumption
10	0.16	Fit for human consumption

Although; the melamine contamination was found in other samples but that was within the safest permissible levels (Figure 2). However, sample 5, 6 and 7 was found with no melamine contamination.



**Figure 2:** Comparison of unpacked Samples with Permissible Levels

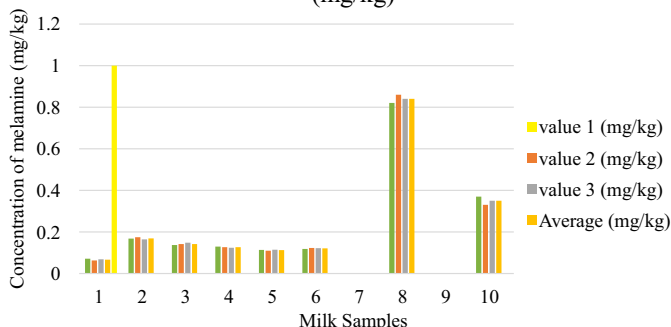
3 readings of each sample were taken by ELISA reader at 450nm and calculated an average value out of it. Table 3 shows different readings of melamine in milk of packed samples. Figure 3 showed melamine levels (mg/kg) in 10 selected samples.

**Table 3:** Melamine levels(mg/kg)in packed samples

Samples	Value 1 (mg/kg)	Value 2 (mg/kg)	Value 3 (mg/kg)	Average (mg/kg) Mean $\pm$ SD
1	0.071	0.063	0.069	0.067 $\pm$ 0.004
2	0.168	0.175	0.164	0.169 $\pm$ 0.006
3	0.137	0.142	0.148	0.142 $\pm$ 0.006
4	0.13	0.127	0.124	0.127 $\pm$ 0.003
5	0.114	0.110	0.115	0.113 $\pm$ 0.002
6	0.118	0.123	0.122	0.121 $\pm$ 0.002
7	NOT DETECTED			
8	0.82	0.86	0.84	0.84 $\pm$ 0.020
9	NOT DETECTED			
10	0.37	0.33	0.35	0.35 $\pm$ 0.020

The permissible level or the level up to which the consumption of melamine in milk is safe is observed to be 2.5mg/kg by European Commission 2009 [16].

Melamine level detected in packed milk samples (mg/kg)



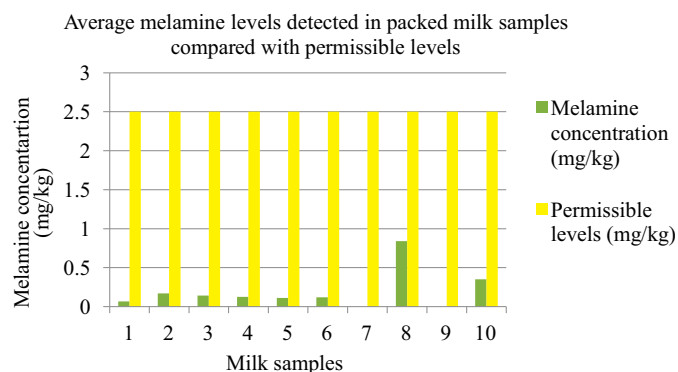
**Figure 3:** Comparison of Values of Packed Samples in mg/kg

By comparing packed milk samples with permissible levels of EU, 2009, it was noted that all milk samples were safe for human consumption (Table 4).

**Table 4:** Results of Packed Milk Samples

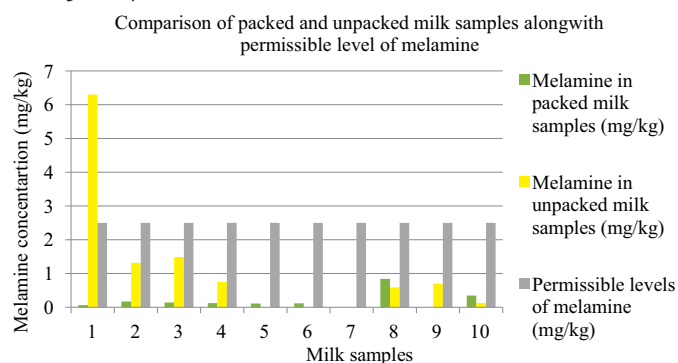
Samples	Average level of Melamine in packed samples (mg/kg )	Results
1	0.067	Fit for human consumption
2	0.169	Fit for human consumption
3	0.142	Fit for human consumption
4	0.127	Fit for human consumption
5	0.113	Fit for human consumption
6	0.121	Fit for human consumption
7	0	Fit for human consumption
8	0.84	Fit for human consumption
9	0	Fit for human consumption
10	0.35	Fit for human consumption

Packed milk has lower levels of melamine as compared to the unpacked milk samples (Figure 4).



**Figure 4:** Comparison of packed samples with permissible level

Figure 5 depicted the comparison of melamine concentration in unpacked and packed milk samples along with permissible levels.



**Figure 5:** Comparison of Packed and Unpacked Milk Samples

## DISCUSSION

An article reviewed advancements in melamine detection, highlighting the evolution from traditional methods to innovative electrochemical sensor technologies [17]. Another study presented a rapid immunochromatographic assay using luminescent quantum dot beads for sensitive melamine detection in milk. [18]. In a study, 300 samples of milk and dairy products that were bought from well-known Turkish merchants were analyzed using the technique. While 2% of cheese, 8% of milk powder, and 44% of yogurt samples had melamine at levels of 121,  $694 \pm 146$ , and  $294 \pm 98$   $\mu\text{g/kg}$ , respectively, infant formulae and pasteurized UHT milk did not contain melamine. These results fell short of the thresholds established by EU law and the Codex Alimentarius Commission [18]. These results showed that packed samples were within permissible levels established by the Codex Alimentarius Commission (2010) and EU regulations (European Commission, 2002, 2009; 1 mg/kg for infant formula, 2.5 mg/kg for dairy goods)[19]. In a study conducted by Gouri, 2025, five distinct brands of protein powders underwent FTIR analysis, resulting in spectra for each sample. Eighty percent of the samples indicated the presence of melamine. To prevent melamine contamination and safeguard consumer safety, stringent

quality control protocols and routine testing are recommended [20]. The contamination of one unpacked sample found beyond permissible levels is risky and smaller quantities found were due to contamination during the processing of dairy items, while the larger quantities were due to intentional inclusion. Eating foods with these minimal amounts of melamine poses no health risk to consumers. Despite progress, obstacles remain, such as the intricate and variable nature of milk composition, the significant expenses associated with advanced technology, the requirement for specialized knowledge, and the absence of uniform protocols[21].

## CONCLUSIONS

Melamine was absent in packaged milk samples and within the acceptable limits set by the European Commission in 2009; however, one sample of unpackaged milk revealed a significant presence of melamine. Additionally, some unpackaged milk samples showed minimal or no detectable levels of melamine. Therefore, this survey indicates that consumers of unpackaged milk face a certain risk of melamine exposure, while packaged milk presents a lower or negligible risk.

## Authors Contribution

Conceptualization: NZ

Methodology: MKS

Formal analysis: AJ, MS, SN

Writing, review and editing: NZ, AS

All authors have read and agreed to the published version of the manuscript.

## Conflicts of Interest

All the authors declare no conflict of interest.

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