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Original Article

Screening of Melamine in Milk and Milk-based Products Using Enzyme-linked Immunosorbent Assay

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ABSTRACT

Background and Objectives: Melamine is an organic nitrogen used as an adulterant to increase nitrogen and protein contents in milks. Enzyme-linked immunosorbent assay has verified as a good alternative for rapid screening of melamine and other food additives instead of the laborious chromatography techniques.

Materials and Methods: In the present study, MaxSignal enzyme-linked immunosorbent assay kit was used to screen melamine in 30 samples, including milks, infant formulas, chocolates, tea whiteners and pet foods. The kit was based on a competitive colorimetric enzyme-linked immunosorbent assay.

Results: Melamine was detected in the range of 0.05–0.69 ppm in infant formula/milk powder samples and 0.001–0.042 ppm in liquid milk samples, which were under the set limits of Codex Alimentarius, 2012. Codex Alimentarius Commission adopted a maximum melamine level of 1 ppm for powdered infant formulas and of 2.5 ppm for other foods and animal foods. The commission has now set a maximum limit of 0.15 ppm for melamine in liquid milks. Further protein analyses of milk and infant formula samples were also carried out to verify the presence of melamine.

Conclusions: It has been seen that enzyme-linked immunosorbent assay could effectively be used as a screening tool for detecting melamine in a wide range of food samples. Whereas, complementary techniques such as high-performance liquid chromatography, gas chromatography/mass spectrometry can be adopted as verification methods for regulatory compliance.

Keywords: Melamine, ELISA, Antibody, Kjeldahl method, HPLC, GC/MS

Introduction

Milk is highly nutritious and source of energy providing lactose, fats, proteins, calcium, vitamins and minerals for human consumption (2). Adulteration includes intentional addition, substitution of inferior substances or removal of any valuable components from foods for personal benefits (25), while food fraud includes intentional addition of unhealthy components. This has been defined as economic motivated adulteration, which is fraudulent, intentional addition of a substance for increasing market values, gaining higher profits or decreasing production costs (27). Substandard milk is sold in majority of areas within Pakistan, where people are unable to access healthy foods. A number of adulterants are added in milk, including water, detergent, cane sugar, starch, rice flour, formalin, sodium chloride, urea, hydrogen peroxide, melamine, cyanuric acid, boric acid, ammonium sulfate, vegetable oil, caustic soda, glucose, arrowroot, hypochlorite, salicylic acid and sorbitol (2, 25). Melamine (2,4,6-triamino-1,3,5-triazine) is an organic, nitrogen-rich industrial chemical. The chemical is a trimer of cyanimide, NC-NH2, containing 67% nitrogen. The compound chemical formula is $C_3H_6N_6$ (22, 7). Hydrolyzed melamine is degraded into ammeline, ammelide and cyanuric acid (Araujo et al. 2012). It is widely used for the production of melamine resins by reacting with formaldehyde (13). Furthermore, melamine is used for making plastics, decorative laminates, dinnerware, moulding compounds, coatings, flame retardant additives and adhesives (33). In addition to its multifarious industrial uses, melamine has been increasingly used in food and milk products as a synthetic source of proteins. Because of its high nitrogen contents (66%), it is illegally added to milk products, infant formulas, animal foods and nitrogen fertilizers as protein booster (4, 35). Spiking packaged milk with melamine has become a ubiquitous practice, as companies use this to increase market values, augment nitrogen contents of milks

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and decrease product costs (38). Although melamine is not harmful at low levels, a tolerable daily intake (TDI) limit of 0.2 mg/kg has been set based on dose-response assessment of sub-chronic rat studies. Moreover, safety limits of 1 mg/kg melamine in infant formulas and 2.5 mg/kg in other foods have been established (5).

Studies on melamine migration from tableware have verified that leaching of the chemical is affected by various factors such as acidity, temperature and pH. Assays have shown that melamine migration is well below the specific migration limit (SML) of 30 mg/kg by the European Commission Directive. However, such a long-term exposure to low-dose melamine can be harmful to young children (2, 19). Melamine became a subject of health concerns early in this century, when it was reported as the cause of death for children and pets. The first incident of melamine poisoning in pets was reported in the Republic of Korea, 2004. In 2007, ingestion of pet food contaminated with melamine and cyanuric acid resulted in more than 100 pet deaths in North America (24). In 2008, another major incident of food safety violation was made public in China. The scandal involved milks and infant formulas with other food materials highly contaminated with melamine. The incident affected nearly 294,000 Chinese infants and young children with six reported deaths and 50,000 hospitalized as a result of kidney stones, hematuria, hydronephrosis and renal failure (14, 10, 4). Countries worldwide reacted to this incident in a variety of ways from taking no actions to banning all imports of milk and dairy products from China. Melamine and cyanuric acid form a complex as a result of hydrogen bonding and co-ingestion of these chemicals result in formation of renal stones (23, 26). Renal problems precipitated because of melamine can include indirect effects on cardiac disorders (6). Urinary tract (UT) calculi were detected in ultrasonography of the children fed with melamine-contaminated milk products (37). Ultrasound examinations on 25 Chinese patients affected during the 2008 melamine incident showed presence of calculi in kidneys and uteri (31). Children exposed to high levels of melamine were reported seven times more susceptible to urinary stones, compared to those exposed to melamine-free formulas (11). A study has verified that melamine induces nephrolithiasis, which leads to chronic inflammation, dysplasia and urothelial carcinoma (20). Moreover, melamine intoxication has been associated with risks of developing heart diseases.

In 2010, a milk adulteration issue became public in Pakistan, when Pakistan Standards and Quality Control Authority (PSQCA) chairman confirmed the presence of melamine and other harmful adulterants in packaged milks (The News, 2010). Due to the alarming situation of unhygienic foods sold in local markets and no inspection and regulation systems, it is highly likely that the National Food Safety Legislation has not been enforced properly and PSQCA functioning includes defects. Every year, thousands of people and young children lose their lives because of malnutrition and deadly diseases such as gastroenteritis and food poisoning. Food adulteration and contamination are the major issues, which need to be addressed through appropriate implementation of food laws within the country (28, 12). To control, screen and identify health problems caused by this chemical, a number of technical methods have been used in studies. High-performance liquid chromatography (HPLC), ion chromatography, enzyme-linked immunosorbent assay (ELISA) and spectrometry are the most commonly used methods. Chromatography methods such as gas chromatography/mass spectrometry (GC/MS), liquid chromatography/ mass spectrometry (LC/MS) and capillary zone electrophoresis (CZE) are widely used for the identification and quantification of melamine. Fluorescent nanoparticles of quantum dots are used for biological imaging in various techniques (6, 34). Immunoassays are good alternatives as they provide quick, cost-effective analysis methods. These methods can be used for on-site or in-field screening of melamine from food samples (9). Regarding health hazards associated with melamine, strictest standards of acceptable levels of contaminants within food products should be applied. Competitive national food safety systems should be established to monitor, prevent and detect illegal activities. Comprehensive studies should be carried out to prevent contamination of melamine and its analogs in food industries (32). The current study can facilitate this process in Pakistan by focusing on detection of melamine quantity within milk products readily, which serves as the first milestone in regulation of melamine within the food products from local markets. Increases in import of milk-based products with lacks of regulatory practices in food sectors of Pakistan emerge investigation of such screening methods that could ensure melamine limits in food products of the market. Therefore, the major aims of the present study were to screen melamine levels and their relationships with protein contents in various packaged milk samples and milk-based infant formulas available in Pakistan local markets. To achieve these aims, ELISA based method was used for the assessment of melamine in various food products.

Materials and Methods

Materials and Methods

Samples were analyzed for screening melamine, total protein, non-casein nitrogen (NCN) and non-protein nitrogen (NPN) using ELISA. Thirty samples were totally analyzed, including branded liquid milks, powder milks (tea whiteners), infant formulas, pet foods and chocolates collected from local markets of Lahore, Pakistan. MaxSignal Melamine ELISA Test Kit (catalog no. 1077)

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was purchased from BIOO Scientific, USA. The test kit was a competitive enzyme immunoassay for the quantitative analysis of melamine with capacity of 96 determinations. Chemical and reagents used in the sample preparation and assay such as extraction buffers, melamine standards, wash solutions, antibody mixes, HRP conjugate substrates and stop solutions were provided by the manufacturer. All the chemicals were used based on the guidelines provided by the manufacturer.

Sample preparation for milk, infant formula, chocolate and feed

Various quantities (1 ml for milk, 1 g of infant formula or chocolate and 0.5 g of feed) of the samples were collected. Various quantities of buffers were used for various samples as shown in Table 1.

Table 1. Quantities of the reagents used in sample preparation

Reagent	Milk	Infant formula	Chocolate	Feed
Melamine Sample Extraction Buffer	4mL	4mL	5mL	5mL
Melamine Clean Up Buffer	250µl	250µl	250µl	250µl
Melamine Sample Balance Buffer	250µl	100µl	250µl	250µl

Melamine sample extraction buffer was added to the sample in a tube and mixed for 5 s. Then, cleanup buffer was added to the tube and vortexed at the maximum speed for 3 min. Sample tube was centrifuged at 4,000 g for 10 min and 250 µl of clear supernatant were transferred into another tube and diluted with 1× melamine sample balance buffer. Diluted extract was vortexed for 15 s and re-centrifuged at 4,000 g for 2 min. The clear supernatant was collected in another tube and stored. Then, 50 µl of the supernatant were used for the assay.

Enzyme-linked immunosorbent assay procedure

Briefly, 50 µl of each melamine standard in duplicate were added into various plate wells from low to high concentrations (e.g. 5, 20, 50, 100 and 200 ng/ml). Two wells were left blank with no standards. After adding standards, 50 µl of each sample in duplicate were added into various sample wells. Then, 100 µl of the melamine antibody mix were added to each well and mixed gently for 1 min. Plate was incubated at room temperature for 30 min. After incubation, plate was washed three times with 250 µl of the wash solution. After the last wash, plate was inverted and gently tapped on a paper towel to remove excess reagents. Then, 100 µl of TMB substrate were added into each well and mixed gently. Plate was incubated at room temperature for 20 min . After the incubation, 100 µl of the stop solution were added into each well to stop the enzyme reaction. Following addition of the stop buffer, plate was read at 450 nm using microplate reader (BIO-RAD Model 680, USA). Reading was carried out twice.

Melamine concentration calculation

Relative absorbance from each reference standard was used to generate standard curve against reference standard concentration (ng/ml). The relative absorbance (%) was calculated by the following formula.

$$\text{Relative absorbance (\%)} = \frac{\text{absorbance of standard (sample)} \times 100}{\text{Absorbance of zero standard}}$$

Analysis of the samples through protein estimation

Nine milk and nine infant formula samples were analyzed using protein estimation method. Total protein, NCN and NPN (%) were assessed using official methods of analysis by AOAC 2016 (1).

Results and Discussion

Enzyme-linked immunosorbent assay results

In the present study, a competitive enzyme-linked immunosorbent assay (cELISA) was used for milk, infant formula, tea whitener, chocolate and pet food samples. The assay was carried out to find melamine adulteration. Further protein estimation analyses of milk and infant formula samples were carried out to validate the results.

Calibration curve

Five melamine standards with concentrations of 5, 20, 50, 100, 200 ng/ml were used. Relative absorbance was calculated based on the formula as previously described (Figure 1).

Enzyme-linked immunosorbent assay plates during assay

All 30 samples were loaded in duplicate into the ELISA plate. The ELISA was carried out using MaxSignal Melamine Test Kit (catalog no. 1077). Blue color was resulted from enzyme-substrate reactions and yellow color from adding stop solution (Figures 2a, b).

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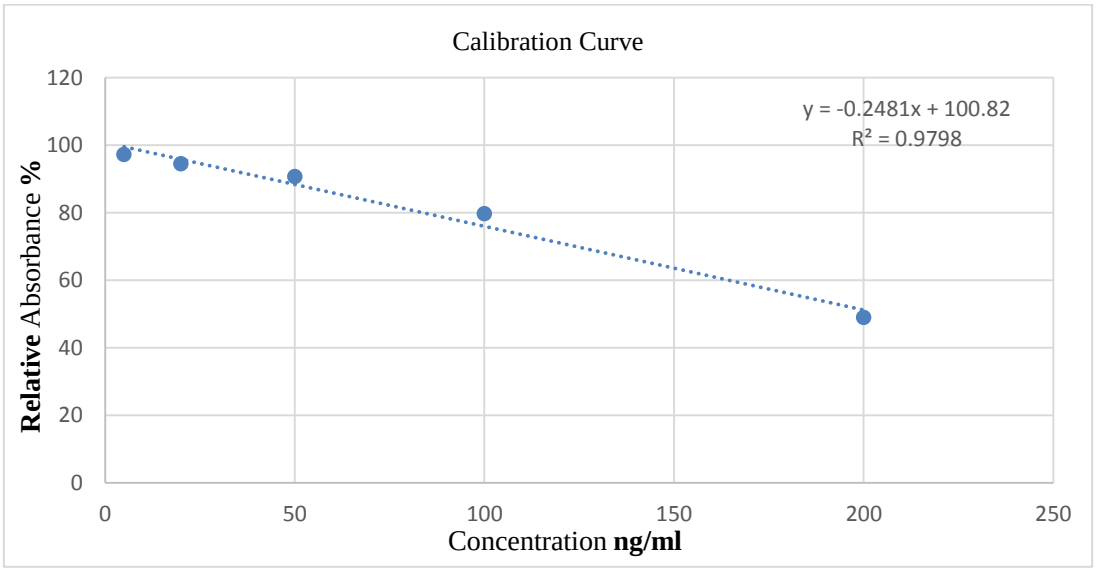
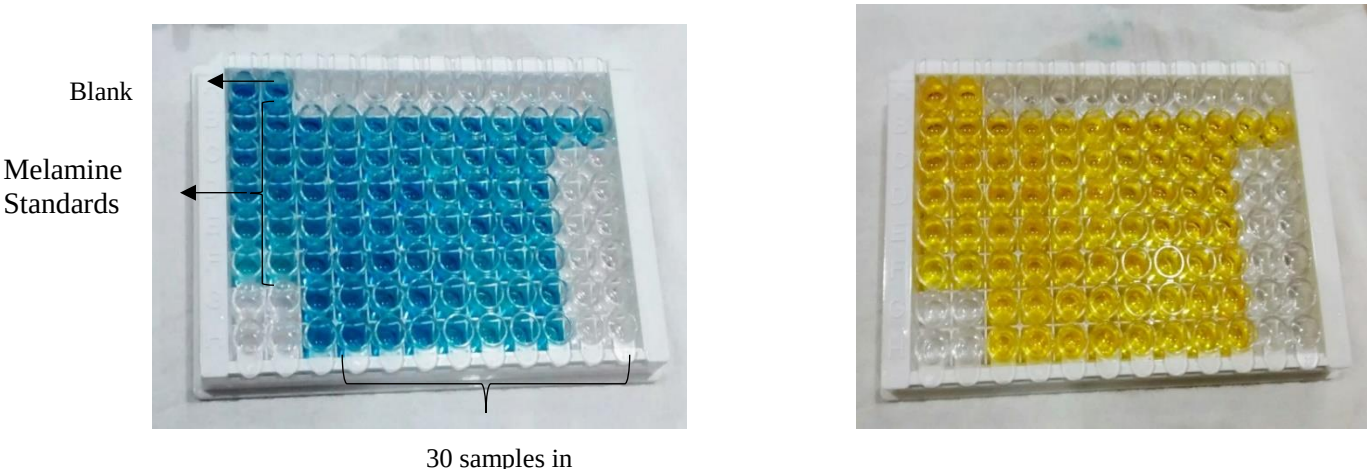


Figure 1. Calibration curves of five melamine standards used in the assay



(a) **(b)**

Figure 2. (a) ELISA plate during reaction, (b) ELISA plate after reaction

Enzyme-linked immunosorbent assay plates during assay

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Protein analysis of milk and infant formula samples validated the presence of melamine. Comparison of melamine, total protein, NPN and NCN estimation results of the infant formula/milk powder samples is shown in Figure 3.

Comparison of melamine, total protein, NPN and NCN estimation results of the liquid milk samples is presented in Figure 4.

Melamine was detected in the range of 0.05–0.69 ppm in infant formula/milk powder samples of Nestle Buniad and Meiji, respectively, as well as 0.001–0.042 ppm in milk samples of Milkville and Good Milk, respectively. These results were similar to those of a previous study by Suhaimi et al. (2011). In their study, ELISA and LC/MS were used for melamine screening in raw milk and animal food samples. Melamine was not detected in raw milk samples using ELISA, while 4.5% of the food samples contained melamine in the range of 1–5 ppm.

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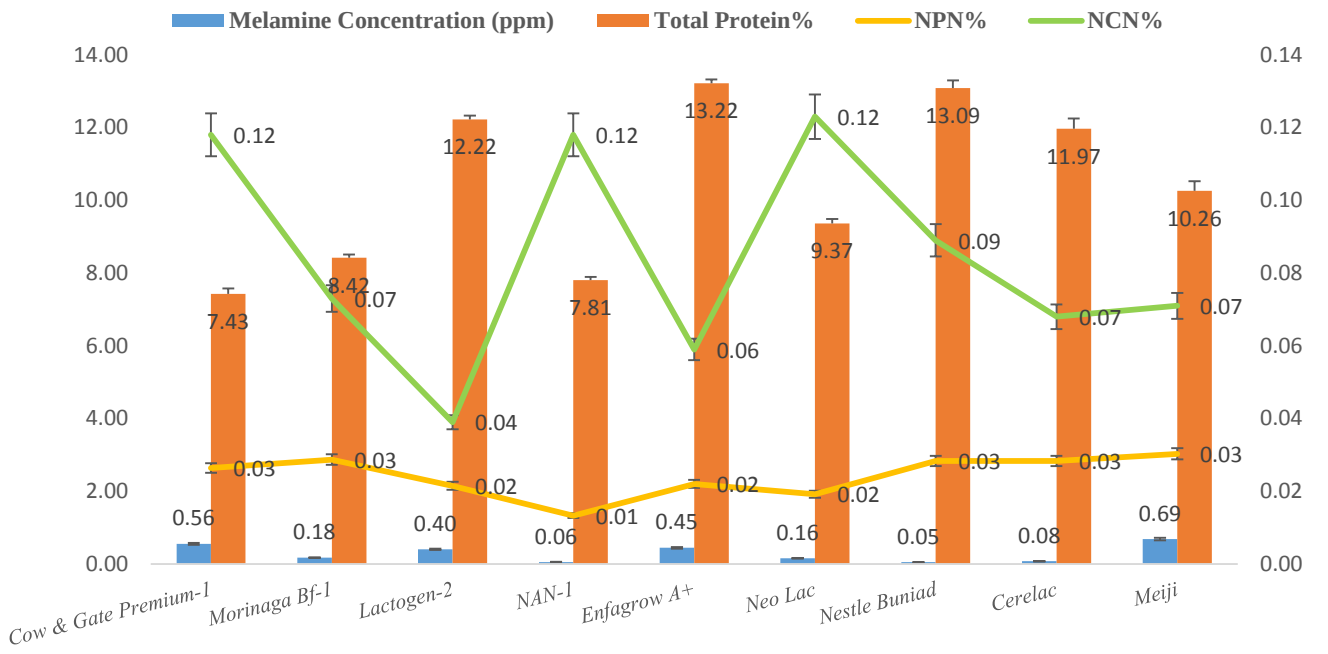


Figure 3. Comparison of melamine, protein NPN and NCN estimation results of the infant formula/milk powder samples

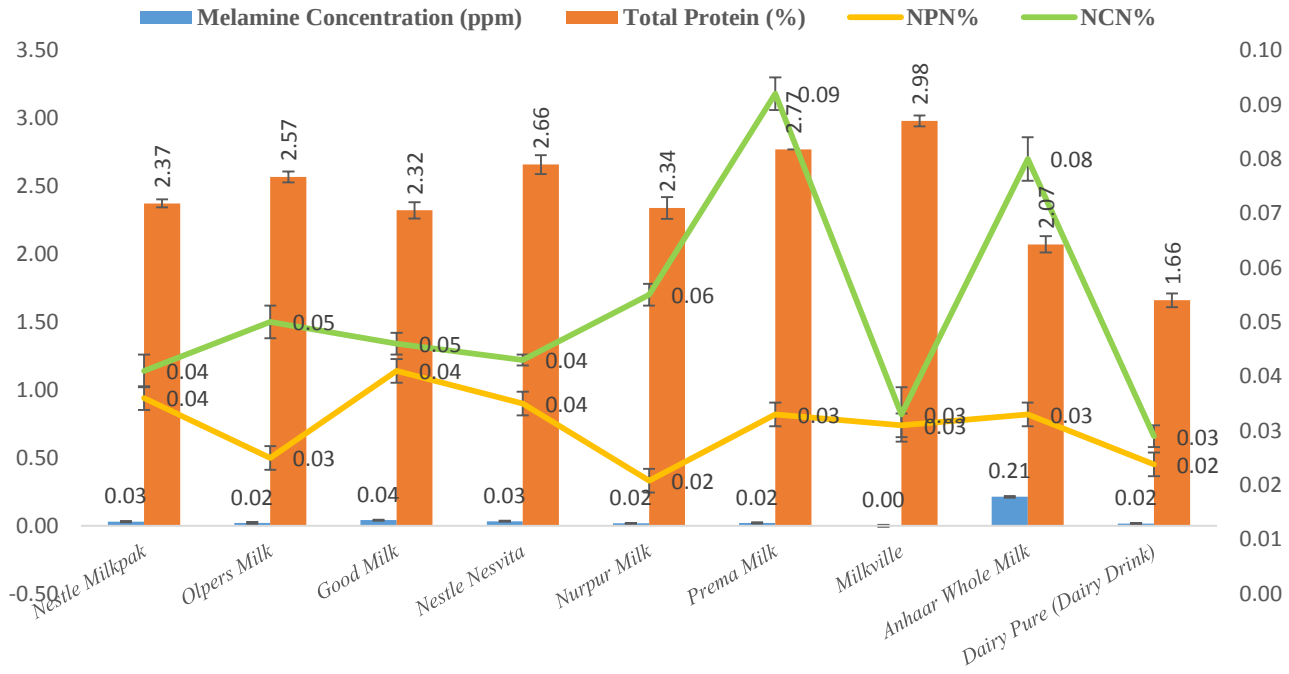


Figure 4. Comparison of melamine, protein, NPN and NCN estimation results of the liquid milk samples

Samples with higher melamine levels were subjected to crude protein analysis using Kjeldhal method; as in the present study. A similar technique was used in a local study; in which, competitive ELISA method was used to detect melamine in 84 commercially available pet food samples. Out of all positive samples, 53% of the samples contained melamine beyond the set limits (Iffat et al., 2015). Gerber (9, 8) has reported that ELISA kits were good alternatives for quantitative analysis of melamine in food products. The researcher used commercial ELISA kits to screen melamine in infant formula, milk, animal food and wheat samples. Specificity, cross-reactivity and ability to detect melamine of the two kits were assessed. In general, ELISA was reported as a good detection method because of its simplicity and low costs. All these studies used ELISA to verify presence of melamine in milk samples using various methodologies. However, all the studies provided similar results, reporting that melamine

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was found in the analyzed samples. Competitive ELISA has been used in a number of studies. Various studies have been carried on the development of assays for melamine detection using various antibodies (17). Melamine specific antibodies are produced for screening of melamine in food samples. Lei et al. (2011) used this method for the detection of melamine in milk. Plate coating antigens were prepared by three haptens of melamine with various spacer arms. Efficiency of the assay was more than that in the present study due to the low detection range (0.01–0.1 ppm), compared to 0.2 ppm. Melamine specific antibodies were coated in 96-well microtiter plates and standard ELISA protocol was used for the assay using melamine standards, goat anti-rabbit horseradish peroxidase (HRP) and 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Sun et al. (2013) used a similar technique in their study; in which, they produced melamine specific antibodies for ELISA. Melamine in tea whiteners and chocolates is presented in Table 2 and melamine in food samples is shown in Table 3. Linearity of the calibration standard was close to 0.97 and the range of melamine concentration was 0.001–1.163 ppm (Figures 3 and 4, Tables 2 and 3). This range was less than the set limits of Codex Alimentarius 2012 (5).

Yin et al. (2010) developed an indirect competitive ELISA (cELISA) based on monoclonal antibodies to melamine, which were prepared by linking melamine haptens to carrier proteins using carbodiimide method. Limits of the detection included 0.1 mg/l for milk, 0.2 mg/kg for milk powder and 0.5 mg/l for feeds, which were quite close to 0.2 ppm limit of detection of ELISA in the current study using melamine antibody provided by the manufacturer. Low-cross reactivities of the melamine to ammelide, cyanurate and ammeline were detected in the two studies. A monoclonal-antibody based inhibition ELISA was developed by Zhou et al. (2012) for the detection of melamine in milk and pet food samples. The antibody was produced through immunization of rats by complete or incomplete Freund's adjuvant (CFA/IFA). In general, 0.01 ng/ml limit of detection (LOD) was achieved. In various studies, efficiency of the competitive ELISA was enhanced using chemiluminescent or fluorescence antibodies. Chemiluminescent substrate was used in a study by Li et al. (2014). They developed melamine-specific polyclonal antibodies. The ELISA was carried out based on a general protocol, except that chemiluminescent substrate was used and the intensity of chemiluminescence was quantified. The antibodies showed no cross reactivity with other drugs and limit of detection was as low as 1 ng/ml. Wang et al. (2014) used this method in their study. They developed polyclonal antibodies by coupling various haptens of melamine with bovine serum albumin (BSA) and immunizing rabbits with this mixture. Three fluorescein-labelled melamine tracers were prepared and their structural effects on the assay were studied. Use of fluorescent nanoparticles or quantum dots has also been discussed in various studies. Li et al. (2010) reported the effectiveness of this method. These kits were capable for qualitative and quantitative field detections of melamine. A similar method was used in a previous study; in which, secondary-antibody conjugated quantum dots were used to develop an indirect competitive fluorescence-linked immunoassay. Results of icFLISA were similar to those of cELISA (34). Studies have revealed that ELISA serves as an effective melamine screening method (18).

Table 2. Melamine concentrations (ppm) in tea whiteners and chocolates

Sr. No.	Tea whiteners	Concentration (ppm)	Chocolates	Concentration (ppm)
1	Haleeb All Max (Tea Whitener)	0.156 ± 0.021	Cadbury Dairy milk (Chocolate)	0.475 ± 0.050
2	Nestle Everyday (Tea Whitener)	0.027 ± 0.005	Snickers (Chocolate)	0.179 ± 0.023
3	Tarang (Tea Whitener)	0.124 ± 0.009	Toblerone (Chocolate)	0.379 ± 0.040
4	TeaMax (Tea Whitener)	0.047 ± 0.002	Mars (Chocolate)	0.208 ± 0.035

Table 3. Mean melamine concentrations (ppb) in pet foods

Sr. No.	Pet Feeds	Concentration (ppm)
1	Smart heart (Adult Cat food)	1.163 ± 0.09
2	Me-O (Cat Food)	0.136 ± 0.05

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Protein content determination through Kjeldahl method with ELISA is also a good analytical method. However, development of further appropriate protein estimation method is necessary to detect multiple protein adulterants in food products (21). This method can be used for the detection of melamine in a wide range of samples such as chocolates, feeds, fish/meats and milks. The MaxSignal ELISA kit was able to detect melamine with concentrations as low as 0.2 ppm. However, validity of the method can be improved if verification assays such as GC/MS and HPLC coupled to MS are used. Recent developments in this technique with use of fluorescent nanoparticles have detected adulterants much simpler and quicker. On-site melamine detection techniques can also help rapid screening of melamine in food products. This method has verified useful as it can be used with various modifications for the quantitative screening of melamine.

Conclusion

As a result of rapid increases in food adulterations worldwide, a number of melamine screening methods are used nowadays, including chromatography, spectrometry and ion separation. Of these methods, ELISA is an easy cost-effective method. Owing to the commercial availability of ELISA kits, melamine detection has become easy. The MaxSignal ELISA Test Kit used in this study was able to detect 0.001–0.042 ppm melamine in milk, 0.05–0.69 ppm in infant formula, 0.179–0.475 ppm in chocolate and 0.136–1.163 ppm in pet food samples. Melamine antibody used in the assay showed a complete cross-reactivity to the target analytic. However, a less cross-reactivity (> 15%) was detected within melamine analogs (cyanurate, ammeline and ammelide). Linearity of the calibration standard was close to 0.97 and the range of melamine concentration was 0.001–1.163 ppm. The total assay was carried out within 30 min. However, validity of the method can greatly be improved if verification assays such as GC/MS and HPLC coupled with MS are used. Recent developments in this technique using fluorescent nanoparticles have made detection of adulterants much simpler and quicker. Furthermore, on-site melamine detection techniques have provided rapid screening of melamine in food products. In conclusion, ELISA can be used as a reliable cost-effective method with various modifications for the quantitative screening of melamine.

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