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Detection of Melamine Using Commercial Enzyme-Linked Immunosorbent Assay Technology

ERIC A. E. GARBER*

Division of Bioanalytical Chemistry, Office of Regulatory Science, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, Maryland 20740, USA

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ABSTRACT

Recent cases of adulteration with melamine have led to the need for rapid and reliable screening methods. To meet this need, commercial enzyme-linked immunosorbent assay (ELISA) test kits for the detection of triazines were evaluated. The recently released Melamine Plate kit (Abraxis, Warminster, Pa.) displayed a limit of detection of 9 ng/ml for melamine in phosphate-buffered saline (PBS) and approximately 1 μg/ml for melamine added to dog food. An atrazine ELISA test kit produced by Abraxis required 0.2 mg/ml to generate a response more than four times the standard deviation from background. In contrast, with the EnviroGard Triazine Plate kit (Strategic Diagnostics, Inc., Newark, Del.), 1.5 mg/ml melamine in PBS generated a signal only one standard deviation from background, which was insufficient to define a limit of detection. Extraction based on dilution with 105 mM sodium phosphate/75 mM NaCl/2.5% nonfat milk/0.05% Tween 20 (UD) enabled detection of fivefold less melamine in dog food than did use of the procedure recommended by the manufacturer, which entailed extraction into 60% methanol, sonication, centrifugation, filtration, and further dilution into 10% methanol/PBS. Using the Abraxis Melamine ELISA, both extraction protocols yielded identical results with a dog food sample adulterated with melamine. The recovery of melamine spiked into gravy from dog food using UD was 74% ± 4%. In conclusion, the recently released Abraxis ELISA for melamine proved to be a useful alternative to more cumbersome methods.

The discovery of melamine in pet food, animal feed, and protein sources including wheat gluten, rice protein concentrate, and corn gluten created an urgent need for rapid methods for detecting melamine in food *(1, 2, 8, 10, 14).*

Melamine (2,4,6-triamino-1,3,5-triazine) is used in the production of plastics, in finishers for paper, in fertilizer, as a flame retardant, and in the manufacture of wrinkle-free textiles. The use of melamine as a nonprotein nitrogen source for cattle was studied by Newton and Utley *(9).* Although the melamine was digested, most of the nitrogen was eliminated in the urine and not assimilated by microbes in the rumen. Melamine is classified by the World Health Organization as not posing a health risk *(13).* The use of melamine-formaldehyde resins in the production of molded plastics and as coatings in contact with food is approved by the U.S. Food and Drug Administration (FDA) as long as the yield of chloroform-soluble extractives does not exceed 0.5 mg/in^2 of food contact surface (21 CFR 177.1460).

The triamino structure of melamine is both an advantage and disadvantage in the development of analytical methods. By controlling the pH and counter ions present, it is possible to adjust the chromatographic properties of melamine. In addition, under acidic conditions, melamine exhibits a strong ultraviolet absorbance, which makes it amendable to methods employing UV detection. However, the complex composition of food makes both UV detection and acidic conditions problematic. Such problems can be circumvented by extracting the melamine from the food prior to acidification. This was the approach Ishiwata et al. *(7)* used to study the transfer of melamine into hot, acidic beverages (limit of quantitation [LOQ], 50 ng/ml) by liquid chromatography. An alternative approach taken to enable the use of C-18 chromatography in tandem with electrospray mass spectrometry involved extending the elution profile by including tridecafluoroheptanoic acid to generate ion pairs *(11).* The limit of detection (LOD) and LOQ using this ion pair approach were 0.01 and 0.1 mg/kg, respectively. Another approach that employed C-18 chromatography to detect melamine entailed using 20% concentrated ammonium hydroxide in acetonitrile as the mobile phase to detect melamine in meat and eggs with an LOD of 0.02 mg/kg *(4).* Trimethylsilyl derivatization provided an alternative method for handling the three amino groups *(12).* According to this method originally developed by FDA's Forensic Chemistry Center, the pet food sample is extracted using methanol followed by sonication, centrifugation, filtration, and drying. The sample is then silanized using bis(trimethylsilyl)trifluoro-acetamide and detected using gas chromatography/mass spectrometry with an LOD of 10 μg/ml and a recovery from dry pet food of 87%.

Commercial ELISA test kits were examined as a potential solution to meet the need for high-throughput screening of samples for the presence of melamine. In addition to a commercial ELISA for the detection of melamine, two test kits for triazines (i.e., the herbicide atrazine) were examined for cross-reactivity. All three ELISAs de-

* Author for correspondence. Tel: 301-436-2224; Fax: 301-436-2332; E-mail: eric.garber@fda.hhs.gov.

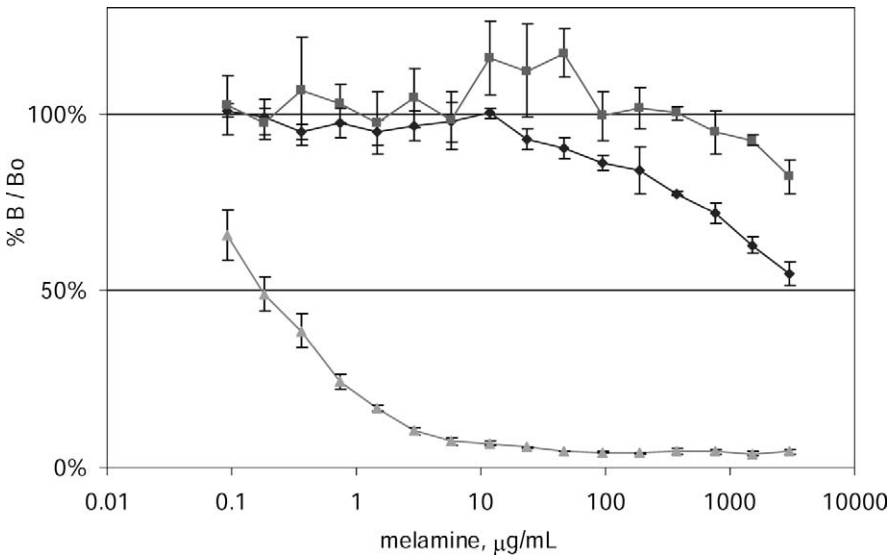

FIGURE 1. *Detection of melamine in PBS using the Strategic Diagnostics, Inc., EnviroGard Triazaine assay (—■—); Abraxis Atrazine ELISA (—◆—); and Abraxis Melamine ELISA (—▲—). The measured responses were plotted based on the concentration of melamine in the analytical sample. The samples analyzed using the Abraxis Atrazine ELISA were not diluted in half prior to placing 50 μl into each well of the microtiter plate; thus, the concentrations of melamine and the solvent milieu were identical for all assays. The data points represent the average ± SD of three samples.*

tected melamine. However, only the ELISA specifically manufactured for the detection of melamine displayed an LOD comparable with, if not better than, those reported in most published methods. Furthermore, the melamine ELISA did not require extensive sample preparation.

MATERIALS AND METHODS

Melamine and phosphate-buffered saline (PBS; catalog no. P3813) were acquired from Sigma-Aldrich Chemical Company (St. Louis, Mo.). Dog food (dry and canned beef stew) was purchased locally. Gravy from canned "country stew dog food" was prepared by removing the meat chunks and storing the remainder at −20°C. The gravy was used as-is with no attempt to remove the vegetable matter. Dry dog food was ground immediately prior to use and stored in airtight containers. An investigatory sample of dog food suspected of containing melamine was supplied as a puree.

ELISA test kits. Abraxis (Westminster, Pa.) Melamine Plate kit (catalog no. 5005B), Abraxis Atrazine ELISA (product no. 520005), and Strategic Diagnostics, Inc. (Newark, Del.) EnviroGard Triazine Plate kit (product no. 7211000) were purchased from the manufacturers. The decision to examine these specific atrazine and triazine ELISAs versus analogous products produced by other suppliers was based on the cross-reaction properties reported by the manufacturers (either in supplied documentation or orally), which indicated a likelihood of cross-reactivity. A commercial ELISA produced by Beacon Analytical Systems, Inc. (Portland, Maine) was not examined due to a reported cross-reactivity with melamine of <0.005% by the test kit manufacturer.

The ELISA test kits were initially used according to the recommendations of the manufacturer. Subsequently, changes that improved sensitivity or eliminated unnecessary steps were also evaluated. One step found to be unnecessary was the manufacturer's recommendation that pet food samples be filtered through G-6 glass filters following centrifugation prior to analysis using the melamine ELISA.

All three ELISAs use a competitive design and thus display a decrease in response when antigen is present in the sample; this is expressed as the percent response (% B/B_0) defined as the response generated by a sample (B) versus the response generated by a control sample lacking melamine (B_0). The LOD was defined as the concentration of melamine necessary to generate a response greater than four times the standard deviation (SD) from background.

RESULTS

Detection of melamine using ELISA test kits. Figure 1 compares the results obtained from the analysis of melamine in PBS using ELISA test kits for the detection of melamine, atrazine, and triazines.

All three ELISAs detected melamine. However, the test kit for the detection of triazines required ≥1,500 μg/ml melamine to display a positive response 1 SD from background, insufficient to enable the assignment of a reliable LOD value.

The atrazine ELISA generated responses significantly different from background for PBS samples containing ≥23 μg/ml melamine with an LOD of 0.2 mg/ml. Identical results were obtained whether or not the PBS samples were diluted twofold prior to analysis (Fig. 2). This indicates that the test kit manufacturer's recommendation of a twofold dilution of the analytical sample could be omitted for samples prepared in PBS. Omission of the twofold dilution improved the LOD when expressed as the concentration of melamine in the original sample.

The melamine ELISA generated positive responses with all samples containing ≥9 ng/ml melamine. The LOD values for melamine in the analytical samples prepared from PBS, 105 mM sodium phosphate/75 mM NaCl/2.5% nonfat milk/0.05% Tween 20 (UD), gravy from dog food, and dry dog food were 9, 28, 9 ng/ml, and either 9 or 25 ng/g depending on the method of extraction, respectively (see Table 1). The test kit standards reproducibly displayed LOD values of <20 ng/ml.

The responses generated by the melamine ELISA deviated from exponential trendlines at melamine concentrations >750 ng/ml, with 3 μg/ml melamine having a % B/B_0 value of 10%; both characteristic of the onset of saturation. As such, the test kit was appropriate for the analysis of samples containing between 30 ng/ml (>LOD) and 750 ng/ml melamine, a range that encompassed the test kit standards.

Figure 3 examines the effectiveness of the melamine ELISA to detect melamine spiked into dry dog food using the above two extraction procedures. The first procedure

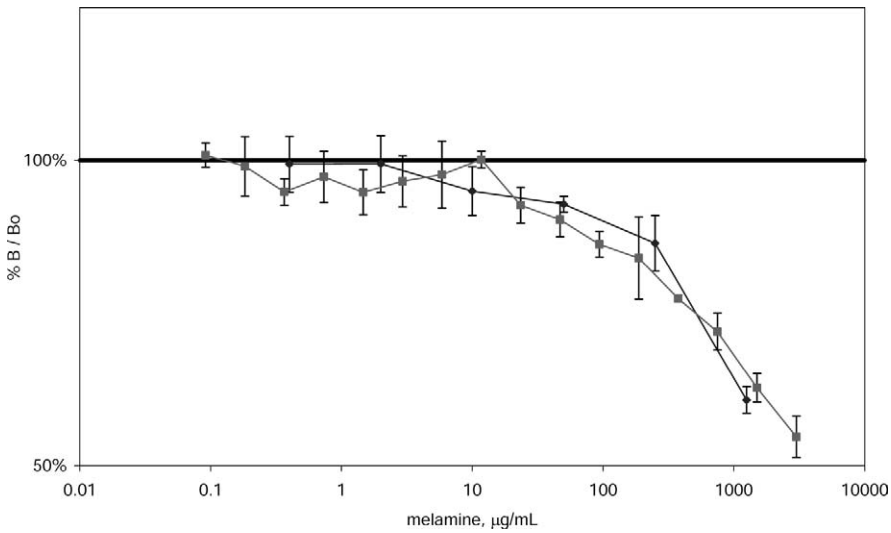

FIGURE 2. *Analysis of melamine in PBS using the Abraxis Atrazine ELISA. Samples were analyzed either according to the test kit protocol, which included a twofold dilution prior to analysis (—◆—), or omitting the additional dilution step (—■—). The data (average ± SD,* n = *3) were plotted versus the final concentration of analyte in the well of the microtiter plate.*

was recommended by the test kit manufacturer for pet food and entailed extraction of the ground sample with 60% methanol, vortexing, sonication, centrifugation, and dilution into 10% methanol, resulting in a 200-fold dilution. The inclusion of a filtration step following centrifugation was omitted as described in ''Materials and Methods.'' The second protocol entailed mixing the ground sample 1:40 with UD with no further processing. As indicated by Figure 3A, the UD procedure was more sensitive when the two methods were compared based on the concentration of melamine in the original, spiked sample. However, the results from the two extraction procedures overlapped when the data were plotted based on the concentration of melamine in the analytical sample following extraction and preparation (Fig. 3B). Thus, the increased sensitivity of the UD method was entirely due to the sample being diluted only 40-fold and not 200-fold. An additional advantage of the UD method was the simplicity of sample preparation, which required minimal time and labor.

Analysis of dog food containing melamine. An investigatory sample of dog food, reported to contain melamine, was obtained. Aliquots of the sample were analyzed using the melamine ELISA according to both the manufacturer's recommended extraction protocol for pet food and the UD procedure. The melamine content was determined to be 1.2 ± 0.7 mg/ml ($n = 3$) and 1.1 ± 0.6 mg/ml ($n = 3$) using the two methods. The agreement between the two methods was consistent with the previously presented data (Fig. 3), which indicated that the only significant difference between the two methods was the fivefold greater dilution associated with the recommended protocol.

Recovery of melamine. Figure 4 compares the detection using the melamine ELISA of melamine in PBS, in

TABLE 1. *LODs for melamine in PBS and dog food*

		Background			LOD[a]			
	Diluent	OD_{450}	SD	Bkgd–4D	OD_{450}	SD	Initial µg/ml[b]	Diluted ng/ml[b]
PBS	PBS	1.81	0.04	1.66	1.628	0.009	0.37	9[c]
PBS	UD[d]	1.57	0.08	1.25	1.221	0.018	1.1	28
Dog food gravy	UD	1.2	0.02	1.12	1.11[e]	0.061[e]	0.37	9
Dry dog food	UD[f]	1.16	0.02	1.07	1.07[g]	[g]	[g]	9[g]
Dry dog food	UD[h]	0.93	0.02	0.85	0.86	0.09	1	25

[a] LOD, lowest concentration of melamine that generated a response more than four times the standard deviation from the background (Bkgd–4D).

[b] Concentration prior to (initial) and after (diluted) 40-fold dilution with PBS or UD.

[c] Another series of analyses gave an average background response of 1.676 ± 0.046 ($n = 6$) corresponding to an LOD of 8 ng/ml. Test kit standards yielded backgrounds of 1.20 ± 0.01, $n = 3$, and 1.549 ± 0.063, corresponding to calculated LODs of 9 and 17 ng/ml.

[d] UD, 105 mM sodium phosphate/75 mM NaCl/2.5% skim milk/0.1% Tween 20, pH 6.8.

[e] 1.1 µg/ml (initial)/28 ng/ml (diluted) melamine in gravy gave a response of 0.987 ± 0.052; at 3.3 µg/ml (initial)/83 ng/ml (diluted), the response was 0.731 ± 0.012.

[f] Extracted according to the manufacturer's recommended procedure, which entailed a 200-fold dilution of the initial sample.

[g] Extrapolation based on the trendline $y = 6.2251(\exp - 6.52486x)$, $R^2 = 0.9413$, where $x = \% B/B_0$ and $y =$ µg/g yielded an LOD of 9 ng/g. Of actual samples, one with a response ≤LOD had a response of 0.97, equivalent to an initial concentration of 10 µg/g and diluted at 50 ng/g.

[h] Extracted according to UD protocol, which entailed a 40-fold dilution with UD. All samples ran in triplicate unless specified otherwise.

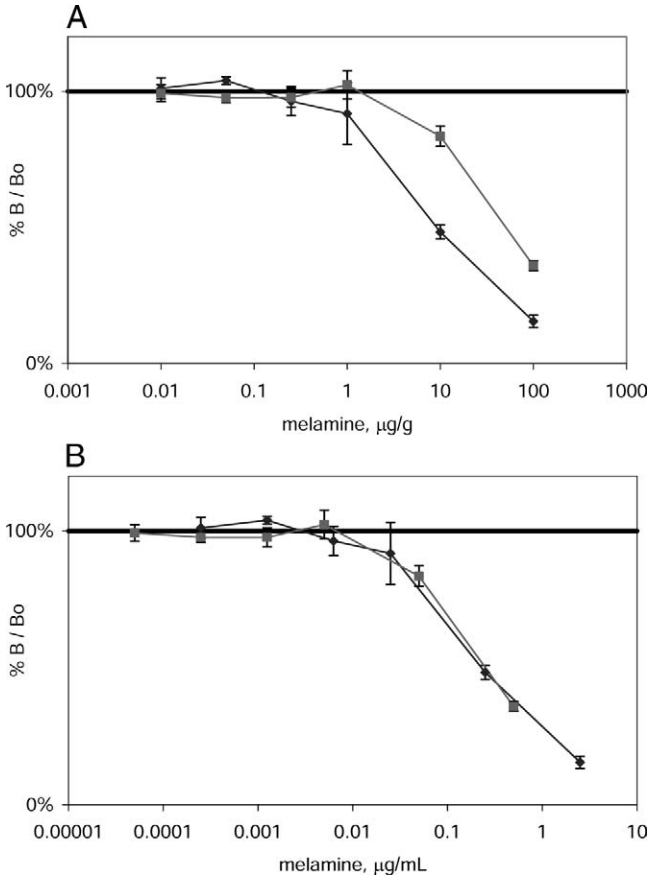

FIGURE 3. *Comparison of extraction protocols for the analysis of melamine spiked into dry dog food. Samples of dry dog food spiked with varying amounts of melamine were analyzed using the Melamine ELISA manufactured by Abraxis either according to the recommended protocol, which entailed extraction of the ground dog food with 60% methanol, vortexing, sonication, centrifugation, and dilution into 10% methanol (—■—) or mixed with UD buffer (—♦—). Figure 3A depicts the* % B/B_0 *plotted based on the initial concentration of melamine spiked into the dog food, and Figure 3B depicts the same data plotted based on the concentration of melamine in the analytical sample added to the microtiter plate well. The data points represent the average ± SD of three samples.*

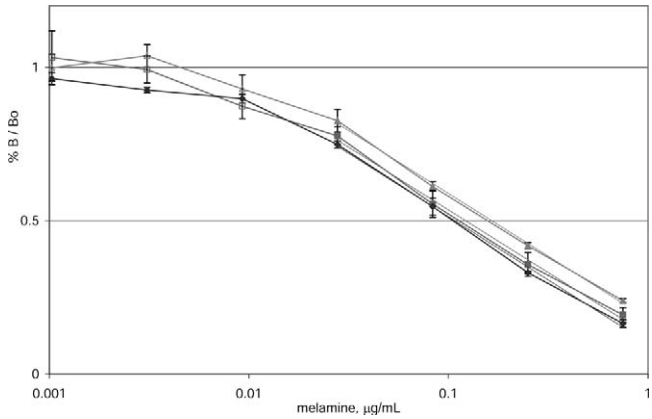

FIGURE 4. *Detection of melamine in PBS and dog food gravy. —♦—, PBS spiked with melamine and diluted 40-fold with PBS; the dashed line depicts the trendline described by* y = *−0.179 ln*(x) + *0.1012 (*R^2 = *0.9973). —■—, PBS spiked with melamine and diluted 40-fold with UD; the dashed line depicts the trendline described by* y = *−0.1773 ln*(x) + *0.1273 (*R^2 = *0.9957). —▲—, Dog food gravy spiked with melamine and diluted 40-fold with UD; the dashed line depicts the trendline described by* y = *−0.1778 ln*(x) + *0.1798 (*R^2 = *0.9983). The abscissa reflects the concentration of melamine in the analytical sample following dilution. The data points represent the average ± SD of three samples.*

PBS subsequently diluted 40-fold with UD, and in spiked into dog food gravy subsequently diluted 40-fold with UD. Recoveries were calculated based on the responses generated by samples within the linear region of the ELISA, 28 to 750 ng/ml. The recovery of melamine spiked into gravy diluted with UD was 74% ± 4% (n = 4 concentrations, each the average of three samples) when compared with the responses generated by melamine dissolved in PBS diluted with UD. The percent recovery of melamine in PBS diluted with UD versus PBS was 88% ± 8% (n = 4 concentrations, each the average of three samples).

The gravy samples and PBS samples, both sets diluted 40-fold with UD, generated parallel curves (see Fig. 4) described by trendlines with equations of $y = -0.1778 \ln(x) + 0.1798$ ($R^2 = 0.9983$) and $y = -0.1773 \ln(x) + 0.1273$ ($R^2 = 0.9957$), where y = % B/B_0 and x = µg/ml melamine, respectively. Rearranging the equations to better illustrate the parallel responses yielded $y = 2.7366 \exp(-5.6158x)$ ($R^2 = 0.9983$) and $y = 2.0266 \exp(-5.6165x)$ ($R^2 = 0.9957$), where y = µg/ml melamine and x = % B/B_0. In contrast, the equation that described the detection of melamine in PBS was $y = 1.7484 \exp(-5.5705x)$ ($R^2 = 0.9973$). The occurrence of identical slopes for the gravy and PBS samples diluted in UD was consistent with the minimization of matrix effects by the 40-fold dilution. Furthermore, the nonparallel trendline for melamine in PBS reflected the differences between PBS and UD as a solvent.

DISCUSSION

All three of the ELISA test kits successfully detected melamine. The LOD values varied with the test kit with the melamine ELISA having an LOD of 9 ng/ml. All three test kits would have been able to detect the 200 mg/g melamine contaminating the wheat gluten used to make pet food and animal feed *(3).* However, only the melamine ELISA detected melamine at concentrations sufficiently low enough to surpass other validated methods. As such, the Abraxis ELISA for Melamine provides a rapid, high-throughput alternative for the screening of samples for the presence of melamine. Furthermore, an LOD of 9 ng/ml in the analytical sample meant the ELISA was compatible with inclusion of a 40-fold dilution with UD prior to analysis, conditions that obviate food matrix effects, including false positives that may result from high levels of wheat lectin *(5, 6).*

The effects of large concentrations of analyte are often a concern when working with immunology-based biosensors because the affinity constants of the antibodies and the amount of productive complexes necessary to generate a discernable signal are often orders of magnitude less than

the target quantity of analyte. Immunoassays that employ a sandwich design and do not include a step to remove free antigen, which may compete with complexed antigen for the detector, are subject to a hook effect. Besides eliminating the ability to quantitate analyte, hook effects can result in false negatives unless the signal-to-noise is sufficiently large that the decrease in response does not approach background. All three of the test kits examined involved the use of a competitive ELISA design in which the target analyte (i.e., melamine) in the test sample competes with a labeled antigen, supplied with the test kit, to bind with the capture antibody. Thus, all three assays should display a saturation response with no hook effect at high concentrations of analyte. This was confirmed using 108 μg/ml atrazine (data not shown) and 3 mg/ml melamine, the former orders of magnitude greater than the LODs of the triazine and atrazine ELISAs. As a consequence of not undergoing a hook effect, all three assays are amendable to a configuration in which a single incubation step is used for all reagents, including the sample. Such a format is amendable to high throughput.

The observation that dilution with PBS compared with UD resulted in different trendline slopes describing the detection of melamine represented by −5.571 versus −5.616 and −5.617 may provide a useful approach for assay optimization and troubleshooting the analysis of difficult food samples. Food samples often entail a combination of multiple factors requiring optimization (i.e., pH, ionic strength, dielectric, etc.). By standardizing how specific solvent effectors alter it, the slope may provide a tool for deciding which modification(s) to make to optimize an assay. Furthermore, the ability to quantify differences may facilitate troubleshooting subtle features and provide an indicator for future reference purposes when comparing kits.

In conclusion, the Abraxis ELISA for Melamine proved to be a reliable, sensitive method for screening samples for the presence of melamine. In addition, the Abraxis ELISA for Melamine displayed a sensitivity comparable with or better than those of the more cumbersome methods currently employed.

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