



Melamine testing in pet food

Automated and sensitive high throughput ELISA testing

About Vet Food Agro Diagnostics Sdn Bhd

Vet Food Agro Diagnostics Sdn Bhd (VFAD) is a service laboratory based in Malaysia, which provides a wide range of screening and testing services to the veterinary, food, agriculture and agro-based industries across Southeast Asia. VFAD works very closely with local government authorities, such as the Malaysian Department of Veterinary Services (DVS), on the control and prevention of infectious animal diseases, as well as a variety of food safety programs. This work has led to numerous awards and recognitions, as well as development of a number of patent-pending inventions.

Increased awareness of and interest in determining melamine contamination in various animal feed and human food products, led Vet Food Agro Diagnostics Sdn Bhd to develop and validate an automated enzyme-linked immunosorbent assay (ELISA) using the AgraQuant[®] Melamine ELISA Test Kit from Romer Labs[®].

Introduction

Melamine (1,3,5-triazine-2,4,6-triamine, Figure 1) is a white crystalline compound used in the manufacture of products such as plastics, glues and flame retardants.

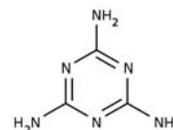


Figure 1 Structural formula of melamine

In 2007, scientists determined a link between melamine contamination in animal feed, and subsequently infant formula, and serious illness and mortality in pets and human infants. Pet owners reported symptoms commonly associated with renal failure, which could have arisen from the digestion of melamine. It became apparent that, due to its high nitrogen content, melamine had been added to food products as an extender. Since protein concentrations are typically measured

by the analysis of total nitrogen, this gave the appearance of higher protein levels.

Tecan's liquid handling and detection instruments provide an excellent solution for simple, fast and reliable screening of melamine in various samples, and VFAD uses the Tecan Freedom EVO[®] 150 liquid handling platform to automate the AgraQuant Melamine ELISA Test Kit. Validation of VFAD's automated system shows comparable sensitivity between the ELISA method and LC/MSMS analysis, with good reproducibility.

Materials and methods

Materials

AgraQuant Melamine ELISA Test Kit

Instruments

Tecan Freedom EVO 150 liquid handling platform

Tecan Sunrise[™] absorbance reader

Tecan HydroFlex[™] plate washer

Platform configuration

In addition to liquid handling (LiHa) and robotic manipulator (RoMa) arms, the Freedom EVO 150 platform is equipped with a Sunrise absorbance reader, a HydroFlex plate washer and a barcode scanner, plus two hotels and a six-position room temperature incubator. Reagent troughs, a microplate carrier and microfuge tube racks are located in the loading area of the workstation.



Figure 2 Overview of the Freedom EVO 150 configuration



Figure 3 Detailed view of the loading area (left); reader and washer are integrated on the right hand side of the platform (right)

Procedure

The AgraQuant Melamine ELISA involves two procedures – sample extraction and an ELISA assay – and is performed according to the manufacturer's instructions. This application note describes the analysis of pet food (wet or dry), but the assay can also be used to measure melamine in milk, milk powder and wheat gluten.

Pet food is homogenized, then extracted with methanol:water (60:40 v/v). After centrifugation, the clear upper layer is filtered, and the filtrate collected and diluted 1:20 with sample diluent in preparation for the ELISA assay.

AgraQuant Melamine ELISA plates are prepared from strips and loaded into the hotel. Four standards and up to 40 samples can be analyzed per plate, per run, without compromising the reliability and robustness of the assay.

Standards and extracted samples (150 µl) are pipetted into antibody-coated microwells, enzyme-conjugated melamine (50 µl) is added to each well, and the solution mixed three times by drawing up and redispensing the liquid. After incubation in darkness at room temperature for 30 minutes, the plate is transferred to the HydroFlex washer and washed four times with wash buffer. Substrate (100 µl) is dispensed into each microplate strip, and room temperature incubation performed for a further 30 minutes. The intensity of the blue color produced is inversely proportional to the melamine concentration in the sample. Stop solution (100 µl) is added, causing a color change from blue to yellow, and the microplate is transferred to the Sunrise microplate reader for absorption measurement using an absorbance filter of 450 nm.

Validation results

VFAD validated the automated protocol to meet the manufacturer's requirements for the AgraQuant Melamine ELISA Test Kit.

Standard curves (Figure 4) were generated from the test kit on several occasions. The average r^2 value was 0.989, which is above the minimum value of 0.985 specified in the assay Certificate of Performance (CoP).

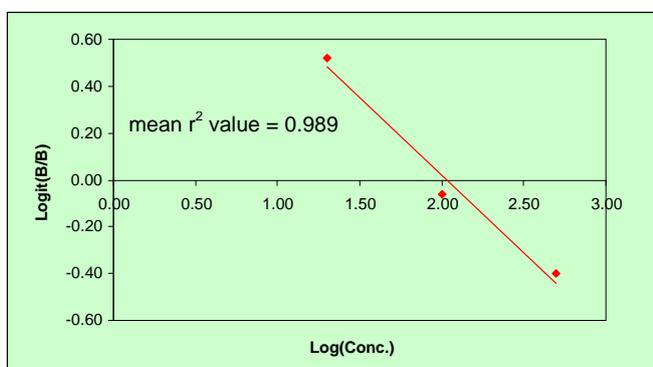


Figure 4 Evaluation of standard curves

A key aspect of the validation study was the exclusion of cross contamination between antibody-coated microplate wells. Melamine standard (positive control, 100 μ l of 100 ppb in 1 mL) and sample diluent (negative control) were added to the microwells, in a chequerboard pattern, on four test strips. Melamine was not detected in any of the negative control wells. The percentage CV of the positive control wells was 6.0 % (Table 2).

	Melamine 10 ppb (added to plate)
Mean concentration (ppb)	11.7
Standard deviation (ppb)	0.7
CV (%)	6.0

Table 2 Cross contamination check

Animal feed samples were spiked with melamine at five different concentrations and analyzed by AgraQuant Melamine ELISA and an in-house LC-MS/MS method. The data obtained by ELISA was compared to that obtained by LC-MS/MS analysis by plotting the concentrations determined

by the two methods against one another (Figure 5). This showed a high degree of correlation, with an r^2 value of 0.988.

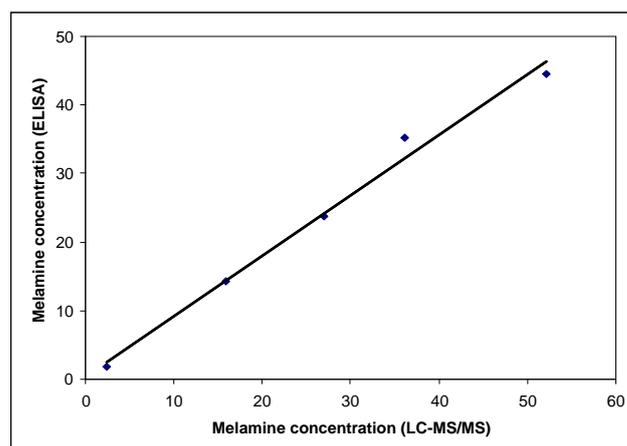


Figure 5 Comparison of melamine detection by ELISA vs. an LC-MS/MS method

Conclusions

The Tecan Freedom EVO 150 allows automation of the AgraQuant Melamine ELISA Test Kit, providing an excellent solution for simple, fast and reliable screening of melamine. The entire process is fully automated and independent of operator intervention, from the introduction of samples into the microwells right through to analysis and exporting of data. Although the kit manufacturer recommends analyzing a maximum of 20 samples per batch, due to the critical time constraints of the assay, the speed and efficiency of the automated protocol has allowed VFAD to double the number of samples that can be processed per run, without losing any sensitivity.

Acknowledgements

We would like to thank Dr. Tan Do Yew at Vet Food Agro Diagnostics Sdn Bhd for generously sharing his expertise in automation of the AgraQuant Melamine ELISA, and for preparing the manuscript for this application note.

References

1. AgraQuant Melamine Assay Kit Insert and Certificate of Performance.
2. Application Note – AgraQuant Melamine ELISA COKAQ9300

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