Evaluation of Rapid ELISA Assay System for Detection of Melamine Residuals in Milk

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Abstract

This paper describes how melamine screening from milk samples can be easily performed using simple ELISA assay with high sensitivity. The performance of two commercial melamine ELISA assays was evaluated with basic ELISA system including a microplate washer, dispenser and a photometer from Thermo Fisher Scientific. This ELISA system can also be easily fully automated using for example Thermo Scientific Catalyst Express robotics and supporting software. With this instrument combination it is possible to detect below 10 µg/kg melamine residuals from milk samples.

Introduction

Melamine is an industrial chemical most commonly used as a fire retardant in dry powder extinguishers due to its release of nitrogen gas when burned or charred. Melamine has been used illegally by adding it to food products in order to increase the apparent protein concentration. Standard tests such as the Kjeldahl and Dumas tests estimate protein content by measuring the nitrogen content, so nitrogen rich melamine increases the nitrogen content leading to overestimation of the protein content. Due to the well known melamine scandal in China, there is need for rapid test systems that detect melamine residuals in milk with sufficiently low concentrations.

The most common techniques to detect melamine are liquid chromatography (LC) alone or combined with tandem mass spectrometry (LC/MS/MS). Alternatively, gas chromatography combined with tandem mass spectrometry (GC/MS/MS) can also be used. Chromatographic methods are rather expensive, require special instrumentation and are laborious. Therefore a simple high throughput screening assay for melamine with reasonable costs is strongly needed. Classic ELISA type immunoassays are very good and easy solutions for this need: they can be performed in microplates where large amount of samples can be analysed simultaneously, they require only low cost instrumentation and the running costs are rather affordable.

Methods

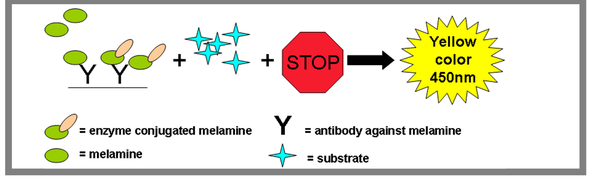
Two different commercially available melamine ELISA assay kits were used in this study:

1. AgraQuant® Melamine Sensitive Assay (cat.no COKAQ9400), Romer Labs Singapore Pte Ltd, Singapore

2. Abraxis Melamine Plate Kit (cat.no 50005B), Abraxis LLC, US

Assays of both kits have the same operational principle of a competitive ELISA assay (Figure 1).

FIGURE 1. General principle of the competitive melamine ELISA assay. Enzyme conjugated melamine competes with the melamine from the sample for binding to melamine antibody. When melamine is not present in the sample, all antibodies are occupied with enzyme conjugate, leading to high enzymatic activity in the colour formation reaction and therefore high absorbance. The enzyme activity and absorbance values decrease according to increasing amount of the unlabeled melamine from the unknown sample.



Because milk samples contaminated with melamine were not available, milk samples were spiked with melamine using pure melamine (Sigma-Aldhrich, cat no. M2659). Three types of milk products were selected for the study: Natural full milk with 3.5 % of milk fat, fat free milk and natural milk powder. Artificial milk was prepared from the milk powder by mixing 20 g of milk powder with 100 ml of distilled water.

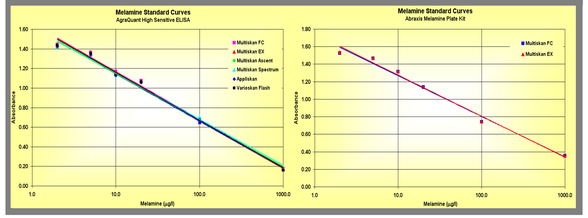
The milk samples were first centrifugated (1500 x g, 10 min, 10 °C). Then 200 µl of the milk serum under the fat layer was mixed with 800 µl of assay diluent. That dilution was pipetted on the plate. The ELISA assays were performed according to the kit instructions. HRP (horseradish peroxidase)-melamine conjugate, chromogenic HRP substrate and stop solution additions were done with Thermo Scientific Multidrop Combi microplate dispenser. Microplate washing was performed with Thermo Scientific Wellwash AC microplate washer. Photometric absorbance readings at 450nm were performed with six different Thermo Scientific microplate photometers or multitechnology readers: Multiskan FC (microplate photometer), Multiskan EX (microplate photometer), Multiskan Ascent (microplate photometer), Multiskan Spectrum (microplate spectrophotometer), Appliskan (filter based multitechnology reader) and Varioskan Flash (monochromator based multitechnology reader).

Measurement and calculation protocols were created either with Thermo Scientific SkanIt Software or Thermo Scientific Ascent Software, depending on the used microplate photometer model. Both softwares include calculations that automatically created the melamine standard curve and calculated the melamine concentrations of the spiked milk samples.

Results

Melamine standard curves generated with AgraQuant kit were measured with all six different microplate photometer models When standard curves of Abraxis kit were measured, only two microplate readers were used because based on the AgraQuant data it was obvious that the reader made no difference in the results. The assay standard curves of both ELISA kits are shown in figure 2. Limit of Detection for the assay was calculated based on the calibrator data and data from zero control samples using standard IUPAC 3*SD method. The results are presented in table 1.

FIGURE 2. Standard curves of AgraQuant Sensitive Melamine sssay and Abraxis Melamine Plate Kit assay. Absorbance values were fitted using linear regression fit and Log-lin transformation.



The measurement range of the assays fits perfectly to the area where all photometers perform very well and therefore the assays can be easily done with any of the readers. The detection limit of the assay is heavily dependent on the accuracy and precision of the photometric reading, but according to this data, all instruments are also performing identically and sufficiently well to achieve good assay sensitivity. The marginal differences seen in the LOD values between instruments are too small to be relevant.

It is well possible to detect below 10 µg/l melamine concentrations with both kits. This sensitivity equals the required sensitivity for LC/MS/MS in Chinese melamine testing standard. This clearly shows the power of ELISA assay for a purpose like melamine screening.

TABLE 1. Limits of Detection (LOD) of AgraQuant Sensitive Melamine Assay kit and Abraxis Melamine Plate Kit with different Thermo Scientific microplate photometers.

Photometer	Limit of Detection (melamine µg/l)	
	AgraQuant Sensitive Melamine Assay Kit	Abraxis Melamine Plate Kit
Multiskan FC	7.6	9.4
Multiskan EX	7.6	9.4
Multiskan Ascent	7.7	
Multiskan Spectrum	7.7	
Appliskan	7.8	
Varioskan Flash	7.6	

A group of different milk samples spiked with pure melamine was analyzed using both ELISA kits. These measurements were done with all different photometer types but only the results from Multiskan FC measurements are shown in Table 2. Results with all other photometers were fully identical with the results from Multiskan FC.

The results show also a similar behaviour of these two kits. Rather good recovery efficiency (< 10% difference) was obtained with both kits, even Abraxis kit showed generally a bit smaller differences to spiked concentrations. AgraQuant kit showed a weak tendency to produce a bit too low result values but both kits have a common tendency to underestimate those samples that have high melamine concentration. The differences are anyhow rather small and obtained accuracy can surely be improved with more experience about performing the assays. Even with this accuracy level, these results show clearly that melamine ELISA assays are well suitable for screening purposes.

TABLE 2. The results of the melamine ELISA assays with spiked milk samples. The measurement was done with two replicates of each sample and the recovery efficiency was calculated from the average values.

Sample	Spiked concentration (µg/l in well)	Measured concentration with AgraQuant Kit	Recovery (%)	Measured concentration with Abraxis Kit	Recovery (%)
Full milk	20	18.0	89.8	21.3	106.3
Full milk	100	101	100.6	111	111.5
Full milk	500	539	107.7	478	95.6
Full milk	1 000	872	87.2	929	92.9
Fat free milk	20	17.8	89.2	22.0	109.9
Fat free milk	100	85	85.0	115	115.1
Fat free milk	500	483	96.5	549	109.8
Fat free milk	1 000	811	81.1	755	75.5
Milk Powder	20	not tested	NA	20.2	100.9
Milk Powder	100	not tested	NA	85	85.1
		Average recovery:	**92.2**	**Average recovery:**	**100.3**

Conclusions

Based on these tests it can be concluded that:

- both of these melamine ELISA kits are well suitable for screening of melamine residuals in dairy products
- sensitivity of these ELISA assays is about equal to mass spectrometric analyses

These ELISA assays are easier to perform than chromatographic and spectrometric analysis, and a large scale screening with thousands of samples can easily be done with reasonable costs. These assays show some tendency to underestimate the melamine concentrations in milk samples. It is anyhow well within acceptable levels for melamine screening where mainly the existence of melamine (simple yes/no answer) is to be detected. When these assays are used for screening, it is still recommended that all positive samples would be confirmed with LC/MS/MS or GC/MS/MS system to verify the exact melamine concentration.

This ELISA system is ideal for the laboratories that require small / medium throughput melamine assay capacity. This instrumentation can be used either manually where assay plates are moved by the user, or the fully automated ELISA system can be built by connecting these instruments into the robotic system, such as Thermo Scientific Catalyst Express.

Further Information

For further information about Thermo Scientific microplate instruments, please contact:

Thermo Fisher Scientific
PO. Box 100, FI-01621 Vantaa, Finland
Email: info.microplateinstruments@thermofisher.com
Web: http://www.thermo.com/mpi
http://www.thermo.com/readingroom

For further information about ELISA kits for melamine assays, please refer to the following web pages:
Romer Labs
http://www.romerlabs.com/pdts_kits.html
Abraxis LLC
http://www.abraxiskits.com/product_veterinary.htm

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