Application Note:

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High Sensitivity ELISA Assays for the Detection of Melamine Residuals in Milk

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This note describes how melamine screening from milk samples can be easily performed using simple ELISA assay with high sensitivity. The total assay time is about one hour and only standard ELISA instrumentation is needed. The required instrumentation is Thermo Scientific Multiskan FC microplate photometer (or any other Multiskan photometer) with Thermo Scientific Wellwash AC microplate washer (Wellwash 4 Mk 2 can also be used) and Thermo Scientific Multidrop Combi microplate dispenser. In addition, the ELISA system can easily be fully automated using, as an example, the Thermo Scientific Catalyst Express robotics and supporting software. With this instrument combination it is possible to detect below 10 µg/kg melamine residuals in milk samples.

Introduction

Background

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 content. Standard tests such as the Kjeldahl and Dumas tests estimate protein content by measuring the nitrogen content, therefore nitrogen-rich melamine increases the nitrogen content leading to overestimation of the protein content. The Association of Analytical Communities (AOAC) International, a scientific association that sets standards for analytical methods, lists the Kjeldahl and Dumas techniques as the standard methods for measuring protein content in food.

Melamine can cause fatal kidney stones, especially when combined with cyanuric acid due to the formation of an insoluble melamine cyanurate. It has also been explained that when melamine and cyanuric acid are absorbed into the bloodstream, they concentrate and interact in the urine-filled renal microtubules, then crystallize and form large numbers of round yellow crystals, which in turn block and damage the renal cells that line the tubes, causing the kidneys to malfunction.

The most common techniques to detect melamine are liquid chromatography (LC) or combined with tandem mass spectrometry (LC/MS/MS). Alternatively, gas chromatography combined with tandem mass spectrometry (GC/MS/MS) can also be used. Chinese authorities have just released the National Standard of Melamine test. The requested detection limit for the LC assay is 2 mg/kg, for LC/MS/MS 0.01 mg/kg and for GC/MS/MS 0.005 mg/kg (the General Administration of Quality Supervision, the National Standardization Committee, GB/T22388-2008, "Raw milk and dairy products in the detection of melamine").

Chromatographic methods are rather expensive, require special instrumentation and are laborous. Therefore a simple high throughput screening assay for melamine with reasonable costs is urgently 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 simultaneously analyzed and require low instrumentation costs and affordable running prices.

Even if there has not been a vital need for melamine testing in milk products, there are a couple of commercial ELISA kit manufacturers that have melamine assay kits in their portfolio. Melamine testing has been needed in some earlier cases where melamine has been added to animal food. Melamine is also a metabolite of cyromazine (a cyclopropyl derivative of melamine that is used as pesticide). Melamine is therefore formed in the body of mammals who have ingested cyromazine and melamine testing can be used to detect cyromazine residuals.

Assay Principle

This melamine ELISA assay study was performed with two ELISA assay kits from two different manufacturers. Both kits use the same competitive ELISA operational principle. These ELISA assay kits include melamine antibody coated microplates, horseradish peroxidase (HRP) conjugated melamine and chromogenic HRP substrate. Both unknown samples and HRP conjugated melamine are added into the microplate well coated with a melamine antibody. Then the HRP conjugated and free melamine are bound to the antibody according to the competition principle: the binding ratio is the same as the concentration ratio. As a result, the amount of bound HRP conjugated melamine is conversely dependent on the amount of the free melamine. After the binding phase, unbound material is removed with the microplate washer and chromogenic HRP substrate is added. HRP enzyme activity in this step is directly proportional to the amount of bound HRP conjugated melamine (and therefore conversely dependent on the melamine concentration in the sample). As a last step, the HRP enzyme reaction is stopped after a certain incubation time and the amount of the coloured dye formed in the HRP enzyme reaction is measured with the microplate photometer using 450 nm measurement wavelength. The general principle of these competitive ELISA assays is shown in Figure 1.

Materials and Methods

ELISA Kits

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

Instrumentation

Centrifugation of the melamine spiked milk samples during sample preparation was performed with a Thermo Scientific IEC MicroCL 17R centrifuge at 10° C temperature. HRP-melamine conjugate, chromogenic HRP substrate and stop solution additions were done with Multidrop® Combi microplate dispenser. Microplate washing was performed with Wellwash® AC microplate washer. Photometric absorbance readings were performed with six different microplate photometer 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).

Samples and Sample Preparation

Because melamine contaminated milk samples were not available, milk samples were spiked with melamine using pure melamine (Sigma-Aldhrich, cat no. M2659) and different milk products from the local grocery store. Three different types of milk products were selected for the study: Natural full milk with 3.5 % of milk fat, fat free milk wherefrom all the fat has been removed and natural milk powder. Artificial milk used in these tests was prepared from the milk powder by mixing 20 g of milk powder with 100 ml of distilled water. A group of spiked milk samples was prepared by mixing melamine stock solution (2 mg/ml dissolved in distilled water) with different types of milk products. With all spiked milk products (full milk, fat free milk, and artificial milk from milk powder) two different samples were analyzed: Milk spiked with 20 and 100 µg/l melamine in the final assay well. In addition, milk samples spiked with 500 and 1,000 µg/l melamine in the assay well were used with full and fat free milk. All these spiked samples were measured with an Abraxis Melamine Plate kit. Only spiked full and fat free milk samples were measured with the AgraQuant Sensitive Assay kit because all results with the Abraxis kit showed no differences whether milk or milk powder was used.

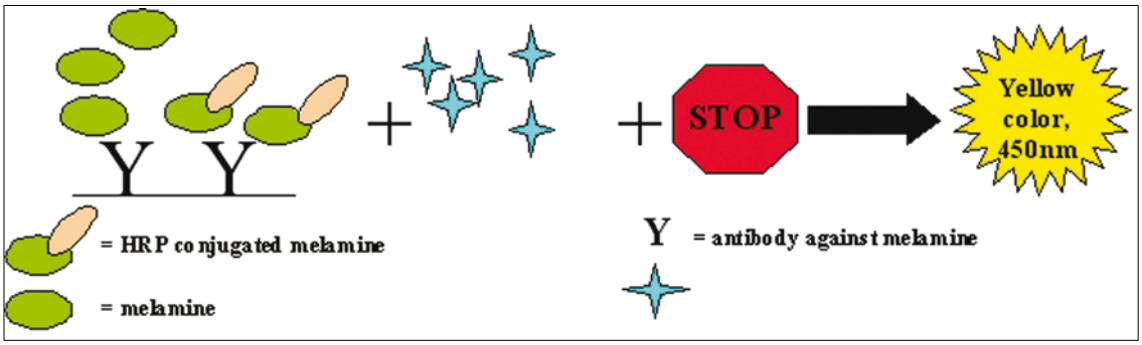

Figure 1. General principle of the competitive ELISA assay. Enzyme conjugated melamine competes with the melamine from the sample for binding to melamine antibody. When melamine is not present in the assay all antibodies are occupied with enzyme conjugate, leading to high enzyme activity in the color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

When the AgraQuant kit from the Romer Lab was used, the spiked milk samples were prepared for the ELISA assay as described in the kit instructions.

1. Approximately 1 ml of spiked milk samples were added into a clean test tube.
2. The samples were centrifuged with the speed of 1500 g at 10° C for 10 minutes.
3. Aliquots of 200 µl of milk serum below the fat layer were transferred into a clean test tube.
4. 800 µL of assay diluent was added into the milk serum and diluted samples were mixed carefully.

The samples were ready for ELISA testing.

Sample preparation for Melamine Plate Kit assay was performed based on information provided separately from Abraxis LLC, and spiked milk samples were prepared quite differently than described in the kit insert. This modified sample preparation was used to improve the sensitivity of the assay with spiked milk samples. If milk samples are prepared according to original instructions in the kit insert, remarkably lower melamine sensitivity will be observed because the dilution factor of the samples is very different in these two sample preparation protocols. This difference is not seen when the assay sensitivity is calculated from the calibrator data only. The sample preparation procedure was a follows.

1. Approximately 1 ml of spiked milk was pipetted to a microcentrifuge tube.
2. Samples were centrifuged at 10,000 g for 5 minutes at 10° C. This process separated the sample into three separate layers.
3. A 250 µl aliquot was removed from the middle layer into a new test tube.
4. The 250µl aliquot was diluted 1:3 by adding 500 µl of 10 % MeOH/20mM PBS solution.

The spiked samples were then ready to be analyzed using Abraxis Melamine Plate Kit.

ELISA Assay Procedure

Both ELISA assay kits were used according to the instructions from the manufacturer. Briefly, the following procedure was used.

1. Either 100 (Abraxis kit) or 150 µl (AgraQuant kit) of melamine standard or spiked sample was added into the antibody-coated well.
   Note: These kits include only calibrators of 20, 100 and 1000 µg/l but for this study additional calibrators with concentrations of 2, 5 and 10 µg/l were prepared by diluting the highest calibrator from the kits with water.
2. HRP-melamine conjugate was added (50 µl) to each well with Multidrop Combi.
3. The plate was mixed for a short time and incubated for 30 minutes at room temperature.
4. All unbound sample was removed by washing the plate with distilled water. Washing was repeated four times with 300 µl volume using Wellwash AC.
5. An aliquot of 100 µl of the HRP-substrate was added into each well with another Multidrop Combi.
6. The plate was incubated at room temperature for 20 (AgraQuant kit) or 30 (Abraxis kit) minutes.
7. The enzyme reaction was stopped by adding 100 µl of stop solution into each well using a third Multidrop Combi.
8. The absorbance was read with a microplate photometer using 450 nm wavelength.

Microplate Reader Measurement and Calculation Protocols

Appropriate measurement and calculation protocols were created with either Thermo Scientific SkanIt Software or Thermo Scientific Ascent Software, depending on the microplate photometer model used. The example below shows how the protocol was created for Multiskan FC. A similar protocol was used for all other models.

1. A microplate layout was created to define different sample types, calibrators, controls and unknown samples. A sample without any melamine was defined to be the zero level control (giving the highest absorbance). The layout structure is shown in Figure 2.
2. A measurement step using a 450 nm filter was added to measure the absorbances (Figure 3.).
3. Calculations of the results can be performed with SkanIt Software. The calculation is based on generating the calibration curve and determining the concentrations of the unknowns based on the fitted curve. The calibration

	01	02	03	04	05	06	07	08	09	10	11	12
A	Zero_0001 Control 0	Zero_0001 Control 0	Un_0002 Unknown	Un_0002 Unknown	Un_0010 Unknown	Un_0010 Unknown	Un_0018 Unknown	Un_0018 Unknown	Un_0026 Unknown	Un_0026 Unknown	Un_0034 Unknown	Un_0034 Unknown
B	Cal_0001 Calibrator 2 ug/l	Cal_0001 Calibrator 2 ug/l	Un_0003 Unknown	Un_0003 Unknown	Un_0011 Unknown	Un_0011 Unknown	Un_0019 Unknown	Un_0019 Unknown	Un_0027 Unknown	Un_0027 Unknown	Un_0035 Unknown	Un_0035 Unknown
C	Cal_0002 Calibrator 5 ug/l	Cal_0002 Calibrator 5 ug/l	Un_0004 Unknown	Un_0004 Unknown	Un_0012 Unknown	Un_0012 Unknown	Un_0020 Unknown	Un_0020 Unknown	Un_0028 Unknown	Un_0028 Unknown	Un_0036 Unknown	Un_0036 Unknown
D	Cal_0003 Calibrator 10 ug/l	Cal_0003 Calibrator 10 ug/l	Un_0005 Unknown	Un_0005 Unknown	Un_0013 Unknown	Un_0013 Unknown	Un_0021 Unknown	Un_0021 Unknown	Un_0029 Unknown	Un_0029 Unknown	Un_0037 Unknown	Un_0037 Unknown
E	Cal_0004 Calibrator 20 ug/l	Cal_0004 Calibrator 20 ug/l	Un_0006 Unknown	Un_0006 Unknown	Un_0014 Unknown	Un_0014 Unknown	Un_0022 Unknown	Un_0022 Unknown	Un_0030 Unknown	Un_0030 Unknown	Un_0038 Unknown	Un_0038 Unknown
F	Cal_0005 Calibrator 100 ug/l	Cal_0005 Calibrator 100 ug/l	Un_0007 Unknown	Un_0007 Unknown	Un_0015 Unknown	Un_0015 Unknown	Un_0023 Unknown	Un_0023 Unknown	Un_0031 Unknown	Un_0031 Unknown	Un_0039 Unknown	Un_0039 Unknown
G	Cal_0006 Calibrator 1000 ug/l	Cal_0006 Calibrator 1000 ug/l	Un_0008 Unknown	Un_0008 Unknown	Un_0016 Unknown	Un_0016 Unknown	Un_0024 Unknown	Un_0024 Unknown	Un_0032 Unknown	Un_0032 Unknown	Un_0040 Unknown	Un_0040 Unknown
H	Un_0001 Unknown	Un_0001 Unknown	Un_0009 Unknown	Un_0009 Unknown	Un_0017 Unknown	Un_0017 Unknown	Un_0025 Unknown	Un_0025 Unknown	Un_0033 Unknown	Un_0033 Unknown	Un_0041 Unknown	Un_0041 Unknown

Figure 2. Microplate layout used in melamine ELISA assays.

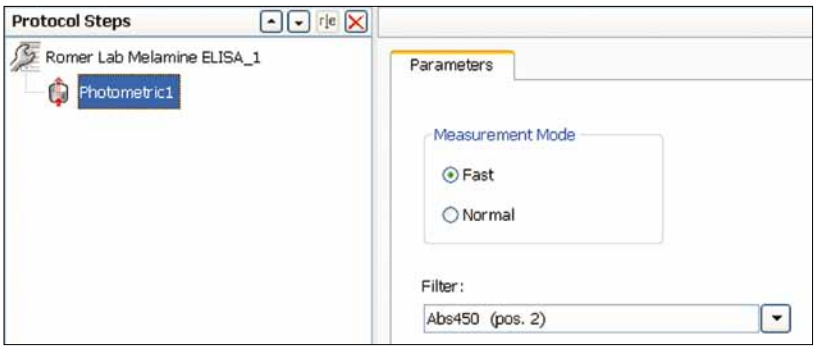

Figure 3. Setting the photometric measurement with SkanIt Software for Multiskan FC.

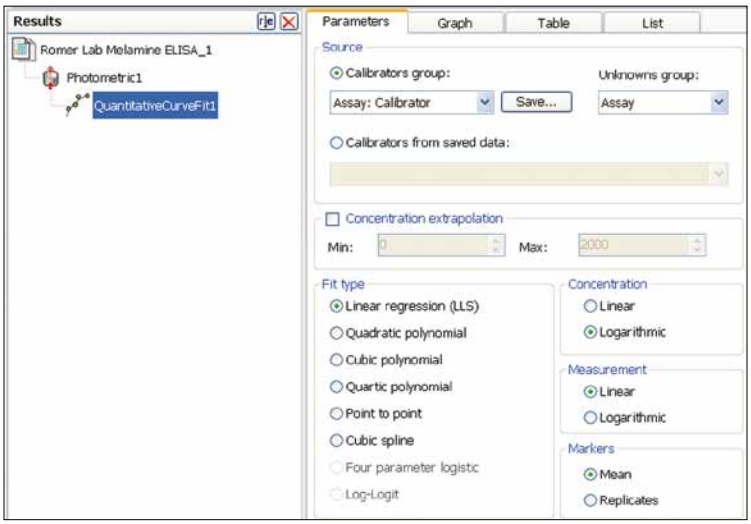

Figure 4. Curve fit calculation parameters used in the test.

curve can be calculated based on either measured absorbances or normalized values where absorbance of the zero control has been set to 100 %. The type curve fitting used was a linear regression using Log-Lin transformation and scaling. The parameters for the curve fitting are shown in Figure 4.

Results and Discussion

Melamine Calibration Curves and Assay Sensitivities

Melamine calibration curves generated with the AgraQuant kit were measured with all six different microplate photometer models, and the curves are shown in Figure 5. When calibration curves of the Abraxis kit were measured, only two microplate readers were used (Multiskan FC and Multiskan EX) because based on the AgraQuant data it was obvious that the reader made no difference in the results. The calibration curves with the Abraxis kit are shown in Figure 6. The limit of detection (LOD) for this assay was calculated based on the calibrator data and data from the zero control samples using the standard IUPAC 3*SD method. The results are collected into Table I.

As seen from the calibration curves there are no differences between the microplate instrument models. The measurement range used in these assays fits perfectly to the area where all photometers perform very well and therefore 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 also performed identically and sufficiently well to obtain good assay sensitivity. The marginal differences seen in the LOD values between instruments are too small to be relevant.

These results show that with both ELISA kits it is possible to easily detect below 10 µg/l melamine concentrations. This sensitivity is the same that is the required sensitivity for LC/MS/MS in the Chinese melamine testing standard. This clearly shows the power of ELISA testing for purposes such as melamine screening.

Determination of Melamine from Spiked Milk Samples

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

These results also show the similar behavior of these two kits. Rather good recovery efficiency (< 10

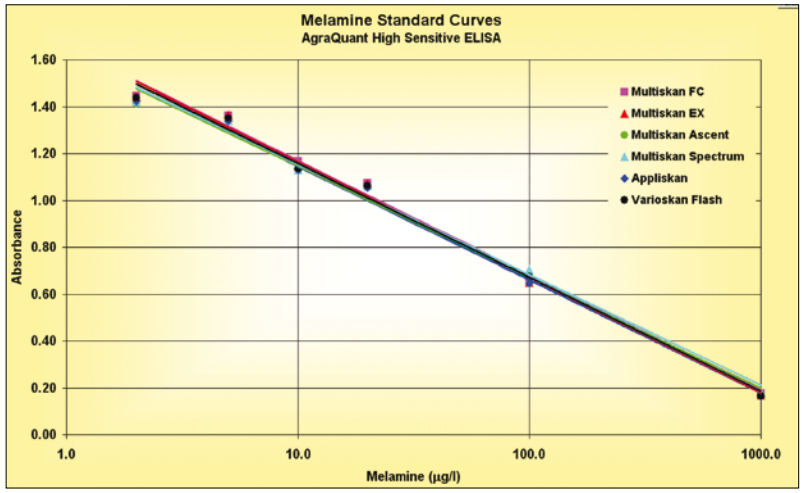

Figure 5. Calibration curves of AgraQuant Sensitive Melamine assay. Absorbance values were fitted using linear regression fit and Log-Lin transformation.

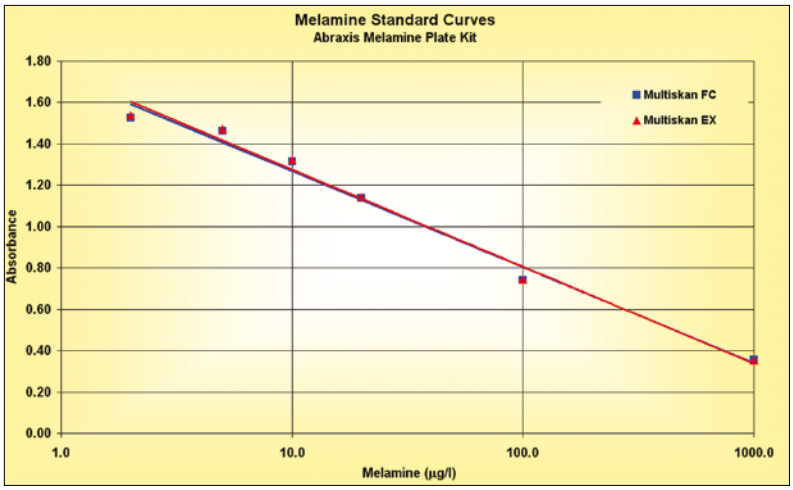

Figure 6. Calibration curves of Abraxis Melamine Plate Kit assay. Absorbance values were fitted using linear regression fit and Log-Lin transformation.

Photometer	Limit of Detection (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	not tested
Multiskan Spectrum	7.7	not tested
Appliskan	7.8	not tested
Varioskan Flash	7.8	not tested

Table I. Limits of Detection of AgraQuant Sensitive Melamine Assay kit and Abraxis Melamine Plate Kit with different Thermo Scientific microplate instruments.

% difference) was obtained with both kits. The Abraxis kit showed generally a bit smaller differences to spiked concentrations. The AgraQuant kit showed in these tests a weak tendency to produce a little 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 on performing the assays. Even with this accuracy level, these results show clearly that ELISA assays are well suitable for screening purposes.

Conclusion

Based on these tests it can be concluded that both of these melamine ELISA kits are well suitable for screening dairy products for melamine residuals. Assay sensitivity is about equal to mass spectrometric analyses but assays with these ELISA kits are easier to perform so a large scale screening with thousands of samples is easily done with reasonable costs. These assays show some tendency to underestimate the melamine concentrations in milk samples. This is considered 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 be confirmed with LC/MS/MS or GC/MS/MS systems to verify the exact melamine concentration.

Sample type	Spiked concentration (µg/L in well)	Measured concentration with AgraQuant Kit	Recovery (%)	Measured concentration with Abraxis Kit	Recovery (%)
Full milk	20	18	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	1000	872	87.2	929	92.9
Fat free milk	20	17.8	89.2	22	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	1000	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**

Table II. 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 also calculated from the average values.

Further Information

For further information about Multiskan FC, please refer to the following web pages:

- www.thermo.com/readingroom
- www.thermo.com/mpi

For further information about ELISA kits for melamine assays, please refer to the following web pages:

- Romer Lab www.romerlabs.com/pdts_kits.html
- Abraxis LLC www.abraxiskits.com/product_pesticides.htm

In addition to these offices, Thermo Fisher Scientific maintains a network of representative organizations throughout the world.

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