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Application Note 221

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Rapid Determination of Melamine in Liquid Milk and Milk Powder by HPLC on the Acclaim Mixed-Mode WCX-1 Column with UV Detection

INTRODUCTION

Melamine (2,4,6-triamino-1,3,5-triazine, structure shown in Figure 1) is a chemical used in some plastics and fertilizer products. Recent investigations of death and health problems of babies in China have revealed that some baby foods (milk powder) have been contaminated by melamine. Some manufacturers illegally used melamine as an adulterant to increase the apparent protein content. Melamine was also used as an adulterant to increase the apparent protein content of animal feeds and there were news reports that melamine was found in eggs obtained from some markets.

The reported methods for quantitative determination of melamine include enzyme immunoassay (EIA), gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS), and high-performance liquid chromatography (HPLC) with UV detection.[1-4] Standard methods enacted by the Chinese government for determining melamine in raw milk and dairy products included HPLC-UV, LC-MS, and GC-MS methods.[5] However, the high cost of operation and maintenance of GC/LC-MS systems as well as the labor intensive derivatization that GC-MS requires limits their use in milk product factories. The HPLC-UV method therefore is presently the popular choice for most factories. Because numerous batches of raw milk had to be monitored, another standard HPLC-UV method for rapidly determining melamine in raw milk was soon recommended;[6] however, its application is limited in the analysis of liquid milk products.

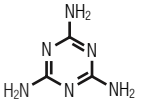

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Figure 1. Structure of melamine.

In this Application Note (AN), we developed a simple HPLC method for rapid analysis of melamine in both liquid milk and milk powder samples. The separation was performed on the Acclaim® Mixed-Mode WCX-1 column[7] and UltiMate® 3000 HPLC system with UV detection using an acetate buffer and acetonitrile mobile phase. A sample analysis is completed within 10 min. The Acclaim Mixed-Mode WCX-1 column features a new mixed-mode silica-based packing material that incorporates both hydrophobic and weak cation-exchange properties, and demonstrates great potential for separating samples that contain a mixture of ionic and neutral compounds. Using an Acclaim Mixed Mode WCX-1 column with an UltiMate 3000 system allows a fast analysis of both liquid and powdered milk for melamine.

EQUIPMENT

Dionex UltiMate 3000 HPLC system consisting of:

- HPG 3400A pump
- WPS 3000TSL autosampler
- TCC-3000 thermostatted column compartment
- VWD-3400RS UV-vis Detector

Dionex Summit® UVD-340U Photodiode Array Detector

Chromeleon® 6.80 SP5 Chromatography Management Software

Kudos® SK3200LH Ultrasonic generator, Kudos Ultrasonic Instrumental Co., Shanghai, China

Mettler Toledo AL-204 Electoral o balance, Mettler-Toledo (Shanghai) Co., Shanghai, China

Anke® TGL-16B centrifuge, Anting Scientific Instrumental Factory, Shanghai, China

IKA® MS1 Minishaker, IKA Works, Guangzhou, China

REGENTS AND STANDARDS

Water, from Milli-Q® Gradient A 10

Methanol (CH_3OH), HPLC grade, Fisher

Acetonitrile (CH_3CN), HPLC grade, Fisher

Ammonium acetate (NH_4Ac), analytical grade, SCRC, China

Acetic acid (HAc), analytical grade, SCRC, China

Sodium 1-octane sulfate (98%), Baker Analyzed HPLC Reagent, USA

Melamine (99.0%), HPLC grade, Fluka

CHROMATOGRAPHIC CONDITIONS

Guard Column:	Acclaim® Mixed-Mode WCX-1, 5 µm, 4.3 × 10 mm, P/N 068354, with guard column holder, P/N 59526
Analytical Column:	Acclaim Mixed-Mode WCX-1, 5 µm, 4.6 × 250 mm, P/N 068352
Column Temp.:	30 °C
Mobile Phase:	Acetate buffer (mixture of 700 mL of 10 mM HAc and 300 mL of 10 mM NH_4Ac, ~ pH 4.3) – CH_3CN (8 : 2, v/v)
Flow Rate:	1.0 mL/min
Inj. Volume:	20 µL
UV detection:	Absorbance at 240 nm

PREPARATION OF STANDARDS

Stock Standard Solution

Accurately weigh ~100 mg of melamine, dissolve in a 100 mL volumetric flask with aqueous methanol (50%, v/v). The melamine concentration is 1000 µg/mL

Working Standard Solutions

Prepare seven working standard solutions for calibration by adding defined volumes of the stock standard solution and diluting with the acetate buffer used in the mobile phase. The concentrations of melamine are 0.05, 0.1, 0.2, 1.0, 5.0, 20, and 40 µg/mL, respectively.

PREPARATION OF SAMPLES

Milk Powder Sample

Put an accurately weighed ~1 g of dried sample into a 15 mL centrifuge tube, and then add 10 mL water. After 1 min of vortex shaking, put in an ultrasonic bath for 30 min. Add 1 mL dilute HAc (3%, v/v) and store the solution at 4 °C for at least 30 min. After 15 min of centrifugation (setting = rpm ≥ 10000), move the supernatant to a 10-mL volumetric flask, and add water to the mark. Prior to injection, filter the solution through a 0.2 µm filter (Millex-HV).

Liquid Milk Sample

Directly add 1 mL dilute HAc (3%, v/v) to an accurately measured 10 mL of liquid milk sample in a 15 mL centrifuge tube and store the solution at 4 °C for at least 30 min. The remainder of the procedure is the same as that for milk powder.

Spiked Milk Powder and Liquid Milk Samples

Add 40 µL of the stock standard solution of melamine to the 15 mL centrifuge tubes together with the accurately weighed ~1 g of dried sample, and together with the accurately measured 10 mL of liquid milk sample, respectively. The remainder of the sample preparation procedure is the same as that for the milk powder and liquid milk samples.

RESULTS AND DISCUSSION

Optimized Procedure for Preparing Milk and Milk Powder Samples

The common procedure for preparing milk and milk powder samples for melamine consists of two steps, sample extraction and cleaning the sample extract on an activated SPE column; and then drying the cleaned extract with N_2 at 50 °C.[5] A simpler preparation procedure for use with an ion-exchange (IEX) analysis method was reported;[6] however, it is only for liquid milk samples. Therefore it is necessary to find an efficient and simple way to prepare both milk and milk powder samples.

The optimized procedure in this AN is simple and efficient, and does not require clean up by SPE and sample drying. It requires only precipitation with dilute acetic acid, subsequent centrifugation and filtration, and is suitable for both liquid milk and milk powder products. Additionally, this procedure is also suitable for the reversed-phase ion-pair chromatography method (RP-PIC). Figure 2 shows that this sample preparation method yields good chromatography for melamine using either the Acclaim Mixed-Mode WCX-1 column method or the RP-PIC method with the Acclaim 120 C18 column, as no interfering matrix peaks elute in the retention time range of melamine.

Optimized Chromatographic Conditions

Melamine is a hydrophilic compound that is poorly retained on a typical RP column (e.g. C18 or C8 column). Most RP methods for melamine use an ion-pairing reagent. With an ion-pairing reagent in the mobile phase, e.g. octane sulfate, melamine is well retained. However, the ion-pairing reagent may coat the RP stationary phase, changing the retention property of RP column, which may not be desired if the column is used for other methods. The RP-PIC method is also not compatible with MS detection. We therefore attempted to separate the cationic melamine on the Acclaim Mixed-Mode WCX-1 column using an ammonium acetate buffer as the eluent.

The Acclaim Mixed-Mode WCX-1 column features a mixed mode silica-based packing material that incorporates both hydrophobic and weak cation-exchange properties. Mobile phase pH affects the charge and hydrophobicity of the stationary phase. At a pH below the pKa of the stationary phase carboxylate group, the cation-exchange functionality is OFF so that hydrophobic interaction is the primary retention mechanism. At a pH

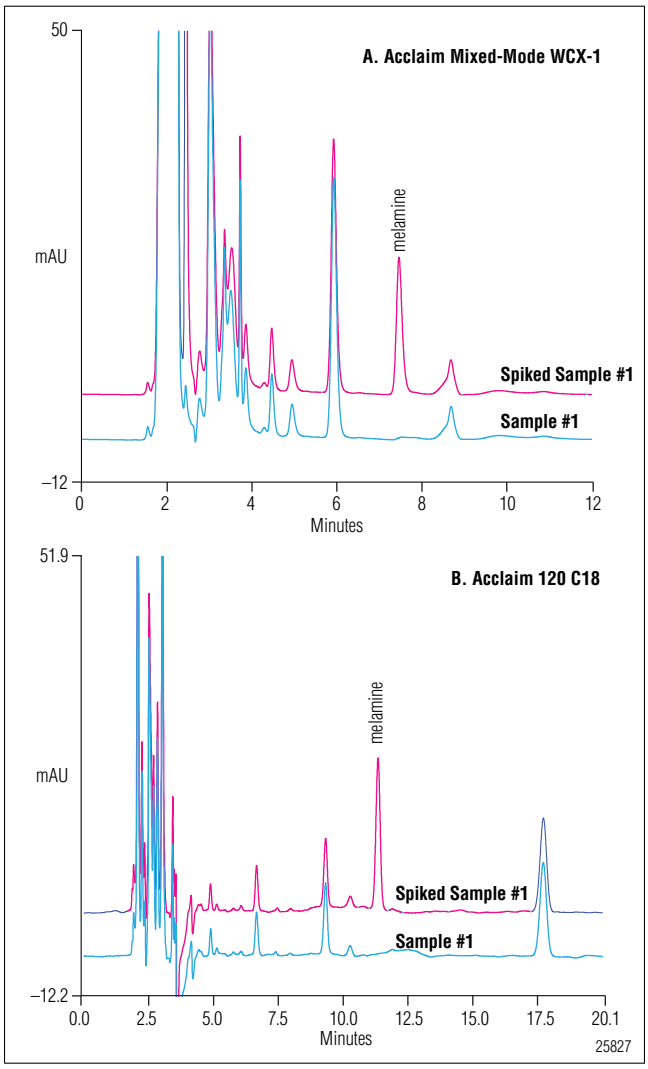

Figure 2. Chromatograms of a milk sample and the same sample spiked with melamine on (A) an Acclaim Mixed-Mode WCX-1 column, and (B) an Acclaim 120 C18 column. Chromatograms: 1, sample #1; and 2, sample #1 spiked with 4 µg/mL melamine. Melamine is detected by absorbance at 240 nm.

above the pKa of the stationary phase carboxylate group, the cation-exchange functionality is ON so that both cation-exchange and hydrophobic interaction contribute to retention depending on the structures of analytes. Our experiments revealed that melamine does not have good retention when the pH is lower than 3.5 and higher than 5.0. Ionic strength is crucial for changing retention of charged molecules. An increase in ionic strength results in a retention decrease for melamine based on its basicity. Hydrophobic retention is markedly affected by the organic modifier composition of the mobile phase. In general, all types of molecules (acids, bases, and neutrals) are less

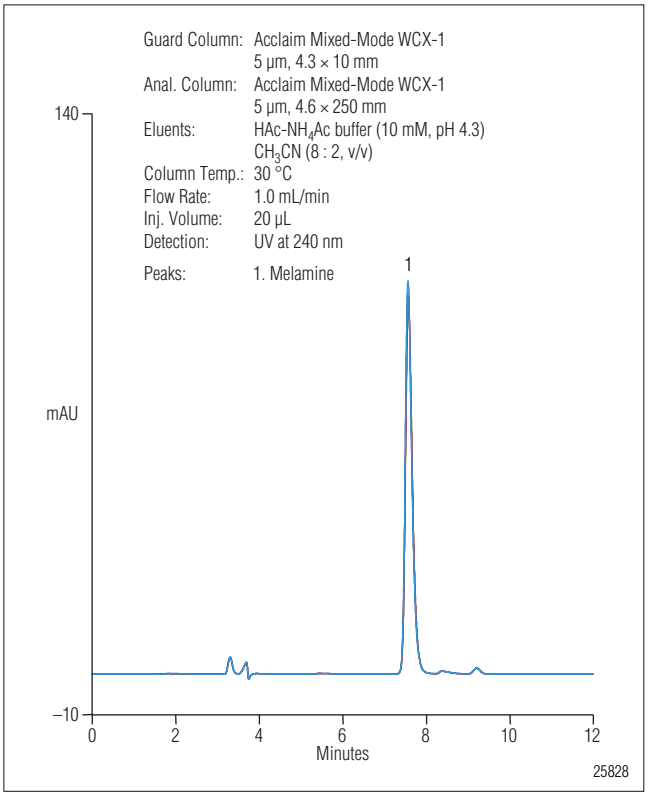

Figure 3. Overlay of chromatograms of five consecutive injections of a 20 µg/mL melamine standard.

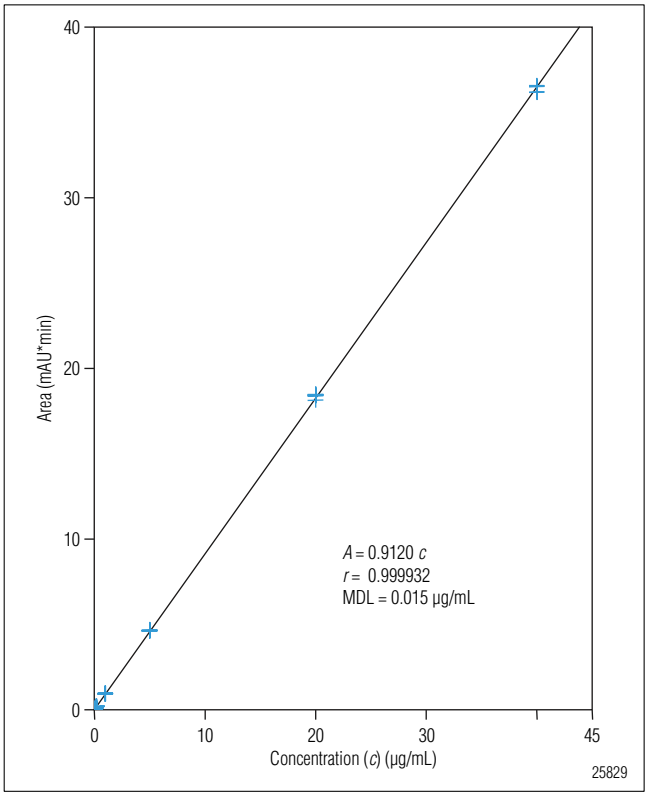

Figure 4. Calibration curve for melamine.

retained with an increase in the organic content of the mobile phase, though to different extents, when other conditions (e.g. ionic strength, pH, temperature, etc.) remain constant.[7]

The optimized mobile phase for these samples is 10 mM ammonium acetate pH 4.3, 20% CH_3CN. For more complex samples, a higher buffer capacity may be required. In these situations, increase the concentration of ammonium acetate. This may result in a decrease in melamine retention time, but it may be possible to restore it by decreasing the percent of CH_3CN in the mobile phase.

Chromatographic Performance

Figure 3 shows overlay chromatograms of five consecutive injections of a 20 µg/mL melamine standard. Note the good reproducibility of retention time and peak area. Calibration linearity for melamine was investigated by making five replicate injections of each standard prepared at seven different concentrations. The external standard method was used to establish the calibration curve and to quantify melamine in samples. As shown in Figure 4, excellent linearity was achieved throughout the range from 0.05 to 40 µg/mL. The linearity equation of melamine is as follows, with the curve forced through the origin.

$$A = 0.9120\ c$$

Here, A stands peak area, and c stands for melamine concentration (µg/mL). The correlation coefficient (r) is 0.9999.

Method reproducibility was estimated by making ten consecutive injections of a 1 µg/mL standard. The RSD for retention time was 0.037, and the RSD for peak area was 0.472.

The detection limit of melamine was calculated using the equation:

$$\text{Detection limit} = S t_{(n-1,\ 1-\alpha = 0.99)}$$

S = standard deviation of replicate analyses

n = number of replicates

$t_{(n-1,\ 1-\alpha = 0.99)}$ = Student's value for the 99% confidence level with n–1 degrees of freedom

Using the same 10 injections of 1 µg/mL standard the calculated MDL was 0.015 µg/mL.

Sample Analysis

Five samples, including three milk powder samples (#1 - #3) and two liquid milk samples (#4 and #5), were analyzed using the sample preparation method and WCX chromatography method described in this AN. No melamine was detected in #1. Melamine was found in the other milk and milk powder samples. The results were summarized in Table 1 and Figure 5 shows the chromatograms of these samples. We also spiked samples #1 and #4 with melamine before sample preparation and found that melamine was sufficiently recovered from both.

Comparison of Sample Preparation and Analysis Methods

The three milk powder samples, #1–#3, and three additional milk powder samples, #6– #8, were prepared with the sample preparation method presented in the application note and the procedure for milk powder samples that uses an activated SPE column (detailed in reference 5). These samples were then analyzed using an Acclaim 120 C18 column under the chromatographic conditions in Ref. 5 and on the Acclaim Mixed-Mode WCX-1 column under the chromatographic conditions described in this AN. This allowed an evaluation of both the simplified sample preparation procedure and the chromatography procedure in this AN. Table 2 shows the results for all six samples prepared using both sample preparation procedures and analyzed by both chromatography methods. The results show good agreement for all six samples using either of the two sample preparation methods or chromatography procedures.

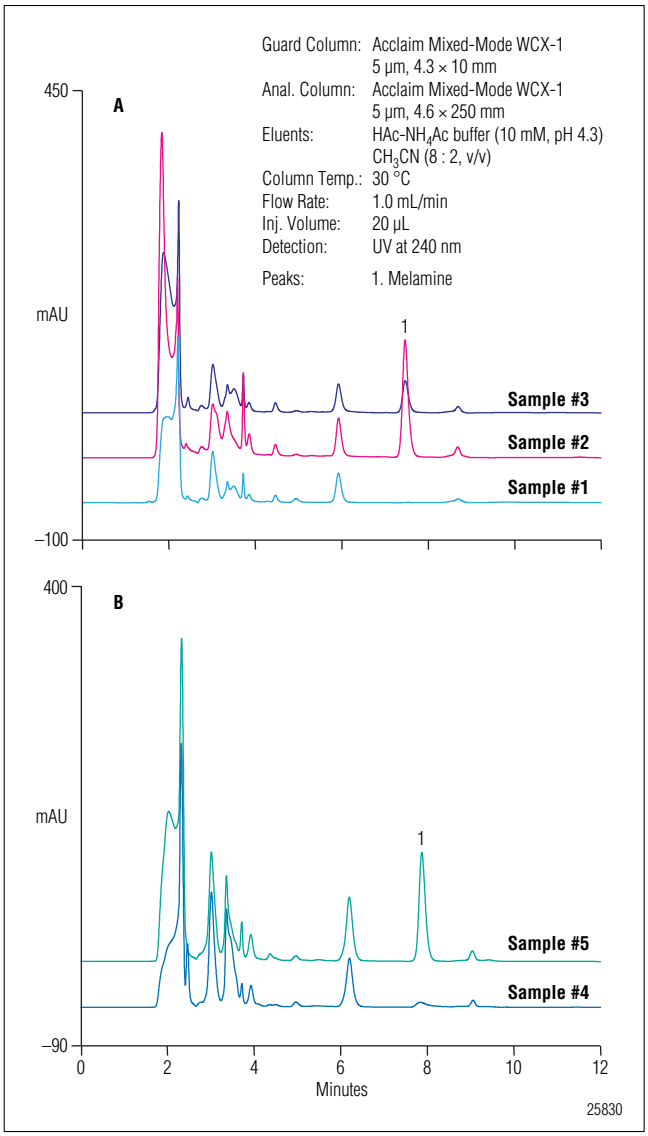

Figure 5. Chromatograms of (A) milk powder samples #1– #3, and (B) liquid milk samples #4 and #5.

Table 1.Sample Analysis Results

	Milk Powder						Liquid Milk				
	#1				#2	#3	#4				#5
	Detected (mg/Kg)	Added (mg/Kg)	Found (mg/Kg)	Recovery (%)	Detected (mg/Kg)	Detected (mg/Kg)	Detected (mg/L)	Added (mg/L)	Found (mg/L)	Recovery (%)	(mg/L)
Melamine	n.a.	40	35	88	262	67	1.8	4.0	3.6	90	22

Note: * Injections were made for each sample

** Found = Measured value of spiked sample - Measured value of sample

The complicated matrix of milk products may sometimes yield in a false positive for melamine. An efficient way to determine if the peak is melamine is by comparison of the peak's UV spectrum to that of melamine. When we analyzed sample #8, using the sample preparation method described in Ref. 5 and the Mixed-Mode WCX-1 column method, a small peak with retention time near that of melamine was found, and labeled as melamine with a concentration was 3.3 mg/Kg. The other sample preparation method and other chromatography method did not detect melamine in this sample. We reanalyzed the sample after substituting the VWD UV-Vis detector with a photodiode array detector. Comparison of the UV spectra, shown in Figure 6 revealed that the peak was not melamine. Using a photodiode array detector for this analysis will help reduce the possibility of false positives for melamine.

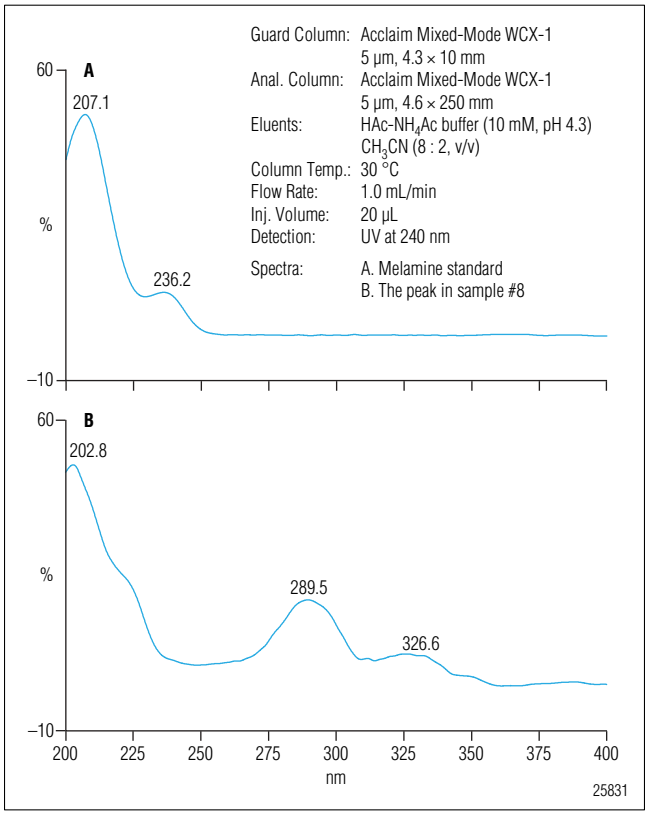

Figure 6. UV-spectra of (A) melamine standard and (B) the putative melamine peak in sample #8.

CONCLUSION

This application note describes an efficient and simple method for preparing liquid milk and milk powder samples coupled to an HPLC method for rapid analysis of melamine in these samples. The Acclaim Mixed-mode WCX-1 column exhibits good retention of melamine, using ammonium acetate buffer and acetonitrile as the mobile phase. This mobile phase should make this method compatible with MS detection.

Table 2. Comparison of Milk Powder Sample Analysis Results Using Different Sample Preparation Methods and HPLC Analysis Methods

	Melamine Concentration using the RP-PIC method (mg/Kg)[1]		Melamine Concentration using the Acclaim Mixed-Mode WCX-1 Column (mg/Kg)[2]	
Sample #	Prepared following the description in this AN	Prepared following the description in Ref. 5	Prepared following the description in this AN	Prepared following the description in Ref. 5
-	n.a.	n.a.	n.a.	n.a.
-	274	229	262	229
-	67	68	67	68
-	2.2	2.3	2.4	2.8
-	n.a.	n.a.	n.a.	n.a.
-	n.a.	n.a.	n.a.	n.a.

Note: 1. Using the chromatographic conditions described in Ref. 5.
2. Using the chromatographic conditions described in this AN.

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