

A Fast Method for Determination of Melamine in Liquid Milk and Milk Powder by HPLC With UV Detection

Melamine discovered in baby formula and products containing milk has led to one of the largest worldwide food recalls in history. Some manufacturers in China illegally added melamine, an ingredient used in plastics manufacturing, to increase the apparent protein content of low-quality milk. This chemical, shown in *Figure 1*, can form an insoluble compound in the body, causing kidney stones that can be deadly in infants.

Factories that produce milk products or use milk products imported from China will need to begin testing for melamine. Melamine has been quantified using enzyme immunoassay (EIA), GC-MS, LC-MS, and HPLC with UV detection.¹⁻⁴ The Chinese government has approved four methods for melamine testing in milk. Initially three methods were released, including GC-MS, LC-MS, and HPLC-UV methods.⁵ An HPLC method using a strong cation-exchange (SCX) analytical column and UV detection for liquid milk only was released more recently.⁶ Derivatization of milk for GC analysis is labor intensive, and the cost of operation of MS detection can be high. The HPLC-UV method is laborious, requiring solid-phase extraction (SPE) followed by solvent evaporation. The ion-pairing compound required for this reversed-phase (RP) separation can increase noise and damage col-

umns, and is incompatible with MS detection. The SCX separation has difficulties with interferences, especially when analyzing powdered milk or milk with additives. A simpler, more robust method for testing a wider variety of milk products is necessary.

This article compares results of experiments using the current C18 method and a new, rapid HPLC method performed using an Acclaim[®] Mixed-Mode WCX-1 column (Dionex Corp., Sunnyvale, CA).⁷ The column features a mixed-mode silica-based packing material that incorporates both hydrophobic and weak cation-exchange properties, and demonstrates high potential for separating samples that contain a mixture of ionic and neutral compounds, without requiring ion-pairing compounds. Melamine analysis of either liquid or powdered milk is completed within 10 min, about half the time required for the C18 separation. The background noise is significantly reduced using the WCX-1 column, and the mobile phase is also compatible with MS detection. A new sample preparation method that eliminates the SPE and evaporation steps is also demonstrated.

Experimental Instrumentation

An UltiMate[®] 3000 HPLC system (Dionex) was used for all separations, and consisted of an HPG-3400A pump, WPS-3000TSL autosampler, TCC-3000 thermostated column compartment, and VWD-3400RS UV-VIS detector (all from Dionex). Chromeleon[®] 6.80 SP5 Chromatography Management Software (Dionex) was used for instrument control and data collection and processing. Samples were prepared using an IKA[®] MS1 Minishaker (IKA Works, Guangzhou, China), Kudos[®] SK3200LH ultrasonic generator (Kudos Ultrasonic Instrumental Co., Shanghai, China), and Anke[®] TGL-16B centrifuge (Anting Scientific Instrumental Factory, Shanghai, China). Solid-phase extraction was performed using a strata[™]-x-c SCX

SPE column (Phenomenex, Torrance, CA), and sample drying was performed using an SE-506 nitrogen purge instrument (Shine Tech., Beijing, China).

Chromatographic conditions

Chromatographic conditions are given in *Table 1*.

Reagents and standards

Deionized water was produced using a Milli-Q[®] Gradient A 10 (Millipore, Bedford, MA). HPLC-grade methanol (CH₃OH) and acetonitrile (CH₃CN) were obtained from Fisher Scientific (Pittsburgh, PA). Analytical-grade ammonium acetate (NH₄Ac), acetic acid (HAc), citric acid (C₆H₈O₇ · H₂O), and ammonia solution (25%–28%) were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Sodium 1-octane sulfate (98%) was a Baker Analyzed HPLC Reagent obtained from J.T. Baker (Phillipsburg, NJ). Nitrogen (N₂, 99.999%) was from Lumin Gas Works (Shanghai, China). HPLC-grade melamine (99.0%), used as a standard, was acquired from Fluka (Milwaukee, WI).

Samples

Eight powdered milk samples and two liquid milk samples suspected of containing melamine were obtained from manufacturers.

Sample preparation: solid-phase extraction method

Milk powder samples #1–5 (approximately 2 g of each) were accurately weighed into 50-mL centrifuge tubes, and 15 mL of aqueous trichloroacetic acid (1%, v/v) and 5 mL acetonitrile were added to each. After 1 min of vortex shaking, samples were placed in an ultrasonic bath for 30 min, and then shaken again for 10 min. After 10 min of centrifugation (setting = rpm ≥10,000), the supernatants were passed through filter paper into 25-mL volumetric

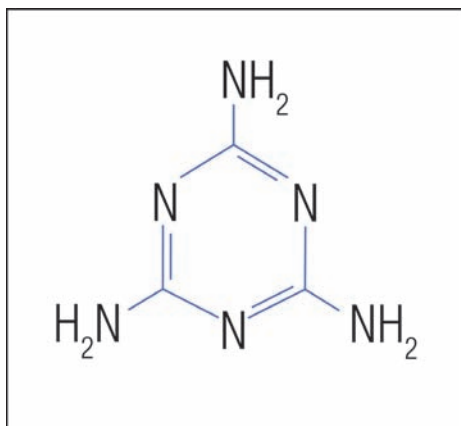


Figure 1 Structure of melamine.

Table 1 Chromatographic conditions

	C18 method	WCX-1 method
Guard column	Acclaim 120 C18, 5 μ m, 4.3 \times 10 mm	Acclaim Mixed-Mode WCX-1, 5 μ m, 4.3 \times 10 mm
Analytical column	Acclaim 120 C18, 5 μ m, 4.6 \times 250 mm	Acclaim Mixed-Mode WCX-1, 5 μ m, 4.6 \times 250 mm
Column temp.	40 °C	30 °C
Mobile phase	Citric acid, sodium 1-octane sulfonate buffer (adjusted to pH 3.0 with 1 M NaOH)/CH ₃ CN (92:8, v/v)	NH ₄ Ac buffer (10 mM, pH 4.3)/CH ₃ CN (8:2, v/v)
Flow rate	1.0 mL/min	1.0 mL/min
Injection vol.	20 μ L	20 μ L
UV detection	Absorbance at 240 nm	Absorbance at 240 nm

flasks. Samples were brought to volume with 1% aqueous trichloroacetic acid.

Prior to use, the SPE column was activated by passing 3 mL CH₃OH and 5 mL H₂O through in turn. Sample extracts were diluted with deionized (DI) water (5 mL of extract and 5 mL of water), and transferred onto the activated SCX SPE column. The SPE column was washed with 3 mL methanol and 3 mL water, respectively; then samples were eluted with 6 mL of aminated methanol solution (mixture of 5 mL ammonia solution and 95 mL methanol). The collected eluents were dried with N₂ at 50 °C. The residues were dissolved in 1 mL of mobile phase and vortex mixed for 1 min. Prior to injection, solutions were filtered through a 0.2- μ m Millex[®]-HV filter (Millipore).

Sample preparation: new method

Milk powder samples #6–8 (approximately 1 g each) were accurately weighed into 15-mL centrifuge tubes, and 10 mL water was added to each. After 1 min of vortex shaking, each was placed in an ultrasonic bath for 30 min. To each was added 1 mL dilute HAc (3%, v/v), and sample solutions were stored at 4 °C for at least 30 min. After 15 min of centrifugation (setting = rpm \geq 10,000), supernatants were transferred to 10-mL volumetric flasks and were brought to volume with DI water. Prior to injection, the solutions were filtered through a 0.2- μ m Millex-HV filter.

Liquid milk samples #9–10 (approximately 10 mL) were accurately measured into 15-mL centrifuge tubes, diluted with 1 mL dilute HAc (3%, v/v), and stored at 4 °C for at least 30 min. The remainder of the procedure was the same as that for milk powder samples #6–8.

Spiked milk powder and liquid milk sample preparation

A 1000- μ g/mL melamine stock standard was prepared by accurately weighing approximately 100 mg of melamine into a 100-mL volumetric flask and bringing to volume with aqueous methanol (50%, v/v). The stock standard was diluted appropriately to prepare working standards with concentrations of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 20, 25, 40, 50, and 100 μ g/mL for calibration.

For milk powder samples #1–5, spiked samples were prepared by adding 20 μ L of the stock standard solution of melamine to the 50-mL centrifuge tubes together with the weighed sample, and then following the first procedure above. For milk powder samples #6–8, spiked samples were prepared by adding 40 μ L of the stock standard solution of melamine to the 15-mL centrifuge tubes together with the weighed sample, and then following the second procedure above. For liquid milk samples #9–10, spiked samples were prepared by adding 40 μ L of the stock standard solution of melamine to the 15-mL centrifuge tubes together with the measured sample, and then following the second procedure above.

Results and discussion

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, such as octane sulfate. With an ion-pairing reagent in the mobile phase, melamine is well retained. However, the ion-pairing reagent may coat the RP sta-

tionary phase, changing the retention property of the RP column, which may not be desirable if the column is used for other methods. The RP-PIC (paired ion chromatography) method is also not compatible with MS detection. Therefore, separation of the cationic melamine was attempted on the Acclaim Mixed-Mode WCX-1 column using an ammonium acetate buffer as the eluent to prevent column damage and make the method compatible with MS.

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 pK_a of the stationary phase carboxylate group, the hydrophobic interaction is the primary retention mechanism. At a pH above the pK_a of the stationary phase carboxylate group, both cation-exchange and hydrophobic interactions contribute to retention, depending on the structures of analytes. Experiments revealed that melamine is poorly retained when the pH is lower than 3.5. At a pH higher than 5.0, melamine becomes deprotonated and is also poorly retained.

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 alkalinity. 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 retained with an increase in the organic content of the mobile phase when other conditions (e.g., ionic strength, pH, temperature, etc.) remain constant.

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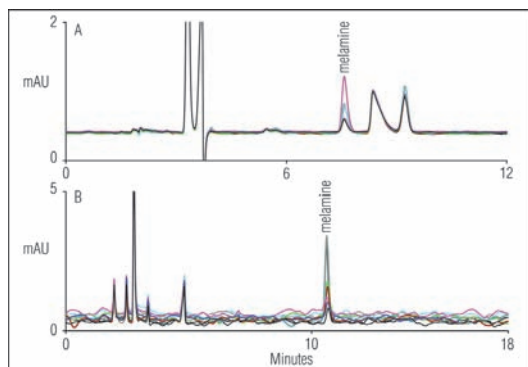
DETERMINATION OF MELAMINE *continued*

Figure 2 Chromatograms of five injections each of three different levels of standards on a) Acclaim Mixed-Mode WCX-1 column (0.05, 0.1, and 0.2 $\mu\text{g/L}$), and b) Acclaim 120 C18 column (0.1, 0.2, and 0.5 $\mu\text{g/L}$). The baseline of chromatogram b is much noisier.

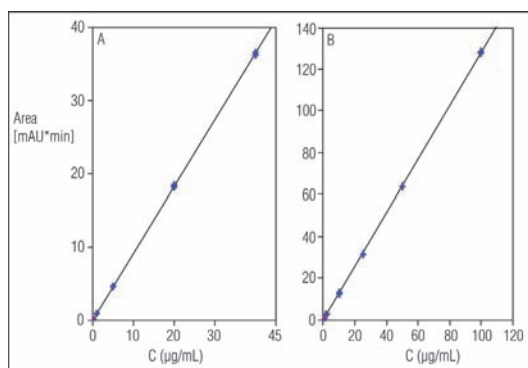


Figure 3 Calibration curves for a) Acclaim Mixed-Mode WCX-1 column, and b) Acclaim 120 C18 column.

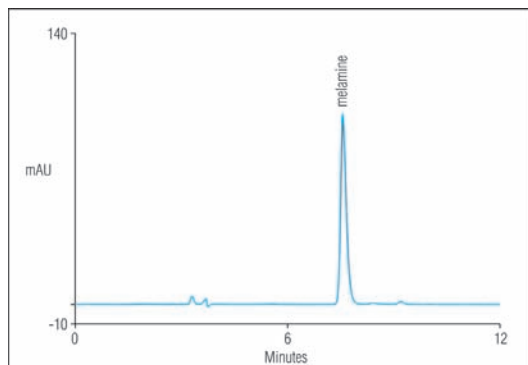


Figure 4 Overlay of chromatograms of five consecutive injections of a 20- $\mu\text{g/mL}$ melamine standard on the WCX-1 column.

The optimized mobile phase used for these samples is 80% 10 mM ammonium acetate, pH 4.3, 20% CH_3CN . For more complex samples, a higher buffer capacity may be required. In these situations, the concentration of ammonium acetate can be increased. This may result in a decrease in melamine retention time, but retention time might be restored by decreasing the percent of CH_3CN in the mobile phase.

Table 2 Comparison of milk powder sample analysis results using different HPLC methods*

Sample #	Melamine concentration using the Acclaim Mixed-Mode WCX-1 column** ($\mu\text{g/mL}$)	Melamine concentration using the RP-PIC method*** ($\mu\text{g/mL}$)
1	0.61	0.45
2	3.6	3.5
3	8.6	7.8
4	0.44	0.33
5	0.45	0.39

*The samples were prepared following the procedure described in Ref. 5.

**Under the WCX-1 chromatographic conditions described in this article.

***Under the chromatographic conditions described in Ref. 5.

Chromatographic performance

Figure 2 shows a comparison of multiple injections of different concentration standards on the WCX-1 column (chromatogram a) and the C18 column (chromatogram b). The baseline of the C18 separation is much noisier than the WCX-1 method, possibly due to the ion-pairing compound. Although there are two solvent peaks in the WCX-1 chromatogram with retention times close to the melamine, they do not interfere.

Calibration linearity for melamine was investigated by making five replicate injections of standards 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 3, excellent linearity was achieved throughout the range from 0.05 to 40 $\mu\text{g/mL}$ on the WCX-1 column and throughout the range from 0.2 to 100 $\mu\text{g/mL}$ on the C18 column. The linearity equations of melamine are as follows, with the curve forced through the origin:

$$A = 0.9120 c \text{ for the WCX-1 column}$$

$$A = 1.2788 c \text{ for the C18 column}$$

Here, A stands for peak area, and c stands for melamine concentration ($\mu\text{g/mL}$). The correlation coefficient (r) is 0.999932 for the WCX-1 method and 0.999961 for the C18 method.

Figure 4 shows an overlay of chromatograms from five consecutive injections of a 20- $\mu\text{g/mL}$ melamine standard on the WCX-1 column. Note the good reproducibility of retention time and peak area.

Method detection limits (MDLs) for the two melamine separations were calculated by using $S/N = 3$. The calculated MDL values are 0.028 $\mu\text{g/mL}$ for the WCX-1 method and 0.17 $\mu\text{g/mL}$ for the C18 method, demonstrating the lower noise and higher sensitivity of the WCX-1 separation.

Comparison of analysis results

Table 2 lists the analysis results of five different types of milk powder samples (#1–5) obtained from a manufacturer. The samples were prepared according to the SPE procedure specified in Ref. 6, and separations were performed on an Acclaim 120

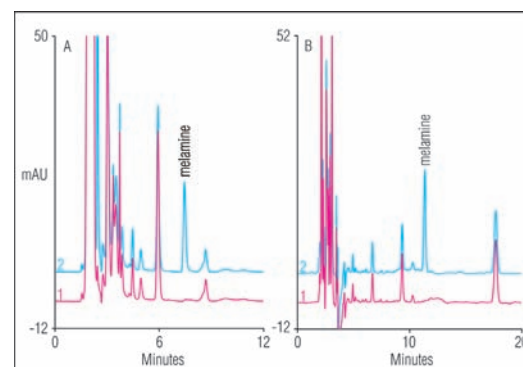


Figure 5 Chromatograms of a milk sample and the same sample spiked with melamine on a) Acclaim Mixed-Mode WCX-1 column, and b) Acclaim 120 C18 column. Chromatograms: 1) sample #6, and 2) sample #6 spiked with 4 $\mu\text{g/mL}$ melamine. Melamine is detected by absorbance at 240 nm.

Table 3 Sample analysis results

	Milk powder				Liquid milk						
	#6		#7	#8	#9		#10				
Melamine	Detected ($\mu\text{g/mL}$)	Added ($\mu\text{g/mL}$)	Found ($\mu\text{g/mL}$)	Recovery (%)	Detected ($\mu\text{g/mL}$)	Detected ($\mu\text{g/mL}$)	Detected ($\mu\text{g/mL}$)	Added ($\mu\text{g/mL}$)	Found ($\mu\text{g/mL}$)	Recovery (%)	Detected ($\mu\text{g/mL}$)
	NA	4.0	3.5	88	25.3	7.2	1.8	4.0	5.4	90	22

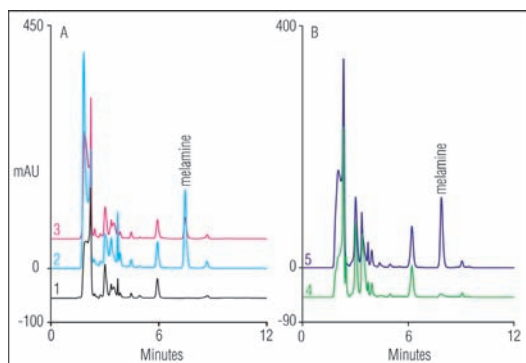


Figure 6 Chromatograms of a) milk powder samples #6, #7, and #8, and b) liquid milk samples #9 and #10, separated on the WCX-1 column. Samples were prepared using the new sample preparation method. The chromatogram of sample #6 clearly shows the lack of interfering peaks in real samples using the new sample preparation method.

C18 column under the chromatographic conditions in Ref. 6 (RP-PIC method) and on the Acclaim Mixed-Mode WCX-1 column under the chromatographic conditions described above. The results are similar for all five samples.

Sample analysis

Five samples, including three milk powder samples (#6–8) and two liquid milk samples (#9 and #10), were analyzed using the sample preparation method described in this article. Figure 5 compares separation of sample #6 on the WCX-1 and C18 columns. No melamine was detected in #6; thus spiked samples were also compared. Both columns produced good separations with no interfering peaks using the new sample preparation method.

Melamine was found in all the other milk and milk powder samples. The analytical results produced using the new sample preparation method and the WCX-1 column are summarized in Table 3. Figure 6 shows overlays of chromatograms of samples #6–10 separated using the WCX-1 column. Samples #6 and #10 were also

spiked with melamine before sample preparation, and both demonstrated sufficient recovery, generally better than the recovery from the C18 column separation method.

Conclusion

This paper compares the currently approved HPLC-UV method to 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 allows faster separations and exhibits good retention of melamine, using ammonium acetate buffer and acetonitrile as the mobile phase. This mobile phase prevents column damage caused by ion-pairing reagents and also makes the separation method compatible with MS detection. Background noise is lower using the WCX-1 method, enabling lower detection limits. The newly developed sample preparation method is less labor intensive and produces higher recoveries.

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