Quantitative Liquid Chromatography Analysis of Melamine in Dairy Products Using Agilent's 1120 Compact LC and 1200 Rapid Resolution LC

Application Note

Food Safety

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Abstract

In this application, three different LC methods are developed for the determination of melamine in dairy products. The first is a modification of the U.S. FDA method [1]. An Agilent LC system (1120 or 1200) is used with a ZORBAX SB-C8 LC column to run in reversed-phase ion-pair mode for routine quantitation of melamine. The second method is targeted for high throughput using an Agilent Rapid Resolution LC (RRLC) system (1200SL) to speed melamine analysis by more than three times with a Rapid Resolution High Throughput (RRHT) column. The third is an alternative ion-exchange LC method where a ZORBAX 300SCX column is employed to successfully retain melamine using a simple mobile phase of buffered water/acetonitrile without the presence of ion-pair reagent. Due to the complexity of dairy product matrices, a cleanup step using solid phase extraction (SPE) is required for the above methods. The Agilent SampliQ SCX, a mixed-mode polymer SPE cartridge with combined reversed-phase and strong cation exchange properties, is used to successfully remove matrix interferences.

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Introduction

In March 2007, imported pet food ingredients contaminated with melamine caused renal failure in dogs and cats across the United States. Once again, this compound is in the news as an illicit adulterant in milk and milk products. The same contaminant is now being detected in other food products that contain milk imported from China and as global concern rises, widespread testing for melamine is proceeding.

The published analytical approaches include LC, LC/MS, and GC/MS. The LC method is being used for quantitative analyses of melamine. Liquid chromatographic separation of this small polar compound can be achieved by reversed-phase ion-pair liquid chromatography. The U.S. FDA developed this methodology for melamine in pet food in 2007. With a slight modification of the proportion of mobile phase, the method can be successfully applied to separate melamine from a variety of dairy product matrices.

The disadvantage of conventional HPLC is time and solvent consumption. The Agilent 1200 Series Rapid Resolution LC system is designed for highest throughput without loss of resolution or with better resolution in combination with the Agilent ZORBAX RRHT columns. In this application note, the conventional HPLC method is transferred from a 4.6 mm × 250 mm, 5 µm ZORBAX SB-C8 column to a 4.6 mm × 50 mm, 1.8 µm RRHT ZORBAX SB-C8 column with equivalent results, and the LC run time is shortened from almost 20 minutes to 6 minutes.

An alternative approach for liquid chromatographic separation of this small polar compound is ion exchange chromatography. Agilent ZORBAX 300SCX is an ionic bonded-phase column packing used for cation exchange high-performance liquid chromatography. This packing consists of an aromatic sulfonic acid moiety covalently bonded to ZORBAX porous silica. This column is successfully applied to retain melamine using a simple mobile phase of buffered water/acetonitrile without the presence of ion-pair reagent.

For complex dairy product matrices, it is necessary to remove interferences such as protein, sugar, and fat before LC injection. Solid-phase extraction (SPE) is a simple way to clean up the complex matrix extract. SampliQ is a new family of SPE cartridges from Agilent with a wide range of sorbent chemistries. Among this family, the mixed-mode SampliQ Strong Cation Exchange (SCX) cartridge is a sulfonic acid-modified divinyl benzene polymer with both ion exchange and reversed-phase retention properties. This makes the SampliQ SCX very effective for cleanup after solvent extraction.

Experimental

Standard Preparation

A stock solution of melamine at 1,000 µg/mL is prepared in methanol by sonication. Dilutions in mobile phase are made up at 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 µg/mL concentrations.

Sample Preparation

The sample preparation process is a modification of the China national standard [2].

Sample Extraction Procedure

For liquid milk, milk powder, yogurt, ice cream, and creamy candy samples:

- Weigh 2 ± 0.01 g of sample and add to a 50-mL centrifuge tube, add 15 mL of 5% trichloroacetic acid in water and 5 mL of acetonitrile, then cap.
- Sonicate for 10 min and then place samples on vertical shaker for 10 min. Centrifuge for 10 min at 4000 rpm.
- Wet filter paper with 5% trichloroacetic acid in water, then filter the supernatant into a 25.0-mL volumetric flask, and bring to volume with 5% trichloroacetic acid in water.
- Transfer a 5.0-mL aliquot of the extract into a glass tube, and then add 5.0 mL purified water. Vortex to mix thoroughly.

For cheese, cream, and chocolate samples:

- Weigh 2 ± 0.01 g of sample, grind with 8~12 g of sea sand in a mortar, and then transfer into a 50-mL centrifuge tube.
- Wash the used mortar with 5 mL of 5% trichloroacetic acid in water three times, transfer washings into a 50-mL centrifuge tube, and then add 5 mL of acetonitrile.
- Proceed with the sonication and other steps as described in the previous preocedure..
- If the sample is very fatty, defat the extract using liquid-liquid extraction with hexane saturated with 5% trichloroacetic acid in water before cleanup by SPE.

Sample Cleanup Procedure

A SampliQ SCX SPE cartridge (p/n 5982-3236, 3 mL, 60 mg, or p/n 5982-3267, 6 mL, 150 mg) can be used to clean up sample extracts; the latter is used in this application note. All SPE elution steps, including conditioning, sample load, washing, and the final elution, are performed with a flow rate of less than 1 mL/min except for drying the cartridge by applying vacuum.

- Condition the SPE cartridge with 5 mL of methanol followed by 6 mL of water.
- Load the above sample extract to the conditioned cartridge. Wash the cartridge with 5 mL of water followed by 5 mL of methanol.
- Dry the cartridge by applying vacuum, and then elute with 5 mL of 5% ammonium hydroxide in methanol.
- Evaporate the eluate to dryness under a stream of nitrogen at approximately 50 °C.
- Reconstitute the dried extract in 1.0 mL of mobile phase, vortex for 1 min, and filter through a 0.2-µm regenerated cellulose membrane filter (p/n 5064-8222) into a glass LC vial.

Instrumentation and Conditions

Conventional HPLC method using 1120 Compact LC or 1200 LC:

- Agilent 1120 Compact LC system with gradient pump (degasser inside), autosampler, column compartment, and variable wavelength detector (VWD) or equivalent 1200 Series components
- EZChrom Elite Compact software or ChemStation software (Ver. B.04.01 or later)

Column	ZORBAX SB-C8 (also known as ZORBAX Rx-C8), 4.6 mm × 250 mm, 5 µm (p/n 880975-906)
Buffer	10 mM citric acid, 10 mM sodium octane sulfonate, adjusted to pH 3.0
Mobile phase	92:8 buffer:acetonitrile
Flow rate	1.5 mL/min
Injection volume	20 µL
Column temperature	30 °C
Detection wavelength	240 nm
Run time	20 min

High-Throughput Method Using 1200SL RRLC:

- Agilent 1200SL Series binary pump, degasser, wellplate sampler, thermostatted column compartment and diode array detector (DAD)
- ChemStation software (Ver. B.04.01 or later)

Column	ZORBAX SB-C8 RRHT, 4.6 mm × 50 mm, 1.8 µm (p/n 827975-906)
Buffer	10 mM citric acid, 10 mM sodium octane sulfonate, adjusted to pH 3.0
Mobile phase	92:8 buffer:acetonitrile
Flow rate	1.5 mL/min
Injection volume	8 µL
Column temperature	30 °C
Detection wavelength	240 nm
Run time	6 min

Ion Exchange Chromatography Method with 1120 Compact LC or 1200 LC:

- Agilent 1200 Series binary pump, degasser, wellplate sampler, thermostatted column compartment and variable wavelength detector (VWD) or equivalent 1120 Series components
- EZChrom Elite Compact software or ChemStation software (Ver. B.04.01 or later)

Column	ZORBAX 300SCX, 4.6 mm × 150 mm, 5 µm (p/n 883952-704)
Buffer	50 mM ammonium formate solution, adjust to pH 3.0 with formic acid
Mobile phase	15:85 buffer:acetonitrile
Flow rate	1.0 mL/min
Injection volume	10 µL
Column temperature	30 °C
Detection wavelength	240 nm
Run time	5.5 min

Results and Discussion

Separation of Melamine in Dairy Products by Reversed-Phase Ion-Pair LC

Melamine is not retained by reversed-phase LC and thus elutes with the solvent and unretained matrix interferences. However, using an ion-pairing reagent with reversed-phase chromatography, melamine can be well retained and separated from interferences. Figure 1 (a) is the chromatogram of melamine standard by reversed-phase ion-pair LC. Figure 1 (b) is the chromatogram of a positive yogurt sample after cleanup with the Agilent SampliQ SCX SPE cartridge.

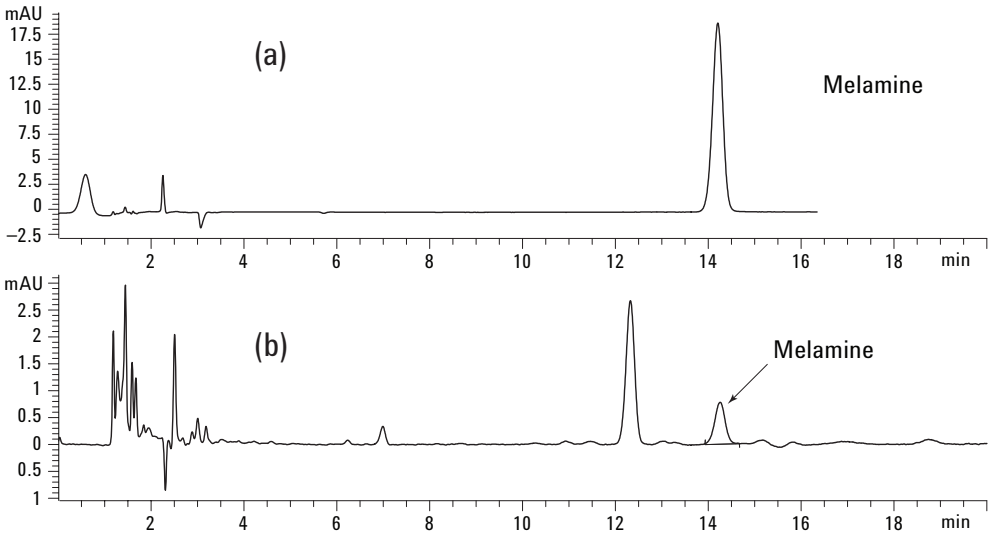

Figure 1. Separation of (a) 20 µg/mL melamine standard, and (b) positive yogurt sample after cleanup by SampliQ SCX SPE cartridge. Retention time of melamine is 14.2 minutes.

High-Throughput Analysis by Agilent 1200SL RRLC with RRHT Column

With the Agilent 1200 Series RRLC system, high throughput is possible. In combination with the Agilent ZORBAX RRHT columns, excellent chromatographic resolution can be achieved at much shorter run times than with a conventional LC system. A RRLC method is developed to dramatically increase the sample throughput for the determination of melamine in dairy products. Figure 2 (a) is the chromatogram of a melamine standard by the RRLC method with the retention time of melamine at 2.8 minutes.

Figure 2 (b) is the chromatogram of the same yogurt sample in Figure 1 (b). In order to make sure that the column is clear for the next injection, the total run time is extended to 6 minutes. The high throughput RRLC method is applied in the variety of dairy products matrices, including yogurt, liquid milk, and milk powder to demonstrate that the same resolution is achieved as with the conventional HPLC method. The calibration curve for the RRLC method is shown in Figure 3. The calibration includes 0.8, 2.0, 20.0, 40.0, and 80.0 µg/mL. The instrumental LOD (limit of detection) of the RRLC method is 0.03 µg/mL.

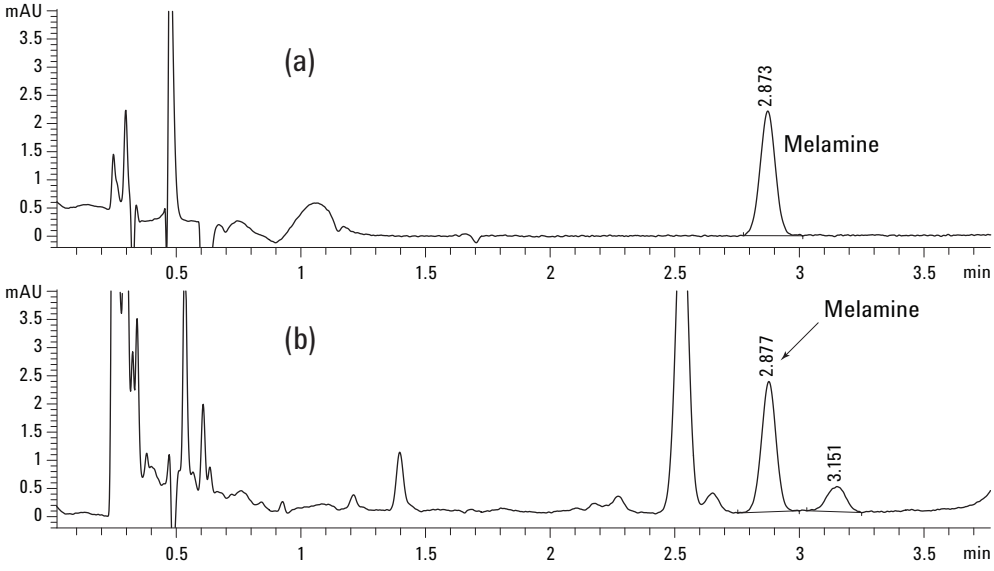

Figure 2. Separation of (a) 0.8 µg/mL melamine standard, and (b) positive yogurt sample after cleanup by SampliQ SCX SPE cartridge. Retention time of melamine is 2.8 minutes.

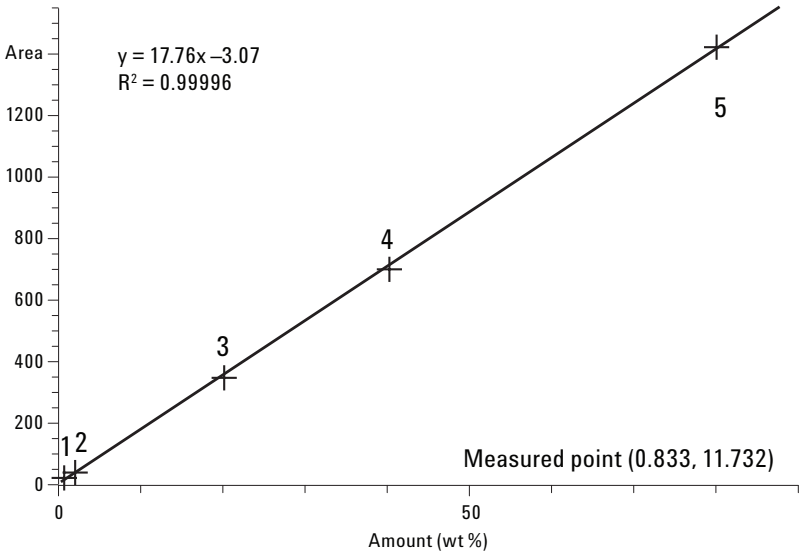

Figure 3. Calibration curve of RRLC method.

Ion Exchange Chromatographic Method

An alternative to ion-pair reversed-phase chromatography for melamine is ion exchange chromatography (IEC). The Agilent ZORBAX 300SCX is used for cation exchange high-performance liquid chromatography (HPLC). This column is employed to separate melamine in dairy product matrices with sufficient retention to separate matrix interferences. Figure 4 shows the separation of melamine from interferences without the SPE cleanup step. Generally, the total run time of the ion exchange chromatography is only 5.5 minutes with an LOD of 0.05 µg/mL, as shown in Figure 5. The calibration curve for the IEC method is shown in Figure 6. The calibration points include 0.5, 1.0, 5.0, 10, 50, and 100 µg/mL. Although the separation is shown to be interference free for raw milk and liquid milk without any additive, it is still recommended that the cleanup step be included to ensure robust methodology for running many samples and samples of different matrices.

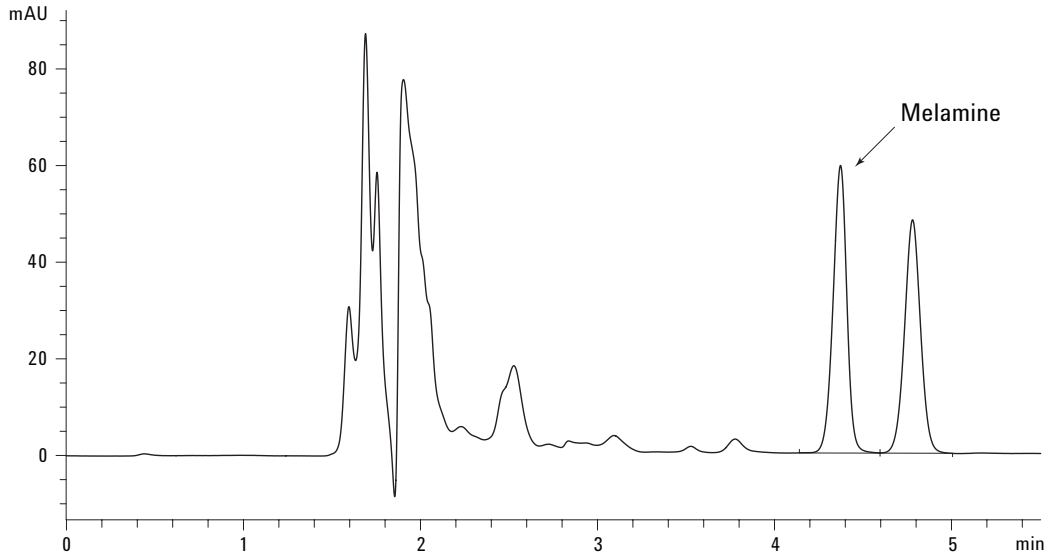

Figure 4. Separation of melamine in milk powder sample by IEC without cleanup by SPE.

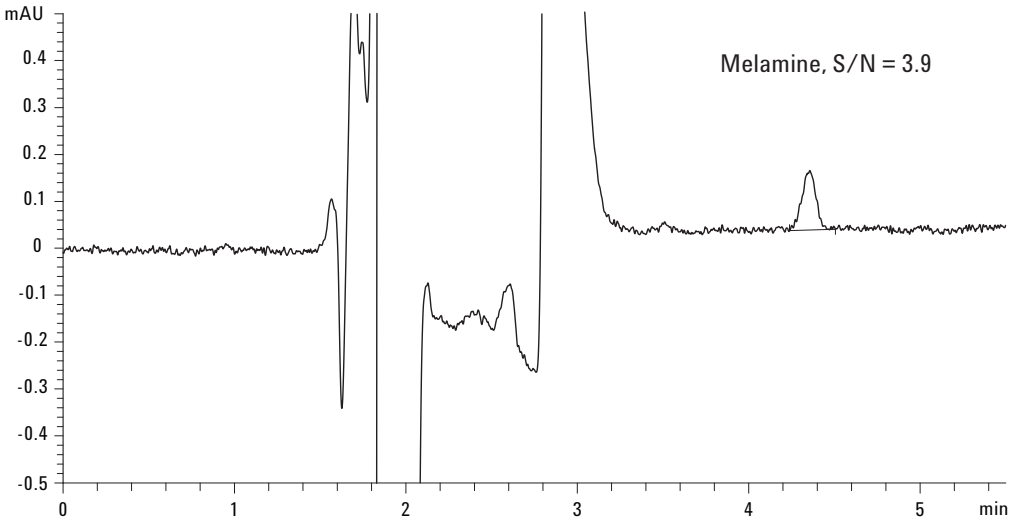

Figure 5. Limit of detection (LOD) for melamine at the concentration of 0.05 µg/mL.

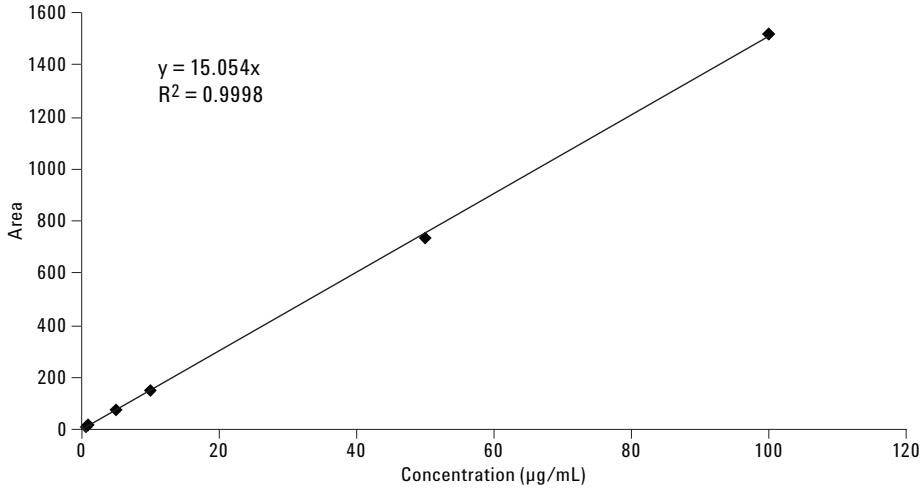

Figure 6. Calibration curve of IEC method.

Conclusions

Three approaches are described in this application note; the first is a reversed-phase ion-pair LC method employing Agilent 1120 compact LC or 1200 HPLC with an SB-C8 column. The second is a high-throughput method, which reduces the LC run time from 20 minutes to 6 minutes using the Agilent 1200 RRLC with the ZORBAX RRHT SB-C8 column. The last is an IEC method using the Agilent ZORBAX 300SCX column. Each successfully separates melamine from matrix interferences and provides identification by retention time and quantitative results. The results of this study, including sample cleanup with SampliQ SCX SPE cartridges and the three separation approaches, show that a complete solution from Agilent for the determination of melamine in dairy products is provided. The reversed-phase ion-pair method is based on the FDA and China national standards. However, the IEC method is simple, quick, sensitive, and robust. With this method, melamine can be successfully retained using a simple mobile phase without the presence of an ion-pair reagent.

References

1. Updated FCC Developmental Melamine Quantitation (HPLC-UV), April 2, 2007
2. GB/T 22388-2008 Determination of melamine in raw milk and dairy products, October 7, 2008

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Published in the USA
October 23, 2008
5989-9949EN

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