Quick UHPLC/MS analysis of Melamine and Its Analogues from Powdered Infant Milk Using Polymeric Solid Phase Extraction

Daniel Tran, Agilent Technologies, 25200 Commercentre Drive, Lake Forest, CA 92630

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Introduction

Since the infant milk scandal in 2007, several methods have been released for the analysis of melamine from dairy products. However, a comprehensive method for the analysis of the related compounds (ammeline, ammelide, and cyanuric acid) is difficult to find. The US FDA has a comprehensive method but the lengthy sample preparation steps may not be practical in a production laboratory where low sample preparation time is critical. The hydrophilic nature of the analytes, suggests a HILC LC column would be the most promising and most procedures use a HILIC LC method. HILIC has many pitfalls concerned with reproducibility and high organic solvent usage.

An alternative simple method was developed using Captiva ND plates, This quick and reliable method utilizes a reversed phase LC column and limits the preparation time to a minimum, while maintaining quantification of melamine at regulatory levels. Using Captiva ND plates a simple and quick analysis of melamine and its analogues was developed. Good linearity was achieved for each analyte and as well as good accuracy and precision. A comparison of diluted samples to samples processed through the Captiva ND was also investigated, demonstrating better sensitivity is achieved by using the Captiva ND plate

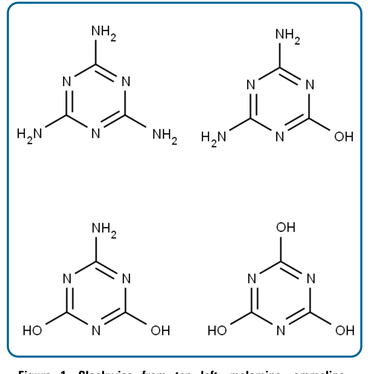


Figure 1. Clockwise from top left, melamine, ammeline, ammelide, and cyanuric acid respectively

Experimental

Standards

Standards were ordered from Chromadex and made into 1.0 mg/mL stock solutions. Melamine and cyanuric acid were prepared in water. Ammeline and ammelide were prepared in a 2% ammonium hydroxide solution. All four compounds were mixed together to a concentration of 100µg/ml in 2% Ammonium Hydroxide. Ammonium Hydroxide is required to keep all compounds in solution.

Powdered infant milk was prefortified to concentrations ranging from 0.1 µg/g to 10 µg/g of melamine and its analogues.

Sample Preparation

1. Weigh out 1 ± 0.01 g powdered infant milk
2. The milk was then spiked with analytes to 1 µg/g
3. Add 20 mL H2O
4. Vortex or shake, there should be no remaining powder
5. Transfer 2, 1mL aliquots of milk to test tubes
6. To one add 2mL 0.1N HCL (will be applied to Plexa PCX)
7. To the other sample add 2mL 0.1N sodium hydroxide (will be applied to Plexa PAX)
8. Let sit for 30 mins.

	Plexa PCX	Plexa PAX
Condition	1. 3mL MeOH 2. 3mL H2O	1. 3mL MeOH 2. 3mL H2O
Load	Sample pretreated w/ 0.1N HCL	Sample pretreated w/ 0.1N NaOH
Wash	1. 3mL 2% formic acid in H2O 2. 3mL 50:50 MeOH:ACN	1. 3mL H2O 2. 3mL MeOH
Elute	3mL 5% Ammonium Hydroxide in 50:50 MeOH:ACN	5% Acetic Acid in MeOH
Evaporate	Reconstitute in 500ul 50:50 MeOH:ACN	

Table 1. SPE Method

LC Conditions

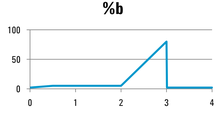
Column – Pursuit XRs Ultra 2.8 Diphenyl 100x2.0mm
Instrument: Agilent 1290/6460 QQQ

Mobile Phase
A: 0.1% Aqueous Formic Acid
B: MeOH

Pump Program
Flow rate 400 µL/ min.

t_0	A: 98%, B: 2%
$t_{0.5-2.0}$	A: 95%, B: 5%
$t_{2.01-3.00}$	A: 20%, B: 80%
$t_{3.01-4.00}$	A: 98%, B: 2%

Run Time = 4:00 minutes.



MS Conditions

MS Conditions	
Source	Agilent JetStream ESI+
Gas Temp	300 °C
Gas Flow	5 L/min
Nebulizer	20 psi
Sheath Gas Temp	275 °C
Sheath Gas Flow	7L/min
Capillary	+3500/-2000

Compound	Precursor Ion	Product Ion	Fragment	Collision Energy	Polarity
Melamine	127	85.1	100	18	+
Cyanuric Acid	128	42.1	60	14	-
Ammeline	128	69.1	140	34	+
Ammelide	127	84	100	6	-

Results and Discussion

Although this method requires a separate preparation for cyanuric acid, a single reversed phase LC run can be used for all compounds. Figure 2 shows the separation achieved using a reversed phase column. The melamine-cyanuric acid pair as well as the ammeline-ammelide pair show baseline separation.

The samples treated with HCL were applied to Plexa PCX cartridges. From those cartridges melamine, ammeline, and ammelide were extracted. Cyanuric acid could only be extracted on an anion exchange cartridge. Those samples were pretreated with NaOH and extracted on the Plexa PAX cartridges. All compounds were reliably detected down to 0.2 µg/g.

Results and Discussion

Figure 2. baseline separation of the melamine-cyanuric acid pair and the ammeline-ammelide pair at 10ng/ml

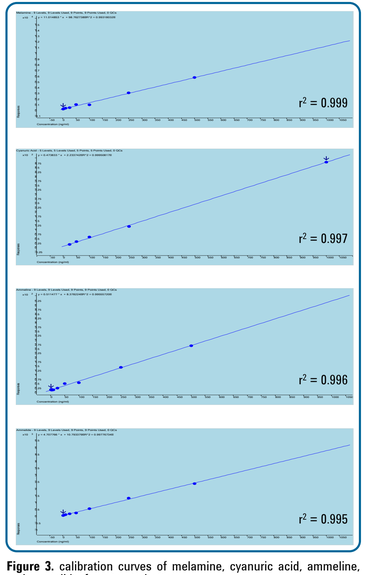


Figure 3. calibration curves of melamine, cyanuric acid, ammeline, and ammelide, from top to bottom.

All compounds showed linearity from 0.5 µg/g - 10µg/g with a R^2 above 0.995. A minimum of 5 levels were used for the calibration curves.

	Average % Recovery ± RSD	
	SPE	**Captiva**
Melamine	100 ± 7.9	94 ± 12.4
Cyanuric Acid	117 ± 5.9	n/a
Ammeline	114 ± 6.8	n/a
Ammelide	112 ± 5.8	n/a

Table 2. Recoveries of melamine and its analogues from fortified powdered infant formula compared to Captiva ND method, 1.0 µg/g (n=6)

Table 2 lists the relative recoveries of melamine and its analogues following sample preparation with Plexa PCX & Plexa PAX. All analytes extracted with Plexa PCX showed relative recoveries within 14% of true value with RSDs below 8%. Cyanuric acid, extracted from Plexa PAX, showed relative recovery within 17% of true value with a RSD of less than 6%.

Analyte sensitivity increased across the board for all compounds. Figure 4 demonstrates a 3 fold sensitivity increase for melamine compared to a similar sample prepared via Captiva ND, both were prefortified to 1.0µg/g.

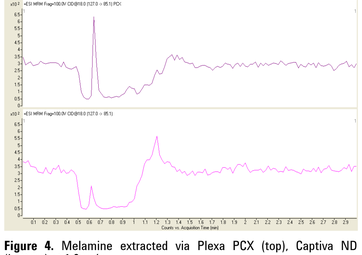


Figure 4. Melamine extracted via Plexa PCX (top), Captiva ND (bottom) at 1.0µg/g

Quick analysis of melamine and its analogues were accomplished down to the regulatory levels of 1µg/g (2) and below. The previous Captiva ND method was sensitivity enough only for melamine, maintaining a signal-to-noise ratio > 5:1 required by the US FDA at the 1µg/g level (1).

Conclusions

- This method couples generic polymeric SPE methods & reversed phase LC to reduce the complexity involved with the current FDA method, employing complex sample preparation & HILIC LC.
- The two tiered approach using Plexa PCX & Plexa PAX allows for the quick analysis of melamine and its analogues down to and below regulatory levels without sacrificing sensitivity and ease of use.
- At regulatory levels melamine, ammeline, and ammelide showed relative recoveries within 14% of true value with RSDs below 8%. Cyanuric acid showed a relative recovery within 17% of true value with a RSD of less than 6%.
- Sensitivity was maintained below the regulatory level of 1µg/g. Ammeline, ammelide, and cyanuric acid maintained an SNR above 5:1 down to the 0.1µg/g level. Melamine was capable of maintaining that SNR down to 0.5 µg/g.

References

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2. Chinese Ministry of Health (2011). China sets limits of melamine levels tolerable in food products, 21 April, 2011 (http://english.gov.cn/2011-04/21/content_1849392.htm)
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