

Simple and Rapid Screening of Melamine in Milk Products with High Resolution Accurate Mass Benchtop Orbitrap LC/MS

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Overview

Purpose: To develop a rapid and simple liquid chromatography mass spectrometry (LC/MS) method employing high resolution, accurate mass capability for analyzing melamine in three milk products at sub-ppm (mg/kg) or ppb (µg/kg) level without using solid phase extraction and with only minimal LC separation.

Methods: Milk products were extracted with dilute formic acid followed by protein removal with acetonitrile. The melamine was separated with a fast 1-minute isocratic run on an anion exchange column operated in Hydrophilic Interaction Chromatography (HILIC) mode, and detected using a Thermo Scientific Exactive benchtop Orbitrap-powered mass spectrometer operated at the resolution of 50,000.

Results: The Exactive™ mass spectrometer can detect the melamine standard to 0.1 ppb (0.5 pg on column) and with a linearity response range up to 100 ppb. The milk extracts show substantial ion suppression but with consistent response in each of the three sample matrices. Melamine can be detected in the final extract from each matrix at <1 ppb, which corresponds to <44 ppb (in coffee cream), <65 ppb (in infant formula) and <110 ppb (in instant coffee mix).

Introduction

While generally for industrial use, melamine, a nitrogen-rich, white crystal, was added to many milk products in order to falsify protein levels. Young children who consumed the contaminated milk products were found to have kidney stones and renal failure. This caused a melamine-contaminated milk scandal in China in 2008. The contaminated milk products were found soon afterward in many other countries and regions, causing a widespread concerns and demands for monitoring melamine in various milk products.

Different countries vary in setting the Maximum Residue Limit (MRL) for melamine, but generally follow the United States Food and Drug Administration (US FDA) MRL of 1 ppm for infant formula and 2.5 ppm for other milk products¹. Most food testing labs employ mass spectrometry based methods, particularly liquid chromatography tandem mass spectrometry (LC-MS/MS), for detecting sub-ppm to low ppb levels of melamine.

Milk is a complex matrix containing soluble protein, sugars, and fat, with additional nutrients such as vitamins and minerals in the infant formula. Sample cleanup is critical and two approaches are generally used. First, the dilute-and-shoot in which the milk products are dissolved in dilute acid, followed by protein precipitation with acetonitrile. US FDA uses a similar method for reporting limit of quantitation (LOQ) of 250 ppb on LC-MS/MS. For more sensitive detection by LC-MS/MS, additional solid phase extraction (SPE) is required.

The second complication in analyzing melamine with LC/MS is that melamine, being a strong polar small molecule, cannot be retained in conventional reverse-phase HPLC. Ion pairing or HILIC mode are used.

In this study, we evaluate a simple and rapid LC/MS method to screen trace level melamine in milk products by utilizing a new benchtop high resolution, accurate mass Orbitrap mass spectrometer. The sample preparation uses dilute-and-shoot. Analysis is fast and requires minimal LC separation.

Methods

Samples:

Concentrated infant formula, instant coffee mix (3-in-1 with coffee, creamer and sugar) and liquid coffee creamer (non flavored) were purchased from local supermarket.

Sample Preparation:

Milk samples were extracted with 2.5% Formic Acid followed by protein removal with acetonitrile following the US FDA published procedures and shown in Figure 2. The total dilution factor as a result of sample preparation was given in Table 1.

LC Conditions:

LC: Thermo Scientific Accela Liquid Chromatography System
 Column: Thermo Scientific Hypersil BioBasic AX 50x3 mm, 5 µm
 Eluent: 95:5 MeCN (0.1% FA) Water (0.1% FA) isocratic at 500 µL/min
 Injection: 5 µL (with loop)
 Run time: 1 minute

FIGURE 1. Melamine (C₃H₆N₆)

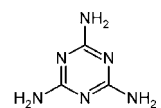


FIGURE 2. Sample Preparation Flowchart

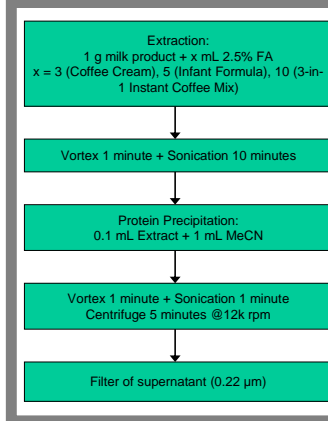


Table 1. Total Dilution Factor

Infant Formula	Coffee Cream	3-in-1 Coffee Mix
65	44	110

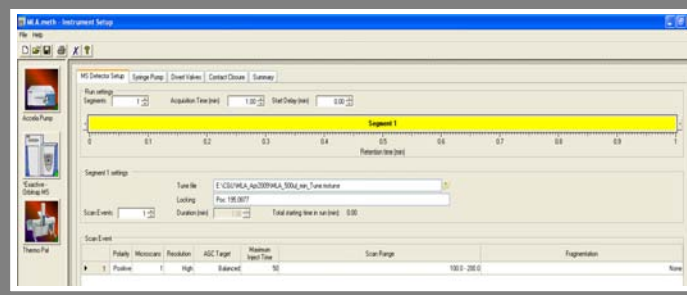
FIGURE 3. Exactive and LC module



Mass Spectrometer Conditions:

Mass Spectrometer: Exactive benchtop high resolution, accurate mass system (Figure 3)
 MS Parameter setting: See Figure 4
 Resolution: High (R=50,000)
 Lockmass: m/z 195.0877 (Caffeine)
 Ion Source: Electrospray ionization (ESI) +3.5 KV
 Vaporizer Temp: 300 °C
 Tube lens: 94 V
 Sheath/Aux Gas: 30/10 unit with N₂
 Capillary Temp: 270 °C

FIGURE 4. MS Parameter Settings



Results and Discussion

The goal of this study is to explore high resolution mass benchtop mass spectrometry to develop a simple and rapid LC/MS method based on accurate mass determination to test melamine in milk products with a detection limit lower than 250 ppb (reporting LOQ of US FDA method for infant formula with LC-MS/MS using a triple stage quadrupole mass spectrometer). The sample preparation followed the dilute-and-shoot approach without the use of a laborious and time-consuming SPE procedure.

Other than the conventional ion source tuning, the Exactive was simple to setup with resolution being the only parameter to adjusted to high (R=50,000). The m/z 195.0877 of caffeine was used as lock mass because caffeine was conveniently present in the tuning solution. After each tuning, the residue caffeine peak can be observed for at least a couple of days. That additional strong caffeine peak can be found in coffee samples. Under these conditions, melamine (m/z 127.0727) can be unambiguously identified with mass accuracy better than 3 ppm. A 3-ppm mass accuracy window filter was thus used for data processing.

The Exactive mass spectrometer sensitivity and linear response range were evaluated with the melamine standard solution. Figure 5 shows the chromatogram and accurate mass spectra of a representative 0.1 ppb solution. Figure 6 shows a representative calibration curve demonstrating a linear response from 0.1 to 100 ppb.

Figure 5. Chromatogram and Spectra of 0.1 ppb (5 pg on column) Melamine Standard

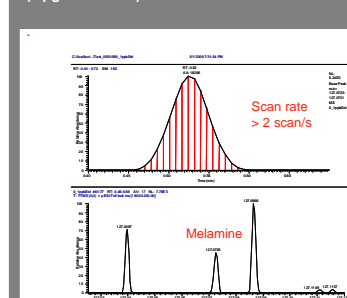
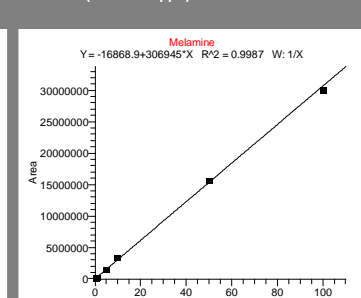


Figure 6. Calibration Curve of Melamine Standard Solution (0.1 to 100 ppb)



Milk samples were found to have a strong matrix effect that results in severe ion suppression. Preliminary experiments with loop injection without any LC separation failed to detect 1 ppb melamine spiked in any of the three matrices even with further 5x dilution of the sample matrices. Thus it is decided that a simple LC separation is required.

The current LC separation employed an anion exchange column running with strong organic phase (95% v/v MeCN) in 1 minute, creating a HILIC condition² that separated the melamine (R.T. ~0.54-0.6 min) from the major interference species eluted either in the void volume (0.35-0.4 min) or after the melamine. An isocratic run was chosen to eliminate the column equilibration time between each injection, thus increasing the sample throughput.

Figure 7 shows the comparison of 1 ppb melamine spiked in a mobile phase (Neat) and in three extracted sample matrices. As shown, 1 ppb spikes can be detected. Based on the dilution factor from sample preparation in Table 1, the detection of 1 ppb spike corresponds to 65, 44, and 110 ppb in infant formula, coffee cream and 3-in-1 Instant Coffee Mix, respectively.

The responses of 1 ppb melamine in matrices are only 30-50% of that in Neat, but found to be consistent in each sample matrices in the 1-10 ppb range evaluated.

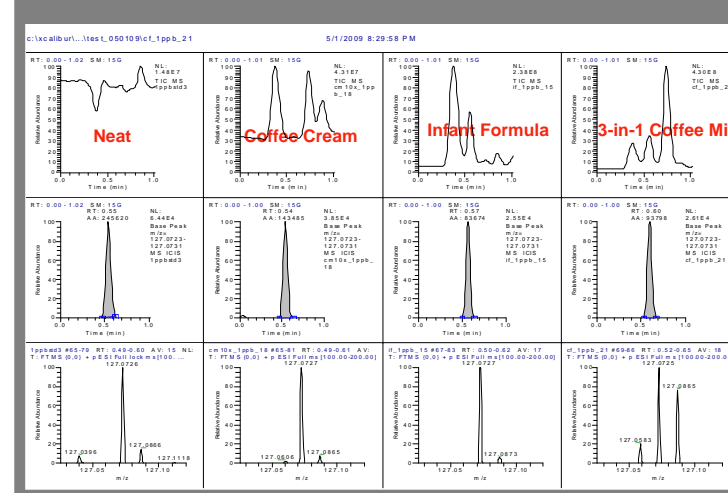
Table 2. Melamine Response Factor (RF) in Sample Matrix Compared to Neat Standard (= 1) and RSD% (n=3)

	Coffee Cream	Infant Formula	3-in-1 Coffee Mix
RF	0.50	0.31	0.34
RSD%	8.0%	7.2%	3.4%

The average response factor (RF) values from spiking 1, 5 and 10 ppb in each of three sample matrices are given in Table 2. A constant response factor makes it possible to use standard addition method for melamine quantitation.

The overall recovery was also evaluated by spiking 300 ppb in three milk products before extraction. The recovery values were found to be 75-91%.

FIGURE 7. Comparison of 1 ppb Melamine in standard (Neat) and spiked in extract sample matrix (top: TIC; middle: chromatographic peak, bottom: mass spectra).



Conclusions

- The high resolution, accurate mass Exactive mass spectrometer is shown to be sensitive in detecting <0.1 ppb melamine (0.5 pg on column) in neat standard and response is linear from 0.1 to 100 ppb. The error for mass accuracy is < 2-3 ppm with lock mass.
- Milk samples prepared by dilute-and-shoot have shown severe ion suppression that has been reduced with a simple and rapid HILIC LC separation (1 minute run), after which a consistent response factor of 0.3-0.5 for each sample matrix can be used for quantitation.
- Quantitation limits are less than 44, 65, and 110 ppb for coffee cream, infant formula and 3-in-1 instant coffee mix, respectively, exceeding the requirements of 100-250 ppb LOQ such as by US FDA (250 ppb in infant formula).

References

- <http://www.cfsan.fda.gov/~frf/lib4421.html>, Determination of Melamine and Cyanuric Acid Residues in Infant Formula using LC-MS/MS, Laboratory Information Bulletin No. 4421, October 2008, US FDA
- Varels, P., Beck, J., Wang, K., and Ghosh, D., Analysis of Melamine and Cyanuric Acid in Food Matrices by LC-MS/MS, Thermo Fisher Scientific App. Note 424.