Automation of Solid Phase Extraction (SPE) Prior to Analysis of Infant Formula for Melamine

Keywords: Beverage, FDA Method LIB No. 4422, Food, GB/T 22388-2008, GX-271 ASPEC, HILIC, Infant Formula, Milk, Strata™-X-C, Solid phase extraction (SPE)

Introduction

Melamine (CAS No. 108-78-1) is an organic base and a trimer of cyanamide with a 1, 3, 5-triazine skeleton (Figure 1). It is used for the manufacture of a variety of products including plastics, fire retardants, housewares, adhesives, cleaning products and colorants (pigment yellow 150).

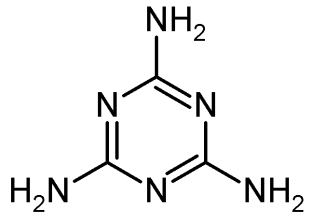

Figure 1. Chemical Structure of Melamine

Melamine is sometimes illegally added to food products to increase the apparent protein content. Kjeldahl and Dumas are standard tests that estimate protein levels by measuring the nitrogen content of the food product. The addition of melamine can falsely elevate the estimated protein level when using these tests (Snyder, 2007).

In 2007, a pet food recall was initiated by several pet food manufacturers when serious illness and deaths occurred in animals that had consumed pet food contaminated with melamine. In late 2008, some 300,000 infants in China were sickened, and at least six died, after consuming infant formula that had been deliberately adulterated with melamine (Fountain, 2009). As a result, the U.S. Food and Drug Administration (FDA) developed a method for the determination of melamine in foods (Smoker and Krynitsky, 2008) and a safety threshold of 1 ppm of melamine in infant formula was established (Associated Press, 2008).

Current methods for the determination of melamine in foods, like infant formula, use solid phase extraction (SPE) followed by triple quadrupole tandem mass spectrometry (LC/MS-MS). These methods are time consuming and complex. The purpose of this study was to determine if the SPE step used in FDA Method LIB No. 4422 for the analysis of melamine in foods could be readily automated to aid in providing a more simplified, more robust and less time consuming method for the extraction of melamine from infant formula prior to analysis. The SPE method in FDA LIB No, 4422 was modified and optimized utilizing the Gilson GX-271 ASPEC™ automated SPE system (Figure 2). The method was evaluated for performance using HPLC with a zwitterionic HILIC LC column.

Figure 2. Gilson GX-271 ASPEC System with 406 Single Syringe Pump (Part no. 2614007)

Experimental Conditions

Materials

All solvents were distilled in glass suitable for GC, HPLC, pesticide residues analysis and spectrophotometry. HPLC solvents were obtained from J.T. Baker or Fisher. All reagents were ACS grade quality or better. Melamine was purchased from Aldrich (Part no. M2659). Infant formula was purchased from the local grocery (Similac® brand) in both powder and liquid forms.

Preparation of Standards and Samples Prior to SPE

For liquid milk, weigh 5 g into a 50 mL conical Falcon™ tube. For powdered milk, weigh 1 g sample and add 4 mL of distilled water into a 50 mL Falcon tube.

Preparation of melamine stock solution: 1 mg/mL of melamine in 1:1 Acetonitrile (ACN): Water. Preparation of daily use melamine working standard: Dilute stock solution to 100 µg/mL in ACN containing 2% diethylamine (DEA).

- Add the desired amount of native melamine from the working standard (100 µg/mL) to the sample. Bring the sample volume up to 2 mL total using 2% DEA in ACN. This will drastically reduce precipitation in the liquid milk that can clog the SPE cartridges.
- Add 18 mL of extraction solution (1:1 ACN: Water)
- Vortex for 15–20 seconds until smooth
- Shake for 20 minutes at 700 rpm
- Centrifuge to separate the precipitant from the liquid
- Decant the liquid and transfer to test tubes for SPE cleanup

SPE Hardware

The Gilson GX-271 ASPEC System was configured as follows:

Description	Part Numbers
GX-271 ASPEC w/ Single 406 Syringe Pump	2614007
10 mL Syringe	25025345
SPE Pressure Reg. Assembly and plumbing package for gas + 10 mL Plumbing Package	25051376, 2644703 and 2644701
221x1.5x1.1 BV Tapered Probe and Guide Assembly for 1.5 mm Probes	27067374 and 26046228
Rinse Stations	26034551 and 26034555
Code 61 Rack with glass or plastic bottles	2954715, 2954663 or 2954662
Locator Tray for five 20-Series Racks	26041033
DEC Accessory Kit for 3 mL SPE Cartridges	2604702
Rack Code 343 for 80 13x100 mm Tubes	260440025
Safety Shield Assembly, GX27X	2604706
TRILUTION® LH Software Package	21063020, 210630R20 and ORACLE10GXE

SPE Method Optimization

The manual SPE method described in FDA Laboratory Information Bulletin LIB No. 4422 was optimized for automation utilizing the Gilson GX-271 ASPEC System. This system, in conjunction with Gilson TRILUTION® LH software, allows one to readily evaluate different SPE conditions such as types of solvents used, flow rates for conditioning, loading, washing and eluting, and volumes for each SPE task (Figures 3 and 4). SPE cartridges from four different manufacturers were evaluated, including the Waters Oasis™ MCX Cartridge (6 mL, 150mg, 60 micron) used in the FDA method listed above. Although the FDA method calls for the use of this cartridge, we determined through automated method development that the Phenomenex Strata™-X-C cartridge was more efficient and produced cleaner extracts prior to analysis using a HILIC LC column.

Figure 3. Wash Task Page in TRILUTION LH Method Builder. The open fields for all method tasks allows for easy method development with no manual intervention.

Figure 4. Gilson Mobile Rack. This rack allows for easy method development. Several types of SPE cartridges can be placed on the rack and eluted into different fraction collection tubes.

Solid Phase Extraction (SPE) Protocol

The final SPE procedure chosen used 3 mL Phenomenex Strata™ -X- C (200 mg, 33 micron) Cartridges. The cartridges were sealed using Gilson 3 mL Sealing Caps.

The SPE protocol is entirely automated using the Gilson GX-271 ASPEC system. The SPE steps are summarized with the schematic provided in the GX-271 ASPEC control software, TRILUTION LH (Figure 5).

Figure 5. TRILUTION LH SPE Tasks for Extraction of Melamine from Infant Formula

The details of each step are as follows:

- Initialization Step: Gilson Mobile SPE Racks are moved above the waste rack (Figure 4) and probe rinsed
- Condition SPE cartridge with 5 mL of ACN at a flow rate of 8 mL/min
- Condition SPE Cartridge with 5 mL of 4% formic acid/water at a flow rate of 8 mL/min
- Load 6 mL of sample extract at a flow rate of 2 mL/min
- Wash cartridge with 5 mL of ACN at a flow rate of 8 mL/min
- Wash cartridge with 5 mL of 0.2% DEA in ACN at a flow rate of 8 mL/min
- Move the Gilson Mobile SPE Rack over the collection tubes
- Elute the analytes of interest with 4 mL of 2% DEA in ACN
- Evaporate to dryness
- Dissolve in 500 uL of 2% DEA in ACN and analyze via HPLC

HPLC Analysis

HPLC Analysis was performed using a Gilson 322 HPLC Pump with a Merck SeQuant zwitterionic ZIC®-HILIC column (150 mm x 2.1mm, 5 um, 200Å, PEEK). Separation was accomplished using a binary gradient as follows:

- Mobile Phase A: 5% 10 mM Ammonium acetate: 95% ACN
- Mobile Phase B: 10 mM Ammonium acetate dissolved in 50:50 ACN/Water
    - Pump Program
        - 1–10 min: 100% A at 0.3 mL/min
        - 10.1–15 min: 50% A at 0.6 mL/min
        - 15.1–20 min: 100% A at 0.6 mL/min
        - 20.1–30 min: 100% A at 0.3 mL/min

The detection system was a Gilson 155 UV/VIS Detector at a wavelength of 230/232nm, sensitivity 0.005 AUFS.

Results

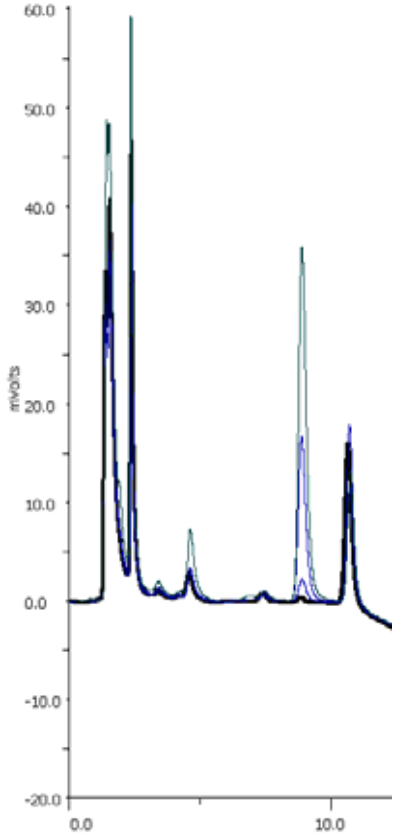

Figure 6. Chromatogram of a melamine extract from milk-based infant formula (liquid milk). Melamine elutes at 9.2 min. The overlay shows melamine levels at 100 ppb, 500 ppb and 1000 ppb.

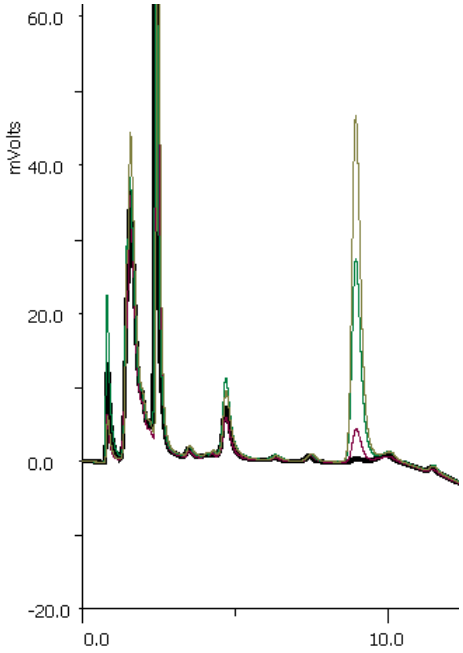

Figure 7. Chromatogram of a melamine extract from milk-based infant formula (powdered milk). Melamine elutes at 9.2 min. The overlay shows melamine levels at 100 ppb, 500 ppb and 1000 ppb.

Table 1. Coefficient of variation (CV) for melamine spiked into milk-based liquid infant formula. Reproducibility was measured for 5 days (n=3).

Melamine (ppb)	CV (%)
10	N/A
100	4
500	10
1000	3

Table 2. Coefficient of variation (CV) for melamine spiked into powdered milk-based infant formula. Reproducibility was measured over 5 days (n=3).

Melamine (ppb)	CV (%)
10	4
100	4
500	5
1000	6

Conclusion

Automation of the SPE protocols for melamine extraction used in FDA Method LIB No. 4422 produced good results, with an LOQ of approximately 100 ppb for liquid milk infant formula and 50 ppb for powdered milk infant formula. Results were reproducible from day to day with CVs of 4 to 10%. There was no carryover observed between samples. Optimization and automation of the SPE method employing a mixed mode cation exchange SPE cartridge and HILIC analysis (Merck, 2009; Nest Group, 2009) was able to detect melamine below the FDA safety threshold of 1 ppm allowable in infant milk.

In early experiments, the extraction of liquid milk produced a large percentage of suspended precipitant which caused SPE cartridges to easily clog. Upon diluting the sample with 2% DEA in ACN to a total volume of 2 mL and reducing the solvent volume to 18 mL we were able to eliminate the precipitation and resolve the clogging issue.

Automation of the SPE process allows one to easily optimize extraction conditions, reduce potential errors that may occur during manual extractions, increase lab efficiency and increase sample throughput. Although HILIC LC with UV detection was used in this study, the automated SPE method for melamine is readily adaptable for use with other SPE protocols and detection methods such as LC/MS-MS.

The Gilson GX-271 ASPEC and the GX-274 ASPEC, which is able to process four samples in parallel, are currently used for the automation of SPE extraction of melamine in raw milk and dairy products by several laboratories using Method GB/T 22388-2008 (AQSIQ and SAC – China, 2008; Chen, X-H, 2009; FLEXNEWS, 2008). This method utilizes a mixed mode SPE column (cation exchange/C8). Methanol and water are used for condition and wash steps with elution of melamine with 5% ammonia in methanol. Analysis is by LC/MS-MS, HPLC with UV or GC/MS.

Future studies will evaluate SPE protocols for the analysis of both melamine and cyanuric acid in different matrices utilizing LC/MS.

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