Method of Detection of Melamine in Milk and Milk Products

(USFDA Method)

A. Principle

A liquid chromatography triple quadrupole tandem mass spectrometry (LC-MS/MS) method for residues of melamine consists of an initial extraction with 2.5% aqueous formic acid, followed by a series of filtration, centrifugation, and dilution steps. The method is used for detection of both Melamine and Cyanuric Acid using HILIC LC Column. Melamine is detected in positive ion mode and cyanuric acid in negative ion mode. The extracts are analyzed by LC-MS/MS. Analyte concentrations are calculated using external standard calibration with a standard curve prepared in a pre-fortified control matrix which has been carried through the extraction procedure.

B. Chemicals and Reagents

(i) *Melamine (MEL).* CAS #: 108-78-1.
(ii) *Acetonitrile (ACN.)* LC grade.
(iii) *Formic acid.* Reagent grade >95%.
(iv) *Water.* LC grade, or purified by Millipore Milli-Q system to>18 M-ohm resistivity, or equivalent.
(v) *Ammonium Formate.* Purity> 97%.

C. Preparation of Solutions

(i) *0.1% Formic acid in water.* 1mL formic acid is transferred to 1L graduated flask and diluted to volume with LC water.
(ii) *Mobile Phase A.* 0.1% Formic acid in Acetonitrile (5:95 v/v). Mix 50 mL of 0.1% formic acid in water with 950 mL ACN in a 1 L solvent bottle.

(iii) *Mobile Phase B.* 20 mM Ammonium Formate in Acetonitrile (50:50 v/v). Mix 500 mL of 20 mM ammonium formate and 500 mL of acetonitrile in a 1 L solvent bottle.

(iv) *2.5% Formic acid in water.* 25 mL formic acid is transferred to 1 L volumetric flask and diluted to volume with LC grade water.

(v) *20mM Ammonium formate.* 0.63 gm of ammonium formate is weighed and dissolved in 0.5 L LC grade water.

D. Equipment

(i) *Liquid chromatograph.* Binary LC pump is recommended for accurate mixing at low flow rate and rapid response to mobile phase gradient.

(ii) *Liquid chromatography column.* ZIC-HILIC, 2.1 X 150mm, 5µm, 200 A

(iii) *Mass Spectrometer.* Triple quadrupole capable of meeting system suitability.

(iv) *Centrifuge.* Capable of 4000 RPM with 50 mL tubes.

(v) *Microcentrifuge.* Capable of 13,000 RPM with 1.5 or 2 mL tubes.

(vi) *Mixers and shakers*. Single and multi tube vortex mixers (VWR), platform shaker.

(vii) *Utrasonic bath.* Including timer and heater

(viii) *Centrifuge tubes*. 50mL disposable polypropylene with caps, with graducations from 5 to 50 mL and 1.5 mL microcentrifuge tubes.

(ix) *Syringe Filters*. Polyvinylidene fluoride (PVDF), 13mm, 0.22um

(x) *Syringes.* Three mL polypropylene.

E. Procedure

(i) Standard Preparation

Individual stock solutions, Melamine, approximately 100 µg/mL. Weigh approximately 10 mg of standard using a weigh boat to nearest 0.1 mg and transfer to a 100 mL glass volumetric flask. Add 70 mL 0.1% formic acid in

water and sonicate for 10 minutes. Maintain the volume as 100mL with 0.1% formic acid in water and mix thoroughly. Calculate exact concentration, correcting for purity.

Standard mixture dilution, 50 µg/mL is used for fortification and matrix calibration standards. Using volumetric pipets, transfer 5.00 mL of each stock standard into a 20 mL glass scintillation vial.

(ii) Sample Preparation

1. Sample powder (2.0 ± 0.1gm) is weighed in a 50 mL polypropylene centrifuge tube.
2. Pre-fortify control and matrix calibration standards.
3. 14 mL of 2.5% Formic acid in water is added to samples. Tube is tightly sealed. Dissolve sample by shaking for 15-30 seconds (vortex as needed), then sonicate in ultrasonic bath and mix on multi vortex mixer for 30 minutes each.
4. Centrifuge at 4000 rpm (3750 gm) for 10 minutes at room temperature.
5. Approximately 1.4 mL of the supernatant is transferred into a 1.5 mL micro centrifuge tube.
6. Centrifuge at 13,200 rpm (16100 gm) for 30 minutes.
7. Load aqueous extract into a plastic 3 mL syringe and force through a 13mm, 0.22um PVDF filter into a micro centrifuge tube. (Note: some formulations may require some force, or two filtration steps to obtain a clear solution before the next step.). Possible stopping point: aqueous extracts can be stored at 5-10°C for future dilutions.
8. Vortex mix for 30 seconds and centrifuge at 13200 rpm (16100gm) for 30 minutes.
9. Supernatant is transferred to a 2 mL autosampler vial, avoiding the precipitate.

F. Instrumental Analysis

The column is equilibrated in Mobile Phase A at 0.4 mL/min for 30-60 min.

It is necessary to evaluate system suitability, solvent blank (1x) and mixed standard are injected at 7.0 ng/mL (3-4x).
Data should meet the signal-to-noise and ion ratio criteria before continuing.

It is recommended to inject the standards and sample in following sequence: (i) solvent blank (Mobile Phase A), (ii) extracted matrix standards from 0.25 to 5 µg/g, (iii) solvent blank, (iv) control extracts, (v) post-fortified extracts and solvent standards for calculation of recoveries and matrix effects, (vi) solvent blank, (vii) unknown samples, and (viii) continuous calibration standards (an extracted matrix standard as well as solvent standard at 7 ng/mL), to verify that instrument response was maintained during the run.

G. Calculations

Use external standard calibration. The calibration curve should not include the origin, but does include a matrix blank with a concentration of 0. Export the processed data into Microsoft Excel or equivalent spreadsheet program for further calculations:

Recovery (%) = calculated from extracted calibration curve
Matrix effect (%) = 100 x Post-fortified sample / solvent standard (same cone)

The limit of quantification (LOQ) for each analyte is defined as the concentration of the lowest calibration standard used, or the lowest calibration standard which shows > 10-fold higher response than background signals in negative control sample.

H. Calculations for Confirmatory Analysis

Calculate ion ratios as percent relative abundances. The Melamine ion ratio is m/z 68/85.