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LPS-005

Determination of Melamine in Various Milk-Containing Products

by Liquid/Liquid Extraction and Cation Exchange Solid Phase Extraction to Prepare Samples, and Electrospray Positive Ionization Liquid Chromatography Tandem Mass Spectrometry to Quantitate Melamine

Bureau of Chemical Safety
Food Directorate
Health Products and Food Branch

A PAHO/WHO Collaborating Center for Food Contamination Monitoring

World Health Organization

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Canada

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1.0 Principle and Scope

This work instruction describes the analytical method used for the extraction and quantitation of melamine in liquid and powdered infant formula. It may also be used to extract melamine in milk, yogurt beverages and various products containing milk powder.

The method uses liquid/liquid extraction and cation exchange solid phase extraction to prepare samples, and electrospray positive ionization liquid chromatography tandem mass spectrometry to quantitate melamine.

2.0 Definitions

ACN: Acetonitrile.

Analytical batch: The group of samples, blanks, QC samples that one analyst can prepare at one time.

DCM: Dichloromethane.

Duplicate Sample: Sample that is extracted, processed, and analysed twice in the same analytical batch. A duplicate sample is used to monitor the precision of the analytical method.

HCl: Hydrochloric acid.

HILIC: Hydrophobic interaction liquid chromatography.

Internal Performance Standard: A compound with physical-chemical properties as close to the analyte as possible that is added to all sample and blank extracts, and all calibration standards, just prior to instrumental analysis. This standard corrects for variations in final sample and standard volumes, variations in injection volumes, and matrix effects.

Internal recovery standard: A compound with physical-chemical properties as close to the analyte as possible that is added to all sample and blank extracts, and all calibration standards, prior to extraction (i.e. at the beginning of sample processing). This standard monitors losses of analyte during sample processing.

LC-MS/MS: Liquid chromatography tandem mass spectrometry.

MEL: Melamine (1,3,5-triazine-2,4,6-triamine).

*MEL: $^{15}N_3$,$^{13}C_3$-melamine.

^{13}C-MEL: $^{13}C_3$-melamine.

MeOH: Methanol.

Milli-Q water: De-ionized laboratory water passed through Milli-Q filter system to achieve a resistivity of 18 MΩ.

NH_4OH: Ammonium hydroxide.

Personal laboratory notebook: A bound notebook used for general recording of information and data by one specific individual.

QC: Quality control.

QC Calibration check standard: A standard solution made from an entirely independent source of analyte than what is used to prepare the calibration standards. The QC calibration check standard is used to monitor the accuracy of the instrumental calibration.

QC liquid formula: A liquid infant formula product with known amount of MEL. Analysed frequently as an in-house reference material to monitor analytical method performance.

QC powdered formula: A powdered infant formula product with known amount of MEL. Analysed frequently as an in-house reference material to monitor analytical method performance.

Reagent blank: Blank sample that contains no matrix. It is processed and analysed in exactly the same manner as other samples. The reagent blank is used to monitor background levels of analyte that contaminate sample during processing.

SPE: Solid phase extraction.

Solution binder: Binder containing records of all standard solutions made for the melamine project.

UPLC: Ultra-high performance liquid chromatograph

3.0 Equipment and Supplies

3.1 Equipment

3.1.1 Centrifuge: Eppendorf 5804R and/or 5810R, refrigerated centrifuge.

3.1.2 Rotary mixer: Cole Palmer, Roto-torque, model 7637.

3.1.3 Vortex shaker: Vortex 2 Genie, Scientific Industries.

3.1.4 SPE manifold: Supelco, 12-port glass SPE manifold with valve for vacuum attachment.

3.1.5 Laboratory Nitrogen Generator (Parker Analytical Gas System; Model # 75-880)

3.1.6 Nitrogen evaporator: Organomation, The Myer N-Evap.

3.1.7 Sonicator, Branson model: 5210 or equivalent

3.1.8 Waters Acquity UPLC

3.1.9 Various Eppendorf Research Single Channel variable pipettors ranging from 10-5000uL; VWR.

3.1.10 Balance (top loading), Ohaus, model:TS4KD or equivalent

3.1.11 Balance (analytical), Mettler Toledo, model: AE 200 or equivalent

3.1.12 Waters Tandem Mass Spectrometer: Premier Triple Quadrupole MS/MS (Manchester, UK)

3.1.12.1 Masslynx 4.1 Datasystem (Manchester, UK)

3.1.12.2 Ionization Mode: positive ion electrospray

3.2 Materials

3.2.1 General Laboratory Glassware and Containers: Assorted beakers, class A volumetric flasks, culture tubes, and storage bottles with teflon-capped liners.

3.2.2 Disposable polypropylene tips for pipettors (3.1.8)

3.2.3 Pasture Pipettes

3.2.4 Scoopula

3.2.5 Centrifuge tubes: 50mL polypropylene conical tubes: Corning, 50mL, Cat. No. 05-538-55A; Fisher.

3.2.6 Centrifuge tubes: 15mL polypropylene conical tubes: Corning, 15mL, Cat. No. 05-538-53D, Fisher.

3.2.7 SPE cation exchange cartridges Oasis MCX, 6 cc, 150 mg, 30µm; Cat. No 186 000 256, Waters.

3.2.8 Syringe Filters; Acrodisc Syringe Filters, nylon 0.2µm pore size x 13mm; Pall, Life Science, Cat. No. 28143-985; VWR.

3.2.9 Disposable Syringes: BD 1mL tuberculin syringe with slip tip (for syringe filters); Cat. No. BD309602; VWR.

3.2.10 Weight boat

3.2.11 Auto sampler vials: 2ml, amber DP vials, 12 x 32mm with blue screw caps target DP with septa; Cat. No. C58002 W/C580053B; Chromatographic Specialties.

3.2.12 10 mL disposable glass culture tubes

3.2.13 HPLC Solvent Filters: 0.20µm pore size x 47mm Nylon 66; Supelco Inc., Bellefonte, PA

3.2.14 HILIC Column: 2.1 x 100 mm Acquity UPLC BEH HILIC column, 1.7 µm, (Waters Assoc.), part no. 186003461

3.2.15 Disposable polyethylene transfer pipettes.

3.3 Chemicals

3.3.1 Hydrochloric acid (37.4%, ACS Reagent, Sigma).

3.3.2 Dichloromethane (high purity, OmniSolv, EMD).

3.3.3 Methanol (high purity, OmniSolv, EMD).

3.3.4 Ammonium hydroxide (30%, Baker).

3.3.5 Acetonitrile (high purity, OmniSolv, EMD).

3.3.6 Melamine = 99% purity. 5 g of crystals from Sigma-Aldrich, Cat. No. 240818.

3.3.7 Melamine. 98% purity. 100 µg/mL stock solution in water from Cambridge Isotope Laboratories (CIL), catalogue number ULM-8156-1.2.

3.3.8 $^{15}N_3$,$^{13}C_3$-Melamine. $^{15}N_3$ at 98% purity; $^{13}C_3$ at 99% purity. 100 µg/mL solution in water from Cambridge Isotope Laboratories, catalogue number CNLM-8150-1.2. For use as an internal performance standard.

3.3.9 $^{13}C_3$-Melamine. $^{13}C_3$ at 99% purity. Synthesized according to the method described in "Preparation of [$^{13}C_3$]-melamine and [$^{13}C_3$]-cyanuric acid and their application to the analysis of melamine and cyanuric acid in meat and pet food using liquid chromatography-tandem mass spectrometry" Varelis et al. 2008, Food Additives and Contaminants 10:1210-1217. For use as an internal recovery standard.

3.3.10 Desolvation and cone gas: Nitrogen: supplied by a Laboratory Nitrogen Generator (Parker Analytical Gas System; Model # 75-880).

3.3.11 Collision gas: Argon (ultra pure).

3.3.12 Nitrogen gas (ultra pure).

3.3.13 Ammonium formate (Sigma, Cat. No. F-2004)

3.3.14 Concentrated Formic Acid (>96% A.C.S. Reagent Grade, Sigma, Cat. No. 251364-500G).

3.3.15 LC-MS grade water (OmniSolv, EMD Chem. Inc.)

3.3.16 Milli-Q water.

3.4 Solutions

3.4.1 Sample extraction acid solution, 1.0 N HCl.

3.4.1.1 Using a 100 mL graduated cylinder, measure 83.0 mL of concentrated HCl; transfer into a 1 L volumetric flask which contains approximately 500 mL Milli-Q water.

3.4.1.2 Make up to volume with Milli-Q water and mix the solution.

3.4.1.3 Label the flask with name (HCl 1N), date prepared. Store at room temperature, the solution may be used for up to 1 year.

3.4.2 SPE wash solution, 0.1 N HCl.

3.4.2.1 Using a 100 mL graduated cylinder, measure 100.0 mL 1.0 N HCl (prepared in 3.4.1); transfer into a 1 L volumetric flask which contains approximately 500 mL Milli-Q water.

3.4.2.1 Make up to volume with Milli-Q water and mix the solution.

3.4.2.2 Label the flask with name (HCl 0.1 N), date prepared. Store at room temperature, the solution may be used for up to 1 year.

3.4.3 SPE elution solution, 5% NH_4OH in MeOH (v/v).

3.4.3.1 Using a 100 mL graduated cylinder, measure 50 mL of NH_4OH (30%); transfer into a 1 L Erlenmeyer flask which contains 500ml of MeOH.

3.4.3.2 Add 450 ml more of MeOH and mix the solution.

3.4.3.3 Label the flask with name (5% NH_4OH in MeOH (v/v)), date prepared. Store at room temperature, solution may be used for up to 1 month.

3.4.4 Reconstitution Solution, ACN:H_2O 90:10 (v/v).

3.4.4.1 Using a 500 mL graduated cylinder, measure 500 mL then 400 mL of ACN (total 900 mL); transfer into a 1 L Erlenmeyer flask.

3.4.4.2 Add 100 mL Milli-Q water and mix the solution.

3.4.4.3 Label the flask with name (90:10 (v/v) ACN:H_2O), date prepared. Store at room temperature, solution may be used for up to 1 month.

3.4.5 Internal Performance Standard Solution; 500 pg/µL *MEL in ACN:H_2O; 90:10 (v/v).

3.4.5.1 Using a 2-20 µL adjustable pipette, transfer 7.5 µL of 100 µg/mL *MEL (Cambridge solution listed in 3.3.8) into a 2 mL autosampler vial.

3.4.5.2 Add 1350.0 µL ACN and 142.5 µL Milli Q water to the vial using a 100-1000 µL and a 20-200 µL adjustable pipettes.

3.4.5.3 Place screw cap on the vial, mix the solution. This solution may be stored at room temperature or it may be refrigerated. The solution may be used for up to 1 month.

3.4.6 Stock Internal Recovery Standard Solution; 100 µg/mL $^{13}C_3$-MEL ACN:H_2O; 90:10 (v/v).

3.4.6.1 On an analytical balance, accurately weight 1.00 mg $^{13}C_3$-MEL crystals (synthesized in-house, see 3.3.9) into a 10 mL volumetric flask.

3.4.6.2 Add approximately 9 mL Milli-Q water.

3.4.6.3 Sonicate the solution for approximately 30 minutes or until the crystals have completely dissolved.

3.4.6.4 Make up to volume with Milli-Q water and mix the solution.

3.4.6.5 This solution may be stored at room temperature or it may be refrigerated. The solution may be used for up to 6 months.

**note: this $^{13}C_3$-MEL is not pure; therefore the concentrations calculated are based upon the weight of the product as opposed to only $^{13}C_3$-MEL .*

3.4.7 Intermediate Internal Recovery Standard Solution; 500 pg/µL $^{13}C_3$-MEL in 90:10 (v/v) ACN:H_2O.

3.4.7.1 Using a 10-100 µL adjustable pipette, transfer 50 µL of the stock Internal Recovery Standard Solution prepared in 3.4.6 into a 10 mL volumetric flask.

3.4.7.2 Make up to volume with 90:10 (v/v) ACN:H_2O (see 3.4.4) and mix the solution.

3.4.7.3 This solution may be stored at room temperature or it may be refrigerated. The solution may be used for up to 1 month.

3.4.8 Melamine (native); 10 µg/mL Intermediate solution in 90:10 (v/v) ACN:H_2O.

3.4.8.1 Using a 10-100 µL adjustable pipette, transfer 100 µL of 100 µg/mL melamine stock solution purchased from CIL (see 3.3.7) into a 2 mL autosampler vial.

3.4.8.2 Using a 100-1000 µL pipette, add 900 µL of ACN to the vial.

3.4.8.3 Place screw cap on the vial, mix the solution. This solution may be stored at room temperature or it may be refrigerated. The solution may be used for up to 1 month.

3.4.9 Melamine (native); 1 µg/mL Intermediate solution in 90:10 (v/v) ACN:H_2O.

3.4.9.1 Using a 2-20 µL adjustable pipette, transfer 10 µL of 100 µg/ml melamine stock solution purchased from CIL (see 3.3.7) into a 2 mL autosampler vial.

3.4.9.2 Using a 10-100 µL adjustable pipette, add 90 µL Milli-Q water.

3.4.9.3 Using a 100-1000 µL adjustable pipette, add 900 µL of ACN.

3.4.9.4 Place screw cap on the vial, mix the solution. This solution may be stored at room temperature or it may be refrigerated. The solution may be used for up to 1 month.

3.4.10 Melamine (native); 0.02 µg/mL Intermediate solution in 90:10 (v/v) ACN:H_2O.

3.4.10.1 Using a 2-20 µL adjustable pipette, transfer 20 µL of 1 µg/mL melamine intermediate solution prepared in 3.4.9 into a 2 mL autosampler vial.

3.4.10.2 Using a 100-1000 µL adjustable pipette, add 980 µL of 90:10 (v/v) ACN:H_2O (see 3.4.4) to the vial.

3.4.10.3 Place screw cap on the vial, mix the solution. This solution may be stored at room temperature or it may be refrigerated. The solution may be used for up to 1 month.

3.4.11 Melamine Calibration Curve Solutions (Final volumes of all calibration curve solutions are 10.0 mL).

	Melamine (native); 0.02 µg/mL 90:10 (v/v) ACN:H_2O	Melamine (native); 1.0 µg/mL 90:10 (v/v) ACN:H_2O	Melamine (native); 10.0 µg/mL 90:10 (v/v) ACN:H_2O	500 pg/µl *MEL in ACN:H_2O; 90:10 (v/v)	500 pg/µL $^{13}C_3$-MEL ACN:H_2O; 90:10 (v/v)	90:10 ACN:H_2O
Melamine Concentration	prepared in 3.4.10	prepared in 3.4.9	prepared in 3.4.8	prepared in 3.4.5	prepared in 3.4.7	prepared in 3.4.4
(pg/µL)	(µL)	(µL)	(µL)	(µL)	(µL)	(µL)
0.100	50			100	100	100
0.500	250			100	100	100
1.00	500			100	100	100
5.00		50		100	100	100
10.0		100		100	100	100
50.0			50	100	100	100
100			100	100	100	100

3.4.12 Melamine (native) 1.26 g/L stock solution in H_2O.

3.4.12.1 On an analytical balance, accurately weight 0.63 g of native melamine crystals (purchased from Sigma-Aldrich, see 3.3.6) on a weight boat.

3.4.12.2 Transfer the crystal in a 500 mL volumetric flask.

3.4.12.3 Do a quantitative transfer by adding a small amount of Milli-Q water to the weight boat and transfer the rinsing to the 500 mL volumetric flask, repeat 2 times. Add approximately 480 mL Milli-Q water, hand shake the solution.

3.4.12.4 Sonicate the solution for approximately 30 minutes or until the crystals have completely dissolved. Make up to volume with Milli-Q water.

3.4.12.5 This solution may be stored at room temperature or it may be refrigerated. The solution may be used for up to 6 months.

3.4.13 Melamine (native) 12.6 µg/mL intermediate solution in H_2O.

3.4.13.1 Using a 100-1000 µL adjustable pipette, transfer 1.0 mL of 1.26 g/L solution prepared in 3.4.12 in a 100 mL volumetric flask.

3.4.13.2 Make up to volume with Milli-Q water, mix the solution.

3.4.13.3 This solution may be stored at room temperature or it may be refrigerated. The solution may be used for up to 6 months.

3.4.14 Melamine (native) 1.26 µg/mL diluted solution in 90:10 (v/v) ACN:H_2O.

3.4.14.1 Using a 10-100 µL adjustable pipette, transfer 100 µL of 12.6 µg/mL solution prepared in 3.4.13 in a 2 mL autosampler vial.

3.4.14.2 Add 900 µL of ACN, cap and mix the solution.

3.4.14.3 This solution may be stored at room temperature or it may be refrigerated. The solution may be used for up to 1 month.

3.4.15 QC calibration check standard

50 pg/µL MEL, 5 pg/µL *MEL, and 50 pg/µL $^{13}C_3$-MEL in 90:10 (v/v) ACN:H_2O prepared in a 2.0 mL autosampler vial.

QC Reference	Melamine (native) 1.26 µg/mL in ACN:H2O; 90:10 (v/v)	500 pg/µL *MEL in ACN:H2O; 90:10 (v/v)	500 pg/µL 13C3-MEL in 90:10 (v/v) ACN:H2O	90:10 (v/v) ACN:H2O
Melamine Concentration 50 pg/µL	prepared in 3.4.14	prepared in 3.4.5	prepared in 3.4.7	prepared in 3.4.4
	(µL)	(µL)	(µL)	(µL)
	39.7	10	100	850.3

This solution may be stored at room temperature or it may be refrigerated. This solution was freshly prepared with each analytical batch.

3.4.16 Ammonium formate stock solution, 1 M: Dissolve 63.06 g of ammonium formate in 1L of LC-MS grade water. The solution is stored at room temperature and may be used for up to 1 year.

3.4.17 UPLC mobile phases

3.4.17.1 Mobile Phase A: aqueous solution of 0.5 mM ammonium formate and 0.01% (v/v) formic acid:

Add 500µL of 1 M aqueous ammonium formate (3.4.16) and 100µL of formic acid in 1 litre of LC-MS grade water.

3.4.17.2 Mobile phase B: 0.01% (v/v) formic acid in acetonitrile:

Add 100µL of concentrated formic acid to 1 L acetonitrile.

4.0 Health and Safety

Standard laboratory protective clothing and eye protection are required during sample processing and analysis.

5.0 Responsibilities

It is the responsibility of the analyst preparing the melamine samples to ensure that it is done according to this work instruction.

6.0 Procedure

This procedure was adapted from the analytical method described in "*Determination and confirmation of melamine residues in catfish, trout, tilapia, salmon, and shrimp by liquid chromatography with tandem mass spectrometry*" Andersen et al., Journal of Agricultural and Food Chemistry, 2008, 56(12):4340-4347.

6.1 Sampling

A sampling plan was established to ensure a true representation of availability and / or consumption of products consumed within the Canadian population. The sampling plan must be fully documented in accordance standard operating procedures.

6.2 Glassware Cleaning

Most glassware used is disposable and requires no cleaning, however, when some glassware requires cleaning (e.g. volumetric flask) it is done according to the following procedure:

6.2.1 Use warm tap water, and laboratory detergent for manual washing of glassware.

6.2.2 Wash glassware by manually brushing using a laboratory cleaning brush.

6.2.3 Thoroughly rinse all glassware with warm tap water followed by 3 rinses of distilled water.

6.2.4 Rinse the glassware with ethanol and allow the glassware to air dry.

6.3 Sample preparation

6.3.1 Weigh out approximately 5.00 g of sample; dispensing with a pasture pipette or disposable polyethylene transfer pipette for liquid samples, or a scoopula for solid samples, into a 50 mL falcon tube (3.2.5).

Note: ensure required QC samples are included in the analytical batch (see 8.1).

6.3.2 Record exact mass in personal laboratory notebook.

6.3.3 Add 25 µL of 100 µg/mL Internal Recovery Standard Solution (3.4.6) to all samples including reagent blanks.

6.3.4 For all solid samples let stand for 1 hour. For all liquid samples let stand for 30 minutes.

6.4 Sample extraction

6.4.4 Using a 25 mL graduated cylinder add 24.0 mL of Milli-Q water to all samples and blanks.

6.4.5 Use a 10-1000 µL pipette to accurately add 1.0 mL of 1.0 N HCl to all samples and blanks.

6.4.6 Tightly cap, hand shake, and then mix all samples and blanks on a vortexer for 5 seconds.

6.4.7 Rotary mix all samples and blanks for 10 minutes on setting 5 (equivalent to 50 rpm).

6.4.8 Centrifuge all samples and blanks for 10 minutes at 3200 – 5500 g at 4°C.

6.4.9 Use a 500-5000 µL pipette to take a 5.00 mL aliquot of the aqueous layer supernatant. Avoid any solid material present.

6.4.10 Transfer the 5.00 mL aliquot in a 15 mL polypropylene centrifuge tube.

6.4.11 Using a pipette, add 10 mL DCM to the 5.00 mL aliquot.

6.4.12 Tightly cap, and then shake by hand all samples and blanks.

6.4.13 Rotary mix all samples and blanks for 10 minutes on setting 5 (equivalent to 50 rpm).

6.4.14 Centrifuge all samples and blanks for 10 minutes at 3200 – 5500 g at 4°C.

6.4.15 Using a pasteur pipette or disposable polyethylene transfer pipette, remove the entire top aqueous layer (i.e. top layer).

6.4.16 Place the collected aqueous layer into a 10 mL glass tube.

6.4.17 Using a 500-5000 µL pipette, add 2.50 mL Milli-Q water to the remaining DCM and re-extract by repeating steps 6.4.12 to 6.6.14.

6.4.18 Using a pasteur pipette or disposable polyethylene transfer pipette, remove the entire aqueous layer (ie. the top layer) and combine with the previous 5.00 mL aqueous layer from sec. 6.4.16. Keep the pipette to transfer the extract to the SPE cartridge.

note: Samples and blanks can be placed in the refrigerator at 4°C at this point and stored overnight.

6.5 Sample Clean-up

6.5.1 Condition each Oasis MCX SPE cartridge.

6.5.1.1 Using a pipette, add 5.0 mL MeOH to the SPE cartridge. Let drain through cartridge using gravity only. Do not let the cartridge run dry.

6.5.1.2 Using a pipette, add 5.0 mL H_2O to the SPE cartridge. Let drain through cartridge using gravity only. Do not let the cartridge run dry.

6.5.2 Using a glass pasteur pipette or disposable polyethylene transfer pipette, add entire sample or blank extract to the SPE cartridge. Let drain through cartridge using gravity only. Do not let the cartridge run dry.

Note: sample must be at room temperature before proceeding with this step.

6.5.3 Once sample or blank extract is completely loaded, wash SPE cartridge.

6.5.3.1 Using a pipette, add 5.0 mL HCl (0.1 N) to each sample's glass tube and then transfer to the SPE cartridge. Let drain through cartridge using gravity only. Do not let the cartridge run dry.

6.5.3.2 Using a pipette, add 2.0 mL MeOH to the SPE cartridge. Let drain through cartridge using gravity only.

6.5.4 Dry the cartridge by pushing air through the cartridge using an empty disposable syringe and adaptor.

6.5.5 Elute analytes from the SPE cartridge by adding 5.0 mL NH_4OH (5% v/v in MeOH). Collect each sample in a 10 mL glass tube.

6.5.6 Repeat drying step (6.5.4).

6.6 Preparation of extract for LC-MS/MS analysis

6.6.1 Dry the SPE eluate under N_2 in a 55°C water bath.

6.6.2 Using a 100-1000 µL pipette reconstitute the dried extract in 1.0 mL 90:10 (v/v) ACN:H_2O.

6.6.3 Mix all samples and blanks on a vortexer for 5 seconds (low setting).

6.6.4 Filter solution through a 0.2 µm nylon syringe filter using a disposable syringe into an autosampler vial.

6.6.5 Using a 10-100 µL pipette, take a 100 µL aliquot of the filtered extract and place in an autosampler vial.

6.6.6 Using a 2-20 µL pipette, add 10 µL of 500 pg/µL solution prepared in 3.4.5 so the final concentration of *MEL equals 5 pg/µL when prepared to the final volume (6.6.7).

6.6.7 Using a 100-1000 µL pipette, add 890 µL 90:10 (v/v) ACN:H_2O so the final volume of all extracts equals 1.00 mL

6.6.8 Tightly cap, and then mix all samples and blanks on a vortexer for 5 seconds.

6.6.9 In order to avoid issues with MEL solubility, samples and blanks are stored at room temperature until LC-MS/MS analysis.

6.7 Sample analysis by LC-MS/MS

6.7.1 UPLC-MS/MS Conditions and Parameters

6.7.1.1 UPLC conditions

- Mobile Phase composition (see 3.4.17):

  Mobile phase A: 0.5 mM ammonium formate and 0.01 % formic acid in water.

  Mobile phase B: 0.01% formic acid in actonitrile.

- UPLC column: 2.1 x 100mm Acquity UPLC BEH HILIC column, 1.7 µm particle size (Waters)
- Column flow: 0.17 mL/min
- Injection volume: 5.0 µL
- Column temperature: 40 °C
- Autosampler tray temperature: 20°C
- Melamine elution time: approximately 5.2 minutes
- Gradient:

Time	Flow Rate	% A	% B
Initial	0.17	10	90
2	0.17	10	90
4	0.17	40	60
10	0.17	40	60
10.5	0.25	10	90
12.5	0.25	10	90
12.6	0.17	10	90
15	0.17	10	90

6.7.1.2 MS/MS conditions

- Ionization mode: positive ion electrospray
- Capillary voltage: 3.5 kV
- Cone voltage: 30 V
- Source temperature: 120°C
- Desolvation temperature: 400°C
- Cone gas (N_2) flow: 50 L/hr
- Desolvation gas (N_2) flow: 900 L/hr
- Collision gas (Ar) pressure: 9.8 x 10-3 mbar (0.5 mL/min)
- Ion energies for both quadrupole analyzers: 0.3 V
- Resolution: baseline unit resolution for both quadrupole mass analysers
- Multiplier voltage: 650 V
- Multiple reaction monitoring (MRM) conditions:

Precursor Ion	Product Ion	Dwell time (sec)	Cone Volt. (V)	Collision energy (EV)
127	43	0.06	30	24
127	68	0.06	30	25
127	85	0.06	30	17
130	44	0.06	30	25
130	87	0.06	30	18
133	45	0.06	30	25
133	89	0.06	30	18

6.7.2 Transitions used for melamine determination in multiple reaction monitoring mode.

6.7.2.1 Quantitation transition of MEL: m/z 127 → m/z 85; primary transition for MEL.

6.7.2.2 Confirmation transition of MEL: m/z 127 → m/z 68.

6.7.2.3 Secondary confirmation transition of MEL: m/z 127 → m/z 43.

6.7.2.4 Quantitation transition of *MEL: m/z 133 → m/z 89; primary transition for *MEL.

6.7.2.5 Confirmation transition of *MEL: m/z 133 → m/z 45

6.7.2.6 Quantitation transition of ^{13}C-MEL: m/z 130 → m/z 87; primary transition for ^{13}C-MEL.

6.7.2.7 Confirmation transition of ^{13}C-MEL: m/z 130 → m/z 44

6.8 Data Analysis

6.8.1 Criteria for Quantification

6.8.1.1 Ratio of the peak height of the quantitation transition to the peak height of the confirmation transition for MEL and *MEL transition pairs must be within a set tolerance of the mean ratio from all calibration standards from the particular analytical run.

6.8.1.1.1 If the concentration of MEL in the extract is = 10 pg/μL, the tolerance is ± 20% of the mean ratio from the standards.

6.8.1.1.2 If the concentration of MEL in the extract is < 10 pg/μL, the tolerance is ± 25% of the mean ratio from the standards.

6.8.1.2 The retention time of MEL and *MEL in all samples and blanks must be within 0.3 minutes of the average retention time of MEL and *MEL in calibration standards. The retention time of MEL and *MEL within a sample must not differ more than 0.05 min.

6.8.1.3 Each peak must have a signal to noise greater than 9 to 1.

6.8.1.4 The correlation coefficient of the calibration curve for the quantitation transition of native MEL associated with each analytical batch of samples must be = 0.99.

6.8.2 Calibration Curve

6.8.2.1 All calibration curves were constructed using the TargetLynx (associated with MassLynx 4.1) software.

6.8.2.2 The MEL response factor was calculated as the ratio of the peak height of the MEL quantitation transition (6.7.2.1) to the peak height of the *MEL quantitation transition (6.7.2.4).

6.8.2.3 A 7-point calibration curve was developed using the following standard solutions: 0.10, 0.50, 1.0, 5.0, 10, 50, and 100 pg/µL MEL (see 3.4.11). A 1/X weighted linear curve was used to plot the MEL response factor versus the MEL concentration in the calibration standards.

6.8.3 Sample and Blank Data Analysis

6.8.3.1 Response factors were calculated for MEL using the TargetLynx software, as described in 6.8.2.2.

6.8.3.2 Concentrations (pg/µL) of MEL in sample and blank extracts were calculated in TargetLynx using the calibration curve equation generated in 6.8.2.3.

6.8.3.3 Concentrations (ng/g sample) of MEL in samples and blank extracts were calculated in Microsoft Excel using the following equation:

<u>***IF SAMPLE IS SOLID (eg. powdered milk sample)</u>

$$MEL_{sample} = \frac{(MEL_{extract}) \times (volume_{extract}) \times DF_{1(solid)} \times DF_2}{(mass_{sample}) \times (1000\,pg/ng)}$$

where:

MEL_{sample} = concentration of MEL in sample in ng/g

$MEL_{extract}$ = concentration of MEL in extract (calculated in 6.8.3.2) in pg/µL

volume $_{extract}$ = 1000 µL

DF$_{1\ (solid)}$ = first dilution factor = 5 (for solid samples only)

DF$_2$ = second dilution factor = 10

mass $_{sample}$ = mass of sample analysed in g

<u>***IF SAMPLE IS LIQUID (eg. liquid milk sample)</u>

$$MEL_{sample} = \frac{(MEL_{extract}) \times (volume_{extract}) \times DF_{1(liquid)} \times DF_2}{(mass_{sample}) \times (1000\, pg / ng)}$$

where:

MEL $_{sample}$ = concentration of MEL in sample in ng/g

MEL $_{extract}$ = concentration of MEL in extract (calculated in 6.8.3.2) in pg/µL

volume $_{extract}$ = 1000 µL

DF$_{1\ (liquid)}$ = first dilution factor = 6 (for liquid samples only)

DF$_2$ = second dilution factor = 10

mass $_{sample}$ = mass of sample analysed in g

6.8.3.4 Concentrations of MEL in blanks were calculated on a ng/g basis assuming 5.00 g of sample equivalent.

6.8.3.5 Blank-corrected concentrations (ng/g sample) of MEL in samples were calculated in Microsoft Excel using the following equation:

$$MEL_{sample,corr} = MEL_{sample} - MEL_{blank}$$

where:

MEL $_{sample}$ = concentration of MEL in sample in ng/g

MEL $_{blank}$ = concentration of MEL in reagent blank in ng/g

(both calculated in 6.8.3.3.)

6.8.4 Recovery calculations

6.8.4.1 ***IF SAMPLE IS SOLID (eg. powdered milk sample)

Concentrations (pg/µL) of ^{13}C-MEL in sample and blank extracts were calculated in TargetLynx using the calibration curve equation generated in 6.8.2.3

Percent recoveries of internal recovery standard are calculated in Microsoft Excel using the following equation:

$$^{13}C-MEL_{recovery\ solid} = 100 * \left(\frac{^{13}C-MEL_{sample}}{average\ ^{13}C-MEL_{standards}} \right)$$

where:

^{13}C-MEL $_{recovery}$ = % recovery of internal recovery standard in sample

^{13}C-MEL $_{sample}$ = concentration of internal recovery standard in sample extract

average ^{13}C-MEL $_{standard}$ = average concentration of internal recovery standard in all calibration standards run in the analysis of that particular analytical batch.

6.8.4.2 **IF SAMPLE IS LIQUID (eg. liquid milk sample)

Concentrations (pg/µL) of ^{13}C-MEL in sample and blank extracts were calculated in TargetLynx using the calibration curve equation generated in 6.8.2.2

Percent recoveries of internal recovery standard are calculated in Microsoft Excel using the following equation:

$$^{13}C-MEL_{recovery\ \ liquid} = 100 * \left(\frac{^{13}C-MEL_{sample}}{average\ ^{13}C-MEL_{standard}} \right) * 1.2$$

where:

^{13}C-MEL $_{recovery}$ = % recovery of internal recovery standard in sample

^{13}C-MEL $_{sample}$ = concentration of internal recovery standard in sample extract

average ^{13}C-MEL $_{standard}$ = average concentration of internal recovery standard in all calibration standards run in the analysis of that particular analytical batch.

*1.2= factor taken into account due to additional dilution of liquid samples.

7.0 Record Keeping

7.1 All information concerning sample preparation, standard preparation, instrument conditions, sample calculations, etc., must be written in a personal laboratory notebook.

7.2 All melamine standards prepared will be described in the solutions binder.

8.0 Quality Control and Quality Assurance

8.1 The following QC measures are taken when analysing samples:

QC Measured	***Method Used***	***Reference/condition***	***Acceptance Criteria****
Background	reagent blank	analysed in each batch	< limit of detection
Recovery	addition of internal recovery standard	see 6.3.3	n/a
	QC calib check std	see 3.4.15 analysed once per sequence	±20% of the theoretical concentration
	QC liquid formula	see 8.3 analysed every 20 samples	±20% of the mean MEL concentration
Accuracy	QC powdered formula	see 8.3 analysed every 20 samples	±20% of the mean MEL concentration
Precision	duplicate sample	see 8.2 one duplicate pair per 20 samples	RPD < 20%
Calibration curve	correlation coefficient	see 6.8.2.3	0.99

** if acceptance criteria are not met, appropriate corrective action must be taken*

8.2 Duplicate Sample

The relative percent difference of the duplicate measurements of MEL in sample (ng/g) must be below 20%. Calculate this difference as:

$$RPD = 100 \times \frac{\left(MEL_{sample,corr} - MEL_{duplicate,corr}\right)}{\left(\frac{MEL_{sample,corr} + MEL_{duplicate,corr}}{2}\right)}$$

where:

RPD = relative percent difference

$MEL_{sample,corr}$ = blank-corrected concentration of MEL in sample

$MEL_{duplicate,corr}$ = blank-corrected concentration of MEL in duplicate

8.3 QC Samples -- formula

Additional QC samples are selected to monitor accuracy and precision:

- one QC liquid formula with melamine levels > limit of quantitation is selected as a QC when an analytical batch of liquid samples is being analysed. The same liquid formula is used as the liquid QC sample throughout the study.
- one QC powdered formula with melamine levels > limit of quantitation is selected as a QC when an analytical batch of powdered (solid) samples is being analysed. The same powdered formula is used as the powdered QC sample throughout the study.

9.0 Associated Documents: