Food and Chemical Toxicology 80 (2015) 310–318

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Investigation of melamine and cyanuric acid deposition in pig tissues using LC-MS/MS methods

Andriy Tkachenko [a], James Clark [b], Natalie Knutson [b], Betzy Wallace [c], Malgorzata Bomba [c], Michele Yacopucci [c], Blaine Rhodes [d], Sarah M. Nemser [a], Jake Guag [a], Renate Reimschuessel [a,*]

[a] United States Food and Drug Administration, Center for Veterinary Medicine, Office of Research, 8401 Muirkirk Rd., Laurel, MD 20708, USA
[b] State Laboratory, Arizona Department of Health Services, 250 N. 17th Avenue, Phoenix, AZ 85007, USA
[c] New Hampshire State Public Health Laboratory, 29 Hazen Drive, Concord, NH 03301, USA
[d] Washington State Public Health Laboratory, 1610 N.E. 150th St., Shoreline, WA 98155, USA

ARTICLE INFO

Article history:
Received 18 August 2014
Accepted 9 March 2015
Available online 25 March 2015

Keywords:
Mass spectrometry
Melamine
Cyanuric acid
Residues
Pig

ABSTRACT

Four LC-MS/MS methods were developed to quantify melamine (MEL) and cyanuric acid (CYA) in various pig tissues at or above the level of concern (2.5 mg/kg). Pigs treated with 200 mg/kg bw/day CYA daily for 7 days did not accumulate significant residue concentrations in muscle, liver or kidney. Pigs treated with 200 mg/kg bw MEL daily for 7 or 28 days had MEL residues in muscles (3–13 ppm), liver (2.8–14.1 ppm) and kidney (9.4–27.2 ppm). Treatment with MEL and CYA at 100 mg/kg bw of each triazine daily for 7 days resulted in MEL (26–59 ppm in muscle, 30–49 ppm in liver and 367–6300 ppm in kidney) and CYA (1.8–5.8 ppm in muscle, 2.6–6.5 ppm in liver and 303–7100 ppm in kidney). Treatment with MEL and CYA at 1, 3 or 10 mg/kg bw/day for 7 days did not result in residues greater than the level of concern in all tissues tested. Pigs dosed with 33 mg/kg bw/day of MEL + CYA for 7 days contained residues above the level of concern only in kidney. Deposition of MEL and CYA depends on the tissue type (muscles, liver and kidney), dosage and whether the triazines are given alone or in combination.

1. Introduction

During the 2007 pet food recall in the United States, animal feeds destined for farm pigs, poultry and fish were contaminated with melamine and related compounds (MRC). In several states contaminated ingredients and scraps from the production of pet food were mixed with grain and bakery meal to make swine and chicken feed, with the scraps typically comprising up to 15% (USFDA, 2007). Edible tissues from animals that ate the contaminated feed in 2007 contained insignificant amounts of MRC. However, questions arose as to the depositions of MRC in edible tissues of animals if fed higher concentrations of the chemicals. During the 2008 infant formula recall in China, hundreds of tons of human food were found to be contaminated with melamine due to intentional adulteration of milk. Although Chinese authorities urged manufacturers and distributors to destroy all contaminated products, hundreds of tons of melamine-contaminated dairy products were still discovered years later according to Chinese and international public media (China Daily, 2010; CNN, 2011; New York Times, 2009, 2010, 2011; The Telegraph, 2011; Reuters, 2014). Discoveries of such large amounts of melamine-contaminated products after the fact suggest the possibility that some of those products may be diluted and fed to farm animals intentionally for economically motivated reasons.

The carry-over of MRC from feed to edible tissues of animals has been investigated in pig, chicken, lamb, sheep and fish (Andersen et al., 2011; Cruywagen et al., 2011; Li et al., 2010; Lv et al., 2010; USFDA, 2007; Wang et al., 2014). The data reported suggest that

Abbreviations: ACN, acetonitrile; ACUC, Animal Care and Use Committee; APCI, atmospheric pressure chemical ionization; bw, body weight; CYA, cyanuric acid; ESI, electrospray ionization; LC-MS/MS, liquid chromatography tandem mass spectrometry; LOD, limit of method detection; LOQ, limit of method quantitation; MEL, melamine; MRC, melamine and related compounds; MRM, multiple reaction monitoring; NOAEL, no observable adverse effect level; N/R, not reported; PVDF, polyvinylidene fluoride; PTFE, polytetrafluoroethylene; RPM, rotations per minute; RSD%, relative standard deviation percent; DOE, United States Department of Energy; USFDA, United States Food and Drug Administration; ZIC-HILIC, zwitterionic hydrophilic interaction liquid chromatography.

* Corresponding author. Center for Veterinary Medicine, U.S. FDA, 8401 Muirkirk Rd, Laurel, MD 20708, USA. Tel.: +1 240 402 5404; fax: (301)-210-4685 (USA).
E-mail address: renate.reimschuessel@fda.hhs.gov (R. Reimschuessel).

http://dx.doi.org/10.1016/j.fct.2015.03.007

multiple factors determine MRC depositions in tissues and more data are needed to fully evaluate human health risks associated with consumption of products derived from animals exposed to MRC.

In this study we investigated the deposition of melamine (MEL) and cyanuric acid (CYA) in various tissues of pigs dosed with various concentrations and combinations of these two chemicals. The major goal of this study is twofold. First, to develop LC-MS/MS methods that could be used by laboratories with various equipment platforms which are able to quantify MEL and CYA in various pig tissues at the level of concern (2.5 mg/kg; USFDA, 2008), and secondly, to determine deposition of MEL and CYA in the loin, ham, liver and kidney of pigs that had been exposed to these chemicals alone or in combination for 7 or 28 days during a previous NOAEL (No Observable Adverse Effect Level) study (Stine et al., 2011).

2. Methods

2.1. Animals, husbandry and experimental design

Loin, ham, liver and kidney used in this study were obtained from animals used in a previous NOAEL study where the formation of renal crystals was used as the key parameter to evaluate the toxic effect of the melamine and cyanuric acid exposure (Stine et al., 2011). The reader is referred to the NOAEL study communication for a detailed description of animal treatment and the experimental design. Briefly, weanling cross-bred barrows (20–26 kg body weight) were obtained from a local producer and fed a standard corn and soybean diet during a 2-week acclimatization period. In the 7 day study, pigs were randomly assigned to treatment groups of 0, 1.0, 3.3, 10, 33, or 100 mg/kg bw/day of MEL and CYA each or 200 mg/kg bw/day of either compound individually (MEL or CYA; n = 2 each treatment). Pigs were given the test compound(s) for 7 consecutive days and sacrificed on the 8th day. In the 28 day study, pigs were randomly assigned to 0, 1.0, or 3.3 mg/kg bw/day of MEL + CYA or 200 mg/kg bw/day MEL alone (n = 12 control, n = 8 other treatments). Pigs were given the test compound(s) for 28 consecutive days and sacrificed on the 29th day. MEL and/or CYA were mixed with retail chocolate pudding in amounts tabulated from daily weight measurements to ensure accurate dosing. The number of pigs used in the study was based on both the need to address objectives of the preliminary risk assessment and the need to adhere to the Animal Care and Use Committee (ACUC) guidelines for reducing the number of large mammals used in research. Animal research was conducted at a laboratory certified by the Association for Assessment and Accreditation of Laboratory Animal Care International. The experimental protocol was approved by the ACUC at the Office of Research, Center for Veterinary Medicine, USFDA, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (1996) and the Animal Welfare Act of 1966 (P.L. 89–544), as amended.

2.2. Preparation and analysis of tissues

The kidney, stomach, bladder, urine and blood were analyzed for presence of crystals, blood urea nitrogen, urine pH, blood creatinine, kidney weight and other parameters, and the results were reported in a previous communication (Stine et al., 2011). Loin, ham and liver were pre-cut into 5–10 g cubes and divided into four portions (70–150 g) for LC-MS/MS analysis in each collaborating laboratory. Loin, ham and liver from pigs with IDs 1466 and 1470 were excluded from the study as the labels had come off in the freezer. A portion (approx. 50%) of kidney from each experimental pig was archived. The rest of each kidney was homogenized with dry ice using a blender (Waring Commercial) to obtain a homogeneous powder and divided into four equal portions (15–50 g) for LC-MS/MS analysis in each of four collaborating laboratories.

2.3. LC-MS/MS analysis

2.3.1. Laboratory #1

The procedure was modified from previously reported method (Heller and Nochetto, 2008). Loin or ham (2 g) was weighed into a 50 mL polypropylene centrifuge tube. Then, 20 mL of 2.5% aqueous formic acid was added and the mixture was homogenized for 20 s with a homogenizer (Omni International) using a metal probe. Each sample was spiked with internal standards to obtain 400 ng/g each of melamine-$^{13}C^{15}N$ and cyanuric acid-$^{13}C^{15}N$ and sonicated for 30 min. Samples were mixed on a multi-tube vortex mixer for 30 min and centrifuged at 3750 *g* for 30 min at 4 °C. Supernatant (1.8 mL) was transferred in a 2 mL tube with 0.2 mL of hexane, vortexed for 20 s and centrifuged at 16,000 *g* for 30 min. Top (hexane) layer was removed by pipetting and approximately 0.5 mL aliquot was filtered through a glass fiber syringe filter (0.7 or 1.0 μm pore size). Filtrate (0.05 mL) was added to 0.95 mL of acetonitrile (ACN), vortexed for 15 s and centrifuged for 30 min at 16,000 *g* to precipitate particulate matter. Supernatant (0.9 mL) was filtered through a polyvinylidene fluoride (PVDF) syringe filter (0.2 μm pore size) in such a way that the first 0.80 mL was discarded (to wash out possible contaminants) and only the last 0.1 mL was placed into a 350 μL autosampler vial for LC-MS/MS analysis. Liver and kidney were processed using the same procedure as for loin and ham, but the defatting step with hexane was omitted.

Extracts were automatically injected (5 μL) and chromatographed on a guarded analytical column (SeQuant, ZIC-HILIC, 150 × 2.1 mm 5 μm) at 30 °C using Shimadzu Prominence LC coupled with an AB Sciex API-4000 triple-Q mass spectrometer. CYA and MEL eluted at 4.2 min and 7.5 min, respectively, under gradient elution of mobile phase A (95% of ACN and 5% of 0.1% aqueous formic acid) and B (50% of 20 mM ammonium formate and 50% of ACN). Mobile phase A was 100% from 0 to 4 min. Concentration of mobile phase B (50% of 20 mM ammonium formate and 50% of ACN) gradually changed from 0 to 75% for the next 5 min (from 4 to 9 min). Flow was diverted to waste between 0–3.5 min and 10–14 min. Mass spectral data were acquired in electrospray ionization (ESI) with multiple reaction monitoring (MRM) mode using negative polarity for CYA and positive for MEL. Peaks were integrated and values corrected to internal standards to compensate for matrix effect, losses during extraction and instrument variability using Analyst 1.5.1 software. Concentration of analytes was calculated based on calibration curve standards prepared in solvent. Linear regression was used to quantify MEL for up to 2.5 μg/g and CYA for up to 3.5 μg/g in samples. An exponential regression was used for a calibration curve to quantify MEL between 2.5 and 3.5 μg/g in samples.

2.3.2. Laboratory #2

The procedure was adapted in part from the previously reported method (Filigenzi et al., 2008). Muscles, liver and kidney were homogenized using a Robot Coupe after freezing with liquid nitrogen or dry ice. Each sample (1 g) was weighed out in a polypropylene centrifuge tube and spiked with internal standards at a final concentration of 500 ng/g each of melamine-^{13}C and cyanuric acid-^{13}C. An aliquot of 25 mL of extraction solvent (50% ACN, 40% water and 10% diethylamine) was added and the mixture was homogenized for 1 min using an Ultra-Turrax T-25 tissue homogenizer. The extract was centrifuged at 800 RPM (5 min, 25 °C) and 2.5 mL of the supernatant was transferred to 5.5 mL of ACN. After vortex mixing (20 s), the mixture was centrifuged at 1500 RPM for 5 min at 25 °C. Approximately 4.5 mL of supernatant was filtered through a 1.0 μm PTFE syringe filter and 4 mL of filtrate was transferred to a glass tube for evaporation to dryness under a stream of nitrogen in the TurboVap water bath at 60 °C. The residue was reconstituted with 1 mL of ACN/water (9:1) mixture, vortexed for 20 s and sonicated for 2 min. The extract was then filtered through a 0.45 μm PVDF syringe filter into an autosampler vial and analyzed on Agilent 1100 LC coupled with an AB Sciex API-4000 triple-Q mass spectrometer. A 5 μL aliquot was injected and chromatographed on the guarded analytical column (Phenomenex, HILIC 100Å, 2.6 μm, 2.1 mm × 100 mm) at 30 °C. CYA eluted at 0.7 min and MEL at 2.1 min under gradient elution from 100% to 90% of mobile phase A over the first 3 minutes. Mobile phase A comprised 10 mM aqueous ammonium acetate buffer (pH 5) and mobile phase B comprised 100% of ACN. Mass spectral data were acquired in atmospheric pressure chemical ionization (APCI) MRM mode with negative polarity for CYA and positive for MEL. The ratio of quantitation and confirmation ions for unknown samples was ± 30% of the mean ratio for the calibration curve standards prepared in solvent. Peaks were integrated and values were corrected to internal standards to compensate for matrix effect, losses during extraction and instrument variability using Analyst 1.5.1 software.

2.3.3. Laboratory #3

The procedure was adapted from previously reported methods (Filigenzi et al., 2008; Turnipseed et al., 2008). Loin, ham or liver was pre-cut into chunks, blended using a Waring blender followed by a Tekmar tissumizer, weighed (2 g) into a polypropylene centrifuge tube and spiked with internal standards to obtain 1 μg/g each of melamine-^{13}C and cyanuric acid-^{13}C. The mixture was vortexed after adding 1 mL of water. An aliquot of 7 mL of extraction solvent (5:5:0.1, ACN:water:diethylamine) was added and the mixture was centrifuged at 5000 RPM after shaking for 10 min. Supernatant (3 mL) was mixed with 3 mL of water and 3 mL of hexane, shaken for 10 min and centrifuged at 5000 RPM for 10 min. The top layer, containing hexane, was removed and 500 μL of the aqueous extract was filtered through Millipore membrane (3000 nominal molecular weight limit units), using an Amicon Ultra-0.5 filtration unit at 14,000 RPM for 30 min. The filtrate (300 μL) was dried in a glass tube at 55°C, dissolved in 300 μL of mobile phase A, and injected (2 μL) for LC-MS/MS analysis.

The kidney was processed using the same procedure as for loin, ham and liver, but only 1 g of kidney was used. Extraction solvents used for kidney processing were reduced in equal proportions. Extracts were analyzed on a guarded analytical column (Nest Group, ZIC-HILIC, 150 × 2.1 mm 5 μm) at 30 °C. Mobile phase A was a mixture of ACN (95%) and 0.1% aqueous formic acid (5%). Mobile phase B was a mixture of ACN (50%) and 20 mM ammonium formate (50%). Mass spectral data were acquired using Varian 1200-MS (for some loin) or Waters Quattro Micro triple-Q (for some loin, ham, liver and kidney) mass spectrometers in ESI mode with positive polarity for MEL and negative for CYA.

Calibrator and quality control (QC) spiked samples were prepared in blank matrices purchased from local supermarkets or butcher shops. Calibrator values and QC samples were required to be ± 20% of nominal (spiked) value. The ratio of quantitation and confirmation ions for unknown samples was ± 20% of the mean ratio for the calibration standards. If the lowest or highest calibrator did not pass the

requirement, the reportable range for unknown samples was adjusted accordingly. QC samples at low and high levels were analyzed approximately for every 10 samples.

2.3.4. Laboratory #4

The procedure was adapted from previously reported methods (Filigenzi et al., 2008; Xia et al., 2010). Loin, ham or liver samples (1 g) were weighed out in a 50 mL polypropylene centrifuge tube and spiked with internal standards to obtain 0.6 μg/g of each melamine-^{13}C and cyanuric acid-^{13}C. ACN–water mixture (9:1) was added and the content was homogenized for 20 s using the Ultra Turrax T-25 homogenizer. Concentrated formic acid was added to obtain a mixture (10 mL) of ACN, water and 2.5% formic acid. The mixture was vortexed for 20 s and placed overnight at 4 °C. Sample was sonicated for 30 min and centrifuged at 7000 RPM for 10 min. Supernatant (1 mL) was cleaned on a Strata-X Polymeric Reverse Phase Solid Phase Extraction cartridge using the Zymarc RapidTrace workstation. Analytes were eluted with an ACN–water (9:1) mixture and placed in the Turbovap LV for evaporation with a stream of nitrogen at 65 °C. Dried sample was reconstituted in 1 mL of ACN–water (9:1) mixture, sonicated for 10 min and transferred to an autosampler vial for LC-MS/MS analysis. Sample was chromatographed on HILIC column (Kinetex, 150 × 2.10 mm, 2.6 μm) at 30 °C. CYA eluted at 1.16 min and MEL at 2.6 min under gradient elution of mobile phase A (100 mM ammonium acetate, pH 5.8) from 8% to 50% (from 0 to 2 min) and from 50% to 2% (from 2.1 to 4.1 min). Mobile phase B was 100% ACN. Concentration of analytes was calculated based on calibration curves prepared in solvent.

Kidney was processed differently. Specifically, 2 g of kidney was spiked with internal standards as indicated previously and homogenized for 20 s in the extraction solvent containing 2.5% formic acid in an ACN–water (9:1) mixture using the Ultra Turrax T-25 homogenizer. Homogenized kidney was vortexed for 20 s, sonicated for 30 min and centrifuged at 4000 RPM for 15 min. Supernatant (2 mL) was transferred in a 2 mL tube and centrifuged at 16,000 *g* for 30 min. Supernatant (1 mL) was transferred into a 15 mL Falcon tube with 3 mL of ACN, vortexed for 30 s and centrifuged at 7800 RPM for 15 min. The extract was transferred (avoiding the pellet) into a 16 × 100 mm disposable glass culture tube and dried under a stream of nitrogen as indicated previously. Dried sample was reconstituted in 1 mL of ACN–water (9:1) mixture and sonicated for 10 min. Extract was defatted with 1 mL of hexane, dried under a stream of nitrogen at 65 °C, reconstituted in 1 mL of ACN–water (9:1) mixture, centrifuged at 14,000 RPM and analyzed with LC-MS/MS. Sample was chromatographed on ZIC-HILIC column (SeQuant, 150 × 2.1 mm, 5 μm) at 30 °C. CYA eluted at 5.5 min and MEL at 8.0 min under gradient elution of mobile phase A (95% of ACN and 5% of 0.1% aqueous formic acid) from 8% to 50% (from 0 to 2 min) and from 50% to 2% (from 2.1 to 4.1 min). Mobile phase B was 100% ACN. Concentration of analytes was calculated based on the calibration curve prepared in kidney.

Processed extracts of loin, ham, liver or kidney were automatically injected (20 μL) and analyzed on Agilent 1290 Infinity LC coupled with Agilent 6430 triple-Q mass spectrometer. Mass spectral data were acquired in ESI mode with negative polarity for CYA and positive for MEL. Peaks were integrated and values corrected to internal standards using MassHunter software.

2.4. Validation of LC/MS/MS methods

Prior to analyzing experimental samples, the four LC-MS/MS methods, which differed from each other, were validated within each laboratory (in-house validation) and through an external validation exercise using pig muscles.

The in-house validation was performed most extensively for pig muscles (loin or ham) and was assumed to be acceptable for other matrices provided that the methods included application of internal standards (to compensate for matrix effect and losses during extraction) and performance criteria of methods met the criteria outlined below.

For the external validation exercise, loin was purchased in a local supermarket, homogenized with dry ice, spiked with MEL and/or CYA and shipped for analysis to each of the four participating laboratories and one commercial laboratory. The commercial laboratory used a previously reported method (Smoker and Krynitsky, 2008) and their results were used for confirmation. The test samples also included incurred loin obtained from a pig dosed with combination of MEL + CYA at 33 mg/kg bw/day each for 7 days. Performance of each LC-MS/MS method was assessed using z-sore analysis as previously reported (Breidbach et al., 2010), but with some modifications. Specifically, the assigned values for spiked samples were determined by a formulation approach based on knowing proportions of tissue, solvent and analytes used during the spiking (Thompson et al., 2006). The assigned values for incurred loin were determined by consensus as the average of five values reported by four participants and one commercial laboratory (see Supplementary Table S1). All participants were blinded to the analyte levels in the test samples and were not allowed to reveal or discuss results with each other until the final submission of results to organizers.

Inter-laboratory comparison of results obtained during analysis of loin, ham, liver and kidney in experimental pigs (incurred samples) was used as a complementary approach to evaluate performance of the LC-MS/MS methods used. The sample analysis by four laboratories was synchronized in such a way that each of the four tissue types (loin, ham, liver and kidney) was analyzed one tissue type at a time. Each laboratory submitted results for one tissue type prior to analysis of the next type. Samples were shipped and analyzed in the following order: loin, ham, liver and kidney. Participating laboratories were not allowed to discuss the LC-MS/MS results among each other until the final results for all tissues (loin, ham, liver and kidney) were submitted to the organizers.

3. Results

3.1. Validation of LC-MS/MS methods

3.1.1. The in-house method validation

In laboratory #1 a 12-point calibration curve typically resulted in the correlation coefficient value (R^2) ≥ 0.997 for either MEL or CYA. Concentration of analytes was calculated based on a calibration curve prepared in solvent (95% of ACN and 5% of 0.1% aqueous formic acid) with MEL and CYA concentrations equivalent to 1.45–3500 ng/g in matrix. The limit of method quantitation (LOQ) was the lowest point in the calibration curve with signal to noise ratio ≥5. Inter-day accuracy and precision of the method were determined by analysis of five replicates for each spike level (0.3, 1.0 and 3 μg/g) on three different days. In loin, inter-day accuracy ranged between 102–104% for CYA and 98–102% for MEL and precision (relative standard deviation percent, RSD%) ranged between 4–5% for CYA and 7–10% for MEL. In kidney, inter-day accuracy ranged between 100–101% for CYA and 99–100% for MEL and inter-day precision (RSD%) ranged between 4–7% for CYA and 8–12% for MEL. Reagent blank spiked with internal standards, negative control matrix and negative control matrix spiked with internal standards were included as QC samples and confirmed no carryover.

In laboratory #2 a 7-point calibration curve in solvent typically resulted in R^2 ≥ 0.995 (linear regression) for either MEL or CYA. The LOQ was the lowest point (with signal to noise ratio ≥5) in the calibration curve in solvent ranging from 10 to 2000 ng/mL of each MEL and CYA (equivalent to 200–40,000 ng/g in matrix). Recoveries of MEL and CYA were within the range of 81–92% for pig muscles spiked at 200 ppb (n = 3) and 1000 ppb (n = 3) on three separate days. Blank solvent, blank solvent spiked with internal standards, blank matrix and blank matrix spiked with internal standards were included during sample analysis and confirmed no carryover. The instrument stability was monitored by re-running one of the higher calibration curve standards (typically the 500 ng/mL standard) at the end of each analysis using ±10% acceptance criterion of initial response.

In laboratory #3 a 6-point calibration curve in matrix typically resulted in R^2 ≥ 0.990 (linear regression; 1/x weighted) for either MEL or CYA. The LOQ was the lowest point in the calibration curve prepared in matrix ranging from 300 to 10,000 ng/mL of each MEL and CYA. Blank solvent, blank solvent spiked with internal standards, blank matrix and blank matrix spiked with internal standards were included during sample analysis and confirmed no carryover. Recoveries of MEL and CYA were within the range of 92–106% for pig muscles spiked at 300, 500, 1000, 3000, 5000 and 10,000 ng/g and analyzed on four separate days. The inter-day precision for 500 and 5000 ng/g of MEL spikes in pig muscles was 11.8% and 7.4%, respectively. For 500 and 5000 ng/g CYA spikes the inter-day precision in pig muscles was 10.1% and 8.2%, respectively.

In laboratory #4 an 8-point calibration curve in solvent was used for ham, loin and liver and typically resulted in R^2 ≥ 0.995 for either MEL or CYA. A 6-point calibration curve in matrix was used for quantitation of both analytes in kidney and typically resulted in R^2 ≥ 0.990. Blank solvent, blank solvent spiked with internal standards, blank matrix and blank matrix spiked with internal standards were included during sample analysis and confirmed no carryover. To determine the method accuracy and precision, negative control samples were spiked and analyzed on different days (ham on eight days, loin on six days and liver on five days). The inter-day accuracy of the method to determine MEL at levels of 0.6 and 1.2 mg/kg ranged between 97–135% for ham, 90–158% for loin and

76–113% for liver. The inter-day accuracy of the method to determine CYA at levels of 0.6 and 1.2 mg/kg ranged between 96–142% for ham, 83–124% for loin and 92–119% for liver. The inter-day precision (RSD%) of the method to determine MEL at levels of 0.6 and 1.2 mg/kg ranged between 5.3–9.4% for ham, 13.7–15.2% for loin and 15.3–8.0% for liver. The inter-day RSD% of the method to determine CYA at levels of 0.6 and 1.2 mg/kg ranged between 13.6–17.0% for ham, 13.7–14.9% for loin and 8.4–3.4% for liver.

3.1.2. Validation of methods through the external exercise and analysis of experimental pigs

Results obtained during the external method validation exercise (see Supplementary Table S1) were similar between four participants and the commercial laboratory. The *z*-scores calculated (not shown) for all reported values were ≤2 and, therefore, all four methods from participating laboratories were considered as those which are suitable to determine MEL and CYA in pig muscles at or above the level of concern.

In general, inter-laboratory comparison of results for loin, liver, kidney and ham in experimental pigs (Tables 1, 2, 3 and Supplementary Table S2) were in a good agreement for both analytes. Specifically, the conclusions regarding MEL and CYA residues outlined in the sections below were confirmed by results obtained by each of the four methods (laboratories).

In summary, based on data obtained during (a) in-house method validation, (b) the external validation exercise and (c) inter-laboratory comparison of results for experimental pigs, all methods used were concluded to be suitable to determine MEL and CYA in pig muscles, liver and kidney at or above the level of concern (2.5 mg/kg).

3.2. Residues of MEL and CYA in experimental pigs

Depositions of MEL and CYA in loin (Table 1) were almost identical to those in ham (Supplementary Table S1). Pigs treated with 200 mg/kg bw/day CYA daily for 7 days did not accumulate significant residue concentrations in loin, liver, kidney and ham (Tables 1, 2, 3 and Supplementary Table S2). Treatment with 200 mg/kg bw/day MEL for 7 days caused residues of MEL in loin (7.3–10.4 ppm), ham (7.8–9.9 ppm), liver (6.4–11 ppm) and kidney (20.3–27.2 ppm). Treatment with 200 mg/kg bw/day MEL for a longer period of time (28 days) caused residues of MEL in loin (3.1–15 ppm), ham (4.2–13 ppm), liver (2.8–14.1 ppm) and kidney (9.4–24 ppm) at levels similar to when treated just for 7 days. In one out of ten pigs treated with 200 mg/kg bw/day MEL, not only MEL, but also significant levels of CYA (2.7–6.0 ppm) were found in the kidney (Table 3).

Pigs fed 1, 3 or 10 mg/kg bw/day of MEL + CYA did not have residues above the level of concern in any tissue analyzed. Pigs fed 33 mg/kg bw/day of MEL + CYA contained residues above the level of concern in kidney only ranging from 31 to 269 ppm for MEL and 48 to 276 ppm for CYA.

Treatment with a combination of MEL + CYA at 100 mg/kg bw/day of each for 7 days caused residues of both MEL (32–50 ppm in loin, 26–59 ppm in ham, 30–49 ppm in liver and 367–6300 ppm in kidney) and CYA (1.8–5.7 ppm in loin, 1.6–5.6 ppm in ham, 2.6–6.5 ppm in liver and 303–7100 ppm in kidney). Residue levels of MEL and CYA in some kidneys were 100–1000 magnitude higher than in muscles (loin and ham) and liver. In summary, pigs can accumulate MEL and/or CYA at levels above the concern if fed high concentrations of MEL alone or the combination of MEL + CYA. Deposition of MEL and CYA in tissues depends on type of pig tissue (muscles, liver and kidney), amount of MEL and CYA given and type of administration (MEL and CYA alone or in combination).

4. Discussion

4.1. LC-MS/MS method performance

There are a number of LC-MS/MS methods reported in quantitation of MEL and CYA using different types, brands and combinations of liquid chromatographs, mass spectrometers and sample processing equipment (e.g. automatic sample extraction units, filtering centrifuges and others). Previously reported methods also differ significantly in their limits of detection, targeted matrices and the depth and degree of the method validation schemes used by analysts. The methods developed by each laboratory in this study were modified versions of previously reported procedures. The method development strategies chosen by participants were based on both the equipment availability and objectives of the study. Since the degree of method modifications varied among laboratories, the in-house method validation strategies and validation criteria chosen by each laboratory differed as well and depended solely on the discretion of analysts. The study organizers recognized the challenges associated with optimizing four different methods. There are, however, advantages to this approach in a regulatory setting. By concurrently developing methods that can be used by various government and state laboratories with different equipment platforms, we expanded the capabilities and capacities to conduct testing for significant food contaminants.

The LC-MS/MS method used in laboratory #1 was simplified when used for liver and kidney versus the method used for loin and ham. Specifically, the defatting step with hexane was found to be unnecessary for liver and kidney and, therefore, omitted for these samples. The mass spectrometry in laboratory #2 used the APCI mode allowing investigators to address a wider range of analyte concentrations (up to 40 ppm) in samples. Laboratory #3 analyzed some loin samples using Varian 1200-MS triple-Q mass spectrometer and due to unforeseen circumstances had to switch to another mass spectrometer to analyze the rest of the samples. The method was successfully transferred to Waters Quattro Micro triple-Q mass spectrometer without compromising the accuracy and precision of the method. The changes of the method introduced in laboratory #4 for liver and kidney versus loin and ham were targeted to improve the accuracy and precision of the method performance.

Comparison of inter-laboratory results for loin, ham, liver and kidney in experimental pigs was used as an additional approach to evaluate the four LC-MS/MS methods. Importantly, loin, ham and liver were not pre-homogenized but pre-cut into 5–10 g cubes prior to dividing into four portions (80–150 g) and shipping to the four laboratories for analysis. Since fat, protein and blood contents do vary even within the same type of tissue, some inter-laboratory variations of results were expected for loin, ham and liver.

The performance of the four methods was evaluated by comparing results for each individual pig rather than for each dosing group because biological variability in the accumulation of MEL and CYA was found among pigs treated identically. For example, four participants reported that loin from pig ID 237 contained 4.92, 3.98, 3.08 and 4.17 ppm of MEL (19% RSD), whereas loin from pig ID 352, which was treated the same way as the pig with ID 237, contained more than twice as much MEL (12.56, 10.80, 15.02 and 10.20 ppm; 18% RSD). In general, inter-laboratory variability of MEL and CYA residues reported at or above the level of concern in loin, ham and liver varied with RSD from 7 to 30%. Inter-laboratory variability of results was higher for kidneys with concentrations of MEL and CYA 100–1000 times exceeding the level of concern (2.5 mg/kg). Since LC-MS/MS methods were required to address concentrations close to the level of concern, all four methods developed for kidney were also considered acceptable as those which met objectives of the study. Since melamine cyanurate crystals in kidneys could have been unevenly distributed in the very heavily dosed animals, this could

Table 1
Concentration of MEL and CYA found in loin of each individual pig by four participating laboratories.

Exposure (days)	Dose (mg/kg bw/day)	Pig ID	MEL (ppm)				CYA (ppm)			
			Lab. #1	Lab. #2	Lab. #3	Lab. #4	Lab. #1	Lab. #2	Lab. #3	Lab. #4
7	0 MEL + CYA	13	<LOD (0.05)	<LOD (0.08)	<LOD (0.05)	0.09	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
7	0 MEL + CYA	1466	E	E	E	E	E	E	E	E
7	1 MEL + CYA	19	0.07	<LOD (0.08)	<LOD (0.05)	0.56	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.15)
7	1 MEL + CYA	1472	0.06	<LOQ (0.2)	<LOQ (0.3)	0.27	<LOD (0.14)	<LOD (0.06)	<LOQ (0.3)	<LOD (0.15)
7	3.3 MEL + CYA	20	0.18	<LOD (0.08)	<LOD (0.05)	0.18	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.05)
7	3.3 MEL + CYA	1469	0.20	0.27	<LOQ (0.3)	0.17	<LOQ (0.24)	<LOD (0.06)	<LOD (0.11)	<LOD (0.11)
7	10 MEL + CYA	16[b]	0.37	0.24	0.47	1.29	<LOQ (0.24)	<LOD (0.06)	<LOD (0.11)	0.12
7	10 MEL + CYA	1465[b]	0.49	0.51	0.64	0.48	0.07	0.26	0.36	<LOD (0.11)
7	33 MEL + CYA	14[b]	1.88	1.05	1.64	2.49	<LOD (0.047)	<LOD (0.06)	<LOD (0.11)	<LOD (0.11)
7	33 MEL + CYA	1471[b]	1.24	1.13	1.45	1.74	<LOD (0.05)	0.21	<LOQ (0.3)	<LOD (0.15)
7	100 MEL + CYA	15[b]	**49.96**	**32.80**	**37.66**	**38.55**	**5.72**	**5.14**	**3.80**	**2.58**
7	100 MEL + CYA	1468[b]	**36.88**	**32.40**	**32.35**	**31.86**	2.21	**2.72**	1.82	2.19
7	200 MEL	18	**9.89**	**7.28**	**10.35**	**9.50**	<LOD(0.047)	<LOD (0.06)	<LOD (0.11)	<LOD (0.15)
7	200 MEL	1470[b]	E	E	E	E	E	E	E	E
7	200 CYA	17	0.08	<LOQ (0.2)	<LOD (0.05)	0.36	0.24	<LOD (0.06)	<LOD (0.11)	<LOD (0.15)
7	200 CYA	1467	0.07	0.25	<LOD (0.05)	0.06	0.11	0.39	0.54	<LOD (0.11)
28	0 MEL + CYA	89	<LOD (0.05)	<LOD (0.08)	<LOQ (0.3)	0.08	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
28	0 MEL + CYA	90	<LOD (0.05)	<LOD (0.08)	<LOD (0.05)	0.07	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
28	0 MEL + CYA	91	<LOD (0.05)	<LOD (0.08)	<LOD (0.05)	0.07	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
28	0 MEL + CYA	95	<LOD (0.05)	<LOD (0.08)	<LOD (0.05)	0.10	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
28	0 MEL + CYA	242	<LOD (0.05)	<LOD (0.08)	<LOD (0.05)	0.28	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.15)
28	0 MEL + CYA	243	<LOQ (0.03)	<LOD (0.08)	<LOD (0.05)	<LOD (0.02)	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.11)
28	0 MEL + CYA	246	<LOD (0.05)	N/R[a]	N/R[a]	N/R[a]	<LOD (0.14)	N/R[a]	N/R[a]	N/R[a]
28	0 MEL + CYA	247	<LOD (0.05)	<LOD (0.08)	<LOD (0.05)	<LOD (0.02)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.11)
28	0 MEL + CYA	345	0.08	<LOQ (0.2)	<LOD (0.05)	0.13	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
28	0 MEL + CYA	348	<LOD (0.05)	<LOD (0.08)	<LOD (0.05)	0.18	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.11)
28	0 MEL + CYA	351	<LOQ (0.03)	<LOD (0.08)	<LOD (0.05)	0.51	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.15)
28	0 MEL + CYA	353	<LOD (0.05)	<LOQ (0.2)	<LOD (0.05)	<LOD (0.02)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.11)
28	1 MEL + CYA	88	0.07	<LOD (0.08)	<LOQ (0.3)	0.12	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
28	1 MEL + CYA	94	0.07	<LOD (0.08)	<LOQ (0.3)	0.13	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
28	1 MEL + CYA	98	0.06	<LOD (0.08)	<LOQ (0.3)	0.12	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
28	1 MEL + CYA	99	0.08	<LOD (0.08)	<LOQ (0.3)	0.14	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
28	1 MEL + CYA	238	0.10	<LOD (0.08)	<LOD (0.05)	0.09	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.05)
28	1 MEL + CYA	239	0.15	<LOD (0.08)	<LOD (0.05)	0.15	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.11)
28	1 MEL + CYA	241	0.03	<LOD (0.08)	<LOD (0.05)	<LOD (0.06)	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.05)
28	1 MEL + CYA	245	0.08	<LOD (0.08)	<LOQ (0.3)	<LOQ (0.04)	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.11)
28	3.3 MEL + CYA	92	0.17	<LOQ (0.2)	<LOQ (0.3)	0.21	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
28	3.3 MEL + CYA	93	0.13	<LOQ (0.2)	<LOQ (0.3)	0.17	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
28	3.3 MEL + CYA	97	0.33	0.23	<LOQ (0.3)	0.38	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
28	3.3 MEL + CYA	234	0.12	<LOQ (0.2)	<LOD (0.05)	0.17	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.12)
28	3.3 MEL + CYA	235	0.29	0.25	<LOQ (0.3)	0.25	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.05)
28	3.3 MEL + CYA	236	0.15	<LOD (0.08)	<LOD (0.05)	0.42	<LOQ (0.24)	<LOD (0.06)	<LOD (0.11)	<LOD (0.15)
28	3.3 MEL + CYA	240	0.16	<LOQ (0.2)	<LOQ (0.3)	0.11	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.11)
28	3.3 MEL + CYA	249	0.25	<LOQ (0.2)	<LOQ (0.3)	0.23	<LOD (0.14)	<LOD (0.06)	<LOD (0.11)	<LOD (0.05)
28	200 MEL	237	**4.92**	**3.98**	**3.08**	**4.17**	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.15)
28	200 MEL	343	**8.62**	**8.12**	**9.95**	**7.61**	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.15)
28	200 MEL	344	**10.16**	**9.16**	**9.71**	**8.56**	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.11)
28	200 MEL	346	**12.98**	**9.70**	**9.94**	**10.60**	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.15)
28	200 MEL	347	**7.59**	**5.90**	**7.03**	**6.67**	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.05)
28	200 MEL	350	**9.36**	**8.28**	**8.22**	**7.91**	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.15)
28	200 MEL	352	**12.56**	**10.80**	**15.02**	**10.20**	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.05)
28	200 MEL	354	**6.41**	**5.28**	**5.00**	**4.90**	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.15)

LOD – limit of method detection. LOQ – limit of method quantitation. E – excluded from the study because labels had come off in the freezer. N/R – not reported.

[a] Samples were concluded as free of both MEL and CYA based on results obtained in laboratory #1, and laboratories #2, #3 and #4 used the samples for LC-MS/MS method development and validation.

[b] Crystals were found in renal medulla, cortex and urine sediment (Stine et al., 2011). Concentrations exceeding the level of concern are in bold.

Table 2
Concentration of MEL and CYA found in liver of each individual pig by four participating laboratories.

Exposure (days)	Dose (mg/kg bw/day)	Pig ID	MEL (ppm)				CYA (ppm)			
			Lab. #1	Lab. #2	Lab. #3	Lab. #4	Lab. #1	Lab. #2	Lab. #3	Lab. #4
7	0 MEL + CYA	13	0.01	<LOD (0.08)	<LOD (0.05)	<LOD (0.22)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.08)
7	0 MEL + CYA	1466	E	E	E	E	E	E	E	E
7	1 MEL + CYA	19	0.05	<LOD (0.08)	<LOQ (0.3)	<LOD (0.07)	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.16)
7	1 MEL + CYA	1472	0.01	<LOD (0.08)	<LOD (0.05)	<LOD (0.07)	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.16)
7	3.3 MEL + CYA	20	0.14	<LOQ (0.2)	0.31	0.18	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.16)
7	3.3 MEL + CYA	1469	0.16	<LOD (0.08)	<LOQ (0.3)	0.29	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.08)
7	10 MEL + CYA	16[b]	0.40	0.32	0.51	0.35	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.16)
7	10 MEL + CYA	1465[b]	0.41	<LOQ (0.2)	0.40	0.44	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.16)
7	33 MEL + CYA	14[b]	2.03	1.65	1.96	1.74	0.06	<LOD (0.06)	<LOD (0.11)	<LOD (0.04)
7	33 MEL + CYA	1471[b]	1.40	1.17	1.31	1.26	0.10	0.28	<LOD (0.11)	<LOD (0.04)
7	100 MEL + CYA	15[b]	**49.39**	**44.80**	**46.69**	**38.82**	**4.52**	**6.48**	**4.36**	**3.54**
7	100 MEL + CYA	1468[b]	**38.30**	**38.40**	**41.50**	**29.47**	**2.67**	**2.92**	**2.57**	**2.75**
7	200 MEL	18	**11.06**	**10.02**	**10.00**	**6.35**	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.04)
7	200 MEL	1470[b]	E	E	E	E	E	E	E	E
7	200 CYA	17	0.15	<LOD (0.08)	<LOD (0.05)	<LOD (0.07)	0.34	0.30	0.41	<LOD (0.16)
7	200 CYA	1467	0.04	<LOD (0.08)	<LOD (0.05)	<LOQ (0.09)	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.14)
28	0 MEL + CYA	89	<LOD (0.01)	<LOD (0.08)	<LOD (0.05)	0.09	0.10	<LOQ (0.2)	<LOQ (0.3)	<LOD (0.14)
28	0 MEL + CYA	90	<LOD (0.01)	<LOD (0.08)	<LOD (0.05)	<LOD (0.05)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.14)
28	0 MEL + CYA	91	<LOD (0.01)	<LOD (0.08)	<LOD (0.05)	<LOQ (0.09)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.14)
28	0 MEL + CYA	95	<LOD (0.01)	<LOD (0.08)	<LOD (0.05)	<LOD (0.22)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.08)
28	0 MEL + CYA	242	<LOD (0.01)	<LOD (0.08)	<LOD (0.05)	<LOD (0.07)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.16)
28	0 MEL + CYA	243	<LOD (0.01)	<LOD (0.08)	<LOD (0.05)	<LOD (0.05)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.14)
28	0 MEL + CYA	246	<LOD (0.01)	<LOD (0.08)	<LOD (0.05)	0.34	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	0.27
28	0 MEL + CYA	247	<LOD (0.01)	<LOD (0.08)	<LOD (0.05)	<LOD (0.05)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.14)
28	0 MEL + CYA	345	0.05	<LOD (0.08)	<LOD (0.05)	<LOD (0.22)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.08)
28	0 MEL + CYA	348	<LOD (0.01)	N/R[a]	N/R[a]	N/R[a]	<LOD (0.05)	N/R[a]	N/R[a]	N/R[a]
28	0 MEL + CYA	351	<LOD (0.01)	<LOD (0.08)	<LOD (0.05)	<LOD (0.07)	<LOD (0.02)	<LOD (0.06)	<LOD (0.11)	<LOD (0.16)
28	0 MEL + CYA	353	<LOD (0.01)	<LOD (0.08)	<LOD (0.05)	<LOD (0.05)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.14)
28	1 MEL + CYA	88	0.04	<LOD (0.08)	<LOQ (0.3)	<LOD (0.22)	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.08)
28	1 MEL + CYA	94	0.05	<LOD (0.08)	<LOD (0.05)	<LOD (0.22)	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.08)
28	1 MEL + CYA	98	0.04	<LOD (0.08)	<LOD (0.05)	<LOD (0.22)	<LOD (0.02)	<LOD (0.06)	<LOD (0.11)	<LOD (0.08)
28	1 MEL + CYA	99	0.05	<LOD (0.08)	<LOD (0.05)	<LOD (0.22)	<LOD (0.02)	<LOD (0.06)	<LOD (0.11)	<LOD (0.08)
28	1 MEL + CYA	238	0.08	<LOD (0.08)	<LOD (0.05)	0.10	<LOD (0.02)	<LOD (0.06)	<LOD (0.11)	<LOD (0.14)
28	1 MEL + CYA	239	0.11	<LOD (0.08)	<LOD (0.05)	<LOD (0.22)	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.08)
28	1 MEL + CYA	241	0.01	<LOD (0.08)	<LOD (0.05)	<LOD (0.05)	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.14)
28	1 MEL + CYA	245	0.06	<LOD (0.08)	<LOD (0.05)	0.09	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.14)
28	3.3 MEL + CYA	92	0.15	<LOD (0.08)	<LOQ (0.3)	0.17	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	0.46
28	3.3 MEL + CYA	93	0.10	<LOD (0.08)	<LOQ (0.3)	0.14	<LOD (0.02)	<LOD (0.06)	<LOD (0.11)	<LOD (0.16)
28	3.3 MEL + CYA	97	0.31	0.28	<LOQ (O.3)	0.33	0.03	<LOD (0.06)	<LOD (0.11)	<LOD (0.16)
28	3.3 MEL + CYA	234	0.09	<LOD (0.08)	<LOQ (O.3)	<LOD (0.22)	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.08)
28	3.3 MEL + CYA	235	0.24	<LOQ (0.2)	<LOQ (O.3)	0.30	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.16)
28	3.3 MEL + CYA	236	0.11	<LOD (0.08)	<LOQ (O.3)	0.14	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.16)
28	3.3 MEL + CYA	240	0.12	<LOQ (0.2)	<LOQ (O.3)	<LOD (0.22)	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.08)
28	3.3 MEL + CYA	249	0.21	<LOQ (0.2)	<LOQ (O.3)	0.26	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.16)
28	200 MEL	237	**3.66**	**4.52**	**4.10**	**2.75**	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.04)
28	200 MEL	343	**7.67**	**8.78**	**9.48**	**7.62**	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.04)
28	200 MEL	344	**9.56**	**11.10**	**11.11**	**7.92**	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.04)
28	200 MEL	346	**11.32**	**14.14**	**12.58**	**10.49**	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.04)
28	200 MEL	347	**6.46**	**6.66**	**7.70**	**5.87**	<LOD (0.03)	<LOQ (0.2)	<LOD (0.11)	<LOD (0.04)
28	200 MEL	350	**7.83**	**9.02**	**9.76**	**7.38**	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.04)
28	200 MEL	352	**11.45**	**13.14**	**12.15**	**9.68**	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.04)
28	200 MEL	354	**5.25**	**6.52**	**6.54**	**4.86**	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	<LOD (0.04)

LOD – limit of method detection. LOQ – limit of method quantitation. E – excluded from the study because labels had come off in the freezer. N/R – not reported.

[a] Samples were concluded as free of both MEL and CYA based on results obtained in laboratory #1, and laboratories #2, #3 and #4 used the samples for LC-MS/MS method development and validation.

[b] Crystals were found in renal medulla, cortex and urine sediment (Stine et al., 2011). Concentrations exceeding the level of concern are in bold.

Table 3
Concentration of MEL and CYA found in kidney of each individual pig by four participating laboratories.

Exposure (days)	Dose (mg/kg bw/day)	Pig ID	MEL (ppm)				CYA (ppm)			
			Lab. #1	Lab. #2	Lab. #3	Lab. #4	Lab. #1	Lab. #2	Lab. #3	Lab. #4
7	0 MEL + CYA	13	0.04	<LOD (0.08)	<LOD (0.05)	0.03	<LOD (0.05)	<LOQ (0.2)	<LOD (0.11)	0.02
7	0 MEL + CYA	1466	0.05	0.36	<LOD (0.05)	0.43	<LOQ (0.03)	<LOQ (0.2)	<LOD (0.11)	0.39
7	1 MEL + CYA	19	0.10	0.49	<LOQ (0.3)	0.17	0.03	0.56	<LOD (0.11)	0.08
7	1 MEL + CYA	1472	0.13	0.27	<LOD (0.05)	0.16	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	0.04
7	3.3 MEL + CYA	20	0.28	<LOD (0.08)	<LOQ (0.3)	0.42	0.04	<LOQ (0.2)	<LOD (0.11)	0.14
7	3.3 MEL + CYA	1469	0.29	0.28	<LOQ (0.3)	0.29	<LOD (0.03)	<LOQ (0.2)	<LOD (0.11)	<LOQ (0.12)
7	10 MEL + CYA	16[b]	0.58	0.57	0.51	0.72	<LOD (0.03)	0.27	<LOD (0.11)	0.12
7	10 MEL + CYA	1465[b]	1.30	1.00	0.87	0.97	0.36	<LOQ (0.2)	<LOQ (0.3)	0.24
7	33 MEL + CYA	14[b]	**108.40**	**252.00**	**269.27**	**129.25**	**145.60**	**276.00**	**272.31**	**121.55**
7	33 MEL + CYA	1471[b]	**62.60**	**149.20**	**30.49**	**49.78**	**76.20**	**158.20**	**61.76**	**48.30**
7	100 MEL + CYA	15[b]	**1448.78**	**5550.00**	**3905.76**	**429.21**	**1284.74**	**5800.00**	**4681.44**	**378.42**
7	100 MEL + CYA	1468[b]	**1373.39**	**6300.00**	**1695.36**	**367.38**	**1246.16**	**7100.00**	**2673.60**	**303.01**
7	200 MEL	18	**20.25**	**20.60**	**23.72**	**27.15**	0.05	0.34	<LOQ (0.3)	1.48
7	200 MEL	1470[b]	**17.92**	**15.50**	**15.49**	**18.90**	**4.03**	**5.16**	**2.66**	**5.95**
7	200 CYA	17	0.10	0.36	<LOD (0.05)	0.12	1.40	1.69	1.17	1.29
7	200 CYA	1467	0.08	0.39	<LOD (0.05)	0.11	<LOD (0.03)	0.38	<LOD (0.11)	<LOQ (0.12)
28	0 MEL + CYA	89	0.02	<LOQ (0.2)	<LOQ (0.3)	0.05	0.38	0.57	<LOQ (0.3)	0.40
28	0 MEL + CYA	90	0.01	<LOQ (0.2)	<LOD (0.05)	0.21	0.21	0.41	<LOD (0.11)	0.34
28	0 MEL + CYA	91	0.03	0.24	<LOD (0.05)	0.02	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOQ (0.02)
28	0 MEL + CYA	95	<LOD (0.01)	0.24	<LOD (0.05)	0.04	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOQ (0.02)
28	0 MEL + CYA	242	<LOD (0.01)	<LOQ (0.2)	<LOD (0.05)	<LOQ (0.03)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.01)
28	0 MEL + CYA	243	0.01	<LOQ (0.2)	<LOD (0.05)	<LOQ (0.03)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOD (0.01)
28	0 MEL + CYA	246	0.05	<LOQ (0.2)	<LOD (0.05)	0.16	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	0.14
28	0 MEL + CYA	247	<LOD (0.01)	<LOQ (0.2)	<LOD (0.05)	<LOQ (0.03)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOQ (0.02)
28	0 MEL + CYA	345	0.12	0.69	<LOQ (0.5)	1.45	<LOD (0.05)	<LOD (0.06)	<LOQ (0.3)	0.03
28	0 MEL + CYA	348	<LOD (0.01)	N/R[a]	N/R[a]	N/R[a]	<LOD (0.05)	N/R[a]	N/R[a]	N/R[a]
28	0 MEL + CYA	351	0.12	0.23	<LOQ (0.5)	0.11	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	0.02
28	0 MEL + CYA	353	<LOD (0.01)	<LOQ (0.2)	<LOD (0.05)	<LOQ (0.03)	<LOD (0.05)	<LOD (0.06)	<LOD (0.11)	<LOQ (0.02)
28	1 MEL + CYA	88	0.21	0.32	<LOQ (0.3)	0.27	<LOD (0.03)	0.26	<LOD (0.11)	0.07
28	1 MEL + CYA	94	0.13	0.33	<LOD (0.05)	1.97	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	1.59
28	1 MEL + CYA	98	0.14	0.33	<LOQ (0.3)	0.58	<LOD (0.03)	<LOQ (0.2)	<LOQ (0.3)	0.38
28	1 MEL + CYA	99	0.11	0.31	<LOQ (0.3)	0.43	<LOD (0.03)	<LOQ (0.2)	<LOD (0.11)	0.28
28	1 MEL + CYA	238	0.04	<LOQ (0.2)	<LOQ (0.3)	0.98	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	0.83
28	1 MEL + CYA	239	0.21	0.27	<LOQ (0.3)	0.28	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	0.22
28	1 MEL + CYA	241	0.32	0.33	0.31	0.28	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	0.02
28	1 MEL + CYA	245	0.12	<LOQ (0.2)	<LOQ (0.5)	0.33	0.03	<LOD (0.06)	<LOD (0.11)	0.21
28	3.3 MEL + CYA	92	0.25	0.42	<LOQ (0.3)	0.34	<LOD (0.03)	<LOQ (0.2)	<LOD (0.11)	0.12
28	3.3 MEL + CYA	93	0.21	0.39	<LOQ (0.3)	0.33	<LOD (0.03)	<LOQ (0.2)	<LOD (0.11)	0.11
28	3.3 MEL + CYA	97	0.47	0.63	0.57	0.60	<LOD (0.03)	<LOQ (0.2)	<LOQ (0.3)	0.07
28	3.3 MEL + CYA	234	0.23	0.39	<LOQ (0.3)	0.30	0.04	<LOQ (0.2)	<LOQ (0.3)	<LOQ (0.02)
28	3.3 MEL + CYA	235	0.70	0.82	0.71	0.80	0.13	0.25	<LOQ (0.3)	0.17
28	3.3 MEL + CYA	236	0.17	0.39	<LOQ (0.3)	0.39	<LOD (0.03)	<LOQ (0.2)	<LOD (0.11)	0.20
28	3.3 MEL + CYA	240	0.24	0.26	<LOQ (0.3)	0.32	0.03	<LOD (0.06)	<LOQ (0.3)	0.11
28	3.3 MEL + CYA	249	0.34	0.41	<LOQ (0.5)	0.34	<LOD (0.03)	<LOD (0.06)	<LOD (0.11)	<LOQ (0.11)
28	200 MEL	237	**9.99**	**9.72**	**9.41**	**11.70**	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	0.20
28	200 MEL	343	**15.58**	**14.10**	**16.16**	**16.35**	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	0.06
28	200 MEL	344	**17.58**	**14.80**	**18.45**	**23.05**	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	<LOQ (0.11)
28	200 MEL	346	**22.07**	**19.54**	**23.29**	**24.01**	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	0.15
28	200 MEL	347	**16.73**	**14.60**	**16.60**	**14.30**	<LOQ (0.03)	<LOQ (0.2)	<LOD (0.11)	0.17
28	200 MEL	350	**16.04**	**13.48**	**15.77**	**14.88**	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	0.08
28	200 MEL	352	**20.99**	**17.82**	**21.24**	**18.69**	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	0.05
28	200 MEL	354	**12.49**	**11.78**	**12.31**	**12.76**	<LOQ (0.03)	<LOD (0.06)	<LOD (0.11)	0.16

LOD – limit of method detection. LOQ – limit of method quantitation. N/R – not reported.

[a] Samples were concluded as free of both MEL and CYA based on results obtained in laboratory #1, and laboratories #2, #3 and #4 used the samples for LC-MS/MS method development and validation.

[b] Crystals were found in renal medulla, cortex and urine sediment (Stine et al., 2011). Concentrations exceeding the level of concern are in bold.

have contributed to the variability. It is unlikely that poor solubility of crystals caused this variation. Three laboratories used extraction solvents which were shown to dissolve melamine–cyanurate complex previously (Heller and Nochetto, 2008; Filigenzi et al., 2008; Turnipseed et al., 2008). One laboratory used slightly modified version of the solvent which was previously shown to dissolve melamine–cyanurate complex. The high inter-laboratory variability of results for high residue kidneys could also have occurred due to analytical and technical errors associated with multiple dilutions of samples containing internal standards.

4.2. Residues of MEL and CYA in experimental pigs

This study shows that MEL or CYA can be deposited in edible tissues of pigs only when the pigs are given very high concentrations over multiple days. Specifically, MEL and/or CYA were deposited in loin, ham, liver and kidney at levels above concern when pigs were fed only MEL at 200 ppm bw/day or a combination of MEL + CYA each at 100 ppm bw/day. Pigs given less than 33 mg/kg bw daily for 7 days did not develop residues above the level of concern (2.5 ppm) in any tissue tested. However, kidneys of pigs fed 33 ppm bw/day contained residues of both analytes above the level of concern. Results were similar between all four laboratories using four different LC-MS/MS methods, which provides a high degree of confidence regarding the conclusions drawn.

Residues of MEL and CYA in loin (Table 1) were almost identical to those in ham (Supplementary Table S1) and very similar to those in liver (Table 2). This is consistent with the previously reported data for lambs (Lv et al., 2010) and pigs (Li et al., 2010; Wang et al., 2014), but contradicts the data reported by Cruywagen and coworkers (2011) who found that MEL residues in sheep muscles were similar to those in kidney and significantly higher than in liver. Some differences in deposition of MEL and CYA found in this study versus in other studies may be explained by differences in the dosing matrices used and the consumption of water by the test animals (Sprando et al., 2012). In this study MEL and CYA were administered as a mixture with pudding and water was supplied to the animals *ad libitum*.

Residue levels of MEL and CYA in some kidneys were 100–1000 times higher than in muscles and liver, confirming conclusions reported for these same kidneys in our previous communication (Stine et al., 2011). High concentrations of MEL and CYA in renal tissue are not surprising as they are excreted by the kidney and concentrate there. The renal melamine-cyanurate crystal formation in animals dosed with both triazines caused marked accumulation of the analytes in the kidney, as expected.

One of the pigs dosed with only MEL had residues of CYA in kidney at levels above the level of concern (Pig ID 1470, Table 3). This same pig contained visible melamine cyanurate crystals in the renal medulla, cortex and urine sediment (Stine et al., 2011). This finding supports our previous results that the crystals' CYA was being measured as a residue (Stine et al., 2011). Interestingly, the ratio of MEL to CYA in those crystals micro-dissected from the kidney was approximately 1:1 (Stine et al., 2011), whereas the ratio of MEL to CYA in the kidney was approximately 4:1 (see pig ID # 1470, Table 3). This suggests that significant portion of MEL in the kidney was not associated with crystals and the amount of crystals formed was likely limited by availability of CYA. Since the feed had been found to be free of CYA, MEL was likely converted to CYA by the gut bacteria (Seffernick et al., 2010; Wackett et al., 2002; Zheng et al., 2013) or, less likely, by the test animal's metabolism. Finding CYA in a pig dosed with MEL in this study is consistent with previous reports in rats and fish where crystals were found in a few of the animals dosed with MEL alone (Jacob et al., 2011; Reimschuessel et al., 2009; Stine et al., 2012; Stine et al., 2014).

Treating pigs with 200 mg/kg MEL for 28 days resulted in residues of MEL at levels similar to levels when treated for only 7 days. This suggests that an equilibrium has likely occurred by approximately 7 days, with absorption and excretion remaining similar.

In summary, treatment with combinations of MEL + CYA at 1, 3, 10 or 33 mg/kg bw/day or CYA alone at 200 mg/kg bw/day did not result in residues greater than the level of concern in edible muscle or liver (Supplementary Table S3).

Disclaimers

The views expressed in this article are those of the authors and may not reflect the official policy of the Department of Health and Human Services, the U.S. Food and Drug Administration, or the U.S. Government.

Conflict of interests

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

Sally Hartman, Nina French, Jason Mihalic and Olgica Ceric are thanked for assistance in organizing this project, insightful discussions and analysis support.

Cynthia Stine, Charles Gieseker, Eric Evans, Tamara Mayer, Nicholas Hasbrouck, Elizabeth Tall, Jamie Boehmer, Gonçalo Gamboa da Costa and Jeffrey Ward are thanked for providing tissues from their previous NOAEL study.

USFDA-FERN and FSIS-FERN Program Offices are acknowledged for assistance in organizing this project.

This project was supported in part by an appointment to the Research Participation Program in the Center for Veterinary Medicine administered by the Oak Ridge Institute for Science and education through an interagency agreement between DOE and USFDA.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.fct.2015.03.007.

References

Andersen, W.C., Turnipseed, S.B., Karbiwnyk, C.M., Evans, E., Hasbrouck, N., Mayer, T.D., et al., 2011. Bioaccumulation of melamine in catfish muscle following continuous, low-dose, oral administration. J. Agric. Food Chem. 59 (7), 3111–3117.

Breidbach, A., Bouten, K., Kröger, K., Ulberth, F., 2010. Capabilities of laboratories to determine melamine in food-results of an international proficiency test. Anal. Bioanal. Chem. 396 (1), 503–510.

China Daily, 2010. Six arrested in melamine-tainted milk scandal. Available from: http://www.chinadaily.com.cn/china/2010-08/23/content_11186225.htm (accessed 12.30.13.).

CNN, 2011. China seizes 26 tons of melamine-tainted milk. Available from: http://www.cnn.com/2011/WORLD/asiapcf/04/27/china.tainted.milk/ (accessed 12.30.13.). By Helena Hong on.

Cruywagen, C.W., van de Vyver, W.F., Stander, M.A., 2011. Quantification of melamine absorption, distribution to tissues, and excretion by sheep. J. Anim. Sci. 89 (7), 2164–2169.

Filigenzi, M.S., Puschner, B., Aston, L.S., Poppenga, R.H., 2008. Diagnostic determination of melamine and related compounds in kidney tissue by liquid chromatography/tandem mass spectrometry. J. Agric. Food Chem. 56, 7593–7599.

Heller, D., Nochetto, C., 2008. Simultaneous determination and confirmation of melamine and cyanuric acid in animal feed by zwitterionic hydrophilic interaction chromatography and tandem mass spectrometry. Rapid Commun. Mass Spectrom. 22 (22), 3624–3632.

Jacob, C.C., Reimschuessel, R., Von Tungeln, L.S., Olson, G.R., Warbritton, A.R., Hattan, D.G., et al., 2011. Dose-response assessment of nephrotoxicity from a 7-day combined exposure to melamine and cyanuric acid in F344 rats. Toxicol. Sci. 119, 391–397.

Li, M., Gong, L., Wu, H., Zhang, L., Shang, B., Chen, Y., 2010. Residue depletion of melamine in pigs exposed to melamine contaminated feed. J. Anim. Vet. Adv. 9, 2089–2093.

Lv, X., Wang, J., Wu, L., Qiu, J., Li, J., Wu, Z., et al., 2010. Tissue deposition and residue depletion in lambs exposed to melamine and cyanuric acid-contaminated diets. J. Agric. Food Chem. 58 (2), 943–948.

New York Times, 2009. China arrests 3 for selling tainted milk powder. Available from: http://www.nytimes.com/2009/12/12/world/asia/12milk.html (accessed 12.30.13.). By David Barboza on.

New York Times, 2010. Discovery of melamine-tainted milk shuts Shanghai dairy. Available from: http://www.nytimes.com/2010/01/02/world/asia/02milk.html?_r=0 (accessed 12.30.13.). By David Barboza on.

New York Times, 2011. China: inquiry on tainted milk powder. Available from: http://www.nytimes.com/2011/01/15/world/asia/15briefs-Milk.html?ref=melamine (accessed 12.30.13.). By Edward Wong on.

Reimschuessel, R., Evans, E.R., Stine, C.B., Stine, C., Mayer, T., Hasbrouck, N., et al., 2009. Fish – a non-mammalian model for determining a "No Observable Effect Level" (NOEL) for crystal formation in kidneys after exposure to melamine and cyanuric acid. In: Programs and Abstracts of the 238th National Meeting of the American Chemical Society. American Chemical Society, Washington, D.C.

Reuters, 2014. China seizes 12 tonnes of melamine tainted yoghurt candy. Reporting by Ben Blanchard. Available from: http://www.reuters.com/article/2014/07/31/us-china-food-yoghurt-idUSKBN0G015W20140731 (accessed on 08.06.14.). Editing by Robert Birsel on.

Seffernick, J.L., Dodge, A.G., Sadowsky, M.J., Bumpus, J.A., Wackett, L.P., 2010. Bacterial ammeline metabolism via guanine deaminase. J. Bacteriol. 192 (4), 1106–1112.

Smoker, M., Krynitsky, A.J., 2008. Interim Method for Determination of Melamine and Cyanuric Acid Residues in Foods using LC-MS/MS: Version 1.0. US FDA/CFSAN Laboratory Information Bulletin LIB No. 4422.

Sprando, R.L., Reimschuessel, R., Stine, C.B., Black, T., Olejnik, N., Scott, M., et al., 2012. Timing and route of exposure affects crystal formation in melamine and cyanuric exposed male and female rats: gavage vs. feeding. Food Chem. Toxicol. 50 (12), 4389–4397.

Stine, C., Reimschuessel, R., Gieseker, C., Evans, E., Mayer, T., Hasbrouck, N., et al., 2011. A No Observable Adverse Effects Level (NOAEL) for pigs fed melamine and cyanuric acid. Regul. Toxicol. Pharmacol. 60 (3), 363–372.

Stine, C., Reimschuessel, R., Keltner, Z., Nochetto, C., Black, T., Olejnik, N., et al., 2014. Reproductive toxicity in rats with crystal nephropathy following high doses of oral melamine or cyanuric acid. Food Chem. Toxicol. 68, 142–153.

Stine, C.B., Nochetto, C.B., Evans, E.R., Gieseker, C.M., Mayer, T.D., Hasbrouck, N.R., et al., 2012. Depletion of melamine and cyanuric acid in serum from catfish *Ictalurus punctatus* and rainbow trout *Onchorhynchus mykiss*. Food Chem. Toxicol. 50 (10), 3426–3432.

The Telegraph, 2011. Tainted milk powder seized in Chinese warehouses. Available from: http://www.telegraph.co.uk/news/worldnews/asia/china/8476073/Tainted-milk-powder-seized-in-Chinese-warehouses.html (accessed 12.30.13.). By Peter Foster on.

Thompson, M., Ellison, S., Wood, R., 2006. The international harmonized protocol for the proficiency testing of analytical chemistry laboratories (IUPAC Technical Report). Pure Appl. Chem. 78, 145–196.

Turnipseed, S., Casey, C., Nochetto, C., Heller, D., 2008. Determination of melamine and cyanuric acid residues in infant formula using LC-MS/MS. Laboratory information bulletin No. 4421, vol. 24. USFDA. Available from: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071637.htm (accessed 07.01.14.).

USFDA, 2007. Interim melamine and analogues safety/risk assessment. Available from: http://www.fda.gov/ScienceResearch/SpecialTopics/PeerReviewofScientificInformationandAssessments/ucm155012.htm (accessed 12.31.13.).

USFDA, 2008. FDA issues interim safety and risk assessment of melamine and melamine-related compounds in food. Available from: http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2008/ucm116960.htm (accessed 12.15.14.).

Wackett, L.P., Sadowsky, M.J., Martinez, B., Shapir, N., 2002. Biodegradation of atrazine and related s-triazine compounds: from enzymes to field studies. Appl. Microbiol. Biotechnol. 58 (1), 39–45.

Wang, W., Chen, H., Yu, B., Mao, X., Chen, D., 2014. Tissue deposition and residue depletion of melamine in fattening pigs following oral administration. Food Addit. Contam. 31 (1), 7–14.

Xia, J., Zhou, N., Zhou, C., Chen, B., Wu, Y., Yao, S., 2010. Simultaneous determination of melamine and related compounds by hydrophilic interaction liquid chromatography-electrospray mass spectrometry. J. Sep. Sci. 33 (17–18), 2688–2697.

Zheng, X., Zhao, A., Xie, G., Chi, Y., Zhao, L., Li, H., et al., 2013. Melamine-induced renal toxicity is mediated by the gut microbiota. Sci. Transl. Med. 5, 172ra22.