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Determination and Confirmation of Melamine Residues in Catfish, Trout, Tilapia, Salmon, and Shrimp by Liquid Chromatography with Tandem Mass Spectrometry

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Pet and food animal (hogs, chicken, and fish) feeds were recently found to be contaminated with melamine (MEL). A quantitative and confirmatory method is presented to determine MEL residues in edible tissues from fish fed this contaminant. Edible tissues were extracted with acidic acetonitrile, defatted with dichloromethane, and cleaned up using mixed-mode cation exchange solid-phase extraction cartridges. Extracts were analyzed by liquid chromatography with tandem mass spectrometry with hydrophilic interaction chromatography and electrospray ionization in positive ion mode. Fish and shrimp tissues were fortified with 10−500 μg/kg (ppb) of MEL with an average recovery of 63.8% (21.5% relative standard deviation, $n = 121$). Incurred fish tissues were generated by feeding fish up to 400 mg/kg of MEL or a combination of MEL and the related triazine cyanuric acid (CYA). MEL and CYA are known to form an insoluble complex in the kidneys, which may lead to renal failure. Fifty-five treated catfish, trout, tilapia, and salmon were analyzed after withdrawal times of 1−14 days. MEL residues were found in edible tissues from all of the fish with concentrations ranging from 0.011 to 210 mg/kg (ppm). Incurred shrimp and a survey of market seafood products were also analyzed as part of this study.

KEYWORDS: Melamine; cyanuric acid; fish; shrimp; LC-MS/MS; incurred muscle

INTRODUCTION

In the spring of 2007, pet food, wheat flour, and other protein-based food commodities were found to be contaminated with melamine (MEL), a triazine-based industrial chemical used in the manufacture of plastics, flame retardants, and other products. MEL is not approved by the U.S. Food and Drug Administration as a food additive for use in human or animal feed; however, the presence of this nitrogen-rich chemical in food commodities, such as wheat gluten and rice protein, artificially skews the results of protein analysis, causing the food to test as a more protein-rich product. MEL in combination with another triazine, cyanuric acid (CYA), resulted in the formation of insoluble melamine cyanurate (MEL-CYA) crystals in kidneys, causing renal failure in hundreds of cats and dogs in the United States who consumed adulterated feed (*1–4*).

MEL also has been found in feeds of animals raised for human consumption, including chickens, hogs, and fish (*5, 6*). Because these animals were fed MEL-contaminated feeds, it is important to monitor edible tissues from these animals for MEL residues that may enter the human food supply. Analytical methods are available to determine MEL in a variety of matrixes including tableware (*7*), soil (*8*), plant matter (*9, 10*), and, more recently, animal feeds and grains (*11–16*); however, there are relatively few methods for the determination of MEL in animal matrixes, such as tissues and biological fluids. In two of these methods, MEL was determined by liquid chromatography with ultraviolet detection in bovine muscle (*17*) and porcine plasma (*18*). Two methods based on liquid chromatography with tandem mass spectrometry (LC-MS/MS) recently have been published. Brown et al. analyzed MEL and CYA residues in kidney tissue and urine from cats and dogs affected by contaminated pet food (*1*). The Brown method was only evaluated above 5000 μg/kg, likely due to high levels found in the kidneys and urine of affected animals. Filigenzi et al. validated a method to determine MEL residues in porcine muscle using LC-MS/MS with a limit of detection (LOD) of 1.7 μg/kg (*19*). In our laboratory, we

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have successfully adapted the Filigenzi extraction method for the determination of MEL in catfish muscle (*20*). Others are working on methods to monitor for residues of CYA (*21*) in fish and shrimp, as well as both MEL and CYA (*22*) in fish and pork tissues. In the present study, our method was validated for multiple fish species and shrimp and used to analyze muscle samples from catfish, trout, tilapia, or salmon that had been deliberately fed MEL, CYA, or the combination of MEL and CYA (*23*). Incurred shrimp that were given feed containing MEL at three different levels were also analyzed. Finally, a survey of over 100 market fish was also conducted as part of this study. This paper represents the first method developed for the analysis of MEL in fish and shrimp edible tissues and the first determination of MEL in muscle tissues of fish deliberately fed MEL and the combination of MEL and CYA.

MATERIALS AND METHODS

Reagents. MEL was obtained from Sigma-Aldrich (99+% purity, St. Louis, MO). $^{15}N_3$-labeled MEL internal standard was used to compare recoveries in select experiments. This labeled standard was graciously provided by researchers from the FDA National Center for Toxiological Research in Jefferson, Arkansas. High-purity chromatographic and spectrophotometric grade acetonitrile and methanol were used. Dichloromethane was liquid chromatographic grade. All water used was deionized and purified to 18.2 MΩ cm (Millipore, Bedford, MA). Concentrated hydrochloric acid and ammonium hydroxide were ACS grade, and ammonium formate was reagent grade. Solutions used in the procedure include 50/50 (v/v) acetonitrile/water, 1.0 and 0.1 M hydrochloric acid in water, 2% (v/v) ammonium hydroxide in water, 5% (v/v) ammonium hydroxide in methanol, 20 mM ammonium formate in water, and 95/5 (v/v) acetonitrile/20 mM ammonium formate.

Standard Solutions. A 100.0 μg/mL stock solution of MEL was prepared by dissolving 10.00 mg of MEL in 100.0 mL of the 2% ammonium hydroxide solution. The solution was sonicated for 20−30 min until crystals of MEL were no longer visible. The stock solution was stable for up to 3 months when stored at room temperature. Intermediate solutions of 10.0 and 1.0 μg/mL MEL were made by taking appropriate aliquots of the stock solution and diluting to volume in water. Fresh intermediate solutions were prepared from the stock solution every week. MEL calibration standards were prepared once every 1−2 days in concentrations of 10, 25, 50, 100, 500, 1000, and 2000 ng/mL. These calibrants were prepared by diluting appropriate aliquots of the 1 and 10 μg/mL MEL intermediate solutions to 5 mL with 95/5 acetonitrile/ammonium formate.

Sample Preparation. Thawed fish filets were cut into 3−5 cm cubes (approximate size). Trout and salmon muscle were processed with skin intact (scales removed); catfish and tilapia were muscle only. Shells were removed from shrimp tail muscles before processing; however, the tails were not "deveined". Samples were blended with dry ice in a blender/homogenizer with pulsed action until contents were uniform and had the consistency of a fine powder. The homogenate was allowed to degas in the freezer overnight and then was tightly sealed until analysis. Control tissues were used for method validation. These consisted of farm-raised catfish, farm-raised rainbow trout, wild-caught salmon, and wild pink shrimp purchased locally. Aquacultured tilapia was provided by the FDA Center for Veterinary Medicine Aquaculture Research Facility. Control tissues were analyzed by the method, and no MEL residue was detected. To generate validation data, 5.0 g portions of thawed tissue homogenate were fortified by spiking with appropriate volumes of the 1 or 10 μg/mL MEL solution to produce samples containing 10, 25, 50, 100, or 500 ng/g of MEL. Samples were allowed to sit at room temperature for at least 15 min before proceeding with extraction.

Incurred Tissues. Catfish (*Ictalurus punctatus*) [725−2675 g of body weight (BW)], trout (*Oncorhynchus mykiss*) (650−1100 g), tilapia (*Oreochromis species*) (350−1550 g), and salmon (*Salmo salar*) (1000−2450 g) were fed varying quantities of MEL, CYA, and the combination of MEL and CYA. Catfish, trout, and salmon were fed by intragastric tubing, and tilapia were dosed orally using capsules embedded in gel food. The target dose was 400 mg/kg BW of MEL or CYA or a combined dose of 400 mg/kg BW of MEL + 400 mg/kg BW of CYA; detailed dosing data are described elsewhere (*23*). One group of tilapia was given lower doses of MEL + CYA (approximately 3−20 mg/kg BW) to try to develop preliminary data on crystal formation thresholds. Fish were typically dosed once daily for 3 days and then euthanatized 1−14 days after dosing. Shrimp were fed feed containing 50 or 100 mg/kg of MEL for 14 days and then were harvested. Fish and shrimp were fileted or shelled as above and then blended with dry ice according to the sample preparation method.

Extraction Procedure. Homogenized muscle or muscle plus skin (5.0 g) was weighed into a 50 mL polypropylene centrifuge tube. To this was added 24 mL of a 50/50 (v/v) solution of acetonitrile/water and 1 mL of 1.0 N hydrochloric acid. The sample was capped, shaken vigorously for 30 s, and then vortex mixed for 1 min. The sample was centrifuged at 4000 rpm for 5 min at 5 °C. Breaking through the solid fat layer at the top of the sample with the tip of a pipet, a 5 mL aliquot of supernatant was removed to a 15 mL polypropylene centrifuge tube. The sample portion that remained in the 50 mL tube was discarded. Dichloromethane (10 mL) was added to the contents of the 15 mL tube, and the sample was shaken for 2 min. The sample was centrifuged at 4000 rpm for 5 min at 5 °C. A portion (2.5 mL) of the upper aqueous layer was carefully removed to a glass culture tube. Water (2.5 mL) was added to the dichloromethane layer, and the sample was re-extracted by shaking for 1 min. The polypropylene tube was again centrifuged at 4000 rpm for 5 min at 5 °C, and the entire upper aqueous layer was removed and combined with the first aqueous extract in the glass culture tube. This combined extract was vortex mixed for 5 s.

An Oasis MCX solid-phase extraction (SPE) cartridge (150 mg, 6 mL, Waters Corp., Milford, MA) was used to cleanup sample extracts. The SPE cartridge was conditioned with methanol (5 mL) followed by water (5 mL). The sample was applied to the conditioned cartridge and allowed to elute by gravity. The cartridge was washed with 5 mL of 0.1 N HCl, followed by 2 mL of methanol. The cartridge was dried by applying vacuum for 1 min. The column was eluted into a glass culture tube using 5 mL of 5% ammonium hydroxide in methanol. Using a commercial evaporator (Turbo-Vap LV, Zymark, Hopkinton, MA), the eluate was evaporated to dryness in a water bath at 55 °C under blowing nitrogen at 15 psi for 20 min. The dried extract was reconstituted in 1.0 mL of 95/5 acetonitrile/ammonium formate (20 mM), vortex mixed for 15 s, and filtered through a 0.2 μm nylon syringe filter (Acrodisc 13 mm, Pall Life Sciences, East Hills, NY) into a glass LC vial.

LC-MS/MS Quantification and Confirmation. The LC-MS/MS consisted of a Thermo (San Jose, CA) TSQ Quantum triple quadrupole mass spectrometer coupled to a Thermo Surveyor LC-MS pump and autosampler. A metal needle sample kit was installed on the electrospray source; the orientation of the spray to the orifice was set at the second notch (approximately 62° offset). XCaliber V2.0 software was used to acquire and analyze the data. The column was an Atlantis HILIC Silica column, 3 μm, 3.0 mm × 50 mm (Waters Corp.).

The LC-MS/MS was operated in positive ion mode and tuned by flowing a MEL standard solution (10 ng/μL) at a rate of 10 μL/min using a syringe pump into a stream of 350 μL/min 75/25 acetonitrile/ammonium formate (20 mM) via a T-union. The combined stream was introduced into the electrospray interface. Selected reaction monitoring (SRM) was performed on the protonated molecule for MEL using the following general parameters: source spray voltage, 5 kV; capillary temperature, 270 °C; sheath gas (nitrogen), 14 (arbitrary units); auxiliary gas (nitrogen), 0 (arbitrary units); Q1 peak width, $m/z = 0.7$; Q3 peak width, $m/z = 0.7$; collision gas, 1.5 torr argon; scan width, $m/z = 1$; and scan time, 0.5 s. To optimize the signal for the m/z 127 precursor ion, the electrospray skimmer potential was set to 20 V. Two SRM transitions of m/z 127 → 85 (collision energy = 7 V) and m/z 127 → 68 (collision energy = 23 V) were monitored.

The LC mobile phase program consisted of a binary gradient of acetonitrile and 20 mM aqueous ammonium formate. The composition started out at 95% acetonitrile and decreased linearly to 50% acetonitrile over 5 min. The mobile phase was then returned to 95% acetonitrile between 5 and 7 min, and the column was re-equilibrated for 5 min.

Table 1. Recovery of MEL from Fortified Tissue and Negative Controls

fortification level (μg/kg) (ppb)	recovery (%)	RSD[a] (%)	no. of samples confirmed/ analyzed	no. of days of analysis	recovery with $^{15}N_3$-MEL (%)	RSD with $^{15}N_3$-MEL (%)	samples analyzed with $^{15}N_3$-MEL
			catfish				
control	0	0	0/12	8			
10	90.3	5.1	3/3	1			
25	78.2	17.9	7/8	6			
50	74.5	20.5	10/10	7			
100	73.1	3.2	5/5	1			
500	62.6	6.3	3/3	1			
			trout				
control	0	0	0/5	5			
25	60.2	23.9	5/5	5			
50	61.0	20.1	8/8	6			
			tilapia				
control	0	0	0/10	7			
25	71.5	8.3	8/8	6			
50	68.0	10.5	7/8	6			
			salmon				
control	0	0	0/4	4	0	0	2
25	66.0	19.6	8/8	4	113.3	7.0	6
50	59.6	17.9	17/17	5	102.4	10.5	6
			shrimp				
control	0	0	1/10	4	0	0	1
10	65.7	10.9	4/6	2			
25	77.8	2.3	2/3	1			
50	53.0	15.1	22/22	4	97.4	11.2	8
100	52.2	11.2	6/6	2			
500	49.9	6.7	6/6	2			

[a] The residual standard deviation was calculated from all extraction recovery data. In most cases, data were obtained on more than a single day of analysis.

The flow rate was 350 μL/min. The column was kept in an insulated compartment, but the temperature was not controlled. The injection volume was 10 μL, and the needle was flushed with 400 μL of 50/50 water/methanol between samples. The LC flow was diverted to waste for the first 0.14 min and again at 6 min.

Quantitative data were obtained by comparison of the area counts of the chromatographic peak observed for the *m/z* 127 → 85 SRM transition to the calibration curve generated for that transition from MEL standards with concentrations ranging from 10 to 2000 ng/mL. For confirmation, peak area counts from the *m/z* 127 → 85 and *m/z* 127 → 68 SRM transitions were generated, and the resulting chromatographic peaks were integrated. Relative abundances were calculated from these peak areas and compared to contemporary standards. To be confirmed, the retention time of MEL found in samples had to match within 5% of that for standards analyzed on the same day, and the relative abundance of the two transitions had to match within 10% of that for the standards (*24*).

RESULTS AND DISCUSSION

The MEL extraction was a modification of one developed to extract MEL from pork tissues developed by Filigenzi and coworkers (*19*). In Filigenzi et al., 10 g samples of pork tissue were homogenized with acetonitrile and water and then centrifuged. A portion of the supernatant was then acidified with hydrochloric acid and subsequently cleaned up with liquid−liquid extraction and SPE. An internal standard was also added to sample extracts. In the method described in this paper, higher extraction recoveries of MEL from fish tissue resulted from acidifying the sample in the initial aqueous acetonitrile extraction. The tissue and extraction solution were externally agitated using a vortex mixer. By avoiding the use of immersion homogenizers, sample throughput was increased, and a potential source of cross-contamination was eliminated. In addition, a smaller sample size (5 g) and the elimination of internal standard also reduced solvent volumes and reduced analysis costs. It should be noted that the acidic acetonitrile extraction solution used in this method most likely extracts free MEL from muscle and is likely unable to dissolve crystals of the MEL-CYA complex that might be found in kidney.

MEL was analyzed by LC-MS/MS with hydrophilic interaction chromatography (HILIC). The predominant species in the positive electrospray ionization of this compound is the MH^+ ion at *m/z* 127; sodium or potassium adducts were not observed. The protonated molecule can be dissociated into product ions at *m/z* 85 ($C_2N_4H_5^+$) and *m/z* 68 ($C_2N_3H_2^+$) (*25*). Using these ion transitions for SRM allows for quantitative and qualitative analysis of MEL residues. Because of its high polarity, MEL is not well-retained on traditional reversed-phase LC columns. HILIC columns have been found to be an effective tool in separating MEL from analogous compounds and background material (*22, 26*).

MEL was extracted from fortified muscle with an overall average recovery of 63.8% [21.5% relative standard deviation (RSD), $n = 121$] over the concentration range 10−500 μg/kg (ppb). Individual recoveries for each species and fortification level are presented in **Table 1**. Average recoveries for each species were 74.8% (17.7% RSD, $n = 28$) for catfish, 60.7% (20.6% RSD, $n = 13$) for trout, 70.0% (9.7% RSD, $n = 15$) for tilapia, 61.6% (18.7% RSD, $n = 25$) for salmon, and 54.9% (17.3% RSD, $n = 40$) for shrimp. In **Table 1**, RSD values for most fortification levels were calculated from recovery data generated from multiple days of analysis. Standards were run with each day of analysis. For a few fortification levels, extractions were only performed on a single day, and RSD values are correspondingly low; for example, five catfish samples fortified with 100 μg/kg of MEL had a same-day RSD of 3.2%. Higher RSD values were obtained for samples analyzed on multiple days; for example, eight trout samples fortified with 50 μg/kg of MEL were analyzed on six nonconsecutive days, with a resulting interday RSD of 20.1%. The method LOD was calculated from the standard deviation of the 10 μg/kg catfish

Table 2. Selected Relative Abundance Data[a]

samples	% relative abundance *m/z* 127 → 68 transition relative to the *m/z* 127→85 transition
day 1	
solvent standards (average, $n = 7$)	76.5
catfish fortified with 500 μg/kg MEL	76.1,75.3,76.9
catfish fortified with 100 μg/kg MEL	75.4,75.5,75.0,84.4,74.9,
catfish fortified with 50 μg/kg MEL	79.1,78.6,77.7
day 2	
solvent standards (average, $n = 6$)	73.9
catfish fortified with 25 μg/kg MEL	72.5,82.7,83.0
catfish fortified with 10 μg/kg MEL	73.3,81.5,82.5
day 3	
solvent standards (average, $n = 7$)	76.8
trout fortified with 25 μg/kg MEL	85.9
trout fortified with 50 μg/kg MEL	76.1,83.3
incurred trout T10	80.2,80.5,81.4,80.1,78.7
incurred trout T11	77.9,78.3,78.7,72.6,79.2
incurred catfish C18	76.6,81.8,74.0
day 4	
solvent standards (average, $n = 13$)	34.8
salmon fortified with 25 μg/kg MEL	33.9,29.7,34.0
salmon fortified with 50 μg/kg MEL	39.4,36.2,35.8
shrimp fortified with 50 μg/kg MEL	32.9,34.8,40.9
incurred salmon S25	39.5,38.6,39.1
incurred salmon S24	37.9,36.4,39.0
incurred shrimp SH2	38.5,38.0,39.6
incurred shrimp SH3	33.9,35.3,36.2

[a] Day 1, 5/3/07; day 2, 5/4/07; day 3, 6/1/07; and day 4, 8/13/07. Tuning parameters were reoptimized between day 3 and day 4.

spikes multiplied by the Student's *t* value at the 99% confidence level and was found to be 3.2 μg/kg. The limit of quantification (LOQ) was 10 times the standard deviation of the 10 μg/kg catfish spikes or 4.7 μg/kg.

Several salmon and shrimp samples were additionally fortified with 50 μg/kg of $^{15}N_3$-labeled MEL internal standard to correct for extraction recoveries as shown in **Table 1**. Internal standard was added to the thawed muscle homogenate at least 15 min prior to the addition of extraction solution. Average recoveries for these samples were 107.9% (9.9% RSD, $n = 12$) for salmon and 97.4% (11.2% RSD, $n = 8$) for shrimp. While the use of internal standard did significantly increase the apparent recovery of MEL for these samples, our initial difficulty in obtaining the standard by custom synthesis and its high cost made its use throughout the entire study prohibitive. In addition, because the levels of MEL found in incurred tissues varied widely, the use of internal standard was less useful when sample extracts had to be diluted.

Selected observed relative abundance data for the two transitions are summarized in **Table 2** for analyses on four different, nonconsecutive days. As shown, the relative peak areas of the two transitions varied throughout the study due to changes in optimal tuning parameters (day 4 as compared to days 1−3); however, the ratios were stable within the day that the samples were analyzed. Selected SRM chromatograms for tilapia are presented in **Figure 1**. MEL was confirmed in 122 of the 127 total fortified samples with abundance ratios within 10% of those observed for the standards. Of the five samples that did not confirm, one had an abundance ratio that varied from that of the standards by 22%, while the others were different by only 11−14%. Because there are only two MEL transitions available to monitor, the confirmation criteria are necessarily strict (*24*). MEL was confirmed in one of 41 control tissues; however, the level found (3.0 μg/kg) was below that of the method detection level (3.2 μg/kg). MEL was not detected in any of the method blanks.

The standard curve for solvent-based MEL calibration standards was linear over the range from 10 to 2000 μg/kg. Calibration curves were generated on each of 22 analysis days from either 10−1000 or 10−2000 μg/kg with correlation coefficients of $R^2 = 0.9966$ or above. Several control catfish sample extracts were spiked with MEL to determine the extent of ion suppression. Recoveries for these end spikes ranged from 87.6 to 97.8%, indicating that ion suppression was not significant and that the use of solvent-based calibration standards was justified.

Incurred Fish Tissues. Unless indicated, catfish, trout, and salmon were dosed with approximately 400 mg/kg BW of MEL or CYA or 400 mg/kg BW each of MEL and CYA (MEL + CYA) once a day for 3 days as summarized in **Table 3**. Tilapia had to be trained to eat gel food containing the contaminants; therefore, it was more difficult to obtain a consistent dose over the 3 days of feeding. As a result, tilapia were dosed with 300−400 mg/kg BW of MEL or lower quantities of MEL + CYA. Microscopic analysis has shown that fish that were fed the MEL + CYA combination dose developed MEL-CYA renal crystals with morphology similar to those found in cat and dog contaminated pet food renal failure cases (*1, 23, 27*). Fish that were dosed with only MEL or CYA did not generally form renal crystals. MEL-CYA crystals were not observed in the fish muscle or skin by microscopic analysis. It was assumed that MEL extracted from the fish muscles was therefore uncomplexed or free MEL. Additional details about fish dosing and pathology can be found in Reimschuessel et al. (*23*). Analysis of these same fish tissues for CYA residues has recently been completed (*21*).

Whereas the method was validated by analyzing fortified tissue samples with MEL concentrations in the parts per billion range (10−500 μg/kg), many of the incurred sample extracts had MEL concentrations in the parts per million range. These incurred extracts had to be diluted and reanalyzed to ensure that the MEL concentration measured was within the linear range of the calibration curve. Dilution factors ranged from 1:4 to 1:200 to cover the wide range of MEL concentrations found in the incurred fish. Adequate analysis precision for three or five replicates of each incurred fish was found with the residual standard deviation for each fish being 17% or below, as shown in **Table 3**.

Control Fish. One or two of each species of the nondosed incursion study fish were analyzed as controls. Although no MEL was found in the nondosed catfish or tilapia, low concentrations of MEL were found in muscle tissues of the nondosed trout and salmon at levels ranging from 0.04 to 0.12 mg/kg. These fish were not deliberately fed MEL; however, 0.5 (approximate) and 6.7 mg/kg of MEL was subsequently confirmed (*28*) in the respective commercial control trout and salmon feed, which had been given to these fish for 6 months prior to starting the study. The fact that the method detected residues in these fish demonstrates both the sensitivity of this method and the fact that residues do accumulate in fish when consuming feed with lower concentrations of MEL. It should be noted that these trout and salmon were not used as controls for method validation.

MEL Only Fish. MEL residues were confirmed in muscles from the exposed fish. The concentrations found ranged from 81−210 mg/kg for catfish, 34−80 mg/kg for trout, 0.02−177 mg/kg for tilapia, and 58 to 94 mg/kg for salmon. Because it

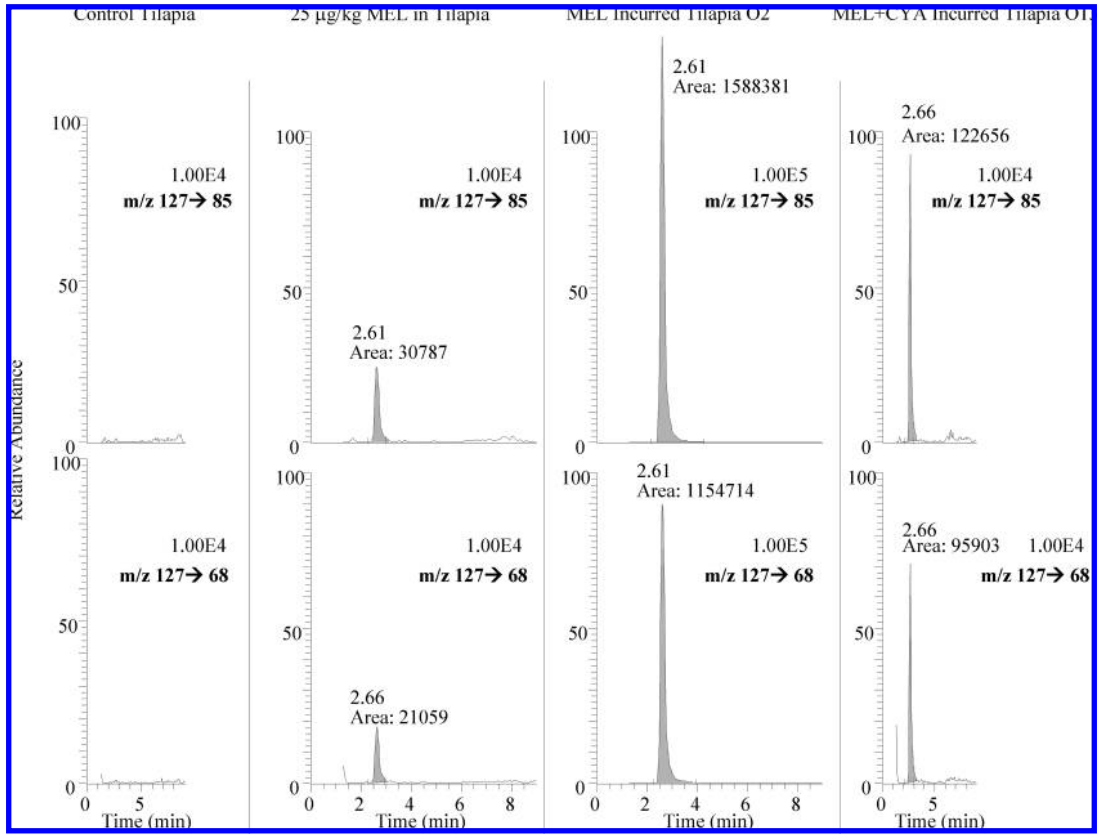

Figure 1. SRM chromatograms for control tilapia, tilapia fortified with 25 μg/kg (ppb) of MEL, MEL incurred tilapia O2 [177 mg/kg (ppm) found in fish, diluted sample], and MEL + CYA incurred tilapia O13 (0.085 mg/kg found in fish).

was not uncommon for tilapia to spit out some of the dosed gel food, it is unclear if the lower levels of MEL found for the two 6 day withdrawal fish (O3 and O4) are a result of a smaller ingested MEL dose or of the ability of tilapia to excrete MEL more quickly than could the other species (**Table 3**).

CYA Only Fish. MEL residues were also confirmed in some fish that had been dosed only with CYA. In catfish, 0.006−0.012 mg/kg of MEL was found in muscle of three fish dosed with an average of 440 mg/kg BW of CYA over 3 days with withdrawal times of 1, 3, and 6 days. It is interesting to note that although no MEL was detected in the catfish feed, measurable quantities of MEL were found in muscles of catfish dosed with CYA. Three salmon dosed with an average of 410 mg/kg BW of CYA had MEL muscle concentrations of 0.08, 6.2, and 0.02 mg/kg for withdrawal times of 1, 6, and 10 days, respectively. Salmon S22, with a MEL muscle concentration of 6.2 mg/kg, was found to have a significant number of MEL-CYA crystals present in the kidney tissues. The salmon in this study were exposed to low levels of MEL in the contaminated commercial salmon feed; however, tissues from the control salmon were found to have significantly lower (0.08−0.12 mg/kg) concentrations of MEL in the muscle than what was found in S22. S22 also had an abdominal neoplasm, which may have reduced normal renal MEL excretion (*23*). One tilapia and one trout that were dosed with CYA under similar conditions were also analyzed by the method; however, MEL residues were not detected in either sample.

MEL + CYA Fish. MEL was confirmed in the muscle of fish that received the combined dose MEL + CYA but generally at lower levels than those fed MEL alone. Significant variation was sometimes found between two fish at the same withdrawal time. For example, for two trout fed MEL + CYA, at 1 day withdrawal, trout T22 had 12.8 mg/kg MEL in the tissue, while trout T23 had 116.8 mg/kg of MEL. T22 had more MEL-CYA crystals in the kidney than T23, perhaps reducing the availability of MEL for accumulation in muscle.

At 14 days after dosing, MEL was found in the tissues of all fish at concentrations of 0.07−21.8 mg/kg, indicating that MEL has a significant residence time in muscle. It should be noted that while the 14 day catfish and trout were dosed with approximately 440 mg/kg BW of MEL + CYA, the salmon received only 210 mg/kg BW and the tilapia only 40 mg/kg BW. Two salmon (S25 and S31) that were dosed with a higher level of MEL + CYA (approximately 380 mg/kg BW) for the longer withdrawal study died at 7 and 11 days following exposure.

Low Dose MEL + CYA. Several tilapia were fed lower dosages (3−17 mg/kg BW) of MEL + CYA, to begin to establish a lower limit of biological effects. Tilapia O19, O20, and O21 consumed 3−4 mg/kg BW each of MEL and CYA on each of 1, 2, or 3 days, respectively, and were sacrificed 1 day after dosing. None of these fish developed renal crystals; however, measurable (0.37−0.84 mg/kg) quantities of MEL were found in the tissues. Crystals were found in the kidneys of tilapia that received more than 7 mg/kg BW of MEL + CYA on each of 3 days.

Incurred Shrimp. Muscle (including gut/vein) from shrimp exposed to a constant dose of MEL in feed was also evaluated by this method. MEL was blended with feed ingredients and then pelleted. Shrimp had regular access to the food over a 14 day period. Shrimp were divided into three groups, with each group receiving either control feed, feed containing 50 mg/kg of MEL, or feed containing 100 mg/kg of MEL. Each group contained approximately 160 g of tail muscle. As shown in

Table 3. Incurred Fish Data

fish ID	contaminant (1×/day for 3 days)	dose of MEL (mg MEL/kg of BW)	withdrawal time (days)	kidney crystals[a]	MEL concn found (mg/kg) (ppm)	% RSD (replicates analyzed)
			catfish			
C15	MEL	451	1		193.7	13.3 (5)
C16	MEL	471	1		210.1	10.7 (5)
C11	MEL	299[b]	3		128.4	5.3 (5)
C12	MEL	479[b]	3		80.5	1.5 (5)
C13	MEL	308[b]	6		129.6	11.3 (5)
C14	MEL	418[b]	6		93.1	4.6 (5)
C21	MEL + CYA	444	1	4	32.5	4.2 (3)
C18	MEL + CYA	475	3	4	28.7	7.1 (3)
C22	MEL + CYA	435	6	4	20.9	12.5 (3)
C23	MEL + CYA	456	14	4	13.4	11.9 (3)
C24	MEL + CYA	440	14	4	17.2	2.0 (3)
C19	CYA		1		0.012	(1)
C17	CYA		3		0.009	(1)
C20	CYA		6		0.006	(1)
			trout			
T13	"control"[c]	~0.5 mg/kg in feed			0.038	7.2 (3)
T18	"control"[c]	~0.5 mg/kg in feed			0.036	5.9 (3)
T7	MEL	396	1		72.8	4.9 (5)
T8	MEL	432	1		60.5	4.3 (5)
T9	MEL	427	3		65.6	6.6 (5)
T10	MEL	402	3		80.3	10.9 (5)
T11	MEL	405	6		34.3	7.3 (5)
T12	MEL	408	6		40.8	7.5 (5)
T22	MEL + CYA	417	1	4	12.8	7.0 (3)
T23	MEL + CYA	428	1	3	116.8	4.5 (3)
T16	MEL + CYA	390	3	4	35.0	17.1 (3)
T17	MEL + CYA	397	3	3	114.9	2.9 (3)
T24	MEL + CYA	418	6	4	22.6	12.8 (3)
T25	MEL + CYA	407	6	4	37.0	5.6 (3)
T26	MEL + CYA	425	14	4	8.6	2.5 (3)
T27	MEL + CYA	443	14	4	21.8	4.4 (3)
			tilapia			
O5	MEL	371[b]	1		35.1	8.2 (5)
O6	MEL	385	1		43.2	6.4 (5)
O1	MEL	456	3		80.4	14.6 (5)
O2	MEL	409	3		177.0	3.2 (5)
O3	MEL	300	6		0.017	11.4 (5)
O4	MEL	321	6		4.5	12.6 (5)
O13	MEL + CYA	53[d]	1	1	0.085	3.2 (3)
O14	MEL + CYA	52[b]	1	4	32.4	2.7 (3)
O9	MEL + CYA	114	3	4	3.8	3.0 (3)
O10	MEL + CYA	216[b]	3	2	0.011	13.3 (3)
O18	MEL + CYA	49	6	4	16.1	1.3 (3)
O26	MEL + CYA	39	14	4	4.9	3.5 (3)
O27	MEL + CYA	42	14	4	0.072	7.2 (3)
O19	MEL + CYA (1 day only)	3	1		0.390	1.4 (3)
O20	MEL + CYA (2 days only)	3	1		0.840	1.3 (3)
O21	MEL + CYA	4	1		0.370	3.0 (3)
O22	MEL + CYA	12	1	1	1.388	7.3 (3)
O23	MEL + CYA	7	3	1	0.175	3.7 (3)
O24	MEL + CYA	14	3	2	0.036	4.0 (3)
O25	MEL + CYA	17	3	3	0.081	4.8 (3)
			salmon			
S26	"control"[c]	6.7 mg/kg in feed			0.080	16.2 (3)
S27	"control"[c]	6.7 mg/kg in feed			0.118	1.9 (3)
S28	MEL	407	1		78.0	3.0 (3)
S20	MEL	416	6		93.5	1.0 (3)
S21	MEL	421	10		58.3	6.7 (3)
S30	MEL + CYA	403	1		41.7	2.4 (3)
S32	MEL + CYA	394	3	4	6.5	11.2 (3)
S24	MEL + CYA	391	6	4	78.8	6.0 (3)
S25	MEL + CYA	376	7	4	68.6	9.3 (3)
S31	MEL + CYA	388	11	4	31.7	3.6 (3)
S33	MEL + CYA	208	14	4	13.4	8.4 (3)
S34	MEL + CYA	211	14	4	20.2	8.5 (3)
S29	CYA		1		0.083	(1)
S22	CYA		6	3	6.2	3.2 (3)
S23	CYA		10		0.019	(1)
			shrimp			
SH1	"control"[c]	>100 mg/kg in feed		N/A	0.041	9.4 (3)
SH2	MEL	50 mg/kg in feed		N/A	0.217	8.3 (3)
SH3	MEL	100 mg/kg in feed		N/A	0.051	4.7 (3)

[a] Key: 1, very few; 2, few; 3, many; and 4, very many. [b] Only a partial dose of MEL was received. [c] These were nondosed incursion study fish or shrimp and were not used as negative control tissues for method validation. [d] Most of the MEL dose was not eaten.

Table 3, MEL was found at levels of 41, 217, and 51 μg/kg in the shrimp fed with control, 50 mg/kg, and 100 mg/kg feed, respectively. In a later analysis of the control shrimp feed, MEL was confirmed at a concentration in excess of 100 mg/kg (*28*). MEL has been reported to be used as a binder in some fish and shrimp feeds (*5*). The shrimp dosed with feed containing 100 mg/kg of MEL were observed to eat less than either the control or the 50 mg/kg groups.

Fish Survey. A total of 105 market-ready shrimp, catfish, tilapia, salmon, eel, and other types of fish were analyzed by this method. Thirty-three samples (31.4%) were found to have MEL concentrations above the method detection level. Ten of these samples (9.5%) had MEL concentrations in excess of 50 μg/kg, with a range of concentrations from 51 to 237 μg/kg (ppb). A preliminary risk assessment conducted by the Food and Drug Administration concluded that human exposures to MEL residues through the consumption of contaminated fish is not likely to present a health risk when the residue level does not exceed 50 μg/kg (*29*). Although tumor formation has been shown in some animal studies from significantly higher levels of exposure to MEL, other toxicological studies have concluded that MEL, by itself, is relatively nontoxic to mammals (*30, 31*). The combination of MEL and CYA, however, has been shown to induce renal crystals (*23, 27*), suggesting that the presence of the two triazines in animal and human food supplies may present a greater health concern than does exposure to MEL alone.

In summary, a method was validated for the determination of MEL residues in fish and shrimp muscle. The average recovery of MEL was 63.8% ($n = 121$, without internal standard) averaged over the five species studied, and the method LOD was 3.2 μg/kg (ppb). The method was applied to the analysis of incurred tissues as well as to market-ready fish and shrimp samples. High concentrations of MEL residues were found to reside in edible tissues from fish that were deliberately dosed with high levels of MEL. Furthermore, fish raised on contaminated feed containing low levels of MEL (6.7 mg/kg) were found to have edible tissue residue levels exceeding 50 μg/kg. Of the market-ready samples, nearly 10% were found to have residues of MEL exceeding 50 μg/kg. It is important to monitor both animal feeds and tissues to prevent violative residues from entering the human food supply.

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