JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Quantitative and Confirmatory Analyses of Malachite Green and Leucomalachite Green Residues in Fish and Shrimp

WENDY C. ANDERSEN,* SHERRI B. TURNIPSEED, AND JOSÉ E. ROYBAL

Animal Drugs Research Center, Food and Drug Administration, Denver Federal Center, P.O. Box 25087, Denver, Colorado 80225-0087

Liquid chromatographic methods are presented for the quantitative and confirmatory determination of malachite green (MG) and leucomalachite green (LMG) for channel catfish, rainbow trout, tilapia, basa, Atlantic salmon, and tiger shrimp. Residues were extracted from tissues with ammonium acetate buffer and acetonitrile and isolated by partitioning into dichloromethane. LMG was quantitatively oxidized to the chromic MG with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone. Extracts were analyzed for total MG by liquid chromatography with both visible detection (LC-VIS) at 618 nm for routine screening and ion trap mass spectrometry (LC-MS^n) with no discharge-atmospheric pressure chemical ionization for residue confirmation. The method was validated in each species fortified with LMG at 1, 2, 4, and 10 ng/g (ppb), and average recoveries ranged from 85.9 to 93.9%. Quantitative data were consistent for the two detection methods, with measured method detection limits of 1.0 ng/g for LC-VIS and 0.25 ng/g for LC-MS^n. Incurred tissues from catfish, trout, tilapia, and salmon that had been treated with MG were also extracted and analyzed as part of this study.

KEYWORDS: Malachite green; leucomalachite green; LC-VIS; LC-MS^n; ND-APCI; fish; shrimp

INTRODUCTION

Malachite green (MG) is a triphenylmethane dye that has received considerable attention for its effective, yet illegal, use as an inexpensive topical fungicide and parasiticide in the aquaculture industry. MG is readily absorbed by fish and metabolically reduced to the lipophilic leucomalachite green (LMG), which is known to have a long residence time in edible fish tissues (*1*). The majority of persistent residues present in fish are therefore in the form of LMG. Studies have shown that MG and LMG are potential mutagens (*2*−*4*), and for this reason, MG is not permitted for use as an aquaculture veterinary drug in the United States, Europe, or Canada. Regardless, numerous incidences of MG misuse in aquaculture have occurred (*5*), and analytical methods are needed to monitor for low levels of this drug in fish tissue. The European Commission requires that methods be able to determine the sum of MG and LMG residues at the minimum performance limit of 2 ng/g (*6*). MG and LMG residues are quantitatively determined and confirmed at a minimum level of 1 ng/g under current U.S. Food and Drug Administration sample testing protocols.

Because MG has a strong chromophore at 618 nm and is positively charged, many analytical methods take advantage of these characteristics by using residue detection schemes that are based on liquid chromatography with visible absorbance (LC-VIS) or mass spectrometric detection (LC-MS). Because the dye is found primarily as the colorless reduced leuco form in fish tissue, oxidation is required to convert the residue to MG for visible analysis. Several methods are available to quantify MG and LMG residues in fish tissue at or below 2 ng/g. Most rely on the use of a lead oxide column reactor to convert LMG to MG, enabling LC-VIS (*7*−*10*) or LC-MS (*7*, *11*, *12*). The manually prepared lead oxide reactor can be plagued by problems, however, including rapid depletion and peak broadening, which lead to a decrease in method sensitivity. Other methods have been described to individually measure MG and LMG residues by isotope dilution LC-MS (*13*), LC-tandem MS (*14*, *15*), and LC with separate detection of MG (VIS) and LMG (fluorescence) (*16*).

The present study describes the development and validation of a method for the quantitative and confirmatory determination of total MG and LMG residues in fish using LC-VIS and LC-MS^n with no-discharge atmospheric pressure chemical ionization (ND-APCI) and ion trap detection (*17*, *18*). As compared to visible spectroscopic techniques, mass spectrometry methods provide greater sensitivity and concurrent residue confirmation for the detection of MG and LMG; however, reliable and robust methods are also needed to routinely screen numerous laboratory samples without straining the resources of high-demand LC-MS instruments. This method incorporates an in situ oxidation of LMG to MG with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), allowing the determination of a single total MG residue that results from the presence of LMG and/or MG in fish tissues (*17*−*19*). DDQ rapidly and effectively oxidizes leucotriphenylmethane dyes to their chromic analogues (*20*, *21*) and eliminates the need for a lead oxide reactor. A slight variation to this extraction procedure was previously validated for LMG and MG in salmon (*17*, *18*). The current study contains additional data demonstrating that the modified extraction procedure produces

10.1021/jf0532258
Published on Web 06/06/2006

equivalent results for salmon analysis and can also be used to analyze a broad variety of fish species.

MATERIALS AND METHODS

Reagents. Reference standards of MG (oxalate) and LMG were obtained from Sigma-Aldrich (St. Louis, MO). High-purity chromatographic and spectrophotometric grade acetonitrile and methanol were used; dichloromethane was high purity grade; all water used was deionized and purified to 18.2 MΩ cm with a Milli Q Plus water system (Millipore, Bedford, MA). Glacial acetic acid was ACS reagent grade; chromatographic grade alumina (80−200 mesh, basic) was obtained from EMD Chemicals (Gibbstown, NJ); ammonium acetate (anhydrous, 98%) and reagent grade diethylene glycol were used; DDQ (98%), formic acid (ACS grade, 96%), hydroxylamine hydrochloride (HAH, ACS reagent grade, 99.8%), and *p*-toluenesulfonic acid (*p*-TSA, monohydrate, 98.5%) were purchased from Sigma-Aldrich. Solid phase extraction (SPE) columns were neutral alumina (Bakerbond, 1000 mg, JT Baker, Phillipsburg, NJ) and propylsulfonic acid (Bond Elut LRC, 500 mg, Varian, Inc., Palo Alto, CA). Ammonium acetate (0.1 M) buffer was prepared by dissolving 7.7 g of ammonium acetate in 1000 mL of water and then adjusting the solution to pH 4.5 by adding 8 mL of acetic acid and 5 mL of 1 M *p*-TSA. DDQ stock solution (0.01 M) was prepared by adding 0.227 g of DDQ to a 100 mL volumetric flask and making it up to volume with acetonitrile. The solution was stored tightly capped in the refrigerator (4−8 °C) for up to 1 month. The DDQ working solution (0.001 M) was prepared by aliquoting 5 mL of the DDQ stock solution into a 50 mL volumetric flask and diluting to the mark with acetonitrile; this solution was stored at room temperature and prepared fresh weekly. Mobile phase A was a 50:50 mixture (by volume) of ammonium acetate buffer and acetonitrile, which was filtered through a 0.45 μm PVDF membrane before use. Mobile phase B was 100% acetonitrile. Mobile phase A was used as 95% of the LC-VIS mobile phase, to dilute calibration standards and to elute SPE columns.

Standard Solutions. *Stock Solutions (100 μg/mL).* Ten (10.0) mg of MG was weighed into a 100 mL volumetric flask and diluted to volume with methanol. The mass of MG was corrected for purity and for the MG−oxalate product, which contains two molecules of MG for one molecule of the MG−oxalate dimer complex. Ten milligrams of LMG (corrected for purity) was weighed into a low-actinic 100 mL volumetric flask and diluted to volume with methanol. Stock solutions were stored at room temperature, protected from light, and freshly prepared every 6 months.

Intermediate Solution I (1.0 μg/mL). One milliliter of each stock solution was pipetted into separate 100 mL volumetric flasks and diluted to volume with methanol. These solutions were freshly prepared monthly and stored at room temperature.

Intermediate Solution II (0.1 μg/mL). One milliliter of each intermediate solution I was pipetted into a 15 mL graduated glass centrifuge tube and diluted to 10 mL with methanol. These solutions were freshly prepared weekly and stored at room temperature.

Calibrant Solutions. A series of MG calibrants were prepared with concentrations of 1, 2, 4, 10, and 20 ng/g by aliquoting 50, 100, or 200 μL of MG intermediate solutions into individual 15 mL graduated glass or disposable polypropylene centrifuge tubes and diluting each to 5.0 mL with mobile phase A. Calibrants were vortex mixed and used to generate a six-point (zero included) MG standard curve. Calibration standards were prepared every 1−2 days.

LC-VIS. The LC-VIS system consisted of an Agilent 1100 with programmable diode array detector (DAD) (Avondale, PA). The column was an Alltima C18, 3 μm, 4.6 mm × 150 mm with a guard cartridge (5 μm, 4.6 mm × 7.5 mm) of the same phase (Alltech Associates, Deerfield, IL) and a ColumnSaver prefilter (0.5 μm, MacMod Analytical Inc., Chadds Ford, PA). The LC was operated isocratically with a mobile phase consisting of 95:5 mobile phase A/mobile phase B (equivalent to 47.5% ammonium acetate buffer and 52.5% acetonitrile), a flow rate of 1.0 mL/min, and a column temperature of 35 °C. All injections were 100 μL. An automated needle wash of mobile phase A was used prior to injection. The DAD was set at an absorbance wavelength of 618 nm (4.0 nm bandwidth) with a reference of 725 nm (8 nm bandwidth) using a tungsten lamp. At the end of each day, the column was flushed with 100% methanol for 30 min.

ND-APCI LC-MS^n. The LC-MS^n consisted of an Agilent 1100 LC interfaced to a ThermoElectron (San Jose, CA) DECA-XP Plus Ion Trap MS with an APCI source. XCaliber (Version 1.3) was the software used to operate the LC and MS. The column was a YMC phenyl 3-4-5 cartridge column, 3 μm, 120 Å, 4.0 mm × 50 mm, with a guard cartridge insert (4.0 mm × 20 mm) of the same phase (Waters Corp., Milford, MA).

The LC-MS was tuned by flowing MG standard solution (0.5 ng/μL MG in 50:50 water/methanol) at a rate of 10 μL/min using a syringe pump into a stream of 700 μL/min 63:37 0.1% formic acid/ACN via a T fitting. Typical MS parameters for ND-APCI were determined to be as follows: corona discharge, 0 μA; vaporizer temperature, 400 °C; capillary temperature, 220 °C; capillary voltage, 40 V; sheath gas, 70; and auxiliary gas, 40. The number of prescans was set to two, and the maximum inject time was set to 500 ms for MS^2 scans. The MS acquisition programs consisted of a MS^2 scan of *m*/*z* 329 with an isolation width of 2 amu, a relative collision energy = 48 or 50%, an activation $Q = 0.25$ (radio frequency parameter), an activation time = 30 ms, and a mass range = *m*/*z* 150−350.

The LC program was isocratic (63:37 0.1% formic acid/ACN) for the first 10 min, followed by a quick gradient to 100% ACN from 10 to 10.5 min, a column wash of 100% ACN from 10.5 to 12 min, a ramp back to 63:37 0.1% formic acid/ACN from 12 to 12.5 min, and equilibration at that composition for 2.5 min. The column oven was maintained at 30 °C. The mobile phase flow was 700 μL/min. Ten microliter injections were made with a needle wash of water or methanol. The divert valve was switched to the MS at 1 min and to waste at 9.8 min.

Residue Confirmation and Quantification by ND-APCI LC-MS^n. For qualitative assessment, individual ion transition chromatograms (*m*/*z* 329, 313−315, 284−286, 251, and 208) were generated and the resulting chromatographic peaks were integrated. Relative abundances were calculated from these peak areas and compared to contemporary standards. For quantitative assessment, the area counts of the MG peak from the total ion chromatogram of the *m*/*z* 329 product ion scan, not the extracted ion chromatograms, were used. A calibration curve was generated from the total ion current chromatogram with the same standards as were used for the LC-VIS analyses.

Sample Preparation. Thawed fish fillets were cut into 3−5 cm cubes and placed in a freezer (−20 to −30 °C) until use. Trout and salmon tissue were processed with skin intact (scales removed). Shells were removed from shrimp before processing. 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. In this study, aquacultured fillets of fresh rainbow trout, tilapia, and Atlantic salmon and imported frozen basa fillets and headless tiger shrimp were purchased at a local market. Frozen channel catfish fillets were provided from the FDA Gulf Coast Seafood Laboratory. To generate validation data, 5.0 g portions of thawed tissue homogenate were fortified by spiking with 50, 100, or 200 μL of LMG intermediate solutions to produce samples containing 1, 2, 4, or 10 ng/g of LMG. Samples fortified with MG at 2 ng/g were generated by spiking 5.0 g of tissue with 100 μL of the MG intermediate solution II. Samples were allowed to sit at room temperature for at least 15 min before proceeding with extraction.

Incurred Tissues. One of each live catfish, trout, tilapia, and salmon fish was placed into individual water baths containing 10 μg/L (ppb) MG for 1 h. The fish were then returned to clean water and sampled 16−24 h after MG exposure. One of each species of unexposed control fish was also sampled. Fish were filleted (skin was left intact on the trout and salmon), frozen, and blended with dry ice according to sample preparation method above.

Extraction Procedure. The extraction procedure was a modification of that reported by Andersen et al. (*17*). Fish tissue composite (5.0 g) was weighed into a 50 mL polypropylene centrifuge tube and allowed to thaw. The sample was vortex mixed for 30 s with ammonium acetate buffer (5 mL), HAH solution (1 mL, 0.25 g/mL), and *p*-TSA solution (100 μL, 1 M). Acetonitrile (25 mL) was added, and the sample was

shaken vigorously for 30 s. The sample was then defatted by adding 10 g of alumina, vigorously shaking for an additional 15 s, and then centrifuging for 5 min at 4000 rpm (2730 rcf) at 0 °C. The supernatant was decanted into a 250 mL separatory funnel containing water (50 mL) and diethylene glycol (2 mL). Solids were re-extracted with an additional 25 mL of acetonitrile, vortex mixed for 30 s, shaken vigorously for 30 s, and then centrifuged for 5 min. The supernatant was combined with the first extract in the separatory funnel. Dichloromethane (25 mL) was added, and after inversion to release pressure, the sample was liquid−liquid extracted for 30 s. Phases were allowed to separate for a maximum of 10 min (salmon may require up to 15 min to separate) (*17*). The lower dichloromethane layer was collected into a 150 mL glass pear-shaped boiling flask. The aqueous phase was re-extracted with an additional 25 mL of dichloromethane, and the organic phase was combined with the first extract after 10 min of phase separation. The extract was evaporated to dryness under reduced pressure while heating the flask in a water bath set at 50 °C. Acetonitrile (3 mL) was added to the oily residue, and the flask was swirled to dissolve the residue. At this point, the sample could be stoppered and stored overnight at room temperature and protected from light. DDQ solution (3 mL, 0.001 M) was added to flask, and the oxidation reaction was allowed to proceed for 30 min with periodic sample agitation. The oxidized sample was applied to an alumina SPE cartridge (conditioned with 5 mL of methanol and 5 mL of acetonitrile) that was positioned above a PRS SPE cartridge (conditioned with 5 mL of methanol and 5 mL of acetonitrile) and having a 5 mL reservoir of acetonitrile. The sample was eluted under vacuum with a flow rate of approximately 4 mL/min. The boiling flask was rinsed twice with acetonitrile (5 mL each), and these rinsings were sequentially applied to the alumina cartridge. After all of the solvent had eluted, the alumina cartridge was removed and discarded and the PRS cartridge was washed with acetonitrile (5 mL) and partially dried for 2−3 s under vacuum. The PRS cartridge was eluted by gravity into a 15 mL graduated centrifuge tube with 4 mL of mobile phase A. The eluate was diluted to 5.0 mL with mobile phase A and transferred to a chromatographic vial for analysis by LC-VIS. An additional portion of the sample was transferred to a second vial for simultaneous confirmation by ND-APCI LC-MS^n or stored in the refrigerator (ca. 4 °C) for several days for later residue confirmation.

RESULTS AND DISCUSSION

The extraction method presented herein is a modification of one recently validated for LMG and MG residue determination in salmon (*17*). Two changes were made to the original salmon method to ensure high residue recovery of LMG and MG in catfish. Most importantly, the quantity of alumina used to defat the tissue in the initial extraction was increased from 6 to 10 g. When 6 g of alumina was used for the extraction of LMG residues in catfish, recoveries of 10 4 ng/g spikes ranged from only 33 to 59%. Recoveries were dramatically higher when 10 g of alumina was used in the extraction (**Table 1**). The use of alumina is necessary to ensure high recoveries of MG from fatty fish tissues (*17*). In the second method change, the phase separation time allowed for liquid−liquid extraction was reduced from 15 to 10 min. Low recoveries were observed for catfish with long phase separation times. The lower fat content in the samples resulting from the increase in alumina appeared to better de-emulsify the extracts. Catfish, trout, tilapia, basa, and shrimp samples all separated within a few minutes during the liquid−liquid extraction, making separation times longer than 10 min unnecessary. In the current study, 15 fortified salmon samples were extracted using 10 g of alumina with an average recovery of 92.7% (10.0% RSD) (**Table 1**). These results are comparable to the 95.4% average recovery (11.1% RSD, $n = 35$) obtained from the earlier study (*17*). In addition, 10 incurred salmon samples were extracted using either 6 or 10 g of alumina. The five samples extracted with 6 g of alumina had an average combined MG/LMG concentration of 29.7 ng/g (6.4% RSD), as compared with the five 10 g alumina samples, which had an average combined MG/LMG concentration of 26.4 ng/g (3.2% RSD). Slight differences between these numbers are likely attributable to the analyses occurring 5 weeks apart. These results indicate that the current method can be used for the analysis of catfish, trout, tilapia, basa, shrimp, and salmon. Moreover, salmon extracted with the increased quantity of alumina did not have the previously seen problems either with emulsions (organic and aqueous phases separated within 10 min) or with lower recoveries associated with color changes during DDQ oxidation (*17*). In unpublished work, the method also was successfully used for the extraction of LMG in eel and broadhead clarias fish.

Table 1. Recovery and Confirmation of MG in LMG-Fortified Fish and Shrimp

LMG concn (ng/g)	LC-VIS average MG recovery (RSD, %)	no. of samples by LC-VIS	LC-MS^n confirmed/ analyzed	LC-MS^n recovery (RSD, %)
		catfish		
1	82.4 (6.8)	10	5/5	92.2 (12.8)
2	89.1 (7.2)	22	7/7	97.6 (9.9)
4	82.2 (5.1)	5	5/5	102.4 (4.0)
10	84.2 (11.1)	10	5/5	98.0 (17.3)
		trout		
1	87.7 (6.2)	5	5/5	88.5 (4.2)
2	89.1 (5.0)	17	7/7	83.4 (13.3)
4	86.7 (5.8)	5	5/5	81.6 (11.1)
10	84.9 (2.0)	5	5/5	86.0 (5.8)
		tilapia		
1	102.6 (5.2)	10	10/10	82.3 (15.4)
2	94.1 (6.8)	17	12/12	94.0 (14.6)
4	84.3 (3.4)	5	3/3	75.0 (11.8)
10	85.4 (0.6)	5	3/3	78.7 (21.4)
		basa		
1	91.5 (6.6)	5	5/5	88.6 (12.4)
2	76.1 (2.5)	5	5/5	88.2 (6.2)
4	98.5 (5.8)	5	5/5	109.4 (6.3)
10	95.9 (6.3)	6	6/6	111.9 (6.6)
		salmon		
2	95.1 (10.9)	7	2/2	115.9 (10.7)
4	91.2 (9.8)	6	2/2	83.1 (5.4)
10	88.6 (8.5)	2	2/2	75.5 (11.2)
		shrimp		
1	95.5 (8.6)	8	10/10	80.4 (20.8)
2	91.4 (5.2)	15	10/10	95.3 (8.2)
4	87.1 (1.2)	5	3/3	96.1 (6.8)
10	79.4 (8.0)	5	3/3	90.7 (5.4)

Residue Quantification by LC-VIS. The average recoveries of MG from fish and shrimp samples fortified with LMG are shown in **Table 1**. Overall recoveries were 85.9% (8.5% RSD, $n = 47$) for catfish, 87.8% (5.1% RSD, $n = 32$) for rainbow trout, 93.9% (8.9% RSD, $n = 37$) for tilapia, 90.8% (11.0% RSD, $n = 21$) for basa, 92.7% (10.0% RSD, $n = 15$) for salmon, and 89.9% (8.4% RSD, $n = 33$) for shrimp. Correlation coefficients (r^2) for the calibration standards were greater than 0.995. Typical chromatograms are shown in **Figure 1** for a 2 ng/g MG standard, catfish fortified with 2 ng/g LMG, and control fish. The aquacultured salmon and trout samples typically had some background peaks near the retention time of MG in the chromatogram (*17*); however, catfish, tilapia, basa, and shrimp were free from interference.

MG was not detected in any of two reagent blanks, nine control tilapia samples, eight control shrimp samples, or four control salmon samples. An interference peak was found in one of 11 control catfish samples and in one of eight control trout samples, but the interference peaks were less than three times

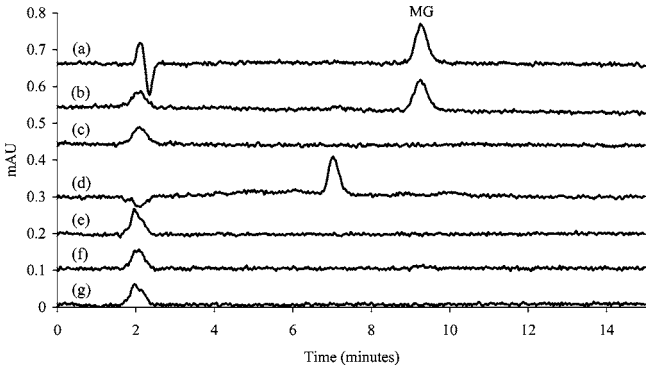

Figure 1. LC-VIS chromatograms: a, 2 ppb MG standard; b, 2 ppb LMG spike (recovered as MG) in catfish; c, control catfish; d, control trout; e, control tilapia; f, control basa; and g, control shrimp.

Table 2. LC-VIS Day to Day Reproducibility of LMG Spikes

	recovery of 2 ng/g spikes (%)						
	SP1	SP2	SP3	SP4	SP5	average (%)	RSD (%)
				catfish			
day 1	83.3	94.3	94.3	99.7	95.6	93.4	6.5
day 2	86.0	87.2	84.9	83.0	86.5	85.5	1.9
day 3	92.3	86.2	86.8	80.3	90.7	87.3	5.3
				trout			
day 1	85.5	87.0	78.1	88.9	91.5	86.2	5.9
day 2	90.1	85.5	90.9	88.5	89.1	88.8	2.3
day 3	86.8	90.2	87.7	88.4	90.7	88.8	1.9
				tilapia			
day 1	91.3	89.9	83.4	95.0	83.6	88.6	5.7
day 2	107.0	99.1	98.5	99.5	94.6	99.7	4.5
day 3	100.2	96.2	95.8	95.2	95.8	96.6	2.1
				shrimp			
day 1	95.3	88.4	85.1	86.9	88.5	88.8	4.4
day 2	91.7	103.9	97.4	93.8	88.5	95.1	6.2
day 3	87.9	88.5	92.0	91.5	91.2	90.2	2.1

the noise and could not be quantified by the LC-VIS method. MG was found in all of 10 control basa samples at a level that was below the detection limit of the LC-VIS method. By LC-MS^n, the average background level of MG found in the basa samples was 0.30 ng/g. This quantity was subtracted from all basa recovery data presented in **Table 1**. In an attempt to eliminate this source of background contamination, nearly 30 basa samples obtained from retail and other sources were tested during the course of this study; however, a sample was not found that did not have a background level of MG. As in the full salmon validation (*17*), the LC-VIS method detection limit was designated as 1 ng/g in accordance with the lowest calibration and spike levels. The average signal-to-noise ratio for 38 1 ng/g spikes in fish was 3.8. The day to day reproducibility of the method was measured by extracting five fish samples fortified with 2 ng/g of LMG on each of 3 days, as shown in **Table 2**. The recoveries for these interday samples varied by less than 7% (% RSD, $n = 15$) for each of the species studied.

The emphasis of the validation study was to determine LMG residues since this metabolite is expected to be the major compound found in tissue (*1*). Several MG spikes were also included in the study to determine how the extraction and DDQ oxidation might affect residual MG that may also be present in the tissue. Five samples each of catfish, trout, tilapia, and shrimp were fortified at a concentration of 2 ng/g MG, extracted, and analyzed. Average recoveries of MG were 64.4 ± 5.7% for catfish, 78.1 ± 10.8% for trout, 77.9 ± 3.4% for tilapia, and 66.9 ± 5.2% for shrimp. Previous studies also found satisfactory yet lower recoveries for MG as compared to LMG (*17*, *18*, *22*).

Residue Confirmation by ND-APCI LC-MS^n. This LC-MS^n method was developed to provide confirmation of the analysis of LMG residues. To achieve the most efficient use of laboratory resources, the same extracts prepared for the LC-VIS method were used for LC-MS analysis. Most sample extracts were refrigerated and analyzed within 5 days of the extraction. Some refrigerated samples were analyzed 15−60 days after the extraction. MG residues were confirmed in all cases. Storage of the sample extracts in the freezer (−20 to −30 °C) resulted in rapid sample degradation and is not recommended.

MG is a charged (not protonated) species in solution with a molecular ion at *m*/*z* 329. ND-APCI has been shown to be a very sensitive and selective technique for the analysis of MG (*12*, *13*, *18*, *23*). The use of ion trap detection allows for full collection of product scan data, further increasing the analytical selectivity of the method. The product ions include *m*/*z* 314 ($M^+ - CH_3$), *m*/*z* 313 ($M^+ - H - CH_3$), *m*/*z* 285 ($M^+ - NC_2H_6$), *m*/*z* 251 ($M^+ - C_6H_6$), *m*/*z* 237 ($M^+ - C_6H_5 - CH_3$), and *m*/*z* 208 ($M^+ - C_6H_5NC_2H_6$). High collision energy was needed to obtain significant abundance of these ions. The amount of MG that could be detected and yield an adequate product ion spectrum was less than 1 pg. For an animal drug residue to be positively confirmed, several criteria must be met; FDA guidelines for these criteria have been published (*24*). The retention time must match (within 5%) that of a standard. When compared to an MG standard, the product ion spectra must be visually similar with a minimum of unexplained background ions, and the relevant product ions should have similar relative abundances. To determine these ratios, extracted ion chromatograms were generated for *m*/*z* 329, *m*/*z* 313−315, *m*/*z* 284−286, *m*/*z* 251, and *m*/*z* 208, the peaks were integrated, and the areas obtained were compared to that for the largest ion in the product spectrum (*m*/*z* 329). Examples of the of MS^2 total ion chromatograms obtained from the analyses of tilapia samples (extracts from control, 2 ng/g fortified, and incurred fish) are shown in **Figure 2**. The extracted ion chromatograms and MS^2 spectrum from a sample of retail basa contaminated with MG are illustrated in **Figure 3**.

Table 3 consists of retention time and relative abundance data that were obtained for catfish, tilapia, and shrimp. These results are representative of the data collected for the other species. For any one day's analysis, the variance of the retention times and relative abundances is much less than what is required by the confirmation criteria. Chromatographic retention and relative abundances of the product ions can vary somewhat over time. At one point during the study, the pressure in the ion trap increased, which changed the relative abundances of the product ions. Lowering the collision energy slightly to a value of 48% for these analyses kept the ratios generally consistent with the rest of the validation data. For brevity, the qualitative results are presented as averages and standard deviations for each type of sample analyzed in a day. It is important to note, however, that each individual sample was evaluated to determine if confirmation criteria were met.

MG was confirmed in all samples fortified with LMG over the 1−10 ng/g range and in all extracts from fish that had been dosed with MG. While MG was not confirmed in any of the salmon control tissue extracts analyzed previously (*18*), the residue was found at very low levels (<0.25 ng/g) in a small percentage of the control extracts generated for this validation study. MG was confirmed in two of the six control catfish

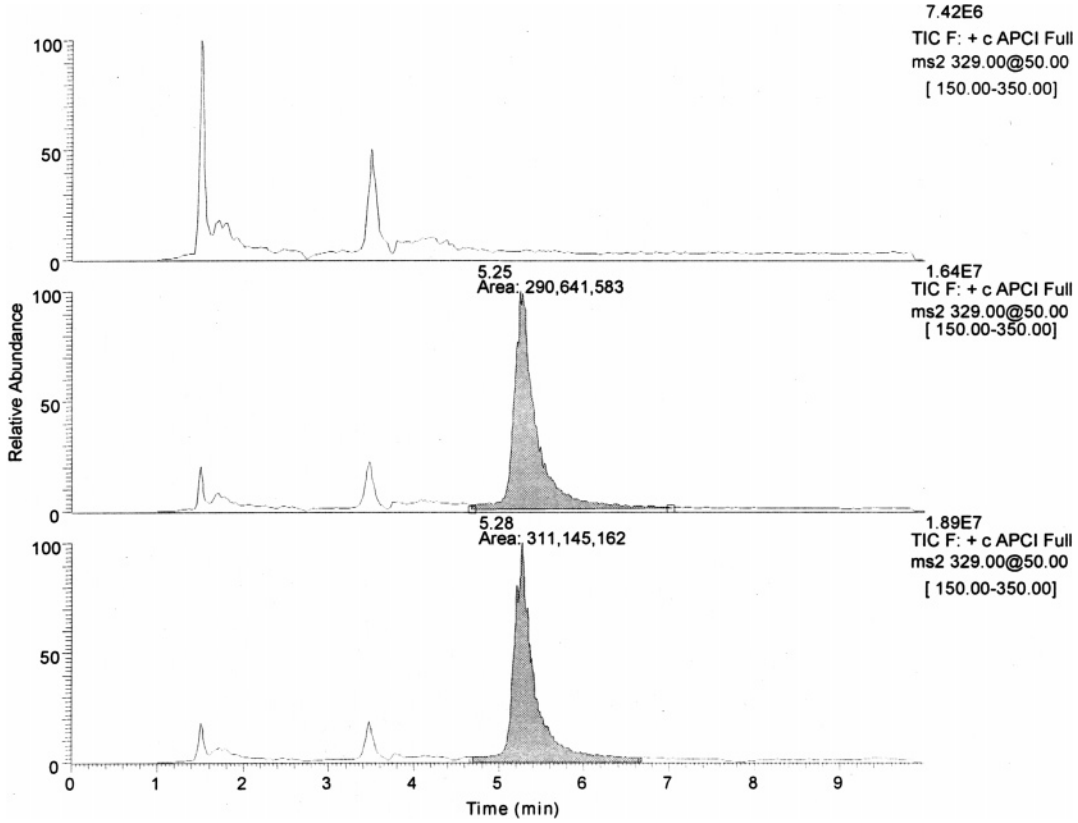

Figure 2. LC-MS^n total ion current chromatograms from MS^2 of *m*/*z* 329. Comparison of tilapia control (top), a 2 ng/g spike of the same tilapia tissue (middle), and tilapia that had been dosed with MG (bottom). MG elutes at approximately 5.3 min.

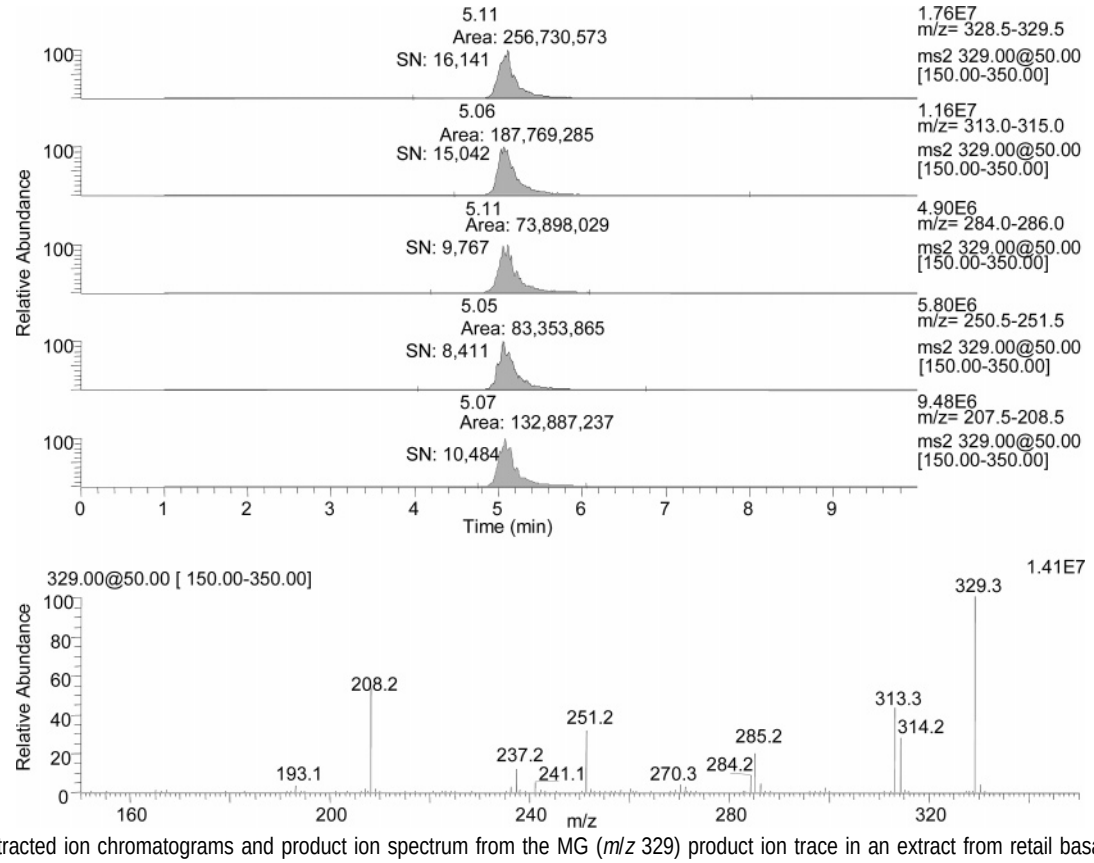

Figure 3. Extracted ion chromatograms and product ion spectrum from the MG (*m*/*z* 329) product ion trace in an extract from retail basa (diluted 1:5). Extracted ion ranges (from top to bottom): *m*/*z* 329, *m*/*z* 313–315, *m*/*z* 284–286, *m*/*z* 251, and *m*/*z* 208.

extracts, one of nine tilapia samples, and one of eight shrimp extracts. MG was not found in any of the trout or salmon control tissues. Although MG was detected in one of these catfish samples by LC-VIS, the concentration was below the detection limit of that method. MG was not present in any of the solvent blanks (injected between standards and fish extracts in every

Table 3. LC-MS^n Relative Abundance Data

			% relative abundance				
sample type	N	retention time	m/z 329	m/z 313–315	m/z 284–286	m/z 251	m/z 208
			catfish				
standards day 1[a]	8	5.21 ± 0.05	100	52 ± 5	20 ± 1	22 ± 2	37 ± 3
1 ng/g fortified	5	5.24 ± 0.02	100	56 ± 6	21 ± 2	23 ± 2	38 ± 4
2 ng/g fortified	5	5.25 ± 0.02	100	55 ± 5	21 ± 2	22 ± 1	37 ± 3
10 ng/g fortified	5	5.23 ± 0.03	100	56 ± 6	21 ± 2	23 ± 2	39 ± 4
standards day 2	7	5.13 ± 0.03	100	79 ± 8	33 ± 3	35 ± 4	56 ± 5
4 ng/g fortified	5	5.12 ± 0.03	100	73 ± 5	29 ± 2	32 ± 3	51 ± 5
standards day 3	7	5.30 ± 0.06	100	52 ± 6	23 ± 3	24 ± 2	38 ± 5
incurred	5	5.25 ± 0.03	100	52 ± 4	21 ± 1	23 ± 2	36 ± 2
2 ng/g fortified	2	5.25 ± 0.01	100	54 ± 6	21 ± 1	23 ± 1	37 ± 2
			tilapia				
standards day 1	8	5.30 ± 0.03	100	53 ± 3	22 ± 2	21 ± 8	38 ± 4
2 ng/g fortified	10	5.31 ± 0.03	100	50 ± 4	21 ± 2	23 ± 2	36 ± 3
standards day 2	8	5.33 ± 0.03	100	55 ± 7	22 ± 3	24 ± 3	38 ± 4
1 ng/g fortified	5	5.35 ± 0.02	100	57 ± 3	24 ± 2	25 ± 2	40 ± 2
4 ng/g fortified	3	5.33 ± 0.01	100	55 ± 7	23 ± 2	24 ± 4	38 ± 5
10 ng/g fortified	3	5.31 ± 0.02	100	61 ± 6	24 ± 1	27 ± 4	43 ± 4
standards day 3	7	5.30 ± 0.02	100	45 ± 5	18 ± 1	21 ± 3	32 ± 3
incurred	5	5.30 ± 0.02	100	46 ± 4	20 ± 2	21 ± 2	31 ± 6
2 ng/g fortified	2	5.29 ± 0.05	100	44 ± 2	19 ± 2	28 ± 1	31 ± 1
standards day 4[a]	8	5.19 ± 0.05	100	58 ± 4	22 ± 2	23 ± 2	40 ± 3
1 ng/g fortified	5	5.26 ± 0.02	100	59 ± 6	22 ± 3	24 ± 3	41 ± 4
			shrimp				
standards day 1	8	5.34 ± 0.04	100	70 ± 5	29 ± 4	31 ± 3	49 ± 5
1 ng/g fortified	5	5.33 ± 0.03	100	68 ± 3	28 ± 4	29 ± 5	49 ± 2
4 ng/g fortified	3	5.32 ± 0.01	100	68 ± 11	28 ± 5	31 ± 4	46 ± 6
10 ng/g fortified	3	5.34 ± 0.03	100	69 ± 6	29 ± 3	29 ± 4	50 ± 5
standards day 2	8	5.29 ± 0.03	100	62 ± 6	25 ± 2	28 ± 3	42 ± 4
2 ng/g fortified	10	5.26 ± 0.03	100	62 ± 6	26 ± 3	28 ± 3	46 ± 4
standards day 3[a]	8	5.19 ± 0.05	100	58 ± 4	22 ± 2	23 ± 2	40 ± 3
1 ng/g fortified	5	5.26 ± 0.02	100	59 ± 6	22 ± 3	24 ± 3	41 ± 4

[a] On these days, the relative collision energy = 48%.

LC-MS^n sequence), indicating that instrument carryover was not an issue. Also, MG was not found in any method reagent blanks tested. As with the LC-VIS analyses, MG was detected by LC-MS^n in all extracts of basa purchased at the local market. MG was confirmed in nine of 10 of these basa extracts and detected, but not confirmed, in the tenth sample.

Residue Quantification by ND-APCI LC-MS^n. Although LC-VIS was selected as the routine method for residue screening and quantification in this study, both LC-VIS and LC-MS^n with ND-APCI methods provided comparable quantitative results, as shown in **Table 1**. The percent recovery was determined by measuring the amount of MG in the sample (peak area from the total ion chromatograms) and comparing this amount to a calibration curve generated using standards in solvent. The standard curves were linear ($r^2 > 0.995$) in this range of 1−20 ng/g. For samples with low quantities of MG (1 ng/g or lower), more accurate quantification was obtained by using a limited calibration range, with the 20 ng/g standard excluded. The recoveries by LC-MS^n at all fortification levels ranged from 75 to 116%, with relative standard deviations of 21% or less. With a few exceptions, the two detection methods give comparable results with average recoveries equivalent within the margin of error (RSD) for each method. The precision for the LC-VIS determination was better than for the LC-MS^n method, particularly at the 1 ng/g fortification level. However, these results indicate that LC-MS^n is an acceptable alternative quantification method offering advantages in cases where the determination of MG residues at levels lower than the 1 ng/g LC-VIS detection limit is required. For example, in salmon samples fortified with LMG at 0.25 ng/g, MG ions were previously detected and confirmed by LC-MS^n with a recovery of 70% and an RSD of 12%; MG was detected but not confirmed in salmon fortified with LMG at 0.1 ng/g (*18*). In the current study, the LC-MS^n method was used to estimate the low amount of MG residues in the retail basa, which was used as a "control" tissue. It was found that this fish contained an average of 0.30 ng/g of total LMG/MG residue ($n = 9$ samples extracted and analyzed over 5 days, RSD = 30%). This quantity was subtracted from the calculated amount of residue found by both LC-VIS and LC-MS^n in fortified samples.

Table 4. Total LMG and MG Residues Recovered from Incurred Fish ($n = 5$ Replicates)

incurred fish	depuration time (h)	LMG/MG found by LC-VIS (ng/g) (%RSD)	LMG/MG found by LC-MS^n (ng/g) (%RSD)
catfish	16	32.2 (6.8)	31.3 (8.7)
tilapia	16.25	1.9 (7.2)	2.1 (14.2)
trout	16.5	27.1 (5.2)	28.6 (3.8)
salmon	24	26.4 (3.2)	27.4 (7.3)
basa	*a*	64.3 (5.8)	64.7 (11.3)

[a] Positive retail sample found to contain MG.

Incurred Tissues. Catfish, tilapia, trout, and salmon exposed to 10 μg/L MG in water for 1 h were extracted after depuration times of 16, 16.25, 16.5, and 24 h, respectively. The average ($n = 5$) sum of MG and LMG residues found in the tissues by both LC-VIS and LC-MS^n is shown in **Table 4**. The concentration of residue found in each species by the two different methods varied by less than 10%. Higher concentration tissue extracts were appropriately diluted with mobile phase A to conform to the 1−20 ng/g calibration range. The calculated concentration of these diluted (1:2) incurred samples was within 1−4% of that of the concentrated extracts, demonstrating the linearity of the method. Five replicates of a commercially obtained sample of basa that tested positive for MG were also analyzed. An average concentration of 64 ng/g of LMG/MG residues was found in this "incurred" basa sample by both LC-VIS and LC-MS^n (**Table 4**).

Conclusion. Two methods have been presented to determine the sum of LMG and MG residues in fish and shrimp with method detection limits of at least 1.0 ng/g (ppb). Both methods rely on the in situ conversion of LMG to MG using the oxidizing agent DDQ. LMG residues in catfish, trout, tilapia, basa, salmon, and shrimp were validated over the concentration range of 1.0−10.0 ng/g, with overall recoveries (as MG) of 86−94% with RSDs of 11% or less. ND-APCI LC-MS^n was used for both residue confirmation and as an alternate quantitation method with a method detection limit of at least 0.25 ng/g.

ACKNOWLEDGMENT

We gratefully thank Renate Reimschuessel and Charles Gieseker from the U.S. Food and Drug Administration Center for Veterinary Medicine for providing the incurred fish tissues used in this study and Steven Plakas of the U.S. Food and Drug Administration Gulf Coast Seafood Laboratory for providing the catfish.

LITERATURE CITED

(1) Plakas, S. M.; Doerge, D. R.; Turnipseed, S. B. Disposition and metabolism of malachite green and other therapeutic dyes in fish. In *Xenobiotics in Fish*; Beconi-Barker, M., Gingerich, W. H., Smith, D. J., Eds.; Plenum Press: New York, 1999; pp 149−166.

(2) Culp, S. J.; Beland, F. A. Malachite green: A toxicological review. *J. Am. Coll. Toxicol.* **1996**, *15*, 219−238.
(3) Culp, S. J.; Blankenship, L. R.; Kusewitt, D. F.; Doerge, D. R.; Mulligan, L. T.; Beland, F. A. Toxicity and metabolism of malachite green and leucomalachite green during short-term feeding to Fischer 344 rats and B6C3F1 mice. *Chem.-Biol. Interact.* **1999**, *122*, 153−170.
(4) Mittelstaedt, R. A.; Mei, N.; Webb, P. J.; Shaddock, J. G.; Dobrovolsky, V. N.; McGarrity, L. J.; Morris, S. M.; Chen, T.; Beland, F. A.; Greenlees, K. J.; Heflich, R. H. Genotoxicity of malachite green and leucomalachite green in female Big Blue B6C3F1 mice. *Mutat. Res.* **2004**, *561*, 127−138.
(5) Veterinary Residues Committee. Annual Report on Surveillance for Veterinary Residues in Food in the UK for 2001, 2002, and 2003, available at http://www.vet-residues-committee.gov.uk/.
(6) Commission Decision 2004/25/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. *Off. J. Eur. Union* **2004**, *L6*, 38−39.
(7) Bergwerff, A. A.; Scherpenisse, P. Determination of residues of malachite green in aquatic animals. *J. Chromatogr. B* **2003**, *788*, 351−359.
(8) Brandt, A.-M.; Laerke, S. Determination of malachite green and leucomalachite green by HPLC with postcolumn oxidation. Proceedings of the EuroResidue V Conference, Noordwijkerhout, The Netherlands, 2004; pp 358−362.
(9) Plakas, S. M.; El Said, K. R.; Stehly, G. R.; Roybal, J. E. Optimization of a liquid chromatographic method for the determination of malachite green and its metabolites in fish tissues. *J. AOAC Int.* **1995**, *78*, 1388−1394.
(10) Tarbin, J. A.; Barnes, K. A.; Bygrave, J.; Farrington, W. H. H. Screening and confirmation of triphenylmethane dyes and their leuco metabolites in trout muscle using HPLC-vis and ESP-LC-MS. *Analyst* **1998**, *123*, 2567−2571.
(11) Scherpenisse, P.; Bergwerff, A. A. Determination of residues of malachite green in finfish by liquid chromatography tandem mass spectrometry. *Anal. Chim. Acta* **2005**, *529*, 173−177.
(12) Valle, L.; Diaz, C.; Zanocco, A. L.; Richter, P. Determination of the sum of malachite green and leucomalachite green in salmon muscle by liquid chromatography-atmospheric pressure chemical ionisation-mass spectrometry. *J. Chromatogr. A* **2005**, *1067*, 101−105.
(13) Doerge, D. R.; Churchwell, M. I.; Gehring, T. A.; Pu, Y. M.; Plakas, S. M. Analysis of malachite green and metabolites in fish using liquid chromatography atmospheric pressure chemical ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 1625−1634.
(14) Van Rhijn, J. A.; Mulder, P. P. J.; van Baardewijk, F.; te Brinke, E. M.; Lasaroms, J. J. P. Confirmatory analysis of traces of malachite green and its main metabolite leucomalachite green in muscle tissue of Atlantic salmon. Proceedings of the EuroResidue V Conference, Noordwijkerhout, The Netherlands, 2004; pp 808−813.
(15) Van de Riet, J. M.; Murphy, C. J.; Pearce, J. N.; Potter, R. A.; Burns, B. G. Determination of malachite green and leucomalachite green in a variety of aquacultured products by liquid chromatography with tandem mass spectrometry detection. *J. AOAC Int.* **2005**, *88*, 744−749.
(16) Mitrowska, K.; Posyniak, A.; Zmudzki, J. Determination of malachite green and leucomalachite green in carp muscle by liquid chromatography with visible and fluorescence detection. *J. Chromatogr. A* **2005**, *1089*, 187−192.
(17) Andersen, W. C.; Roybal, J. E.; Turnipseed, S. B. Liquid chromatographic determination of malachite green and leucomalachite green (LMG) residues in salmon with in situ LMG oxidation. *J. AOAC Int.* **2005**, *88*, 1292−1298.
(18) Turnipseed, S. B.; Andersen, W. C.; Roybal, J. E. Determination and confirmation of malachite green and leucomalachite green residues in salmon using liquid chromatography with no-discharge atmospheric pressure chemical ionization. *J. AOAC Int.* **2005**, *88*, 1312−1317.
(19) Van Rhijn, J. A.; Mulder, P. P. J.; van Baardewijk, F.; te Brinke, E. M.; Lasaroms, J. J. P. Confirmatory analysis of traces of malachite green and leucomalachite green in muscle tissue of Atlantic salmon. Poster presented at the EuroResidue V Conference on Residues of Veterinary Drugs in Food, Noordwijkerhout, The Netherlands, May 10−12, 2004.
(20) Cho, B. P.; Yang, T.; Blankenship, L. R.; Moody, J. D.; Churchwell, M.; Beland, F. A.; Culp, S. J. Synthesis and characterization of N-demethylated metabolites of malachite green and leucomalachite green. *Chem. Res. Toxicol.* **2003**, *16*, 285−294.
(21) Lewis, E. S.; Perry, J. M.; Grinstein, R. H. Rates and isotope effects in the quinone oxidation of leuco triphenylmethane dyes. *J. Am. Chem. Soc.* **1970**, *92*, 899−905.
(22) Roybal, J. E.; Pfenning, A. P.; Munns, R. K.; Holland, D. C.; Hurlbut, J. A.; Long, A. R. Determination of malachite green and its metabolite, leucomalachite green, in catfish (Ictalurus punctatus) tissue by liquid chromatography with visible detection. *J. AOAC Int.* **1995**, *78*, 453−457.
(23) Turnipseed, S. B.; Andersen, W. C.; Karbiwnyk, C. M.; Roybal, J. E.; Miller, K. E. No-discharge atmospheric pressure chemical ionization: Evaluation and application to the analysis of animal drug residues in complex matrices. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1231−1239.
(24) U.S. Food and Drug Administration. Guideline for industry: Mass spectrometry for confirmation of the identity of animal drug residues. *Fed. Regist.* **2003**, *68*, 25617−25618.

Received for review December 23, 2005. Revised manuscript received April 23, 2006. Accepted April 24, 2006.

JF0532258