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HPLC Determination and MS Confirmation of Malachite Green, Gentian Violet, and Their Leuco Metabolite Residues in Channel Catfish Muscle

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Residues of malachite green (MG), gentian violet (GV), and their leuco metabolites in channel catfish muscle were individually determined by HPLC using diode array and fluorescence detectors and confirmed by tandem mass spectrometry. This detection scheme obviates a PbO_2 reactor that converts leuco forms to chromatic forms for absorbance detection, therefore eliminating uncertainties in oxidant depletion and data integrity. Extraction was performed once in pH 3 McIlvaine buffer and acetonitrile, followed by cleanup using a polymeric strong cation-exchange column. Liquid−liquid extraction was excluded to provide an environmentally responsible and relatively rapid protocol. Spectrometric limits of detection (LOD; S/N = 3) for MG (λ = 620 nm) and GV (λ = 588 nm) were 0.38 and 0.26 ng/g with 44.5−49.2% and 92.2−101.4% recoveries (1−10 ng/g, n = 6), respectively. Fluorometric LOD (S/N = 3) for LMG and LGV (λ_{ex} = 266 nm, λ_{em} = 360 nm) were 0.10 and 0.09 ng/g with 74.3−84.5% and 80.6−86.5% recoveries (1−10 ng/g, n = 6), respectively. This simplified protocol saves costs and meets the sensitivity requirements set by the Food and Drug Administration and the European Union.

KEYWORDS: Malachite green; gentian violet; leucomalachite green; leucogentian violet; triphenylmethane; catfish; HPLC-MS

INTRODUCTION

Malachite green (MG) and gentian violet (GV) are triphenylmethane dyes (**Figure 1**) that have been used in aquaculture since 1936 as fungicides, ectoparasiticides, and antiseptics (*1*). Absorbed readily by fish, they are reduced rapidly and extensively by intestinal flora to leucomalachite green (LMG) and leucogentian violet (LGV) metabolites (*2*) that persist in catfish muscle with half-lives of about 10 days and even longer in fat and organs (*3*,*4*). Based on documented mutagenicity (*5*) and carcinogenicity (*6*) in mammals, MG and GV are banned in many countries for aquacultural use but, due to their low cost and high efficacy, may still be used illegally. So far, their safe levels in fish have not been established. In the European Union (EU), monitoring methodologies must meet the European minimum required performance limit (MRPL) at 2 ng/g for the sum of parent drugs and their leuco forms. In the U.S., the Food and Drug Administration (FDA) stipulates a minimum sensitivity of 1 ng/g for regulatory testing. Catfish aquaculture is growing rapidly in Asia and North America. Channel catfish, *Ictalurus punctatus*, is the leading species of the U.S. aquacultural output. In 2008, the U.S. Department of Agriculture (USDA) Food Safety Inspection Service' jurisdiction was extended by law to cultivated catfish.

To detect any banned drugs, the technical challenge is very low limits of detection (LODs). To achieve 1−2 ng/g LOD for the sum of parent drugs and their metabolites in a complex matrix such as fish muscle, meticulous sample preparation must be performed to isolate trace amount of target analytes. Typically, extraction must be performed once or multiple times, and cleanup must rely upon both liquid−liquid partitioning (LLP) and solid-phase extraction (SPE) (*7*−*9*). To achieve better cleanup and enrichment, more than one type of cartridge may be needed (*8*, *9*). Unfortunately, thorough cleanup usually consumes more chemicals, requires more steps, and hence lowers productivity. Furthermore, dichloromethane, the most common LLP solvent for triphenylmethane extraction, is harmful to human health. In this work, sample preparation was simplified at the cost of reduced but acceptable recoveries: extraction was performed only once, and cleanup was done using one SPE cartridge without LLP. The result is an environmentally responsible protocol that saves chemical and disposal costs and improves sample throughput.

The majority of current quantitative methods are based on high-performance liquid chromatography (HPLC). To take advantage of parent dyes' characteristic strong absorption in the orange-red region, many quantitative schemes relied upon prior oxidation to convert leuco forms back to chromatic forms. The oxidants used so far include PbO_2 (*10*−*12*), iodine (*13*), and dichlorodicyanobenzoquinone (*14*). Alternatively, oxidation can be carried out electrochemically (*15*). Mass spectrometric (MS) detection is mainly used for confirmation (*9*, *12*, *16*) but can also be developed as a quantitative scheme based on isotope dilution (*17*, *18*) or on a single MG peak which was confirmed by an earlier desmethyl-MG

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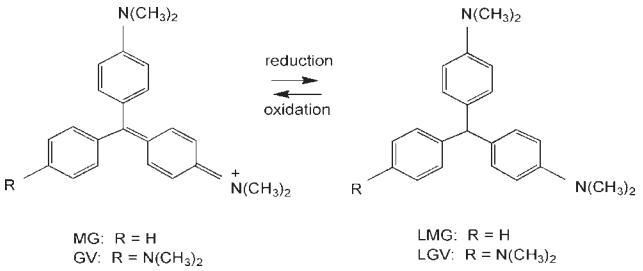

Figure 1. Structures of MG, GV, LMG, and LGV.

peak (*m*/*z* 313) (*19*). Oxidation was performed even with MS detection due to MG's improved detectability over LMG (*7*, *16*). An oxidation reactor inevitably causes band broadening and cross-contamination, and limited oxidant life causes concern on reaction completion and data integrity. Though band broadening can be ameliorated using a smaller reactor, shortened lifetime demands periodic quality check that compromises productivity (*19*). PbO_2 can further oxidize MG and GV to mono- and dimethylation products (*20*) and even solvents leading to interference peaks. To overcome such problems, Mitrowska et al. developed a simultaneous detection scheme for MG and LMG that rendered an oxidation reactor unnecessary (*20*). While MG was determined by visible spectrometry at 620 nm, LMG was independently determined by fluorometry at λ_{ex} = 265 nm and λ_{em} = 360 nm. This effective scheme was extended to GV and LGV in this work.

MATERIALS AND METHODS

Reagents. Research grade MG oxalate (97%) and GV (95%) were purchased from Crescent Chemical (Islandia, NY). LMG (99%) and LGV (99%), L-ascorbic acid (99+% reagent grade), and *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD) (95%) were purchased from Sigma-Aldrich (St. Louis, MO). Reagent grade sodium chloride, citric acid monohydrate, ammonium acetate, sodium acetate trihydrate, sodium phosphate, glacial acetic acid, and HPLC and spectrophotometric grade acetonitrile were from J. T. Baker Chemical (Phillipsburg, NJ). Neutral alumina (80−200 mesh, reagent grade) was from Fisher Scientific (Pittsburgh, PA). *p*-Toluenesulfonic acid monohydrate (*p*-TSA) (99%) was from ACROS Organics (Morris Plains, NJ). Reagent grade ammonium hydroxide (28−30% NH_3) was from GFS Chemicals (Powell, OH).

Standard and Reagent Solutions. *p*-TSA solution (1 M) was prepared in water; 1 mg/mL TMPD solution was prepared in methanol. McIlvaine buffer at pH 3.0 was prepared by mixing 18.9 mL of 0.2 M sodium hydrogen phosphate and 81.1 mL of 0.1 M citric acid. A 0.01 M acetate buffer was prepared by dissolving 0.771 g of ammonium acetate in ∼0.9 L of water, adjusting pH to 4.1 with acetic acid, and diluting with water to 1.0 L. SPE eluting solvent was freshly prepared before use by mixing 4.3 mL of 29% (m/v) ammonium hydroxide and 5.0 mL of 0.5 mg/mL L-ascorbic acid in 5:95 (v/v) methanol-acetonitrile, and filling to 100 mL with acetonitrile. The HPLC mobile phase consisted of a 20:80 (v/v) mixture of 0.01 M acetate buffer and acetonitrile. A Barnstead E-pure system (Dubuque, IA) was used to prepare deionized water for the above solutions.

Stock Solutions (*100 μg/mL*). MG, LMG, GV, and LGV (10.0 mg each) were weighed, with purity and molecular formula corrected, into individual 100 mL volumetric flasks, dissolved in acetonitrile, and filled to mark. These stock solutions were stable in amber bottles at 4 °C for up to 6 months.

Working Solution 1 (*1.0 μg/mL*). MG, LMG, GV, and LGV stock solutions, 1.0 mL each, were pipetted into a 100 mL volumetric flask, diluted to mark with acetonitrile, and mixed well. This combined working solution was prepared weekly.

Working Solution 2 (*0.1 μg/mL*). Working solution 1 (1.0 mL) was pipetted into a 10 mL volumetric flask, diluted to mark with acetonitrile, and mixed well. Preparation was also performed weekly.

Calibration Standards. A series was prepared daily by pipetting 0, 20, 50, 100, and 200 μL aliquots of working solution 2 and 30, 40, 50, 60, and 80 μL aliquots of working solution 1 into ten 5 mL volumetric flasks. Each was diluted to mark with 30:70 (v/v) acetate buffer−methanol and mixed thoroughly. Based on a 5 g sample weight, these solutions corresponded to 0, 0.4, 1, 2, 4, 6, 8, 10, 12, and 16 ng/g each of MG, LMG, GV, and LGV.

Extraction and Cleanup. Skinless fillets of channel catfish, *I. punctatus*, were purchased from local food stores, homogenized with a food processor, and immediately stored at −20 °C in polyethylene bags of suitable sizes. Portions of 5.00 ± 0.02 g of thawed muscle were weighed into 50 mL polypropylene centrifuge tubes; each was spiked at a desired level by adding a certain volume of a suitable working solution, vortex mixing for 30 s, and allowed to stand in the dark for 10 min. Extraction was carried out by adding 4 mL of pH 3 McIlvaine buffer, 100 μL of 1 M *p*-TSA, and 100 μL of 1 mg/mL TMPD and vortex mixing for 30 s. To each tube was added 25 mL of acetonitrile, followed by 1 min vortex mixing; then, 5 g of NaCl was added followed by 20 s vortex mixing. Finally, the tubes were centrifuged at 3176*g* for 5 min under 25 °C. Four layers formed from bottom up: excess salt, an aqueous layer, a solid pellet, and an analyte-laden organic layer.

Oasis MCX SPE columns (150 mg/6 mL) (Waters, Milford, MA) were installed on a vacuum manifold. On the top of MCX sorbent bed, roughly 2 g of neutral alumina was added to form a second bed. Two sequential 3−5 mL aliquots of acetonitrile were added quickly to this bed using a disposable transfer pipet. Quick action was necessary to agitate alumina particles to allow air bubbles to escape. Then, the combined sorbent beds were loaded with the organic supernatant (top layer) using a disposable transfer pipet, and the flow rate was adjusted to < 2 mL/min. Columns were then washed with 5 mL of acetonitrile at ∼1 mL/min. Next, each column was removed from the manifold and inverted into a waste beaker. The alumina sorbent was removed by tapping the column against the beaker rim or by introducing compressed air or nitrogen into the syringe tip; residual alumina particles were removed using an acetonitrile wash bottle. Finally, the analytes were eluted with 6 mL of freshly made SPE eluent into 15 mL screw-capped polypropylene tubes. The eluates were evaporated to dryness under N_2 in a 40 °C water bath; the residues were readily dissolved by vortex mixing in 1 mL of 30:70 (v/v) 0.01 M acetate buffer−methanol for HPLC analysis.

HPLC-DAD-Fluorescence. The LC system was an Agilent Model 1100 that consisted of a binary pump, a vacuum degasser, an automatic injector, a column oven, a G1325A diode-array detector (DAD), and a Jasco FP-1520 fluorescence detector (Jasco, Tokyo, Japan). The system operation and data processing were controlled by ChemStation software. A Prodigy ODS-3 C18 (3 μm, 150 mm × 4.6 mm) column (Phenomenex, Torrance, CA) was placed in a 30 °C oven and run isocratically at 1.0 mL/min flow rate. Injection volume was 100 μL. The DAD was set at 620 nm for MG or 588 nm for GV. Excitation and emission wavelengths of the fluorescence detector were set at 266 and 360 nm, respectively.

HPLC-MS/MS. For confirmation, a Prodigy ODS-3 C18 (3 μm, 100 mm × 2.0 mm) column (Phenomenex) was used with the same mobile phase at 0.4 mL/min flow rate. The detector was an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada) with an electrospray ionization (ESI) source. The MS operation and data processing were controlled by Analyst 1.5 software. The turbo-ion spray potential was set at +5000 V, and the source temperature was set at 525 °C. Samples (10.0 μL) were injected under 300 mbar with 0.1 min duration. The spectrometer was operated in the positive mode, and analyses were performed in select reaction monitoring (SRM) mode. Two transitions were monitored for each analyte as shown in **Table 1**, for both of which the dwell time was set at 50 ms.

RESULTS AND DISCUSSION

Extraction and Cleanup. Due to strong binding of triphenylmethane dyes to fish tissue, effective extraction must rely upon acidic media to denature proteins. As reported previously (*7*), a pH 3 McIlvaine buffer−acetonitrile mixture served this purpose well, which was aided by *p*-TSA, a strong yet nonoxidizing acid. TMPD was added to reduce analyte demethylation. To improve throughput, extraction was performed only once at the cost of recovery. NaCl was added to enhance analyte partitioning into acetonitrile based on salting-out effect; salts that generate excessive solvation heat should be avoided due to concern of analyte

Table 1. MS/MS Detector Conditions[a]

analyte	Q1	Q3	dwell time (ms)	DP (V)	FP (V)	CE (V)	CXP (V)
MG	329.3	313.2	50	41	140	49	10
	329.3	208.1	50	41	140	47	20
GV	372.3	356.3	50	31	125	51	12
	372.3	340.2	50	31	125	71	10
LMG	331.3	239.2	50	31	120	43	18
	331.3	316.2	50	31	120	29	10
LGV	374.3	358.3	50	36	140	41	12
	374.3	239.1	50	36	140	49	24

[a] DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision exit potential.

degradation. Salt provided an extra advantage in this case: the higher density of NaCl-saturated aqueous phase caused formation of a solid tissue layer during centrifugation that physically separated the top organic layer from the aqueous layer, making it very convenient to transfer the analyte-laden supernatant using a disposable transfer pipet.

Cleanup is crucial to achieve 1−2 ng/g sensitivity for these banned drugs in highly complex tissue matrices. The majority of current methods include meticulous cleanup that relied on both LLP and SPE (*7*−*9*, *12*, *19*−*21*). LLP is highly effective to exclude polar interfering components; unfortunately, dichloromethane, the most prominent solvent for extraction of MG, GV, and their leuco forms, is known as a health concern. LLP is notoriously tedious; any prolonged procedure inevitably led to degradation and loss of target analytes especially MG (*7*), the least stable entity among the four. To speed up sample preparation and make the method user-friendly, LLP was eliminated from the protocol, and a single SPE column was used for cleanup. Under such restraints, several sorbent types were tested and compared. C18 and Oasis hydrophilic−lipophilic balance (HLB) sorbents failed to retain polar parent drugs, leading to unsatisfactory recoveries. Due to very strong adsorption, activated carbon yielded low recovery as expected.

Multiple amine groups on these drugs, when protonated, should make retention possible on a strong cation-exchange sorbent (*22*). However, use of a silica-based strong cation-exchange column resulted in turbid eluates and a noisy background in the fluorescence chromatogram, likely due to the leaching and detection of benzenesulfonic acid residues released under alkaline elution conditions. Fortunately, when an Oasis MCX polymeric column was used, the eluates became clear, and the fluorescence background decreased dramatically. Synthesized from HLB copolymer, this sorbent is stable in a broader pH range than its silica counterpart. Furthermore, combination of HLB sorbent and an extra benzene ring renders the reversed-phase mechanism an important role in retaining these triphenylmethane dyes. However, when used alone as is, two problems were encountered: defatting especially of those polar lipids was compromised (*21*), and considerable loss of parent drugs was visibly observed during acetonitrile washing. This situation can be avoided by adding a polar alumina column (*8*, *9*, *21*). Extra cost aside, coupling two columns in tandem using an adaptor captures air in between, which may affect flow pattern and data consistency unless caution is exercised. In this work, a polar sorbent bed was formed of ∼2 g of neutral alumina above the MCX sorbent. This top bed selectively retained polar parent drugs during loading and then slowly released them during acetonitrile washing to the MCX sorbent bed below. In practice, only 5 mL of acetonitrile was used at a relatively fast flow rate (1 mL/min) to minimize analyte loss. This dual-bed approach significantly improved recoveries from ∼20% to ∼48% for MG and from ∼20% to ∼95% for GV (**Table 2**). The amount of alumina was not critical, but a bubble-free bed was a prerequisite for reproducible flow pattern and recovery; so, agitation must be introduced by fast action as described above.

Table 2. Recoveries of MG and GV (by HPLC-DAD) and LMG and LGV (by HPLC-Fluorescence) Spiked in Catfish Muscle at 1, 2, and 10 ng/g

		recovery (%)						average	RSD
analyte	ng/g	1	2	3	4	5	6	(%)	(%)
MG	10	43.5	42.9	46.7	42.9	48.2	42.7	44.5	5.3
	2	41.9	48.2	40.3	53.1	53.9	51.1	48.1	12.0
	1	43.3	51.1	54.5	47.5	50.2	48.7	49.2	7.6
GV	10	98.4	95.9	101.1	97.8	92.2	101.1	97.7	3.4
	2	85.7	93.4	94.4	91.2	95.1	93.4	92.2	3.7
	1	103.2	98.7	94.6	100.0	108.0	103.9	101.4	4.6
LMG	10	68.7	76.1	76.2	77.4	77.3	70.1	74.3	5.2
	2	75.7	83.7	75.6	82.9	85.0	85.9	81.5	5.7
	1	83.2	88.2	78.7	78.0	89.6	86.6	84.0	5.8
LGV	10	76.8	81.2	82.0	83.4	82.7	77.4	80.6	3.5
	2	81.7	88.3	81.1	86.9	92.6	84.8	85.9	5.0
	1	87.2	93.0	85.3	71.7	93.6	88.4	86.5	9.2

This sample preparation protocol was relatively robust, as evidenced by consistent recovery in the 1−10 ng/g range. Among four analytes, the most polar MG yielded the lowest recovery. Several machanisms have been suggested so far to account for this observation. First of all, MG binds to fish tissue more strongly than LMG, resulting in very slow extraction (*19*). Second, MG undergoes photooxidative demethylation during sample preparation as evidenced by decreasing recoveries with time and the lack of corresponding increase in LMG peak height (*7*). MG was also found to convert to LMG during evaporation (*18*). Finally, though irrelevant to this discussion, MG and GV also suffer worse matrix suppression effects than their leuco counterparts in mass spectrometry (*17*). Because of contrasting polarities between parents and their leuco forms, it is difficult to design an extraction-cleanup protocol that would work for all analytes with equal recovery efficiencies. One tailored to parent drugs usually sacrifices the recoveries of their leuco metabolites and *vice versa*. Because of extensive MG-to-LMG conversion by intestinal flora (*2*), leuco metabolites are the dominant residue forms in fish tissue. Consequently, it is commonly accepted that leuco metabolites should be the focus of monitoring programs (*3*, *4*), and their recoveries are far more relevant than those of parent drugs. Accordingly, emphasis was given to leuco forms in this extraction and cleanup protocol. Despite its relative simplicity that reduced assay time by about one-third from typical procedures, this protocol yielded excellent recoveries for GV and both leuco forms and a lower but acceptable recovery for MG. Ammoniated acetonitrile was chosen as eluent over ammoniated methanol due to better MG stability, in which ascorbic acid was added to minimize demethylation.

An important consideration in absorbance detection is the equilibrium between chromatic and carbinol forms. Because the former dominates (>99%) under acidic (<4) pH (*3*), the basic eluate must be acidified prior to chromatography by evaporating to dryness under nitrogen at 40 °C and dissolving the residues in 30:70 (v/v) pH 4.1 acetate buffer−methanol. During drying, the previously reported temperature ranged from ambient (*7*) to 60 °C (*18*). Beyond this range, analyte loss becomes a concern. Stability of MG and LMG solutions was studied previously: both are very stable in acetonitrile (*23*), but photooxidative demethylation occurs in aqueous media, especially for LMG at room temperature (*24*).

Quantitation by HPLC-DAD-Fluorescence. Detection of both MG and LMG in their native states obviated an oxidation reactor

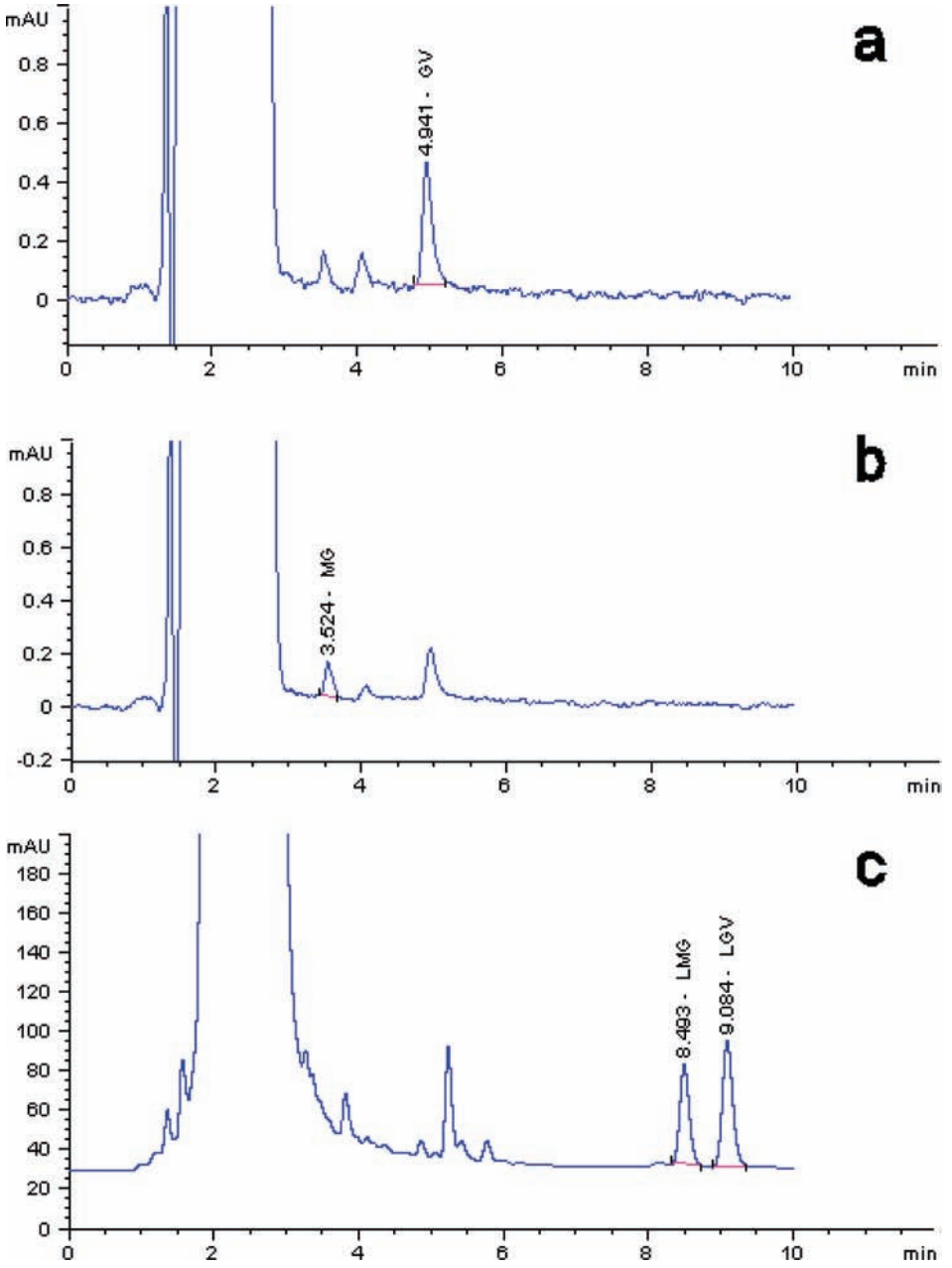

Figure 2. Chromatograms of MG, GV, LMG, and LGV in catfish muscle spiked at 1 ng/g. (**a**) DAD, λ = 588 nm; (**b**) DAD, λ = 620 nm; and (**c**) fluorescence detector, λ_{ex} = 266 nm and λ_{em} = 360 nm.

and all the related problems. As previously reported (*20*), simultaneous determination of MG and LMG could be accomplished using two spectrometric techniques: MG by absorption spectrometry based on a conjugated benzene ring system and LMG by fluorometry based on multiple unconjugated benzene rings. In this work, this simultaneous determination scheme was extended to GV and LGV, which, due to an additional dimethylamine group, behave somewhat differently from MG and LMG in extraction and cleanup precesses. Besides, GV's absorption peak shifts 27 nm to the blue (ε = 118400 M^{-1} cm^{-1} at 590 nm) relative to that of MG (ε = 137200 M^{-1} cm^{-1} at 617 nm) (*25*). DAD chromatograms of MG and GV are shown in **Figure 2a**,**b**, and a fluorescence chromatogram of LMG and LGV is shown in **Figure 2c**. Overall, satisfactory quantitation was achieved for all entities (**Table 3**): the resulting LODs were well below FDA's 1 ng/g required sensitivity and EU's 2 ng/g MRPL, and good linear response (r > 0.9990) was observed in the 0.5−16.0 ng/g range. Leuco metabolites had even better LODs (~0.1 ng/g) than their parent drugs. Structurally, three nonconjugated benzene rings make leuco forms good candidates for fluorescence detection which, unlike absorbance, is measured against a zero background leading to inherently higher sensitivity.

Table 3. Quantitative Performance of HPLC/Fluorescence/DAD on Spiked Samples

detector	analyte	r (0.5−16.0 ng/g)	LOD (ng/g, S/N = 3)
DAD	MG	0.9998	0.38
	GV	0.9994	0.26
fluorescence	LMG	0.9999	0.10
	LGV	0.9994	0.09

Confirmation by HPLC-MS/MS. Confirmation was based on matching retention times and product ion abundances between spiked samples and reagent standards. **Figure 3** shows an LC-MS/MS SRM chromatogram of MG, GV, LMG, and LGV in catfish muscle, each spiked at 1 ng/g. Two major transition ion pairs for

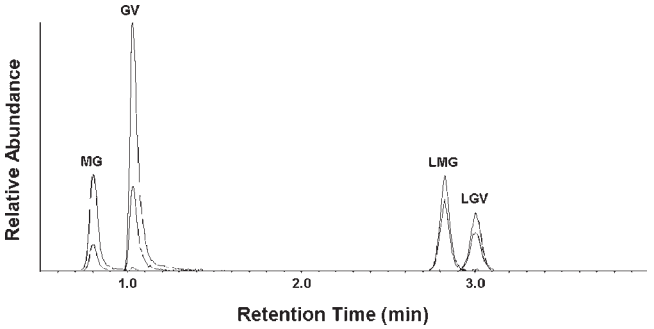

Figure 3. LC-MS/MS SRM chromatogram of MG, GV, LMG, and LGV in catfish muscle, each spiked at 1 ng/g. Two major transition ion pairs were monitored for each analyte, as presented in **Table 1**.

Table 4. Abundance Ratios of Two Transition Ion Pairs of MG, GV, LMG, and LGV in a Reference Standard vs Spiked Samples

		abundance ratio				
			spiked samples (n = 4)			
analyte	transition ion pairs (m/z)	ref std (4 ng/g)	ng/g	average	RSD (%)	difference (%)
MG	(329→208)/(329→313)	0.265	0.5	0.249	2.7	−5.9
			1	0.266	5.0	0.2
			2	0.254	2.2	−4.2
			4	0.261	5.1	−1.4
GV	(372→340)/(372→356)	0.357	0.5	0.346	4.4	−3.2
			1	0.334	5.1	−6.3
			2	0.340	0.9	−4.8
			4	0.340	1.9	−4.8
LMG	(331→316)/(331→239)	0.720	0.5	0.745	4.0	3.5
			1	0.784	1.0	8.9
			2	0.776	0.9	7.7
			4	0.772	0.3	7.2
LGV	(374→239)/(374→358)	0.716	0.5	0.673	8.3	−6.0
			1	0.685	5.1	−4.4
			2	0.651	4.2	−9.1
			4	0.665	2.9	−7.1

each analyte are presented in **Table 1** with certain operational parameters. In **Table 4**, the abundance ratios of two major transition ion pairs are calculated and compared between samples spiked at 0.5, 1, 2, or 4 ng/g (n = 4) and a reference standard (in solvents only) at 4 ng/g. Well-matched (within ±10%) ratios and retention times confirmed the identity of a specific target analyte. Quantitative performance of HPLC-ESI-MS/MS may be compromised by matrix effects (*26*), though exact mechanisms are still uncertain. This technique was, therefore, only employed for confirmation in this work. The clean background in **Figure 3** demonstrates the quality of sample preparation. The high S/N of major transition peaks of all four analytes enabled confirmation even at 0.5 ng/g, well below the detection limits needed by the FDA and the EU.

ABBREVIATIONS USED

ACS, American Chemical Society; CE, collision energy; CXP, collision exit potential; DAD, diode array detector; DP, declustering potential; EP, entrance potential; ESI, electrospray ionization; EU, European Union; FDA, Food and Drug Administration; GV, gentian violet; HLB, hydrophilic−lipophilic balance; HPLC, high-performance liquid chromatography; LGV, leucogentian violet; LLP, liquid−liquid partitioning; LMG, leucomalachite green; LOD, limit of detection; MG, malachite green; MRL, maximum residue limit; MRPL, minimum required performance limit; MS, mass spectrometry; RSD, relative standard deviation; SD, standard deviation; S/N, signal-to-noise ratio; SPE, solid-phase extraction; SRM, select reaction monitoring; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine dihydrochloride; TSA, toluenesulfonic acid; USDA, United States Department of Agriculture; UV, ultraviolet.

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