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Confirmation of malachite green, gentian violet and their leuco analogs in catfish and trout tissue by high-performance liquid chromatography utilizing electrochemistry with ultraviolet-visible diode array detection and fluorescence detection

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Abstract

A sensitive analytical procedure for the confirmation of residues of malachite green (MG), gentian violet (GV) and their leuco analogs (LMG and LGV) in catfish and trout tissue at 10 ng/g is described. Frozen (−20°C) fish fillets were cut into small pieces and homogenized in Waring blendors. The compounds of interest were extracted from 20-g amounts of homogenized fish tissue with acetonitrile–buffer, partitioned against methylene chloride, and isolated with tandem neutral alumina and propylsulfonic acid cation-exchange solid-phase extraction cartridges. Samples of 100 μl (0.8 g equiv.) were chromatographed isocratically in 10 min using an acetonitrile–buffer mobile phase on a short-chain deactivated (SCD) reversed-phase column (150×4.6 mm I.D.) in-line with a post-column oxidation coulometric electrochemical cell (EC), a UV–Vis diode array detector and a fluorescence detector. © 1997 Elsevier Science B.V.

Keywords: Catfish; Trout; Electrochemical oxidation; Malachite green; Gentian violet; Leucomalachite green; Leucogentian violet

1. Introduction

Malachite green (MG) and gentian violet (GV) (Fig. 1), are on the Food and Drug Administration's (FDA's) priority list for fish drugs that need analytical methods development. Although not approved by the FDA for use in the aquaculture industry, MG has been used since the early 1930s [1] to combat ecto-parasites and control fungus on fish eggs, fingerlings, and adult fish because of its anti-parasitic and anti-microbial properties. MG and GV are similar structurally and related to other triphenylmethane dyes such as rosaniline which has been linked to increased risk of human bladder cancer. The leuco form of rosaniline induces renal, hepatic, and lung tumors in mice [2]. In a number of species including man, it has been shown that the intestinal microflora systems [3] are capable of converting GV to the leuco form (LGV). The FDA, therefore, has need of a sensitive analytical method for the confirmation of residues of MG-GV and their analogs, LMG-LGV, in catfish and trout for monitoring illicit use and for potential use in enforcement proceedings.

Several methods have been reported in the sci-

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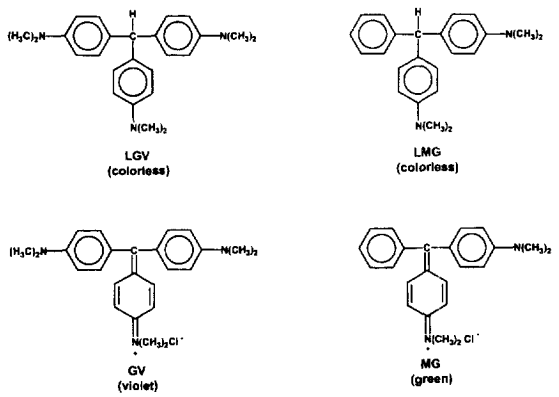

Fig. 1. Chemical structures of leucomalachite green (LMG), leucogentian violet (LGV), malachite green (MG), and gentian violet (GV).

entific literature for LMG-MG and for LGV-GV analyses in a variety of matrices. Most of these methods employed liquid chromatography with UV-vis detection or electrochemical detection [4–8]. In 1991, Allen and Meinertz [9] reported a HPLC method for separating the leuco and chromatic forms of two triphenylmethane dyes, LMG-MG and LGV-GV. The leuco form was oxidized to the chromatic form with an in-line post-column cartridge packed with 10% PbO_2–Celite 545 with subsequent detection of both forms by visible spectrophotometry. Their method eliminated the need to split the sample to assay the leuco forms by difference before and after complete oxidation. No methods were found in the literature for the simultaneous analyses of all four compounds in catfish or trout tissues. However, Roybal et al. [10] reported a method for the analysis of MG and its metabolite (LMG) in catfish tissue. Roybal's procedure employing a HPLC with PbO_2 post-column oxidation (LMG→MG) and visible detection was modified for our application for analyses of LMG, LGV, MG, and GV in edible catfish and trout samples [11]. Liquid chromatography combined with atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS) and/or particle beam liquid chromatography–mass spectrometry (PB-LC–MS) are excellent confirmatory techniques [12,13] but are not prevalent in laboratories due to their expense. Although less expensive than LC–MS, confirmation by gas chromatography mass spectrometry (GC–MS) [14,15] is applicable only to the volatile leuco forms and not to the chromatic forms of the dyes.

2. Application

An analytical procedure is described for the concurrent analyses of LMG, LGV, MG, and GV in catfish or trout. The information gathered from an in-line coulometric EC cell, an UV–Vis diode array detector, and a fluorescence detector combine to form the basis for the confirmation of LMG, LGV, MG, and GV at the 10 ng/g level. These detectors are available in many laboratories, whereas LC–APCI-MS and PB-LC–MS are not due to their expense. This method, therefore, may be applicable for detection and confirmation of residues of MG-GV and their analogs LMG-LGV in catfish and trout in order to monitor for their illicit use and for potential use in enforcement proceedings.

3. Experimental

3.1. Chemicals

LMG, LGV, and malachite green oxalate were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). GV was obtained from Hilton-Davis (Cincinnati, OH, USA) and had previously been assayed to contain 94.8% GV, 4.3% methyl violet (MV) and 0.5% LGV. All were used as received. Hydroxylamine hydrochloride was from Mallinckrodt (Chesterfield, MO, USA) and was AR grade. Basic alumina, Brockman activity I, and di(ethylene glycol) were purchased from Fisher Scientific (Springfield, NJ, USA). The *p*-toluene sulfonic acid (*p*-TSA) and ammonium acetate were purchased from Fluka (Ronkonkoma, NY, USA). The glacial acetic acid and the HPLC grade acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA).

3.2. Fish samples

3.2.1. Preparation

Several pounds of catfish and trout fillets were purchased at a local market and stored at −20°C.

The catfish and trout fillets were cut into small pieces and homogenized in Waring blendors. The homogenized catfish and trout were stored in separate zip-lock plastic bags until required for processing and analysis.

3.2.2. Extraction

Four 20.0-g fish samples were weighed into individual 250-ml Falcon polypropylene tubes obtained from Becton Dickinson Labware (Lincoln Park, NJ, USA). Three ml of aqueous 0.25 g/ml hydroxylamine hydrochloride, 5 ml of aqueous 0.05 *M* *p*-TSA and 20 ml of aqueous 0.1 *M* ammonium acetate (adjusted to pH 4.5 with glacial acetic acid) were added to each sample. These were homogenized for 1 min at 20 000 rpm using a Tekmar (Cincinnati, OH, USA) Ultra-Turrax T25 tissuemizer equipped with a S25N-18G dispensing tool. Acetonitrile (90 ml) was added to each and the samples were homogenized for an additional 10 s. The Falcon tubes were capped and shaken vigorously by hand for 1 min. Basic alumina (20 g) was added and the tubes were again shaken vigorously for 1 min. The four tubes were centrifuged (centrifuge speed was not critical) and the supernatants were decanted into 250-ml separatory funnels. Acetonitrile (30 ml) was added to the Falcon tubes and the samples were extracted, centrifuged and decanted again into the separatory funnels.

3.2.3. Liquid–liquid partition

To the combined supernatants in the separatory funnels, 100 ml of deionized distilled water, 50 ml of methylene chloride, and 2 ml of di(ethylene glycol) were added to each. The separatory funnels were then shaken vigorously by hand for 1 min and left to stand for the phases to separate. The bottom layer of each was collected in a 500-ml round-bottom flask containing several boiling chips. An additional 50 ml of methylene chloride were added to the separatory funnels which were again shaken for 1 min. The methylene chloride layers were added to their respective 500-ml round-bottom flask. These samples were then concentrated under vacuum to approximately 2–5 ml using a Büchi (Flawil, Switzerland) rotary evaporator at 65°C. The samples can be reserved overnight in the dark at this point.

3.2.4. Solid-phase extraction

J.T. Baker (Phillipsburg, NJ, USA) 6-ml (1000 mg) neutral alumina cartridges and Varian (Harbor City, CA, USA) 2.8-ml (500 mg) Bond Elut PRS cartridges were prewashed with 5 ml acetonitrile. The alumina cartridge was then placed on top of the PRS cartridge using a Bond Elut adapter. This assembly was then attached to an Alltech (Deerfield, IL, USA) solid-phase extraction vacuum manifold. The sample flow control valves were replaced with 15-gauge needles to reduce dead volume. A 2-ml amount of methylene chloride was added to each 500-ml sample flask which was then swirled to dissolve the residue. A 5-ml amount of acetonitrile was added to the flask prior to the addition of the sample extracts to the cartridge assemblies. The flasks were rinsed with an additional 2×5 ml of acetonitrile which were also applied to the cartridge assemblies. Finally, a 5-ml amount of acetonitrile was rinsed through each cartridge. All wash fractions and the alumina cartridges were discarded. A 2-ml volume of deionized water was rinsed through each PRS cartridge followed by a 1-ml volume of acetonitrile–0.1 *M* ammonium acetate buffer (50:50) adjusted to pH 4.5 with glacial acetic acid. These fractions were also discarded. The LMG, MG, LGV, and GV residues were eluted from the PRS cartridges with 2 ml of the above eluting acetonitrile–buffer and collected in graduated 2.5-ml centrifuge tubes containing 0.5 ml of 2.5 mg/ml hydroxylamine hydrochloride in water. Samples, standards, and controls were each contained in 2.5 ml of a mixture of 40% acetonitrile–buffer. All fish extract samples were 8 g equiv/ml (i.e., 0.8 g equiv./100 μl injection).

3.3. Liquid chromatography

The liquid chromatograph consisted of a Waters (Milford, MA, USA) Model 600 multisolvent delivery system, a Model 996 photodiode array detector, a Model 474 fluorescence detector, an ESA (Bedford, MA, USA) electrochemical cell (EC) Model 5010, a PC using Waters Millennium software version 2.1, and a Rheodyne (Cotati, CA, USA) Model 7125 injector with a 250-μl loop. The EC cell preceded the diode array and fluorescence detectors and was set at +0.9 V using the ESA Coulochem Model

5100A controller. The diode array detector was operated from 250 to 800 nm at 1.2 nm resolution. The fluorescence detector excitation and emission wavelengths were: E_X=265 nm and E_M=360 nm, respectively. Chromatographic separation was on a 20×2.0 mm I.D. pellicular C_{18} guard column with a SynChrom (Lafayette, IN, USA) 5 μm SynChropak 150×4.6 mm I.D. SCD-100 analytical column. The mobile phase was 55:45 acetonitrile–buffer. The LC buffer was prepared by adding 0.4 g ammonium acetate and 1 ml of triethylamine (TEA) to approximately 400 ml of water. The pH was then adjusted to 3.6 with glacial acetic acid. This was diluted with water to 450 ml and added to 550 ml of acetonitrile. The flow-rate was 2 ml/min at a pressure of 138 bar. All injections were 100 μl. All fish extract samples were 8 g equiv./ml (i.e., 0.8 g equiv./100 μl injection).

4. Results and discussion

Fig. 2 illustrates a three-chromatogram overlay of an 8 ng/component/100 μl inj. mixed standard, a 10 ng/g (8 ng/0.8 g equiv. inj.) of fortified catfish tissue sample and the corresponding control catfish sample. Each signal was that of the 588-nm chromatographic channel of the diode array detector. The retention times (t_R) of LMG, LGV, MG, and GV were approximately 4.0, 4.3, 5.2, and 7.9 min, respectively. The LMG and LGV were detected in the visible 588-nm chromatographic channel due to their post-column oxidation by the EC cell at +0.9 V to their chromatic forms, MG and GV, respectively.

Fig. 3 is the corresponding diode array UV-Vis spectrum from 250 to 800 nm of each of these four peaks at the 10 ng/g level. The top spectrum in each section is that of the 8 ng/component/100 μl inj. mixed standard. The middle spectrum is that of the 10 ng/g fortified catfish sample and the lower spectrum is from the control catfish chromatogram at the peak retention times. Fig. 3A and Fig. 3C are similar because the LMG and MG were both detected as the chromatic MG form. Fig. 3B and Fig. 3D are also similar because the LGV and GV were both detected as the chromatic GV form.

Fig. 4 illustrates a three-chromatogram overlay of an 8 ng/component/100 μl inj. mixed standard, a 10 ng/g fortified trout tissue sample and the corresponding control trout sample. Each signal is that of the 588-nm chromatographic channel of the diode array detector. The unknown peak at t_R=6.1 min did not interfere with detection of the MG peak at t_R=5.2 min.

Fig. 5 is the corresponding UV-Vis spectrum from 250 to 800 nm of each of these four peaks at the 10 ng/g level. Fig. 5A–D each contain the spectra of the 8 ng/component/100 μl inj. mixed standard, the 10 ng/g fortified trout sample, and the control trout sample.

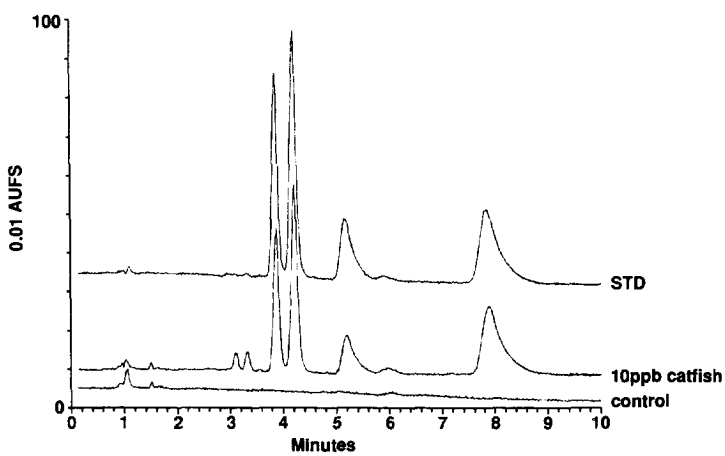

Fig. 2. Three chromatograms from the 588-nm channel overlaid: an 8 ng/component/100 μl inj. mixed standard, a 10 ng/g fortified catfish tissue sample and the corresponding control catfish sample.

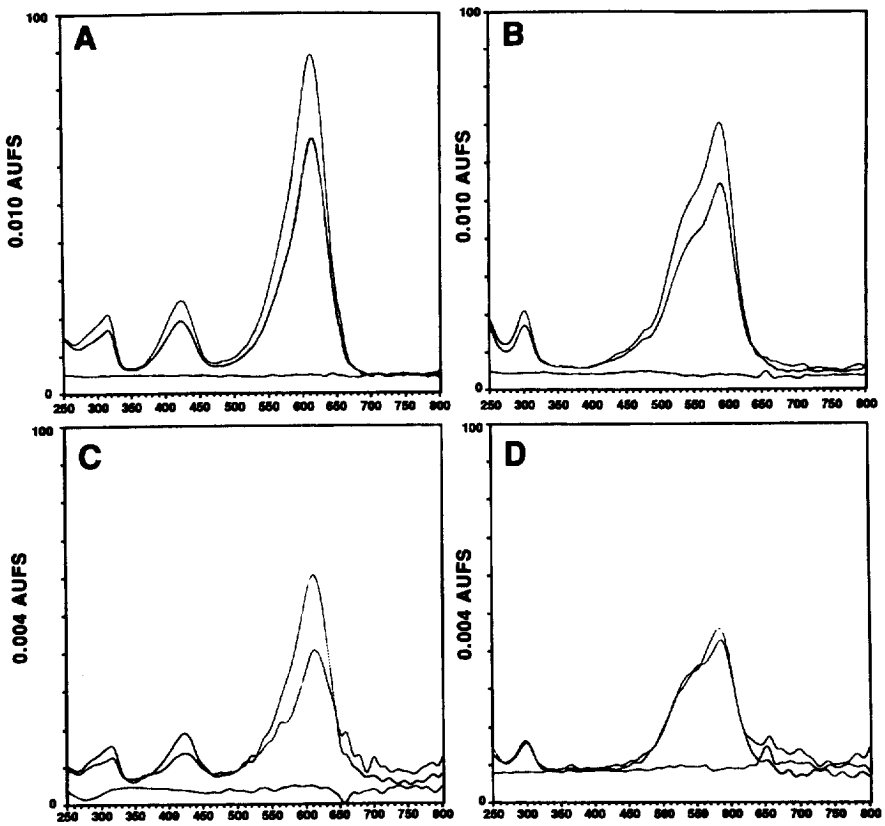

Fig. 3. (A), (B), (C) and (D) each contain the spectra of: in descending order, the 8 ng/component/100 μl inj. mixed standard, the 10 ng/g fortified catfish sample, and the control catfish sample for the LMG, LGV, MG, and GV peaks at t_R values of 4.0, 4.3, 5.2, and 7.9 min, respectively. (A) Contains the spectra of LMG oxidized to MG and (B) contains the spectra of LGV oxidized to GV. (C) and (D) are the spectra of MG and GV, respectively.

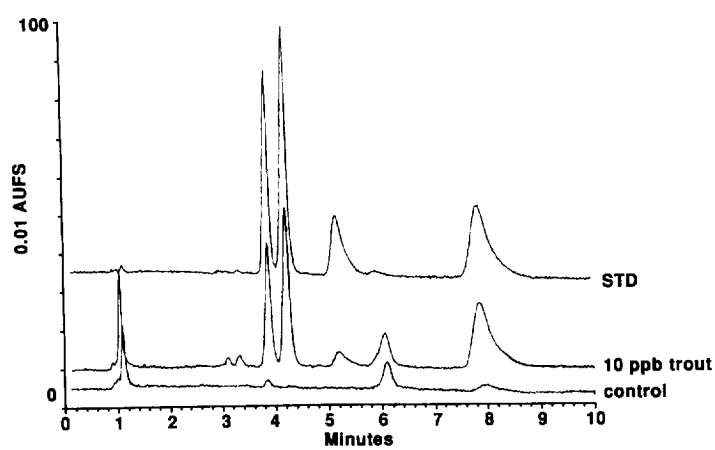

Fig. 4. Three chromatograms from the 588-nm channel overlaid: an 8 ng/component/100 μl inj. mixed standard, a 10 ng/g fortified trout tissue sample and the corresponding control trout sample.

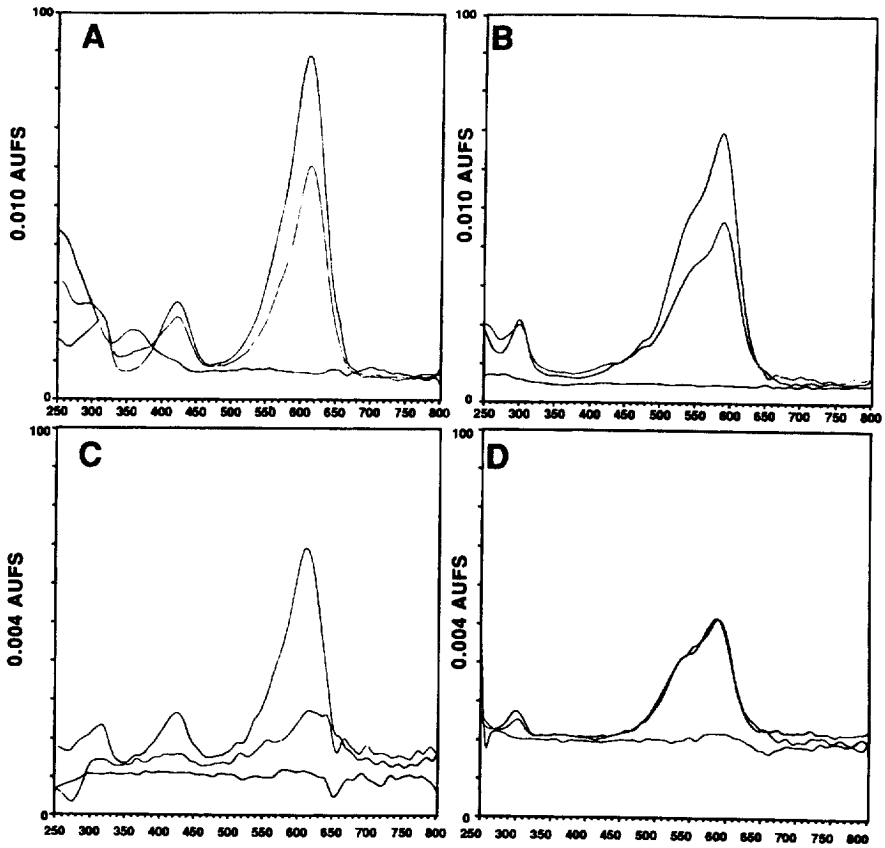

Fig. 5. (A), (B), (C) and (D) each contain the spectra of: in descending order, the 8 ng/component/100 μl inj. mixed standard, the 10 ng/g fortified trout sample, and the control trout sample for the LMG, LGV, MG, and GV peaks at t_R values of 4.0, 4.3, 5.2, and 7.9 min, respectively. (A) Contains the spectra of LMG oxidized to MG and (B) contains the spectra of LGV oxidized to GV. (C) and (D) are the spectra of MG and GV, respectively.

Fig. 6 is that of a 10 ng/g fortified catfish tissue sample following simultaneously the 588-nm channel and the fluorescence channel under the conditions of the EC off and EC on (i.e., two injections). The PbO_2 post-column oxidation column described by Roybal was replaced with a coulometric EC cell. This allowed the chromatograms to be overlaid without shifting the retention times because the flow dynamics were the same under nonoxidative conditions (EC off) or oxidative conditions (EC on). The lower trace with the EC off illustrated the natural fluorescence of LMG (t_R=4.0) and LGV (t_R=4.3) in a 10 ng/g fortified catfish matrix. When the coulometric EC was turned on, both leuco forms were completely oxidized to their nonfluorescing chromatic forms and thus vanished from the fluorescence channel (lower trace). This disappearance was balanced by the appearance of their chromatic forms in the diode array 588-nm channel (upper trace). This upper trace with the EC on is the 588-nm chromatographic channel discussed earlier. With the EC off, the upper trace also illustrated that the fluorescent leuco forms have no response in the visible 588-nm channel. The responses of the chromatic MG and GV were essentially unaffected by the state of the EC cell.

Fig. 7 is the corresponding 10 ng/g fortified trout tissue sample. The 588-nm channel and the fluorescence channel were again collected under the conditions of the EC off and EC on.

The confirmation of malachite green, gentian violet and their leuco analogs in catfish and trout

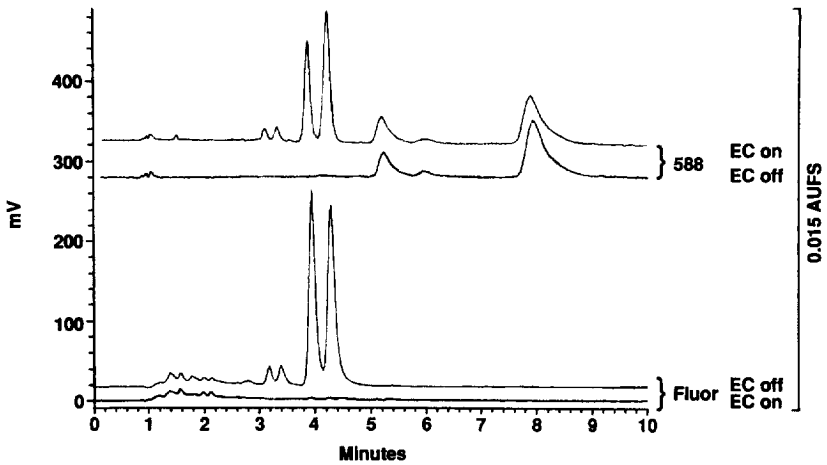

Fig. 6. Chromatograms of a 10 ng/g fortified catfish tissue sample followed simultaneously in the 588-nm channel and the fluorescence channel under the conditions of: EC off and EC on. With the EC off the natural fluorescence of LMG and LGV were observed.

tissue at the 10 ng/g level therefore consisted of the following: (1) the correct LC retention times, (2) the observation of the natural fluorescence of LMG and LGV when the EC was off, (3) the absence of the LMG and LGV peaks in the 588-nm channel when the EC was off, (4) the fluorescence of LMG and LGV disappeared when the EC was on, (5) the appearance of LMG→MG and LGV→GV in the 588-nm diode array channel at 4.0 and 4.3 min when the EC was on, (6) the correct UV-Vis spectra maxima for all four peaks, and (7) the overall shapes of the spectra matched that of the admixture standard. All seven conditions contributed to the confirmation of LMG, and LGV at the 10 ng/g level in catfish and trout. Confirmation of MG and GV was by: (1), (6) and (7).

Figs. 8 and 9 are each three chromatograms overlaid from the fluorescence channel. The upper trace is the 8 ng/component/100 μl inj. mixed standard. The center trace is that of a 10 ng/g

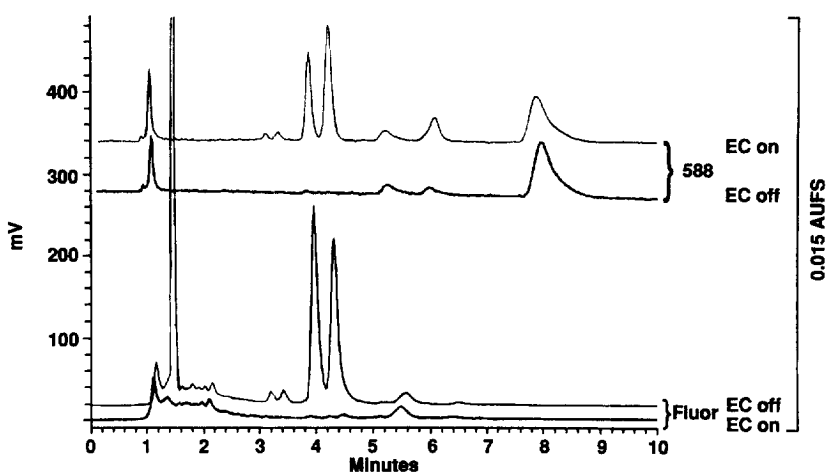

Fig. 7. Chromatograms of a 10 ng/g fortified trout tissue sample followed simultaneously in the 588-nm channel and the fluorescence channel under the conditions of: EC off and EC on. With the EC off the natural fluorescence of LMG and LGV were observed.

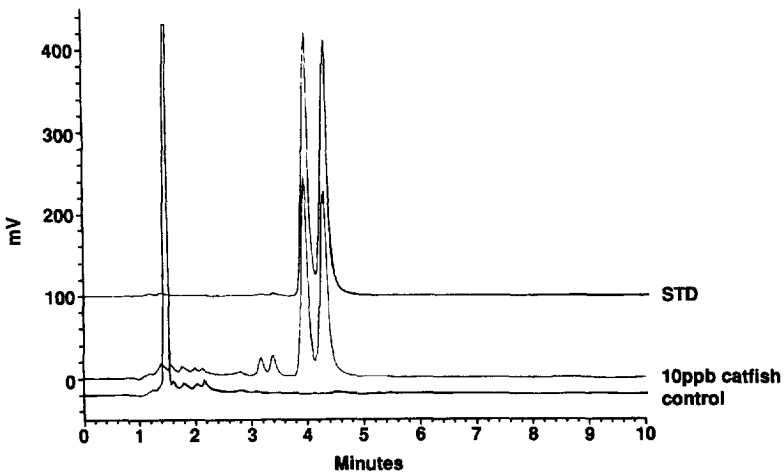

Fig. 8. Three chromatograms overlaid from the fluorescence channel: an 8 ng/component/100 μl inj. mixed standard, a 10 ng/g fortified catfish tissue sample and the corresponding control catfish sample. Peaks at 4.0 and 4.3 are LMG and LGV, respectively.

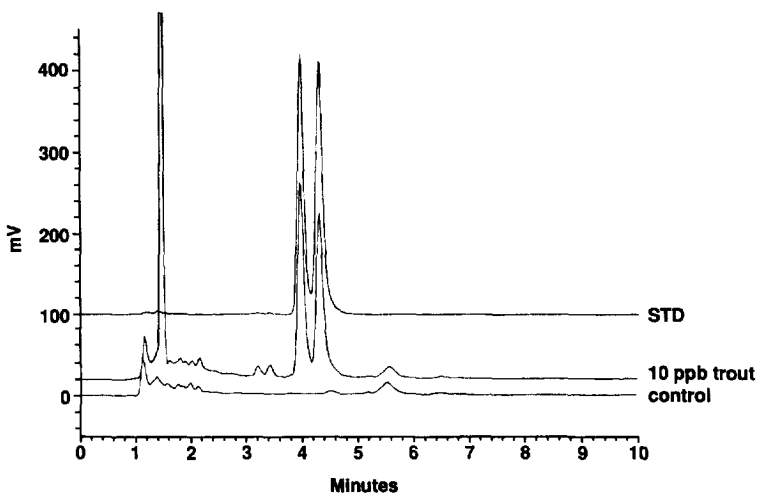

Fig. 9. Three chromatograms overlaid from the fluorescence channel: an 8 ng/component/100 μl inj. mixed standard, a 10 ng/g fortified trout tissue sample and the corresponding control trout sample. Peaks at 4.0 and 4.3 are LMG and LGV, respectively.

fortified fish tissue (catfish or trout) and the lower trace is the corresponding control fish tissue. Each figure illustrated there was no difficulty in assaying LMG and LGV by fluorescence detection at the 10 ng/g level.

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