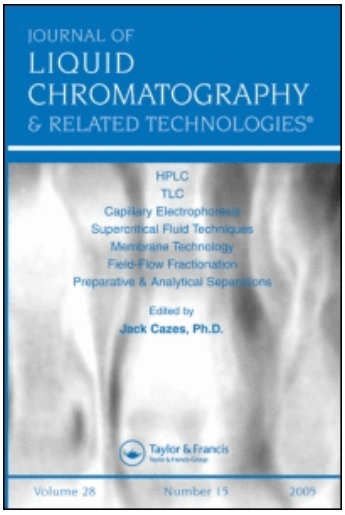

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POST-COLUMN ELECTROCHEMICAL OXIDATION OF LEUCO MALACHITE GREEN FOR THE ANALYSIS OF RAINBOW TROUT FLESH USING HPLC WITH ABSORBANCE DETECTION

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ABSTRACT

Malachite green is a dye used in aquaculture as a parasiticide. A method is described for the quantitation of residues of malachite green and its colourless reduced form, leuco malachite green, in the flesh of rainbow trout. The analytes are extracted with solvent and purified using a C_{18} solid phase extraction cartridge. Determination of both compounds by reversed phase HPLC with absorbance detection at 610 nm is achieved following post-column oxidation of the leuco form to malachite green using an electrochemical detector cell. The limits of detection for malachite green and leuco malachite are 6 ng/g and 3 ng/g, respectively. At concentrations from 25-200 ng/g, recoveries in the range 73-87% were achieved for malachite green and 89-98% for leuco malachite green. Oxidation of leuco malachite green using activated charcoal is also described as as an alternate manner of determining total malachite green.

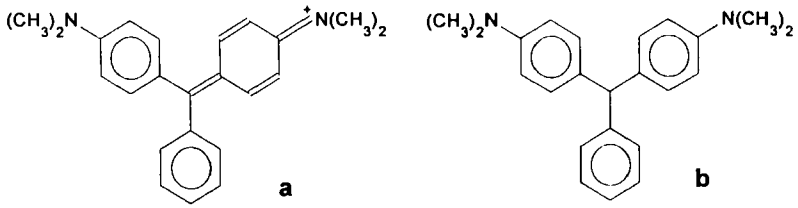

Figure 1. Structures of (a) malachite green, (b) leuco malachite green

INTRODUCTION

Malachite green oxalate (C.I. 42000) is a triarylmethane dye commonly applied to newly laid fish eggs to inhibit fungal and protozoal infections.[1] The reduction of malachite green (**MG**) to its colourless leuco base (**LMG**) is a facile process which has been reported to occur in fish flesh.[2] To monitor good aquacultural practice and prevent exposure of consumers to residues of these suspected carcinogens[1], an efficient method for the detection of malachite green residues in rainbow trout was required (see Figure 1).

Methods employing TLC[3] or HPLC[4] have previously been described for the detection of **MG** itself in fish. Determination of **MG** alone was considered unsuitable for monitoring residues in fish flesh. It has been found that up to 90% of the total malachite green species present in fish were in the leuco form.[2] This is corroborated by the authors' observations. Several HPLC methods for determining both **MG** and **LMG**, in fish flesh,[2,5-8] plasma,[9] and in waters,[10,11] have also been published. While diol, cyano, or cation exchange solid phase extraction (SPE) cartridges, alone, were found to be effective for the relatively simple concentration and clean-up of the analytes during the analysis of water or plasma, methods for analysis of fish tissue have employed either solvent partitioning and washing to separate interferences,[7] or a combination of alumina and cation exchange SPE.[8]

For GC/MS confirmation of **LMG** in catfish tissue, a third clean-up on a cyano column was added.[12] Due to losses of the less polar leuco form into the organic phase, recoveries of **LMG** were often less than 30% in our hands when a solvent partitioning clean-up was attempted. To obtain high yields for both forms of the analyte simultaneously, a simple and efficient SPE protocol using a single C_{18} cartridge was developed and is described here.

Previous HPLC methods for the determination of these compounds have taken advantage of the strong and distinctive visible absorption of **MG** for detection and quantitation of both it and **LMG** (after oxidation to **MG**). In methods where the analytes are individually determined, oxidation of **LMG** can be performed in a post-column reactor containing lead dioxide. As an alternative to the use of absorbance, electrochemical detection has been described in the analysis of water.[11] We found that in our analysis of fish tissue extracts the selectivity and stability of the absorbance detector operating at 610 nm gave the best results for reliable quantitation. However, the use of a coulometrically efficient elecrochemical cell to oxidize **LMG** to **MG** between the outlet of the HPLC column and the inlet of the visible detector was found to be an effective way to overcome the problems associated with packing and maintaining a PbO_2 post-column reactor, while avoiding the band-broadening such reactors are liable to produce.

In previous procedures in which both compounds have been analysed without separation, the leuco form has generally been oxidised to the coloured form by oxidation with a lead (IV) oxide slurry after sample clean-up. The extent of this oxidation tends to be rather variable, depending upon the age, activity, and rate of addition of the oxidant.[1] We also found that the PbO_2 could further oxidise **MG** to a derivative which was not easily quantitated. The degree of further oxidation was found to differ between standards and samples (perhaps due to sacrificial oxidation of other components of the fish extract). Consequently, the use of PbO_2 slurries gave recoveries of **LMG** which were low and variable. Our observations, on the behavior of the carbon electrode in the amperometric detector, led to some investigations which demonstrate that activated charcoal may be superior to lead dioxide for pre-separation oxidation of **LMG** to **MG**.

MATERIALS AND METHODS

Standards and Reagents

L.R grade standards were obtained from E. Merck (Melbourne, Australia). Stock standards of **LMG** and **MG** were prepared fresh weekly in methanol at approximately 0.5 mg/mL and stored below -10°C. Working standards were a 1:125 dilution of the stock in water to give 4 μg/mL. HPLC standards (a 1:40 dilution of working standard in HPLC mobile phase) had a final concentration of 0.1 μg/mL.

Acetonitrile, methanol, and dichloromethane (HPLC grade) were obtained from Mallinkrodt. Water was purified with a Waters Milli-Q system. Perchloric acid (70%) was May and Baker Pronalys (AR) grade and pentane sulfonic acid (HPLC grade) was from Sigma. Bakerbond C_{18} cartridges (500 mg) (J.T.Baker) were employed for SPE clean-ups. Activated charcoal (decolourising powder) was supplied by Ajax Chemicals (Melbourne, Australia).

Chromatographic System

The HPLC system consisted of an LKB 2248 pump / LKB 2157 auto sampler coupled to a 25 cm x 4.6mm Alltech 5μm Econosphere C18 column. The mobile phase was 0.01M pentane sulfonic acid in acetonitrile containing 6% 0.05M aqueous phosphoric acid. It could be recycled 2-3 times, then renewed. Absorbance at 610nm was measured using an Activon Linear UVIS 204 variable wavelength detector. An ESA Coulochem model 5100A electrochemical detector with ESA 5010 analytical cell operating at a potential of 0.45V relative to the in-built proprietary reference electrode was coupled between the HPLC column and the visible detector.

Selection of the operating potential was acheived by performing repeated 10 microlitre injections of standard solutions of **LMG** or **MG** in mobile phase at a flow rate of 0.1mL/min after removal of the analytical column. The response of the visible detector was recorded at a range of potentials at the electrode of the electrochemical detector to determine an optimum voltage for the analysis.

Sample Extraction

Each fish to be tested was filleted and the flesh blended in a food processor before weighing 9g of tissue into a 50 mL polypropylene centrifuge tube. After homogenising for 1 minute at high speed with 2 mL dichloromethane, 16 mL acetonitrile, and 1 mL 0.4M perchloric acid in acetonitrile, the extraction was continued in the dark for 3 hours at 60 rpm on a rotary mixer .

After centrifuging at 3200 rpm for five minutes, 15 mL of the supernatant was transferred to a graduated glass tube and evaporated to 5 mL at 60°C using nitrogen. This extract was diluted with 20 mL of water, before being loaded

onto a C_{18} SPE cartridge which had been pre-conditioned with 10 mL of 20% acetonitrile in H_2O. The cartridge was dried with a stream of air for 10 minutes and the analytes were slowly eluted with 2 mL of HPLC mobile phase of which 25 μL was injected into the HPLC.

Alternative Oxidation and Clean-up using Activated Charcoal

As an alternative to the SPE clean-up, after centrifuging the initial dichloromethane/acetonitrile/perchloric acid extract of the sample, 15mL of supernatant was transferred to a graduated glass tube and evaporated to approximately 0.5 mL at 60°C using nitrogen. This extract was diluted to 2 mL with HPLC mobile phase, in preparation for clean-up and oxidation with charcoal conditioned as follows.

Approximately 1g of finely powdered charcoal was shaken vigorously with 40 mL of 5M nitric acid for 1 minute. After standing for five minutes the acid was removed by centrifuging at 1500 rpm. The remaining paste was rinsed twice with 40 mL of water and then with HPLC mobile phase before being diluted to 20mL with mobile phase.

To oxidise **LMG** in the sample extracts, 100 μL aliquots from the stirred charcoal slurry (5 mg of carbon) were swiftly pipetted into all sample tubes. The samples were then vortexed for 30 seconds, centrifuged at 2000 rpm for 1 minute and 25 μL aliquots analysed by HPLC.

RESULTS AND DISCUSSION

The electrode in the ESA 5010 analytical cell is a porous carbon frit through which the column eluant flows. This design results in efficient oxidation of almost all the analyte passing through the electrode. Consequently, excellent sensitivity can be acheived in subsequent Absorbance detection (at 610 nm) of **LMG** which has been oxidized to **MG**. An oxidation potential of 0.45V was chosen for the analysis of samples in this study since it gave maximal oxidation of **LMG** whilst minimizing the degradation of **MG**. As can be seen from the data in Figure 2, at an applied potential of 0.45V the oxidation efficiency for **LMG** is at a maximum of 57%, while only 3% of **MG** is degraded.

The use of a coulometrically efficient electrochemical cell at the exit of the HPLC column was found to be a more reliable oxidation method for **LMG** than pre-column treatment with lead dioxide. The chromatographic separation of

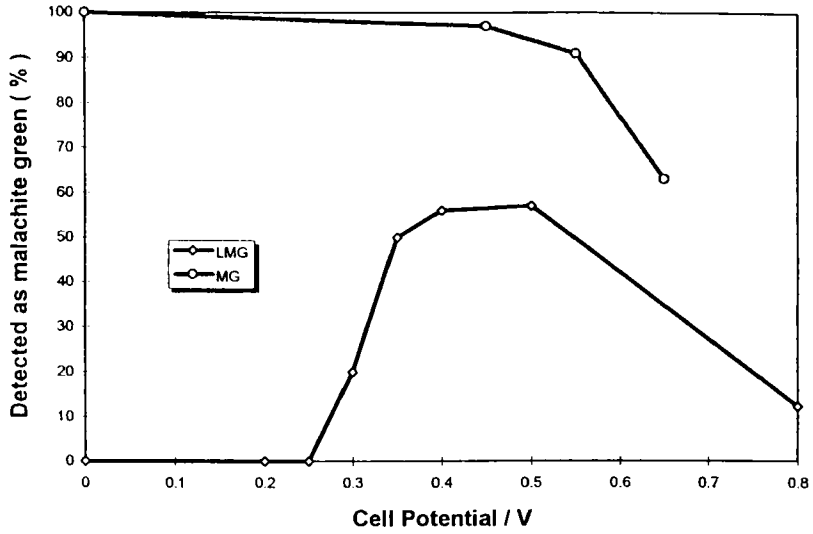

Figure 2. Response of MG and LMG to change in cell potential. Visible response at 610nm following direct injection of malachite green (MG) or leuco-malachite green (LMG) solutions (0.1 μg/mL) into the electrochemical detector.

LMG from other oxidizable co-extractives in the fish extracts, prior to its oxidation enhances the consistency of the oxidation. As oxidation is now fully automated the method requires fewer manipulative steps and this can be expected to increase repeatability of results as well as save time. In addition, the unreliable and unpleasant use of PbO_2, a suspected carcinogen, is avoided. Another benefit of the use of the electrochemical cell for oxidation is that it allows speciation of the two major forms of malachite green by chromatographically separating them prior to detection.

Sample Extraction

A range of extraction systems was examined, including several solvent/ homogenisation and percolation techniques. Their effectiveness appeared to be limited by the strong binding of the dyes to the fish flesh, and its oil content (10-15%) which causes emulsion formation. Long extraction times have been shown to be necessary for extraction of malachite green residues from fish tissue.[4] Three hours was found to be sufficient for fortified samples with the system described here. Acidification of the extraction solvent was vital for good yields.

In studies of various eluants for the HPLC system, it was found the analytes were strongly retained against elution by even traditionally powerful reverse phase solvents such as methanol, acetonitrile, acetone, and dichloromethane. This distinctive selectivity of the HPLC mobile phase for **MG** and **LMG** forms the basis of our SPE clean up. Use of mobile phase as the eluant means that samples are immediately ready for injection and since the SPE cartridge used has a similar chemistry to the HPLC column (ODS), interferences likely to cause column damage are removed.

Analysis of Fish Tissue

Rainbow trout samples were analysed using this method both before and after fortification with the analytes (Figure 3). Apart from one batch of fish (discussed below), no significant interferences were observed in the traces produced by the visible detector at 610nm. Both compounds were readily detected at spiking levels down to 25 ng/g (ppb) with coefficients of variation below 10% at this level (Table 1). Based on three times the standard deviation of the results for the lowest fortification level studied (an approximation of the standard deviation of the blank), the limit of detection for the method is 3 ng/g for MG and 6ng/g for LMG. The limit of quantition for MG and LMG is 6ng/g and 12 ng/g respectively.[13,14]

Chromatographic performance was good. More than 1500 injections of standards and samples were performed during method development, with no decrease in column performance. Maintenance was limited to weekly washing with methanol and 1:1 methanol/dichloromethane and occasional regeneration of the electrochemical cell using 5M nitric acid. Both the visible response and the electrochemical oxidation were consistent and linear within a batch .

Confirmation of Positive Results

The detector wavelength used confers a fairly high degree of selectivity on the analysis when coupled with retention time information. However, the on-line electrochemical oxidation allows additional confidence in the identity of a peak eluting at the correct retention time for **LMG**. If the output of the electrochemical cell is monitored during the analysis, current flow reflecting the passage of an electro-active species (**LMG**) will be observed shortly prior to detection of the compound at the visible detector. This would not occur if the peak was due to an already coloured co-extractive. Secondly, the size of the suspected **LMG** peak (in the visible chromatogram) should vary in a similar manner to that of a leuco standard when the electrode potential is manually

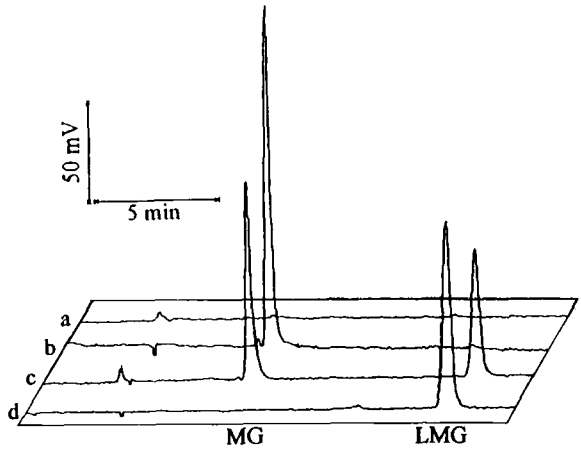

Figure 3. Representative chromatograms; (a) blank rainbow trout sample, (b) 100 ppb MG std, (c) rainbow trout sample fortified at 50 ppb each MG and LMG, (d) 50 ppb LMG std

Table 1

Recovery of Leuco Malachite Green (LMG) and Malachite Green (MG) from Fortified Rainbow Trout Flesh Using the Electrochemical Method

	MG Spiked Samples Spiking Level (ppb)				LMG Spiked Samples Spiking Level (ppb)		
	25	**50**	**100**	**200**	**25**	**100**	**200**
No. of samples	7	6	5	6	7	6	6
Recovery %	87	73	76	84	98	95	89
C.V %	8.0	6.5	12.5	5.8	4.0	7.4	6.9

altered. This would provide strong confirmation of the identity of the unknown as it would seem very unlikely that any co-extracted compound would have the same retention time, hydrodynamic voltammogram, and coloured oxidation product as leuco malachite green.

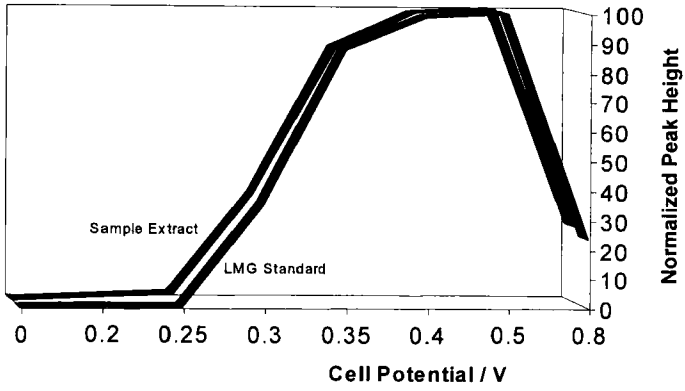

Figure 4. Oxidation of (a) leuco malachite green standard and (b) unfortified rainbow trout sample using the HPLC electrochemical cell.

The usefulness of this technique was strikingly demonstrated on one batch of fish samples that were obtained for use in validation. When a blank sample was analysed, it contained what appeared to be a large interference at the retention time of **LMG**. The sample was re-analysed at a range of electrode potentials, and the results are shown in Figure 4.

When the sample was injected 8 times with different applied voltages in the electrochemical cell, the 'interference' showed an identical hydrodynamic voltammogram to that of a **LMG** std, as well as having the same retention time on two different HPLC columns. It therefore appears that the 'interference' was incurred **LMG**, present in some of the samples at more than 100 ng/g.

Use of Activated Charcoal for Oxidation

A decrease in the efficiency of the oxidation of **LMG** by the electrochemical cell after processing several batches of samples was observed as a reduction in the peak height of standards. This was probably due to poisoning of the carbon electrode by contaminants in the fish extract, as it could be reversed by washing the electrochemical oxidation cell with 10mL of 5M nitric acid. After this cleaning process it was necessary to re-condition the cell with several injections of **LMG** standard, otherwise oxidation would occur in the absence of an applied potential.

Table 2

Oxidation of LMG (200 ng/mL Solution) Using Charcoal

Sample	Charcoal/mg	% Oxidation Efficiency
LMG Standard	1[a]	99
LMG Standard	5[a]	87
LMG Standard	5[b]	0
LMG Spiked fish extract	1[a]	60
LMG Spiked fish extract	5[a]	91

[a] Activated with 5M nitric acid as described in Experimental Section.
[b] Not activated.

Table 3

Effect of Treatment with Acid-Activated Charcoal on Malachite Green*

	Charcoal/mg	% Malachite Green Loss
MG Standard	1	0
MG Standard	5	3
MG Standard	25	43

* 140 ng/mL solution.

The observation of oxidation of **LMG** by a freshly washed carbon electrode in the absence of an applied voltage, led us to examine the use of activated charcoal treated with 5M HNO_3 as an alternative method to PbO_2 slurries for pre-HPLC oxidation. It was found that the leuco base was very efficiently oxidised to **MG** by charcoal slurries freshly activated with nitric acid (Table 2). The use of 5 mg of charcoal gave almost complete oxidation of **LMG** in both standards and sample extracts, without compromising the recovery of **MG**. Charcoal prepared three hours prior to use had only 50% of the activity of the fresh material. This method results in minimal destruction of **MG** as shown in Table 3. It also appears to simultaneously remove many interferences from the sample extract and could form the basis of a simpler and more repeatable method of quantitation of total malachite green residues than current procedures based on lead dioxide slurries.

CONCLUSIONS

Leuco malachite green and malachite green can be simultaneously and independantly determined in rainbow trout flesh, in a fast, efficient, and cost effective procedure. The method described offers repeatable recoveries of more than 75% and 89% in the range 25-200 ng/g for **MG** and **LMG** with detection limits of 6 and 3 ng/g, respectively. Excellent confirmation of the identity of **LMG** residues can be obtained using its electrochemical oxidation profile. This convenient and effective on-line oxidation using an electrochemical cell, can also be expected to be applicable in other liquid chromatographic applications where oxidation of analytes is required prior to detection by absorbance or fluorescence.

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