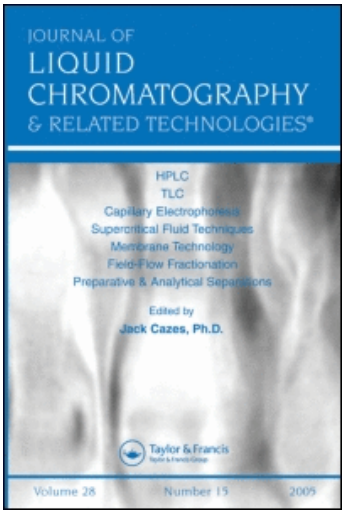

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A Time and Cost-Effective Assay for the Determination of Residues of Malachite Green in Fish Tissues by HPLC

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A TIME AND COST-EFFECTIVE ASSAY FOR THE DETERMINATION OF RESIDUES OF MALACHITE GREEN IN FISH TISSUES BY HPLC

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ABSTRACT

A time and cost-effective method for the extraction and determination of residues of malachite green in fish tissue, muscle and liver, by HPLC is presented.

The calibration curves were linear, and the recovery of malachite green was 101-116 %. The detection limits were 1 and 10 ng/g for malachite green in muscle and liver, respectively.

A longer time of sample exposure to the extraction fluids was required to extract malachite green from samples from treated fish than from samples to which a standard amount of malachite green had been added. We suggest that samples be exposed to the extraction solvents overnight for extraction of residues of malachite green from fish tissue.

INTRODUCTION

Malachite green is an organic dye used extensively as a fungicide and ectoparasiticide in fish farming throughout much of the world. Alderman (1) has given a detailed review

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of malachite green and its chemical and physical properties as they relate to aquaculture. In addition its mode of action, toxicity and fate are considered (1).

To be able to monitor residues of malachite green in farmed fish a simple, sensitive, and time-efficient method for the determination of malachite green is desirable. A number of methods for the determination of malachite green in fish tissue have been published. Thin layer chromatographic methods have been presented by M. Beeke (2), and Edelhäuser & Klein (3). HPLC methods have been described by Klein & Edelhäuser (4) and Bauer et al. (5).

In previous studies, we discovered that brief exposure to the extraction solvent was not sufficient to extract malachite green from samples taken from fish treated with the chemical. If the samples were exposed to the extraction solvents for a longer period of time, higher levels of malachite green were measured in the samples.

The purpose of the present study was to 1) develop a procedure for the extraction of malachite green from the tissue of fish treated with malachite green, and 2) develop a time and cost-effective, yet sensitive, assay for the determination of the residues of malachite green so extracted from fish tissue.

MATERIALS AND METHODS

Materials and Reagents

Muscle and liver tissues of rainbow trout (*Salmo gairdneri*) served as samples.

All chemicals and solvents were of analytical or HPLC grade. The malachite green used in the experiments was Malachite green oxalate salt (C.I. 42000) from Sigma Chemical Company.

The standard solution of malachite green was prepared in water.

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 400 solvent delivery system, an ISS 100 sampling system equipped with a Lauda RMT6 cooler (14 °C) from Messgeräte Werk Lauda, (Lauda-Königshafen, Germany), and a LC 95 UV detector (Perkin Elmer, Norwalk, Conn., USA). The detector was operated at 615 nm. The integration was carried out by use of the software programme Omega-2 (Perkin-Elmer) in an Olivetti M 300 PC connected to a Star LC24-10 printer. The analytical column (stainless steel, 15 cm x 4.6 mm I.D.) and guard column (stainless steel, 5.0 x 3.0 mm I.D.) were packed with 5 µm particles of PLRP-S polymer adsorbent (Polymer Laboratories, Amherst, MA, USA).

The mobile phase was 0.02 M phosphoric acid-acetonitrile-tetrahydrofurane (49:40:11) at a flow rate of 1.0 ml/min. Aliquots of 25 µl were injected onto the column for the determination of malachite green.

Sample Preparation and Clean-up (Fig. 1)

The tissue sample was ground, and 3 g muscle or liver was weighed into a 50 ml centrifuge tube with screw cap (NUNC). One ml CH_3COOH and 6 ml CH_3CN-$CHCl_3$ (9:1) were added. The sample was mechanically mixed to remove aggregates, followed by mixing using a whirlimixer, whereafter the sample was left over-night.

The sample was then mixed in a whirlimixer, and the homogenate centrifuged for 5 min. at 4000 rpm. Five ml of the supernatant was transferred to a centrifuge tube and 3 ml hexane added. The sample was mixed, and centrifuged for approx. 20 sec. at 3000 rpm. The hexane was discharged, and 1 ml 5 M NaCl, 3 ml dietylether, and 2 ml hexane, were added. The sample was then mixed for 10 sec., and centrifuged for 10 sec. The organic phase was transferred to a conical test tube, and evaporated

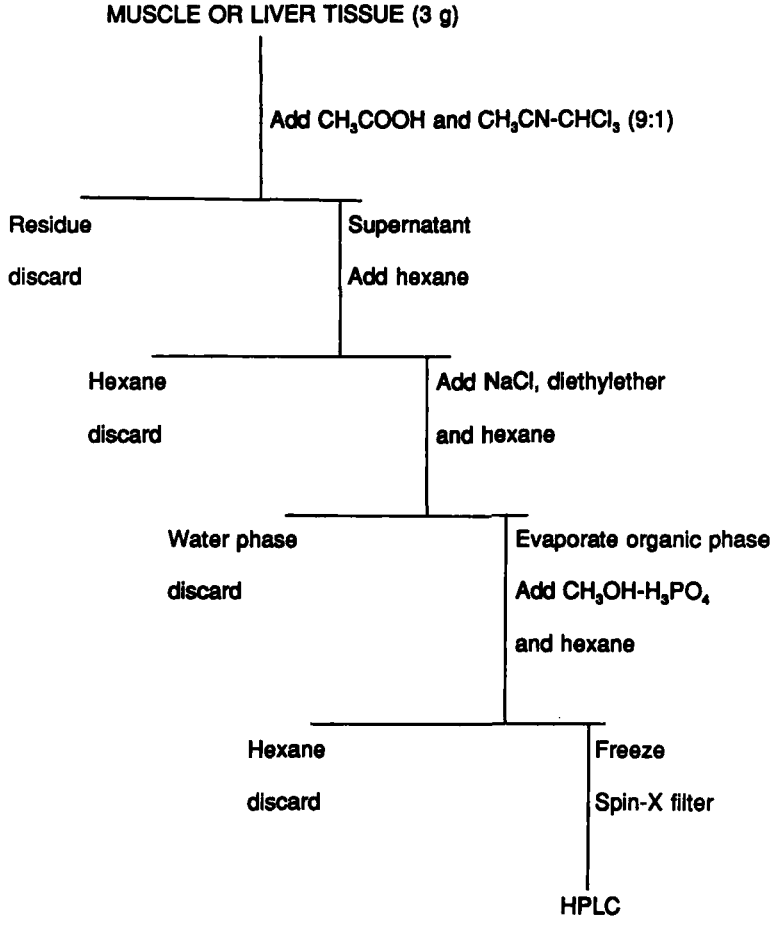


FIGURE 1.

Extraction and clean-up procedure for malachite green from fish muscle and liver.

on a heating block (60 °C) under a stream of nitrogen. 250 µl CH_3OH-0.02 M H_3PO_4 (70:30) were added, and the sample was mixed until the residue was completely dissolved. One ml hexane was added to the sample, which was mixed and centrifuged for 2 min. at 3000 rpm. The hexane was discharged, and the sample was put in a freezer (-20 °C) for 5 min. The extract was transferred to a Costar^R spin-X^{TM} centrifuge filter unit (low type) with 0.45 µm cellulose acetate binding, and centrifuged for 2 min. Aliquotes of 25 µl of the filtrate were injected onto the HPLC.

Calibration Curves and Recovery Studies.

The calibration curves for malachite green were made by spiking tissue samples (muscle and liver), with standard solutions of malachite green to yield 1, 2, 3, 5, 10, 15, 25, 50, 100, 150, 200 and 400 ng malachite green per gram in muscle, and 10, 15, 25, 50, 100, 150, 200, and 400 ng/g in liver samples. Duplicate samples were used. The recovery rates were determined by comparing results of analysis of spiked tissues (muscle and liver) to those of standard solutions.

The linearity of the standard curves for malachite green in muscle and liver was tested using peak height measurements.

Test of extraction time.

The efficacy of the extraction of malachite green from tissue after standard addition was tested by adding 150 µl of a standard malachite green solution containing 0.5 µg/ml to each sample to give 25 ng malachite green per gram sample. The standard was well mixed with the sample, and the sample was then left for 15 min. before starting extraction. The samples were then mixed with solvents for 1 min., and then either held at the room temperature, when the time of exposure to the extraction solvents was varied from immediate removal to over-night, or in an ultrasonic bath,

exposure time then ranging from 5 to 20 minutes. Three parallels were performed of each test.

The extraction time needed was also tested on "real" samples, i.e. samples from fish actually treated with malachite green. The exposure time was varied as for the samples to which standard solution had been added. Two parallels were performed of each test.

RESULTS AND DISCUSSION

Chromatograms of clean muscle and liver samples, spiked samples, and real samples are shown in Figure 2.

The standard curves were linear in the investigated areas, 1-400 ng/g and 10-400 ng/g for malachite green in muscle and liver, respectively, while the corresponding correlation coefficients were r=0.9966 and r=0.9987, respectively.

The recovery of malachite green from muscle and liver tissue varied from 101 to 116 %, with a standard deviation ranging from 2.5 to 8.0 (Table 1). The detection limit for malachite green in muscle and liver was 1 ng/g and 10 ng/g tissue, respectively. However, in liver, detection of 5 ng/g was possible, but recovery (approx. 60 %) was lower than at higher concentrations. Recovery might also vary more at lower concentrations.

When developing the present method, we tested the effect of varying the duration of exposure of the sample to the extraction chemicals, on the extraction of malachite green from fish muscle. Results are shown in Table 2.

For samples to which standard had been added, it seemed that exposure to solvents for 30 min. to 1 hour at room temperature, or for a few minutes in an

TABLE 1.

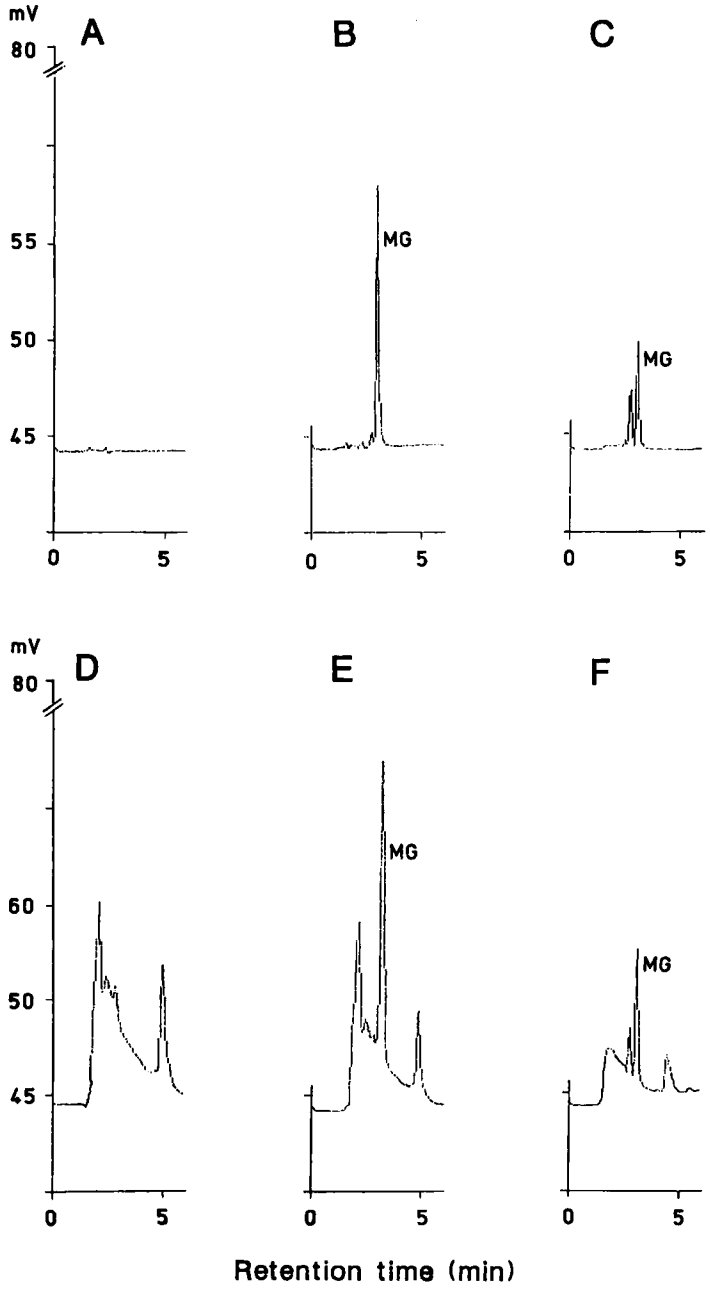
Recovery of Malachite Green from Spiked Samples of Fish Muscle and Liver Tissue.

Tissue	No. of samples	Amount in spiked samples (µg/g)	Recovery % Mean	Recovery % SD
Muscle (3 g)	8	0.15	101	8.0
	8	0.01	116	2.5
Liver (3 g)	8	0.15	109	4.7
	7	0.01	102	2.8

TABLE 2.

Tests of the Period of Time Needed for Exposure of the Samples to the Extraction Solvent for Extraction of Malachite Green from Fish Muscle.
The tests were performed both on samples to which a standard amount of 25 ng/g malachite green per gram fish had been added, and on "real" samples, i.e. samples of a fish treated with malachite green. Three parallels were performed of each test after standard addition, and two parallels of each test on the real samples.

Time at room temperature	Time in ultrasonic bath	Average content of malachite green (ng/g): Samples with standard addition (ng/g)	Average content of malachite green (ng/g): "Real" samples
0		21	
10 min		22	3.8
30 min		24	4.0
1 hour		27	5.7
3 hours			6.1
over night		26	8.9
	5 min	26	3.9
	10 min	24	4.0
	15 min	24	3.6
	20 min	27	4.3



ultrasonic bath, was sufficient for the extraction of malachite green from the fish tissue. But when the experiments were performed on "real" samples from fish that had been actually exposed to malachite green, a longer sample exposure period was needed to extract the malachite green (Table 2). Exposure to the solvents over-night is necessary to extract malachite green from samples from treated fish.

Of methods described previously, those of Klein & Edelhäuser (4), and Bauer et al. (5), are sensitive, both with detection limits of 1 ng/g. But they are more time-consuming per sample and the consumption of chemicals is greater than with the present method.

The method presented in this paper should be useful for most work on residues of malachite green in farmed fish, being sensitive and time-effective, as well as sparing on chemicals. A technician could easily deal with 20 samples a day. In addition to

FIGURE 2.

Chromatograms of extracts from 3 g muscle and liver for the determination of malachite green (25 µl injected onto the HPLC).

A - Unspiked muscle tissue.

B - Muscle tissue spiked with 100 ng malachite green per gram sample.

C - Sample of muscle tissue of trout treated with malachite green. The sample containing 41 ng/g.

D - Unspiked liver tissue.

E - Liver tissue spiked with 100 ng malachite green per gram sample.

F - Sample of liver tissue from trout treated with malachite green. The sample containing 51 ng/g.

trout, we have tested the method on samples from salmon and eel, and it seems to be well suited for samples from different fish species. The present method can be used to determine malachite green residues, nor only in muscle and liver, but also in other fish tissues, such as skin.

ACKNOWLEDGEMENTS

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