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Determination of residues of malachite green in aquatic animals

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Abstract

Residues of malachite green (MG) were extracted from homogenized animal tissues with a mixture of McIlvaine buffer (pH 3.0)–acetonitrile, and purified over an aromatic sulfonic acid solid-phase extraction column followed by HPLC or LC–ESI-MS–MS analysis. Ascorbic acid and *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine dihydrochloride were added to reduce de-methylation of the dye. Responses were recorded at 620 nm (HPLC) or by multiple-reaction-monitoring (LC–MS–MS) after post-column oxidation using PbO_2. MG and its primary metabolite leuco-malachite green (LMG) were successfully determined at 2.5–2000 μg/kg in catfish, eel, rainbow trout, salmon, tropical prawns and turbot, with a limit of detection at 1 μg/kg (HPLC) and 0.2 μg/kg (LC–MS–MS) for both MG and LMG. Recoveries for LMG were between 86±15% (prawn) and 105±14% (eel). Freeze–thawing cycles, and storage at 4 °C and −20 °C affected the recovery of both MG and LMG. Analyses of eel, trout and (processed) salmon field samples collected at local retailers, fish-market and -shops demonstrated trace levels of MG-residues.

Keywords: Residue analysis; Malachite green

1. Introduction

Malachite green (MG), originally used as a dyeing agent of textiles [1], was introduced as an ecto-parasiticide, fungicide and antiseptic in aquaculture in 1933 [2]. The broad fungicidal and anti-parasitical spectrum, and its efficacy in treating trout suffering from proliferative kidney disease (PKD) has made the drug very popular among fish culturists [1]. After administration, MG is prevalently reduced into leuco-malachite green (LMG) in channel catfish [3] and rainbow trout [4], and deposited in fatty tissue of the fish.

The potential carcinogenic, genotoxic, mutagenic and teratogenic properties were demonstrated in many animal species and cell lines [5]. For that reason, the USA placed MG under scrutiny in 1978, only permitted to specified national fish hatcheries that produced fish for restoration of depleted stocks. As MG is not registered as a veterinary drug, the administration of this antibiotic is not allowed in the European Union either. The ban on its use and its potential adverse effects in humans, necessitated a robust and reliable analytical method for determination of residues of MG in tissues derived from (cultured) aquatic animals, which can be used in the control of this drug.

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Several analytical approaches for the determination of residues of malachite green have been published. Methods include, for example, HPLC equipped with a post-column unit for oxidation of LMG and with either an absorbance or MS detector for detection in trout (eggs, fry and muscle), catfish (muscle and plasma) [4,6,7] and in trout muscle [8]. The reactors (from 10×2 to 32×4 mm) used in these studies were filled with either 100% lead(IV)oxide [7,8] or with a mixture of 10% lead(IV)oxide with celite [4,6]. Other methods include GC–MS of LMG in catfish [9] or LC–APCI-MS in catfish and trout [10] without the use of a reactor for the conversion of LMG. This study aimed at the development and validation of a procedure for sample processing and of an HPLC analysis method for determination of residues of MG in finfish with aquacultural interest and in prawns. Furthermore, the use of LC–ESI-MS–MS analysis was explored for confirmation of detected residues of this drug in such matrices.

2. Experimental

2.1. Reagents and chemicals

All chemicals used were of analytical grade unless stated otherwise. Acetic acid, acetonitrile, ascorbic acid, citric acid monohydrate, lead(IV)oxide, di-sodium hydrogen phosphate dihydrate, were purchased from Merck (Darmstadt, Germany). Ammonium hydroxide 25% (m/v), dichloromethane, methanol (HPLC grade), sodium acetate, sodium perchlorate monohydrate, *para*-toluenesulfonic acid (*p*-TSA) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Brilliant green and *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD) were from Aldrich (Steinheim, Germany), and celite from Acros (Geel, Belgium). *N*,*N*-Dimethylformamide was bought from Rathburn (Walkerburn, UK). Leuco-malachite green and 1-pentanesulfonic acid (sodium salt) were delivered by Sigma (St. Louis, MO, USA) and malachite green oxalate (Vetrenal reference standard) by Riedel-de-Haën (Seelze, Germany). Aromatic sulfonic acid-bonded SPE columns (3 ml; 500 mg) were purchased from J.T. Baker. Water was of Milli-Q quality (Millipore, Bedford, USA).

A McIlvaine solution at pH 3.0 was prepared by mixing 18.9 ml 0.2 *M* sodium hydrogen phosphate and 81.1 ml 0.1 *M* citric acid, whereas these volumes were 62.5 and 37.5 ml, respectively, to obtain a McIlvaine solution at pH 6.0. The SPE-eluent was prepared just before use and consisted of a mixture of 2.5 ml 25% (m/v) ammonium hydroxide, 2.5 ml 1.0 mg/ml methanolic ascorbic acid and 45 ml methanol. The sample-solvent was composed, just before use, from 2.5 ml 1.0 mg/ml methanolic ascorbic acid, 20 ml 50 m*M* sodium perchlorate containing 25 m*M* sodium acetate and 25 m*M* 1-pentanesulfonic acid adjusted to pH 4.0 with acetic acid, and 27.5 ml acetonitrile. Chemicals and solutions containing the dyes were protected from light.

Fish were bought at the fish market and local stores. Blank trout were collected from aquaria at Utrecht University (The Netherlands). Tropical prawns were collected by the Dutch Inspectorate for Health Protection and Veterinary Public Health.

2.2. Apparatus

Screw-capped polypropylene tubes (50 ml and 6 ml) were supplied by Sarstedt (Numbrecht, Germany). Series of SPE columns equipped with 75-ml reservoir adapters were processed simultaneously using a vacuum manifold (J.T. Baker). Samples were dried using an evaporation manifold from Pierce (Rockford, IL, USA).

2.3. Sample preparation

Field and blank samples were homogenized using a household blender (Moulinette, Gouda, The Netherlands). Homogenized blank tissue samples were spiked with MG and LMG at levels indicated in the text, and incubated 15 min at ambient temperature prior to extraction and further processing as described below. A number of such samples referred to as quality controls, were prepared and used to monitor the extraction procedure and to determine the accuracy of the method as described in the text. Samples were processed immediately or stored as indicated in the text.

Volumes of 2 ml McIlvaine solution at pH 3.0, 100 μl 1 *M* *p*-TSA and 50 μl 1 mg/ml methanolic TMPD were vortex-mixed with 2.0 g homogenate

weighed into a 50-ml screw-capped polypropylene tube. Then, 12 ml acetonitrile were added and, after vortex-mixing, tissues were extracted for 15 min on a platform shaker operating at 500 rpm. The suspension was centrifuged for 5 min at 3400 *g* at 15 °C, and the supernatant was collected in a 50-ml screw-capped polypropylene tube. Residual material was mixed with 2 ml McIlvaine solution at pH 6.0 followed by addition of 12 ml acetonitrile. The suspension was then mixed for 15 min on a platform shaker operating at 500 rpm followed by centrifugation for 5 min at 3400 *g* at 15 °C. Combined supernatants were vortex-mixed with 6 ml dichloromethane and centrifuged for 5 min at 3400 *g* at 15 °C. After removal of the lower water-phase, the sample was passed under reduced pressure applied to the outlet, through an SPE column at a flow-rate of 0.5 ml/min, which were run dry. The SPE column was washed with 1.5 ml methanol and dried in a stream of N_2(g) for 10 min. Residues of MG were then eluted with 3 ml SPE-eluent (see Section 2.1) into a 6-ml screw-capped polypropylene tube and dried under a stream of N_2(g) at ambient temperature. Residual material was vortex-mixed after addition of 20 μl 10 μg/ml methanolic brilliant green and 980 μl sample-solvent (see Section 2.1) for 20 s, and centrifuged for 5 min at 3400 *g* before HPLC or LC–MS–MS analysis.

2.4. HPLC analysis

Aliquots of 50 μl were analyzed on an HPLC system, which consisted of an HPLC pump (type P100; Thermoquest, San José, CA, USA) operating at a flow-rate of 0.6 ml/min, an autosampler (type Promis; Spark-Holland, Emmen, The Netherlands) equipped with a 100-μl stainless steel loop, and a light absorbance detector (type 785; Applied Biosystems, Foster City, CA, USA) monitoring the effluent at λ=620 nm using a Tungsten light source. The mobile phase comprised a mixture of 50 m*M* sodium perchlorate containing 25 m*M* sodium acetate and 25 m*M* 1-pentanesulfonic acid adjusted to pH 4.0 with acetic acid, and acetonitrile in a ratio of 2:3 (v/v). The mobile phase was leaded through a pre-column oxidation reactor (50×4.6 mm) filled with lead(IV)oxide and celite in a weight ratio of 1:1. This reactor was placed between the pump and the autosampler. The Inertsil ODS-2 HPLC column (100×3.0 mm) was preceded by a guard-column (10×2.1 mm) packed with 8-μm reversed-phase material for such columns (Varian, Bergen op Zoom, The Netherlands). Before detection at λ=620 nm, the effluent was passed through a post-column reactor (20×2 mm) packed with lead(IV)oxide and celite in a weight ratio of 3:1 to convert the colorless leuco-form into the chromophore.

Chromatograms were recorded and processed by Chromquest software package version 2.51 (Thermoquest).

2.5. LC–MS–MS analysis

For LC–MS–MS analysis, 10-μl aliquots were injected on an HPLC system consisting of two HPLC pumps (PE200 series, Applied Biosystems), an autosampler (PE 200 series, Applied Biosystems) and an API-365 MS detector (Applied Biosystems). The LC–MS–MS was controlled by Analyst software package (version 1.1). The HPLC column was a Phenomenex Luna C_{18} (50×2 mm, Phenomenex, Torrance, USA) with guard column (SecurityGuard C_{18}, 4×2 mm, Phenomenex). The mobile phase was a mixture of 50 m*M* ammonium acetate, pH 4.4, and acetonitrile (2:3, v/v) flowed at 200 μl/min. Before the eluent was introduced into the ionization chamber for MS detection of the analytes, the effluent was passed through a post-column reactor filled with lead(IV)oxide and celite (see Section 2.4).

The MS was equipped with an ESI interface operating at an ionization voltage of +5500 V and a source temperature of 400 °C. The entrance, declustering and focusing potentials were set at −9, 40 and 180 V, respectively. Tandem MS analysis was performed using the multi-reaction-monitoring (MRM) mode. Collision energy (CE) was optimized for each product-ion trace measured. The following traces were monitored: m/z 329.3→m/z 165.0 (CE 75 V), m/z 329.3→m/z 208.0 (CE 55 V), m/z 329.3→m/z 313.3 (CE 45 V) and for the internal standard m/z 385.0→m/z 341.0 (CE 50 V) (Fig. 1).

2.6. Data evaluation

Concentration data of standard solutions were regressed on peak areas of the signals divided by that

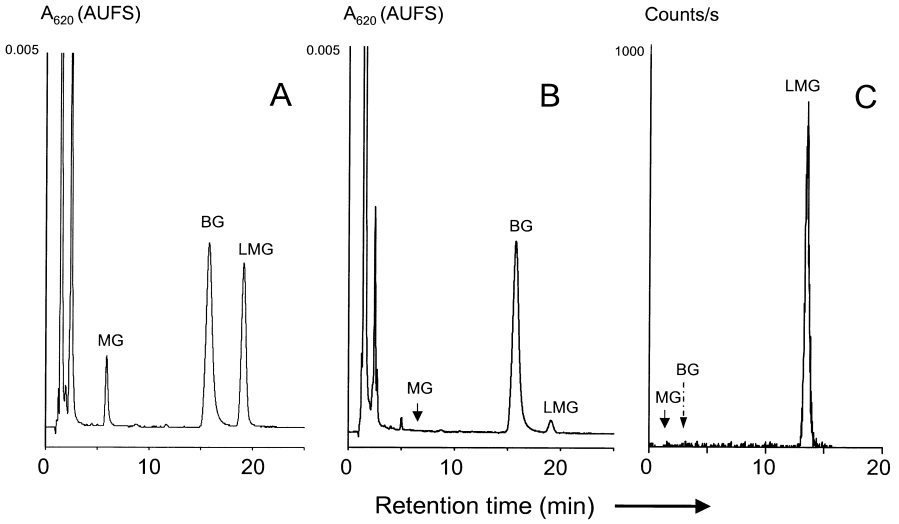

Fig. 1. Typical elution HPLC profiles of MG residues extracted from (A) salmon spiked at 20 μg/kg LMG and MG each; (B) residue-incurred salmon fillet (2.9 μg/kg). Analysis of the residue-incurred salmon was repeated using the LC–MS–MS system as shown in (C); the profile shows the monitoring of the $m/z = 329.5 \rightarrow m/z$ 313.3 fragmentation. The elution positions of MG, LMG and the internal standard brilliant green (BG) are indicated. Note: BG is not detected in the $m/z = 329.5 \rightarrow m/z$ 313.3 trace (C) and its position is therefore depicted as an under broken arrow.

of brilliant green, by least-square regression analysis. The content of residues of MG were calculated by interpolation of the standard curve after division of peak area of the analyte by that of brilliant green.

3. Results and discussion

Standard curves of the regressed concentration points gave squared correlation coefficients better than 0.9999 for each MG and LMG separate and as a mixture. The time interval between the moment of spiking and moment of sample processing had a dramatic influence on the recovery of MG, viz. 81, 63, 60, 54 and 45% for intervals of 1, 15 and 30 min, and 1 and 2 h, respectively. Since no corresponding signal for LMG was observed, MG was apparently not reduced in vitro into LMG during this incubation. In contrast, little effect was observed on the recovery of LMG, viz. 100, 96, 93, 93 and 95% for intervals of 1, 15 and 30 min, and 1 and 2 h, respectively. Furthermore, inspection of the liquid extraction process learned that homogenization using ultraturrax or the household blender gave similar and best recoveries of the MG residues compared to ultrasonification and no support at all (results not shown).

A close inspection of chromatograms of processed fish fillet samples, showed significant satellite peaks (up to 15% of the parent peak area) suggesting degradation of MG and especially of LMG. This degradation is most probably de-methylation of the dye [10–12]. Comparison of the analysis of spiked samples with that of the standards revealed that this breakdown originated partly from the working-up procedure, as suggested earlier [10]. In this study, it was found that addition of TMPD and ascorbic acid to the laboratory samples could largely reduce this degradation in all tested tissues.

The accuracy of the test was optimized in the method development for the lipophilic LMG derivative and not for the hydrophilic parent molecule, as LMG is the prevalent residue observed long after administration of MG [13,14]. In tracing illegal use of MG, LMG seems therefore the best indicator molecule in analysis strategies, as suggested before [3]. Recoveries determined at various levels in catfish, eel, trout, turbot and south-Asian prawns reflected this choice (Table 1). Namely, whereas in the case of MG, recoveries ranged from 43.8±1.9%

Table 1
Averaged recoveries (%) and standard deviation (%) of MG and LMG from spiked catfish, eel, rainbow trout and turbot muscle tissues, and from prawns at different spiking levels, as determined by HPLC analysis at λ 620 nm

Concentration (μg/kg)	Aquatic animal species									
	Catfish		Eel		Trout		Turbot		Prawn	
	MG	LMG	MG	LMG	MG	LMG	MG	LMG	MG	LMG
2.5	53±2 (5)[a]	103±2 (5)[a]	63±4 (6)	105±14 (6)	48±5 (6)	96±2 (6)	Np	Np	59±5 (6)	86±15 (5)[b]
5.0	52.3±1.8 (3)	92±5 (3)	61.5±1.1 (3)	88±7 (3)	43.8±1.9 (3)	91.7±1.4 (3)	61.4±1.7 (3)	93±3 (3)	62±10 (3)	96±15 (3)
10	Np[c]	Np	Np	Np	52±5 (3)	91.4±0.5 (3)	62±3 (5)	92±4 (5)	Np	Np
20	53±3 (2)[a]	92.4±0.7 (2)	Np	Np	Np	Np	64±3 (6)	70±3 (6)	Np	Np
50	Np	Np	Np	Np	48±4 (3)	89±3 (3)	Np	Np	Np	Np
100	58±3 (2)[a]	94.0±0.9 (2)[b]	67.7±1.3 (3)	89.1±0.8 (3)	53±3 (6)	94±2 (6)	56±8 (3)	88±8 (3)	62±3 (3)	95±3 (3)
500	Np	Np	Np	Np	53.4±1.7 (3)	91.1±1.3 (3)	Np	Np	Np	Np
1000	Np	Np	Np	Np	57±5 (3)	91±3 (3)	Np	Np	Np	Np
2000	56.6±0.7 (3)	93±2 (3)	63±3 (3)	91±3 (3)	49±2 (6)	90±2 (6)	Np	Np	63±4 (3)	94±6 (3)

The number of samples is given within brackets.
[a] The number of samples is reduced because the processing of a single sample failed.
[b] The series contained a single outlier as assessed by a *Q*-test.
[c] Np, analysis not performed for this sample.

(at 5 μg/kg in rainbow trout) to 67.7% (at 100 μg/kg in eel), recoveries of LMG were between 86±15% (at 2.5 μg/kg in prawn) and 105±14% (at 2.5 μg/kg in eel). Recoveries from non-processed salmon fillets were in the same range, namely 57±4 and 95±5% for MG and LMG, respectively. These recoveries from smoked and canned salmon were 72±4% (MG)/84±2% (LMG) and 87±8% (MG)/94±2% (LMG), respectively.

The limit of detection (LOD) was determined on the basis of the response of blank samples plus three times the standard deviation (SD) compared with that of spiked samples, and was 1 μg/kg for both MG and LMG. This sensitivity is in accordance with those reported for similar HPLC analysis approaches, in which LODs were reported at 2 μg/kg (MG and LMG) [3,8], and at 2 μg/kg (MG) and 1 μg/kg (LMG) [15].

The use of an LC–MS–MS system was then explored for the confirmation of HPLC analysis results. It was found that sensitivity was greatly improved with preservation of accuracy and precision found for the HPLC–Vis approach (results not shown). Conversion of LMG in the cation MG using a PbO_2 reactor in-line significantly supports ESI-MS. Exploiting the MRM mode by monitoring a number of product-ion tracks (see Section 2.5), the LOD was found at 0.2 μg/kg in tissues. This is much better than the LOD reported for particle beam LC–MS at 20 μg/kg [16,17], for GC–MS at 5 μg/kg [9,17], but comparable for LC–ESP-MS at 0.4 μg/kg (MG) and 0.5 μg/kg (LMG) [8]. Isotope dilution LC–APCI-MS was more sensitive for detection of MG, giving LODs at 0.02 μg/kg (MG) and 0.5 μg/kg (LMG) [10].

Robustness of the method, which was apparently omitted in many method descriptions so far, was investigated. For that reason, chromatographic parameters of MG and LMG were determined on three different HPLC columns. Each HPLC column was examined with three mobile phase batches (Table 2). All chromatograms were similar, with best analytical performance for the determination of LMG. Furthermore, samples were processed over different SPE-columns from different suppliers, or with different batch numbers from the same supplier (Table 3). All batches aromatic sulfonic acid type SPE columns obtained from J.T. Baker gave acceptable recoveries, as well as the SCX cartridges purchased from IST and Varian (Bond Elut). The other columns gave rise to unsatisfactory recovery results.

The possible interference of dyes, of which several are used in animals as well [17] on the determination of MG and LMG, was investigated. Samples containing 1 μg/ml MG and 1 μg/ml LMG were fortified with either crystal violet, Janus green, methylene blue, methylene green, thionine or toluidine blue each at 100 μg/ml. The response of

Table 2
Influence of three eluent batches on different HPLC column production batches upon the elution characteristics of MG and LMG

Residue	HPLC column batch number	Number of plates (n)	Resolution (R)	Tailing (A)	Capacity (k')[a]
MG	356526	$2.14\times10^3\pm0.14\times10^3$	1.84±0.05	1.75±0.03	2.62±0.04
	356412	$1.87\times10^3\pm0.11\times10^3$	1.50±0.07	1.72±0.02	2.55±0.02
	356551	$2.0\times10^3\pm0.2\times10^3$	1.78±0.09	1.75±0.02	2.56±0.10
LMG	356526	$6.99\times10^3\pm0.06\times10^3$	Base-line	1.08±0.01	22.7±0.3
	356412	$5.841\times10^3\pm0.005\times10^3$	Base-line	1.05±0.01	21.7±0.3
	356551	$6.48\times10^3\pm0.08\times10^3$	Base-line	1.00±0.005	22.2±0.6

Inertsil ODS-2 HPLC columns (100×3 mm) were tested.
[a] k' was determined as $(t_R-t_0)/t_0$.

samples with and without the addition of non-MG chromophores were compared and showed nil or minor interference (Table 4). Thionine gave a considerable interference at the elution position of LMG at this high spiking level. This colorant is a metabolite of methylene blue and may be used as an antimicrobial agent (antiseptic and antidote) in animals [17]. It must be noted however that this dye was not taken through the clean-up procedure, which may have eliminated it from the test sample.

To determine the inter-day variation, 2-g trout samples were spiked with MG and LMG, stored at −20 °C until the day of analysis. Samples were analyzed on six different days showing very similar results even when another investigator executed the analysis (Table 5). However, analysis of variance (ANOVA) of these data sets revealed a significant difference at 95% reliability for the recoveries found at the different days. The SD for the determination of MG and LMG within days was 3 and 5%, respectively. At the other hand, the SD for the determination of MG and LMG between days was 7 and 10%, respectively.

In the control of illegal veterinary use of the drug, stored aquatic products may have to be sampled. Alternatively, samples may have to be stored until

Table 3
Recovery of MG and LMG using different production batches of SPE columns from a single supplier, and from different suppliers

Producer and column type	Batch no. of column	Recovery (%)±SD (%) of	
		MG	LMG
J.T. Baker, aromatic sulfonic acid	H 40579	48.8±0.6	94±3
J.T. Baker, aromatic sulfonic acid	J 40570	54.6±1.0	96.6±0.7
J.T. Baker, aromatic sulfonic acid	J 51558	54±2	95.4±1.0
J.T. Baker, aromatic sulfonic acid	K 36551	52.8±1.3	91.2±0.5
J.T. Baker, aromatic sulfonic acid	H 49081	50±5	91±4
J.T. Baker, sulfonic acid	E18101	33±3	101±15
IST, SCX	5032405^{E}A	41±6	96±3
Supelco, supelclean LC-SCX	SP 1266 C	Nd[a]	Nd
Merck, SCX	L988333	Nd	Nd
Varian, SCX	173478	48±2	102±3

In each case, the test was carried out in 3-fold. All tested columns were 3 ml/500 mg versions. For this purpose, rainbow trout muscle tissue was spiked at 25 μg/kg MG and 25 μg/kg LMG.
[a] Nd, the recovery of this residue was below the limit of quantification.

Table 4
Interference of several dyes upon the determination of MG and LMG

Potential interference	Relative difference	
	MG (%)	LMG (%)
Crystal violet	7	8
Janus green	3	0.3
Methylene blue	8	5
Methylene green[a]	13	2
Thionine	0.4	17
Toluidine blue	15	8

The relative signal difference is obtained by comparison of the peak heights of MG and of LMG in the absence and in the presence of the possible interferences.

[a] The elution profile of only methylene green showed a peak at the elution position of MG.

analysis. Such conditions may affect the final level in the laboratory sample. Stability of MG and LMG during storage at +4 °C (for 1, 2 and 4 days) and at –20 °C (for 1, 2 and 6 months) was therefore investigated (Tables 6 and 7). Whereas degradation of MG residues was less than 20% within 6 months at −20 °C, residues were apparently very unstable at +4 °C, as only approximately 60% MG and LMG was recovered after 4 days. Freeze–thaw cycles also affected stability of the residues considerably (Table 8).

As some of the products, which are sampled are smoked or canned, thus possibly heat-treated, the influence of a higher temperature on the recovery of MG and LMG was studied as well. Salmon spiked at 35 μg/kg MG and 50 μg/kg LMG was homogenized, and after a withdrawal period of 15 min, heated

Table 6
Retrieval (%) of MG and LMG after storage for 1, 2 and 6 months at −20 °C

Months of storage	Residue			
	MG		LMG	
	50 μg/kg	500 μg/kg	50 μg/kg	500 μg/kg
1	88±2	94±5	93±2	98.0±1.3
2	82±3	90.9±1.6	92.1±0.7	93.7±0.6
6	82.9±1.8	92±5	93±2	90±5

Rainbow trout samples were spiked at 50 and at 500 μg/kg for both MG and LMG in triplicate for each analysis run. Results were corrected for the recovery found for the quality controls spiked at identical levels and analyzed on the same day.

Table 7
Retrieval (%) of MG and LMG after 1, 2 and 4 days of storage at +4 °C

Days of storage	Residue	
	MG	LMG
1	76±3	95.6±1.9
2	68±3	80±2
4	58.6±1.5	60.5±1.6

Rainbow trout samples of 2 g each were spiked at 35 μg/kg MG and 50 μg/kg LMG in triplicate for each day of analysis. Results were corrected for the recovery found for the quality controls spiked at identical levels and analyzed on the same day.

Table 5
Recoveries of MG and LMG from rainbow trout muscle tissues spiked at 35 μg/kg MG and at 50 μg/kg LMG stored at −20 °C

Day of analysis	Recovery (%)±SD (%) of MG		Recovery (%)±SD (%) of LMG	
	Stored sample[a]	QC sample	Stored sample[a]	QC sample
Day 1	54±4	49±6	86±4	97±2
Day 2	55±3	59±4	87±3	95±4
Day 3	53±2	53.2±0.3	90±4	96±2
Day 4	52±2	54±6	84±7	97.5±1.2
Day 5	48±3	54±2	79±5	94±4
Day 6	56.1±1.1	58±3	89±3	99±3
Another investigator[c]	50±2[b]	52±2	79±5	101±8

Six fresh samples at day 1, and otherwise six thawed samples were analyzed together with three freshly prepared quality control samples (QC) spiked at identical MG and LMG concentrations at six different days to monitor the performance of the method. SD, standard deviation.

[a] Recoveries of test samples were not corrected for the recovery of the QC samples.

[b] Number of samples is four instead of six, because of mal-spiking: one sample did not contain a detectable amount of the analyte, whereas the other contained the double amount.

[c] The complete method for analysis of six samples was carried by another investigator in the same research laboratory.

Table 8
Retrieval (%) of MG and LMG after one, two and three freeze–thaw cycles

Number of cycles	Residue	
	MG	LMG
0	109±4	98.6±1.2
1	84±3	86±6
2	76±8	84±6

For this purpose, 18 rainbow trout samples were spiked with MG and LMG at 35 and 50 μg/kg, respectively, and frozen at −20 °C. After 1 day all samples were thawed, six were analyzed and the remainder frozen again. This cycle was repeated twice. Results were corrected for the recovery found for the quality controls spiked at identical levels and analyzed on the same day.

at 70 °C for 20 min. Analyses of these samples showed that heat-treatment affected the recovery of especially MG (29±2%) and to some extent that of LMG (81±1%) compared to recoveries from raw salmon (see above). In contrast, a field sample containing incurred residues at 2.9 μg/kg LMG (see below), showed only 1.9±0.3 μg/kg after this treatment. Surprisingly, spiking after heating gave higher recoveries for MG (81%) in correspondence with those recoveries from canned salmon, which is heated in the can.

Fresh whole trout and eel, and fresh, smoked and canned salmon fillets collected at different local retailers, fish-shops and fish-markets to spread the chance of obtaining a single suspected batch, were investigated. Surprisingly, the analysis uncovered the presence of residues of MG in many fish samples (Table 9). Analysis of rainbow trouts with unknown origin according to the vendors, but most likely cultured, showed 13 out 18 fish contained traces of LMG (at <15 μg/kg) with HPLC–Vis. Eel, which is prevalently cultured for food production, was found to be contaminated as well. Five out of 10 fish contained LMG (at <10 μg/kg). Analysis of fresh salmon fillets showed a single sample to contain 2.9 μg/kg. As expected, results of the analysis of this filet in duplicate by HPLC–Vis (2.86 and 2.96 μg/kg, respectively) and in duplicate by LC–MS–MS (2.91 and 2.93 μg/kg, respectively) revealed excellent similarity. In addition, four out of six other fresh salmon fillets were shown to contain LMG just above LOD (0.20 to 0.26 μg/kg) at the different product-ion tracks using LC–MS–MS. It must be noted that MG residues are very unstable in these refrigerated fresh fillets (see above), suggesting that these levels may have been significantly higher at the time of slaughter. In none of the five smoked salmon samples nor in the eight canned salmon

Table 9
Summary of the results from the analysis of 18 most probably cultured trout, 10 eel and 20 salmon products

Species	LMG (μg/kg)	Species (/product)	LMG (μg/kg)	Species (/product)	LMG (μg/kg)
Trout	n.d.[a]	Trout	n.d	Smoked salmon	n.d. (MS)
Trout	1.3	Trout	2.8	Smoked salmon	n.d. (MS)
Trout	2.1	Eel	1.7	Smoked salmon	0.2 (MS)
Trout	2.1	Eel	9.7	Smoked salmon	n.d. (MS)
Trout	n.d.	Eel	n.d.	Smoked salmon	n.d. (MS)
Trout	n.d.	Eel	n.d.	Smoked salmon	n.d. (MS)
Trout	1.8	Eel	n.d.	Smoked salmon	n.d. (MS)
Trout	n.d.	Eel	7.0	Smoked salmon	n.d. (MS)
Trout	1.6	Eel	n.d.	Canned salmon	n.d. (MS)
Trout	14.9	Eel	1.5	Canned salmon	n.d. (MS)
Trout	1.9	Eel	2.4	Canned salmon	n.d. (MS)
Trout	2.8	Eel	n.d.	Canned salmon	n.d. (MS)
Trout	2.2	Fresh salmon	2.9	Canned salmon	n.d. (MS)
Trout	2.8	Fresh salmon	0.2 (MS)	Canned salmon	n.d. (MS)
Trout	4.2	Fresh salmon	0.2 (MS)	Canned salmon	n.d. (MS)
Trout	1.5	Smoked salmon	0.2 (MS)	Canned salmon	n.d. (MS)

The fish were purchased from local retailers and vendors. Samples were analyzed by HPLC–Vis or by LC–MS/MS as indicated by "MS" in brackets. Only LMG was detected and reported in this table.

[a] n.d., not detected.

samples, could LMG be detected. These products are heated, which impairs recovery (see above).

The described HPLC method has been furnished with numerous data, which is scarcely given in other public method presentations, showing its ability to detect MG in several fish species. The collected data demonstrate that the sample processing and HPLC analysis, backed-up by LC–MS–MS analysis when necessary, is amenable in control and inspection programs to secure food free from this veterinary drug.

4. Notation

CE, collision energy
LMG, leuco-malachite green
LOD, limit of detection
MG, malachite green
PKD, proliferative kidney disease
SCX, strong cation exchanger
SD, standard deviation
SPE, solid-phase extraction
TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine dihydrochloride
p-TSA, *para*-toluenesulfonic acid

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