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Original Research Paper

DETERMINATION OF MALACHITE GREEN AND LEUCOMALACHITE GREEN IN TROUT AND CARP MUSCLE BY LIQUID CHROMATOGRAPHY WITH VISIBLE AND FLUORESCENCE DETECTION

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Summary: A fast and specific method for determination of malachite green (MG) and its major metabolite leucomalachite green (LMG) in trout and carp muscle is described. MG and LMG residues were extracted from fish muscle with an acetonitrile-buffer mixture and isolated by partitioning into dichloromethane. Extracts were then cleaned up on solid-phase extraction (SPE) columns. Chromatographic separation was achieved by using reverse-phase column with an isocratic mobile phase consisting of acetonitrile and acetate buffer (0.01M, pH 4.1). MG was detected with an absorbance detector (λ= 618 nm), while a fluorescence detector (λ_{ex} = 265 nm and λ_{em} = 370 nm) was used for detection of LMG. Both detectors were connected on-line which allowed simultaneous analysis of a sample extract for MG and LMG. The method was validated according to Commission Decision 2002/657/EC. The mean recoveries of MG and LMG from muscle fortified at three levels (2, 3, 4 µg/kg) were 55% and 74%, respectively. Relative standard deviations of the mean at all fortification levels were less than 15% and 13% for MG and LMG, respectively. With the described method 33 samples of fish bought in local shops and fish farms between August 2004 and April 2005 were analysed. Seven samples showed detectable amounts of residues.

Key words: antifungal agents–therapeutic use; rosaniline dyes–chemistry; aniline compounds–chemistry; drug residues–analysis–methods; food analysis; chromatography, liquid; trout; carps; fish

Introduction

Malachite green (MG) is a triphenylmethane dye, originally used as a dyeing agent in the textile industry, but it have been also widely used in aquaculture industry as an anti-fungal, anti microbial and anti-parasitic agent for many decades (1). It is used in the form of bath treatment, either on its own or synergistically with formalin (1, 2). MG is easily absorbed into tissues during waterborne exposure and rapidly transformed to its reduced form, leucomalachite green (LMG). LMG in tissues may be eliminated at a rate that is dependent on the fat content (3, 4). Because of its suspected carcinogenic, mutagenic and teratogenic properties, MG has never been registered as a veterinary drug for fish treatment in the European Union (5). The ban on its use necessitated a robust and reliable analytical method for determination of residues of MG in fish muscle. According to the European Commission, methods for determining MG in fish tissues should meet the minimum required performance limit (MRPL) of 2 µg/kg for the sum of MG and LMG (6). Several analytical approaches for determination of MG residues have been published. For the determination of MG residues, high-performance liquid chromatography (HPLC) with post-column unit for oxidation of LMG and an absorbance detector for the detection of MG has been commonly used. The post-column reactors were filed with lead (IV) oxide (7-11) or 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (12). As an alternative to lead (IV) oxide, an electrochemical cell was used (13). As mass spectrometers

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become more common, methods based on mass spectrometry (MS) have also been reported for the confirmation of suspected MG residues (4, 14-17). However, the post-column reactor has been used with mass spectrometry as well, because detection of MG is more sensitive compared to LMG (4, 16, 17). The use of a fluorescence detector for the detection of LMG has also been reported (13, 18).

Although MS methods provide greater sensitivity and residue confirmation for the detection of MG and LMG in fish, reliable and robust methods are needed to routinely screen numerous laboratory samples without straining the resources of sophisticated LC-MS instruments. In this report, we present a selective, sensitive and relatively fast LC method with visible and fluorescence detection for simultaneous determination of LMG and MG in trout and carp. Because LMG is detected with a fluorescence detector, the post-column oxidation procedure is not needed. The method was validated according to Commission Decision 2002/657/EC (19), it is suitable for routine analysis and provides a detection limit below 1.0 μg/kg. To check if MG is still illegally used in the fish farming industry due to the low cost, easy availability, and high efficacy against fungus, bacteria and parasite, 33 samples of fish collected randomly at different fish farms, fish shops and fish markets were analysed with the presented method.

Materials and methods

Chemicals

Organic solvents used were LC grade and other chemicals were of analytical grade unless stated otherwise. Acetonitrile and methanol were obtained from J.T. Baker (Deventer, the Netherlands), hydroxylamine (HA) hydrochloride, p-toluenesulfonic acid (p-TSA), ammonium acetate (extra pure), triethylamine (TEA), glacial acetic acid and dichloromethane were from Merck (Darmstadt, Germany).

An acetate buffer (0.1 M, pH 4.5) was prepared by dissolving 7.708 g ammonium acetate in about 800 ml of water, adjusting the pH to 4.5 with acetic acid and diluting the solution to 1000 ml. An acetate buffer (0.01 M, pH 4.1) used for the mobile phase was prepared by dissolving 0.771 g ammonium acetate in about 800 ml of water. 2 ml TEA was added before a pH adjustment to 4.1 with acetic acid and dilution to 1000 ml.

Standards and standard solutions

MG oxalate and crystal violet chloride (CV) were purchased from Riedel-de Haën (Seelze, Germany) and LMG and leuco crystal violet (LCV) from Aldrich (Steinheim, Germany). Individual stock solutions of MG, CV, LMG and LCV at 100 μg/ml were prepared in methanol, taking into account the active substances. These solutions were combined and diluted in methanol to prepare an intermediate standard solution of 1 μg/ml. Working standard solutions were prepared by several dilutions of intermediate standards with methanol for recovery experiments and with a mixture of acetate buffer (0.1 M, pH 4.5), acetonitrile and hydroxylamine hydrochloride solution (2.5 mg/ml) (40:40:20, v/v/v) for calibration.

Sample preparation equipment

The instruments used were an Ultra-Turrax T25 (IKA-Labortechnik, Janke & Kunkel, Germany), a temperature controlled Minifuge T centrifuge (Heraeus, Osterode, Germany) and a vacuum rotary evaporator Büchi Model R-205 (Osterode, Germany). For the extraction, a linear shaker Vibromix 314 EVT (Tehtnica, Železniki, Slovenia) was used. Solid-phase extraction was carried out on a vacuum manifold for Visiprep™ (Supelco, Bellefonte, USA).

Sample collection and preparation

33 samples of fish were collected randomly between August 2004 and April 2005 at different fish farms, fish shops and fish markets in Slovenia. Among 33 collected samples, 8 were imported from other EU countries. 13 samples of rainbow trout, 12 samples of brown trout, 6 samples of brook trout and 2 samples of carp were examined in the study. Fish samples (2–3 fish/sample) were filleted and the bones removed. The muscle tissue with skin was homogenized, frozen and stored at –18°C before analysis.

Extraction and clean-up

A homogenized sample (10 g) was weighed into a 50 ml centrifuge tube. The spiked sample was prepared by adding a known amount of working standard solution to the fish muscle. Three milliliters of aqueous 0.25 g/ml HA, 5 ml of aqueous 0.05 M p-TSA and 5 ml of 0.1 M ammonium acetate buffer (pH 4.5) were added to each sample and homogenized for 1 min with an Ultra-Turrax at 13000 rpm. Then 20 ml of acetonitrile were added, the tube was capped and shaken vigorously on a platform shaker for 5 min. The tube was centrifuged at 2000 *g* for 10 min at 20°C. The supernatant was decanted into a 100 ml centrifuge tube. Another 20 ml acetonitrile

were added to the sample pellet and the sample was shaken and centrifuged under the same conditions as before. The supernatants were combined in a 100 ml centrifuge tube.

To the supernatant, 20 ml of deionised water and 20 ml of dichloromethane were added, and the tube was shaken vigorously on a platform shaker for 5 min and centrifuged at 1400 *g* for 10 min at 10°C. The lower dichloromethane layer was transferred into a 500 ml round bottom flask. The extraction with 20 ml of dichloromethane was repeated and the lower layer was transferred into the same flask. The combined dichloromethane extract was then concentrated on a rotary evaporator at 65°C to approximately 5 ml. At this point, the sample was kept overnight in the dark.

J.T Baker neutral alumina (6 ml, 1 g) and Varian Bond Elut PRS-SPE columns (3 ml, 500 mg) were pre-washed with 5 ml acetonitrile. With an adapter, the alumina SPE column was placed on top of the PRS-SPE column. This assembly was then attached to the solid-phase extraction vacuum manifold. To the sample extract in the 500 ml round bottom flask, 2 ml of dichloromethane were added. The flask was swirled to dissolve the residue. 5 ml of acetonitrile were added to the flask prior to pouring the sample extract onto the columns. The flask was rinsed 2 times with 5 ml of acetonitrile, which was also applied to the columns. At this moment the alumina SPE column was discarded and the PRS-SPE column was rinsed with 2 ml of water followed by 1 ml of a mixture of ammonium acetate buffer (0.1 M, pH 4.5) and acetonitrile (50:50, v/v). The MG and LMG were eluted from the PRS-SPE column with 2 ml of the above mixture of buffer and acetonitrile, and collected in a graduated tube containing 0.5 ml of 2.5 mg/ml HA in water. The volume of the eluate was adjusted to 2.5 ml with the mixture of buffer and acetonitrile. The content was mixed well and filtered through a 0.45 μm filter before HPLC analysis.

Liquid chromatography

An Agilent 1100 HPLC system (USA) consisting of a quaternary pump, a vacuum degasser, an automatic injector, a column thermostat, a fluorescence detector (GA1321A) set at λ_{ex} = 265 nm and λ_{em} = 370 nm and Hewlett Packard (Atlanta, USA) LC-95 UV/Vis detector set at 618 nm were used. Both detectors were connected on-line. The ChemStation software controlled the LC system and processed the data.

Three different analytical columns were tested: SynChropak SCD-100, 5 μm, 150x4.6 mm was obtained from Eprogen (Darien, USA); Luna Phenyl-Hexyl, 5 μm, 250x3.0 mm from Phenomenex (Torrance, USA); and PerkinElmer-HS 5 C-18, 5 μm, 150x4.6 mm from PerkinElmer (Boston, USA). The optimal mobile phase was selected by varying the proportion of acetonitrile and pH of 0.01 M acetate buffer.

SynChropak SCD-100 analytical column was selected for the determination of MG and LMG in fish. A C18 guard cartridge (4x3 mm, Phenomenex, Torrance, USA) was used prior to the analytical one. The mobile phase was acetonitrile and ammonium acetate buffer (0.01 M, pH 4.1) (62:38, v/v). The injected volume was 100 μl and the separation of the analytes was accomplished with a flow of 1 ml/min at 27°C. Quantification was performed using the external standard method and was based on peak area.

Method validation

Validation of the procedure was carried out in accordance with Commission Decision 2002/657/EC (19). The linearity of the LC-Vis/FLD response was checked across a wide concentration range from 5 to 40 ng/ml. Concentrations of standards were applied to the abscissa and the corresponding peak areas to the ordinate. The least squares method was used to create the calibration curves, which were evaluated by regression and correlation. Linearity in matrix was also checked. The chromatographic response was recorded from the samples of trout meat with standard additions in the whole range from 1 μg/kg to 5 μg/kg, with five calibration points. The absence of interfering endogenous compounds around the retention times of the analytes was verified with an analysis of blank samples of different carp and trout muscle samples and also with an analysis of blank samples fortified with CV, LCV, MG and LMG at 4 μg/kg. Precision (repeatability and within-laboratory reproducibility) was checked with an analysis of blank samples of trout and carp. Samples were fortified with MG and LMG at 2, 3 and 4 μg/kg. At each level the analyses were performed with six replicates. The analyses of fortified blank samples of trout were repeated on two other days close to each other, with the same instruments, batches of reagents and the same operators and on two other days with the same instruments but with different batches of reagents and different operators. Repeatability and within-laboratory reproducibility were expressed with standard deviation and relative standard deviation. The decision limit (CCα) was de-

termined as the corresponding concentration at the y-intercept of the calibration curve plus 2.33-times the standard deviation of the within-laboratory reproducibility at 2 μg/kg. The detection capability (CCβ) was the corresponding concentration at the CCα plus 1.64 times the standard deviation of the within-laboratory reproducibility at 2 μg/kg.

Results and Discussion

Method development

Most methods published so far are based on the post-column oxidation of LMG to MG and detection of MG using visible light absorption. As mentioned before, oxidation has been commonly performed using PbO_2 (7-11). While the methods that used PbO_2 reactor provide adequate sensitivity and recovery, the manually prepared lead-oxide reactor can be plagued by problems, including rapid depletion and peak broadening, which lead to a decrease in method sensitivity (12). For this reason we avoided the oxidation of LMG to MG and thus both analytes were detected as such. MG and LMG were detected simultaneously, MG with an absorbance detector and LMG with a fluorescence detector. To optimize the analytical procedure the absorption spectrum of the MG solution and fluorescence spectra of the LMG solution was measured. According to the absorbance spectrum, the absorbance detector was set at 618 nm. That wavelength is usually used for detection of MG (7, 8, 11). With regard to previously published methods (13, 18) and to the apex in the recorded fluorescence spectrum, the wavelength of emission was raised from 360 to 370 nm, while the wavelength of excitation was left the same (256 nm). This change improved the sensitivity of the method.

Three different analytical columns were tested for effective resolution of MG, CV, LMG and LCV. CV is a triphenylmethane dye with very similar structure to MG and anti-parasitic and anti-microbial properties (13). On all tested columns MG and CV were separated with baseline resolution, but the separation of LMG and LCV was not easy to achieve. The best resolution of LMG and LCV was achieved on a Phenyl-Hexyl column at 27°C with a mobile phase of 55% acetonitrile and 45% 0.01 M acetate buffer (pH = 3.6) and a flow-rate at 1 ml/min. At this condition, the resolution (R) between LMG and LCV was higher than 2.5, but the retention time was relatively long (t_r for LMG was 22 min). On a SynChropak SCD-100 column at 27°C with 62% acetonitrile and 38% 0.01 M acetate buffer (pH = 4.1) as mobile phase and a flow-rate at 1 ml/min, near-baseline separation between LMG and LCV was achieved (R = 1.4) in less than 11 min (Figure 1). But on a PerkinElmer-HS 5 C-18 column using a different mixture of ammonium acetate buffer (0.01 M) and acetonitrile as the mobile phase, the resolution between LMG and LCV was always less than 1. Because the aim of our study was to develop a fast and economical method, we chose the SynChropak SCD-100 column, which was also used by Rushing and Hansen (13).

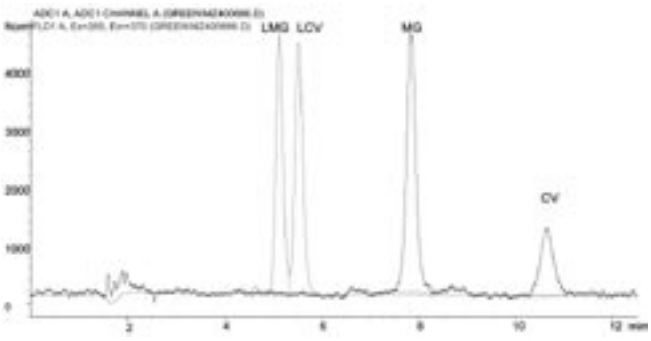

Figure 1: Typical chromatogram of mixed solution (50 ng/mL)

We tested two procedures of sample extraction and sample clean-up. First we followed the procedure of Rushing and Hansen (13). With this procedure, extraction of MG and LMG was performed using a mixture of ammonium acetate buffer and acetonitrile; liquid-liquid extraction into dichloromethane and solid-phase extraction followed. With regard to the original method we decreased the weight of the sample and the volumes of chemicals used for the extraction by a factor of two. Although the method was performed more economically, the consumption of organic solvents was still very high and the time used for sample preparation was long, especially because separatory funnels were used for liquid-liquid extraction. For these reasons we performed liquid-liquid extraction according to the procedure described by Halme and co-workers (4). The sample extraction in this method is performed with smaller volumes of organic solvents. Instead of separatory funnels for liquid-liquid extraction, solvents were separated by centrifugation and the lower layer was transferred using the pipette. All these changes reduced the time used for sample preparation and decreased the recovery of LMG from 90% to 78%, which was still acceptable for the determination of veterinary drug residues in food. The recovery of MG remained the same (around 50–60%).

Method validation

Chromatograms demonstrating the selectivity of the procedure are shown in Figure 2, 3 and 4. In Figure 2 chromatograms of blank sample and spiked sample of trout with LMG and LCV at 4 μg/kg are shown. LMG and LCV were separated with almost baseline resolution. From chromatograms of blank and spiked sample of trout (Figure 3) and blank and spiked sample of carp (Figure 4) with MG and LMG at 4 μg/kg, it is evident that no interfering peaks from endogenous compounds were found at the retention times of the target analytes. Hence the selectivity of the procedure is considered satisfactory.

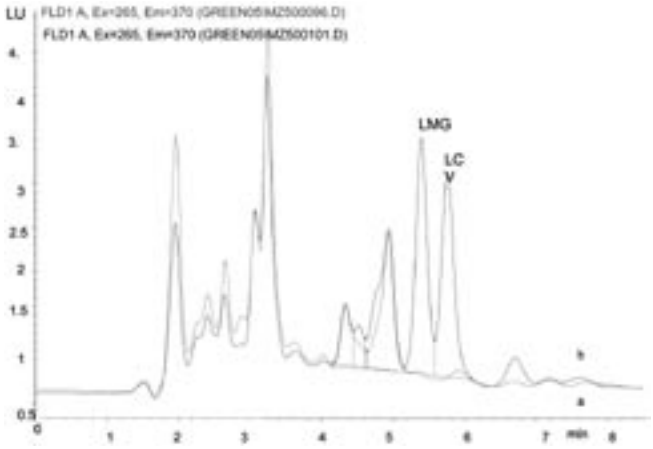

Figure 2: Chromatograms of (a) trout muscle and (b) trout muscle with a standard addition of LMG and LCV (4 μg/kg)

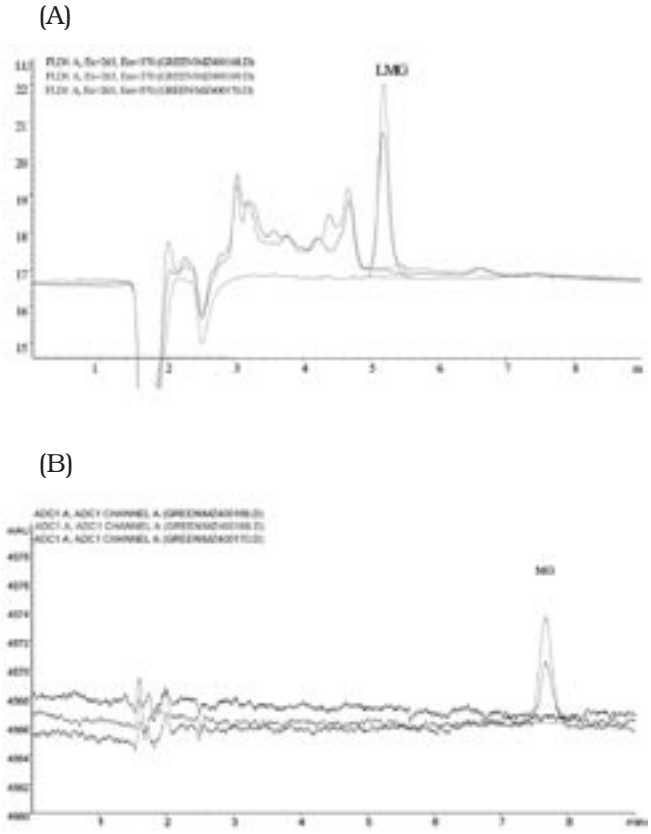

Figure 3: Selectivity for the determination of MG and LMG in trout muscle tissue. (A) LMG: standard mixture 16 ng/mL, blank and blank with standard addition of 4 μg/kg (B) MG: standard mixture 16 ng/mL, blank and blank with standard addition of 4 μg/kg.

The results of the linearity of the LC-Vis/FLD response and matrix calibration curve are reported in Table 1. The standard calibration curves are linear over the range 5-40 ng/ml and the matrix calibration curves were linear over the range 1-5 μg/kg for MG and LMG. The correlation coefficients of the standard and matrix calibration curves were greater than 0.9993 for both MG and LMG.

Table 1: Linearity of MG and LMG determination on standard and matrix level

	Analyte	Slope	Intercept	Correlation coefficient
Standards	MG	6.59	-3.67	0.9995
	LMG	3.49	-1.16	0.9999
Trout muscles	MG	8.56	-3.87	0.9993
	LMG	5.62	1.32	0.9998

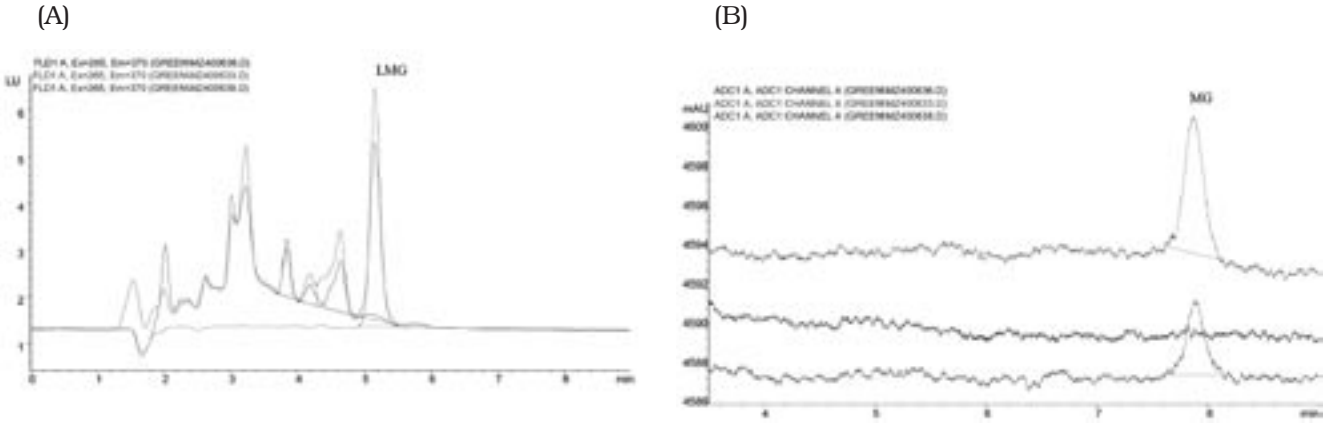

Figure 4: Selectivity for the determination of MG and LMG in carp muscle tissue. (A) LMG: standard mixture 16 ng/mL, blank and blank with standard addition of 4 μg/kg. (B) MG: standard mixture 16 ng/mL, blank and blank with standard addition of 4 μg/kg.

The average recoveries and precision of repeatability and within-laboratory reproducibility at 2, 3 and 4 μg/kg are summarised in Table 2 for trout samples and in Table 3 for carp samples. For MG the average recovery was in the range between 47% and 62%. The recovery for LMG was higher than for MG and ranged between 67% and 78%. In Table 2 and 3 we can see that there were almost no differences between trout and carp samples in recovery and standard deviation of measurements. Accuracy of the method was checked by participating in the proficiency testing programme organized by FAPAS® (Series 2 Round 59, 2004). The z-score lied between -2 and 2, hence the result was considered as satisfactory.

Table 2: Accuracy and precision of MG and LMG determined in fortified trout muscle at three concentration levels (n = 18 at each level)

	Parameter	Fortification level (μg/kg)		
		2	3	4
MG	Repeatability			
	Mean concentration (μg/kg)	1.23	1.83	2.32
	s (μg/kg)[a]	0.15	0.17	0.30
	RSD (%)[b]	11.9	9.2	3.0
	Recovery	62	61	58
	Within-lab reproducibility			
	Mean concentration (μg/kg)	1.10	1.69	2.21
	s (μg/kg)	0.16	0.21	0.26
	RSD (%)	14.5	12.8	11.6
	Recovery	55	57	55
LMG	Repeatability			
	Mean concentration (μg/kg)	1.44	2.02	2.94
	s (μg/kg)	0.17	0.10	0.16
	RSD (%)	12.0	4.6	5.5
	Recovery	72	67	74
	Within-lab reproducibility			
	Mean concentration (μg/kg)	1.54	2.10	2.94
	s (μg/kg)	0.19	0.23	0.18
	RSD (%)	12.1	10.7	6.2
	Recovery	77	72	74

[a] s = Standard deviation
[b] RSD = Relative standard deviation

The CCα's were 0.6 μg/kg for MG and 0.5 μg/kg for LMG. The CCβ's were 1.0 μg/kg and 0.9 μg/kg, respectively. The method thus meets the European Commission Performance requirements of 2 μg/kg. This sensitivity is also in accordance with those reported for similar HPLC analysis approaches (7, 9, 10, 12), besides the method that was published by Mitrowska and co-workers (18). CCα's of that LC-UV/Vis method were lower (0.15 μg/kg for MG and 0.13 μg/kg for LMG), and similar to CCα's of the LC-MS/MS methods (4, 14, 15).

Table 3: Accuracy and precision of MG and LMG determined in fortified carp muscle at three concentration levels (n = 6 at each level)

	Parameter	Fortification level (μg/kg)		
		2	3	4
MG	Repeatability			
	Mean concentration (μg/kg)	0.93	1.45	2.05
	s (μg/kg)[a]	0.10	0.21	0.19
	RSD (%)[b]	10.8	13.6	9.3
	Recovery	47	52	51
LMG	Repeatability			
	Mean concentration (μg/kg)	1.54	2.33	3.01
	s (μg/kg)	0.10	0.05	0.10
	RSD (%)	4.7	2.3	3.3
	Recovery	77	78	75

[a] s = Standard deviation
[b] RSD = Relative standard deviation

Fish Samples Analysis

The method presented was tested on a number of samples collected randomly at different fish farms, fish shops and fish markets. Together with each series of samples a fortified blank sample with MG and LMG at 2 μg/kg to control recovery was analysed. The results were corrected for recovery of respective series and then used as final results. Surprisingly, 7 out of 33 samples contained residues of MG (Table 4), i.e. in excess of CCα, and 1 out of 7 samples containing residues of MG was imported from another EU country. In four samples the sum of MG and LMG was higher than 2 μg/kg (MRPL-value). In all seven samples we detected LMG, but in two of them we also found MG. These two samples of brown trout contained high concentrations of LMG (28 and 18 μg/kg), and therefore the presence of MG was not surprising.

Table 4: Summary of results from the analysis of 33 samples

Species	MG (μg/kg)	LMG (μg/kg)	Species	MG (μg/kg)	LMG (μg/kg)
Brown trout	nd[a]	nd	Brown trout	nd	nd
Rainbow trout	nd	1.5	Carp	nd	nd
Brown trout	nd	nd	Brown trout	nd	2.8
Rainbow trout	nd	0.8	Rainbow trout	nd	nd
Brown trout	0.8	28	Brown trout	nd	nd
Brook trout	nd	nd	Rainbow trout	nd	nd
Brook trout	nd	nd	Brown trout	nd	2.2
Brown trout	nd	nd	Rainbow trout	nd	nd
Brown trout	nd	nd	Rainbow trout	nd	nd
Brook trout	nd	nd	Rainbow trout	nd	nd

Species	MG (μg/kg)	LMG (μg/kg)	Species	MG (μg/kg)	LMG (μg/kg)
Rainbow trout	nd	nd	Rainbow trout	nd	nd
Brook trout	nd	nd	Carp	nd	nd
Rainbow trout	nd	nd	Brown trout	nd	nd
Rainbow trout	nd	nd	Brown trout	2.0	18
Brook trout	nd	nd	Rainbow trout	nd	nd
Rainbow trout	nd	nd	Brook trout	nd	nd
Brown trout	nd	1.2			

[a] nd = not detected

Conclusion

A sensitive and specific method for the determination of MG and LMG residues in trout and carp muscle has been described. The obtained validation results indicate the accordance of the method performance with Commission Decision 2002/657/EC (19). The CCα and CCβ for MG and LMG are below the MRPL of 2 μg/kg. The collected data demonstrate that the sample processing and HPLC analysis is amenable in control and inspection programs to secure food free of this veterinary drug.

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References

1. Srivastava S, Sinha R, Roy D. Toxicological effects of malachite green: review. Aquatic Toxicol 2004; 66: 319-29.

2. Alderman DJ. Malachite green: a review. J Fish Dis 1985; 8: 234-47.

3. Plakas SM, El Said KR, Stehly GR, Gingerich WH, Allen JL. Uptake, tissue distribution, and metabolism of malachite green in chanel catfish (Ictalurus punctus). Can J Fish Aquat Sci 1996; 53: 1427-33.

4. Halme E, Lindfors E, Peltonen K. Determination of malachite green residues in rainbow trout muscle with liquid chromatography and liquid chromatography coupled with tandem mass spectrometry. Food Addit Contam 2004; 21: 641-8.

5. Council Regulation of 26 July 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medical products in foodstuffs of animal origin (2377/90/EC). Off J Eur Commun 1990; L 224: 1-8.

6. Commission Decision of 22 December 2003 amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin (2004/25/EC). Off J Eur Commun 2004; L 6: 38-9.

7. Plakas SM, El Said KR, Stehly GR, Roybal JE. Optimization of liquid chromatographic method for determination of malachite green and its metabolites in fish tissues. J AOAC Int 1995; 78 (6): 1388-93.

8. Allen JL, Gofus JE, Meinertz JR. Determination of malachite green residues in the eggs, fry, and adult muscle tissue of rainbow trout (Oncorhynchus mykiss). J AOAC Int 1994; 77 (3): 553-7.

9. Brandt AM, Lrke S. Determination of malachite green and leucomalachite green by HPLC with post-column oxidation. In: Proceedings of the Euroresidue V. Conference on residues of veterinary drugs in food. Noordwijkerhout: Federation of European Chemical Societies, 2004: 358-62.

10. Hajee CAJ, Haagsma N. Simultaneous determination of malachite green in eel plasma using post-column oxidation. J Chromatogr B 1995; 669: 219-27.

11. Roybal JE, Pfenning AP, Munns RK, Holland DC, Hurlbut JA, Long AR. Determination of malachite green and its metabolite, leucomalachite green, in catfish (Ictalurus punctatus) tissue by liquid chromatography with visible detection. J AOAC Int 1995; 78 (2): 453-7.

12. Andersen WC, Roybal JE, Turnipseed SB. Liquid chromatographic determination of malachite green and leucomalachite green (LMG) residues in salmon with in situ LMG oxidation. J AOAC Int 2005; 88 (5):1292-8.

13. Rushing LG, Hansen Jr. EB. 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. J Chromatogr B 1997; 700: 223–31.

14. Lee KC, Wu JL, Cai Z. Determination of malachite green and leucomalachite green in edible goldfish muscle by liquid chromatography-ion trap mass spectrometry. J Chromatogr B 2006; 843: 247-51.

15. Walle L, Diaz C, Zanocco AL, Richter P. Determination of the sum of malachite green and leucomalachite

green in salmon muscle by liquid chromatography-atmospheric pressure chemical ionisation-mass spectrometry. J Chromatogr A 2005; 1067: 101-5.

16. Scherpenisse P, Bergwerff AA. Determination of residues of malachite green in finfish by liquid chromatography tandem mass spectrometry. Anal Chim Acta 2005; 529: 173-7.

17. Tarbin JA, Barnes KA, Bygrave J, William H, Farrington H. Screening and confirmation of triphenylmethane dyes an their leuco metabolites in trout muscle using HPLC-Vis and ESP-LC-MS. Analyst 1998; 123: 2567-71.

18. Mitrowska K, Posyniak A, Zmudzki J. Determination of malachite green and leucomalachite green in carp muscle by liquid chromatography with visible and fluorescence detection. J Chromatogr A 2005; 1089: 187-92.

19. Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC). Off J Eur Commun 2002; L 221: 8-36.

DOLOČANJE MALAHITNEGA IN LEVKOMALAHITNEGA ZELENILA V MESU POSTRVI IN KRAPOV S TEKOČINSKO KROMATOGRAFIJO S SPEKTROMETRIČNO IN FLUORESCENČNO DETEKCIJO

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Povzetek: Uvedli smo hitro in specifično metodo za določanje malahitnega zelenila (MG) in njegovega glavnega metabolita levkomalahitnega zelenila (LMG) v mesu postrvi in krapov. Ostanke MG in LMG smo iz mesa rib ekstrahirali z mešanico acetonitrila in acetatnega pufra, nato pa iz te mešanice v diklorometan. Ekstrakt smo očistili še z metodo tekoče-trdno. Kromatografsko ločitev smo izvedli na analitski koloni z reverzno fazo z uporabo izokratske mobilne faze, sestavljene iz mešanice acetonitrila in acetatnega pufra (0.01 M, pH 4.1). MG smo zaznali s pomočjo spektrometričnega detektorja (λ = 618 nm), LMG pa s pomočjo fluorescenčnega detektorja (λ_{ex} = 265 nm in λ_{em} = 370 nm). Uporaba obeh detektorjev omogoča istočasno analizo ekstrata vzorca na MG in LMG. Metodo smo validirali po postopku, opisanem v Evropski direktivi 2002/657/EC. Povprečni izkoristek za MG in LMG, dobljen z analizo obogatenih vzorcev mesa rib na treh koncentracijskih nivojih (2, 3, 4 µg/kg), se je gibal med 55 % in 74 %. Relativna standardna deviacija povprečja je bila na vseh treh koncentracijskih nivojih manjša od 15 % za MG in 13 % za LMG. S predstavljeno metodo smo analizirali 33 vzorcev rib, kupljenih v lokalnih trgovinah in na farmah med avgustom 2004 in aprilom 2005. V sedmih vzorcih smo ugotovili prisotnost ostankov MG.

Ključne besede: antimikotiki–uporaba za zdravljenje; rosanilinska barvila–kemija; anilinske spojine–kemija; zdravila, ostanki–analize–metode; kromatografija, tekočinska; hrana, analize; postrv; krapi; ribe