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Determination of malachite green and leucomalachite green in carp muscle by liquid chromatography with visible and fluorescence detection

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Abstract

A liquid chromatography-VIS/FLD method for the analysis of malachite green (MG) and its major metabolite, leucomalachite green (LMG) in carp muscle has been described. The method consists in an extraction with acetonitrile-buffer mixture followed by partioning with dichloromethane. Clean up and isolation were performed on SCX solid phase extraction (SPE) column. Chromatographic separation was achieved by using phenyl-hexyl column with an isocratic mobile phase consisting of acetonitrile and acetate buffer (0.05 M, pH 4.5) (60:40, v/v). Liquid chromatography with absorbance detector ($\lambda = 620$ nm) was used for the determination of MG while LMG was detected by fluorescence detector ($\lambda_{ex} = 265$ nm and $\lambda_{em} = 360$ nm). The both detectors were connected on-line which allowed direct analysis of a sample extract for MG and LMG without the need for any post-column procedure. The whole method has been validated, according to the EU requirements (Commission Decision 2002/657/EC). Specificity, stability, decision limit (CCα), detection capability (CCβ), accuracy and precision were determined. Average recoveries of MG and LMG from muscle fortified at three levels (0.5, 1 and 2 μg/kg) were 62% (range from 60.4 to 63.5%) and 90% (range from 89.0 to 91.5%), respectively. Relative standard deviations (RSD) of recoveries at all fortification levels were less than 10.9 and 8.6% for MG and LMG, respectively. The calculated CCα for MG and LMG were 0.15 and 0.13 μg/kg, and CCβ were 0.37 and 0.32 μg/kg, complying with the minimum required performance limit (MRPL) of 2 μg/kg (sum of MG and LMG).

Keywords: Malachite green; Leucomalachite green; Residues; Carp; Analytical method

1. Introduction

Malachite green (MG) is a cationic triphenylmethane dye which has been used worldwide as fungicide and ectoparasicide in cultured fish eggs, fingerlings and adult fish since 1930s [1]. Due to its potential animal carcinogenicity, mutagenity and teratogenicity [2,3], MG has never been registered as a veterinary medicine in the European Union [4]. Because MG is easily available at low cost and highly effective, there is still concern about its illegal use. In fish, among them in carp, MG is easily absorbed into tissues during waterborne exposure and extensively metabolized to the reduced, colourless compound, leucomalachite green (LMG) Fig. 1. The main metabolite is stored in fat, and so the elimination rate constant of LMG from fish is strongly dependent on the fat content [5,6]. According to the European Commission, methods which can be used for the determination of MG residues in fish muscles should meet a minimum required performance limit (MRPL) of 2 μg/kg for the sum of MG and LMG [7].

Current methods for the determination of MG and LMG in fish tissues are based on liquid chromatography (LC), mainly with visible (VIS) detection. The parent compound has λ_{max} at 620 nm, whereas the leuco form has λ_{max} at 265 nm, making difficult to determine MG and LMG using the same condition [8]. Simultaneous LC-VIS determination of both forms is possible by post-column oxidation of LMG to MG using cartridge containing lead(IV) oxide (PbO_2) [9,10]. Electrochemical oxidation has been used as an alternative to PbO_2 [11]. For confirmatory purposes an analytical procedures utilise

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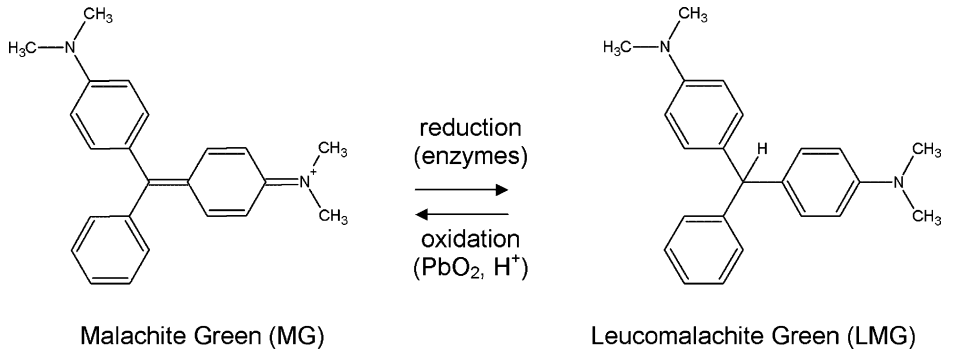

Fig. 1. Structures of MG and LMG.

the detection by mass spectrometry (MS) with liquid or gas chromatography, which does not demand post-column oxidation of LMG [12,13]. However, the PbO_2 reactor has been used with MS, because detection of MG is more sensitive comparing with LMG [8,14]. The use of fluorescence (FLD) detector for LMG determination has been also reported [15]. In our previous paper we described the method which allows direct analysis of MG and LMG in rainbow trout muscle without the need for any post-column procedure.

Although there are many methods to determine MG residues in a variety of fish species, none of the methods has been developed for carp, a very popular fish in Poland and Central and East Europe. This paper reports the development of a selective and sensitive LC method with VIS and FLD detection for the simultaneous analysis of MG and LMG residues in carp muscles. The method is less laborious and more convenient for the determination of MG and LMG from matrix and has been validated according to the quality criteria of Commission Decision 2002/657/EC and the method shows that such sample processing can provide the proper results [16].

2. Experimental

2.1. Reagents and chemicals

All chemicals used were of analytical grade unless stated otherwise. Ammonium hydroxide (25%), acetic acid (99.5%), acetonitrile, acetonitrile (LC grade), dichloromethane and methanol (LC grade) were from J.T. Baker (Deventer, The Netherlands). Ultrapure water was filtered through a Milli-Q system Millipore (Bedford, MA, USA).

The Strata SCX (strong cation-exchange) disposable SPE columns (3 ml, 500 mg) were purchased from Phenomenex (Torrance, CA, USA).

L(+)-ascorbic acid (99%), hydroxylamine hydrochloride, malachite green oxalate, leucomalachite green, *p*-toluenesulfonic acid monohydrate (98.5%), sodium acetate (99%) were obtained from Sigma-Aldrich (Steinheim, Germany). Other substances used for test specificity were obtained from commercial sources.

An acetate buffer (0.05 M, pH 4.5) was prepared by dissolving 4.1 g of sodium acetate in a 1000 ml volumetric flask with about 800 ml of water and completing flask to 1000 ml. The pH was adjusted to 4.5 with acetic acid. This solution was filtered by a 0.45 μm nylon filter.

An ascorbic acid solution (1 mg/ml) was prepared by dissolving 50 mg of L(+)-ascorbic acid in 50 ml of methanol. A hydroxylamine (HA) solution (25%) was prepared by dissolving 25.0 g of hydroxylamine hydrochloride in 100 ml of water.

A *p*-toluenesulfonic acid (*p*-TSA) solution (1 M) was prepared by dissolving 19.0 g of *p*-TSA in 100 ml of water.

2.2. Standard solutions

Individual stock solutions of MG and LMG at 1 mg/ml were prepared in acetonitrile (stable for at least 3 months), taking into account the content of active substances. These solutions were combined and diluted in acetonitrile to prepare an intermediate standard solution of 1 μg/ml (stable for at least 3 months).

For quantification, calibration and recovery experiments working standard solutions (5, 10 and 100 ng/ml) were prepared by serial dilution of the intermediate standard solution with a mixture of acetate buffer (0.05 M, pH 4.5), acetonitrile and ascorbic acid solution (1 mg/ml) (47.5:47.5:5, v/v/v) (stable for at least 1 month). All standard solutions were made in amber volumetric flasks and stored at 4 °C.

2.3. Sample preparation equipment

The instruments used were a Mettler Toledo AX 205 and PR 803 analytical balance (Greifensee, Switzerland), a Metrohm 780 pH meter (Herisau, Switzerland), a Zipperer X1030 homogenizer (Staufen, Germany), a Heraeus Varifuge 3.0R cooling centrifuge (Osterode, Germany), and a IKA Labortechnik MS2 vortex mixer (Wilmington, NC, USA). Solid-phase extraction was carried out on a Baker vacuum manifold for 12 columns equipped with 75 ml reservoir adapters (J.T. Baker, Phillipsburg, NJ, USA). Samples were dried using a Reacti-Therm III heating module and a Reacti-Vap evaporator from Pierce (Rockford, Il, USA)

2.4. Sample preparation

The carps were obtained from the local market. The fish was filleted, the skin and bones were removed, and the muscles were minced and deep-frozen before being analysed.

An accurately weighed 5 g amount of the carp muscle was placed into 50 ml centrifuge tube. A 0.5 ml of HA solution (25%), 0.5 ml of *p*-TSA solution (1 M), 5 ml of acetate buffer (0.05 M, pH 4.5) were added and the sample was homogenized for 1 min at 10 000 rpm using a homogenizer. Then, 20 ml of acetonitrile was added and the homogenization was repeated. The tube was centrifuged at 4000 rpm for 10 min at 10 °C and the supernatant was collected. The extraction with 20 ml of acetonitrile was repeated and followed by centrifugation (with the same conditions as before), and the supernatants were combined. Dichloromethane (10 ml) was added to the supernatant and the sample was vortex-mixed and centrifuged (with the same conditions as before). The organic phase was passed through the SCX SPE column. Before extraction, the column was conditioned with 3 ml of mixture containing acetonitrile and dichloromethane (80:20, v/v). The analyte-containing column was washed with 2 ml of acetonitrile and dried under vacuum for 10 min. MG and LMG residues were eluted with 5 ml of mixture containing acetonitrile and ammonium hydroxide (25%) (90:10, v/v). The eluate was accurately evaporated to dryness under a stream of nitrogen at 50 °C, and the residue was dissolved in 500 μl of mixture of acetate buffer (0.05 M, pH 4.5), acetonitrile and ascorbic acid solution (1 mg/ml) (42.5:42.5:5, v/v/v) and transferred into a vial for chromatographic analysis.

2.5. Liquid chromatography equipment and conditions

The chromatographic system consisted of a Shimadzu Class VP Series high performance liquid chromatograph (Duisburg, Germany) equipped with a quaternary pump, degasser, autosampler and column heater. The absorbance detector was set at 620 nm for MG detection, while the fluorescence detector was set at λ_{ex} = 265 nm and λ_{em} = 360 nm for analysis of LMG. The both detectors were connected online. The CLASS-VP software controlled the LC system and processed the data.

The chromatographic separation was performed with isocratic elution on a Luna phenyl-hexyl (150 mm × 4.6 mm, 5 μm) analytical column (Phenomenex, Torrance, CA, USA). A phenyl-hexyl guard cartridge (40 mm × 2 mm, Phenomenex) was used prior to the analytical one. The mobile phase for LC analyses consisted of acetonitrile and a acetate buffer (0.05 M, pH 4.5) (60:40, v/v). The injected volume was 50 μl and the separation of the analytes was accomplished with flow of 1 ml/min at ambient temperature.

2.6. Method validation

The evaluation of the suitability of the whole procedure for the determination of MG and LMG residues in the carp muscles was carried out in accordance with the Commission Decision 2002/657/EC [16]. Quantification was performed using external standards and was based on peak area.

2.6.1. Specificity

To verify the absence of interfering endogenous compounds around the retention time of analytes, 20 blank samples of different carp muscle were analysed. Besides, known amounts of brilliant green, methylene green, crystal violet, leucocrystal violet, ciprofloxacin and enrofloxacin were spiked into blank carp samples to evaluate possible interferences which may occur in the method.

2.6.2. Accuracy

Three sets, each of six, of blank carp samples were fortified at 0.5, 1 and 2 μg/kg of MG and LMG and analysed. The percentage recovery was calculated as 100 times the measured amount divided by the fortification level.

2.6.3. Precision

Three sets, each of six, of blank carp samples were fortified at 0.5, 1 and 2 μg/kg of MG and LMG. They were analysed on different days close to each other, with the same instruments and the same operators. The standard deviation (SD) and relative standard deviation (RSD) were calculated as repeatability.

Another two sets, each of six, of blank carp samples were fortified at the same levels of MG and LMG as for repeatability determination. They were analysed on different days, with the same instrument and the different operators. The overall SD and RSD were calculated as within-laboratory reproducibility.

2.6.4. Linearity of response

The linearity of the LC-VIS/FLD response was proved with six standard calibration points in the concentration range 0–100 ng/ml of MG and LMG. The standard curves were obtained by plotting the recorded peak area (mAu, %*F*) versus the corresponding concentrations of the standard solutions. The linearity of the standard curves were checked by calculation of the regression line and the correlation coefficient was also calculated.

2.6.5. Matrix calibration curves

Blank carp muscle samples were fortified with working standard solutions of MG and LMG to produce a calibration curves with points equivalent to 0.5, 1, 2, 5 and 10 μg/kg of MG and LMG. All samples were analysed on three different days. The calibration curves were obtained by plotting the recorded peak area (mAu, %*F*) versus the corresponding concentrations of the fortified samples. The linearity of the calibration curves were expressed by the correlation coefficient.

2.6.6. Decision limit and detection capability

The decision limit (CCα) and detection capability (CCβ) were determined by the matrix calibration curve procedure according to the ISO 11843 [17]. CCα was calculated with a statistical certainty of $1-\alpha$ ($\alpha = 0.01$), CCβ was calculated with a statistical certainty of $1-\beta$ ($\beta = 0.05$) to detect the concentration below MRPL.

2.6.7. Stability

The stability of MG and LMG was investigated in standard solutions of analytes (100 ng/ml) in mobile phase with ascorbic acid solution. The standard solutions were stored at three various storage conditions: at 4 °C in amber flasks, at 20 °C in amber flasks and at 20 °C in clear flasks. The concentration of standard solutions was analysed twice and the instrument responses were compared with the peak areas obtained at the moment of solution preparation. The investigation was carried on until 5% of losses of one of the analytes were observed.

The stability of MG and LMG in matrix was investigated in incurred carp samples (10 μg/kg). The material was analysed twice when the samples was fresh and after 1, 2, 6 and 12 months of storing at −20 °C.

3. Results and discussion

3.1. Overview of approach

The experiment intended to develop and validate a simple and sensitive method for the determination of MG and LMG in carp muscle at concentration below 2 μg/kg.

Usually, the determination of LMG is performed by LC with VIS detector using post-column oxidation [9,10]. However, in this approach a further oxidizing of LMG to MG and to other derivatives is possible and other oxidizable coextractives may appear on the chromatogram. The lifetime of the column packed with lead(IV) oxide is not long and its preparation is inconvenient for routine testing. Additionally, the possibility of cross contamination with lead compounds in multiresidue laboratory and problems with utilization may occur. The chromatographic method described in this paper allows the direct analysis of MG and LMG without the need for any post-column procedure. The use of fluorescence detector makes the identification of suspected residues of LMG found in the sample possible in unambiguous way. It is important because LMG exists longer than parent substance in fish muscles after exposing to MG. Typical chromatograms corresponding to a separation under developed conditions is shown in Fig. 2. The obtained peaks are symmetrical and fully separated with the retention times of 7 min for MG and 23 min for LMG, respectively.

3.2. Method validation

The method performance was investigated with respect to various parameters such as specificity, accuracy, precision, decision limit and detection capability.

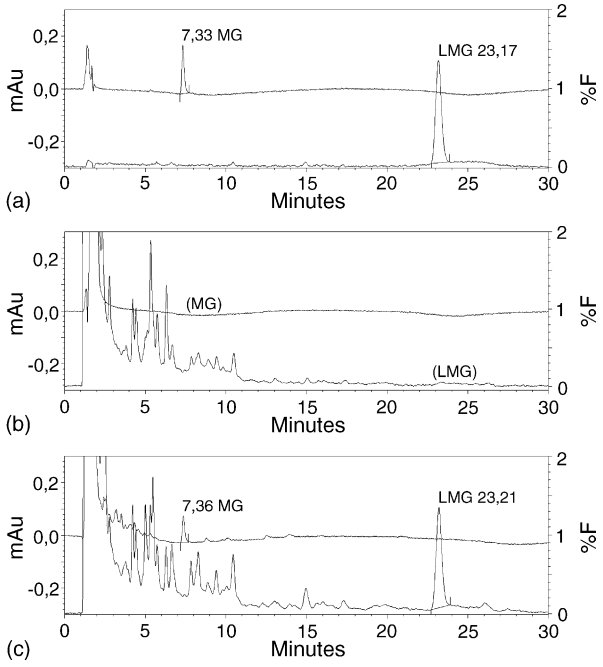

Fig. 2. Typical chromatograms of: (a) a standard solution of MG and LMG at 5 ng/ml; (b) a blank carp muscle; (c) a blank carp muscle fortified with MG and LMG at the level of 0.5 μg/kg.

The specificity was evaluated by the analysis of 20 blank samples of different carp muscles. The chromatogram obtained from the analysis of the blank muscle extract is shown in Fig. 2. No interfering peaks from endogenous compounds were found in the retention time of the target analytes. Additionally, blank carp samples fortified with 2 μg/kg of brilliant green, methylene green, crystal violet, leucocrystal violet, ciprofloxacin, enrofloxacin were analysed. No interferences were observed in the retention windows of MG and LMG in chromatograms of before mentioned substances.

The accuracy, calculated as spike recovery, and precision as repeatability and within-laboratory reproducibility at 0.5, 1 and 2 μg/kg were summarized in Table 1. The results showed good accuracy ranged between 60 and 64% for MG and 89 and 92% for LMG with a good RSD, less than 10.9% under within-laboratory reproducibility.

The results of the linearity of the LC-VIS/FLD response and matrix calibration curve are reported in Table 2. The standard calibration curves were linear over the range 0–100 ng/ml and the matrix calibration curves were linear over the range 0–10 μg/kg for MG and LMG. The correlation coefficients of the standard and matrix calibration curves were above 0.9998 for each MG and LMG.

In the Commission Decision 2002/657/EC, the decision limit means that the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. Likewise detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with statistical certainty of $1-\beta$. The

Table 1
Accuracy and precision of MG and LMG determined in fortified carp muscles at three concentration levels ($n = 18$)

Analyte	Parameter	Fortification level (μg/kg)		
		0.5	1	2
MG	Accuracy			
	Recovery (%)	60.4	63.5	62.8
	Repeatability			
	Mean concentration (μg/kg)	0.30	0.63	1.23
	SD (μg/kg)	0.027	0.055	0.108
	RSD (%)	9.0	8.7	8.8
	Within-lab reproducibility			
	Mean concentration (μg/kg)	0.30	0.64	1.26
	SD (μg/kg)	0.028	0.069	0.097
	RSD (%)	9.4	10.9	7.7
LMG	Accuracy			
	Recovery (%)	89.3	89.0	91.5
	Repeatability			
	Mean concentration (μg/kg)	0.44	0.89	1.83
	SD (μg/kg)	0.032	0.077	0.112
	RSD (%)	7.1	8.6	6.1
	Within-lab reproducibility			
	Mean concentration (μg/kg)	0.45	0.89	1.83
	SD (μg/kg)	0.037	0.068	0.145
	RSD (%)	8.4	7.7	7.9

Table 2
Linearity of MG and LMG determination on standard and matrix level

	Analyte	Slope	Intercept	Correlation coefficient
Standards	MG	4200	−160	0.9999
	LMG	704173	−19112	0.9999
Carp muscles	MG	2672	−279	0.9999
	LMG	656608	−53174	0.9998

values of the CCα and CCβ were determined by the matrix calibration curve procedure according to ISO 11843. The corresponding concentration at the intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept equals the decision limit. The corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit equals the decision limit. The calculated critical concentration of CCα and CCβ are presented in Table 3.

The results of stability MG and LMG in mobile phase with ascorbic acid are reported in Table 4. We observed the degradation of MG and a specially of LMG in the solutions without ascorbic acid (data not shown). This degradation is probably caused by photo-oxidative de-methylation of the dyes [14]. The addition of ascorbic acid to the solution largely

Table 3
CCα and CCβ obtained for MG and LMG in carp muscles

Analyte	CCα (μg/kg)	CCβ (μg/kg)
MG	0.15	0.37
LMG	0.13	0.32

Table 4
Stability of MG and LMG in working standard solution in various storage conditions

Solution	Mobile phase with ascorbic acid
Stability at 4 °C in darkness	
MG	4 Weeks
LMG	4 Weeks
Stability at 20 °C in darkness	
MG	2 Weeks
LMG	1 Week
Stability at 20 °C in daylight	
MG	4 Weeks
LMG	1 Week

reduces the degradation of MG and LMG. In all investigated storage conditions the working standard solution of MG in mobile phase with ascorbic acid was more stable than LMG. As it was found, the storage of working standard solution at 4 °C in amber flasks was the most optimal condition for the stability of the both analytes.

An incurred carp samples (10 μg/kg) stored at −20 °C were analysed twice after 1, 2, 6 and 12 months and the stability of MG and LMG was observed for at least 12 months. After that period, the degradation of the both analytes was less than 10%.

The developed method was checked in proficiency testing programme organized by FAPAS® (series 2 Round 59, 2004) with satisfactory *z*-score result. This result proves good accuracy and reproducibility of the developed method.

4. Conclusions

A sensitive and specific method for the determination of MG and LMG residues in carp muscles has been described. The obtained validation results indicate the accordance of the method performance with the Commission Decision 2002/657/EC. The CCα and CCβ for MG and LMG are below the MRPL of 2 μg/kg. This method is suitable for routine regulatory analysis because the method avoids the use of oxidation step of LMG to MG and can be performed quickly.

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