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A confirmatory analysis of malachite green residues in rainbow trout with liquid chromatography–electrospray tandem mass spectrometry

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Abstract

A quantitative liquid chromatography–tandem mass spectrometric (LC–MS/MS) method has been developed for the determination of malachite green (MG) and its metabolite leucomalachite green (LMG) in fish. Residues were extracted with an acetonitrile–acetate buffer and purified using the automated solid-phase extraction (ASPEC). Residues were analyzed with a reversed-phase LC–MS/MS using a positive-ion electrospray ionisation (ESI). Isotope-labelled leucomalachite green (LMG-D5) was used as an internal standard for the quantification of LMG residues. The related dye, brilliant green (BG) was used as an instrumental standard. Identification and quantification of analytes were based on the ion transitions monitored by multiple reaction monitoring (MRM). The decision limit (CCα) for MG and LMG was 0.13 and 0.16 μg kg^{-1}. The respective detection capabilities (CCβ) were 0.22 and 0.27 μg kg^{-1}. The absolute recovery (repeatability SD_r) was in the range of 58–65% (7.8–11.2%) for MG and 59–68% (9.7–16.9%) for LMG. LMG was quantified also based on the internal standard, giving a recovery (repeatability SD_r) of 103–110% (4.8–9.3%). The method was further evaluated by analyzing a total of 34 fish residue monitoring samples, of which eight samples were found to be non-compliant containing low residues of LMG.

Keywords: Malachite green; Leucomalachite green; Fish; LC–MS/MS; Confirmation

1. Introduction

The use of malachite green (MG) for the control of fungal infections and ectoparasites in fish farming is prohibited in the US and the EU due to its toxicological and potentially carcinogenic nature [1,2]. MG is a triphenylmethane dye, originally used as a dyeing agent in the textile industry but it was also widely used in fish farming industry for many decades. The reason for its popularity derives from its broad antimicrobial spectrum and effectiveness in the prevention and treatment of certain fish diseases compared to other fishery chemicals [3]. Although the use of MG has been prohibited for several years, non-compliant traces of MG residues have still been detected in the residue monitoring schemes performed in the EU Member States [4]. Findings of MG residues in aquaculture products have also been frequently reported in Rapid Alert System for Food and Feed (RASFF) notifications of the European Commission.

As recently stated by the European Commission, any analytical method to be used for the determination of MG residues in meat of aquaculture products has to meet a minimum required performance limit (MRPL) of 2 μg kg^{-1} for the sum of MG and its metabolite leucomalachite green (LMG) [5]. LMG is the reduced form of MG and the most prevalent residue in fish tissues, thus making it the actual target analyte for the monitoring of MG abuse [6–8]. Extraction of MG residues from muscle tissue is usually performed with an acidic buffer and/or with organic solvent after which there is a subsequent liquid–liquid partition step with methylene chloride. Some straightforward methods wholly omit this partition step instead going directly to sample purification [9]. Solid-phase extraction (SPE) has been the most common sample cleanup technique utilised in MG methods.

Due to the strong absorption of MG in the visible range of the spectrum, liquid chromatography (LC) with Vis detection has been used in many applications (e.g. [6,10–15]). However, methods based on mass spectrometry (MS) have become more common for the determination of MG residues. These methods utilise MS ionization techniques such as particle beam with electron ionization [16], atmospheric pressure chemical ioniza-

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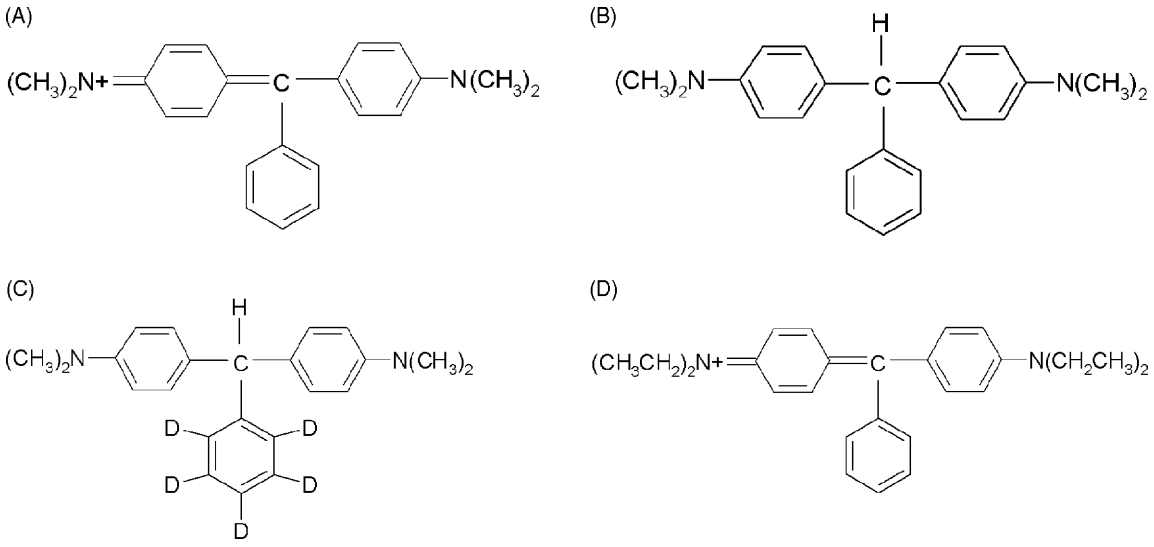

Fig. 1. Structures for malachite green (A), leucomalachite green (B), isotope-labelled leucomalachite green (C) and brilliant green (D).

tion APCI [8] or electrospray ionization ESI [9,17–21]. One gas chromatographic/mass spectrometric (GC–MS) method has also been developed to confirm the presence of the volatile LMG only [22].

In many LC methods, introduction of an in-line post-column oxidation reactor containing lead(IV)oxide (PbO_2) [23] has enabled the simultaneous detection of MG and the colourless LMG metabolite. However, with MS detection, LMG can be determined without the presence of an oxidation reactor [9,21]. In some methods, the oxidation reactor is still included because of its advantages, e.g. in improving the repeatability or sensitivity of the LMG determination [17,18,20]. A previous report from our group described a semi-automated method with a sample preparation procedure suitable for both the quantitative LC–Vis and for the confirmative LC–MS/MS analysis of MG residues [19]. The PbO_2 post-column oxidation reactor for the determination of LMG was used in both detection methods. In this paper we describe a more sensitive, quantitative LC–MS/MS method, which permits the confirmation of LMG by itself without the post-column oxidation. The reliability of the method has been confirmed by a method validation procedure.

2. Experimental

2.1. Chemicals and standard solutions

Organic solvents used were of HPLC grade and other chemicals were of analytical grade. Water was purified via the Milli-Q system (Millipore, Bedford, MA, USA).

MG oxalate Vetranal® (97.4%) was from Riedel-de Haën (Seelze, Germany) and LMG was from Aldrich (Steinheim, Germany). Deuterium-labelled LMG (LMG-D5) was acquired from WITEGA Laboratorien Berlin-Adlershof GmbH (Berlin, Germany) and was used as the internal standard for LMG. The purity of LMG-D5 was >99%, and the enrichment level of LMG-D5 was >98 atom %D, in other words, at least 98% of the molecules of the deuterated compound contained exactly five deuterium atoms. Brilliant green hydrogen sulfate (BG) (≥95%) was obtained from Fluka (Buchs, Switzerland) and was used as the instrumental standard in MS analysis. The structures of the analytes and the standard compounds used in this study are presented in Fig. 1.

Stock standard solutions (500 $\mu g\,ml^{-1}$) were prepared in acetonitrile and renewed once every 2 months. Intermediate standard solutions (500 $ng\,ml^{-1}$) were prepared by diluting the stock standard solutions with acetonitrile at the beginning of each set of analysis. Six standards for calibration were usually prepared in the concentration range of 0.4–3 $ng\,ml^{-1}$ (equivalent to a concentration range of 0.24–1.80 $\mu g\,kg^{-1}$) by diluting the intermediate standard solutions of MG and LMG with a mixture of ammonium acetate (0.1 M pH 4.0) and acetonitrile (40:60) containing methanolic L-(+)-ascorbic acid (10 $\mu g\,ml^{-1}$). Ascorbic acid was added to the standard solutions to prevent the photo-oxidative demethylation of LMG [11]. LMG-D5 and BG were added to all standards at 3.3 $ng\,ml^{-1}$ (equivalent to a concentration of 2 $\mu g\,kg^{-1}$). Standard solutions were stored in a refrigerator and were protected from light.

2.2. Fish samples

More than 95% of the cultured fish in Finland is rainbow trout, the rest being mainly whitefish and char. Rainbow trout was therefore the fish species chosen for method validation. The blank fish sample was bought at the local fish market, and the muscle was cut into small pieces and frozen at −20 °C before use. The unknown fish samples for the study were obtained from freshwater fisheries. They were handled in an identical manner to the blank samples before analysis.

The internal standard LMG-D5 was added to 5 g of an unknown sample at a concentration of 2.0 $\mu g\,kg^{-1}$ before sample preparation. Along with each series of unknown samples, one blank sample and two fortified blank samples at the concentration of 2 $\mu g\,kg^{-1}$ for MG, LMG and LMG-D5 were used as quality control (QC) samples.

Table 1
The MRM parameters used to produce diagnostic ions for the identification

Analyte	Precursor ion	Product ion	Cone voltage (V)	Collision energy (eV)	Relative ion intensity (range)
MG	329	313[a]	55	35	3.4
		208[b]	55	35	(3.1–3.9)[c]
LMG	331	239[a]	38	30	1.5
		316[b]	38	20	(1.3–1.7)[d]
LMG-D5	336	239[a]	38	30	1.6
		321[b]	38	20	(1.3–1.9)[d]
BG	385	341	55	40	

The relative ion intensities were identified based on the area ratios of the diagnostic product ions (the most abundant product ion/the second product ion) obtained from the fortified blank samples of the validation study. In the table, relative ion intensities are expressed as mean values and the observed range is shown in parenthesis.
[a] Ion used for quantification.
[b] Ion used for confirmation.
[c] Meets the maximum permitted tolerance of ±25% for relative ion intensity >20–50% [24].
[d] Meets the maximum permitted tolerance of ±20% for relative ion intensity >50% [24].

2.3. Sample preparation

Samples were prepared as earlier reported [19]. Shortly, samples (5 g) were homogenized with an aqueous ammonium acetate buffer (0.1 M pH 4.0) containing hydroxylamine hydrochloride and *p*-toluenesulfonic acid to optimize the recovery [12,13]. The homogenized samples were extracted with acetonitrile and methylene chloride. However, the extraction procedure differed from the one previously reported [19] in that the partition step with methylene chloride (5 ml) was performed only once. The upper phase was then recovered, concentrated and purified on alumina and propylsulfonic acid SPE columns using the automated solid-phase extraction system (ASPEC, Gilson, Villiers Le Bel, France). Residues were eluted from the PRS column with a mixture of ammonium acetate (0.1 M pH 4.0) and acetonitrile (3 ml 40:60). Ascorbic acid solution (10 μg ml^{-1}) and the instrumental standard BG (3.3 ng ml^{-1}) were added and the sample volume was adjusted to 3 ml with a mixture of acetate buffer and acetonitrile. Finally, the samples were mixed well and filtered before analysis.

2.4. Chromatography

The chromatographic separation was carried out using a Zorbax Eclipse XDB-C18 narrow-bore analytical column (3.5 μm, 150 mm × 2.1 mm i.d., Agilent Technologies, Palo Alto, CA, USA) with an Eclipse XDB-C8 narrow-bore guard column (5 μm, 12.5 mm × 2.1 mm i.d., Agilent Technologies, Palo Alto, CA, USA) in a stainless-steel guard column holder. The mobile phase was a mixture of ammonium acetate (25 mM pH 4.0) and acetonitrile (25:75). The flow-rate of the mobile phase was 200 μl min^{-1} and the system was operated at 40 °C. The injection volume was 10 μl.

2.5. LC–MS/MS analysis

The LC–MS/MS system consisted of a Waters Alliance 2695 Separations Module (Waters, Milford, MA, USA) and a Micromass Quattro Micro tandem mass spectrometer with API source operating in the positive-ion electrospray (ESI) mode (Micromass UK Ltd., Altrincham, Cheshire, UK). The following parameters were used: capillary voltage 2.40 kV; source temperature 150 °C; desolvation temperature 300 °C; N_2 cone gas flow 20 L/h and N_2 desolvation gas flow 500 L/h. Argon was used as the collision gas. Cone voltages and collision energies were determined for each precursor and the product ions separately (Table 1). Ion transitions were monitored with the Multiple Reaction Monitoring (MRM) mode. The LC–MS/MS system was controlled and the data acquired by the MassLynx 4.0 software (Waters).

2.6. Quantification

The quantification of LMG in unknown samples was based on the internal standard (I.S.) method. The response of LMG in the sample was calculated by the ratio of peak area of analyte/(peak area of I.S./added concentration of I.S.) and compared with the calibration curve made with external standards. The correction was automatically calculated by the QuanLynx software (MassLynx 4.0, Waters). The quantification of MG was based on the external standard method with the results being corrected for the mean recovery of MG in QC samples analyzed along with unknown samples. The results were further expressed, when necessary, with uncertainty limits, based on the precision from the validation data.

2.7. Validation

The validation included determination of selectivity, linearity, recovery, accuracy, repeatability, within-laboratory reproducibility, decision limit (CCα) and detection capability (CCβ). The validation was performed following the European Commission Decision 2002/657/EC [24].

3. Results and discussion

A quantitative LC–MS/MS method is presented for the determination of MG and its metabolite LMG in fish muscle as

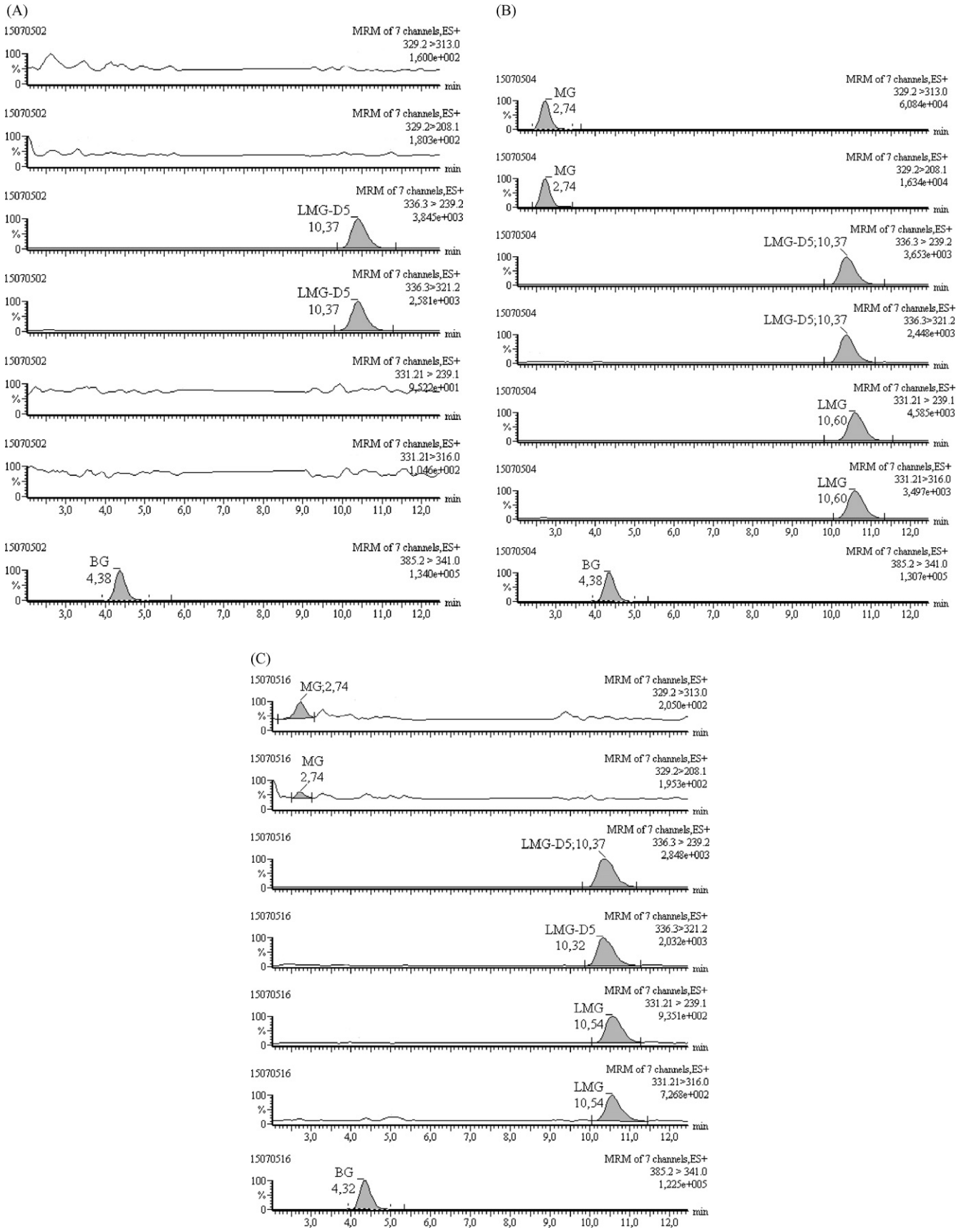

Fig. 2. Ion chromatograms of a blank rainbow trout sample (A), a blank sample fortified at 2 $\mu g\,kg^{-1}$ (B) and an unknown fish sample containing 0.4 $\mu g\,kg^{-1}$ LMG (C). Internal standard (LMG-D5) and instrumental standard (BG) are present in all samples at 2 $\mu g\,kg^{-1}$. Experimental conditions are the same as described in the text.

such. The sensitivity of the method has been markedly improved compared to the previous method [19], as the chromatographic separation was modified by using a different analytical column with a smaller internal volume. The improved sensitivity permitted the identification of LMG via its own product ions, and thus the post-column oxidation with lead(IV)oxide could be omitted. Moreover, the performance of the method was verified by the addition of internal and instrumental standards to control the sample preparation and analyte determination.

The use of an internal standard is valuable in quantitative MS analysis, since this compensates for the losses of analytes in sample preparation and analysis. The isotope-labelled analytes are generally recommended and in the present method, the isotope-labelled LMG-D5 was added to unknown samples before the extraction. This was used for the quantification of the LMG metabolite. The LMG-D5 has been previously used as an internal standard for the determination of both LMG and MG residues with reported accuracies of 103 and 101% [9]. In the present method, the use of LMG-D5 in the quantification of MG was inapplicable due to differences noticed in the behaviour of the analytes in the sample purification and in MS analysis. The quantification of MG was therefore performed by using external standards as previously described (Section 2.6).

Another triphenylmethane dye, BG was added to all standards and the final form of sample extracts to examine the performance of the MS instrument. The added concentration of BG was the same for all standards and samples, so the stability of area response of the monitored ion transition was followed within the analytical run to confirm the success of each run. BG could also be used as an internal standard for MG analysis. BG has been added in another MG method to the purified sample extracts and used as an internal standard in the HPLC or LC–MS/MS analysis of MG and LMG [18,20]. In that method, LMG was converted to MG by PbO_2 post-column oxidation before detection. MG related triphenylmethane dyes such as BG and crystal violet (CV) are also effective but illegal antimicrobials [3], and their use as a standard for MG analysis may thus be problematic, since they may be present in aquaculture samples. Recent findings of CV reported in RASFF notifications indicate that these other triphenylmethane dyes are occasionally detected in aquaculture samples.

The performance of MS instrument may vary during an analytical run, this being reflected in changes in the area responses of analyte mass ions, e.g. in consecutive determinations of a standard solution. The relative ion intensities of the diagnostic product ions, however, remained the same. The analytes also behave differently in the MS analysis depending on their chemical characteristics. In the MS determination, we found that the responses clearly varied more for LMG and LMG-D5 than for MG or BG. This was probably due to ESI ionization which is more suitable for polar, ionic compounds than for non-polar compounds. This was also one reason why LMG-D5 was not used in the quantification of MG in the present method. Matrix effects and adduct formation may also disturb the ESI ionization, but no adduct formation was found to occur while optimizing the MS parameters.

Typical ion chromatograms of a blank rainbow trout sample, a blank sample fortified at 2 μg kg^{-1} and an unknown fish sample are shown in Fig. 2. For the identification, two mass ion transitions and the relative ion intensities of the diagnostic product ions were measured for MG, LMG and internal standard LMG-D5. The most abundant product ion was used for the quantification and the second product ion for the confirmation. For determination of instrumental standard BG, one diagnostic product ion was measured. The relative ion intensities were obtained for every analyte (except for BG) by calculating the area ratios of the diagnostic product ions, based on the determination of the fortified blank samples of the validation study. The MRM parameters and relative ion intensities are presented in Table 1. The identification procedure meets the Commission requirement of four identification points for MG, LMG and LMG-D5 [24].

The selectivity of the method was established by comparing the slopes of calibration curves made with and without matrix. Matrix-matched standards were prepared in the same concentration range as the solvent-based standards but by adding the standard solutions of MG and LMG to a series of purified blank fish extracts (see Section 2.1). Three series of solvent-based and matrix-matched standards were made and analyzed side by side on three different days. Statistical evaluation (two-tailed paired *T*-test, 95% confidence) showed no significant difference in the slopes. As no matrix effect was observed for MS determination, standards without matrix were used for the quantification.

Regression coefficients for the calibration line of MG and LMG were determined from calibrations made with standards without matrix on six different days. Calibration lines were constructed by weighted ($1/x$) linear regression. Data is presented in Table 2. The response showed good linearity in the studied concentration range. The range is rather narrow but nonetheless adequate for the determination of MG residue levels which have generally been below the MRPL (2 μg kg^{-1}) level when used in residue monitoring. When necessary, standards with greater concentrations can be made and the linearity of calibration demonstrated at the relevant concentration range.

The recovery and repeatability of the method were determined by analyzing blank samples fortified at concentration levels 1.0, 1.5 and 2 μg kg^{-1}. In the validation study, six replicate analyses for every concentration level were made on three different days. To determine the absolute recovery of the method, both MG and LMG were quantified based on external standards. The average recovery (repeatability SD_r) calculated from the analyses made on different days was in the range of 58–65% (7.8–11.2%) for MG and 59–68% (9.7–16.9%) for LMG. The

Table 2
Regression coefficients for calibration line ($y = A_0 + A_1x$) of MG and LMG ($n = 6$)

Analyte	A_0[a]	A_1[a]	R^2	SE
MG	20.8 ± 307	3450 ± 964	0.995	178
LMG[b]	−0.04 ± 0.05	1.49 ± 0.16	0.991	26

$A_0 = y$ intercept; A_1 = slope of regression line; x = amount of analyte (ng/ml); y = area response; R^2 = correlation coefficient of regression line; SE = standard error of regression line.

[a] Mean ± SD.

[b] Calibration based on internal standard method.

Table 3
Average recovery (%) and repeatability (%) of MG and LMG from fortified rainbow trout muscle

Fortification (μg/kg)	Average recovery ± SD_r (%)		
	MG	LMG	$LMG_{I.S.quant.}$
1.0	58.0 ± 9.0 (n = 18)	59.4 ± 16.9 (n = 18)	103.3 ± 9.0 (n = 18)
1.5	61.2 ± 7.8 (n = 18)	67.3 ± 10.5 (n = 16)[a]	110.1 ± 4.8 (n = 16)
2.0	64.9 ± 11.2 (n = 17)[a]	67.5 ± 9.7 (n = 15)[a]	103.4 ± 9.3 (n = 15)

SD_r = repeatability standard deviation (%), n = number of samples analyzed. $LMG_{I.S.quant.}$ = quantification was based on the internarnal standard.
[a] Number of samples was reduced because the preparation of a sample or a single analyte failed.

within-laboratory reproducibility (SD_{wlR}) calculated based on the validation data was approx. 21% for MG and 15% for LMG. The repeatability and within-laboratory reproducibility for each concentration level studied were calculated by using single factorial analysis of variances (ANOVA).

LMG is the target analyte in MG residue monitoring, and its determination in samples was optimized by the use of isotope-labelled internal standard LMG-D5 in the quantification. In the validation study, the blank samples were also fortified with LMG-D5 and the LMG results were quantified based on the addition of LMG-D5 to the fortified samples. The average recoveries (repeatability SD_r) representing the accuracy of the method were 103–110% (4.8–9.3%), which we consider as very acceptable. The results of the validation study are presented in Table 3.

The decision limit CCα and the detection capability CCβ were determined by the calibration curve procedure according to ISO 11843 [24]. The CCα was determined as the corresponding concentration at the y-intercept of the calibration curve plus 2.33-times the standard deviation of the within-laboratory reproducibility of the y-intercept. The CCβ was the corresponding concentration at the CCα plus 1.64-times the standard deviation of the within-laboratory reproducibility. The CCα was 0.13 μg kg^{-1} for MG and 0.16 μg kg^{-1} for LMG. The CCβ′s were 0.22 and 0.27 μg kg^{-1}, respectively. The method thus meets the European Commission performance requirements of 2 μg kg^{-1} [5].

The method has been used in performing the national residue monitoring of MG and LMG in Finland. A total of 34 fish muscle samples were analyzed with the method during the early summer 2005. Residues of LMG, that exceeded the CCα, were determined in eight samples. The LMG residue levels in these non-compliant samples were in the concentration range of 0.35–1.54 μg kg^{-1}. No residues of MG above the CCα were found.

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