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Liquid chromatography–tandem mass spectrometry method for the determination of dye residues in aquaculture products: Development and validation

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ABSTRACT

A method is described for the identification and the quantitative determination of the triphenylmethane dyes, malachite green (MG), crystal violet (CV), brilliant green (BG) and leuco malachite green (LMG) and leuco crystal violet (LCV). The analytes were isolated from the matrix by liquid–liquid extraction with acetonitrile. Determination was performed using LC–MS/MS with positive electrospray ionisation. 4 different deuterated internal standards were introduced to improve the quantitative performance of the method. The method has been validated in line with the EU criteria of Commission Decision 2002/657/EC in accordance with the minimum required performance limit (MRPL) set at 2 $\mu g\,kg^{-1}$ for the sum of MG and LMG. For all the monitored compounds, accuracy, intra-day and inter-day precision were determined at each level of fortification (0.5, 0.75, 1.0 and 2.0 $\mu g\,kg^{-1}$). Decision limits CCα and detection capabilities CCβ were calculated according to the standard ISO 11843-2. A study on the applicability of the method was conducted on various aquacultured species with the aim to assess the matrix effects. The presence of residues of leuco brilliant green in fish has also been confirmed from experimental study performed on trout treated with brilliant green, using LTQ-Orbitrap mass spectrometer.

1. Introduction

The triphenylmethane dyes, malachite green (MG) and crystal violet (CV) have been used as antimicrobial, antifungal and antiparasitic agents in aquaculture to treat and prevent fungal and protozoal infections. However, due to their possible carcinogen, mutagen and teratogen effects on animals, these compounds are not registered as veterinary drugs and are authorized neither in EU nor in many other countries all over the world. In exposed fish, MG and CV are extensively metabolized to their reduced leuco forms, leuco malachite green (LMG) and leuco crystal violet (LCV), which are also mutagenic compounds. Another triphenylmethane compound, brilliant green (BG), displays a similar chemical structure and therefore might have similar toxic effects, but no metabolism data are available in the literature for BG. The chemical structures of the compounds are shown in Fig. 1.

In the European Union, analytical methods used to determine these residues in aquaculture products have to be sensitive enough to reach the European minimum required performance limit (MRPL) of 2 $\mu g\,kg^{-1}$ for the sum of MG and LMG [1]. No MPRL was set for other dyes CV or BG. Several published methods have been developed for the analysis of MG, CV, BG and their metabolite in fish. Among the most recent of them, some use an oxidation step to transform the leuco forms in the parent forms allowing to detect the compounds in a unique parent form with liquid chromatography and detection in visible spectrum for the screening step or mass spectrometry detection for the confirmatory step [2–7] Other methods allow the determination of the compounds in their generic form, without post-column oxidation, using in that case liquid chromatography coupled to mass spectrometry for the detection [8–11].

The present study displays a LC–MS/MS method developed for the simultaneous determination and quantification of residues of MG, LMG, CV, LCV and BG in aquaculture products (fish tissues and shrimps). The method allows a simple and fast sample preparation. A study on the applicability of the method was conducted on various matrices to assess the matrix effects. Additionally preparation of treated trouts (*Oncorhynchus mykiss*) with BG was carried out in order to identify the presence of LBG in fish muscle using LTQ-Orbitrap mass spectrometry.

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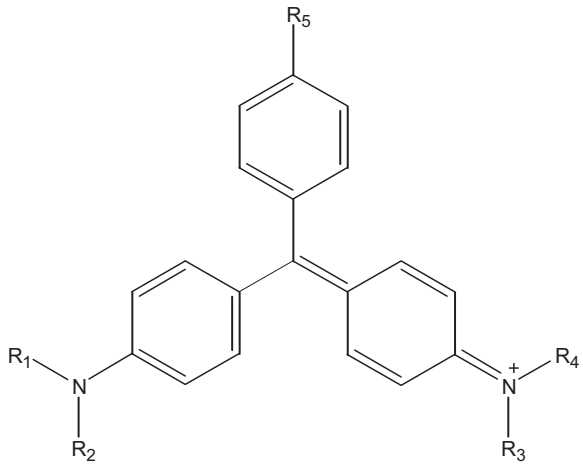
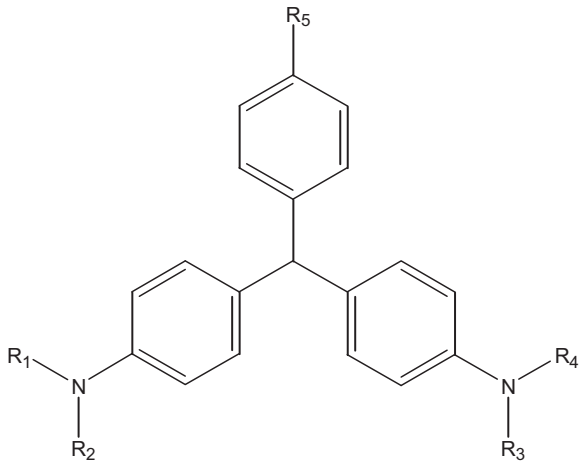


Malachite green: $R_1 = R_2 = R_3 = R_4 = CH_3$; R5 = H
Brilliant green : $R_1 = R_2 = R_3 = R_4 = CH_2CH_3$; R5 = H
Crystal violet : $R_1 = R_2 = R_3 = R_4 = CH_3$; R5 = $N(CH_3)_2$



Leuco malachite green: $R_1 = R_2 = R_3 = R_4 = CH_3$; R5 = H
Leuco brilliant green : $R_1 = R_2 = R_3 = R_4 = CH_2CH_3$; R5 = H
Leuco crystal violet : $R_1 = R_2 = R_3 = R_4 = CH_3$; R5 = $N(CH_3)_2$

Fig. 1. Chemical structures of the triphenylmethane dyes and their leuco forms.

2. Experimental

2.1. Reagents and chemicals

All reagents and solvents used were of analytical grade or HPLC grade. Acetonitrile was supplied by Fisher (Leicestershire, England). Hydroxylamine chlorhydrate and magnesium sulfate anhydrous were obtained from VWR International (Leuven, Belgium). Ammonium formate was obtained from Sigma–Aldrich (St Louis, USA). Ascorbic acid was supplied by Prolabo (Paris, France) and Formic acid (100%) was purchased from Merck (Darmstadt, Germany).

Deionised water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

Malachite green oxalate (CAS 2437-29-8), leuco malachite green (CAS 129-73-7), crystal violet (CAS 548-62-9), leuco crystal violet (CAS 603-48-5), brilliant green (CAS 633-03-4) were obtained from Sigma–Aldrich (St Louis, USA). D5-Malachite green picrate (MG-D5), D5-leuco malachite green (LMG-D5), D6-crystal violet trihydrate (CV-D6) and D6-leuco crystal violet (LCV-D6) were purchased from Witega (Berlin, Germany). Leuco brilliant green was custom made by Atlanchim Pharma (Nantes, France) according to published conditions [12]. A hydroxylamine solution at 9.5 g l^{-1} in deionised water was prepared by dissolving 5 g of hydroxylamine chlorhydrate in deionised water and diluting to 250 ml.

A formic acid solution at 5% in deionised water (v/v) was prepared by diluting 5 ml of formic acid (100%) in a 100 ml volumetric flask containing about 90 ml of deionised water and completing flask to 100 ml with deionised water. An ammonium formate buffer (0.05 M, pH 4.5) was prepared by dissolving 3.15 g of ammonium formate in a 1000 ml volumetric flask with about 900 ml of deionised water, then adding 5 ml of formic acid solution at 5% in water (v/v) and completing the flask to 1000 ml with deionised water. This solution was used as LC mobile phase.

2.2. Standard solutions

Individual stock solutions of each reference compound at 100 μg ml^{-1} were prepared in acetonitrile, taking into account of the content of the active substances. These solutions were stored at −20 °C in ambered flasks (protecting them from light). Mixed intermediate standard solution of malachite green, leuco malachite green, crystal violet, leuco crystal violet, brilliant green at 1 μg ml^{-1} and mixed intermediate solution of deuterated standards at 1 μg ml^{-1} were prepared by diluting stock standard solutions in acetonitrile, respectively. These solutions were stored at −20 °C in ambered flasks and were found stable for at least 1 month. These intermediate solutions were diluted in acetonitrile to prepare working standard mixed solutions (MG, LMG, CV, LCV, and BG) at concentration of 40, 30, 20 and 10 μg l^{-1} and a working deuterated internal standard mixed solution (MG-D5, LMG-D5, CV-D6, and LCV-D6) at 40 μg l^{-1}. These solutions are prepared fresh daily.

2.3. Sample preparation

To avoid any loss due to light exposure, solutions and extracts are all protected from the light during the sample preparation procedure.

Muscle tissues, taken from fish, were homogenized in a domestic food blender and were kept frozen at −20 °C until analysis. The sample (2.00 ± 0.02 g) was fortified at 2 μg kg^{-1} with internal standards by adding 100 μl of deuterated internal standard mix solution (40 μg l^{-1}). Then 500 μl of hydroxylamine solution (9.5 g l^{-1}) was added and the sample was mixed and allowed to stand for 10 min in the dark before extraction. Then 8 ml of acetonitrile and 1 g (±0.1 g) of anhydrous magnesium sulfate were added. The tube was vortex-mixed vigorously for 1 min at maximum speed and was shaken for 10 min with a rotative stirrer at 100 rpm. After agitation, the tube was centrifuged at 2000 × *g* for 5 min at 4 °C. All of the supernatant was taken up by pipetting, transferred into a new clean tube and was evaporated to dryness at 50 °C under a gentle stream of nitrogen. The remaining residue at the bottom of the tube was reconstituted by dissolving in 800 μl of solution of acetonitrile/1 g l^{-1} ascorbic acid (100/1;v/v). The mixture was then transferred into an Eppendorf tube and centrifuged at 20,000 × *g* for 5 min. The extract was filtered through a 0.45 μm PVDF filter into HPLC vial prior to LC–MS/MS analysis.

2.4. Matrix calibration

The calibration standards for calibration curves were prepared using matrix-extracted (fortified prior to extraction) for each run of analysis and were used for quantification. Tissue samples were fortified with MG, LMG, CV, LCV, and BG at levels corresponding to 0.0, 0.5, 1.0, 1.5 and 2.0 μg kg^{-1} by adding either 0 or 100 μl of working standard mix solutions prepared at 10, 20, 30 and 40 μg l^{-1}, respectively. 100 μl of deuterated internal standard mix solution

Table 1
Gradient timetable for the LC–MS/MS analysis (flow rate 250 μl min^{-1}).

Time (min)	% A (ammonium formate buffer)	% B (acetonitrile)	Curve
0	60	40	
1	10	90	Linear
15	10	90	
16	60	40	
20	60	40	

(40 μg l^{-1}) was also added to each sample including the 0.0 μg kg^{-1}. Calibration curves were constructed by plotting the response factor (peak area analyte/internal standard peak area) as a function of analyte concentration on the range 0–2 μg kg^{-1}.

2.5. Liquid chromatography–mass spectrometry

2.5.1. LC–MS/MS analysis (QqQ)

LC analysis was carried out by a Waters 2695 HPLC system (Waters, Saint-Quentin en Yvelines, France) constituted of a quaternary pump, a degasser, an autosampler and a column oven. The chromatographic separation was performed on a reversed-phase HPLC column Symmetry C18 Waters (100 mm × 2.1 mm; 3.5 μm) equipped with a guard column Symmetry C18 Waters (10 mm × 2.1 mm). The mobile phase consisted of [A] ammonium formate buffer (0.05 M, pH 4.5) and [B] acetonitrile. The gradient program is reported in Table 1. The flow rate was 250 μl min^{-1}, the injection volume was 20 μl and the column oven was maintained at 30 °C.

The LC system was coupled to a Quattro LCZ triple quadrupole mass spectrometer (Waters, Saint-Quentin en Yvelines, France) equipped with an electrospray ionisation source (ESI) through a Z-spray interface and controlled by Masslynx software (version 4.5). The mass spectrometer was operated in positive ESI mode. The source conditions were as follows: desolvation temperature: 350 °C, source temperature: 150 °C, nebulization nitrogen gas flow: 80 l h^{-1}, desolvation nitrogen gas flow: 785 l h^{-1}, capillary voltage 3.2 kV. Argon was used as the collision gas at a pressure of 2.3×10^{-3} mbar and the multiplier was set at 650.

The analytes were detected using a multiple reaction monitoring (MRM) mode monitoring two transitions for each compound and one for each deuterated internal standard with dwell times all set at 120 ms, performing two periods of acquisition. Table 2 summarizes the MRM acquisition parameters.

2.5.2. LC-LTQ-Orbitrap analysis (for identification of LBG)

Chromatographic separations were performed on an Accela liquid chromatography U-HPLC system (ThermoFisher, Bremen, Germany) equipped with a Symmetry C18 column (100 mm × 2.1 mm; 3.5 μm particle size) from Waters (Saint-Quentin en Yvelines, France). The column oven temperature was set at 25 °C, the flow rate used was 200 μl min^{-1}, and the injection volume was 20 μl. The mobile phase consisted of [A] ammonium formate buffer (0.05 M, pH 4.5) and [B] acetonitrile. The elution gradient was linearly ramped from 20% to 90% of eluent B over 3 min and hold at 90% for 12 min (3–15 min). Then the elution gradient was linearly ramped down to 20% B over 3 min and maintained for 7 min to allow column conditioning for the next injection.

Mass spectral analysis was carried out on LTQ-Orbitrap mass spectrometer XL MS (ThermoFisher, Bremen, Germany) with an electrospray ionisation probe and operated in the positive ion mode. The instrument was calibrated using the manufacturer's calibration solution (consisting of caffeine, the tetrapeptide MRFA and Ultramark) to reach mass accuracies in the 1–3 ppm range. Parameters of the ion source were as follows: capillary voltage 32 V, ion spray voltage 5 kV, Tube lens 85 V, capillary temperature 350 °C, sheath gas flow 20 (arbitrary units), aux. gas flow 5 (arbitrary units) and sweep gas 0 (arbitrary units). Nitrogen was used as the sheath and auxiliary gas in the ion source. Different experiments were performed on the instrument: (a) full scan FTMS from m/z 100–500 at a resolving power of 60,000 (full width at half maximum), (b) high-energy collision dissociation (HCD) FTMS HCD MS/MS on selected precursor ion with recorded spectrum from m/z 50 to 500 at a resolving power of 60,000 (FWHM), (c) collision induced dissociation (CID) ITMS MS^n at low resolution, using the LTQ mass analyser, on selected precursor ions. CID experiments were performed at normalized energy collision of 35, with activation q_z and time, respectively, set at 0.25 ms and 30 ms.

2.6. Validation study

The LC–MS/MS method was validated as a quantitative confirmatory method according to the EU Decision 2002/657/EC [13]. The criteria applied for identification of each analyte were verified by monitoring relative retention times and relative ion intensities. The process for quantification was based on peak areas response of the most intense MRM transition divided by the peak area response of the corresponding internal standard. The quantitative parameters evaluated in the validation procedure were: specificity, linear-

Table 2
MS/MS parameters for the determination of each compound.

Compound	MRM transitions (m/z)	Collision energy (eV)	Cone voltage (V)	Retention time (min)
Period 1: from 3.10 min to 6.8 min				
Malachite green	329 > 313[a]	35	43	5.1
	329 > 208	35	43	
Crystal violet	372 > 356[a]	40	25	5.6
	372 > 251	35	25	
Brilliant green	385 > 341[a]	35	35	6.0
	385 > 297	50	35	
Malachite green-D5	334 > 318	40	30	5.1
Crystal violet-D6	378 > 362	40	25	5.6
Period 2: from 7.0 min to 15.0 min				
Leuco malachite green	331 > 239[a]	25	25	7.8
	331 > 316	20	25	
Leuco crystal violet	374 > 358[a]	30	25	7.9
	374 > 239	25	25	
Leuco brilliant green	387 > 342[a]	30	25	10.9
	387 > 281	30	25	
Leuco malachite green-D5	336 > 239	25	25	7.8
Leuco crystal violet-D6	380 > 364	35	25	7.9

[a] The most abundant MRM transition.

ity, precision (repeatability and within-laboratory reproducibility), trueness, decision limit (CCα), detection capability (CCβ). The stability of dyes in solutions was also evaluated.

The specificity was tested by analysing 30 samples of different origin/species (trouts, fresh shrimps, boiled shrimps, salmons, and other varied fishes) in order to verify the absence of potential interfering compounds at the retention times of the given analytes.

The rest of the validation study was carried out using trout muscles. Matrix calibration curves were obtained by spiking blank trout muscle samples with the 5 compounds (MG, LMG, CV, LCV, and BG) at five levels 0.0, 0.5, 1.0, 1.5 and 2.0 $\mu g\,kg^{-1}$. To evaluate precision and trueness, six replicates of spiked samples at the four concentration levels of 0.5, 0.75, 1.0 and 2.0 $\mu g\,kg^{-1}$ were prepared and analysed during the same day as the matrix calibration curve. Each series, consisting of a matrix calibration curve and 24 replicates, were prepared on three different days giving a total of 72 replicates over the 3 days. The responses were based on the peak area ratio of the analyte compared to those of its corresponding internal standard.

The linearity of the method was evaluated by a linear regression analysis of the matrix-extracted calibration curves. The precision (intra-day repeatability and inter-day repeatability) was determined from all the 72 analyses of replicates and expressed as relative standard deviation.

The decision limit (CCα) and the detection capability (CCβ) were determined from the matrix-extracted calibration curve according to the standard ISO 11843 [14].

2.7. Stability

The stability of individual intermediate solutions at 1 $\mu g\,ml^{-1}$ in acetonitrile was evaluated according to Croubels et al. [15]. Different conditions of storage were evaluated: at −20 °C solutions kept away from light, at +4 °C solutions kept away from light, and at room temperature solutions kept in light. To evaluate the stability, the original stored working solution is compared with a working solution that is prepared freshly each day of analysis, starting from day 1. The injection sequence is carried out alterning fresh and stored solutions, i.e. first the original stored solution, then the fresh working solution, and so on.

2.8. Matrix effects

Experiments to evaluate matrix effects were conducted according to the protocol applied by Matuszewski et al. [16]. MS/MS peak areas of known amounts of standard solutions of the analytes were compared with those measured from same amount of analytes spiked in a muscle extract, just after the extraction process is completed. Matrix effect (%ME) was calculated as follows:

$$\%ME = \frac{\text{Peak area of post extraction spiked analyte}}{\text{Peak area of standard analyte}} \times 100\%$$

This equation means that no matrix effect is observed when %ME is equal to 100%.

Values greater than 100% indicate a signal enhancement while values lower than 100% indicate a trend in ionisation suppression.

Experiments were carried out to assess the matrix effects for different species of aquaculture products: trout, raw shrimp, boiled shrimp, salmon, tilapia. ME% was calculated with and without internal standard on 6 replicates for each matrix. Blank muscle sample has been processed through extraction and just after the evaporation step the residue at the bottom of the tube was dissolved in a solution of ACN containing 5 $\mu g\,l^{-1}$ of each compound instead of a blank solution of ACN. This extract was compared to a standard solution of the analytes set at 5 $\mu g\,l^{-1}$.

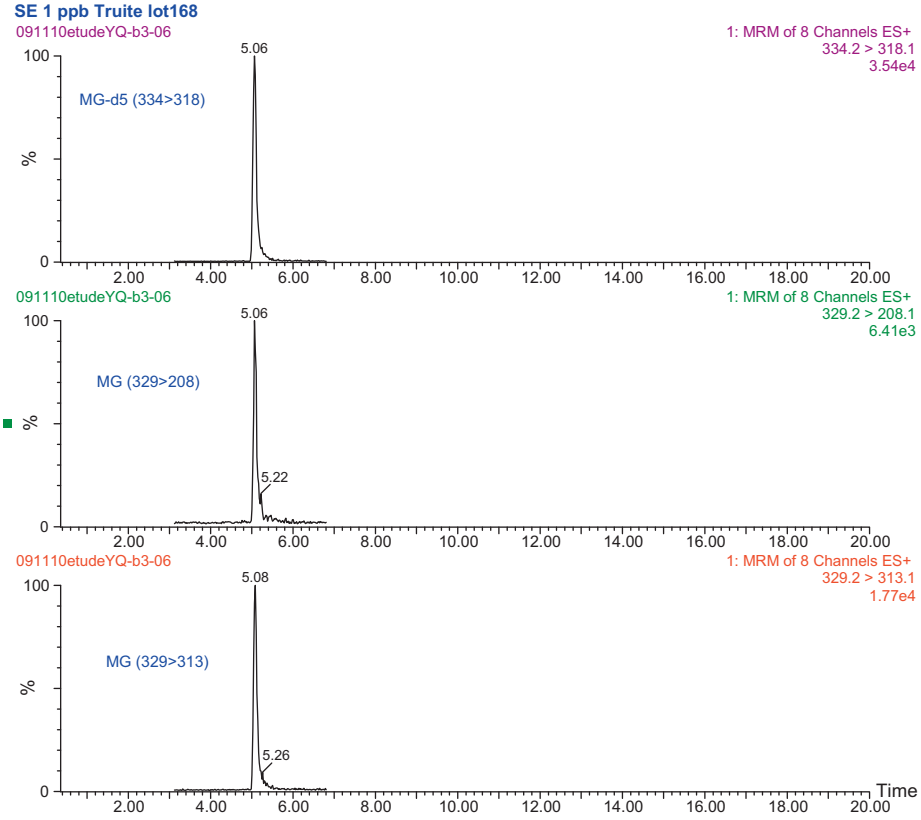


Fig. 2. MRM chromatograms of a trout muscle sample spiked at 1 $\mu g\,kg^{-1}$ with MG, CV, BG, LMG, and LCV and at 2 $\mu g\,kg^{-1}$ with MG-D5, CV-D6, LMG-D5, and LCV-D6.

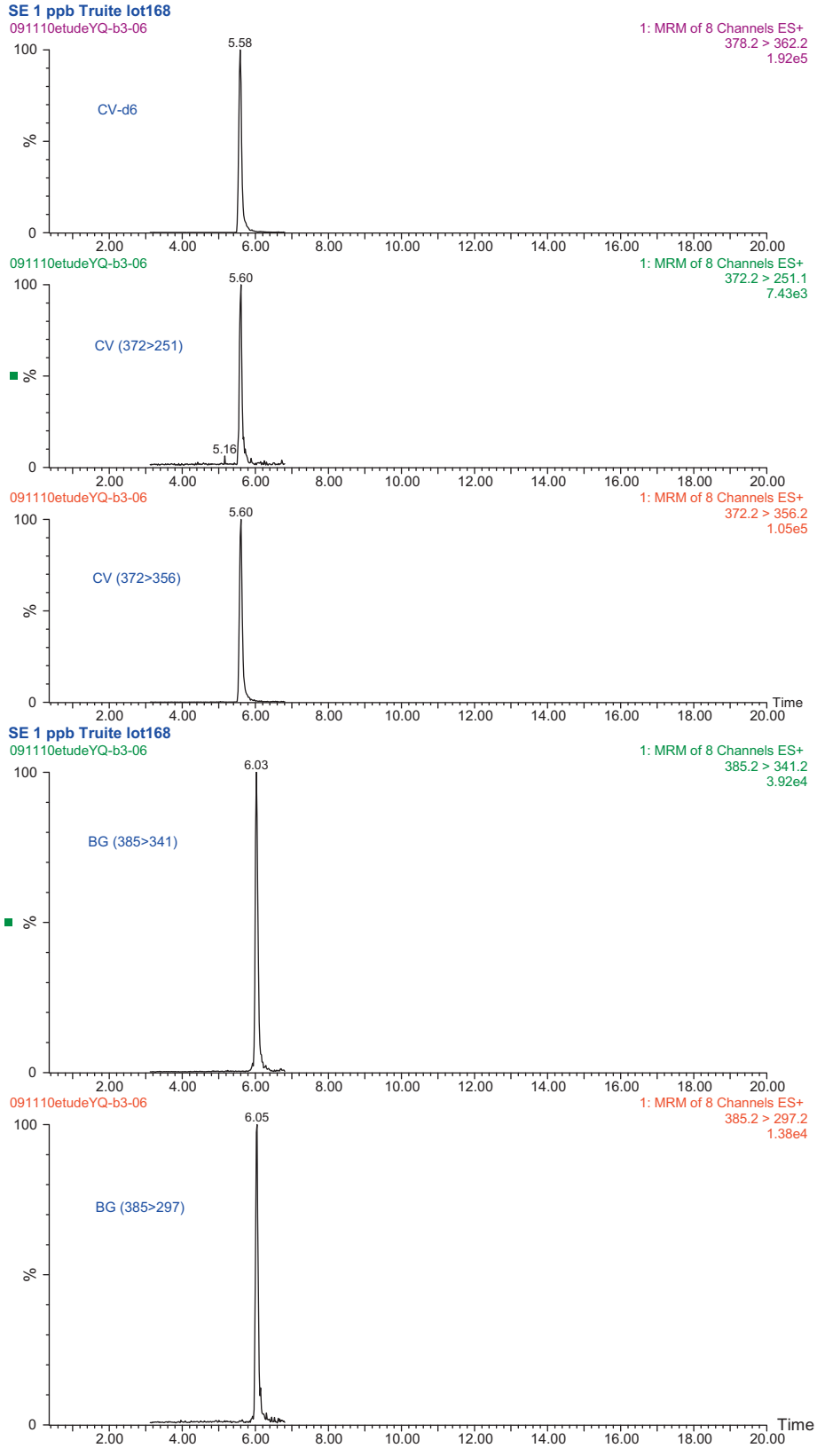
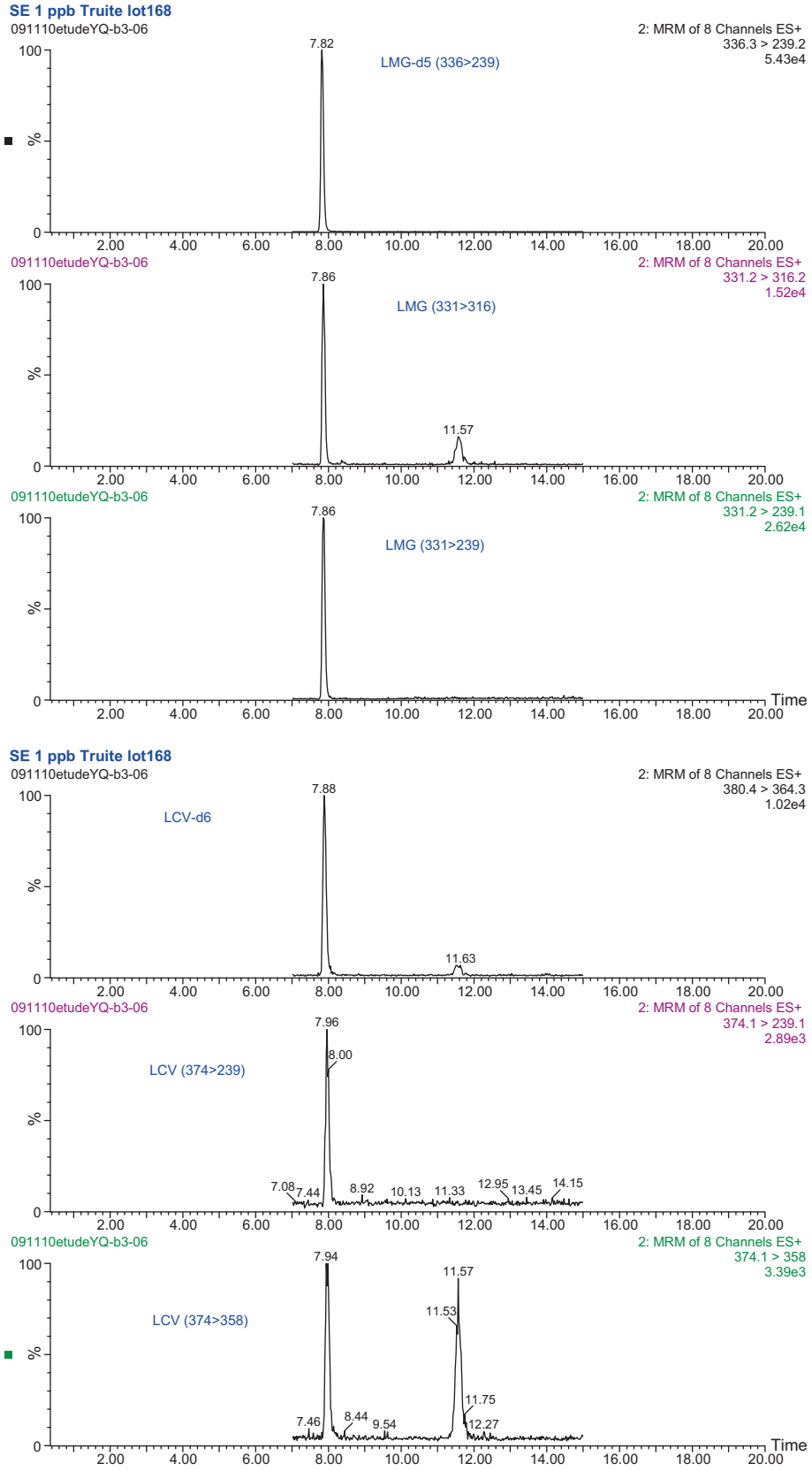


Fig. 2. (*Continued*)



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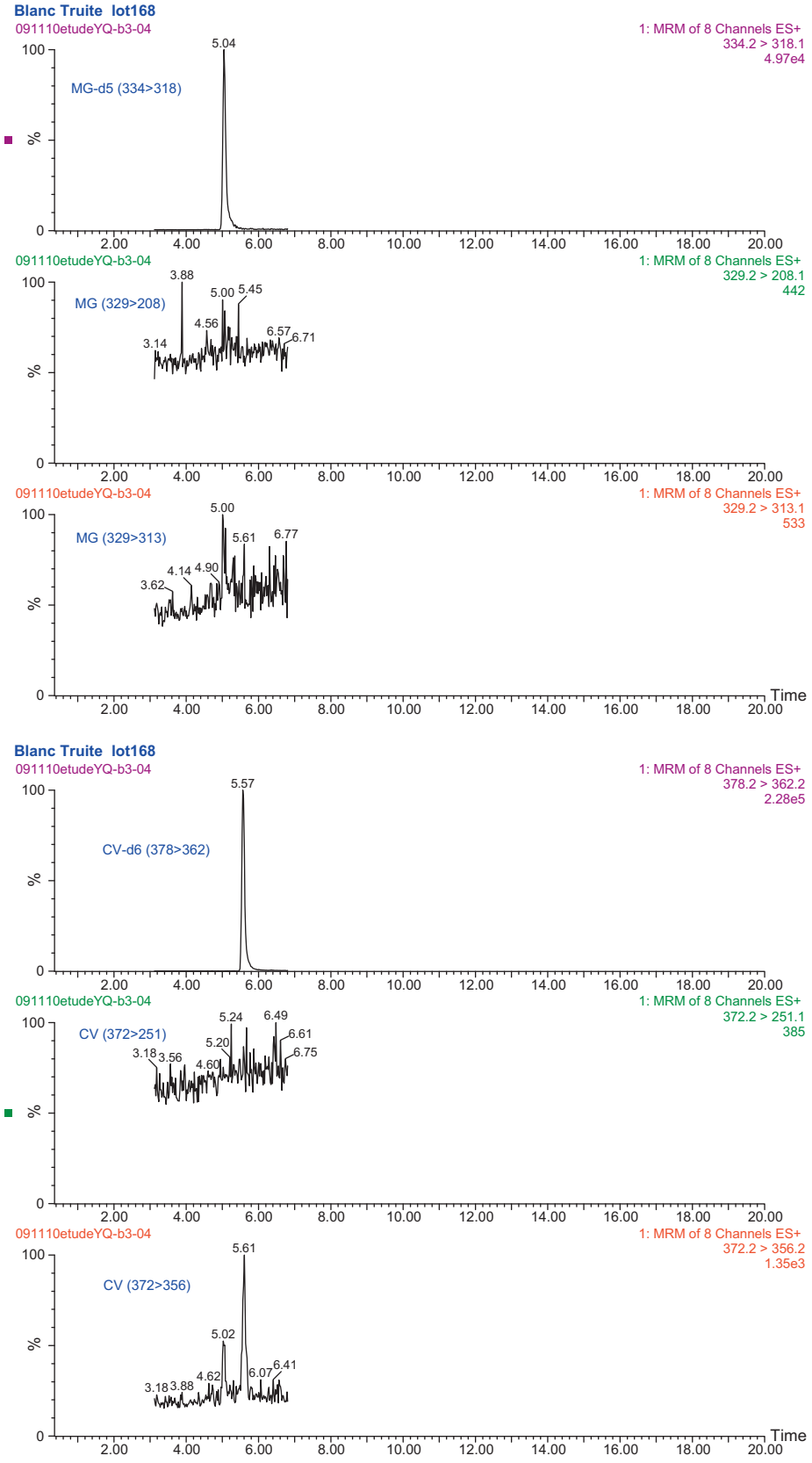

Fig. 3. MRM chromatograms of a negative trout muscle sample spiked at 2 $\mu g\,kg^{-1}$ with MG-D5, CV-D6, LMG-D5, and LCV-D6.

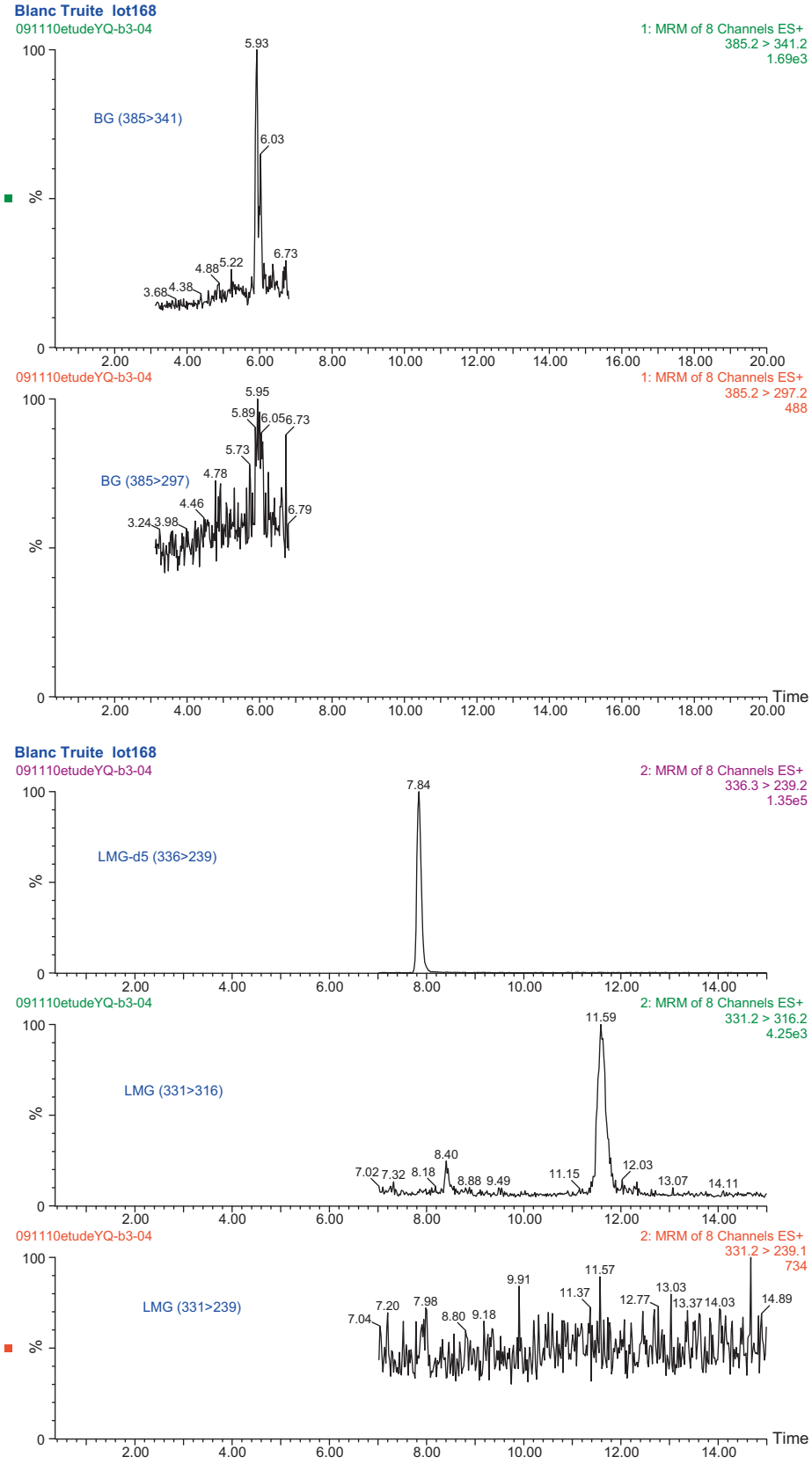

Fig. 3. (*Continued*)

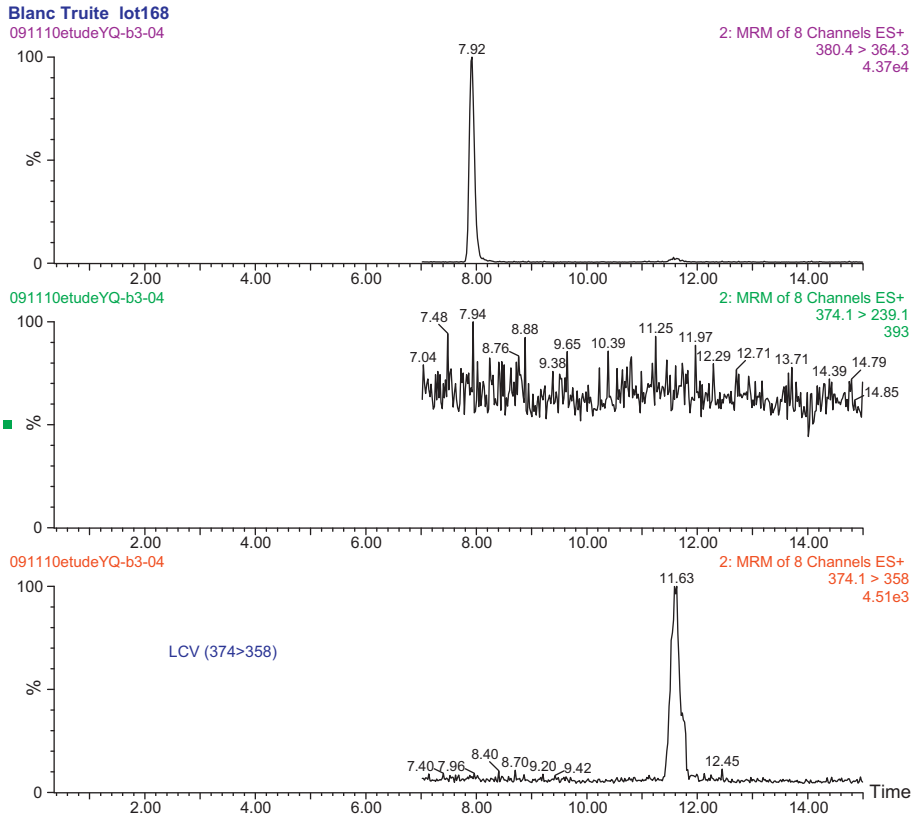

Fig. 3. (*Continued*).

3. Results and discussion

3.1. Method development

The previous LC–MS/MS method developed in our laboratory [17] and used as an experimental model for a mathematical study [18] dealt with the simultaneous confirmation of MG and LMG. In this method, CV was used as the internal standard for the quantification of MG. As CV was found to also be possibly used for curing/disinfecting treatments in aquaculture, a revision of this method was needed and modifications were made to extend the method to the confirmation of both CV and LCV. Another triphenylmethane dye, brilliant green (BG) was also added to the scope of the method.

Table 3
Precision and trueness.

	Fortification level ($\mu g\,kg^{-1}$)	Precision		Trueness
		Intra-day RSD (%) ($n=6$)	Inter-day RSD (%) ($n=18$)	Recovery (%) ($n=18$)
Malachite green	0.5	6.3	6.1	104.4
	0.75	5.3	5.9	105.1
	1	4.9	4.8	103.9
	2	4.8	4.7	100.1
Leuco malachite green	0.5	7.6	7.7	101.7
	0.75	5.6	9.9	101.0
	1	4.7	7.2	101.7
	2	3.0	7.1	104.8
Crystal violet	0.5	3.0	3.1	100.2
	0.75	3.5	4.0	104.3
	1	2.0	1.9	101.7
	2	2.7	2.8	100.4
Leuco crystal violet	0.5	8.1	8.9	100.5
	0.75	6.7	9.2	101.4
	1	8.8	10.6	103.7
	2	7.5	7.6	103.0
Brilliant green	0.5	10.3	9.6	109.8
	0.75	6.8	9.4	104.8
	1	8.6	7.9	105.4
	2	6.8	7.4	100.4

The extraction of dyes from fortified tissues was operated through a liquid/liquid extraction using MeCN with MgSO4 as an extraction solvent. The 1 g of MgSO4 is intended to help removing water mixed to MeCN, allowing a more rapid evaporation step of the supernatant under N_2 stream. MeCN with 1% acetic acid was also tested as en extraction solvent but gave no better recoveries compared to MeCN alone. No further clean-up step was found necessary. Different reconstitution solvents were tried and finally the dried residue was re-dissolved in a mixture of MeCN/1 g l^{-1} ascorbic acid (100/1; v/v), to allow a good peak shape and stability of the extract solution in HPLC autosampler vials.

A first attempt to use only two deuterated internal standards, i.e. MG-D5 and LMG-D5, to quantify MG, CV, BG with MG-D5 and LMG, LCV with LMG-D5 led to unsatisfactory results for accuracy and fidelity of CV analyte. Thus, two other appropriate internal standards, i.e. CV-D6 and LCV-D6, were finally added to improve quantification.

The mobile phase composition was also revised. Initially, 0.1 M ammonium acetate (pH 4.5) was used mixed with MeCN. Finally, ammonium formate was preferred improving chromatographic peaks shape and because of its higher solubility compared to ammonium acetate in MeCN.

According to the Decision 2002/657/EC, four identification points are required to satisfy confirmatory criteria for illegal veterinary drug residues in foodstuffs. So, in multiple reaction monitoring mode (MRM), 1 precursor ion and two product ions were monitored for each dye residue, yielding 4 identification points. Moreover, each of the deuterated internal standards was detected using only 1 MRM transition.

3.2. Method validation

Method specificity was demonstrated by analysing 30 blank samples of different origins collected from trout, shrimp, salmon, tilapia, pangasius and catfish. No interfering peak was detected at the retention time of the target analytes in any of these species. The retention time observed for the analytes prepared in aqueous solution was similar to that of the analytes when prepared spiked in matrix. The relative ion intensities in spiked samples were calculated and were found fully in accordance with the ion ratio tolerance of the Decision 2002/657/EC.

Figs. 2 and 3 show the LC–MS/MS chromatograms of a representative blank trout sample and a trout sample spiked at 1 μg kg^{-1} for each of the analytes. The peaks at 11.57 min in LMG and LCV chromatograms present both in Fig. 2 for the spiked sample and in Fig. 3 for the blank sample were due to the matrix.

The linearity of the method was evaluated by linear regression analysis of the matrix-extracted calibration curves on the range 0–2 μg kg^{-1} using 5 calibration points. Linear regression coefficients r^2 calculated for each analyte were all found higher than 0.98.

Accuracy (precision and trueness) was calculated using six replicates of spiked samples at each concentration level of 0.5, 0.75, 1.0 and 2.0 μg kg^{-1} analysed on the same day as the corresponding matrix calibration curve, and extended during three different days. The accuracy was assessed by calculating trueness and precision. Trueness was expressed as the recovery rate and precision was expressed as the relative standard deviation (RSD%) which was determined through repeatability measurements (intra-day) and within-laboratory reproducibility measurements (inter-day). All values are reported in Table 3. Mean recoveries ($n = 18$) determined for each level of concentrations were all found satisfactory for each analyte. Values ranged from 100% to 109%. The intra-day and inter-day repeatability values for all the analytes were below or equal to 10.3% and 10.6%, respectively.

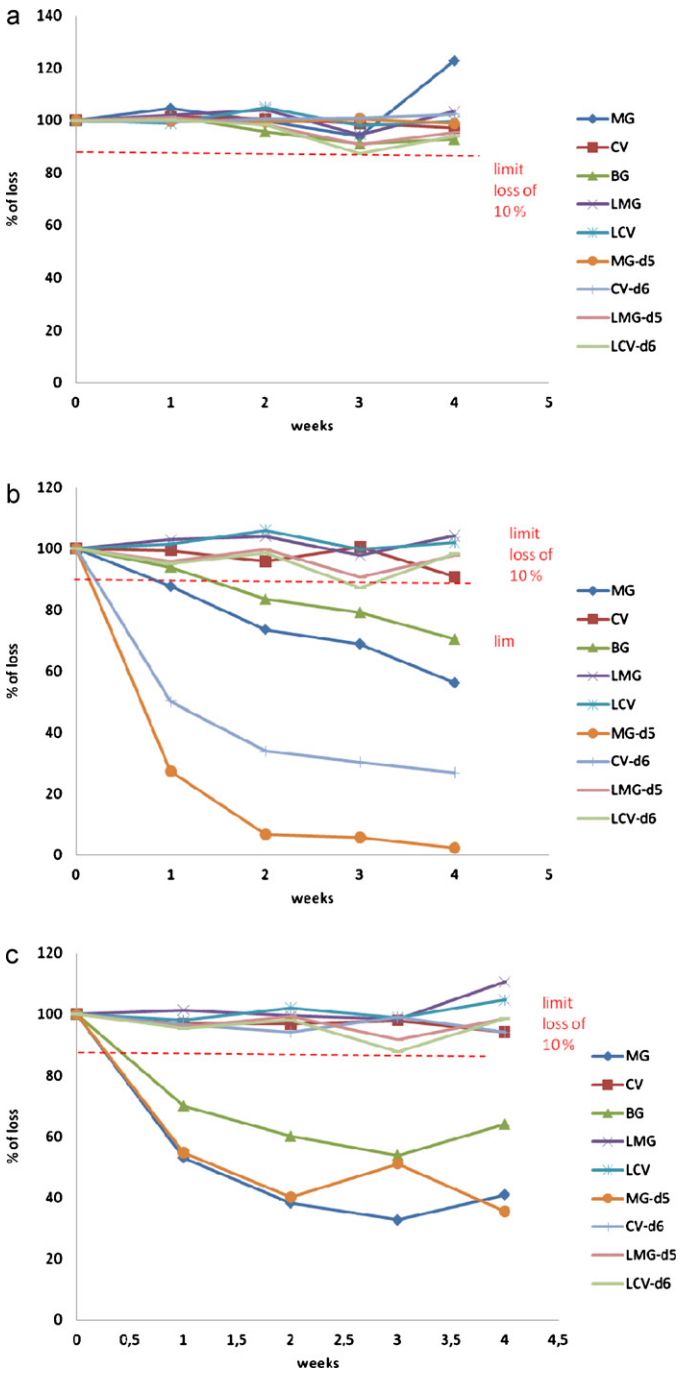

Fig. 4. Stability of individual standard solution at 1 μg ml^{-1} in MeCN (a) stored at −20 °C in the dark, (b) stored at +4 °C in the dark and (c) stored at room temperature and kept in daylight.

The values of the decision limit CCα and of the detection capability CCβ were determined from the matrix calibration curve according to the standard ISO 11843. The calculated values, reported in Table 4 are the median value over the three days. CCα ranged from 0.13 to 0.42 μg kg^{-1} for the targeted analytes. The decision limit CCα means the limit at and above which it can be concluded that a sample is non compliant with an error probability of α. For banned substances, $\alpha = 0.01$ (=1%). For malachite green, the EU MRPL has been established at 2.0 μg kg^{-1} for the sum of malachite green and leuco malachite green. For substances with an MRPL set, CCα and CCβ must always be calculated below the MRPL. In document SANCO/2004/2726-rev4-december 2008 [19], guide-

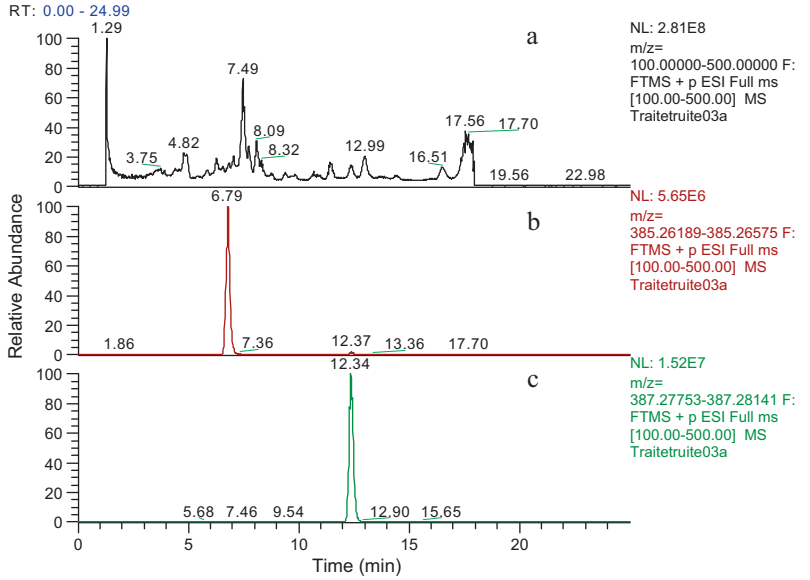

Fig. 5. Full scan FTMS chromatograms of a treated trout sample: (a) total ion chromatogram, (b) extracted ion chromatograms of theoretical masses of BG and (c) extracted ion chromatograms of theoretical masses of LBG (mass accuracy window of 5 ppm).

lines have been drafted by the EU-RLs in charge of veterinary drug residue control, for assessment of non-compliant results of substances for which a sum "maximum residue limit" is established and especially for the particular case of malachite green and leuco malachite green, for which a sum of MRPL has been established. For this particular case of malachite green and leuco malachite green, provided the full identification criteria have passed for the analytes, any result in excess of CCα should then be investigated. To that end, for method which measures separately malachite green and leuco malachite green, it is recommended that each individual CCα should always be less than ½ MRPL, i.e. 1.0 $\mu g\,kg^{-1}$. In the presented method the CCα for malachite green (0.25 $\mu g\,kg^{-1}$) and for leuco malachite green (0.17 $\mu g\,kg^{-1}$) fulfilled these criteria.

3.3. Matrix effects

The matrix effects were determined for the 5 targeted dyes in two different conditions, i.e. with internal standards (using peak area ratios) and also without internal standards (using peak area). The evaluation took place in different aquaculture products (trout, raw shrimp, boiled shrimp, salmon, pangasius, and tilapia). 6 samples of different batches/origins were analysed for each kind of aquaculture products.

The results are displayed in Table 5. The matrix effects calculated without internal standards showed ionisation suppression of about 20%, 10% and 30% for LMG, CV and LCV, respectively, although no ionisation suppression or signal enhancement were observed for MG and BG, for all kinds of matrices.

The results showed also that matrix effects are readily overcome by the use of an appropriate internal standard. Matrix effects are totally eliminated for MG, LMG, CV and BG and are found minimized (<10%) for LCV. The variability of the matrix effect was also studied in terms of aquaculture products with taking into account the 6 samples of different origins for each product. The relative standard deviation (RSD) values obtained for the matrix effect calculated without internal standard were systematically found much

Table 4
Decision limits and detection capabilities.

Analyte	CCα ($\mu g\,kg^{-1}$)	CCβ ($\mu g\,kg^{-1}$)
Malachite green	0.25	0.32
Leuco malachite green	0.17	0.22
Crystal violet	0.13	0.17
Leuco crystal violet	0.42	0.54
Brilliant green	0.23	0.29

Table 5
Matrix effects based on area and area ratios.

	Matrix effect without internal standard		Matrix effect with internal standard integrated	
	ME (%)	RSD (%)	ME (%)	RSD (%)
Malachite green				
Trouts ($n=6$)	88.2	9.8	100.2	1.4
Shrimps ($n=6$)	103.7	2.8	103.0	3.7
Boiled shrimps ($n=6$)	105.1	9.1	101.1	3.4
Salmon ($n=6$)	96.6	6.7	102.6	3.3
Other fishes ($n=6$)	102.1	3.9	100.9	3.2
All-mean ($n=30$)	99.2	9.0	101.6	3.1
Leuco malachite green				
Trouts ($n=6$)	90.8	29.4	96.0	7.3
Shrimps ($n=6$)	80.6	18.9	102.6	5.9
Boiled shrimps ($n=6$)	76.8	15.1	97.2	3.7
Salmon ($n=6$)	87.8	19.9	97.0	2.8
Other fishes ($n=6$)	64.8	29.0	98.1	5.4
All-mean ($n=$ **30**)	80.2	24.5	98.2	5.5
Crystal violet				
Trouts ($n=6$)	83.1	9.8	98.1	1.4
Shrimps ($n=6$)	101.2	2.4	102.2	1.9
Boiled shrimps ($n=6$)	99.5	3.8	99.9	2.3
Salmon ($n=6$)	74.5	14.7	98.4	4.7
Other fishes ($n=6$)	98.6	2.1	100.3	2.5
All-mean ($n=30$)	91.4	13.6	99.8	3.0
Leuco crystal violet				
Trouts ($n=6$)	81.8	29	93.9	6.3
Shrimps ($n=6$)	73.7	16.7	90.1	3.7
Boiled shrimps ($n=6$)	58.2	10.8	81.2	11
Salmon ($n=6$)	73.1	24	90.9	5.6
Other fishes ($n=6$)	59.4	34	95.2	8.4
All-mean ($n=30$)	69.2	26.6	90.3	8.7
Brilliant green				
Trouts ($n=6$)	91.3	7.6	103.9	4.3
Shrimps ($n=6$)	101.6	2.2	100.9	4.5
Boiled shrimps ($n=6$)	103.6	6.3	99.8	4.3
Salmon ($n=6$)	97.7	8.4	103.7	4.7
Other fishes ($n=6$)	100.2	2.1	99	3.3
All-mean ($n=30$)	98.9	7.0	101.5	4.4

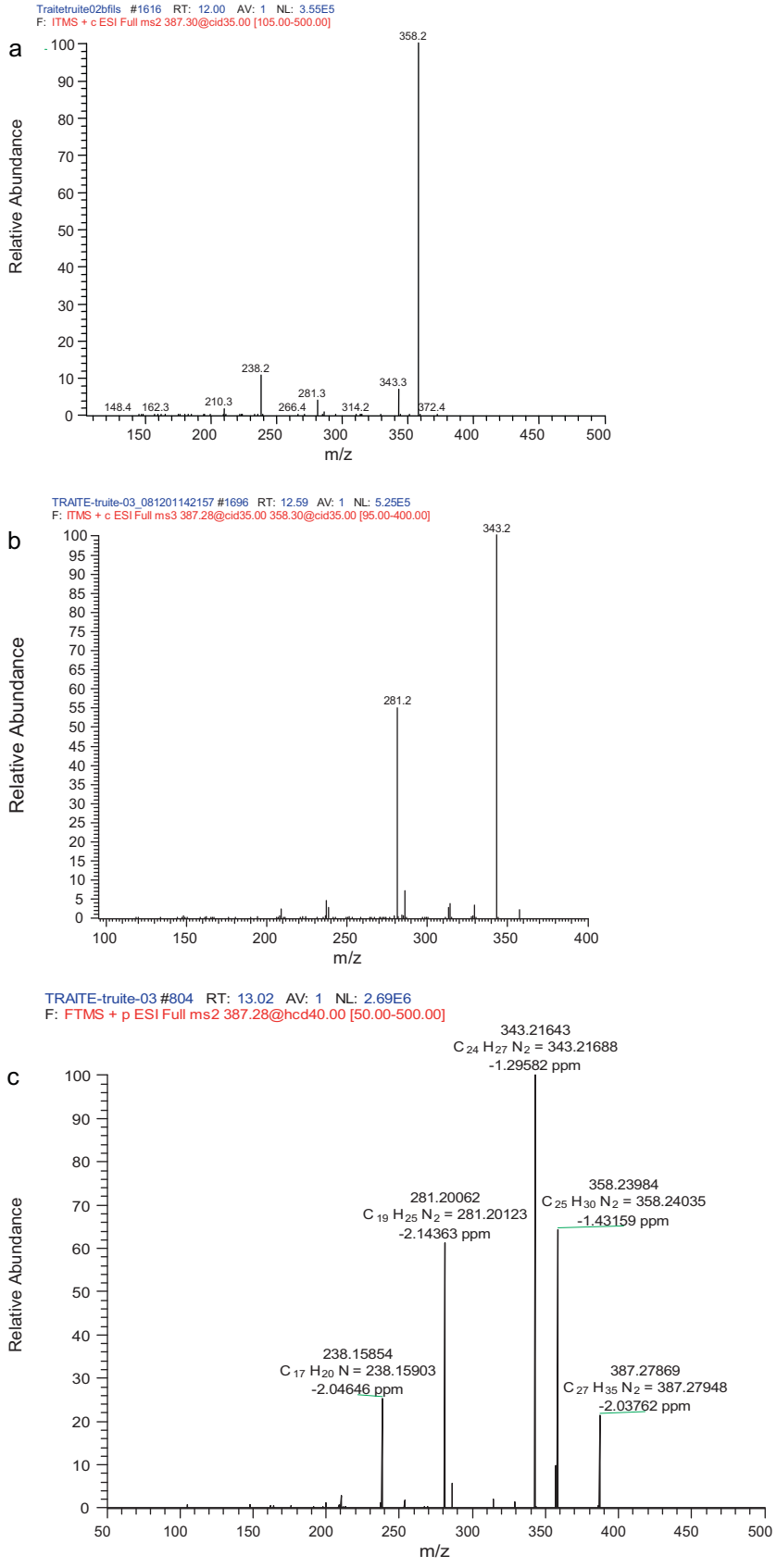

Fig. 6. (a) ITMS CID-full scan MS^2 spectrum of m/z 387 for trout incurred sample. (b) ITMS CID-full scan MS^3 spectrum of m/z 358 from 387 for trout incurred sample. (c) FTMS HCD-full scan MS^2 spectrum of m/z 387 for trout incurred sample.

Table 6
Concentration of BG and LBG in trouts treated with BG.

Animal	Treatment with brilliant green		Brilliant green (MRM 385 > 341)	Leuco brilliant green (MRM 387 > 343)
	Bath at 100 ng ml^{-1}	Bath in clear water before slaughtering		
Trout 1	–	–	–	–
Trout 2	1 h	0	7.9 μg kg^{-1}	7.8 μg kg^{-1}
Trout 3	1 h	1 h	27.4 μg kg^{-1}	18.2 μg kg^{-1}
Trout 4	1 h	2 h	25.4 μg kg^{-1}	14.9 μg kg^{-1}

higher than those calculated using internal standard, especially for LMG and LCV. This experiment made us conclude that the internal standards compensate the ionisation suppression for all analytes except for LCV and therefore dramatically limit the high variability of the signal.

3.4. Stability

The stability of individual solutions of MG, LMG, LCV, CV and BG and of the internal deuterated standards at 1 μg ml^{-1} in MeCN was evaluated after storage at −20 °C in dark conditions (i.e. protected from light), at +4 °C in dark conditions and at +4 °C at room temperature unprotected from light. Fig. 4a–c shows the plots for the measurement of the stability against time.

It was found that all individual solutions are stable after 4 weeks of storage at −20 °C protected from light. Similarly, all leuco solutions LCV, LMG, LCV-D6, LMG-D5, and CV solutions are stable at +4 °C and at room temperature after 4 weeks of storage. On the opposite, the chromic form MG, BG, MG-D5 and CV-D6 are found not stable after storage at +4 °C and at room temperature during shorter periods ranging from 1 to 2 weeks. In conclusion, the chromic forms are demonstrated less stable than the leuco forms.

3.5. Identification of metabolites of brilliant green

There is no such literature data on the metabolism of BG as the one for MG and CV. As brilliant green displays a similar structure compared to MG and CV, it is presumed to also metabolize in its leuco form in vivo. Andersen et al. [7] assume the same hypothesis but they were not able to directly analyse LBG before it converted to BG. Up to now, no exact confirmation of leuco brilliant green was made. To achieve the identification of a possible metabolite of brilliant green in fish tissues, trouts (*O. mykiss*) were exposed to BG. Trouts ($n = 3$) were placed in a water bath with a BG concentration of 100 ng ml^{-1} for 1 h, then were returned to a clean water bath before slaughtering in 3 lots at periods of 0, 1 and 2 h. Muscle samples were collected and processed using the sample preparation described in Section 2.3. The analysis was carried out using an LC-LTQ-Orbitrap mass spectrometer, operated in full scan mode at 60,000 resolution. The high power of resolution and the high mass accuracy of the Orbitrap system allow the analysis of targeted screening based on the extraction of the exact mass of targeted compounds on full-scan LC–HRMS ion chromatogram. As shown in Fig. 5, extracted ion chromatograms of brilliant green (theoretical mass M^+: 385.26382) and of leuco brilliant green (theoretical mass MH^+: 387.27947) in treated fish, both exhibit nice peaks, respectively, at 6.8 min and 12.3 min. The confirmation of the identity of the peak at 12.3 min supposed to be leuco brilliant green was further investigated by means of 2 different fragmentation experiments using collision induced dissociation CID on the linear trap LTQ (Fig. 6a and b) and using the high collision dissociation HCD with detection by the orbital trap (Fig. 6c). The definitive identification of the peak at 12.3 min as being the leuco brilliant green was fully confirmed by comparison with a custom-made reference standard of leuco brilliant green, showing the same retention time, the same fragmentation spectrum both under CID and HCD experiments. In opposition to BG which is green coloured, the leuco-brilliant green reference standard is a yellow oil which needs to be stored under nitrogen as it is easily oxidizable in BG. It is stable in acetonitrile or methanol solutions for at least 40 h as these solutions remain colourless after this time.

The quantification of BG and LBG in the incurred muscles of trouts was further investigated using a LC–MS/MS instrument operated in MRM mode. After 1 h of BG bath treatment the trout muscle contained 7.9 μg kg^{-1} of BG and 7.8 μg kg^{-1} of LBG. After 1 and 2 h of depuration in clean water, the concentration of BG in trout muscle tissue ranged from 25.4 to 27.4 μg kg^{-1} and from 14.9 to 18.2 μg kg^{-1} for LBG, as shown in Table 6. It is assumed that a tissue depletion study should be carried out to precisely establish to what extent leuco brilliant green is persistent in muscle tissue and if it should be designed as the target marker residue for BG treatment.

4. Conclusion

This work shows a simple and easy liquid chromatography–tandem mass spectrometry method suitable for the simultaneous confirmation and quantification of MG, LMG, CV, LCV and BG in fish muscle. This validated method fulfills the criteria required by the Commission Decision 2002/657/EC for trueness and precision of a confirmatory method. An additional work enabled to identify and confirm the presence of LBG in BG treated trout. As this standard is still not commercially available up to now, it is not yet possible to include this analyte in the routine method for detection. Further work should be done to study the depletion of LBG in fish over a longer period to measure the persistence of LBG in fish muscle.

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