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## Research Article

# Simultaneous quantification of antibiotic dyes in aquatic products and feeds by liquid chromatography-tandem mass spectrometry

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## ABSTRACT

A confirmatory and quantitative method based on liquid chromatography-tandem mass spectrometry (LC/MS/MS) has been developed for the determination of low-level residues of three antibiotic dyes and two metabolites in fish muscle and feed. The target compounds include methylene blue (MB), crystal violet (CV), leucocrystal violet (LCV), malachite green (MG), and leucomalachite green (LMG). The procedures involve solvent extraction by 50% McIlvaine's buffer with acetonitrile, followed by solid phase extraction (SPE) with an MCX cartridge. High performance liquid chromatography (HPLC) and positive electrospray ionization (ESI) MS with multiple reaction monitoring of two transition reactions was applied for each compound. The detected ion ratios of MB, CV, LCV, MG, and LMG were 11.8, 34.9, 88.4, 25.6, and 42.0, respectively. The average fortification recoveries of the MB, CV, LCV, MG, and LMG of the level of 0.8 µg/kg tested in fish muscle and feed samples were 99.68, 98.93, 100.49, 100.01, and 100.00%, respectively. The precision of analysis of analytes in fish muscle and feed ranged from 4% to 14% and from 7% to 14%, respectively. The decision limits (CC<sub>α</sub>) were 0.28–0.54 µg/kg, and the detection capabilities (CC<sub>β</sub>) were 0.35–0.67 µg/kg (*n* = 99).

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## 1. Introduction

Triphenylmethane is a colorless solid, which is soluble in nonpolar organic solvents but not in water.

Triphenylmethane has the basic skeleton of synthetic dyes called triarylmethane dyes. Many of the triphenylmethane dyes are pH indicators, and some display fluorescence [1,2]. Triarylmethanes such as methyl green (MeG), malachite green

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(MG), crystal violet (CV), and methylene blue (MB) are potentially carcinogenic and mutagenic [3–5]. MG is not a legal veterinary drug in the European Union (EU) [6]. The leuco-form leucomalachite green (LMG) is an *in vivo* mutagen in transgenic female mouse liver; the mutagenicities of MG and LMG correlate with their tumorigenicities in mice and rats [7].

MG has been widely used as a topical fungicide and anti-protozoal agent in fish farming throughout the world for several decades. However, the half-life of MG in fish muscle is approximately 70 hours, and LMG (metabolite of MG) persists even longer in fish tissue [8]. According to the European Commission, the determination of MG residues in fish muscle must reach a minimum required performance limit of 2 µg/kg for the sum of MG and LMG [9]. The Taiwan Food and Drug Administration has also recommended an analytical method for MG and LMG analysis and has set a detection limit of 0.5 µg/kg for both MG and LMG [10]. CV is also known to be effective in the treatment of fungal infections in fish farming. Consequently, analytical methods for CV, MG, and LMG have been reported in the last few years [11,12]. From a practical standpoint, a quick routine procedure with low detection limit and small sample size is desired.

A number of methods have been reported for the analysis of MG, LMG, CV, and leucocrystal violet (LCV) residues, including visible light detection with oxidation of the leuco-metabolites carried out either pre-column or post-column [13–15]. Other methods developed for the detection of these residues are based on detection by liquid chromatography-tandem mass spectrometry (LC/MS/MS) without oxidation reactions [11,12,16]. Most methods are based on the solvent extraction of MG and LMG from fish tissues using McIlvaine's buffer with acetonitrile [17–19]. Liquid/liquid partitioning and a solid phase extraction (SPE) cartridge were used for sample clean-up [20].

However, MG and CV are easily metabolized in animal tissues, and previous reports have focused on the determination of MG, CV, LMG, and LCV in fish tissue by LC/MS/MS [11,12,16]. Analysis of carcinogenic dye residues in different fish tissue and feed samples is difficult due to the complexity of the matrix and trace concentration levels. A simultaneous and more sensitive method for routine analysis is required. The aim of this study was therefore to develop and validate a method for the simultaneous determination of MB, MG, and CV and their metabolites, LMG and LCV, in trout, eel, grouper, and tilapia muscles and fish feeds.

## 2. Methods

### 2.1. Apparatus

The LC/MS/MS/electrospray ionization (ESI) system consisted of an Agilent Series 1260 high performance liquid chromatography (HPLC) system (Agilent Technologies, Stuttgart, Germany) connected to a Sciex API 4000 plus triple stage quadrupole mass spectrometer (AB Sciex, Chromos, Singapore). HPLC analysis was performed on a C<sub>8</sub> reversed-phase column (4.6 × 150 mm, 5 µm, Eclipse XDB-C<sub>8</sub>; Agilent Technologies) at 30 °C. A fast gradient for chromatographic separation of the analytes was performed using water

containing 0.5 mM ammonium acetate (mobile phase A) and HPLC-grade acetonitrile containing 0.1% formic acid. The running time for each sample was set for 7 minutes and the injection volume was 25 µL.

The MS detection system included an ESI source. The ion source block temperature was set at 650 °C and the electrospray capillary voltage was set at 5500 V. Nitrogen was used as the collision gas at a pressure of 5 mTorr.

### 2.2. Reagents and solutions

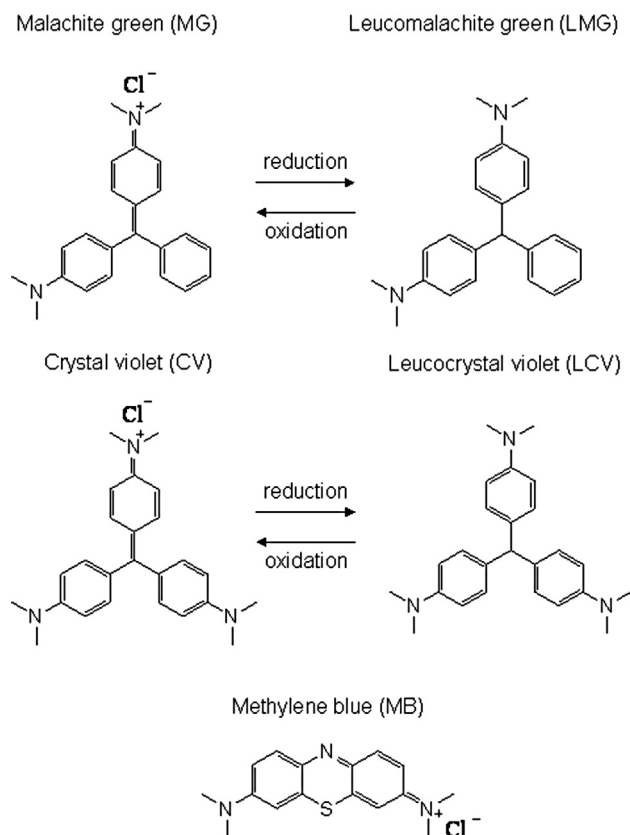
CV chloride (purity > 89.7%) was purchased from Sigma-Aldrich (Buchs, Switzerland). MG oxalate salt (purity > 96.9%) was provided by Sigma-Aldrich (St. Louis, MI, USA). LMG (purity > 98.7%) and LCV (purity > 99.4%) were supplied by Sigma-Aldrich (Milwaukee, WI, USA). Internal standards as Malachite green-d<sub>5</sub> pricate (MG-d<sub>5</sub>) (purity > 98%) and leucomalachite green-d<sub>5</sub> (LMG-d<sub>5</sub>) (purity > 98%) were purchased from Sigma-Aldrich (Munich, Germany). MB hydrate (purity > 96.7%) was provided by Sigma-Aldrich (St. Louis, MO, USA). Individual stock standard solutions were prepared at a concentration of 1000 mg/L in methanol (stable for 3 months). Intermediate single standards solutions of MG, LMG, MG-d<sub>5</sub>, LMG-d<sub>5</sub>, CV, LCV, and MB were prepared in methanol:water (50:50, v/v) at a concentration of 1.0 mg/L and stored in a refrigerator at –25 °C (stable for 1 month). Mixtures of all chemicals were freshly made at six different concentration levels for the preparation of calibration standards. Calibration standards were spiked in fortified sample matrix.

Acetonitrile, methanol, n-hexane, and ethyl acetate were analytical grade and supplied by Merck (Darmstadt, Germany). N,N,N',N'-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD; ≥ 95% purity) was supplied by Sigma-Aldrich (St. Louis, MO, USA). Citric acid, disodium hydrogen phosphate, ammonium acetate, 25% ammonia, acetic acid, and hydrochloric acid were supplied by Merck. Deionized water was obtained using a Millipore purification system (Millipore, Billerica, MA, USA) with a specific resistance of 18.2 MΩ cm. McIlvaine's buffer was prepared by adding 9.36 g citric acid and 1.55 g disodium hydrogen phosphate and diluting to 500 mL with water. The pH was adjusted to 3.0. The elute solvent was freshly prepared by making a solution with 5 mL of 25% ammonia, 50 mL of ethyl acetate, and 45 mL of methanol. TMPD solution was obtained by using 50 mg of TMPD and diluting with methanol to 50 mL. The reconstituted solvent consisted of McIlvaine's buffer:acetonitrile (50:50, v/v).

Cartridges packed with copolymer that contained cation exchange solid phase (Oasis MCX, 60 mg, 3 mL) were supplied by Waters (Dublin, Ireland). Hydrochloric acid (0.1 N) was prepared by diluting 8.24 mL of hydrochloric acid in 1000 mL of water.

### 2.3. Fish muscle and feed samples

Fish muscle of fresh Taiwan tilapia (*Oreochromis hybrids* Tilapia spp) and milk fish (*Chanos chanos*) and feed samples were bought from traditional markets and then stored at –20 °C. Laboratory samples of fish muscle and feed that contained no detectable residues of the analytes were used as negative control and matrix-matched calibration. Fresh feed samples were bought from feed factories directly.



**Fig. 1 – The structures of the five antibiotic dyes used in the study.**

#### 2.4. General procedure

Fish muscle was sliced from dorsal muscle and homogenized using a high speed blender. Feed sample was first prehomogenized with a large volume blender (up to 1 kg capacity), and then over 50 g was taken and homogenized in a high-speed blender (variable speed up to 10,000 rpm). A well-homogenized fish muscle or feed was accurately weighed (1.0 g) into a 50-mL plastic tube. The internal standard consisting of 50  $\mu$ L of MG-d<sub>5</sub> and LMG-d<sub>5</sub> at concentrations of 100  $\mu$ g/L was spiked into the matrix. TMPD solution (50  $\mu$ L) and 10 mL of McIlvaine's buffer:acetonitrile (50:50, v/v) were added. After vortexing for 45 seconds and

sonication for 15 minutes, the pellets were separated by centrifuging for 20 minutes at 2400 g and the supernatant was kept. The pellets were extracted with 5 mL of McIlvaine's buffer:acetonitrile (50:50, v/v), repeating all shaking and centrifuging steps. The supernatants were combined into another 50-mL plastic tube. The sample extracts were further purified by cation exchange SPE using an Oasis MCX cartridge. A total of 15 mL of sample extract was loaded onto the cartridges (preconditioned with 2 mL of methanol, 2 mL of water, and 2 mL of McIlvaine's buffer). The cartridges were washed with 2 mL of 0.1 N hydrochloric acid, 2.5 mL of water, and then dried using a vacuum pump. The cartridges were further washed with 3 mL of 50% methanol solution and 5 mL of *n*-hexane and then dried using a vacuum pump for 5 minutes. The cartridges were eluted with the eluting solvent (5 mL). The eluates were reduced to dryness under a nitrogen stream at 50 °C and reconstituted with 1 mL of McIlvaine's buffer:acetonitrile (50:50, v/v). The final solution was filtered using a 0.22- $\mu$ m filter and the sample was transferred into a HPLC vial. An aliquot (25  $\mu$ L) was injected onto the LC column.

#### 2.5. Matrix-matched calibration

Matrix-matched calibration curves including fish muscle and feed samples were prepared and used for quantification, respectively. Control samples previously tested and shown to contain no residues were prepared as described in 'General procedure'. Control samples were used for each calibration standard level. Fish tissue or feed samples were weighed into 50-mL polypropylene tubes. Blank, calibration curve, and spiked samples were fortified with internal standard at a concentration level of 5  $\mu$ g/L by adding 50  $\mu$ L of 100  $\mu$ g/L mix solution of MG-d<sub>5</sub> and LMG-d<sub>5</sub> before sample pretreatment. Calibration samples were fortified at levels corresponding to 0.5, 0.8, 1.0, 2.0, 5.0, and 10.0  $\mu$ g/L by adding 5, 8, 10, 20, 50, and 100  $\mu$ L of a 100  $\mu$ g/L mix standard solution.

#### 2.6. Method validation

Identification of the MG, LMG, CV, LCV, and MB in the samples was based on the Commission Decision 2002/657/EC criteria [9]. The stability of the stored standard solution of mixed MG, LMG, CV, LCV, and MB was tested under four

**Table 1 – Transition reactions monitored by LC/MS/MS/ESI, retention time, and peak area ratios.**

Analyte	Transition reactions (m/z)		Retention time (min)	Peak area ratio (%)
	Quantitation ion pair	Confirmation ion pair		
MG	329.2 $\rightarrow$ 313.2	329.2 $\rightarrow$ 208.2	3.22	25 $\pm$ 3
LMG	331.2 $\rightarrow$ 239.2	331.2 $\rightarrow$ 316.0	4.81	43 $\pm$ 4
MG-d <sub>5</sub>	334.5 $\rightarrow$ 318.3	—	3.10	—
LMG-d <sub>5</sub>	336.4 $\rightarrow$ 239.3	—	4.76	—
CV	372.4 $\rightarrow$ 356.3	372.4 $\rightarrow$ 340.4	3.59	32 $\pm$ 3
LCV	374.3 $\rightarrow$ 358.4	374.3 $\rightarrow$ 239.1	4.78	90 $\pm$ 3
MB	284.4 $\rightarrow$ 268.2	284.4 $\rightarrow$ 240.4	2.06	10 $\pm$ 2

CV = crystal violet; LC/MS/MS/ESI = liquid chromatography/tandem mass spectrometry/electrospray ionization; LCV = leucocrystal violet; LMG = leucomalachite green; LMG-d<sub>5</sub> = leucomalachite green-d<sub>5</sub>; MB = methylene blue; MG = malachite green; MG-d<sub>5</sub> = malachite green-d<sub>5</sub>.

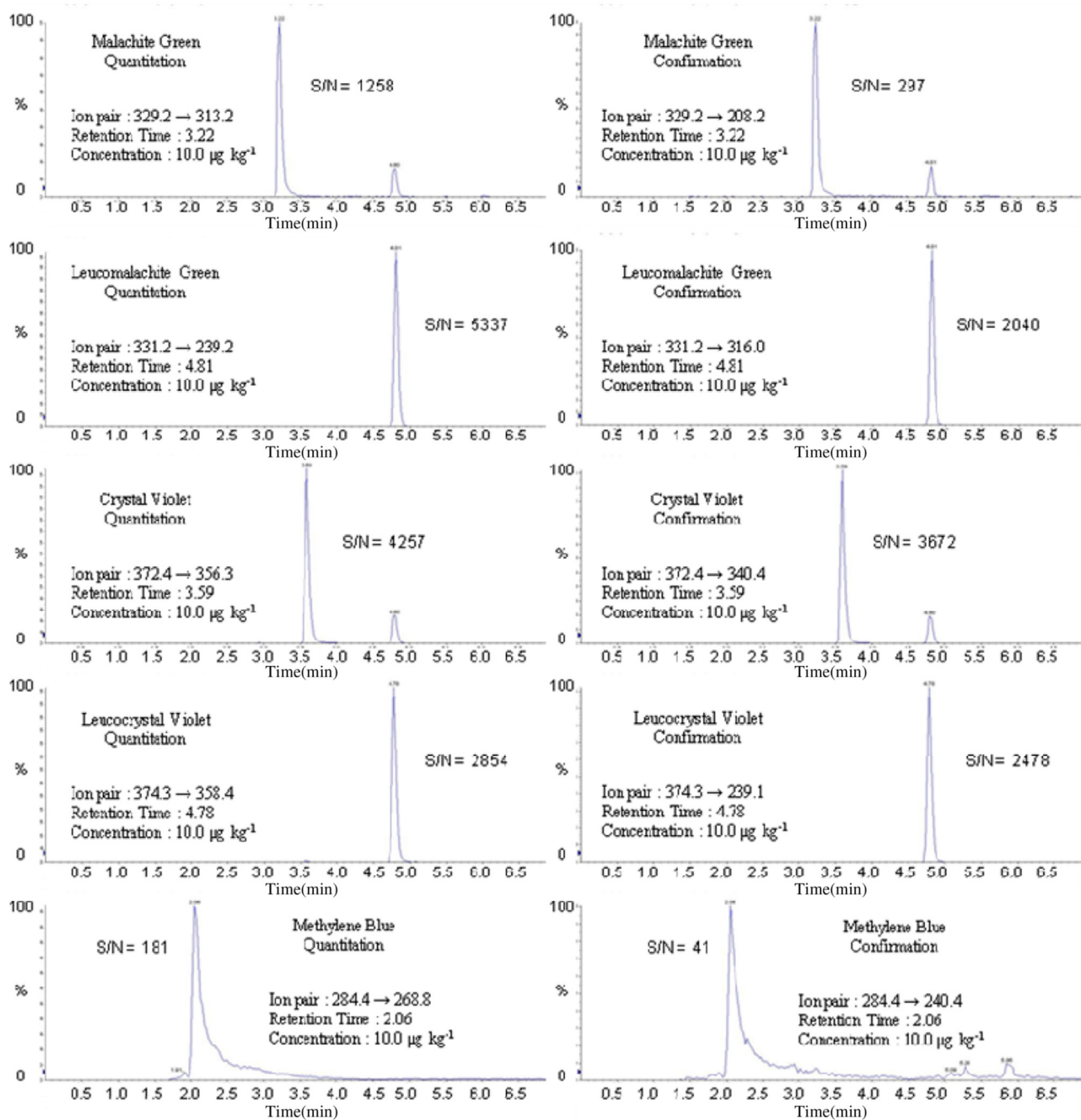


Fig. 2 – The multiple reaction monitoring (MRM) chromatogram for each of the target analytes in the fish muscle extract spiked at 10 µg/kg.

different conditions: storage in the light for 8 hours per day at 25 °C; in darkness at 25 °C; in darkness at 4 °C; and in darkness at –20 °C. The storage time was 30 days. Calculation of the concentration of each analyte residue was performed using freshly prepared analyte solution at the time of analysis. The following formula was applied: Analyte Remaining (%) =  $C_t/C_{\text{fresh}} \times 100\%$ , where  $C_t$  = concentration at time point  $t$ ,  $C_{\text{fresh}}$  = concentration of fresh solution. The maximum storage time and the optimum storing conditions

were recorded. Because there was no certified reference material available, the trueness of measurements was shown by the recovery of additions of seven fortified concentration levels of the analytes to blank fish muscle and feed samples. Recovery was calculated using the following formula: Trueness (%) =  $(C_m/C_f) \times 100\%$ , where  $C_m$  = mean of measurements,  $C_f$  = addition of fortified concentration levels. Recovery data was considered acceptable when the trueness was within  $\pm 10\%$  of the target value. Precision



(intra- and inter-day) was calculated by analysis of blank fish muscle and feed samples fortified with MG, LMG, CV, LCV, and MB at six fortification levels (0.5, 0.8, 1.0, 2.0, 5.0, and 10.0 µg/kg), and the experiments were performed by the same operator in triplicate on the same day and on 12 separate occasions in a month. Ion ratios (peak area of confirmation ion pair/peak area of quantitation ion pair × 100%) of the described MB, CV, LCV, MG, and LMG were  $11.8 \pm 5.0$ ,  $34.9 \pm 4.4$ ,  $88.4 \pm 3.2$ ,  $25.6 \pm 2.6$ , and  $42.0 \pm 4.4$ , respectively ( $n = 99$ ).

The values of decision limit ( $CC\alpha$ ) and detection capability ( $CC\beta$ ) were calculated following the Commission Decision 2002/657/EC guidelines [9].  $CC\alpha$  was calculated as the corresponding concentration of the y-intercept of a calibration curve constructed with blank fish tissue and feed samples, which was spiked with fortified concentrations of MG, LMG, CV, LCV, and MB mixed standard solutions at concentration levels above the minimum required performance concentration levels in equidistant steps, plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept ( $\alpha$ -error is the rate of false noncompliant results and defined as 1%).  $CC\beta$  was calculated as the corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility, which was the mean of the decision limit.  $\beta$ -Error is the rate of false compliant results and defined as 5%. The equation and method for estimating  $CC\alpha$  and  $CC\beta$  refer to ISO 11843-2 (2000) (this is an official document). The calculated equations are shown as follows:

$$CC\alpha = t_{df,1-\alpha} \frac{Sy}{b} \sqrt{1 + \frac{1}{n} + \frac{\bar{x}^2}{J \sum_{i=1}^I (x_i - \bar{x})^2}}$$

$$CC\beta = \delta_{\alpha,\beta} \frac{Sy}{b} \sqrt{1 + \frac{1}{I} + \frac{\bar{x}^2}{J \sum_{i=1}^I (x_i - \bar{x})^2}}$$

( $b$  is the slope of the regression line,  $\bar{x}$  is the mean concentration,  $t$  is the associated t-value,  $\delta$  is the noncentrality parameter,  $Sy$  is the standard error of the estimate,  $J$  is the number of replicates per concentration level for the spiked samples,  $I$  is the number of concentration levels for the spiked

samples: ( $i = 1, 2, \dots, I$ ) and  $\delta_{\alpha,\beta} = t_{df,1-\alpha} + t_{df,1-\beta}$  is the non-centrality parameter ( $\alpha = 1\%$ ,  $\beta = 5\%$ ).

### 3. Results and discussion

#### 3.1. Method development

The LC/MS/MS method was developed to provide confirmatory data for the analysis of fish muscle tissue and feed samples for MG, LMG, CV, LCV, and MB whose structures are shown in Fig. 1. The MS/MS fragmentation conditions were investigated and collision energies were optimized for each individual compound. For a method to be deemed confirmatory, one parent ion and two daughter ions must be monitored (Table 1). This yielded four identification points, which provided a suitable confirmatory method in accordance with 2002/657/EC [21].

HPLC columns and conditions were studied in order to optimize the chromatographic separation in terms of resolution and overall analysis time due to the different properties of compounds under investigation. Eclipse XDB-C<sub>8</sub> (4.6 × 150 mm, 5 µm; Agilent) using an ammonium acetate/acetonitrile mobile phase was subsequently found to give the most reliable result, good peak shape, and nice resolution. Product ion spectra resulting from collision-induced dissociation were examined and suitable ions selected for multiple reaction monitoring (MRM) schemes (Fig. 2).

Numerous HPLC methods for the determination of dyes such as MG, CV, and some of their leuco-metabolites in aquatic products have been proposed. Most of them have applied MS to determine the amount of MG or CV in samples [11,12,14,16,20–23]. Development of simultaneous determination of MB, MG, and CV and their metabolites LMG and LCV in fish muscles and fish feeds by LC/MS/MS is required.

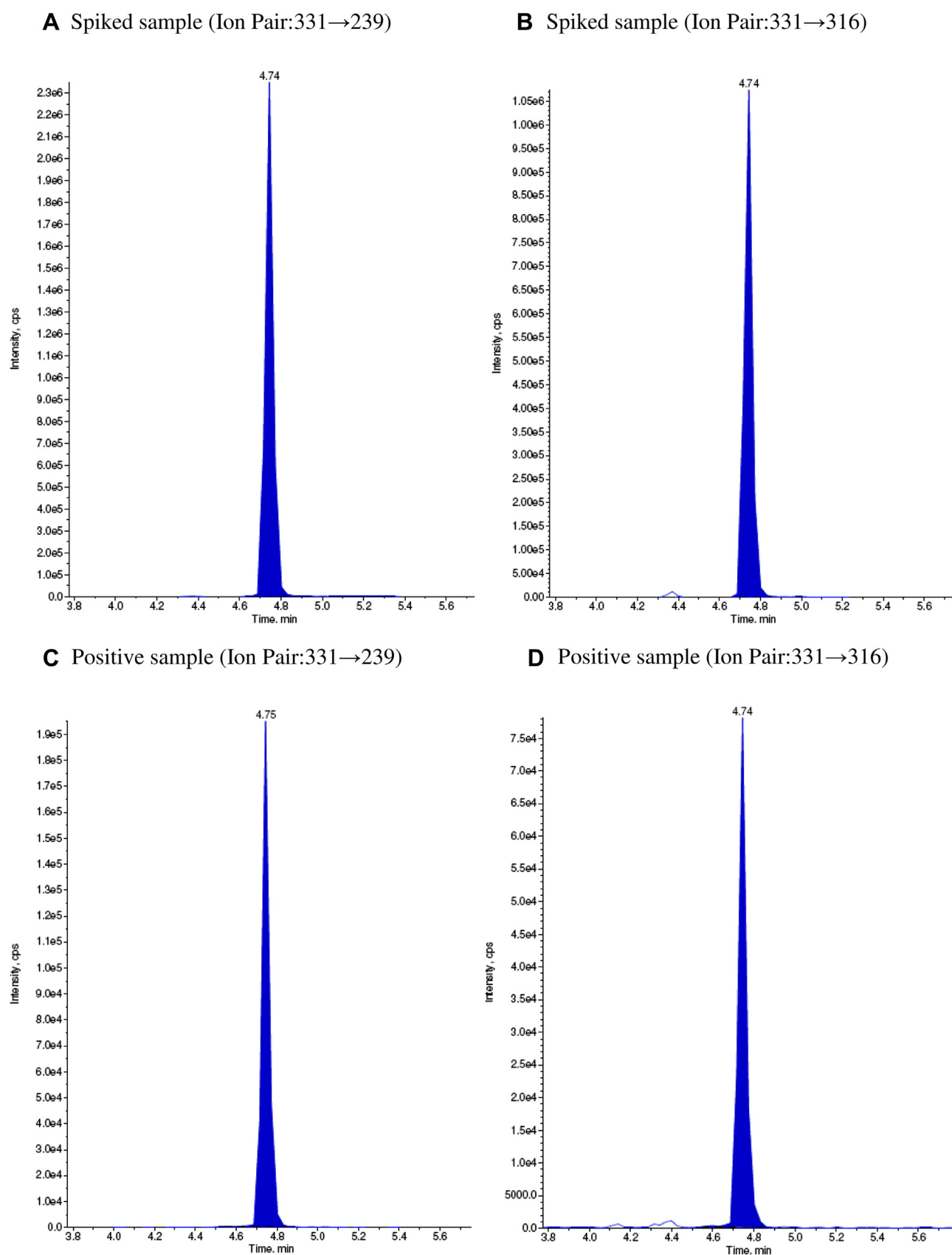
#### 3.2. Method validation

The linearity of the chromatographic response was tested with matrix-matched curves using six concentration levels in the range of 0.5–10.0 µg/L. The linear regression ( $r$ ) for all the calibration curves used in this study were  $\geq 0.995$ .

**Table 2 – Results for repeatability of inter-day and intra-day reproducibilities of malachite green (MG), leucomalachite green (LMG), crystal violet (CV), leucocrystal crystal violet (LCV), and methylene blue (MB) in fish muscle and feed samples.**

Analyte	Matrix	Fortification concentration level (µg/kg)	Intra-day		Inter-day	
			Recovery (%)	RSD* (%)	Recovery (%)	RSD* (%)
MG	Fish muscle ( $n = 79$ )	0.8	101	6.6	103	12.6
LMG		0.8	100	6.1	101	9.7
CV		0.8	99	3.8	106	13.0
LCV		0.8	100	5.1	102	14.0
MB		0.8	100	6.8	102	12.0
MG	Feed ( $n = 20$ )	0.8	100	8.9	97	11.3
LMG		0.8	99	7.0	96	10.1
CV		0.8	95	7.1	93	8.9
LCV		0.8	96	9.3	94	11.1
MB		0.8	97	8.4	96	10.2

\* RSD is relative standard deviation



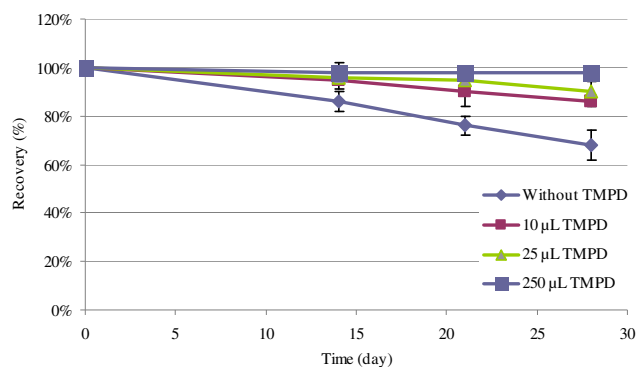
**Fig. 3** – The typical chromatograms of spiked fortified concentration at 10  $\mu\text{g/kg}$  for leucomalachite green (LMG) in the blank feed sample and positive sample (A) quantitation ion pair ( $m/z$ : 331→239) of spiked sample; (B) confirmation ion pair ( $m/z$ : 331→316) of spiked sample; (C) quantitation ion pair ( $m/z$ : 331→239) of positive sample; (D) confirmation ion pair ( $m/z$ : 331→316) of positive sample.

The recovery of the method was determined using fish muscle ( $n = 79$ ) and feed samples ( $n = 20$ ) fortified at  $0.8 \mu\text{g/kg}$  for each dye and metabolite. The mean recoveries of fish muscle ( $n = 99$ ) of analytes, determined during 1 year (Table 2), were 103, 101, 106, 102, and 102% for MG, LMG, CV, LCV, and MB, respectively. The average corrected recoveries of feed ( $n = 99$ ) of analytes were 97, 96, 93, 94, and 96% for MG, LMG, CV, LCV, and MB, respectively. The usefulness of a suitable isotope internal standard was demonstrated in the excellent reproducibilities, and inter-day and intra-day reproducibility was obtained by using the internal standard of MG and LMG (Table 2). Although no isotope internal standard is available for CV, LCV, and MB, an acceptable repeatability about intra-day and inter-day reproducibility was obtained by using matrix-match calibration curves. In the comparison between fish muscle and feed samples (spiked concentration level  $0.8 \mu\text{g/kg}$ ), the recoveries of feed samples were 28–63% of the fish muscle samples (data was not shown). Therefore, the matrix was the same with the calibration curve and the testing sample during the analysis of dyes and metabolites in fish muscle and feed samples. The developed method was evaluated by comparison of results when the method was performed, and the results were then passed through FAPAS proficiency tests. The test result of  $1.765 \mu\text{g/kg}$  of LMG in fish muscle compared with the  $1.99 \mu\text{g/kg}$  FAPAS (Food Analysis Performance Assessment Scheme) assigned value (Report No. 02185, 2012) was  $-0.5$  of the z-score. In real sample testing, it was a positive case in terms of the residue of LMG in feeds. The concentration of LMG was  $3.03 \text{ ppb}$ . The relative ion intensity (46.9%) of LMG in the positive sample was similar to the relative ion intensity (47.0%) of LMG in the spiked sample. The chromatogram is shown in Fig. 3.

$\text{CC}\alpha$  and  $\text{CC}\beta$  were calculated following the calibration curve procedure according to ISO 11843. The calibration curve procedure involved the use of six concentration levels and was performed by the same operator in triplicate on 1 day and on 12 separate occasions within a month. Each spiked concentration of MG, LMG, CV, LCV, and MB was duplicated and determined twice by LC/MS/MS/ESI. The  $\text{CC}\alpha$  and  $\text{CC}\beta$  values are shown in Table 3. The decision limits ( $\text{CC}\alpha$ ) and detection capabilities ( $\text{CC}\beta$ ) were  $0.28\text{--}0.54 \mu\text{g/kg}$  and  $0.35\text{--}0.67 \mu\text{g/kg}$  for MG, LMG, CV, LCV, and MB, respectively.

**Table 3 – Calculated  $\text{CC}\alpha$  and  $\text{CC}\beta$  values of malachite green (MG), leucomalachite green (LMG), crystal violet (CV), leucocrystal crystal violet (LCV), and methylene blue (MB) in fish muscle and feed samples ( $n = 99$ ).**

Analyte	Matrix	$\text{CC}\alpha$ ( $\mu\text{g/kg}$ )	$\text{CC}\beta$ ( $\mu\text{g/kg}$ )
MG	Fish muscle ( $n = 79$ )	$0.29 \pm 0.15$	$0.39 \pm 0.20$
LMG		$0.15 \pm 0.09$	$0.22 \pm 0.13$
CV		$0.32 \pm 0.16$	$0.42 \pm 0.22$
LCV		$0.29 \pm 0.14$	$0.39 \pm 0.19$
MB		$0.41 \pm 0.18$	$0.54 \pm 0.23$
MG	Feed ( $n = 20$ )	$0.47 \pm 0.24$	$0.57 \pm 0.29$
LMG		$0.33 \pm 0.19$	$0.40 \pm 0.23$
CV		$0.50 \pm 0.26$	$0.60 \pm 0.32$
LCV		$0.47 \pm 0.22$	$0.57 \pm 0.27$
MB		$0.59 \pm 0.26$	$0.72 \pm 0.31$



**Fig. 4 – Stability of malachite green (MG) stock standard solution at  $1.0 \text{ mg/L}$  concentration level (stored in a refrigerator at  $-25^\circ\text{C}$ ) with the addition of  $1000 \text{ mg/L}$   $\text{N,N,N',N'}$ -tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD) in 28 days.**

As part of the method validation, the stock solution standard stability was assessed in several ways (stored in a refrigerator at  $-25^\circ\text{C}$ ): (1) without adding TMPD; (2)  $10 \mu\text{L}$ ,  $1000 \mu\text{g/L}$  TMPD; (3)  $25 \mu\text{L}$ ,  $1000 \mu\text{g/L}$  TMPD; (4)  $250 \mu\text{L}$ ,  $1000 \mu\text{g/L}$  TMPD. The stability of the stock standard solution was assessed by the addition of  $250 \mu\text{L}$  of TMPD (concentration level:  $1000 \mu\text{g/L}$ ). It was found that MG was stable for at least 4 weeks with the addition of  $25 \mu\text{L}$  of  $1000 \mu\text{g/L}$  TMPD (Fig. 4).

Based on the acceptable results of the method validation, the method that we described in this study could be executed in the analysis of three antibiotic dyes and two leuco-form metabolites in fish muscle and fish meal. Compared with previous methods [11,12,14,16,20–23], we can determine three antibiotic dyes and two metabolites in one round and get lower limits of quantification ( $0.5 \mu\text{g/kg}$  and  $1.0 \mu\text{g/kg}$ ).

## 4. Conclusions

A relatively stable, fast, and selective LC/MS/MS method for the simultaneous determination of MG, LMG, CV, LCV, and MB in fish muscle and feed samples was developed. There are few published confirmatory methods for the simultaneous determination of MG, LMG, CV, LCV, and MB in fish muscle and feed samples that are validated according to the Commission Decision 2002/657/EC. This study shows that the required sensitivities for MG and LMG were obtained and met the MRPLs (Minimum Required Performance Limits) of  $2 \mu\text{g/kg}$  and  $0.5 \mu\text{g/kg}$  defined by the EU and Taiwan Food and Drug Administration, respectively. Although there is no MRPL set for CV, LCV, and MB, the method is sensitive for CV, LCV, and MB. The method performed very well in terms of accuracy and stability (over 1 year,  $n = 99$ ). The results of this study were satisfactory for the development of a rugged analytical method.

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