



# Analysis of Triphenylmethane Dye Residues and their Leuco-Forms in Frozen Fish by LC-MS/MS, Fish Microbial Quality, and Effect of Immersion in Whole Milk on Dye Removal

Sana Gammoh<sup>1</sup>, Muhammad H. Alu'datt<sup>1</sup>, Mohammad N. Alhamad, Taha Rababah, Zaid A. Ammari, Carole C. Tranchant<sup>2</sup>, Wesam Talafha, and Mohammad AlRosan

**Abstract:** A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was validated and used to quantify crystal violet (CV), leucocrystal violet (LCV), malachite green (MG), leucomalachite green (LMG), and brilliant green (BG) residues in frozen fish (121 samples) from various countries, in order to detect the use of prohibited antibiotic dyes in fish for human consumption. The microbial quality of the fish was also assessed along with the effectiveness of a simple treatment with whole fat milk to reduce the levels of CV and LCV contamination. CV and LCV were the only two residues detected. They were found in farmed *Pangasius* (0.362 to 41.34  $\mu\text{g}/\text{kg}$  and 0.178 to 10.58  $\mu\text{g}/\text{kg}$ , respectively) and *Tilapia* (1.24 to 9.48  $\mu\text{g}/\text{kg}$  and 1.29 to 2.81  $\mu\text{g}/\text{kg}$ ). Based on aerobic plate count (APC), 74%, 59%, and 55% of the samples of *Tilapia* fillets (from China) and *Pangasius* fillets (United Arab Emirates and Vietnam), and 100% and 50% of the skin samples of *Hake* (Argentina and U.S.A.) were of unacceptable microbial quality (APC >  $10^7$  cfu/g). Human pathogens, namely *Escherichia coli*, *Staphylococcus aureus*, and *Vibrio* spp., were detected in most fish. A significant reduction in CV and LCV concentrations by more than a third was achieved after immersing *Pangasius* and *Tilapia* fillets in whole fat milk for 120 minutes. These findings support the necessity of regular inspections and monitoring of CV and other antibiotic dye residues in fish, along with routine assessments of fish microbial quality, in order to protect public health.

**Keywords:** processed fish, antibiotic dyes, leuco metabolites, detection method, decontamination treatment, milk

**Practical Application:** The described LC–MS/MS method can be used to rapidly and simultaneously quantify antibiotic dye residues in frozen fish. CV and LCV were detected in farmed *Pangasius* and *Tilapia* fillets and their concentrations was reduced by more than one third after immersing the fillets in whole milk for 120 min, a treatment which is not intended to replace safe fish farming practices upstream to artificially lower the level of banned dyes in fish. The findings support the necessity of regular inspections and monitoring of CV and other antibiotic dye residues in fish, along with assessments of fish microbial quality, to protect public health.

## Introduction

Cationic triphenylmethane dyes, which include crystal violet (CV, also known as gentian violet), malachite green (MG), and brilliant green (BG), are common veterinary drugs used as anti-fungal, antibacterial, and antiparasitic agents in fish (Balabanova, Popova, & Tchipeva, 2003). Because of their low cost and ease of use, they have been widely used in the aquaculture of food and ornamental fish species since the 1930s (Alderman, 1985; Dowling, Mulder, Duffy, Regan, & Smyth, 2007; Foster & Woodbury, 1936). In various animal intestinal tracts under anaerobic

conditions, CV is reduced metabolically to leucocrystal violet (LCV; Docampo & Moreno, 1989; McDonald & Cerniglia, 1984). Fish tissues readily absorb CV and MG from water, then the dyes undergo bioconversion into non-chromophore (leuco) metabolites, namely LCV and leucomalachite green (LMG), which are persistent forms in tissues (Thompson, Rushing, Gehring, & Lochmann, 1999). The levels of LCV and LMG were found to be mostly dependent on the elimination times and lipid content of the fish (Alderman & Clifton-Hadley, 1993).

For safety reasons, many countries including the United States, Canada, and member states of the European Union (EU) have now banned the use of CV and MG in fish intended for human consumption (Andersen et al., 2009; Verdon et al., 2015). Toxicity concerns related to these dyes include possible carcinogenic and mutagenic effects in humans (Culp & Beland, 1996; Srivastava, Sinha, & Roy, 2004; Stammati et al., 2005; Sudova, Machova, Svobodova, & Vesely, 2007). BG has not been as well studied as CV and MG, but its chemical similarity suggests that it may be comparably toxic to humans and effectively absorbed by fish (Andersen et al., 2009). The European Commission has

JFDS-2018-0914 Submitted 7/7/2018, Accepted 12/12/2018. Authors Gammoh, Alu'datt, Rababah, Talafha, Al-Rousan are with Dept. of Nutrition and Food Technology, Faculty of Agriculture, Jordan Univ. of Science and Technology, P.O. Box 3030, Irbid, 22110, Jordan. Alhamad is with Dept. of Natural Resources and Environment, Faculty of Agriculture, Jordan Univ. of Science and Technology, P.O. Box 3030, Irbid, 22110, Jordan. Ammari is with Dept. of Medicine, Univ. of Toledo Medical Center, Toledo, OH, U.S.A. Tranchant is with School of Food Science, Nutrition and Family Studies, Univ. de Moncton, Moncton, NB, E1A 3E9, Canada. Direct inquiries to author Gammoh (Email: sana\_gammoh@hotmail.com).

established methods for the determination these antibiotic residues and has set a minimum required performance limit (MRPL) of 2  $\mu\text{g}/\text{kg}$  for the sum of MG and LMG residues in the flesh of fish and other seafood products (European Commission, 2004). Although there are no established safe levels for the presence of CV, LCV, MG, LMG, and BG in seafood, it has been suggested that these compounds should be determined at concentrations below  $\mu\text{g}/\text{kg}$  (Xu et al., 2012). For quantitative determination, liquid chromatography–tandem mass spectrometry (LC-MS/MS) is recognized as one of the best techniques (Stolker & Brinkman, 2005). Compared to UV-Vis and fluorescence detection methods, LC-MS/MS allows for simultaneous detection of triphenylmethane dyes, without post-column oxidation and with superior sensitivity and selectivity (Ding et al., 2007). A few LC-MS/MS methods have been proposed for the analysis of CV, LCV, MG, and/or LMG in fish (Ascari et al., 2012; Chen et al., 2013; Giaccone et al., 2018; Hurtaud-Pessel, Couëdor, & Verdon, 2011; Lee et al., 2010; Xu et al., 2012), but none has yet received general acceptance. As confirmed by available reports, the potential for misuse and illegal use of triphenylmethane dyes in fish remains high (Andersen et al., 2009; Giaccone et al., 2018; Lee et al., 2010; Love, Rodman, Neff, & Nachman, 2011), which underscores the need for sensitive detection and widespread testing of fish products sold on various markets.

The removal of these dyes and their metabolites from fish products has received little consideration in the literature. Previous studies focused on the removal of CV and MG from aqueous solutions using biosorbents (Raval, Shah, & Shah, 2017), ionic liquids (Lv, Cui, Zou, & Zheng, 2018), or biological removal (Török et al., 2015). Other studies showed that CV is able to bind to proteins such as bovine serum albumin (BSA; Qin, Yin, & Shen, 2016; Xu et al., 2009) and casein (Santhanalakshmi & Balaji, 2001). Similarly, MG was reported to bind to BSA (Zhang et al., 2009) and lysozyme (Ding et al., 2012). These proteins occur naturally in milk, along with other proteins and nonprotein compounds which can bind a variety of molecules with different affinity through different mechanisms (Livney, 2010). The purpose of the present study was threefold: (i) to survey frozen fish products imported to Jordan for the presence of CV, LCV, MG, LMG, and BG using a validated LC-MS/MS method; (ii) to assess the microbial quality of the fish; and (iii) to assess the effectiveness of a treatment with whole fat milk to reduce the levels of dye residues in contaminated fish.

## Materials and Methods

### Chemicals and reagents

All the chemicals were of analytical-reagent grade and were purchased from Sigma-Aldrich (NJ, U.S.A.) unless otherwise stated. Acetonitrile (ACN), formic acid, and ammonium acetate were of LC-MS grade and hydroxylamine hydrochloride (HAH) was 99% pure. Standard materials for the dyes were CV chloride (46364, VETRANAL, CAS 548-62-9, 97.5% pure), LCV (219215, CAS 603-48-5, 99.5%), MG oxalate (46396, VETRANAL, CAS 2437-29-8, 99.3%), LMG (125660, CAS 129-73-7, 99.9%), LMG- $d_5$  internal standard (34182, CAS 947601-82-3, 99.9%), and BG sulfate (B6756, CAS 633-03-4, 90%).

For each standard (CV, LCV, MG, LMG, and BG), stock solutions were prepared in a serial manner with ACN in amber volumetric flasks. Stock solution A (100  $\mu\text{g}/\text{mL}$ ) was prepared first by dissolving the proper amount of standard in 100 mL of ACN; stock solution B (100 ng/mL) was prepared by diluting

0.1 mL from solution A in 100 mL ACN; and stock solution C (100 pg/mL) was prepared by diluting 0.1 mL solution B with 100 mL ACN. For the internal standard (IS), stock solutions D and E (100  $\mu\text{g}/\text{mL}$  and 1  $\mu\text{g}/\text{mL}$  of LMG- $d_5$  in 100 mL ACN, respectively) were prepared. Four quality control (QC) samples were prepared for each standard: lower limit of quantification (LLOQ) QC (0.01 ng/mL), QC low (1.0 ng/mL), QC medium (40 ng/mL), and QC high (80 ng/mL). To avoid degradation of these compounds due to exposure to light (Pérez-Estrada, Agüera, Hernando, Malato, & Fernández-Alba, 2008), all the standard solutions and the fish extracts were protected from light using amber vials, volumetric flasks, and centrifuge tubes. They were prepared by working in unlighted areas or under dimmed light as additional precautions. Standard solutions were stored in a dark volumetric flask at  $-40^\circ\text{C}$  until use for a maximum of 6 months, except for BG solutions which were only kept for a maximum of 2 months as recommended (Andersen et al., 2009). The solutions were brought to room temperature prior to use.

### Fish sample collection and preparation

Frozen fish samples imported to Jordan from various countries were collected from Jordanian markets. The samples (121 in total) were selected on the basis of species and country of origin: 27 skinless fillets of Pangasius from Vietnam, 27 skinless fillets of Pangasius from the United Arab Emirates (UAE), 27 skinless fillets of Tilapia from China, 20 headed Argentine Hake from Argentina, and 20 headed Pacific Hake from the United States. The samples were placed in sterile bags and transported frozen to the laboratory in an insulated icebox. From each fish or fillet, a composite sample was prepared under aseptic conditions as described by Effkemann (2005) by cutting small cubes of 3 to 5 cm from different parts, followed by homogenizing using a stomacher and storing at  $-20^\circ\text{C}$  in the dark until further analyses. Proximate chemical composition of the fish was determined according to the AOAC (2000) methods for moisture, crude protein, fat, and ash, with triplicate determinations. The nutrient content was expressed in g per 100 g of raw edible parts.

### Sample extraction and clean-up

Sample extraction and clean-up was performed as described by Effkemann (2005) with some modifications. Two grams of homogenized fish samples was transferred into a clean 20 mL glass tube, followed by addition of 100  $\mu\text{L}$  of IS solution E (LMG- $d_5$  1  $\mu\text{g}/\text{mL}$ ), 900  $\mu\text{L}$  of deionized water, and 2 mL of HAH solution (5 g/L) to help prevent the degradation of the dyes being analyzed (Giaccone et al., 2018). The tubes were placed on a vortex-mixer (FINEPCR, South Korea) for 10 min, then 8 mL of ACN were added and the mixture was vortexed for another 10 min, then centrifuged (Universal centrifuge Z 326 K, Hermle, Germany) at  $2,558 \times g$  for 5 min at  $4^\circ\text{C}$ . Two milliliters of the supernatant was withdrawn using a clean disposable syringe and transferred into 2 mL vials using a syringe filter (nylon membrane, diameter 25 mm, pore size 0.45  $\mu\text{m}$ ) (Anaqua Chemicals Supply, Cleveland, OH, U.S.A.).

### LC-MS/MS analyses

LC-MS/MS analyses were carried out using a gradient LC-MS/MS electrospray ionization (ESI) system consisting of an Agilent HPLC system (3200 Series; Agilent Technologies, Stuttgart, Germany) connected to an API 3200 mass spectrometer (AB-SCIEX QTRAP, Ontario, Canada), according to the method

described by Effkemann (2005), with some modifications. Mobile phase A consisted of deionized H<sub>2</sub>O, 10 mM ammonium acetate, and 0.1% formic acid, whereas mobile phase B consisted of ACN/H<sub>2</sub>O (90/10), 10 mM ammonium acetate, and 0.1% formic acid. ACN was used as it was found to be a better choice than methanol to preserve the stability of CV, MG, and their leuco metabolites, whereas formic acid was found to sharpen their elution (Xu et al., 2012). The analyses were performed at a flow rate of 400  $\mu$ L/min. Chromatographic separation was performed on a Synergi 4  $\mu$ m Max-RP 80Å, 50  $\times$  2 mm analytical column (Phenomenex Inc., CO, U.S.A.) at 30 °C. The injection volume of the samples or standard solutions was 10  $\mu$ L. The analyses were conducted using positive-ion electrospray interface (ESI+) in multiple reaction monitoring (MRM) mode, using a three-stage procedure: isocratic liquid chromatography program from 0 to 5 min with 70% of mobile phase A and 30% of mobile phase B; 5 to 14 min with 10% of mobile phase A and 90% of mobile phase B; then 14 to 15 min with 70% of mobile phase A and 30% of mobile phase B. At the retention time of the analytes being measured (5.1, 4.8, 4.1, 8.2, 8.1, and 5.8 min for CV, LCV, MG, LMG, LMG-d<sub>5</sub>, and BG, respectively), the conditions of the ESI interface were as follows: source temperature 150 °C, desolvation temperature 350 °C, desolvation gas (nitrogen) 800 L/h, cone gas (nitrogen) 100 L/h, with argon as the collision gas (SCIEX, 2015).

#### LC-MS/MS method validation

The LC-MS/MS method was validated in compliance with the FDA Guidance for Bioanalytical Method Validation (FDA, 2013) using fresh fish muscle as blank. Blank samples were prepared from local fresh fish caught from the Aqaba coast, Jordan, which had a known history and had not been exposed to triphenylmethane dyes. The linearity of the assay was evaluated using eight-point calibration curves of peak areas versus concentration prepared with CV, LCV, MG, LMG, and BG standards over a concentration range of 0.01 to 100 ng/mL (0.01, 0.1, 1.0, 5.0, 10, 25, 50, and 100 ng/mL), including the LLOQ which was the lowest concentration. Curve linearity was determined by weighted least-square linear regression analysis and was considered to be acceptable when squared correlation coefficients ( $r^2$ ) exceeded 0.999. Specificity of the assay was assessed by spiking blank samples with known amounts of CV, LCV, MG, LMG, BG, and IS LMG-d<sub>5</sub> and by checking for potential significant interference in the chromatograms at the peak regions of the standards. Inter-day accuracy and precision were evaluated on three different days by analyzing blank muscle samples fortified with CV, LCV, MG, LMG, BG, and LMG-d<sub>5</sub> at concentrations of 0.01 ng/mL (LLOQ), 1.0 ng/mL (QC low), 40.0 ng/mL (QC medium), and 80.0 ng/mL (QC high). At each concentration, six replicate analyses were conducted. Accuracy was assessed by performing recovery experiments and calculating the percent deviation from the nominal concentration. According to the FDA guidelines, the mean value should be within  $\pm 15\%$  of the nominal value for all the concentrations, except for the LLOQ, where it should be within  $\pm 20\%$  of the nominal value. Recovery was calculated as the ratio of (measured concentration/fortification concentration)  $\times$  100. Precision and reproducibility were assessed using the relative standard deviation (RSD) of the recovery. The limits of quantification (LLOQ) and limits of detection (LOD) were calculated and defined as a signal-to-noise ratio of 10:1 and 3:1, respectively, and obtained from blank samples. Potential matrix effect, that is, the possible effect of some endogenous fish constituents on the ionization of the analytes being measured, which could suppress

or enhance ionization, was investigated by comparing the peak areas for the analytes in standard solution with the peak areas of the same standard solutions spiked into blank fish.

#### Microbiological analyses

**Preparation of test samples.** Fifty grams of composite sample was mixed with 450 mL of a 2% (v/v) sterile sodium citrate solution under aseptic conditions by placing in a stomacher for 4 minutes. An initial dilution of  $10^{-1}$  was prepared and additional dilutions ( $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$ ) were prepared in 0.1 mL sterile peptone water. All the dilutions were used within 20 min of preparation.

**Aerobic plate count.** Aerobic plate count (APC), an indicator of microbial populations in a product (Maturin & Peeler, 2001), was determined according to the method described by Baylis (2007). A 1-mL aliquot of each dilution (up to  $10^{-6}$ ) was placed in two Petri dishes. Fifteen milliliters of prepared plate count agar at 45 °C was poured onto each plate, then the plates were gently swirled for mixing the sample with agar. The plates were left to solidify and dry. They were then incubated at 30 °C for 48 hr before counting the aerobic microorganisms, expressed as colony-forming units per gram of sample (cfu/g).

**Detection of microorganisms.** *Salmonella* spp. was determined according to the ISO 6579:2002 standard (Thermo Scientific, 2013). Pre-enrichment was conducted using samples diluted in 225 mL of buffered peptone water incubated at 37 °C for 18 hr. Secondary selective enrichment was performed in RVS Broth incubated for 24 hr at 41.5 °C and Muller–Kauffmann Tetrathionate–Novobiocin broth incubated for 24 hr at 37 °C  $\pm$  1 °C, and plating on xylose–lysine–deoxycholate agar and brilliant green agar incubated for 24 hr at 37 °C.

*Escherichia coli* was determined according to the ISO 7251:2005 standard (Thermo Scientific, 2013). Pre-enrichment was conducted with samples diluted in 225 mL lauryl sulfate broth. Secondary selective enrichment was performed by inoculating one loopful of culture on EC broth followed by incubation for 24 hours at 44 °C (or up to 48 hr for negative tubes). Growth and gas production in brilliant green bile broth and indole production in tryptone water (10 mL) following incubation at 44 °C were used for confirmation of *E. coli*.

*Staphylococcus aureus* was determined according to the ISO 6888:2003 standard (Thermo Scientific, 2013). Pre-enrichment was conducted with samples diluted in 225 mL of Giolitti–Cantoni broth sealed with paraffin incubated for 24 hr at 37 °C (up to 48 hr for negative tubes). Secondary selective enrichment was performed with one loopful of inocula onto Baird–Parker agar incubated for 24 hr at 37 °C. Brain heart infusion broth and rabbit plasma were used for confirmation of coagulase-positive *Staphylococci*.

*Vibrio* spp. was determined according to the ISO/TS 21872-1:2007 standard (ISO, 2007), which allows for the detection of potentially enteropathogenic *V. parahaemolyticus* and *V. cholerae*. Pre-enrichment was conducted in 225 mL of alkaline saline peptone water (ASPW) incubated for 6 hr at 37 °C. After incubation, 1 mL of enriched culture was transferred to a test tube containing 10 mL of ASPW and incubated for 18 hr at 41.5 °C, followed by inoculation onto thiosulfate citrate bile salts sucrose agar and incubation for 24 hr at 37 °C.

**Enumeration of microorganisms by spread-plate technique.** Serial dilutions and enumeration by spread-plate technique were performed according to the National Food Safety Standard of the People's Republic of China (2010). Colonies of survivors were counted after incubation and the number of cfu/g

**Table 1—Moisture, protein, fat, and ash composition of frozen Pacific Hake, Argentine Hake, Pangasius, and Tilapia fish imported to Jordan from various countries.**

Fish and country of origin	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Pacific Hake <sup>b</sup> , U.S.A.	79.0	16.08	2.45	1.32
Argentine Hake <sup>b</sup> , Argentina	78.6	15.98	2.30	1.90
Pangasius <sup>a</sup> , Vietnam	82.0	15.20	2.46	2.06
Tilapia <sup>a</sup> , China	79.9	14.75	0.54	1.17
Pangasius <sup>a</sup> , UAE	90.6	6.99	0.68	1.50

Mean concentration values are for three replicates and expressed in percent (g per 100 g of raw edible parts).

<sup>a</sup>Fillet fish.

<sup>b</sup>Headed.

of the original sample was calculated from the number of colonies counted on selected plates.

**Treatment of fish with whole milk.** Fish samples that tested positive for CV and LCV (Pangasius and Tilapia) were treated by immersing the skinless fillets in commercially available whole fat milk (3.5% milk fat, homogenized and pasteurized) for different times (0, 30, 60, 120, and 240 min) at 4 °C in order to determine the effect of this treatment on the concentrations of CV and LCV residues in the flesh. Preliminary trials were also conducted using water as a control treatment. Immersion in water resulted in appreciable loss in the cohesiveness and integrity of fish fillets after the first 30 min of immersion. Our aim was to use a control that did not appreciably affect the texture of the fish. Therefore, fish without any treatment served as its own control (time 0). The dimensions of the frozen fillets ranged from 28 to 33.5 cm (length), 9.2 to 11.5 cm (width), 0.9 to 1.4 cm (where the fillet thickness is the highest), and 0.4 to 0.6 cm (where thickness is the lowest). The thawed fillets were divided up linearly into two halves: one half was used for detection of the dyes and microbiological analyses, whereas the other half was immersed in 350 mL of milk. After treatment, the fillets were rinsed with deionized water for 1 minute and a composite sample was prepared as previously described using different parts of the fillet. Seven samples of fish contaminated with CV and/or LCV were used in this experiment, with three replicates per sample, and monitored for the presence of CV and LCV using LC-MS/MS.

**Statistical analyses.** Data were checked for normality and homogeneity of variance and analyzed using one-way ANOVA performed with the SAS JMP software, version 8.0.1 (SAS 2009 Institute Inc., Cary, NC, U.S.A.). Means were separated using the least significant difference (LSD) test procedure. Pearson correlation coefficients (*r*) were calculated to test the relationship between select variables. For all statistical tests, values of  $P \leq 0.05$  were considered significant.

## Results and Discussion

### Proximate composition of imported frozen fish

The moisture, protein, fat, and ash composition of frozen Pacific Hake, Argentine Hake, Pangasius, and Tilapia is shown in Table 1. Pangasius from the UAE had the highest moisture content (90.6%), whereas Pangasius from Vietnam, Tilapia from China, Pacific Hake, and Argentine Hake had lower contents (82.0%, 79.9%, 79.0%, and 78.6%, respectively). These values are consistent with the average moisture content generally found in fish (ca. 80%), noting that values around 90% are considered extreme (FAO, 1995). In the case of Pangasius (UAE), the very high moisture content was accompanied by very low fat (0.68%), in line with the generally inverse relation between fat and moisture contents in fish (FAO, 1995). All the fish studied are considered lean

fish as their fat contents do not exceed 5% by weight, ranging from 0.54% for Tilapia (China) to 2.5% for Pangasius (Vietnam) and Pacific Hake (U.S.A.). The protein content of UAE Pangasius (7%) was lower than the usual range of protein in fish muscle (15% to 20%; FAO, 1995), whereas the other fish had protein levels near 15%. Low protein levels may be indicative of some fish farming practices, as lower quantity and quality of proteins have been reported for fish fed on waste (Love et al., 2011). Ash content ranged from 1.17% (Tilapia) to 2.06% for Pangasius (Vietnam), which is comparable to the typical ash values reported for fish fillets (1.2% to 1.5%; FAO, 1995) and Pangasius fillets from Vietnam (0.76% to 2.38%; Guimaraes et al., 2016).

### Analytical performance

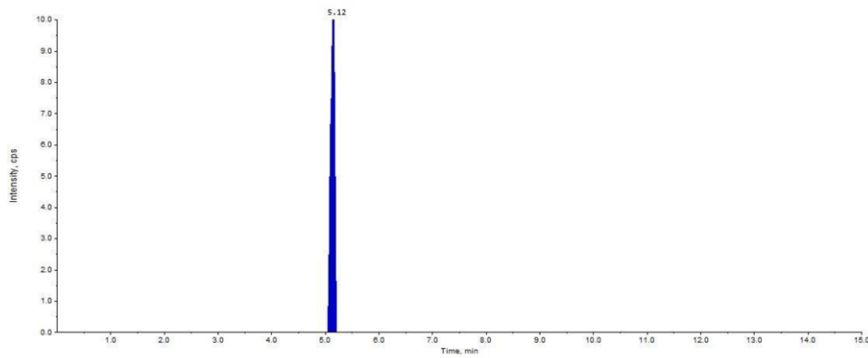
The LC-MS/MS conditions used in this work enabled the accurate and precise measurement of CV, LCV, MG, LMG, and BG in fish, as summarized in Table 2. Moreover, the method was specific to the dyes and leuco metabolites being quantified. Good resolution and reproducibility of the peaks was obtained with all five analytes (average retention times of 5.1, 4.8, 4.1, 8.2, and 5.8 min, respectively), with no interference with fresh fish endogenous constituents at these retention times. Representative chromatograms of the LC-MS/MS response for CV, LCV, MG, LMG, and BG at different concentrations are shown in Figure 1 and Figure S1 to S4, respectively. Matrix effect experiments showed no discernable interference and no enhancement or suppression in response at the retention times of CV, LCV, MG, LMG, and BG (Figure 2).

As shown in Table 2, repeated extraction and analysis of the four QC samples (LLOQ, QC low, QC medium, and QC high) yielded an interassay accuracy ranging from 92.57% to 100.6% (CV), 97.78% to 100.3% (LCV), 99.73% to 101.1% (MG), 97.64% to 100% (LMG), and 88.78% to 104.6% (BG). Interassay precision ranged from 0.16% to 28.64% (CV), 0.49% to 2.52% (LCV), 0.28% to 2.64% (MG), 0.42% to 9.63% (LMG), and 0.24% to 30.91% (BG). For all the analytes being quantified, good linearity was achieved in the range 0.01 to 100 ng/mL with squared correlation coefficients ( $r^2$ ) exceeding 0.999. The average recovery of the dyes in blank samples of fortified fish ranged from 94% to 102% (CV), 96.83% to 102.6% (LCV), 93% to 100.3% (MG), 89.92% to 104% (LMG), and 68.73% to 100% (BG). The method achieved LLOQ and LOD of 9.588 and 2.879 pg/g for CV, 9.544 and 2.866 pg/g for LCV, 9.932 and 2.982 pg/g for MG, 9.406 and 2.824 pg/g for LMG, and 10.272 and 3.085 pg/g for BG.

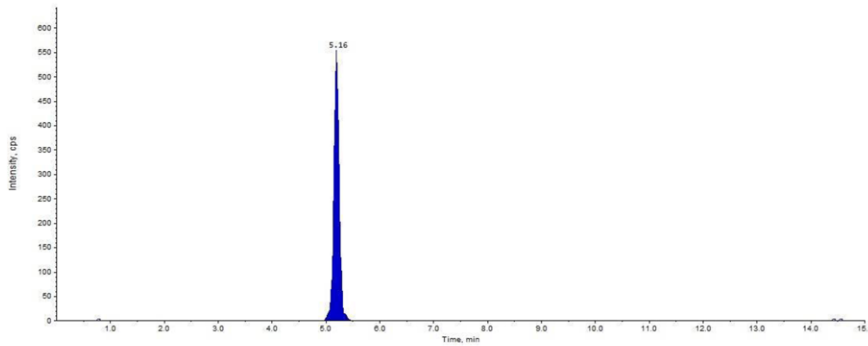
This method is sensitive, relatively rapid, and simple to implement, and can be used to simultaneously detect CV, LCV, MG, LMG, and BG in fish tissues. In this respect, it compares favorably to previously reported LC-MS/MS methods used for quantifying these dyes in fish. Other LC methods based on a combination of high resolution MS (LC-HRMS) and QuEChERS may provide



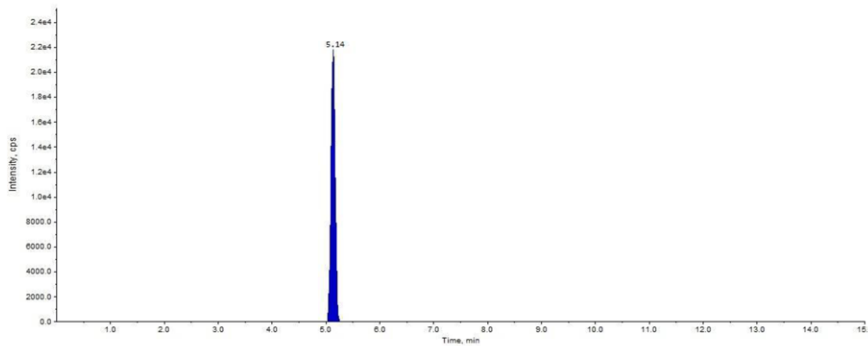
**A QC LLOQ**



**B QC Low**



**C QC Medium**



**D QC High**

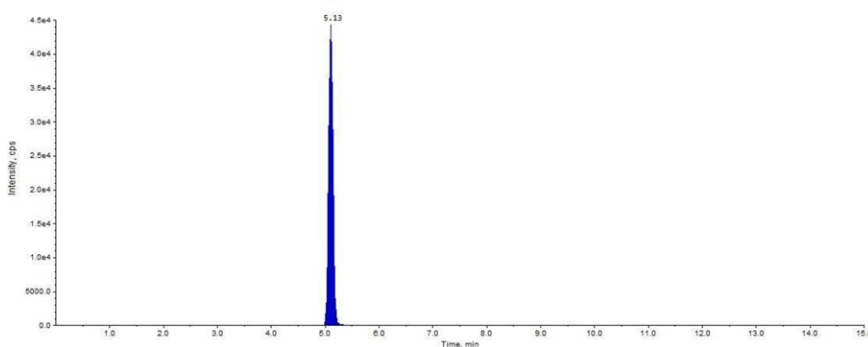
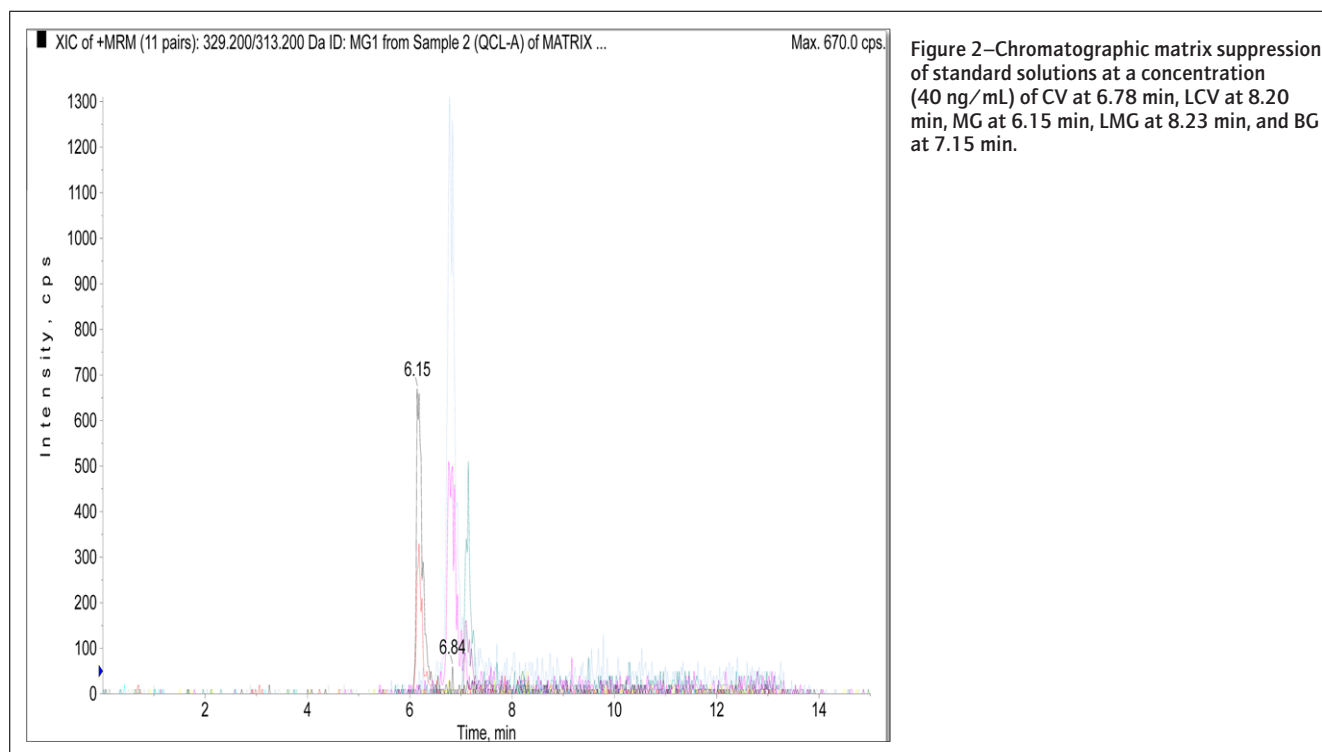


Figure 1—Chromatographic response (LC-MS/MS) for crystal violet (CV) in blank fish samples at retention time 5.1 min: (A) QC LLOQ at 0.01 ng/mL, (B) QC Low at 1.0 ng/mL, (C) QC Medium at 40.0 ng/mL, and (D) QC High at 80.0 ng/mL.

**Table 2—Values of accuracy, precision, linearity, recovery, lower limit of quantification (LLOQ), and limit of detection (LOD) of the LC-MS/MS method for CV, LCV, MG, LMG, and BG.**

Analyte	Concentration of QC samples (ng/mL)	Accuracy (%)	Precision (RSD%)	Linearity ( $r^2$ )	Recovery %	LLOQ (pg/g) (theoretical value 10 pg/g)	LOD (pg/g)
CV	0.01 (LLOQ)	100.0	2.35	0.9997	94.0	9.588	2.879
	1 (QCL)	92.57	28.64		102.0		
	40 (QCM)	97.33	0.43		95.68		
	80 (QCH)	100.6	0.16		98.20		
LCV	0.01 (LLOQ)	100.0	0.87	0.9995	98.66	9.544	2.866
	1 (QCL)	97.78	1.34		102.6		
	40 (QCM)	100.3	0.49		96.83		
	80 (QCH)	99.54	2.52		99.08		
MG	0.01 (LLOQ)	100.0	2.64	0.9997	93.0	9.932	2.982
	1 (QCL)	99.73	2.36		99.72		
	40 (QCM)	99.92	0.41		99.91		
	80 (QCH)	101.1	0.28		100.3		
LMG	0.01 (LLOQ)	100.0	9.63	0.9995	100.3	9.406	2.824
	1 (QCL)	99.87	1.11		104.0		
	40 (QCM)	97.64	4.75		89.92		
	80 (QCH)	99.54	0.42		102.2		
BG	0.01 (LLOQ)	100.0	5.39	0.9995	95.0	10.272	3.085
	1 (QCL)	88.78	30.91		68.73		
	40 (QCM)	99.90	0.24		98.66		
	80 (QCH)	104.6	2.37		100.0		

RSD, relative standard deviation; QCL, QC low; QCM, QC medium; QCH, QC high.



more rapid and sensitive analysis (Giacome et al., 2018), but the high cost of the equipment required tends to limit their use. Our validation findings compare favorably with recovery efficiencies previously obtained by LC-MS/MS and LC-MS for these dyes, which ranged from 73% to 104% (Giacome et al., 2018) and 49% to 104% (Andersen et al., 2009), respectively. LLOQ around 0.01 ng/g is consistent with and slightly lower than sensitivities previously reported for LC-MS methods for the measurement of triphenylmethane dyes in fish. Limits of quantification of 0.5 ng/g

for CV, MG, and their leuco metabolites (Xu et al., 2012) and of 0.21 to 0.37 ng/g for CV, MG, BG, LV, and LMG (Giacome et al., 2018) have been reported for LC-MS/MS methods. With LC-MS, comparable quantification limits of 0.21, 0.39, and 0.75 ng/g for CV, BG, and MG, respectively, were reported by Andersen et al. (2009). These methods were validated by analyzing various fishes that did not include Pangasius, Tilapia, or Hake. Further research could be conducted to assess the method's ruggedness to normal operational conditions.

**Table 3—Occurrence levels of CV and LCV residues<sup>a</sup> in frozen Pangasius, Tilapia, and Hake fish imported to Jordan from various countries.**

Fish and country of origin	No. of samples	No. of positive samples (%)	Concentration of CV ( $\mu\text{g}/\text{kg}$ )								
			Distribution of samples								
			Mean	Range	<0.4	0.4–1	1–5	5–10	10–20	20–30	>30
Pangasius, Vietnam	27	17 (62%)	11.7	0.362–41.34	1	–	5	3	4	3	1
Pangasius, UAE	27	8 (29%)	4.4	0.954–10.55	–	1	4	2	1	–	–
Tilapia, China	27	11 (40%)	4.6	1.24–9.48	–	–	7	4	–	–	–
Argentine Hake, Argentina	20	–	–	–	–	–	–	–	–	–	–
Pacific Hake, U.S.A.	20	–	–	–	–	–	–	–	–	–	–
Total	121	36 (30%)	6.9		1	1	14	9	5	3	1

Fish and country of origin	No. of samples	No. of positive samples (%)	Concentration of LCV ( $\mu\text{g}/\text{kg}$ )								
			Distribution of samples								
			Mean	Range	<0.17	0.17–1	1–5	5–10	10–20	20–30	>30
Pangasius, Vietnam	27	5 (18%)	5.26	0.178–10.58	1	–	2	1	1	–	–
Pangasius, UAE	27	–	–	–	–	–	–	–	–	–	–
Tilapia, China	27	2 (7%)	2.1	1.29–2.81	–	–	2	–	–	–	–
Argentine Hake, Argentina	20	–	–	–	–	–	–	–	–	–	–
Pacific Hake, U.S.A.	20	–	–	–	–	–	–	–	–	–	–
Total	121	7 (5.7%)	3.2	0.178–10.58	1	–	4	1	1	–	–

<sup>a</sup>MG, LMG, and BG residues were not detected.

### CV, LCV, MG, LMG, and BG residues in frozen fish flesh

Unlike most comparable studies, a relatively large number of samples, including four different species (two farmed fish and two wild-caught fish) and five countries of origin were tested in our work. MG, LMG, and BG were not detected in any of the 121 samples, whereas CV and LCV were detected in Pangasius and Tilapia as shown in Table 3. CV was found in 17 (62%) of the Pangasius samples originating from Vietnam, 11 (40%) of the Tilapia samples from China, and 8 (29%) of the Pangasius samples from the UAE. The mean concentrations of CV in these fish were 11.7, 4.4, and 4.6  $\mu\text{g}/\text{kg}$ , respectively. In Pangasius, the residual levels of CV ranged from 0.362 to 41.34  $\mu\text{g}/\text{kg}$ . In Tilapia, they ranged from 1.24 to 9.48  $\mu\text{g}/\text{kg}$ . The highest levels of CV (up to 30  $\mu\text{g}/\text{kg}$  and above) were found in Vietnamese Pangasius. CV and LCV were not detected in Argentine Hake and Pacific Hake. These findings are consistent with the fact that Pangasius and Tilapia are farmed fish, whereas Argentine and Pacific Hake are wild caught.

LCV was detected in five (18%) of the Pangasius samples from Vietnam and two (7%) of the Tilapia samples from China, but was not found in Pangasius from the UAE. In both fish, the average concentration of LCV (5.26 and 2.1  $\mu\text{g}/\text{kg}$ , respectively) was lower than that of CV. In Pangasius, the residual levels of LCV ranged from 0.178 to 10.58  $\mu\text{g}/\text{kg}$ . In Tilapia, they ranged from 1.29 to 2.81  $\mu\text{g}/\text{kg}$ . The fact that no LCV was found in Pangasius from the UAE may be explained by the very low fat content (0.68%) of this fish. Indeed, the leuco forms of CV are lipophilic and may thus be stored in fish fat tissue over long periods of time (Berntssen et al., 2018; Rushing & Thompson, 1997). Likewise, the low fat content of Tilapia (0.58%) may have contributed to the lower residual levels of LCV found in this fish, compared to Vietnamese Pangasius.

The high proportion of Vietnamese Pangasius contaminated with CV and LCV and the high residual levels found in these samples is consistent with the findings of Love et al. (2011). These researchers found that Asian seafood products, including catfish, tilapia, crab, shrimp, and prawns, were most frequently

in violation of veterinary drug residue standards of importing countries, including Canada, the EU, Japan, and the United States. Vietnam had the greatest number of veterinary drug residue violations among exporting countries. In fish, MG and CV were among the top veterinary drugs in violation (Love et al., 2011). Likewise, a survey of 73 fish samples (sea bass, sea bream, mackerel, and salmon) sold in Italy detected the presence of CV and MG in 22 samples (Giaccome et al., 2018). Their leuco metabolites were also detected in the positive samples but at lower concentrations, whereas BG was not detected (Giaccome et al., 2018). In another survey of 263 processed fish (eel, salmon, shrimp, and fish cake) sold in Korea, Lee et al. (2010) detected CV and MG in two samples at levels of 168.4  $\mu\text{g}/\text{kg}$  (CV in eel) and 2.6  $\mu\text{g}/\text{kg}$  (MG in shrimp), respectively. Consistent with our findings, Hashimoto et al. (2012) detected no MG nor LMG residues in samples of tilapia fillets (frozen and fresh) sold in Brazil. These findings support the necessity of regular monitoring of triphenylmethane dye residues in fish worldwide. In emerging countries and markets such as Vietnam, emphasis should also be placed on education/training and on promoting safe fish farming practices, replacing these banned substances by safer and affordable alternatives (Romero, Feijoo, & Navarrete, 2012; Sudova et al., 2007; Watts, Schreier, Lanska, & Hale, 2017).

### Microbial quality of frozen fish

The levels of microorganisms detected in frozen fish samples are summarized in Table 4. APC was used as an indicator of general microbiological contamination and of handling conditions during fish production (FAO, 1995). Mean APC values ranged from  $1.4 \times 10^5$  and  $3.7 \times 10^5$  cfu/g in the flesh (skinless fillets) of Pacific Hake (U.S.A.) and Argentine Hake, respectively, to  $1.7 \times 10^8$  and  $8.9 \times 10^8$  cfu/g in skinless fillets of Tilapia (China) and Pangasius (Vietnam). For frozen fish, APC  $<5 \times 10^5$  cfu/g indicate good microbial quality, whereas counts between  $5 \times 10^5$  and  $10^7$  cfu/g indicate marginally acceptable quality. APC  $\geq 10^7$  cfu/g indicate unacceptable microbial quality (Fan et al., 2009; Gram &

**Table 4—Detection and enumeration of aerobic plate count, *Vibrio* spp., *S. aureus*, and *E. coli*<sup>a</sup> in frozen Pangasius, Tilapia, and Hake fish from various countries.**

Fish and country of origin	No. of samples	Range of aerobic plate count (cfu/g) <sup>a</sup>							Positive (%)	Mean
		10 <sup>5</sup> –10 <sup>6</sup>	10 <sup>6</sup> –10 <sup>7</sup>	10 <sup>7</sup> –10 <sup>8</sup>	10 <sup>8</sup> –10 <sup>9</sup>	10 <sup>9</sup> –10 <sup>10</sup>				
Pangasius, Vietnam	27	4(14.8%)	8(29.6%)	6(22.2%)	1(3.7%)	8(29.6%)		27 (100%)	8.9 10 <sup>8</sup>	
Pangasius, UAE	27	5(18.5%)	6(22.2%)	8(29.6%)	8(29.6%)			27 (100%)	9.0 10 <sup>7</sup>	
Tilapia, China	27	6(22.2%)	1(3.7%)	2(7.4%)	18(66.6%)			20 (100%)	1.7 10 <sup>8</sup>	
Pacific Hake, U.S.A.	20	–	10(50%)	10(50%)	–			20 (100%)	1.4 10 <sup>7</sup>	
Argentine Hake, Argentina	20	10(50%)	10(50%)	–	–			20 (100%)	1.4 10 <sup>5</sup>	
	20	–	8(40%)	10(50%)	–			20 (100%)	1.5 10 <sup>8</sup>	
	20	12(60%)	–	–	–			20 (100%)	3.7 10 <sup>5</sup>	

Fish and country of origin	No. of samples	Range of <i>Vibrio</i> spp. (cfu/g)							Positive (%)	Mean
		10 <sup>1</sup> –10 <sup>2</sup>	10 <sup>2</sup> –10 <sup>3</sup>	10 <sup>3</sup> –10 <sup>4</sup>	10 <sup>4</sup> –10 <sup>5</sup>	10 <sup>5</sup> –10 <sup>6</sup>				
Pangasius, Vietnam	27	13 (48%)	4 (14.8%)	–	–	17 (62.8%)		7.4 10 <sup>2</sup>		
Pangasius, UAE	27	6 (22%)	–	–	–	6 (22%)		5.2 10 <sup>2</sup>		
Tilapia, China	27	2 (7.4%)	–	–	–	2 (7.4%)		2.5 10 <sup>2</sup>		
Pacific Hake, U.S.A.	20	–	2 (10%)	–	–	–		2 (10%)	1.1 10 <sup>2</sup>	
Argentine Hake, Argentina	20	–	–	–	–	–		–	–	
	20	–	–	–	–	–		–	–	
	20	–	–	–	–	–		–	–	
Pangasius, Vietnam	27	–	4 (14.8%)	5 (18.5%)	–	–	<b>Range of <i>S. aureus</i> (cfu/g)</b>	9 (33.3%)	1.7 10 <sup>3</sup>	
Pangasius, UAE	27	–	4 (14.8%)	4 (14.8%)	–	–	–	8 (29.6%)	1.2 10 <sup>3</sup>	
Tilapia, China	27	–	4 (14.8%)	4 (14.8%)	–	–	–	8 (29.6%)	1.3 10 <sup>3</sup>	
Pacific Hake, U.S.A.	20	–	4 (20%)	–	–	–	–	4 (20%)	2.2 10 <sup>2</sup>	
Argentine Hake, Argentina	20	–	–	4 (20%)	–	–	–	–	–	
	20	–	4 (20%)	–	4 (20%)	–	<b>Range of <i>E. coli</i> (cfu/g)</b>	8 (40%)	1.4 10 <sup>4</sup>	
	20	–	–	–	–	–	–	4 (20%)	2.4 10 <sup>2</sup>	
Pangasius, Vietnam	27	–	8(29.6%)	1(3.7%)	–	–	–	9 (33.3%)	4.5 10 <sup>2</sup>	
Pangasius, UAE	27	–	3(11.1%)	4(14.8%)	–	–	–	8 (29.6%)	1.2 10 <sup>3</sup>	
Tilapia, China	27	–	6(22.2%)	3(11.1%)	–	–	–	9 (33.3%)	8.7 10 <sup>2</sup>	
Pacific Hake, U.S.A.	20	–	–	–	–	–	–	–	–	
Argentine Hake, Argentina	20	–	–	–	–	–	–	–	–	
	20	–	–	4(20%)	–	–	–	4(20%)	2.3 10 <sup>3</sup>	
	20	–	–	–	–	–	–	–	–	

<sup>a</sup> *Salmonella* spp. were not detected.

<sup>b</sup> Numbers of samples (and percentages) are indicated in each range.



Dalgaard, 2002). As shown in Table 4, 74%, 59.2%, and 55.5% of the samples of Tilapia (China), Pangasius (UAE), and Pangasius (Vietnam), respectively, had APC exceeding  $10^7$  cfu/g, indicating unacceptable microbial quality. In contrast, all the samples of Argentine and Pacific Hake flesh were of acceptable microbial quality. Their skin, however, was of unacceptable microbial quality for 100% and 50% of the samples of Argentine and Pacific Hake, respectively. Contamination of seawater with sewage, such as the one reported along the Argentine southwest Atlantic shores (Islam & Tanaka, 2004), as well as unhygienic handling and storage practices, may have contributed to the high APC in the skin of the wild-caught Hake fish.

Interestingly, there was no significant relationship between the presence of CV in some samples of farmed fish, namely, Pangasius (Vietnam and UAE) and Tilapia (China), and the values of APC. This suggests that the use of CV in these farmed fish was not sufficient to raise their microbiological quality to acceptable levels. These findings highlight the need for continued efforts and support toward education, training, and the promotion of good hygiene practices in fish farming and handling in all the countries that export fish.

The presence of human pathogens was also investigated and, as for APC, did not show obvious relationship with the presence of CV. *Salmonella* spp. was not detected in any of the samples, indicating that all of them complied with the *Salmonella*-free standard that applies to frozen fish (González-Rodríguez, Sanz, Santos, Otero, & García-López, 2002). In contrast, *Vibrio* spp. were detected in 62.8%, 22%, and 7.4% of the samples of Pangasius (Vietnam), Pangasius (UAE), and Tilapia, respectively, as well as in 10% of the samples of Pacific Hake skin (Table 4). Argentine Hake skin and flesh were found to be free of *Vibrios*. The acceptable limit for *V. parahaemolyticus* in frozen fish is  $10^2$  to  $10^3$  cfu/g, whereas levels  $>10^3$  cfu/g are considered unacceptable for human consumption. This limit was exceeded for 14.8% of the samples of Vietnamese Pangasius. The levels of CV and LCV detected in these samples ranged from 0.4 to 30  $\mu\text{g}/\text{kg}$  and 0.17 to 20  $\mu\text{g}/\text{kg}$ , respectively. *V. parahaemolyticus* occurs naturally in marine and estuary locations. It is frequently linked to food poisoning outbreaks due to the consumption of seafood in Asia, Europe, North America, and other regions (Hara-Kudo & Kumagai, 2014).

*S. aureus* was detected in 33.3%, 29.6%, and 29.6% of the samples of Vietnamese Pangasius, UAE Pangasius, and Tilapia, respectively, as well as in Argentine Hake (40% and 20% of the skin and flesh samples, respectively) and Pacific Hake (20% of the skin samples; Table 4). The acceptable limit for *S. aureus* in frozen fish is  $10^2$  to  $10^4$  cfu/g and levels  $>10^4$  cfu/g are deemed unacceptable for human consumption (Herrera, Santos, Otero, & García-López, 2006). Twenty percent of the Argentine Hake skin samples did not meet this standard. Various fish products including frozen fish have been involved in food poisoning outbreaks resulting from *S. aureus* contamination (Bryan, 1980; Nakano et al., 2004; Sanjeev, Iyer, Rao, & James, 1986). Freshly caught fish is generally free from this pathogen (Bryan, 1980). Contamination with *S. aureus* usually takes place during handling as a result of unsanitary and improper handling, inappropriate storage conditions, and/or cross-contamination (Amuna, 2014). In particular, humans are known carriers of enterotoxigenic *S. aureus* via nose, throat, and skin infections, which can be transmitted to food during handling (Pereira, de Carmo, dos Santos, & Bergdoll, 1994).

*E. coli* was detected in 33.3%, 29.6%, 33.3%, and 20% of the samples of Vietnamese Pangasius, UAE Pangasius, Tilapia, and Argentine Hake skin. These samples did not comply with the

acceptable limit for *E. coli* in frozen fish which is less than  $5 \times 10^2$  cfu/g (González-Rodríguez et al., 2002). Pacific Hake skin and flesh were found to be free of *E. coli*.

### Effect of immersing fish in whole fat milk on the levels of CV and LCV

This study is the first to evaluate the effect of a treatment with whole milk to reduce the levels of CV and LCV residues in fish. Whole fat homogenized milk was used as this food grade liquid has the potential to solubilize and extract hydrophilic and lipophilic dye residues. Homogenized milk contains proteins such as caseins and BSA, which were found to bind to CV (Qin et al., 2016; Santhanalakshmi & Balaji, 2001), as well as lipids in the form of small fat globules stabilized by a membrane which contains proteins (Livney, 2010). Before treatment, the average levels of CV and LCV were 14.76 and 6.12  $\mu\text{g}/\text{kg}$ , respectively, in Pangasius, and 4.40 and 2.30  $\mu\text{g}/\text{kg}$ , respectively, in Tilapia (Table 5). As shown in Table 5, a significant reduction of the CV and LCV residue levels in Pangasius was obtained after treatment for 30 minutes and these levels were further reduced after longer treatment times. After 120 min, a 34.4% and 44.8% reduction was achieved for CV and LCV, respectively, in Pangasius (Table 5 and Table S1). Similarly, for Tilapia, a significant reduction in CV and LCV levels was obtained after 30 minutes, and these levels were reduced by 38.6% and 47%, respectively, after 120 min. It is noteworthy that the physical structure of both Pangasius and Tilapia fillets was maintained after treatment for 120 min. After 240 min, by contrast, some deterioration of the muscle tissue was noticed as the tissue had lost some cohesiveness. Treatment times of 120 min or less are thus recommended for effective reduction of CV and LCV contamination levels in these fish without affecting their texture. In both fish, the reduction incurred with LCV was greater than with CV, which may be related to the different characteristics of both substances, LCV being lipophilic, whereas CV is cationic and water soluble (Rushing & Thompson, 1997; Santhanalakshmi & Balaji, 2001). Thus, CV may be solubilized in the milk serum, possibly bound to some soluble proteins such as caseins, whey proteins and/or BSA. In model systems, the interaction between CV and BSA was found to involve hydrophobic interactions (Qin et al., 2016; Xu et al., 2009) with possible hydrogen bonds and electrostatic interactions (Qin et al., 2016). Hydrophobic and non-hydrophobic interactions may also be at play in the binding of CV to casein (Santhanalakshmi & Balaji, 2001). In contrast, LCV may bind hydrophobically to some soluble proteins, as well as to lipids and proteins in the fat globule membrane.

The efficacy of this simple procedure for reducing the residual levels of CV, LCV, and other banned dyes in fish should be further investigated. Future studies considering different concentrations and composition of milk, or alternative wash solutions, would be useful. It should be noted that while this procedure has the potential to lower the levels of some dye residues in fish, it is not intended to replace safe fish farming practices upstream. It should be reserved for very select applications where there are demonstrated benefits regarding safety. The use of any decontamination treatment should be carefully monitored to detect fraud and illicit use of the banned dyes.

### Conclusion

This study simultaneously assessed the presence of banned triphenylmethane dyes in a diverse sample of imported frozen fish products and the microbiological quality of these products. The LC-MS/MS method used for quantifying these antibiotic dyes

**Table 5—Reduction of crystal violet (CV) and leucocrystal violet (LCV) concentrations in Pangasius and Tilapia fish fillets after immersion in whole fat milk for different times.**

Time (minutes)	Pangasius				Tilapia			
	CV		LCV		CV		LCV	
	Concentration ( $\mu\text{g}/\text{kg}$ ) <sup>a</sup>	Reduction (%)	Concentration ( $\mu\text{g}/\text{kg}$ ) <sup>a</sup>	Reduction (%)	Concentration ( $\mu\text{g}/\text{kg}$ ) <sup>a</sup>	Reduction (%)	Concentration ( $\mu\text{g}/\text{kg}$ ) <sup>a</sup>	Reduction (%)
0	14.76 a	0	6.12 a	0	4.40 a	0	2.30 a	–
30	12.52 ab	15.2	5.14 ab	16.0	3.85 ab	12.5	1.93 ab	16.1
60	10.69 ab	27.6	4.22 ab	31.1	3.34 ab	24.1	1.59 ab	30.9
120	9.68 ab	34.4	3.38 b	44.8	2.70 b	38.6	1.22 b	47.0
240	9.17 b	37.9	3.12 b	49.0	2.51 b	43.0	1.12 b	51.3

<sup>a</sup>In each column, means followed by different letters differ significantly ( $P \leq 0.05$ ) according to LSD comparison.

and their leuco metabolites met the validation criteria of widely recognized guidelines. It is sensitive, rapid, and relatively simple to implement, and can be used to simultaneously detect CV, LCV, MG, LMG, and BG in fish tissue. Although MG, LMG, and BG were not detected in any of the 121 samples tested, CV and LCV were detected in farmed Pangasius and Tilapia. The proportions of samples contaminated with CV and LCV were particularly high for Pangasius from Vietnam and Tilapia from China. There was no obvious relationship between the presence of CV in some fish and the microbial quality of the fish. The presence of CV in a significant number of the farmed fish and the unacceptable microbial quality of many of the fish (both farmed fish and wild-caught fish) highlight the need for continued efforts and support toward training and promoting safe fish farming practices, as well as good hygienic practices in fish farming, catching, and handling. This is especially crucial in emerging countries such as Vietnam and for the protection of public health worldwide. These findings support the necessity of regular inspections and monitoring of CV and other antibiotic dye residues in fish, along with routine assessments of fish microbial quality, in order to protect public health. A significant reduction in CV and LCV concentrations was achieved after immersing fish fillets in whole milk for 120 min. The efficacy of this simple procedure for reducing the residual levels of CV and other banned dyes in fish products and byproducts should be further investigated, noting that it is not intended to replace safe fish farming practices upstream.

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## Authors' Contributions

Gammoh and Alu'datt designed the experiments and contributed to the writing of the manuscript. Rababah designed the microbiological experiments. Al-Rousan conducted the experiments and contributed to the writing of the manuscript. Talafha contributed to the organization of the data and follow up of the experiments. Alhamad performed the statistical analyses. Ammari and Tranchant helped review the literature, interpret the results, and write the manuscript. All the authors read and approved the final manuscript.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1**—Chromatographic response (LC-MS/MS) for leucocrystal violet (LCV) in blank fish samples at retention time 4.78 minutes: (a) QC LLOQ at 0.01 ng/mL, (b) QC Low at 1.0 ng/mL, (c) QC Medium at 40.0 ng/mL, and (d) QC High at 80.0 ng/mL.

**Figure S2**—Chromatographic response (LC-MS/MS) for malachite green (MG) in blank fish samples at retention time 4.1 minutes: (a) QC LLOQ at 0.01 ng/mL, (b) QC Low at 1.0 ng/mL, (c) QC Medium at 40.0 ng/mL, and (d) QC High at 80.0 ng/mL.

**Figure S3**—Chromatographic response (LC-MS/MS) for leucomalachite green (LMG) in blank fish samples at retention time 8.2 minutes: (a) QC LLOQ at 0.01 ng/mL, (b) QC Low at 1.0 ng/mL, (c) QC Medium at 40.0 ng/mL, and (d) QC High at 80.0 ng/mL.

**Figure S4**—Chromatographic response (LC-MS/MS) for brilliant green (BG) in blank fish samples at retention time 5.8 minutes: (a) QC LLOQ at 0.01 ng/mL, (b) QC Low at 1.0 ng/mL, (c) QC Medium at 40.0 ng/mL, and (d) QC High at 80.0 ng/mL.

**Table S1**—Reduction of crystal violet (CV) and leucocrystal violet (LCV) concentrations in *Pangasius* and *Tilapia* fish fillets after immersion in whole fat milk for 120 minutes.