LABORATORY INFORMATION BULLETIN

Triphenylmethane Dye Residue Analysis in Raw and Processed Aquaculture Products by AOAC Official Method of Analysis 2012.25 (CARTS Projects IR01183 and IR01444)

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ABSTRACT

Triphenylmethane dyes and metabolites are known or suspected mutagens and are prohibited in animals intended for human consumption. Despite toxicity, triphenylmethane dyes are used illegally as inexpensive treatments for fungal and parasite infections in aquatic animals. AOAC Official Method of Analysis 2012.25 for the LC-MS/MS determination of malachite green, crystal violet, brilliant green and metabolites leucomalachite green and leucocrystal violet has been validated for a variety of raw finfish including: Arctic char, barramundi, catfish, pompano, salmon, seabream, striped bass, tilapia, and trout; as well as for eel, frog legs, shrimp, scallops, and prepared products including smoked trout, dried shrimp, and highly processed canned eel and dace products. The canned products contained oil, salt, sugar, flavorings, spices, sauces, and/or preservatives. Dyes and metabolites were recovered from seafood products with > 85% accuracy and precision generally < 20% RSD. The method detection limit was typically below 0.25 µg/kg, with a range of 0.03 to 0.60 µg/kg for the analytes in different products. The limit of quantification was < 1.0 µg/kg. Compounds were identified in over 99 % of the 467 fortified and incurred samples tested. These results support the use of AOAC 2012.25 for triphenylmethane dye residue analysis in a wide variety of aquacultured products, regardless of the presence of additional food ingredients or the type of processing.

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INTRODUCTION

Triphenylmethane dye residues are common targets for regulatory seafood monitoring programs since malachite green (MG), crystal violet (CV), and their leuco metabolites (LMG, LCV) are known or suspected carcinogens, mutagens, and/or teratogens, and as such, are not permitted for use in food-producing animals (1). Despite restrictions, these compounds continue to be used illegally in aquaculture because they are effective and inexpensive antifungal and antiparasitic agents. AOAC Method 2012.25 is an Official Method of Analysis (OMA) for the LC-MS/MS quantitative and confirmatory determination of residues of MG, CV, brilliant green (BG) and the metabolites LMG and LCV in aquaculture products (2,3). This method was originally developed for trout (4) and was the subject of an AOAC Collaborative Study (5) and FDA Level Four multi-laboratory validation (6) for salmon, catfish, and shrimp.

The designation of Method 2012.25 as an AOAC OMA for aquaculture products warrants this use of the method for product testing beyond the specific validated matrices. However, some aquaculture products are vastly different from the raw finfish and shrimp matrices evaluated in previous validation studies (4,5). Products that require testing may be cooked by different techniques or processed into prepared foods with breading, seasonings, or sauces. For instance, the Centre for Food Safety (Hong Kong) issued a stop sale order in November 2017 for a canned, fried dace product with salted black beans that was found to contain MG residue (7). Other aquaculture products may not belong to fish or crustacean animal classifications at all (e.g., mollusks or amphibians), yet residues have been identified in these products; for example, CV has been found in aquacultured frog legs (8).

Validation studies rarely span an extended range of animal classifications or include processed foods. In general, performing method validation for every unique matrix is not feasible (6). The quantification of triphenylmethane dyes in complex tissue matrices is particularly challenging due to chemistry differences between the dyes and metabolites, as well as the low residue limits required for regulatory compliance (1). Consequently, an exhaustive evaluation of method performance was undertaken. This *Laboratory Information Bulletin* reports the Denver Laboratory method performance results for AOAC OMA 2012.25 for a wide scope of regulated aquaculture products, including raw finfish (Arctic char, barramundi, catfish, hybrid striped bass, pompano, salmon, seabream, tilapia, and trout), raw non-finfish products (eel, scallops, shrimp, and frog legs), as well as processed products (broiled eel, canned eel, canned dace, dried shrimp, and smoked trout) (9,10).

EXPERIMENTAL

Safety and Caution

Triphenylmethane dyes and leuco compounds are known or suspected carcinogens, mutagens, and/or teratogens. Appropriate personal protection safety measures must be practiced. Triphenylmethane dyes and metabolites are light sensitive and require efforts to reduce background contamination. During the extraction, samples and solutions should be protected from excessive light exposure by storing standard solutions, samples, and extracts in a dark cabinet, in amber colored glass, or covered with foil when not in use. Contamination by black marker ink should be minimized (known to contain crystal violet). Instrument carryover should be minimized

by using an injection needle wash. Injection of a water blanks between samples will also reduce instrument carryover if additional precautions are needed.

Aquaculture Products

Negative control matrix sources of each product were either laboratory control samples that had been previously tested and found to be free of triphenylmethane dye residues by other methodology (11), control sources from the FDA Center for Veterinary Medicine (CVM) Office of Research Aquaculture Program, or products purchased for this study from various retail establishments. Two sources each of aquacultured Atlantic salmon, catfish, and tilapia were tested, and a single source each of farm-raised Arctic char, barramundi, hybrid striped bass, pompano, and yellow fin seabream was tested. Three sources of shrimp were tested. Ten sources of eel were tested ranging from wild-caught raw to highly processed and canned products with oil, salt, sugar, flavorings, spices, sauces, and preservatives (Table 1). Three sources of canned dace were tested. Two were whole fried dace packed with oil, black beans, soy sauce, sugar, spices, and sodium glutamate, and the third source was described as dried dace with black beans, oil, salt, and water. Three sources each of uncooked scallops, uncooked frog legs, and dried shrimp were tested. Two of the dried shrimp products listed only shrimp and salt in the ingredients and one of these was indicated to be raw. The third dried shrimp product listed salt, FD&C Yellow #5 and #6, and sulfur dioxide in the ingredients. Finally, only one matrix source of smoked trout was tested. This product was declared to be brined in salt and cane sugar.

Incurred samples of salmon, catfish, tilapia, and trout were produced by the FDA CVM/OR Aquaculture Program with approval by the FDA/CVM Institutional Animal Care and Use Committee. A salmon, catfish, and tilapia were bathed in individual treatment baths containing a mixture of MG, CV, and BG (each with concentration 2 μg/L). One trout was exposed to a bath containing MG, CV, and BG (each with concentration 4 μg/L). Fish were placed in the exposure tank for 1 h, removed to a clean water tank, and then sacrificed 1 h after the exposure period.

Sample Preparation

Muscle tissue was homogenized with dry ice in a food processor and stored at -20 °C or lower until analysis. Samples were prepared according to how the product would be prepared for consumption. For example, for raw finfish like catfish, tilapia, and barramundi, which are sold as skinless filets, the filets were homogenized without skin. Conversely, Arctic char and salmon commonly are purchased with skin attached, and the product would likely be cooked with the skin remaining attached. Thus, for Arctic char and salmon, the muscle and skin were homogenized together. Frog muscle was removed from the leg bones prior to homogenization; however, bones were not removed from the eel and dace products as these are commonly prepared or consumed whole. Excess sauce and oil was removed from the canned eel and dace products by blotting the fish with a paper towel prior to homogenization. Shells, legs, and heads were removed from raw shrimp prior to homogenization; however, the dried shrimp products were homogenized as received, typically as the whole shrimp with exoskeleton, heads, and legs if all were present. The frozen scallop product was homogenized as the edible portion only, as it was received as such. The raw trout filets were homogenized with their attached skin, as trout are normally cooked with the skin; however, only the filet of the smoked trout product was homogenized as the edible portion.

Table 1. Method accuracy for 10 different types of eel matrix ranging from wild-caught to highly processed canned products.

Eel	Product Description	Additional Ingredients	Method Accuracy (% Recovery[a])				
			MG	LMG	CV	LCV	BG
1	Wild eel, frozen		102.5	99.6	103.5	91.0	110.2
2	Wild-caught, raw		99.7	101.0	107.6	104.0	80.1
3	Broiled eel, frozen		102.9	99.9	101.2	115.6	94.4[b]
4	Broiled Conger eel		101.0	99.5	103.1	94.3	106.0
5	Smoked eel, canned	soybean oil, salt	105.5	102.0	99.1	104.9	87.9
6	Roasted eel, canned	sugar, salt, soy sauce, capsicum, flavor enhancer	103.0	96.4	103.4	94.5	111.0
7	Roasted eel with black beans, canned	fermented bean, sugar, salt, soy sauce, capsicum, and flavor enhancer	99.7	94.8	105.7	98.2	100.7
8	Braised eel with dressing sauce, canned	fermented black beans, soy sauce, salt, sugar, soybean oil, spices, food additives, and flavor enhancer	100.1	95.0	99.4	98.3[b]	87.6
9	Roasted eel with black pepper, canned	black pepper, sugar, salt, soy sauce, and capsicum	96.2	94.4	101.2	96.1	116.3
10	Hot roasted eel, canned	sugar, soy sauce, broad bean paste product, chili paste, preservatives, and color and food additives	97.8	96.2	98.9	87.1	109.5

[a]Average of 9 replicates for eel sources 1-4, average of 6 replicates for sources 5-10. Fortification at the 1.0 µg/kg level. Data collected over 3 days with different calibrant sources per day.
[b]Interday precision 15-18 % RSD; interday precision was below 14% RSD for all other data points.

Chemicals and Reagents

(a) ***Analyte standards*** - Standards of MG oxalate (≥97% purity), LMG (≥98%), CV chloride (≥91%, Vetranal), LCV (≥99%), BG hydrogen sulfate (≥98%), d^5-MG picrate (≥99%, Vetranal), d^5-LMG (≥99%, OEKANAL), d^6-CV trihydrate (≥99%, OEKANAL), and d^6-LCV (≥96%, OEKANAL) were purchased from Sigma-Aldrich (St. Louis, MO).

Individual stock solutions of the analytes and of the stable isotopically labeled internal standards were prepared in ACN with concentrations of approximately 200 µg/mL (corrected for purity and the counter ion). Stock solutions were stored in the dark at -20 °C. The stock solutions had a stated stability of at least 1 year (4). A second set of individual stock solutions of MG, CV, BG, LMG, and LCV was prepared and designated as Initial Calibration Verification (ICV) stock solutions.

Mixed intermediate solutions (1000 ng/mL) of the analytes and of the stable isotopically labeled internal standards were prepared in ACN by combining approximately 500 µL of the individual stock solutions into a 100 mL volumetric flask and diluting to the mark with ACN to yield 1000 ng/mL solutions. Intermediate solutions were stored in the dark at -20 °C. The intermediate solutions had a stated stability of 1 month (4). A second mixed analyte intermediate solution was prepared from the ICV stock solutions as the ICV mixed intermediate solution.

Working solutions were prepared daily from the mixed intermediates by dilution with ACN to yield concentrations of 0, 5, 10, 20, 50, and 100 ng/mL and 40 ng/mL for the internal standard. An ICV working solution with concentration 20 ng/mL was also prepared. The working solutions were kept at room temperature and protected from light by wrapping them in aluminum foil when not in use. Working solutions were prepared according to Table 2.

Table 2: Working standard solutions

Standard	Volume of Mixed Intermediate Solution (µL)	Final Concentration (µg/L)	Final Volume of ACN (mL)
Working Solution 1 (WS 1)	0	0	10
Working Solution 2 (WS 2)	50	5	10
Working Solution 3 (WS 3)	100	10	10
Working Solution 4 (WS 4)	200	20	10
Working Solution 5 (WS 5)[a]	500	50	10
Working Solution 6 (WS 6)	1000	100	10
Working Solution ICV (WS ICV)	200	20	10
Mixed intermediate internal standard (WS-IS)	400	40	10

[a]For the validation, fortified samples at concentration 2 µg/kg were prepared from a working solution (“WS-5-spk”) with concentration 40 µg/L, prepared from 400 µL of the mixed intermediate.

(b) ***Solvents, reagents, and mobile phase solutions*** - LC-MS Optima grade acetonitrile, water, and formic acid were purchased from Fisher Scientific (Waltham, MA). Hydroxylamine hydrochloride (HAH), anhydrous magnesium sulfate ($MgSO_4$), ammonium formate, and ascorbic acid were reagent grade or better. Reagent solutions were prepared monthly according to these procedures:

Hydroxylamine solution in water (9.5 g/L) - 5.0 g of hydroxylamine hydrochloride was dissolved in purified water to a final volume of 250 mL. The final concentration is 9.5 g/L of hydroxylamine (not 9.5 g/L of hydroxylamine hydrochloride).

Ascorbic acid solution in water (1 g/L) - 100 mg of ascorbic acid was dissolved in deionized water to a final volume of 100 mL.

Reconstitution solution (100:1) - prepared by combining 1 mL ascorbic acid solution (1 g/L) with 100 mL acetonitrile.

5% Formic acid in water (5% v/v) - 5 mL concentrated formic acid in deionized water diluted to a final volume of 100 mL.

Mobile Phase A - Ammonium formate buffer (0.05 M, pH 4.5) prepared by adding 3.15 g ammonium formate to 900 mL water, then adding 5 mL of 5% formic acid in water, mixing, and diluting to 1.00 L with water.

Mobile Phase B - Acetonitrile.

Equipment (equivalent equipment may be substituted)

(a) ***Blender/homogenizer*** - RobotCoupe Blixer, homogenizer, 4 quart, model RS1BX4V (RobotCoupe USA, Inc., Ridgeland, MS).

(b) ***Vortex mixer*** - Vortex Genie 2 (Scientific Industries, Bohemia, NY).

(c) ***Multitube platform shaker*** - 2500 rpm setting, (Thermo Fisher Scientific, Waltham, MA), rotary stirrer set to 100 rpm.

(d) ***Centrifuge*** - Refrigerated centrifuge operated at 4°C and 2000 × *g*, Sorvall RC 6+ (Thermo Fisher Scientific), for use with 50 mL polypropylene centrifuge tubes.

(e) ***Microcentrifuge*** - High speed microcentrifuge operated at 14,000 or higher × *g*, Sorvall Legend Micro 21 (Thermo Fisher Scientific), for use with 2.0 mL plastic microcentrifuge tubes. Microcentrifuge can be operated in the range 14,000 to 20,000 x g with acceptable results (5-7).

(f) ***Nitrogen evaporator*** - TurboVap LV heated to 50°C (Biotage, Charlotte, NC). Evaporation tubes - 10–15 mL polypropylene tubes, glass tubes.

(g) ***Syringe filters*** - PVDF 0.45 µm, 13 mm Millex-HV (EMD Millipore Corp., Billerica, MA) used with disposable polypropylene syringes (BD Tuberculin, 1 mL; Thermo Fisher Scientific). Other syringe filters were found to yield satisfactory method performance including: 0.22 µm PVDF and 0.45 µm PTFE filters (5,6).

(h) ***Autosampler vials*** - Glass or polypropylene, with caps. Amber colored vials recommended to protect light sensitive compounds.

LC-MS/MS Instrumentation

Various LC-MS/MS systems have been used to perform the Method 2012.25 analysis (5). In the Denver Laboratory, the Agilent 6490 HPLC-MS/MS system described below was used to collect most of the validation data presented here, though Agilent 6495 (UHPLC), SCIEX 5500 QTrap, and Thermo Quantiva (UHPLC) systems were found to yield comparable results (12). Improved chromatography on UHPLC systems is obtained using specific UHPLC conditions indicated below (2,4,5).

HPLC separation was conducted using an Agilent 1200 HPLC system equipped with a Waters Symmetry C_{18} column (100 x 2.1 mm, 3.5 µm, Waters, Milford, MA). The mobile phase was a mixture of ammonium formate buffer (A) and acetonitrile (B) with a flow rate of 0.25 mL/min under the following gradient conditions: 60 % A decreased to 10 % from 0 to 1 min, then held at 10 % A for 14 min. This was followed by a 4 min post run equilibration with the 60 % A initial composition. The column oven was set to 30 °C, and the autosampler tray was set to 5 °C. The injection volume was 20 µL, and a 5 s needle wash with methanol:water (50:50) was used to minimize carryover. For UHPLC analysis, the gradient program consisted of an 80% to 10% gradient in A from 0 to 3 min, and a hold at 10% A for a total run time of 15 min. This was followed by a 4 min postrun equilibration at 80% A. The injection volume was reduced to 10 µL.

The Agilent 6490 triple quadrupole mass spectrometer had an electrospray Jet Stream source and was operated in positive ion mode with multiple reaction monitoring (MRM). The gas temperature was 220 °C, gas flow was 19 L/min, and the nebulizer was set to 20 psi. The sheath gas heater and flow were 300 °C and 12 L/min, respectively. The capillary voltage was 3.0 kV and the multiplier was set to 200 V (delta EMV). The ion funnel parameters were 200 V for positive high pressure RF and 110 V for positive low pressure RF. Two MRMs were collected for each analyte and one for each internal standard. Additional alternate MRMs were also collected for MG, CV, and LMG, which were not specified by Method 2012.25 (2,3), but were found during automated compound optimization. MRMs and optimized collision energies for all transitions are shown in Table 3. Product ion transitions were collected in a single time segment with wide unit resolution (1 amu), dwell time of 50 ms, fragmentor setting of 380 V, and cell acceleration of 4 V.

Calibration Standards

For quantitative analysis, a set of six matrix-matched extracted calibrants were prepared for each type of matrix. Sample portions (2.0 g) were fortified with analyte and internal standard working solutions, and then extracted per the procedure. Extracted standards were prepared at the following concentrations 0, 0.25, 0.5, 1.0, 2.5, and 5.0 µg/kg (as tissue equivalent) and with 2.0 µg/kg of internal standards, according to Table 4. An extracted ICV standard was also prepared at concentration 1.0 µg/kg. Calibration curves for each analyte were constructed from the response of extracted matrix calibrants as concentration versus the peak area ratio of the quantitative product ion transition to peak area of the corresponding internal standard. d^5-MG was used as the internal standard for both MG and BG.

The extraction results in a 2.5X concentration factor; therefore, a calibrant or sample fortified at 1.0 µg/kg (1.0 ng/g) in matrix will produce an extract with an equivalent concentration of 2.5 µg/L in the LC vial:

2.00 g sample aliquot→0.8 mL final volume extract: 2.5X concentration factor. Sample with 1.00 ng/g (1.00 µg/kg) x 2g/0.8 mL= 2.5 ng/mL (2.5 µg/L) in the vial.

Table 3: Product ion transitions, collision energies, and typical ion ratios for qualification transition relative the quantitative transition

Compound	MRM (m/z)	Collision Energy (eV)	Median Ion Ratio (Qual/Quant, %)	Median Retention Time (min)
MG	329 → 313[a]	40	100	3.5
	329 → 208	48	28	
	329 → 241[b,c]	72	19	
LMG	331 → 239[a]	36	100	5.4
	331 →316	20	41	
	331 → 223[a]	64	32	
CV	372 → 356[a]	44	100	3.9
	372 → 251	35	5	
	372 → 340[a]	64	45	
LCV	374 → 358[a]	44	100	5.5
	374 → 239	30	81	
BG	385 → 341[a]	44	100	4.3
	385 → 297	64	44	
d5-MG	334 → 318	44		3.5
d5-LMG	336 → 239	36		5.4
d6-CV	378 → 362	48		3.9
d6-LCV	380 → 364	32		5.4

[a]Quantitative product ion transition; [b]alternate qualitative transition; [c]200 ms dwell time

Table 4: Preparation of extracted calibrants, negative controls, spikes, and samples

Extracted Calibrants (tissue equivalent concentration)	Tissue Weight (g)	Spiked with
Extracted Calibrant 1 (0 µg/kg)	2.00	100 µL of WS-1 + 100 µL W-IS
Extracted Calibrant 2 (0.25 µg/kg)	2.00	100 µL of WS-2 + 100 µL W-IS
Extracted Calibrant 3 (0.5 µg/kg)	2.00	100 µL of WS-3 + 100 µL W-IS
Extracted Calibrant 4 (1.0 µg/kg)	2.00	100 µL of WS-4 + 100 µL W-IS
Extracted Calibrant 5 (2.5 µg/kg)	2.00	100 µL of WS-5 + 100 µL W-IS
Extracted Calibrant 6 (5.0 µg/kg)	2.00	100 µL of WS-6 + 100 µL W-IS
Extracted ICV Calibrant (1.0 µg/kg)	2.00	100 µL of WS-ICV + 100 µL W-IS

Matrix Spikes and Samples	Weight (g)	Spiked with
Reagent blank		100 µL W-IS (internal std only)
Negative Control or Sample	2.00	100 µL W-IS (internal std only)
Spike fortified at 0.5 µg/kg	2.00	100 µL of WS-3 + 100 µL W-IS
Spike fortified at 1.0 µg/kg	2.00	100 µL of WS-4 + 100 µL W-IS
Spike fortified at 2.0 µg/kg	2.00	100 µL of WS-5-spk[a] + 100 µL W-IS

[a]100 µL of 40 µg/L Working Solution

Example calculation for a spike fortified at 1.0 µg/kg:

0.1 mL of WS	20 µg	1 L	1000 ng	= 1.00 ng
2.00 g of tissue	L	1000 mL	1 µg	g

System Suitability Solvent Standard

A system suitability solvent standard was also prepared to run with each analysis. This solvent standard was prepared at concentration 2.5 ng/mL in vial (1.0 µg/kg tissue equivalent) by combining 125 µL of WS-4 + 125 µL W-IS + 750 µL of the reconstitution solution.

Sample Extraction

Samples were extracted according to the established procedure (2-5,9,10), summarized below:

1. Weigh 2.00 g (± 0.02 g) portions of homogenized tissue into 50 mL disposable centrifuge tubes and let thaw.
2. Fortify samples with 100 µL internal standard working solution (2.0 µg/kg). Prepare calibrants and controls as indicated in Table 4.
3. Allow samples to equilibrate for 15 min while protected from light.
4. Add 500 µL hydroxylamine solution (9.5 g/L) to the samples, vortex mix briefly, and allow samples to stand in the dark for 10 min.
5. Add 8 mL of acetonitrile and 1.0 g (±0.1) of anhydrous magnesium sulfate to each tube. Vortex mix tubes (1 min, maximum speed), then agitate tubes (10 min) using a rotary stirrer or a multitube vortexer (2,500 rpm).
6. Centrifuge the tubes (2000 × *g*, 5 min, 4°C), and transfer all supernatant to a clean tube for evaporation.
7. Evaporate the supernatant to dryness (50°C, 15 psi N_2). For some matrix types (e.g. salmon), the point of dryness may be a viscous oil.
8. Reconstitute the dried extract with 800 µL of reconstitution solution (100:1 acetonitrile: ascorbic acid (1 g/mL)).
9. Vortex mix all samples sufficiently to break up dried extracts; for example, vortex mixing on high speed for 30 s followed by 10 min of mixing on a multitube vortexer ensures complete dissolution of analytes and internal standards.
10. Transfer extracts to microcentrifuge tubes, centrifuge at 14,000 × *g* or higher for 5 min, and filter (PVDF, 0.45 µm) into autosampler vials for LC-MS/MS analysis. Other microcentrifuge speeds and filters are acceptable; see Equipment sections (e) and (g).
11. Analyze by LC-MS/MS.

METHOD VALIDATION

AOAC OMA 2012.25 was previously validated as an FDA Level Four (6) multi-laboratory chemical method of analysis (5). In the Denver Laboratory single laboratory validation, FDA Level Two (6) validation criteria were met by testing negative control matrix blanks and fortified samples at three concentration levels (0.5, 1.0, and 2.0 µg/kg), with at least three replicates tested at each concentration level. For triphenylmethane dye residue analysis, 1.0 µg/kg is the target testing level (TTL). Due to existing method performance data for trout, salmon, catfish, and tilapia finfish (4,5), only one or two sources of other finfish and one source of smoked trout was tested in Denver Laboratory single laboratory validation. For the non-finfish matrix types that had not been studied previously, validation data was collected over three days using at least three sources of each type of matrix.

Method accuracy and precision were expressed as analyte recovery (%) and relative standard deviation (% RSD). Method accuracy (trueness) was determined by calculating the recoveries of analytes in each matrix from a calibration curve based on extracted matrix calibrants with all analyte responses corrected relative to their respective stable isotope-matched internal standard response. For finfish matrix, the precision was determined at each concentration from n = 3 replicates collected on a single day of analysis, whereas, interday precision was determined for the non-finfish matrices with data collected over at least three days ($n \geq 9$). Linearity was determined from the correlation coefficient (R^2) of the linear regression of the six calibrants on the calibration curve. The sensitivity of the method was determined from the standard deviation (sd) of the 0.5 µg/kg fortified samples for each analyte in each matrix type. The method detection limit (MDL) was the sd multiplied by the student's t-value at the 99% confidence level and the limit of quantification (LOQ) was the sd multiplied by 10 (6,13).

Quantitative Analysis and Residue Screening

Concentrations of analytes detected in each matrix were calculated from the linear regression of the extracted calibrant calibration curve in the same matrix. The method is also designed to permit screening using a single matrix-matched extracted calibrant (2,4). For screening, the peak area ratio (analyte:internal standard) of each sample can be compared to the peak area ratio of a single (0.5 or 1.0 µg/kg) extracted matrix calibrant. In this study, the threshold limit for screening was determined for each analyte in each matrix based on the average concentration found for the 1.0 µg/kg fortified samples minus the sd multiplied by the Student's t value at the 95 % confidence interval (one-tailed) (6,13).

Qualitative Analysis

Identification criteria (14) established for residue testing was applied to confirm the identity of analyte residues in tested samples. Analytes were determined to be present in a sample when three conditions were met: 1) the signal to noise ratio was ≥ 3 for both MRM transitions; 2) the retention time was ± 5 % of the average retention time for the corresponding non-zero extracted matrix calibrant samples; and 3) the peak area ratio of the qualified ion:quantification ion was within ±10 % absolute of the corresponding average ratio for the non-zero extracted matrix calibrant samples. In addition to these criteria, it was also required that the peak area response for

the sample must yield a calculated concentration that is greater than the 0.25 µg/kg, which is the concentration of the lowest calibrant.

RESULTS AND DISCUSSION

Quantitative Method Performance

Method accuracy (trueness) was determined by calculating the recoveries of analytes in each matrix from a calibration curve based on extracted matrix calibrants with all analyte responses corrected relative to their respective internal standard response. These results are shown in Table 5 for finfish and in Table 6 for the non-finfish matrix types and processed seafood matrices. Ion chromatograms for processed eel and dace samples are shown in Figures 1-3.

Acceptable method performance for a quantitative residue analysis method with a 1.0 µg/kg target testing level (TTL) is characterized by having analyte recovery between 40% and 120% and an RSD of 22% or less (6). In this report, except for BG in Arctic char and dried shrimp, this performance criteria was met with average analyte recoveries at each concentration level ranging from 86 to 119 % and precision below 18 % RSD. Arctic char at the 0.5 µg/kg fortification level had a 27 % RSD for BG, and at the 2.0 µg/kg fortification level had an average BG recovery of 146%. Overall, across all concentration levels, the average BG recovery in Arctic char was 115 %, but the overall precision (26 % RSD) was still more variable than typical method performance for the other matrix types. Likewise, the precision for BG in dried shrimp matrix was 23 and 24 % RSD for the individual 1.0 and 2.0 µg/kg concentration levels, but the precision overall across all levels was 22 % RSD.

The calibration curves used for all analytes in all matrices had correlation coefficients (R^2) greater than 0.99. For a few analyte/matrix combinations, one of the six calibrants needed to be dropped to achieve $R^2 > 0.99$. For those few curves, R^2 was greater than 0.95 with all six calibrants. AOAC OMA 2012.25 specifies $R^2 \geq 0.95$ as an acceptable measure of linearity (2-4).

Detection Limits

The method detection limit and limit of quantification were calculated for each analyte in each matrix and are reported in Tables 5 and 6. The MDL was usually 0.25 µg/kg or below and the LOQ was 0.5 µg/kg or below, yet higher MDL and/or LOQ were observed for MG, LMG, and BG in some matrices. In all cases, the LOQ of Method 2012.25 was below 1.0 µg/kg in the matrices tested.

Semi-Quantitative Residue Screening

AOAC Method 2012.25 describes a sample analysis strategy wherein samples can be extracted and analyzed concurrent with a negative matrix control and a single extracted matrix calibrant fortified at 0.5 µg/kg rather than using a full set of extracted matrix calibrants to generate a curve for every analysis (2,4). This screening strategy was tested previously, where it was proposed that an analytical response > 70 % above the response of the 0.5 µg/kg matrix-matched extracted calibrant would trigger further testing with a full calibration curve (9). In the current study, a threshold limit was determined for the 1.0 µg/kg TTL for each analyte in each matrix,

based on the calculated concentration of the samples fortified at the 1.0 µg/kg level and the 95 % confidence interval. For most analytes and matrices, the threshold limit was > 0.80 µg/kg. Threshold limits between 0.60 and 0.80 µg/kg were determined for two or three analytes in pompano (LCV, BG), frog legs (LMG, LCV, BG) and dried shrimp (MG, BG). Unexpectedly, two different threshold limits were calculated for BG in Arctic char (0.38 and 0.81 µg/kg) from two sets of Arctic char validation data. The second set was collected to verify that the higher variability, recovery, and detection limits observed for BG in this matrix were repeatable. Poor precision (27-34 % RSD) was observed for BG for both data sets at the lowest fortification level, but at the 1.0 µg/kg fortification level, one set had poor precision and the other was within the expected range. To simplify the screening recommendation to apply across all matrix types, the screening threshold limit is suggested to be arbitrarily lowered to 0.60 µg/kg for all residues in all matrices, except for BG in Arctic char, which should have a threshold limit of 0.35 µg/kg.

When these limits were applied to the data, with few exceptions, the negative control and 0.5 µg/kg fortified samples yielded results below the established threshold limits and would be screened as negatives, while all the 1.0 and 2.0 µg/kg fortified samples yielded responses above the threshold limit (0% false negative rate). The exceptions were four samples fortified at the 0.5 µg/kg level that yielded responses above the threshold for one analyte (MG, CV, or BG). Of these, seabream and scallop false positive screening results were both in samples that were identified as outliers by the Dixon Q test. Two Arctic char samples also had screening results above the 0.35 µg/kg limit for BG (0.39 and 0.57 µg/kg). Of the 66 samples tested at this concentration, with 330 analytes tested (5 analytes per sample), these few samples with incorrect screening results represent a false positive rate of 1.2 %.

Based on these results, Method 2012.25 is suitable for screening samples at the 1.0 µg/kg TTL. The screening strategy would prove useful when the samples to be analyzed consist of several different types of aquaculture products and it becomes time-consuming to prepare full sets of extracted calibrants to match every matrix type.

Qualitative Confirmation of Identity

The identities of residues were confirmed in all but a few fortified and incurred samples using the identification criteria (signal to noise, retention time, and ion ratio) established by the FDA (14). Overall, 2319 of the 2335 analytes (99.3 %) met the criteria for identity confirmation in the 467 fortified and incurred samples tested. Signal to noise and retention time were generally acceptable, but it was the product ion ratio criterion that typically determined identification. In addition to the qualitative product ion transitions indicated in Method 2012.25 (2,4), additional transitions were collected for MG, LMG, and CV and shown in Table 3. The alternate qualitative transitions produced equivalent results for analyte confirmation in positive samples, and either transition could be used as the qualifier (5,9,10). The FDA confirmation criterion for product ion ratio specifies that when only two product ion transitions are collected, the ratio of the two must be within ±10 % (absolute) of the ratio in corresponding standards (non-zero extracted matrix calibrants for this method). When three product ion transitions are acquired, the two ratios for a sample must be within ± 20 % of the ratios for the standards. Even though one quantitative product ion transition and two qualifier transitions were acquired for MG, LMG, and CV, all the product ion ratios were within ± 10% of the ratio for the standards and it was not necessary to use the less rigorous criteria of ± 20%.

Table 5: Method accuracy[a] and detection limits for raw finfish matrix

Fortification Level (µg/kg)	Trueness (as % Recovery) ± %RSD MG	LMG	CV	LCV	BG
			Arctic Char		
0.5	100.3 ± 1.7	102.9 ± 1.5	106.1 ± 3.4	95.8 ± 3.3	93.2 ± 27.2
1.0	92.5 ± 1.5	98.3 ± 3.3	101.5 ± 4.1	92.8 ± 1.0	105.8 ± 8.2
2.0	102.6 ± 1.7	103.8 ± 1.5	105.8 ± 3.7	96.9 ± 4.3	145.7 ± 15.9
MDL (µg/kg)	0.06	0.05	0.12	0.11	0.60
LOQ (µg/kg)	0.08	0.08	0.18	0.16	0.86
			Barramundi		
0.5	96.7 ± 8.8	91.8 ± 3.9	97.7 ± 5.8	97.7 ± 3.2	96.3 ± 7.5
1.0	100.6 ± 3.7	94.2 ± 2.3	100.9 ± 5.7	94.9 ± 3.8	97.2 ± 6.5
2.0	103.6 ± 10.8	98.7 ± 9.8	90.6 ± 10.2	101.3 ± 2.5	92.6 ± 9.2
MDL (µg/kg)	0.30	0.12	0.20	0.11	0.25
LOQ (µg/kg)	0.42	0.18	0.28	0.16	0.36
			Catfish		
0.5	100.6 ± 3.5	95.7 ± 3.6	91.9 ± 6.0	91.4 ± 3.0	110.4 ± 5.6
1.0	95.1 ± 10.3	101.1 ± 4.7	99.5 ± 4.5	97.4 ± 4.8	94.7 ± 8.5
2.0	107.0 ± 8.6	107.7 ± 5.3	101.7 ± 3.0	101.2 ± 1.8	111.2 ± 2.2
MDL (µg/kg)	0.12	0.12	0.19	0.10	0.21
LOQ (µg/kg)	0.18	0.17	0.27	0.14	0.31
			Pompano		
0.5	92.1 ± 7.8	118.9 ± 4.1	111.4 ± 6.4	116.5 ± 4.3	92.9 ± 6.9
1.0	97.3 ± 4.7	110.6 ± 8.3	102.2 ± 5.8	94.9 ± 13.1	94.8 ± 11.0
2.0	101.3 ± 2.9	101.6 ± 9.4	98.0 ± 7.9	102.6 ± 6.4	99.3 ± 7.1
MDL (µg/kg)	0.25	0.17	0.25	0.18	0.23
LOQ (µg/kg)	0.36	0.24	0.36	0.25	0.32
			Salmon		
0.5	111.2 ± 3.3	110.4 ± 0.4	107.1 ± 2.9	105.7 ± 1.4	109.3 ± 10.8
1.0	101.8 ± 0.6	105.3 ± 1.7	107.7 ± 6.3	103.8 ± 0.4	98.7 ± 13.0
2.0	100.8 ± 2.1	106.6 ± 3.5	107.4 ± 2.3	106.3 ± 3.2	98.5 ± 4.5
MDL (µg/kg)	0.06	0.15	0.13	0.13	0.14
LOQ (µg/kg)	0.14	0.45	0.39	0.39	0.42
			Seabream		
0.5	90.7 ± 3.5	98.1 ± 8.3	107.7 ± 5.3	109.8 ± 2.9	89.4 ± 3.0
1.0	101.2 ± 5.4	94.7 ± 1.2	100.1 ± 2.5	102.3 ± 4.3	100.2 ± 5.1
2.0	99.3 ± 3.1	96.7 ± 2.9	97.1 ± 4.0	96.3 ± 4.6	98.0 ± 0.5
MDL (µg/kg)	0.11	0.28	0.20	0.11	0.09
LOQ (µg/kg)	0.16	0.41	0.29	0.16	0.13
			Striped Bass		
0.5	102.8 ± 6.5	105.2 ± 0.8	99.0 ± 2.3	107.8 ± 2.7	103.5 ± 5.4
1.0	96.2 ± 5.0	100.5 ± 3.3	96.3 ± 1.9	112.6 ± 3.9	102.4 ± 3.3
2.0	96.1 ± 2.2	101.5 ± 1.3	102.5 ± 1.8	110.4 ± 3.0	93.9 ± 4.9
MDL (µg/kg)	0.23	0.03	0.08	0.10	0.19
LOQ (µg/kg)	0.33	0.04	0.11	0.15	0.28
			Tilapia		
0.5	105.7 ± 3.1	105.0 ±3.3	106.7 ± 3.1	106.0 ± 2.3	95.0 ± 7.2
1.0	101.2 ± 3.0	101.1 ± 3.7	104.3 ± 4.9	103.1 ± 0.8	99.1 ± 10.0
2.0	99.8 ± 4.9	101.8 ± 3.1	101.0 ± 2.7	101.5 ± 3.8	97.5 ± 3.6
MDL (µg/kg)	0.11	0.12	0.12	0.09	0.24
LOQ (µg/kg)	0.18	0.17	0.17	0.12	0.34
			Trout		
1.0[b]	91.6 ± <0.1	95.88 ± 3.4	98.3 ± 4.3	95.9 ± 7.4	98.6 ± 2.5

[a]Extracted matrix calibrants with internal standard correction; n=3 at each fortification level.
[b]n=2 replicates only for verification; Method 2012.25 was originally validated in trout. Raw trout performance data is consistent with smoked trout (see next page).

Table 6: Accuracy[a] and detection limits for other aquaculture and processed seafood

Fortification Level (μg/kg)	Trueness (as % Recovery) ± %RSD				
	MG	LMG	CV	LCV	BG
Dace[b,c] (canned, fried, fermented, sauced, additives, preservatives)					
0.5	96.9 ± 8.1	95.8[c] ± 6.4	96.1 ± 6.4	98.5 ± 3.5	100.3 ± 10.6
1.0	101.6 ± 6.5	104.2[c] ± 6.0	102.8 ± 7.0	100.9 ± 8.4	104.3 ± 13.7
2.0	100.4 ± 7.7	103.7[c] ± 5.2	101.2 ± 6.7	100.0 ± 6.1	103.7 ± 8.0
MDL (μg/kg)	0.11	0.10	0.09	0.05	0.15
LOQ (μg/kg)	0.39	0.31	0.31	0.17	0.53
Eel[d] (raw and broiled, without seasonings or sauces)					
0.5	99.2 ± 10.9	102.8 ± 4.8	103.6 ± 6.3	109.8 ± 5.8	102.9 ± 13.9
1.0	101.5 ± 5.4	100.0 ± 6.7	103.9 ± 5.7	101.2 ± 13.8	97.7 ± 16.5
2.0	104.8 ± 10.2	98.9 ± 5.1	101.1 ± 3.6	101.9 ± 3.4	101.9 ± 8.8
MDL (μg/kg)	0.16	0.07	0.10	0.09	0.21
LOQ (μg/kg)	0.54	0.25	0.33	0.32	0.71
Eel[e] (canned, smoked, roasted, braised, sauced, additives, preservatives)					
1.0	100.4 ± 7.1	96.4 ± 9.4	101.3 ± 6.1	96.5 ± 12.5	102.1 ± 15.6
Frog Legs[b]					
0.5	107.1 ± 6.4	92.7 ± 11.1	104.9 ± 7.0	91.6 ± 9.2	98.1 ± 13.1
1.0	100.9 ± 4.2	90.5 ± 13.4	100.0 ± 5.4	86.4 ± 15.2	106.5 ± 13.2
2.0	101.9 ± 7.2	89.7 ± 11.9	98.4 ± 4.9	90.7 ± 16.4	105.5 ± 6.4
MDL (μg/kg)	0.10	0.15	0.11	0.12	0.19
LOQ (μg/kg)	0.34	0.51	0.37	0.42	0.64
Scallops[b]					
0.5	109.9 ± 12.3	103.6 ± 3.6	103.4 ± 5.2	104.8 ± 6.4	101.4 ± 10.7
1.0	105.3 ± 5.7	101.7 ± 5.2	102.3 ± 3.5	103.8 ± 6.2	101.5 ± 12.9
2.0	103.2 ± 5.1	97.8 ± 4.7	99.0 ± 5.9	101.1 ± 4.1	102.4 ± 10.3
MDL (μg/kg)	0.04	0.06	0.08	0.10	0.16
LOQ (μg/kg)	0.13	0.19	0.27	0.33	0.54
Shrimp					
0.5	100.3 ± 4.4	107.2 ± 2.8	105.4 ± 3.6	108.5 ± 3.5	104.3 ± 6.2
1.0	105.3 ± 7.0	91.4 ± 5.1	98.0 ± 2.2	88.1 ± 4.6	103.0± 10.5
2.0	104.1 ± 11.8	99.5 ± 3.5	97.8 ± 4.5	100.8 ± 5.6	90.9 ± 11.0
MDL (μg/kg)	0.16	0.11	0.13	0.13	0.22
LOQ (μg/kg)	0.22	0.15	0.19	0.19	0.32
Shrimp[b] (dried, salted, additives, preservatives)					
0.5	94.6 ± 15.5	94.6 ± 6.3	94.1 ± 5.4	100.0 ± 5.0	96.7 ± 17.1
1.0	101.5 ± 17.2	100.8 ± 7.8	101.2 ± 7.8	102.9 ± 8.0	103.7 ± 23.2
2.0	101.6 ± 11.0	99.8 ± 5.5	103.3 ± 5.4	105.2 ± 3.6	95.1 ± 24.0
MDL (μg/kg)	0.21	0.09	0.07	0.07	0.24
LOQ (μg/kg)	0.74	0.30	0.25	0.25	0.83
Trout (smoked, salt and sugar brined)					
0.5	103.9 ± 4.1	102.8 ± 3.1	106.6 ± 3.2	87.6 ± 7.5	101.4 ± 6.4
1.0	96.8 ± 5.1	98.7 ± 4.2	99.0 ± 4.8	94.8 ± 4.6	95.1 ± 4.9
2.0	92.8 ± 0.9	98.1 ± 6.5	100.4 ± 4.2	97.6 ± 8.3	89.4 ± 10.4
MDL (μg/kg)	0.15	0.11	0.12	0.23	0.23
LOQ (μg/kg)	0.21	0.16	0.17	0.33	0.32

[a]Extracted matrix calibrants with internal standard correction; n=3 at each level unless noted.

[b]Three matrix sources; n=9 for the 0.5 and 2.0 μg/kg levels and n=27 for the 1.0 μg/kg level.

[c]Fewer replicates included (n=6 or n=9); dace control source contained LMG.

[d]Four matrix sources; n=9 for 3 sources at 0.5 and 2.0 μg/kg levels and n=36 for 4 sources at 1.0 μg/kg level.

[e]Six canned eel matrix sources; n=6 with n=2 tested per matrix per day for 3 days.

Only one qualifier transition was collected for LCV and BG. While this was adequate for BG, it was problematic for LCV in a few fortified pompano, scallop, and eel samples, where 10 % of the samples did not meet ion ratio criteria at the 1.0 μg/kg fortification level. Background interference was noted for the LCV product ion transitions in some of the control sources for these matrices, which may have skewed the product ion ratio somewhat above 10%. Figure 2 shows the negative control chromatograms for two of the processed eel sources, where background interference can be observed. The average ion ratio for LCV in the calibrants varied greatly over the course of this study (e.g., 62 to 93 %), whereas the ratio for other analytes shifted by 7% or less during the study. In the multi-laboratory study of AOAC Method 2012.25 (5), lack of confirmation of LCV in positive fortified samples accounted for more than half of the samples that did not meet the requirements for identity confirmation. For practical use of this method, it may be prudent to acquire a second qualifier transition for LCV, to permit the ion ratio acceptance criterion of ± 20% (14). All of the non-confirmed samples in pompano, scallop, and eel had a product ion ratio within ± 20% of the ratio for the calibrants for the m/z 374→239 qualifier transition; thus, that product ion ratio would meet the ± 20% criterion if a second product ion ratio was included.

Many of the negative samples met the confirmation criteria for analyte identification. The presence of identifying transitions in many of the negative samples is likely a result of low level system carryover. Calculated concentrations determined from the quantitative transition responses of the negative control samples were typically less than 0.10 μg/kg, and below the respective MDL determined for the analyte in the specific matrix. A few negative control samples had calculated concentrations of MG, CV, or LCV residues ranging from 0.06 to 0.16 μg/kg; concentrations that were above the respective MDL, but below the LOQ for each analyte. Injecting water blanks between samples (2,7), or establishing more rigorous procedures for injection needle wash and/or laboratory contamination control may reduce the number of negative control samples that meet identification criteria for these analytes. To avoid extending the analysis run time with water injections, we applied an additional requirement beyond the established criteria (14) for analyte identification, such that the peak area response for each sample must yield a calculated concentration that is greater than that of the 0.25 μg/kg calibration standard. With this additional criterion applied, residues were not confirmed in the negative control samples.

Matrix Effects

Matrix effects have been thoroughly investigated and described elsewhere (9, 10). To test system suitability and observe the effect of matrix on analyte detection, a solvent calibrant was prepared at the 1.0 μg/kg level and analyzed each day of analysis. Relative to the matrix-matched extracted calibration curve, the calculated concentration of the solvent calibrant was usually > 0.90 μg/kg for MG, LMG, CV and LCV indicating that the extracted matrix calibrants and internal standard correction adequately corrected for matrix effects. The solvent calibrant had a lower response for some analytes compared to the analyte response in eel, dace, and frog matrix (0.6 to 0.8), indicating a higher matrix effect for these types of samples. This was also evident in the negative control chromatograms for these products, which were noisy and had somewhat elevated baselines around the retention time of the analytes (Figures 2 and 3). BG consistently yielded a lower response for the solvent calibrant (0.6 to 0.7 μg/kg) for all matrix types, which likely was an indication of differences between the responses for the d^5-MG internal standard and the BG analyte in matrix.

Matrix Control Sources

One to three control sources of each product were tested in the validation, with at least three sources tested for the non-finfish matrix types to account for the potential greater variance. Each of the three sources was used to prepare a set of extracted calibration standards on a different day, and controls and fortified samples from all the sources were tested on each day to determine if there were differences in analyte recovery when the fortified source did not match the calibrant source. For most matrix types, similar recovery results were achieved regardless of which source was used for the calibrants. For scallops and dried shrimp, higher recoveries were observed for MG (121-139%) and/or BG (120-158%) when the calibrant source did not match one of the fortified sources (10). Across all the sources however, average recoveries for these residues met the expected method performance.

One of the three control sources of dace tested positive for LMG with a calculated concentration of 0.60 μg/kg (± 0.06 μg/kg; Figure 3). The adulterated dace product was purchased from an online marketplace, and was the same brand identified in Hong Kong for MG adulteration in 2015 (15) and in a November 2017 product recall (7). LMG is the metabolic marker for MG exposure in fish, and the metabolite has a long residence time in fish muscle (1). When subjected to the semi-quantitative screening approach (comparison to a 1.0 μg/kg dace calibrant), replicates tested from this source of dace yielded LMG responses of 0.49 to 0.62 μg/kg relative to the single matrix calibrant. Thus, only some of the replicates would have had a response high enough to trigger additional testing according to the screening procedure (> 0.60 μg/kg of the threshold limit). Quantitative analysis of this sample with the full calibration curve yielded an LMG concentration exactly at the threshold (0.60 μg/kg), which was below the TTL of 1.0 μg/kg.

Processed Matrix Studies

Eel - Ten sources of eel were evaluated in this study as described in Table 1. The method accuracy is shown for each analyte in each matrix source. The data represents the average recovery from 3 days of analysis with 2 or 3 replicates tested on each day, and each day having a different matrix source (1, 2, or 3) for the extracted matrix calibrants. The interday precision was 18 % RSD or lower even with the additional contribution from variable source matrix calibrants. The method accuracy ranged from 80 to 116 % overall. The same range in recoveries was observed for the plain eel products (sources 1-4) as was observed for the canned, cooked, and heavily sauced products (sources 5-10). Ion chromatograms of a fortified sample in Source 10 at the 1.0 μg/kg level is shown in Figure 1, and chromatograms of negative control Sources 1 and 8 are shown in Figure 2. The presence of matrix background and/or residual low concentration analyte carryover can clearly be observed in the chromatograms for both negative control samples, with more matrix background present for Source 8 as would be expected for the canned product with additional ingredient components. However, the presence of some interference did not impact the method performance. Similar quantitative results were achieved for MG, LMG, CV, and LCV for both Sources 1 and 8, and BG was within the performance range of the method (110 and 88 %, Table 1). Overall, AOAC OMA 2012.25 performed as expected in eel regardless of the contribution to the matrix background by oils, sugar, salt, spices, fermented products, flavor enhancers or other food additives.

Dace - Raw dace was not available for comparison to the processed products. However, Method 2012.25 performed as expected in canned products regardless of the presence of oil, fermented black beans, soy sauce, sugar, spices, and sodium glutamate. Average analyte recoveries ranged from 95 to 104 %, and the interday precision was 8% or less (Table 6). Ion chromatograms are shown in Figure 3 for the control dace source that was found positive for LMG as well as a different dace source fortified at the 0.5 µg/kg level.

Dried Shrimp - The data presented in Table 6 is based on a 2.0 g sample size. Dried samples introduce more matrix interference to extracts than do raw or cooked samples. Dried shrimp is 20 % water, whereas raw shrimp is approximately 80 % water (16,17). For dried foods, the sample size of the product is commonly reduced to minimize matrix interferences, or water is added to swell the product and assist extraction (18). The effect of sample size for dried shrimp products was studied by comparing the 2.0 g portion with a reduced portion (0.5 g) and a rehydrated portion (1.0 g + 1.0 mL water, 4 hr equilibration). The 0.5 g and the 1.0 g rehydrated portions had analyte recoveries closer to the typical performance range, and they also had acceptable precision (RSD < 22%); whereas, the 2.0 g portion samples yielded greater variability for MG, CV, and BG across all three individual matrix sources. Additional details of these experiments have been published (10). Despite higher variability for MG, CV, and BG, the overall accuracy across the three matrix sources was largely consistent for the different sample sizes. Quantitative validation data collected for the 2.0 g sample portions (Table 6) support the use of this sample size; however, smaller or rehydrated samples portions may also yield acceptable results.

Smoked Trout - Two sets of extracted matrix calibrants were prepared on the same day from a smoked trout matrix and a raw trout matrix. Negative control, fortified, and incurred samples were analyzed with residue concentrations calculated using both of the calibration curves generated from the different matrix calibrants. Method accuracy and precision are shown in Tables 5 and 6 for fortified raw and smoked trout, and the results are indistinguishable for the two matrices. Overall, Method 2012.25 performance in smoked trout is similar to the method performance in raw trout.

Incurred Fish

Analyte concentrations determined for incurred fish samples are summarized in Table 7. The salmon and catfish muscle samples were part of AOAC collaborative study testing, with 14 laboratories reporting similar concentrations (5). Residues were identified as well in the incurred trout sample using a high resolution mass spectrometry screening method (19).

Table 7. Residues determined in incurred[a] fish

Compound	Salmon	Catfish	Tilapia	Trout
Malachite Green	1.8	3.1	0.3	4.4
Crystal Violet	<MDL	0.2	<MDL	<MDL
Brilliant Green	1.8	1.2	0.1	7.3
Leucomalachite Green	0.8	2.7	0.3	2.8
Leucocrystal Violet	0.4	4.3	0.3	2.6

[a]Salmon, catfish, and tilapia were treated with 2 µg/L of MG, CV, and BG for one hour; trout treatment was 4 µg/L of MG, CV, and BG for one hour.

CONCLUSIONS

AOAC Official Method of Analysis 2012.25 has been validated for the quantitative analysis and qualitative confirmation of identity for MG, LMG, CV, LCV, and BG in finfish (Arctic char, barramundi, catfish, hybrid striped bass, pompano, salmon, seabream, tilapia, and trout), aquacultured products (eel, frog legs, scallops, and shrimp) and processed seafood products including canned eel and dace products, dried shrimp, and smoked trout (4,5,9,10). Average analyte recoveries ranged from 86 to 119 % (<18% RSD) in all seafood matrices, using internal standard corrected extracted matrix calibrants to account for matrix effects and extraction losses (some exceptions were noted for BG in Arctic char and dried shrimp). Method performance for the processed canned products was not altered by the presence of oils, salt, sugar, fermented products, or food additives. The method yielded a detection limit below 0.5 µg/kg for all analytes in all matrices, except for BG in Arctic char, which had a calculated MDL of 0.60 µg/kg. The limit of quantification was less than 1.0 µg/kg for all analytes in all matrix types. With Method 2012.25, samples can be screened against a single matrix-matched extracted calibrant prepared at 1.0 µg/kg level, using a screening threshold limit of 0.6 µg/kg for most analytes/matrices (BG in Arctic char requires a lower threshold). LCV residues did not meet the product ion ratio confirmation criterion for identification in 10 % of the pompano, eel, and scallop samples, however, greater than 99 % of the analytes were positively identified in 467 fortified and incurred samples of all matrix types tested.

The fact that the method performed well for many different types of seafood and aquacultured products illustrates the ruggedness and flexibility of this procedure. The few exceptions to adequate method accuracy and precision that have been discussed primarily involve BG, which uses a different stable isotopically labeled analyte (d^5-MG) for internal standard correction. Overall, the results summarized here show that this method should be applicable to a wide variety of aquacultured products without the need for further matrix specific validation.

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Figure 1. Ion chromatograms of dyes and metabolites in eel matrix Source 10 – canned hot roasted eel fortified at 1.0 μg/kg.

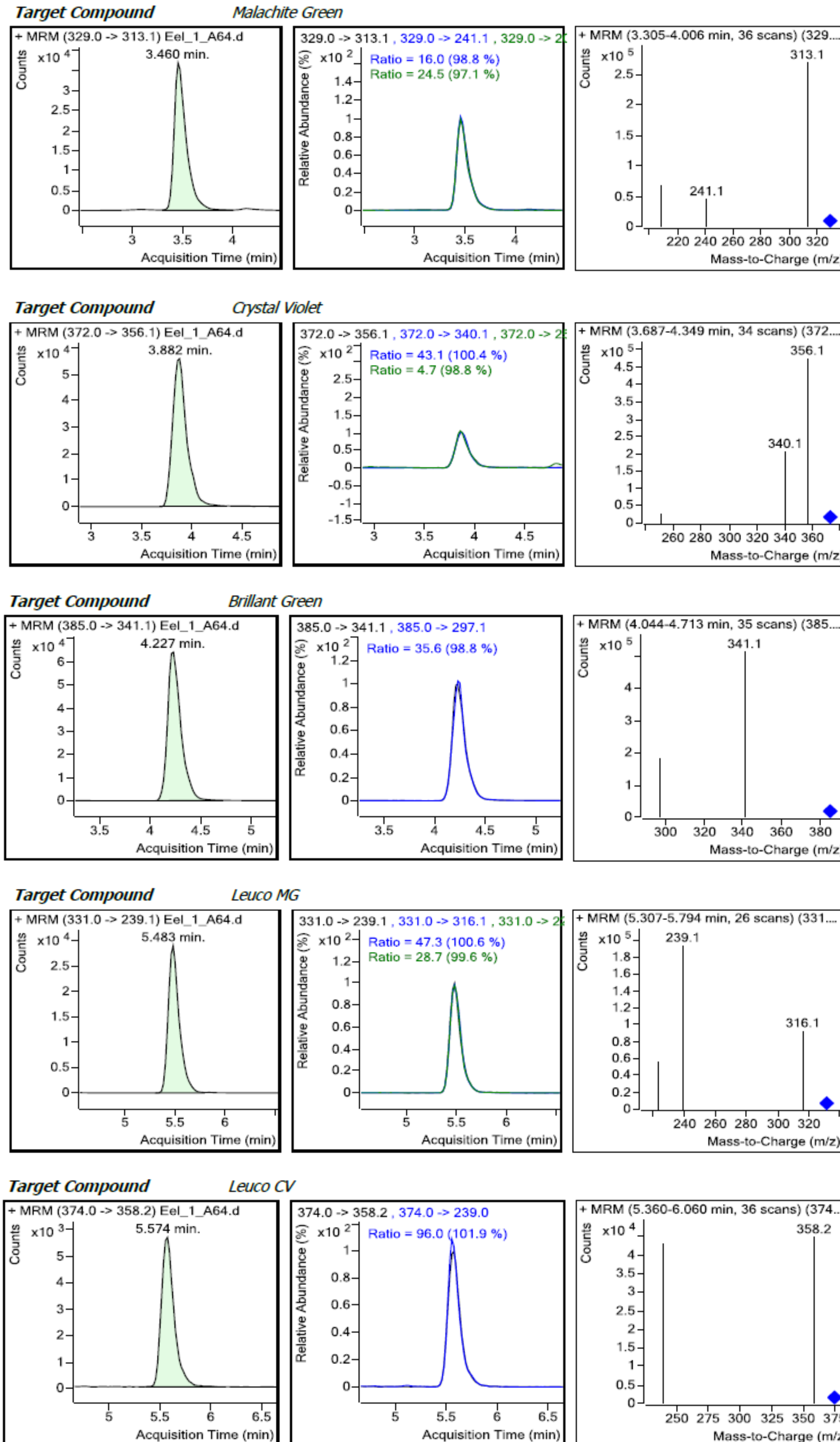

Figure 2. Ion chromatograms of dyes and metabolites in negative control eel matrix - Source 1 is wild frozen eel and Source 8 is canned braised eel with sauce.

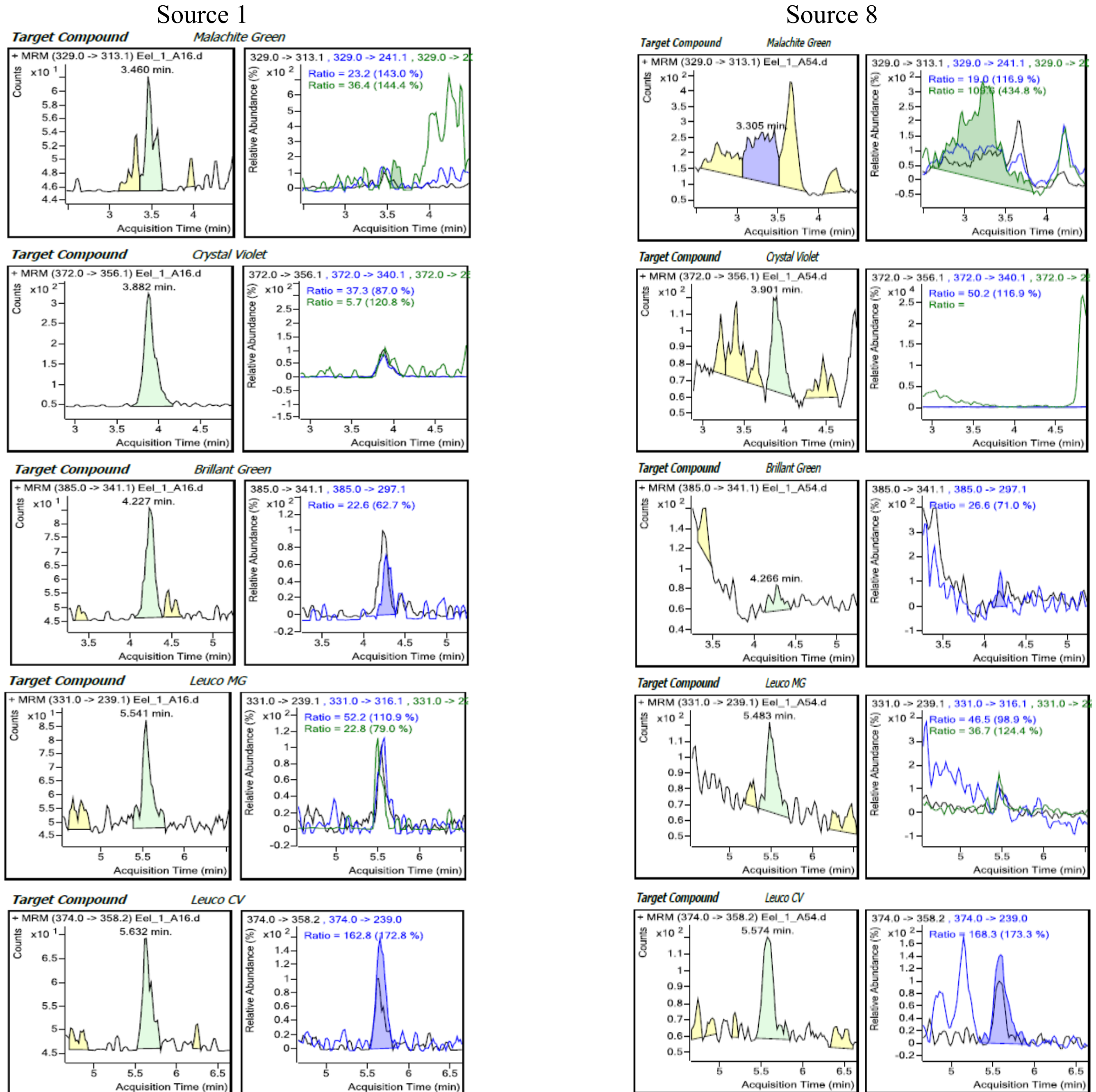

Figure 3. Ion chromatograms of dyes and metabolites in Source 3 control dace (0.60 μg/kg LMG identified) and in a Source 1 dace calibrant at the 0.5 μg/kg level.

Source 3 - Positive

Source 1 - Calibrant