A Simple SPE Method for the Determination of Malachite Green, Crystal Violet and Other Synthetic Dyes in Seafood Coupled with LC-MS/MS Detection

UCT Part Numbers

CSDAU206
CLEAN SCREEN® DAU
200mg / 6mL column

Introduction:

Triphenylmethane dyes (TPMs) are synthetic dyes used for a wide range of industrial applications. However, they are also used in aquaculture production due to their antibacterial, anti-fungal and anti-parasitic properties. They are cheap, very effective and readily available, but due to their toxicity to humans (mutagenic & carcinogenic) the TPMs are banned in the US and EU. In both regions there is a zero tolerance policy regarding their use. As such, the respective authorities (FDA and EU Commission) have established very low detection limits for these compounds in seafood products, namely 1 µg/kg (US) and 2 µg/kg (EU). As the parent dyes are rapidly metabolized to form their leuco- metabolites that predominate and persist in fish tissue, the detection limits are expressed as the sum of parent drug and corresponding leuco-form.

Analyzing TPMs can be difficult due to their instability, which causes problems during sample preparation and instrumental analysis. The TPM dyes readily undergo oxidationreduction reactions and are prone to UV degradation (demethylation). As such, it is important to take these issues into account when developing a method or when doing routine analysis of these compounds. To stabilize the dyes it is common to add an antioxidant prior to sample extraction and in the final sample extract prior to detection. Common antioxidants used include TMPD*, hydroxylamine and ascorbic acid.

Analytical standards solutions also experience stability issues and should be prepared in an aprotic solvent (e.g. acetonitrile) as the leuco metabolites are not stable for a long period of time in H2O and MeOH (even at -20 °C) and oxidize to the non-leuco form. This can in turn affect the reliability of the analytical method by leading to poor recoveries/reproducibility and non-linear calibration curves. It is also preferable to prepare a mixed working standard solution on a regular basis.

Most reported methods for TPM analysis use lengthy sample preparation procedures or do little to no sample cleanup. Most methods include malachite green, leucomalachite green, crystal violet, leucocrystal violet and sometimes the structurally related dye brilliant green. HPLC-UV is often used for their analysis due to the compounds good UV absorbance, but this technique can only detect the parent compounds and not the leuco- metabolites. To include these, an oxidation step needs to be incorporated into the LC system using a post-column reactor to convert the leuco- form to the chromophorous parent form. Alternatively, the use of LC-MS/MS avoids this requirement and is capable of detecting all TPM dyes. It also offers the advantage of simpler sample preparation methodology and the inclusion of additional compounds besides the classical TPM dyes.

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Introduction (Cont.):

Sample preparation approaches for TPM analysis often use time consuming, complicated procedures consisting of numerous steps and requiring large volumes of solvent to evaporate. SPE using strong-cation exchange sorbent is also used and is very effective at removing matrix interferences. However, it is difficult to elute dyes from SCX sorbent as most dyes carry a permanent positive charge (see Figure 1). Most methods using SCX SPE use a high salt concentration (> 1 M) for elution. However this is not very suitable for LC-MS/MS analysis due to the lack of volatility of such high salt concentrations.

This application note uses an alternative elution solvent composed of methanol (MeOH) containing 1% triethylamine (TEA) and 0.5% formic acid (i.e. triethylammonium formate). The positively charged TEA (a quaternary amine) then acts as a counter-ion to release the dyes from the sorbent. No evaporation step is included in the method which avoids any loss that may occur with this step. The eluted extracts can be analyzed directly as TEA is LC-MS/MS compatible (due to its volatility) and has no ion-pairing effect on the dyes as they carry the same charge. Ascorbic acid is used as antioxidant because positively charged hydroxylamine (quaternary amine) will interfere with the retention on the sorbent (same mechanism as elution solvent). The simple method outlined here allows for the rapid analysis of dyes in seafood while achieving good accuracy/precision and low sensitivity.

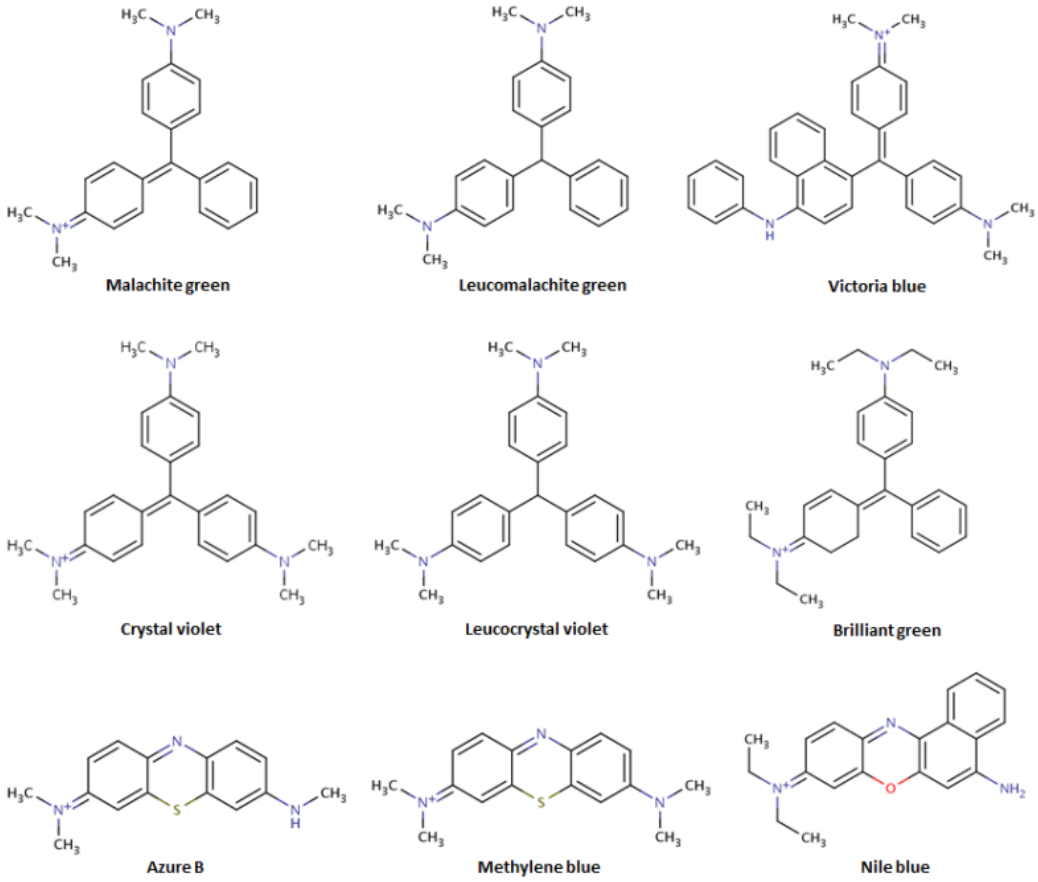

Figure 1. Structure of the 9 dyes included in the method.

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Compounds included in Method	Abbreviation
Malachite Green	MG
Leuco-Malachite Green	LMG
Crystal Violet	CV
Leuco-Crystal Violet	LCV
Nile Blue	NB
Azure B	AB
Methylene Blue	MB
Brilliant Green	BG
Victoria Blue	VB

Procedure:

1. Sample Extraction

a) Weigh 2 ± 0.1g of sample into a 15mL polypropylene centrifuge tube.
b) Add 10 mL of 1% formic acid in acetonitrile and 1mL of 1M ascorbic acid (antioxidant) to each sample.
c) Shake on a SPEX® SamplePrep® GenoGrinder® (or alternative mechanical shaker) or vortex samples for 15 minutes to fully extract the dyes.
d) Centrifuge the samples for 10 min at ≥3000 rcf and 4°C.
e) Transfer the supernatant to a 50mL polypropylene centrifuge tube and add 20mL of McIlvaine's buffer (0.1M, pH 3.5)**.
f) Vortex the samples briefly (1 min) to mix the organic solvent with the buffer.
g) Prior to the SPE step, centrifuge the samples for 5 min at ≥3000 rcf and 4°C. This makes the SPE step easier by separating out any solid particles and/or lipids in the sample.

2. Condition Cartridge

a) Add 3 mL of methanol to SPE cartridge CSDAU256.
b) Add 3 mL of ultrapure water.
c) Add 1 mL of McIlvaine's buffer (0.1M, pH 3.5).
Note: Do not let the cartridge go dry otherwise repeat steps a) through c).

3. SPE Extraction

a) Load supernatant from step 1.g).
b) Adjust vacuum for flow of 1–3 mL per minute.

4. Wash cartridge

a) Add 3 mL of 0.1% formic acid and slowly draw through.
b) Add 3 mL of 0.1% formic acid in MeOH and slowly draw through.
c) Dry under vacuum for 1 minute to remove excess solvent.
Note: A portion of the dyes will stick to the SPE frit due to the hydrocarbon backbone. The wash steps included in the method uses acidified solutions to maintain a positive charge on the dyes, which ensures they are retained on the cation-exchange sorbent.

5. Elute Cartridge

a) Elute the dyes from the SPE cartridge using 3-4 mL elution solvent (1% TEA + 0.5% formic acid in MeOH).
b) Vortex the samples for 2 min and transfer a 1 mL aliquot to an autosampler vial for analysis.

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LC-MS/MS Conditions:

HPLC Conditions	
Instrumentation	Thermo Scientific™ Dionex™ Ultimate™ 3000 LC system
HPLC column	Thermo Scientific™ Accucore™ C18, 100 x 2.1 mm, 2.6 µm **(p/n:17126-102130)**
Guard Column	Accucore™ C18 Defender™, 10 x 2.1 mm, 2.6 µm **(p/n: 17126-012105)**
Run Time	12 min (including re-equilibration time)
Column temp.	40°C
Flow rate	400 µL/min
Injection volume	5 µL
Autosampler temp.	10°C
Wash solvent	MeOH:ultrapure water (1:1, v/v)
Divert valve	Mobile phase was sent to waste (0–3.5 & and 8–12min) to prevent ion source contamination.

Mobile Phase & Gradient:	
A	0.1% formic acid in ultrapure water
B	0.1% formic acid in MeOH

Time (min)	A (%)	B (%)
0.0	90	10
2.0	90	10
3.0	40	60
4.0	40	60
5.0	0	100
8.0	0	100
8.2	90	10
12.0	90	10

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MS Conditions	
Instrumentation	Thermo Scientific™ TSQ Vantage™ tandem mass spectrometer
Ionization mode	**ESI+**
Spray voltage	**4000 V**
Vaporizer temperature	**350°C**
Capillary temperature	**300°C**
Sheath gas pressure	60 arbitrary units
Auxiliary gas	5 arbitrary units
Ion sweep gas	0 arbitrary units
Declustering	0 V
Q1 and Q3 peak	0.2 and 0.7 Da
Collision gas	Argon
Collision gas pressure	2.0 mTorr
Acquisition Method	EZ method (SRM)
Cycle time	0.6 sec
Software	TraceFinderTM version 3.0
Weighting factor applied to 'calibration curves	1/X

SRM Transitions							
Analyte	**t_R (min)**	**Precursor ion**	**Product ion 1**	**CE 1**	**Product ion 2**	**CE 2**	**S-lens (V)**
Azure B	4.74	270.1	212.0	48	254.1	31	100
Methylene Blue	4.77	284.1	252.0	53	268.1	33	100
Crystal Violet	4.93	374.2	239.0	33	358.2	28	100
Malachite Green	5.59	329.1	165.1	59	313.2	37	100
Nile Blue	5.76	318.1	245.1	55	274.1	35	100
Leucomalachite Green	5.98	331.1	223.1	50	239.1	29	100
Leucocrystal Violet	5.99	372.2	340.2	51	356.2	37	100
Brilliant Green	6.19	385.2	297.1	51	341.2	37	100
Victoria Blue	6.27	470.2	333.1	48	349.1	34	145

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Accuracy & precision Data for 9 Dyes Spiked in Salmon Tissue (n=5)									
	MG	**LMG**	**CV**	**LCV**	**NB**	**AB**	**MB**	**BG**	**VB**
1 ppb									
Sample 1	92.2	83.8	88.1	101.7	87.2	94.2	76.5	98.5	98.2
Sample 2	94.0	109.5	83.4	92.8	85.4	97.3	80.0	92.9	90.4
Sample 3	92.6	91.2	83.0	95.5	85.0	96.8	77.6	92.1	91.4
Sample 4	93.5	105.6	94.9	91.3	85.1	94.3	73.3	93.2	92.5
Sample 5	92.4	81.2	83.1	103.0	87.9	96.0	76.8	99.0	93.4
Mean	**92.9**	**94.3**	**86.5**	**96.9**	**86.1**	**95.7**	**76.9**	**95.1**	**93.2**
RSD	**0.83**	**13.52**	**5.97**	**5.44**	**1.58**	**1.51**	**3.13**	**3.51**	**3.26**
10 ppb									
Sample 1	97.1	91.1	92.6	100.4	95.9	96.6	85.6	106.9	104.4
Sample 2	99.7	96.6	99.1	105.9	98.6	98.6	81.8	103.2	104.4
Sample 3	98.7	94.9	98.3	97.6	97.5	99.4	87.0	104.2	102.7
Sample 4	99.9	88.1	91.0	107.3	99.1	97.9	88.2	98.5	92.2
Sample 5	100.0	97.5	98.6	97.4	99.5	93.5	86.5	102.1	104.3
Mean	**99.1**	**93.7**	**95.9**	**101.7**	**98.1**	**97.2**	**85.8**	**103.0**	**101.6**
RSD	**1.24**	**4.24**	**3.99**	**4.56**	**1.49**	**2.39**	**2.85**	**2.98**	**5.21**

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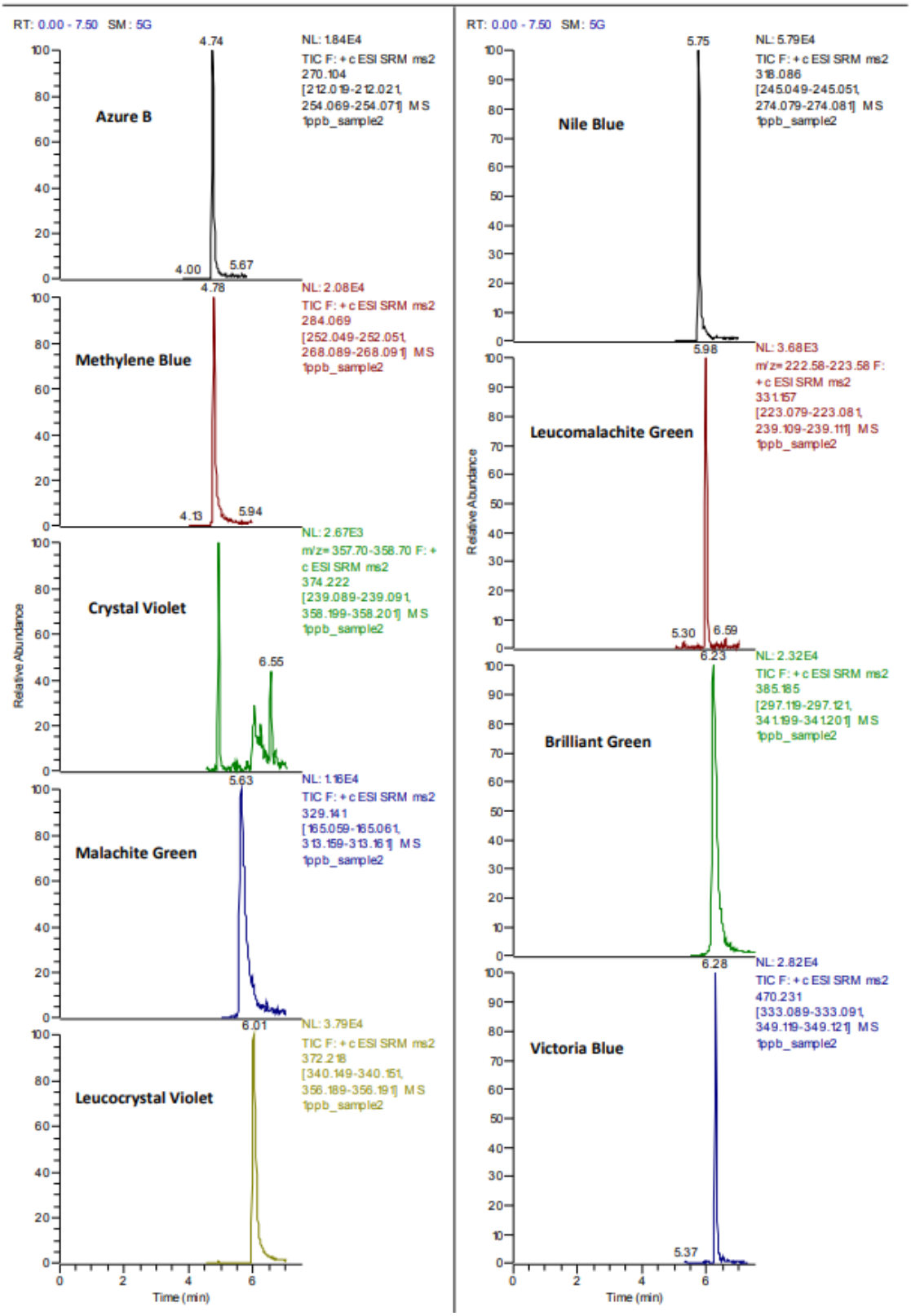

Figure 2. Chromatogram of salmon tissue fortified with the 9 dyes a 1 ng/g.

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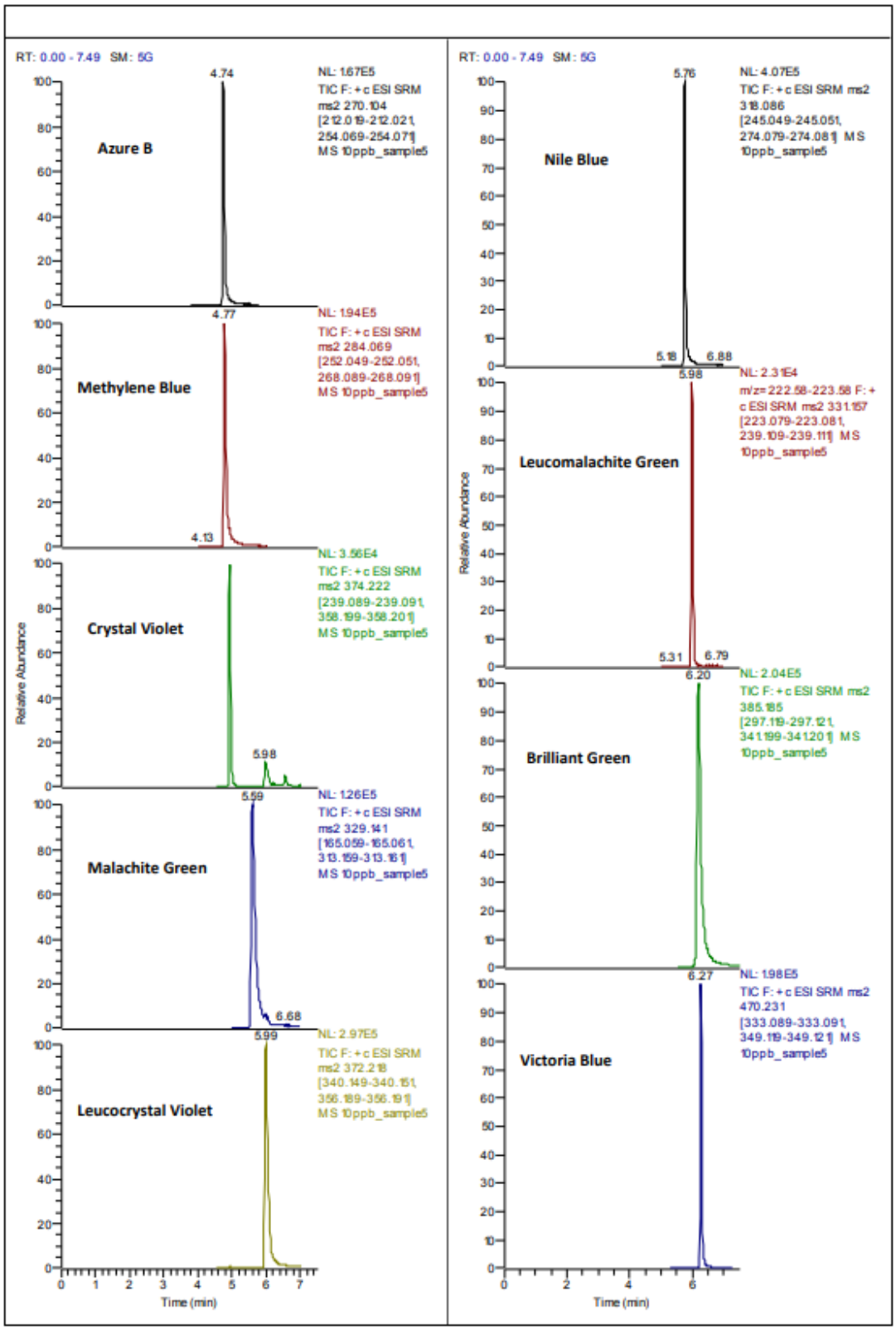

Figure 3. Chromatogram of salmon tissue fortified with the 9 dyes a 10 ng/g.

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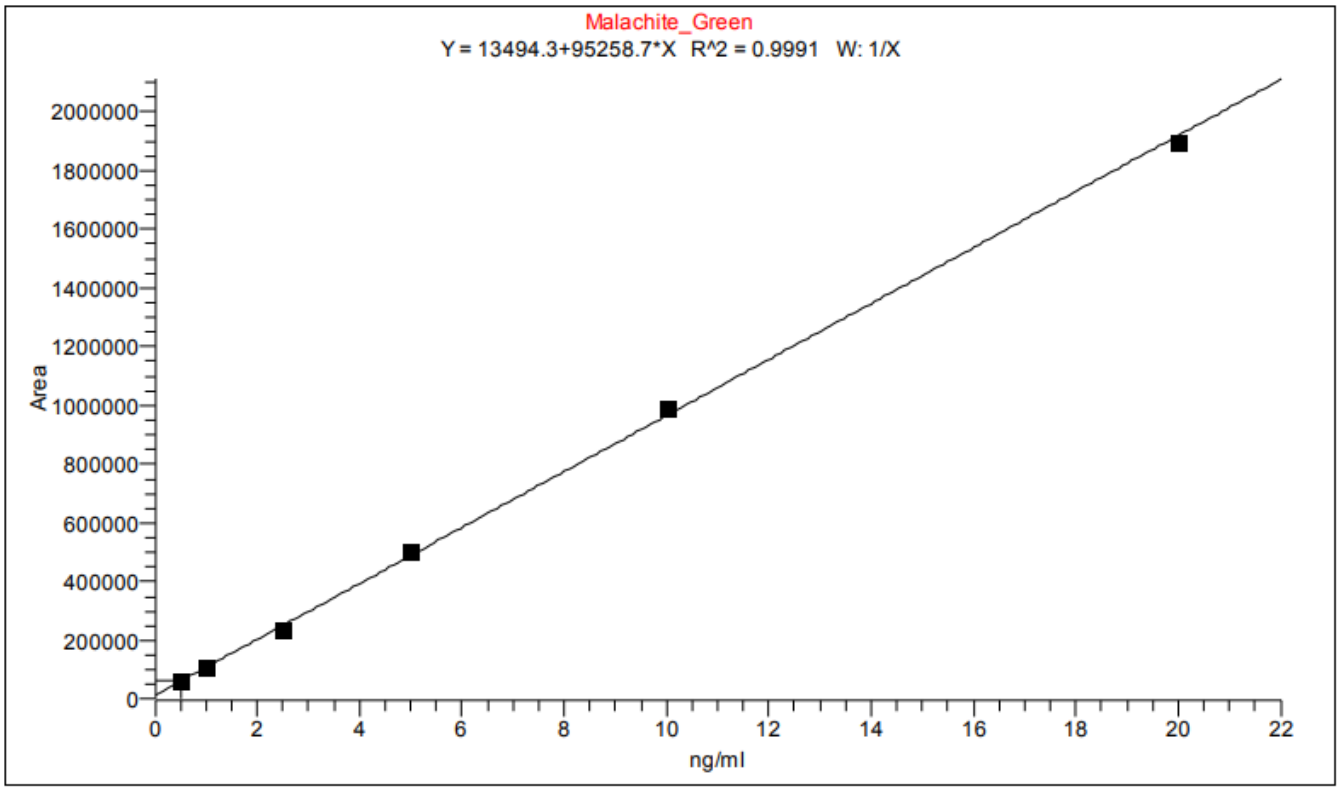

Figure 4. Calibration curve example (malachite green).

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References:

[1] * TMPD: N,N,N',N'-tetramethyl-1,4-phenylenediamine dihydrochloride

[2] **Mcilvaines buffer pH 3.5 – mix 500mL of 0.1M citric acid with 400mL 0.1M disodium hydrogen phosphate. Adjust pH to the correct value (3.5) using the citrate or phosphate solution.

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