LC/MS/MS of Malachite Green and Crystal Violet in Fish with Agilent Bond Elut PCX and Poroshell 120

Application Note

Food Testing & Agriculture

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Abstract

A method for simultaneous determination of malachite green and crystal violet, and their metabolites leuco-malachite green and leuco-crystal violet in fish, was developed and validated. The analytes were extracted by solid phase extraction and quantified by liquid chromatography coupled to electrospray ionization tandem mass spectrometry operating in positive ion multiple reaction monitoring mode. The method provided a 0.5 ng/g limit of quantification for all these compounds in fish. The dynamic calibration ranges for these compounds were obtained from 0.5 to 100 ng/g. The overall recoveries ranged from 96 to 109% with RSD values between 1.7 and 4.5%.

Introduction

Malachite green (MG) and crystal violet (CV) are synthetic pigments mainly used for dyeing. In addition, they are used to treat water-borne infectious diseases, particularly in fish and eels, as they have antibacterial properties. However, MG is a suspected carcinogen and so its use in aquaculture is currently prohibited [1].

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In this work, we used Agilent Bond Elut Plexa PCX solid phase extraction (SPE) cartridges to extract MG, CV and their metabolites leuco-malachite green (LMG) and leuco-crystal violet (LCV) from the flesh of fish, and analysis by LC/MS/MS. Table 1 shows the names and structures of the 4 compounds.

Experimental

Reagents and chemicals

All reagents were MS, HPLC or analytical grade. Methanol, acetonitrile and water were from Honeywell. The standards and chemicals were purchased from Beijing J&K Scientific. The internal standard isotope-labeled malachite green (MG-d5) and isotope-labeled leuco-malachite green (LMG-d6) were purchased from Dr. Ehrenstorfer GmbH. Fish (crucian carp) was purchased from a local supermarket.

Standard solutions (1.0 mg/mL) were made in methanol individually and stored in a freezer at –20 °C. A combined working solution (10 µg/mL) was made in acetonitrile:water (10:90) and also stored at 4 °C. The spiked solutions were then made daily by appropriately diluting the combined working solution in water.

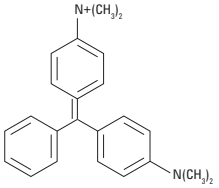
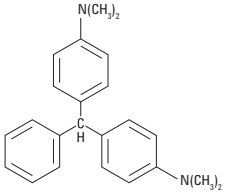
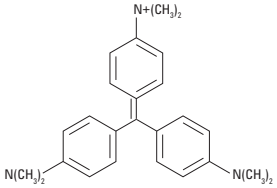
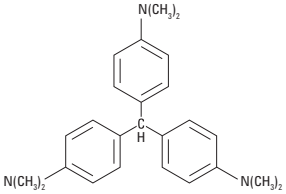
Internal standard solutions were made in methanol individually and stored in the freezer at –20 °C. The standards were mixed and then diluted to 100 ng/mL with methanol before use.

TMPD solution: dissolve 50 mg of N,N,N,N-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD) in methanol and make it to 50 mL.

McIlvaine's buffer: mix 445.5 mL of 0.1 mol/L citric acid solution with 54.5 mL 0.2 mol/L sodium phosphate, dibasic solution.

Elution buffer: mix 50 mL of 5 mol/L ammonium acetate solution (adjust pH to 7.0) with 100 mL ethyl acetate and 350 mL methanol.

Table 1. Compounds used in this study

Compound	CAS no.	Structure
Malachite green	569-64-2	
Leuco-malachite green	129-73-7	
Crystal violet	548-62-9	
Leuco-crystal violet	603-48-5	

Equipment and materials

Agilent 1200 HPLC system

Agilent 6460 Triple Quadrupole LC/MS/MS system

Agilent Bond Elut Plexa PCX cartridges, 60 mg, 3 mL (part number 12108603)

Agilent Poroshell 120 EC-C18 column, narrow bore, 2.1 × 50 mm, 2.7 µm (part number 699775-902)

Agilent Vac Elut 20 Manifold (part number 12234101)

Sample preparation

Sample pretreatment

Cut the meat of fish into small pieces, and homogenize it with the homogenizer. Take 1 g homogenized sample and place in a centrifugation tube. Add 50 µL TMPD solution and 10 mL of McIlvaine's buffer:acetonitrile (1:1 v/v). Vortex 1 min, then centrifugation at 4500 rpm for 5 min and transfer the supernatant to a clean tube. Add 5 mL of McIlvaine's buffer: acetonitrile (1:1 v/v) to the pellet, vortex 1 min, then centrifuge at 4500 rpm for 5 min. Transfer the supernatant and add it to the supernatant from the first extraction. Get the sample solution for SPE.

Solid-phase extraction

The SPE procedure is shown in Figure 1. Agilent Bond Elut Plexa PCX cartridges were preconditioned with 2 mL methanol and then equilibrated with 2 mL 2% formic acid (FA) in water. The sample solution was then loaded onto a cartridge and passed through the cartridge by gravity (about 1 mL/min). The cartridges were washed with 2 mL 2% FA in water, 2 mL methanol and 2 mL hexane. Full vacuum was applied to the cartridge for 3 min to completely dry the resin. The compounds were eluted with 4 mL elution buffer at 1 mL/min. Water was added to the eluate up to 5 mL and vortexed for 10 seconds. The sample was transferred to a 2 mL chromatography vial for analysis.

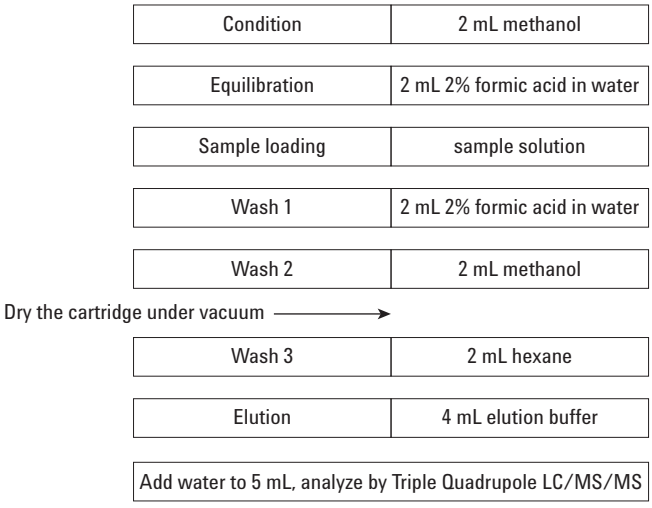


Figure 1. Solid phase extraction procedure to extract antibacterial agents in fish samples

Instrument conditions

HPLC conditions

Column:	Agilent Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 µm (part number 699775-902)
Mobile phase:	Water (5 mM NH_4Ac, A): acetonitrile (0.1% FA, B)
Injection volume:	5 µL
Flow rate:	0.4 mL/min
Temperature:	Ambient

Gradient:

Time (min)	%B
0	30
5	80
6	80
6.5	30
7	30

MS conditions

The standard and internal standard compounds were monitored in the positive mode. The source conditions are shown in Table 2 and the MRM channels are shown in Table 3.

Table 2. MS source parameters

Gas temp:	300 °C
Gas flow:	5 L/min
Nebulizer:	45 psi
Sheath gas temp:	400 °C
Sheath gas flow:	11 L/min
Nozzle voltage:	Positive: 0 V Negative: 0 V
Capillary:	Positive: 3500 V Negative: 3500 V

Table 3. Masses monitored in multiple-reaction-monitoring mode

Analyte	MRM channels (m/z)	Fragmentor (V)	CE (V)
MG	1) 329.3>313.3	175	38
	2) 329.3>208.3		38
CV	1) 372.3>356.2	175	42
	2) 372.3>251.1		36
LMG	1) 331.3>316.2	175	26
	2) 331.3>238.2		16
LCV	1) 374.3>358.3	175	30
	2) 374.3>238.2		26
MG-d5	334.3>318.3	175	38
LMG-d6	337.3>240.2	175	30

Results and Discussion

Linearity and limit of detection

The linearity calibration range for all of the pesticides tested was 0.5 to 100 ng/g. Calibration curves, spiked in matrix blanks, were made at levels of 0.5, 1, 5, 10, and 100 ng/g; the internal standards were made at 10 ng/g. Matrix blanks were created by taking fish through the entire sample preparation procedure. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte/peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). The 0.5 ng/g quantification limits (LOQ) established for all compounds are lower than the MRLs of these compounds in aquatic product. Table 4 shows the linear regression equation and correlation coefficient (R^2) [2].

Table 4. Linearity of antibacterial agents

Compound	Internal standard	Regression equation	R^2
MG	MG-d5	Y=0.0961x+0.0049	1
CV	MG-d5	Y=0.2808x-0.0524	0.999
LMG	LMG-d6	Y=0.0649x+0.0033	1
LCV	LMG-d6	Y=0.05461x-0.0011	1

Recovery and reproducibility

The recovery and reproducibility for the method were determined at 3 levels; fish meat spiked to a concentration of 1, 10, and 50 ng/g. The analysis was performed with 6 replicates at each level. The recovery and reproducibility data are shown in Table 5. The chromatograms of spiked fish extracts (10 ng/g) are shown in Figure 2.

Table 5. Recoveries and reproducibility of antibacterial agents in fish

Compound	Spiked level (ng/g)	Recovery (%)	RSD (n=6)
MG	1	102.1	3.5
	10	102.8	1.8
	50	99.2	1.9
CV	1	96.9	2.1
	10	102.8	2.6
	50	97.4	1.7
LMG	1	103.5	2.6
	10	108.9	3.4
	50	96.7	3.7
LCV	1	99.4	3.8
	10	106.1	3.8
	50	102.7	4.5

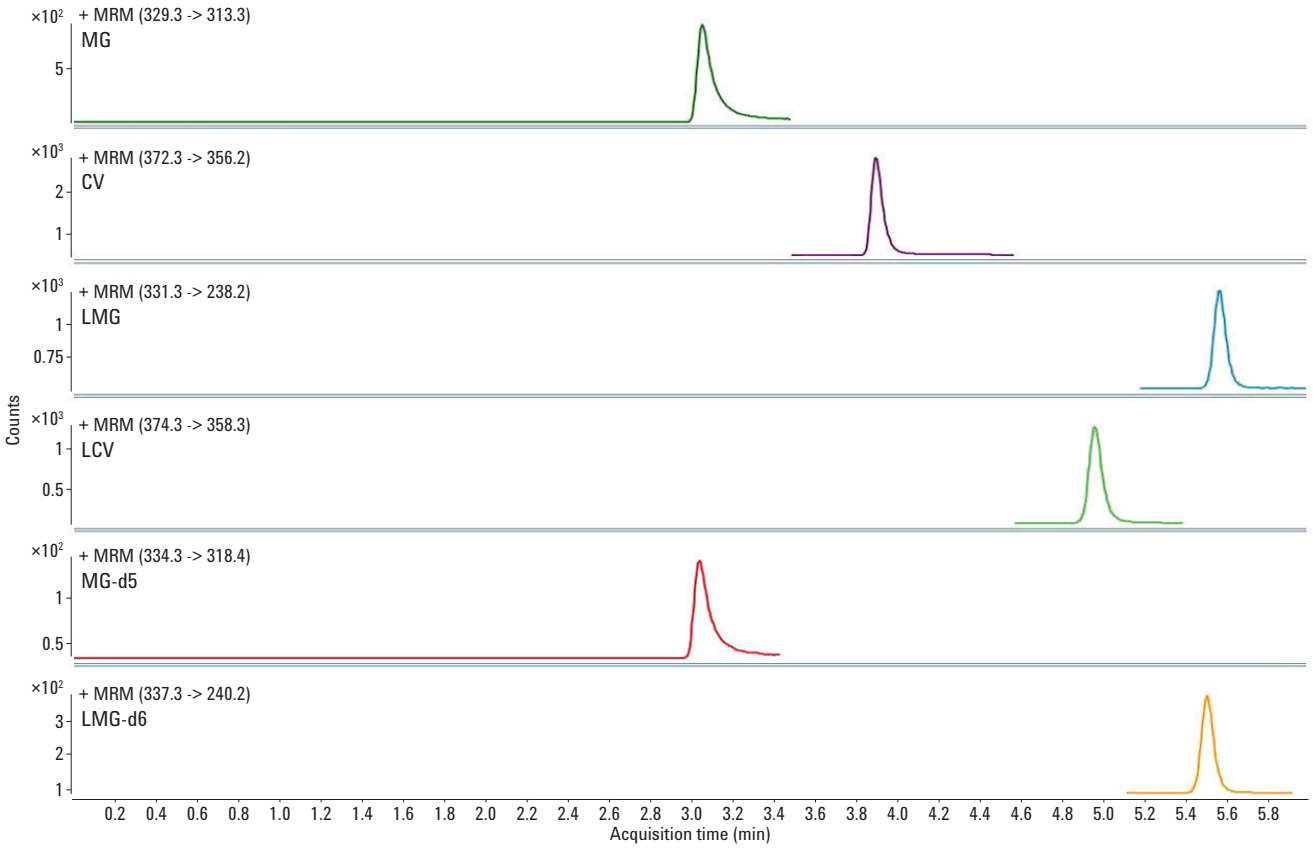


Figure 2. Chromatograms of 10 ng/g spiked sample extracts of antibacterial agents in fish on an Agilent Poroshell 120 EC-C18 column

Conclusion

Malachite green and crystal violet, and their metabolites leuco-malachite green and leuco-crystal violet, were measured simultaneously. The result of this study showed that Agilent Bond Elut Plexa PCX could be used as an effective method for purification and enrichment of dyes in aquatic product such as fish. The recovery and reproducibility results based on matrix spiked standards were acceptable for dye residue determination in fish under regulations. The impurities and matrix effect were minimal and did not interfere with the quantification of any target compound.

References

1. Kazuyuki Yamashita. *LC-MS/MS Analysis of Malachite Green and Crystal Violet using Pursuit XRs*. Application Note, Agilent Technologies, Inc. Publication number SI-01313. (2008)

2. GB/T 19857-2005. *Determination of malachite green and crystal violet residues in aquatic product*. China Standard. www.cn-standard.net.

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