Bundesamt für Verbraucherschutz und Lebensmittelsicherheit

# Validation of a Method for Triphenylmethane Dye Residues in Aquaculture Products by LC-MS/MS

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## Introduction

Malachite green (MG) is a triphenylmethane dye, which was successfully used as veterinary drug in aquaculture for the treatment and prevention of different diseases for many years. However, due to their possible carcinogenic, mutagenic and teratogenic effects, the triphenylmethane dyes are not authorised for use in food-producing animals. In the framework of the German residue control plan, MG, crystal violet (CV) and brilliant green (BG) as well as the metabolites leuco-MG and leuco-CV are analysed routinely. For this purpose a robust and economic method with good performance parameters is required. The presented confirmatory LC-MS/MS-method is able to qualify and quantify 6 TPM substances (Table 1, 3 dyes and their corresponding leuco-forms) with the help of their respective deuterated internal standards with the exception of LBG. Because of the different properties of the dyes and their corresponding leuco-metabolites, an extraction method without clean-up was chosen. The evaporation and reconstitution of the extract solutions could be omitted. The method parameters like the critical concentrations CCα and CCβ, the repeatability, the within-laboratory reproducibility and the recovery are presented.

## Experimental

Table 1: Analytes and their corresponding internal standards

| Analytes | Abbreviation | Retention time (min) | Internal standards |
|---|---|---|---|
| Malachite green | MG | 4.5 | Malachite green-D5 |
| Crystal violet | CV | 5.1 | Crystal violet-D6 |
| Brilliant green | BG | 5.7 | Brilliant green-D5 |
| Leuco malachite green | LMG | 6.4 | Leuco malachite green-D5 |
| Leuco crystal violet | LCV | 6.5 | Leuco crystal violet-D6 |
| Leuco brilliant green | LBG | 9.2 | Leuco crystal violet-D6 |

**Sample preparation**

**Sample and Spiking**

- weigh in 1 g of sample
- add 500 µl of hydroxylamine solution (20 g $l^{-1}$)
- add spike solutions for validation
- wait for 10 min

**Extraction**

- add 8 ml of acetonitrile containing ascorbic acid (1 %; 100/1, v/v)
- vortex sample for approximately 1 min
- add 1 g of anhydrous magnesium sulfate
- shake vigorously by hand and for 10 min by overhead shaker
- centrifuge for 5 min at 3400 g and 5 °C
- centrifuge a part of the supernatant again for 5 min at 20,000 g

**LC-MS/MS-Measurement**

### LC-MS/MS

LC: Agilent Technologies, 1290 Series, Symmetry C18 , Waters (100 mm × 2.0 mm, 3.5 µm) with adequate guard; mobile phase: A = ammonium formate buffer (50 mM, pH 4.5) and B = acetonitrile; gradient program: 0 min = 20 % B, 1 min = 20 % B, 3 min = 90 % B, 10 min = 90 % B, 11 min = 20 % B, 14 min = 20 % B; flow = 0.2 ml/min; oven temperature = 30 °C; injection volume = 10 µl; sampler temperature = 10 °C. Chromatograms of all analytes are shown in Figure 1.
MS/MS: QTRAP 5500 (AB SCIEX), ionisation mode = ESI+; scan type = MRM; dwell time = 100 ms; resolution Q1 and Q3 = unit; gas 1 + 2= 50 psi; curtain gas = 20 psi; ion spray voltage = 5500 V; source = 600 °C.

### Validation

In accordance with Commission Decision 2002/657/EC, the validation of the samples was accomplished with concentrations of 0.5, 1.0, 1.5 and 2.0 µg $kg^{-1}$. The study was designed on the basis of an in-house concept with 8 factor-level combinations (4 factors on two levels, see Table 2). The calculation of the validation experiment data was carried out with the help of the InterVal software (QuoData, Dresden, Germany). The results are shown in Table 3 and Figure 2.

Table 2: Factor-level combinations (runs) with 4 factors

| Run | Species | Storage of extract | Age of column | Operator |
|---|---|---|---|---|
| Run 01 | trout | 24 h | old | Mö |
| Run 02 | trout | 24 h | new | Ho |
| Run 03 | trout | 0 h | old | Ho |
| Run 04 | trout | 0 h | new | Mö |
| Run 05 | shrimp | 24 h | old | Ho |
| Run 06 | shrimp | 24 h | new | Mö |
| Run 07 | shrimp | 0 h | old | Mö |
| Run 08 | shrimp | 0 h | new | Ho |

## Results

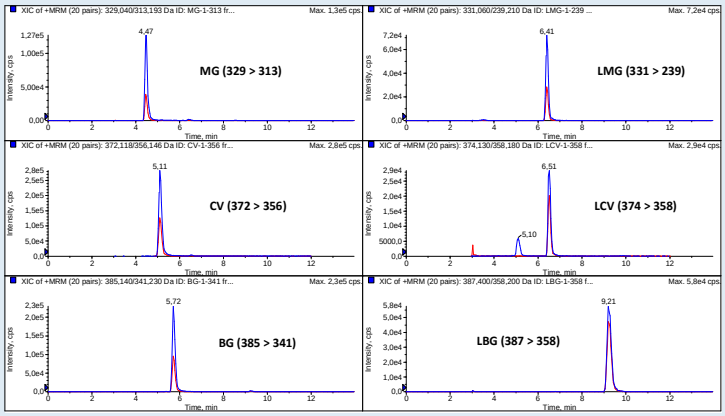


Figure 1: Chromatograms of two transitions of analytes in a trout muscle sample fortified at a level of 1 µg $kg^{-1}$

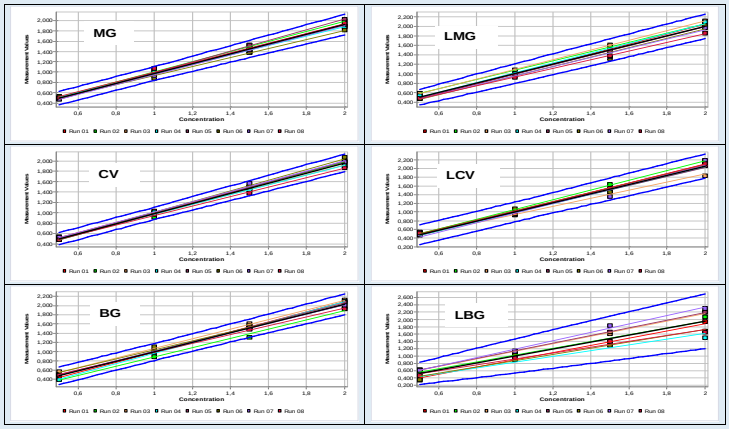


Figure 2: Single curves (runs 1-8), overall curves (black) and prediction intervals (blue) of the measurement values of the analytes MG, CV, BG, LMG, LCV and LBG in the recovery samples of the 8 factor-level combinations of the validation

Table 3: Performance characteristics of the method at a concentration level of 0.75 µg $kg^{-1}$ by matrix calibration

| Analyte | $RSD_r$* [%] | $RSD_{wR}$** [%] | Recovery [%] | CCα [µg $kg^{-1}$] | CCβ [µg $kg^{-1}$] |
|---|---|---|---|---|---|
| MG | 5.9 | 5.9 | 97.8 | 0.64 | 0.73 |
| LMG | 7.1 | 8.3 | 100.2 | 0.67 | 0.80 |
| CV | 5.2 | 5.2 | 99.0 | 0.62 | 0.70 |
| LCV | 10.1 | 10.1 | 98.7 | 0.72 | 0.86 |
| BG | 5.4 | 8.3 | 97.7 | 0.70 | 0.82 |
| LBG | 13.8 | 17.2 | 102.0 | 0.83 | 1.15 |

* $RSD_r$: repeatability; ** $RSD_{wR}$: within-laboratory reproducibility

## Conclusions

The verification of different factors was successful regarding the applicability and the ruggedness of the method. Their influence on the validation parameters was not significant. Quantitation was effected on the basis of matrix calibration curves as well as standard calibration. All validation parameters were satisfying and lay in the required ranges with the exception of CCβ for LBG. The recommendation for CCα to be less than 1 µg $kg^{-1}$ (½ MRPL for the sum of MG and LMG) was fulfilled. This was true also for the other substances. The method can be applied for muscle of aquaculture (fish and crustaceans) in a concentration range around 1 µg $kg^{-1}$.

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