Journal of Chromatography A, 1218 (2011) 1632–1645

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Liquid chromatography–tandem mass spectrometry method for the determination of dye residues in aquaculture products: Development and validation

D. Hurtaud-Pessel*, P. Couëdor, E. Verdon

Anses, French Agency for Food, Environmental and Occupational Health Safety, Fougères Laboratory, Unit Veterinary Drug Residues, EU-RL for Antibiotic and Dye Residues, La Haute Marche, Javené, BP 90203, 35302 Fougères, France

ARTICLE INFO

Article history:
Received 29 November 2010
Received in revised form 14 January 2011
Accepted 19 January 2011
Available online 27 January 2011

Keywords:
Malachite green
Crystal violet
Brilliant green
LC–MS/MS
Validation
LTQ-Orbitrap

ABSTRACT

A method is described for the identification and the quantitative determination of the triphenylmethane dyes, malachite green (MG), crystal violet (CV), brilliant green (BG) and leuco malachite green (LMG) and leuco crystal violet (LCV). The analytes were isolated from the matrix by liquid–liquid extraction with acetonitrile. Determination was performed using LC–MS/MS with positive electrospray ionisation. 4 different deuterated internal standards were introduced to improve the quantitative performance of the method. The method has been validated in line with the EU criteria of Commission Decision 2002/657/EC in accordance with the minimum required performance limit (MRPL) set at 2 $\mu g\,kg^{-1}$ for the sum of MG and LMG. For all the monitored compounds, accuracy, intra-day and inter-day precision were determined at each level of fortification (0.5, 0.75, 1.0 and 2.0 $\mu g\,kg^{-1}$). Decision limits CCα and detection capabilities CCβ were calculated according to the standard ISO 11843-2. A study on the applicability of the method was conducted on various aquacultured species with the aim to assess the matrix effects. The presence of residues of leuco brilliant green in fish has also been confirmed from experimental study performed on trout treated with brilliant green, using LTQ-Orbitrap mass spectrometer.

1. Introduction

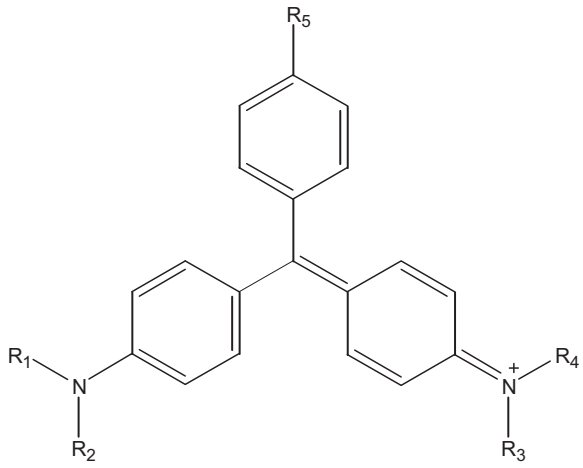
The triphenylmethane dyes, malachite green (MG) and crystal violet (CV) have been used as antimicrobial, antifungal and antiparasitic agents in aquaculture to treat and prevent fungal and protozoal infections. However, due to their possible carcinogen, mutagen and teratogen effects on animals, these compounds are not registered as veterinary drugs and are authorized neither in EU nor in many other countries all over the world. In exposed fish, MG and CV are extensively metabolized to their reduced leuco forms, leuco malachite green (LMG) and leuco crystal violet (LCV), which are also mutagenic compounds. Another triphenylmethane compound, brilliant green (BG), displays a similar chemical structure and therefore might have similar toxic effects, but no metabolism data are available in the literature for BG. The chemical structures of the compounds are shown in Fig. 1.

In the European Union, analytical methods used to determine these residues in aquaculture products have to be sensitive enough to reach the European minimum required performance limit (MRPL) of 2 $\mu g\,kg^{-1}$ for the sum of MG and LMG [1]. No MPRL was set for other dyes CV or BG. Several published methods have been developed for the analysis of MG, CV, BG and their metabolite in fish. Among the most recent of them, some use an oxidation step to transform the leuco forms in the parent forms allowing to detect the compounds in a unique parent form with liquid chromatography and detection in visible spectrum for the screening step or mass spectrometry detection for the confirmatory step [2–7] Other methods allow the determination of the compounds in their generic form, without post-column oxidation, using in that case liquid chromatography coupled to mass spectrometry for the detection [8–11].

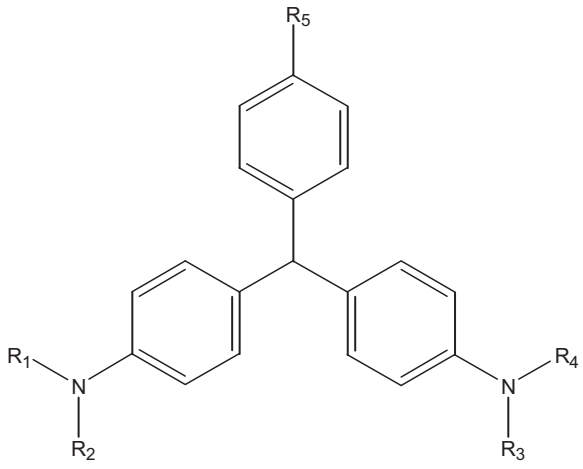
The present study displays a LC–MS/MS method developed for the simultaneous determination and quantification of residues of MG, LMG, CV, LCV and BG in aquaculture products (fish tissues and shrimps). The method allows a simple and fast sample preparation. A study on the applicability of the method was conducted on various matrices to assess the matrix effects. Additionally preparation of treated trouts (*Oncorhynchus mykiss*) with BG was carried out in order to identify the presence of LBG in fish muscle using LTQ-Orbitrap mass spectrometry.

* Corresponding author. Tel.: +33 2 99 94 78 78; fax: +33 2 99 94 78 80.
E-mail address: dominique.pessel@anses.fr (D. Hurtaud-Pessel).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.01.061



Malachite green: $R_1 = R_2 = R_3 = R_4 = CH_3$; R5 = H
Brilliant green : $R_1 = R_2 = R_3 = R_4 = CH_2CH_3$; R5 = H
Crystal violet : $R_1 = R_2 = R_3 = R_4 = CH_3$; R5 = $N(CH_3)_2$



Leuco malachite green: $R_1 = R_2 = R_3 = R_4 = CH_3$; R5 = H
Leuco brilliant green : $R_1 = R_2 = R_3 = R_4 = CH_2CH_3$; R5 = H
Leuco crystal violet : $R_1 = R_2 = R_3 = R_4 = CH_3$; R5 = $N(CH_3)_2$

Fig. 1. Chemical structures of the triphenylmethane dyes and their leuco forms.

2. Experimental

2.1. Reagents and chemicals

All reagents and solvents used were of analytical grade or HPLC grade. Acetonitrile was supplied by Fisher (Leicestershire, England). Hydroxylamine chlorhydrate and magnesium sulfate anhydrous were obtained from VWR International (Leuven, Belgium). Ammonium formate was obtained from Sigma–Aldrich (St Louis, USA). Ascorbic acid was supplied by Prolabo (Paris, France) and Formic acid (100%) was purchased from Merck (Darmstadt, Germany).

Deionised water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

Malachite green oxalate (CAS 2437-29-8), leuco malachite green (CAS 129-73-7), crystal violet (CAS 548-62-9), leuco crystal violet (CAS 603-48-5), brilliant green (CAS 633-03-4) were obtained from Sigma–Aldrich (St Louis, USA). D5-Malachite green picrate (MG-D5), D5-leuco malachite green (LMG-D5), D6-crystal violet trihydrate (CV-D6) and D6-leuco crystal violet (LCV-D6) were purchased from Witega (Berlin, Germany). Leuco brilliant green was custom made by Atlanchim Pharma (Nantes, France) according to published conditions [12]. A hydroxylamine solution at 9.5 g l^{-1} in deionised water was prepared by dissolving 5 g of hydroxylamine chlorhydrate in deionised water and diluting to 250 ml.

A formic acid solution at 5% in deionised water (v/v) was prepared by diluting 5 ml of formic acid (100%) in a 100 ml volumetric flask containing about 90 ml of deionised water and completing flask to 100 ml with deionised water. An ammonium formate buffer (0.05 M, pH 4.5) was prepared by dissolving 3.15 g of ammonium formate in a 1000 ml volumetric flask with about 900 ml of deionised water, then adding 5 ml of formic acid solution at 5% in water (v/v) and completing the flask to 1000 ml with deionised water. This solution was used as LC mobile phase.

2.2. Standard solutions

Individual stock solutions of each reference compound at 100 μg ml^{-1} were prepared in acetonitrile, taking into account of the content of the active substances. These solutions were stored at −20 °C in ambered flasks (protecting them from light). Mixed intermediate standard solution of malachite green, leuco malachite green, crystal violet, leuco crystal violet, brilliant green at 1 μg ml^{-1} and mixed intermediate solution of deuterated standards at 1 μg ml^{-1} were prepared by diluting stock standard solutions in acetonitrile, respectively. These solutions were stored at −20 °C in ambered flasks and were found stable for at least 1 month. These intermediate solutions were diluted in acetonitrile to prepare working standard mixed solutions (MG, LMG, CV, LCV, and BG) at concentration of 40, 30, 20 and 10 μg l^{-1} and a working deuterated internal standard mixed solution (MG-D5, LMG-D5, CV-D6, and LCV-D6) at 40 μg l^{-1}. These solutions are prepared fresh daily.

2.3. Sample preparation

To avoid any loss due to light exposure, solutions and extracts are all protected from the light during the sample preparation procedure.

Muscle tissues, taken from fish, were homogenized in a domestic food blender and were kept frozen at −20 °C until analysis. The sample (2.00 ± 0.02 g) was fortified at 2 μg kg^{-1} with internal standards by adding 100 μl of deuterated internal standard mix solution (40 μg l^{-1}). Then 500 μl of hydroxylamine solution (9.5 g l^{-1}) was added and the sample was mixed and allowed to stand for 10 min in the dark before extraction. Then 8 ml of acetonitrile and 1 g (±0.1 g) of anhydrous magnesium sulfate were added. The tube was vortex-mixed vigorously for 1 min at maximum speed and was shaken for 10 min with a rotative stirrer at 100 rpm. After agitation, the tube was centrifuged at 2000 × *g* for 5 min at 4 °C. All of the supernatant was taken up by pipetting, transferred into a new clean tube and was evaporated to dryness at 50 °C under a gentle stream of nitrogen. The remaining residue at the bottom of the tube was reconstituted by dissolving in 800 μl of solution of acetonitrile/1 g l^{-1} ascorbic acid (100/1;v/v). The mixture was then transferred into an Eppendorf tube and centrifuged at 20,000 × *g* for 5 min. The extract was filtered through a 0.45 μm PVDF filter into HPLC vial prior to LC–MS/MS analysis.

2.4. Matrix calibration

The calibration standards for calibration curves were prepared using matrix-extracted (fortified prior to extraction) for each run of analysis and were used for quantification. Tissue samples were fortified with MG, LMG, CV, LCV, and BG at levels corresponding to 0.0, 0.5, 1.0, 1.5 and 2.0 μg kg^{-1} by adding either 0 or 100 μl of working standard mix solutions prepared at 10, 20, 30 and 40 μg l^{-1}, respectively. 100 μl of deuterated internal standard mix solution