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Determination of malachite green residues in farmed rainbow trout in Iran

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ABSTRACT

This study was carried out to determine the residues of malachite green (MG) in rainbow trout muscle samples ($n = 144$) obtained from trout farms in Iran. A high-performance liquid chromatography method with visible detection at 618 nm has been developed to determine the sum of MG and leucomalachite green (LMG) in the muscle samples. This method is based on in situ oxidation of colorless LMG to chromic MG with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone. The method was validated according to the criteria described in Commission Decision 2002/657/EC. The recoveries for MG and LMG in spiked muscle samples were higher than 70%; and the relative standard deviations were lower than 9.9% for repeatability and 11.5% for within-laboratory reproducibility. The values of decision limit (CCα) and detection capability (CCβ) for MG were 0.16 μg/kg and 0.39 μg/kg, respectively, which are below the minimum required performance limit (MRPL) of 2 μg/kg for sum of MG and LMG. The residues of MG were detected in 48.6% of the rainbow trout muscle samples, ranging between 0.30 and 146.1 μg/kg. Most of the contaminated samples contained MG at the level of 2–10 μg/kg. The results indicated that application of MG in such a level in trout farms could be a potential hazard for public health. Further investigations should be carried out to determine the residues of MG in other farmed fish species in Iran.

1. Introduction

Malachite green (MG) is a cationic triphenylmethane dye originally used as a coloring agent in the textile industry. In spite of its prohibition, MG is still used illegally in aquaculture industry to control ecto-parasites and fungal infections on fish eggs, fingerlings, and adult fish. It is due to low cost, high efficacy, and lack of alternatives (Alderman, 1985; Hashimoto, Paschoal, Queiroz, & Reyes, 2011).

The MG residues in fish originate from its illicit use in fish farms or from environmental pollution as a result of its industrial applications and discharge of the waste water to the rivers and streams without any pre-treatment (Khodabakhshi & Amin, 2012; Pourreza & Elhami, 2010; Schuetze, Heberer, & Juergensen, 2008). The dyestuff is readily absorbed and distributed in different tissues of the exposed fish. It is extensively metabolized and transformed to a reduced colorless compound, leucomalachite green (LMG). It was determined that up to 90% of MG absorbed by the muscle tissue is accumulated as LMG. Due to its lipophilic nature, LMG has a long persistent residence time in fish tissues (Bauer, Dangschat, & Knöppler, 1988; Jiang, Xie, & Liang, 2009; Srivastava, Sinha, & Roy, 2004). Mitrowska, Posyniak, and Zmudzki (2007) demonstrated that LMG is relatively stable during fish cooking even at high temperatures.

MG has never been approved worldwide as a veterinary medicine in animals destined for human consumption due to the potential mutagenic, carcinogenic, and teratogenic properties of the compound and its metabolite, LMG (Sudova, Machova, Svobodova, & Vesely, 2007). Therefore, presence of MG or LMG in foodstuffs, even at low levels, is considered an adulteration and lead to rejection of the product. For this purpose, appropriate analytical methods are needed to detect low levels of these compounds in various foodstuffs. According to the European Commission (2004), the analytical methods should meet a minimum required performance limit (MRPL) of 2 μg/kg for determining the sum of MG and LMG in seafood products.

Conventional analytical methods used for determination of MG in seafood products are liquid chromatography coupled to mass spectrometry or tandem mass spectrometry (Nebot et al., 2013; Tao et al., 2011; Valle, Díaz, Zanocco, & Richter, 2005), enzyme-linked

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immunosorbent assay (Bilandžić, Varenina, Kolanović, Oraić, & Zrnčić, 2012; Xing et al., 2009), and high-performance liquid chromatography (HPLC) coupled to visible range absorption or fluorescence detectors (Bajc, Doganoc, & Gačnik, 2007; Mitrowska, Posyniak, & Zmudzki, 2005). The sum of MG and its biotransformed colorless compound, LMG, can be determined by HPLC with a visible range absorption detector, after in situ oxidation of LMG to MG (Andersen, Turnipseed, & Roybal, 2006). The determination of both compounds together is a good screening method for this kind of residue, since the combined signals can lead to increase in sensitivity and improvement of analyte detectability (Valle et al., 2005). Moreover, the established MRPL pertains to sum of MG and LMG present in flesh of aquaculture products; hence it is not necessary to determine each analyte, individually.

Owing to its rapid growth rate and high nutritional value, rainbow trout (*Oncorhynchus mykiss*) is widely used as a farmed fish in many countries around the world. It is also the main freshwater fish species farmed in Iran, with a production of 95,230 metric ton in 2011 (Annual Fishery Statistics, 2012, pp. 35–40). Referring to the existing scientific literature, no comprehensive study was performed to determine the presence and levels of MG residues in fish and seafood products in Iran. Therefore, this study aimed to determine the MG concentrations in fillets of rainbow trout collected from Iranian trout farms during year 2011.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical grade unless otherwise stated. Methanol (HPLC grade), dichloromethane, acetonitrile (HPLC grade), glacial acetic acid, sodium acetate, L(+)-ascorbic acid (99% purity), and perchloric acid 70% were purchased from Merck (Darmstadt, Germany). MG oxalate and LMG reference standards, and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 98% purity) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Deionized water (Milli-Q Millipore 18.2 MΩ/cm resistivity) was applied throughout this study. Sep-Pak® C18 Vac cartridge (500 mg sorbent, 6 ml) was purchased from Waters Corporation (Dublin, Ireland).

The solutions such as acidic acetonitrile, DDQ (0.001 M), acetate buffer (0.05 M, pH 4.5), and ascorbic acid (1 mg/ml) were prepared as described in previous studies (Andersen et al., 2006; Mitrowska et al., 2007; Van de Riet, Murphy, Pearce, Potter, & Burns, 2005). Individual stock solutions of MG and LMG (1 mg/ml) were prepared by dissolving the solid standard in acetonitrile and stored at −20 °C. These solutions were diluted in acetonitrile to prepare the intermediate standard solutions of 1 μg/ml. Working standard solutions of MG (for calibration and recovery experiments) and LMG (only for recovery experiments) were individually prepared by diluting of intermediate standard solutions in a mixture containing acetonitrile, acetate buffer (0.05 M, pH 4.5), and ascorbic acid (1 mg/ml) (47.5:47.5:5, *v/v/v*). A nine-point (zero included) MG standard calibration curve was prepared at 2, 5, 10, 20, 40, 60, 80, and 100 ng/ml.

2.2. Sample collection and preparation

During year 2011, a total of 144 samples of market-size rainbow trout (250–350 g) were obtained from trout farms located in central, northern, and north-west parts of Iran. The mentioned areas have different environmental and climatic conditions; and produce more than 90% of rainbow trout in Iran market. The samples were transported to the laboratory inside an insulated ice chest. Upon arrival, the fish were eviscerated, beheaded, deboned, skinned, and filleted. The fillets of 3 individual fish from each trout farm were pooled, homogenized, and stored at −20 °C prior to analyses.

2.3. Sample extraction and clean-up

The muscle samples of rainbow trout were extracted and clean-up according to the method of Van de Riet et al. (2005) with a minor modification. The modification was using the DDQ solution to oxidize colorless LMG to its chromic analog, MG. A total of 4.0 g of the homogenized fish sample was accurately weighed and uniformly mixed with 16 ml of acidic acetonitrile in a 50 ml Falcon tube. The homogenate was diluted to 25 ml mark with dichloromethane, shaken for 10 min, and then centrifuged at 1500 rpm for 10 min. Subsequently, the supernatant (10 ml) was transferred to a tube, 3 ml of DDQ solution (0.001 M) was added, and the oxidation reaction was allowed to proceed for 30 min with periodic sample agitation. The oxidized sample was evaporated to ~3 ml under a stream of nitrogen at 40 °C and passed through the solid-phase extraction (SPE) column, which was previously conditioned with 2.5 ml of acetonitrile. The first eluate was collected. The SPE column was then rinsed with 2 ml of acetonitrile. This second eluate was collected and combined with the first. Afterwards, the mixed eluate was evaporated to dryness under a stream of nitrogen at 40 °C. The remaining residue was dissolved in 1 ml of a mixture containing acetonitrile, acetate buffer (0.05 M, pH 4.5), and ascorbic acid (1 mg/ml) (47.5:47.5:5, *v/v/v*). The sample was filtered through a 0.45 μm syringe filter before analysis.

2.4. High-performance liquid chromatography analysis

An Agilent 1260 Infinity HPLC system (Agilent Corporation, USA) equipped with a vacuum degasser, an auto-sampler, a quaternary pump, an analytical guard column (Eclipse XDB-C18, 5 μm particle size, 12.5 mm × 4.6 mm i.d.; Agilent Corporation, USA), and a G1315D diode-array detector (DAD) was used for chromatographic analysis. The DAD was set at an absorbance wavelength of 618 nm for MG detection. The instrument control and data processing was conducted by using ChemStation software. Chromatographic separation was performed on a ZORBAX Eclipse XDB-C18 analytical column (5 μm particle size, 250 mm × 4.6 mm i.d.; Agilent Corporation, USA). The HPLC mobile phase was a mixture of acetate buffer (0.05 M, pH 4.5) and acetonitrile (50:50, *v/v*), and pumped at a flow rate of 1.0 ml/min. The injection volume of the sample or standard solutions was 100 μl.

2.5. Method validation study

Validation of the method for determination of total MG in the rainbow trout muscle was based on the conventional validation approach described in Commission Decision 2002/657/EC (European Commission, 2002).

The linearity of the HPLC-DAD response was studied using nine-point MG standard calibration curve in the concentration range of 0–100 ng/ml. The calibration curve was constructed by plotting the peak areas versus the corresponding concentrations of the standard solutions. The linearity of the calibration curve was evaluated by the correlation coefficient.

To evaluate the specificity of the method, blank samples of rainbow trout muscle ($n = 20$) obtained from different specimens were analyzed. Moreover, the blank samples were spiked with known amounts of crystal violet, leucocrystal violet, sulfamethazine, sulfathiazole, sulfadiazine, and oxytetracycline to evaluate possible interference that may occur in the method. The stability of MG and LMG in rainbow trout muscle was investigated by spiking known amounts (10 μg/kg of MG and 8 μg/kg of LMG) of the analytes to the blank samples. The samples were analyzed when they were fresh and after 1, 2, 4, and 8 weeks of frozen storage at −20 °C.