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Determination of malachite green and leucomalachite green in edible goldfish muscle by liquid chromatography–ion trap mass spectrometry

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Abstract

A liquid chromatography–ion trap mass spectrometry method with three "time segments" has been developed to determine malachite green (MG) and its major metabolite, leucomalachite green (LMG) in edible goldfish muscle. By using the optimized "time segments", MG and LMG as well as the internal standard atrazine-d_5 were analyzed with good sensitivity with positive ESI–MS in a single run. The homogenized fish muscle tissues were extracted with a solution of perchloric acid and acetonitrile, followed by partitioning with dichloromethane. Strata-x polymeric solid-phase extraction column was used for the clean-up process. The determination of MG and LMG was achieved by using a reversed-phase HPLC gradient program coupled with MS/MS in multiple-reaction-monitoring mode. Matrix calibration curves were linear over the ranges of 5–500 ng/ml for MG and 1–100 ng/ml for LMG. Recoveries of the fish tissue extraction at three spiked levels (2, 10 and 30 ng/g for MG as well as 0.4, 2 and 6 ng/g for LMG) were better than 71% and 89%, respectively. Relative standard derivations from six determinations were less than 8%. The method detection limits were 0.13 ng/g for MG and 0.06 ng/g for LMG.

Keywords: LC–ion trap MS; Time segments; Malachite green; Leucomalachite green; Edible goldfish

1. Introduction

Malachite green (MG) is a cationic triphenylmethane dye (Fig. 1a) that has been widely used in aquaculture industry as an anti-fungal, anti-microbial and anti-parasitic agent since 1930s [1–4]. MG is readily absorbed into tissues during waterborne exposure and metabolized rapidly to the reduced and colorless form, leucomalachite green (LMG) (Fig. 1b) in fish [1–4]. LMG in tissues may be eliminated at a rate that is strongly dependent on the fat content [2–4]. The members of the triphenylmethane dyes have shown to cause the human or animal cancers [4–5]. For example, rosaniline has proven to be bladder carcinogens in human while leuco form of rosaniline has found to induce hepatic, renal and lung tumors in mice [6]. With the similar structure as rosaniline, MG has shown toxic to microbial and mammalian cells, promotion of the hepatic tumor formation in rodents and reproductive abnormalities in rabbits and fishes [7].

Because of its potential mutagenity, teratogenicity, genotoxicity and carcinogenicity, MG is not approved as a veterinary drug for fishes by the European Union and the U.S. Food and Drug Administration [7]. However, MG is often illegally used in the fish farming industry due to the low cost, easy availability, high efficacy against fungus, bacteria and parasite [7]. The use of MG has caused environmental contamination and affected human health. Thus, development of specific, sensitive and reliable method is necessary for the determinations of MG and LMG in water and biota tissues [8]. According to the requirement of the European Commission, methods for determining MG in fish tissues should meet the minimum required performance limit of 2 ng/g for the sum of MG and LMG [9].

It was found that up to 90% of the total malachite green in fish existed in the leuco form [10]. Several analytical methods for the determination of MG and LMG in various matrices such as fish tissue [6,11–13] and water [14] have been reported. High-performance liquid chromatography (HPLC) with UV–vis by post-column oxidation of LMG to MG using lead (IV) oxide [11] or with electrochemical detection [12] has been commonly used for the determination of MG and LMG. Some new analyt-

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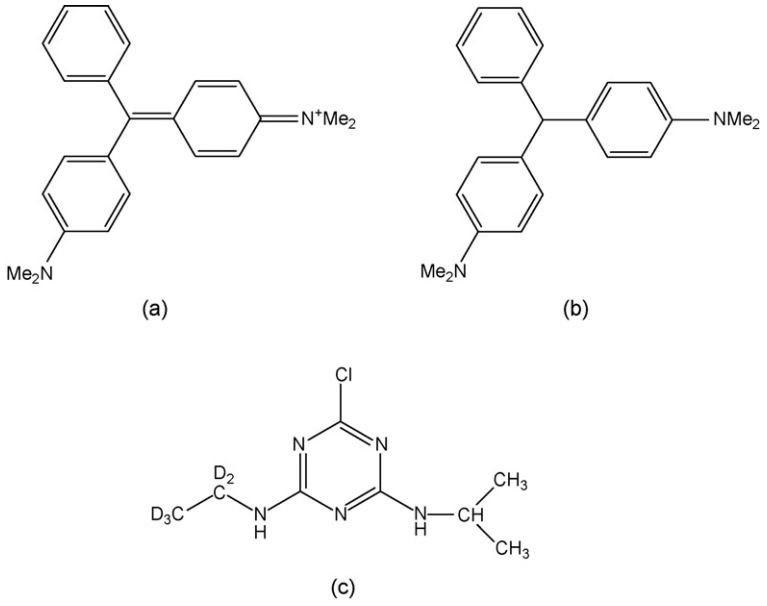

Fig. 1. Chemical structures of malachite green (a), leucomalachite green (b) and the internal standard atrazine-d_5 (c).

ical procedures apply the hyphenated techniques of chromatography with mass spectrometry, for example, GC–MS [13] or LC–APCI–MS [6], for the confirmation of MG and LMG. LC coupled with ion trap MS has become a useful technique for screening and confirming multi-drug residues with high specificity [15]. However, slow scan-speed for several quantitative ions in multiple-reaction monitoring (MRM) mode of the ion trap MS method often affects the sensitivity and repeatability. Although the post-column oxidation method may reduce the number of quantitative ions [9,16–17], levels of LMG could not be directly quantified. This paper describes LC–ion trap MS method development with the use of the time-segments for the simultaneous determination of MG and LMG in edible goldfish muscle tissue without the need of post-column oxidation.

2. Experimental

2.1. Reagents and chemicals

All organic solvents were of HPLC-grade and other chemicals used were of analytical grade all unless stated otherwise. Malachite green oxalate and leucomalachite green were obtained from Sigma–Aldrich (St. Louis, MO, USA) and ascorbic acid was obtained from Merck (Darmstadt, Germany). Atrazine-d_5 (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine-d_5) (Fig. 1c) was obtained from Crescent Chemical Co. Inc. (Augsburg, Germany). Acetonitrile and dichloromethane were purchased from Tedia (Fairfield, USA). Ammonium acetate was obtained from Panreac (Barcelona, Spain) and formic acid was from Acros organics (New Jersey, USA). 60% ACS reagent grade perchloric acid was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Milli-Q water was produced by using a Milli-Q Ultrapure Water Purification Academic System with a water outlet operating at 18.2 Ω from Millipore (Billerica, USA). 5 mM ammonium acetate buffer containing 0.1% formic acid was prepared by filtering through 0.2 μm cellulose acetate filter paper (Alltech, Deerfield, USA). The buffer and organic solution were degassed ultrasonically by a Branson 2510 series Ultrasonic degasser (Danbury, U.S.A.) for 10 min prior to the LC–MS analysis.

MG (500 μg/ml) and LMG (100 μg/ml) stock mixture solutions were each prepared by dissolving 5 mg MG and 1 mg LMG in 10 ml of acetonitrile. The intermediate standard solution of MG (5 μg/ml) and LMG (1 μg/ml) was prepared by diluting the stock solution with acetonitrile. The stock and intermediate solutions were stored in dark at 4 °C for less than 2 months. Working standard solutions for calibration from 5–500 ng/ml for MG and from 1–100 ng/ml for LMG were prepared by serially diluting the intermediate standard solution with a mixture of 5 mM ammonium acetate buffer and acetonitrile (4:6, 0.1% formic acid).

2.2. Fish tissue samples

2.2.1. Preparation

Edible goldfishes (*Carassius auratus*) were purchased from a local market. Individual fish fillets were cut into small pieces and blended in an Extra Fine Blade Blender (Hitachi, Tokyo, Japan) for homogenization. The tissue samples were stored in separate zip-lock plastic bags at −20 °C until the sample preparation and analysis.

2.2.2. Extraction

The fish samples were extracted as described in reference [1] with minor modifications. Five grams of blended tissue were accurately weighed into individual 50-ml Teflon centrifuge tubes. Sixteen milliliters of the acetonitrile containing 250 mg ascorbic acid and 0.8% perchloric acid were added to the sample. Individual sample was each spiked with 10 ng MG and 2 ng LMG, 50 ng MG and 10 ng LMG or 150 ng MG

and 30 ng LMG, for the recovery tests. The sample mixtures were homogenized using a polytron homogenizer (Kinematiza, Kriens-Luceme, Switzerland) for 1 min at 10,000 rpm. Nine milliliters of dichloromethane were then added to the each sample, followed by shaking vigorously in the orbital mixer (Denley) at 350 rpm for 10 min. The sample mixture was centrifuged by Avanti J-25I centrifuge (Beckman, CA, USA) at 4000 rpm for 10 min. Fifteen milliliters of supernatant were collected and preconcentrated to approximately 1 ml by using a TurboVap LV Evaporator (Zymark, Hopkinton, U.S.A.) under a slow stream of dry nitrogen at 40 °C. 80 pg of the internal standard atrazine-d_5 were then added into the sample extract.

2.2.3. Clean-up process

Strata-x 33 μm polymeric column (200 mg/6 ml, phenomenex, CA, USA) was used for the clean-up of the biota sample extract. Solvent was kept at 1–2 ml/min by using a vacuum manifold (Alltech, Deerfield, USA) with water vacuum pump. The SPE column was conditioned with 3 ml of the mixture containing acetonitrile and dichloromethane (6:4), followed by 3 ml of acetonitrile. The sample extract was then added to the cartridge. MG and LMG were eluted with 4 ml of a mixture containing acetonitrile and dichloromethane (6:4). The extract was evaporated to dryness by using the TurboVap LV Evaporator under a slow stream of dry nitrogen at 40 °C and reconstituted with 400 μl of the mixture of 5 mM ammonium acetate buffer and acetonitrile (4:6, 0.1% formic acid). The sample was then centrifuged by a cyclone centrifuge with freezer (Eppendorf, Hamburg, Germany) at 13,000 rpm and 15 °C for 10 min. The supernatant was collected for LC–MS analysis.

2.3. Liquid chromatography

HPLC analysis was performed on Agilent HP1100 series HPLC system equipped with degasser, binary pump, diode array detector and auto-sampler (San Francisco, U.S.A.). A beta-basic C18 column (50 mm × 2 mm, 5 μm, Keystone Scientific Inc., Bellefonte, USA) was used for the separation of MG, LMG and atrazine-d_5. A gradient program was used at a flow rate of 200 μl/min, in which mobile phase A consisted of 5 mM ammonium acetate containing 0.1% formic acid and mobile phase B consisted of acetonitrile containing 0.1% formic acid. The gradient program was applied as follows: 80%A/20%B for 2 min; increased to 20%A/80%B from 2 to 6 min; kept at 20%A/80%B for 9 min. Twenty microliters of each calibration standard and sample extracts were injected onto the HPLC column. Under these conditions, atrazine-d_5, MG and LMG were eluted at 8.8, 9.9 and 11.6 min, respectively.

2.3.1. Mass spectrometry

ESI–MS analysis was performed on an Esquire 4000 ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany) with an electrospray ionization (ESI) interface. The instrument was operated at positive ion mode at an ionization voltage of +4500 V and source temperature of 365 °C. Nitrogen was used as nebulizer gas at 40 psi and drying gas at a flow rate of 9 l/min. The MS analysis was performed with the multiple-reaction-monitoring (MRM) mode. Collision energy was optimized for each compound. Three time segments were used in mass spectrometric acquisition in order to optimize the instrumental parameters for the $[M+H]^+$ ions at *m/z* 329.2 for MG, *m/z* 331.2 for LMG and *m/z* 221.2 for atrazine-d_5. After the MS/MS analysis, fragment ions at *m/z* 208.0 and *m/z* 313.2 for MG (CE 0.55 eV), *m/z* 239.2 and *m/z* 316.2 for LMG (CE 0.60 eV) as well as *m/z* 179.0 for atrazine-d_5 (CE 0.55 eV) were selected as quantitative ions. Signals of the extracted ions of *m/z* 208.0 and *m/z* 313.2 for MG as well as *m/z* 239.2 and *m/z* 316.2 for LMG were combined for the peak integration in order to increase the peak intensity.

3. Results and discussion

The reversed-phase HPLC conditions were optimized in order to separate the analytes from the significant matrix background, to ensure the baseline chromatographic separation of MG, LMG and the internal standard, and to achieve the best peak shape and ESI–MS sensitivity for the analysis. The selection of the LC column, the composition of mobile phases and flow-rate were found to be crucial for the HPLC separation and method detection limits. For the mobile phase conditions, 5 mM ammonium acetate containing 0.1% formic acid and acetonitrile containing 0.1% formic acid were found to well suit for the LC–MS analysis. The use of the buffer system in the mobile phase ensured the stable chromatographic retention times of MG, LMG and the internal standard.

Simultaneous detection of MG, LMG and atrazine-d_5 with the MRM mode was challenging in ion trap mass spectrometry especially when trace analysis is required. When scanning three sets of MRM ions simultaneously, the scan speed of MRM mode in ion trap mass spectrometry was slow so that the sensitivity and repeatability were poor. An alternative approach to reduce the number of MRM ions is to oxidize LMG to MG by using a post-column oxidation reactor so that the MS/MS analysis could be focused on MG [9,16–17]. However, the extent of the LMG oxidation was not consistent, depending on the activity of the oxidant and the different types of samples [10]. In addition, lead (IV) oxide in the post-column could oxidize MG into a derivative that was difficult to be quantitated [10]. By using a program of "time segments", post-column oxidation method was not needed. Ion trap MS with "time segments" had faster scan speed because each quantitative ion in the MRM mode was optimized and detected in its corresponding segment. Along with the LC separation, the method provided good sensitivity and repeatability for the simultaneous determination of MG and LMG. Thus, the "time segments" program was optimized and applied for the MS/MS analyses of the three $[M+H]^+$ ions with different mass spectrometric parameters.

After the baseline separation of MG, LMG and atrazine-d_5 by the optimized LC conditions, it is feasible to develop "time segments" program in order to scan each of the $[M+H]^+$ ions of the analytes and the internal standard in a single LC–MS run. In addition to the waste-switched period of the first 8 min for eliminating the contamination of the ESI ion source, the MS data acquisition was divided into three segments. The segments were subsequently operated for atrazine-d_5 (8–9.6 min),

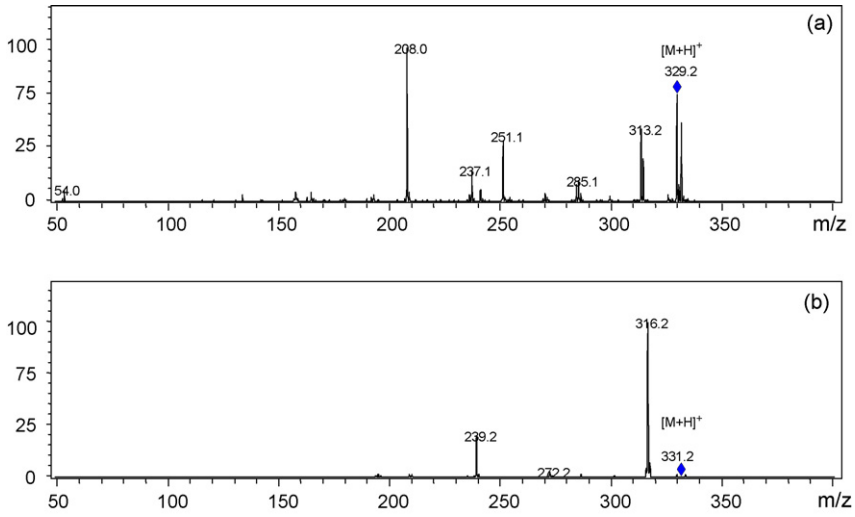

Fig. 2. Positive ion MS/MS spectra obtained from the LC–MS/MS analysis of 1995 pg MG (a) and 405 pg LMG (b).

MG (9.6–10.8 min) and LMG (10.8–15 min). The MS operation conditions for each segment were optimized accordingly. Different mass spectrometric parameters were employed on each time segment to achieve the best sensitivity for the analytes. In particular, the second segment started at 9.6 min when the MS/MS analysis was switched for the determination of MG, and the third segment started at 10.8 min for LMG that was detected at 11.6 min. Fig. 2 shows the MS/MS spectra of 1995 pg MG and 405 pg LMG standards acquired under the optimized conditions. Fragment ions *m/z* 208.0 and *m/z* 313.2 for MG as well as *m/z* 239.2 and *m/z* 316.2 for LMG were selected as quantitative ions. Signals of the extracted ions of *m/z* 208.0 and *m/z* 313.2 for MG as well as *m/z* 239.2 and *m/z* 316.2 for LMG were combined for the integration in order to increase the peak intensity.

By using the "time segments" program, atrazine-d_5, MG and LMG were analyzed simultaneously with good sensitivity. Fig. 3 shows the extracted ion chromatogram from the LC–MS/MS analysis of the spiked edible goldfish containing 4.05 pg atrazine-d_5, 1995 pg MG and 405 pg LMG in a single run. Because the "time segments" program did not involve the change in the ionization mode of ESI, the switching of segments or MS/MS parameters was smooth. Another critical condition for applying the "time segments" program is that the chromatographic retention time of the analytes and the internal standards should be stable. Under the current HPLC conditions, it was found that the retention times only varied within ±0.1 min during the entire method development period. The little variation in the retention time ensured the successful application of the "time segments" program.

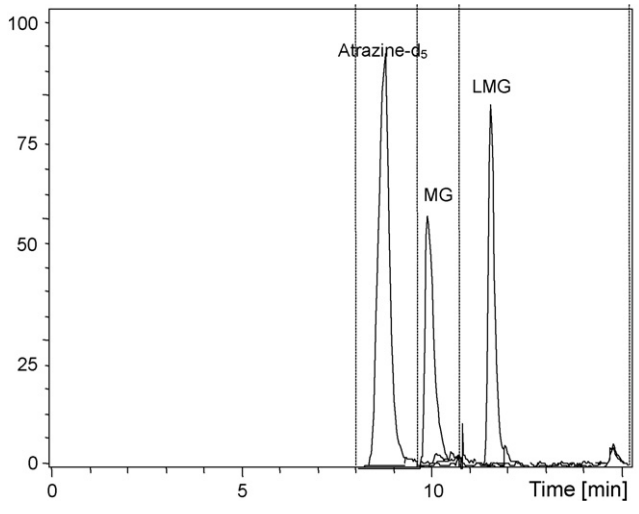

Fig. 3. Extracted MRM ion chromatogram obtained from the LC–ion trap MS/MS analysis of the spiked edible goldfish muscle containing 4.05 pg atrazine-d_5, 1995 pg MG and 405 pg LMG by using the optimized "time segments" program.

Internal standard method was applied for the determination of MG and LMG in order to achieve good analytical method performance. Atrazine-d_5 was selected as the internal standard because of its similar structure to the analytes. Isotopically labeled atrazine was preferred over native atrazine because the native compound is a common herbicide and it might exist in the aquatic environment. The MS/MS fragment ion of the atrazine-d_5 at *m/z* 179.0 was selected as the quantitative ion. The internal standard method provided better accuracy and precision for the trace analysis of MG and LMG in the fish tissues.

The developed LC–ion trap MS was applied for the analysis of the matrix calibration standards. Calibration curves of $y = 0.0035x + 0.0113$ for MG and $y = 0.021x + 0.0023$ for LMG were obtained. By using the least-square regression method, the concentration of the matrix-spiked calibration solution was regressed on peaks of the MG or LMG signals divided by that of the atrazine-d_5 at 4 pg level in the range of 5–500 ng/ml for MG or 1–100 ng/ml for LMG. The concentrations of MG or LMG were calculated by interpolation of the matrix calibration curve after obtaining the peak area ratio of MG or LMG versus that of atrazine. The curves of regressed concentration points over the calibration ranges showed squared correlation coefficients of better than 0.999 for MG and 0.997 for LMG. The limit of detection (LOD) was calculated with S/N ratio better than 3. The LOD for the LC–ESI–MS method was 0.13 ng/g for MG and 0.06 ng/g for LMG.

Table 1
Recovery and precision for MG and LMG determinations in the edible goldfish muscle tissues at three spiked levels

Analyte	Spiked level (ng/g)	n	Found (ng/g)[a]	Recovery (%)	R.S.D. (%)	
					Intra-day	Inter-day
MG	2	6	1.4 ± 0.7	71.7	6.2	10.2
	10	6	11.0 ± 5.6	109.5	6.0	6.1
	30	6	34.0 ± 13.7	113.3	5.4	6.9
LMG	0.4	6	0.4 ± 0.2	89.5	7.7	10.8
	2	6	1.9 ± 0.9	93.4	6.2	7.4
	6	6	6.3 ± 3.0	105.0	6.4	9.8

[a] Mean ± S.D.

The edible goldfish tissue samples were extracted with the solution of 0.8% perchloric acid in acetonitrile (v/v) and 250 mg ascorbic acid was added to the sample to prevent the photo-oxidation demethylation of the LMG during the extraction processes. The analytes were eluted from the strata-x 33 μm polymeric SPE column with a mixture of acetonitrile and dichoromethane (6:4). Under the developed conditions, the SPE clean-up appeared to efficiently remove non-polar interfering compounds that may cause ion suppression and affected LC–MS analytical performance. Results obtained for matrix calibration curves of both MG and LMG showed that the clean-up process could successfully remove the interferences that affected the quantitative analysis. The extracts of edible goldfish tissue and recovery samples were analyzed by the developed LC–ion trap MS method. MG and LMG were not detected in the goldfish tissue samples from the repeated analyses. Therefore, the fish tissue was used for making the spiked standard samples for evaluating the method performance. Five grams of the blended tissue were accurately weighed and spiked with 10 ng MG and 2 ng LMG, 50 ng MG and 10 ng LMG as well as 150 ng MG and 30 ng LMG, respectively. Then, the spiked samples were each prepared by following the sample preparation procedures described in Section 2. The experiment was repeated six times to get the average recovery and precision (R.S.D.) data for each spiked level of MG and LMG. The average recovery (accuracy) at 2, 10 and 30 ng/g of MG as well as 0.4, 2 and 6 ng/g of LMG ranged 71.7–113.3% (R.S.D. 5.4–6.2%) for MG and 89.5–105.0% (R.S.D. 6.2–7.7%) for LMG. For the determination of inter-day stabilities, extracts of spiked samples were stored at −20 °C at dark prior to analysis. The sample extracts were analyzed continuously over 1 week. The obtained inter-day precisions (R.S.D.) ranged from 6.1–10.2% for MG and 7.4–10.8% for LMG. The precision data indicated good repeatability of the analytical method over 1 week (Table 1).

4. Conclusions

A specific and sensitive method by LC–ion trap MS method with three "time segments" in positive ion mode has been developed to determine malachite green (MG) and leucomalachite green (LMG) in edible goldfish muscles with good precision and accuracy. MG and LMG concentrations in the fish tissue samples can be successfully determined by using atrazine-d_5 as internal standard. The strata-x polymeric SPE provided recoveries of better than 71% and 89% for MG and LMG, respectively, in the concentrations of 2, 10, 30 ng/g for MG as well as 0.4, 2 and 6 ng/g for LMG. The method meets the European Commission minimum required performance limits of 2 ng/g for the sum of MG and LMG.

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