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A simplified and small-scale sample preparation technique for determining malachite green and leuco-malachite green in cultured eel

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ABSTRACT

Aninexpensive, simple, and small scale technique of sample preparation followed by high-performance liquid chromatography (HPLC) coupled photo-diode array (PDA) detector for simultaneous quantification of malachite green (MG) and its metabolite, leuco-malachite green (LMG), in cultured eel is described. The HPLC-PDA was performed on a C4column with anisocratic mobile phase. Analytes were extracted from the sample using a handheld ultrasonic homogenizer, and purified by MonoSpin®C18, a centrifugal monolithic SPE spin mini-columns.The proposed method was validated by the analyses of spiked eel samples, resulting recoveries > 95 % with relative standard deviations<2% and total analytical time <15 min/sample (24 samples <4 hours).

KEYWORDS

Malachite green;
Leuco-malachite green;
An international harmonized analytical method;
Centrifugal monolithic SPE spin column.

INTRODUCTION

The present expansion and diversification in the internal seafood trade is nothing short of eye-opening. By way of illustration, approximately 80% of the seafood consumed in the United States is imported from approximately 62 countries[1]. To meet this situation, the development of international harmonized methods to determine harmful substanceresidues in foods is essential in order to guarantee equitable international trade in these foods and ensure food safety for consumers. Whether in industrial nations or developing countries, an international harmonized method for residue monitoring in foods is urgently-needed. The optimal harmonized method for chemical residue monitoring in foods must be easy-to use, small scale, very economical in time and cost, and must cause negligible harm to the environment and analysts.

According to news in 2005, malachite green (MG) was detected in 18 out of 27 live eel or eel products imported from China to Hong Kong local market and food outlets, resulting in a government recall of all remaining products to be destroyed[2]. On August 4, in the same year, Japanese Ministry of Health, Labour and Welfare announced two violation cases: MG has been found in eel products imported from China[3]. MG is a basic organic pigment of the bluish green and is used in the treatment for infectious diseases of ornamental fishes, such as Saprolegniasis caused by a fungus belonging to the genus Saprolegnia and ichthyophthirius disease[4,5]. However, MG has never been registered as a veterinary drug for use to edible cultured eel in many countries because of its potential carcinogenicity, mutagenicity and teratogenicity in mammals[6]. Nevertheless, numerous residues of MG and its metabolite, leuco-malachite green (LMG) in eel prod-

ucts have been reported internationally[7,8], and theyare the most frequentlyprohibited drugs found in eel products[9,10]. Strict monitoring for the residues of MG and LMG is, therefore, important means of guaranteeing food safety of the food supply and manage global health risks.

Although several techniques based on high-performance liquid chromatography (HPLC) for monitoring MG and/or LMG in cultured fish samples including eel products have been reported[11-18], these methods have crucial drawbacks:

Firstly, they involve skilled analytical techniques or several analytical steps in the sample preparation, which are time-/cost-consuming and data-reproducibility lowering, and do not permit the determination of large number of samples.

Secondly, all of the methods consume large quantities of organic solvents in the HPLC mobile phases as well as for extraction and de-proteinization in sample preparation. Risks associated with these solvents extend beyond direct implications for the health of humans and wildlife to affect our environment and the ecosystem in which we all reside. Additionally, incineration for disposal of waste organic solvents has steadily increasing over the past ten-odd years and has spent huge amounts of money. Reducing the use of organic solvents is an important goal in terms of environmental conservation, human health and the economy[19,20].

Thirdly, most of the recent methods are based on LC-MS/MS. The facility is available are limited to part of industrial nations because these are hugely expensive, and the methodologies use complex and specific: technicians require for the system maintenance and results interpretation. These are unavailable in a lot of laboratories for routine analysis, particularly in developing countries.

The present study was developed in such a way that, in idiotproof, low-cost, and small-scale with minimized organic solvent consumption, MG and LMG residues in cultured eel can be determined with higher accuracy and precision.

EXPERIMENTAL

Reagents and apparatus

All chemicals including MG and LMG standards were purchased from Wako Pure Chem. Ltd. (Osaka, Japan). Octane sulfonic acid (OSA, sodium 1-octanesulfonate) was of an ion-paring reagent for HPLC. Acetonitrile and distilled water were of HPLC grade. Octane sulfonic acid (OSA, sodium 1-octanesulfonate) was of an ion-paring reagent for HPLC.

The following apparatuses were used in the sample preparation: handheld ultrasonic-homogenizer (model HOM-100, 2 mm ID probe, Iwaki Glass Co., Ltd., Funabashi, Japan); micro-centrifuge (Biofuge® fresco, Kendo Lab. Products, Hanau, Germany); two type of MonoSpin®as centrifugal monolithic SPE spin mini-column (sample throughput volume ≤ 300 µL), MonoSpinC18 (octadecyl and non-polar functional group) and -SCX (bonded propyl benzene sulfone acid combing both strong cation and non-polarity) (GL Sciences, Inc., Tokyo, Japan). AnInertsil®W300 C4 (5 µmd_p, 150×4.6 mm) (Pore diameter, 30 nm; Pore volume, 1.05 mL/g; Surface area, 150 m^2/g; Carbon load, 3 %) column for HPLC was used (GL Science).

The HPLC system, used for method development, included a model PU-980 pump and DG-980-50-degasser (Jasco Corp., Tokyo, Japan) equipped with a model CO-810 column oven (Thosoh Corp., Tokyo, Japan), as well as a model SPD-M10A $_{VP}$photodiode-array (PDA) detector (Shimadzu Scientific Instruments, Kyoto, Japan).

Preparation of stock standards and working mixed solutions

Stock standard solutions of MG and LMG were prepared by dissolving each compound in acetonitrile followed by water to a concentration of 400 ng/mL. Working mixed standard solutions of these two compounds were prepared by suitably diluting the stock solutions with water. These solutions were kept in a refrigerator (5°C).

Preparation of calibration standards and quality control samples

For method validation studies, calibration standards and quality control samples (QCs), terms defined in the FDA guideline[25], were prepared by spiking appropriate aliquots of the mixed standard solution in blank eel samples. Calibration standards were used to construct

calibration curves from which the concentrations of analytes in unknown monitoring samples are determined practically. QCs used to evaluate the performance of the proposed method. In this study, the standards were prepared in the range of 15–300ng/g for bothanalytes. Three QC levels (For bothanalytes, QC1 = 15ng/g; QC2= 30ng/g; QC3 = 50ng/g) were prepared.

Sample preparation

An accurate 0.1 g sample was taken into a 1.5 mL micro-centrifuge tube and homogenized with 0.6 mL of 80% (v/v) acetonitrile solution (in water) with a handheld ultrasonic-homogenizer for 30 s. After being homogenized, the capped tube was centrifuged at 10,000 g for 5 min. A 100 µL of supernatant liquid was poured to a MonoSpin C18 and, immediately after, the capped mini-column wascentrifuged at 3,000 g for 1 min. The eluate was injected into the HPLC system.

HPLC operating conditions

The analytical column was a Inertsil WP300® C4 (150 × 4.6 mm, 5 µm) column using an isocratic mobile phase of acetonitrile - 0.02 mol/mL OSA (1:1, v/v) at a flow rate of 1.0 mL/min at 50!. PDA detector was operated at 190 - 700 nm: the monitoring wavelengths were adjusted to 261 and 619 nm, which represent maximums for LMG and MG, respectively (Figure 1). For LMG, similar findings (260 – 266 nm)have been reported by the previous papers[17,18,21-24].

Method validation

The performance of the developed method was validated in terms of some parameters from the international guidelines for bio-analytical procedure[25-28].

RESULTS AND DISCUSSION

The aim of this work was to developed a technique for monitoring MG and LMG residues in eel that can be recommended as the international harmonized analytical method

Sample Preparation - Application of Centrifugal Spin mini-Column

In comparison to the previous techniques for determining MG/LMGin fish samples[11-18], the procedure used in this study is very easy and small-scale technique that minimizes organic solvent consumption in the preparation of MG and LMG. The extract obtained by the present operation was purified by subsequent centrifugal monolithic silica spin mini-column, MonoSpin®. The spin mini-column is a monolithic SPE column which is said to be excellent for the small volume sample with easy and quick operation by centrifuge[29].

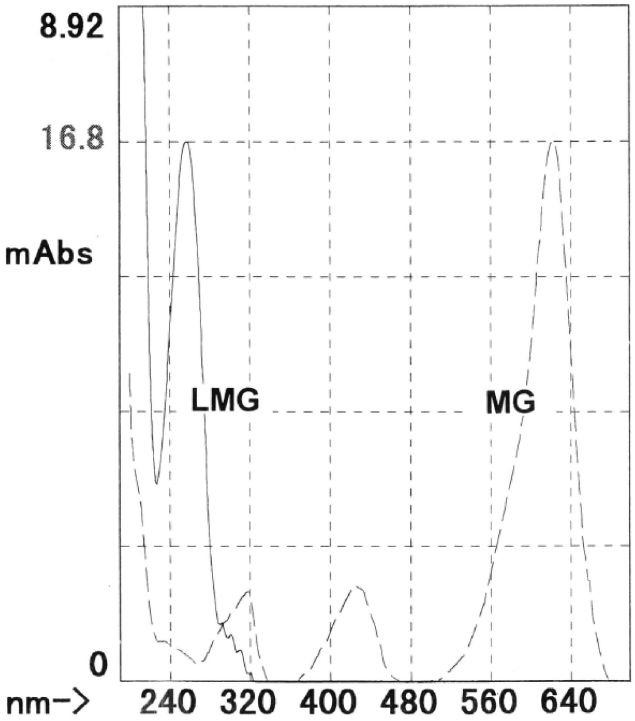

Figure 1 : Typical 1. Typical absorption spectra of peaks for MG (dashed line, max. 619 nm) and LMG (solid line, max. 261 nm) in the HPLC chromatogram

TABLE 1 presents the effect of acetonitrile concentration in the eluent (acetonitrile – water, v/v) on the recoveries of MG and LMG from MonoSpin C18 and –SCX. In this study, a 100 µL portion of a mixed standard solution containing 5 ng of each compound was applied to the spin min-column. The eluate was examined by HPLC. On MonoSpinC18, 70 - 90% acetone solutions as the eluent gave good recoveries for bothanalytes. There were no significant differences in data among 70 - 90 % acetone eluents.

Based on the above findings, eel extracts processed with 0.6 mL of 90 – 70% acetone (5 % interval) were examined. The extract was fortified (50ng/g eel sample of each compound) with a mixed standard solution, and mixed. A 100 µLportion of the extract was applied to MonoSpin C18. The centrifugal accelerationand time were standardized at 3,000 g and 1 min,

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TABLE 1 : Effect of the acetonitrile concentration in the eluent (acetonitrile–water, v/v) on the recoveriesof MG and LMG from MonoSpin® mini-columns

	Acetonitrile concentration (%, v/v) in the eluent										
	0	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
MonoSpin-C18:											
MG	0	0	0	92.3	97.2	99.7	100.1	98.9	102.2	88.6	77.7
LMG	0	0	0	8.4	6.9	36.0	53.4	80.5	100.8	85.1	82.0
MonoSpin-SCX:											
MG	0	83.3	62.5	23.1	22.1	11.1	10.3	7.9	6.5	7.8	5.9
LMG	0	11.1	10.2	2.0	0	0	0	0	0	0	0

Data are averages (%, n=5): a 100 µL portion of a mixed standard solution containing 5 ng of each compound was poured to MonoSpin® mini-column and centrifuged at 3,000 rpm for 1 min. The eluate was injected into the HPLC system

respectively.The eluate was determined by HPLC and the resulting chromatograms were compared with regard to the recoveries and purification efficacy. A 80% acetone as an extraction solution and the MonoSpinC18 eluent gave the best recovery of twoanalytes simultaneously and the most clear chromatogram without interfering peaks.

The present procedure can realize small-scale extraction and easy purification of MG and LMG in quiteshort time and resulted in sufficient recoveries and repeatabilities (TABLE 2).

Figure 1 displays the HPLC traces under the established procedure including the HPLC system. The resulting chromatograms were free of interfering compounds for quantitation and identification of MG and LMG by HPLC, with PDA detector set at 619 and 261 nm (giving maximums for MG and LMG, respectively).The present HPLC analysis accomplished good separations without the need for a gradient system to improve the separation and pre-column washing after analysis. This figure demonstrates that the present method can provide the quantitation and identification of the analytes.

Method validation

Main method validation data

TABLE 2summeries the method validation data for the main performance parameters. The accuracy and precisionare well within the international method acceptance criteria[27,28].

The system-suitability evaluation is an essential parameter of HPLC determination, and it ascertains the strictness of the system used. The suitability was evaluated as the relative standard deviations of peak areas

TABLE 2 : Method validation data

	MG	**LMG**
Linearity (*r*)[a]	0.9979	0.9942
Range (ppb) 15–300		
Recovery test (%):		
Accuacy[b]	98.8	101.1
Precision[c]1.61.9		
Quantitative limit[d](ppb)	6.4	8.9
System suitability[e] (%) :		
Retention time	0.05	0.08
Peak area	0.81	0.56

[a]*r* is the correlation coefficient ($p<0.01$) for calibration curve; [b]Average recoveries from 18 replicates (=six replicates at three QC levels (15,30, and 50 ng/g for MG and LMG)); [c] Values are relative standard deviations (RSD, n= 18); [d]Quatitative limit as the concentration of analyte giving a signal-to-noise ratio = 10; [e] Data as the relative standard deviations calculated for 20 replicate injections of the prepared eluate for an eel sample spiked with MG/LMG (each 30 ng/g).

and retention times calculated for 20 replicate injections of a spiked eel sample (30 ng/g of each compound). The values for MG and LMG were estimated to be < 1.0 % for peak areas and < 0.1 % for retention times, respectively.

The other validation findings are as follows:

Specificity and selectivity

The application of the proposed procedure to 10 blank eel samples demonstrated that no interference peak was presented around the retention times for MG and LMG in any of the sample examined.

The present HPLC-PDA system easily confirmed the peak identity of target compound. Bothanalytes were identified in an eel sample by their retention times and absorption spectra. The MG and LMG spectra ob-

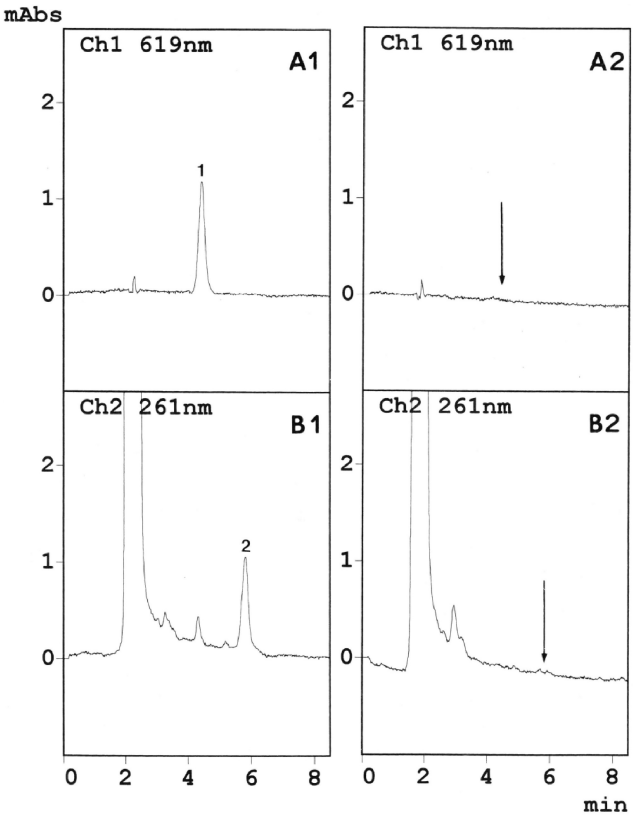

Figure 2 : Chromatograms obtained from the HPLC system for a spiked eel sample (each compound 50 ng/g) (A1 and B1) and a blank eel sample (A2 and B2). Peaks, 1= MG (retention time, Rt= 4.4 min); 2= LMG (Rt= 5.9 min).Arrows indicate the retention times of MG (A2) and LMG (B2), respectively

tained from the eelsample were practically identical to those of the standards.Because of the complete separations, PDA detection at trace levels is fully available. It is, therefore, instructive to demonstrate purification effectiveness of the sample preparation. The system did not require the use of MS/MS, which is very expensive and is not available in a lot of laboratories for routine analysis.

Robustness

In this test, some HPLC parameters were performed using a spiked (30 ng/gof each compound) eel sample obtained under the established procedure.

Changes of ±5% units of the flow rate (1.0 mL/min) and the column temperature (50°C) were determined. The effect on the peak areas and the validations in the retention times were evaluated. Changes of ±5% of the flow rate and the column temperature had no effect on the peak areas, whereas the variations in the retention times were obtained with the flow rate and the column temperature. Normal retention times for MG and LMGwere 4.4 and 5.9 min, respectively. At +5% the flow rate, the three retention times were decreased, ranging between 1.0 and 6.1 % and at -5%, the times were increased ranging between 4.5 and 7.8 %. By changing the column temperature by +5%, decreasing retention times obtained were 2.0–7.7 %, however, no significant variations were observed with -5%. During these studies, both the target compounds were separated.

Cost and time performance

The total time and budget required for the analysis of a single sample was <15 min and approximately269Rs (US $ 4.4) as of 23April, 2014, respectively. For sequential analyses, a batch of 24 samples could be analyzed in approximately3.5h. These findings became term required for the routine assay. The short analytical time not only increased the sample throughput for analysis but also positively affected the cost.

CONCLUSIONS

An idiotproofoperatingsample preparation followed by HPLC-PDA method for simultaneous determination of MG and LMG in cultured eel has been successfully established. The present procedure provided an easy-to-use, rapid, and space-saving and resulted in high recovery and repeatability with considerable saving of analysis time/cost. In particular, the present sample preparing technique may be proposed as an international harmonized method for extraction and cleanup of MG and LMG from the eel.

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