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Article

An Isocratic Toxic Chemical-Free Mobile Phase HPLC-PDA Analysis of Malachite Green and Leuco-Malachite Green

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Abstract: This paper describes a reserved-phase high-performance liquid chromatographic (HPLC) method for detecting malachite green (MG) and leuco-malachite green (LMG) using an isocratic toxic organic solvent/reagent-free mobile phase. Chromatographic separations were performed an Inertsil® WP300 C4 with 0.02 mol/L octane sulfonic acid–ethanol mobile phase and a photodiode-array detector. The total run time was <5 min. The system suitability was well within the international acceptance criteria. A harmless method for simultaneously detecting MG and LMG was developed and may be further applied to the quantification in foods.

Keywords: internal harmonized analytical method; malachite green; leuco-malachite green; high-performance liquid chromatography; photo-diode array

1. Introduction

Malachite green (MG) is a basic organic bluish green pigment and is used in the treatment of infectious diseases of ornamental fishes, such as Saprolegniasis caused by a fungus belonging to the genus Saprolegnia and ichthyophthirius disease [1,2]. However, MG is prohibited (*i.e.*, it has never been registered) as a veterinary drug for use to edible culture fishes in many countries including the United State, Canada, EU, Norway and Japan because of its potential carcinogenicity, mutagenicity and teratogenicity in mammals [3]. Despite its illegality, numerous reports of MG misuse in aquaculture have been reported in the US and internationally [4], and residues of MG and its metabolite, leuco-malachite green (LMG) are the most prohibited compounds found in aquaculture products [5,6]. Strict monitoring for the presence of MG and LMG in aquaculture products is, therefore, an important means of guaranteeing food safety of the food supply and managing global health risks.

Depending on the recent expansion and diversification in the international food trade, the development of international harmonized methods to determine chemical residues in foods is essential 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 must be easy-to-use, economical in time and cost, and must cause no harm to the environment and analyst. Although several high-performance liquid chromatographic (HPLC) methods have been developed for the monitoring MG and LMG with different detection modes [7–13], these methods have crucial drawbacks.

Firstly, all of the methods consume large quantities of toxic organic solvents, acetonitrile and/or methanol [14], in the mobile phases. Risks associated with these solvents extend beyond direct implications for the health of humans and wildlife to affecting our environment and the ecosystem in which we all reside. Eliminating the use of toxic solvents and reagents is an important goal in terms of environmental conservation, human health and the economy [15,16]. Secondly, most of the recent methods are based on LC-MS/MS. The availability of the LC-MS/MS system is limited to industrial nations because these are hugely expensive, and the methodologies are complex and specific. These are unavailable in a lot of laboratories for routine analysis, particularly in developing countries. No optimal method that satisfies the aforementioned requirements has yet been identified.

As the first examination problem in the establishment of an international harmonized method for the residue monitoring of MG and LMG, this paper describes isocratic mobile phase HPLC conditions to detect MG and LMG without toxic solvent/reagent consumption.

2. Experimental Section

2.1. Chemicals and Reagents

All chemicals including malachite green (MG) and leuco-malachite green (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. Ethanol (nontoxic class, the human or environmental toxicity is negligible) [14] and distilled water were of HPLC grade. Octane sulfonic acid (OSA, sodium 1-octanesulfonate) (also nontoxic class) was of an ion-paring reagent for HPLC.

2.2. Equipments

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).

The following four types of non-polar sorbent columns (5 μm d_P; 4.6 mm i.d.; 150 mm length) for HPLC analysis were used: Inertsil® ODS-4; InertSustain® C18; Inertsil HILIC (diol); Inertsil WP300 C4 (GL Sciences, Tokyo, Japan). Table 1 lists the particle physical specifications.

Table 1. Physical/chemical specifications of the reversed-phase columns [a] used and chromatographic malachite green (MG) and leuco-malachite green (LMG) separations obtained under the high-performance liquid chromatographic (HPLC) conditions examined [b].

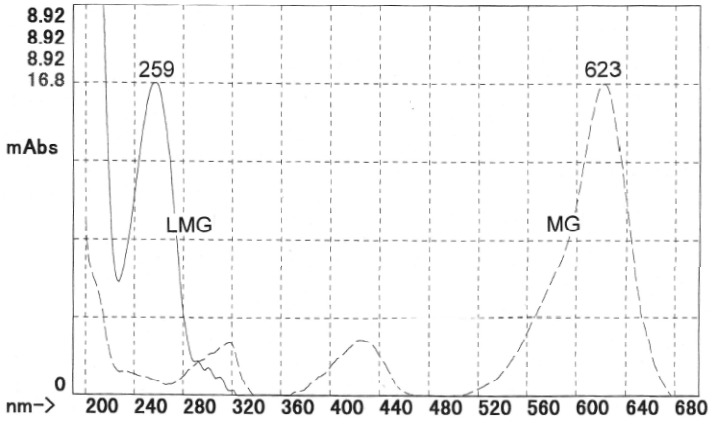
Column		Pore Diameter	Pore Volume	Surface Area	Carbon Load	HPLC Target Compounds			
						Ethanol-Water		Ethanol-OSA [c]	
Silica Type	Trade Name	(nm)	(mL/g)	(m^2/g)	(%)	Separation	Peak Forms	Separation	Peak Forms
(a) C18	Inertsil ODS-4	10	1.05	450	11	N-LMG [d]	-	N-LMG	-
(b) C18	InertSustain C18	10	0.85	350	14	N-LMG	-	N-LMG	-
(c) diol	Inertsil HILIC	10	1.05	450	20	N-LMG	-	N-LMG	-
(d) C4	Inertsil WP300 C4	30	1.05	150	3	N-MG [e]	-	Separated	Symmetrical/ Sharp

[a] i.d. = 4.6 mm; length = 150 mm; d_p = 5 μm. [b] Isocratic mobile phase of ethanol–water or −0.02 mol/L octane sulfonic acid (OSA), ethanol concentrations in the mobile phase ≤50%; flow-rates ≥0.75 mL/min; column temperatures ≥30 °C; HPLC retention times ≤15 min. [c] 0.02 mol/L octane sulfonic acid. [d] No LMG was eluted. [e] No MG was eluted.

2.3. Operating Conditions

The analytical column was a Inertsil WP300 C4 (150 × 4.6 mm, 5 μM) column a 0.02 mol/mL OSA–ethanol (55:45, *v*/*v*) at a flow rate of 1.0 mL/min at 50 °C. PDA detector was operated at 190–700 nm: the monitoring wavelengths were adjusted to 259 and 623 nm, which represent maximums for LMG and MG, respectively (Figure 1). A similar maximum wavelength for LMG (at 266 nm) has been reported by one other study group [12,13].

Figure 1. Typical absorption spectra of peaks for MG (dashed line) and LMG (solid line) in the HPLC chromatogram.



2.4. Preparation of Stock Standards and Working Mixed Solutions

Stock standard solutions of MG and LMG were prepared by dissolving each compound in ethanol followed by water to a concentration of 200 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).

2.5. HPLC Validation

2.5.1. Linearity

The calibration curve was generated by plotting peak areas ranging from 10 to 200 ng/mL *versus* their concentrations. The linearity was assessed from the linear regression with its correlation coefficient.

2.5.2. Detection Limit

The detection limit should correspond to the concentration of the signal-to-noise ratio. The value was defined as the lowest concentration level resulting in a peak area of three times the baseline noise.

2.5.3. Robustness

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.

2.5.4. System Suitability Test

The HPLC system suitability 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 and retention times calculated for 20 replicate injections of a mixed standard solution (100 ng/mL of MG and 50 ng/mL of LMG, respectively).

3. Results and Discussion

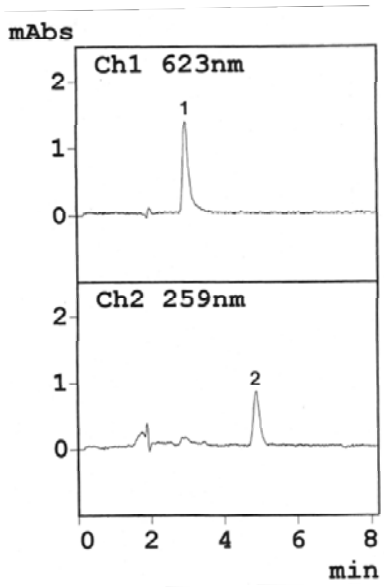
3.1. Optimum HPLC Conditions

In the present study, four types of non-polar sorbent columns (C18, diol, and C4) (Table 1) were tested to achieve the separation with an isocratic toxic solvent-free mobile phase. The examined HPLC retention times were ≤15 min (Table 1). Because the HPLC separations were performed serially, the time/run was critical for routine residue monitoring. The short run time not only increased the sample for analysis but also affected the method-development time.

The four columns were compared with regard to the separation between MG and LMG and the sharpness of peaks obtained upon injection of equal amounts. The chromatographic separations within the conditions ranges examined are also presented in Table 1.

The complete separation of the two compounds and their symmetrical peaks were obtained by column (d) and 0.02 mol/L OSA–ethanol (55:45, *v*/*v*) mobile phase with column temperature of 50 °C and flow rate of 1.0 mL/min. Figure 2 displays the resulting chromatogram obtained from the HPLC. The two target peaks are clearly distinguished at 2.95 and 4.84 min, respectively. The present HPLC-PDA analysis accomplished optimum separation in a short time without the need for a gradient system to improve the separation and pre-column washing after an analysis. From the data shown in Table 1, it is difficult to prove the criterial parameter in the column with regard to the retentions of the target compounds and their peak forms.

Figure 2. Typical chromatograms of a standard mixture (MG 100 ng/mL and LMG 50 ng/mL) obtained from the HPLC system. Peaks, 1 = MG (retention time, R_t = 2.95 min); 2 = LMG (R_t = 4.84 min).



3.2. HPLC Validation

3.2.1. Main Validation Data

Table 2 summarizes the validation data for the main performance parameters (linearity, range, detection limit, and system suitability). The system suitability values were well within the international acceptance limits [17].

Table 2. Chromatographic method validation data.

	MG	LMG	Acceptance Limit [a]
Linearity (*r*) [b]	0.9995	0.9993	≥0.999
Range (ng/mL)	10–200		
Detection limit [c] (ng/mL)	1.94	1.56	
System suitability			
Injection precision(%) [d]			
retention time	0.32	0.69	≤1
peak area	0.66	0.15	≤1
Tailing factor (TF)	1.33	0.96	≤2

[a] FDA guidelines [17]. [b] *r* is the correlation coefficient ($p < 0.01$) for calibration curve. [c] Detection limit as the concentration of analyte giving a signal-to-noise ratio = 3. [d] Data as the relative standard deviations calculated for 20 replicate injections of a mixed standard solution (100 ng/mL of MG and 50 ng/mL of LMG, respectively).

3.2.2. Robustness

Changes of ±5% of the flow rate and the column temperature had no effect on the peak areas; the variations in the retention times were obtained with the flow rate and the column temperature. Normal retention times for MG and LMG were 2.95 and 4.84 min, respectively. At +5% of the flow rate, the retention times decreased, ranging between 4.2% and 4.7% and at −5%, the times were increased ranging between 2.7% and 3.2%. By changing the column temperature by +5%, decreasing retention times obtained were 1.8%–3.79% and at −5%, the times were increased ranging between 1.7% and 2.0%. During these studies, both target compounds were completely separated.

4. Conclusions

In the present paper, an HPLC-PDA method for detecting MG and LMG using an isocratic toxic solvent-free mobile phase has been successfully established. The 0.02 mol/L OSA–ethanol mobile phase method, which has never been applied before, is harmlessness to the environment and to humans and has a short run time and high sensitivity. The HPLC conditions may be proposed as an international harmonized method for detecting MG and LMG. For the quantification in various foods, the proposed HPLC method will be applicable by performing a suitable sample preparation technique.

Conflicts of Interest

The author declares no conflict of interest.

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