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Development and Validation of Analytical Methodologies for the Determination of malachite green (MG), leucomalachite green (LMG), crystal violet (CV) and leucocrystal violet (LCV) residues in Fish by using LC-MSMS

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ABSTRACT

A sensitive method for the determination, confirmation and validation of the sum of malachite green (MG), leucomalachite green (LMG) and sum of crystal violet (CV) and leucocrystal violet in fish muscle has been developed. It is based on liquid–liquid extraction by acetonitrile followed by liquid chromatography– tandem mass spectrometry (LC–MSMS) with electrospray ion source (ESI) in positive mode for determination of (malachite green, leuco malachite green, crystal violet, and leuco crystal violet). The method is simple, fast and inexpensive for simultaneous analysis of selected compounds. Fish samples were homogenated with shaker in mixture of acetonitrile, Mcllvain buffer, TMPD and p-TSA then followed by centrifugation; the supernatant was evaporated then reconstituted by acetonitrile perchloric acid. Quantitation and confirmation of each compound was done by liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI) in positive -ion mode, the mass spectrometer was operated in multiple reactions monitoring (MRM) mode. Two different MRM are used for confirmation. In compliance with Decision 2002/657/EC, which lays down guidelines and procedures for validating methods, validation of the method was carried out. The following parameters were determined: limit of detection (CCa), detection capability (CCβ), linearity, precision, precision, selectivity, specificity and effect of the matrix. The decision limits (CCα) for MG, LMG, CV and LCV were 1.2µg/kg. The respective detection capabilities (CCβ) were 1.5, 1.4, 1.4 and 1.3µg/kg. Typical recoveries (intermediate precision) in fish samples, for MG, CV, LMG and LCV for 2.0 mg/kg level fortified samples using the optimized procedure were in the range 84%, 87%, 88% and 100%, respectively. The findings indicate the suitability of the method for detecting MG, CV and their metabolites (LMG and LCV) simultaneously in fish.

Keywords: Validation, LC- MSMS, malachite green (MG), leucomalachite green (LMG), crystal violet (CV) and leucocrystal violet (LCV)

1. Introduction

Illegal dyes are prohibited compounds (malachite green, crystal violet and their metabolites leucomalachite green and leucocrystal violet). These dyes form enormousness effectiveness in treatment of aquaculture as their action is antifungal, antibacterial and antiparasitic agents. Veterinary drugs have adversely effect on both animals and human health when it used improperly illustrated by the poor handling and management of veterinary drugs. In addition, the deficiency of abuse, misunderstanding, misuse of facilities and storage. There were numerous unseemly practices and attitudes connected with improper drug handling and management issues in the professionals, awareness problems in the community and easy accessibility of the drugs in the black markets that can potentially affect the drug effectiveness. Generally, about 63.9% of the respondents showed that they had no enough knowledge to enhance the handling and management of drugs starting from

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acquisition to end user to assure the quality, safety and effectiveness of veterinary drugs (Hurtaud, Couëdor, Verdon, and Dowell, 2011).

Studies have shown that both MG and CV and their metabolites can be mutagenic and carcinogenic. (Mittelstaedt *et al.* 2004; Stammati *et al.* 2005; Turnipseed *et al.* 2005); therefore, their use is not allowed as veterinary medicine in fish and shrimp breeding (Arroyo *et al.* 2008; Yuan *et al.* 2009). Most compounds are rapidly reduced to the no-chromophore metabolites leucomalachite green (LMG) and leucocrystal violet (LCV), which are persistent forms in the tissue, when MG and CV are consumed by fish. (Rushing and Thompson 1997) as shown in figure (1).

The aim of this study was to develop and validate a sensitive, simple and multi-class LC-MS/MS for the simultaneous determination of residues of to determine the amount of the illegal dyes in aquaculture by using HPLC MSMS. Illegal dyes are class of veterinary drugs; malachite green, leuco malachite green, crystal violet and leuco crystal violet are illegal dyes hence, they used as fungicide, ectoparaticide and antiseptic. The minimum required performance limited in European is 2 µg/Kg. If it exceeds limit it will be carcinogenic and toxic. This method depends on liquid, liquid extraction that based on the molecular weight by positive-ion mode which depends on two factors proton affinity and energy transfer. The method will be done by extraction sample from fish by using acetonitrile, p-Toluenesulfonic acid and Tetramethyl-p-phenylenediamine dihydrochloride. Spike, stander in matrix and calibration mixture solutions that have known amount of standard (illegal dyes), hence they are the controls that will be prepared with different concentration to be sure that the beaks in the result are true. This research is done to control using of illegal dyes in Egyptian local markets and providing reliable data about the actual level of the contamination regarding these illegal dyes in fish then using these data to calculate the risk exposure.

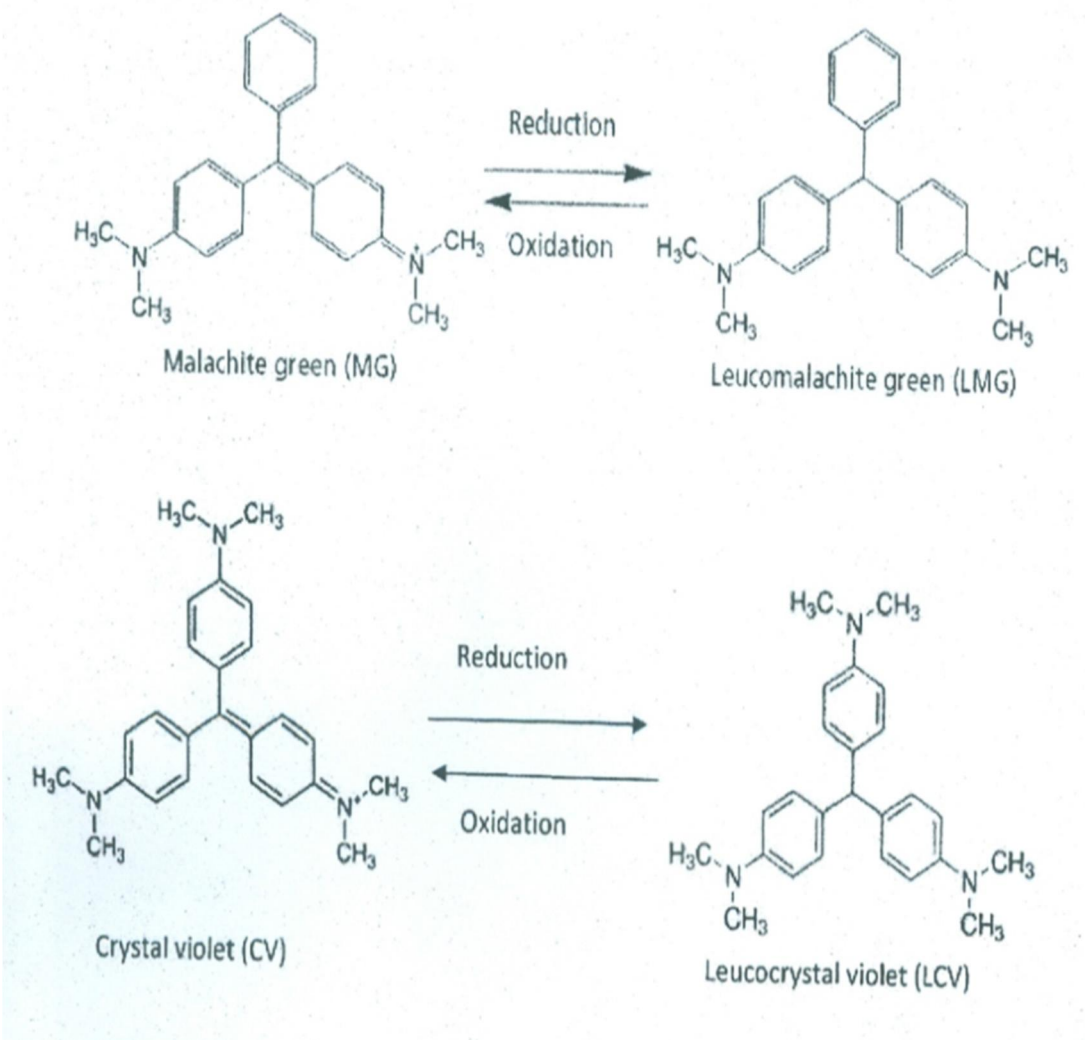

Fig. 1: Structures and conversion of MG and LMG; CV and LCV

2. Materials and Methods

Chemicals and reagents

All the solvents (Acetonitrile and methanol) used were HPLC grade, MG, LMG, CV, and LCV were purchased from Dr Ehrenstorfer (Augsburg, Germany). All reference standard materials were with experimental purity > 94%. Citric acid, formic acid and Ammonium hydroxide solution 30 % used were (Riedel-deHaen, ≥ 99%). De-ionized water (DIW) is obtained by a Millipore water purification system.

0.1 M citric acid

19.2 g citric acid was dissolved in water and completed to a total volume of 1 L.

p-Toluenesulfonic acid (*p*-TSA) monohydrate, Sigma-Aldrich

***p*-TSA 1 M:** 19 g p-TSA hydrate was dissolved in water and completed to a total volume of 100 L.
N,N,N',N'-Tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) was purchased from Sigma-Aldrich.

TMPD (1 mg/mL): 10 mg TMPD was dissolved in 10 mL methanol.

Sodium hydrogen phosphate (Na2HPO4) was purchased from Sigma-Aldrich.

McIlvaine buffer pH 3.0

100 mL 0.2 M sodium hydrogen phosphate was mixed with 430 mL 0.1 M citric acid.

Ammonium formate buffer: was prepared at 0.05 mol/L and the pH was adjusted to 3.8 with formic acid.

Standard and reagent solutions

Standard solutions of MG, LMG, CV and LCV (1 mg/mL) were prepared in methanol and stored at _20°C. The pool of intermediate solution and fortification solution were prepared on the day of use.

Sample preparation

Samples were kept in the refrigerator at -10°C during transport until sample treatment and analysis, fish tissue must be processed long enough to produce a homogeneous blend of tissue, but not long enough to become warm. 2.0g ± 0.1 (W) fish sample was weighed in 50ml Plastic tube (i.e. fortify with 200 µl from 10 ppb in case of spiked sample to get an expected level at 1µg/kg for illegal dyes compounds), 2 mL McIlvaine buffer, 50 µL 1 mg/mL TMPD, 50 µL 1M *p*-TSA and 10.0 ml acetonitrile were added, samples were shaked well by shaker for 1 min, then centrifuged at 4000 rpm for 10 min at 4˚C. The supernatant were taken and evaporated by evaporator at 40 ˚C, then reconstituted by 2 ml (acetonitrile perchloric acid 0.08% by taking 800 µl perchloric acid and completed into 1 litre acetonitrile). The sample were filtered using disposable acrodisc 0.45 um coupled with 5 ml plastic syringe in amber vial and 25 µl of the sample were injected into LC-MS/MS system.

Apparatus and conditions

The LC/MS/MS system was an Agilent model 1200 coupled to an API 4000 triple quadrupole of Applied Biosystems using C18 column. 25 µl was injected from each concentrated sample into HPLC-MS/MS system. Liquid chromatography separation was carried out on C 18 column ZORBAX Eclipse XDBC18 4.6 mm x 150 mm, 5 µm particle sizes. The mobile phase A [10mM ammonium formate solutions in the MeOH: HO_2 (1: 9, v/v)], and solvent B (methanol). A gradient elution program was at 0.3 ml/ min flow rate. The ESI source was used in the positive mode for detection of MG, CV, LMG and LCV. The nitrogen was used as nebulizer gas, curtain gas, heater gas and collision gas according to manufacturer's setting; source temperature was 600 °C, ion spray potential

5500 V, decluster potential and collision energy were optimized using a Harvard apparatus syringe pump.

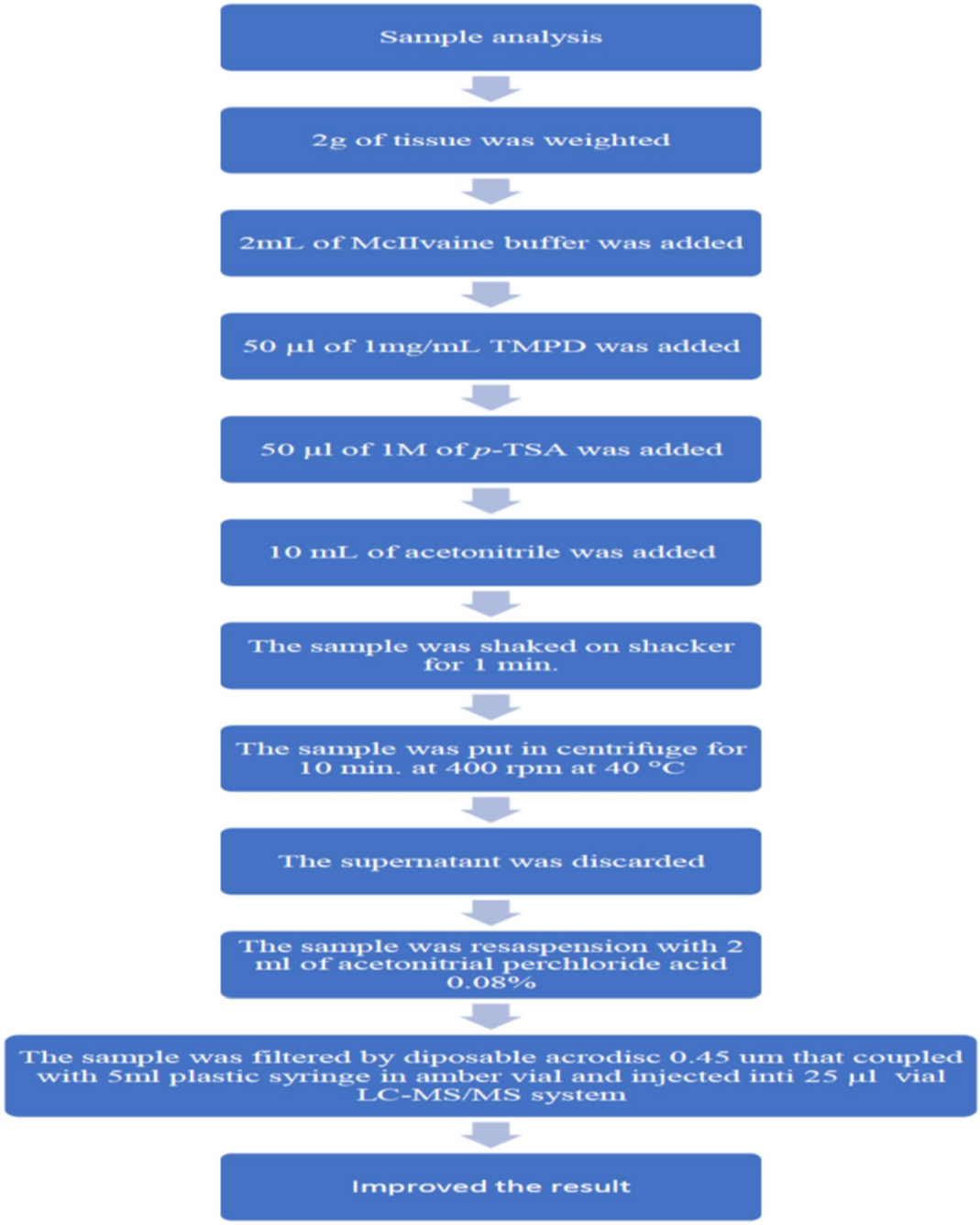

Figure 8: Flow chart represents the sample analysis

Fig. 8: Flow chart represents the sample analysis

Table 1: HPLC Program

	Total time (min)	Flow rate (µl/min)	Mobile phase A (%)	Mobile phase B (%)
0	0	400	60.0	40
1	2.00	400	60.0	40
2	10.00	400	0.0	100
3	26.00	400	0.0	100
4	26.10	400	60.0	40
5	30.00	400	60.0	40

Injection volume was 25 µl

Table 2: optimized compounds (parent ions and daughter ions), declustering potential (DP), and collision cell exit potential (CXP).

Analyte	Parent ion	Daughter ion	DP (V)	CE (V)	CXP (V)
Malachite green	329.30	313.0	96	57	20
		208.2	96	47	12
Crystal violet	372.20	340.4	70	70	10
		356.4	70	70	10
Leuco malachite green	331.26	239.0	36	41	14
		223.0	36	71	12
Leucocrystal violet	374.20	238.3	50	50	10
		358.3	50	50	10

The analytes was performed by using electro spray ionization positive mode (ESI+) with (MRM) multiple reactions monitoring mode. Each compound has two daughter ions for identification as shown in table (2).

Method validation

The validation parameter was determined according to ISO 17025-2017 and the validation criteria described in Commission Decision (2002/657/EC) of the European Community. The minimum required performance limit (MRPL) was the 2 µg/kg.

Decision limit (CCα) and detection capability (CCβ)

The decision limits (CCα) and detection capabilities (CCβ) were calculated as described in the Commission Decision 2002/657/EC. The determination of the decision limits (CCα) was obtained by analysing at least 20 blank materials per matrix fortified with the analyte (s) at the permitted limit. The concentration at the permitted limit plus 1,64 times the corresponding standard deviation equal the decision limit($\alpha = 5$ %). the analysis and extraction of 20 blank samples fortified at level of concentrations 1MRPL and the detection capabilities (CCβ) was obtained by analysing at least 20 blank materials per matrix fortified with the analyte (s) at the decision limit. Analyse the samples and identify the analytes. The value of the decision limit plus 1,64 times the standard deviation of the within-laboratory reproducibility of the measured content equals the detection capability ($\beta = 5$ %).

Linearity

The study of linearity consisted of injection of standard solutions at concentrations 0.25MRPL, 0.50MRPL, 1.00MRPL, 2.0MRPL and 5.0MRPL on 3 different days. The linearity was calculated using the correlation coefficient (r) of the line by Student t test at 99% significance.

Repeatability and reproducibility

The repeatability of the method was determined by the extraction of 24 replicates of fish at the following concentration levels: 6 blank samples fortified at 0.5 µg kg^{-1}, 6 blank samples fortified at 1.0 µg kg^{-1}, 6 blank samples fortified at 1.5 µg kg^{-1}, and 6 blank samples fortified at 2.0 µg $kg^{-}1$. This experiment was repeated three days. Reproducibility was the same test as repeatability but made with another analyst.

Selectivity

6 replicates, to determine the selectivity of this process, at levels 0.5 MRPL, 1.0 MRPL,1.5 MRPL, and 2 MRPL were analysed for a total of 24 samples analysed.

Method uncertainty

The combined uncertainty was calculated by the intermediate precision (reproducibility) and the bias then multiply by factor (k=2) to obtain the expanded uncertainty.

3. Result and Discussion

To provide confirmatory data for the analysis of fish samples for MG, LMG, CV, LCV, and LMG whose structures are shown in Figure1, the LC/MS/MS method was developed. It is important to track one parent ion and two daughter ions for a system to be considered confirmatory. This yielded four identification points which, in accordance with 2002/657/EC: 2002, provided an effective confirmatory process. For each individual compound, the MS/MS fragmentation conditions were investigated and collision energies were optimised. Product ion spectra resulting from collision-induced dissociation have been investigated and sufficient ions have been chosen for multiple reaction monitoring (MRM) systems (Table 1). The precursor and daughter ions obtained as a result are in good agreement with previous results (Dowling *et al.*, 2007), suggesting that compounds have been correctly described.

In order to optimize the chromatographic separation in terms of resolution and overall analysis time due to the different properties of compounds, the conditions of HPLC columns were studied and a gradient separation was developed, that provided good resolution and good chromatograms asm shown in (Table 1). In solvent blank, reagent blank, matrix blank, there were no large peaks that indicated that the experiment for the respective compounds was conducted in a contamination-free condition. In compliance with Decision 2002/657/EC, which lays down guidelines and procedures for validating methods, validation of the method was carried out. The following parameters were determined: decision limit (CCα), detection capacity (CCβ), linearity, precision, selectivity, specificity and matrix effect. The decision limits (CCa) and capabilities for detection (CCβ) are shown in the table (3). For all substances, CC alpha values were less than MRPL, which suggests that the method was modified for the purpose. This research was used for the analysis of dyes during the year. The results show that the method is stable and appropriate for routine quality control operations to detect MG, CV and their metabolites simultaneously.

Table 3: Accuracy, precision values, decision limit (CC_α), detection capability (CC_β) and measurement uncertainty (MU) obtained for the studied of MG, CV and its metabolities residues in fish.

Compound	Fortification level µg Kg^{-1}	Accuracy	Reapitability		Reproducibility		R2	CCα µg Kg^{-1}	CCβ µg Kg^{-1}	MU%
		Percentage recovery	Mean conc.	CV	Mean conc.	CV				
MG	0.5	80.00	0.42	13.0	0.40	11.9	0.999	1.2	1.5	33
	1.0	87.00	1.03	6.70	0.89	15.5				
	1.5	83.00	1.35	15.0	1.24	15.0				
	2.0	85.00	1.76	9.70	1.69	9.90				
LMG	0.5	100.0	0.53	9.20	0.50	9.00	0.989	1.2	1.4	33
	1.0	83.00	0.86	4.90	0.83	7.10				
	1.5	79.00	1.29	10.7	1.20	12.5				
	2.0	86.00	1.92	4.96	1.72	15.5				
CV	0.5	88.00	0.46	15.2	0.44	16.0	0.984	1.2	1.4	33
	1.0	98.00	1.08	16.0	0.98	15.0				
	1.5	83.00	1.30	11.5	1.24	11.0				
	2.0	82.00	1.67	8.70	1.63	8.30				
LCV	0.5	110.0	0.53	7.90	0.55	11.8	0.988	1.2	1.3	33
	1.0	96.00	1.10	8.89	0.96	14.6				
	1.5	96.00	1.45	6.56	1.44	12.3				
	2.0	98.00	2.00	8.20	1.96	11.3				

4. Conclusion

This paper describes an LC-MS/MS method for simultaneous determination of the sum of malachite green (MG), leucomalachite green (LMG) and sum of crystal violet (CV) and leucocrystal violet in fish muscle. The method is simple, fast and it does not require any cleanup procedure, allowing thus high sample throughput. The use of matrix-matched standards is recommended to eliminate the matrix effect. The method demonstrated excellent performance parameters. Recoveries of all compounds, at the levels of fortification of 0.5, 1, 1.5, and 2 µg kg^{-1}, ranged between 70 % and

120 %. LOQs is 0.5 µg kg−1. The method allows for the determination of trace levels of these compounds in fish samples.

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