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Development of new TLC method for determination of malachite green residues in raw fish

[1]Abou-Raya S.H., A.R. Shalaby[2], M. E. Abdel-Aziz[1] and M.T. El-Sayed[2]

[1]Food Science Department, Faculty of Agriculture, Cairo University, Giza, Egypt
[2]Food Science and Technology Dept., National Research Centre, Dokki, Cairo, Egypt

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ABSTRACT

A simple, rapid, accurate of Thin Layer Chromatographic (TLC) method for determination of malachite green (MG) and leuco-malachite green (LMG) residues in raw fish is established. MG and LMG residues were extracted from raw fish by ammonium acetate buffer, methylene chloride and separated by partitioning into methylene chloride. Leuco-malachite green was quantitatively oxidized to the chromic malachite green by reaction with 2,3dichloro- 5, 6-dicyano-1,4 benzoquinone. Samples were then cleaned-up by prepared column containing silica and alumina. The suggested cleaning solution (hexane: isopropanol: ethanol) (6/3/1V/V/V) effectively eliminated the interfering substances with MG giving high resolution on TLC. The extract was washed by a mixture of acidified methanol/ acidified ACN 1:1 v/v. The washed extract was dried. The total MG on TLC gave green color at visible light by naked eyes. This property was used to determine MG and LMG on TLC without fluorescence by densitometer. Using a mobile-phase of Acetonitrile: methanol: ammonium acetate (0.1M): formic acid 0.1% (5: 2: 2: 0.5, v/v/v/v) and a silica gel thin-layer, the total MG was selectively determined by densitometry at 620 nm. The average recoveries for raw samples were between 62.44 and 75.30% for MG and from 66.24 to77.65% for LMG with comparative standard deviations less than 10%.

Key words: Fish, Malachite green, Extraction, Separation, TLC.

Introduction

Aquaculture production, the fastest growing systems, is one of the most important food protein sources in developing countries. However, fish and seafood can be easily infected with certain diseases which need to be cured using veterinary drugs. Moreover, the approved substances are limited. This leads to use some of unapproved substances including fungicides, ectoparasiticides and disinfectants. Malachite green (MG) is the main compound generally used in fish farms because of its low cost and high efficacy (El-ghayaty *et al.,* 2016). The hazard of MG is classified as class II where shows a significant health hazard to humans through consuming the contaminated fish with MG residues (Hidayah *et al.,* 2013). It is of magnitude to state that MG is heat stable; and thus can't be broken down easily by routine fish cooking (Mitrowska *et al.,* 2007). Due to its potential carcinogenicity, mutagenity and teratogenicity (Culp and Beland, 1996 and Fessard *et al.,* 1999), MG is not registered for veterinary use in many countries as the European Union, United States, Canada (Council Regulation, 1990). However, since its low cost, availability, efficiency, MG is still used illegally in aquaculture. MG can easily penetrate fish tissue during the exposure to contaminated water. It is extensively metabolized to the reduced colorless compounds, LMG, which is also a suspected mutagen and teratogen (Culp *et al.,* 1999 and Mittelstaedt *et al.*, 2004).

It is of importance to state that potential hazard of illegal use of unapproved drugs (MG), and also the misuse of approved components will be threatened consumer health. Actually, the public health attention associated with MG residues in addition to other contaminants have been motivated to find an institution integrating with suitable analytical methods to determine MG in real seafood samples. It is fortunate that, United States and European Union have been set the maximum residue limits of sum MG and LMG in foods by zero allowances policy (Hidayah *et al.,* 2013). However, it is of importance to mention that according to the European Commission, method that can be used for

Corresponding Author: Abou-Raya S.H., Food Science Department, Faculty of Agriculture, Cairo University, Giza, Egypt. E-mail: aborayasalah1947@yahoo.com

determination MG residues in fish muscle must be fit to meet the minimum required performance limit (MRPL) as 2 µg kg^{-1} for the sum of MG and LMG (Commission Decision, 2004/25/EC). Lower limit of total MG and LMG have been established by FDA, 1 µg Kg^{1} (Collette, 2006).

There are many different methods to determine MG and LMG in fish. The common used method is basing on liquid chromatography (LC), mainly with visible (VIS) detection. The origin component (MG) has λmax at 620 nm, whereas the leuco form has λmax at 265 nm (Tarbin *et al.,*1998) Liquid chromatography with tandem masses spectrometry (LC-MS/MS) and Gas Chromatography masses spectrometry (GC-MS) are also used (Hidayah *et al.,* 2013). Mass spectrometry (MS) detection for MG and LMG residues was preferred for determining and confirmation the analysis (Dowling *et al*., 2007 and Turnipseed *et al.,* 2006). These methods often require complicated operations of pretreatment and expensive apparatuses, as well as large efforts and long time to be done. So, some alternative methods with quick, reliable and simple pretreatments have to be developed for detecting illegal residues of MG and LMG in the products of aquatic food. There are no available studies used or developed TLC techniques in MG determination. So, the present work was an attempt to develop an accurate, precision and sensitive (TLC) method for determining MG and LMG residues in raw fish.

Materials and Methods

1. Chemicals and supplies

Standards of MG and LMG were supplied by Sigma (St Louis, MO, USA). All chemicals and solvents (formylic acid, Methanol; MeOH, acetonitrile; ACN, dichloromethane; DCM and ethyl acetate, 2, 3-dichloro-5, 6-dicyano-1, 4-benzoquinone; DDQ and alumina) were of reagent grade and double distilled water was used. Glass wool and a disposable Pastier pipette (5mm ID) were used. Thin Layer Chromatography (TLC) plates (20 X 20 cm aluminum sheets pre-coated with 0.25mm silica gel G60) were purchased from E. Merck, Germany.

2. Apparatus

Cooling centrifuge, (sigma 3-18ks Germany) ultrasonic bath (Buhler, Hechingen, Germany), homogenizer and, vortex (Mechanical Precyzyjna, Model type ST-2) were used for sample preparation. The developed TLC plates were scanned by CAMAG-TLC Scanner.

3. Standard solution

Stock standard solution of MG and LMG mixture was prepared by dissolving 10 mg of each in 20 ml of acidified (0.001% formic acid) ACN to obtain a final concentration of 1 mg ml^{-1}. Mixture stock solution was put in amber glass to prevent the photo-degradation and stored at -20 ºC. Stock solutions were diluted with acidified DCM to give the working standard solution.

4. Fortification of samples

The fish samples were spiked with MG and LMG standard at 5 levels of 2, 4, 6, 8, and 10ng g^{-1} of each compound per gram fish muscle. Fortified samples were allowed to stand at 4'C for 1 hour before analysis.

5. Malachite green analysis

5.1. Sample extraction

Extraction was performed by the method of Andersen et al., (2006) with some modification. Fresh Tilapia fish muscle (10 g) was homogenized and transferred into a clean50 ml glass centrifuged tube. Five milliliters buffer of ammonium acetate was added followed by 1 ml of HAH solution and 100µL of p-TSA solution. The centrifuge tube was capped and mixed vigorously for 30 seconds.

Twenty milliliters of dichloromethane was added and shaken vigorously for 30 seconds. The mixture was, sonicated (10 min) and then centrifuged at 5000 rpm (10 min).The extraction process was repeated twice. The supernatant was collected and evaporated under vacuum at 45 °C. Five milliliters of 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone, (DDQ) dissolved in dichloromethane (DCM) (0.005 M) was added to oxidized the residue of MG. The dissolved residue was left in the dark for 30 min with periodic sample agitation and the volume was applied to mini-column.

5.2. Clean up techniques

Pasteur pipettes (ID 4 mm) was used as mini-column where a small glass wool plug was inserted in the restricted end and packed with silica gel (ca25 mm height). Alumina (ca15 mm height) layers were then added to the column and the dissolved residue was applied into mini column to collect a clear elute.

The effect of the cleaning solution was examined using 2 mini-columns. Both columns were loaded with 10 ml of spiked sample extract containing 100ng of LMG. Without cleaning, the first column was directly eluted with 5 ml (Me OH: ACN, 1:1 v/v). Whereas, 10 ml of the suggested cleaning solution (hexan: isopropanol :ethanol, 6:3:1 v/v) was applied into the second column before the elution step. The eluents were evaporated at 40 ºC and quantitatively transferred to 1.5ml vials and then dried using nitrogen. Vials were dissolved with known volume and 10, 20µL were applied on TLC plate.

5.3. Separation of MG

One-dimensional TLC technique was used to separate the Malachite Green. The Malachite Green standard and the fish extracts were applied 2 cm from the base of the TLC plate and at 1 cm intervals using a micro syringe10, 20, 50, 80 and 100 l of each fish extract.

Twelve suggested mobile phases were tested for their efficiency in MG separation (Table 1). The selected developing system (Acetonitrile: methanol: ammonium acetate (0.1M): formic acid 0.1%, 5: 2: 2: 0.5, v/v/v/v) was used for validation procedure

The plate was developed for 10 cm and then eliminated from the jar and dried at room temperature before interpretation.

Table 1: Various solvent systems were proposed and tested.

No.	Compositions	Ratio	R_f
1	Ethanol: acetonitrile: methanol: acetic Acid (.1N)	2: 8: 1 :1	0.21
2	Ethanol: acetonitrile: methanol: acetic Acid (.1N)	2.5 :7: 1.7 :1	0.25
3	Ethanol: acetonitrile: methanol: acetic Acid (.1N)	3: 7: 2 :1.5	0.32
4	Ethanol: acetonitrile: methanol: acetic Acid (.1N)	4 :7: 3 :1	0.48
5	Acetonitrile: methanol: ammonium acetate (0.1M)	10: 2: 1	0.27
6	Acetonitrile: methanol: ammonium acetate (0.1M)	8: 2: 1	0.29
7	Acetonitrile: methanol: ammonium acetate (0.1M)	6: 4: 1	0.60
8	Acetonitrile: methanol: ammonium acetate (0.1M) : formic acid 0.1%	5: 2: 2: 0.5	0.61
9	Ethyl acetate: acetonitrile: methanol: formic acid 0.1%	7: 5: 2: 2	0.49
10	Ethyl acetate: acetonitrile: methanol: formic acid 0.1%	5: 3: 2: 2	0.38
11	Ethyl acetate: acetonitrile: methanol: formic acid 0.1%	7: 3: 1.5: 2	0.36
12	Ethyl acetate: acetonitrile: methanol: formic acid 0.1%	4: 6: 3: 2	0.56

R_f value = (distance to center of spot) / (distance to solvent front)

5.4. Interpretation of the chromatogram and Quantification

The plate was examined under visible light (620 nm) to characterize the presence of MG in the sample. The developed TLC plate was placed under a CAMAG TLC Scanner 3 and the absorbance value for each separated spot was recorded at wavelength of 620 nm.

6. Linearity of response

The linearity was proved with 5 standard calibration points in the concentration range 10-100 ng ml-1 of MG and LMG each. The standard curves were obtained by planning the recorded peak area versus the concentrations of the standard solutions. The linearity of the standard curves was checked by sum each of the regression line and correlation coefficient.

7. Recovery test

Recoveries (n = 3) of MG and LMG from raw fish muscle samples fortified at 2, 4, 6, 8 and 10 ng g^{-1}. The calculation was achieved by comparing the densitometer peak areas of each sample to the areas of the same standard solution

8. Validation of procedure

The performance features of methods were determined by the criteria followed by Commission Decision 2002/ 657/EC for qualitative screening methods (EC, 2002). The limit of quantification (LOQ) obtained by adding 10times for the standard deviation of 20blank samples to the mean of blank value. Response linearity was calculated from the five point calibration curve (10, 20, 50, 80 and 100 ng).

Results and Discussion

1. Separation and identification of MG

Cleaning step is important in removing the interferences with retention of the analyses on the sorbent. The suggested cleaning solution (hexane: isopropanol: ethanol) (6/3/1 V/V/V) effectively eliminated the interfering substances with MG giving high resolution on TLC. Also, it is of significance to find a suitable solvent system that has an ability to separate MG from impurities on TLC. In general, solvent systems for TLC can be chosen by consultation to the literatures (Shalaby, 1996). There are no available literatures concerned with determination/detection of MG residues in food by TLC method; which can assist for selecting the suitable solvent system. On the other hand, it was mentioned that micro-circular technique and microscope-slide layers (Shalaby, 1996) is the best way to try to find favorably one - or more component solvents for separating. So, micro-circular technique was used to find a suitable solvent system for separating MG on TLC. Methanol, acetonitrile, chloroform, hexane, isopropyl alcohol, ethanol, acetone and water were tested for their ability to separate MG on TLC silica gel. As well as, various solvent systems were proposed and tested for their separation ability of MG. The patterns of MG spots on TLC plates using the suggested mobile phases are illustrated in (Fig.1).

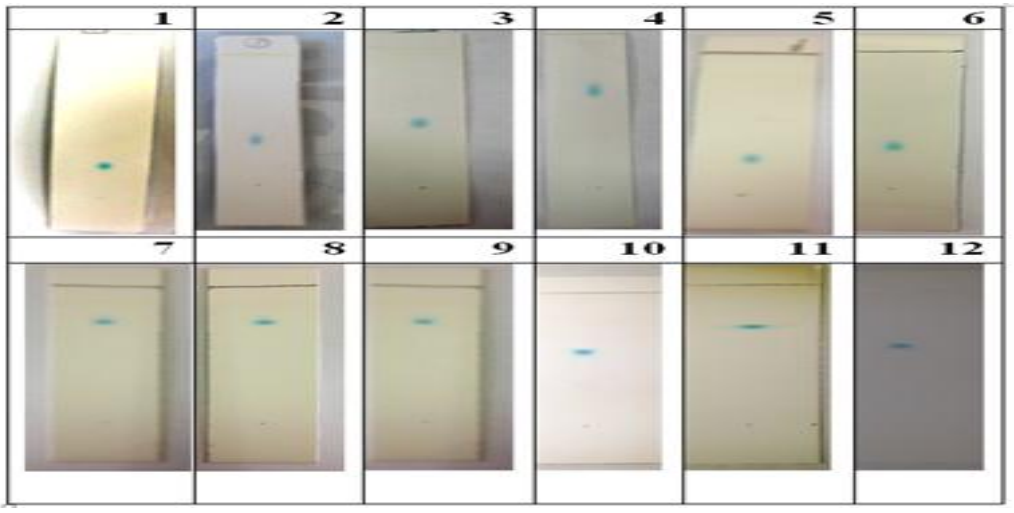

Fig. 1: TLC patterns of MG separation using 12 examined developing systems.

The developing system no 8 was the best one where it gave clear and compact separated spot. Likewise, it had the higher R_f value (0.61) which could allow to separate more impurities found in fish samples. Consequently, overlapping with MG spot would be avoided.

2. Validation of the TLC method

Validation of the method was performed using increment raw fish spiked with 2,4,6,8, 10 ng g^{-1}.

2.1. Limit of quantification

The limit of quantification (LOQ) was 0.2 ng g^{-1} for total MG in raw fish which confirmed the Minimum Required Performance Limit (MRPL) (Commission Decision, 2004/25/EC).

2.2. Linearity

Table (2) shows the linearity parameters of both MG and LMG standards at 5 different concentrations. Linear calibration standard curves were obtained. The correlation coefficients were higher than 0.989 for both MG and LMG which reflected how the method was good. The shape of densitometer chromatogram were sharp and pure (Fig.2) which referred to a good resolution.

Accordingly, the sensitivity of MG identification on TLC was established. It is of interest to point out that a very high sensitivity could be obtained by the established method. Change of 70.3 units per 1ng MG change referred to change of 0.1 ng MG (20 ng g^{-1}) which was easily detected using densitometer.

Table 2: Linearity of densitometer response of different concentrations of MG and LMG.

Analyte	Slope	Intercept	Correlation coefficient (r^2)	Equation
MG	3.8631	89.597	0.989	y = 3.8631x + 89.597
LMG	3.2428	59.593	0.990	y = 3.2428x + 59.593

X= ng of MG and LMG on TLC, Y=response of the densitometer (peak area).

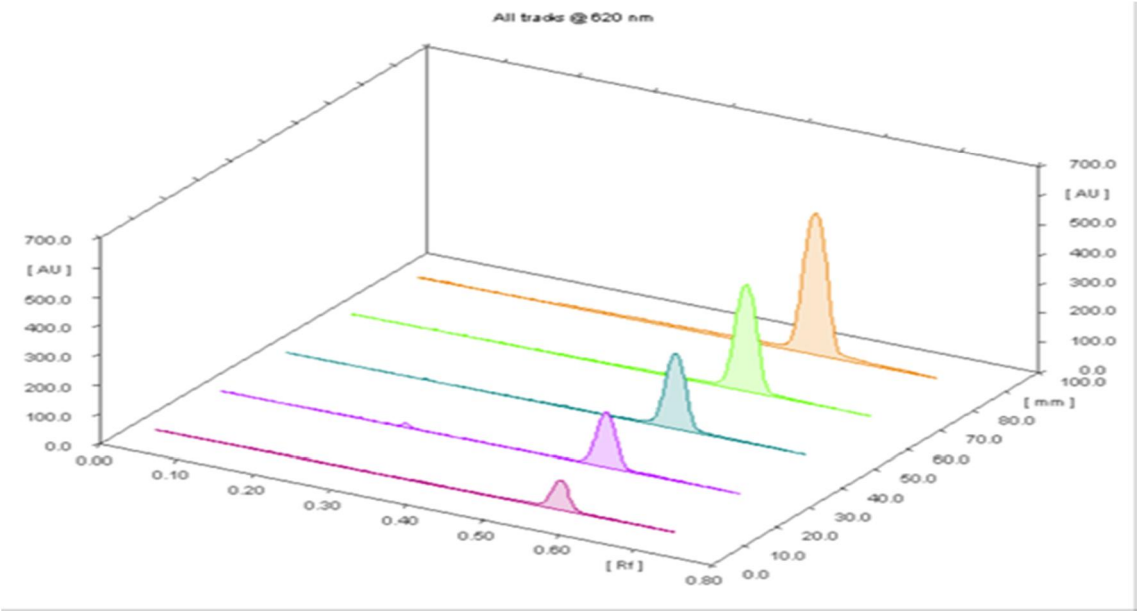

Fig. 2: Densitometric profile of chromatographic standard of MG (from 10 ng to 100 ng) on silica gel TLC plate.

2.4. Recovery

The recovery of MG and LMG total in spiked raw fish muscle at 2, 4, 6, 8, 10 ng g^{-1} are illustrated in Table (3). The average recoveries for raw samples were between 62.44 and 75.30% for MG and from 66.24 to 77.65% for LMG with relative standard deviation lower than 10%.

Table 3: Recoveries (n= 3) of MG and LMG from fortified fish muscles samples at 5 levels.

Spiked concentrations (ng)	Recovery (%) Mean±(SD)		RSD %	
	MG	LMG	MG	LMG
20	62.44±4.80	66.24±4.94	7.69	7.45
40	69.54±1.52	70.93±3.95	2.19	5.57
60	71.22±2.19	73.30±2.24	3.07	3.06
80	74.20±1.57	76.75±1.52	2.12	1.99
100	75.30±2.21	77.66±2.26	2.94	2.91

SD = Standard deviation, RSD = Relative standard deviation

2.5. Precision and accuracy

Precision is the reproducibility of multiple measurements. It is usually described by the standard deviation, standard error, or confidence interval. A common description of precision is by standard deviation or comparative standard deviation (variation coefficient, C.V.) of a set of replicate results (Shalaby, 1996). The precision related to the error inside the laboratory of the method (repeatability) or to the error between the laboratories of the method (reproducibility). The repeatability of the used TLC method is presented in Table (4). Three different amounts of MG (20, 40 and 80 ng, representing concentrations of 4, 8 and 16 µg kg-1 sample were analyzed, where ten replicates each amount were used, developed and determined by densitometry. The obtained results indicated that a relative standard deviation (RSD) was less than 10% (6.36%). It represents the combined error of TLC resolution, application and apparatus detection and quantification. Generally, it was noticed that by increasing the concentrations of MG, the calculated RDS was decreased.

Table 4: Precision and accuracy of MG determined in fortified fish muscle at three concentration levels (n= 10).

Amounts applied (ng)	Range (ng)	Mean±(SD)	RSD %
20	17.35: 20.54	19.13±1.15	6.05
40	37.23: 40.88	38.34±1.40	3.65
80	76.21: 80.60	77.90±1.82	2.33

SD = Standard deviation, RSD = Relative standard deviation

Generally, accuracy and precision are two important factors to consider when taking data measurements. The accuracy inverts how close a measurement is to a known or accepted value, where, precision inverts how reproducible measurements are, even if they are far from the accepted value.

Conclusion

From the above mentioned results, it could be concluded that the developed method for MG determination characterized by its simplicity, accuracy and applicability. Also, the used cleaning solution (hexane: isopropanol: ethanol, 6/3/1 V/V/V) increased the purity of the separated MG on TLC and no interference was observed.

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