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Research Article
Impact of Cooking on Malachite Green and Leucomalachite Green Residues Existing in Tilapia Fish

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Abstract

Introduction: Food safety is determined to assure that food safety hazards present in the food have been eliminated, reduced to an established acceptable level or prevented from exceeding the acceptable level. Fish and seafood suffering from certain diseases need to be treated by MG, which metabolized to LMG. Such fish are cooked before its consumption may cause health problems. Many analytical methods for determining MG and LMG residues were required expensive apparatuses, large efforts and long time to done. **Objective:** Establishing a rapid and accurate Thin Layer Chromatographic (TLC) method for determining MG and LMG in fish and to follow up the effects of various cooking methods on MG and LMG residues in treated fish. **Methodology:** Thin-layer chromatography, roasting, frying and microwaving were used in this study. **Results:** Limit of quantification (LOQ) for MG and LMG was 0.2 ng g^{-1}. Standard curves were linear for MG and LMG with correlation coefficients of 0.9913. No interferences occurred for MG and LMG on the developed TLC of blank samples. Average recoveries of raw, roasted, fried and microwaving samples were given. The losses observed in LMG were 18, 22 and 30% for roasting, frying and microwaving, respectively. The relative standard deviations were fewer than 15% for malachite green and fewer than 12% for leucomalachite green. The decrease of cooked samples weights of raw muscle, during roasting, frying and microwaving was observed. **Conclusion:** It could be conclusion that high temperatures and cooking processes do not ensure a full elimination of both malachite green and leucomalachite green which may be present in Tilapia fish.

Key words: Malachite green, leucomalachite green, Tilapia, cooking effects

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Food safety is determined to assure that food safety hazards present in the food have been eliminated, reduced to an established acceptable level or prevented from exceeding the acceptable level. Farms of fish are systems growing fast for food protein production especially in developing countries. It considered a main exporter of protein in those countries. However, fish from aquiculture are suffering from different diseases which need to be treated by veterinary drugs. The number of compounds approved for their use to treat fish and seafood is rather limited. The main compounds used in aquaculture farms are OTC and MG, so there are still serious problems with their residues in fish and environment pollution due to the abundant and illegal use of such hazards which threaten the public health.

Malachite Green (MG) is a cationic triphenylmethane dye, which has been used worldwide as fungicide and ectoparasicide in cultured fish eggs, finger lings and adult fish[1] since 1930s. The MG is not approved for using in numerous states such as the nations of European Union, America and others[2] that is because MG is a main reason in carcinogenicity, mutagenicity and teratogenicity diseases[3,4]. The MG is the most effective drugs which are available at low cost, so there is still concerned about its illegal use. The absorption of MG into fish tissues was easy where exposed to waterborne extensively. The adsorbed malachite green turned to color less material carcinogenic, leucomalachite green (LMG)[5,6]. It is of interest to know if residues of both MG and LMG could be eliminated or reduced by cooking methods. Although fishes are usually cooked before consumption, there is required to more results about the impact of cooking on residues of MG and LMG to evaluate the risks to the consumer for saving the consumers from such hazards[7]. Now a days there are multiple methods for analyzing the fish for MG residues given in different manuscripts[8-11], but they are required expensive apparatuses, as well as large efforts and long time to done. Although analysis of MG/LMG by surface-enhanced resonance Raman scattering[12], electrochemical biosensor[13], a lateral flow immunoassay[14] and using magnetic molecularly imprinted polymers[15], still needing expensive apparatuses, long time or large efforts.

Most risk occurring by veterinary drug residues in fishes linked with their levels in raw muscles, which may contain on such residues since consumed after cooking. Since, the information about the impact of concoction on veterinary drug residues is still scanty, the risk to the consumer from dietary exposure to these residues is not well known. Generally, the problems that will solve are (1) The problem lies in spread of veterinary drug residues in fishes that causing many diseases to consumer and (2) All methods used to determine MG and LMG need to expensive apparatuses, large efforts and long time to done. So, the aim of this study is to establish a rapid, accurate and economic Thin Layer Chromatographic (TLC) method for determining MG and LMG in fish and to follow up the effects of various cooking methods on MG and LMG residues in fish.

MATERIALS AND METHODS

Chemicals and reagents: All solvents (acetonitrile, ACN, dichloromethane, DCM, formic acid, butylated hydroxytoluene; BHT) and chemicals (Anhydrous sodium sulfate, ammonium acetate hydroxylamine hydrochloride, HAH, p-toluene sulfonic acid, p-TSA and 2,3-dichloro-5, 6-dicyano-1,4-benzoquinone, DDQ) used during this study were of analytical grade. Water was purified before use in a Milli-Q system (Millipore, Bedford, MA, USA). Thin-layer chromatography (TLC) plates (20×20 cm aluminum sheets precoated with 0.25 mm silica gel G. 60) were purchased from Macherey-Nagel, Duren, Germany.

Acidified ACN, DCM and Milli-RQ water were prepared by diluting 1 mL of formic acid in 999 mL of each solvent.

Standard solution: Stock standard solutions of MG and LMG were prepared separately by dissolving 10 mg in 10 mL of acidified ACN to obtain a final concentration of 1 mg mL^{-1}. Two milliliters each of MG and LMG were mixed to give mixture stock solution of 500 μg mL^{-1}. Each stock standard solution was put in amber glass to prevent the photo-degradation and stored at -20°C. Stock solutions were diluted with acidified ACN to give a series of intermediate standard solutions. Series of working standard solution were also prepared using either acidified ACN or acidified DCM. All standard solutions made in amber volumetric flasks and stored at 4°C.

Apparatus: Refrigerator centrifuge, ultrasonic bath (Buhler, Hechingen, Germany) and homogenizer (Mechanika Precyzyjna, Model type ST-2) were used for sample preparation. The developed TLC plates were scanned by CAMAG TLC Scanner 3 (Shimadzu CS-9000 chromatogram scanner).

Samples: Fourteen Tilapia fishes with a median weight of 400 g were placed in a tank containing aerated water. After 7 days, 12 fishes were exposed to a bath of 2 mg L^{-1} MG for 2 h (temperature was from 17-18°C and pH was from

7.6-8.0. The treated fish were taken from the tank, eviscerated, beheaded, deboned, skinned, filleted and their muscles were collected separately. The remaining two Tilapia were kept as controls, where their muscles were used as the blank sample material. Portions of incurred sample material were mixed with suitable amount of blank sample material to obtain levels about 200 ng g^{-1} of MG and LMG. All muscles were homogenized, mixed and formed[16] into 100 g.

Cooking procedures

Roasting: Three portions (100 g each) of Tilapia samples were placed on a metal tray and cooked in the center of an electric oven (J. P. Selecta, S. A. (Spain), w: 2000, v: 230, Hz: 50, serial No. 0361031) at 200°C for the specified time (4, 8 and 12 min), one sample was removed at each time, allowed to cool at room temperature, minced and blended with the resultant juice and analyzed in triplicate.

Frying: Three portions (100 g each) of Tilapia samples were placed separately on frying pan containing oil at 160-180°C and cooked for a specified time (4, 6 and 8 min, i.e., 2, 3 and 4 min at each surface), removed, allowed to cool and analyzed in triplicate..

Microwaving: Three portions (100 g each) of Tilapia muscle samples were placed into glass petri dishes and cooked in a Gold-star Microwave Oven (Model ER-535 MD) (2450 MHz) at six power level. The samples were withdrawn after 0.5, 1 and 2 min, one sample was removed at each time, allowed to cool at room temperature, minced and blended with the resultant juice and analyzed in triplicate.

Determination of MG and LMG: The determination of MG and LMG in cooked and raw muscle was performed by TLC method. This method was validated and regularly checked in proficiency tests.

Sample extraction: Accurately weigh 5.0 g of homogenized tissue and put into a 50 mL centrifuge tube. Add 5 mL of ammonium acetate buffer, 1 mL of hydroxylamine hydrochloride (HAH) solution and 100 µL of p-toluene sulfonic acid (p-TSA) solution to the sample and mix by vortex for 30 sec. Add 20 mL of dichloromethane, cap and shake vigorously for 30 sec. Centrifuge at 0°C for 5 min at 4000 rpm and collect the supernatant into round flask. Re-extract the residues twice. Evaporate the extract to dryness under reduced pressure and at 50°C. Add 3 mL dichloromethane containing DDQ (0.001 M mL) to the dry oily residue, swirl for dissolving the residue and left in the dark for 30 min with periodic sample agitation. The solution was passed through alumina column, washed with 2 mL of BHT solution in ACN (0.01 mg mL^{-1}), filtered and focused using nitrogen.

TLC chromatography: One dimensional TLC technique was used to separate the MG under investigation. The standard and the extracts of fortified samples of MG and LMG were applied 2 cm from the base of the TLC plate and at 2 cm intervals using a micro syringe. The plate was developed in water: Acidified acetonitrile (1:5 v/v) for 15 cm. The plate was removed from the jar and allowed to dry at room temperature before scanning.

Recovery test: The recoveries (n = 3) of MG and LMG from blank Tilapia muscle samples fortified at 100 ng g^{-1} for the raw samples (5 g) and for the samples cooked by roasting for 6 min, frying for 4 min and microwaving for 1 min were determined.

Linearity of response: The linearity was proved with seven standard calibration points in the concentration range 5-100 ng mL^{-1} of MG and LMG. The standard curves were obtained by plotting the recorded peak area versus the corresponding concentrations of the standard solutions. The linearity of the standard curves was checked by calculation of the regression line and the correlation coefficient.

RESULTS AND DISCUSSION

Validation of the analytical method: The TLC method was used for analyzing MG and LMG in fish. The validation of the method was performed using a lot of raw and cooked muscles fortified at 100 ng g^{-1}. The limit of quantification (LOQ) was 0.2 ng g^{-1} for MG and LMG in fish sample which conform the Minimum Required Performance Limit (MRPL). By using range of 5-100 ng for MG and LMG, linear calibration standard curves were obtained with correlation coefficients of 0.9913 for both MG and LMG. It was observed that no any interference on MG and LMG spots on the developed TLC chromatogram of blank muscle samples from the control Tilapia fish. The recoveries of both malachite green and leucomalachite green from raw and cooked fish muscle samples were given in Table 1. The average recoveries for raw, roasted, fried and microwaving samples were between 83 and 59% for MG and from 89-70% for LMG with relative standard deviations fewer than 10%. Although, TLC method use for determining MG and LMG, all workers used a high-performance liquid chromatography or other higher apparatus[7,17,18]. However, TLC method is satisfactory for use in the present study.

Table 1: Recoveries (n = 3) of MG and LMG from raw and cooked samples determined in fortified Tilapia muscles at level of 100 ng g^{-1}

	MG		LMG	
Samples	Mean±SD (μg kg^{-1})	Recovery (%)	Mean±SD (μg kg^{-1})	Recovery (%)
Raw	63.8±7.3	83	92.5±8.9	89
Roasted[a]	51.6±7.7	67	85.5±9.0	82
Fried[b]	57.5±6.0	61	77.2±7.1	78
Microwaving[c]	61.1±5.5	59	52.4±9.1	70

[a]For 6 min, [b]For 4 min, [c]For 1 min

Table 2: Recoveries (n = 5) of MG and LMG from fortified fish muscles samples at 3 levels

	Recovery (%)	
Fortification level (ng g^{-1})	MG	LMG
25	75±3	60±2
50	81±4	72±4
100	86±5	79±3

Homogeneity of raw samples: Adequate homogeneity of raw Tilapia muscles which extend to each cooking methods was tested for analyzing results[19]. The standard deviation in a lot of the fish samples not exceeds the allowable fraction of the relative standard deviation. Average level of MG in incurred samples was 189 ng g^{-1} with relative standard deviation fewer than 15%, whereas, the level of LMG was 102 ng g^{-1} with relative standard deviation fewer than 12%. However, there are wide range of the results indicating uneven distribution of MG in the fish farms where may be stopped the flow of water, inadequate aeration and defect in the cleaning[20].

Effect of MG/MG levels on its recovery: Table 2 showed the effect of different concentrations of MG and LMG in fortified Tilapia muscles on recoveries levels were tested. Tilapia muscles were fortified at three levels (25, 50 and 100 ng g^{-1}). The average recoveries of MG were 79, 81 and 86%, respectively, with Relative Standard Deviations (RSD) ranged from 2-5%. The average recoveries of LMG were lower than that recorded for MG and RSD ranged from 2-4%. Similar findings were obtained where the reduction in malachite green level was different[20] according to the difference in the time, concentration, methods of application of MG and its purity and the varying concentration of residual impurities[21].

Effect of different cooking methods on MG/LMG contents: Changes in levels of MG and LMG residues during cooking were detected. The levels detected in raw muscles were adjusted for changing the sample weight resulting from the cooking procedures to give their real concentrations Table 3. Fish muscles subjected to cooking either via frying, roasting or microwaving. The results showed that cooking had effect in reducing the level of residues, where there were a significant reduction percentages in MG, while LMG residues showed lower reduction percentages. By calculation geometrically the internal temperature in the centre of the cooked sample was monitored for each cooking method. It was noticed that internal temperature in the fish muscles did not high above 100°C of any cooking method, as this temperature was not preserved for more than 12 min. The highest temperatures obtained were 99.6°C through frying, followed by 98.4°C during microwaving, then 96.4°C during roasting treatments Table 3. In contrary and for the exterior surfaces, the maximum temperature in the frying (150°C) was higher than any of the other processes and the required time for frying was 8 min. The weights shortage of all cooked samples was observed where the decrease of cooked samples weights ranged from 12-17, 11-35 and 8-53% of the initial weight of 5 g of raw Tilapia muscle, during roasting, frying and microwaving, respectively.

The obtained results showed that in roasted muscles MG level reduced by 48.4% in 12 min. The decrease of MG in fried samples was 51.6% in 8 min. The decrement of MG in cooked muscles by microwaving was fast and great, where MG was reduced by 80.8% in 2 min (Table 3). Concerning LMG, it was found to be more stable in cooked samples than MG. It seems that the time of cooking did not affect the LMG levels in cooked muscles, where the levels of LMG were reduced during different cooking methods to very small levels. For example roasting and frying methods reduced by 26.2 and 34.8%, respectively. However, it was observed that leucomalachite green is unstable only during microwaving where the greatest loss of LMG was 57.2% in 2 min. The lack of LMG through cooking by microwave did not linked with temperature but may be caused by time of cooking.

The fluids resulted from roasting fishes did not contain any residues of MG and LMG. Also, no juices were observed during the frying and microwaving muscles. The possibility of lack of both MG and LMG by leaching out of cooked Tilapia meat was excluded.

Table 3: Effect of cooking on MG and LMG residues in Tilapia muscle (5 g each)

			MG		LMG	
Cooking methods and times	[a]Temperature (°C)	Mass reduction±SD (%)	[b]Total±SD (ng)	Reduction (%)	[b]Total±SD (ng)	Reduction (%)
Roasting (min)						
0	3.8	0	663.0±21.7	0	485.6±11.6	0
4	89.7	12±1	538.0±17.7	18.9	402.5±08.6	9.5
8	94.8	15±0	448.5±13.1	32.4	317.2±15.3	12.4
12	96.4	17±4	342.1±11.6	48.4	345.4±14.8	26.2
Frying (min)						
0	3.7	0	783.1±34.6	0	535.7±28.5	0
4	90.9	11±0	626.7±24.9	20.0	402.5±07.8	12.7
6	96.4	24±0	511.1±27.2	34.7	377.2±25.7	27.1
8	99.6	35±1	379.1±13.7	51.6	332.1±16.5	34.8
Microwaving (min)						
0	3.8	0	652.1±24.5	0	518.1±06.8	0
0.5	89.0	9±2	493.8±17.2	34.3	384.8±23.9	28.5
1.0	97.5	34±5	337.2±14.4	58.3	267.8±15.0	40.1
2.0	98.4	53±3	125.5±12.5	80.8	193.2±20.1	57.2

[a]Internal temperatures in geometric centre of cooked Tilapia muscles, [b]Mean of three replicates

CONCLUSION

It could be concluded that the different factors affecting MG residue after cooking process were the time of cooking and temperature which play main floor in MG residue decrease. Microwaving was the only cooking procedure that caused a lack of LMG (57.2% in 2 min). However, both a high temperatures and cooking processes do not ensure a complete destruction of those hazards which may be existing in fish.

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