# Malachite green residues in farmed fish and the effect of some different cooking ways on it

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## Abstract

This study was carried out to determine the residues of malachite green (MG) in *Tilapia nilotica* (N=40) and *Mugil cephalus* (N=40) fish muscle tissues by using Enzyme Immune Assay (EIA). Samples of farmed fish were obtained from Ismailia and Port- said markets. MG residues were detected in *Tilapia nilotica* in one sample (2.5%) above the permissible limits. Residues in six samples (15%) were under the permissible limits and were not detected in 33 samples (82.5%). In *Mugil* fish, MG was detected in two samples (5%) above permissible and detected in nine samples (22.5%) under permissible limits but was not detected at all in 29 samples (72.5%). The effects of various cooking methods (boiling, frying and microwaving) on residues of malachite green (MG) in *Tilapia nilotica* and *Mugil cephalus* were investigated. The MG residues in cooked fish were determined by EIA. The results showed that in muscles cooked by boiling, MG reduced by 50% in *Tilapia nilotica* after boiling for 15 minutes, while residues were reduced by 30% in the *Mugil*. Frying in deep oil reduced MG by 45% and 55% in *Tilapia nilotica* and *mugil cephalus*, respectively. Microwave reduced MG by 30% and 29% after two minutes, 40% and 35% after three minutes and 50% and 46% after 5 minutes in *Tilapia nilotica* and *Mugil cephalus*, respectively. The results in this study showed that the high temperature does not guarantee a full breakdown of residue of MG and leucomalachite green (LMG) which may occur in *Tilapia nilotica* and *Mugil cephalus* fish muscles. In conclusion, the overall results showed that MG residues exceeded the Egyptian maximum permissible limits in 2.5 - 5% of the contaminted samples analyzed from the two different locations (Ismailia and Port- said markets). Since this study is limited to farmed fish *Tilapia nilotica* and *mugil cephalus,* more investigations should be carried out to determine the residues of MG in other farmed fish species.

**Keywords:** Malachite Green – farm fishes – chemical residues – cooking by microwave – boiling – frying

## Introduction

Aquaculture is currently the largest single source of fish supply in Egypt accounting for almost 65 percent of the total fish production of the country with over 99 percent produced from privately owned farms. As a result aquaculture is considered as the only viable option for reducing the gap between production and consumption of fish in Egypt. *Tilapia nilotica* has become the most important aquaculture species with a total harvest of about 390 280 tonnes, more

than 55 percent of the total aquaculture harvest in 2009 and the annual production of farmed *Mugil cephalus* is approximately 209 980 tonnes accounting for approximately 30 percent of the total aquaculture production in 2009 **(GAFRD, 2010 and Macfadyen *et al.*, 2011)**. The use of chemicals agents has helped in preventing and controlling the disease in aquaculture products.

Malachite green (MG), one of the most used chemical agents to meet those purposes has been used since 1933 **(Rahman *et al.*, 2005)**. However, MG is classified as a Class II Health Hazard and shows a significant health risk to humans through consumption of the fish that contain MG residues. In addition, MG is temperature stable and thus may not be degraded during routine fish processing **(Mitrowska *et al.*, 2007).** MG is a cationic triphenylmethane dye commercially available as the oxalate and hydrochloride salts. It is a metallic-looking crystal and dissolves in water easily as a blue-green solution. It is widely used in large scale in aquaculture as a parasiticide in food and other indusatries for one or more purposes, because of its controlling effect on fungal attacks, protozoan infections and helminthes on a wide variety of fish and other aquatic organisms.

MG and leucomalachite green (LMG) are both toxic to aquatic organisms and humans. MG is rapidly reduced into LMG and deposited in the fatty tissue of the fish with little MG remaining. LMG is very toxic to aquatic organisms as it is deposited in fatty tissue and remained for more than ten months after treatment **(Jiang *et al.*, 2009)**. LMG is found in high concentration in liver and gall bladder **(Sudova *et al.*, 2007)**. Furthermore, LMG will be slowly oxidized back to MG during storage or freezing of fish tissues **(Stammati *et al.*, 2005)**. Previous study demonstrated that this dye can be easily absorbed by fish tissues when it is entering water cycles and was reduced to LMG which is more persistent than MG **(Bauer *et al.*, 1988)**. These compounds may influence the immune and reproductive systems. It is also carcinogenic, mutagenic, teratogenic, induces chromosomal fractures and also reduces fertility in fish such as rainbow trout. MG sometimes acts as a respiratory enzyme poison and may damage the cell ability to produce energy for metabolic processes in fish tissues **(Srivastava *et al.*, 2004; Mitrowska *et al.*, 2005; Stammati *et al.*, 2005)**.

MG also has been found to be effective against white spot disease and ciliates **(Wong and Cheung, 2009)** and other disease in fish, fish eggs and crayfish **(Sudova *et al.*, 2007).** It is act as anti-parasitic, anti-fungal, anti-protozoan and plays a role in controlling skin and gill flukes **(Alderman, 1985 and 2002; Gerundo *et al.*, 1991; Liu *et al.*, 2009).** In African aquaculture, it has been used against infection by bacteria and protozoans **(Rintamaki-Kinnunen and Valtonen, 1997),** cestodes, trematodes, nematodes, crustaceans, etc. **(Hecht and Endemann, 1998).** Aquaculture industries have been using malachite green extensively as a topical

treatment by bath or flush methods without paying attention to the fact that topically applied therapeutants might also be absorbed systemically and produce significant internal effects. On the other hand, it is also used as a food colouring agent, food additive, a medical disinfectant and anthelminthic as well as a dye in silk, wool, jute, leather, cotton, paper and acrylic industries **(Culp and Beland, 1996)**.

The use of MG in food products has been prohibited in USA and European countries since 1983 **(Jiang *et al.*, 2009).** In 2002 the European Commission approved decision No 2002/657/EC in which stated that the minimum required performance limits (MRPLs) for total MG and LMG concentration was set at 2 μg/kg **(Sudova *et al.*, 2007)**.

In 2002, the largest numbers of positive tests of MG residue in aquaculture products were observed in Ireland followed by France, Austria and United Kingdom. However, in 2003, the number of positive results of MG residue decreased from 112 to 81 cases. Most of them are observed in United Kingdom, followed by France, Ireland and Austria **(Sudova *et al.*, 2007)**. In other cases, Hong Kong has imported fishes, crabs, eels and other aquaculture products from Taiwan and China in 2005, although their Health Department has found a trace of MG residues in the products. Furthermore, United States Food and Drug Administration (FDA) has detected the MG residues in imported seafood from China in year 2006. Consequently, the Food and Drug Administration has blocked the importation of several types of seafood in June 2007 **(Jiang *et al.*, 2009)**.

Although MG is heat stable its residue could be affected by cooking methods. The effects of various cooking methods on residues of malachite green (MG) and its major metabolite, leucomalachite green (LMG), in incurred carp muscles were investigated by **(Mitrowska *et al.*, 2007 and Farag *et al.*, 2012).**

The aim of this study was to detect trace amounts of MG residue in Egyptian farmed *Tilapia nilotica* and *Mugil cephalus* also to study the effects of various cooking methods (boiling, frying and microwaving) on residues of malachite green (MG) in examined fish.

## Material and methods

**Samples collection:**

A total of 80 fish samples of *Tilapia nilotica* and *Mugil cephalus*, (40 each) wear collected from fish markets at Ismailia and port-said governorates from January to August (2015) then transferred directly to the laboratory in an ice box to be investigated without delay.

**Reagents:**

Most of the reagents were contained in the Enzyme Immune Assay (EIA) test kit. Perchloric acid, Acetonitrile, Methanol and dichloromethane were of analytical grade. Malachite green standard solutions used for the calibration curve and spiked

samples at levels of 4 ppb, 2 ppb, 1 ppb, 0.5 ppb, 0.25 ppb and 0.125 ppb were all included in the EIA test kit.

**Apparatus:**

Microtiter plate spectrophotometer (450 nm), centrifuge and vortex mixer were used for the analyses.

**Sample preparation:**

Two grams of homogenised fish samples were placed in polypropylene tube, and 8 ml Perchloric acid/Acetonitrile solution (0.8 ml Perchloric acid diluted to one litre with acetonitrile) added. Tubes were vortex mixed for one minute then 4.5 ml dichloromethane (CH2Cl2) were added and vortex for ten minutes. The mixture was centrifuged at 4000rpm for five minutes at room temperature. Five ml of the supernatant were pipetted into a glass and evaporated to dryness at 50˚C. The residue was dissolved in 25µl methanol (100%) and 215µl sample dilution buffer. Dissolved residue was vortex for several minutes and centrifuged for ten minutes at 4000rpm at room temperature then 50µl of the solution were pipetted into the respective wells of the EIA plate.

**Experimental**

**Effect of cooking:**

Twenty five grams of 18 each of Tilapia nilotica and Mugil fish samples spiked with 4 ppb standard solution of malachite green (Sigma) fish, then 3 samples of each were cooked by boiling for 15 minutes, frying in deep oil and microwave for 2, 3 and 5 minutes, while 3 samples were left uncooked for comparison. The spiked samples either cooked or not were extracted in the same way mentioned before.

**EIA testing:**

All extracted samples, were subjected to EIA testing using EuroProxima Malachite Green EIA Kit (5161MG/LMG) as indicated by the manufacturer literature. The standard and samples were analysed in duplicate. To the marked microwells, 50µl of standards or samples were added. Then 25µl of diluted conjugate (malachite green-HRP) and 25µl of diluted antibody were added and after mixing gently by rocking the plate manually, the contents were incubated at 4˚C for 30 minutes. The liquid was poured out of the wells and after removal of liquid completely, all wells were filled with rinsing buffer (250µl). After rinsing, the buffer was also discarded; washing was repeated two more times. Then, 100µl of substrate were added into each well. After mixing thoroughly and incubating at room temperature in dark for 15 minutes, 100µl of stop solution was added and mixed well then, the absorbance was read at 450nm. The kit detects malachite green with 100% efficiency while, that for leuco-malachite green is 70%. The limit of detection of the test after extraction was 0.2ppb.

**Evaluation:**

In order to obtain the MG concentration in ppb present in the samples the concentration were read from the calibration curve for MG. For the construction of the calibration curve, the mean of the absorbance values obtained for the standards was divided by the absorbance value of the zero standard and multiplied by 100 (percentage maximum absorbance). The absorbance is inversely proportional to the MG.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard}} \times 100 = \% \text{ maximal absorbance}$$

**Calibration curve:**

The values (% maximal absorbance) calculated for the standards were plotted (on the Y-axis) versus the Malachite Green equivalent concentration (ppb) on a logarithmic X-axis. The calibration curve was virtually linear in the 4 – 0.125 ppb range **Fig (1).**

**Statistical analysis:**

A descriptive statistical analysis was performed to estimate the mean, minimum, maximum and standard error using the MEANS procedure of the Statistical Analysis System software **(SAS, 2004).**

## Results and discussion

**Sample screening:**

In the present experiment, the samples were collected from the farmed fish in Ismailia and Port-said governorate. A total of 80 fish samples of *Tilapia nilotica* and *Mugil cephalus,* and 40 each were analysed for level of malachite green by EIA. The assessment of MG residual levels in examined samples are shown in **Tables (1)** and **(2).** The samples collected from two different places. From Ismallia governorate we collected 15 samples of *Tilapia nilotica* and 20 samples of *mugil* and from Port-said 25 samples of *Tilapia nilotica* and 20 samples of *mugil.* MG residues were detected in *Tilapia nilotica* in one sample (2.5%) above the permissible limits, in six samples (15%) under the permissible limits and was not detected in 33 samples (82.5%). In *Mugil cephalus* fish MG was detected in two samples above the permissible limits (5%), detected in nine samples (22.5%) under the permissible limits but not detected at all in 29 samples (72.5%). The mean values of MG levels were 0.3125 ±0.11 ppb and 0.475 ±0.12 ppb in *Tilapia nilotica* and *Mugil cephalus* samples, respectively. Higher results have been obtained by several authors. For instance, **Bilandžić *et al.*, (2012**) determined malachite green in 18.1% of 42 carp (*Cyprinus carpio*) and 30 rainbow trout (Oncorhynchus mykiss) collected from fish farms in Croatia from 2009 to 2011 by EIA. Results from routine monitoring investigations at the Institute of Ichthyology in Cuxhaven carried out in 2005 yielded 14 positive detects out of 166 investigated fish tissue samples. The highest residues were measured in caviar of

trouts from Sweden (619 µg/kg) **(Laves, 2005a)** and in an eel sample from China (3911 µg/kg) **(Laves, 2005b)**. In 2005, investigations by the Hong Kong Health Department revealed that freshwater fish, crabs and other aquaculture products from China contained MG **(Xiaomin, 2005)**. Hong Kong's Food & Environmental Hygiene Department confirmed that 11 of 14 eel-based products tested from local supermarkets contained high levels of MG up to 4,500 µg/kg and up to 900 µg/kg for fresh water fish The Hong Kong Health Department also released further test results showing that eight types of freshwater fish from China also contained malachite green, including grass carp, mandarin carp, milk fish, snakehead fish and California perch **(Xiaomin, 2005)**. However, **Hashimoto, *et al.* (2012)** analysed *Tilapia* samples (n = 20) commercialized in Campinas, SP, Brazil. None of the samples presented detectable levels of MG or LMG residues.

**Cooking effect:**

In present study fish samples either *Tilapia nilotica* or *Mugil* were subjected to three cooking methods boiling, frying and microwave as shown in **Tables (3)** and **(4).** The mean value of MG levels in uncooked, boiled and fried *Tilapia* samples were 4 ± 0.11 ppb, 2.03± 0.11 ppb and 2.3± 0.11 ppb, respectively. However in *Mugil*, the mean value of MG levels in uncooked, boiled and fried samples were 4.1 ±0.11 ppb, 2.87 ±0.11 ppb and 1.84 ±0.11 ppb, respectively. Thus MG was reduced by 50% and 30% in *Tilapia nilotica* and *Mugil cephalus*, respectively after boiling for 15 minutes. Frying in deep oil for ten minutes reduced MG by 45% and 55% in *Tilapia nilotica* and *Mugil cephalus*, respectively. Frying was more effective in reduction of MG than boiling in Mugil, while boiling was more effective in *Tilapia nilotica*. These differences may be due to higher content of fat in *Mugil* than *Tilapia nilotica* as *Mugil* fat content ranged from 9% to 13% **(Mostafa and Salem, 2015),** while fat content in *tilapia nilotica* about 2% **(Salihu-Lasisi *et al*., 2013).** MG and LMG are high in fatty fish whereas the distribution of MG depends on the fat content in the fish tissue **(Jiang *et al*., 2009).** The mean values of MG levels in microwaved *Tilapia* samples were 2.8 ±0.02 ppb, 2.4 ±0.02 and 2 ±0.02 after two, three and five minutes, respectively, while these values were 2.84 ±0.02 ppb, 2.6 ±0.02 and 2.21 ±0.02 after two, three and five minutes, respectively, in *Mugil cephalus* samples. Microwave reduced MG by 30% and 29% after two minutes, 40% and 35% after three minutes and 50% and 46% after five minutes in *Tilapia nilotica* and *Mugil cephalus*, respectively. A study of the effects of cooking on MG has shown that boiling in water has little effect on the level of LMG, while reduced the MG by 54% in carp muscle. However, microwave cooking of fish resulted in some decomposition of LMG about 40%, while MG reduced by 60% **(Mitrowska *et al*., 2007)**, while **Farag *et al*., (2012)** found that the levels of malachite green in the scalding (boiling) and microwave treated samples were 52.51% and 59.98% respectively in the

examined fresh *Oreochromis niloticus* samples. The lesser variation between the reduction effect of scalding (boiling) and microwave may be explained as malachite green is stable at 100°C and the internal temperature of the meat by cooking (boiling and microwave) did not rise above 100°C, and this cooking involved not more than 15 minutes where the highest achieved temperature were 96.4 °C and 99.6°C during boiling and microwave treatment respectively **(Mitrowska *et al.*, 2007).** Greater reduction or elimination of malachite green from fish needs higher temperature and more time than the normal cooking conditions. So cooking by high temperature was not a guarantee for full and complete breakdown of the malachite green residues which may occur in fish. Our results revealed that boiling is the best way in controlling MG in Tilapia while frying is the best in *Mugil cephalus*.

**Table (1): Occurrence level of MG residues in *tilapia nilotica*.**

| Governorate | Sample No. | +ve | | above (MRPL) | | ≤(MRPL) | | ND | | Min ppb | Max ppb | Mean ±SE ppb |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % | No. | % | | | |
| Ismailia | 15 | 3 | 20 | 1 | 6.6 | 2 | 13.3 | 12 | 80 | 0 | 2.5 | 0.413 ±0.11 |
| Port-said | 25 | 4 | 16 | 0 | 0 | 4 | 16 | 21 | 84 | 0 | 1.8 | 0.252 ±0.11 |
| Total | 40 | 7 | 17.5 | 1 | 2.5 | 6 | 15 | 33 | 82.5 | 0 | 2.5 | 0.3125 ±0.11 |

ppb = Part per billion. ND = Non detectable level (< 0.2 ppb). Min. = Minimum. Max. = Maximum. SE = Standard error.

**Table (2): Occurrence level of MG residues in *Mugil cephalus.***

| Governorate | Sample No. | +ve | | above (MRPL) | | ≤(MRPL) | | Not detected | | Min ppb | Max ppb | Mean ±SE ppb |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % | No | % | | | |
| Ismailia | 20 | 6 | 30 | 1 | 5 | 5 | 25 | 14 | 70 | 0 | 2.1 | 0.53 ±0.12 |
| Port-said | 20 | 5 | 25 | 1 | 5 | 4 | 20 | 15 | 75 | 0 | 2.3 | 0.425 ±0.12 |
| Total | 40 | 11 | 27.5 | 2 | 5 | 9 | 22.5 | 29 | 72.5 | 0 | 2.3 | 0.475 ±0.12 |

ppb = Part per billion. ND = Non detectable level (< 0.2 ppb). Min. = Minimum. Max. = Maximum. SE = Standard error.

**Table (3): MG reduction after boiling and frying of spiked *Tilapia nilotica* and *Mugil cephalus* (mean of three trials).**

| Fish type | Level of MG before cooking | | | Level of MG after boiling for 15 minutes | | | | Level of MG after frying in deep oil for 10 minutes | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Min ppb | Max ppb | Mean ppb | Min ppb | Max ppb | Mean ppb | % of reduction | Min ppb | Max ppb | Mean ppb | % of reduction |
| *Tilapia nilotica* | 3.8 | 4.1 | 4±0.1 | 2 | 2.1 | 2.03 | 50 | 2.25 | 2.35 | 2.3 | 45% |
| *Mugil cephalus* | 3.7 | 4.2 | 4.1 | 2.85 | 2.89 | 2.87 | 30 | 1.8 | 1.85 | 1.84 | 55% |

ppb = Part per billion. ND = Non detectable level (< 0.2 ppb). Min. = Minimum. Max. = Maximum. SE = 0.11.

**Table (4): MG reduction after microwave of spiked *Tilapia nilotica* and *Mugil cephalus* (mean of three trials).**

| Fish type | Level of MG after Microwave | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 minutes | | | | 3 minutes | | | | 5 minutes | | | |
| | Min ppb | Max ppb | Mean ppb | Reducti-on % | Min ppb | Max ppb | Mean ppb | Reducti-on % | Min ppb | Max ppb | Mean ppb | Reducti-on % |
| *Tilapia nilotica* | 2.78 | 2.85 | 2.8 | 30 | 2.3 | 2.6 | 2.4 | 40 | 1.9 | 2.1 | 2 | 50 |
| *Mugil cephalus* | 2.78 | 2.85 | 2.84 | 29 | 2.55 | 2.65 | 2.6 | 35 | 2.1 | 2.24 | 2.21 | 46 |

ppb = Part per billion. ND = Non detectable level (< 0.2 ppb). Min. = Minimum. Max. = Maximum. SE = 0.02.

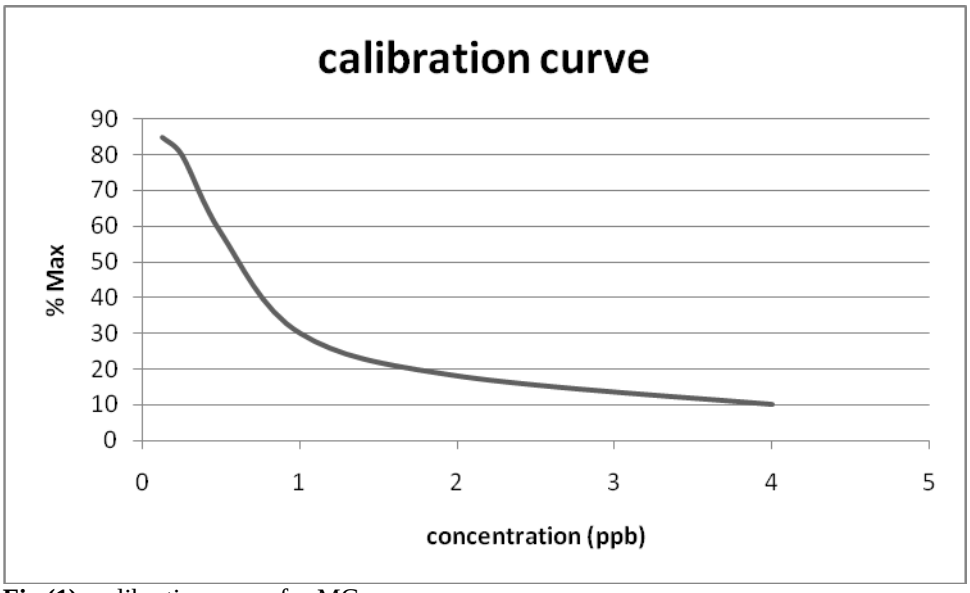


**Fig (1):** calibration curve for MG.

## Conclusions

MG and LMG residue remains for a long time in edible fish tissues and it may pose toxicity and be harmful to human health when consumers eat contaminated fish. The sum of MG and its metabolite LMG aggregate concentration was set at 2 µg/kg, stated as the minimum required performance limit (MPRL) that permitted in aquaculture industry either followed the EU or CODEX limits. The findings of this investigation gave no evidence for the illegal use of MG in Egypt, but these results do not exclude the possibility of misuse of these potentially harmful chemicals in future. Therefore, there is need to routinely monitor theses chemicals as food quality and health control measures. Our results showed that the high temperature does not guarantee a full breakdown of residue of MG and LMG which may occur in *Tilapia nilotica* and *Mugil cephalus* muscles.

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