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DETERMINATION OF MALACHITE GREEN RESIDUES IN FARMED FISH IN EL-FAYOUM GOVERNORATE

By

Jehan R. Daoud[1], Jehan M. Ouf[2], Mohamed A. Maky[1] and Abd - Elrahman S. Ali[*3]

[1] Department of Food Hygiene and Control, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt.

[2] Department of Food Hygiene, Animal Health Research Institute, Dokki, Giza.

[3] Department of Food Hygiene, Animal Health Research Institute (El- Fayoum branch laboratory).

*Corresponding author: abdelrahmanali515@gmail.com.

ABSTRACT

Malachite green (MG) is still being used as an antiparasitic and antifungal agent in aquaculture. This is due to the fact that many farmer owners are not aware of the potential genotoxic and carcinogenic properties of MG. One hundred fish samples were collected from different fish farms at El- Fayoum governorate (Egypt), and the incidence of Malachite green (MG) residue was determined by using an enzyme-linked Immunosorbant assay (ELISA). The data obtained in the present work proved that 59 samples of fish out of 100 (59%) showed the presence of malachite green residues in *Oreochromis niloticus* and *Mugil cephalus* samples with an incidence of 28 (56%) and 31 (62%). The mean values of malachite green levels in the examined *Oreochromis niloticus* and *Mugil cephalus* samples were 1.603 ±0.165 ppb and 1.244 ±0.114 ppb. Heat treatment has high effect on malachite green residues, as mean values before and after frying were 2.49±0.234 and 0.94±0.277, for roasting 2.49±0.234 and 1.62±0.245 and for microwaving 2.49±0.234 and 0.62±0.233 respectively, with reduction percent for frying, roasting and microwaving were 62.25 %, 34.94 % and 75.1 % respectively. The results revealed that, freezing had trivial effect on malachite green residues, as reduction percent after freezing for three months was 0.8 %. Statistical analysis by using One-Way ANOVA test revealed that there are highly significant differences ($p<0.01$) between different mean values and different reduction percent recovered from frying, roasting and microwaving. Comparing the results of malachite green residues in examined *Oreochromis niloticus* and *Mugil cephalus* samples with Commission Regulation (EU, 2004) for maximum residues limits, it was clear that 16 (16%) of examined samples were more than MRLs which is 2 µg/kg.

Keywords:

Malachite Green-ELISA- Heat Treatment (Frying-Roasting - Microwaving)- Freezing.

INTRODUCTION

Malachite green is a cationic triphenylmethane dye commercially available as the oxalate and hydrochloride salts. It is a metallic-looking crystal and dissolves in water easily forming a blue-green solution. It is widely used in large scale in aquaculture as a parasiticide in food and other industries 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 **(El-ghayaty *et al.,* 2016)**.

Due to effectiveness of malachite green and relatively low cost, it is a procurable agent for freshwater fish farmers. However, its safety, and that of its metabolite leucomalachite green (LMG), has never been established. In fish tissue, malachite green is rapidly metabolized to the reduced,colourless compound,leucomalachite green.The major metabolite, leucomalachite green, is retained in fish muscle and fat much longer due to its lipophilic nature, and therefore the majority of the intake of malachite green would be in the leuco form **(Stammati *et al.,* 2005; Mitrowska *et al.,* 2008).**

Malachite green 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 toxic to aquatic organisms and humans. It 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).** It 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 theimmune and reproductive systems. It is also carcinogenic, mutagenic and 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).**

It 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 acts as anti-parasitic, anti-fungal, and anti-protozoan and plays a role in controlling skin and gill flukes **(Alderman, 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, and a medical disinfectant and anthelminthic as well as a dye in silk, wool, jute, leather, cotton, paper and acrylic industries **(Culp and Beland, 1996).** It's usage in food products have 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 2µg/kg**.** In 2002, it added that, 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).**

Fish containing MG and its major metabolic, leucomalachite green cause significant health hazard for humans who eat contaminated fish. They have mutagenic, carcinogenic and teratogenic effects based on its structural similarity to known carcinogens, for example cause bladder cancer and liver tumor in human **(Culp *et al.,* 2002).**The aim of this study was assessment of malachite green residues in fish meat of farmed fish (*Oreochromis niloticus* and *Mugil cephalus*) in EL-Fayoum city by enzyme linked Immunosorbant assay (ELISA), studying the effect of different heat treatment (frying, roasting and microwaving) and freezing storage on malachite green residues in examined fish.

MATERIAL AND METHODS

Samples collection:

A total of one hundred fish samples of *Oreochromis niloticus* and *Mugil cephalus*, (fifty each) were collected from different fish markets at El- Fayoum governorate. The collected samples were subsequently rapidly transported under complete aseptic condition to laboratory of the Department of Food hygiene, Animal Health Research Institute in Dokki, Giza. Each sample weigh 300-350 g was analyzed for determination of malachite green residues. Each positive sample which contains malachite green residues above the permissible limit was divided into four parts, forming four groups. The first group was treated with frying, the second with roasting, the third with microwaving, and the fourth kept at freezing storage for three months to study the effect of different heat treatment (frying, roasting and microwaving) and freezing storage on malachite green residues.

Reagents:

Most of the reagents were contained in the Enzyme-linked Immunosorbant assay (ELISA) test kit. Perchloric acid, Acetonitrile, Methanol and dichloromethane were of analytical grade. Malachite green standard solutions used for the calibration curve at levels of 4.05 ppb, 1.35 ppb, 0.45 ppb, 0.15 ppb, 0.05 ppb and 0 ppb were all included in the ELISA test kit.

Apparatus:

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

Sample preparation: method according to BIOMATIC Malachite Green ELISA Kit (Catalog Number: EKC40009)**.**

1.00±0.05g of the homogenized sample was weighed, and put in 10 mL centrifugal tube. 200 µL of Extractant, 1g Alumina-N and 4 ml of acetonitrile were added. The mixture was shacked for 2 min. Then it was centrifuged at 4000 rpm for 5 min. 2ml of supernatant was transferred into a new centrifugal tube, and 5 µL of Oxidant was added, then they were shacked properly for 10 second. The sample was dried by blowing nitrogen gas at 65°C. 50 µL of methanol was added, the mixture was shacked lightly; and 450 µL of sample diluent was added, and they were shacked properly for 30 seconds. 50 µL of sample was taken for further analysis.

Experimental work:

Heat treatment:

Frying:

Positive samples with malachite green residues above the permissible limit 2 µg/kg were placedseparatelyonfrying pancontainingoil at 160-180°Cfrying for15minutes.

After cooling, the processed samples were subjected for the assessment of malachite green level by ELISA.

Roasting:

Positive samples with malachite green residues above the permissible limit 2 µg/kg were placed on metal tray and cooked by chewing for 15 minutes. After cooling, the processed samples were subjected for the assessment of malachite green level by ELISA.

Microwaving:

Positive samples with malachite green residues above the permissible limit 2 µg/kg were placed in microwave at 220 °C for 20 minutes. After cooling, the processed samples were subjected for the assessment of malachite green level by ELISA.

Effect of freezing storage on malachite green residues:

Muscle samples proved to contain malachite green residues above the permissible limit 2µg/kg were kept at -20°C and examined for the presence of malachite green residues after three months.

ELISA testing:

All extracted samples, were subjected to ELISA testing using BIOMATIC Malachite Green ELISA Kit (Catalog Number: EKC40009): as indicated by the manufacturer literature. All reagents and samples were brought to room temperature (20~25°C) before use.

All samples and standards were assayed in duplicate. All reagents and samples were prepared as directed in the previous sections. The numbers of wells to be used were determined and any remaining wells and the desiccant were put back into the pouch and sealed the Ziploc, stored unused wells at 2-8°C. Fifty µL of Standard or Sample were added per well.

Then 50 µL of HRP-conjugate and 50 µL were added of antibody to each well. The microtiter plate was covered with a new adhesive strip and mixed well, and then incubated for 30 min at 25℃. Each well was aspirated and washed, repeating the process four times. Each well was washed by filling with 250 µL of Wash buffer (1x) and let to stand for 30 seconds, complete

removal of liquid at each step is essential for good performance. After the last wash, any remaining liquid was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels. 100 µL of TMB-substrate was added to each well, mixed well and incubated for 15 minutes at 25°C. 50 µL of stop solution was added to each well, the plate gently taped to ensure thorough mixing. The optical density of each well was determined within 5 min, using a microplate reader set to 450 nm (Recommend to read the OD value at the dual-wavelength: 450/630 nm within 5 min). The limit of detection of the test after extraction was 0.3 ppb.

Calculations:

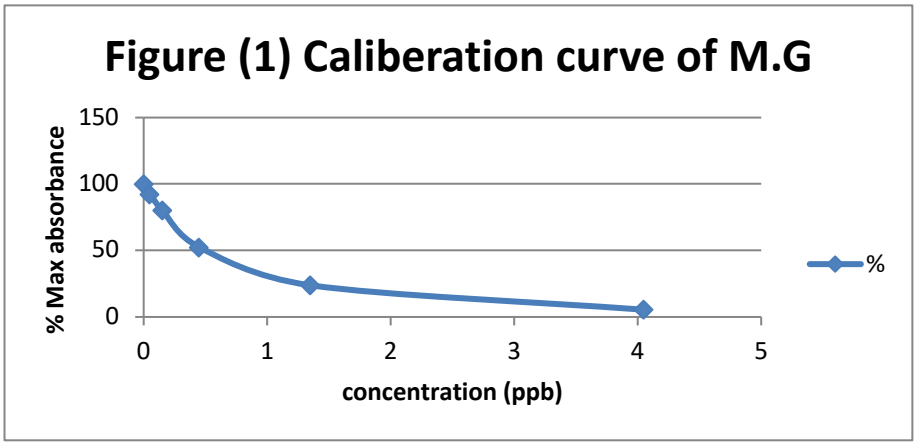
In order to obtain the MG concentration in ppb present in the samples, the concentration was read from the calibration curve for MG. For the construction of the calibration curve, the mean of the absorbance values obtained for the standards was divided by the absorbance value of the zero standard and 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 calculated values (% maximal absorbance) for the standards were plotted (on the Y-axis) versus theMalachiteGreenequivalent concentration(ppb) on a logarithmicX-axis.

The calibration curve was virtually linear in the 4 -0.125 ppb range Fig. (1).



Statistical analysis:

The statistical analysis was performed using chi-square test, t-test and ANOVA test of the SPSS software version 20 for windows (SPSS Inc., Chicago, IL, USA) to compare mean values and the significance was tested as $\alpha = 0.05$.

RESULTS AND DISCUSSION

In the present work, the samples were collected from farms at EL-Fayoum governorate. A total of one hundred fish samples of *Oreochromis niloticus* and *Mugil cephalus,* (fifty of each) were analysed for the level of malachite green residues by ELISA. Incidence of malachite green residual levels in the examined *Oreochromis niloticus* and *Mugil cephalus* samples were shown in (Table 1). Malachite green was detected in the examined *Oreochromis niloticus* and *mugil cephalus* samples with an incidence of 28 (56%) and 31 (62%), with an incidence of 59 (59%) of all the samples. This result was less than that recorded by **Schuetze *et al.,* (2008) and Barani and Tajik (2017)** who recorded an incidence of 83.31% and 61% in farmed fish, and higher than the results recorded by **Rasmussen (2007); Bilandžić *et al.,* (2012); Farag *et al.,* (2012); Fu *et al.,* (2013); Adel *et al.,* (2016) and El-ghayaty *et al.,* (2016)** who recorded an incidence of 55.55%, 18.1%, 18.66%, 56.1%, 58.4% and 17.5% in farmed fish respectively. In (Table 2) the mean values of malachite green levels in the examined *Oreochromis niloticus* and *Mugil cephalus* samples were 1.603 ±0.165 ppb and 1.244 ±0.114 ppb. Higher results were obtained by **Xiaomin, (2005)** (900-4500 µg/kg), while lower results were obtained by **Bilandžić *et al.,* (2012)** (0.231 µg/kg). MG residue was identified in various food products in Egypt; and it was more prevalent in Ismailia and Port- said markets **(El-ghayaty *et al.,* 2016)** and at Kafr El-Sheikh farmed fish **(Farag *et al.,* 2012).** In India; it was more prevalent in foodstuffs from rural markets than those from urban markets **(Tripathi *et al.,*2007).** In Slovenia, 7 out of 33 trout samples, obtained from fish farms and markets, contained residues of MG **(Bajc *et al.,*2007).** In a survey conducted in Croatia, MG residue was detected in 18.1% of farmed fish samples over a three-year period **(Bilandzic *et al.,* 2012)**. In a recent study in China, 56.1% of freshwater fish samples contained residues of MG, ranging between 0.5 and 148 mg/kg **(Fu *et al.,* 2013).** In (Table 3) malachite green residues were detected less than maximum residue limits (MRLs) in *Oreochromis niloticus* and *Mugil cephalus* in 38 (76%) and 46 (92%) samples and more than

maximum residue limits (MRLs) in 12 (24%) and 4 (8%) samples. In 2002, the Directive 2002/657/EC was published, which deals with the performance of analytical methods and interpretation of the results, and defines the maximum residue limits (MRL) and minimum required performance limit (MRPL) applicable to the determination of contaminants in food. Directive **European Commission, (2004)** adds an MRPL equal to 2 μg kg^{-1} for the sum of MG and LMG in aquaculture products to the previous directive. Comparing the results of malachite green residues in all examined samples (n=100) with **Commission Regulation (EU, 2004)** for maximum residues limits, it is clear that 84 samples (84%) were less than permissible limit, while 16 samples (16%) were more than permissible limit which is 2μg/kg. From the public health significant, the fish which exceed the permissible limits of malachite green according to **European Commission (2004) (2 μg/kg)** consider unfit for human consumption and cause significant health hazard for humans who eat contaminated fish **(Culp *et al.,* 2002).** On the other hand, FAO Directive 07/2005/QD-BTS also includes MG in the list of banished chemical compounds used in aquaculture **(FAO, 2005) (Hashimoto *et al.,* 2011)**. In (Table 4) frequency distribution of examined *Oreochromis niloticus* and *Mugil cephalus* samples, showed that out of 100 samples were detected for malachite green residues 10 (20%), 11 (22%), 6 (12%), 16 (32%), 12 (24%) and 4 (8%) 0.3 to < 1ppb, 1 to < 2ppb and ≥ 2ppb respectively. Out of 100 samples 21 (21%), 22 (22%) and 16 (16%) were detected formalachitegreen residues(0.3to<1ppb,1 to<2ppband≥2ppb) respectively.

From (Table 5) concerning the frying effect on malachite green residues in positive samples above MRLs showed that frying has high effect on malachite green residues, as mean values before and after frying were 2.49±0.234 and 0.94 ± 0.277 respectively, with reduction percent of 62.25 %. These results approximately agree with the results recorded by **Mitrowska *et al.* (2007);El-ghayaty*et al.*(2016)and Shalaby *et al.*(2017)** who recorded a reduction percentage of frying 49 %, 50 % and 51.6 % respectively. From (Table 6) concerning the roasting effect on malachite green residues in positive samples above MRLs showed that roasting has high effect on malachite green residues, as mean values before and after roasting were 2.49 ± 0.234 and 1.62 ± 0.245 respectively, with reduction percent of 34.94 %. This result was less than the result recorded by **Shalaby *et al.* (2017)** who recorded the reduction percent of roasting on malachite green residues of 48.4 %. From (Table 7) concerning the microwaving effect on malachite green residues in positive samples above MRLs showed that microwaving has

highly effect on malachite green residues, as mean values before and after microwaving were 2.49 ± 0.234 and 0.62 ± 0.233, with reduction percent of 75.1 %. This result was less than the results recorded by **Mitrowska *et al.*, (2007); Shalaby *et al.*,(2017)** who recorded a reduction percentage of microwaving 97 % and 80.8 %, and more than the results recorded by **Farag *et al.*, (2012);** who recorded a reduction percentage of microwaving 59.98 %. (Table 8) showed the correlation between the different mean values of malachite green residues samples recovered from frying, roasting and microwaving which were 0.94 ± 0.277, 1.62 ± 0.245 and 0.62 ± 0.233 respectively with least significant difference of 0.17, statistical analysis by using One-Way ANOVA test revealed that there are highly significant differences ($p<0.01$) between different mean values recovered from frying, roasting and microwaving. (Table 9) showed the correlation between the different reduction percent of malachite green residues samples recovered from frying, roasting and microwaving were 62.16 %, 34.17 % and 75.35 respectively with least significant difference of 7.5, statistical analysis by using One-Way ANOVA test revealed that there are highly significant differences ($p<0.01$) between different reduction percent recovered from frying, roasting and microwaving. This indicates that, the heat treatment reduces the levels of malachite green but not eliminates it from fish muscle. Also, there are greater differences between the reduction percent of malachite green residues by frying, roasting and microwaving. From (Table 10) concerning the freezing effect for three months on malachite green residues in positive samples above MRLs it is showed that, freezing has trivial effect on malachite green residues, as mean values before and after freezing were 2.49 ±0.234 and 2.47±0.232 respectively, with reduction percent of 0.8 %. Appropriate MRLs need to be set by the regulatory body in the country and should be followed and enforced. Fish farmers should have awareness about the best fish practices to prevent infection and avoid use of malachite green. Fish and fish products should be used of good known source. Organic fish farming may be encouraged by providing appropriate incentives to the farmers in form of subsidy. Education programs should be improved to raise the awareness for workers, processors and handler.

Good manufacturing practice (GMP) should be followed in order to assure safety and quality of fish and fish products.

Table (1): Incidence of malachite green residues in examined *Oreochromis niloticus* and *Mugil cephalus* fish samples.

Type of sample	Total number	Positive		ND	
		No	%	No	%
Oreochromis niloticus	**50**	**28**	**56***	**22**	**44***
Mugil cephalus	**50**	**31**	**62***	**19**	**38***
Total	**100**	**59**	**59**	**41**	**41**
χ^2 value	**0.37^{NS}**				
P value	**0.54**				

^{NS} non-significant

*** There are no significant differences (P > 0.05) between *Oreochromis niloticus* and *Mugil cephalus* fish samples.**

Table (2): Statistical analytical results of malachite green residues (ppb) recovered from fresh *Oreochromis niloticus* and *Mugil cephalus* samples.

Items	Samples (n=50)		Total (n=100)
	Oreochromis niloticus	***Mugil cephalus***	
Min.	**< 0.3**	**< 0.3**	**< 0.3**
Max.	**2.84**	**2.81**	**2.84**
Mean	**1.603***	**1.244***	**1.414**
S.E.	**0.165**	**0.114**	**0.1**
t- test	**-1.82^{NS}**		
P- value	**0.074**		

^{NS} non-significant

*** There are no significant differences (P > 0.05) between the mean of *Oreochromis niloticus* and *Mugil cephalus* fish samples.**

Table (3): Incidence of malachite green residues in examined Samples of *Oreochromis niloticus* and *Mugil cephalus*.

Examined samples	Less than MRLs		More than MRLs	
	No	%	No	%
Oreochromis niloticus	**38**	**76***	**12**	**24***
Mugil cephalus	**46**	**92***	**4**	**8***
Total	**84**	**84**	**16**	**16**
χ^2 value	**4.76***			
P value	**0.029**			

***There are significant differences (P < 0.05) between less and more than MRLs of *Oreochromis niloticus* and *Mugil cephalus* fish samples. Maximum Residue Limits (MRLs) in µg/kg: according to Commission Regulation (EU) (2004) (2 µg/kg).**

Table(4): Frequency distribution ofmalachite green levels (expressed as ppb) for *Oreochromis niloticus* and *Mugil cephalus* samples (n=100).

Levels range (ppb)	*Oreochromis niloticus*		*Mugil cephalus*		Total	
	No	%	No	%	No	%
< 0.3 ppb	22	44	19	38	41	41
0.3 to< 1 ppb	10	20	11	22	21	21
1 to< 2 ppb	6	12	16	32	22	22
≥ 2 ppb	12	24	4	8	16	16

Table (5): Correlation between mean values of malachite green residues before and after frying (n=16).

Items	Frying	
	Before	After
Min.	2.13	0.55
Max.	2.84	1.3
Mean	2.49**	0.94**
Standard deviation	0.234	0.277
Reduction %	62.25 %	
t- test	17.08**	
P- value	0.000	

**** Highly significant**

**** There are highly significant differences (P < 0.01) between means before and after frying.**

Table (6): Correlation between mean values of malachite green residues before and after roasting (n=16).

Items	Roasting	
	Before	After
Min.	2.13	1.2
Max.	2.84	2.1
Mean	2.49**	1.62**
Standard deviation	0.234	0.245
Reduction %	34.94 %	
t- test	10.2**	
P- value	0.000	

**** Highly significant.**

**** There are highly significant differences (P < 0.01) between means before and after roasting.**

Table (7): Correlation between mean values of malachite green residues before and after microwaving (n=16).

Items	Microwaving	
	Before	After
Min.	2.13	0.3
Max.	2.84	1.12
Mean	2.49**	0.62**
Standard deviation	0.234	0.233
Reduction %	75.1 %	
t- test	22.61**	
P- value	0.000	

**** Highly significant**

****There are highly significant differences(P<0.01) between means before and after microwaving.**

Table (8): Correlation between the different mean values of malachite green residues samples recovered from (frying, roasting and microwaving) (n=16).

Items	Frying	Roasting	Microwaving
Mean	0.94	1.62	0.62
LSD	0.17		
Calculated F	72.02**		
P- value	0.000		

LSD: Least significant difference

**** Highly significant by using one way ANOVA test**

**** There are highly significant differences (P < 0.01) between different means recovered from (frying, roasting and microwaving).**

Table (9): Correlation between the different reduction percent of malachite green residues samples recovered from (frying, roasting and microwaving) (n=16).

Items	Frying	Roasting	Microwaving
Reduction %	62.16	34.17	75.35
LSD	7.5		
Calculated F	62.98**		
P- value	0.000		

LSD: Least significant difference

**** Highly significant by using one-way ANOVA test**

**** There are highly significant differences (P < 0.01) between different reduction percent recovered from (frying, roasting and microwaving).**

Table (10): Correlation between mean values of malachite green residues before and after freezing (n=16).

Items	Freezing	
	Before	After
Min.	2.13	2.13
Max.	2.84	2.84
Mean	2.49	2.47
Standard deviation	0.234	0.232
Reduction %	0.8 %	

CONCLUSIONS

The data obtained in present work proved that malachite green was detected in the examined samples with an incidence of 59 (59%) of all the samples. Malachite green was detected in the examined *Oreochromis niloticus* and *Mugil cephalus samples* with an incidence of 28 (56%) and 31 (62%) respectively. Heat treatment had highly effect on malachite green residues, as reduction percent after frying, roasting and microwaving were 62.25 %, 34.94 % and 75.1 % respectively. The results revealed that, freezing had trivial effect on malachite green residues, as reduction percent after freezing three months 0.8%. Fifty nine percent of examined samples contained malachite green residues which indicate obvious use of malachite green in fish farming. Malachite green residue remains for a long time in edible fish tissues and it may pose toxicity and be harmful to human health. Malachite green should be banned and completely prohibited in farmed fish so we are in need for stricter regulation due to carcinogenicity and their potential harmful effect on human health. Educational programs should be improved to raise the awareness for workers, processors and handler.

Hazard Analysis Critical Control Point (HACCP) system should be strictly applied.

Hygienic practice should be strictly followed and enforced to make the fish meat safer for human consumption. Careful periodically malachite green residues examination of fish and fish products. Fish and fish products should be used of good known source. Much more concerns must be given to the cooking regime by efficient cooking of fish meat immediately before eating.

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