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Comparative Study on Determination of Histamine Using HPLC and ELISA in Marine Fish and Control of Excessive Histamine in Fish Fillet by Natural Oils

Reyad R. Shawish[1], Zakaria Hassan[1], Walaa Abd-Elmegid[2]

[1]Department of Food Hygiene and Control, Faculty of Veterinary Medicine, University of Sadat City, Egypt.

[2]Department of nutrition, Faculty of medicine, Benha University, Egypt.

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Correspondence to:
zico76us@yahoo.com

Abstract

Our a complete of 60 samples of fresh marine fishes represented by Sardine, Saurus, Barboni and Chrysophyres were collected at different times from various fish markets in Alexandria governorate, Egypt and examined for determination of their levels of histamine by HPLC from one side and ELISA from the other side to study the accuracy of the two techniques. Results showed that all of specimens contained detectable value of histamine but the highest unacceptable sample in sardine fish and lowest unacceptable sample in Chrysophyres fish and there is no significant differences ($P<0.01$) as indicated by HPLC and ELISA test for detection of histamine. Further, some trials to control the formation of such serious histamine level (mg %) were treated by using 1% essential oils (Rosemary oil, Garlic oil and Lemon oil) by the use of HPLC. The outcomes indicated that the development of histamine formation become simply decreased by the percentage of 15.2% ,47.8 and 58.2% with addition of Rosemary oil (1%) for 2 ,4 days and 6 days ,respectively; 9.9 % , 35.3% and 45.7% after addition of garlic oil(1%) for two ,four days and six days , respectively and the best reduction degree in histamine development changed into recorded through the addition of Lemon oil (1%) 2.1% , 21.5% and 29.3% for two ,four days and six days ,respectively. In conclusion, such results can propose that the essential oil treatment to the fish fillets might be helpful in reduction of histamine formation so that it will produce fish meat of excessive best and protection.

1. INTRODUCTION

Histamine fish poisoning is a foodborne chemical intoxication as a result of bacterially spoiled fish or seafood fish. Histamine fish poisoning outbreaks which include Sardine, Saurus, Barboni and Chrysophyres, which contain high quantities of histidine of their muscle were implicated in histamine fish poisoning. (Phuvasate and Su, 2010; Hungerford, 2010). The growth of bacterial species which includes Morganella morganii, Proteus vulgaris, Klebsiella pneumoniae and Enterobacter aerogenes which produce histidine decarboxylase causes the formation of histamine in fish at some stage in storage. This foremost toxin, histamine, causes mild to extreme allergies. Histamine is warmth-resistant and solid, thus it couldn't be removed by means of freezing, cooking or smoking methods. The price of histamine manufacturing in fish increases whilst uncovered to higher temperatures. At gift, the pleasant exercise to prevent histamine fish poisoning is soaking fish in ice or bloodless seawater at once after catching and retaining fish at 4°C. However, histamine-producing microorganism can nonetheless develop slowly underneath low temperature (Phuvasate and Su, 2010; Sangcharoen et al., 2009). The ailment is an essential trouble for food safety, public fitness subject and global trade. This prevalence has stimulated food producers, distributors, eating places and all meals handlers to be greater cautious approximately the safety of food.

The manipulate of biogenic amines formation is specially targeted on the controlling the growth of biogenic amines forming microorganism due to the fact histamine is warmness stable (Kurt, S. and O. Zorba, 2009). It isn't always detectable organoleptically by way of even skilled panelists (Tapingkae, W., et al, 2010). once shaped, histamine is difficult to break with the aid of the usage of methods which include freezing, cooking, curing, or smoking (Etkind, P., et al, 1987). but, there are some techniques capable of degrade histamine which includes the gamma irradiation (Kim, J.H.H. et al, 2004) and application of essential oils to degrade the histamine (Nader., et al,2016).

This study aimed to determination of their levels of histamine by HPLC from one side and ELISA from the other side to study the accuracy of the two techniques and investigate the role of certain essential oils (Rosemary oil ,Garlic oil and Lemon oil) as natural bioactive compounds to reduce the concentrations of histamine in sardine fish fillets.

2. MATERIAL AND METHODS

2.1. Collection of samples:

Sixty random samples of fresh marine fishes represented by Sardine, Saurus, Barboni and Chrysophyres (15 of each) were collected at different times from various fish markets in Alexandria governorate, Egypt. All collected samples were examined for determination of their levels of histamine by HPLC from one side and ELISA from the other side to study the accuracy of the two techniques. Further, some trials to control the formation of such serious histamine in marine fishes were considered.

2.1.1. Determination of histamine by HPLC:

Histamine contents in the examined samples were determined according to the protocol recommended by Krause *et al.* (1995) and Pinho *et al.* (2001).

2.1.1.1. Reagents preparation:

Dansyl chloride solution: 500mg of dansyl chloride were dissolved in 100 ml acetone

Standard solutions: Stock standard solutions of histamine were prepared by disseverment of 25 mg of histamine-2HCl in 25 ml distilled water.

2.1.1.2. Extraction of samples:

Twenty five grams of each sample was blended with 125 ml of 5% Tri-chloroacetic acid for 3 min using a warning blender then filtration was achieved using filter paper Whatman No1. Thus, 10 ml of the filtrate were transferred into a suitable glass tube with 4g NaCl and 1 ml of 50 % NaOH. The filtrate was extracted three times (2min each) by using 5 ml n-butanol: chloroform (1:1 v/v) and the upper clear layer was transferred to 100 ml separating funnel by using disposable Pasteur pipette. To combine the organic extracts (upper layer), 15 ml of n-heptane was added in separating funnel and extracted three times with 1.0 ml portions of 0.2 NHCl, the HCl layer was collected in a glass Stoppard tube. Solution was evaporated just to dryness using water bath at 95°C with aid of a gentle current of air.

2.1.1.3. Formation of dansylamines:

One hundred µl of each stock standard solution (or sample extract) were transferred to 50ml vial and dried under vacuum. About 0.5 ml of saturated NaHCO3 solution was added to the residue of the sample extract (or the standard). Vial was stoppered and carefully mixed to prevent loss- due to spattering. Carefully, 1.0 ml dansyl chloride solution was added and mixed thoroughly using vortex mixer. The reaction mixture was incubated at 55°C for 45 min. Accurately, 10 ml of distilled water were added to the reaction mixture, then vial was stoppered and shaked vigorously using vortex mixer, the extraction of dansylated biogenic amine was carried out using 5ml of diethyl ether for 3times again vial was stoppered, shaked for 11.0 min and the ether layers were collected in a culture tube using disposable Pasteur pipette. The combined ether extracts were carefully evaporated at 35°C in dry bath with aid of current air. The obtained dry material was dissolved in 1ml methanol and 10µl were injected in HPLC.

2.1.1.4. Apparatus HPLC conditions:

High performance liquid chromatography (HPLC) used for dansylamines determination was an Agilent 1100 HPLC system, Agilen Technologies, Waldbronn, Germany, equipped with quaternary pump model G 1311A, UV detector (Model G 1314A) set at 254nm wavelength, auto sampler (model G1329A VP-ODS) and Shim pack (150× 4.6 mm) column (Shimadzu, Kyoto, Japan) was used for biogenic amines separation. Data were integrated and recorded using Chemstation Software program.

2.1.2. Determination of histamine by ELISA:

2.1.2.1. Intended use and principle of the test:

This enzyme immunoassay is for the quantitative determination of histamine in plasma and urine. In combination with supplementary kit (available for purchase separately, cat. No. BA E-1100). First, histamine is quantitively acylated. The subsequent competitive ELISA kit uses the microtiter plate format. The antigen is bound to the

solid phase of the microtiter plate. The acylated standards controls, samples, and the solid phase bound analyte compete for a fixed number of antiserum binding site. After the system is in equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase is detected by an anti-rabbit IgG- peroxidase conjugate using TMB as a substrate. The reaction is monitored at 450 nm. Quantification of unknown samples is achieved by comparing their absorbance with a reference curve prepared with known standard concentrations.

2.1.2.2. Test procedure:

All reagents and samples were allowed to reach room temperature prior to use. Measurement in duplicates is recommended.

2.1.2.3. Preparation of reagents:

- Wash Buffer:

20 ml Wash Buffer Concentrate was diluted with distilled water to a final volume of 1,000 ml, and stored up to 6 months at 4-8ºC.

- Acylation Diluent:

The Acylation Diluent has a freezing point of 18.5°C. To ensure that the Acylation Diluent is liquid when being used, it must be ensured that the Acylation Diluent has reached room temperature and forms a homogeneous, crystal-free solution before being used. Alternative the Acylation Diluent can be stored at room temperature (20 – 25°C) separate from the other kit components.

- Acylation Reagent:

Each vial was reconstituted with 1.25 ml Acylation Diluent. The Acylation Reagent has to be newly prepared prior to the assay (not longer than 1 hour in advance). If more than 1.25 ml is needed, the contents of 2 or 3 vials were pooled and mix thoroughly.

2.1.2.4. Sample preparation and acylation:

Pipette 25 µL of standards, 25 µL of controls, 25 µL of plasma samples, 10 µL of fish samples, or 50 µL of supernatant from the release test* into the respective wells of the Reaction Plate.25 µL of Acylation Buffer were added to all wells.25 µL of Acylation Reagent were added to all wells.Incubated for 45 min at RT (20-25°C) on a shaker (approx. 600 rpm).200 µL of distilled water were added to all wells.Incubated for 15 min. at RT (20-25°C) on a shaker (approx. 600 rpm).25 µL of the prepared standards, controls, and samples were taken for the Histamine ELISA.

* For the release test the Histamine Release supplementary kit (available for purchase separately, cat. no. BA E-1100) has to be used.

2.1.2.5. Histamine ELISA:

25 µL of the acylated standards, controls, and samples were pipetted into the appropriate wells of the Histamine Microtiter Strips.100 µL of the Histamine Antiserum were pipetted into all wells and cover plate with adhesive foil.Incubated for 3 hours at RT (20-25°C) on a shaker (approx. 600 rpm).

Alternatively: shake the Histamine Micro titer Strips briefly by hand and incubate for 15 – 20 hours at 2 – 8°C.

The foil was removed. The contents of the wells was discarded or aspirated and each well was washed 4 times thoroughly with 300 µL Wash Buffer. Blotted dry by tapping the inverted plate on absorbent material.100 µL of the Enzyme Conjugate was pipetted into all wells.Incubated for 30 min at RT (20-25°C) on a shaker (approx. 600 rpm).

The contents of well were discarded or aspirated and each well was washed 4 times thoroughly with 300 µL Wash Buffer. Blotted dry by tapping the inverted plate on absorbent material. 100 µL of the Substrate were pipetted into all wells and incubate for 20-30 min at RT (20-25°C) on a shaker (approx. 600 rpm). Avoid exposure to direct sunlight.100 µL of the Stop Solution were piptted to each well and shake the microtiter plate to ensure a homogeneous distribution of the solution. The absorbance of the solution in the wells was read within 10 minutes, using a microplate reader set to 450 nm with a reference wavelength between 620 nm and 650 nm.

1.2.6. Calculation of results:

	Concentration of the standards					
Standard	A	B	C	D	E	F
Histamine (ng/mL = µg/L)	0	0.5	1.5	5	15	50
Histamine (nmol/L)	0	4.5	13.5	45	135	450
Conversion:	Histamine (ng/mL) x 9 = Histamine (nmol/L)					

The calibration curve is obtained by plotting the absorbance readings (calculate the mean absorbance) of the standards (linear, y-axis) against the corresponding standard concentrations (logarithmic, x- axis).Anon-

linear regression was used for curve fitting (e.g., spline, 4- parameter, akima).The concentrations of the plasma samples and the controls can be read directly from the standard curve.

1.2.7. Quality control

It is recommended to use control samples according to state and federal regulations. Use controls at both normal and pathological levels. The kit controls, or other commercially available controls, should fall within established confidence limits. The confidence limits of the kit controls are printed on the QC- Report.

1.2.7.1. Calibration

The binding of the antisera and the enzyme conjugates and the activity of the enzyme used are temperature dependent, and the extinction values may vary if a thermostat is not used. The higher the temperature, the higher the extinction values will be. The extinction values also depend on the incubation times. The optimal temperature during the Enzyme Immunoassay is between 20-25°C.

In cases of overflow, the absorbance of the solution in the wells was read within 10 minutes, using a microplate reader set to 405 nm.

Control of histamine levels (mg %) in the examined samples of sardine fillets

Within the respect of this study the guidelines adopted by (Khalafalla *et al*.2015) were carried out with some modifications. Accordingly, 60 samples of sardine fillets were bought from 5 different sources (supermarkets and fish markets) then classified into groups (5 samples for each point) as follow:

The 1 group was represented as control group without any additives and stored at 4°C for 2, 4 and 6 days.

The 2 group was immersed in Rosemary oil (1%) and stored in refrigerator at 4°C for 2, 4 and 6 days.

The 3 group was immersed in Garlic oil (1%) and stored in refrigerator at 4°C for 2, 4 and 6 days.

The 4 group was immersed in Lemon oil (1%) and stored in refrigerator at 4°C for 2, 4 and 6 days.

For obtaining essential oils 1%, using tween 80 to dissolve the oil in distilled water according to (Hood *et al*., 2003)

Actually, 50 grams of each sample were immersed in 100 ml of each essential oil for 15 minutes. All controlled and treated samples were analyzed by HPLC for determination of their levels of histamine (mg %) at zero, 2, 4 and 6 days according to the protocol recommended by (Krause *et al*. 1995) and (Pinho *et al*. 2001).

Sensory Analyses

Sensory evaluations of the samples were performed as described by Schormüller (1968) and were conducted by five previously trained panelists, who were asked to evaluate appearance, odour, flavour and texture by using a form. According to the scoring table, a total score of sensory attributes of 15 means first quality, scores from 14.9 to 13 indicate second quality, scores from 12.9 to 11.0 indicate third quality and scores from 10.9 to 6.0 indicate fourth quality and a score of 6 or less corresponds to spoiled products.

3.RESULTS AND DISCUSSION

The results given in Table (1) show that the results obtained by the HPLC are some wheat similar to ELISA method. The mean value histamine level in sardine fish are 29.06 ± 2.14 and 28.79 ± 1.96 by HPLC and ELISA, respectively but in saurus fish are 23.83 ± 1.75 and 24.46 ± 2.02 by HPLC and ELISA, respectively and in case of Barboni fish are14.59 ± 1.32 and 14.25 ± 1.58 by HPLC and ELISA, respectively .finally the histamine level in Chrysophyres are 9.91 ± 0.86 and 9.72 ± 0.81 by HPLC and ELISA , respectively . Non-significant differences ($P<0.01$) as indicated by ANOVA test. These results were lower than results reported by authors [Zaman, M.Z. et al, 2009]. Histamine content in all positive salted fish samples exceeded the maximum allowable level of 50 ppm designed by US FDA [Food and Drug Administration (FDA), 1998].

Table (1): Statistical analytical results of histamine levels (mg/kg) in the examined samples of marine fish by using HPLC and ELISA techniques (n=15).

Technique / Fish	HPLC			ELISA		
	Min	Max	Mean ± S.E*	Min	Max	Mean ± S.E*
Sardine	7.2	49.5	29.06 ± 2.14	7.5	48.7	28.79 ± 1.96 NS
Saurus	5.9	41.6	23.83 ± 1.75	5.6	42.0	24.46 ± 2.02
Barboni	2.6	28.1	14.59 ± 1.32	2.2	28.5	14.25 ± 1.58
Chrysophyres	1.7	20.3	9.91 ± 0.86	1.4	20.9	9.72 ± 0.81

S.E* = standard error of mean

NS = Non significant differences (P<0.01) as indicated by ANOVA test.

Table (2): Acceptability of the examined samples of marine fish based on their levels of histamine (n=15).

Fish species	Maximum Permissible Limit (mg %)*	Accepted samples		Unaccepted Samples	
		No.	%	No.	%
Sardine	20	9	60	6	40
Saurus		11	73.33	4	26.67
Barboni		12	80	3	20
Chrysophyres		14	93.33	1	6.67

* Maximum Residual Limit stipulated by Egyptian Organization for Standardization "EOS" (2005).

Table (3): Effect of addition of essential oils (1%) on hisatmine levels (mg%) in the examined samples of sardine fillets (n=5).

Storage time (4°C)	Min	Max	Mean ± S.E*	Progression %
1. Control:				
Zero time	4.5	43.8	21.53 ± 0.96	
2 days	6.1	49.5	27.66 ± 1.15	28.5
4 days	15.2	61.4	36.95 ± 1.38	71.6
6 days	17.6	65.9	39.71 ± 1.52	84.4
2. Rosemary oil (1%):				
Zero time	4.5	43.8	21.53 ± 0.96	
2 days	5.8	46.1	24.80 ± 1.05	15.2
4 days	11.3	55.4	31.82 ± 1.21	47.8
6 days	12.9	61.7	34.07 ± 1.29	58.2
3. Garlic oil (1%):				
Zero time	4.5	43.8	21.53 ± 0.96	
2 days	5.2	44.9	23.67 ± 1.01	9.9
4 days	9.7	52.6	29.14 ± 1.13	35.3
6 days	10.4	55.1	31.39 ± 1.42	45.7
4. Lemon oil (1%):				
Zero time	4.5	43.8	21.53 ± 0.96	
2 days	4.9	44.7	21.98 ± 0.99	2.1
4 days	7.6	48.2	26.15 ± 1.04	21.5
6 days	8.8	50.4	27.83 ± 1.20	29.3

Biogenic amine poisoning is related with the intake of fish from Scombroid family such as tuna and mackerel However, certain non-scombroid fish are also implicated in histamine poisoning including Sardine, Saurus, Barboni and Chrysophyres, [McLauchlin, J.,et al 2006].

Table (2) reported the acceptability of examined marines fish samples according to the Egyptian Standard Specification (2005) as follow: 60% , 73.33%, 80% and 93.33% of examined Sardine, Saurus, Barboni and Chryosphers fishes samples were accepted according to Egyptian Standard specification so the highest unacceptable sample in sardine fish and lowest unacceptable sample in Chrysophyres fish.

Historically, biogenic amine formation in food has been averted, typically by using proscribing microbial boom via chilling and freezing and cooking. However, for plenty fishing primarily based populations, such measures are not realistic consequently, secondary manage measures to prevent biogenic amine formation in ingredients or to lessen their levels once shaped need to be taken into consideration as options. Such tactics to restriction microbial increase may additionally include hydrostatic pressures, irradiation, controlled surroundings packaging, or using meals additives.

Histamine might also probably be degraded by using the usage of bacterial amine oxidase or amine-negative microorganism only some may be fee- effective and realistic for use in populations (Aishath,et al.2010).

Table (4): Changes in sensory traits of control and essential oil treated sardine fillets (n=5).

Character Storage time	Appearance (5)	odor (5)	Texture (5)	Flavor (5)	Overall (20)	Judgment
1. Control:						
Zero time	5	5	5	5	20	Excellent
2 days	4.2	3.8	4.0	3.6	15.6	Good
4 days	2.8	2.6	3.2	2.8	11.4	Middle
6 days	2.6	2.4	2.0	2.2	9.2	Poor
2. Rosemary oil:						
Zero time	5	5	5	5	20	Excellent
2 days	4.2	4.0	4.4	3.8	16.4	Good
4 days	3.4	3.2	3.6	3.6	13.8	Middle
6 days	2.8	2.6	3.0	2.8	11.2	Middle
3. Garlic oil:						
Zero time	5	5	5	5	20	Excellent
2 days	4.4	4.0	4.4	4.2	17.0	Good
4 days	3.8	3.4	3.6	3.6	14.4	Middle
6 days	3.0	2.6	3.2	2.8	11.6	Middle
4. Lemon oil:						
Zero time	5	5	5	5	20	Excellent
2 days	4.6	4.4	4.6	4.6	18.2	Very good
4 days	4.2	3.6	4.0	3.4	15.2	Good
6 days	3.2	3.2	3.6	3.0	13.0	Middle

Essential oils and their additives commonly used as flavoring in the meals enterprise also present some antibacterial, antifungal and antioxidant properties (Tajkarimi, M.M. et al, 2010).

The end result achieved in table (3) declared the impact of Essential oils (1%) on histamine degrees (mg%) within the examined fish fillet samples. In which the mean value of histamine level in the control group at the zero time is 21.53 ± 0.96, while 27.66 ± 1.15 , 36.95 ± 1.38 and 39.71 ± 1.52 after 2, 4 and 6 days (Storage time at4°C), respectively.

The addition of Rosemary oil (1%) decreased histamine tiers to mean value of 24.80 ± 1.05 , 31.82 ± 1.21and 34.07 ± 1.29 after 2, 4 and 6 days (Storage time at4°C), respectively. even as by means of addition of garlic oil (1%) the progression of histamine ranges decreased to 23.67 ± 1.01 , 29.14 ± 1.13 and 31.39 ± 1.42 after 2, 4 and 6 days (Storage time at 4°C), respectively. Furthermore, the addition of Lemon oil (1%) cleared the highest reduction in histamine levels to the mean value of 21.98 ± 0.99 , 26.15 ± 1.04 and 27.83 ± 1.20 in 2, 4 and 6 days days (Storage time at 4°C), respectively. Nearly similar results were reported by [Abu-Salem *et al*., 2011] who reported that histamine was reduced from 21±0.261 mg/kg to 19.08±0.158 mg/kg in luncheon roll meat samples and Nader, et al, 2016 reported that the that the progression of histamine formation changed into actually reduced through the share of 13.2% and 23.9% after addition of garlic oil (1%) for three days and seven days (garage time at 4°C), respectively in frozen fish fillets.

Table (3) nominated the discount% of histamine ranges within the tested fish fillet samples, where in the progression of histamine formation turned into absolutely decreased with the aid of the proportion of 15.2% ,47.8 and 58.2% with addition of Rosemary oil (1%) for 2 ,4 days and 6 days ,respectively; 9.9 % , 35.3% and 45.7% after addition of garlic oil(1%) for 2 ,4 days and 6 days , respectively and the highest reduction level in histamine progression was recorded by the addition of Lemon oil (1%) 2.1% , 21.5% and 29.3% for 2 ,4 days and 6 days ,respectively. Özogul et al. 2011, analyzing the effect of rosemary and sage tea extracts on BA formation in vacuum-packed sardine fillets saved at 3 °C, found that their content material typically multiplied in all treatments with increasing storage time, and that putrescine and cadaverine were the most abundant BAs formed in sardine muscle.

Antimicrobial effect of garlic may be defined by using of enzymes as cysteine proteinases and alcohol dehydrogenases. These organizations of

enzymes are observed in a huge type of infectious organisms inclusive of microorganism, fungi and viruses and this presents a scientific basis for fast-spectrum antimicrobial interest of garlic. It is unlikely that bacteria might develop resistance to allicin because this would require editing several enzymes that make their survival and interest possible (Zheng, W. and S.Y. Wang, 2001.

The Lemon oil reveals a broad spectrum of inhibition in opposition to many bacterial strains even diluted at 1%. They thus confirmed that the dithymoquinone found in this oil has a enormous antimicrobial effect on microorganism (Gram high quality and bad) [Aljabre, S.H.A.,et al,2005].

The essential oil treatment to the fish fillets inhibited the microbiological homes all through the storage periods. all the treatments have been effective in retarding fish sensory deterioration, exhibited a superb impact, causing low histamine content, in particular histamine, putrescine, cadaverine. These consequences indicated that vital oil might be a capability software for extending the shelf existence and keeping the first-class of the fish fillets (Luyun, C.,et al,2015).

As conclusion, the two techniques by HPLC and ELISA are similar for detection of histamine level and the usage of the essential oil treatment of frozen fish fillets is a useful and low value technique to reduce the formation of histamine in order that essential oils can be used as herbal food preservatives with the freezing technique, it is going to be helpful in reduction of histamine formation that allows you to produce fish meat of high quality and safety.

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