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Evaluation of Some Methods of Histamine Determination to be used in HACCP System Implementation of Sardine Canning Process.

Ahmed M Ayesh^{1,2}, Hussein A Murad², Yousef Y Sultan^{2*}, Ahmed M Hegazy³, and Wael A Bazaraa³.

¹Biology Dept, Faculty of Sciences & Arts.-Khulais, King Abdulaziz Univ., Jeddah, Saudi Arabia.

²Food toxins and contaminants Dept., National Research Centre, Cairo, Egypt.

³Food Science Dept., Faculty of Agric. Cairo Univ., Egypt.

ABSTRACT

Histamine is the most important chemical hazards in fish and fishery products, where it is the main cause of histamine poisoning. The HACCP approach offers the advantage of in process control of histamine formation. This avoids the need for large scale terminal analysis of finished products which is time consuming and beyond the capabilities of many small canneries. In HACCP system, most monitoring procedures for critical control points should be done rapidly because the relation to on line processes. So, ELISA (Ridascreen^RHistamine), ion exchange (Rida^RQuick Histamine) and TLC techniques for histamine determination in sardine were evaluated and compared with HPLC methods as the most sensitive one (Detection limit, 1.2 ppm). Statistically, there were no significant differences between the results of TLC technique and those obtained by HPLC. In addition to histamine measurement, TLC also characterized by the possibility to analyse histamine potentiators (cadaverine, putrescine, tyramine) in 12 sardine samples at the same time on one plate. Although ELISA and ion exchange methods were characterized by simplicity and relatively low cost, its data were significantly higher than HPLC results. Receiving, sterilization and incubation period steps were established as CCPs during sardine canning. ELISA was recommended for the determination of histamine in sardine receiving step just as a screening technique. More study is required to identify an accurate and rapid technique for monitoring the histamine levels during sardine canning process. HPLC as well as TLC methods can be used in the verification step to ensure the efficiency of HACCP plan.

Keywords: Histamine, Sardine, HACCP, HPLC, CCPs.

**Corresponding author*

INTRODUCTION

Histamine is formed from the amino acid histidine by bacterial histidine decarboxylase (Taylor *et al.*, 1984, Rawles *et al.*, 1996 and Hungerford, 2010). It does not have odour or colour to indicate its presence in food (Stratton *et al.*, 1991). A toxic level of histamine may be found before the fish appears spoiled or is organoleptically unacceptable (López-Sabter *et al.*, 1994). Fish that commonly cause histamine fish poisoning (HFP) include scombroid fish like mackerel (*Scomber spp.*), tuna (*Thunnus spp.*), and nonscombroid fish like sardines (*Sardinella spp.*), sockeye salmon (*Oncorhynchus nerka*), amberjack (*Seriola spp.*) (Taylor, 1986, Müller *et al.*, 1992 and Gessner *et al.*, 1996). Histamine is the main compound responsible for this intoxication, but other biogenic amines (potentiators) such as putrescine, cadaverine and tyramine increase histamine toxicity (Taylor *et al.*, 1984).

Many countries have set guidelines for maximum permitted levels of histamine in fish. However, levels of possible potentiators in food and the ability of mast cell to secrete histamine can effect on the guidelines establishment (Lehane and Olley, 2000). It is extremely stable, thus it cannot be easily removed or destroyed by cooking, retorting or freezing. Therefore, monitoring of histamine is a critical task in seafood industry. To control histamine formation, the HACCP guideline for histamine is established at 50 mg kg⁻¹ fish muscle (FDA, 1995). Fish containing histamine above this level should be discarded and may not be used for human consumption (Kim *et al.*, 2002 and Tahmouzi *et al.*, 2011).

A HACCP system is designed to identify hazards, establish critical control points and critical limits, monitor procedures and keep records for procedures. Among many hazards identified in seafood products, histamine is a major cause of seafood related illnesses in the USA (FDA, 1998, CDC, 2000 and Koşe *et al.*, 2011). Most monitoring procedures for critical control points should be done rapidly because the relation to on line process. The rapidity, simplicity and efficacy of marine toxins make them useful for HACCP system implementation (NACMCF, 1998, Jabber and Loishy, 1999, FAO/WHO, 2001 and Hungerford, 2010). This is largely the result of the introduction of international standards of food hygiene and the application of risk analysis and hazard analysis critical control points (HACCP) principles (Lehane and Olley, 2000).

The TLC method can be effectively used in the fish industry to detect biogenic amines, especially tyramine, putrescine and cadaverine which can potentiate histamine toxicity in fish and fishery products (Tao *et al.*, 2011). High Performance liquid chromatography (HPLC) is a suitable technique that allows simultaneous analysis of histamine and other biogenic amines in food (Mietz and Karmas, 1977 and Shakila *et al.*, 2001). Rapid testing methods are available for histamine detection, for example, commercial competitive ELISA kit ALERT (qualitative) and veratox (quantitative) from Neogen, USA and ELISA system, Australia. There are also several enzyme immunoassay tests including Histamine test kit (quantitative) from Immunotech, France, which is approved by the AOAC (Price, 1999).

Several companies have produced test kits which have been advertised as rapid, easy to use and capable of providing accurate results at low cost, but little comparative data is available. So, the aim of the present study was to compare the reference method (HPLC) with TLC and the available rapid methods (ELISA and ion exchange kits) for histamine determination in sardine fish. Depending on such comparison, their ability to use at the established CCPs during canning sardine process was discussed. The last aim was to establish a suggested plan of HACCP system which can be used during fish processes.

MATERIALS AND METHODS

Samples

Fresh sardine samples were obtained from retail local market in Cairo government. Sardine samples were divided into two portions. The first one represented fresh sardine which used for the comparison between the tested methods. The second portion was stored overnight at ambient temperature (25±2°C) to increase the possibility of biogenic amines formation. Fresh and stored sardine were canned in the pilot of Agriculture Research Centre, Giza, Egypt. Samples were taken from different steps of the canning process. Such steps were A) raw material B) precooking C) sterilization and D) after draining of filling solution (Figure 1)

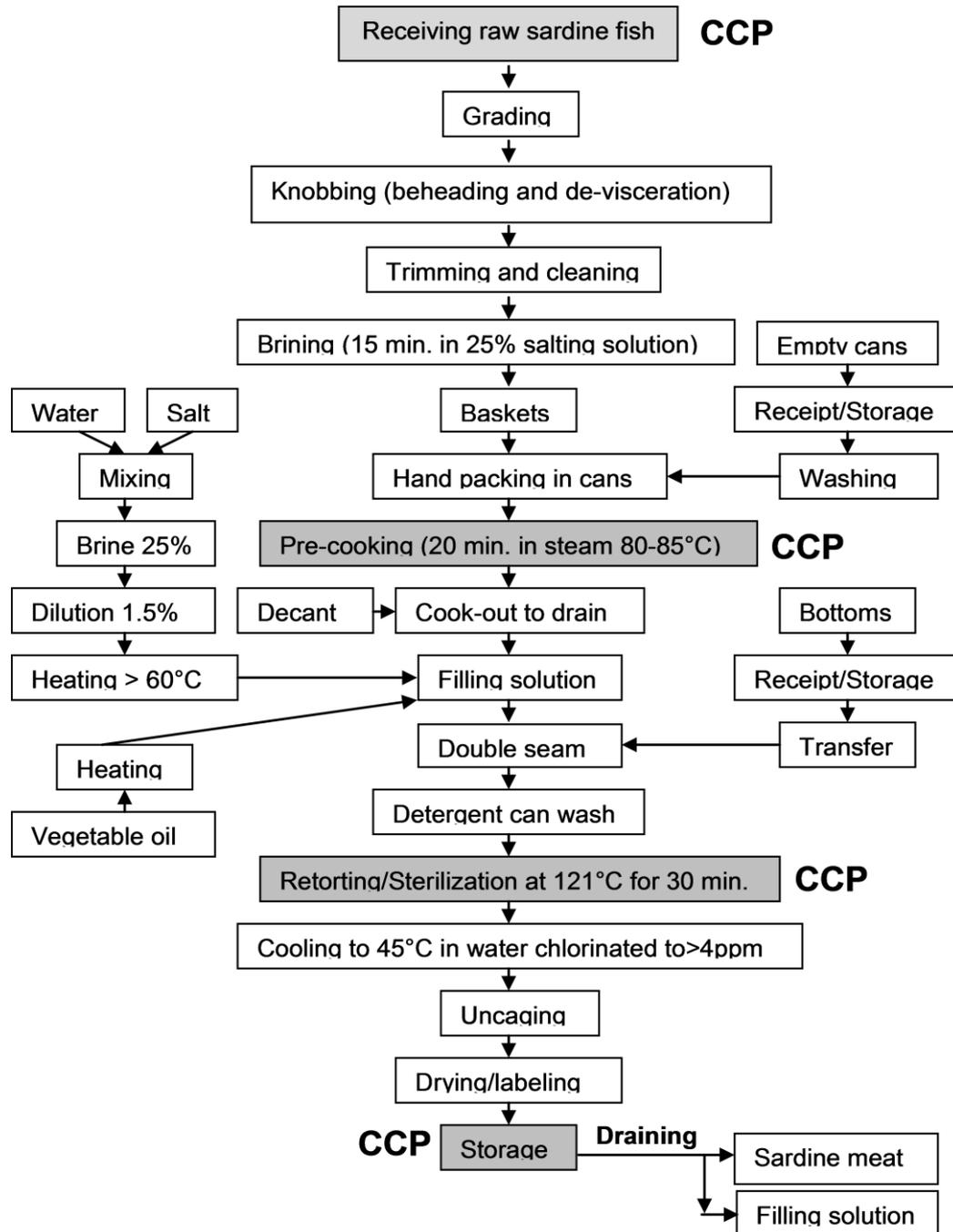


Figure 1: Flow sheet diagram of sardine canning

Analytical Methods

The authentic biogenic amines (Histamine-2HCl, cadaverine- 2HCl, Putrescine-2HCl and tyramine-HCl) and dansyl chloride (5-[Dimethylamino] naphthalene-1-sulfonyl chloride) were obtained from sigma Co., Saint Louis, MO., USA.

Biogenic amines were extracted, dansylated for TLC and HPLC determination according to Mietz and Karmas (1977), Maijala and Eerola (1993), Ayes *et al.*, (2012), Sultan and Marrez, (2014).

HPLC analysis

An Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) model G1311A equipped with UV detector model G1314A set at 254 nm wavelength, autosampler model G1329A and VP-ODS Shimpack (150x4.6 mm, 5µm) column (Shimadzu, Kyoto, Japan) was used for biogenic amines quantitative analysis. Data were integrated and recorded using chemstation Software program (Agilent Technologies, Waldbronn, Germany). A gradient program was used for the separation of biogenic amines (Table 1).

Table 1: A gradient program for the separation of biogenic amines

Time (min.)	Flow rate ml/min	Solvent		
		0.02 N acetic acid%	Methanol%	Acetonitrile%
0	1	60	20	20
10	1	20	40	40
15	1	15	35	50
20	1	60	20	20
25	1	60	20	20

Determination of amines by TLC densitometer

One-dimensional TLC carried out the chromatographic separation to separate the histamine and other studied dansylamines. Ten microliters of standard dansylamine and the methanolic sample extract were spotted on the TLC plates (aluminium sheets 20x20 cm, Merk, Darmstadt, Germany). The plate was developed by chloroform: benzene: triethylamine (6:4.5:1). The resulting zones were examined and marked under long ultraviolet wavelength (365). The marked areas were determined using CS-9000 Dual wavelength flying spot scanning densitometer (SHIMADZU, Japan) using wavelength 254nm. Standard curve of each dansylamine was used to calculate the concentrations of biogenic amines in the tested samples.

The quantitative analysis of histamine in fish samples by kits

Based on ELISA technique, histamine was analyzed using Ridascreen^R Histamine (R-biopharm, Darmstadt-Germany) kit. Preparation of fish samples and test procedure were carried out according to the instruction of R- biopharm (2004). Rida^R Quick Histamine kit was represented ion exchange technique. Extraction, clean up and test procedure of histamine determination were carried out according to the instruction of R-biopharm (2003). Samples absorbance of both procedures was measured using MRX Microplate Reader (Dynatech, Virginia, USA). The results were calculated using RIDARSOFT software.

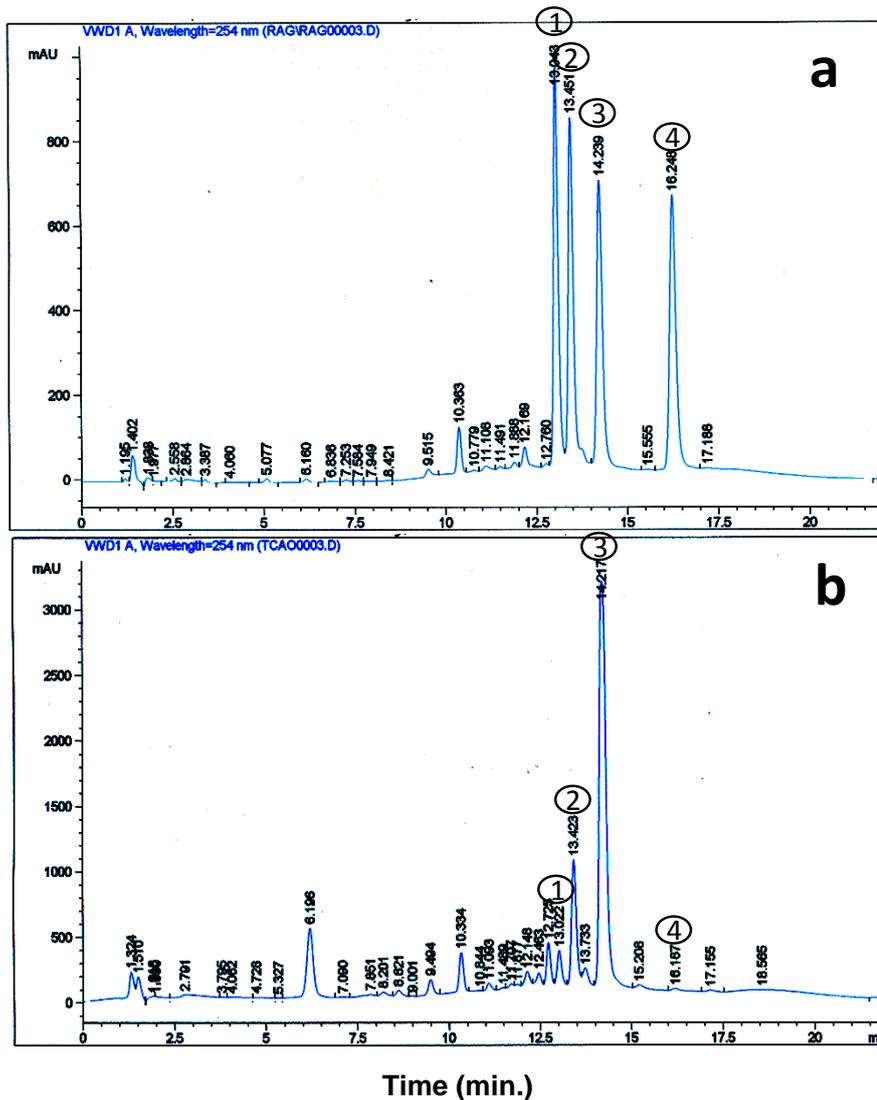
RESULTS AND DISCUSSION

Biogenic amines determination by HPLC

Histamine and its potentiators (putrescine, cadaverine and tyramine) in both reference and sardine sample were well separated in 17 min total run time with sharp and symmetric peaks (Figure 2 a and b). In general, histamine in both type of sardine samples (fresh and stored) represented the domain one followed by cadaverine, putrescine and tyramine.

The use of HPLC in the separation and quantitation of biogenic amines from fish and fish products was reported by Yen and Hsieh, (1991), Hwang *et al.*, (1995), Veciana-Nogués *et al.*, (1995), Özogul *et al.*, (2002) and Cinquina *et al.*, (2004). They agreed upon that HPLC gave excellent results in terms of accuracy, precision, reproducibility and high resolution. Therefore, HPLC was chosen to be used as the reference method. Shakila *et al.*, (2001) indicated that HPLC method for the determination of biogenic amines was more efficient, more sensitive and more reproducible than TLC.

Figure 2: HPLC chromatogram for biogenic amines in a) standard and b) sardine sample (1.putrescine, 2.cadaverine, 3.histamine and 4.tyramine).



Biogenic amines determination by TLC:

Precoated silica gel G 60 TLC plates were found to offer a neat and reproducible resolution of different amines. Figure 3 shows the TLC separation of the studied biogenic amines (histamine, putrescine, cadaverine and tyramine). Results indicated a good resolution of the tested amines on TLC plate, and the R_f values were 0.39, 0.51, 0.77 and 0.89 for the spots of putrescine, cadaverine, histamine and tyramine, respectively. Detection of amines under the long wave UV light (365 nm) showed bright coloured fluorescent spots. Histamine appeared as yellowish, tyramine as green and other amines (putrescine and cadaverine) as greenish blue spots. The spots of putrescine, cadaverine and histamine were clearly observed under the UV light in all sardine samples. However, tyramine was not detected in the same samples on TLC plates. The distinctly amine spots on the TLC plates were neatly scanned in the following order of separation putrescine, cadaverine, histamine and tyramine as clear peaks (Figure 4 a, b). The chromatogram of these amines on TLC was very close in shape to that obtained by HPLC. Also, values of these amines obtained by TLC were very close to those determined by HPLC (Figure 5). Analysis of variance of biogenic amines in sardine samples determined by both HPLC and TLC methods at different steps of canning process indicated that no significance different was observed regarding to method type (Table 2).

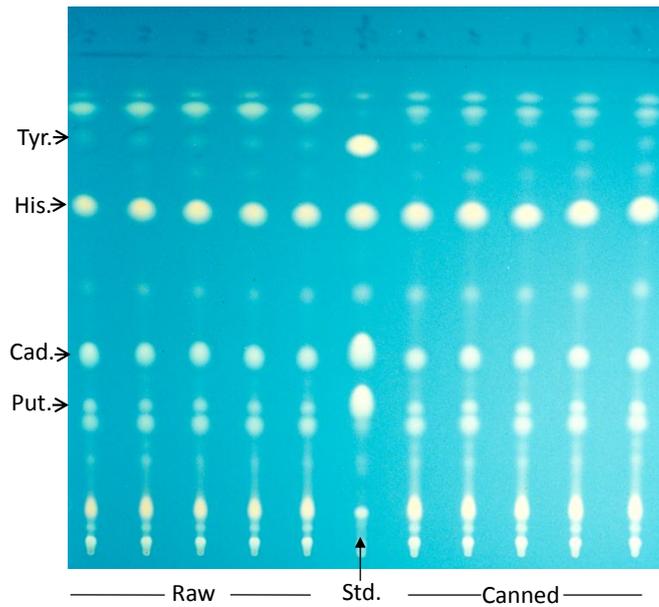
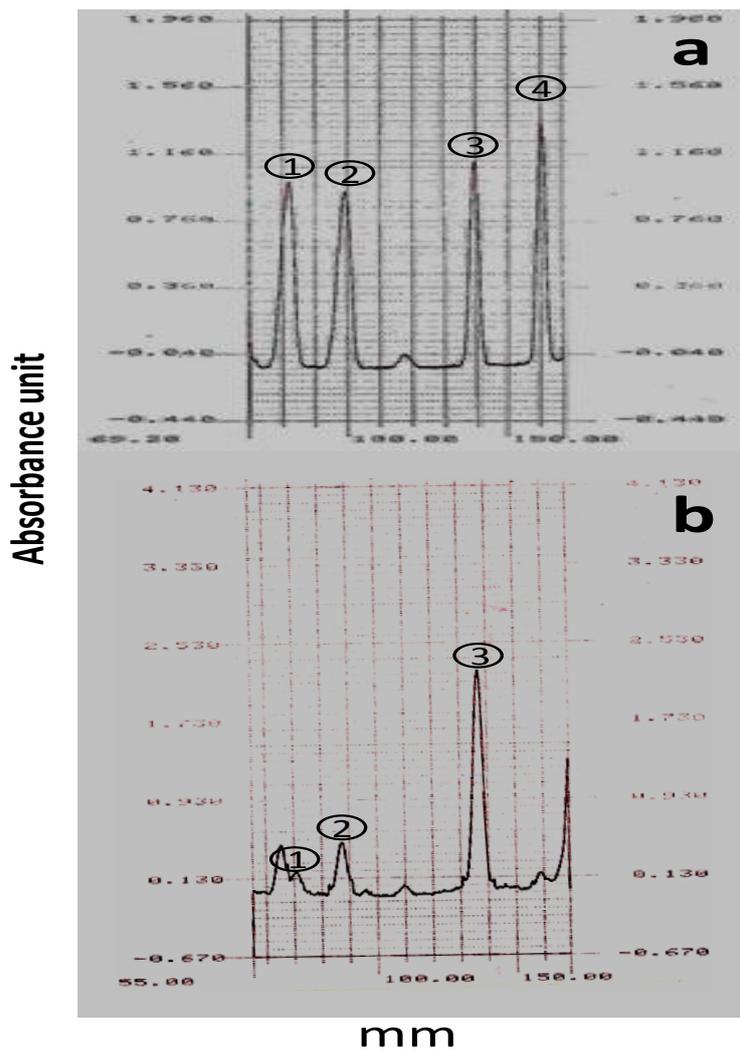


Figure 3: TLC separation of biogenic amines from raw and stored canned sardine samples. Separated spots were visualized using long wave UV (365 nm).

Figure 4: TLC chromatogram for biogenic amines in a) standard and b) sardine sample (1.putrescine, 2.cadaverine, 3.histamine and 4.tyramine).



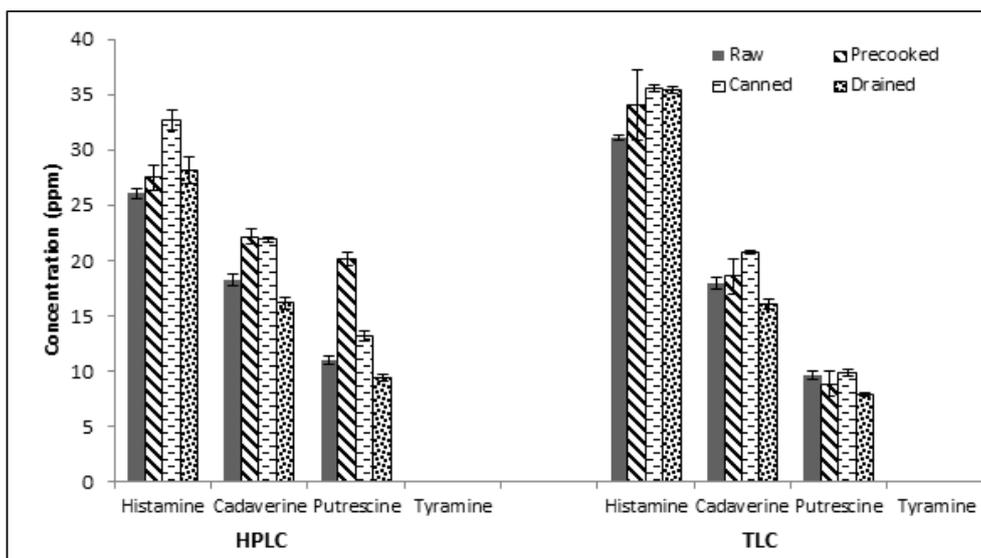


Figure 5: Concentration of biogenic amines in fresh sardine samples at different canning steps using TLC and HPLC. * Bars indicate mean values \pm SE.

Table 2. Analysis of variance of the effect of canning step, biogenic amine type, method type and their interaction on concentration of the biogenic amines on fresh sardine.

Effect	SS	DF	MS	F	P
Intercept	22679.66	1	22679.66	11878.45	0.000000
Treatments	137.95	3	45.98	24.08	0.000000
Biogenic amine type	12476.90	3	4158.97	2178.25	0.000000
Method type	0.06	1	0.06	0.03	0.855813
Treatment x Biogenic type	163.54	9	18.17	9.52	0.000000
treatment x Method type	42.51	3	14.17	7.42	0.000243
biogenic type x Method type	302.03	3	100.68	52.73	0.000000
Treatment x Biogenic type x Method type	82.43	9	9.16	4.80	0.000067
Error	122.20	64	1.91		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

Comparison between different methods for histamine determination in sardine samples

Figure 6: Determination of histamine content (ppm) in fresh sardine fish by different methods. *Bars indicate mean values \pm SE. Different letters show significant differences (P<0.05) within each sample type.

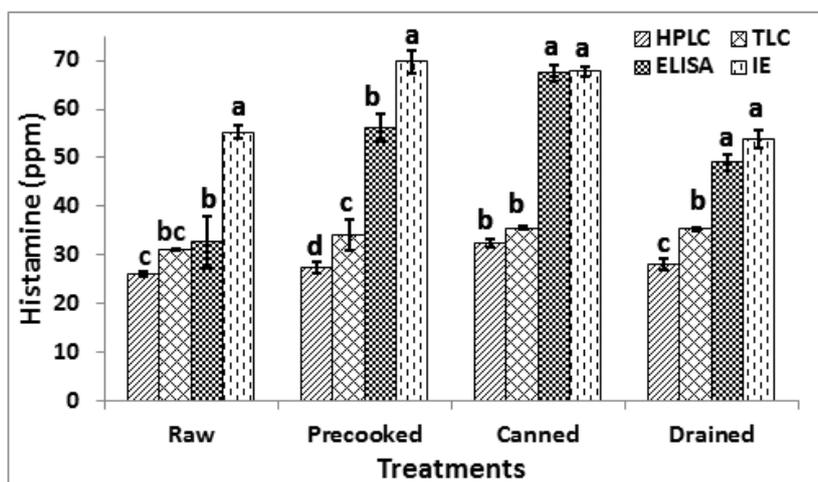


Figure 6 represents histamine levels in sardine samples at different processing steps using different analysis techniques. In general, TLC techniques in samples from all steps gave close values of histamine to those obtained by HPLC. However, histamine values were doubled using ELISA (Rida^Rscreen) and ion exchange (Rida^Rquick) kits especially in heat treated samples (50-70 ppm). ELISA was suitable to determine histamine just in raw samples. So, TLC as well as ELISA methods were the methods of choice at the receiving step of raw sardine. These results were not in agreement with Rahimi *et al.*, (2012) who used Rida^Rscreen kit as an authorized method to determine histamine in canned tuna fish samples in range 17 to 210 mg 100g⁻¹.

Chevrier *et al.*, (1986) reported that direct enzyme immunoassay method for quantifying histamine was a sensitive, specific, rapid and accurate, but involved many steps. Also, Aygün *et al.*, (1999) demonstrated that the ELISA was suitable for the determination of histamine in cheese. Likewise, Serrar *et al.*, (1995) cleared that values for histamine content in some fishery products were obtained by ELISA within 5 h and it was closely related with HPLC method. Recently, some of biogenic amines, such as putrescine, cadaverine, spermidine and histamine were determined in fish as quality indices by ion-exchange chromatography. This technique was simple, rapid and useful for routine checks in repetitive analyses (Cinquina *et al.*, 2004).

Table 3 compares between the studied methods (Ridascreen^RHistamine, Rida^RQuick Histamine and TLC) for histamine determination with HPLC method. High Performance liquid chromatography was the most sensitive (1.2 ppm) method among the tested ones. Also, HPLC as well as TLC can be successfully used in the quantification of biogenic amines such as putrescine, cadaverine and tyramine during histamine determination. Ion exchange (Rida^RQuick Histamine) and ELISA (Ridascreen^RHistamine) techniques were simple, use lower volume of solvents and cost effective (190 and 120 LE) when compared with HPLC method. In these two methods (ELISA and Ion exchange), samples could be run in parallel in the same microtitre plate. Therefore, a great reduction in analysis time could be achieved. Data in Table 3 revealed that TLC method had close sensitivity (3-4 ppm) to ELISA method (2.5-7.5 ppm). Although, TLC method consumed relatively large amount of unsafe solvents and required long sample preparation it characterized by the possibility to analyse 12 samples at the same time on one plate.

Table 3: General comparison of the tested methods for histamine determination

Comparison element	Ridascreen ^R histamine	Rida ^R Quick histamine	TLC method	HPLC method
Principle of test	Quantitative enzyme immunoassay	Quantitative colorimetric assay	TLC-densitometer	HPLC-UV detector
Detection limit	2.5-7.5 ppm	20 ppm	3-4 ppm	1.2 ppm
Extraction	Phosphate buffer (pH 7.2), Tween 20	Water and isopropanol	Water, TCA, NaOH, NaCl, chloroform, butanol, Heptan, HCl, NaHCO ₃ , acetone, dansyl cholrid and diethyl ether	Water, TCA, NaOH, NaCl, chloroform, butanol, Heptan, HCl, NaHCO ₃ , acetone, dansyl cholrid and diethyl ether
Filtration	None	None	Quick and easy	Quick and easy
Supplied reagents	Standards, conjugate, antibody, substrate, chromogen and stop reagents	Standards, elution buffer, 2 color reagents, washing buffer	-	-
Solvents volume	-	Low (10 ml)	Medium (120 ml)	Medium (100 ml)
Safety of reagent	Safe	Safe	Unsafe	Unsafe
Preparation steps	Simple	Simple	Long	Long
Incubation times	2 hr	None	45 min.	45 min.
Total test time	3 hr/sample	20 min/sample	6 hr	6 hr
Reading results	ELISA reader at 450 nm	ELISA reader at 450 nm	Densitometr at 254 nm	UV detector at 254 nm
Cost of sample	120 LE	190 LE	160 LE	300 LE

Determination of critical control points (CCPs)

The development of a HACCP system for a seafood establishment begins with the construction of a flow-chart for the entire process. This chart should begin with the acquisition of raw materials and include all the required steps (ICMSF, 1988).

Histamine formation in fish is one of the important chemical hazards. So, histamine content in sardine fish was determined by HPLC throughout canning process to establish the CCPS (Table 4). Significant change ($P < 0.05$) was observed after sterilization step, where histamine content increased by 18.5% in canned sardine fish (Table 5). This observation was confirmed after sterilization of samples contained high amount of histamine (stored fish). Around 50% increase in histamine level was noticed in canned samples of stored fish. Such increased was probably due to the increase of period between the double seaming and the come-up time of the retort. This period was enough to histamine formation by bacterial histidine decarboxylase. Our results were very close to those reported by Veciana-Nogués *et al.*, (1989) and Ganwiak *et al.*, (1991). They studied the effect of the thermal sterilization on histamine content in canned fish and found that sterilization step increase the amount of histamine due to protein breakdown. Pan (1985) reported that during thermal processing (116°C), scombroid fish of good quality did not show an increase in histamine content. However, bonito with an initial histamine content of 37.5 and 85.5 ppm increased to 53.5 and 193.5 ppm, respectively. He also indicated the optimal temperature for histidine decarboxylase was 55°C , and for mackerel muscle alkaline protease was 60°C . Therefore, it may be possible that during the come-up time in thermal processing, there was further degradation by endogenous and microbial enzymes to produce histamine. Also, Zee *et al.*, (1983), López-Sabter *et al.*, (1994) and Veciana-Nogues *et al.*, (1997) noticed that the level of histamine remained constant in thermally processed fish due to its heat stability. These results didn't agree with those reported by Sims *et al.*, (1992) who reported a decrease in histamine, putrescine and cadaverine after heat treatment.

From the previous results concerning the determination of histamine in sardine fish by HPLC, the critical control points were established on the flow chart of the sardine process (Figure 1).

Table 5: Determination of histamine (ppm) by HPLC in the major steps during canning sardine to identify CCPs

Treatment	Fresh sardine	Stored sardine
Raw	*26.03 _b ±0.43	146.60 _b ±3.46
Precooked	27.5 _b ±1.10	158.86 _b ±1.84
Canned	32.63 _a ±0.89	240.36 _a ±10.63
Drained	28.13 _b ±1.14	161.0 _b ±8.34

*Means followed by different subscripts within column are statistically different ($P < 0.05$).

Establishment HACCP plan in sardine canning process

Table 4 shows the HACCP plan worksheet of sardine canning process. The main topics in this plan included processing steps, hazards, CCPs, critical limits, the preventive measures monitoring, corrective action and records.

A sensory assessment (appearance, odour) of the raw material immediately before processing is practical for ensuring that no spoiled fish enter the processing area. Also, it is important to monitor time/temperature conditions during fish handling and processing. Using an adequate quality of ice or other cooling media at the time of delivery reduce the histamine formation.

Receiving step was identified as a CCP, whereas the histamine content in the received fish should be under the permissible limit (50 ppm) established by FDA (2001). Any deviation above this limit, the fish lot will be rejected. Ráidascreen[®] Histamine or TLC methods can be used to monitor the histamine content in this step especially in case of high number of samples analysis. Corrective action is performed by either rejecting the fish lot or subdividing the lot and analysing each portion alone. Portion containing >50 ppm histamine should be rejected. Receiving records included the results of receiving temperature, sensory examination, and visual observation on the adequacy of ice and histamine levels.



Table 4: HACCP plan worksheet-Sardine canning process

Processing step	Hazard	CCP	Critical limit	Preventative measures	Monitoring	Corrective action	Record
Receiving	-Histamine -Decomposed fish	Yes	Histamine < 50 ppm	-Control supply source -Have the supplier provide a product temperature history	-Measure temperature upon receipt -Visual inspection -Histamine testing by *QE or TLC methods	-Subdivide lot, re-examine portion of the lot for histamine by QE or TLC method reject portions of ≥50 ppm histamine	-Report of Visual inspection, temperature and histamine analysis
Knobbing and de-viseration	-Histamine -Discoloured Flesh	No	-	-Control time of fish cleaning and hygienic practices -GMP	-	-	-
Brining	-Temperature -Salt conc. -Salt purity	No	-	-Control temperature and salt concentration	-	-	-
Packing	-Defected empty Cans -Over filled cans	No	-	-Select can supplier -Set up empty can sampling plan and specification required and train workers on container integrity -Calibrate the used balance	-	-	-
Precooking	-Under cooking	No	-	-Control time and temperature of precooking	-	-	-
Filling solution and seaming	-Over filling -Defect double seam	No	-	-Calibrate balance used -Adjustment of seamer -Test run before use -Train QC/seam mechanic	-	-	-
Sterilizing	-Improper processing resulting in outgrowth of microbes and toxins -Histamine increasing -Improper pressure resulting in physical defects in cans	Yes	-Sterilization Temperature, pressure and time	-Train retort operators -Establish process schedule -Calibration of the retort -Close surveillance of operation (by QC/QA)	-Time, pressure and temperature -Histamine testing	-Reprocess the under processed cans	-Time and temperature -Histamine report
Cooling	-Post process contamination	No	-	-Restrict area -Traffic control -GHP (use of chlorinated cooling water)	-	-	-
Incubation period	-Cans swelling	Yes	-Abnormal appearance	-GMP and GHP	-Visual inspection of cans	-Reject effected cans	-Percentage of defect cans

QE: Quantitative ELISA (Ridascreen[®] histamine) QC: Quality Control QA: Quality Assurance GHP: Good Hygienic Practices GMP: Good Manufacturing practices

The second CCP was sterilization step where, histamine level was higher than found in precooked step. Therefore, it is possible to start up with raw fish with accepted histamine level, but end with unacceptable product (Pan, 1985). Also, any improper processing may result in outgrowth of microbes and toxins formation. Although Rida^RQuick histamine is a rapid test (20 min) and suitable to monitor the histamine level in this step, it couldn't be used as a result of its false positive results.

The incubation period was also defined as CCP and considered as an evaluation procedure of all the previous steps to ensure the safety of end products. Visual inspection of can shape is an important practice to identify the defected cans. Such cans will be rejected in corrective action step. Good hygienic practices (GHP) as well as good manufacturing practices (GMP) are the preventative measures in can storage (e.g. control of the relative humidity of storage).

Verification step is defined as those activities, other than monitoring, that determine the validity of the HACCP plan and that the system is operating biogenic amines by HPLC as well as TLC techniques in the final product can verify the efficiency of HACCP system in food industry.

CONCLUSION

Many rapid kits based on different techniques for histamine determination are available in markets. However, nature of sample can effect on the results of determination. In the present study, it was recommended to be used Ridascreen^RHistamine just in the receiving step of raw sardine. However, Ridaquick^Rhistamine could not be used in HACCP system of sardine canning. So, more studies are required to test more available kits that characterized by rapidity and accuracy. The guidance levels of histamine in fish should decrease and re-establish according to the existence of other biogenic amines that act as potentiators of histamine toxicity. Also, new specification of histamine levels in canned sardine should be established according to processing step. HPLC or TLC was recommended to be used in the verification step of HACCP system in sardine canning process. The suggested HACCP plan work sheets for sardine canning process are applicable in food industries.

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