Isolation and Identification of Histamine-forming bacteria in frozen Skipjack tuna (*Katsuwonus pelamis*)

Koohdar V. A.[1]*; Razavilar V.[1]; Motalebi A. A.[2]; Mosakhani F.[3]; Valinassab T.[2]

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Abstract

In this study a series of experiments were carried out to detect and identify histamine-forming bacteria and analyze histamine content for evaluation of current harvesting and post harvesting procedures. The target fish was Skipjack (*Katsuwonus pelamis*) in which the samples were collected from Oman Sea waters harvested by gillnet or purse seine methods. Bacteriological isolates and the amount of histamine were obtained from the muscle around the gills. The obtained results indicated that the average of total and psychrophilic counts were 7.2×10^6 and 2.9×10^6 CFU/g, respectively. Histamine-forming bacteria occurred on a low scale of total bacterial load with the mean of 2.8×10^2 CFU/g. Diverse bacterial isolates were identified as histamine-forming bacteria. Amongst them, *Proteus* spp. with the highest abundance in samples contributed 24.5% followed by *Clostridium perfringens* (22.5%), *Klebsiella* spp. (15.0%), *Enterobacter* spp. (11.5%) and the other isolates (26.5%). In comparison with USFDA standard, the amount of histamine in 22.2 and 42.2% of the examined samples were 20- 50ppm, and >50ppm, respectively. Therefore, there are seafood safety risks in the current harvesting and post harvesting methods used in skipjack industry and proper preventional methods for histamine formation are recommended.

Keywords: Skipjack, *Katsuwonus pelamis*, Histamine, Bacteria, Oman Sea

1-Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, P.O.Box: 14155-775, Tehran, Iran.
2-Iranian Fisheries Research Organization, P.O.Box: 14155-6116, Tehran, Iran.
3-Faculty of Veterinary Medicine, Islamic Azad University, Karaj Branch, P.O.Box: 31485-313, Karaj, Iran.
* Corresponding author's email: valiollah.kohdar@kiau.ac.ir

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Introduction

Fish and seafood are one of the most important protein sources than other foods in many parts of the world; but fish is a highly perishable food, which spoils soon after death, if not preserved properly (Motalebi, 2010). Consumption of spoiled fish results in the outbreaks of food poisoning such as scombroid poisoning. Scombroid poisoning also called histamine poisoning is worldwide intoxication, but since it is a rather mild illness it is usually incompletely recorded in most countries (Ahmed, 1991). It is known as a poisoning related with some types of dark meat consumption belonging to Scombroidae and Scombrosocidae families which contain very large amounts of free histidine generally in their muscle tissues (Mlcneryey et al., 1996). Histidine is converted to histamine by microbial histidine decarboxylase enzyme. Consumption of spoiled fresh, frozen fish and tinned fish products which contain unusually high levels of histamine which results in the outbreaks of histamine fish poisoning is one such food poisoning (Çaklý and Kýþla, 2003; Choudhury et al., 2008). Freshly caught fish have histamine levels of less than 2ppm. Fish containing histamine levels greater than 20ppm cause adverse symptoms in people. According to the Food and Drug Administration (FDA), histamine levels of between 20 and 50ppm indicate that the fish has deteriorated. The FDA "action level" for histamine in raw, frozen or canned tuna is 50ppm (FDA, 1998; Codori and Marinopoulos, 2010).

A large and diverse group of bacteria have been reported to be responsible for the histamine found in fish and most of them are considered to be enterobacteria (Middlebrooks et al., 1988). In general, the amino acid decarboxylase enzymes, especially histidine decarboxylase, can be found in some species of Enterobacteriaceae, *Lactobacillus, Pseudomonas, Vibrio, Clostridium* and *Photobacterium* (Taylor, 1986; Ababouch, 1991; Lehane and Olley, 2000). Yoshinaga and Frank (1982) suggested that the diversity of bacteria with histidine decarboxylase activity observed in scombroids can be attributed to the type of seafood, fish species, duration and temperature storage condition. Also there are some other factors such as feeding habits, geographical position, fishing gear, season, water temperature and salinity, the way the product is handled after harvest and market environs which can affect composition and type of histamine-forming bacteria in fish.

The Skipjack is distributed in all parts of the Oman Sea both Iranian and Oman waters (Assadi and Dehghani, 1997), and the main fishing methods in these areas are drift gillnet, long-line and purse-seine with the large-scale fishing of industrial purse seiners. It is noteworthy to mention that the caught fishes cannot immediately be collected after entangling and they remain inside the water for a while with considerable duration for further transferring on board to be cooled before being frozen and stored. If these delays are prolonged, some post mortem decomposition and accumulation of histamine can occur in the fish (Yoshinaga and Frank, 1982). Other factors such as unsuitable handling, post-catching contamination, inadequate chill-storage procedures, inadequate freezing and

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thawing procedures also affect the probability of histamine accumulation. These kinds of experiments can be used for evaluation of current harvesting and post harvesting methods in skipjack tuna industry. The main objectives of this study were to identify the histamine- forming bacteria and to determine the amount of histamine in frozen skipjack.

Materials and methods

Forty-five specimens of frozen skipjack tuna were randomly obtained from the fish canning company. All samples were harvested from Oman Sea (Chabahar port). Each specimen was gutted and then cut into small pieces. Since the tissues of gill and gut are considered as the major source of histamine-forming bacteria in fish (Lopez-Sabater et al., 1996), 0.5-1 kg of muscle near the gills were collected aseptically. Samples were covered with ice and immediately (<an hour) transported to the microbiology laboratory and were preserved frozen until analysis. After thawing at room temperature, samples were skinned and deboned aseptically, and the flesh were homogenized and blended in warning commercial blender model 35BL40 (8011P), without any adding of liquid. The homogenate was diluted in sterile 0.1% peptone (Yoshinaga and Frank, 1982), and inoculated on duplicate plates of trypticase soy agar (TSA) for mesophilic and psychrophilic counts after incubation at 35°C for 24h and 20°C for 5 days, respectively. The Enterobacteriaceae family was enumerated in violet red bile dextrose agar (VRBA) after incubation at 37°C for 24h (Lopez-Sabater et al., 1996).

For the enumeration of histamine-forming bacteria, 1ml of serially diluted homogenates was poured on to 4 plates containing Niven's medium (Niven et al., 1981) and 2 plates containing modified Niven's medium (Yoshinaga and Frank, 1982). Two plates containing Niven's medium were incubated at 37°C for 48h, 2 other plates were incubated at 20°C for 5 days (Lopez-Sabater et al., 1996), and the final 2 plates containing the modified Niven's medium were incubated at 37°C for 48h in an anaerobic condition (Yoshinaga and Frank, 1982). The colonies with purple halo around Niven's medium and pink halo around modified Niven's medium were enumerated. These positive colonies were aseptically isolated and streaked on trypticase soy agar slants supplemented with 0.1% L-histidine-Hcl with pH= 6.0 and incubated at 37°C for 24h. Isolates were stored at a temperature of 2°C until used for bacterial species identification (Lopez-Sabater et al., 1996). Niven-positive isolates were gram stained and examined under oil immersion. Gram-positive isolates were identified by catalase test, carbohydrate fermentation, colony morphology and various biochemical tests (Holt et al., 1994). Gram-negative rods were identified by differential media and carbohydrate and biochemical properties as described by Berge's manual of determinative bacteriology (Holt et al., 1994). All isolated strains were confirmed as histamine- forming bacteria by the methods described by Smith et al. (1982) and Yoshinaga and Frank (1982). The muscle samples were analyzed for amount of histamine by the enzymic method. Enzyme Linked Immonosurbant Assay (ELISA) method was used for this purpose. Marcobal et al. (2005) reported

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that ELISA method for histamine determination has a good correlation with high performance liquid chromatography (HPLC) analysis. The Veratox® histamine test was from NEOGEN Corporation. It is commonly used for the quantitative analysis of histamine in scombroids. From point of statistical analysis, histamine content, the mean values of histamine-forming bacteria and histamine-forming Enterobacteriaceae counts were compared using a one-way analysis of variance (ANOVA). Statistical analyses were done using the SPSS software (ver. 16), and all significant levels were considered at the level of $p<0.05$.

Results

The mean counts of mesophilic and psychrophilic in 45 samples of skipjack were 7.2×10^{6} and 2.9×10^{6} CFU/g, respectively. The mean of histamine-forming bacterial count was about 2.8×10^{2} CFU/g and it was 0.004% and 0.009% of the total and psychrophilic bacterial loads, respectively (Fig. 1). Enterobacteriaceae counts were between 0.0 to 3.1×10^{4} CFU/g and the average of it was 1.4×10^{3} CFU/g. Figure 2 shows histamine concentration and the mean values of histamine-forming enterobacteriaceae count in studied samples.

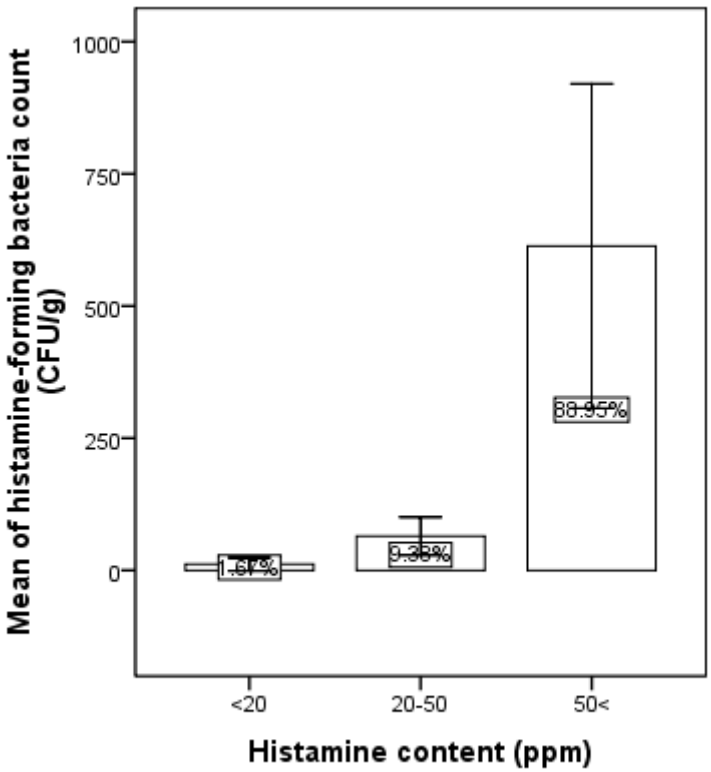

Figure 1: Histamine content (ELIZA method) and the mean values of histamine-forming bacterial count in frozen skipjack; n=45

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Fourteen bacterial strains with histidine decarboxylase activity were isolated and then tested for their ability to produce histamine, of which 8 strains (57.14%) of these tentative isolates showed positive results (Table 1). Sixteen of the total 45 samples of frozen skipjacks contained less than 20ppm amount of histamine; but this amount was 20-50ppm and more than 50ppm in 10 and 19 samples, respectively. Tables 2 and 3 show histamine concentration and the type of bacteria isolated in samples with a histamine content of more than 20 ppm. There was significant difference in histamine contents in samples with different numbers of histamine- forming bacteria; so that the samples with high quantities of histamine-forming bacteria had significantly higher levels of histamine than other samples ($p<0.05$). The same result was achieved for samples with different numbers of histamine- forming enterobacteriaceae ($p<0.05$).

Table 1: Histamine-forming bacteria isolated from frozen skipjack; n=33

Bacterial species	No. of tentative histamine-forming bacteria	Frequency (%)	Confirmed histamine-forming bacteria
Aeromonas hydrophila	250	2.0	n
Citrobacter freundii	1000	8.0	n
Clostridium perfringens	2807	22.5	y
Enterobacter aerogenes	915	7.3	y
Enterobacter cloacae	530	4.2	y
Escherichia coli	518	4.2	n
Klebsiella oxytoca	230	1.8	y
Klebsiella pneumoniae	1640	13.1	y
Morganella morgan	315	2.5	y
Proteus mirabilis	1695	13.6	y
Proteus vulgaris	1360	11.0	y
Pseudomonas aeruginosa	111	0.9	n
Pseudomonas fluorescens	760	6.1	n
Serratia marcescens	355	2.8	n
Total	12486	)0	

y= yes, n=no

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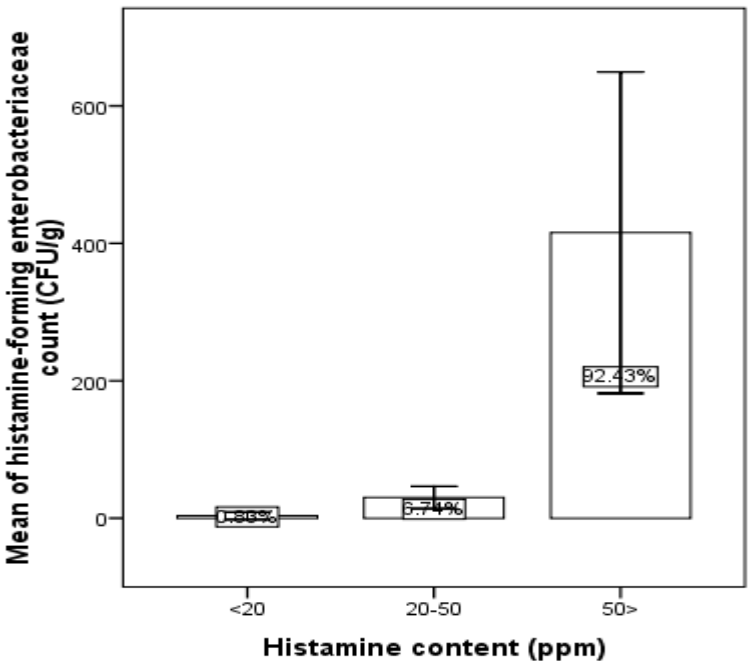

Figure 2: Histamine content (ELIZA method) and the mean values of histamine-forming enterobacteriaceae count in frozen skipjack; n=45

Table 2: Bacterial isolates, histamine-forming bacterial count, and histamine-forming enterobacteriaceae count in 10 samples with histamine concentration of 20–50 ppm

Sample	Histamine-forming bacterial count (CFU/g)	Histamine-forming Enterobacteriaceae count (CFU/g)	Histamine content (ppm)	Bacterial isolates
1	80	80	21.0	*C. freundii, E. coli, K. oxytoca*
2	25	25	33.1	*C. freundii, K. oxytoca, S. marcescens*
3	65	50	35.9	*Cl. perfringens, E. aerogenes, E. coli*
4	37	10	23.2	*Cl. perfringens, E.coli, P. aeruginosa*
5	45	30	42.5	*Cl. Perfringens, K. pneumoniae*
6	190	40	45.6	*Cl. Perfringens, K. pneumoniae, P. mirabilis, S. marcescens*
7	25	25	26.5	*E. cloacae*
8	95	30	44.8	*E. cloacae*
9	25	15	33.4	*K. pneumoniae, P fluorescens*
10	60	0	24.7	

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Table 3: Bacterial isolates, histamine-forming bacterial count, and histamine-forming enterobacteriaceae count in 19 samples with histamine concentration of more than 50 ppm

Sample	Histamine forming bacterial count (CFU/g)	Histamine-forming Enterobacteriaceae count (CFU/g)	Histamine content (ppm)	Bacterial isolates
1	2160	1500	163.7	*A. hydrophila,C. freundii,Cl. Perfringens, E. aerogenes, E. coli, P. mirabilis, P. vulgaris*
2	120	110	120.0	*A. hydrophila, C. freundii, E. aerogenes, K. pneumoniae, P. mirabilis*
3	485	445	153.8	*A. hydrophila, E. aerogenes, E. cloacae, E. coli, K. pneumoniae, K. oxytoca, P. mirabilis, P. vulgaris*
4	300	130	98.2	*C. freundii, Cl. Perfringens, E. aerogenes, K. neumoniae*
5	2050	1500	169.5	*C. freundii, Cl. Perfringens, E. aerogenes, E. cloacae, K. pneumoniae, P. fluorescens, P. mirabilis, P. vulgaris*
6	420	220	175.5	*C. freundii, Cl. Perfringens,E. aerogenes, K. pneumoniae, M. morganii, P. fluorescens, P. vulgaris*
7	1400	850	142.0	*C. freundii, Cl. Perfringens, E. aerogenes, P. fluorescens, P. mirabilis, P. vulgaris, S. marcescens*
8	455	340	44.8	*C. freundii, Cl. perfringens, E. cloacae, E. coli, K. pneumoniae, S. marcescens*
9	95	45	88.5	*C. freundii, Cl. Perfringens, K. pneumoniae, P. fluorescens*
10	290	100	153.8	*C. freundii,Cl. Perfringens,M. morganii, P. mirabilis*
11	1150	900	108.7	*Cl. Perfringens, E. aerogenes, E. coli, K. pneumoniae, M. morganii, P. mirabilis, S. marcescens*
12	570	290	123.2	*Cl. perfringens, E. aerogenes, E-coli, K. pneumoniae, P. fluorescens, S. marcescens*
13	910	810	197.0	*Cl. perfringens, E. aerogenes, K. pneumoniae, P. mirabilis, P. vulgaris*
14	185	100	78.2	*Cl. Perfringens, E. aerogenes, S. marcescens*
15	220	160	144.0	*Cl. perfringens, E. cloacae, K. pneumoniae, P. aeruginosa, P. fluorescens, P. mirabilis*
16	270	85	64.6	*Cl. perfringens, E. coli, k. pneumoniae, P. aeruginosa*
17	365	190	87.6	*Cl. Perfringens, E. coli, P. fluorescens , P. mirabilis, P. vulgaris, S. marcescens*
18	130	130	76.2	*E. cloacae, E. coli, K. pneumoniae*
19	80	80	64.2	*E. cloacae, P. mirabilis*

Discussion

Histamine content was significantly ($p<0.05$) higher in samples with high histamine-forming bacterial count (Fig. 1). The average histamine-forming bacterial count was 0.004% of the average total bacterial count, and we achieved the same results as the Lopez-Sabater et al. (1996) report related to the incidence of

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histamine-forming bacteria in which their estimation was < 0.1 % of the total bacterial load. Meanwhile, Ababouch et al. (1991)'s measurement of this value was about 0.97% of total flora. Some other studies found higher values and it was reported that histamine-forming bacteria in skipjack and jack mackerel enumerated to about 31 and 13.4% of total bacterial load, respectively (Omura et al., 1978; Yoshinaga and Frank, 1982). However, it should be considered that these investigations were carried out with spoiled fish.

Significant relationship ($p<0.05$) was observed between histamine content and Enterobacteriaceae count (Fig. 2). The average Enterobacteriaceae count of 42.2% of the samples having high histamine-forming bacterial count and containing more than 50ppm amount of histamine, was more than 3.3×10^3 CFU/g. Enterobacteriaceae species are the most important histamine-forming bacteria in tuna fish (Frank et al., 1985). In this investigation 66.2% and 22.5% of histamine-forming bacteria belonged to Enterobacteriaceae and *Clostridium perfringens*, respectively. Lopez-Sabater et al. (1996) reported that 83% of histamine-forming bacteria belonged to the Enterobacteriaceae family. Lopez-Sabater et al. (1994) reported that all isolates with histidine-decarboxylase activity isolated in their investigation were gram-negative and from the 40 strains isolated from Niven's medium, 77.5% belonged to the Enterobacteriaceae family. However, it is noteworthy to mention that only aerobic histamine-forming bacteria could be isolated in their investigations meanwhile in the present study, both aerobic and anaerobic were found. Based on the Yoshinaga and Frank (1982) study, 50% of the isolated histamine-producing bacteria were *Clostridium perfringens*.

All bacterial species with histidine decarboxylase activity isolated in this study (Table 1) have previously been reported by other researchers (Omura et al., 1978; Yoshinaga and Frank, 1982; Taylor and Speckhard, 1983; Frank et al., 1985; Middlebrooks et al., 1988; Lopez-Sabater et al., 1994; Kim, 2001; Tsai et al. 2004; Choudhury et al., 2008). Behling and Taylor (1982) indicated that the histamine-producing bacteria could be divided into two categories: a) those species capable of producing large quantities of histamine (>100mg/100ml) in tuna infusion broth (TFIB) during a short time (<24h) incubation at a temperature above 15°C and b) those capable of producing low histamine (<25mg/100ml) in TFIB with a long time incubation (≥48h) at a temperature ≥30°C. The prevalence frequency for different bacterial species indicated that *Proteus* spp. (24.5%), *Clostridium perfringens* (22.5%), *Klebsiella* spp. (15.0%), *Enterobacter* spp. (11.6%), and *Morganella morganii* (2.5%) had the highest amount of histamine-forming bacteria which belong to the category of prolific histamine producers, and samples with high concentrations of histamine, contained various numbers of these organisms (Tables 2 and 3). On the other hand, other species consisting of *Citrobacter freundii* with 8.0% prevalence, *Pseudomonas* spp. (7.0 %), *E. coli* (4.1%), *Seratia marcescens* (2.8%) and *Aeromonas*

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hydrophila (2.0%) can be categorized as slow-producers of the histamine group. Histamine intake ranged within 8-40, 40-100 and >100mg/100g which may cause slight, intermediate and intensive poisoning, respectively (Parente et al., 2001; Önal, 2007). The obtained results showed that of 45 examined samples, 35.6%, 22.2% and 42.2% of them contained less than 20ppm, 20- 50ppm, and more than 50ppm amount of histamine, respectively. It can be concluded that there are sea food safety risks in the usual fishing method in the Oman Sea and post-fishing procedures used in skipjack canning industry in study areas. Since the prolific histamine forming bacteria were mesophilic and typically occur as a result of post-fishing contamination, good hygienic practices and proper cooling of tuna (with emphasis on skipjack) after catching and during transportation is recommended.

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جداسازی وشناسایی باکتریهای تولید کننده هیستامین در ماهی هوور مسقطی (*Katsuwonus pelamis*) منجمد مورد استفاده در تولید کنسرو

ولی اله کوهدار[1*]، ودود رضویلر[1]، عباسعلی مطلبی[2]، فرهاد موسی خانی[3]، تورج ولی نسب[2]

چکیده

دراین مطالعه، مجموعه ای از آزمایشات برای جداسازی وشناسایی باکتریهای تولید کننده هیستامین و آنالیز میزان هیستامین، جهت ارزیابی روشهای صید و پس از صید انجام شد. ماهی هوور مسقطی (*Katsuwonus pelamis*) صید شده ازآبهای دریای عمان با روشهای تورگوشگیر و تورگردان پیاله ای برای این منظور استفاده شد. جداسازی سویه های باکتریها و تعیین میزان هیستامین، بااستفاده از عضلات اطراف آبشش ها انجام شد. نتایج حاصله نشان داد که میانگین شمارش کلی باکتریها (TPC) و شمارش سرمادوست ها به ترتیب $۷/۲\times۱۰^{6}$ و $۲/۹\times۱۰^{6}$ CFU/g بود. باکتریهای تولید کننده هیستامین به نسبت پایینی در مقایسه با کل باکتریها شمارش شدند. باکتریهای متنوعی به عنوان باکتریهای تولید کننده هیستامین شناسایی شدند. از میان آنها، گونه های پروتئوس با بیشترین فراوانی (۲۴/۵٪) و پس از آن کلستریدیوم پرفرینجنس (۲۲/۵٪)، گونه های کلبسیلا (۱۵/۰٪)، گونه های انتروباکتر (۱۱/۵٪) و سایر باکتریها (۲۶/۵٪) در تولید هیستامین نقش داشتند. میزان هیستامین در نمونه های مورد آزمون متفاوت بود و ۳۵/۶، ۲۲/۲ و ۴۲/۲ درصد نمونه ها به ترتیب حاوی مقادیر < ۲۰ ppm، ۲۰–۵۰ ppm و > ۵۰ppm هیستامین بودند؛ بنابراین مخاطرات سلامتی درخصوص روشهای صید وپس از صید موجود در مورد تون ماهی هوور مسقطی وجود دارد و روشهای پیشگیری مناسب جهت جلوگیری از تولید هیستامین پیشنهاد می شود.

واژگان کلیدی: هوور مسقطی، *Katsuwonus pelamis* ، هیستامین، باکتری، دریای عمان

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*آدرس پست الکترونیکی نویسنده مسئول: valiollah.kohdar@kiau.ac.ir

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