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The Relationship of Histamine Content in European Pilchard (*Sardina pilchardus*) with Freshness, Temperature, and Storage Duration

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ABSTRACT

Histamine food poisoning, stemming from the consumption of certain histamine-rich fish species, such as tuna, mackerel, European pilchards, and herring, is one of the major public health issues worldwide. The present study aimed to evaluate the histamine content in fresh European pilchard (*Sardina pilchardus* Walbaum, 1792) of Mehdia, a coastal city in the north of Morocco. Three randomly selected batches of fresh European pilchards, each weighing 20kg, were obtained from different boats upon landing. The evolution of histamine production was monitored every 8 hours for 6 days, with one batch stored at 0°C and the other at 10°C. The organoleptic characteristics were examined considering sensory evaluation according to the rating system of European Council Regulation No. 2406/96 as common marketing standards for certain fishery products and the quality index method (QIM). The histamine content in European pilchard flesh was determined using the fluorometric method. The results indicated that the average histamine content did not exceed 5 ppm during storage at 0°C. The freshness ratings were highest during the first 3 days, corresponding to QIM values of 0 to 10 at 0°C. On days 4 and 5, the freshness ratings were on quality A, corresponding to QIM values of 11 and 12, and on the last day, they were on quality B, corresponding to a QIM value of 15 with preservation of the organoleptic quality. Statistically, a significant correlation was found between the European pilchard's freshness and the storage duration. In contrast, this correlation between the histamine content and the storage duration was insignificant. At 10°C, the average histamine content exceeded the regulatory limit in force (100 ppm) after 32 hours of storage, and spoilage occurred on day 3. Statistical analysis revealed a strong correlation between the histamine content, storage temperature, the degree of freshness, and the duration of storage. The extra freshness quality index of European pilchard guarantees a very large margin of safety regarding histamine and can be consumed without risk.

Keywords: Degree freshness, European pilchard, Histamine, Sardine, Storage, Temperature

INTRODUCTION

Histamine is a biogenic amine responsible for several allergic and inflammatory phenomena (Oktariani et al., 2022; Bose et al., 2023). It is formed as a result of the enzymatic activity of histidine decarboxylase produced by some bacteria, such as *Morganella morganii*, *Raoultella ornithinolytica*, *Raoultella planticola*, *Proteus vulgaris*, *Proteus mirabilis*, *Klebsiella sp*., *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundi*, *Serratia liquefaciens*, and *Serratia fonticola* (Tao et al., 2022; Ginigaddarage et al., 2023; Rachmawati et al., 2023), which is accentuated by poor storage conditions (Emborg and Dalgaard, 2008; Abuhlega and Ali, 2022). This leads to the decarboxylation of L-histidine, which is naturally present in the flesh of many species of fish (García-Ruiz et al., 2011). Consumption of fish containing high levels of histamine causes histamine or scombroid poisoning (Colombo et al., 2017). The World Health Organization (WHO) estimates that around 600 million people experience food intoxication each year, resulting in the annual loss of 420,000 lives (WHO, 2022). This significantly impedes socio-economic development as it negatively affects the health systems, national economies, tourism, and commercial exchanges (WHO, 2022). Internationally, Dalgaard et al. (2008) reported thousands of outbreaks, incidents, and cases of food poisoning had been reported in Japan, Denmark, the United Kingdom, and Taiwan between 1986 and 2005. In France, an average of 11 outbreaks of histamine food poisoning affected around 67 cases per year between 2008 and 2019 (ANSES, 2021). In Morocco, the Poison Control and Pharmacovigilance Center (CAPM) recorded 14344 outbreaks, incidents, and cases of food poisoning between 2013 and 2020, with an annual number of outbreaks varying from 226 in 2020 to 2,887 in 2015. Food-borne diseases occupy the second etiology, with 15.7% of all poisonings. Poisoning caused by fish accounts for 10.6% of these food poisonings (Poison Control and Pharmacovigilance Center of Morocco, 2021).

On the regulatory level, the toxic dose of histamine in fish is not yet known accurately (Emborg and Dalgaard, 2008; Hungerford, 2021). According to ANSES (2021), the histamine level below 50 ppm is generally considered non-toxic. From 50 to 200 mg/kg, the food can present a potential risk of toxicity, especially for sensitive people. Histamine

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levels within a range of 200-1000 mg/kg suggest a probable risk of toxicity. Beyond 1000 mg/kg, the fish is considered toxic.

International regulations define a maximal limit for histamine in fish to determine the suitability of fish consumption for humans (Debeer et al., 2021). The Moroccan Ministerial Decree n° 293-19 (Official Bulletin, 2019) and European Commission regulation n° 2073/2005 (EC, 2005) have aligned histamine as a safety microbiological criterion, setting concentration limits for nine fish samples. Accordingly, the average content must not exceed 100 mg/kg, two samples may exceed 100 mg/kg but not reach 200 mg/kg, and no sample should exceed 200 mg/kg. At the Codex Alimentarius Commission, histamine study has been extensively discussed across various committees. This includes the analysis of epidemiological data, the evaluation of public health risks caused by histamine, the review of sampling plans, and applied protection (FAO/WHO, 2013). In the United States, the Food and Drug Administration (FDA) has proposed a very severe lowering of the histamine limit from 50 to 35 ppm as a limit of decomposition in fish far exceeding internationally recognized Codex guidelines and standards (FDA, 2021), which is 100 ppm. This proposal limit may represent a major obstacle to international trade in fishery products.

Histamine in seafood is a very complex issue for the fisheries sector. Epidemiological and economic data, foreign exchange inputs generated by this sector and its contribution to food, economic and social security, discussions within the Codex Alimentarius, and the new regulatory requirements proposed by the FDA show that it is still important to study this health hazard. The first link in the value chain is storage, which is the intermediate step between harvesting and processing or consuming seafood products. The control of histamine during this stage is crucial to avoid any bacterial contamination that may increase the histamine level. In this context, this experimental study aimed to contribute to better control of the danger of histamine in the European pilchard (*Sardina pilchardus*, Walbaum, 1792) of Mehdia (a coastal city located in the north of Morocco) through monitoring of the freshness and kinetics of histamine production in the fresh European pilchard during 6 days of storage at 0°C and at 10°C. In this regard, the relationship of histamine with freshness, histamine content, temperature, and duration of storage, as well as the ability of the frosting to control histamine, were examined.

MATERIALS AND METHODS

Ethical approval

This study was conducted according to the guidelines of the Agronomic and Veterinary Institute Hassan II, Rabat, Morocco.

Study location

The study was carried out in the laboratory of the Food Safety Unit of the Agronomic and Veterinary Institute Hassan II, Rabat, Morocco.

Samples

The study focused on evaluating fresh European pilchard (*Sardina pilchardus*) landed in the fishing port of Mehdia in northern Morocco. For this purpose, three randomly selected batches of fresh European pilchards, each weighing 20 kg, were taken from the landing of three different boats. These batches were promptly placed into insulated boxes containing ice and transferred immediately to the laboratory. In the laboratory, each batch was divided into two groups (A and B). Hygienic and cold conditions (0°C) were ensured. Group A was preserved under melting ice in a refrigerator at 0°C, and group B was preserved under melting ice in a refrigerator at 10°C. The investigated European pilchard had an average weight of 25.60 ± 6.2 g and an average length of 17.67 ± 0.76 cm.

To comprehensively assess the alteration in each group, six fish per group were collected every 8 hours over 6 days, with the first sample collected 9 hours after the initial catch. This resulted in 102 samples, with 51 samples from the group stored at 0°C and 51 samples from the group stored at 10°C.

Temperature measurement

The core temperature of each sample was taken by a calibrated thermometer with an interchangeable probe (Testo 110, Forbach, France) on six fish pieces every 8 hours. The arithmetic average of the temperature values was calculated.

Organoleptic evaluation of freshness

The sensory evaluation was carried out on whole fish using two methods by a panel of three veterinary inspectors experienced in the matter. The rating system of European Council Regulation (EC) N° 2406/96 (1996) establishes common marketing standards for certain fishery products. Hence, three classes of freshness are defined, namely first quality extra (E), second quality A, and third quality B or not admitted. To better interpret the results of this study, an appreciation score of freshness from 0 to 3 was assigned to each of the characteristics evaluated during its evaluation

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(skin and skin mucus, flesh consistency, gill covers, shape and color of eye, color, smell, and mucus of gills). The total sum of the appreciation scores was then calculated.

The quality index method (QIM) consists of assigning a score of demerits from 0 to 3 for each character (general appearance (Surface, stiffness, flesh firmness), eye (clarity cornea, pupil, shape), cover bloodiness, gills (color and smell), abdomen post girl, flesh appearance), the total sum of scores was calculated when the QIM value is high, the fish is spoiled (Triqui and Bouchriti, 2003).

Determination of histamine content

Lerke and Bell fluorimetry method is a quantitative histamine assay technique used in several laboratories and known for its accuracy, repeatability, simplicity, and speed. This method is based on the extraction, purification, and then determination of histamine using specific chemical solutions for each step (Lerke and Bell, 1976; Rachidi et al., 2011). Histamine was extracted from 10g of flesh well mixed with 90 ml of trichloroacetic acid (TCA), then separated by chromatography on an ion exchange column (Amberlite CG50) and eluted with hydrochloric acid (HCl, 0.7 Normality). The dosage was carried out by fluorometry after complexation with orthophthalaldehyde (OPA). Histamine concentrations were read using a Trilogy^{TM} fluorometer (Turner Designs Instrument, Model 7200-000, California, USA) by fluorescence at emission and excitation lengths wave of 450 and 360 nm, respectively.

Statistical analysis

The obtained results were analyzed by the R-Studio statistical software, a linear regression analysis software that allows the relationship existing between a dependent variable Y (average content of histamine), and independent variables (Freshness index [IF] and duration of storage) in each storage temperature. The intensity of the relationship given is appreciated by the determination index (R^2). This index is between 0 and 1. The relationship is weak when it is close to 0 and strong when it is close to 1. The significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

The results showed a significant variation in the evolution of the mean histamine content and freshness index during storage for both protocols ($p < 0.05$). The average histamine content varied from 2.19 ppm on day 4 when stored at 0°C to 1988.32 ppm on day 3 when stored at 10°C. The freshness index evolved from extra quality to quality B on day 6 of storage at 0°C without crossing the organoleptic rejection threshold and towards organoleptic rejection on day 3 of storage at 10°C.

The first protocol for fish stored at 0°C

During storage at 0°C, the fish were stored under melting ice at 0°C for 6 days. Temperature monitoring results varied between 0.1°C and 1°C. The average temperature was 0.49°C. The mean histamine content and degree of freshness are represented in Table 1. Each value is the average of three different batches.

Table 1. Freshness index, quality index method, and histamine means contents for European pilchard stored at 0°C

Sampling time (hours)	Freshness index	Appreciation score of freshness	QIM	Histamine means contents (ppm)
0	E	27	0	2.77
8	E	27	0	4.22
16	E	26	0	3.86
24	E	26	0	3.14
32	E	25	4	2.26
40	E	25	4	3.72
48	E	25	9	4.35
56	E	25	9	4.09
64	E	25	10	3.22
72	A	23	11	3.21
80	A	23	12	4.22
88	A	21	12	2.19
96	A	21	12	3.2
104	A	20	12	3.6
112	A	18	12	3.31
120	B	14	15	5.02
128	B	14	15	4.95

E, A, B, Not admitted: Freshness index according to European Council Regulation (EC) No 2406/96: E (first quality extra), A (second quality), B (third quality). Not admitted: Spoiled, QIM: Quality index method

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The organoleptic evaluation of European pilchard stored at 0°C indicated that the loss of freshness was very slow, and it did not reach the level of organoleptic rejection after day 6. The organoleptic characteristics examined over time did not lose their quality simultaneously. The loss of abdomen integrity, shape, and loss of shine of the eyes began from day 3 by going through very distinct shades with the quality maintenance of the flesh, smell, and rigidity until day 6 of storage. Thus, the European Pilchard kept its freshness in class E (extra, the best one) for the first 3 days, with a corresponding QIM of 0 to 10. It then transitioned to class A on days 4 and 5 with a QIM between 11 and 12. Finally, class B had a QIM of 15 on the last day without reaching the organoleptic rejection threshold. There was a strong correlation between the QIM, the freshness index, and the duration of storage, with a determination coefficient of 0.85 for the freshness index and 0.9 for the QIM. Figure 1 shows a graphical representation of the evolution of the freshness index, the QIM, and the histamine level in European Pilchard stored at 0°C. QIM values have been reported in previous studies for organoleptic rejection from fish kept under ice (0°C); a QIM value of 15 was used for salmon and sea bream (Huidobro et al., 2000; Sveinsdottir et al., 2002). A lower QIM value was reported for hake (*Merluccius merluccius*) kept at 4°C and cod fillets under melting ice (Baixas-Nogueras et al., 2003; Cardenas et al., 2007). Considering the precision of prediction of the quality index methods, which are between days 1 and 3 (Baixas-Nogueras et al., 2003), it can be concluded that the Mehdia European Pilchard, stored under ice at 0°C can retain its organoleptic characteristics for 9 days. Comparable intervals between 9 and 12 days have been reported in other studies (Perera et al., 2020), and even longer shelf lives have been found in some cases, more than 16 days under salt-added ice (Losada et al., 2004).

Histamine content remained almost stable throughout 6 days of storage and varied between 2.19 and 5.02 ppm. This concentration was well below the regulatory limit in force. Moreover, the coefficient of determination value of 0.09 showed a very low relationship between the histamine content and the duration of storage when it was kept at 0°C. The statistical analysis yielded a linear regression equation in the form of Y (histamine content) = 1.55IFB+2.87. This equation showed that the duration of storage has no impact on the histamine content at this temperature. The coefficient of determination was equal to 0.09, near 0, reflecting the weak relationship between the two variables (Figure 2). The analysis reveals that when the fish maintained an Extra class freshness, there was no significant increase in histamine content. However, as the fish freshness transitioned from class A to class B, the histamine content exhibited a slight increase of 1.55 ppm, representing a minimal change.

The results of storage at 0°C suggested that histamine production in flesh European pilchard could be stopped by the fish frosting. This is consistent with a study conducted by Perera et al.(2020) and contrary to the study by Mohamed et al. (2022), indicating that the histamine production in flesh European pilchard was only delayed and histamine content could increase during storage in ice (0°C). These results are consistent with a study on the occurrence of histamine in canned European Pilchard marketed in Morocco, revealing that the average histamine content did not exceed 5.14 ppm. The study further indicated that canned sardines sold in the northern coastal area of Morocco, corresponding to the current area studied in this work, had the lowest risk with an average histamine content of 2.11 ppm (El Hariri et al., 2017). Mejrhit et al.(2018) previously found superior results in fresh Moroccan sardines with a concentration of up to 200 ppm, indicating a significant improvement in hygiene conditions, handling, and awareness-raising of professionals in the sector, as well as the evolution of fishing and processing techniques.

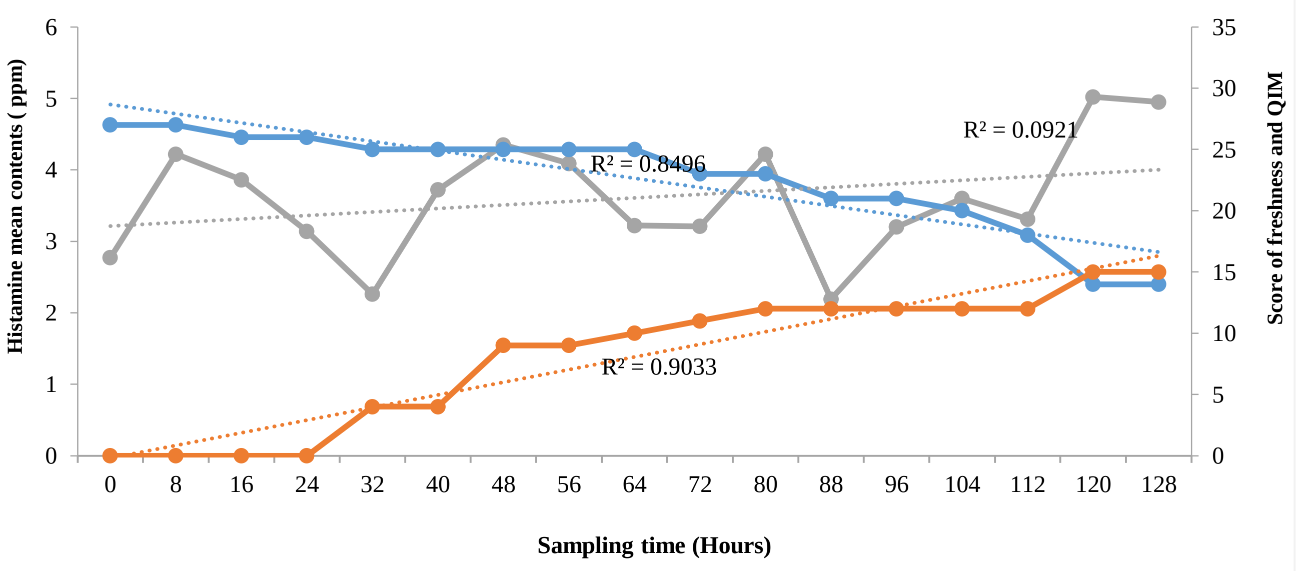

Figure 1. Evolution of freshness index, quality index method, and histamine mean contents of European pilchard stored at 0°C. Gray: Histamine mean content (ppm), Blue: Appreciation score of freshness, Orange: Quality index method (QIM)

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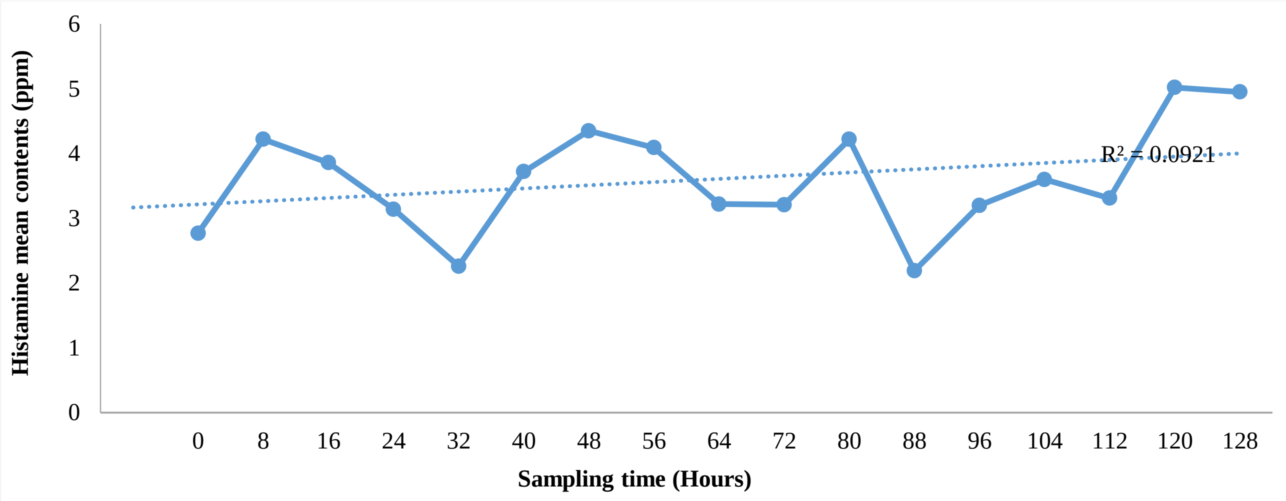

Figure 2. Histamine means contents of European pilchard stored at 0°C (ppm)

The second protocol for fish stored at 10°C

The fish were stored at 10°C for 6 days during this protocol. The temperature varied between 8.2°C and 10.7°C. The average temperature was 9.81°C. The results of the mean histamine content, freshness index, and QIM obtained are presented in Table 2. At storage at 10°C, the European pilchard began to lose its freshness from the 16th hour and passed rapidly to class A. Then, the fish reached the threshold of organoleptic rejection from the third day of storage, when there was a sour smell of putrefaction and an injury to the abdomen and eyes. The organoleptic rejection point corresponded to a QIM of 15. The evolution of the freshness index and QIM of the European pilchard stored at 10°C are represented in Figure 3. The evolution of histamine content of European pilchard stored at 10°C is represented in Figure 4. The graph shows a rapid change in the histamine content after 32 hours, reaching high concentrations of 824.74 ppm. The histamine level increased massively and reached an overwhelming value of 1988 ppm on the third day of storage.

Statistical analysis concluded a linear regression equation in the form Y = 554.57 IFB+30.11H (time). This equation showed that duration of storage has an impact on the histamine content. For each 8 hours, there was an increase of 30.11 ppm. As shown in Figure 5, the evolution of the histamine content as a function of duration of storage has a coefficient of determination equal to 0.87, which was close to 1, reflecting the strong relationship between the two variables. When the fish had an Extra freshness quality, the histamine content did not increase. When the fish freshness changed from class A to class B, the histamine content increased by 554.57 ppm, contrary to the 0°C storage temperature, where the histamine content increased by 1.55 ppm only. Thus, there was a strong association between histamine content and degree of freshness according to duration and storage temperature. This association was already reported by several studies involving European pilchard (Visciano et al., 2007; Mohamed et al., 2022).

Table 2. Freshness index, quality index method, and histamine mean contents for European pilchard stored at 10°C

Sampling time (hours)	Freshness index	Appreciation score of freshness	QIM	Histamine means contents. (ppm)
0	E	27	0	27.94
8	E	26	1	66.29
16	E	25	2	75.42
24	A	21	5	53.75
32	A	18	5	824.74
40	B	16	7	1022.72
48	B	15	8	2002.19
56	B	14	12	1852.42
64	B	14	15	1988.32
72	Not admitted	12	16	--
80	Not admitted	9	19	--
88	Not admitted	5	25	--
96	Not admitted	2	28	--
104	Not admitted	0	28	--
112	Not admitted	0	28	--
120	Not admitted	0	28	--
128	Not admitted	0	28	--

E, A, B, Not admitted: Freshness index according to European Council Regulation (EC) No 2406/96: E (first quality extra), A (second quality) B (third quality). Not admitted: Spoiled, QIM: Quality index method.

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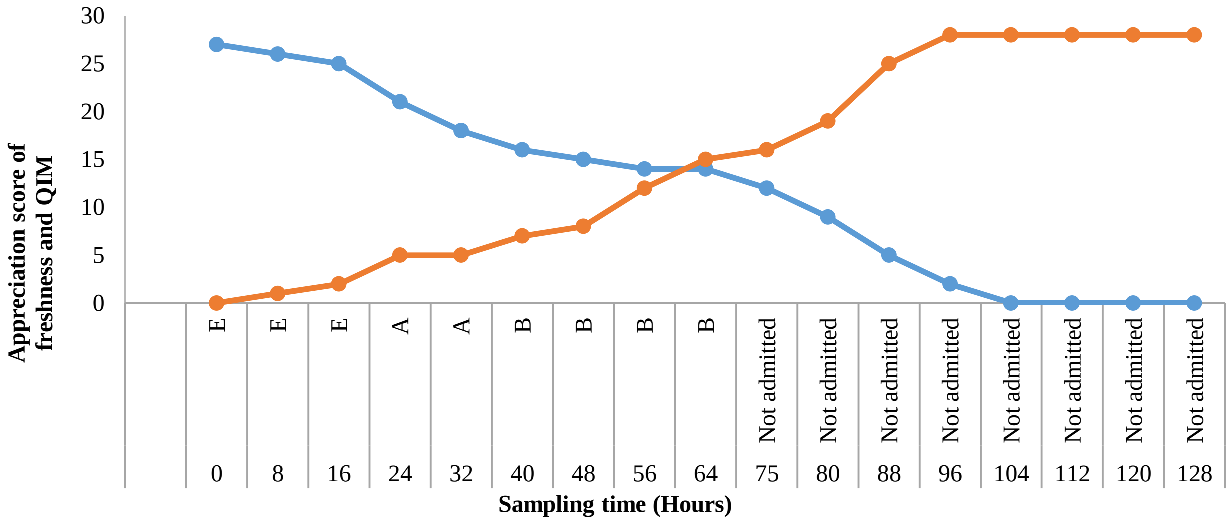

Figure 3. Evolution of the freshness index and quality index method of the European pilchard stored at 10°C. Blue: Appreciation of score of freshness, Orange: Quality index method. E, A, B, Not admitted: Freshness index according to European Council Regulation (EC) No 2406/96: E (first quality extra), A (second quality) B (third quality). Not admitted: Spoiled, QIM: Quality index method.

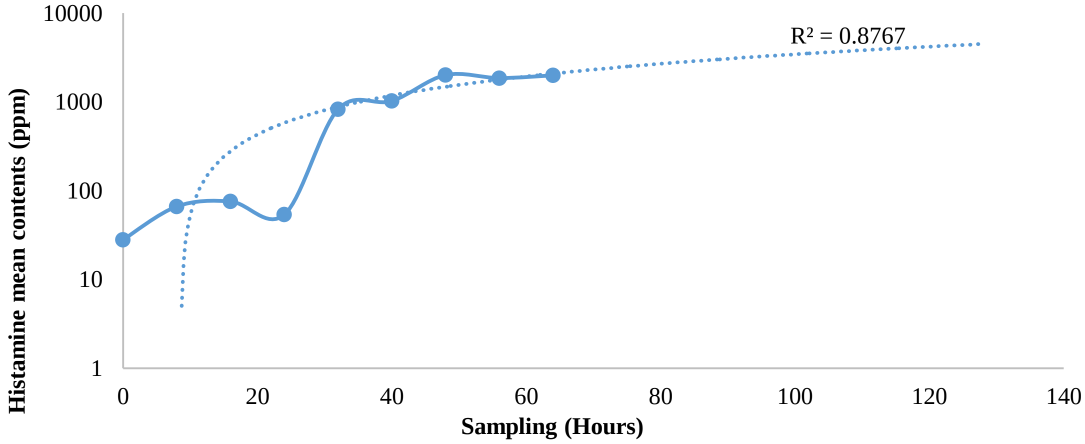

Figure 4. Histamine means contents of European pilchard stored at 10°C (ppm)

Comparative study of both protocols

The evolution of histamine and freshness is highly variable during storage for both protocols. Figure 5 indicates the difference in the evolution of the histamine content for each protocol. The average histamine content remained relatively constant at 0°C, while at 10°C, the average histamine content evolved rapidly between the 32 and 56 hours of storage and then slowed down to the end of storage.

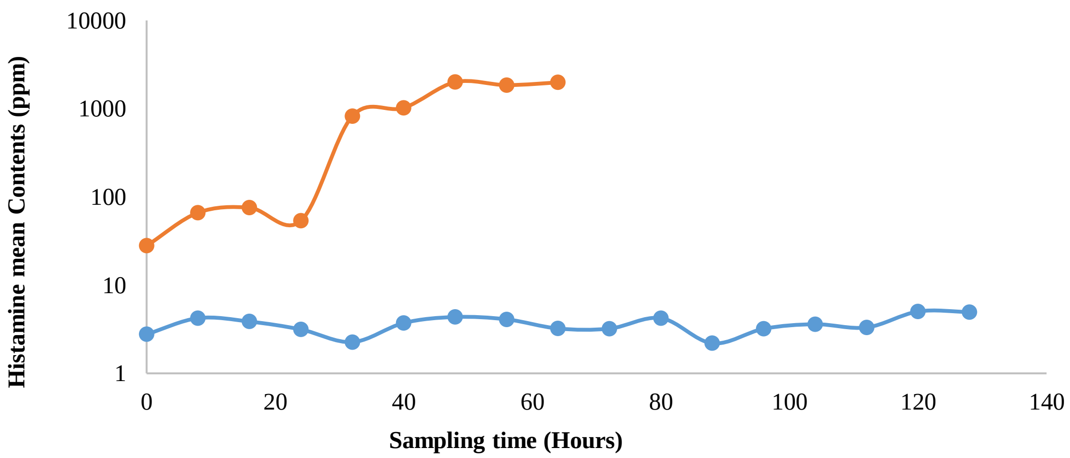

Figure 5. Histamine mean contents of European pilchard stored at 0 and 10°C (ppm). Blue: Storage at 0°C, Orange: Storage at 10°C.

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CONCLUSION

The storage at 0°C kept the organoleptic characteristics of the fresh European pilchard in very good condition for up to 6 days. The mean histamine content did not increase during storage at 0°C, unlike storage at 10°C, where the histamine content could easily exceed the acceptability threshold. A strong association was found between histamine content and degree of freshness according to duration and storage temperature. The storage temperature is critically important for the histamine content in flesh fish as well as for the degree of freshness over a given shelf life. When the fish has an extra freshness class, the histamine content does not increase. It can be concluded that fresh European Pilchard, which has an extra freshness quality, guarantees a very large margin of safety with regard to histamine and that it can be consumed without any risk. These results highlight the importance and suitability of frosting and the control of the storage phase in managing the risk of histamine in fishery products by involving all stakeholders in the sector, mainly the competent health authorities and food business operators. Further research is suggested to be conducted regarding the bacteria forming histamine in European pilchard, toxic doses of histamine, and the other risk management factors, such as the level of consumption specific to certain categories of consumers.

DECLARATIONS

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Authors' contributions

R. Khatouf conceptualized, conducted the research, and wrote the manuscript, Said Dahani, Oleya El Hariri, and N. Bouchriti conceptualized, analyzed data, supervised the research, and revised the final form. All authors read and approved the final version of the manuscript.

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Availability of data and materials

The data of the current study are available regarding the reasonable request from the authors.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors before publication in this journal.

Competing interests

The authors had no conflict of interest to declare.

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