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A Prevalence Study of Histamine and Histamine Producing Bacteria in Two Commercial Tropical Marine Fish Sold in Trinidad, West Indies

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Abstract

Background: Scombroid poisoning is responsible for the highest morbidity worldwide of any fish related food poisoning. However, there is little information available on this potential hazard to the fish consuming population of the Caribbean. This study focused on quantifying the levels of scombroid toxin (histamine) and selected histamine producing bacteria in two popularly consumed marine fish (carite, *Scomberomorus brasiliensis* and king fish, *Scomberomorus cavalla*) in Trinidad, West Indies.

Methods: A total of 78 fish were sampled at five different market types throughout the island. A commercial histamine kit was used to quantify histamine levels and the overall microbiological quality was evaluated from aerobic plate counts of fish tissue. Secondly, the role of fish and market types was investigated as potential sources of histamine producing bacteria (HPB).

Results and conclusion: Of the fish sampled, 98.7% had histamine levels within USFDA acceptable limits of ≤ 50ppm. Overall, histamine levels were significantly higher in carite than in kingfish. One carite sampled from a wholesale market was in violation of the limit with a histamine level of 57 ppm. A significant relationship was observed between sensory characteristics and histamine levels. Fish tissue and gills were the main sources of histamine producing bacteria. There was a significant ($p = 0.05$) association between market type and the number of histamine producing bacterial types, with the highest morphological diversity of HPB present in fish from landing sites and retail markets. These market types also had the highest proportion of bacterial isolates positive for potential histamine production (12.9% each) as compared to supermarkets (11.3%), wholesale markets (8.1%) and mongers (6.5%).

Keywords: Histamine; Histamine producing bacteria; *Scomberomorus brasiliensis*

Introduction

Consumer concern about the safety of the food they eat has been increasing, highlighted by a number of "food scares" in recent years [1]. The globalization of food trade and increasing problems worldwide with emerging and re-emerging food borne diseases have increased the risk of rapid spread of infectious agents [2,3]. Consumers' demand for fresh, healthy food is increasing around the world [4] with fish being no exception. There is numerous health hazards associated with the consumption of fish such as puffer fish poisoning [5], *anisakis simplex*, ciguatera poisoning, scombroid poisoning etc [6].

Scombroid poisoning is associated with consumption of spoilt fish [7] and is responsible for the highest morbidity worldwide of any fish related food poisoning [8]. This condition arises after eating fish containing high levels of histamine which results from bacterial conversion of the amino acid, histidine to histamine [9].

Ingestion of food containing small amounts of histamine has little effect on humans, but in large amounts histamine (>50 mg/100g) can bring about scombroid fish poisoning. Histamine is considered as an indicator of earlier microbial decomposition of seafood and a guidance level of 50ppm is considered as the chemical index for fish spoilage [10,11]. Fish from the scombroid family are most susceptible to high histamine level because of the presence of high concentrations of histidine [12]. The largest outbreak (2656 cases) was recorded in Japan in 1973. Since then, the worldwide network for harvesting, processing and distributing fish products has recognized histamine poisoning as a global problem [13].

Although scombroid fish is very popular in the Caribbean, there is a dearth of limited published literature on scombroid poisoning in this region. Scombroid poisoning is the second major type of poisoning affecting people in the Eastern Caribbean due to consumption of spoiled *Acanthocybium solandri* (wahoo), *Scomberomorus cavalla* (king fish or king mackerel) and *S. maculatus* (cero) [14]. Despite this report, there are suggestions that the level of scombroid poisoning in official statistics of countries may be underreported because this conditioning can be mis-diagnosed for other food borne illness [15] or categorized as non-specific food poisoning.

In Trinidad and Tobago, carite (*Scomberomorus brasiliensis*) and kingfish (*Scomberomorus cavalla*) are two of the more popular marine fish species caught and consumed locally. Both are members of the *Scomberesocidae* family [14]. However, there is no published data on scombroid poisoning or the risk faced by consumers' in Trinidad.

The objectives of this study were to (ONE) determine histamine

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levels in carite and kingfish sold by different types of retail outlets in Trinidad and (TWO) investigate the occurrence of histamine producing bacteria on fish and their possible sources of contamination.

Materials and Methods

Sampling

A sample size of 78 fish was determined assuming standard deviations of equal number to 3.090 ($V=\Sigma (\mu i - \mu)^2 / G$ of 4.000) [16,17]. Equal numbers or carite and kingfish (39 each) were collected from five market types sampled on three separate occasions over an 8 month period (January-August, 2010).

Fish were sampled from five different market types: supermarkets, fish mongers and fish vendors, landing sites, wholesale markets. Each site was sampled 3 times. All samples were randomly chosen. Fish were placed in a cooler containing ice and transported back to lab. A sensory evaluation of whole fish was conducted, by scoring sensory characteristics including texture, smell, colour etc. of the various parts of the body [18]. The evaluation was done by one trained individual to ensure consistency and the average score of all characteristic was used as a quality indicator.

Meat cutting scissors and forceps was flame sterilized and used to remove sections of fish tissue. For whole fish, samples (~10 g) were taken from the head (proximal ventral fin); body (anus) and tail (tail fin) and macerated into 1 composite (~30 g) sample using a mortar and pestle [19]. For fish slice samples, sections of slice were taken and macerated in the same manner. The macerated tissue was used for aerobic plate count and the remaining stored at -20°C for histamine analysis.

Aerobic plate count

10 g macerated fish tissue was aseptically weighed and stomached for 120 s on high with 10 ml of buffered saline solution. 80 ml of buffered saline were added to the homogenate and mixed to make a 1:9 dilution. This mixture was then plated onto Plate Count Agar using the following dilutions: 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}. Plates were incubated for 24 hrs at 37°C before colonies were counted and total aerobic plate counts calculated [19].

Histamine analysis

The samples were tested using the Max Signal histamine enzyme kit (Bio Scientific, Austin, TX). This is a colorimetric enzymatic assay for the determination of histamine in fresh fish/seafood, fish meal, wine and milk. The protocol specified by the kit was used.

Histamine producing bacteria analysis

Based on the results obtained from the prevalence study, a detailed investigation was conducted on the occurrence of histamine producing bacteria and their possible sources of contamination of carite. 10 carite were sampled from all market types (2 fish per market) for histamine analysis and microbiological evaluation. Swabs of environmental contact surfaces as well as samples of the water supply were taken from the various markets during sampling. Swabs of gills and intestines of the fish were also taken immediately on reaching the laboratory. All swabs were placed in 10ml of sterile buffered saline solution. Fish tissue (muscle and skin) was sampled from the head and body and made into a composite sample. 10 g was aseptically weighed and stomached for 120 s on high with 10 ml of buffered saline solution. 1 ml of fish tissue homogenate and samples of the stock saline swabs and water supply were each added to 9 ml of histidine broth (enrichment) and incubated for 37°C for 24 hrs. The respective histidine broths were serially diluted using 9.9 ml of sterile saline and plated onto (Tryptic Soy Agar) TSA, using a modified surface plating method [20,21].

Representative colonies of each colony type were picked of TSA plates and streaked onto Niven's agar and incubated at 35°C for 48 hrs [21]. Niven's positive isolates (purple colonies) were picked off and incubated in (brain heart infusion broth) BHI for 24 hrs at 37°C. 50% glycerol was added to BHI tubes and isolates stored in freezer at -20°C. Isolates stored in BHI and glycerol were plated on to Nutrient agar (Oxoid) and incubated at 37°C for 24 hrs. Bacterial DNA was extracted and purified using the PrepMan® Ultra Sample Preparation (Applied Biosystems Inc., USA). The partial 16S rRNA gene (~1500 bp) of isolates was amplified by polymerase chain reaction (PCR) using a TC-512 thermal cycler (Techne, UK). PCR amplification (25 µL) was performed using universal eubacterial 27F and 1492R primers. Each reaction tube contained PCR grade water, 5X Buffer (Green GoTaq Flexi) , 25 mM $MgCl_2$, 10 pmol of each primer, 25 mM of each dNTPs, 0.125U of GoTaq DNA polymerase, and template DNA (~10 ng). Amplifications were carried out for 30 cycles (95°C for 45 s, 55°C for 45 s, and 72°C for 1m 30 s) with an initial denaturation of 96°C for 5 min and a final extension of 72°C for 5 min.

After confirmation of the expected amplicon size, the PCR products were sent to Macrogen Inc, Seoul, Korea for sequencing. An approximately 800 bp region of the 16S rRNA gene sequences was used to identify isolates by comparison to sequences in GenBank database (http://www.ncbi.nlm.nih.gov/sites/enterz?db=Nucleotide) using BLAST (Basic Local Alignment Search Tool; National Center for Biotechnology Information, Bethesda, MS, USA) (http://www.ncbi.nlm.nih.gov/BLAST/).

Statistical analysis

Statistical analysis was done using the statistical program SPSS software Version 17 (IBM, New York, USA). ANOVA was used for statistical analysis of histamine data collected and Post Hoc test performed where means were different. Pearson correlation was used to determine if there was a significant relationship between sensory evaluation and histamine. Pearson Chi-Square and Tukey HSD were used to analyze the significance of sources of contamination of histamine producing bacteria (HPB). Significance level was at a 0.05 level.

Results

Aerobic plate counts (APC) of fish varied significantly ($p < 0.001$) among market types (Figure 1). The retail markets had the highest

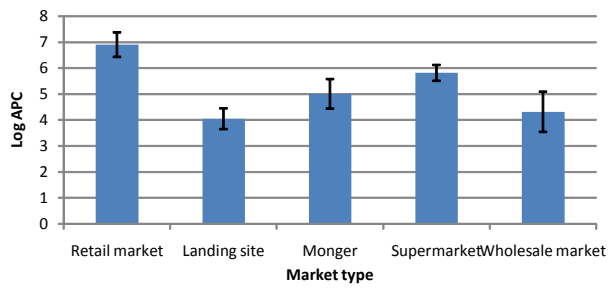


Figure 1: Aerobic plate counts of fish from different market types. Note: values are means of both carite and kingfish.

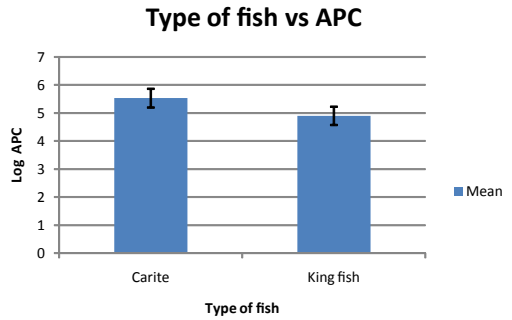
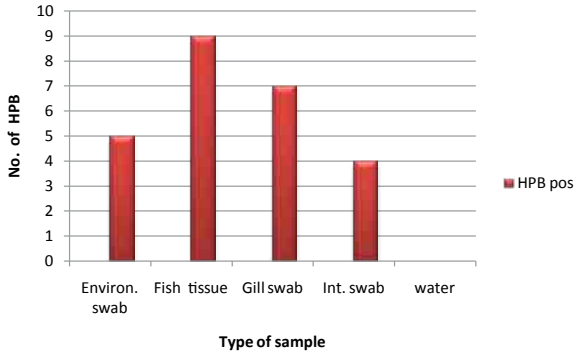


Figure 2: Aerobic plate counts of carite and kingfish (n=39).note: values are means of five market types.



Note: Data is for all market types

Figure 3: Number of different histamine producing bacteria in fish, environment and water samples.

overall APC ($6.9x10^7$ CFU/g) followed by supermarkets ($5.8x10^7$ CFU/g), wholesale markets ($4.3x10^7$ CFU/g) , mongers ($5x10^7$ CFU/g) and landing sites ($4x10^7$ CFU/g). The Tukey analysis showed that the fish markets had APC significantly ($p < 0.15$) higher than landing sites, wholesale markets and mongers. Supermarkets had higher ($p \leq 0.05$) APC than landing sites and wholesale markets. All samples exceeded the minimum recommended acceptable limit ($m=5x10^5$ CFU/g) for good quality seafood. However, all counts were within the maximum recommended limits for marginally acceptable quality ($M=10^7$) [22].

The APC did not vary significantly ($p > 0.05$) between carite (5.534×10^7 CFU/g) and kingfish (4.894×10^7 CFU/g) (Figure 2). Market type significantly influenced the number of HPB positive and negative sample isolates ($p < 0.05$) from fish samples (Figure 3). Fish tissue had the highest number of different types of HPB (nine), followed by gills (seven), environmental contact surfaces (five) and intestines (four). All water samples for all market types were negative for the presence of aerobic counts. There was a significant ($p = 0.05$) relationship between market type and the number of histamine producing bacterial types (Figure 4). The highest number of positive HPB was from landing sites and retail markets (eight), followed by supermarkets (seven), wholesale market (five) and the least at mongers (four). These market types also had the highest proportion (12.9% each) of TSA bacterial isolates positive for potential histamine production based on growth characteristics on Niven's agar as compared to supermarkets (11.3%), wholesale markets (8.1%) and mongers (6.5%). Most of the bacteria isolated were Gram negative rods (Table1). 88.9% belonged to the class Proteobacteria and 77.7% belong to the genus Enterobacteriaceae. The majority of bacteria identified are potentially harmful to humans causing nosocomial or zoonotic diseases. *Klebsiella pneumonia, Aeromonas spp.* and *Morganella morganii* are three known histamine producers that were detected.

Market type significantly ($p = 0.011$) influenced histamine levels in fish (Figure 5). The lowest level of histamine observed was at the landing sites (mean = 13.67 ± 1.79; range = 3.3 - 29.51 ppm). The highest level was observed for the wholesale market (mean = 27.54 ± 3.46; range = 7.67 – 57 ppm). Moderate histamine levels were detected in fish samples from fish monger (mean = 16.98 ± 2.45; range = 6.03 - 28.05 ppm), retail market (mean = 18.83 ± 2.11; range = 4.94 - 41 ppm) and supermarket (mean = 18.83 ± 1.34; range = 6.25 - 44.25 ppm) respectively. One fish from the wholesale market had a histamine level of 57 ppm, which was in violation of the USFDA limit of 50ppm. Histamine level varied significantly ($p < 0.001$) with fish type (Figure 6). Carite had histamine levels (mean=26.59 ppm) that was more than twice the levels found in kingfish (mean=11.747 ppm).

There was a significant correlation ($r = 0.481$; $p = 0.017$) between sensory evaluation scores and histamine level (Figure 7). The general trend was that the lower the sensory score the higher the histamine level. The lower the score signifies a high level of spoilage of fish. One fish with a sensory score of 3 had a histamine level of 57 ppm.

Discussion

The results of the study generally showed the expected trend of increasing bacterial counts with movement up the supply chain

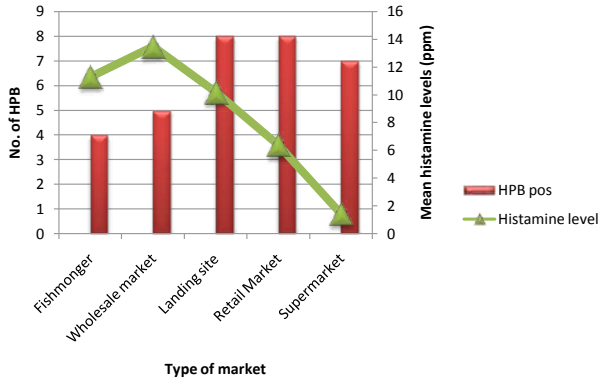


Figure 4: Number of positive hpb and histamine level in carite in market resample (n=8).

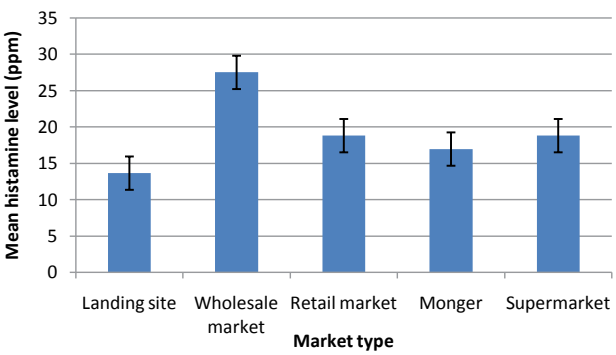


Figure 5: Histamine levels at the different market types. Note: values are means of both carite and kingfish.

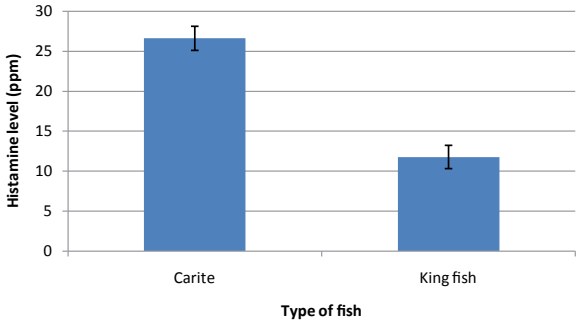


Figure 6: Histamine levels in two fish sampled at 5 different market types.

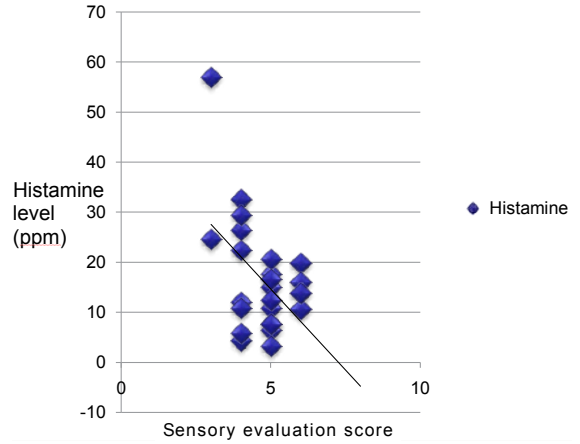


Figure 7: Scatter plot comparing sensory evaluation score to histamine level (r =-0.481)

(retail markets, fish mongers and supermarkets) (Figure 1). This is not surprising since fish higher up the chain are subjected to more handling and storage processes, resulting in more contamination events and time for heterotrophic bacteria to multiply. The highest APC was found in samples from retail markets, which could be due to poor sanitation and handling practices as was reported in a similar study in Taiwan [16]. The relatively high APC found in fish from supermarkets is suggestive that significant multiplication of bacteria may be taking place after harvest and wholesale marketing. However, better storage and handling at the supermarkets may have resulted in lower APC levels than retail markets. The relatively low counts at the landing sites and wholesale markets was not surprising since these are at the lower end of the supply chain and bacteria may not have had a lot of time to multiply. A study done at landing sites in Kenya reported an APC of 6.16 log cfu/g in fish sampled, which is slightly more than what was detected in this study [23]. However, it was surprising that fish from mongers also had relatively low APCs since it is a common practice to keep fish on top of tables. The lower counts may be due to rapid turnover of fish since sampling was conducted during a high demand period (Lent) and the mongers are normally small suppliers with a limited number of fish. The APC did not vary significantly ($p > 0.05$) between carite and kingfish (Figure 2). This could be due to the fact that both fish are generally marketed together and may have been subjected to similar handling and storage conditions.

Differences in the number of histamine producing bacterial types (Figure 4) as well as proportion of TSA isolates with potential histamine production capability in fish from different market types could be reflective of the nature of contamination taking place due to handling and processing of fish. However, the relative diversity and proportion of HPB in fish found at landing sites was surprising. This could be due to natural occurrence of HPB in fish, which can comprise 1% of fish normal microflora [24]. It should also be noted that there was a significant relationship between sources of contamination and histamine producing bacterial types (Figure 3). The relatively high diversity HPB isolates obtained from fish tissue and gills supports previous suggestions that these components are major sources of these bacteria [25]. Environmental contact surfaces including table tops, equipment and fish intestines also had HPB, but the morphological types were less varied. This suggests that, although post catching factors can be important in contamination of fish with HPB, pre-catching factors were more significant. It was significant that water used in sanitation, practices does not appear to be a source of HPB in contrast to other countries in which the water may be an additional source of these bacteria [26].

Identification of bacteria from Niven's agar (Table 1) showed the presence of several histamine producing species, such as *Morganella morganii, Klebsiella pneumoniae, Aeromonas spp., Proteus mirabilis, Enterobacter aerogenes and Citrobacter freundii* [27- 30]. There appears to be selectivity of these bacteria to specific environments since they were found in different fish tissues or in the environment. Although Niven's agar is known to sometimes give false negative of positive results, it was significant that most of the positive isolates identified are known histamine producers [31].

Market type significantly ($p = 0.011$) influenced histamine levels in fish (Figure 5). The landing sites had fish with expectedly, the lowest histamine levels. Although all fish from the landing sites had histamine levels within acceptable limits, the levels were higher when compared to a study done at landing sites in Sri Lanka (range = 0.223-9.4 ppm) [32]. The presence of histamine in fish at landing sites in this study could be due to factors such as ocean temperature and practices of fishermen locally. Gillnetting is the most common method of capture for carite and kingfish in Trinidad and Tobago, where water temperatures would normally exceed 20°C. After being caught, fish may remain in nets at this elevated temperature for several hours and may die before it leaves the water [13]. In addition, when the catch is brought on to the fishing vessel and put on ice the rate of cooling, which depends on the size of the catch and on the size of the individual fish [13], may not be rapid enough to prevent additional deterioration and histamine production. However, it must be noted that most fishermen in Trinidad and Tobago do not put fish immediately on ice when they dock at the landing site, further delaying that critical chilling period. In addition, contamination with histamine producing bacteria can occur in fish during the off-loading process from boats [28].

Fish from landing sites are usually sold to wholesale vendors. In this study, histamine levels in fish from wholesale markets ranged from 7.67-57 ppm. Contrary to the findings of this study, relatively low levels of histamine (>1 ppm) were reported in fish sold at wholesale markets in Peru [33]. It should be noted that most fish had histamine levels similar to that found at landing sites with the exception of one carite with a level of 57 ppm. This was a surprising finding as fish sold at wholesale markets are expected to have a fast turn-over and no processing of fish is done. Possible reasons for the high histamine level in this fish could be poor handling, poor sanitation and temperature abuse at the landing sites.

Fish sold at wholesale markets are usually distributed to retail

Isolate ID	Identified isolate	% similarity	Source	Gram stain	Comments	References
N3	*Klebsiella pneumoniae*	100	fish gills and intestines	*Gram negative rod*	Cause Pneumonia. Strong histamine producer. Found in environmental contaminant.	[41]
N5	*Lactococcus garvieae*	100	fish intestines	Gram positive cocci	Zoonotic disease. Found in fish, cattle, and humans.	[42]
N8	*Citrobacter freundii*	100	fish tissue	Gram negative bacilli	Nosocomial infections. Found in food, and the intestinal tracts of animals and humans, environment.	[43]
N9	*Enterobacter aerogenes*	100	environment	Gram negative rods	Nosocomial infection. Found in human G.I. tract.	[44]
N11	*Aeromonas spp.*	100	fish tissue	Gram negative rods	Weak histamine producer. Found on refrigerated fish.	[45]
N19	*Morganella morganii*	100	environment and fish tissue	Gram negative rods	Nosocomial infection and strong histamine producer. Found in environment, intestinal tracts of humans, mammals, and reptiles as normal flora.	[46]
N26	*Enterobacteriaceae bacterium*	100	fish gills	Gram negative rods	Saprophytes and others being plant and animal parasites. Found in environment.	[47]
N27	*Proteus mirabilis*	100	fish intestines	Gram negative rods	Urinary tract infections and septic lesions. Found in intestines of humans and a wide variety of animals, as well as in manure, soil, and polluted waters.	[47]
N37	*Enterobacter* spp.	100	fish tissue and environment	Gram negative rods	Nosocomial infection. Found in feces of man and other animals, sewage, soil, water, and dairy products.	[44]

Table 1: Identity of bacteria isolated from niven's agar for both the market re-sample and storage experiments.

vendors, mongers and supermarkets. All three market types had moderate levels of histamine. A greater exposure to contamination and variable storage conditions could account for the higher histamine levels found compared to landing sites. A common practice among local retail vendors and mongers are exposure of fish to elevated temperatures for a prolong period of time. Fish are usually kept on display on table tops of vending stalls at ambient temperatures until being sold to the consumer. Whole fish are typically stored ungutted until sold to the consumer. The gut itself may be a source of contamination, particularly if chilling is delayed [34]. Also most vendors displayed poor sanitation and hygiene practices which could account for contamination of histamine producing bacteria (HPB).

It should be noted that certain fish types belonging to the *Scombridae* and *Scomberesocidae* families, like carite and kingfish, have been known to have high histamine levels [12]. However, in this study, carite had histamine levels more than twice ($p < 0.001$) that of kingfish (Figure 6). Also the fish that was in violation of the USFDA standards with a histamine level of 57 ppm was carite. Differences in pre-catch factors between both fish species may account for this variation. Fish feeding habit, geographical location, season and ocean temperature may influence the character of the microflora, and hence histamine production capability [24,35,36]. Differences in free histidine levels may also be a factor, but this needs to be confirmed since there is little information in the literature for the levels of this amino acid in the two fish species investigated. Since free histidine in the fish muscle is the substrate for microbial decarboxylation to produce histamine, species difference in histidine content has a large effect on the potential hazards of poor handling practices [37]. The free histidine can vary with season. This has been documented in herrings, with the highest level observed in the summer [38].

Some scombroid fish such as mackerel show quite marked fish to fish and seasonal variations in chemical composition and susceptibility to spoilage by various microbiological, enzymic and auto-oxidatve processes [39]. Therefore it is possible that certain fish in an apparently homogenous batch will develop all factors necessary to induce scombroid poisoning. This could also be another reason why there was variation between the carite and kingfish caught in the same batch. The significant increases in histamine level with decreasing quality based on sensory score (Figure 7) is suggestive histidine arising from proteolysis is being converted to histamine [13] hence the use of fish texture as a spoilage indicator [40].

Conclusion

Histamine levels in fish were generally within acceptable limits of 50 ppm. Only a small percent of fish (1.3%) exceeded this limit. Market type significantly influenced histamine levels in fish with relatively low levels found at landing sites compared to mongers, retail markets and supermarkets. Although mean levels in wholesale markets was overall highest, removal of the single fish (carite) that violated acceptable limits resulted mean levels being similar to landing sites.

Carite had histamine level that was more than twice the levels found in kingfish. Sensory evaluation was a better predictor of histamine levels in fish as compared to APC. APC indicated that carite and kingfish sold at all market types generally have marginal microbiological quality with all samples exceeding 5 X 10^5 CFU/g.

There was a significant relationship between sources of contamination and histamine producing bacteria. Fish tissue and gills were the major sources of HPB. HPB were isolated environmental contact surfaces and fish intestine however in moderate amounts. Water supply at all market types was not responsible for contamination of fish with HPB.

There was a significant relationship between market type and presence of histamine producing bacteria in fish. The highest percentage of positive HPB was isolated from landing sites and retail markets.

Acknowledgements

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