

BIOGENIC AMINE ANALYSIS OF FRESH AND STORED BLUEFISH
(*POMATOMUS SALTATRIX*) AND MICROBIOLOGICAL SURVEY OF
HISTAMINE-FORMING BACTERIA

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Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Food Science and Technology

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August 1998

Blacksburg, Virginia

Key words: biogenic amines, histamine, putrescine, cadaverine, bluefish, *Morganella
morganii*

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ABSTRACT

Changes in histamine, putrescine, and cadaverine concentrations in fresh and stored bluefish (*Pomatomus saltatrix*) were determined using a new HPLC method. The HPLC method utilized a 5.0% (w/v) trichloroacetic acid (TCA) extraction, pre-column fluorecamine derivitization, and fluorescence detection. The derivatives were stable over 24 h. The 5% TCA extraction produced percent recoveries of 98.6%, 98.7, and 100.0% for histamine, cadaverine, and putrescine respectively. The HPLC process including extraction, derivatization, and HPLC analyses was conducted in less than 45 minutes.

Fresh bluefish was found to contain between <1 ppm and 99 ppm histamine, and no cadaverine or putrescine. Fresh bluefish fillets were stored at 5°, 10°, and 15° C until sensory rejection. Fresh bluefish fillets inoculated with *Morganella morganii* were also stored at the same conditions. Histamine levels as high as 2200 ppm were observed in the inoculated fish stored at 15° C. Overall, histamine achieved higher levels in the bluefish pieces inoculated with *Morganella morganii*. Histamine was present in greater amounts than putrescine and cadaverine in the bluefish samples. Histamine levels at each temperature exceeded the 50 ppm advisory level established by the FDA before 100%

sensory rejection. Putrescine levels increased at each temperature during storage. Cadaverine was present only in uninoculated bluefish stored at 15° C. Consumer risk from histamine poisoning seems to be the greatest in those fish stored at 5°C where acceptance levels were higher and histamine levels above 100 ppm were observed.

The presence of histamine-forming bacteria in fish-processing facilities was studied. Environmental sampling techniques were conducted in the Hampton Roads area of Virginia in fish-processing facilities that regularly handle scombroid fish or other fish which are known to accumulate histamine levels greater than 50 ppm. Surfaces that come into contact with the fish were swabbed and the histamine-forming bacteria from these areas were identified. One isolate each of *Klebsiella ozaenae* and *Vibrio alginolyticus*, and two isolates of *Aeromonas* sp. were found in the processing facilities. The study concluded that histamine-forming bacteria do not make up a large part of the microflora associated with fish-processing facilities. Fishing vessels were also sampled and no histamine-forming bacteria were identified.

ACKNOWLEDGEMENTS

I would like to thank Dr. George J. Flick, my major advisor, for his guidance and support during the completion of my thesis research. I would also like to thank my committee members, Dr. Merle D. Pierson and Dr. Harold M. McNair for their direction. I would like to thank Tatiana Lorca, Troy Wilkerson, and Fletcher Arritt for assistance in running samples. Additionally, I would like to recognize the faculty and staff of the Food Science Department for their help and advice during my studies.

I would like to extend a heartfelt thanks to my family for their love and patience throughout my educational endeavors. My deepest gratitude is to Kristine Savill for her unconditional love and support through these last few years. Without the support of those people closest to me, I would not have been able to complete such a task.

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SECTION I: LITERATURE REVIEW

A. Introduction to Biogenic Amines

1. *Structure and Function of Biogenic Amines*

Biogenic amines are thought to play a causative role in histamine poisoning or scombroid poisoning. In order to explain the mechanisms behind histamine related illnesses, it is important to understand the structure and function of the group of molecules known as biogenic amines. Amines are defined as basic nitrogenous compounds in which one, two, or three atoms of hydrogen are replaced by alkyl or aryl groups (Shalaby, 1996). The amines are noted as monoamines, those with one amine group on the molecule, or polyamines, those with more than one amine group on the molecule. Amines are weakly basic, but they are the strongest bases that are found in significant quantities under physiological conditions (Carey, 1992).

The amines of interest when dealing with histamine poisoning are known as biogenic amines. Biogenic amines are designated as biogenic because they are derived from the action of living organisms (Shalaby, 1996). The most common biogenic amines present in foods are histamine, putrescine, cadaverine, tyramine, tryptamine, β -phenylethylamine, spermine, and spermidine (Shalaby, 1996).

Biogenic amines play an important role in many common physiological activities in living things. The diamine putrescine, and polyamines spermidine and spermine, are present almost universally in animals and plants (Smith, 1980). These polyamines are important in the regulation of DNA, RNA, protein synthesis, and membrane function (Smith, 1980). Amines are also responsible for a number of pharmacological reactions in the human body; many of these reactions are symptoms of amine toxications.

According to Shalaby (1996), pharmacological reactions of histamine include liberation of adrenaline and noradrenaline, excitation of the smooth muscles of the intestine and respiratory tract, and stimulation of sensory and motor neurons. Tyramine ingestion is known to cause migraines, increase blood sugar, and increase respiration. Putrescine and cadaverine are known to cause hypotension, bradycardia, and potentiate the toxicity of other amines (Shalaby, 1996).

2. *Biogenic Amine Formation*

Biogenic amines are generally formed by decarboxylation of free amino acids in tissue or by amination and transamination of aldehydes and ketones (Sillo Santos, 1996). The decarboxylation of amino acids into biogenic amines in foods and food products occur due to the action of certain decarboxylase enzymes. These enzymes, either native in the raw material or produced in the food or food product by bacteria, are specific for the formation of each individual biogenic amine (Brink *et al.*, 1990).

There have been two identified methods of action for amino acid decarboxylation, a pyridoxal phosphate dependent reaction and a non-pyridoxal phosphate dependent reaction (Eitenmiller *et al.*, 1984). Pyridoxal phosphate is joined in a Schiff base linkage to the amino group of a lysyl residue to form the active site of the enzyme. The carbonyl group of the pyridoxal phosphate reacts readily with amino acids to form this Schiff base intermediate. The Schiff base intermediate is then decarboxylated to yield water, the corresponding amine, and the original pyridoxal phosphate moiety. The non-pyridoxal phosphate decarboxylation uses a pyruvoyl residue instead of pyridoxal-5-phosphate as the active site on the enzyme. This pyruvoyl residue acts in a similar manner to the

pyrodoxial-5-phosphate reaction of decarboxylation of an amino acid (Eitenmiller *et al.*, 1984).

In addition to the necessary enzymes being present in the food, there must also be adequate amounts of the precursor or progenitor compound present in order to form biogenic amines. It should be noted that in order for the decarboxylase enzymes to act on amino acids in tissue, it is necessary for these amino acids to be free and unattached from larger protein molecules. Proteolysis, either autolytic or bacterial, may play a significant role in the availability of amino acids as substrates for decarboxylase enzymes and production of the biogenic amines (Shalaby, 1996). Each specific biogenic amine is formed from a specific progenitor amino acid compound (Table 1).

Table 1: The main biogenic amines of interest in foods and the corresponding precursors.

PRECURSOR AMINO ACID	BIOGENIC AMINE
Histidine	Histamine
Tyrosine	Tyramine
Hydroxytryptophan	Serotonin
Tryptophan	Tryptamine
Lysine	Cadaverine
Ornithine	Putrescine
Arginine	Spermidine
Arginine	Spermine

3. Biogenic Amine Detoxification and Diamine Oxidase

In order to fully understand the mechanism behind histamine or scombroid poisoning, it is necessary to gain an understanding of the human body's metabolism and breakdown of the biogenic amines present in foods. Two enzymes primarily conduct the metabolizing of histamine in the body: diamine oxidase and histamine-N-methyltransferase (Hui *et al.*, 1985).

Diamine oxidases are a general class of enzymes that are central to polyamine metabolism in most organisms (Equi *et al.*, 1991). It is also noted that the term monoamine oxidase is used to describe to enzymes responsible for the metabolism of biogenic amines. Monoamine oxidase is commonly used to describe the class of enzymes responsible for the breakdown of certain monoamines, namely tyramine, 2-phenylethylamine, tryptamine, serotonin, and dopamine. The function of diamine oxidases are to catalyze the oxidative deamination of a range of polyamines for which there are generally no reliable or convenient non-enzymatic methods available (Equi *et al.*, 1991). Orally administered histamine is predominantly metabolized by the diamine oxidase pathway in humans. This diamine oxidase enzyme is present in high concentrations in the mature intestinal mucosa of humans and other mammalian species (Luk *et al.*, 1980). The diamine oxidase pathway yields the end product imidazoleacetic acid riboside, a much less toxic compound than histamine, and one that is readily excreted in urine (Taylor, 1990).

Histidine-N-methyltransferase is also responsible for the metabolism of histamine in the human body. The end product for this pathway of histamine metabolism is N-methylimidazoleacetic acid, which is readily excreted in the urine (Taylor, 1990). Histidine-N-methyltransferase differs from diamine oxidase in that histidine-N-methyltransferase is specific for one compound, histamine, whereas the diamine oxidase enzyme acts on histamine and a variety of other diamine substrates. It should be noted that histidine-N-methyltransferase dominates histamine metabolism in all human tissues except the intestinal tract (Taylor, 1990).

It is believed by some that other compounds present in foods may somehow enhance absorption of histamine or reduce the effectiveness of the histamine metabolism pathways: diamine oxidase and histamine-N-methyltransferase. According to Taylor (1990), humans can tolerate 150 mg of histamine orally with only mild or no adverse reactions. However, humans eating spoiled tuna containing in excess of 50 mg/100 g will always develop more serious symptoms of histamine poisoning. After completing a study using smoked mackerel fillets linked to a scombrototoxin outbreak, Ijomah *et al.* (1990) concluded that the correlation between histamine dose and response alone are too poor to explain scombrototoxicosis simply as histamine intoxication.

It has been suggested that other biogenic amines may play a synergistic role with histamine in scombrototoxin occurrences. Spoiled fish and other foods may possess inhibitors of diamine oxidase and histamine N-methyltransferase that interfere with the detoxification of histamine, and thus, potentiate the toxicity of histamine (Taylor, 1990). It is thought that the biogenic amines putrescine and cadaverine may interfere with histamine metabolism by competing for the same enzyme: diamine oxidase. Taylor also reported that putrescine, cadaverine, and tyramine will inhibit both diamine oxidase and histamine N-methyltransferase, and increase the intestinal absorption and urinary excretion of unmetabolized histamine. The preoral toxicity of histamine in the guinea pig was shown to be potentiated by simultaneous administration of cadaverine (Bjeldanes *et al.*, 1978). In the same study, it was found that the relative levels of cadaverine and histamine that showed potentiation of histamine toxication in the guinea pig were of the same order of magnitude as those present in toxic fish. Observations on urinary excretions of histamine and histamine breakdown products showed inhibition of the

histamine metabolism process diamine oxidase by putrescine and cadaverine, and inhibition of both diamine oxidase and histamine N-methyltransferase was achieved with intake of β -phenylethylamine (Hui *et al.*, 1985). Cadaverine was found to enhance the absorption of unmetabolized histamine in perfused intestinal segments by inhibiting conversion of histamine to its less toxic metabolites (Lyons *et al.*, 1983).

Besides the effect of certain biogenic amines present in food on the activity of diamine oxidase and histamine N-methyltransferase metabolism, it should be noted that some medications or drug therapies might have an adverse reaction on histamine metabolism. Monoamine oxidase inhibitors are often prescribed to patients with psychological disorders such as depression. Inhibitors of diamine oxidases are known to possess antimalarial, antitrypanosomal, antibacterial, and antifungal properties together with possible cancer chemotherapy (Equi *et al.*, 1991). Early evidence showed that histamine poisoning might occur when histamine metabolism is blocked by monoamine oxidase inhibitors (Ijomah *et al.*, 1991). Ionizid therapy has been implicated as a factor in several outbreaks of histamine poisoning (Taylor, 1990). The pharmacological inhibitors of histamine metabolism: aminoguanidine, isoniazid, quinacrine, and cimetidine were found to be more potent and have a longer duration than the biogenic amines putrescine, tyramine, β -phenylethylamine and cadaverine (Hui *et al.*, 1985).

There exists several assays that are used to monitor diamine oxidases and their activities. A procedure using radioactive substrates and liquid scintillation counting was used to determine oxidase activity (Okuyama *et al.*, 1961). A kinetic assay procedure was used to monitor formation of putrescine and cadaverine derivatives, and was found suitable for systematic characterization of the molecular recognition process by these

enzymes (Equi *et al.*, 1991). A sensitive assay for diamine oxidase activity using high-performance liquid chromatography has been developed (Biondi *et al.*, 1984).

In many of the studies, urinary metabolite observation is used to determine diamine oxidase and histamine-N-methyltransferase activity *in vivo*. It is thought that observation of histamine metabolites versus unmetabolized histamine will give an indication of performance of histamine metabolism. Hui *et al.* (1985) used this method to observe the effects of possible histamine poisoning potentiators on histamine metabolism in the guinea pig.

B. Histamine Poisoning

1. Etiology of Histamine Poisoning

Although it is known that biogenic amines are naturally occurring in food and food products, it is also thought that ingestion of large amounts of some of these biogenic amines can lead to illness. It has been suggested for quite some time that ingestion of certain amounts of histamine may result in what is known as histamine poisoning (Merson, 1973; Motil *et al.*, 1978).

Histamine poisoning can result from the consumption of foods, typically certain types of fish and cheeses, that contain unusually high levels of histamine (Taylor *et al.*, 1989). Histamine poisoning in fish is referred to as “scombroid fish poisoning” because spoiled fish of the families *scombroidae* and *scomberesocidae*, such as tuna and mackerel, are often implicated in histamine poisoning outbreaks. There have been outbreaks associated with other, non-scombroid fish including mahi mahi, bluefish, and swordfish. Since symptoms of histamine poisoning resemble those of scombroid

poisoning, and since scombrototoxin outbreaks have been associated with fish with high histamine levels, histamine has been labeled a scombrototoxin.

There has been some debate about how much of a role histamine plays in scombroid poisoning. Many studies point to the role of other biogenic amines present in fish (cadaverine, putrescine, and spermine) and their enhancement of histamine effects in scombroid poisoning (Bjeldanes *et al.*, 1978; Taylor *et al.*, 1979; Ijomah *et al.*, 1992). In a volunteer study conducted by Clifford *et al.* (1989), it was tentatively concluded that histamine alone is unlikely to be the causative agent in scombrototoxicosis. Other studies point to the role of endogenous histamine rather than dietary histamine as the main cause behind scombroid poisoning. Clifford *et al.* (1991) and Ijomah *et al.* (1991) suggested that there might be an unknown toxin present in the spoiled fish that acts as a mast cell degranulator which releases endogenous histamine, and that antihistamines are effective in scombroid poisonings because they act on this endogenous histamine. Pure histamine itself has produced symptoms similar to those from scombroid poisoning (Motil *et al.*, 1979). Recent studies have reaffirmed the belief that histamine is the toxin responsible for scombroid poisoning (Morrow *et al.*, 1991). For now it is widely accepted that histamine is the cause of scombroid poisoning.

2. Description of Scombroid Poisoning

The earliest recorded incident of scombroid poisoning occurred in 1828 following consumption of bonito by a group of British sailors (Henderson, 1830). The first reported United States outbreak of scombroid poisoning in a nationally distributed canned product occurred in 1973. In February of 1973, scombroid fish poisoning occurred in 232

persons who had eaten from either of two lots of commercially canned tuna (Merson *et al.*, 1974). Cases occurred in four states, with no reported hospitalizations or deaths.

The onset of symptoms of scombroid poisoning varies, but it occurs more rapidly than other food-borne illnesses. Features of the first fifty scombroid fish poisoning incidents reported in Britain documented incubation periods from 10 minutes to two hours (Gilbert *et al.*, 1980). In a volunteer study on histamine poisoning, onset of typical histamine poisoning symptoms appeared from 30 to 90 minutes after ingestion of the histamine (Motil *et al.*, 1979). Hughes observed symptom onset times to be between 10 and 30 minutes to be characteristic for scombroid poisoning (1991).

Histamine poisoning itself is typically a rather mild illness with a wide variety of symptoms involving the skin, the gastrointestinal tract, and the nervous system (Taylor *et al.*, 1989). Because of the wide range in effects of histamine in the body (Table 1), there are great many symptoms that have been associated with histamine poisoning. It is most common for patients to demonstrate only a few of the symptoms, many of which are associated with other food-borne illnesses and food allergies, thus increasing the difficulty of the diagnosis of histamine poisoning. Histamine poisoning can be distinguished from a food allergy by (1) the lack of a previous history of allergic reactions to implicated foods, (2) an attack rate of nearly 100% in group outbreaks of histamine intoxication, and (3) the detection of high levels of histamine in incriminated food (Taylor, 1990).

Table 2: Reported symptoms of histamine poisoning according to bodily system affected (Taylor *et al.*, 1989).

CUTANEOUS SYMPTOMS	GASTROINTESTINAL SYMPTOMS	NEUROLOGICAL SYMPTOMS	OTHER SYMPTOMS
rash urticaria edema facial flushing neck flushing	nausea vomiting diarrhea abdominal cramps	facial, neck flushing headache palpitations tingling, itching oral burning sensation	hypotension bronchospasm shock

The wide variety of symptoms of histamine poisoning can be explained by the powerful biological action of the chemical histamine. Although mast cells and blood basophils contain large amounts of histamine stored in granules, the effect of histamine does not appear unless special reactions (i.e. an allergic reaction) release it into the blood stream (Shalaby, 1996). Histamine exerts its effects by binding to receptors on cellular membranes, which are found in the cardiovascular system, and in various secretory glands (Joosten, 1988).

Typically, the course of the disease is not life threatening. There have been on occasion, serious complications to occur in individuals with pre-existing cardiac and respiratory conditions (Borysiewicz *et al.*, 1981). A young child with a history of bronchial asthma suffered respiratory collapse after ingesting fish with a high histamine level (Russell *et al.*, 1986).

Duration of the disease differs between individual cases. One individual who developed scombroid poisoning after ingesting marlin with a high histamine level reported the duration of case study of an individual ingesting marlin with a high histamine level had a relief in symptoms after approximately three hours (Morrow *et al.*,

1991). In a study of histamine ingestion among volunteers fed 100 mg doses of histamine, all had histamine poisoning symptoms subside within one hour of ingestion (Motil *et al.*, 1979). In an outbreak of histamine poisoning from commercially canned tuna containing between 68 and 280 mg per 100 grams of fish, the duration of symptoms was no longer than eight hours among the 95 patients interviewed (Merson *et al.*, 1974). In an incident of scombroid poisoning due to the ingestion of sailfish, twelve victims showed symptoms and recovered within eight hours (Hwang, 1995).

Treatment of scombroid poisoning includes the administration of antihistamines. These treatments typically shorten the duration of the illness. H1 agonists such as diphenhydramine and chlorpheniramine are the usual choices for treatment (Taylor, 1989). A patient in a volunteer study sought professional attention after ingestion of marlin with a high histamine level, 50 mg of diphenhydramine was administered intramuscularly and the symptoms subsided in 30 minutes (Morrow *et al.*, 1991). Studies with susceptible volunteers predisposed with either placebo or H1 agonist (chlorpheniramine 4 mg) demonstrated convincingly, that the antihistamine can abolish vomiting and diarrhea associated with the ingestion of 50 g of scombrototoxic fish (Ijomah *et al.*, 1991).

3. Prevention of Histamine Formation

Prevention of high levels of histamine in foods seems to involve two distinct aspects: microbial flora and storage temperatures. Biogenic amine levels in fresh fish are generally very low. Levels of histamine, putrescine, and cadaverine usually increase during spoilage of fish and meat whereas levels of spermine and spermidine decrease during this process (Brink *et al.*, 1988).

The microbial flora on fish or other non-fermented food products is generally a more difficult factor to control than storage temperatures. In the Fish and Fisheries Products Hazards and Controls Guide, (Anonymous, 1996) a guide developed by the U.S. Food and Drug Administration for the fisheries industry, recommendations are made for the safe handling of scombroid type fish or related suspect species. It is recommended that the internal temperature of fish is brought to 50° F within six hours of death, and product temperature should be brought to near freezing temperatures within an additional eight hours. There are many studies showing different optimal temperatures for histamine formation in foods (Davidek *et al.*, 1995). Variations in optimal temperatures for histamine formation are probably linked to differences in the microbial flora present on the fish. It is generally accepted that storage of fish below 40° F is the best way to prevent histamine formation (Taylor, 1990; FDA, 1995). In the formation of histamine in fermented products, it is necessary to control the microbiological flora in the product. Control of histamine formation in cheese is tied to reduction of the numbers of histamine-producing bacteria in raw milk (Taylor, 1990).

Histamine formation in fish is generally a greater problem in fresh fish rather than in frozen fish. This is due to a greater chance of temperature abuse and mishandling associated with transportation of fresh fish. Formation of decarboxylating enzymes by microbial flora and subsequent formation of histamine can occur during these temperature abuse periods. It is important to recognize that histamine can exist in processed, heat-treated product (Merson *et al.*, 1974; Ienestea, 1971) due to mishandling. Some histamine is lost in heat processing, but the losses are not considered sufficient to

create safe product when canned (Arnold *et al.*, 1978). Therefore, it is generally accepted that histamine is a heat-stable compound.

C. Bacteria Responsible for Histamine Production

1. *Species with Decarboxylase Activity*

Histamine is formed the decarboxylation of the amino acid histidine found in some food products (Frank, 1985). This decarboxylation is catalyzed by the enzyme histidine decarboxylase (Ababouch *et al.*, 1991). This enzyme, histidine decarboxylase, is not widely distributed among bacteria, but is found in Enterobacteriaceae, *Clostridium*, and *Lactobacillus* among others (Table 3) (Shalaby, 1996). Bacteria from the family *Enterobacteriaceae* are largely responsible for histamine production in fish and meats, whereas the *Lactobacillus* species contribute to high histamine levels in cheeses (Taylor, 1990).

Histamine forming bacteria in meat products other than seafood products include *Pediococcus*, *Streptococcus*, and *Micrococcus*. Amine-producing coliforms such as *Escherichia coli* and *Klebsiella oxytoca* and related bacteria like *Morganella morganii* and *Edwardsiella* spp. as well as the lactic acid bacteria *Lactobacillus brevis*, *L. buchneri*, *L. divergens*, and *L. hilgardii* have been isolated from meat and meat products (Shalaby, 1991).

There are a number of organisms responsible for histamine production in cheese products. Some histamine-forming bacteria are used as starter culture in the dairy industry, such as *Streptococcus lactis* and *Lactobacillus helveticus* (Stratton *et al.*, 1991). Other histamine-forming bacteria that have been isolated from cheeses include *Streptococcus faecium*, *Streptococcus mitis*, *Lactobacillus bulgaris*, *Lactobacillus*

plantarum, and propionibacteria (Edwards and Sandine, 1981). Sumner *et al.* (1985) identified a histamine producing strain of *Lactobacillus buchneri* from Swiss cheese capable of producing 42 mg of histamine/100 mL of medium.

Histamine forming bacteria isolated from sardine include *Morganella morganii*, *Proteus vulgaris*, *Proteus mirabilis*, *Providencia stuartii*, and other unidentified *Proteus* sp. (Ababouch *et al.*, 1991). *Morganella morganii* and *Citrobacter freundii* were isolated from frozen tuna (Taylor and Speckhard, 1983). The histamine formers *Morganella morganii*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Enterobacter aerogenes* were all isolated from tuna that was destined for canning (Lopez-Sabater, 1994). *Morganella morganii*, *Proteus vulgaris*, and *Hafnia alvei* are histamine formers and were isolated from spoiled skipjack tuna (Arnold *et al.*, 1980).

Table 3: Histamine producing bacteria and their source of isolation.

BACTERIA	SOURCE OF ISOLATION
<i>Citrobacter freundii</i>	Tuna, mackerel
<i>Clostridium perfringens</i>	Skipjack Tuna
<i>Edwardsiella</i> spp.	Mahi mahi
<i>Enterobacter aerogenes</i>	Skipjack tuna, tuna, mahi mahi
<i>Enterobacter cloacae</i>	Tuna, bonito, mahi mahi
<i>Hafnia alvei</i>	Skipjack tuna, jack mackerel
<i>Klebsiella oxytoca</i>	Tuna
<i>Klebsiella pneumoniae</i>	Skipjack tuna, tuna, bonito, mahi mahi
<i>Klebsiella</i> spp.	Skipjack tuna, jack mackerel
<i>Morganella morganii</i> (<i>Proteus morganii</i>)	Skipjack tuna, jack mackerel, sardine, tuna, mahi mahi, mackerel
<i>Plesiomonas shigelloids</i>	Tuna
<i>Proteus mirabilis</i>	Mackerel, mahi mahi, sardine, skipjack
<i>Proteus vulgaris</i>	Skipjack tuna, sardine
<i>Proteus</i> spp.	Skipjack tuna, sardine, mahi mahi
<i>Providencia stuartii</i>	Sardine, skipjack tuna
<i>Serratia fonticola</i>	Tuna, bonito
<i>Serratia liquefaciens</i>	Tuna
<i>Serratia marcesens</i>	Tuna, mackerel
<i>Serratia plymuthica</i>	Tuna
<i>Vibrio alginolyticus</i>	Skipjack tuna, mahi mahi

2. Isolation Techniques and Growth Media

There have been several media developed to help in the isolation of histamine-producing bacteria from food sources. The most common type of medium used in differential plating of histamine-forming bacteria is Niven's medium. Moeller (1956) first used a pH-based differential medium to examine decarboxylase activities of several bacteria species. Niven's medium contains 0.5% tryptone, 0.5% yeast extract, 2.7% L-histidine, 0.5% NaCl, 2.0% agar, and 0.0006% bromocresol purple, adjusted to pH of 5.3 (Niven *et al.*, 1981). A change in pH of the medium results from the conversion of the amino acid histidine to histamine. The differentiation in the medium is derived from a color change provided by bromocresol purple pH indicator dye. A dark purple area will

surround the histamine-producing colonies on the plate. This plating method has proven successful in many cases of isolation of histamine-producing organisms. In testing his medium for effectiveness in isolation, Niven (1981) found 28 histamine-forming bacteria on decomposed mahi mahi. Niven's medium was used to isolate 55 histamine-producing bacteria from sardines stored at ambient temperature (Ababouch *et al.*, 1991). Niven's agar was used to isolate 28 histamine-forming bacteria from frozen tuna (Taylor and Speckhard, 1983). Some studies indicate that Niven's agar may give false positive reactions because other alkaline products besides histamine are produced by bacteria on the medium (Ababouch *et al.*, 1991). A study was conducted on the performance of Niven's agar and three modified versions which varied in amount of histidine, amount of glucose, and the use of phenol red as an indicator dye (Chen *et al.*, 1989). In that study, the original Niven's formula performed better than the other modified agars with higher detection rates, and lower rates of false positive and false negative results. It should be mentioned that there have been some isolation problems associated with Niven's agar. Of 184 colonies appearing purple on Niven's agar, only 68 showed a positive result in four other biochemical tests used to confirm histidine decarboxylase activity, thus giving a false-positive rate of 63.1% (Lopez-Sabater *et al.*, 1996).

Much work has been done on developing quantitative methods for observing histamine production by different species of bacteria. Many of the studies involve selective media, differential media, or media with substrate added in the form of an amino acid progenitor compound. A media comprised of trypticase soy broth containing 2% substrate (amino acids histidine, ornithine and lysine, all in monohydrochloride form) was used to determine histamine, cadaverine and putrescine production by various

bacterial species and strains, using thin layer chromatography for quantitation. (Frank *et al.*, 1985). A skipjack infusion broth, comprised of ground skipjack tuna flesh, artificial seawater, and 1% D-glucose, was used along with thin layer chromatography to observe histamine production in bacterial species (Arnold *et al.*, 1980). In a study of histamine detection medium, it was found that the trypticase soy agar supplemented with 2.0% histidine provided similar quantitative results to tuna-infusion type media, and is superior because of ease of preparation, consistent composition, and low price (Taylor and Woychik, 1982). Often times original detection with Niven's agar is followed up with quantitative analysis of histamine production to alleviate false positive or weak histamine formers picked up by Niven's medium (Lopez-Sabater, 1996).

3. Occurrence and Control of Histamine Forming Bacteria

It should be noted that not all bacterial species or strains within a species produce the same amounts of histamine. Of the histamine-forming bacterial species isolated from fish or fish products listed above, only three have been linked to histamine poisoning incidences. *Morganella morganii* (Kawabata *et al.*, 1956), *Klebsiella pneumoniae* (Taylor *et al.*, 1979), and *Hafnia alvei* (Havelka, 1967) were all isolated from fish that were incriminated in a histamine-poisoning outbreak.

Observations of histamine production by bacterial species or strains have shown that while many bacteria have decarboxylase enzymes, few can produce the histamine in large amounts. *Klebsiella oxytoca* and *Morganella morganii* developed large amounts of histamine, 1415 and 2765 ppm on average, respectively, in culture broth after 18 h at 37° C (Lopez-Sabater, 1996). In a study of 112 bacterial strains representing 38 species thought to be histamine producers in food, only eight strains of *Morganella morganii* and

3 strains of *Enterobacter aerogenes* were found capable of forming sufficient histamine in scombroid fish products to elicit food poisoning outbreaks (Taylor *et al.*, 1979).

Lopez-Sabater (1994) sampled tuna fish destined for canning and found *Morganella morganii* as the most frequent and active histamine former, along with other strong histamine formers: *Klebsiella oxytoca*, *Klebsiella pneumoniae*, some strains of *Enterobacter aerogenes* and *Enterobacter cloacae*.

There have been studies conducted on reduction of histamine-forming bacteria in food products. Spices have been reported to have antimicrobial activity on food spoilage bacteria. If microbial populations could be reduced, the formation of histamine on foods could be reduced. Clove and cinnamon were found to be effective against microbial growth and biogenic amine formation, with amine formation inhibited at lower concentrations than those required for growth inhibition (Wendakoon and Sakaguchi, 1992). Findings by Wendakoon and Sakaguchi in 1993 show that clove in combination with NaCl inhibited the growth of *Enterobacter aerogenes* and histamine formation in mackerel flesh.

Many species of fish have been sampled to determine the percentage of histamine-forming bacteria present in microflora on spoiled and fresh fish. Omura *et al.*, (1978) found 44 histamine-forming isolates from a total of 470 from spoiled skipjack tuna and jack mackerel. The proportion of histamine-forming bacteria accounted for less than 0.1% of the total bacterial load sampled from scombroid fish species in a Barcelona Market (Lopez-Sabater, 1996). Ababouch *et al.* (1991) found 55 isolates of 568 total from sardines to be positive histamine-formers.

D. Occurrences of Amines in Foods

1. *Types of Foods Associated with Elevated Histamine Levels*

Virtually all foods that contain proteins or free amino acids and are subject to conditions enabling microbial or biochemical activity can be expected to contain biogenic amines (Table 4) (Silla Santos, 1996). Scombroid fishes and some non-scombroid fishes (such as sardine, marlin, mahi mahi, and herring), as well as other foods such as meat products, cheese, wine, beer, vegetables, fruits, nuts, and chocolate, are a risk for containing toxic levels biogenic amines (Brink *et al.*, 1990; Shalaby, 1996). Conditions within the food such as protein content, pH, salt concentration, sugar concentration, and temperature all have effects on the amounts of biogenic amines present. The microbiological flora present on the product will have an effect on the levels of biogenic amines in foods.

Table 4: Amines common in different types of foods.

FOOD	AMINES FOUND
Fishes	Histamine, tyramine, cadaverine, putrescine, agmatine, spermine, spermidine
Cheeses	Histamine, cadaverine, putrescine, tyramine, beta-phenylethylamine, tyrtamine
Meat and meat products	Histamine, cadaverine putrescine, tyramine, beta-phenylethylamine, tyrtamine
Fermented vegetables	Histamine, cadaverine, putrescine, tyramine, tryptamine
Fermented soy products	Histamine, cadaverine, putrescine, tyramine, tryptamine

2. Fish and Fish Products Associated with Biogenic Amines

Generally, fish and fish products have received the largest amount of attention in terms of biogenic amine content. The fish associated with histamine formation contain red muscles which are rich in free histidine varying from 1 g/kg in herring to as much as 15 g/kg in tuna that may be converted to histamine by the decarboxylase process (Shalaby, 1996). The free amino acid content in marine species is generally high in comparison to other products because they function as osmoregulators in the marine species (Rawles *et al.*, 1996). It is this available substrate that allows the histidine decarboxylase enzymes provided by the bacteria to create high levels of histamine.

Outbreaks of histamine poisoning have occurred in a number of different species. Bartholomew *et al.* (1987) tracked 258 suspected outbreaks of histamine poisoning in

Great Britain from 1976-1986 and observed 111 of those outbreaks were from mackerel, 73 were from tuna, 37 were from sardines or pilchards, and 37 were from other species including salmon, crab, and anchovies. A large outbreak in the United States occurred in four states in people that consumed cans of commercially canned tuna fish (Merson *et al.*, 1974). A scombroid poisoning outbreak occurred in western Taiwan from consumption of sailfish (Hwang *et al.*, 1995). Obviously, species responsible for regional outbreaks will be those regularly consumed by or available to that population, but as transportation and shipping systems become more advanced, it can be expected that a wider variety of species will be responsible for histamine outbreaks. In the Fish and Fisheries Products Hazards and Controls Guide published by the Food and Drug Administration (Anonymous, 1996), a tentative list of species processed or handled by companies in the U.S., and suspect to histamine formation, was compiled (Table 5).

Table 5: The tentative list of species with potential for histamine development.
(Anonymous, 1996)

MARKET NAMES	LATIN NAMES	MARKET NAMES	LATIN NAMES
Amberjack or Yellowtail	<i>Seriola spp.</i>	Atka Mackerel	<i>Pleurogrammus monopterygius</i>
Anchovy	<i>Anchoa spp.</i>	Chub mackerel	<i>Scomber spp.</i>
Bluefish	<i>Pomatomus saltatrix</i>	Jack mackerel	<i>Trachurus spp.</i>
Bonito	<i>Cybiosarda elegans</i>	Spanish mackerel	<i>Scomberomorus spp.</i>
Escolar, Gemfish, or Snake mackerel	<i>Lepidocybium flavobrunneum</i>	Mahi mahi	<i>Coryphaena spp.</i>
Herring	<i>Etrumeus teres</i>	Marlin	<i>Makaira spp.</i>
Sea or Sild herring	<i>Ilisha spp.</i>	Pilchard or Sardine	<i>Tetrapturus spp.</i>
Thread herring	<i>Clupea spp.</i>		<i>Sardina pilchardus</i>
Jack	<i>Opisthonema spp.</i>	Sardine	<i>Sardinops spp.</i>
	<i>Caranx spp.</i>		<i>Harengula spp.</i>
	<i>Oligoplites saurus</i>	Saury	<i>Sardinella spp.</i>
	<i>Seriola rivoliana</i>		<i>Cololabis saira</i>
	<i>Urapsis secunda</i>		<i>Scomberresox saurus</i>
Blue runner	<i>Selene spp.</i>	Shad	<i>Alosa spp.</i>
Jack crevalle	<i>Caranx crysos</i>	Gizzard shad	<i>Dorosoma spp.</i>
Rainbow runner	<i>Alectis indica</i>		<i>Nematalosa</i>
Roosterfish	<i>Elagatis bipinnulata</i>		<i>vlaminghi</i>
	<i>Nematistius</i>	Sprat or bristling	<i>Sprattus spp.</i>
Jobfish	<i>pectoralis</i>	Trevally	<i>Caranx sexfasciatus</i>
	<i>Aphareus spp.</i>	Tuna	<i>Allothunnus fallai</i>
Kahawai	<i>Pristipomoides spp.</i>		<i>Auxis spp.</i>
Mackerel	<i>Arripis spp.</i>		<i>Euthynnus spp.</i>
	<i>Gasterochisma</i>		<i>Katsuwonus pelamis</i>
	<i>melampus</i>		<i>Thunnus tonggol</i>
	<i>Grammatorcynus</i>		<i>Thunnus alalunga</i>
	<i>spp.</i>		<i>Thunnus albacares</i>
	<i>Rastrelliger</i>		<i>Thunnus atlanticus</i>
	<i>kanagurta</i>		<i>Thunnus obesus</i>
	<i>Scomber scombrus</i>		<i>Thunnus thynnus</i>

3. Levels of Biogenic Amines in Fish

There have been many studies on the histamine formation in stored fish, and many studies on the biogenic amine content of fresh fish. Before storage studies on fish products can be conducted, it is necessary to document levels of histamine and other biogenic amines in the fresh product or commercially processed product. Levels in safe products can then be compared to biogenic amine levels in products that have been linked to outbreaks of histamine poisoning. When reporting levels of biogenic amines in food products, the term milligram percent is often used. This term, meaning milligrams per 100 grams of fish, is being replaced with a parts per million conversion. The parts per million (ppm) measurement is used by the Food and Drug Administration in their guideline of a defect action level of 50 ppm histamine in any fish product. In this paper, both the ppm measurement and the mg/100g (milligram percent) measurement will be used to make comparisons of histamine levels easier.

The biogenic amine levels in fresh fish are considered to be negligible. It is thought that temperature abuse and decomposition after capture leads to the formation of biogenic amines in fish. Only traces of histamine and tyramine were found in fresh anchovies (Brink *et al.*, 1990). In a study on the sensory quality of tuna and histamine formation, histamine was not detected until day 12 in sections stored at 0° C, meaning that histamine was present only in very small amounts at the beginning of the study (Lopez-Sabater *et al.*, 1995). A storage study of fillets of herring and haddock was conducted to evaluate amine production, and showed that amine levels were 0 mg/100g when the fish were purchased (Fernandez-Salguero and Mackie, 1987).

Many studies have found that biogenic amine levels, specifically histamine, in processed fish products are higher than levels in freshly caught fish. Ababouch *et al.* (1986) observed histamine levels in 248 samples of commercially processed tuna, mackerel, and sardines in Morocco. Ababouch found concentrations ranging from 0.01 mg/100 g (0.1 ppm) to 694 mg/100 g (6940 ppm) in the samples with a mean concentration of 9.75 mg/100 g (97.5 ppm) in the sardines, 13.74 mg/100 g (137.4 ppm) in mackerel, and 9.86 mg/100 g (98.6 ppm) in tuna. A similar study conducted by Taylor *et al.* (1978) observed mean histamine concentrations in commercially processed fish. In 100 samples each of three fish species, the observed histamine level was 3.58 mg/100 g (35.8 ppm) in chunk light tuna, 1.50 mg/100 g (15 ppm) in albacore tuna, and 2.56 mg/100 g (25.6 ppm) in mackerel. A study on canned fish in Spain revealed that the samples had very low levels of histamine present, below 0.5 mg/100 g or 5 ppm with putrescine and cadaverine ranging from 0 to 1.5 mg/100 g (15 ppm) (Fernandez-Salguero and Mackie, 1987).

Although fresh fish generally has a low concentration of biogenic amines in the flesh, it is possible for the fish to develop very high amounts of amines, especially histamine. This is due to the large amount of substrate (free amino acids) available for the decarboxylation enzymes to convert to amines. Brink *et al.* (1990) found 800 mg/100 g (8000 ppm) and 300 mg/100 g (3000 ppm) in spoiled tuna and spoiled mackerel respectively. An analysis on canned tuna implicated in a scombroid poisoning outbreak determined the histamine level in the cans to be as high as 300 mg/100 g (3000 ppm), with cadaverine as high as 21 mg/100 g (210 ppm), and putrescine as high as 5.6 mg/100 g (56 ppm). Spanish mackerel developed 25 mg/100 g (250 ppm) histamine, 150 mg/100

g (1500 ppm) cadaverine, and 90 mg/100 g (900 ppm) putrescine when stored at 30° C for 48 hrs (Middlebrooks, *et al.*, 1988). When sailfish implicated in a histamine outbreak was analyzed for biogenic amine content, it was found to contain an average of 168 mg/100 g (1680 ppm) of histamine, and 14.5 mg/100 g cadaverine (145 ppm) (Hwang *et al.*, 1995).

It is generally accepted that fermented fish products contain higher amounts of biogenic amines than do non-fermented fish products (Fardiaz and Markakis, 1979). Fermented fish products from Malaysia, such as cinaluk and belacan, showed histamine levels greater than 50 mg/100 g (500 ppm), compared to cured fish with histamine levels from 1 mg/100 g (10 ppm) to 85 mg/100 g (850 ppm) (Mohd and Saari, 1989). Fermented fish paste is a condiment made from small shrimp or fish, and used for rice dishes in Southeast Asia. Fardiaz and Markakis (1979) detected concentrations of histamine as high as 64 mg/100 g (640 ppm) and 58 mg/100 g (580 ppm) from fish paste made of prawn and shrimp respectively. In the same study, histamine content of 40.4 mg/100 g (404.0 ppm) was detected in fish paste made from anchovy.

Changes in biogenic amine concentrations during ripening of anchovies were observed to see if there were significant differences between the ripened product and raw product. According to Veciana-Nogues *et al.* (1996), the ripening process had little influence on the formation of amines, and therefore the amount of amines in the final product depends primarily on the levels of these substances in the raw product. It was found that anchovies packed in brine had a more stable shelf life, than anchovies packed in oil.

Fermented sardine with rice bran is a traditional Japanese food produced in a barrel for a period of 6 months to one year. A study by Yatsunami *et al.* (1994) found that putrescine, histamine, and tyramine content increased over the period of storage. Bacteria isolated from the raw sardines were halotolerant and halophilic isolates of *Staphylococcus*, *Micrococcus*, *Vibrio*, *Pseudomonas* III/IV, and *Pseudomonas* III/IV-H. The histamine-producing isolates from the final fermented products included *Staphylococcus*, *Micrococcus*, and *Vibrio*.

The presence of amines in seafood products is not limited to finfish or finfish products. Hollingworth *et al.* (1991) analyzed vacuum-packed, pasteurized imitation crabmeat for amine content. Histamine, cadaverine, and putrescine were all present in the meat with concentrations of 2.9 mg/100 g (29 ppm), 28.6 mg/100 g (286 ppm), and 12.9 mg/100 g (129 ppm) after 40 days of storage at 22° C. This amount was produced by the lengthy storage time; the fresh product contained 1 ppm, 17 ppm, and 4 ppm for histamine, cadaverine, and putrescine respectively. Only traces of histamine was detected in stored squid and octopus, but 36.9 mg/100 g (369 ppm) of cadaverine could be produced after storage of the squid for 48 hours at 25° C. In the octopus, 9.2 mg/100 g (92 ppm) of cadaverine and 1.5 mg/100 g (15 ppm) of putrescine were detected after storage for 48 hours at 25° C (Takagi *et al.*, 1971).

In a study on the storage of adductor muscle in scallop, agmatine, putrescine, and cadaverine levels increased markedly, reaching 14.21 mg/100 g (142.1 ppm), 19.28 mg/100 g (192.8 ppm), and 20.29 mg/100 g (212.9 ppm) respectively as decomposition progressed (Yamanaka, 1989). Wotton, *et al.* (1989), conducted a study on amine levels in Asian seafood products. Dried squid from the Philippines contained up to 33.3 mg/100

g (333 ppm) histamine and 218.3 mg/100 g (2183 ppm) putrescine respectively. Shrimp paste, also from the Philippines, was analyzed and found to contain 3.9 mg/100g (39 ppm) histamine and 8.5 mg/100 g (85 ppm) cadaverine.

4. Biogenic Amines in Meat and Meat Products

The biogenic amines tyramine, cadaverine, putrescine, spermine, and spermidine have been reported in meat and meat products (Edwards *et al.*, 1983). Fresh pork samples are known to contain spermine and spermidine with traces of other amines (Shalaby, 1996). Shalaby also reported that formation of biogenic amines in pork is temperature and time dependent. Histamine, cadaverine, and putrescine levels increased as spermidine and spermine levels decreased; and the formation of histamine, cadaverine, and putrescine occurred in greater amounts at pork stored at 30°C, than pork stored at 4°C. Spermine and spermidine was detected in fresh beef and pork at an average level of 0.3 mg/100 g (3.0 ppm) spermidine and 3.35 mg/100 g (33.5 ppm) spermine in fresh pork; and .31 mg/100 g (3.1 ppm) spermidine and 3.98 mg/100 g (39.8 ppm) spermine in fresh beef (Hernandez-Jover *et al.*, 1997).

There stands a greater chance of biogenic amine formation in fermented meat products over fresh meat, specifically when there is a lengthy aging process or natural microflora are relied upon for the fermentation. Semi-dry sausages are fermented for short periods of time often with lactic acid cultures added, while dry sausages are allowed to ferment from the action of natural microflora for a longer period of time (Genigeorgis, 1976). Hernandez-Jover *et al.* (1997) and Eerola, *et al.* (1996) studied the effect of starter cultures on biogenic amine formation in fermented sausage. They found that plate count of the starters (*Lactobacillus plantarum* plus *Micrococcus carnosus*, or

Pediococcus pentosaceus plus *Micrococcus carnosus*) increased while levels of histamine-forming bacteria (Enterobacteriaceae and pseudomonads) decreased. The reduction in histamine-forming bacteria decreased amine production, however, it did not prevent it. They postulated that background flora from the raw material had a strong influence on the biogenic amine formation during ripening. Hernandez-Jover *et al.*, (1997) found that with 40% of the ripened meat products had total biogenic amine levels above 30.0 mg/100 g (300 ppm) and that concentrations of tyramine, cadaverine, putrescine, and histamine varied greatly in the products, even among samples from the same commercial brand.

5. Biogenic Amines in Cheeses and Other Fermented Products

After fish, cheese is the most commonly implicated food item associated with histamine poisoning. The first reported case occurred in the Netherlands in 1967 from gouda cheese (Stratton *et al.*, 1991; Taylor 1990). Cheeses usually do not contain high levels of biogenic amines but sometimes high levels may be formed (Joosten and Stadhouders, 1987). During the ripening of cheeses, caesin is slowly degraded by proteolytic enzymes, leading to an increase of free amino acids content (Shalaby, 1996), which act as precursors for the formation of biogenic amines. As with other fermented foods, cheeses that have lengthy ripening times are at a greater risk for the formation of biogenic amines. Other factors that contribute to the formation of biogenic amines in cheeses include storage temperatures and sanitation procedures used in the manufacturing.

Amines have been found to be widespread in other types of fermented foods. Amines specifically histamine and tyramine have been found in fermented soybean

products such as miso, soy sauce, and tempe (Stratton *et al.*, 1991). Fermented vegetables, such as sauerkraut, use undefined spontaneous microflora in the fermentation process, and are at risk for developing high levels of biogenic amines (Shalaby, 1996). Other fermented foods that have been associated with high biogenic amine levels include beers and wines (Davidek and Davidek, 1995).

E. Controlled Storage of Fish

1. Time and Temperature Combinations

There has been much research involving the controlled storage of fish, which are susceptible to the formation of biogenic amines. The two major parameters in these storage studies are time and temperature. Freshly caught sardines (*Sardina pilchardus*) which contain high levels of bacteria on the skin were stored at ambient temperature (25-28° C) and on ice (Ababouch *et al.*, 1991). Histamine, cadaverine, and putrescine reached levels of 235.0 mg/100 g (2350 ppm), 105.0 mg/100 g (1050 ppm), and 30.0 mg/100 g (300 ppm) respectively when stored at ambient temperatures for 24 hours. In the fish stored on ice, the histamine and cadaverine reached similar levels, but after 8 days of storage. Putrescine formation on ice was insignificant. Lund and Klausen (1986) stored mackerel and herring, both vacuum packaged, at temperatures of 2° C and 10° C. They found that the amine contents in the fish stored at 10° C were 2-20 times higher at the time of sensory rejection as compared with samples stored at 2° C. Skipjack tuna (*Katsuwonus pelamis*) was stored at 11 different temperatures ranging from 15.6° C to 48.9° C to determine the optimum temperature for histamine formation in the fish (Frank *et al.*, 1981). After incubation for 24 hrs at each temperature, the optimum temperature

for histamine production was 37.8° C (100° F). At this temperature, 472 mg/100 g (4720 ppm) to 643 mg/100 g (6430 ppm) of histamine was found in all samples.

In 1983, a nomogram was developed by Frank *et al.* to estimate histamine formation in skipjack tuna at elevated temperatures. To construct the nomogram, Frank *et al.* measured histamine formation in whole skipjack tuna incubated at five temperatures between 70° C and 100° C. The nomogram was constructed to show the relationship between the histamine content and incubation time for the given temperature, and could serve as a guide to estimate spoilage in scombroid fish.

In a study by Yamanaka *et al.* (1986), sardine meat was stored at 20° C and 5° C and observed for up to ten days. The sardines stored at 20° C were able to develop 387 mg/100 g (3870 ppm) in 3 days, whereas the sardines stored at 5° C took ten days to produce 241 mg/100 g (2410 ppm). Final putrescine and cadaverine concentrations were similar in sardines stored at 20° C for 3 days and sardines stored at 5° C for ten days.

2. Other Factors that Contribute to Biogenic Amine Formation

In the above studies, there is an obvious correlation between storage temperatures and storage times. There are however, other factors that contribute to histamine formation in fish. A study was conducted to observe differences in formation of histamine in fish harvested at different times throughout the year (Smith *et al.*, 1980). It was found that fish obtained during the summer months of July and September developed higher histamine amounts when stored in chilled sea-water, than those fish harvested in the months of November and March. This change in histamine production may have resulted from differences in the microbiological flora present on the fish, with greater amounts of histamine-forming bacteria possibly present in the warmer weather. Research

has been conducted on the differences in histamine formation between the dark and white muscles of mackerel during storage (Wendakoon *et al.*, 1989). They found the amine levels in the dark muscle were always much higher and more rapid than in the white muscle. Spoilage indicated by volatile base nitrogen during the storage indicated that the dark muscle spoiled faster than the white muscle. Fernandez-Salguero and Mackie (1987) compared amine formation in whole gutted fish to formation in fillets of both haddock (*Melanogrammus aeglefinus*) and herring (*Clupea harengus*). They observed that the formation of the amines: histamine, cadaverine, and putrescine, occurred more rapidly in the haddock fillets than the whole gutted fish and that ungutted herring spoiled more rapidly than herring fillets.

F. Amine Formation as an Indicator of Freshness in Seafoods

The potential use of amine concentration as an indicator of freshness in finfish and shellfish has been discussed for some time. Much of the early work involving amines and freshness used the concentration of total volatile bases, which although nonspecific, has been found an useful indices of spoilage of fish during chilling (Ritchie and Mackie, 1980). Trimethylamine has been determined specifically, mostly by a procedure involving the formation of the picrate salt, and its concentration in the flesh has been shown to be equally useful in quality assessment (Shewan *et al.*, 1971).

Other amines have been found to be possible indicators of spoilage in fish. A study on amines in skipjack tuna confirmed repeatability of judgements by trained panelists and the existence of a “cut-off” point for sample acceptance, which related to specific levels of putrescine and cadaverine in products which had experienced putrefactive decomposition (Sims *et al.*, 1992). Fernandez-Salguero and Mackie (1987)

found that spermidine and spermine are minor components and change only slightly during the storage of fish. They reported that putrescine and cadaverine levels increase steadily once bacterial spoilage has set in, and thus, are potentially useful indices of poor quality fish. In a study on polyamines and amino acids in stored scallops, it was determined that putrescine and ornithine appeared to be useful as potential indicators for freshness of scallop adductor muscle (Yamanaka, 1989). Another biogenic amine, agmatine, was useful as a potential indicator of spoilage in common squid (*Todarodes pacificus*; Yamanaka *et al.*, 1987).

Generally, histamine alone is not useful as an indicator of spoilage. While the presence of histamine in fish is an indication that decomposition has taken place, it is possible to have spoiled fish without formation of large amounts of histamine.

Yamanaka *et al.* (1986) reported that putrescine, cadaverine, tyramine, and tryptamine increased in the sardine and saury pike meat as decomposition progressed, regardless of storage temperature, but the histamine formation varied greatly, and was not as useful as a chemical index for decomposition of fish.

Besides individual biogenic amines serving as quality indices, several researchers have published formulas composed of a number of amine levels, rather than one single amine to serve as a chemical index of quality. An index (Fig. 1) published by Mietz and Karmas (1977) correctly classified samples 90.0% of the time versus an 83.2% correct classification by organoleptic means. This same index correlated well with sensory evaluation for canned tuna of various qualities (Hui and Taylor, 1983).

Figure 1. Chemical quality index (Mietz and Karmas, 1977).

$$\text{Quality index} = \frac{(\text{ppm putrescine} + \text{ppm cadaverine} + \text{ppm histamine})}{(1 + \text{ppm spermidine} + \text{ppm spermine})}$$

Veciana-Nogues *et al.* (1997) proposed an index for tuna quality assessment calculated from the sum of contents of histamine, tyramine, cadaverine, and putrescine, which showed good correlation with both time of storage and organoleptic assessment. In their experimentation, this index performed superior to trimethylamine values in samples for the hygienic quality estimation of tuna.

Other studies disagree with the use of biogenic amine levels in determination of decomposition. A study of decomposition for raw surimi and flaked artificial crab meat showed that total volatile bases and total volatile acids have more potential for being indicators of decomposition than do ethanol, putrescine, cadaverine, and histamine levels (Hollingworth *et al.*, 1990).

G. Recommended Limits of Amine Content

There have been many suggested levels for amine content in foods. The difficulty surrounding determination of amine limits, stems from the disagreement among researchers on the toxicity that amines can develop. When determining the toxicity of amines in food products, one must consider not one particular amine, but other factors including the presence of other amines in the food, the amount of food eaten, and the individual's susceptibility to the amines.

Simple oral ingestion of 8-40 mg histamine can cause slight formation of symptoms, over 40 mg of histamine can cause a moderate development of histamine poisoning, and over 100 mg can cause severe onset of histamine poisoning (Askar and Treptow, 1986). Whereas 10-80 mg tyramine can cause toxic swelling and over 100 mg may cause migraine.

Some researchers have suggested a limit on total biogenic amine content. Ayres *et al.* (1980) stated that consuming more than 40 mg of biogenic amines per meal could result in toxicity. In 1991, Spanjer and Van Roode recommended a maximum total level of histamine, cadaverine, and putrescine to be 30 mg/100 g (300 ppm) in fish and a maximum total level of histamine, cadaverine, putrescine, and tyramine in cheese to be 90 mg/100 g (900 ppm) before amine toxicity is present.

Most of the recommendations for amine levels in foods relate to the biogenic amine content, specifically histamine, of fish. A study by Clifford *et al.* (1989) showed that histamine alone was less toxic than an equal amount of histamine consumed with fish. This occurrence most likely results from the presence of other synergistic amines that contribute to the toxicity of histamine (Bjeldanes *et al.*, 1978). Bartholomew *et al.* (1987) reviewed 250 cases of scombroid poisoning in Great Britain from 1976-1986 and observed that the presence of greater than 20 mg/100 g (200 ppm) in fish is a good indication of its potential to cause scombrototoxic fish poisoning. It should be noted however, that in these studies over half of the reported scombrototoxic incidences in that period of time were associated with fish containing 5 mg/100 g (50 ppm) or less. Taylor (1990) reported that humans can tolerate a dose of 150 mg of histamine orally with mild or no adverse reactions. But, humans eating spoiled tuna containing in excess of 50 mg/100 g (500 ppm) will always develop more serious symptoms.

Many countries have regulated the maximum amount of histamine in some products. Switzerland considered a 10 mg histamine per liter of wine as a permissible limit (Shalaby, 1996). The European Union has recently established regulations for species of fish belonging to the *Scombridae* and *Clupeidae* families and fixed a three-class

plan for maximum allowable levels of histamine in fresh fish ($n=9$; $c=2$; $m=100$ ppm; $M=200$ ppm) and enzymatically ripened fish products ($n=9$; $c=2$; $m=200$ ppm; $M=400$ ppm) where n is the number of units to be analyzed from each lot, m and M are the histamine tolerances, and c is the number of units allowed to contain a histamine level higher than m but lower than M (Anon., 1991).

The US Food and Drug Administration (FDA, 1982) established regulations for canned albacore, skipjack, and yellowfin tunas and consider 20 mg histamine per 100 g (200 ppm) as an indication of prior mishandling “decomposed” and 50 mg histamine per 100 g of fish (500 ppm) as an indication of a potential health hazard. These regulations were expanded to include mahi mahi (dolphin fish).

In September of 1996, The U.S. Food and Drug Administration published a “Fish and Fishery Products Hazards and Controls Guide” (Anonymous, 1996). This publication serves as a guide to processors of fish and fishery products on the implementation of a Hazard Analysis Critical Control Point (HACCP) system of food safety monitoring into each plant as stipulated by the FDA regulation (21 CFR 123). In this manual, scombroid poisoning is discussed along with preventative measures. The official FDA stance as of September 1996 on the acceptable levels of histamine in fish and fishery products is spelled out in this manual. The Food and Drug Administration states that most illness-causing fish have contained histamine concentrations above 20 mg/100 g (200 ppm), often above 50 mg/100 g (500 ppm), but they set a guidance level of 5 mg/100 g fish (100 ppm). The mindset behind the establishment of a 5 mg/100 g (50 ppm) guidance level is that if 5 mg/100 g histamine exist in one part of the fish, there

may be 50 mg/100 g (500 ppm) in another part of the fish. No matter how this limit was derived, there is sure to be much debate about this limit in the future.

H. Methods of Biogenic Amine Detection

1. *Introduction to Biogenic Amine Analysis*

It should be noted that levels of biogenic amines present in fish are difficult to compare between studies because of a wide variety of analytical techniques. Limits of detection and percent recovery vary between the many analytical methods used to evaluate amine content. Just like analytical methods can vary, extraction methods can be equally numerous. The various analytical techniques used in the quantification of biogenic amines from food products include thin-layer chromatography, amino acid analyzers, liquid chromatography, high-pressure liquid chromatography, gas chromatography, capillary zone electrophoresis, and other enzymatic tests.

2. *Fluorometric Procedure*

The fluorometric method is the official AOAC method for determination of histamine in fish. The method involves extraction of the fish with methanol, separation of histamine from amino acids by passing the extract through a liquid chromatography (LC) ion-exchange column, and reaction with *o*-phthalaldehyde to yield a fluorophore under controlled conditions (AOAC Methods, 1980). Detection of histamine by LC is difficult because of the lack of ultraviolet absorbance and fluorescence. For this reason, *o*-phthalaldehyde is added post-column to the eluent and reacts with histamine to form a fluorescing complex that enables detection of the molecules. Drawbacks to the fluorometric method are the slow speed of analysis (maximum of four or five samples per hour) and the strict handling of reagents necessary for proper quantitation.

There are two other variations on this method. These methods differ in the cleaning of the sample before the fluorometric reaction takes place. The officially accepted method uses a methanol extraction followed by a separation in an anion exchange resin LC column, whereas an alternative method uses a 10% trichloroacetic acid extraction followed by separation in a cationic exchange resin LC column for cleanup. This alternative method is the accepted method of histamine analysis in the Federal Republic of Germany (Lerke and Bell, 1976). The third modification of the fluorometric method uses sequential extractions to remove interfering substances instead of ion-exchange resins to isolate the histamine before derivitization with *o*-phthalaldehyde. This method is the officially accepted method of histamine analysis in the United Kingdom (Taylor *et al.*, 1978).

3. *Thin-Layer Chromatography*

Thin-layer chromatography (TLC) is a low-cost alternative to other expensive lab techniques in the determination of biogenic amines in foods. Spinelli *et al.* (1974) developed a TLC method that could determine low levels of spermine, spermidine, putrescine, cadaverine, and histamine. This method uses fluorescent derivatives of the amines formed with dansyl chloride (5-(dimethylamino)-1-naphthalene sulfonyl chloride) for detection (Abdel-Monem and Ohno, 1975). Shalaby (1995) described the use of TLC with a multiple development technique to resolve the dansyl derivatives of histamine, cadaverine, putrescine, phenylethylamine, tyramine, tryptamine, spermine, and spermidine from fish, cheese, and meat samples. Shalaby described this semiquantitative method as simple, versatile, inexpensive, and useful in routine control. The procedure could detect as little as 5 or 10 ng of dansyl derivatives of the amines within 2 hours.

4. High-Performance Liquid Chromatography

There have been quite a few high-performance liquid chromatography (HPLC) methods published for use in separating, isolating, and quantifying biogenic amine levels in food products. Advantages of HPLC include enhanced sensitivity, but most importantly, the ability to simultaneously determine amounts of several different amines in food products. Drawbacks of HPLC methods include expensive machinery, and the need for those machines to be operated by trained personnel. The HPLC methods differ from each other in extraction chemicals, derivitizing agents, column types, separation solvents, and detectors.

Biogenic amines in red wines were determined by the use of on-column derivitization with *o*-phthalaldehyde (Busto *et al.*, 1997). Solid phase extraction was used as a cleanup step before injection and enabled the researchers to recover histamine, cadaverine, putrescine, ethylamine, tyramine, methylamine, isoamylamine, and phenylamine from the red wines with detection limits between 100 and 300 µg per Liter of wine. The derivitization of the amines when using *o*-phthalaldehyde is instantaneous, but results in an unstable derivative. To prevent breakdown of the derivative, post-column derivitization that requires additional pumping mechanisms is used. This method used on-column derivitization in an attempt to avoid the necessity of other equipment.

Some HPLC techniques use benzoyl derivatives of amines for separation. Yen and Hsieh, (1991) separated and quantified eight different amines including histamine, cadaverine, and putrescine from canned fish using benzoyl derivitization followed by

reversed phase HPLC. This method used 6% trichloroacetic acid as the extraction solvent. In 1997, Hornero-Mendez and Garrido-Fernandez used the benzoyl derivative method to analyze biogenic amines in fermented vegetable brines. They obtained detection limits between 5.6 and 54.40 pg and identified nine different biogenic amines in the samples. The major drawback to the use of benzoyl chloride as a derivitizing agent is the fact that derivitization alone takes about twenty minutes, whereas other derivitizing steps are instantaneous.

Dansyl chloride (5-dimethylaminonaphthalene-1-sulphonylchloride) is another derivitizing agent typically used to analyze biogenic amines with reverse phase HPLC. A method using dansyl chloride to derivitize amines was used to simultaneously quantify histamine, putrescine, cadaverine, spermidine, and spermine in fish (Mietz and Karmas, 1977). The concentrations of the biogenic amines were entered in a formula used as a quality index for canned tuna. A modification of this method used gradient elution to speed the analysis, and a solvent mixture of methanol and water instead of the more expensive acetonitrile as the solvents for separation (Rosier and Peteghem, 1988). These methods are effective, but derivitization time is 60 minutes at a temperature of 55° C lengthens sample time.

Ion-pair reversed-phase chromatography has become popular in the analysis of amines from food products. A sensitive reversed-phase ion-pairing HPLC method was used in determining the concentration of polyamines in rat liver. The dansyl-derivitized amines were separated on a reversed phase column with acetonitrile, water, and 5 mM octanesulfonic acid (sodium salt) as the solvents (Danner *et al.*, 1994). Hernandez-Jover *et al.* (1996) used ion-pair chromatography to separate twelve biogenic amines from meat

products. The method was linear for each amine between 0.25 and 10 mg/L, and recoveries ranged between 88 and 104%.

5. *Gas-Liquid Chromatography*

A Gas-Liquid Chromatography (GLC) procedure was developed to quantify levels of putrescine and cadaverine using their perfluoropropionyl derivatives followed by detection with an electron capture or nitrogen-specific detector (Steruszkiewicz and Bond, 1981). With this method they could quantify less than 1 µg diamine/g tissue. Yamamoto *et al.* (1982) developed a gas-liquid chromatographic method for the quantitative determination of putrescine, cadaverine, spermine, and spermidine in foods. The amines were separated from foods by elution through a cation-exchange resin column and then conversion to their (ethyloxy) carbonyl derivatives by the reaction with ethyl chloroformate in aqueous medium before application to the gas chromatograph with a flame ionization detector.

Substituted putrescine and cadaverine analogues were separated by gas chromatography using chiral and achiral stationary phases (Gaget *et al.*, 1987). The influence of the nature of the substituent on the retention behavior and on the resolution of the enantiomers was studied. When the chain length between the two amino groups was lengthened, no clear resolution was obtained of the monosubstituted cadaverine analogues as their N,N'-perfluoracyl derivatives.

Cold on-column injection for the analysis of putrescine and cadaverine was studied by Bonilla *et al.*, 1997. The cold on-column injection technique was used in conjunction with a new base-deactivated fused silica capillary column for direct analysis of putrescine and cadaverine. Excellent resolution of cadaverine, putrescine, and

heptylamine was obtained. Multiple injections gave reproducibilities of 1% for peak areas and 0.03% for retention times.

6. *Capillary Zone Electrophoresis*

Wang *et al.* (1994) reported on the use of capillary zone electrophoresis for the routine determination of biogenic amines in fresh fish samples. They obtained electrophoregrams of fluorescamine-derivatized histamine, cadaverine, and putrescine in methanol and trichloroacetic acid under hydrostatic injection techniques. Efficient peak separation was achieved within 7 minutes of injection, but reproducibility of the injection varied. Reproducibility was improved while maintaining efficient peak resolution by using an electrokinetic injection technique

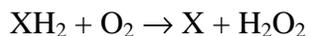
7. *Enzyme Tests*

Lerke *et al.* (1983) developed a two-step, rapid enzyme method to analyze histamine content of fresh or heat-processed fish. First, diamine oxidase catalyzed the breakdown of histamine with formation of hydrogen peroxide. Next, the hydrogen peroxide is detected by the formation of crystal violet from leuco base in the presence of peroxidase. Sumner and Taylor (1989) observed histamine production among dairy-related bacteria using this same method.

8. *Enzyme Electrode*

The joining of an electrochemical sensor and an enzymatic layer forms enzyme electrodes. Enzyme electrodes for oxidases are obtained by coupling an enzymatic film of immobilized oxidase with an oxygen electrode (Clark Electrode) (Gouygou *et al.*, 1992).

Oxidase catalyzed reaction:



where XH_2 is the reduced form of the substrate and X is the oxidized form. A decrease in oxygen concentration, due to enzymatic reaction in the film, is monitored amperometrically by the Clark electrode (Romette *et al.*, 1982).

9. Rapid Testing

There exists other rapid testing procedures for the quantification of biogenic amines in food products. Test strips were obtained from an immobilized enzyme film containing both lentil seeds diamine oxidase and horse radish peroxidase (Gougouy *et al.*, 1992). The films were made by coentrapping the molecules of enzyme and an inactive matrix protein (gelatin or bovine serum albumin) with a bifunctional reagent (glutaraldehyde). Hall *et al.* (1995) reported a solid-phase assay (dipstick test) for histamine in tuna based on the coupling of diamine oxidase to a peroxidase/dye system. The assay was linear to 1.0 mM histamine, and the minimum detectable concentration was 0.07 mM, which corresponds to 2.3 mg/100 g (23 ppm) in tuna extracts. Determinations in fresh and spoiled tuna gave results comparable to the modified AOAC fluorometric method.

In 1996, the Neogen Corporation began to distribute the Alert® for histamine rapid test for determination of histamine in raw, frozen, and canned tuna. The test utilizes ion chromatography for the purification and isolation of histamine from methanol extracted, suspect fish followed by detection with a diazo dye. The Alert® for histamine permits detection of <20, ≥ 20, but <50, ≥ 50 but <100, and ≥100 ppm concentrations of histamine in fish (Neogen Corporation, 1996). In 1998, the company marketed newer

version of the Alert® for histamine test, known as the Verotox® for histamine. This ELISA-based test kit is quantitative for histamine levels in fish and fish products.

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SECTION II: HISTAMINE, PUTRESCINE, AND CADAVERINE DETERMINATION IN TUNA USING PRECOLUMN FLUORESCAMINE DERIVATIZATION IN RP-HPLC

A. Abstract

Histamine, putrescine, and cadaverine belong to a class of compounds collectively known as biogenic amines. The biogenic amines are produced from the decarboxylation of the amino acids histidine, lysine, and arginine found in certain species of fish. The biogenic amines, specifically histamine, can produce severe allergic reactions when consumed at levels as low as 50 ppm. For this reason a rapid and selective method for the determination of biogenic amines in fish is essential.

The goal of this study was two part. The first objective was to evaluate and optimize the use of fluorescamine for biogenic amine derivatization, for both fluorescence and standard UV detection. The effects of reaction pH, molar excess of fluorescamine, derivative stability, and detector response were evaluated. The second objective was to optimize the selective extraction of the biogenic amines from tuna and perform a study of biogenic amine formation as a function of time for tuna (*Thunnus* sp.) stored at 12°C. A second storage study was conducted with bluefish (*Pomatomus saltatrix*); another fish species which is thought to accumulate biogenic amines. At a pH of 8.9 and a 2.5 molar excess of fluorescamine, the reaction proceeded instantaneously to completion. The derivatives were stable over a 24 hour time period. Trichloroacetic acid (TCA) in a 5.0% (w/v) aqueous solution produced the greatest percent recoveries, 98.6% (% RSD 0.70) for histamine, 98.7% (% RSD 1.25) for putrescine, and 100.0% (% RSD 0.77) for cadaverine. The levels of histamine increased from 29 µg/g in fresh tuna to 1845 µg/g in tuna stored at 12° for 10 days. The levels of putrescine and cadaverine were

both less than 100 ng/g in fresh tuna and reached levels of 359 and 655 $\mu\text{g/g}$ respectively during the 10 day storage period. Levels of histamine in fresh bluefish increased from 34 $\mu\text{g/g}$ to 2200 $\mu\text{g/g}$ in fish stored at 15°C for three days. Levels of putrescine and cadaverine in the fresh bluefish were both less than 100 ng/g and levels of putrescine increased to 97 $\mu\text{g/g}$ by day 3. Cadaverine levels remained below detectable limits throughout the 3 day storage period. The entire process including extraction, derivatization, and HPLC analyses was conducted in less than 45 minutes. This method can readily be adapted for both trace analysis < 1.0 ppm or higher concentrations up to 2,000 ppm.

B. Introduction

The biogenic amines histamine, putrescine, and cadaverine occur in a wide variety of food and beverages such as cheese, seafood, and wine [1,2, and 3]. Levels as low as 50 ppm [$\mu\text{g/g}$] have been associated with scombroid poisoning [4]. Scombroid poisoning is largely associated with the formation of histamine, producing severe allergic reactions when consumed. However, research [5] has shown that putrescine and cadaverine may enhance the symptoms associated with histamine poisoning. Therefore, the detection of each of these biogenic amines is essential. The biogenic amines histamine, putrescine, and cadaverine are produced from the microbial decarboxylation of the amino acids histamine, lysine, and arginine [6] shown in figure 1. The formation of the biogenic amines is accelerated at elevated temperatures, associated with improper harvesting and food handling.

HPLC and GC methods have been employed, aimed at the detection of each of these biogenic amines. These methods include: HPLC-UV based derivatization with

dansyl chloride [7]; 5-dimethylaminonaphtalene-sulphonyl [8]; HPLC-fluorescence based derivatization with fluorescamine [9] and ortho-phthaldialdehyde [OPA] [10]; and gas chromatography analysis [11]. Each of these methods can be used for the detection of biogenic amines. However, the HPLC-UV based derivatives generally require a long reaction period (> 1 hour), lack selectivity, and are characterized by poor sensitivity. The methods using GC analysis have excellent sensitivity and do not require derivatization. However, the length of time required to separate the biogenic amines in a complex mixture are often long and the typical extraction solvents such as trichloroacetic acid (TCA) are not compatible for standard GC injections. Due to the higher boiling points of the biogenic amines, GC headspace is not well suited for the analysis of biogenic amines.

At optimal conditions, fluorescamine reacts instantaneously, and is selective for the primary amine groups found on the biogenic amines [12]. The research in this study also indicated that fluorescamine derivatization produced a chromophore as well as a fluorophore, making it suitable for both UV and fluorescence detection.

C. Materials and Methods

1. *Equipment*

A Perkin Elmer series 410 LC pump (Perkin Elmer Corporation, Norwalk, CT) equipped with a Perkin Elmer Diode Array detector (DAD) and a Hitachi Model F100 Fluorescence Spectrophotometer (Hitachi Instruments, Inc., San Jose, CA), and a Hewlett-Packard Model 1100 pump (Hewlett-Packard Co., Wilmington, DE) equipped with a Perkin Elmer (DAD) were used with IB-Sil 100 x 4.6 mm 5.0 μm (C18) RP-HPLC columns obtained from Phenomenex (Torrance, CA). Histamine, putrescine, and

cadaverine standards, and fluorescamine were obtained from Sigma Chemical Co. (St. Louis, MO).

2. *Sample Extraction and Derivatization*

Ten grams of homogenated tuna were extracted with 60 mL of 5.0% TCA in a polypropylene flask. The extract was collected under vacuum filtration. One hundred μ l of the TCA extract was brought to a pH of 8.9 using 0.1 M borate buffer. Five hundred μ l of 4.0 mM of fluorescamine in acetonitrile was added to the mixture and agitated for 30 seconds.

3. *HPLC Conditions*

Five μ l of the derivatized extract was injected at a flow rate of 1.5 mL/min using the following gradient: 0.02M phosphate buffer [pH 7.2]/Acetonitrile; 80/20 held isocratic for 2.0 minutes; then programed to 50/50 in 5.0 minutes. For fluorescence detection, an excitation wavelength of 390 nm and an emission wavelength of 475 nm was used. For UV detection, a wavelength of 275 nm was used.

D. Results

1. *Molar Excess and pH Effects on Biogenic Amine Derivatization and Detection*

Previous research (10) indicated that fluorescamine derivatization produced 2 or more peaks for both putrescine and cadaverine. Less than a 2 molar excess of fluorescamine produced both a 1:1 and a 2:1 diamine:fluorescamine derivative. Secondary peaks were associated with both cadaverine and putrescine with less than a 2 molar excess of fluorescamine as shown in Table 1. The effects of pH on the derivative reaction rate and peak area response were evaluated for histamine, putrescine, and cadaverine at a pH of 2.85, 5.50, 7.20, and 8.90. A reaction using fluorescamine did not

occur at a pH of 2.85. Secondary peaks were associated with putrescine and cadaverine at a pH of 5.50 and 7.20. At a pH of 8.90 a 2:1 [diamine:fluorescamine] reaction was observed, which was characterized by an instantaneous yellow coloration. The results of the pH effects are summarized in Table 2 for putrescine and the derivative reaction is illustrated in Figure 2.

The effects of the mobile phase pH were also evaluated. At lower pH levels, the peak area response and the retention times varied greatly for all of the amines. These effects were confirmed by derivatizing the biogenic amine standards at optimal conditions and then treating them with buffers ranging from a pH of 2.85 to 8.90. Larger peak area responses were observed at the higher pH's. No differences were noted between the peak area response of the derivatives eluted with the pH 7.20 and pH 8.90 buffers, therefore, the pH 7.20 buffer was selected to extend the HPLC column life. It is suspected that the derivatives experienced an accelerated rate of degradation at the lower pH levels resulting in a shift of retention times. The degradation process occurred immediately, therefore a stability test was not established for the biogenic amines conditioned at lower pH levels. The effects of pH on peak area and retention time for cadaverine are shown in Table 3.

2. Derivative Stability Using Fluorescamine

The derivative stability over a 36 h period at room temperature was studied under optimal derivatization conditions (pH = 8.9, 2 molar excess). Each of the biogenic amines were stable over a 24 hour time period. After 24 hours, histamine experienced the fastest rate of derivative breakdown as shown in Figure 4. This stability indicates that

samples derivatized with fluorescamine are well suited for HPLC systems using autosamplers when optimum conditions are used.

3. Extraction of Tuna Using 5.0% TCA

A variety of extracting solvents were tested, with 5.0% (w/v) trichloroacetic acid (TCA) producing the greatest percent recoveries of standards injected into the fresh tuna samples. The trace levels of biogenic amines already present in the tuna were taken into account when calculating the percent recoveries. The percent recoveries ranged from 98.6 to 100.0 % with % RSD values between 0.70 and 0.91 as shown in Table 4. One caution should be observed when derivatizing the biogenic amines after TCA extraction. The 5.0% TCA solution has a pH of approximately 1.0. In order to insure that the derivatization process goes to completion, the extract must be first buffered to a pH of 8.9.

4. Application of UV Detector for Biogenic Amine Analysis

Performing a background check with the photo diode array (PDA) indicated that the presence of other UV absorbing components was not a problem with the separation of the biogenic amines in the tuna. Therefore, an analysis was also conducted using UV absorption for biogenic amine derivatized with fluorescamine. Figure 4 shows the comparison of the UV and fluorescence chromatographs.

5. Levels of Biogenic Amines Found in Stored Tuna and Bluefish

The levels of biogenic amines increased dramatically over a 10 day period in tuna stored at 12°C. Histamine experienced the highest concentration levels in both the fresh tuna (29 µg/g) and tuna stored for 10 days (1845 µg/g), indicating that histamine could

serve as a good indicator of tuna quality. Figure 5 shows representative chromatograms of the biogenic amines in fresh tuna and the tuna stored at 12° C for 10 days.

A second storage study (Table 5) was conducted with fresh bluefish (*Pomatomus saltatrix*) to observe biogenic amine levels. Fresh fish were stored at 15°C and levels of histamine, cadaverine, and putrescine were measured daily. As in the stored tuna, histamine accumulated in the greatest amounts, with 34 µg/g present in fresh fish and 2200 ppm in the fish stored for 3 days. Neither putrescine nor cadaverine were present in the fresh fish, however, 97 µg/g of putrescine was found in bluefish stored for three days at 15°C.

E. Conclusions

The biogenic amines histamine, putrescine, and cadaverine can be successfully detected in tuna and bluefish in the range of 1.0 to 2200 µg/g using precolumn fluorescamine derivatization. The analysis including extraction with 5% (w/v) TCA, derivatization, and RP-HPLC can be conducted in a short time period, 45 minutes or less. However, certain precautions must be observed. First, a minimum of a 2 molar excess of fluorescamine is required for the complete reaction. Second, the pH effects of the extraction solvent, mobile phase, and reaction mixture must be considered. The results obtained in this study indicated that a pH of 8.9 was optimum for biogenic amine derivatization producing a 2:1 diamine:fluorescamine reaction product. In order to achieve reproducible results, the mobile phase required a buffer with a pH range of 7.20 to 8.90.

Fluorescamine proved to be highly selective for the primary amine groups and the reaction was instantaneous at a pH of 8.90. In addition to providing a fluorophor [EX

390nm, EM 574 nm], fluorescamine also provides a strong chromophore with a maximum absorbance at 275 nm. However, unlike fluorescence detection, the use of an ultraviolet (UV) detector will result in a solvent peak for fluorescamine. This fluorescamine peak was not a problem in separating the three biogenic amines of interest from other components found in the fish samples. Therefore, the use of a PDA or UV detector, with limits of detection at approximately 1.0 ppm, would be practical when a fluorescence detector is unavailable. Larger injection volumes could be used to improve detection limits. The biogenic amines were stable over a 24 hour time period at room temperature when derivatized with fluorescamine at a pH of 8.90. This level of stability indicates that this method can be used for routine analysis or quality control where an autosampler is used. In addition, the stability of the reaction products simplifies the preparation process. Series of samples can be prepared at a single time without the concern of degradation.

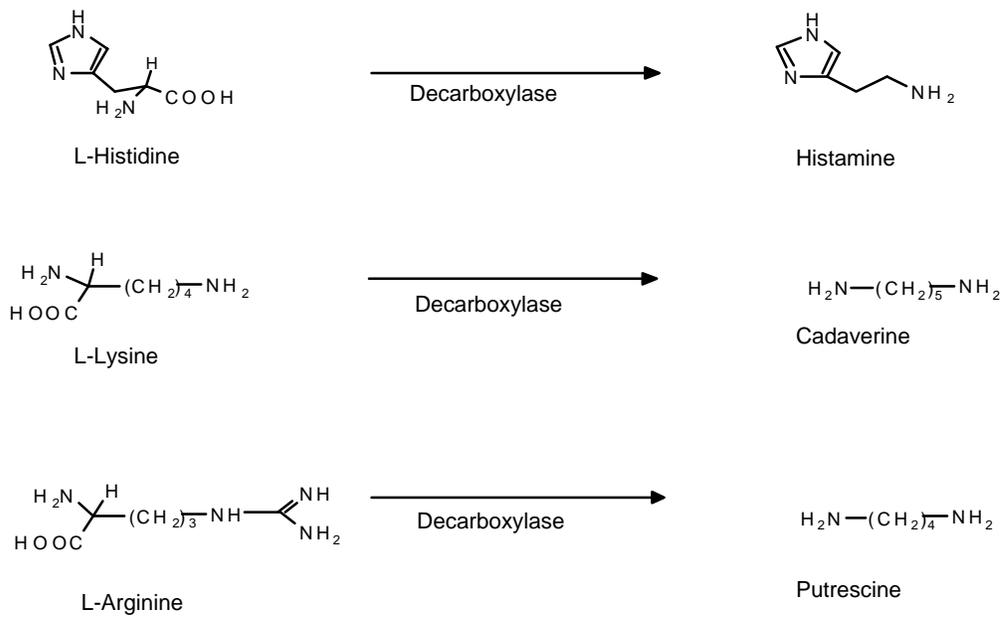


Figure 1. The formation of biogenic amines from the decarboxylation of amino acids.

Table 1. The effects of fluorescamine concentration on the reaction products of putrescine at a 7.6×10^{-4} molar concentration.

Fluorescamine Concentration mM	Theoretical Excess	Putrescine (2:1) ^a Area $\times 10^6$	Putrescine (1:1) ^a Area $\times 10^4$
1.2	< 1.0	24.1	87.0
2.0	1.3	28.9	50.0
3.0	2.0	30.2	0
4.0	2.6	30.2	0

^a diamine:fluorescamine

Table 2. The effects of pH on the peak areas of histamine, putrescine, and cadaverine using fluorescamine at a 2.5 molar excess.

Derivative	pH 2.85		pH 5.50		pH 7.20		pH 8.90	
	Area x10 ⁶	RSD ^a	Area x10 ⁶	RSD	Area x10 ⁶	RSD	Area x10 ⁶	RSD
Histamine (1:1)	0	NA ^b	14.1	1.72%	17.1	0.98%	19.2	0.79%
Putrescine (2:1)	0	NA	0.73	2.11%	15.5	0.86%	20.2	0.73%
Putrescine (1:1)	0	NA	3.44	1.98%	2.85	0.91%	0	NA
Cadaverine (2:1)	0	NA	0.61	1.83%	15.4	0.93%	19.0	0.82%

^a relative standard deviation

^b not applicable

Table 3. Mobile phase effects in HPLC on derivatized biogenic amines using fluorescamine and 0.02 molar buffer concentrations: cadaverine shown (n=3).

pH of Mobile Phase	Cadaverine Average Area x10 ⁶	Relative Standard Deviation	Cadaverine Average Retention Time (min)	Relative Standard Deviation
2.85	3.2	23.7%	3.25	18.6%
5.50	12.3	9.8%	3.21, 6.60	6.51%, 11.37%
7.20	18.9	1.30%	6.63	0.69%
8.90	19.0	0.62%	6.65	0.62%

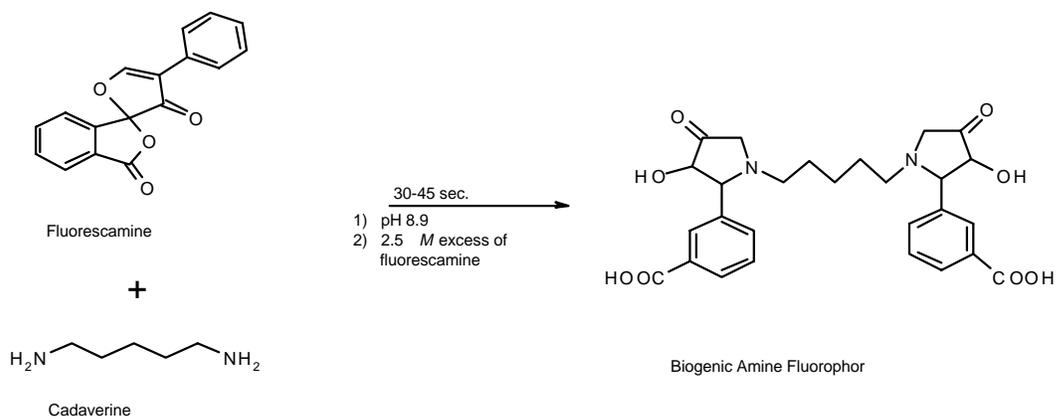


Figure 2. The derivatization of biogenic amines with fluorescamine at optimal conditions: cadaverine shown.

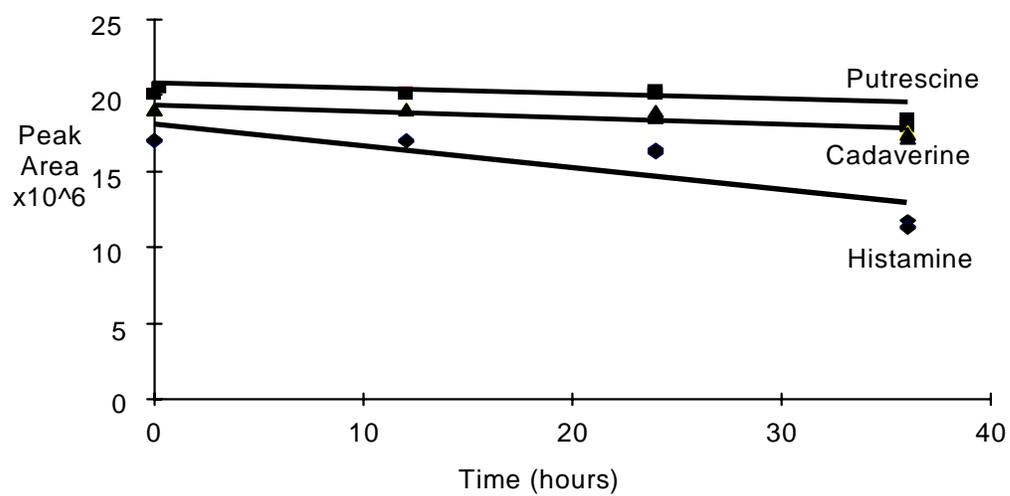


Figure 3. Derivative stability of biogenic amines over a 36 hour time period at room temperature using a 2.5 molar excess of fluorecamine at a reaction pH of 8.9.

Table 4. % Recoveries of tuna injected with biogenic amine standards using 5.0% TCA for extraction.

Standard	Standard Area $\times 10^6$	Spiked Tuna Area $\times 10^6$	% Recovery	% RSD n=3
Histamine	0.71	0.70	98.6	0.91
Putrescine	9.93	9.80	98.7	1.25
Cadaverine	6.20	6.20	100.0	0.70

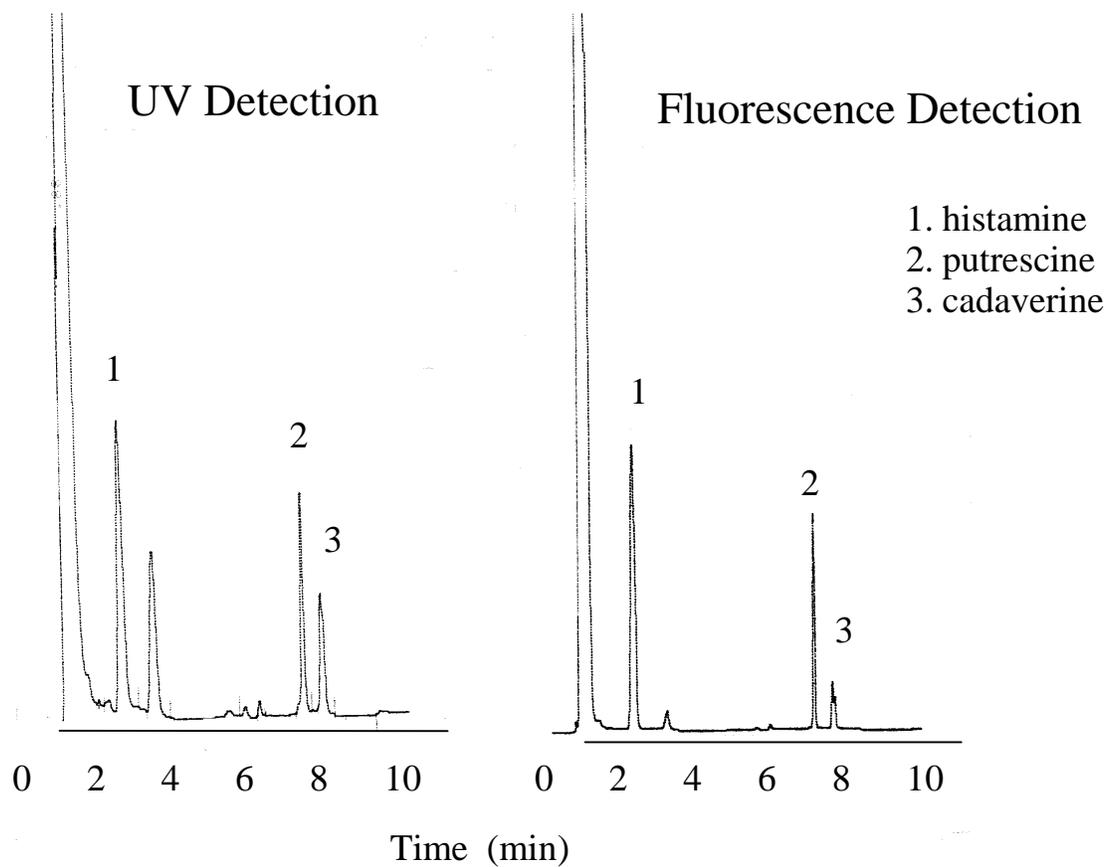


Figure 4. Comparison of UV detection at 275 nm and Fluorescence detection EX 390 and EM 475 using fluorescamine for biogenic amine derivatization in tuna stored at 12°C for 3 days.

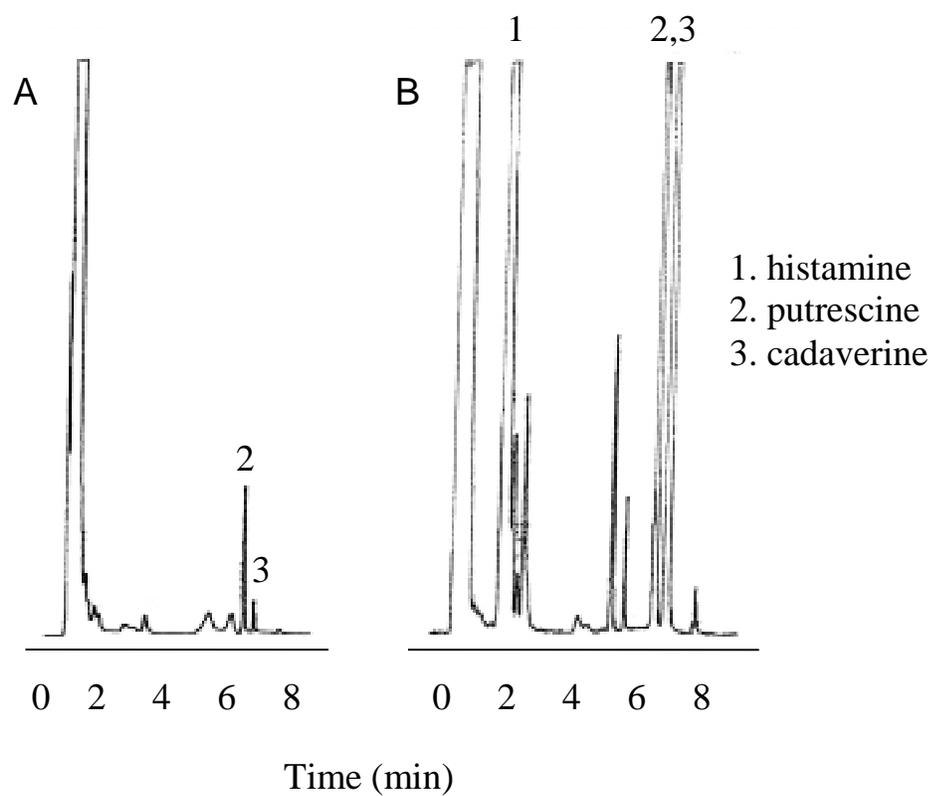


Figure 5. HPLC chromatograms of (A) fresh tuna and (B) tuna stored for 10 days at 12°C using 5.0% TCA extraction and fluorescamine derivatization.

Table 5. Biogenic amine levels in fresh bluefish stored at 15° C.

Day	0	1	2	3
Histamine (µg/g)	34	286	2154	2200
Putrescine (µg/g)	ND	10	64	97
Cadaverine (µg/g)	ND	ND	ND	ND

^a biogenic amine not detected

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SECTION III: BIOGENIC AMINE SURVEY AND ORGANOLEPTIC CHANGES IN FRESH, STORED, AND TEMPERATURE ABUSED BLUEFISH (*POMATOMUS SALTATRIX*).

A. Abstract

Changes in histamine, putrescine, and cadaverine concentrations in bluefish filets (*Pomatomus saltatrix*) stored at 5, 10 and 15° C were determined using high-performance liquid chromatography (HPLC). An organoleptic assessment was conducted simultaneously with the biogenic amine analyses. The histamine levels found in fresh bluefish obtained from wholesale seafood distributors ranged between <1 ppm and 99 ppm. Histamine was produced in fish filets in all three storage temperatures, although the greatest accumulation was observed in fish stored at 15° C which developed histamine levels as high as 2200 ppm. Overall, histamine achieved higher levels in bluefish pieces inoculated with *Morganella morganii*. Histamine was present in greater amounts than putrescine and cadaverine in the bluefish samples. Histamine levels at each temperature exceeded the 50 ppm advisory level established by the FDA before 100% sensory rejection. Putrescine and cadaverine were not found in fresh bluefish. Putrescine levels increased at each temperature during storage. Cadaverine was present only in uninoculated bluefish stored at 15° C. Standard plate counts increased during storage of fish at all temperatures, but the correlation between histamine levels and standard plate count was not significant. Consumer risk from histamine poisoning seems to be the greatest in those fish stored at 5°C where acceptance levels were higher and histamine levels above 100 ppm were observed.

B. Introduction

The ingestion of relatively large amounts of histamine, sometimes present in decomposed fish of certain species, is thought to cause an illness known as histamine poisoning or scombroid poisoning (11). Histamine poisoning in fish is often referred to as “scombroid poisoning” because spoiled fish of the families *scombroidae* and *scomberesocidae*, such as tuna and mackerel, are often implicated in histamine poisoning outbreaks. There have, however, been outbreaks associated with other non-scombroid species such as mahi mahi, bluefish, and sailfish (9,16).

Histamine belongs to a group of chemical compounds known biogenic amines. Biogenic amines are generally formed by decarboxylation of free amino acids in tissue or by amination and transamination of aldehydes and ketones (13). The major source of biogenic amines in fish is the decarboxylation of amino acids by certain decarboxylase enzymes. These enzymes, either native in the raw material or produced in the fish by bacteria, are specific for the formation of each individual biogenic amine (4). Most researchers agree that the majority of biogenic amines present in fish are a result of exogenous decomposition of amino acids by bacteria possessing decarboxylase enzymes (19).

Most of the bacteria that produce histamine in fish and fish products belong to the family Enterobacteriaceae (6). Bacterial species known to possess the ability to convert histidine to histamine include *Enterobacter aerogenes*, *Enterobacter cloacae*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus vulgaris*, and *Vibrio alginolyticus* (6). Despite the ability of many species of bacteria to produce histamine,

only *Morganella morganii*, *Klebsiella pneumoniae*, and *Hafnia alvei* have been isolated from fish causing scombroid poisoning (18).

In addition to histamine levels in fish, concentrations of other biogenic amines may be of importance in scombroid poisoning. The biogenic amines putrescine and cadaverine are thought to play a synergistic role with histamine in the occurrence of scombroid poisoning. It is possible that this potentiation of histamine stems from the inhibition of the histamine detoxification processes in the body by cadaverine and putrescine (16).

This work was conducted to observe levels of histamine, putrescine, and cadaverine in stored bluefish (*Pomatomus saltatrix*) using a new HPLC method. Because of the high histidine level present in bluefish tissue, there is a possibility of histamine development in the species. A known histamine-forming bacteria species, *Morganella morganii*, was inoculated onto a portion of the stored bluefish pieces in order to observe the bacteria's production of histamine. Sensory data was accumulated, along with the chemical analysis, to study the risk of consumer acceptance of bluefish containing high levels of histamine.

C. Materials and Methods

1. Storage of Fish

Fresh bluefish (*Pomatomus saltatrix*) was obtained from wholesale markets in Hampton, VA. After purchase the fish was transported on ice to the Food Science and Technology Department at Virginia Polytechnic Institute and State University in Blacksburg, VA. Upon arrival in Blacksburg, the whole bluefish were filleted and cut into samples approximately 50 g in weight. The samples were placed in sterile Whirl-

Pak bags prior to storage. The bags were placed on trays and then incubated at 5°, 10°, and 15° C for up to seven days or until 100% sensory rejection. Samples were taken daily for sensory and chemical analysis.

2. Inoculation Procedure

The *Morganella morganii* organisms used in this study were ATCC: 25830, batch 95-02SV obtained from American Type Culture Collection, Rockville, MD. One ml of the *M. morganii* stock culture was placed in 9 ml Trypticase Soy broth (TSB; Difco Laboratories, MI) tube and incubated for 24 hr at 37° C. After incubation, 1 ml of the 24 hr culture was inoculated into a second TSB tube and incubated for four hours at 37° C. A four hour incubation was selected after growth curve measurements confirmed that the inoculum was in the log phase of growth. The growth curve for *M. morganii* was derived from hourly absorbance readings (660 nm) of culture tube (TSB; incubated at 37° C) turbidity and simultaneous plating (Trypticase Soy agar; TSA) of the incubating culture. A 0.1 ml aliquot of the 4 hr *M. morganii* culture (10^6 CFU/ml) was dispensed onto the fish pieces using a sterile pipette. After inoculation, both the inoculated samples and uninoculated control samples were stored at the previously described conditions.

3. Sensory Analysis

Sensory analysis was conducted for each temperature during the storage periods until spoilage was detected by 100% of the panelists. The sensory panel consisted of at least 12 volunteers experienced in seafood products. The panelists were presented with a fresh reference during each day of analysis and were asked to make an acceptance decision based on the appearance and smell of each sample. The fresh references consisted of thawed bluefish muscle tissue, kept frozen until needed for sensory analysis, that were obtained from the same lot as the test sample. A new reference sample was thawed on each day of analysis and discarded after that sensory session.

4. Chemical Analysis

Biogenic amine analysis for histamine, cadaverine, and putrescine was conducted using the following extraction and high-performance liquid chromatography procedure. Fish pieces (50 g) to be analyzed were homogenized using a Waring blender. A 10 g portion of the homogenate was extracted three times with 20 ml of 5% w/v trichloroacetic acid (Fisher Scientific, NJ). After centrifugation (6000 x g, 10° C) for ten minutes, the extracts were combined and filtered through glass wool. The sample pH was adjusted to 8.9 using 0.1 M borate buffer (Fisher Scientific, NJ). The sample was then derivatized with 4.0 mM fluorescamine (Sigma Chemicals, MO) in acetonitrile and agitated for 30 s.

The derivatized sample was analyzed with a Perkin Elmer series 410 LC pump equipped with a Phenomenex IB-Sil 100 x 4.6 mm 5.0 µm (C18) RP-HPLC column followed by detection with a Hitachi model F100 Fluorescence Spectrophotometer. Five µl of the derivatized extract was injected at a flow rate of 1.5 ml/min using a gradient consisting of 0.02M phosphate buffer [pH 7.2]/acetonitrile; 80/20 for 2.0 min to 50/50 in five minutes (mobile phase chemicals obtained from Fisher Scientific, NJ). For the fluorescence detection, an excitation wavelength of 390 nm and an emission wavelength of 475 nm was used.

5. Standard Plate Counts

Standard plate counts were conducted daily on samples prior to biogenic amine analysis. A 9 cm² area of each sample was swabbed with a sterile calcium alginate tipped swab. The swab tip was broken off into a 9 ml 0.01% peptone blank, and then serially diluted. The dilutions were plated onto TSA and incubated for 48 hrs at 37°.

6. *Statistical Analysis*

All statistical tests were performed using the SAS statistical program (SAS Institute Inc., SAS Campus Drive, Cary, NC). Logistic regression was performed on the sensory data. A split plot design with mixed models was used to analyze the biogenic amine levels.

D. Results and Discussion

1. *Biogenic Amine Formation*

The method of analysis used for the determination of histamine, cadaverine, and putrescine from the fresh, stored, and temperature abused bluefish was high-performance liquid chromatography (HPLC). Using derivatization with fluorescamine, reverse-phase HPLC, gradient elution, and fluorescence detection, the analytes of interest were simultaneously separated and quantified. This method of analysis provided a relatively quick and accurate measurement of histamine, cadaverine, and putrescine in the bluefish samples.

The histamine levels found in fresh bluefish obtained from wholesale seafood distributors ranged between <1 ppm and 99 ppm. Some of the fish obtained from the distributors was well above the FDA established 50 ppm guidance level (1). The high levels of variability between fish samples can be attributed to many factors including a range of storage temperatures, different harvest times, harvest methods, and differences in microbial flora present on the fish.

Histamine was found in the uninoculated bluefish samples stored at 5° C and 10° C (Tables 1 and 2). The histamine concentrations of those samples were quite variable, and did not appreciably increase over time. Histamine levels in the uninoculated fish stored at 5° C ranged between 18 and 100 ppm, and between 12 and 52 ppm in the uninoculated fish stored at 10° C. Some of this variability may be attributed to histamine formation prior to sampling collection. Histamine levels were found to increase in the uninoculated samples stored at 15° C, reaching 938 ppm in the samples stored for three

days (Table 3). This concentration of histamine suggests the presence of histamine-forming bacteria.

The fish samples inoculated with *M. morganii*, and stored at 5° C, had histamine concentrations ranging from 16 to 116 ppm, but no definite increase over time was observed (Table 5). Histamine levels did increase in inoculated samples at both 10° C and 15° C accumulating 338 and 2200 ppm respectively. This data is in agreement with previous studies concluding that most of the bacteria responsible for high levels of histamine in fish are mesophilic bacteria belonging to the *Enterobacteriaceae* (6). Optimum temperature for histamine production in Skipjack tuna was found to be 37.8° C (7).

Fresh bluefish did not contain detectable levels of putrescine or cadaverine. Putrescine formation did occur during storage at all temperatures, and each increasing increment of temperature showed a significant rise in putrescine production ($p < 0.0001$). Putrescine levels of 30 ppm or greater were found at all storage temperatures. Overall, putrescine levels increased with time, reaching a maximum level of 150 ppm in *M. morganii* inoculated samples stored at 10° C (Table 5). Cadaverine, however, was found only in uninoculated fish stored at 15° C, in which 50 ppm was present on the third day of storage (Table 3). In past research, positive correlation was found between expert sensory decisions for “accept/reject” quality and levels of putrescine and cadaverine in the product (14).

2. Bacteriological Effects

Overall, there was a significantly greater production of histamine in those bluefish samples that were inoculated with *M. morganii* ($p < 0.0001$). This would be expected from histidine-decarboxylase enzymes produced by the bacteria. *M. morganii* is well documented as a histamine-producing organism and has been isolated from fish responsible for scombrototoxic incidences (3,10). Greater differences in histamine formation between inoculated and uninoculated bluefish were observed in fish stored at higher temperatures ($p < 0.0001$). This indicates that histamine-decarboxylase enzyme activity, enzyme production, or both were greater at the higher temperatures.

Putrescine production was greater in those samples inoculated with *M. morganii* than the control samples ($p < 0.025$). This suggests that *M. morganii* contains the enzymes necessary for production of putrescine. The presence of cadaverine in the control samples stored at 15° C and not in the inoculated samples stored at 15° C could be the result of competitive inhibition of cadaverine-producing organisms by *M. morganella*.

The standard plate counts follow a general increasing trend in all of the storage temperatures. The highest counts were found in the samples stored for seven days at 5° C (Tables 1 and 4). Counts of $8.0 * 10^9$ CFU/cm² and $1.3 * 10^9$ CFU/cm² were found in the uninoculated and inoculated samples, respectively. A correlation analysis was conducted on histamine levels and standard plate counts. There was no significant correlation found between the change in histamine concentrations and the change in total plate count at each temperature ($p < 0.64$ at 5° C, $p < 0.90$ at 10° C, and $p < 0.24$ at 15°C). This indicates that either histamine-forming bacteria made up only a small part of the flora or that enzyme production or activity was affected by temperature.

3. *Sensory Analysis*

The sensory analysis was conducted simultaneously with biogenic amine analysis to determine whether a population may be at risk for accepting bluefish with histamine levels greater than 50 ppm. As expected, the percentage of sensory volunteers accepting the fish decreased with each successive day of storage. Overall, the inoculated bluefish had lower percentages of acceptance values ($p < 0.0001$). It is difficult to ascertain if this decrease in acceptance was the result of higher levels of histamine present in the inoculated fish or if it was the result of other decomposition products. In past studies, histamine alone has not been found as an acceptable indicator of fish spoilage. Foods containing unusually high levels of histamine may not appear outwardly spoiled (17). Frank *et al.* (6) reported that skipjack tuna judged decomposed by sensory analysis frequently had histamine levels of less than 50 ppm. Increased microbial loads may have caused increases in volatile amines, which have been widely used as freshness indicators in some species of marine fish and result in strong odors during fish decomposition (5). Acceptance levels were higher in those fish stored at lower temperatures (Tables 1 and 2) and the values for percent acceptance were significantly different between each storage temperature ($p < 0.0001$).

4. *Consumer Risk*

The greatest amounts of histamine accumulated in inoculated bluefish stored at 15° C (Table 6). Histamine levels on storage days two and three exceeded 2000 ppm, compared to levels less than 1000 ppm in the uninoculated samples. However, these levels occurred when there was a zero percent acceptance. Inoculated bluefish stored for one day at 15° C (Table 5), contained histamine levels above 300 ppm, still a possible

illness hazard, but the percent acceptance was below 30%. In inoculated bluefish stored at 10° C (Table 4), histamine levels only exceeded 100 ppm after being judged unacceptable by 90% of the panelists. Histamine levels in uninoculated fish stored at 10° C (Table 3) never exceeded 100 ppm.

Histamine levels at 5° C were variable, but failed to reach the levels observed in spoiled fish at the 15° C storage conditions (Tables 1 and 2). Levels of histamine were often above the 50 ppm advisory level established by the FDA (1), and in some cases above 100 ppm in both the inoculated and uninoculated bluefish. Sensory acceptance was higher in the fish stored at the lowest temperature, indicating a greater likelihood of consumer ingestion of bluefish that have been subjected to these storage temperatures. Since the minimum dose of histamine to cause illness is unknown, it is impossible to predict if this fish would have caused illness in consumers willing to accept the product.

Putrescine and cadaverine are thought to augment scombrototoxic incidences by interfering with histamine metabolism in the human intestine (8). If this is the case, it is important to understand levels of other biogenic amines, besides histamine, that occur in fish. This study concluded that putrescine formation up to 150 ppm was possible in bluefish, and that cadaverine levels of 50 ppm were possible in cases of severe decomposition.

In this study, histamine was found in all bluefish, including the fresh fish. This shows that this particular fish species is at risk for development of histamine. The study concluded that although putrescine was not present in fresh samples, it could be formed during storage temperatures as low as 5° C. Cadaverine formation was only evident at

the highest storage temperature of 15°C. This research shows that bluefish may contain the biogenic amines histamine, putrescine, and cadaverine.

TABLE 1. Sensorial acceptance and histamine, putrescine, and cadaverine levels during 5° C storage of uninoculated bluefish.

time (days)	standard plate count	% acceptance	histamine ^a	putrescine ^a	cadaverine ^a
1	4.1E4	74	58 +/- 28	ND ^b	ND
2	2.0E4	96	50 +/- 43	ND	ND
3	1.8E4	79	45 +/- 63	ND	ND
4	5.3E4	77	42 +/- 43	9 +/- 8	ND
5	1.3E9	57	100 +/- 11	8 +/- 11	ND
6	4.7E7	37	18 +/- 10	14 +/- 12	ND
7	8.0E9	25	25 +/- 44	30 +/- 8	ND

^a Measured in ppm

^b ND, not detected

TABLE 2. Sensorial acceptance and histamine, putrescine, and cadaverine levels during 10° C storage of uninoculated bluefish.

time (days)	standard plate count	% acceptance	histamine ^a	putrescine ^a	cadaverine ^a
1	5.4E4	62	52 +/- 33	ND ^b	ND
2	5.4E5	47	41 +/- 7	ND	ND
3	1.1E8	18	44 +/- 21	34 +/- 40	ND
4	2.6E7	5	12 +/- 14	104 +/- 85	ND
5	4.2E7	0	33 +/- 37	99 +/- 107	ND

^a Measured in ppm

^b ND, not detected

TABLE 3. Sensorial acceptance and histamine, putrescine, and cadaverine levels during 15° C storage of uninoculated bluefish.

time (days)	sandard plate count	% acceptance	histamine ^a	putrescine ^a	cadaverine ^a
1	2.8E6	44	35 +/- 26	ND ^b	ND
2	7.3E6	12	55 +/- 39	51 +/- 39	ND
3	4.2E7	0	938 +/- 200	96 +/- 58	50 +/- 62

^a Measured in ppm

^b ND, not detected

TABLE 4. Sensorial acceptance and histamine, putrescine, and cadaverine levels during 5° C storage of *Morganella morganii* inoculated bluefish.

time (days)	standard plate count	% acceptance	histamine ^a	putrescine ^a	cadaverine ^a
1	3.5E4	77	102 +/- 10	ND ^b	ND
2	1.2E6	95	69 +/- 74	ND	ND
3	1.9E4	79	69 +/- 43	9 +/- 12	ND
4	3.6E5	57	47 +/- 57	83 +/- 72	ND
5	6.5E8	52	116 +/- 0	8 +/- 11	ND
6	2.6E8	19	80 +/- 36	13 +/- 12	ND
7	1.3E9	19	16 +/- 15	77 +/- 8	ND

^a Measured in ppm

^b ND, not detected

TABLE 5. Sensorial acceptance and histamine, putrescine, and cadaverine levels during 10° C storage of *Morganella morganii* inoculated bluefish.

time (days)	standard plate count	% acceptance	histamine ^a	putrescine ^a	cadaverine ^a
1	1.9E5	52	43 +/- 17	ND ^b	ND
2	7.6E5	37	54 +/- 13	ND	ND
3	2.0E7	24	31 +/- 12	35 +/- 20	ND
4	2.0E7	3	338 +/- 13	150 +/- 4	ND

^a Measured in ppm

^b ND, not detected

TABLE 6. Sensorial acceptance and histamine, putrescine, and cadaverine levels during 15° C storage of *Morganella morganii* inoculated bluefish.

time (days)	standard plate counts	% acceptance	histamine ^a	putrescine ^a	cadaverine ^a
1	7.4E6	26	286 +/- 261	10 +/- 7	ND ^b
2	1.6E8	0	2154 +/- 10	64 +/- 10	ND
3	5.0E6	0	2200 +/- 0	97 +/- 0	ND

^a Measured in ppm

^b ND, not detected

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SECTION IV: ISOLATION OF HISTAMINE-PRODUCING BACTERIA FROM FISH-PROCESSING FACILITIES AND FISHING VESSELS.

A. Abstract

The presence of histamine-forming bacteria in fish-processing facilities was studied. Environmental sampling techniques were conducted in the Hampton Roads area of Virginia in fish-processing facilities that regularly handle scombroid fish or other fish which are could potentially accumulate histamine levels greater than 50 ppm. Surfaces that come into contact with the fish were swabbed and the histamine-forming isolates from these areas were identified. One isolate each of *Klebsiella ozaenae* and *Vibrio alginolyticus*, and two isolates of *Aeromonas* sp. were found in the processing facilities. The study concluded that histamine-forming bacteria do not make up a large part of the microflora associated with fish-processing facilities. Fishing vessels were also sampled and no histamine-forming bacteria were identified.

B. Introduction

Histamine is thought to play a causative role in scombroid poisoning. Scombroid poisoning is a clinical syndrome seen in persons ingesting fresh or processed scombroid fish such as tuna, saury, bonito, and mackerel (Motil and Scrimshaw, 1979). Symptoms include a sharp peppery taste in the mouth, flushing of the face and neck, and a feeling of feverishness (Bartholomew *et al.*, 1987). The onset of symptoms is within 10 min to 2 h of consuming the toxic fish (Bartholomew *et al.*, 1987).

Histamine and biogenic amines in general, are formed by decarboxylation of free amino acids in tissue or by amination and transamination of aldehydes and ketones (Silla Santos, 1996). The decarboxylation of the amino acid histidine into histamine in fish occurs due to the action of histidine-decarboxylase. This enzyme is either endogenous in the raw material or produced in the fish by microflora associated with the product (Brink *et al.*, 1990).

It is known that different bacteria vary significantly in either the quantity of decarboxylase they produce and/or the specific activity (turnover number) of those decarboxylases (Wendakoon and Sakaguchi, 1992). The group of bacteria primarily responsible for the decomposition of the scombroid fish, with the ability to decarboxylate histidine to form histamine is *Enterobacteriaceae* (Frank *et al.*, 1985). The histamine-producing bacteria isolated from fish causing scombroid poisoning are *Morganella morganii*, *Klebsiella pneumoniae*, and *Hafnia alvei* (Ababouch *et al.*, 1991).

Histamine-producing bacteria have been considered part of the normal microflora of fish (Yoshinaga and Frank, 1982). This may be the case for some histamine-producing bacteria such as *Vibrio* sp. that are naturally occurring in marine environments. The

circumstances under which scombroid fish would acquire bacteria such as *P. morgani* and *P. pneumoniae* which are not part of the normal microflora of seawater has never been adequately explained (Taylor and Speckhard, 1983).

The purpose of this study was to conduct environmental sampling for histidine-decarboxylase producing bacteria in fish-processing facilities that regularly handle scombroid fish or other fish that could potentially accumulate high levels of histamine. In addition to sampling the processing facilities, catching vessels were also sampled to ascertain if histamine-producing bacteria maintained a presence in that environment.

C. Materials and Methods

1. *Environmental Sampling Protocol*

The fish-processing facilities sampled were wholesale fish houses in the Hampton Roads area of Virginia. Three such fish houses, which receive, process, or pack scombroid fish or other histamine-producing fish, were surveyed. Each of the three facilities was sampled during processing operations in the colder months of January, February, and March, and then again in the warmer months of May, June, or July. By sampling during different times of the year, it was possible to monitor the seasonal changes in microflora. Commercial fishing vessels were also sampled for the histamine forming-bacteria, since they are a possible source of post-harvest contamination.

Sampling locations in the processing and packing facilities were those surfaces that would be expected to come into contact with the fish (Table 1). The surface sampling technique consisted of swabbing a 10 cm² area with a sterile calcium alginate tipped swab dipped in a 10 ml neutralizing buffer solution (Difco Laboratories, Detroit, MI). After sufficient swabbing of the desired surface, the swab was aseptically placed

back into the tube containing the neutralizing buffer, the tip was broken, and the cap replaced. Each surface was swabbed twice. The tubes were transported at 7° C to the Food Science and Technology building at Virginia Polytechnic and State University in Blacksburg, Virginia for examination.

2. Identification of Histamine-Forming Isolates

The tubes containing the neutralizing buffer and sampling swabs were spread plated within eight hours of swabbing onto Trypticase Soy agar (TSA) and incubated for 48 h at 37° C. From a countable plate from each location, a visual description of each different colony was recorded, the colony coded, and then subcultured onto a TSA slant. Each slant was incubated for 24 h at 37°C. The colonies were checked for histidine-decarboxylase activity by streaking a Niven's (Niven *et al.*, 1981) plate from each slant. After incubation of the Niven's agar plates for 48 h at 37° C, the histidine decarboxylase positive organisms were further characterized by gram stain. All histamine-producing gram negative rods were identified using the API20E Enterobacteriaceae identification test strip (bioMerieux Vitek, Inc., Hazelwood, MO).

D. Results and Discussion

The occurrence of histamine-forming bacteria in the fish processing facilities was found to be very low. Only four gram-negative, histamine-forming isolates were found during the environmental sampling of the three fish-processing facilities. Three of the four histamine-forming isolates were found in the warmer months of April and June. The isolated organisms are identified in Table 2, along with the corresponding location where each bacterium was isolated. There were no histamine-forming bacteria isolated from any of the fishing vessels sampled.

Histamine-forming *Klebsiella* sp. have been isolated from skipjack tuna and jack mackerel (Omura *et al.*, 1982). *Klebsiella ozaenae* was found in the knife dip in one of the processing facilities (Table 2). This occurrence is hard to explain because the dip was reported to contain a chlorine solution. It was not known how often the sanitizing solution was replaced, and it is entirely possible that either the concentration of chlorine was not sufficient to eliminate the biological load present on the knives or the microorganism was a recent introduction into the dip.

The histamine-forming bacteria *Vibrio alginolyticus* was found on the processing floor during one of the sampling trips (Table 2). This species has been previously reported as a histamine-former and has been frequently isolated from fish and marine environments in general (Yoshinga and Frank, 1982). Isolates of *Aeromonas* sp. positive for histamine-production were found in a warm water hand dip at one facility and on a fish carton at another facility (Table 2). These organisms are ubiquitous to the marine environment so their presence is not surprising.

It can be concluded that histamine-forming bacteria make up a small part of microbiological flora of these fish-processing facilities. The bacteria *Morganella morganii*, *Klebsiella pneumoniae*, and *Hafnia alvei*, which have been isolated from fish implicated in scombroid poisoning incidences, and are generally considered the most prolific histamine-producing bacteria, were not found during this study. Also, improved standard sanitation operating procedures (sSOP) in the processing facilities should further reduce the incidences of histamine-forming bacteria.

Table 1. Surface swabbed locations in fish processing facilities and fishing vessels.

cutting tables worker's gloves fish cartons fillet knives knife dips scales loading docks warm water hand dips cutting boards	processing floors ice room doors pallets ice boxes air curtain for cold rooms ice shovels conveyor belts deck of fishing vessels inside hull of fishing vessels
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Table 2. Histamine-forming bacteria found in fish processing facilities and the corresponding location of each organism.

organism	location	month of isolation
Klebsiella ozaenae Vibrio alginolyticus Aeromonas sp. Aeromonas sp.	knife dip processing floor warm water hand dip fish carton	January April June June

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