Free Amino Acids in Dark- and White-muscle Fish as Determined by O-phthaldialdehyde Precolumn Derivatization

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ABSTRACT: Free amino acids in fish reflect microbial spoilage, and are precursors of biogenic amines, factors of health concern and indicators of fish decomposition. The objective of this research was to quantify levels of free histidine, lysine, ornithine and glutamine, which may become originators of highly undesirable histamine, cadaverine and putrescine in fish tissue. Liquid chromatography using o-phthaldialdehyde (OPA) pre-column derivatization, gradient elution and a C18 column were used for separation. Histidine was higher in white tissue of mahi-mahi and tuna than in red tissue. No significant difference in lysine levels was found between the two tissues of tuna. Glutamine and ornithine were higher in red tissue of mahi-mahi and tuna. Red snapper had higher levels of lysine than histidine.

Key Words: free amino acids, OPA precolumn derivatization, HPLC, dark-muscle fish, white-muscle fish

Introduction

IT IS WELL ESTABLISHED THAT FREE AMINO ACIDS PLAY A VERY important role in bacterial growth and spoilage of fish (Jay and Kontou 1967; Ingram and Dainty 1971; Jay 1992). Amino acids are broken down during bacterial spoilage to ammonia, a major total volatile base-nitrogen (TVB-N) compound, and other malodorous compounds such as putrescine, cadaverine, and hydrogen sulfide (Ingram and Dainty 1971; Hultin 1984; Finne 1992; Pivarnik and others 1998; Koutsoumanis and others (1999). It is these end products that consumers generally perceive as indicative of fish putrefaction (Finne 1992; Wheaton and Lawson 1985). Thus, microbial spoilage results in extensive deteriorative changes in fish that eventually renders the fish unmarketable (Liston and others 1963).

Deteriorative changes in fish are indicated by several physicochemical factors. Among them are production of biogenic amines due to microbial decarboxylase activity on free amino acids. Biogenic amines are considered indicators of fish spoilage with economic and human health effects (Mietz and Karmas 1978; Finne 1992; Beljaars and others 1998). Histamine, one of the biogenic amines, is known to be the causative factor of scombroid poisoning (Arnold and Brown 1978), and levels greater than 5 mg/100 g imply fish decomposition (Price 1995). Evidence also shows that cadaverine and putrescine are potentiators of histaminosis (Taylor and Eitenmiller 1986), and have the potential to cause illness even in the absence of histamine (FDA 1998). The direct precursors of histamine, cadaverine and putrescine are histidine, lysine and ornithine, respectively, and glutamine is considered an indirect precursor of putrescine (Sikorski and others 1990). The breakdown of proteins in the later stage of spoilage contributes additional amino acids for continued bacterial activity (Ingram and Dainty 1971). Bramstedt (1962) showed that fish proteolytic enzymes and peptidases play important roles in the biochemical spoilage of fish during the first few days postmortem. These enzymes cause an increase in lysine during iced storage and transportation of fish (Shewan and Jones 1957). Bramstedt (1962) observed that the pattern of free amino acids in fish was different for each species, which makes it possible to investigate the influence of factors such as storage and spoilage on the composition of free amino acids.

The importance of amino acids in fish has been well established from the perspective of nutrition (Nettleton 1985; Cowey 1994; Mambrini and Kaushik 1995), fish meal production (Espe and others 1992), fish flavors (Yuko and others 1994), and bacterial and chemical spoilage (Stansby and Olcott 1963; Osterhaug and others 1963). Few studies are available on the role amino acids play in assessing fish freshness and spoilage. Shewan and Jones (1957) showed that among the chemical changes that satisfy the conditions for defining a quality index, total volatile base and lysine are possible candidates. In cod stored at 0 °C, these two indices changed distinctly as storage (spoilage) progressed.

A major difference between the white-muscle and dark-muscle fish is the high level of free histidine consistently found in dark-muscle fish (Lukton and Olcott 1958; Abe 1983). Ito (1957), Konosu and others (1974) and Stockemer (1982) described the large quantities of free histidine as characteristic of fatty fish, while nonfatty fish such as flounder (Konosu and others 1974; Antoine and others 1999) contain little or no histidine in the free state. Work on tuna and mahi-mahi showed that the white tissue in these dark-muscle fish contains greater levels of histidine than the red tissue (Antoine and others 1999). This characteristically large quantity of histidine is the basis for implication of dark-muscle fish in scombroid poisoning (Takagi and others 1969), which is not a problem in white-muscle fish such as grouper, flounder, American red snapper, and so on (FDA 1998). Kimata and Kawai (1953) demonstrated that dark-muscle fish distinguish themselves from white by the formation of large amounts of histamine during bacterial spoilage. The production of high levels of histamine is dependent on temperature, and level and availability of free histidine (Voight and Eitenmiller 1978; Silva and others 1998), and the presence of the relevant de-

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carboxylases (Hierro and others 1999).

Given the importance of free amino acids in fish quality and deterioration, the objective of this research was to quantify the levels of free histidine, lysine, and ornithine in the red and white tissue of fish using an HPLC method for amino acid analysis (Antoine and others 1999). These amino acids were chosen because they are direct precursors of biogenic amines, which are indicators of fish quality and decomposition. Glutamine was quantified because of intrinsic analytical interest which necessitated resolution of its peak from that of histidine, and because glutamine has been reported to be an indirect precursor of putrescine via the glutamate-ornithine pathway (Gale 1940; Hood and Lyman 1950; Ory and others 1953; Morris and Koffron 1969; Morris and others 1970; Rodwell 1977; Umbarger 1978) even though it may be considered minor in some microorganisms (White and others 1964).

Materials and methods

Fish samples and preparation

Fresh boneless fillets of mahi-mahi (*Coryphaena hippurus*), yellowfin tuna (*Thunnus albacares*) (1.5 to 2.5 kg each for 4 fillets), and American red snapper (*Lutjanus campechanus*) (340 to 450 g each for 5 fillets) were purchased from a local store (Northwest Seafood Inc., Gainesville, Fla., U.S.A.). The fillets were all unmatched (from separate fish). The fish were caught in the fall of 1998. Mahi-mahi and yellowfin tuna were caught at the tropical waters of the Caribbean, and the American red snapper from the Florida Gulf coast. The purveyors purchased the fish from their Miami wholesale supplier on the day the fish were landed. The fish were all bled and degutted on deck, stored in ice, and brought to Gainesville. Fish were purchased immediately upon arrival in Gainesville, skinned, stored in ice, and brought to the laboratory for immediate use. In the case of the dark-muscle fish (tuna and mahi-mahi), a sharp knife was used to carefully separate the red and white tissues. Each tissue type was then cut into several portions, chopped, and separately homogenized. Four replicate samples of each tissue type were taken from each fillet for analysis.

Ten g of fish was put into a mason jar followed by 40 mL of extracting solvent (75% methanol in distilled deionized water) and blended for 2 min using a Hamilton Beach Blend Master 14 blender (Hamilton/Proctor-Silex, Inc., Glen Allen, Va., U.S.A.). The extract was then transferred to a 100-mL volumetric flask and the jar was rinsed three times with 15 mL of the extracting solvent. The rinsings were added to the flask which was then brought to a standard volume of 100 mL and stored for 60 min (or overnight) at 4 °C. The content of the flask was transferred to a centrifuge tube and centrifuged at 27,000 × g (15,000 rpm) for 40 min using an IEC refrigerated centrifuge model B20A (Intl. Equipment Co., Needham Heights, Mass., U.S.A.). The supernatant was filtered using an Acrodisc PTFE 0.2-micron filter membrane (Gelman Sciences, Ann Arbor, Mich., U.S.A.), diluted as required, and treated as was the standard solution (Figure 1). When necessary, filtered sample extracts were stored in a freezer at −20 °C.

Reagents

Methanol (HPLC grade), glacial acetic acid and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.); tetrahydrofuran (HPLC grade) and 2-mercaptoethanol were obtained from Mallinckrodt Speciality Chemicals Co. (Paris, Ky., U.S.A.); while amino acid standards, sodium tetraborate (anhydrous), and o-phthaldialdehyde were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Absolute alcohol was obtained from Aaper Alcohol and Chemical Co. (Shelbyville, Ky., U.S.A.), and distilled deionized water was obtained from a Photronix Reagent Grade Water System (Medway, Mass., U.S.A.).

Mobile phase preparation

Mobile phase A was made up of 0.05 M acetate buffer (pH 5.5) prepared from analytical grade acetic acid, methanol and tetrahydrofuran (80:19:1). The pH of the acetate buffer was adjusted using a 10 N NaOH solution. Mobile phase B was made of methanol and 0.05 M acetate buffer (pH 5.5; 80:20). The mobile phases were filtered using Supor®-200 47 mm 0.2 µm filter membrane (Gelman Sciences, Ann Arbor, Much., U.S.A.) and degassed by sparging for 5 min with helium.

Instrumentation

The method of Antoine and others (1999) was slightly modified. A Beckman reversed-phase HPLC column (Ultrasphere ODS 5 µm particle size, 4.6 mm x 25 cm; Fullerton, Ca-

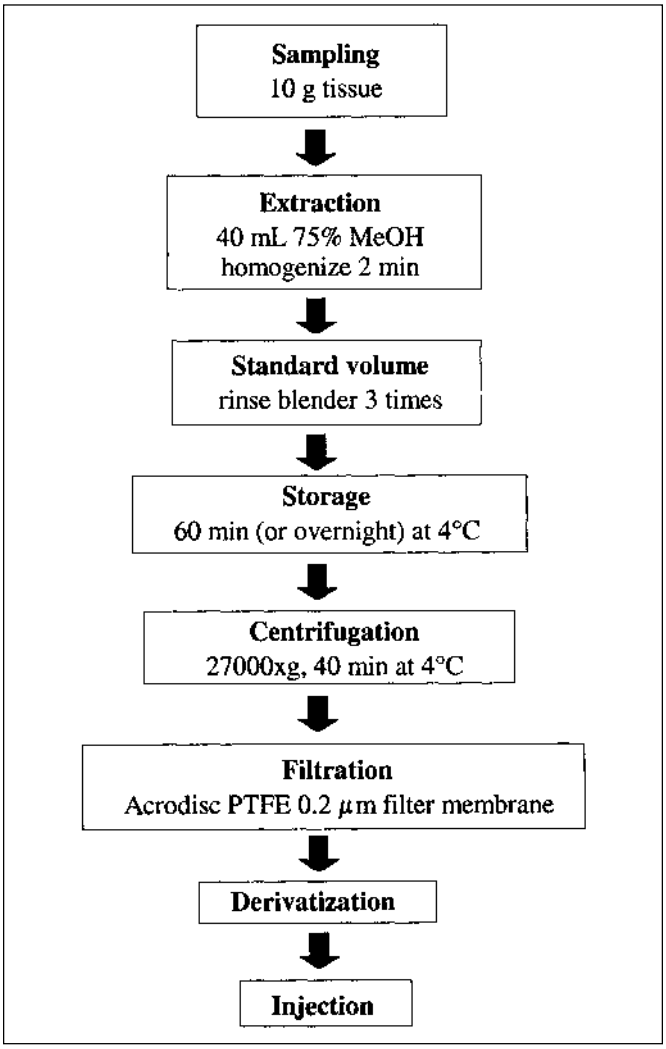

Figure 1—Flow chart showing sample preparation for LC analysis of precolumn OPA-amino acid derivatives

lif., U.S.A.) was used without a guard column. Gradient elution was generated using a Bio-Rad model 2800 solvent delivery system (Hercules, Calif., U.S.A.) equipped with dual pumps. A Rheodyne injection valve (Model 7125-081) containing a 20-µL sample loop was used for injection of samples. BioRad Value Chrom Software Version 4 1988-1994, was used for controlling the gradients and flow rate (1.5 mL/min) of the mobile phases. A Perkin-Elmer LC 240 fluorescence detector (Buckinghamshire, England) fitted with a 7-µL flow cell was used with the excitation monochrometer set at 340 nm and the emission monochrometer at 430 nm. Other detector settings were: response time, 0.7 sec; and an attenuation factor of 256. A Spectra-Physics SP 4092 integrator (San Jose, Calif., U.S.A.) was used to generate chromatograms. Integrator attenuation was 1024 and the chart speed 0.5 cm/min.

OPA-thiol reagent preparation

OPA-thiol reagent was made up at least 24 h before use by dissolving 27 mg o-phthaldialdehyde in 500 µL absolute alcohol. Then 5 mL of 0.1 M sodium tetraborate (pH 9.5) was added, followed by 50 µL mercaptoethanol. The mixture was thoroughly mixed and stored in the dark in a tightly closed container. The OPA-thiol reagent can be kept for several days with periodic additions of 20 µL mercaptoethanol (Joseph and Marsden 1986; Miles and Leong 1992).

Amino acid standards

Stock solutions of histidine, lysine, glutamine and ornithine (2.5 mmoles) were prepared by dissolving the proper amount of each amino acid in 0.05 M sodium phosphate buffer (pH 5.5). Use of acetate buffer (0.05 M, pH 5.5) resulted in decreased fluorescence signal. Each stock solution was then used to prepare working solutions from which a calibration curve was constructed.

Amino acid derivatization

To 100 µL of amino acid standard, or diluted supernatant, 400 µL of OPA was added and mixed thoroughly using a vortex. Sodium phosphate buffer (0.05 M, pH 5.5) was used for the blank, to dilute all supernatants, and for the preparation of standards. Exactly 2 min after mixing, the sample was manually injected and the gradient run started. It was important to keep the time between mixing and injecting into the HPLC consistent (Joseph and Marsden 1986) because of the limited stability of the OPT-amino acid derivatives. The concentration of each amino acid of interest was measured using an external calibration curve. Attempts to use an internal standard proved difficult because of coelutions.

Statistics: Statistical analysis used paired t-test, and two-sample t-test for comparing treatments (Ott 1992).

Results and Discussion

THE LOWEST DETECTABLE LEVELS (2 × THE NOISE LEVEL) FOR the standard amino acids of interest were 35.1 pmol/mL for glutamine, 81 pmol/mL for histidine, 40.6 pmol/mL for lysine, and 30.1 pmol/mL for ornithine. The inter-day (day to day) assays of the amino acid standards were reproducible with a coefficient of variation from 1.0 to 11.5%, and intra-day (within-day) assays of 1.2 to 6.5%. Three different spike levels were used for a recovery study. The average recoveries for the amino acids of interest were 91.5%, 84%, 84.9%, and 80% for histidine, lysine, glutamine, and ornithine, respectively. The Association of Biochemical Resources Foundation (ABRF) considers that a 90 to 95% recovery (70% in some cases) is expected for OPA precolumn derivatization using RP HPLC, depending on the amount analyzed, and the instrument used (West and others 1996). A chromatogram of the amino acid standards of interest is shown in Figure 2. Calibration concentrations ranged from 50 to 400 pmol/mL for glutamine, and 100 to 800 pmol/mL for histidine, lysine, and ornithine.

The levels of the 4 amino acids of interest for the 3 fish species studied are shown in Table 1. In the dark-muscle fish, mahi-mahi and tuna, greater levels of histidine were found in the white tissue than in the dark (red) tissue ($p < 0.01$) (Table 1). This result corresponds with that of Lukton and Olcott (1958), and Abe (1983). The difference in the histidine level between the white and red tissues was greater in yellowfin tuna ($p < 0.01$) than in mahi-mahi ($p < 0.002$). Histidine levels in the white tissue of mahi-mahi was significantly lower ($p < 0.002$) than that in the white tissue of yellowfin tuna. Unlike the dark-muscle fish, histidine levels in American red snapper (a white-muscle fish) were very low (Table 1), which is unique to white-muscle fish (Ito 1957; Konosu and others 1974).

Histidine is important because of its nutritional and physiological roles in fish. Shiau and others (1997) found a positive correlation between histidine and the body weight of milkfish (*Chanos chanos*). In addition, histidine is the precursor of histamine which is found primarily in temperature-abused dark-muscle fish and is of major safety concern (Arnold and Brown 1978; Taylor 1988; Garrett and Hudak-Roos 1990; FDA 1994; Anonymous 1995).

Considerable variations in the levels of some free amino acids can be observed from the same fish species. Hibiki and Simidu (1959) reported a histidine level of 1010 mg/100 g in bigeye tuna *(Parathunnus mebachi* also called *Thunnus obesus*), while Lukton and Olcott (1958) reported a level of 481.1 mg/100 g (30.5 to 31.5 µmol/g) in the white tissue of the same species. Yoshinaga and Frank (1982) reported that histidine is uniformly distributed in fresh skipjack tuna (*Katsuwonus pelamis*) at about 564 to 611 mg/100 g. However, Abe (1983) obtained free L-histidine levels of 1389 mg/100 g (83.4 to 92.8 mmol/g) in the white tissue and 268.5 mg/100 g

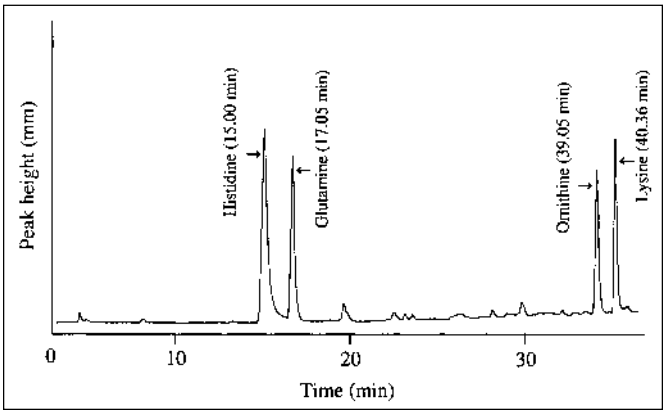

Figure 2—Chromatogram of standard amino acids using binary mobile phases. Mobile phase A was 80:19:1 of 50 mM acetate buffer (pH 5.5), methanol and tetrahydrofuran. Mobile phase B was comprised of 80:20 methanol and 50 mM acetate buffer (pH 5.5). Gradient program used was: 0 to 5% B in 5 min, 5 to 8% B in 5 min, 8 to 40% B in 10 min, 40 to 100% B in 24 min. Flow rate was 1.5 mL/min. Injection volume was 20 µL.

(11.0 to 28.5 µmol) in the red tissue of skipjack tuna. Mukundan and others (1979) also found that the levels of L-histidine in the white tissue of tuna (*Euthynnus affinis*) were more than twice that of the red tissue. Ito (1957) found 563 and 296 mg/100 g of histidine in the white and red tissue of mackerel (*Scomber japonicus*), respectively. Sakaguchi and others (1982), looking at free amino acids in yellowtail (*Seriola quinqueradiata*), obtained more than 1000 mg/100 g of histidine in the white tissue. They reported that red tissue had 28 mg/100 g of histidine, which was far less than the previously reported data (200 to 400 mg/100 g) for some dark-muscle fish.

Lukton and Olcott (1958) summarized that large variations in the imidazole content, including histidine, were observed in different fish of the same species. They also consistently observed that the white tissue contained more of the imidazole compounds than the red tissue. Table 1 data show large variations in histidine content from individual fish of the same species. Such variations are a result of several factors which include feeding, sex, stage of maturity (Sakaguchi and Kawai 1970; Fletcher and others 1995; Shiau and others 1997), and postharvest storage period (Shewan and Jones 1957; Bramstedt 1962). Overall, the data give an idea of the range of the free amino acids that can be found in these fish species. These findings support the concept of a 'range' rather than an 'average value,' as suggested by Stansby (1962), as a better way for describing the nutrients in fish.

Lysine levels in the red tissue of yellowfin tuna were greater, though not significantly, than in the white tissue. Mukundan and others (1979), however, found that the level of lysine in white tissue of tuna (*Euthynnus affinis*) was more than twice that in the red tissue. In mahi-mahi, lysine level in white tissue was greater ($p < 0.01$) than in red tissue, and also greater ($p < 0.05$) than that found in red snapper. However, there was no significant difference in the level of lysine in the white tissue of yellowfin tuna and mahi-mahi, nor was there any significant difference between the white tissue of yellowfin tuna and red snapper. Ito (1957) found similar levels of lysine, 22 mg/100 g, in both tissue types of mackerel (*Scomber japonicus*). Konosu and others (1974) obtained 54 mg/100 g of free lysine from jack mackerel-muscle extracts. But in common mackerel, they found 93 mg/100 g of lysine, while in flounder the level was 17 mg/100 g. Sakaguchi and others (1982) obtained 21 mg/100 g of lysine in white tissue of yellowtail (*Seriola quinqueradiata*) and 5 mg/100 g in red tissue. These studies showed considerable variations in the levels of free lysine found in dark-muscle species.

Ornithine levels in the white tissue of mahi-mahi were lower ($p < 0.01$) than in red tissue (Table 1). In yellowfin tuna, however, the levels in white tissue were similar to those in the red tissue. Ornithine levels in white tissue of mahi-mahi were significantly greater ($p < 0.002$) than in red snapper, but in tuna, the levels were not significantly greater than those in red snapper. It seems that ornithine levels in fish, except for the red tissue in dark-muscle fish, are generally low. For example, Konosu and others (1974) found 3 mg/100 g of ornithine in flounder, and similar values were found by Antoine and others (1999).

Glutamine levels in the red tissue of mahi-mahi were not significantly different from those in yellowfin tuna and red snapper. However, the levels in the red tissue of yellowfin tuna were greater ($p < 0.02$) than those in red snapper. The fact that we were able to quantify glutamine content in the red tissue, but not the white tissue, indicates a difference in the glutamine levels between red and white tissue. It may also mean that the higher levels of histidine in the white tissue make it difficult to resolve the lower level of glutamine. Our observation is different from that of Ito (1957) who claimed that histidine level was the only difference between red and white tissue of Scombridae species. Van der Boon and others (1992), working with goldfish, found that anoxia caused the levels of glutamine in the white tissue to decrease. As such, anoxia can be another factor, in addition to those listed above, that accounts for the low levels of glutamine in the white tissue.

The fact that glutamine was not detected in the white tissue of mahi-mahi and tuna was due to the very low levels present, which would have required excessive overloading of the column. In addition, the large preceding and adjacent histidine peak reduced the resolution of the low levels of glutamine. It was this problem of quantifying low levels of glutamine (< 1 mg/100 g) in the white tissue which prompted us to use acetate buffer as the mobile phase. This problem seems unique to the dark-muscle fish (Figure 3) because of the great difference in the levels of histidine and glutamine in these fish, and the fact that they elute adjacent to each other. Despite this uniqueness, it is possible to measure glutamine of slightly higher levels in the red tissue (Table 1) of these fish species. Glutamine content in red snapper was easily mea-

Table 1. Free amino acid content[a] (mg/100g) in mahi-mahi (*C. hippurus*), yellowfin tuna (*T. Albacares*), and American red snapper (*Lutjanus campechanus*).

Mahi-mahi

	Histidine	Glutamine	Lysine	Ornithine
White tissue				
1	310.9±12	n/d[b]	13.4±1.9	4.3±0.3
2	334.0±17.5	n/d	11.1±1.0	2.7±0.1
3	298.9±18.8	n/d	15.1±1.8	3.2±0.1
4	289.5 ±20.5	n/d	15.8±2.1	3.6±0.3
Red tissue				
1	223.9±11.3	2.0±0.1	11±1.5	5.5±0.5
2	252.7±18.1	1.4±0.1	10±0.8	3.5±0.3
3	232.3±17.6	1.7±0.1	11.8±0.7	4.3±0.2
4	184.6±18.3	1.9±0.1	10.5±0.8	4.2±0.3

Yellowfin tuna

	Histidine	Glutamine	Lysine	Ornithine
White tissue				
1	870.9±58.1	n/d	14.3±0.9	1.6±0.1
2	1032.4±36.3	n/d	7.0±0.5	1.0±0.03
3	1039.2±23.3	n/d	13.5±1.0	1.2 ±0.1
4	884.0±98.2	n/d	8.2±0.9	0.8±005
Red tissue				
1	475.1±13.1	2.1±0.2	30.9±1.6	5.1±0.4
2	385.4±22.9	1.8±0.1	8.6±0.3	1.9±0.2
3	307.3±38.3	n/d	16.7±0.5	2.3 ±0.2
4	347.1±45	3.5±0.2	37.5±3.1	7.2±0.4

American red snapper (*Lutjanus campechanus*).

	Histidine	Glutamine	Lysine	Ornithine
1	2.55±0.1	0.9±.05	3.9±0.16	0.6±0.03
2	2.31±0.2	0.9±.1	3.2±0.54	0.6±0.04
3	2.71±0.4	1.4±0.08	18.7±1.97	1.4±0.08
4	5.14±0.7	0.8±0.08	8.8±0.81	1.7±0.24
5	3.49±0.3	0.7±0.04	7.7±0.31	0.9± 0.04
6	3.77±0.3	1.3±0.1	2.4±0.19	0.5±0.02

[a]Mean ± standard deviation from 4 samples.[b] n/d - not detected

sured, even though its levels were lower than in the red tissue of dark-muscle fish. This was possible because of the resolution of the glutamine peak from the lower levels of the preceding adjacent histidine peak (Figure 3).

The higher levels of glutamine found in red tissue, compared to the white, may be indicative of the high physical activity of these migratory species. As Stansby (1962) explained, active movement and metabolic efficiency greatly contribute to variations in the levels of fish constituents.

Conclusion

THE RESULTS SHOW THAT HISTIDINE LEVELS IN MAHI-MAHI and tuna are significantly higher in the white tissue than in the red tissue. Thus, there is a greater propensity for higher levels of histamine formation in the white tissue of these species. Lysine levels in mahi-mahi are significantly higher in white tissue than in red tissue, while in tuna, the levels are higher in red tissue although not significantly. Because lysine levels in both tissue types are much lower than those of histidine, cadaverine levels in these species are generally much lower than those of histamine. Similarly, the low levels of glutamine and ornithine in mahi-mahi, tuna and American red snapper help explain in part why there are very low levels of putrescine formation in these species. Lysine levels in American red snapper are significantly higher than histidine, therefore cadaverine levels are expected to be greater than levels of histamine, and the low histidine levels are why American red snapper and similar species are not potential sources of histamine intoxication.

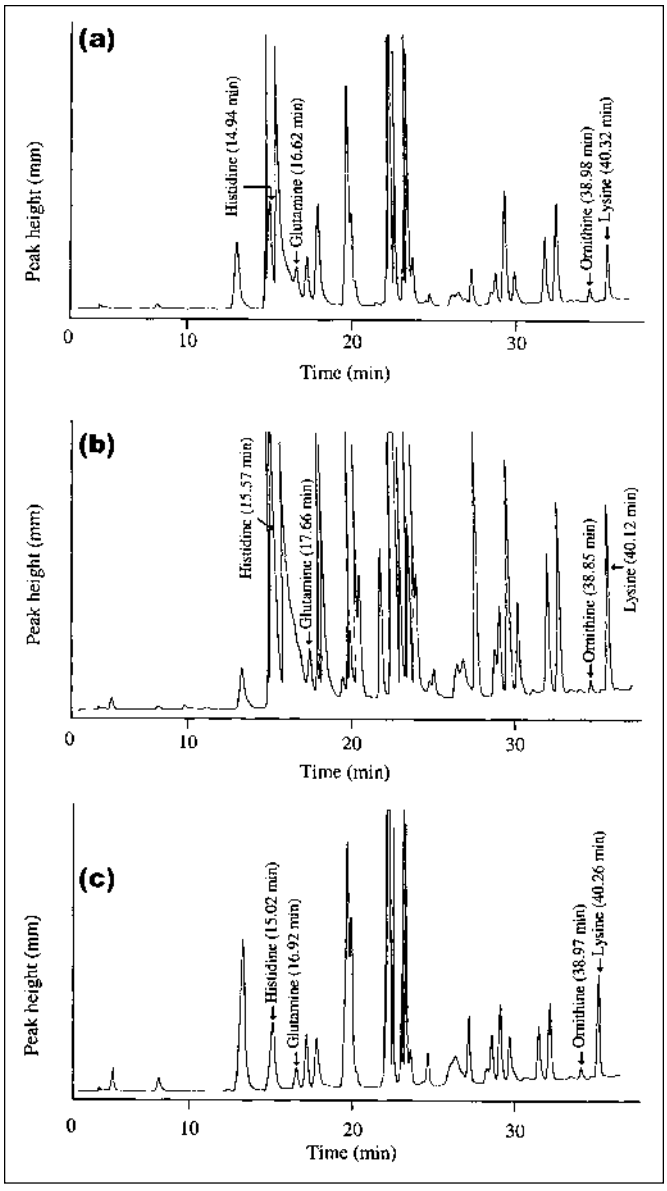

Figure 3—Chromatogram of free amino acids in red muscle of mahi-mahi (a), red muscle of yellowfin tuna (b), and American red snapper (c). Conditions are the same as described in Figure 2.

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