

# HPLC Method for Analysis of Free Amino Acids in Fish Using *o*-Phthaldialdehyde Precolumn Derivatization<sup>†</sup>

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Precolumn derivatization applying *o*-phthaldialdehyde (OPA) was used to analyze free lysine, histidine, and ornithine, precursors of the respective biogenic amines cadaverine, histamine, and putrescine, which are considered indicators of fish quality and safety. This method uses 75% methanol to eliminate the use of strong acids as the extraction solution. Each analysis took 35 min, was reproducible, and allowed separation of primary amino acids in fish samples. A binary solvent delivery system coupled with a fluorescence detector and an Ultrasphere ODS column were utilized for HPLC separation. Linearity of the calibration curves was very good ( $r^2 = 0.99$ ) for the amino acids of interest. Minimum concentrations of detection were 40 pmol/mL for histidine and lysine and 70 pmol/mL for ornithine. Average recoveries were 72% for lysine, 93% for histidine, and 98% for ornithine. This method used solvent gradient elution to study the levels of these analytes in mahi-mahi, bigeye tuna, and flounder.

**Keywords:** *Free amino acids; o-phthaldialdehyde (OPA); HPLC method; precolumn derivatization; fish*

## INTRODUCTION

Some free amino acids contribute to the rapid development of biogenic amines in fish (Lambert and Moss, 1973), which are considered indicators of fish spoilage (Mietz and Karmas, 1978; Finne, 1992). For example, histidine, lysine, and ornithine are the direct precursors of histamine, cadaverine, and putrescine, respectively (Sikorski et al., 1990). Histamine is shown to be the causative factor in scombroid poisoning (Arnold and Brown, 1978), and putrescine and cadaverine are said to potentiate scombroid intoxication (Taylor, 1988; Lutten et al., 1992). As such, understanding of the levels and distribution of free amino acids in fish may explain the formation of biogenic amines and their distribution during decomposition (Kirschbaum et al., 1994).

Methods for amino acid analysis usually require extensive preparatory work (Lindroth and Mopper, 1979) and often involve the use of picric or trichloroacetic acid (TCA) as extracting solvent (Konosu et al., 1974; Sakaguchi et al., 1982). However, Lukton and Olcott (1958) showed that 80% methanol is an effective extracting solvent for free amino acids. Joseph and Marsden (1986) also stated that methanol is an effective extracting solvent for free amino acids, but their HPLC method was not effective in separating some amino acids of interest in dark-muscle fish.

Umagat et al. (1982) explained that the pH of the mobile phase influences the sensitivity and separation of amino acids. Snyder et al. (1997) illustrated the

influence of pH on peak elution, retention time, and order of elution. In our initial work, changes in the pH, for example, affected the order and resolution of histidine and glutamine peaks, whereas ornithine and lysine consistently eluted in the same order but elution times shifted. Roth (1971) and Simons and Johnson (1977) showed that fluorescence intensity varied when the pH of the mobile phase was adjusted between 5 and 12. However, Chen et al. (1982) claimed that even though a high pH favors the *o*-phthaldialdehyde (OPA) fluorogenic reaction, it has not been established that a high pH is needed to observe fluorescence of the product. Jones et al. (1981) effectively made use of pH adjustment to improve resolution. Chan (1985) recommended that optimal resolution can be achieved by correct selection of mobile phase pH, organic modifier, and appropriate gradient profile. Stuart et al. (1979) showed that consistent buffer preparation is crucial to ensure reproducible separations. It is clear that pH is a powerful parameter for adjusting peak resolution and the order and time of elution.

Lindroth and Mopper (1979) explained that HPLC gradient elution of amino acids is preferred because of the wide range of polarities of their derivatives and the expediency of time. The type of elution used depends on the objective of the analysis. Hodgkin (1979) investigated the use of acetonitrile, methanol, and tetrahydrofuran (THF) (individually and in combination) in amino acid analysis and determined that the best combination of organic solvents is methanol and THF. He explained that THF decreases the retention time, thereby decreasing the analysis time and improving peak resolution. Stuart et al. (1979) found that acetonitrile/water and methanol/water were not effective in taurine elution. Jones et al. (1981) later confirmed Hodgkin's (1979) findings and pointed out that a starting solvent mixture containing THF effectively resolved most amino acids.

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Umagat et al. (1982) also reported the need for ternary solvent mixtures in obtaining very good amino acid peak resolutions. The authors using methanol/water experienced difficulties in obtaining complete separation of glutamine from histidine and broad glutamine peaks in fish samples. Inclusion of THF in the mobile phase did improve their separation and peak symmetry.

Roth (1971) first showed that OPA derivatives of most primary amino acids in sodium tetraborate buffer (pH 9.5) yield the strongest fluorescence in the presence of mercaptoethanol—a strong reducing agent. In the absence of the reducing reagent, OPA reacts with amino acids but results in nonfluorescent products. For fluorometry, OPA is more sensitive and easier to use than fluorescamine and ninhydrin (Roth, 1971). OPA is 1 order of magnitude more sensitive than fluorescamine and 10 times more sensitive than ninhydrin (Chan, 1985; Joseph and Marsden, 1986). However, sensitivity depends on the equipment used (Lee and Drescher, 1978), as well as the reducing reagent itself (Stuart and Hill, 1984). Use of a particular method for amino acid analysis depends on availability of instrumentation and purpose of analysis. The method chosen should be highly specific, show high sensitivity and chromatographic selectivity, be reproducible, and be simple to operate (Umagat et al., 1982).

Several researchers have shown that the type of reducing agent used affects the sensitivity and stability of OPA amino acid derivatives (Stuart and Hill, 1984). Simons and Johnson (1977) showed the differences in fluorescence intensity using mercaptoethanol and ethanethiol. The ethanethiol adduct consistently gave 6–7% more fluorescence than the mercaptoethanol adduct, and it is more responsive to changes in the solvent polarity. Stuart et al. (1979) explained that in the presence of OPA, ethanethiol forms more stable fluorescent derivatives with amino acids than mercaptoethanol. Hill et al. (1979) said that the OPA amino acid ethanethiol derivative had greater fluorescence stability because ethanethiol lacked the 2-hydroxy group present in mercaptoethanol. Nakamura and Tamura (1981) showed that the relative fluorescence intensities for mercaptopropionic acid were even greater than for ethanethiol and that the fluorogenic reaction gave intense fluorescence for aliphatic thiols. They claimed that the carboxylic group of mercaptopropionic acid has some stabilizing effect on the OPA adduct. Kucera and Umagat (1983) also showed that mercaptopropionic acid gave very good fluorescence intensity and stability. However, we used mercaptoethanol because it is the conventional reducing agent widely used by many researchers (Roth, 1971; Umagat et al., 1982; Chan, 1985), it adequately served our sensitivity needs, and the greater sensitivity obtained from mercaptopropionic acid and ethanethiol was not essential for our analyses, given the high levels of the free amino acids in fish. Furthermore, the OPA amino acid mercaptoethanol adduct is not responsive to changes in solvent polarity as is the ethanethiol adduct (Simons and Johnson, 1977), a possible factor in fish samples rich in basic amino acids. In addition, the use of phosphate buffer seems to augment the adduct stability during analysis. Stuart et al. (1979) pointed out the importance of maintaining an adequate concentration of  $\text{Na}^+/\text{PO}_4^-$  in the phosphate buffer to achieve adequate resolution of taurine, tyrosine,  $\beta$ -alanine, and arginine.

Because amino acids are of diverse polarities and functional groups, their analysis can be problematic (Umagat et al., 1982). In addition, fish samples contain other substances that can interfere with analysis. Stuart et al. (1979) showed that sodium ion concentration influenced the behavior and resolution of the OPA amino acid thiol derivatives. Hill et al. (1982) demonstrated that a change in buffer concentration leads to a change in retention time. An increase in the buffer concentration leads to an increase in retention time, and a sufficiently high  $\text{Na}^+/\text{PO}_4^-$  concentration enables a stable retention time. Snyder et al. (1997) recommended that, ideally, samples should be prepared in mobile phase to maintain good peak shapes. Therefore, the objective of this study was to develop a method that would enable the timely and reproducible analysis of free amino acids in complex fish matrixes.

## MATERIALS AND METHODS

**Reagents.** Methanol (HPLC grade), sodium phosphate monobasic, sodium hydroxide, and sodium tetraborate were purchased from Fisher Scientific (Fair Lawn, NJ). THF (HPLC grade, stored under inert gas) and 2-mercaptoethanol were purchased from Mallinckrodt (Mallinckrodt Speciality Chemicals Co., Paris, KY). Amino acid standards (histidine, ornithine, and lysine) and OPA were bought from Sigma (St. Louis, MO), and absolute alcohol was from Aaper Alcohol (Aaper Alcohol and Chemical Co., Shelbyville, KY). Distilled deionized water was obtained from a Photronix reagent grade water system (Photronix Corp., Medway, MA).

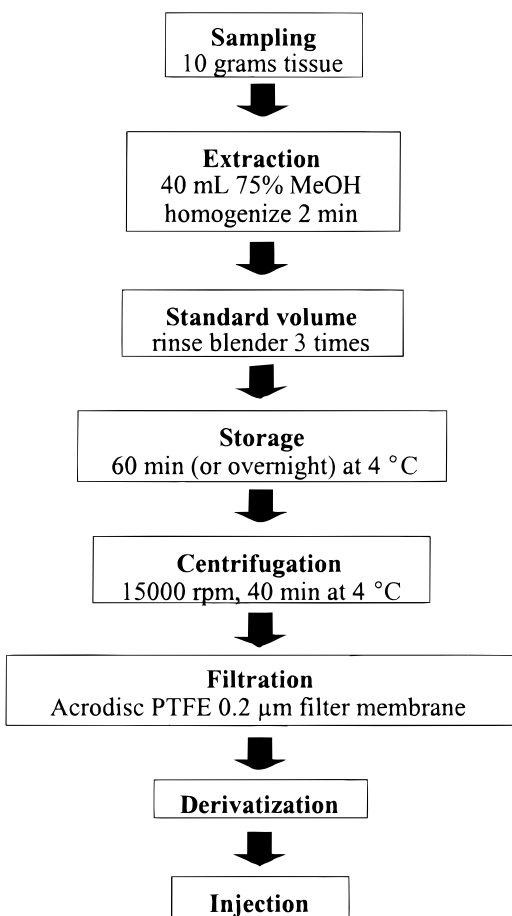
**Buffer Preparation.** Mobile phase A was made of 0.05 M sodium phosphate buffer (pH 5.5), methanol, and THF (80:19:1), respectively. Mobile phase B was made of 80% methanol and 20% of the 0.05 M  $\text{NaH}_2\text{PO}_4$  buffer. The pH of the phosphate buffer was adjusted to 5.5 using 1 N NaOH. The mobile phases were filtered using Supor(R)-200 47 mm, 0.2  $\mu\text{m}$ , filter membranes (Gelman Sciences, Ann Arbor, MI) and degassed by sparging with helium for 5 min.

**OPA Thiol Reagent Preparation.** OPA thiol (sometimes called OPT) reagent was prepared 24 h before use by dissolving 27 mg of *o*-phthalaldehyde in 500  $\mu\text{L}$  of absolute alcohol. Then 5 mL of 0.1 M sodium tetraborate ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) (pH 9.5) was added, followed by 50  $\mu\text{L}$  of mercaptoethanol. The mixture was thoroughly mixed and stored in a tightly closed container in the dark. The reagent can be kept for several days with periodic additions of 20  $\mu\text{L}$  of mercaptoethanol to maintain the yield of OPT amino acid derivatives (Joseph and Marsden, 1986; Miles and Leong, 1992).

**Amino Acid Standards.** A stock solution was prepared by dissolving the equivalent of 2500 nmol of each amino acid in 0.05 M  $\text{NaH}_2\text{PO}_4$  buffer (pH 5.5). This stock solution was then used to prepare a working solution from which a calibration curve was prepared.

**Samples.** Four fresh fillets (3.5–5 lb) each of mahi-mahi (*Coryphaena hippurus*) and bigeye tuna (*Parathunnas mebachii*) and five fillets (12–16 oz) of flounder (common flounder) were purchased from Northwest Seafood Inc. (Gainesville, FL). The fillets were packed in ice and taken to the laboratory. In the case of the dark-meat fish, a sharp knife was used to carefully separate the dark and white tissues from the fillets. Each was then cut into several portions, chopped, and homogenized. Four replicate samples of each fillet were taken for analysis. When necessary, filtered sample extracts were stored in a freezer at  $-20^\circ\text{C}$ .

**Sample Preparation.** Ten grams of fish and 40 mL of extracting solvent (75% methanol in distilled deionized water) were added to a Mason jar and blended for 2 min at the liquefy setting of a Hamilton Beach Blend Master 14 blender (Hamilton/Proctor-Silex, Inc., Glen Allen, VA). The homogenate was then transferred to a 100 mL volumetric flask, and the jar was rinsed three times with 15 mL of the extracting solvent. The washings were added to the flask, which was then brought



**Figure 1.** Sample preparation flowchart for HPLC analysis.

up to volume and stored for 60 min (or overnight) at 4 °C. The contents of the flask were transferred to a 50 mL centrifuge tube and centrifuged at 15000 rpm for 40 min at 4 °C using an IEC refrigerated centrifuge model B20A (International Equipment Co., Needham Heights, MA). The supernatant was filtered using an Acrodisc PTFE 0.2  $\mu\text{m}$  filter membrane (Gelman Sciences), diluted as required, and treated as per standard solution (Figure 1).

**Instrumentation.** This method is based on that of Joseph and Marsden (1986) with several modifications. The HPLC column, an Ultrasphere ODS 5  $\mu\text{m}$  particle size, 4.6 mm  $\times$  25 cm (Beckman Instruments, Inc., Fullerton, CA), was used without a guard column. Gradient elution was generated using a Bio-Rad solvent delivery system, model 2800 (Bio-Rad Laboratories, Hercules, CA) equipped with dual pumps and a Rheodyne injection valve (Model 7125-081) containing a 20  $\mu\text{L}$  sample loop. Bio-Rad Value Chrom software version 4, 1988–1994, was used for controlling the gradients and flow rate of the two degassed mobile phases. A Spectroflow 980 programmable fluorescence detector (ABI Analytical, Inc., Kratos Division, Ramsey, NJ) fitted with a 5  $\mu\text{L}$  flow cell was used with an excitation monochromator setting at 330 nm and an emission cutoff filter of 418 nm. Other detector settings are 0.1 PMT signal, 10% zero offset, 1.0 s response (rise time units), and  $10^{-3}$  A full-scale output range. A Spectra-Physics SP 4092 integrator (San Jose, CA) was used to generate chromatograms.

**Amino Acid Derivatization.** To 100  $\mu\text{L}$  of amino acid standard or diluted sample supernatant was added 400  $\mu\text{L}$  of OPT, followed by thorough mixing using a vortex. Exactly 2 min after mixing, the sample was manually injected onto the column of the HPLC system and the gradient run started. It was important that the time between mixing and injecting into the HPLC be consistent (Joseph and Marsden, 1986; Lindroth and Mopper, 1979) because of the limited stability of the OPT amino acid derivatives. Sodium phosphate buffer was used as

a blank, and the concentration of each amino acid of interest was measured using an external calibration curve.

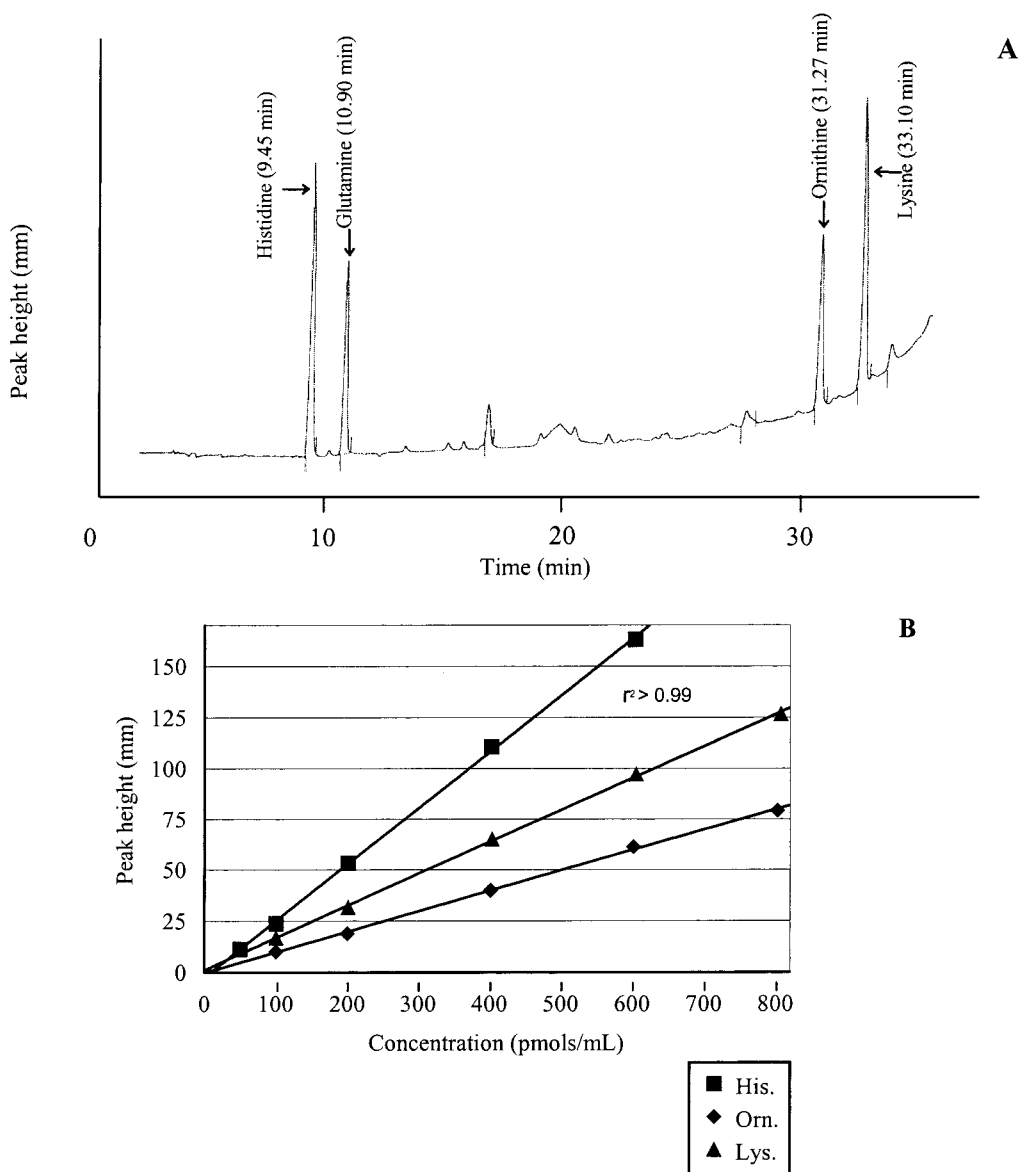
Data analysis was carried out using the SAS (SAS Institute Inc., Raleigh, NC) general linear models procedure.

## RESULTS

The lowest detected levels (2 time the noise level) of the amino acid standards of interest were 40 pmol/mL for histidine and lysine and 70 pmol/mL for ornithine. The interassay (day-to-day) of the amino acid standards was reproducible with a coefficient of variation (CV) of 4.1–8.7%, and the intra-assay (within-day) had a CV of 0.8–3.1%.

A chromatogram of the amino acid standards of interest is shown in Figure 2A. Figure 2B shows the linearity obtained for the calibration curves of these three amino acids. Calibration concentrations ranged from 50 to 600 pmol/mL for histidine and from 100 to 800 pmol/mL for lysine and ornithine. Attempts to use an internal standard (norleucine,  $\epsilon$ -aminocaproic acid, and glycyltryptophan) proved to be frustrating because they coeluted with other peaks.

Three different spike levels were used for the recovery study (Table 1). Volumes of standard solutions, ranging from 0.5 to 2 mL, were added to 10 g of minced fish, and the mixture was homogenized, extracted, and brought to a standard volume (100 mL). Recovery studies using the mahi-mahi species served as a good model because of its complex matrix of high lipid levels and the widely different levels and large number of free amino acids. All standard solutions and sample dilutions were prepared using 0.05 M  $\text{NaH}_2\text{PO}_4$  (pH 5.5). The Association of Biochemical Resources Facility (ABRF) considers that a 90–95% recovery, and 70% in some cases, is expected for OPA precolumn derivatization using RP-HPLC, depending on the amount analyzed, the instrument, and the operator (West et al., 1996). The recovery levels obtained for lysine may be due to the instability of the two forms of its OPA derivatives (Simons and Johnson, 1978). Hill et al. (1982) and Simons and Johnson (1977) discussed the possible internal quenching of the fluorescence of each lysine–OPA–mercaptoethanol structure. Turnell and Cooper (1982) reported on the half-lives of several OPA–amino acid–mercaptoethanol derivatives. They showed that lysine has a half-life of 31.4 min, which is less than its retention time. Umagat et al. (1982) claimed that at pH < 6, detection of lysine is problematic. They reported improvement in the stability of lysine–OPA–derivatives when sodium dodecyl sulfate (SDS) solution (1% v/v) was added to the amino acid samples. However, we did not use SDS in our experiment, which adds to the number of steps in the process of a simplified method. We observed that at pH 6.2, lower sensitivity and poor peak symmetry were obtained for the amino acids of interest, particularly lysine. At pH 5.5 and room temperature (21 °C) we obtained the best peak symmetry and sensitivity for the analytes of interest in the fish samples. At higher temperatures (30, 35, and 40 °C), we observed reduced sensitivity for the amino acids of interest. Cronin et al. (1979) reported a similar decrease in fluorescence for amino acids with one hydrogen atom attached to the amino  $\alpha$ -carbon. Roth (1970) showed that for lysine fluorescent increases from pH 5 to pH 6, is maximum at pH 6–7, and then decreases at pH > 7, and different amino acids have different pH values at which fluorescence is maximum. In foods, lysine, which



**Figure 2.** Chromatogram of standard amino acids using binary mobile phases (A). Mobile phase A was 80:19:1 of 50 mM sodium phosphate buffer (pH 5.5), methanol, and tetrahydrofuran. Mobile phase B was composed of 80:20 of methanol and 50 mM sodium phosphate buffer (pH 5.5). Gradient program used was as follows: 0–15% B in 5 min, 15–50% B in 5 min, and 50–100% B in 24 min. Flow rate was 1.5 mL/min, and injection volume was 20  $\mu$ L. Calibration curves for histidine, lysine, and ornithine are also shown (B).

**Table 1. Percent Recovery of Amino Acid Standards from Fish Samples**

amino acid	spike amount, nmol/g (mg/100 g)	% recovery <sup>a</sup>
histidine	62.5 ( $9.7 \times 10^{-4}$ )	92.8 $\pm$ 7.2
	125.0 ( $1.94 \times 10^{-3}$ )	94.4 $\pm$ 8.8
	250.0 ( $3.88 \times 10^{-3}$ )	92.6 $\pm$ 11.3
ornithine	25.0 ( $4.22 \times 10^{-4}$ )	95.2 $\pm$ 4.8
	50.0 ( $8.43 \times 10^{-4}$ )	95.7 $\pm$ 8.6
	100.0 ( $1.69 \times 10^{-3}$ )	103.5 $\pm$ 2.5
lysine	25.0 ( $3.66 \times 10^{-4}$ )	65.6 $\pm$ 3.0
	50.0 ( $7.3 \times 10^{-4}$ )	70.3 $\pm$ 2.1
	100.0 ( $1.46 \times 10^{-3}$ )	82.3 $\pm$ 4.7

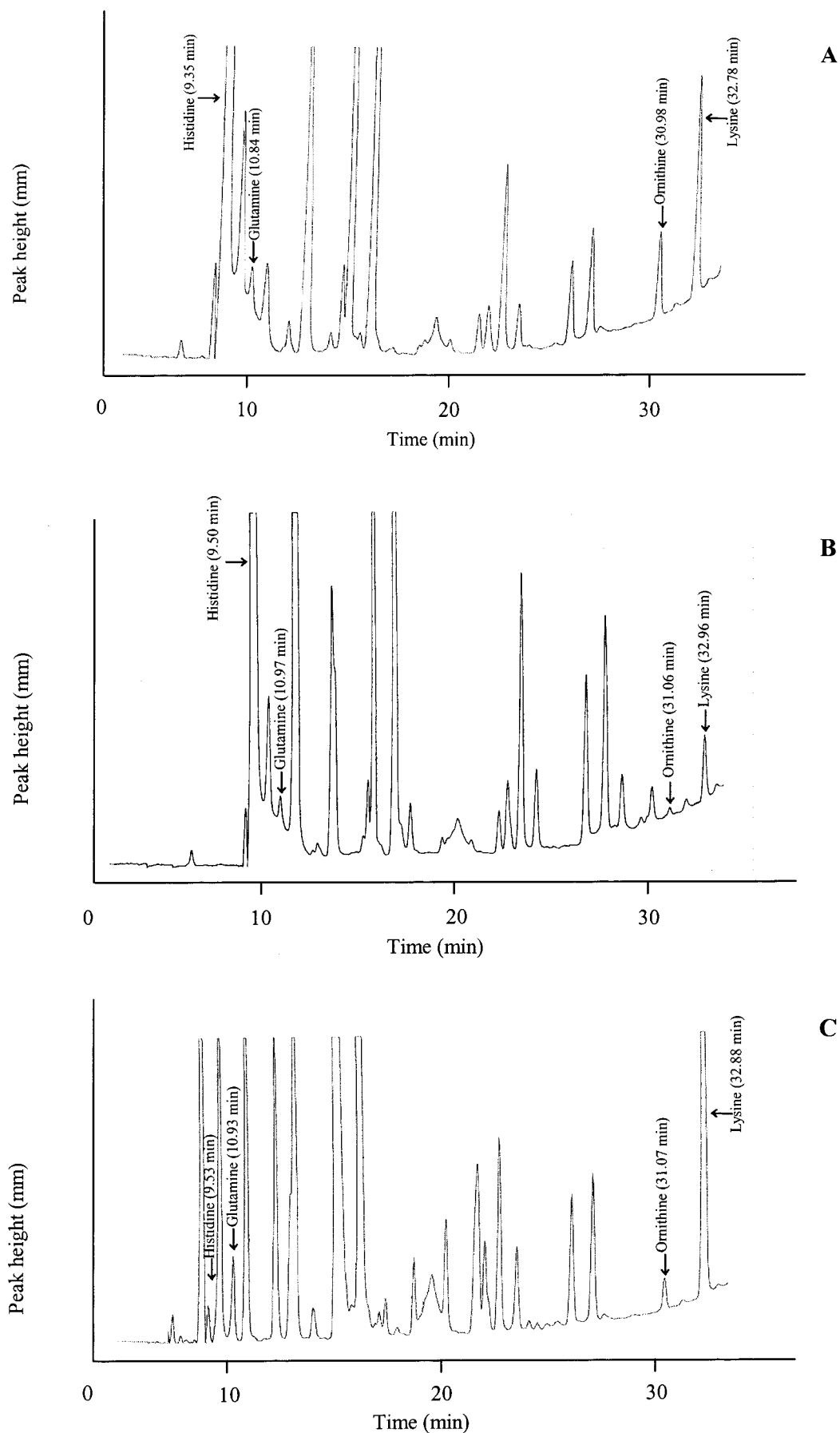
<sup>a</sup> Mean  $\pm$  standard deviation from four samples taken from each fish, each sample analyzed in duplicate.

contains an  $\epsilon$ -amino group, reacts with reducing sugars and carbonyl groups produced from lipid oxidation (Belitz and Grosch, 1987; Damodaran, 1996). The *N*-deoxyketosyl-amino acid derivatives so formed are the initial step of Maillard reactions which form at room

temperature (Kirk, 1979). Due to its reactivity, lysine is lost during processing of foods (Belitz and Grosch, 1987).

Three different fish species were analyzed (Figure 3) to demonstrate the feasibility of using this method for analysis of fish. The levels of the three amino acids found in the fish samples are shown in Table 2. In dark-muscle fish, mahi-mahi and tuna, significantly higher ( $P < 0.05$ ) levels of histidine were obtained from white tissue than from red tissue. The histidine in the white tissue was significantly greater ( $P < 0.05$ ) in tuna than in mahi-mahi, whereas the difference in the red tissue for both had a value of  $P = 0.058$ . Flounder, a white-muscle fish, had very low levels of histidine.

Lysine levels in mahi-mahi were significantly greater ( $P < 0.05$ ) in the white tissue than in the red, but in tuna, the lysine level was higher in the red tissue than the white tissue, although the difference was not significant. Lysine levels were significantly higher ( $P < 0.05$ ) in the white and red tissues of mahi-mahi than



**Figure 3.** Chromatogram of free amino acids in (A) mahi-mahi, (B) bigeye tuna, and (C) flounder samples. Conditions are the same as described in Figure 2A.

**Table 2. Free Amino Acid Content<sup>a</sup> (Milligrams per 100 g) in Mahi-mahi (*C. hippurus*), Bigeye Tuna (*P. mebachii*), and Flounder**

	mahi-mahi			bigeye tuna		flounder		
	histidine	lysine	ornithine	histidine	lysine	histidine	lysine	ornithine
	White Tissue							
1	541.4 ± 23.9	35.8 ± 1.9	25.9 ± 1.8	595.5 ± 59.3	5.9 ± 0.3	1.0 ± 0.0	23.6 ± 2.1	2.2 ± 0.1
2	414.9 ± 25.4	71.6 ± 3.8	48.7 ± 4.1	708.0 ± 44.1	3.5 ± 0.2	0.9 ± 0.3	30.6 ± 1.5	4.2 ± 1.8
3	251.8 ± 12.7	65.0 ± 6.6	13.9 ± 1.1	554.7 ± 131.2	12.9 ± 2.9	1.3 ± 0.2	15.0 ± 0.3	1.4 ± 0.4
4	498.5 ± 76.7	39.1 ± 2.4	10.8 ± 1.4	481.9 ± 39.9	9.0 ± 0.4	1.5 ± 0.1	9.3 ± 0.6	0.7 ± 0.1
						1.5 ± 0.1	7.4 ± 0.3	0.6 ± 0.1
	Red Tissue							
1	367.8 ± 25.7	23.9 ± 0.9	16.7 ± 0.7	478.1 ± 42.6	12.0 ± 0.9			
2	213.8 ± 12.0	36.9 ± 7.7	24.9 ± 1.2	490.1 ± 41.0	8.6 ± 0.2			
3	182.9 ± 18.4	28.9 ± 1.4	9.5 ± 2.1	220.9 ± 27.2	12.3 ± 1.1			
4	278.5 ± 14.9	18.1 ± 1.2	8.5 ± 1.6	398.0 ± 35.1	13.5 ± 1.3			

<sup>a</sup> Mean ± standard deviation from four samples taken from each fish, each sample analyzed in duplicate.

in the respective tissues of bigeye tuna. In mahi-mahi, the lysine levels were significantly higher in the white tissue than in flounder. The lysine level in flounder was higher than that in white tissue of bigeye tuna, although not significantly. Lysine levels in flounder, relative to the histidine, were very high.

Ornithine levels in the white tissue of mahi-mahi were not significantly higher than those in the red tissue. In tuna, the ornithine levels were too low to be measured without excessively loading the column. In flounder, the ornithine levels were significantly lower than those in the white tissue of mahi-mahi.

## DISCUSSION

Hibiki and Simidu (1959) obtained 1010 mg/100 g of histidine in bigeye tuna. It was not clear if they measured combined or free histidine in this dark-muscle fish. Lukton and Olcott (1958) using ninhydrin as well as the Pauly diazotized sulfanilic acid reaction obtained 481.1 mg/100 g of free histidine in white tissue of bigeye tuna. They used 1% picric acid as the extracting solution but also noted that the use of an 80% methanol extracting solution gave similar quantitative results. The above values demonstrated that considerable variations in amino acid levels can be obtained from the same fish species. Lukton and Olcott (1958) concluded that large variations in the imidazole content, including histidine, were observed with different fish of the same species but consistently observed that white tissue contained more imidazole compounds than red tissue. Our data also showed large variations from fish to fish of the same species. Such variations are possibly a result of several factors including differences in feeding, season, sex, and stage of maturity (Sakaguchi and Kawai, 1970; Fletcher et al., 1995). Overall, the data give an idea of the levels of the free amino acids that can be found in the species.

Yoshinaga and Frank (1982) reported that histidine is uniformly distributed in fresh skipjack tuna (*Katsuwonus pelamis*) at concentrations of 564–611 mg/100 g. However, Abe (1983) obtained free L-histidine levels of 1389 mg/100 g in white tissue of skipjack tuna and 268.5 mg/100 g in red tissue. They used 1% picric acid extraction solution and found that the overall L-histidine content in the white tissue of dark-muscle fish ranged from 15.8 to 92.8 mmol/g. Our data showed higher levels of histidine in the white tissue of tuna. Mukundan et al. (1979), using the standard microbiological assay, found that the levels of L-histidine and lysine in the white tissue of tuna (*Euthynnus affinis*) were twice as

great as that of red tissue. We found that histidine and lysine levels in the white tissue of mahi-mahi were consistently greater than those in the red tissue, but this was not so for tuna. Ito (1957) used the microbiological assay and an aqueous alcoholic extract and found 563 and 296 mg/100 g of histidine in white and red tissues, respectively, for mackerel (*Scomber japonicus*). However, similar levels of lysine (22 mg/100 g) were found in both tissue types. Konosu et al. (1974) used TCA extraction solution and obtained 289 and 54 mg/100 g of free histidine and lysine, respectively, from jack mackerel muscle extracts. However, the common mackerel had 676 and 93 mg/100 g of histidine and lysine, respectively, and 5 mg/100 g of ornithine.

Sakaguchi et al. (1982), looking at free amino acids in yellowtail tuna (*Seriola quinqueradiata*), obtained >1000 mg/100 g of histidine and ~21 mg/100 g of lysine in white tissues. They claimed red tissue had 28 mg/100 g of histidine, which was far less than previously reported for some of the dark-muscle fish (200–400 mg/100 g). Their reported lysine level was <5 mg/100 g in red tissue.

Takagi et al. (1969) demonstrated that histamine formation was greater in white tissue, where histidine levels were higher than in red tissue. They concluded that the degree of histamine formation tends to be governed by the histidine content but is not proportional to the loss of histidine. Fletcher et al. (1995) also found large variations in histidine and histamine levels in fish caught simultaneously at the same place and subjected to the same storage regime.

The level of histidine is very low in flounder because it is not a dark-muscle fish. However, relative to histidine, the level of lysine is very high. Konosu et al. (1974) found 1 mg/100 g histidine, 17 mg/100 g lysine, and 3 mg/100 g ornithine in flounder, which are similar to our data.

## CONCLUSIONS

The method reported in this paper is simple in its preparatory and determinative stages, has a relatively short analysis time, and is reproducible. The recovery levels are satisfactory for the amino acids studied and thus enable the qualitative and quantitative determination of free amino acids in complex fish matrixes. The histidine levels in mahi-mahi and tuna were higher in white tissue than in red tissue. In mahi-mahi, lysine levels were higher in the white tissue than in red tissue,

but the pattern was not clear for tuna. Flounder had very low levels of histidine and ornithine and high levels of lysine.

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