Fishery Technology
2010, Vol. 47(2) pp: 161 - 166

Comparative Evaluation of Non-Protein Nitrogenous Compounds in Fishes of Fresh and Brackish Water Environments

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Quantitative comparison of the non-protein nitrogen (NPN) fractions *viz.*, alpha-amino nitrogen, peptides, trimethylamineoxide (TMAO) and free amino acids from muscles of reared fishes of fresh and brackish water environments is attempted. Compared to fishes from the brackish water environments, those from the fresh water showed higher NPN content. Among the fresh water fishes, *Labeo rohita* accounted for maximum NPN content *viz.*, 17.11% of the total nitrogen. Minimum values of peptides were recorded for *Hypophthalmichthys molitrix* and maximum for *Labeo rohita*. TMAO could not be detected in any fresh water fish. Maximum values for alpha-amino nitrogen were obtained for *Liza macrolepis* and *Labeo rohita*. *Etroplus suratensis*, collected from brackish water and fresh water environments showed significantly different peptide and TMAO values ($p<0.05$). In the case of free amino acids in the reared fishes, taurine, histidine, lysine and glycine were predominant. The results indicate that NPN fractions, TMAO, peptides and free amino acids show variations among species based on their habitats.

Key words: Alpha-amino nitrogen, glycine, non-protein nitrogenous compounds, taurine, trimethylamine oxide

The deterioration of fish is related to changes of the macro-components (fats and proteins) as well as the micro-components (NPN compounds and enzymes). Volatile compounds derived from nitrogenous compounds and off flavour compounds from fat oxidation contribute to quality deterioration in fish. Non-protein nitrogenous (NPN) compounds are used as quality parameters for fish as they equip the fish with essential sensory characterestics such as smell and taste (Iida *et al.*, 1992). These compounds are even responsible for deterioration of fresh sea foods, as they serve as substrate for typical spoilage organisms (Fraser & Sumar, 1998). The microbiological spoilage of fish is determined by extrinsic and intrinsic parameters and among intrinsic factors, NPN plays a major role (Gram & Huss, 1996). NPN compounds include different components like free amino acids, peptides, nucleotides, guanidine compounds, quaternary ammonium compounds and urea (Finne, 1992). Individual components of NPN form their own pools in the body of animals, whose amount and nature greatly depend on species, body size, season, environmental factors, nutritional status and the type of muscle. These differences in both the amount and nature of the muscle components are responsible for the difference in flavour in fresh fish as well as in the subsequent spoilage patterns (Kawai, 1996).

NPN varies from 9–18% of the total nitrogen in teleosts, 33-38% in elasmobranchs and 20-25% in crustaceans and cephalopods (Belitz & Grosch, 1987). Even though attempts have been made to classify the NPN compounds of marine fish in India (Velankar & Govindan, 1958; Joshi *et al.*, 1953; Suseela *et al.*, 1999), no efforts have been made so far to compare the profiles of these compounds in fishes of fresh and brackish water environments. The present work was carried out to quantify the non-protein nitrogenous

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compounds *viz.*, alpha amino nitrogen (AAN), peptides, trimethylamine oxide (TMAO) and free amino acids from fishes of fresh and brackish water environments. A database on the quantity of non-protein nitrogenous compounds in fishes at fresh condition is lacking. Knowledge on these lines is also beneficial to isolate many of these compounds for nutraceutical purposes. Non-protein nitrogenous compounds bear a rich flavour and if isolated can be applied for bland sea foods. Quantification of the non-protein nitrogenous compounds from fishes of fresh and brackish water environments aimed in the present investigation can help to build up a database in this line.

Materials and Methods

All the chemicals and reagents used were of analytical grade and were procured from Sigma (MO, USA) and Merck (India). Fresh water fishes, *Catla catla, Labeo rohita, Hypophthalmichthys molitrix* and *Etroplus suratensis* were collected from the farms of Pampa, Alappuzha, and Kumarakom, Kerala by cast netting during the months of December and January. Brackish water fishes, *Liza macrolepis* and *Etroplus suratensis* were procured from Matsyafed farm, Malippuram, Kerala during the same period. The fishes selected were of commercial importance as they are being widely used for fish culture due to their fast growth rate and easy adaptability to environment. The common names of these fishes, their family names and average length and weight of six fishes collected in each category are shown in Table 1. All the fishes were caught in live condition and killed by hitting on the head. The muscle extracts of similar sized fishes were prepared in pre-rigor condition, in 10% trichloroacetic acid (TCA) solution at the collection point itself immediately after catch.

Total nitrogen content of the fish muscle was estimated as per AOAC, (2000). The muscle samples were digested with sulphuric acid and the clear samples were distilled in a Kjeldahl's distillation unit. The amount of ammonia liberated was determined by titration with 0.01N sulphuric acid. The crude protein was derived from the total nitrogen content. NPN content was determined by estimating nitrogen in the TCA extract by using Kjeldahl distillation method (Alongo *et al.*, 1994). The extract was prepared from 10g of fish meat after extracting with 10% TCA and making up the volume to 100 ml using 10% TCA at room temperature. Amount of peptides was estimated using the Biuret method (Gornall *et al.*, 1949) and alpha amino nitrogen by the EBC (European Brewing Convention) Ninhydrin method (EBC, 1975). Briefly, ninhydrin reagent was added to a diluted portion of the TCA extract and heated for 16 min in a stoppered test tube. The test tube was cooled and the solution was made up to a known volume. Absorbance of the solution was read at 570 nm in a UV visible spectrophotometer. The content of trimethylamine oxide – nitrogen (TMAO-N) was determined by the method of Bystedt *et al.* (1959) using titanous chloride as the reducing agent.

The content of free amino acids in the TCA extracts was estimated (Alongo *et al.*, 1994) using a Shimadzu HPLC with an LC 10 AS binary pump, column oven and a post-column derivitization system. The column used was Shimpak Na type 4.6 x 250 mm column packed with strongly acidic cation exchange resin *i.e.* styrene divinyl benzene co-polymer with sulphinic group. A 10% TCA extract of the minced fish muscle was prepared and the TCA was removed by flash evaporation (Fresno *et al.*, 1997). The resulting residue was reconstituted in 0.05M HCl and filtered through Millipore 0.45 μm filter before injecting to the Shimadzu HPLC, 10AS model.

Statistical analysis was done using SPSS version 12.0. Data were subjected to one-way ANOVA by setting level of significance at 5%. Post-hoc test was done using Duncan's method.

Results and Discussion

The total nitrogen and total NPN content, the contents of the major NPN compounds *viz.*, peptides, TMAO and AAN in the fresh samples of reared fishes analysed from fresh and brackish waters are given in Table 2. Among the fresh water fishes analyzed, *Labeo rohita* showed maximum NPN content *i.e.*17.1% of the total nitrogen, which was significantly higher compared to that of *Catla catla* and *Hypophthalmichthys molitrix* ($p<0.05$). *Etroplus suratensis* collected from both fresh water and brackish water environments also showed significantly different NPN contents ($p<0.05$). The lowest value was obtained in the case of *Liza macrolepis*, where NPN was only 8.26%. In the present study, fresh water fishes showed higher NPN content than those from the brackish waters. Non-protein nitrogen content has direct correlation for the flavour of fish. The different fishes might have exhibited different NPN content, owing to the different nitrogenous components possessed by the muscle tissues.

Among the major NPN compounds studied, peptides formed the dominating class. There was significant difference in peptide contents between fresh water fishes and the brackish water fishes as shown in Table 2 ($p<0.05$). Their contents ranged from 345.21 mg/100g in *Hypophthalmichthys molitrix* to 612.02 mg/100g in *Labeo rohita* both of which are fresh water species. *Etroplus suratensis* from the fresh waters contained significantly higher peptide contents than their brackish water counterparts ($p<0.05$). Suseela *et al.* (1999) extensively studied the distribution of NPN compounds in various commercially important fishes of Indian waters and showed that peptide contents varied from 300 mg/100g in fresh and

Table 1. Fishes collected from different environments

Sl. No.	Common name	Scientific name	Family	Average length*(cm)	Weight* (g)
1.	Catla	*Catla catla*	Cyprinidae	24.5 ± 1.8	211 ± 1.9
2.	Rohu	*Labeo rohita*	Cyprinidae	21.7 ± 0.7	198 ± 1.8
3.	Silver carp	*Hypophthalmichthys molitrix*	Cyprinidae	19.8 ± 1.4	175 ± 1.5
4.	Pearl spot	*Etroplus suratensis*	Cichlidae	15.7 ± 1.6	87 ± 1.5
5.	Pearl spot	*Etroplus suratensis*	Cichlidae	13.2 ± 0.8	68 ± 1.4
6.	Mullet	*Liza macrolepis*	Mugilidae	19.0 ± 1.5	169 ± 1.6

*Represents the average length/weight ± SD for n=6 species. Fishes 1-4 were fresh water species and 5,6 were brackish water species. All fishes were collected during the post monsoon season.

Table 2. Total nitrogen and non-protein nitrogenous compounds in fishes from different environments***

Species	Total N (g/100g)	Total NPN (g/100g)	Peptides (mg/100g)	TMAO (mg N/100g)	AAN (mg N/100g)
*Catla catla**	2.80 ± 0.05^{b}	0.40 ± 0.06^{c}	421.21 ± 2.33^{c}	ND	26.25 ± 0.79^{c}
*Labeo rohita**	2.63 ± 0.06^{a}	0.45 ± 0.03^{d}	612.17 ± 3.16^{f}	ND	28.52 ± 0.47^{d}
*Hypophthalmichthys molitrix**	2.85 ± 0.03^{c}	0.39 ± 0.05^{c}	345.52 ± 3.65^{a}	ND	24.01 ± 0.25^{a}
*Etroplus suratensis**	3.03 ± 0.05^{d}	0.25 ± 0.03^{a}	463.27 ± 4.24^{d}	11.85 ± 0.22^{a}	24.20 ± 1.06^{a,b}
*Etroplus suratensis***	3.10 ± 0.10e	0.29 ± 0.06^{b}	372.34 ± 2.52^{b}	48.04 ± 1.05^{c}	25.17 ± 0.39^{b,c}
*Liza macrolepis***	3.02 ± 0.08^{d}	0.25 ± 0.03^{a}	530.09 ± 5.30^{e}	21.60 ± 0.89^{b}	34.22 ± 0.47^{e}

* Fishes from fresh waters

** Fishes from brackish waters

*** Values are mean of six fishes of the same species ± SD; Values with different superscripts in the same column indicate significant difference ($p<0.05$)

ND : Not Detected

Table 3. Major free amino acids in fishes from different environments

Species	Free amino acids (mg/100g)***					
	Glycine	Taurine	Histidine	Lysine	Alanine	Glutamic acid
*Catla catla**	32.23 ± 2.35^{a}	42.66 ± 2.08^{a}	110.33 ± 2.08^{c}	46.35 ± 1.73^{a}	ND	ND
*Labeo rohita**	39.62 ± 1.62^{b}	70.25 ± 2.34^{b}	140.84 ± 1.28^{e}	102.84 ± 1.15^{c}	ND	ND
*Hypophthalmichthys molitrix**	120.41 ± 1.48^{c}	152.34 ± 1.47^{c}	132.52 ± 1.40^{d}	90.24 ± 1.92^{b}	ND	ND
*Etroplus suratensis**	360.27 ± 2.57^{e}	205.43 ± 2.18^{d}	55.33 ± 0.57^{a,b}	ND	280.64 ± 2.71^{b}	204.16 ± 2.83^{b}
*Etroplus suratensis***	160.33 ± 1.82^{d}	310.87 ± 2.67^{f}	58.36 ± 2.81^{b}	ND	55.23 ± 1.28^{a}	190.57 ± 2.14^{a}
*Liza macrolepis***	120.72 ± 2.71^{c}	290.26 ± 2.11^{e}	55.29 ± 2.17^{a}	115.72 ± 1.45^{d}	ND	ND

* Fishes from fresh waters
** Fishes from brackish waters
***Values are mean of six fishes of the same species ± SD; Values with different superscripts in each column indicate significant difference ($p<0.05$).
ND : Not Detected

brackish water fishes to 1775 mg/100g in marine fishes. The values obtained in the present work also correlate well with the already reported values.

The presence of TMAO could not be detected in any of the fresh water fishes except *Etroplus suratensis* (Table 2). In the brackish water fishes, TMAO was 21.60 mg N/100g in *Liza macrolepis* and 48.04 mg N/100g in *Etroplus suratensis*. *Etroplus suratensis* from the brackish waters contained significantly higher fraction of TMAO than those from the fresh waters ($p<0.05$). The comparatively higher contents of TMAO among the brackish water fishes could be attributed to the role it plays in osmoregulation (Suseela *et al.*, 1999). TMAO values in the range of 9-68 mg N/100g for fish and 63-78 mg N/100g for crustaceans and cephalopods have been reported (Velankar & Govindan, 1958). The highest concentrations of TMAO were found in the muscle of elasmobranchs and deep sea teleosts, where upto 230 mmoles N/kg wet weight has been reported (Anthoni *et al.*, 1990).

Among the reared fishes from fresh and brackish water, maximum values for AAN were obtained for *Liza macrolepis*. The AAN contents varied significantly between fresh water fishes and brackish water fishes as shown in Table 2 ($p<0.05$). However there was no significant difference in AAN contents in *Etroplus suratensis* collected from fresh and brackish waters. The higher values of AAN obtained for *Liza macrolepis*, *Labeo rohita and Etroplus suratensis* could be related with the increased level of the basic free amino acids present in them. The free AAN contributes to 40% of the total NPN in invertebrates (Velankar & Govidan, 1958). The AAN in teleosts ranges from 17-81mg N/100g, whereas in crustaceans upto 281 mg N/100g has been reported. The high AAN values observed could also be attributed to the environmental conditions of the fish (McCoid *et al.*, 1984).

The free amino acid content of reared fishes from fresh and brackish waters is shown in Table 3. In the fresh water fishes, glycine, taurine, histidine and lysine were the major free amino acids. Of the four fresh water fishes analysed, significantly higher amounts of glycine and taurine were observed in *Etroplus suratensis* and histidine and lysine in *Labeo rohita* ($p<0.05$). This is in accordance with previous reports which confirmed the high amounts of taurine in muscles of fishes (Sakaguchi *et al.*, 1984). The amount of histidine varied (55-140 mg/100g) significantly among all the fresh water fishes ($p<0.05$). Earlier reports have shown the free

histidine contents in the muscles of *Hypophthalmichthys* species varying from 127 mg/100g to 247 mg/100g (Yudaev, 1960). The free amino acids, histidine and glycine in the white muscles of fish play a role in maintaining osmotic homeostasis (Hegab & Hanke, 1983). Histidine metabolism results when an excess amount of the amino acid becomes available through the splitting of peptides or proteins containing histidine (Bramstedt,1961; Shimizu & Hibiki, 1955). Even among the brackish water fishes *Etroplus suratensis* and *Liza macrolepis*, taurine, glycine and histidine were the major free amino acids observed. The level of free amino acids varied significantly between the two fishes analysed ($p<0.05$). While *Liza macrolepis* contained a significant amount of lysine ($p<0.05$), glutamic acid and alanine were not detected in its muscle tissues.

Variations in free amino acids were also observed between the reared fishes of fresh and brackish water environments (Table 3). While *Etroplus suratensis* from brackish waters showed a significantly higher taurine content, the same species from fresh waters exhibited significantly higher glycine, alanine and glutamic acid levels ($p<0.05$). Lysine was significantly higher in *Liza macrolepis* from the brackish waters than in *Catla catla, Labeo rohita* and *Hypophthalmichthys molitrix* from the fresh waters ($p<0.05$). However, histidine levels were not found to be significantly different between fresh water reared *Etroplus suratensis* and *Liza macrolepis*. Glycine levels were not significantly different between *Hypophthalmichthys molitrix* and *Liza macrolepis*.

In the present investigation it was found that many fishes from different habitats are good sources of non-protein nitrogenous compounds. The NPN fractions, TMAO nitrogen, peptides and the amount of free amino acids show variations among species based on their habitats. However, variety of fishes have to be analyzed to have a clear understanding on the quality, quantity and behaviour of NPN compounds in muscle tissues of fishes from different environments.

The authors are thankul to Dr. K. Devadasan, former Director, Central Institute of Fisheries Technology, Cochin, India for the facilities provided at the Institute to carry out this work. Permission granted by Dr B. Meenakumari, Director, CIFT, Cochin to publish the paper is gratefully acknowledged.

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