

Alterations in Biogenic Amines Content During Storage in Frequently Consumed Fishes in Iran

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The aim of this study was to monitor changes in biogenic amines (BAs) in frequently consumed fishes during storage (1-14 days) at ambient temperature. Levels of five BAs including Histamine (HIS), Putrescine (PUT), Cadaverine (CAD), Tryptamine (TRP), and Spermine (SPM) were determined in four popular fish species marketed in North West of Iran during August, 2012. All the samples were kept at ambient temperature for 14 days and subjected to quantitative measurements of BAs on days 1, 7 and 14 after the catch using HPLC. The concentration of all BAs increased during the storage time except for HIS and PUT in shrimps. In fresh fishes, HIS values ranged between 23.97 ± 2.90 mg·kg⁻¹ to 46.53 ± 5.96 mg·kg⁻¹ which was within recommended levels. However, HIS levels in all the samples crossed the acceptable limit either on day 7 or 14. Also, shrimp samples had relatively higher values of SPM in all three selected time points. In addition, there was a statistically significant ($P < 0.001$) increase in TRP contents during the experimental period. The current study revealed that even in fresh fish samples high amounts of BAs exists. Also it was concluded that BAs could be considered as sensitive sanitation and quality control indices.

Key words: Biogenic amine, Histamine, Putrescine, Cadaverine, Tryptamine, Spermine.

By description, biogenic amines (BAs), also known as biologically active amines, are low molecular weight aliphatic, aromatic or heterocyclic organic compounds that are naturally formed in food by microbial decarboxylation of amino acids or amination and transamination of aldehydes and ketones¹⁻⁴. Chemical structures of some BA related to fish samples are given in Fig. 1.

These compounds are described as biogenic for the reason that they are produced by action of living organisms⁵. These BAs, cause

undesirable organoleptic effects in food stuffs and may possess considerable public health concerns including food poisoning⁶⁻⁸. These adverse health effects are augmented when consumed in large quantities or when human detoxification processes are insufficient^{9,10}. Therefore, various pathophysiological effects are expected if large amounts of the BA are ingested¹¹⁻¹³.

BAs are produced in course of microbial, plant, and animal metabolisms¹⁴. They can also an important influencing factor in many cellular processes in living organism such as growth regulation [spermine (SPM), spermidine (SPR) and cadaverine (CAD)], neural transmission (catecholamines and serotonin) and as mediators of inflammation [histamine (HIS) and tyramine (TYR)]¹⁵. HIS and TYR, which are the most

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important BAs, can cause poisoning following ingestion of food containing high levels of these amines. On the contrary, putrescine (PUT) and CAD, have no documented adverse health threat. However, these amines may enhance biotoxicity of both HIS and TYR through inhibition of these amines oxidizing enzymes¹⁶⁻¹⁹. Furthermore, biogenic polyamines, such as PUT, SPM, SPR and CAD may react with nitrite to form carcinogenic nitrosoamines^{20,21}. Thus, monitoring levels of BAs in foodstuffs have critical importance in reference to human health and safety of various food products.

Although plenty of food may contain variable contents of BAs, high BAs concentrations is reported from fish and fish products, cheese, beers, sausages and fermented vegetables²²⁻²⁶. Many tissues of fishes contain high amounts of free amino acids to maintain osmotic balances²⁷. This facilitates a higher production of BA in this food which results in high probability of intoxications such as HIS poisoning²⁸. The levels of BAs in fish tissues are influenced by various factors including muscle type, the native microflora, harvesting and postharvest management practices²⁹⁻³¹.

Fishes, as an important part of a healthy diet, are very rich sources for nutritive components including high quality proteins. The main health concern regarding this valuable food source is very rapid spoilage which starts right after the catch³². The process involves many metabolic and autolytic interactions between fish tissue and its rapidly changing microbial flora during storage period. Therefore, monitoring of microbial related biomarkers such as BAs are an important part of fish related quality control measures. Recently, assessments of the BAs have been considered as a sensitive and important determinant of fish spoilage and its freshness³³. Therefore, it seems reasonable that BAs are good markers to determine the degree of spoilage within fish samples in areas where a long distance between harvesting and marketing exists. The current study was conducted to assess levels of some important BAs in four species of mostly consumed fishes in North West of Iran which lies far from south of the country where the fish are caught, harvested and then transported to the region.

MATERIALS AND METHODS

Materials

Histamine dihydrochloride (98.5%), tryptamine hydrochloride (98%), were obtained from Acros organic (USA), cadaverine dihydrochloride (99%) and spermine tetrahydrochloride (97%) were purchased from Sigma Aldrich (Switzerland). 1,4- diaminobutan dihydrochloride (99%) and 1,7- diaminoheptan (98%), Phtaldialdehyd and 3 Mercaptopropionic acid (99%) used for preparation of *o*-phthalaldehyde (OPA), Tetrahydrofuran, Methanol, Ethanol, Trichloroacetic acid (TCA), Disodium hydrogen phosphate dodeca hydrate and Natriumdihydrogen phosphate, all in HPLC grade, were obtained from Merck chemicals (Germany). Other reagents used were analytical grade. Deionized water was produced from the shahid ghazi pharmaceutical (Tabriz, Iran).

Sampling

Four fish species were used in this study as follows: Mackerel (*scombromorus commerson*), Salmon trout (*salmo trutta*), Shark (*carcharhinus brachyurus*) and shrimp (*penaeus semisulcatus*) were purchased from local markets in Tabriz, Iran, during August 2012. All the fishes selected were harvested within last 24±12 hours. According to food basket analysis conducted by prefectural fisheries department, these four species are the most frequently consumed fishes in the region. From each species, total of fifteen samples were randomly selected and kept in three groups on ambient temperature. The fishes in first group were collected in sterile bags, kept in cold boxes and immediately transferred to the laboratory. The samples in the other two groups were collected one and two weeks later in a same manner. All the samples were cleaned thoroughly and fillets portions were carefully excised and grounded in a food blender with stainless cutters for 3 minutes to produce a fish meat homogenate.

Preparation of standards and derivatizing agent

Stock solutions of the five BAs (HIS, PUT, CAD, SPM and tryptamine [TRP]) were prepared one by one to concentrations of 1 mg·ml⁻¹ in 0.1 mol·l⁻¹ HCl. A working solution was prepared by diluting 1 ml of each stock solution in 0.1 mol·l⁻¹ HCl to a final volume of 10 ml. The OPA solution (5 ml) was prepared by transferred 25 mg of

phtaldialdehyde into a test tube and then added 4.5 ml methanol, 500 μ l borate buffers and 50 μ g 3-mercaptopropionic acids. Then, the solution was mixed well in a vortex and kept at 4 c in refrigerator prior to use as derivatizing solution agent.

Extraction and derivatization

BAs extraction from the samples was carried out according to the procedure described by Mah *et al.* (23). For extraction of BAs from the sample, a 3 g of each fish meat homogenate was transferred into a centrifuge tube. Then, 10 ml of 5% (w/v) TCA was added to the tube. The mixture was homogenized for 10 minute. Then, the slurry was centrifuged at 5000 g for 10 min at 4°C. The supernatant was separated and the residue was extracted again with the similar volume of the TCA solution. The supernatants were filtered with Whatman paper No.1 and the resultant volume was adjusted to 25 ml using TCA. Derivatization of BA was performed by pre-column *o*-phthalaldehyde. A 50 μ l of the filtrate was transferred into a micro tube, and mixed with same volume of the derivatizing solution, 100 μ l borate buffer and 50 μ l distilled water. Then, the solution was vortexed thoroughly and incubated for 2 minutes at room temperature. Finally, derivitization was stopped using 50 μ l 0.7 mol·l⁻¹.

Determination of biogenic amines

Quantification of the BAs was performed using a reverse phase HPLC following a method developed by Nemati *et al.* (34). The system contained a spectrofluorometric detector (RF-551), pump (K100, KNAUER), degasser (Biotech, model 2003) and chromgate software (EZchrom Elite) was employed. A KNAUER spherimage-80 C18 column (250 mm length – 4 mm internal diameter) was used for separation as a stationary phase. Phosphate buffer (0.0125 mol·l⁻¹; solvent A) and methanol (97% methanol+ 3% THF; solvent B) were used as mobile phases. The program of gradient elution was set for a linear gradient, as shown in Table 1. The flow rate was set at 1.0 ml·min⁻¹. The samples were monitored at 330 nm (excitation wavelength, λ_{ex}) and 460 nm (emission wavelength, λ_{em}) with a sample injector volume of 20 μ l.

Statistical analysis

Data were summarized using Mean (SD). To assess the Interaction effect, two ways ANOVA with repeated measures was used. The assumption of Sphericity was tested using Maucly test and

based on the results Greenhouse-Geisser Test or Sphericity Assumed based Test was used. To investigate Within Group effect or comparing the measurement effect within each group, a series of repeated measures analyses were performed in each type of fish followed by Sidak post hoc tests. To test the between group i.e. comparing the fishes in each measurement, a series of one way ANOVA were performed followed by Sidak post hoc tests. In this case the Levene's homogeneity of variances tests were performed and confirmed this assumption. All analyses were performed using SPSS 13 Software (SPSS Inc., IL, Chicago, USA).

RESULTS

In the current study, we first determined the contents of selected BAs right after arrival in local markets. Typical HPLC chromatograms of BAs from standard solution and fish sample are shown in Fig. 2. The contents of the BAs in testing fishes are shown in Table 2. On day 1 HIS values ranged between 23.97 \pm 2.90 mg·kg⁻¹ in sharks to 46.53 \pm 5.96 mg·kg⁻¹ in shrimps. On the other hand, the HIS levels in all fresh samples were less than 50 mg/kg, which is within acceptable levels of consumer health recommended by US FDA [35]. In Day 7 the HIS levels in all samples were increased as compared to Day 1. The most abundant increase was in shrimps by 1.38 fold. These values were higher than the US FDA upper limit in all species except mackerels. The increasing trend of HIS contents was maintained on the Day 14 except for the shrimps. Besides, there were statistically significant differences (P<0.001) in HIS values between all species tested expect for mackerel as compared to shrimp.

The highest PUT levels on Day 1 were observed in sharks (67.39 \pm 11.34 mg·kg⁻¹) with

Table 1. Gradient elution for biogenic amines analysis

Time (min)	A (%)	B (%)
0	70	30
4	60	40
7	50	50
17	50	50
20	20	80
22	10	90
26	70	30
35	70	30

Table 2. Changes in biogenic amine (Histamin, Putrescine, Cadaverine, Spermine and Tryptamine) levels (mg/kg) in fish samples during ambient temperature storage

	Trout		Mackerel		Shark		Shrimp		Between Group		Interaction Test results
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Test results		
	HIS 1	32.67 a α	5.07	24.33 a α	7.14	23.97 a α	2.90	46.53 a α	5.96	F(3,19)= 18.5, P<0.001	
HIS 7	56.59 b α	4.54	34.45 a α	17.99	76.79 b α	10.84	64.36 b α,γ	2.24	F(3,19)= 13.6, P<0.001		
HIS14	315.11 c α	42.55	55.54 b α,β	13.24	122.19 c α,β	36.49	50.53 a,b α,γ,δ	3.88	F(3,19)= 92.1, P<0.001		
Within Group	Test results		F(1,4)= 179.9, P<0.001 #		F(2,8)= 9.1, P=0.009 \$		F(2,8)= 30.8, P<0.001 \$		F(2,8)= 21.3, P=0.001 \$		
PUT 1	37.91 a α	3.58	34.22 a \acute{a}	12.06	67.39 α	11.34	52.99 a \acute{a}	16.38	F(3,19)= 8.3, F(6,32)= 25.3, P<0.001 \$	P=0.001	
PUT 7	50.60 a α	7.35	72.49 b α	9.75	76.49 α	28.63	273.38 b α	66.67	F(3,19)= 40.0, P<0.001		
PUT 14	277.50 b α	81.05	186.98 b α	60.15	101.52 β	27.95	116.85 c β	21.10	F(3,19)= 11.3, P<0.001		
Within Group	Test results		F(1,4)= 42.8, P=0.003 #		F(2,8)= 21.2, P=0.001 \$		F(2,8)= 2.4, P=0.158 \$		F(2,8)= 38.8, P<0.001 \$		
CAD 1	25.57 a α	8.53	19.43 a α	4.72	25.82 a β	6.32	59.02 a β	20.93	F(3,19)= 11.3, P<0.001		
F(4,18)= 15.0, P<0.001 #											
CAD 7	31.05 a α	4.35	44.61 b β	7.39	28.14 a α	7.63	57.70 a β	16.12	F(3,19)= 9.4, P=0.001		
CAD 14	202.34 b α	55.13	216.65 c α	81.87	40.76 b α	8.62	88.40 b β	9.79	F(3,19)= 15.0, P<0.001		
Within Group	Test results		F(1,4)= 51.3, P=0.002 #		F(1,4)= 23.9, P=0.008 #		F(2,8)= 9.7, P=0.007 \$		F(2,8)= 4.8, P=0.042 \$		
SPM 1	362.83 a α	89.11	242.47 a α	45.04	321.74 a β	67.47	419.27 a β	68.60	F(3,19)= 5.8, P=0.007		
SPM 7	835.98 b α	135.28	684.51 b α	245.56	432.77 a α	116.86	1009.51 b β	124.87	F(4,20)= 0.8, P=0.529 #		
SPM 14	1226.99 c	226.16	962.61 b	554.60	1015.37 b	297.11	1271.82 b	569.62	F(3,19)= 11.1, P<0.001		
Within Group	Test results		F(2,8)= 39.6, P<0.001 \$		F(2,8)= 4.9, P=0.040 \$		F(2,8)= 20.8, P=0.001 \$		F(1,4)= 8.9, P=0.037 #		
TRP 1	4.57 a α	3.00	6.69 a α	2.99	5.13 a α	2.07	9.72 a α	2.37	F(3,19)= 3.8, P=0.030		
TRP 7	15.98 b α	5.23	14.85 b α	2.94	12.44 b α	6.42	42.67 b α	9.72	F(3,19)= 14.5, P<0.001 #		
TRP 14	106.30 c α	25.22	76.14 c α,β	12.64	39.90 c β	9.08	55.22 c β	25.51	F(3,19)= 23.5, P<0.001		
Within Group	Test results		F(1,4)= 61.9, P=0.001 #		F(1,4)= 176.5, P<0.001 #		F(2,8)= 40.0, P<0.001 \$		F(2,8)= 84.3, P=0.013 \$		

#: Greenhouse-Geisser Test, \$: Sphericity Assumed based Test

Similar letters in each column or row show non-significant differences within Group (a, b, c) or between group (α , β , γ , δ) in that column or row.

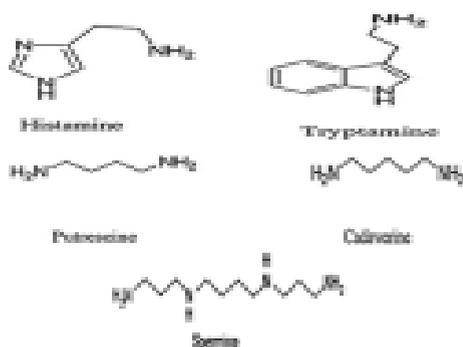


Fig. 1: Chemical structures of some biogenic amines related to fish samples

significant difference in comparison with the other three species. We also found that storage time had a potent effect on level of this amine. On day 14, salmon trout samples had the biggest increase in PUT levels as compared to the others ($277.50 \text{ mg}\cdot\text{kg}^{-1}$, $P=0.003$). Interestingly, PUT levels in shrimp samples were remarkably increased on day 7 but subsequently decreased on day 14.

There was a significant difference among CAD levels between all species on the three days ($P<0.001$) with the highest values detected in the shrimps. Similarly to PUT, the highest CAD levels were determined in salmon trout and mackerel fishes on day 14.

We further analyzed SPM as another spoilage indicator. It was found that shrimp samples

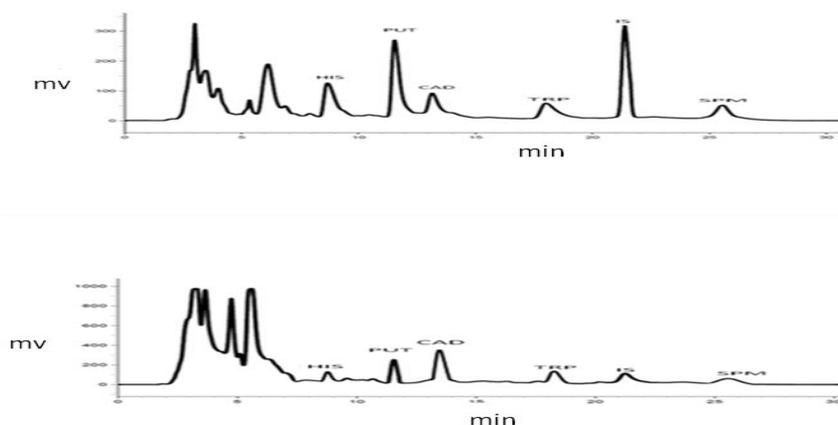


Fig. 2. Typical HPLC chromatograms of biogenic amines in standard solution and in fish sample. (A) Standard solution, (B) sample. Histamine (HIS), Putrescine (PUT), Cadaverine (CAD), Spermine (SPM), Tryptamine (TRP)

had relatively higher values of the amine in all three selected time points. Also, all the samples had the highest SPM on Day 14 ranging from $1015.37\pm 297.11 \text{ mg}\cdot\text{kg}^{-1}$ in shark samples to $1271.82\pm 569.62 \text{ mg}\cdot\text{kg}^{-1}$ in the shrimps.

Finally, TRP as a heterocyclic BA was subjected to analysis. It was observed that all the four selected fish samples experienced a statistically significant increase in TRP levels as storage duration increased ($p\leq 0.01$).

DISCUSSION

Among many BAs, HIS has received special attention by many researchers mainly

caused by its potent toxicological effects on humans which is defined as scombroid intoxication (36, 37). The occurrence of the disease is positively correlated with contents of the amine for each kg of fish consumed. It has been reported that minimum of $50 \text{ mg}\cdot\text{kg}^{-1}$ is required to induce intoxication in the adults. The minimum detected range of HIS in the selected fish samples was $23.97\pm 2.90 \text{ mg}\cdot\text{kg}^{-1}$ which is out of range required for the disease. But, this amount was not reached to cut off limit suggested by FDA, European Union regulation and Australia with $50 \text{ mg}\cdot\text{kg}^{-1}$, $100 \text{ mg}\cdot\text{kg}^{-1}$ and $200 \text{ mg}\cdot\text{kg}^{-1}$, respectively²⁷. On the contrary to our findings, other authors have reported negligible HIS, in fresh fish samples^{38, 39}. The

discrepancy must be attributed to the differences in the composition and load of microorganisms in the fish species or storage conditions. It has been well documented that production of the amine was enhanced as the time between harvesting and consumption increased. In our study, the HIS increased to maximum value, at day 14 after storage at 7 C. this correlated between storage time and formation of HIS was supported by other researchers³³. It is worthy to notice that HIS in shrimp was remarkably decreased on day 14. Decreases in HIS may be explained by limited quantities of histidine, a precursor of HIS, in shrimp or in relationship with growth of microorganisms presenting histaminolytic activity⁴⁰⁻⁴³.

Quantification of PUT and CAD are important quality index of fish and fishery products, Moreover, these two BAs are known as compounds enhancing HIS toxicity⁴⁴. Until a recommended cut off is set for PUT and CAD, these BAs may be used just for their sensory attribution⁴⁵. The amount of these two BAs was increased along with storage time except for PUT values in shrimps on Day 14. These changes are mostly attributed to levels of available free amino acids. It is well known that concentrations of free amino changes during storage period⁴⁶. This alteration is not identical for all marine sources. Yuzuru Otsuka *et al.*⁴⁷ reported that during 15 days storage on ice levels of arginine concentration decreased while ornithine increased in kuro shrimps as precursors of PUT. Therefore, it is speculated that the decreased levels of PUT on day 14 in shrimp samples may be related to depleted levels of arginine within muscle tissues of the tested shrimps. Therefore, this limits using of this BA as a freshness index for shrimps.

We also found an accelerated accumulation of PUT and CAD in salmon trouts during the storage period. The observation could be explained by the fact that salmon trouts were not eviscerated after sampling to simulate the usual practice in local stores. As suggested, skins, gills and intestinal tracts are the main sources of proteolytic bacteria in many fish samples. Thus, the findings were consistent with reports from fresh Skipjack tuna³⁷ and Albacore⁴⁸. SPM, like CAD and PUT has no known noxious effect, but it may convert to carcinogenic nitrosoamines by reacting with nitrite. In agreement with previous reports^{49,50}, we determined a remarkable amount of SPM during

the storage time for all fish species. This is maybe because SPM is a naturally produced compound of living cells.

Little is known on toxic effect of TRP. We found a significantly low production of this BA as compared to the other amines tested. The findings were in good agreement with other reports^{51,52}. However, it has been suggested that this BA is mainly a psychoactive compound with potency to elicit hallucination. It also induces visual disturbances, hearing deficits, dizziness and symptoms of food borne gastro enteritis⁵³. As for other BA its biologic activity augments when mono amine oxidase inhibitors including pain killers and alcohol are consumed. Therefore, determination of its toxic level and upper limit of oral tolerable dose is necessary.

Spoilage of seafood depends on species, geographical location that the fish inhabit, water and air temperature and microorganisms in the water^{54, 55}, and type of the caching vessel used. Besides, there is a seasonal variation in type and speed of spoilage due to fluctuating environmental conditions. Many food quality monitoring systems including the Hazard Analysis and critical control point has focused on HIS and other BAs as quality control measures. Judging from the findings of the current study and recent similar reports from the others^{41, 56}, It is thought that the levels of BAs are strongly correlated, in a time dependent manner with a degree of spoilage and decomposition in many fish samples. This was held true for CAD and PUT, except for the shrimps tested. However, accumulation of the BAs within fishes is dependent on many factors in particular specie of the fish. Therefore, more investigations are necessary to clarify which compound is suitable to be considered as a sanitation index for each fish.

CONCLUSION

The current study revealed that even in fresh fish samples high amounts of BA exists. The levels of this compound are different among various fish species and there is a time dependent change in value of these amines. It was concluded that BA could be considered as sensitive sanitation and quality control indices with some exceptions in particular when fish and maybe other marine food sources have been transported for long

distances or kept for a relatively long time. It is important to notice that decomposition of marine food sources occurs quite frequently right after caching via action of a diverse microbial flora, and endogenous enzymes indicating a vital need for early eviscerating the intestinal tract together with ice chilling and other appropriate preservation methods.

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