

## Characterisation of Biogenic Amines in Fish Collected from Sarawak Using Gas Chromatography

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### ABSTRACT

Determination of five biogenic amines (heptylamine, histamine, tyramine, cadaverine and spermidine) in fish was optimised and validated using gas chromatography – flame ionisation detector (GC-FID) followed by confirmation using mass spectrometry (MS). The biogenic amines were derivatised using BSA (N, O-bis(trimethylsilyl) acetamide) + TMCS (trimethylchlorosilane) as a derivatisation agent. The linear working range was between 0.9995 – 0.9999. The limit of detection (LODs) were in the range of 1.20 – 2.90 µg/mL. The efficiency of recovery for every biogenic amines, which ranged between 98.41 – 116.39%, indicated that analytical procedure can be used to extract biogenic amines in fish. Using GC-FID, the concentration of five biogenic amines were simultaneously determined in fresh and salted fish samples such as mackerel (*Scomberomorus guttatus*), sardine (*Sardinella gibbosa*), whiptail (*Himantura walga*), gourami (*Trichogaster pectoralis*) and toli shad (*Tenuialosa toli*). Histamine is found in fresh mackerel (*S. guttatus*) and sardine (*S. gibbosa*) at concentration of 5.96 and 2.69 mg/kg, respectively. Salted sardine (*S. gibbosa*) has histamine concentration of 8.95 mg/kg. All histamine concentrations detected were below 50 mg/kg (FDA regulation) which is below the permissible threshold associated with scombroid poisoning. Cadaverine was detected in fresh sardine (*S. gibbosa*), whiptail stingray (*H. walga*) and salted gourami (*T. pectoralis*) with concentration of 4.96, 146.39 and 18.80 mg/kg, respectively. None of them has biogenic amines, and histamine within FDA regulation levels (below 50 mg/kg).

Keywords: Biogenic amines, fish, gas chromatography, limit of detection, recovery

### INTRODUCTION

Biogenic amines are nitrogenous compounds which are usually low in molecular weight and have aliphatic, aromatic or heterocyclic chemical structures. Spermidine, cadaverine, putrescine and spermine are aliphatic structures whilst tyramine and phenethylamine are in aromatic form, and histamine and tryptamine are in the heterocyclic structure (Kalac, 2009; Kim *et al.*, 2009; Mohamed *et al.*, 2009; Rabie & Toliba, 2013). Furthermore, those which can be classified such as monoamines are tyramine and phenylethylamine, diamines are putrescine and cadaverine and polyamines for spermidine and spermine based on the number of amine groups (Spano *et al.*, 2010). Biogenic amines can be found in food that contain protein such as fish, meat, cheese, vegetables and wines (Lorenzo *et al.*, 2007). Biogenic amines can be shaped by means of amino acids decarboxylation which relies on the presence of a particular bacterial strain, or by amination and transamination of ketones and aldehydes (Rivas *et al.*, 2008; Linares *et al.*, 2011; Zhai *et al.*, 2012). The factors that influence biogenic amines

accumulation in food, are distribution and storage conditions, food physico-chemical parameters (pH, NaCl and ripening temperature), raw material quality, manufacturing processes, presence of decarboxylase-positive microorganisms and free amino acids and (Pons-Sanchez-Cascado *et al.*, 2006; Linares *et al.*, 2012).

Consumption of histamine by humans, especially at concentrations higher than 500 mg/kg, can lead to a serious issue of histamine poisoning (scombroid poisoning) (Gonzaga *et al.*, 2009). The FDA has also determined, histamine concentration at below 50 mg/kg in fish could be consumed (FDA, 2011). Histamine is one of the amines implicated in the toxicity of food (Zaman *et al.*, 2010), however at low levels histamine is not toxic; the presence of cadaverine and putrescine which have five times higher levels than histamine will contribute to histamine toxicity (Emborg & Dalgaard, 2006). High concentrations of tyramine can cause intoxication. Cheese reaction with symptoms like histamine toxicity (Naila *et al.*, 2010) can cause heart failure and brain

haemorrhage (Standarova *et al.*, 2008). The reaction between cadaverine, putrescine, spermidine and spermine with nitrite can lead to the synthesis of carcinogenic nitrosamines (Aflaki *et al.*, 2015).

Analytical approaches for biogenic amines analysis are aimed to (1) modify the current methods or develop new methods; (2) determine the concentration of biogenic amines in products from other countries using valid methods; (3) analyse biogenic amines used to control the effectiveness of methods developed in food storage, food preparation and packaging to decrease production and accumulation of biogenic amines; and (4) understand the relation between the levels of biogenic amines and biogenic amine-producing microorganisms (Bedia, 2013). The reason for the detection of biogenic amines in food is to indicate food quality and potential toxicity (Onal, 2007).

Various analytical techniques have been employed to determine biogenic amines, such as, thin layer chromatography, high performance liquid chromatography, gas chromatography and capillary electrophoresis (CE) (Bricio *et al.*, 2004; Awan *et al.*, 2008; Tao *et al.*, 2011; Aflaki *et al.*, 2015). These involve the application of chemical reagents such as perchloric acid, trichloroacetic acid, hydrochloric acid and organic solvents for the purpose of extraction of amines from food and beverages (Karovicova & Kohajdova, 2005; Jia *et al.*, 2011; Sagratini *et al.*, 2012; Lazaro *et al.*, 2013).

Fish is an important protein source and also highly perishable. During the deterioration process in fish, bacterial decarboxylases catalyse will convert amino acids to biogenic amines. Biogenic amines concentration relies on the species of fish, temperature, degree of microbiological contamination and storage time. The time and temperature are the main factors for the biogenic amines formation during handling and storage of fresh fish. Bacteria which is associated with biogenic amines are similarly present in a saltwater environment. The formation of biogenic amines in fish such as sardine, tuna, herring and anchovies has been determined (FDA, 2011; Chong *et al.*, 2014; Aflaki *et al.*, 2015). Histamine fish poisoning known as scombroid poisoning is seafood toxicity related to the improper storage of fish. The term "scombroid" is derived from the family Scombridae such as, tuna and mackerel. Non-

scombroid fish such as mahi-mahi (*Coryphaena* spp.), sardines (*Sardinella* spp.), herring (*Clupea* spp.) and swordfish (*Xiphias gladius*) are also able to cause scombroid poisoning in humans (Visciano *et al.*, 2012).

## MATERIALS AND METHODS

### Chemicals and Reagents

Heptylamine, histamine, tyramine, cadaverine, spermidine and BSA+TMCS (N,O – bis (trimethylsilyl) acetamide and trimethylchlorosilane) were purchased from Sigma-Aldrich. HPLC grade water, methanol and dichloromethane were acquired from the laboratory.

### Preparation of Standard Solutions

Stock solution (500 µg/mL) of a mixture from heptylamine, histamine, tyramine, cadaverine and spermidine was prepared in HPLC grade water. The stock solution was derivatised and stored in the refrigerator. Standard solutions (25 – 150 µg/mL), obtained by dilution of stock solution, were analysed by GC and calibration curves acquired.

### Fish and Fish Products

Samples of fresh and salted fish such as sardine (*S. gibbosa*), mackerel (*S. guttatus*), gourami (*T. pectoralis*), whiptail stingray (*H. walga*) and toli shad (*T. toli*) were purchased from Riyal market, Kota Samarahan.

### The Procedure of Recovery Study and Derivatisation

Recovery study was performed to validate the analytical procedure. Precisely 100 µg/mL of biogenic amines standard was spiked into 5 g of fish muscle and placed into an erlenmeyer flask containing methanol 50% (v/v). The solution was homogenized by sonicator for twenty minutes and was placed in a 45°C water bath for 45 minutes. The extract was cooled to 30°C then filtered with filter paper into 20 mL vial. Exactly 200 µL of the supernatant was put into vial 1 mL then derivatised. Precisely 200 µL aliquot of a standard solution or sample in a vial was evaporated with nitrogen gas. 100 µL BSA+TMCS was added into the vial and heated at 80°C for 20 minutes. After it had cooled, the derivatised solution was evaporated with nitrogen gas and the residue was re-dissolved in

100  $\mu$ L dichloromethane. 1  $\mu$ L of the supernatant was injected into GC.

### Gas Chromatography Flame Ion Detector and Mass Spectrometer

This study uses two different detectors of gas chromatography: flame ionisation detector (FID) and mass spectrometer (MS). One of the GC validated was FID, whereas MS used to identify and confirm the structure of derivatised biogenic amines standard. GC-FID is an instrument that lacks the capability to confirm peak identity where it relies on the comparison between the retention time of standard used with the retention time of sample analysed. Thus, MS was applied in this study to ensure that the derivatising agent has bonded with the analyte, and it can be determined whether the derivatising procedure was a success or otherwise. Analysis of biogenic amines was optimised under similar temperature. The temperature programme was 110°C for 2 minutes and increased to 190°C at the rate of 5°C/min maintained for 2 minutes. Derivatised biogenic amines were analysed using a GC, where a HP-5 phenyl methyl siloxane (30 m x 0.25 mm x 0.25  $\mu$ m) silica capillary column was installed in a Hewlett Packard 6890 and equipped with a FID. Carrier gas was hydrogen. Confirmation of peaks and retention times were obtained using GC-FID, derivatised biogenic amines were identified with GC-MS where it was performed on a capillary BPX-5 column (30 meter x 0.25 mm x 0.25  $\mu$ m).

## RESULTS AND DISCUSSION

### Method Validation

Method validation was carried out by determining the specificity, linearity, LOD, LOQ, accuracy, precision and recovery. Results are listed in Table 1. Good specificity was obtained and can be seen in Figure 2, where only five derivatised biogenic amines appeared in

GC-FID chromatogram without impurities peaks and the identity of five peaks were confirmed using GC-MS. Linearity of the calibration curves was established by analysing five concentrations of a mixture of biogenic amines (25–150  $\mu$ g/mL). Good linearity was acquired between peak area and concentration where the value was  $R^2$ : 0.9995- 0.9999.

LOD was ascertained from the lowest amine concentration required to give a signal to noise ratio of three (3 SD/N), while LOQ was ascertained with a signal to noise ratio of ten (10 SD/N) (Gosetti *et al.*, 2007). The precision and accuracy of the technique was evaluated by analysing six times the mixture of derivatised biogenic amines at 150  $\mu$ g/mL on the same day (intra-assay). Good reproducibility was obtained where RSD (SD\*100/mean) below 2% were found (Table 1). A recovery study was performed to validate the analytical procedure, where a concentration at 100  $\mu$ g/mL of a biogenic amines mixture solution was spiked into fish muscle. The solvent was methanol 50% (v/v) whereas the derivatising agent was a mixture of BSA+TMCS. The recovery is estimated as  $R = (C_{\text{spiked}} - C_{\text{sample}}) / C_{\text{added}}$ , where  $C_{\text{spiked}}$  is the level or amount in spiked sample and  $C_{\text{sample}}$  is the level or amount in the sample before to spike and  $C_{\text{added}}$  is the level or amount of enhanced standard. Satisfactory recovery for biogenic amines was obtained ranged between (98.41–116.39%) (Table 1).

### Analysis of Fish and Fish Products

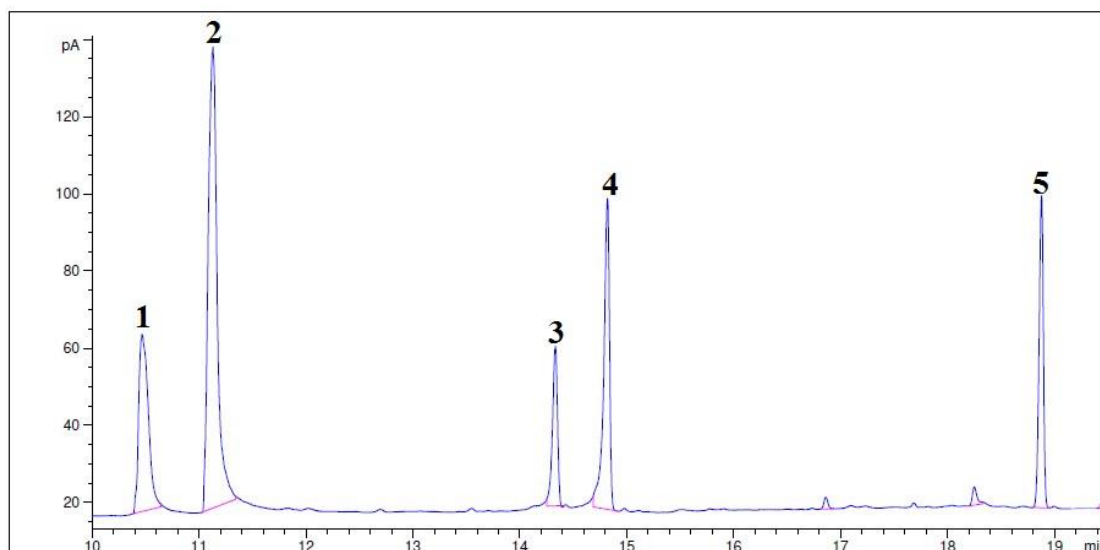
Identification of peaks in samples was based on the comparison of retention time between the retention time of derivatised biogenic amines standard with the retention time of sample.

Table 2 shows that all fresh fish concentration contain biogenic amines. The levels of histamine, tyramine and spermidine were found in mackerel

**Table 1.** The values of linearity, LOD, LOQ, intra-assay and recovery studies.

Biogenic amine	$R^2$	LOD ( $\mu$ g/mL)	LOQ ( $\mu$ g/mL)	Intra-assay (%RSD) (n = 6)	Recovery (%)
Heptylamine	0.9995	2.44	8.13	0.92	102.73
Histamine	0.9999	1.46	4.85	0.78	105.71
Tyramine	0.9999	2.90	9.65	2.01	98.41
Cadaverine	0.9998	2.03	6.77	1.36	109.09
Spermidine	0.9998	1.20	3.98	0.79	116.39

$R^2$ : square of regression coefficient; LOD: limit of detection; LOQ: limit of quantification; RSD: relative standard deviation.



**Figure 1.** GC-FID chromatogram for concentration at 100  $\mu\text{g/mL}$ , (1) heptylamine – 10.45 min, (2) histamine – 11.12 min, (3) tyramine – 14.32 min, (4) cadaverine – 14.88 min and (5) spermidine – 18.89 min.

(*S. guttatus*) with concentration at 5.96, 103.29 and 7.38 mg/kg, respectively. Sardine (*S. gibbosa*) contained the levels of heptylamine, histamine, tyramine, cadaverine and spermidine with concentration at 6.08, 2.69, 106.95, 4.96, 4.04 mg/kg, respectively. Cadaverine and spermidine concentration were found in whiptail stingray (*H. walga*) at 146.39 and  $6.18 \pm 0.24$  mg/kg, respectively; while gourami (*T. pectoralis*) contained the levels of heptylamine, tyramine and spermidine with concentration at 15.11, 135.24 and 19.97 mg/kg, respectively. Toli shad (*T. toli*) had only two biogenic amines such as, tyramine and spermidine, with concentration at  $46.53 \pm 3.94$  and  $11.30 \pm 0.76$  mg/kg, respectively.

According to Gonzaga *et al.* (2009), mackerel (*S. japonicus peruanus*) is a common fish that contain biogenic amines, particularly histamine, whereby the histamine concentration is at 86 mg/kg and based on the FDA regulation, this fish cannot be consumed. Low levels of histamine are also found in mackerel (*S. guttatus*) and sardine (*Dussumieria acuta*) where the concentration is at 15.8 and 12.6 mg/kg, respectively (Aflaki *et al.*, 2015). Significantly reduced levels of biogenic amines are found in salted fish where toli shad (*T. toli*), mackerel (*S. guttatus*) and whiptail stingray (*H. walga*) only contained spermidine with concentration at 8.75,

19.08 and 45.49 mg/kg, respectively. Sardine (*S. gibbosa*) and gourami (*T. pectoralis*) only contained histamine and cadaverine with concentration at 8.95 and 18.80 mg/kg, respectively. The reduction of biogenic amines level in salted fish samples has also shown the influence of salt as a preservative that can control the production of biogenic amines in food, particularly fish. Low level of biogenic amines was also detected by Saaid *et al.* (2009). Histamine, tyramine and spermidine were found in salted gourami with concentration at 3.5, 5.9 and 3.7 mg/kg, respectively. Only histamine and spermidine were found in salted mackerel with concentration at 111.8 and 3.6 mg/kg, respectively. Even though histamine concentration in salted mackerel was beyond the limit of FDA (50 mg/kg), without the presence of cadaverine and putrescine, the histamine toxicity in this sample can be neglected. Kose *et al.* (2012) detected histamine, cadaverine, tyramine and spermidine in mackerel with concentration at 1.9, 1.4, 3.7 and 26.4 mg/kg, respectively.

There is a distinction between fresh fish and salted fish samples, whereby the amine levels in each salted fish had been reduced owing to the preservative or salt. Preservatives is one of the methods that can be applied to control the production or accumulation of biogenic amines (Naila *et al.*, 2010). Good quality fish must

**Table 2.** Biogenic amines concentration in fish and fish products (n = 6).

No.	Sample type	Concentration of biogenic amines (mg/kg)				
		HEP	HIS	TYR	CAD	SPD
Fresh Fish						
1	Mackerel ( <i>S. guttatus</i> )	n.d.	5.96±0.72	103.29±4.8	n.d.	7.38±0.75
2	Sardine ( <i>S. gibbosa</i> )	6.08±0.22	2.69±0.75	106.95±1.9	4.96±0.26	4.04±0.28
3	Whiptail stingray ( <i>H. walga</i> )	n.d.	n.d.	n.d.	146.39±3.0	6.18±0.24
4	Toli shad ( <i>T. toli</i> )	n.d.	n.d.	46.53±3.94	n.d.	11.30±0.76
5	Gourami ( <i>T. pectoralis</i> )	15.11±0.38	n.d.	135.24±6.8	n.d.	19.97±1.70
Salted Fish						
6	Mackerel ( <i>S. guttatus</i> )	n.d.	n.d.	n.d.	n.d.	19.08±0.28
7	Sardine ( <i>S. gibbosa</i> )	n.d.	8.95±0.38	n.d.	n.d.	n.d.
8	Whiptail stingray ( <i>H. walga</i> )	n.d.	n.d.	n.d.	n.d.	45.29±1.11
9	Toli shad ( <i>T. toli</i> )	n.d.	n.d.	n.d.	n.d.	8.75±0.34
10	Gourami ( <i>T. pectoralis</i> )	n.d.	n.d.	n.d.	18.80±0.97	n.d.

n.d.: non-detected; HEP: heptylamine; HIS: histamine; TYR: tyramine; CAD: cadaverine; SPD: spermidine.

contain less than 10 mg/kg of histamine, 30–50 mg/kg can be considered as decomposition (Oguri *et al.*, 2007). Consuming 40 mg biogenic amines or more per meal is considered toxic for human. Not all biogenic amines are toxic, yet the levels of histamine and tyramine must be considered (Saaid *et al.*, 2009).

The FDA has suggested histamine content of fish should be <50 mg/kg and considered safe for human consumption, 50–1000 mg/kg is probably toxic and >1000 mg/kg is considered toxic and not safe for consumption (Proestos *et al.*, 2008). Tyramine level 100–800 mg/kg is admissible but over 800 mg/kg is considered poisonous (Saaid *et al.*, 2009).

## CONCLUSION

All samples such as fresh and salted fish have biogenic amines, salting is one of the factors which can influence or control the biogenic amines level in fish. Although there are no reports yet on cases involving such poisoning, it is recommended to moderate consumption of fish containing biogenic amines exceeding levels set by the FDA. It can be concluded methanol 50% (v/v) may be used as a solvent to extract biogenic amines from food, particularly fish. Also a mixture of BSA + TMCS can be applied as a derivatise agent. Good validation method such as specificity, linearity, LOQ, precision and accuracy has showed that column (capillary HP-5) and temperature programme employed in GC-FID can be used. Furthermore, GC-MC (capillary BPX-5 column) can also be applied to

identify the structure of derivatised biogenic amines.

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