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Research Paper

Fish Analysis Containing Biogenic Amines Using Gas Chromatography Flame Ionization Detector

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Abstract

Biogenic amines generally can be found in fish due to amines in fish undergoing a degradation process. According to the United States Food and Drug Administration (FDA), biogenic amines in fish and fish products can cause harm to consumers if consumed more than 50 μ g/mL. Thus, it is important to analyze them. Five biogenic amines such as heptylamine, histamine, tyramine, cadaverine and spermidine were extracted using soaking method with methanol 50% (v/v), afterward they were detected in fish and fish products using gas chromatography – flame ionisation detector (GC-FID) and the biogenic amines structures were confirmed using mass spectrometry (MS). The detection limits (DLs) were range at 1.20 – 2.90 μ g/mL. Histamine was detected in fish and fish products such as sardine (Sardinella gibosa) and mackerel (Scomberomorus guttatus) at concentration of 5.96 and 2.69 μ g/mL, respectively, whereas cadaverine was found in sardine (Sardinella gibosa) at concentration of 4.96 μ g/mL. Histamine concentrations in this study were detected below 50 μ g/mL which is below the permissible threshold associated with scombroid poisoing.

Keywords

Biogenic amines, gas chromatography, fish, detection limit

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1. INTRODUCTION

Biogenic amines are compounds that contain nitrogenous and having low molecular weight and based on chemical structures can be categorized such as aliphatic, aromatic and heterocyclic. They can also be classified based on the number of amine groups such as monoamines are tyramine and phenylethylamine, diamines are putrescine and cadaverine and polyamines for spermidine and spermine (Liu et al., 2020). Biogenic amines generally can be found in food that containing protein such as meat, fish, milk, vegetables, yoghurt and their products. The presence of biogenic amines in protein foods can be shaped through amino acids decarboxylation with the presence of a particular bacterial straion, or by amination and transamination of ketones and aldehydes (Gama and Rocha, 2020).

Several factors considered as main factors that increase biogenic amines accumulation in food such manufacturing processes, water activity, acidulant and sweetening reagents food physico-chemical parameters (pH, NaCl and ripening temperature) and presence of decarboxylase-posotive microorganisms and free amino acids. All of these factors influence the bacteria population in fish (Hidalgo et al., 2016). Histamine is a special

issue compared to other biogenic amines. Studies have reported that histamine is a chemical compound that naturally found in human body but when ingested in high quantity from food, it can cause histamine poisoning. The FDA also stated that histamine concentration safely consumed at below 50 µg/mL (Food et al., 2011). Histamine poisoning or called Scombroid poisoning is the most common food borne diseases related to fish consumption and related to Scombroidae family such as mackerel, sardine, tuna, blue fish and mahi-mahi (Qiao et al., 2020). Several approaches have been applied in order to determine biogenic amines, such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC) (Aflaki et al., 2015). Biogenic amines detection are imperative and there are some reasons to analyse biogenic amines in food such as to modify the current methods, to determine the biogenic amines concentration form other countries using valid techniques and to indicate food quality and potential toxicity.

The detector in chromatography instruments has an important role to convert the analyte analysed into a signal that can be measured where the signal related to concentration of the analyte. However, biogenic amines have disadvantages when analysed using chromatography approaches such as low volatil-

Figure 1. The chemical structure of biogenic amines used in this study.

ity and lack of chromophores. These conditions causing biogenic amines need to be derivatized before analysed using liquid and gas chromatography. The derivatizing reagents used also influenced by the detectors applied. Dansyl-, dabsyl, benzoyl- and chloride, O-phthaldialdehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC), 6-aminoquinolyl-N-hydroxysuccinimidy (AQC) (Munir and Badri, 2020) are common derivative reagents used by researchers.

Primary amines are suitable and react easily with O-phthaldialdehyde (OPA) and their reaction can be swift with the addition of specific reagents such as N-acetylcysteine or 2-mercaptoethanol. The derivatization step completes below 2 min in a mixture of borate buffer (pH 6-8). Although this derivatization step is very simple but this in not stable owing to several conditions such as temperature and OPA cannot be exposed too long in heat area (de la Torre and Conte-Júnior, 2013). Benzoyl and dansyl chloride can be a solution owing to they are more stable than O-phthaldialdehyde (OPA) and can be used to primary and secondary amines (Lkhagva et al., 2020).

Thus, gas chromatography has become a better method than HPLC based on its sensitivity, high resolution and selectivity. Nevertheless, GC analysis displays several difficulties owing to the polarity of these compounds is very high and they also are not easy to vaporize. Almost all researchers used derivatizing reagent before analyses using GC and column 5MS becomes the best choice to detect biogenic amines eventhough several columns can be used and reliable. The use of derivatizing agent such as acylation, alkylation and silvlation are also considered and suitable for biogenic amines in order to increase the sensitivity of biogenic amines so they can be easily detected using GC. Therefore, derivatization procedure need to be done in order to alter the properties of analyte such as to decrease polarities and to increase volatile properties of biogenic amines and also to improve the selectivity, sensitivity and resolution of GC analysis (Papageorgiou et al., 2018a).

Fish and their products are food that widely consumed due to the nutrients inside their body are needed for human muscle grotwth. Nevertheless, several conditions can convert protein to biogenic amines which they are very harm for human. In this study five biogenic amines analysed (Hep, His, Tyr, Cad and Spd) (Figure 1) and they are the most biogenic amines found in fish and fish products. This study used GC-FID in order to analyse biogenic amines in fish and the use of GC-MS applied in order to confirmed the structure of biogenic amines.

2. EXPERIMENTAL SECTION

2.1 Materials

The mixed standard solutions of BAs was prepared from the following reagents: Heptylamine 99% (HEP), histamine dihydrochloride 99% (HIS), cadaverine dihydrochloride 99% (CAD), tyramine hydrochloride 97% (TYR), spermidine trihydrochloride 97% (SPD) and BSA+TMCS (N,O – bis (trimethlsilyl) acetamide and trimethylchlorosilane) were purchased from Sigma-Aldrich. Other chemicals: Dichloromethane (DCM), methanol, acetone and HPLC grade water were also purchased from Sigma - Aldrich. Fish and fish products: Fresh and salted fish such as sardine (Sardinella gibosa) and mackerel (Scomberomorus guttatus) were purchased from Jenderam market, Dengkil, Malaysia. Standard (stock) solution of BAs was prepared at a concentration around 10000 µg/mL in HPLC grade water. A mixture of stock solution was derivatized and stored in the refrigerator at $4^{\rm o}$ C and was further diluted for experiments and validation purposes.

2.2 Methods

2.2.1 Sample preparation and derivatization procedure

The preparation and derivatization methods were adapted from Jia et al. (2020) with several modifications. Fish and fish products weighed approximately 10 g and homogenized in a glass beaker using soaking method with 50 ml of methanol 50%(v/v), where sample was soaked using methanol solution for 20 minutes. Afterwards, the extract was filtered with filter paper into 10 mL vial. Exactly 100 μL of the supernatant was put into 1 mL vial in order to derivatize. Precisely 100 μL of a mixed standard solutions or samples in a vial was evaporated with nitrogen gas. 10 μL BSA+TMCS was added into the vial and heated at 80°C for 10 minutes. Then, cooled at 30oC and the derivatized solution was evaporated using nitrogen gas and the residue was dissolved in 50 μL dichloromethane. 1 μL of the solution was injected into GC.

2.2.2 Chromatographic procedures

Gas chromatography (GC) unit equipped with two different detectors: Flame ionization detector (FID) and mass spectrometer (MS). FID used in order to acquire quantitative analysis whereas MS applied in order to verify the structure of biogenic amines derivatized. This procedure was followed Espalha et al. (2019) with some modifications. Both of detectors were optimized under similar temperature in order to analyse the samples. The temperature program was 110°C for 2 minutes and increased to 190°C at the rate of 5°C/min maintained for 3 minutes. Separation was achieved using a HP-5 phenyl methyl siloxane (30m, 0.25mm and 0.25µm) silica capillary column installed in a Hewlett Packard 6890 equipped with FID. Carrier gas was hydrogen. Whereas

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analyses derivatised biogenic amines using MS was performed on a capillary BPX-5 column (30m, 0.25mm and 0.25µm).

3. RESULTS AND DISCUSSION

3.1 Analytical Characteristic of The Method

The isolation of biogenic amines from fish are imperative step before analysed using GC. This is for ensuring the accuracy in determining the content of biogenic amines prior to analysis using chromatography techniques. Distilled water is commonly used to extract biogenic amines from fish samples owing to the solubility properties of biogenic amines. Nevertheless, according to some studies the use of acidic solvent can be considered such as hydrochloric acid (HCl) and trichloroacetic acid (TCA). Acid can be a good choice but the characteristics of the matrix should be considered. Although hydrochloric acid generally used yet it is not suitable for fish owing to the difficulties related to occasional sample turbidity (Dadáková et al. (2009); Munir and Badri (2020)). According to some researchers, organic solvents such as methanol and distilled water presented satisfactory result to various of foods (Richard et al., 2008). In this study, every fish sample analyzed using two different method of replicates, such as biological replicate and analytical replicate. Biological replicate means three fish samples with similar species prepared and extracted differently. Afterwards, the extracted fish underwent derivatization step and every sample injected 6 times using GC and called analytical replicate.

The validation of this study was verified by determining the detection limit (DL) and quantification limit (QL), linear range, accuracy (recovery) and precision (repeatability and reproducibility). Results are displayed in Table 1. Linearity was established by injecting various concentrations of a mixed biogenic amines (25 – 150 μ g/mL). Linearity in this study was satisfactory and acquired between peak area and analyte concentration (R² : 0.9995 – 0.9999).

Detection limit was ascertained from the lowest amine concentration required to give a signal to noise ratio of three (3 SD/N) whereas quantification limit was determined with a signal to noise ratio of ten (10 SD/N). The accuracy and precision were evaluated by injecting a mixed biogenic amines six times on the same day (repeatability) and over ten days (reproducibility), respectively. Good repeatability and reproducibility of the retention times (RSD $\leq 0.48\%$) and peak area (RSD $\leq 2.01\%$) were found. Area and retention times of the biogenic amines were stable and consistently reproducible. Recovery study was carried out by spiking 100 $\mu g/mL$ of a mixed biogenic amines to fish sample. The mixture was derivatized and injected into GC. The recovery was estimated as Recovery = (C spiked - C sample) / C sample, where C spiked is the level or amount in spiked sample and C sample is the level or amount in the sample prior spiked and C added is the level or amount of enhanced standard. Good recovery for biogenic amines was acquired (98.4 - 116.4%) (Table

According to the data in Table 1, the relative standard deviation range for the repeatability (same – day) precision of area and tR from 0.53-1.74% and 0.13-0.22%, respectively and for

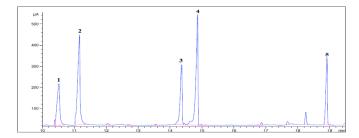


Figure 2. Separation of standard mixture of biogenic amines in GC-FID chromatogram for concentration at 100 μ g/mL. Peaks numbering: (1) HEP – 10.45 min, (2) HIS – 11.12, (3) TRY – 14.32 min (4) CAD – 14.88 min and (5) SPD – 18.89 min.

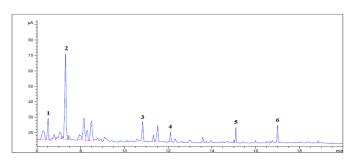


Figure 3. Gas chromatogram analysed by GC/FID for derivatized blank. Peak numbering: (1) 5.45 min, (2) 7.31 min, (3) 10.93, (4) 12.11 min, (5) 15.11 and (6) 17.01.

the reproducibility (between - day) precision the relative standard deviation of area and tR from 0.78-2.01% and 0.11-0.48%, respectively. The RSD results were indicating a good standard of precision. Furthermore, the analytical approach was successful and can be applied to detect biogenic amines in fish and their products. Table 2 shows the comparsion of detection limit of other studies with current study.

3.2 Analysis of fish and fish products

Fish is an important food due to high content of protein needed by human. Nevertheless, histamine intoxication caused by the accumulation of biogenic amines in fish are high. According to several studies, Scombroid fish species such as mackerel, bonito and tuna as well as non-scombroid species such as sardines, salmon, herring, mahi-mahi and anchovies containing high concentration of histidine in their flesh (de la Torre and Conte-Júnior, 2013), thus having possibility converting to histamine.

Biogenic amines determination in fish samples were based on comparison between the retention times of mixed biogenic amines standard with samples retention time. In order to find the concentration of biogenic amines in food samples was based on the biogenic amines derivatized standard where fitted by linear regression analysis. Baseline separation on chromatogram from five biogenic amines derivatized acquired below 20 min. According to Figure 2, no interfering peaks appeared in gas chromatogram at the retention times of the analytes, whereas

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Table 1. The values of linearity, detection and quantification limit (LOD & LOQ), RSD and recovery study.

ВА	\mathbb{R}^2	LOD (µg/mL)	LOQ (μg/mL)	Repeatability (% RSD) (n = 6)		1		Recovery study (%)
				Area	tR	Area	tR	
HEP	0.9995	2.44	8.18	0.53	0.15	0.92	0.32	102.73
HIS	0.9999	1.46	4.85	0.77	0.08	0.78	0.11	105.71
TYR	0.9999	2.9	9.65	1.74	0.17	2.01	0.27	98.41
CAD	0.9998	2.03	6.77	0.92	0.22	1.36	0.48	109.09
SPD	0.9998	1.2	3.98	0.65	0.13	0.79	0.19	116.39

R2: square of regression coefficient; LOD: limit of detection; LOQ: limit of quantification; tR: retention time; RSD: relative standard deviation.

Table 2. The values detection limit of other studies and current study.

Sample	Analyte	Detection limit (μg/mL)	References	
Canned fish	Histamine and Tyramine	3 and 4 (μg/mL)	(Alizadeh et al., 2017)	
Canned fish	Cadaverine, Histamine, Putrescine and Tyramine	2.98 – 45.3 (μg/L)	(Huang et al., 2016)	
Salted fish	Nitrosamine	0.1 (μg/L)	(Qiu et al., 2017)	
Cheese	Cadaverine, Histamine, Putrescine and Tyramine	5.9 – 14 (μg/mL)	(Mohammadi et al., 2017)	
Juice	Cadaverine, Histamine, Putrescine and Tyramine	$4-10 (\mu g/L)$	(Cunha et al., 2017)	
Wine	Butylamine, Cadaverine, Histamine and Tyramine	0.009 – 0.859 (μg/L)	(Papageorgiou et al., 2018b)	
Beer	Cadaverine, Histamine, Putrescine and Tyramine	$0.3 - 2.9 (\mu g/L)$	(Almeida et al., 2012)	
Fish and fish products	Cadaverine, Heptylamine, Histamine, Spermidine and Tyramine	1.20 – 2.90 (µg/mL)	Current study	

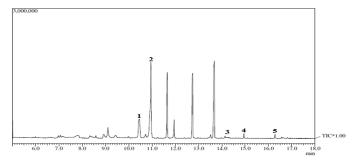


Figure 4. Gas chromatogram analysed by GC/MS for concentration of derivatized 100 μ g/mL biogenic amines standard.

derivatised blank was also analysed in order to compare to derivatized biogenic amines (Figure 3) and showing different retention times with biogenic amines. Peaks in Figure 2 were confirmed by GC/MS. Several peaks appeared but only 5 peaks were confirmed as biogenic amines (Figure 4) according to mass spectrometry library and others peaks were identified as by-products due to reaction between derivatizing agent and biogenic amines standard itself.

Histamine content in fish safely consumed below 50 μ g/mL. Nevertheless, histamine alone is not dangerous at low level, yet the presence of other biogenic amines such as cadaverine and putrescine at concentration higher than histamine can increase the toxicity of histamine itself. Whereas tyramine is the most abundant biogenic amines found in fresh mackerel and sardine in this study. Tyramine is generally the major biogenic amines found in fish beside histamine (Emborg and Dalgaard (2006); Food et al. (2011); Riebroy et al. (2004)). Figure 5 and Figure 6 show FID chromatogram for fresh sardine 3 and fresh mackerel 2. Although several peaks appeared but all of them were confirmed by GC/MS and the peaks were identified according to

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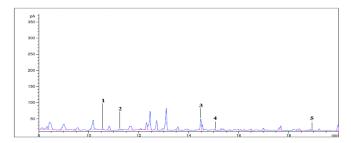


Figure 5. Gas chromatogram analysed by GC/FID for fresh sardine 3. Peaks numbering: (1) HEP – 10.45 min, (2) HIS – 11.12, (3) TRY – 14.42 min (4) CAD – 15.01 min and (5) SPD – 18.89 min.

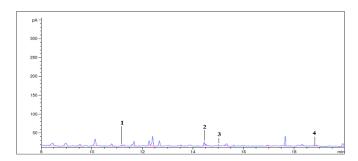


Figure 6. Gas chromatogram analysed by GC/FID for fresh mackerel 2. Peaks numbering: (1) HIS – 11.12, (2) TRY – 14.42 min (3) CAD – 15.01 min and (4) SPD – 18.89 min.

mass spectrometry library.

FDA has released a regulation for controlling fish processing according to hazard analysis critical control points (HACCP), such as, 1. Fish exposed to environment with temperature above 28°C need to be stored in fridge at 4°C as soon as possible after catch, 2. Fish exposed to environment with temperature below 28°C need to be stored in fridge at 4°C as soon as possible after harvest and 3. Fish that grilled before chilling must be stored in fridge at 4°C not more than 12 h from the time of death (Food et al., 2011). Good Manufacturing Practice (GMP) stated that consuming tyramine is acceptable if the level between 100 -800 μg/mL and over 1000 μg/mL can cause toxicity. It reported that tyramine concentrations can alter on storage even at fridge temperature. Tyramine alone at high concentration can cause an intoxication known as the cheese reaction which has similar symptoms to histamine poisoning. Small amount of spermidine was found in this study owing to this amine plays an important role in live fish metabolism and cellular growth. Spermidine can also be dangerous for human if consumed over 600 µg/mL (Saaid et al., 2009).

Five biogenic amines such as CAD, HEP, HIS, SPD and TYR were found in Sardine 3 and showed level 4.96 $\mu g/mL$, 5.76 $\mu g/mL$, 1.12 $\mu g/mL$, 4.04 $\mu g/mL$ and 106.95 $\mu g/mL$, respectively. CAD and SPD were not found in Sardine 1, whereas SPD and TYR were not found in Sardine 2. High histamine concentration was found

in Sardine 2 compared to others but the concentration is below FDA regulation (50 $\mu g/mL$). High histamine concentration was also detected in mackerel with 5.96 $\mu g/mL$, yet it was acceptable according to the FDA regulation. Not all biogenic amines in this study detected in fresh mackerel. Furthermore, the concentration of TYR in mackerel 1 (103.73 $\mu g/mL$) was strongly high but the concentration is decrease significantly in salted mackerel 1 owing to the addition of salt. The method was applied for the detection of biogenic amines in 12 fish samples. The food samples tested and the concentration or biogenic amines found are presented in Table 3.

Biogenic amine levels in salted fish products showing different significant compared to fresh fish. This happens owing to the addition of salt. Salt is one of the preservative methods that can be used to control biogenic amines accumulation in fish. Shalaby (1996) reported that sodium may limit the biogenic amines accumulation and sodium hexametaphosphate at 2% has ability to postpone histamine production. While Mah and Hwang (2009) reported the use of salt in anchovy product can reduce biogenic amine levels. Good quality fish according to the Compliance Policy Guideline No. 540.525 must contain histamine below 10 $\mu g/mL$, where histamine concentration at 30 $\mu g/mL$ considered deteriorate whereas histamine concentration above 50 $\mu g/mL$ is considered dangerous and the foods cannot be consumed due to the toxicity (Food et al. (2011); Oguri et al. (2007)).

4. CONCLUSIONS

The method used in this study is suitable and giving satisfactory results for the determination of five biogenic amines in fish and fish products using GC-FID and MS technique. The peak resolution is very good and no interferences were found. Thus, the use of BSA + TMCS can be used as a derivatizing agent. The use of salt also can be considered as a preservative method to control biogenic amines accumulation if fish. This study also indicates that the biogenic amines in fresh fish samples do not present a health risk if consumed owing to low level of histamine and tyramine. Thus there are no reports yet on cases involving such histamine poisoning. There is possibility they are not consumed every day causing the lack of reported poisoning cases. It is also recommended to controlling fish consumption that containing histamine beyond FDA limitation. Furthermore, consuming food that containing high biogenic amines on a regular basis is not recommended.

5. ACKNOWLEDGEMENT

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Table 3. Biogenic amines concentration detected in fish samples (n = 6).

C 1	C 1 .	Concentration (µg/mL)				
Sample no.	Sample type	CAD	HEP	HIS	SPD	TYR
Fresh fish (S						
1	1 Sardine 1		6.08	1.99	nd	27.95
2	Sardine 2	1.67	1.33	2.69	nd	nd
3	Sardine 3	4.96	5.76	1.12	4.04	106.95
M	2.21	4.39	1.93	1.35	44.95	
Fresh fish (Mackerel)						
4	Mackerel 1	nd	nd	5.96	nd	103.73
5	5 Mackerel 2		nd	1.24	7.34	12.76
6	6 Mackerel 3		nd	nd	2.98	59.92
Mean		0.48	-	2.4	3.44	58.8
Salted fish (S						
7	Sardine 1		2.07	nd	nd	nd
8	8 Sardine 2		nd	1.11	nd	nd
9	9 Sardine 3		1.75	nd	nd	11.17
M	0.59	1.27	0.37	-	3.72	
Salted fish (I						
10	10 Mackerel 1		nd	2.11	nd	19.58
11	11 Mackerel 2		nd	nd	2.12	nd
12	12 Mackerel 3		nd	nd	nd	nd
Mean		-		0.7	0.7	6.53

nd: not detected.

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