DETERMINATION OF BIOGENIC AMINES IN FISH BASED PRODUCTS USING LIQUID CHROMATOGRAPHIC AND CAPILLARY ELECTROPHORESIS

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2018

DETERMINATION OF BIOGENIC AMINES IN FISH BASED PRODUCTS USING LIQUID CHROMATOGRAPHIC AND CAPILLARY ELECTROPHORESIS

by

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Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

April 2018

DEDICATION

This work is dedicated in loving memory of…

My father, Bakar Bin Mat whom we missed and loved, and my mother, Timah binti Ahmad, who is my hero and my role model as independent, hardworking, persevering women. Her unconditional love and strong will are inspiring and remembered dearly in our hearts. May Allah Ta'ala shower HIS Mercy on them and placed them in the highest place in Jannah. Ameen. Mother…I miss you so much!

My love and gratitude to my husband and children, Aizad, Ainil and Hakimi. I truly hope the spirit and energy put together in this challenge to gain knowledge will encourage my children to pursue their own dreams.

My sisters and brothers, Kak Yah, Kak Ina, Hasiah, Marhaini, Abang In and Abang Oman, Kak Zahrah and Kak Nah, thank you for being very supportive as always.

ACKNOWLEDGEMENT

Learning is a continuous process as we improvised. The more we understand, the less we know. Knowledge rests not upon truth alone but upon error also. Those who learned to collaborate and improvise most effectively have prevailed.

My sincere gratitude goes to Associate Professor Dr. Fazilah Ariffin for your guidance and commitment in this study. Your understanding and continuous support to facilitate the research work are greatly appreciated. A special thanks to Professor Bahruddin Saad for many constructive comments, innovative ideas and suggestions in perfecting the research article and thesis. Your generosity and kindness to your students are heartwarming and forever remembered. Being part of learning team members under your guidance has helped me tremendously. My gratitude and appreciation to Dr. Ahmed Makahleh for your guidance and continuous support in the laboratory work. Thank you for your advice and encouragement in making this research collaboration study successful with fruitful findings. A sincere gratitude to Professor Dr. Gulam Rusul Rahmat Ali for your advice and suggestion in the project proposal at the beginning of the study.

Last but not least, to the Laboratory Staffs, Encik Mohd. Zamri Rosidi, Encik Fauzan Mat Puzi at School of Chemical Sciences, Puan Mazura at School of Industrial Technology, Universiti Sains Malaysia; my dear friends, Bahiyah, Hui Fang, Shifa, Baleeqh, Marwan, Syazana, Liza, Wanie and Nadhi, thank you so much for always be there, together through thick and thin. Knowing you and working together as a team have been a wonderful experience.

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LIST OF ABBREVIATIONS

AC	Alternating current
AGM	Agmatine
ANOVA	Analysis of variance
AOAC International	The Official Method of Analysis of AOAC International
BA	Biogenic amines
BGE	Background electrolytes
C^4D	Capacitively coupled contactless conductivity detection
CAD	Cadaverine
CE	Capillary electrophoresis
CZE	Capillary zone electrophoresis
DAD	Diode array detector
DNA	Deoxyribonucleic acid
DNS-Cl	Dansyl chloride
DoF	Department of Fisheries
EFSA	European Food Safety Authority
EIA	ELISA Kit Histamine Food analysis
ELISA	Enzyme linked immunosorbent assays
EME	Electro membrane extraction
EOF	Electro-osmotic flow
EU	European Union
FDA	Food and Drug Administration

FIA	Flow injection analysis
FITC	Fluoresceine isothiocianate isomer
GC-MS	Gas chromatography - mass spectrometry
HCl	Hydrochloric acid
HIS	Histamine
IC	Ion chromatography
kV	Kilo Volt
LC	Liquid chromatography
LCMS/MS	Liquid chromatography tandem mass spectrometry
LDN	Labor Diagnostika Nord
LIF	Laser induced flourescence
LOD	Limit of detection
LOQ	Limit of quantitation
MOH	Ministry of Health
MSG	Mono sodium glutamate
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
PAD	Pulsed amperometric detection
PHE	2-Phenylethylamine
PUT	Putrescine
RP-HPLC	Reversed phase - high performance liquid chromatography

RSD	Relative standard deviation
SD	Standard deviation
SLM	Supported liquid membrane
SPD	Spermidine
SPM	Spermine
SPSS	Statistical Package Social Science
TCA	Trichloroacetic acid
TFP	Traditional fish products
TRP	Tryptamine
TYR	Tyramine
USA	United States of America
UV-Vis	Ultra-Violet visible

PENENTUAN BIOGENIK AMINA DALAM PRODUK BERASASKAN IKAN MENGGUNAKAN KROMATOGRAFI CECAIR DAN KAPILARI ELEKTROFORESIS

ABSTRAK

Suatu kajian menentukan paras biogenik amina (BA) dalam produk berasaskan ikan telah dijalankan menggunakan dua kaedah piawai, fasa terbalik-kromatografi cecair prestasi tinggi/UV-Vis (RP-HPLC/UV-Vis) dan kapilari elektroforesis dengan konduktor kapasitor berpasangan tanpa sentuh (CE-C^4D). Objektif kajian adalah untuk menentukan paras BA di dalam produk perikanan tempatan bagi memastikan keselamatan makanan menggunakan kaedah piawai yang sensitif, mudah dan murah. Dengan menggunakan kaedah RP-HPLC, putresina (PUT), kadaverina (CAD), histamina (HIS) dan tiramina (TYR) dianalisa dalam 15 produk *belacan* tempatan. Sebanyak 53% daripada produk *belacan* mengandungi HIS di atas paras 50 mg kg^{-1} had selamat (purata, 57.63 mg kg^{-1}) tetapi masih di bawah paras 200 mg kg^{-1} had limit bagi penolakan produk fermentasi berasaskan ikan di pasaran antarabangsa. Kandungan garam (NaCl%) adalah melebihi 15% di dalam produk *belacan* dan peratus kelembapan kurang daripada 40% daripada 80% sampel *belacan* yang dianalisa, mengikut peraturan di dalam Akta Makanan 1983/ Peraturan Makanan 1985. Kaedah RP-HPLC/UV-Vis yang dibangunkan ini didapati sensitif dan tepat dengan had pengesanan kualitatif (LOD) 0.01 µg mL^{-1} dan had pengesanan kuantitatif (LOQ) di dalam julat 0.04 - 0.05 µg mL^{-1} bagi PUT, CAD, HIS dan TYR. Kaedah RP-HPLC/UV-Vis yang digunapakai memerlukan proses pre-derivatisasi yang panjang serta reagen yang mahal. Sebagai alternatif, suatu kaedah baru CE-C^4D dibangunkan untuk menganalisis BA di dalam makanan. Paras kandungan lapan BA

seperti HIS, PUT, CAD, TYR, spermina (SPM), spermidina (SPD), triptina (TRP) and 2-feniletilamina (PHE) telah ditentukan di dalam suatu kaedah analisis secara langsung menggunakan CE-C^4D dalam jangkamasa 19 minit. Pengoptimuman kaedah CE-C^4D di-capai melalui penggunaaan 0.4 M asid d-malik sebagai elektrolit pembawa, pH 2.11, suhu 24 ^oC dengan penggunaan voltan 20 kV; ketetapan C^4D pada frekuensi 600 kHz dan 50% amplitud. Keluk kalibrasi adalah linear pada 2 - 200 µg mL^{-1} (r^2 = 0.993 - 0.999) dengan LOD, 0.26 - 0.58 µg mL^{-1} dan LOQ, 0.79 - 1.69 µg mL^{-1}. Ujian perolehan kembali (74.30 – 119.96%), ujian pengulangan harian (intra-day) dan antara-hari (inter-day) diukur sebagai peratus sisihan piawai relatif (%RSD) dengan nilai di antara 3.13 - 20.0 %RSD. Menggunakan kaedah baru CE-C^4D, sebanyak 88% daripada 41 produk perikanan mengandungi HIS kurang daripada 50 mg kg^{-1} had dibenarkan. Produk seperti *ikan pekasam* (72.0 mg kg^{-1}), *ikan kurau* dan *gelama* masin (130.79 dan 96.56 mg kg^{-1}), sotong kering (92.02 mg kg^{-1}) dan *otak udang* (54.50 mg kg^{-1}) mengandungi HIS melebihi 50 mg kg^{-1} had selamat.

DETERMINATION OF BIOGENIC AMINES IN FISH BASED PRODUCTS USING LIQUID CHROMATOGRAPHIC AND CAPILLARY ELECTROPHORESIS

ABSTRACT

Determination of biogenic amines (BA) in fish based products were carried out using two standard methods, reversed phase - high performance liquid chromatography/UV-Vis (RP-HPLC/UV-Vis) and capillary electrophoresis with capacitively coupled contactless conductivity detection (CE-C^4D). The objective of the study was to determine the level of BA in local fish based products for safety assurance using standardised method yet sensitive, simple and at low cost. Using RP-HPLC method, putrescine (PUT), cadaverine (CAD), histamine (HIS) and tyramine (TYR) were analysed in 15 local *belacan* products. About 53% of *belacan* products contain HIS above 50 mg kg^{-1} safe limit (means, 57.63 mg kg^{-1}) but below 200 mg kg^{-1} 'rejected level' for quality assessment in fermented fisheries products for international market. Salt content (NaCl) are above 15% in *belacan* products and moisture percentage are less than 40% in 80% of *belacan* samples, under the regulation for *belacan* products in Food Act 1983/Food Regulation 1985. The RP-HPLC/UV-Vis optimised method was sensitive and accurate with qualitative detection limit (LOD) of 0.01 µg mL^{-1} and quantitative detection limit (LOQ) ranging from 0.04 - 0.05 µg mL^{-1} for CAD, PUT, HIS and TYR. However, HPLC method using UV-Vis detector required expensive derivatisation reagent and laborious procedures. Hence, a new method using CE-C^4D was developed as alternative for BA analysis in food. Eight BA namely HIS, PUT, CAD, TYR, spermine (SPM), spermidine (SPD), tryptamine (TRP) and 2-phenylethylamine (PHE) were determined using a CE-

C^4D method in one analysis with direct detection within 19 minutes. Method optimisation for eight BA separation were obtained using 0.4 M d-malic acid as background electrolyte, pH 2.11, temperature 24 ^oC with applied voltage of 20 kV; C^4D input signal at frequency 600 kHz and 50% amplitude. The calibration was linear from 2 - 200 µg mL^{-1} (r^2 = 0.993-0.999) with LOD, 0.26 - 0.58 µg mL^{-1} and LOQ, 0.79 - 1.69 µg mL^{-1}. Recovery analysis (74.30 - 119.96%), intra-day and inter-day repeatability measured as percent Relative Standard Deviation (%RSD) are ranging from 3.13 – 20.0 %. Using the new CE-C^4D method, about 88% from 41 products tested contain HIS less than 50 mg kg^{-1} permitted level. Products such as *ikan pekasam* (72.0 mg kg^{-1}), salted *ikan kurau* and *gelama* (130.79 and 96.56 mg kg^{-1}), dried cuttlefish (92.02 mg kg^{-1}) and *otak udang* (54.50 mg kg^{-1}) contain HIS above 50 mg kg^{-1} safe limit.

CHAPTER 1

INTRODUCTION

1.1 OVERALL VIEW

Biogenic amines (BA) are low molecular weight nitrogenous compound that are synthesised and degraded as part of cellular metabolism activities by microorganisms in a living cells (Silla Santos, 1996; Halász et al., 1994). BA is a trace element required in a variety of normal cell functions involving cell growth, cell differentiation and as a receptor function (Baron & Stasolla, 2008). They also play important role in DNA replication, gene expression, protein synthesis, stabilisation of lipids, brain development, nerve growth and regeneration. On the contrary, more often, they have been associated with food intoxication following the consumption of certain food containing high accumulation of BA, in particular histamine.

Histamine (HIS), putrescine (PUT), cadaverine (CAD), tyramine (TYR), tryptamine (TRP), 2-phenylethylamine (PHE), spermine (SPM), and spermidine (SPD) are accepted to be the most important BA occurring in foods (Shalaby, 1996). HIS, CAD, PUT are formed from histidine, lysine, ornithine and arginine whereas SPM and SPD are formed from PUT. The concentration and type of BA found in food are influenced by the food composition, in particular amino acids, microbial flora and other parameters which allow bacterial growth during food processing and storage such as food treatment, food additives, temperature, moisture, ripening process and packaging (Carelli et al., 2007; Draisci et al., 1998).

The most toxic BA that has been linked with several incidences of food poisoning is HIS. Consumption of spoiled food was the common cause for HIS poisoning. For people with diminished HIS detoxification enzymes (diamine oxidase) in their bodies, ingestion of HIS above 50 mg kg^{-1} in food may lead to food poisoning (FDA USA, 2001). High HIS in fish (fresh or processed products) is a problem of freshness or deficiency in the cold storage (Halåsz et al., 1994) whereas PUT, CAD, and SPD are useful as quality indices for the decomposition of fish (Zhao et al., 2007; Benner, 2003). High HIS was often followed by an elevation in other BAs such as CAD, PUT and TYR (Silla Santos, 1996; Brink et al., 1990). Depreciation of the organoleptic properties of foodstuff is often associated with high PUT, CAD, SPD and TYR. Thus, they are useful indicator of quality and acceptability in certain foods (Ruiz-Capillas & Jiménez-Colmenero, 2004; Shalaby, 1996).

Fish and fish based products have been categorised as high risk food to contain BA (EFSA, 2011). Although fish and fish based products are known as healthy source of high quality protein, vitamins, mineral and polyunsaturated fatty acids, fish proteins are broken down rapidly under normal environmental temperature that generate BA formation. With fresh fish, the consumer is inclined to judge the freshness of fish visually and to be mindful of the hygienic and refrigeration conditions of the fish. However, sensory and physical evaluations such as visual appearance, smell or physical touch does not give much indication on HIS content in food. In processed fish based products, the waiting duration and conditions before processing, any unhygienic practice may cause substantial BA accumulation in the products. Information on BA content adds another important database for guidelines on the safety and quality assessment of fish products. A

sensitive and simple method, low cost with green technology in sample preparation and analysis is highly needed for rapid screening to ensure products quality and food safety.

1.2 PROBLEM STATEMENT

There is limited data on the levels of BA in traditional processed fish based products such as a shrimp paste *belacan*, fish sausage *keropok lekor*, fish ball, fish cake and other salted and dried or fermented products. With growing consumer interest and market demand worldwide, BA analysis is of prime importance for quality control of products and food safety. Common analytical method for the determination of BA is HPLC whereby derivatisation of the BA is necessary before UV/fluorescence detection as they exhibit limited absorption capacity. Expensive derivatisation reagent, high consumption of the organic solvent for the mobile phase can be a problem for routine analysis with large sample number. The mobile phase used during HPLC analysis can generate significant amount of wastes that requires proper disposal. Food sample is well known as a complex matrix. The official HPLC methods are often encountered with difficulty in getting a clean baseline separation for all BA analytes important in food in a single analysis. Sample pre-treatment or purification was normally performed followed by derivatisation reaction to enhance the sensitivity and detection limit prior to analysis using UV/fluorescence detection. Although HPLC method offer good sensitivity and accuracy in detection, it is very costly, time consuming and laborious.

Alternatively, by using CE-C^4D method, the sample extraction process was kept minimum but with acceptable sensitivity and reproducibility. The contactless conductivity detector or capacitively coupled contactless conductivity detection (C^4D) is known for its

simplicity and low cost. With high resolving power of CE, this electrochemical method offers high separation efficiency of multiple analytes in a single analysis. C^4D offer direct detection of charged analytes (cation) based on the measurable conductivity activity of analytes passing through a detector (C^4D) placed around a capillary tube. Hence, the hypothesis of this study is a CE-C^4D method can be used to detect BA in food sample under suitable experimental condition.

1.3 OBJECTIVES OF STUDY

Hence, the main objectives of the study are:

i) To determine the level of important BAs (HIS, CAD, PUT and TYR) in shrimp paste products obtained from the Northern States of Peninsular Malaysia by RP-HPLC.

ii) To develop alternative method for BA analysis in food using CE-C^4D.

iii) To determine BA HIS, PUT, CAD, SPD, SPM, TYR, TRP, 2-PHE content in other fish based products by CE-C^4D.

CHAPTER 2

LITERATURE REVIEW

2.1 Overall view of fishing industry in Malaysia

Fisheries and fish based food industries are the backbone industry in Malaysia. Fish and fish based products are an important source of protein for Malaysians whereby fish contributes about 60-70% of animal protein intake. Per capita fish consumption in 2003 was about 51.4 kg per year as reported by Ministry of Agriculture Malaysia in 2004. The value is expected to increase to 56 kg per year in 2010. The marine fish landing in 2013 was estimated worth RM11 billion with 1.5 million metric tonnes production [Department of Fisheries (DoF), 2014]. About 16% from the total marine productions were processed into value added products such as fish sausage (keropok lekor), fish crackers (keropok), fish cake, fish ball, etc. Processed marine food industries are dominated mainly by a small and medium scale family enterprise with a very few large producers cater for the export market. The people in this industry are either directly or indirectly involve in capture fisheries or related industry. Apart from value addition in food quality, the processed food industry arises from the need to utilize the abundance source of low value pelagic fishes available from by-catch, excess catch or unmarketable varieties available at lower price.

About 70% from fish landings are consumed fresh, whereas 30% productions are processed into various products such as trash fish and fish flour (51.8%); fish, prawn and cuttlefish crackers and satay (17.4%), and surimi products (11.5%) including fish ball, fish cake and similar products (DoF, 2010). Preserved products such as shrimp paste,

shrimp sauce, fish sauce, salted dried fish, dried anchovies, dried shrimp and cuttlefish contribute about 13.4% from total fisheries production (DoF, 2010). These are local favorite foods but with scarce information on BA content.

Keropok lekor or fish sausage and crackers are important source of protein for Malaysians. Good *keropok lekor* normally contained as much as 90% fish. They are consumed regularly as a snack or light meal for breakfast and evening tea. The fish sausage or crackers are made basically from two main ingredients; fish, shrimps or squid flesh and tapioca or sago flour and seasoned with salt, sugar and monosodium glutamate (MSG). Fish such as 'Ikan parang' (*Chirocentrus dorab*), 'ikan tamban beluru' (*Clupea leiogaster*) and 'ikan selayang' (*Decapterus macrosoma*) are preferred although other low value pelagic fishes such as ikan kerisi (threadfin bream), ikan lolong bara (ox-eyed scad) and others are also used in fish sausage. These small-scale industries are located mainly in the coastal areas of Kelantan, Terengganu, Pahang, Johor and Kedah (DoF, 2014). In 2010, there are about two thousand operators actively involved in the production of *keropok lekor* estimated about 30,000 to 60,000 tonnes a month. A large proportion are sold domestically with small amount are exported to Brunei and the Middle East (Yeap & Thong, 2010).

2.2 Food safety regulations in Malaysia

Food safety is regulated under Food Act, 1983 and Food Regulations, 1985 which covers the importation of foods into Malaysia as well as food quality control on local products. Under Food Act, 1985, fish sausage/crackers should contain fish protein not less than 15%. No regulation is set on the limit of BAs content in fish based-products.

However, many exporting countries are bound by the international regulation set by their importing countries which follow the Food and Drug Administration (FDA USA, 2001) or European Union (EU, 2005). In Malaysia, the Food Regulations was applied primarily in monitoring quality and food safety by issuance of health certificate for export to the EU countries which covers the preliminary, warranty, labelling, food additive & nutrient supplements, packages for food, incidental constituent, standards & particular labelling requirements [Ministry of Health, (MOH), 2015]. Table 2.1. Summaries the levels of biogenic amines in different types of food (page 8).

2.3 Toxicity of histamine (HIS) and other biogenic amines (BA)

Under Food Safety Regulation, HIS is the only BA with safe limit of 50 mg kg^{-1} (FDA USA, 2001). A hazardous level of HIS has been suggested at 500 mg kg^{-1} (FDA USA, 2001) whereas the European Union Regulations stated that the critical level of HIS is ranging from 100 to 200 mg kg^{-1} for fresh, processed, preserved or fermented products (EU, 2005). Higher levels (200 mg kg^{-1}) are recommended by other countries such as South Africa, Italy, Canadian Food Inspection Agency (2010), Australia, Germany, Finland and Switzerland (Carelli et al., 2007; Yong, Wen & Xiao, 2007; Auerswald et al., 2006; Lange et al., 2002; Vesciana-Nogues et al., 1997). High HIS intake has caused life threatening intoxication whereas ingestion of lower amount may cause food intolerance and food allergy. Food poisoning caused by HIS toxicity is manifested by clinical symptoms ranging from mild allergic reactions characterised by itching and skin rashes to more serious life threatening situation such as vomiting, diarrhea, fever, hypertensive crisis, difficulty in breathing, shock and death.

Table 2.1: Summaries of the levels of biogenic amines (BA) in different types of food.

Sample type	BA content (mean or range, mg kg^{-1})	Method of analysis	LOD & LOD	References
1. Fermented and packaged fish products (China)	Light cured horse mackerel: PUT, 64.5; CAD, 244; HIS, 21.3; TYR, 62.8; 2-PHE, 57.6; TRP, 15.4; SPM-ND, SPD-ND; Total BA, 466 Canned sardine: PUT, 1.75; CAD, 1.82; HIS, 7.46; TYR, ND; PHE, ND; TRP, 6.33; SPM, 0.4, SPD, ND; Total BA, 17.8.	RP-HPLC/ Fluorescence	0.02-0.24 mg kg^{-1}	Zhai et al. (2012)
2. Fish and fish products (Korea)	Salted mackerel: PUT, ND; CAD, 2.0; HIS, 0.5; TYR, 6.0; PHE, ND; TRP, 1.6; SPM, 2.0; SPD, 5.5 Canned mackerel: PUT, 2.3; CAD, 7.8; HIS, 1.4; TYR, 4.7; PHE, 1.2; ND, TRP, 3.2; SPM, 1.8; SPD, 4.3. Canned tuna: PUT, 1.8; CAD, 1.7; HIS, 1.4; TYR, 3.2; PHE, 0.2; TRP, 1.8; SPD, 3.0; SPM 4.4.	HPLC-PDA	0.02-0.07 mg kg^{-1}	Park et al. (2010)
3. Salted escolar roe (Taiwan)	PUT, 21.8; CAD, 17.2; HIS, 6.2; TYR, 24.8; PHE, 12.7; SPD, 51.3; SPM, 40.7.	RP-HPLC/UV-Vis	-	Hwang et al. (2012)
4. Salted fish products (Turkey and European Countries)	Anchovies in brine: PUT, ND-84.7; CAD, <0.67-142; HIS, <0.85-422; TYR, 2.0-62.6; PHE, <1.51-9.2; TRP, <1.8-44.4; SPM, 0.8-2.9; SPD, 0.1-25.8 *Lakerda* Turkish and Greek salted fish: PUT, 0.4-66.9; CAD, <0.67-112; HIS, 0.6-293; TYR, 0.6-523; PHE, 0.1-162; TRP, <1.8-9.4; SPM, <0.72-5.3; SPD, 5.6-79.2	HPLC-PDA	-	Koral et al. (2013)

Table 2.1. Continued

5.	HIS in anchovies products (Australia)	Anchovies in oil (n=45): HIS, ND-28.9%; <200 (68.9%), >200 (2.2%), Salted dried anchovies (n=34) – HIS, 55.9%; <200, 41.2%; >200, 2.9%.	HPLC/ Fluorescence	-	NSW Food Authority (2010).
6.	Selected Malaysian foods	Fish sauce *Budu*: PUT, 28.4-64.9; HIS, 99-373; TYR, 58-853; TRP, ND-318; SPD, 3.0-7.0. Shrimp sauce *cincalok*: PUT, 10.6-803; HIS, 6.8-258; TYR, 156-681; SPD, ND-2.2 Shrimp paste *belacan*: PUT, 658; HIS, 31.2; TYR, 242, TRP, ND; SPD, ND. *Ikan pekasam*: PUT, 416; HIS, 195; TYR, 369; TRP, 64.9; SPD, ND. Dried anchovies: PUT, 5.7; HIS, 3.2; TYR, 30.5; TRP, ND: SPD, 61.8	HPLC/UV-Vis	LOD – 4.43-7.43 µg L^{-1}, LOQ – 14.8-24.5 µg L^{-1} for PUT, TYR HIS, TRP, SPD.	Saaid et al. (2009)
7.	*Keropok lekor* (Malaysian fish sausage).	PUT, 4.72; CAD, 5.88; HIS, 6.97 (1-2 h cooling stage at room temperature)	HPLC/ Fluorescent	-	Norkhaizura et al. (2009)
8.	Traditional fish products (TFP) from Turkey.	TFP (n=59): HIS, 10.2% sample > 50, 27.1% < 30, 55.9% < 10 mg kg^{-1}.	ELISA (Food EIA), LDN, Germany.	Sensitivity <2.5 Quantitative, 2.5-250 mg kg^{-1}	Köse et al. (2011)
9.	Fish sauce *Budu*	HIS, 62.5-393; PUT, 5.6-243; CAD, 187 – 705 mg kg^{-1}.	HPLC/ PDA	LOD - HIS, 0.16; PUT, 0.09; CAD, 0.07 mg kg^{-1} LOQ – HIS, 0.53; PUT, 0.31; CAD, 0.24 mg kg^{-1}	Zaman et al. (2010)

Note; LOD - Limit of detection; LOQ - Limit of quantitation

HIS poisoning is often linked to tuna spoilage hence HIS toxicity is referred to as 'scombroid poisoning' (Hungerford, 2010; Lehane & Olley, 2000). Apart from tuna, HIS poisoning has been relating to intake of sardine, mackerel, bonito, mahi-mahi, blue fish, amberjack, anchovy, herring, marlin, trevally, oilfish, jack, shad and jobfish (McLauchlin et al., 2006). About 118 incidences of HIS poisoning were diagnosed from 463 cases of food poisoning in the United State of America from 1998 to 2002 (Lynch et al., 2006). The critical limit for HIS concentration that cause food poisoning varies between individual. Several reported cases showed that HIS poisoning occurred to several people after eating tuna burgers in a restaurant containing HIS level of 213 mg kg^{-1} (Becker et al., 2001). In another incidence, HIS poisoning was reported originating from eating canned anchovies containing 365 mg kg^{-1} HIS (NSW Food Authority, 2010).

Toxicity or food poisoning associated with other amines have rarely been reported. TYR is a vasoactive amine involved in hypertensive crisis (McCabe-Sellers, Staggs & Bogle, 2006) whereas PUT and CAD potentiate HIS toxicity (Shalaby, 1996; Hålasz et al., 1994). The toxic effect of HIS is increase in the presence of PUT and CAD due to their inhibition activities on HIS metabolizing enzymes in small intestine to detoxify the HIS from the body (Lehane & Olley, 2000; Arnold & Brown, 1978). PUT and CAD are reported to be carcinogenic when converted to nitrosamine (Warthesen et al., 1975; Bills et al., 1973). However, the information was insufficient regarding the risk assessment of these amines in foods or the limit for adverse health effects [European Food Safety Authority (EFSA), 2011]. PUT was found as the best indicator of decomposition for penaeid shrimp. The production of CAD followed a similar pattern to PUT in several

shrimp species (Benner et al., 2003) and in white prawn *Penaeus indicus* (Zhao et al., 2007).

2.4 Methods for biogenic amines analysis

Analysis of food is continuously requiring the development of more robust, efficient, sensitive, and cost-effective analytical method. Various methods have been used for BA analysis in complex food matrix. Several methods such as a reversed phase - high performance liquid chromatography (RP-HPLC), thin layer chromatography, gas chromatography-mass spectrometry, to name a few, are used for BA analysis (Awan, Fleet & Thomas, 2008; Moret et al., 2005; Lange, Thomas & Wittmann, 2002). Many commercial instruments available for chemical analysis are equipped with fluorescence/UV-vis detector. With fluorescence and UV-vis detection, derivatisation procedures (pre or post-column) need to be applied as BA lack of chromophores. There is other alternative whereby, derivatisation procedures are not used such as HPLC with conductometric (Cinquina et al., 2004), capillary zone electrophoresis (CZE) with amperometric detection (Sun, Yang & Wang, 2003) and Capillary electrophoresis with capacitively coupled contactless conductivity detection, CE-C^4D (Liu et al., 2014). BA analytes are detected based on the conductivity activity (measurement of an electrical activity) of charged analytes in a capillary passing through a detector which will be converted into a measureable signal (peak) that determined the strength or concentration of analytes using a suitable carrier (liquid/aqueous solution).

2.5 Reversed Phase - High Performance Liquid Chromatography (RP-HPLC)

A reversed phase-high performance liquid chromatography has been most generally used for determination of BA in different kind of foods. The reversed phase describes the use of a polar mobile phase and a non-polar (hydrophobic) stationary phase, as opposite to the normal chromatographic mode. RP-HPLC involves the separations of molecules on the basis of hydrophobicity. Chromatography separation processes occur within the column (stationary phase-hydrophobic) through the mobile phase, whereby the difference in the retention mechanism for molecules absorption or adsorption resulted in the molecules of interest being eluted out in the mobile phase (consisting of an aqueous blend of water, with a miscible polar organic solvent, such as acetonitrile or methanol). A C18–bonded silica (sometimes called ODS) or C8 column are the most popular type of reversed-phase HPLC packing used in BA analysis. An isocratic elution condition for the mobile phase can be program whereby the concentration of organic solvent in mobile phase is constant or with gradient elution program, the amount of organic solvent is increased gradually over a period of time. Biogenic amines as polar compounds will be eluted (separated) in order of its hydrophobic nature in the stationary phase whereas other solutes (proteins, polypeptides) will be absorpted to the hydrophobic surface of the column.

2.6 Capillary Electrophoresis (CE)

Since CE is the main analytical tool used in the studies, a brief introduction to this technique is presented. The CE technique can be traced back to the 1960s where separations was conducted in a small capillary tube filled with an electrolyte, with a strong

electric field applied along the capillary. The modern CE method basically comprises a group of similar related techniques but with different mechanisms of separation or detection. These techniques include capillary zone electrophoresis (CZE), capillary isoelectric focusing, capillary gel electrophoresis, micellar electrokinetic capillary chromatography, capillary isotachophoresis, and capillary electro chromatography. Traditional slab-gel electrophoresis is the CE-analog which has been extensively used for the separations of larger molecules or biological macromolecules such as oligonucleotides, DNA fragments and proteins.

The CZE is the most commonly used method for separation of both anionic and cationic solutes. This CE technique is also known as free solution capillary electrophoresis. The charged ions are separated by differences in their rates of migration through a fused, open or coated silica capillary. The capillary is filled with an electrolyte solution whereby both ends of the capillary platinum electrodes are immersed into a carrier electrolyte or known as the background electrolyte (BGE). A suitable detector is attached near the outlet (Fritz & Gjerde, 1987).

Modern CE consists of a high-voltage power supply, two buffer reservoirs, a capillary and a detector (Figure 2.1). The basic set-up was modernised with automation function such auto sampler, sample and capillary temperature control, programmable power supply, multiple injection devices, multiple auto detectors, fraction collection and computer interfacing software program.

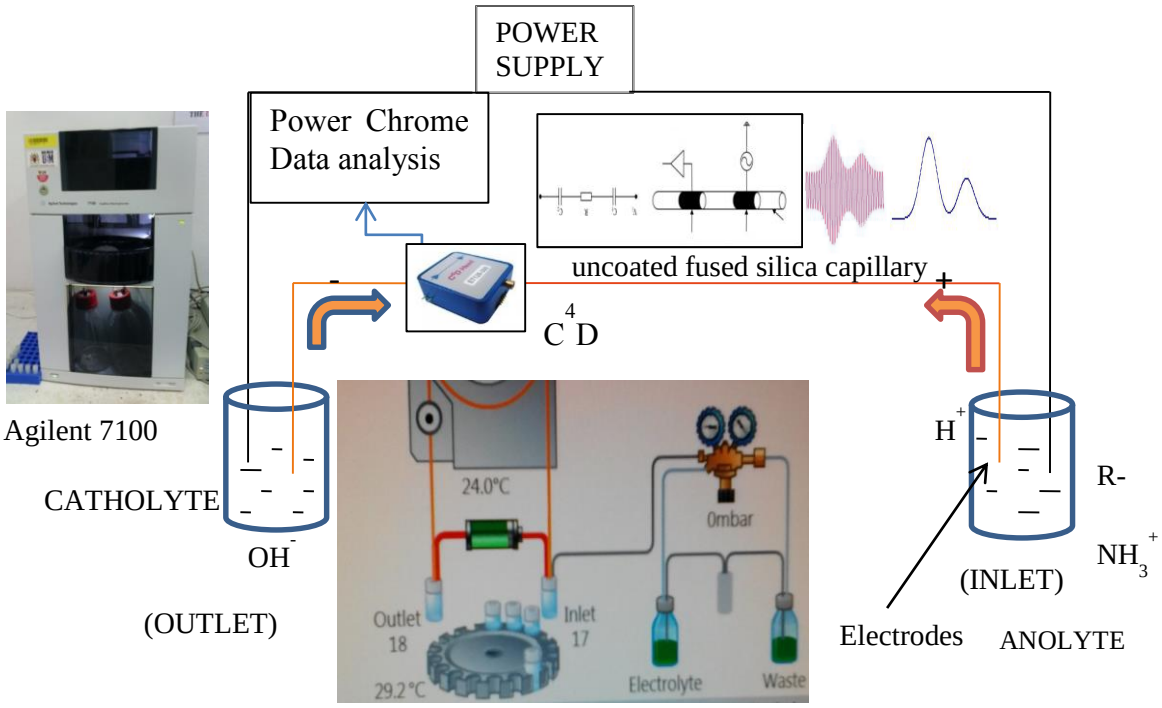

Figure 2.1: Basic setup of a CE-C^{4}D (Agilent 7100, Hanover, Germany)

Under high voltage power supply, the inner surface of a fused silica capillary tube which is covered with silanol groups (Si-OH), will become ionised to SiO^-. The negatively charged surface is counter balanced by positive ions (H^+) from the BGE, forming the double layer as shown in Figure 2.2. BA in an acidic aqueous solution at (pH >2) will become ionised.

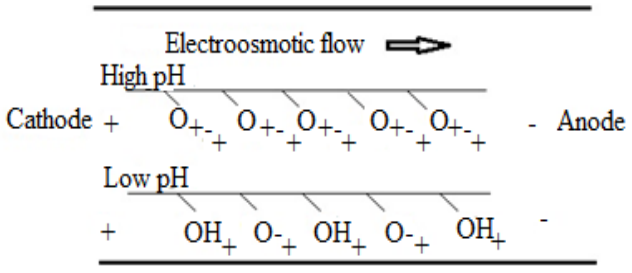

Figure 2.2: Effect of pH on the electro-osmotic flow (Beckman Coulter, Inc.)

Cations (H^+ charged ion) are attracted towards the cathode and their speed is supported by the electro-osmotic flow. Anions (OH^- charged ion), although electrophoretically attracted towards the anode, their movement are swept towards the cathode together with much stronger electro-osmotic flow of BGE. Under these conditions, cations with highest charge/mass ratio will migrate first followed by cations with lower ratios, anions with lower charge/mass ratio and anions with greater charge/mass ratio respectively. A conductivity activity (an electrical signal of charged ion (H^+) exchange between the capillary silanol group (SiO^-) and BGE (H^+ donor) and analytes of interest (protonated amines) within the capillary surface group will be detected and converted into measurable signal (peak area). A successful CE separation of complex analytes normally requires detailed studies of the most suitable BGE compositions.

2.7 Capacitively Coupled Contactless Conductivity Detection (C^4D)

The C^4D represents a simple and practical electrochemical detection technique which was first introduced for analysis of low molecular weight organic and inorganic compound in water. Contactless conductivity detector was created to overcome the problem of electrode fouling that is common with the normal conductivity detection. The C^4D used in this study was obtained from eDAQ Pty. Ltd. (Denistone, AU). It consists of two metal electrodes (1-2 mm in size, 2-3 mm in gap between them) that are placed around the separation capillary made from non-conducting material (e.g. fused silica, polyether ether ketone or Teflon), hence a conductivity activity of charged analytes inside the capillary will be measured. When an alternating current (AC) voltage is applied to the excitation electrode (first electrode), the current passes through the capillary wall, the gap between the electrodes or inside capillary, and then back to the second electrode.

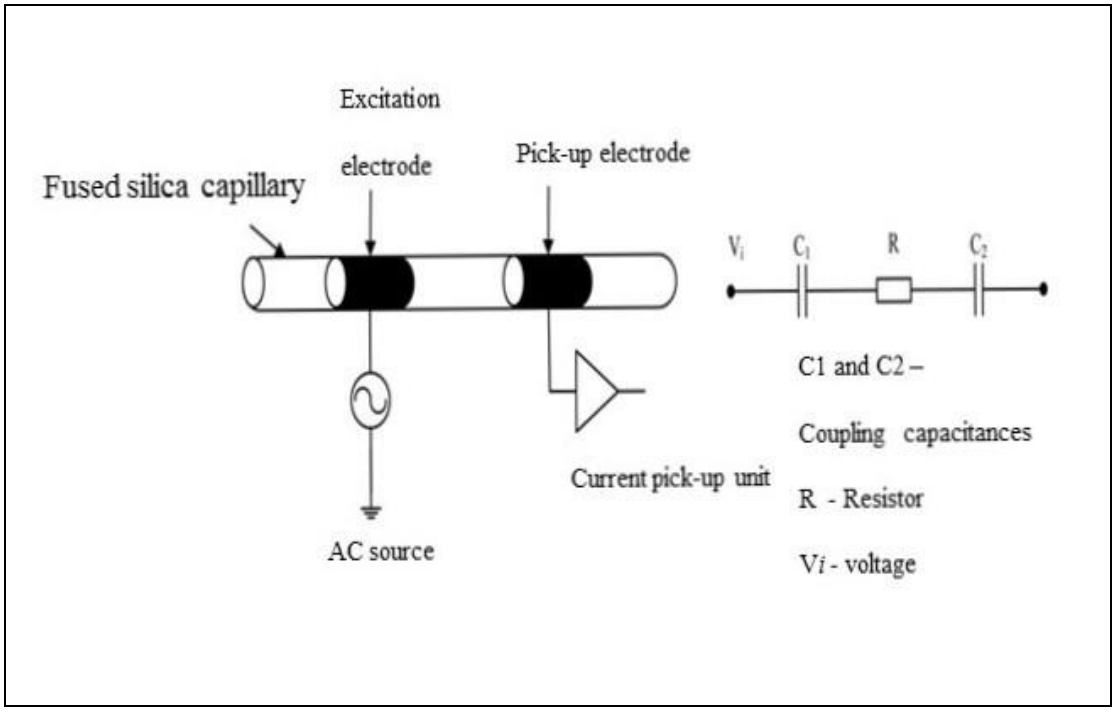

Figure 2.3: Schematic drawing of C^4D detector with attachment to fused silica capillary tube in capillary electrophoresis (Lyu et al., 2016).

The detected current from the second pick-up electrode (rectifier) is different than the one initially produced to the first excitation electrode (amplifier). The ions in the solution have different electrophoretic mobilities and thus, they pass through the detection region in different rhythm. Continuous recording of the current will display a series of peaks, which correspond to the ionic concentration or conductivity value of the ionic solution inside the capillary between the electrodes. Hence, changes in the conductivity of the solution inside the capillary between the electrodes can be monitored and measured (website:http://www.edaq.com/c4d-contactless-conductivity-introduction).

Initial studies on application of C^4D have been focused on inorganic species. However, it becomes apparent that this detector is also well suited for organic ions such as amino acids (Kuban & Hauser, 2004), sulfonic acids (Zemann et al., 1998), quaternary

amines (Gubits & Shcmid, 2004; Zemann et al., 1998), carboxylic acids and different classes of amines (Gong & Hauser, 2005; 2006); basic drugs (Tanyanyiwa & Hauser, 2004) and polysaccharides. CE-C^4D has been used for determination of different organic amines such as short chain aliphatic amines, secondary and tertiary amines, diamines, aromatic and non-aromatic enantiomers (Gong, 2008). Figure 2.4 shows molecular structures of eight important BA found in food.

The success of BA analysis in saliva was demonstrated using CZE-C^4D through a series of procedures (Liu et al., 2014). The saliva sample was initially purified and pre-concentrated using two electrochemical separation process after Electro Membrane Extraction via Supported Liquid Membrane (CZE-EME-SLM). The CE-C^4D was proven suitable for determination of SLM selectivity and separation of amines from complex biological fluid such as a saliva. The CE-C^4D has also been successfully applied for the simultaneous separation and detection of several basic drugs (nortriptyline, haloperidol and loperamine), inorganic cations, proteins and amino acids. However, the analysis of BA using this method in complex matrices such as in a food has not been reported. Table 2.2 Summaries the CE methods for determination of BA with various detector.

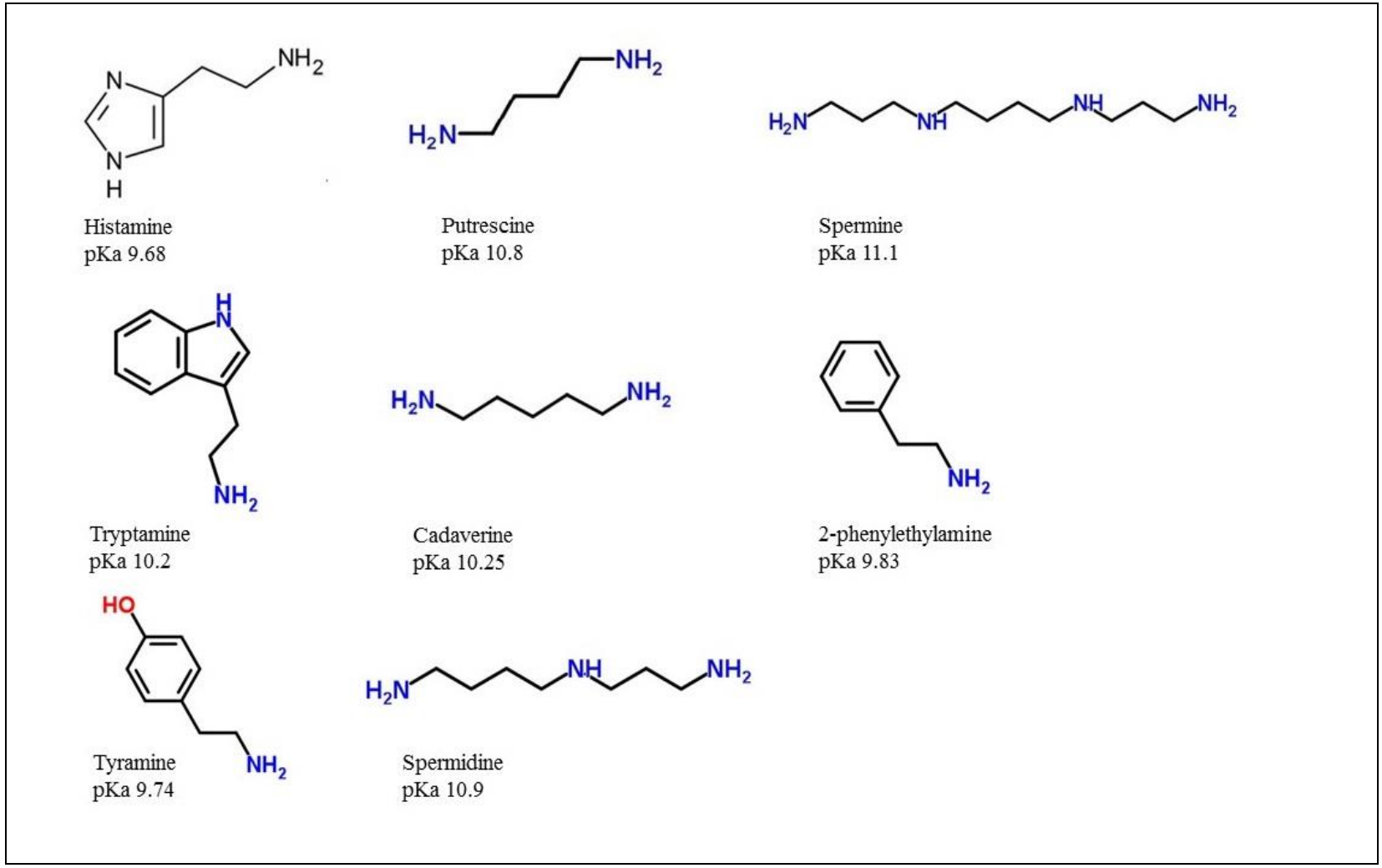

Figure 2.4: Molecular structures of important biogenic amines in food.

Table 2.2: Summaries of capillary electrophoresis (CE) methods for determination of biogenic amines (BA) with various detector.

Method	Sample	BA	LOD and LOQ	Advantage/ disadvantage	References
1. CE-tandem mass spectrometry (MS/MS)	Beer and wine	SPD, SPM, PUT, CAD, HIS, PHE, TRP, TYR, Urocanic acid (UCA)	LOD, 1-2 µg L^{-1} LOQ, 3-8 µg L^{-1}	Fast, direct detection, high sensitivity, expensive	Daniel & Bezerra (2015)
2. CE - LIF	Beer	CAD, PUT, HIS, TYR, TRP, PHE, …	LOD, 1-7.6 µg L^{-1} LOQ- 3.4-25.2 µg L^{-1}	Require derivatisa-tion (FITC)	Cortecero-Ramirez et al. (2007)
3. CE-UV/Vis (DAD)	Wine	PUT, CAD, HIS, TYR, PHE…	LOD, 0.05-0.18 µm L^{-1} LOQ, 0.18-0.6 µm L^{-1}	Sample clean-up with SPE before indirect detection by binding with $CuSO_4$	Arce, Rios & Valcárcel (1998)
4. CE-Ampero-metry	Milk	PUT, CAD, SPM, SPD	LOD, 1-4x10^{-7} M	Expensive (gold electrode)	Sun, Yang & Wang (2003)
5. CE-Contact Conductivity detection	Wine, beer, cheese	CAD, PUT, AGM, HIS, TRP and TYR	LOD, 2-5 µmol L^{-1} LOQ, 5-15 µmol L^{-1}	Electrode fouling	Kvasnička & Voldrich (2006)
6. CE-C^4D	Saliva	PUT, CAD, SPM, SPD	LOD- 1.4-7.0 ng mL^{-1} LOQ- 4.7-23.3 ng mL^{-1}	Laborious sample pre-treatment (EME-SLM), puri-fication and pre-concentration	Liu et al. (2014)

Note* - Limit of detection (LOD)
Limit of quantitation (LOQ)

CHAPTER 3

BIOGENIC AMINE ANALYSIS IN SHRIMP PASTE *BELACAN* OBTAINED FROM THE NORTHERN STATES OF PENINSULAR MALAYSIA

3.1 Introduction

Biogenic amines (BA) accumulation in fermented foods are influenced by many factors. In fermented fisheries products such as shrimp paste *belacan*, the freshness and the cleanliness of raw ingredients have a strong influence on the growth of spoilage microbes at the early stage of fermentation. Many common contaminants and normal flora of aquatic life found in fermented products, to some extent are BA-forming bacteria including some of the microbes involved in fermentation process. Thus, fermented foods normally contain a considerable amount of BA which can only be controlled through strict hygienic practices, use of fresh quality raw materials and where possible, additional microbial controls (EFSA, 2011). Common perception that highly salted preserved food is free from high BA accumulation is not entirely true as certain microbes are not totally killed or suppressed. Under suitable environmental conditions, over time, and with an optimal temperature or pH, microbial growth and proliferation will lead to BA accumulation and deterioration in food quality.

Fermented and preserved fish based products such as fish sauce, shrimp paste and fish paste have been produced and consumed largely within South East Asian countries. Shrimp paste is known as *belacan* in Malay language in Malaysia, Brunei and Singapore, *terasi* in Indonesia, *kapi* in Thailand, *bagoong alamang* in Philippines, *mamtom* or *mamruoc* in Vietnam, *nappi* in Bangladesh or *Saewoojeot* in Korea. It is made from a

fresh tiny shrimp called ‘geragau’ in Malay language or *Acetes* species or *mysid*. *Belacan* or shrimp paste is consumed almost on daily basis by Malaysians as well as people in the South East Asian region. *Belacan* is commonly added in cooking dish as food enhancer but most commonly consumed as appetizer known as *sambal belacan*, with addition of chillies, lime and some other ingredients that is taken as a dipping condiment on a regular basis.

High BA content in salted fermented fisheries products have been reported (Visciano et al., 2012; Kuda et al., 2011; Tsai et al., 2005; Mah et al., 2002). Several authors (Rosma et al., 2009; Stute et al., 2002) have reported high level of BA in fish sauce predominated by HIS, PUT, CAD and TYR. The HIS, PUT and CAD were found above the permitted level (1000 mg kg^{-1}) of safe products in fish sauce with maximum of 1220, 1257 and 1429 mg kg^{-1}, respectively (Tsai et al. 2006). In conclusion, fermented products from Taiwan such as fish sauce, fish paste and shrimp paste contain high level of HIS with an average of 394, 263 and 382 mg kg^{-1} (ppm), respectively. However, the HIS level varies between *belacan* products with 26% of fermented products contained more than 500 ppm of HIS (Tsai et al., 2005).

High PUT (658 mg kg^{-1}) and TYR (243 mg kg^{-1}) with HIS (31.2 mg kg^{-1}) below 50 mg kg^{-1} safe level was reported in local *belacan* products (Saaid et al. 2009). Many researchers have documented high BA content in certain fermented fish products including in local fish sauce *budu* (Zaman et al., 2010; 2009; Rosma et al., 2009). About 58% of unprocessed *budu* obtained from 12 producers in Malaysia were found to contain HIS above the safe level of 500 mg kg^{-1} (Rosma et al., 2009). Similarly, local products *budu* were reported to contain high HIS, PUT, CAD and TYR with maximum value of

1220, 1257, 1429 and 1178 mg kg^{-1}, respectively (Zaman et al., 2009). Different profile of BA was reported in different types of products but detailed information on the presence of BA in local *belacan* is still not thoroughly investigated partly due to the perception that highly salted (15% NaCl and above) dry formed fermented products are less prone to contain high BA than liquid form fermented sauce.

Although there were earlier studies on BA in *belacan* (Karim, 2013; Saaid et al., 2009), these activities were scarce. Hence, the objective of this study was to determine the level of important BA (HIS, CAD, PUT and TYR) in *belacan* by local producers from the Northern States of Peninsular Malaysia (Perlis, Kedah and Pulau Pinang) as well as products from other states found in local shops. The samples were obtained from *belacan* producing area (e.g., Kuala Perlis, Tanjung Dawai, Kuala Kedah, Balik Pulau) within the Northern region including several products from Selangor, Melaka and Perak. The BAs were analysed using a conventional standard method based on reversed phase - high performance liquid chromatography (RP-HPLC). Moisture, salt content (NaCl) and pH of these samples were also determined.

3.2 Materials and methods

For sodium chloride (NaCI) analysis, silver nitrate standard solution (0.1 M) and ammonium thiocyanate (NH_4SCN) standard solution (0.1 M) were obtained from R&M Chemicals (Malaysia). Saturated solution of ferric indicator was prepared using ferric ammonium sulphate ($FeNH_4(SO_4)_2.12H_2O$) (Merck, Germany) in deionised water (Nano-pure Diamond, Millipore, USA). For HPLC analysis, all chemicals and solvents used were of analytical and chromatographic grade, respectively. Standard putrescine dihydro

chloride, histamine dihydrochloride, cadaverine dihydrochloride, tyramine hydrochloride and dansyl chloride (DNS-Cl) were obtained from Sigma Aldrich (St. Louis, USA). Acetone and sodium hydroxide were from System Q'Rec (Malaysia), glutamic acid monosodium monohydrate was from Acros Organics (Switzeland). Sodium hydrogen carbonate ($NaHCO_3$) was from BDH Chemicals (England), hydrochloric acid (HCl) was from Q'rec (Malaysia) and trichloro- acetic acid (TCA) was from R & M Chemicals (Malaysia). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Fisher Chemical, USA), deionised water was obtained in the Laboratory (Nanopure Diamond, Millipore, USA) with purity critical value of 18.2 megohms (MΩ-cm) and used throughout the study.

Analysis of BA was carried out using HITACHI high performance liquid chromatography (HPLC, Tokyo, Japan) consisting of L-6200 Intelligent Pump with L-4250 UV-Vis detector (Plate 3.1) and manual injection loading using syringe injector (20 µL). The separation was achieved using ODS Hypersil HPLC column (LC-18, 25 cm x 4.6 mm, 5 µm size particle) (Thermo Scientific). Data was analysed using EDAQ PowerChrom 280 Ver. 2.7.2 software. The isocratic elution program was used with acetonitrile: water: methanol (60:25:15 v/v) as mobile phase at a flow rate of 1.0 mL min^{-1}. The mobile phase was filtered using membrane filter and degassed in an ultrasonic bath for 15 min prior to use. The detector was set at 254 nm.

Plate 3.1: HPLC unit used for the analysis of BA in *belacan*

3.2.1 A pH, sodium chloride (NaCl) and moisture analysis

10 g of sample in three replicates were homogenised in 10 mL of deionised water (pH 7.0) and the pH was measured using pH meter (OHAUS Starter 3100, NJ, US) (See appendix C- Plate 3.3). Three independent values were obtained from each sample, and means and standard deviations were calculated.

For NaCl analysis, shrimp paste sample was cut into small pieces using a sterile scalpel blade and about 1 g of sample was weighed into 250 mL Erlenmeyer flask. About 50 mL, 0.1 M $AgNO_3$ was added into the flask followed by 20 mL concentrated nitric acid (HNO_3). The sample in solution was thoroughly mixed by gentle shaking and boiled on sand bath until all solids, except AgCl, dissolved (about 15 min). The sample was cooled at room temperature, then was added with H_2O (50 mL) and 5 mL ferric indicator, and titrated with 0.1 M ammonium thiocyanate (NH_4SCN) until the mixed solution become permanent light brown (AOAC, 1995). The excess silver nitrate ($AgNO_3$) that was added