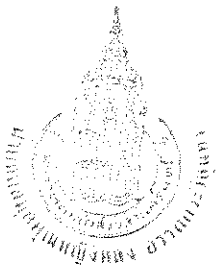

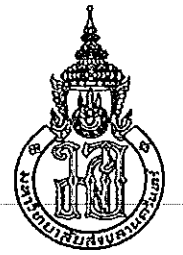

Development of the Gas Chromatographic Techniques for Seafood Freshness Determination (Dimethylamine and Trimethylamine)

Pornpimol Boonchai

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Analytical Chemistry

Prince of Songkla University

2007

Thesis Title	Development of the Gas Chromatographic Techniques for Seafood Freshness Determination (Dimethylamine and Trimethylamine)
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Major Program	Analytical Chemistry

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Dean of Graduate School

ชื่อวิทยานิพนธ์	การพัฒนาเทคนิคแก๊สโครมาโทกราฟีสำหรับการตรวจความสดของอาหารทะเล (ไดเมธิลามีนและไตรเมธิลามีน)
ผู้เขียน	นางสาวพรพิมล บุญชัย
สาขาวิชา	เคมีวิเคราะห์
ปีการศึกษา	2550

บทคัดย่อ

ตรวจวัดความสดของอาหารทะเลโดยการวิเคราะห์ปริมาณสารไดเมธิลามีนและไตรเมธิลามีนด้วยเทคนิคเฮดสเปซแก๊สโครมาโทกราฟีร่วมกับตัวตรวจวัดชนิดไนโตรเจนฟอสฟอรัส และใช้คาปิลารีคอลัมน์ชนิดเอชพี เอฟเอฟเอพี (HP-FFAP) ความยาว 25 เมตร ขนาดเส้นผ่านศูนย์กลางภายใน 0.32 มิลลิเมตร และความหนาของฟิล์ม 0.52 ไมโครเมตร สภาวะที่เหมาะสมของเครื่องแก๊สโครมาโทกราฟเป็นดังนี้ อัตราการไหลของแก๊สพา (ฮีเลียม) 3 มิลลิลิตรต่อนาที อุณหภูมิหัวฉีด 120 องศาเซลเซียส และอุณหภูมิตัวตรวจวัด 220 องศาเซลเซียส โปรแกรมอุณหภูมิคอลัมน์ อุณหภูมิเริ่มต้น 120 องศาเซลเซียส จนถึง 160 องศาเซลเซียส ด้วยอัตรา 30 องศาเซลเซียสต่อนาที และคงอุณหภูมิสุดท้ายไว้ 1 นาที และสภาวะที่เหมาะสมของเทคนิคเฮดสเปซคืออุณหภูมิที่ทำให้ตัวอย่างเข้าสู่สมดุล 70 องศาเซลเซียส โดยใช้เวลาที่เข้าสู่สมดุลเป็นเวลา 20 นาที และอัตราส่วนเฟส 2 โดยใช้ ขวดแก้วขนาด 10 มิลลิลิตร และเพิ่มสัญญาณให้สูงขึ้นโดยการเติมเกลือโซเดียมคาร์บอเนต 1.5 กรัม

สภาวะที่เหมาะสมของเครื่องแก๊สโครมาโทกราฟและเทคนิคเฮดสเปซให้ขีดจำกัดการตรวจวัดความสดของอาหารทะเลสารไดเมธิลามีนและไตรเมธิลามีนที่ 2.7 และ 0.30 นาโนกรัมต่อมิลลิลิตรตามลำดับ โดยมีช่วงความเป็นเส้นตรงของไดเมธิลามีน 2.7×10^{-3} ถึง 250 ไมโครกรัมต่อมิลลิลิตร สำหรับไตรเมธิลามีน อยู่ในช่วง 0.30×10^{-3} ถึง 50 ไมโครกรัมต่อมิลลิลิตร ด้วยค่าสหสัมพันธ์เชิงปริมาณ (coefficient of determination, R^2) มากกว่า 0.99 ความแม่นยำดีมากโดยให้ค่าความเบี่ยงเบนมาตรฐานสัมพัทธ์น้อยกว่า 4 เปอร์เซ็นต์

วิธีการวิเคราะห์ในงานวิจัยนี้นำไปใช้ตรวจวัดความสดของอาหารทะเล (ไดเมธิลามีนและไตรเมธิลามีน) ในตัวอย่าง ปลาทู ปลากะพง กุ้งกุลาดำและกุ้งขาว พบว่าให้ค่าเปอร์เซ็นต์การได้กลับคืนสำหรับสารไดเมธิลามีนและไตรเมธิลามีนในช่วง 53 ถึง 70 เปอร์เซ็นต์โดยมีค่าความเบี่ยงเบนมาตรฐานสัมพัทธ์น้อยกว่า 11.8 เปอร์เซ็นต์ จากการศึกษาพบว่าความเข้มข้นของไดเมธิลามีนและไตรเมธิลามีนในตัวอย่างปลาทู ปลากะพง กุ้งกุลาดำและกุ้งขาว อยู่ในช่วงไม่สามารถ

ตรวจวัดได้ถึง 7.69 มิลลิกรัมต่อหนึ่งร้อยกรัมของตัวอย่าง ซึ่งพบว่าต่ำกว่าข้อกำหนดของ EU ที่กำหนดให้อาหารทะเลสดมีไตรเมธิลามีนไม่เกิน 12 มิลลิกรัมต่อหนึ่งร้อยกรัมของตัวอย่าง

ข้อดีของวิธีวิเคราะห์ไดเมธิลามีนและไตรเมธิลามีนในงานวิจัยนี้เป็นวิธีที่ง่ายและทำได้รวดเร็ว ใช้ปริมาณตัวอย่างน้อย ปราศจากการใช้ตัวทำละลายอินทรีย์และค่าใช้จ่ายน้อย สามารถนำไปประยุกต์ใช้ในการตรวจวัดความสดของอาหารทะเล

Thesis Title	Development of the Gas Chromatographic Techniques for Seafood Freshness Determination (Dimethylamine and Trimethylamine)
Author	Miss Pornpimol Boonchai
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Academic Year	2007

Abstract

Headspace gas chromatographic technique with nitrogen phosphorous detector (HS-GC-NPD) and HP-FFAP capillary column, were investigated for freshness determination in fish and shrimp samples by analyzing dimethylamine (DMA) and trimethylamine (TMA). A capillary column (a 25 m × 0.32 mm i.d. × 0.52 μm film thickness) was used for separation. The optimum GC-NPD conditions were investigated and are as follows: flow rate of carrier gas (helium gas) 3 mL min^{-1}, injector and detector temperature 120°C and 220°C, respectively, column temperature programming 120°C increasing to 160°C with the temperature ramp rate of 30°C min^{-1} and kept at final temperature for 1 min. The optimum conditions for headspace technique are equilibration times 20 min, equilibration temperature 70°C, phase ratio 2, vial size 10 mL and 1.5 g of Na_2CO_3 was added in sample for increasing sensitivity of the analytes.

At optimum HS-GC-NPD conditions the limit of detection were 2.7, 0.30 ng mL^{-1} for DMA and TMA, respectively. The linear dynamic range were 2.7×10^{-3}-250 μg mL^{-1} for DMA and 0.30×10^{-3}-50 μg mL^{-1} for TMA with a coefficient of determination (R^2) greater than 0.99. Excellent precision was obtained with relative standard deviation (%RSD) less than 4%.

The proposed method was applied to test DMA and TMA in Indian mackerel, Sea bass, Giant tiger shrimp and White shrimp samples. The recoveries ranged from 53 to 70% for both analytes with %RSD less than 11.8% (n=5). The concentrations of DMA and TMA in these samples were in the range ND-7.69 mg per

100 g by matrix match calibration curve. These are acceptable since they are less than the EU acceptable quality and safety limit (TMA, 12 mg per 100 g).

The proposed method showed advantages over the conventional method *i.e.,* rapid and simple, small sample size, solvent free and cost effective for freshness (DMA and TMA) analysis in seafood and can be applied for freshness determination.

Acknowledgements

The completion of this thesis would be impossible without the help of many people, whom I would like to thank.

I express my sincere thanks to my advisor Associate Professor Dr. Proespichaya Kanatharana and co-advisor Associate Professor Dr. Panote Thavarungkul for their valuable advices and suggestions throughout the course of this work.

I would also like to thank:

The examination committee members of this thesis for their valuable time;

Higher Education Development Project: the Center for Innovation in Chemistry: Post Graduate Education and Research Program in Chemistry (PERCH-CIC), funded by the Royal Thai Government, for the scholarship and research supporting;

The Chemistry Department and Graduate school, Prince of Songkla University;

My parents, my sister, my brother and my best friends for their loves and attentions throughout my life;

Staffs of the Department of Chemistry for their helps in some technical aspects of this thesis;

And finally, friendship of members in Analytical and Environmental Chemistry/Trace Analysis Research Unit and Biophysics Research Unit: Biosensors and Biocurrents who helped me in innumerable ways during these years.

Pornpimol Boonchai

The Relevant of the Research Work to Thailand

Development of the Gas Chromatographic Techniques for Seafood Freshness Determination (Dimethylamine and Trimethylamine) is a Master of Science Thesis in Analytical Chemistry. It is a basic research which creates a new knowledge to develop country.

This technique can be applied for quantitative analysis of target analyte by several governmental and private organizations in Thailand which are the Ministry of Public Health, the former Ministry of Comerce, the Ministry of Industry, the Ministry of Agriculture and Cooperatives and the Ministry of Education.

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List of Abbreviations

AOAC	Association of Official Agricultural Chemists
AsA	Ascorbic acid
ASTM	American Society for Testing and Materials
ATP	Adenosine-5'-triphosphate
BSC	Benzensulfonyl chloride
CuO	Copper oxide
DMA	Dimethylamine
2, 4-DNFB	2, 4-Dinitro fluorobenzene
Dns-Cl	Dansyl chloride
EPA	Environmental Protection Agency
EU	European Union
$Fe(CO)_5$	Iron pentacarbonyl
Fe_2O_3	Iron (III) oxide
FE-SEM	Field emission-scanning electron microscopy
FIA	Flow injection analysis
FITC	Fluorescein isothiocyanate isomer I
FMO3	Flavin-containing monooxgenase type 3
FMOC	9-fluorenyl- methylchloroformate
GC	Gas chromatograpy
GC-NPD	Gas chromatograph with nitrogen phosphorous detector
H_3BO_3	Boric acid
HETP	Height equivalent to a theoretical plate
HPLC	High performance liquid chromatography
HS	Headspace
HS-SDME	Headspace single drop microextraction
HS-SPME	Headspace solid phase microextraction
IMP	Inosine monophosphate
In_2O_3	Indium oxide
*iso*BOC	Isobutyloxycarbonylation

List of Abbreviations (Continued)

LDR	Linear dynamic range
LEDs	Light emitting diodes
LIF	Laser-induced fluorescence
LLE	Liquid liquid extraction
LOD	Limit of detection
MgO	Magnesium oxide
MOCVD	Metallorganic chemical vapour deposition
MSD	Mass spectrometric detector
$Na_2B_4O_7$	Sodium tetraborate
ND	Not detectable
NPA-OSu	*N*-hydroxysuccinimidyl 4,3,2'-naphthapyrone-4-acetate
NPD	Nitrogen phosphorous detector
NS	Not sampling
OPA	*O*-phthalaldehyde
PDMS	Polydimethylsiloxane
PPIA	2-(2-phenyl-1H-phenanthro-[9,10-d]imidazole-1-yl)-acetic acid
ppm	Parts per million
PDMS/DVB	Polydimethylsiloxane/divinylbenzene
PECVD	Plasma enhanced chemical vapour deposition
PVP	Polyvinylpyrrolidone
QIM	Quality index method
R^2	Coefficient of determination
RF	Radio frequency
RSD	Relative standard deviation
SAMF	6-Oxy-(*N*-succinimidyl acetate)-9-(2'-methoxycarbonyl) fluorescein
SAX	Strong anion-exchanger
SIBA	*N*-succinimidyl benzoate
SIPA	*N*-hydroxysuccinimidyl phenylacetate

List of Abbreviations (Continued)

SnO_2	Tin oxide
SPE	Solid phase extraction
SPME	Solid phase microextraction
SSO	Specific spoilage organism
TCA	Trichloroacetic acid
TiO_2	Titanium dioxide
TMA	Trimethylamine
TMAO	Trimethylamine oxide
TVB-N	Total volatile basic nitrogen
TVC	Total viable counts
US EPA	Unite State Environmental Protection Agency
VOCs	Volatile organic compounds
UV	Ultraviolet
WCOT	Wall-coated open tubular
Y_2O_3	Yttrium oxide
ZnO	Zinc oxide

CHAPTER 1

Introduction

1.1 Introduction

Thailand is one of the world exporters of seafood. The United State of America is the most important market for Thai seafood exporter, with 30.95% of the total exports, followed by Japan, 28.47%, Europe, 8.54%, Asia, 5.49% and other 26.55% (www.foodmarketexchange.com, 2002; Ministry of Agriculture and Cooperatives, 2002).

Freshness is one of the most important criteria for quality of aquaculture products including fish and shrimp. Physical parameters *i.e.*, color, smell, texture could not be used as a scientific indication for fresh fish. However, two aliphatic amines, dimethylamine (DMA) and trimethylamine (TMA) can be used for freshness indication. Most marine fish species and fishery products have metabolize processes, trimethylamine oxide (TMAO) content in seafood product is reduced by bacterial enzymes (trimethylamine oxidase) to trimethylamine (TMA), whereas endogenous enzymes (TMAO dimethylase) reduce TMAO to dimethylamine (DMA) and formaldehyde are shown in Figure 1.1. The concentration of the two amines, TMA and DMA, increases when the freshness decreases. Trimethylamine was used as a quality index of fish spoilage (Pacquit *et al.*, 2006) and the regulation of this compound in fish products varies from country to country. The EU acceptable quality and safety limits are set at 12 mg per 100 g for TMA (The European Commission Council Regulation No. 91/493/EEC, 1991). For DMA, although there is no specific limit but it can react with nitrile to form a nitroso-dimethylamine (carcinogen compound).

The Association of Official Agricultural Chemists (AOAC) recommends a method for the determination of trimethylamine based on extraction of the amine in toluene and subsequent reactions with picric acid (AOAC, 1990). Other developed methods are still quite complicated and time consuming. Therefore, this

work proposed an alternative simple technique for seafood freshness *i.e.*, TMA and DMA analysis.

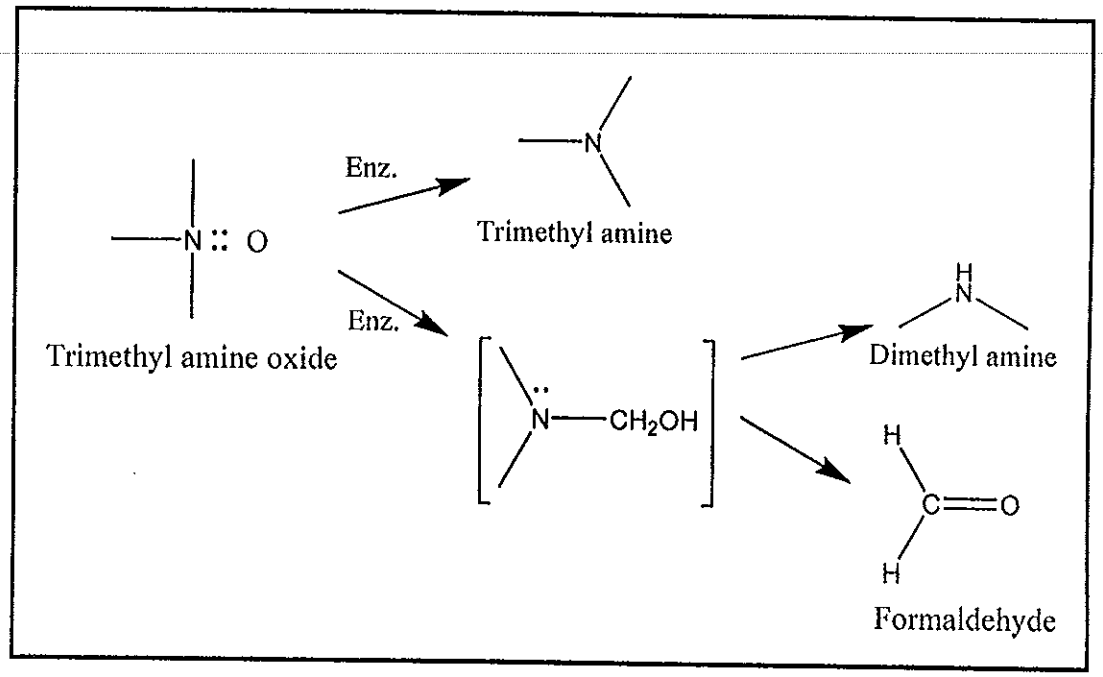


Figure 1.1 Mechanisms of trimethylamine and dimethylamine (Lindsay, 1996)

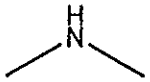
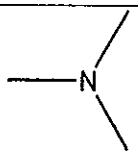
1.2 Background

1.2.1 Chemical identification

Dimethylamine, DMA is a secondary amine. The molecule consists of a nitrogen atom with two methyl substituents and one proton. Dimethylamine is a base and the pKa of the ammonium salt is 10.73. Dimethylamine reacts with acids to form salts.

Trimethylamine, TMA is a tertiary amine. The molecule consists of a nitrogen atom with three methyl substituents. Trimethylamine is a base and the pKa of the ammonium salt is 9.79. Chemical identification of DMA and TMA are shown in Table 1.1.

Table 1.1 Chemical identification of DMA and TMA

Properties	DMA	TMA
Molecular formula	C_2H_7N, CH_3NHCH_3	C_3H_9N, $CH_3N(CH_3)_2$
Synonyms	Aminomethylmethane N-methylmethanamine	Dimethylaminomethane N,N-dimethylmethamine
Structure	 dimethylamine	 trimethylamine
CAS number	124-40-3	75-50-3

Source: Mackay *et al.*, 2006

1.2.2 Physical and chemical properties

Dimethylamine is an organic amine compound, colorless, liquefied and flammable gas with an ammonia and fishlike odor. Dimethylamine is generally used as a solution in water at concentrations up to around 40%. Dimethylamine is used as dehairing agent in tanning, in dyes, in rubber accelerators, in soaps and cleaning compounds and as an agricultural fungicide. In industry dimethylamine is converted to dimethylformamide and the surfactant lauryl dimethylamine oxide. It is a raw material in the production of many pharmaceuticals such as diphenhydramine and also that of the chemical weapon tabun. DMA undergoes nitrosation under weak acid conditions to give dimethylnitrosamine. This is the animal carcinogen that detected and quantified in human urine samples and it may also arise from nitrosation of DMA by nitrogen oxides present in acid rain in highly industrilized countries (Zhang *et al.* 1998).

Trimethylamine is a colorless, hygroscopic, and flammable simple amine with a typical fishy odour in low concentrations and an ammonia like odor in higher concentrations. Trimethylamine has a boiling point of 2.9°C and is a gas at

room temperature. Trimethylamine is a product of decomposition of plants and animals and nitrogenous base and its positively charged cation is called trimethylammonium cation. A common salt of trimethylamine is trimethylammonium chloride, a hygroscopic colorless solid. It is the substance mainly responsible for the fishy odor often associated with fouling fish, bacterial vagina infections, and bad breath. It is also associated with taking large doses of choline and carnitine. Moreover trimethylamine is used in the chemical synthesis of choline, tetramethylammonium hydroxide, plant growth regulators, strongly basic anion exchange resins, and dye leveling agents. Trimethylaminuria is a genetic disorder in which the body is unable to metabolize trimethylamine from food sources. Patients develop a characteristic fish odour of their sweat, urine, and breath after the consumption of cholinerich foods. Trimethylaminuria is an autosomal recessive disorder involving a trimethylamine oxidase deficiency. Physical and chemical properties of DMA and TMA are shown in Table 1.2.

Table 1.2 Physical and chemical properties of DMA and TMA

Properties	DMA	TMA
Molecular weight (g mol^{-1})	45.084	59.110
Density (g/cm^3 at 20°C)	0.6556	0.6356
Color	Colorless	Colorless
Vapour pressure (Pa at 25°C)	206180	219000
Solubility in water (g/m^3)	620000 (25°C)	890000 (30°C)
Melting point (°C)	−92.18	-117.1
Boiling point (°C)	6.88	2.87
Log K_{ow}*	-0.38	0.20

Source: Mackay *et al.*, 2006

*Log K_{ow}: Partition coefficients octanol-water

1.3 Methods to evaluate fish quality

The quality of fish or fishery products can be evaluated based on freshness indicators *i.e.,* sensory, microbial, volatile compounds, lipid oxidation, Adenosine-5'-triphosphate (ATP), K-value and physical.

1.3.1 Sensory evaluation

Sensory evaluation is defined as the scientific discipline used to evoke, measure, analyze and interpret characteristics of food as perceived by the senses of sight, smell, taste, touch and hearing. Sensory tests can be divided into three groups: discriminating tests, which indicate whether there is a difference between samples; descriptive tests; and affective tests (Ólafsdóttir *et al.*, 1997). Characteristic sensory changes occur in the appearance, odour, taste and texture of fish when they deteriorated. Sensory evaluation is important for assessment of the freshness and quality and commonly used in the fish sector and fish inspection services (Luten and Martinsdottir, 1997). The quality index method (QIM) is a grading system for estimating the freshness and quality of seafood, which has been demonstrated to be rapid for many fish species (Larsen *et al.*, 1992). QIM is composed of precise descriptions of quality parameters may be used to predict the remaining shelf life of fish (Luten and Martinsdottir, 1997). The QIM is based on selecting a number of qualities attributes characteristics for a particular species and allocating scores to each attribute depending on the state of freshness or quality of the selected food item. The scores are assigned in whole numbers ranging from 0 to 3 for fresh. The most commonly used attributes for fish are the appearance of eyes, skin and gills together with odour and texture (Sveinsdottir *et al.*, 2003).

1.3.2 Microbial methods

The activity of microorganisms is the main factor limiting the shelf life of fresh fish. An estimation of the total viable counts (TVC) is used as an acceptability index in standards, guidelines and specifications (Ólafsdóttir *et al.*,

1997). During chill storage, psychrotolerant microorganisms are selected differential counting of these microorganisms. More recently, the bacterium *Shewanella putrefaciens*, which produces hydrogen sulphide was used to determined the specific spoilage organism (SSO) of some chilled fresh fish. Most marine fish spoilage bacteria reduce trimethylamine oxide to trimethylamine (TMA). Microbial methods can provide useful measures of fish freshness. However, the most promising results have been achieved with relatively slow detection methods such as plate count and other growth techniques that involve a period of incubation. At the point of sensory rejection, the TVC of fish products are typically 10^7-10^8 cfu/g (Anon, 1995).

1.3.3 Volatile compounds

Odour is one of the most important parameters used to evaluate fish freshness. Measurements of characteristic volatile compounds *i.e.*, trimethylamine, dimethylamine and ammonia can be used to monitor the freshness or spoilage stage of fish. Classical chemical methods for the analysis of total volatile bases have been used for the determination of fish freshness in the industry (Ólafsdóttir *et al.*, 1997). Many method were used for volatile compounds analysis *i.e.*, spectrophotometry, gas chromatograpy (GC), high performance liquid chromatography (HPLC) and sensor. Moreover, the sample preparation techniques *i.e.*, clean-up samples were used for increasing the sensitivity of analytes.

1.3.4 Lipid oxidation

The highly unsaturated lipid of fish is easily oxidized, resulting in alterations in smell, taste, texture, colour and nutritional value. Oxidation starts immediately after catch, but becomes particularly important for shelf life only at temperatures < 0°C, the oxidation becomes the major spoilage factor rather than microbial activity (Ólafsdóttir *et al.*, 1997). The initiation of lipid oxidation arises from various early postmortem changes in fish tissues. These changes include the accumulation of active oxygen species, the activation of haemoproteins, an increase in free iron and the consumption of antioxidants (Hultin, 1994). The loss of fatty acids

and antioxidants can be measured by GC and HPLC (Erickson, 1993). Lipid oxidations are suitable for some species of fish, to use more than one method, especially when comparing difference types of fish products. In addition, the instability of the various oxidation products makes the results difficult to interpret and extremely misleading (Ólafsdóttir *et al.*, 1997).

1.3.5 Adenosine-5'-triphosphate (ATP)

The degradation of ATP could be used as the indication for the freshness of fish according for the K value (the ratio of the sum of inosine and hypoxanthine concentration to the total concentration of ATP metabolite). Since after the fish death, ATP is rapidly degraded to inosine monophosphate (IMP) by endogenous enzymes (autolysis). The further degradation of IMP to inosine and hypoxanthine is much slower, and is catalysed mainly by endogenous IMP phosphohydrolase and inosine ribohydrolase, with a contribution from bacterial enzymes as storage time increases. The degradation of ATP was found to parallel the perceived loss of freshness of fish as determined by trained analysts. ATP as a chemical indicator of freshness but ATP alone cannot be used because it is so rapidly converted to IMP. Concentrations of its intermediate degradation products rise and fall making them unreliable indexes of freshness. As a result, attention has focused on inosine and hypoxanthine, the terminal catabolites of ATP. Inosine accumulates in some species of fish whereas hypoxanthine accumulates in others as terminal catabolites.

A fresh fish will have a low K value. There is abundant evidence in the literature to suggest that the K value is a reliable indicator of freshness that is applicable for frozen fish, smoked fish and fish stored under modified atmospheres (Gill, 1995). It varies between species to species since it has differences in rates of ATP degradation. It also varies with postmortem time and temperature storage conditions, handling conditions (Hattula, 1995).

1.3.6 K-value measurement

The K value is used as a freshness index which the fresh fish will have a low K value. It varies between species to species that owing differences rates of ATP degradation. It also varies with postmortem time and temperature storage conditions (Hattula, 1995). There is abundant evidence in the literature suggested that the K value is a reliable indicator of freshness that is applicable for frozen fish, smoked fish and fish stored under modified atmospheres (Gill, 1995). However this technique is not widely used in industry because it was time consuming and expensive involved in the measurements.

1.3.7 Physical measurements

Physical changes in fish that result in the freshness are mainly related to structure and colour. Texture measurements can be used to determine structural changes. The instruments used are texturometers fitted with a wide variety of accessories for the different types of analyses. The texture of whole fish muscle is difficult to measure because it lacks of a uniform structure, difficult to prepare of standard size samples and variety of sample preparation procedures (Chamberlain and Kow, 1994).

1.4 Determination of dimethylamine and trimethylamine

Aliphatic amines are found in many different matrices such as in waste water, air and food. Especially food matrices are seafood samples, its have volatile amine *i.e.,* dimethylamine and trimethylamine. These are produced by microbial degradation from trimethylamine oxide (TMAO) (Sadok *et al.*, 1996) and used as an indicator for freshness in fish and fishery samples (Ólafsdóttir *et al.*, 1997).

The determination of trimethylamine and dimethylamine in seafood are reported by spectrophotometry (Loughran and Diamond, 2000), capillary electrophoresis (Timm and Jørgensen, 2002), sensors (Pacquit *et al.*, 2006; Zhao *et al.*, 2002), liquid chromatography (Teerlink *et al.*, 1997) and gas chromatography

(Krzymien and Elias, 1990; Veciana-Nogues *et al.*, 1996; Sukpeng, 2001; Béné *et al.*, 2001). Generally, the analytical method chosen involves the use of a separation technique with a suitable detector for identification and quantification of dimethylamine and trimethylamine in the samples.

1.4.1 Spectrophotometric detection

During the part few years, spectrophotometric detection was used in a flow injection analysis (FIA) system for determination of trimethylamine in seafood samples. Direct spectrophotometric measurement at maximum wavelength of 635 nm was carried out. Interferences from other volatile amines present in the extract were suppressed by formaldehyde (Sadok *et al.*, 1996). The forming of colour complexes was usually used to improve trimethylamine and dimethylamine absorbtivity. The linear range between 0-200 μmol L^{-1} (r=0.999) with an RSD of 1.15% (n = 10) at 50 μmol L^{-1} trimethylamine and a limit of detection of 6 μmol L^{-1} (= 0.53 μg TMA g^{-1} of wet tissue under these conditions and in this study) were reported (Sadok *et al.*, 1996). The recovery of TMA from muscle tissue extracts were in the range 99.62-100.46% by spiking extracts of different fish at 50 μmol L^{-1} (Sadok *et al.*, 1996).

Loughran and Diamond (2000) reported the increasing in colour of a sensitive acidochromic dye and absorbance center on the calix[4]arene-based dye at 500-510 nm, calix[4]arene dye can be greatly enhanced thought the formation of Li^{+}-dye complex, which enables it to detect lower volatile bases such as trimethylamine, dimethylamine and ammonia from fish samples. In 2003, Mohammed-Ziegler and coworker reported the increasing absorbtivity by the formation of a complex with chromogenic bridged calixarenes. Namieśnik and coworker (2003) reported the amines determination by derivatization method to increase the absorptive in ultraviolet (UV). Recently, Armenta and coworker (2006) developed a rapid method for determination of trimethylamine in fish and cephalopod samples by extracting with trichloroacetic acid (TCA) followed the on-line vapour phase generation fourier transform infrared spectrophotometry.

The advantages of spectroscopic methods are their ability to provide rapid analysis and simultaneous evaluation of several parameters. These techniques

have been introduced into seafood analysis. Fluorescence spectra can be used to indicate whether a fish has been frozen, and the intensity of the fluorescence from fish muscle reported the use of absorbance spectroscopy in the UV-visible range to determine the freshness of yellow fin tuna. Some disadvantages are spectroscopic methods have so far not proven sufficient to characterite fully the properties of fresh fish, need further developments in instrumentation and techniques for evaluation of spectral data (Ólafsdóttir *et al.*, 1997).

1.4.2 Sensor

Modern analysts are continuing to develop sensor systems that are simpler, less expensive and more suitable for personal and domestic applications. During the past decade, much effort has been paid to develop various DMA and TMA sensors, most of which are based on the DMA and TMA induced electrical conductivity changes of semiconductor metal oxides *i.e.*, In_2O_3 (Takao *et al.*, 1995), ZnO (Kwon *et al.*, 1998; Tang *et al.*, 2000), TiO_2-Fe_2O_3 (Dai, 1998), SnO_2 (Zhao *et al.*, 2000; Niranjan *et al.*, 2002; Hammond *et al.*, 2002), CuO (Hammond *et al.*, 2002) and Y_2O_3 (Zhang *et al.*, 2005). The enhancement of sensitivity and selectivity of DMA and TMA gas sensor on semiconductor metal oxides have been reported *i.e.*, In_2O_3 doped with 5 mol% MgO (In_2O_3-MgO (5 mol%)) and the sensor loaded with 3.0 wt.% Pt exhibits the highest DMA sensitivity at 300°C and TMA could be detected more sensitively at 500°C. Higher sensitivity to DMA than TMA was due to the difference in oxidation process between DMA and TMA over the sensor material (Takao *et al.*, 1995), the TiO_2-Fe_2O_3 sensing thin films by depositing a ceramic substrates using $TiCl_4$ and $Fe(CO)_5$ as the precursors by means of plasma enhanced chemical vapour deposition (PECVD) technique. The gas sensing characteristics of the thin film sensors was measured for TMA (Dai, 1998). While Kwon and coworker (1998) improved the sensitivity and selectivity by the ZnO based thin film sensor was fabricated by a radio frequency (RF) magnetron sputtering method for TMA that responds well to the deterioration of a mackerel during storage (Kwon *et al.*, 1998). The nanosized ZnO was used to modified polyvinylpyrrolidone (PVP) with different molar ratios of Zn^{2+}: PVP prepared by a sol-gel method and characterized by field

emission-scanning electron microscopy (FE-SEM) for exhibited fairly excellent sensitivity, selectivity to TMA (Tang *et al.*, 2006). The other TMA gas sensor used the SnO_2-based by using metalorganic chemical vapour deposition (MOCVD) technique at 290°C gave the stability at 140 times (as long as 21 months) for detection freshness of fish and with little interference of NH_3 (Zhao *et al.*, 2000). Moreover, SnO_2 was modified with thorium (Zhao *et al.*, 2000; Niranjan *et al.*, 2002) to increase the oxidation state of SnO_2 film and the electron concentration in the positive space charge region was depleted (Zhao *et al.*, 2000) and at 225°C gave a maximum sensitivity of 1500 towords 800 ppm of TMA (Niranjan *et al.*, 2002). The catalytic chemiluminescent trimethylamine (TMA) sensor was developed by using the nanosized of Y_2O_3. In 1999, Saja and coworker developed TMA sensors based on thin films of lutetium bisphthalocyanine derivatives with a controlled thickness and designed for the evaluation of vapours produced by the decomposition of fish and seafood. The thickness and structure of the films have a marked influence on both the global responses and on the kinetic behaviour of the sensors when exposed to the TMA vapours. The stability was 96 hours when examined by continual introduction of TMA into the sensor. The linear ranges were 60-42,000 ppm and the detection limit was 10 ppm (Zhang *et al.*, 2005). Most of semiconducting metal oxide sensors are sensitive and stable. However, these techniques used high temperature condition and long time for modification of metal oxides and less application in real samples.

In another work, a highly sensitive sensor that does not need heating apparatus is more adaptable for real-time and on-line fish freshness assay *i.e.*, chemical sensor. Zhao and coworker developed a TMA vapour probe based on the sensitive membrane of chitosan which encapsulated pimelic acid on piezoelectric quartz crystal has been prepared for the assay of fish freshness. The response of the TMA probe is rapid and completely reversible at normal temperature and relatively high humidity. The probe has an exponential responses to TMA in the concentration range of 5-200 ppm, and the %RSD were less than 5 (Zhao *et al.*, 2002).

In addition, TMA gas sensor was constructed based on the biological element *i.e.*, a flavin-containing monooxgenase type 3 (FMO3, one of xenobiotic metabolizing enzymes) (Mistubayashi and Hashimoto, 2002; Mitsubayashi *et al.*, 2004) and a reaction unit with both gas and liquid cells separated by a porous poly

(tatrafluoroethylene) diaphragm membrane (pore size: 30-60 μm, thickness: 0.20 mm). A substrate regeneration cycle was applied to the FMO3 immobilized device in order to amplify the output signal by coupling the monooxygenase with a reducing reagent system of ascorbic acid (AsA) in phosphate buffer. The linear dynamic ranges were 0.52-105 ppm (Mistubayashi and Hashimoto, 2002), 1.0-50.0 mmol L^{-1} (Mitsubayashi *et al.*, 2004). The %RSD less than 3.29 (n=10) (Mistubayashi and Hashimoto, 2002) and good reproducibility (4.39%, n=5) (Mitsubayashi *et al.*, 2004) for fish freshness determination (Mitsubayashi *et al.*, 2004).

Pacquit and coworker (2006) developed a colorimetric sensor with the potential for real-time monitoring of fish freshness. This on-package sensor immobilized chemoreactive dye formulation and developed light emitting diodes (LEDs)-based reflectance colorimeter. The sensor response was found to correlate with bacterial growth patterns in fish samples. In 2007 they developed a smart packing for the monitoring of fish spoilage in the headspace of packaged fish. When fish spoils it releases a variety of basic volatile amines which are detectable with appropriate pH indicating sensors. These are prepared by entrapping within a polymer matrix a pH sensitive dye that responds, through visible color changes to the spoilage volatile compounds that contribute to a quantity known as total volatile basic nitrogen (TVB-N).

1.4.3 Capillary electrophoresis

Capillary electrophoresis technique with indirect UV detection have been applied for analytes of ammonia, dimethylamine, trimethylamine and trimethylamine-N-oxide in aqueous fish extracts. The detection limit for ammonia, dimethylamine, trimethylamine and trimethylamine-N-oxide was less than 0.04 mM (Timm and Jørgensen, 2002).

The potential of capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection for the separation and determination of dimethylamine (DMA) and other low-molecular-mass amines involving precolumn derivatization with fluorescein isothiocyanate isomer I (FITC) was investigated by Debek-Zlotorzynska and Maruszak (1998). They studied different variables that affect

derivatization (pH, FITC concentration, reaction time and temperature) and separation (buffer concentration, addition of various organic modifiers, applied voltage and length of capillary). The linearity, reproducibility and reliability of the method were reported. The estimated instrumental detection limit for a 2-s pressure injection of the FITC-DMA derivative was 50 pg mL^{-1} (10^{-9} M), excitation and emission wavelengths of 488 nm and 520 nm, respectively. However, for practical reasons, a minimum of 5 ng mL^{-1} DMA, they recommended to the derivatization (Debek-Zlotorzynska and Maruszak, 1998).

1.4.4 High performance liquid chromatography

The chemical characteristics of aliphatic amines, *i.e.,* DMA and TMA differ from those of amino acids due to its low molecular mass, high basicity and high volatility, which produces loss of analyte in diluted alkaline solutions. Thus it was necessary to modify the usual order of reagent addition of the derivatizing reagent (Rodríguez López *et al.*, 1996; Teerlink *et al.*, 1997; Busto *et al.*, 1997; Liu *et al.*, 2001; Meseguer Lloret *et al.*, 2002; Meseguer Lloret *et al.*, 2004; Cháfer-Pericás *et al.*, 2004; Cao *et al.*, 2005; Herráez *et al.*, 2006; You *et al.*, 2006; Xian-En *et al.*, 2007) to avoid the loss of DMA and TMA. The derivatization of aliphatic amines before analysis has been applied for determination of all analytes by using derivatizing agents *i.e.,* 9-fluorenyl-methylchloroformate (FMOC) (Rodríguez López *et al.*, 1996; Teerlink *et al.*, 1997; Herráez-Hernández *et al.*, 2006; Cháfer-Pericás *et al.*, 2004), *O*-phthalaldehyde (OPA) (Busto *et al.*, 1997), *N*-hydroxysuccinimidyl 4,3,2'-naphthapyrone-4-acetate (NPA-OSu)(Liu *et al.*, 2001), dansyl chloride (Dns-Cl) (Meseguer Lloret *et al.*, 2002; Meseguer Lloret *et al.*, 2004), 6-Oxy-(N-succinimidyl acetate)-9-(2'-methoxycarbonyl) fluorescein (SAMF) (Cao *et al.*, 2005) and 2-(2-phenyl-1H-phenanthro-[9,10-d]imidazole-1-yl)-acetic acid (PPIA) (You *et al.*, 2006; Xian-En *et al.*, 2007). The stability of derivative based on slightly basic medium; borate buffer at pH 8.0 (20°C, 5.70 min) (Rodríguez López *et al.*, 1996), pH 9.0 (Busto *et al.*, 1997; Cháfer-Pericás *et al.*, 2004) and pH 9.5 (Teerlink *et al.*, 1997); sodium borate buffer at pH 8.50 (Liu *et al.*, 2001); acetate-carbonate buffer at pH 9.5 (100°C, 10 min) (Meseguer Lloret *et al.*, 2002); acetone-hydrogencarbonate buffer at

pH 9.5 (Meseguer Lloret *et al.*, 2004) and H_3BO_3-$Na_2B_4O_7$ buffer pH 8.0 (Cao *et al.*, 2005). The derivatization of analyte reacts at room temperature (Liu *et al.*, 2001; Cao *et al.*, 2005; Xian-En *et al.*, 2007). In addition, the derivatization couple with the solid phase extraction (SPE) for pretreatment of analytes before analysis (Cháfer-Pericás *et al.*, 2004).

Reverse phase HPLC is the most popular approach owing to the hydrophobic interaction of the molecules with C_{18} or C_8 stationary phase. Many reports used C_{18} column (Rodríguez López *et al.*, 1996; Teerlink *et al.*, 1997; Busto *et al.*, 1997; Meseguer Lloret *et al.*, 2002; Meseguer Lloret *et al.*, 2004; Cháfer-Pericás *et al.*, 2004; Cao *et al.*, 2005; Herráez-Hernández *et al.*, 2006) and C_8 column (Liu *et al.*, 2001; You *et al.*, 2006; Xian-En *et al.*, 2007) for separation of amine derivatives. Both isocratic and gradient elution were described on the reports.

The most popular detector used to identify aliphatic amines include the UV spectrophotometric detection (Rodríguez López *et al.*, 1996; Meseguer Lloret *et al.*, 2002; Meseguer Lloret *et al.*, 2004; Cháfer-Pericás *et al.*, 2004), fluorescence detector (Teerlink *et al.*, 1997; Busto *et al.*, 1997; Liu *et al.*, 2001; Meseguer Lloret *et al.*, 2002; Meseguer Lloret *et al.*, 2004; Cao *et al.*, 2005; You *et al.*, 2006; Xian-En *et al.*, 2007), chemiluminescence detector (Meseguer Lloret *et al.*, 2004) and mass spectrometry (Xian-En *et al.*, 2007). The aliphatic amines derivative showed a fluorescence signal with excitation and emission wavelength *i.e.*, dimethylamine derivative (FMOC-DMA) of 265 and 310 nm, respectively (Rodríguez López *et al.*, 1996), the NPA-OSu derivatives at 353 nm and 422 nm, respectively (Liu *et al.*, 2001), the Dns derivatives at 350 nm and 530 nm, respectively (Meseguer Lloret *et al.*, 2002), SAMF derivatives at 484 nm and 516 nm, respectively (Cao *et al.*, 2005) and the PPIA derivatives at 260 nm and 380 nm, respectively (You *et al.*, 2006). In UV detection, the aliphatic amines derivatives were detected based on the derivertizing agent *i.e.,* dimethylamine derivative (FMOC-DMA) at 264 nm (Rodríguez López *et al.*, 1996), the Dns derivatives at 333 nm (Meseguer Lloret *et al.*, 2002) and FMOC-TMA at 262 nm (Cháfer-Pericás *et al.*, 2004).

The HPLC technique was used to determine aliphatic amines in a variety of matrices *i.e.,* serum, urine (Teerlink *et al.*, 1997), lake water (Liu *et al.*, 2001; Cao *et al.*, 2005), waste water, biological sample (You *et al.*, 2006; Xian-En *et*

al., 2007), red wine (Busto *et al.*, 1997; Cao *et al.*, 2005), white wine, cheese (Cao *et al.*, 2005), real water (Meseguer Lloret *et al.*, 2004), real environmental water (Meseguer Lloret *et al.*, 2002), ground water (Rodríguez López *et al.*, 1996) and sea water (Cháfer-Pericás *et al.*, 2004).

Performance of HPLC technique for determination of aliphatic amines showed the linearity ranges were 0.5-15 mg L^{-1} (Busto *et al.*, 1997), 10 to 250 mg L^{-1} (Meseguer Lloret *et al.*, 2002), 0.25-10.0 μg mL^{-1} (Cháfer-Pericás *et al.*, 2004) and 1-10 μg mL^{-1} (Herráez-Hernández *et al.*, 2006). The detection limits were 4.5×10^{-7} M (Rodríguez López *et al.*, 1996), 0.1 μmol L^{-1} (Teerlink *et al.*, 1997), 100-300 μg L^{-1} (Busto *et al.*, 1997), 1.0 fmol (Liu *et al.*, 2001), 2 μg L^{-1} for fluorescence detection and 3 μg L^{-1} for UV detection (Meseguer Lloret *et al.*, 2002), 0.15-0.9 μg L^{-1} (Meseguer Lloret *et al.*, 2004), 5-50 ng mL^{-1} (Cháfer-Pericás *et al.*, 2004), 2-320 fmol (Cao *et al.*, 2005), 250 ng mL^{-1}(Herráez-Hernández *et al.*, 2006) and 3.1-18.2 fmol (You *et al.*, 2006). The recoveries were 98.5% (Rodríguez López *et al.*, 1996), 99-107% (Teerlink *et al.*, 1997), 65-105% (Busto *et al.*, 1997), 37-108% (Meseguer Lloret *et al.*, 2002), 78-114% (Meseguer Lloret *et al.*, 2004), 95-106% (Cao *et al.*, 2005) and 86.6 to 105.1% (You *et al.*, 2006) with %RSD less than 2.7% (You *et al.*, 2006), less than 4 (Rodríguez López *et al.*, 1996), less than 5 (Busto *et al.*, 1997; Cao *et al.*, 2005), less than 6 (Teerlink *et al.*, 1997), 2 to 15% (Meseguer Lloret *et al.*, 2002), 3 to 15% (Meseguer Lloret *et al.*, 2004). However, these techniques require long reaction time, have complicated complex polymer preparation step and the peak widening (Rodríguez López *et al.*, 1996).

1.4.5 Gas chromatography

Gas chromatography (GC) is widely used in the analysis of amines owing to its simplicity, high resolving power, good sensitivity, short analysis time and relative low cost. In some cases, determination of amines can be carried out by direct injection of a sample into the pre-column. In general, amines are separated using strongly basic stationary phases. Detection limit can be improved by analyte preconcentration or by the use of more sensitive and selective detectors. Typical GC detectors used in amine determination include nitrogen phosphorous detector (NPD),

flame ionization detector (FID), and mass spectrometric detector (MSD) (Namieśnik *et al.*, 2003). GC is also the method of choice for analysis of many volatile component in food.

Recently GC technique for determination of aliphatic amines with various derivatizing reagents have been developed (Sacher *et al.*, 1997; Kim *et al.*, 1997; Zhao *et al.*, 2002; Zhao *et al.*, 2003). The derivatizing agents such as benzensulfonyl chloride (BSC) (Sacher *et al.*, 1997; Zhao *et al.*, 2003), 2, 4-dinitro fluorobenzene (2, 4-DNFB) (Sacher *et al.*, 1997), isobutyloxycarbonylation (*iso*BOC) (Kim *et al.*, 1997), *N*-hydroxysuccinimidyl phenylacetate (SIPA) (Zhao *et al.*, 2002), *N*-succinimidyl benzoate (SIBA) (Zhao *et al.*, 2003) were used to form complexes with aliphatic amines in varieties of medium *i.e.,* sodium phosphate buffer at pH 12.0 and pretreatment by solid phase extraction (Kim *et al.*, 1997), in aqueous sodium hydroxide solution and adjustment with hydrochloric acid for pH 5.5 at 80°C for 30 min (Sacher *et al.*, 1997), in aqueous solution modified with H_3BO_3-$Na_2B_4O_7$ at room temperature for 72 min (pH 8.5) and dichloromethane (Zhao *et al.*, 2002), in borate buffer (pH 8.8) at 60°C for 22 min and they extracted all analyte by headspace solid phase microextration (Zhao *et al.*, 2003). Then, detected the analyte by gas chromatography.

The most applied GC methods use capillary columns based on the following stationary phases *i.e.*, 14% cyanopropylphenyl (Zhao *et al.*, 2002; Zhao *et al.*, 2003), 5% phenyl 95% methylpolysiloxane (Sacher *et al.*, 1997; Zhao *et al.*, 2003), 5% phenyl 1% vinyl (Kim *et al.*, 1997; Li *et al.*, 2004; Kaykhaii *et al.*, 2005) and 50% phenyl-50% methyl (Kim *et al.*, 1997). The packed glass column used Chromosorb 103 for improvement of peak shape by decreasing tailing peak of dimethylamine and trimethylamine separation. Alkalinization with KOH and organic solvent extraction before direct injection to GC was done to prevent the damaging of the stationary phase by acidic solution (Krzymien and Elias, 1990).

The separation of DMA, TMA and other aliphatic amines required a relatively wide range of temperature programming. Detector used to identify DMA, TMA and other aliphatic amines include flame ionization detector (FID) (Vciana-Nogues *et al.*, 1996; Kim *et al.*, 1997; Chien *et al.*, 2000; Sukpeng, 2001; Béné *et al.*, 2001; Zhao *et al.*, 2002; Namieśnik *et al.*, 2003; Zhao *et al.*, 2003; Kaykhaii *et al.*,

2005), nitrogen phosphorous detector (Krzymien and Elias, 1990) and mass spectrometer (MS) (Kim *et al.*, 1997; Sacher *et al.*, 1997; Zhao *et al.*, 2002; Zhao *et al.*, 2003). The reported detection limits of aliphatic amines for GC technique were 0.110 and 0.167 mg nitrogen per 100 g (Vciana-Nogues *et al.*, 1996), 500 ng L^{-1} and 100 ng L^{-1} (Sacher *et al.*, 1997), 0.1 pmol (Zhao *et al.*, 2002), 4.27 ng L^{-1} (Zhao *et al.*, 2003), 20 and 0.2 μg L^{-1} (Li *et al.*, 2004) and 2.5 μg L^{-1} (Kaykhaii *et al.*, 2005). The linear ranges were from 0.2 to 12 ppm (Kim *et al.*, 1997), 1-50 μmol (Zhao *et al.*, 2002), 100-200 μg L^{-1} (Zhao *et al.*, 2003) and 0.04-0.8 mg (Chien *et al.*, 2000) with %RSD less than 10 (Sacher *et al.*, 1997) and 12 (Kaykhaii *et al.*, 2005).

The derivatization procedure of aliphatic amines is complicated and time consuming (Zhao *et al.*, 2003). The reaction of derivertizing agent with aliphatic amines proceeds in organic media that cannot be used as derivatizing agent of aliphatic amines in water media. Although the developed two-phase reaction system overcomes the drawback, excess reagent must be removed before analyses, which enhance the sampling time (Zhao *et al.*, 2003). Some report gave the poor recovery (10-30%) (Sacher *et al.*, 1997)

Determination of DMA and TMA by GC technique is more suitable than HPLC technique because GC methods obtain better limit of detections, although they depend on the pretreatment step, the instrumental conditions and the sample matrix that obtained. Table 1.3 summarizes the application of GC to analyse the aliphatic amine.

Table 1.3 Applications of GC to the determination of aliphatic amines

Sample	Pre-treatment	Detection	References
Fish	LLE	NPD	Krzymien and Elias, 1990
Fish	LLE	FID	Vciana-Nogues *et al.*, 1996
Waste and surface water	Derivatization with 2, 4-DNFB and BSC	MS	Sacher *et al.*, 1997
Saliva samples	Derivatization with *iso*BOC and SPE	FID	Kim *et al.*, 1997
Airborne	SPME	FID	Chien *et al.*, 2000
Frozen seafood	HS	FID	Sukpeng, 2001
Fish	SPME	FID	Béné *et al.*, 2001
Lake water	Derivatization with SIPA	FID	Zhao *et al.*, 2002
Lake water	Derivatization with SIBA and HS-SPME	FID	Zhao *et al.*, 2003
Air	SPME	FID	Namieśnik *et al.*, 2003
Tap and river water	HS-SDME	FID	Kaykhaii *et al.*, 2005

1.5 Sample preparation

Sample extraction and clean-up procedures are necessary for reliable and accurate results. Samples must usually be processed in order to isolate and concentrate organic analyte from the sample matrix and provide a suitable sample extractant for analysis. Sample preparation techniques reported on for aliphatic amines are liquid liquid extraction (LLE) (Krzymien and Elias, 1990; Vciana-Nogues *et al.*, 1996), headspace single drop microextraction (HS-SDME) (Kaykhaii *et al.*,

2005), derivatization (Sacher *et al.*, 1997; Zhao *et al.*, 2002), derivatization combined with solid phase extraction (SPE) (Kim *et al.*, 1997), solid phase microextration (Chien *et al.*, 2000; Béné *et al.*, 2001; Zhao *et al.*, 2003; Namieśnik *et al.*, 2003). Sample matrices are gas, air (Namieśnik *et al.*, 2003) and airborne (Chien *et al.*, 2000), liquid samples included saliva (Kim *et al.*, 1997), lake water (Zhao *et al.*, 2002; Zhao *et al.*, 2003), tap and river water (Kaykhaii *et al.*, 2005), waste and surface water (Sacher *et al.*, 1997), solid samples are fish (Krzymien and Elias, 1990; Vciana-Nogues *et al.*, 1996; Béné *et al.*, 2001).

1.5.1 Liquid liquid extraction (LLE)

Liquid liquid extraction (LLE) or solvent extraction is based on the partition of organic compounds between the aqueous sample and an immiscible organic solvent (Christian, 1994). LLE were used to extract trimethylamine and dimethylamine from biological and environmental matrices. There are few reports on the use of LLE to extract DMA and TMA from fish, shellfish, serum, urine and environmental water samples. For fish, 10 g was extracted with toluene or benzene after 65% KOH and 0.6 N perchloric acid neutralization of fish extract prior to the analysis by GC-FID. The detection limits were 0.038, 0.110 mg nitrogen per 100 g for TMA, DMA, respectively. Recovery was 98.11% with 6.39% relative standard deviation (RSD) (Veciana-Nogues *et al.*, 1996). However, LLE has some disadvantages, *i.e.*, large amount of sample, complicated or variety of extract and clean-up steps and toxic solvent.

1.5.2 Headspace solvent microextraction or headspace single drop microextraction (HS-SDME)

Headspace solvent microextraction or headspace single drop microextraction (HS-SDME) has a few reports. It was used to extract amines in tap and river water sample, 1 μL drop of benzyl alcohol containing 2-butanone as an internal standard was suspended from the tip of a microsyringe needle over the headspace of stirred sample solution for extraction. The drop was then retracted and

injected directly into GC-FID. The detection limit was 2.5 μg L^{-1} with %RSD less than 12 (Kaykhaii *et al.*, 2005). This technique enriches and quantifies traces level of aliphatic amine in water samples, however, there is no report on the application in solid sample.

1.5.3 Solid phase extraction and derivatization

Solid phase extraction (SPE) is one of the most popular techniques for sample extraction of various food and baverages such as wine, beer, fish and meat. SPE can be used to extract and clean-up or used for derivatization. Aliphatic amine structure contains polar groups and are able to interact with many solid phase. In the clean-up step for determining biogenic amine in red wine, two solid phase extraction cartridges, a non polar (C_{18}) and strong anion-exchanger (SAX) were used to remove large amount of polyphenols from samples and derivatization of amine with *o*-phthalaldehyde (OPA) before analysis by HPLC (Busto *et al.*, 1997). There are many research works reporting on the sample pretreatment simultaneously with derivatization on C_{18}-SPE cartridges before analyse by HPLC (Maris *et al.*, 1999; Wang *et al.*, 2000; Meseguer Lloret *et al.*, 2002; Meseguer Lloret *et al.*, 2004; Cháfer-Pericás *et al.*, 2004). Overall recoveries were from 54 to 120% with RSD less than 5%. In addition, chromosorb P were used to extract but gave low recovery, 30% (Kim *et al.*, 1997).

In another work, the sample pretreatment for waste and surface water was done by directly derivatized with benzenosulfonyl chloride extracted with dichloromethane in basic medium (Sacher *et al.*, 1997). Direct derivatization with acridine-9-acetyl-*N*-hydroxy succinimide (Maris *et al.*, 1999), SIPA has also been done in waste water before analysed by HPLC (Zhao *et al.*, 2002). Recoveries were from 94 to 115 with %RSD less than 3.5 have been reported. These techniques are complicated and time consuming.

1.5.4 Solid phase microextraction

Solid phase microextraction (SPME) utilizes solid rod of fused-silica and coated with adsorbent polymer. SPME can be conducted as a direct extraction in which the coated fiber is immersed in aqueous samples (Scheppers Wercinski and Pawliszyn, 1999). It is based on the partition of the analyte between the extraction phase and the matrix (Mitra, 2003). The basic principle of this approach is to use a small amount of the extracting phase, usually less than 1 μL for extraction of analytes from sample matrix. This technique combines extraction, concentration and sample introduction in one step. The extracting phase can be either high molecular weight polymer liquid, similar in nature to stationary phase in chromatography, or it can be a solid sorbent, typically of a high porosity to increase the surface area available for adsorption (Pawliszyn, 1999). Polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber was used to extract dimethylamine and trimethylamine in fish samples and analysed by GC-FID (Béné *et al.*, 2001; Namieśnik *et al.*, 2003). PDMS fiber was also used to extract trimethylamine in airborne with the mean recovery of 100.4% (Chien *et al.*, 2000). This technique gave high recovery of amine in airborne.

1.5.5 Static headspace

Static headspace extraction is typically used for analysis of volatile compound in many matrices where complete extraction of the analytes is not required. The sample (solid or liquid) is placed in a headspace vial that is sealed with septum and aluminum crimp cap. After heating for a given period of time at moderated temperature (60-80°C), a volume of vapour phase in equilibrium with the solid or aqueous phase is injected into gas chromatograph system. This technique is very simple, rapid, solvent free and cost effective. Sukpeng (2001) determined dimethylamine and trimethylamine in frozen seafood by headspace gas chromatography with flame ionization detector and packed glass columns (Chromosorb 103, Carbopack B). The detection limits were 0.17 and 0.16 mgN L^{-1} for dimethylamine and trimethylamine, respectively. However, large amounts of

samples were used so, the stationary phase was easily damage and gave low sensitivity.

Since the analysis of dimethylamine and trimethylamine in fish and shrimp samples is important for quality index of freshness that has an impact for the export of the country. Therefore, a simple technique to evaluate these compounds is needed. This work focused on the development of a gas chromatographic technique with static headspace for freshness determination (dimethylamine and trimethylamine) in fish and shrimp samples. The method consisted of the optimization of headspace gas chromatography to enhance the sensitivity and selectivity.

1.6 Objective

The objective of this work is the development of headspace gas chromatography with nitrogen phosphorous detector (HS-GC-NPD) for qualitative and quantitative analysis of dimethylamine and trimethylamine in fish and shrimp samples to test the seafood freshness.

CHAPTER 2

Experimental

2.1 Materials

- Dimethylamine, DMA (40% in water, Fluka, Switzerland)
- Trimethylamine, TMA (45% in water, Fluka, Switzerland)
- Sodium chloride (NaCl, AR grade, Merck, Germany)
- Sodium carbonate (Na_2CO_3, AR grade, Merck, Germany)
- Sodium sulfate (Na_2SO_4, AR grade, Merck, Germany)
- Potassium chloride (KCl, AR grade, Merck, Germany)
- Ultra pure water (Synthesis in Laboratory by Maxima, ELGA, England)
- Quartz wool (Perkin Elmer, USA)
- Helium gas, Ultra high purity, 99.9999% (TIG, Thailand)
- Hydrogen gas, Ultra high purity, 99.9999% (TIG, Thailand)
- Air, zero gas 99.995% (TIG, Thailand)
- Stainless steel water bath laboratory-built with thermostat (Gallenkamb, UK)
- Microliter pipette, (Gilson, France), various size and tips
- Crimp and decrimp (Shimadzu, Japan)
- Propylene rubber cap
- Aluminium cap

2.2 Instruments and Apparatus

- Gas Chromatograph Auto System XL equipped with nitrogen phosphorus detector (GC-NPD) (Perkin Elmer, USA)

- Computer system model intel inside Pentium digital venturis, Turbochrom software (Perkin Elmer, USA)

- Capillary Column: HP-FFAP, 25 m x 0.32 mm i.d. x 0.52 μm film thickness of nitroterephthalic acid modified polyethylene glycol (Agilient, USA)

- Gastight syringe, 1.0 mL (Hamiton, Supelco, USA)

- Syringe cleaner (Hamiton, Switzerland)

- Vortex (Genei Scientific Industries Inc., USA)

2.3 Glasswares

- Volumetric flask 25, 50,100 and 250 mL (Pyrex, USA)

- Headspace vial, 10, 25 and 60 mL (Shimadzu, Japan) with propylene rubber and aluminium crimp cap

- Beaker 50, 100, 250, 500 and 1000 mL (Pyrex, USA)

- Measurement pipette 10, 25 mL (Pyrex, USA)

- Spatula

2.4 Analysis system

DMA and TMA in fish and shrimp samples were analyzed by headspace gas chromatographic technique with nitrogen phosphorus detector (HS-GC-NPD). The analysis system (Figure 2.1) consists of a stainless steel water bath (13 cm × 75 cm × 15 cm), a heating unit and a thermostat for the headspace technique. The analysis is performed by a Gas Chromatograph Auto System XL equipped with nitrogen phosphorus detector (Perkin Elmer, USA) and capillary Column: HP-FFAP, 25 m × 0.32 mm i.d. × 0.52 μm film thickness of nitroterephthalic acid modified polyethylene glycol (Agilient, USA).

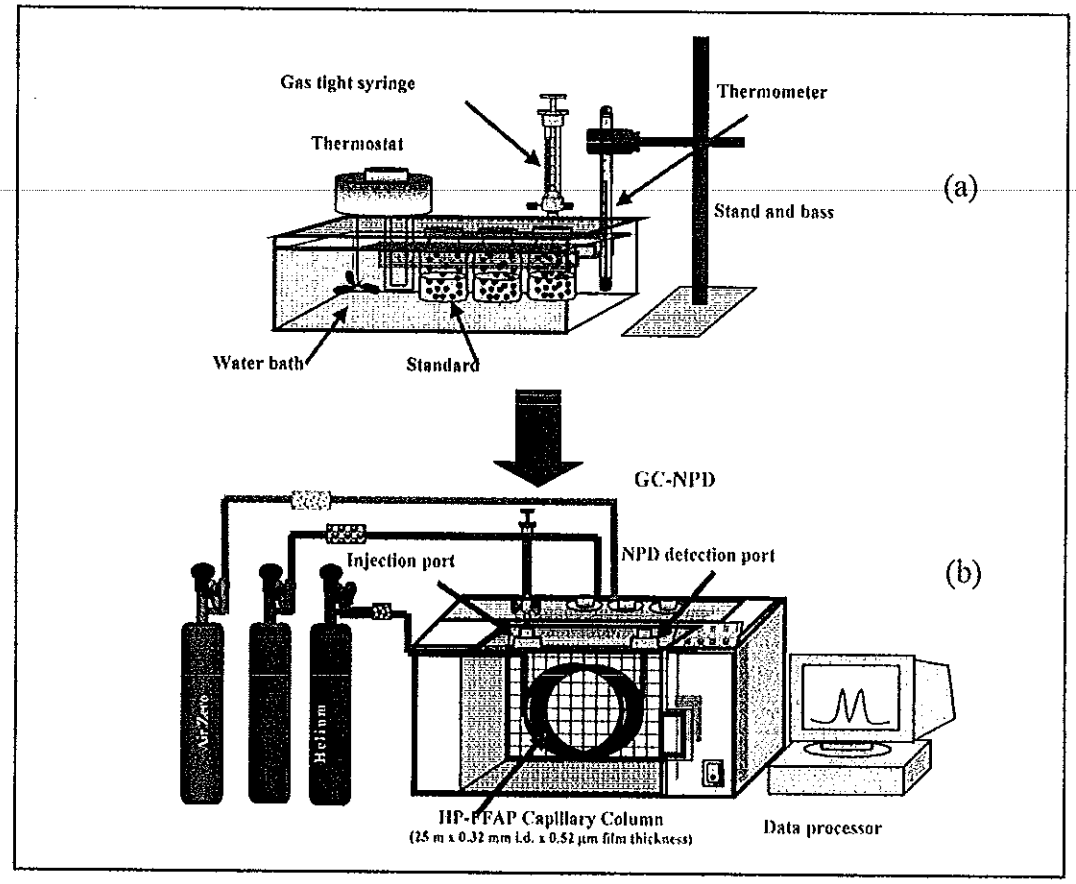


Figure 2.1 Analysis system for dimethylamine and trimethylamine, (a) Headspace (b) Gas Chromatography with Nitrogen Phosphorus Detector, (HS-GC-NPD)

2.5 Preparation of standard solutions

2.5.1 DMA standard stock solution

Standard stock solution of DMA, 1000 µg mL^{-1}, was prepared by diluting 703 µL DMA (with a micro liter pipette) by ultra pure water in a 250 mL volumetric flask.

samples were used so, the stationary phase was easily damage and gave low sensitivity.

Since the analysis of dimethylamine and trimethylamine in fish and shrimp samples is important for quality index of freshness that has an impact for the export of the country. Therefore, a simple technique to evaluate these compounds is needed. This work focused on the development of a gas chromatographic technique with static headspace for freshness determination (dimethylamine and trimethylamine) in fish and shrimp samples. The method consisted of the optimization of headspace gas chromatography to enhance the sensitivity and selectivity.

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CHAPTER 2

Experimental

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- Sodium sulfate (Na_2SO_4, AR grade, Merck, Germany)
- Potassium chloride (KCl, AR grade, Merck, Germany)
- Ultra pure water (Synthesis in Laboratory by Maxima, ELGA, England)
- Quartz wool (Perkin Elmer, USA)
- Helium gas, Ultra high purity, 99.9999% (TIG, Thailand)
- Hydrogen gas, Ultra high purity, 99.9999% (TIG, Thailand)
- Air, zero gas 99.995% (TIG, Thailand)
- Stainless steel water bath laboratory-built with thermostat (Gallenkamb, UK)
- Microliter pipette, (Gilson, France), various size and tips
- Crimp and decrimp (Shimadzu, Japan)
- Propylene rubber cap
- Aluminium cap

2.2 Instruments and Apparatus

- Gas Chromatograph Auto System XL equipped with nitrogen phosphorus detector (GC-NPD) (Perkin Elmer, USA)

2.5.2 TMA standard stock solution

Standard stock solution of TMA, 1000 μg mL^{-1}, was prepared by diluting 669 μL TMA (with a micro liter pipette) by ultra pure water in a 250 mL volumetric flask.

2.5.3 DMA and TMA standard solution

Standard working solution of DMA and TMA were prepared from standard stock solutions in ultra pure water to obtain the mixture with concentrations of 100 μg mL^{-1} and 1 μg mL^{-1} respectively. The solution was used for the investigation of optimum conditions.

2.6 Optimization of the GC-NPD conditions

Conditions of the GC-NPD system under investigation were carrier gas flow rate, column temperature, injector temperature, detector temperature and fuel (hydrogen) and oxidant gases (air) flow rates. In these studies, 20 mL mixture of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) working standard solution in 60 mL sealed vial was placed in a laboratory-built water bath at 60°C. After 20 minutes, a gas-tight syringe was used to transfer 0.5 mL of the vapour (gas) phase and inject into GC-NPD system for analysis. Five replicates were done for each studied value. When an optimum condition was found it was used in the optimization of the following parameter as in the sequence in this section. The optimum value is the one giving the highest response except stated otherwise.

2.6.1 Carrier gas flow rate

Optimization was carried out by varying the flow rate of helium carrier gas at 2.6, 2.8, 3.0, 3.2, 3.4 and 3.6 mL min^{-1}. Other parameters were set as recommended by the Auto System XL-GC instrument manual, column temperature

program was 120°C, temperature ramp rate 30°C min^{-1} to 180°C, hold 1 min, injector and detector temperatures were 120°C and 220°C. The retention times, peak heights and peak areas from the chromatograms were used to calculate the plate number and height equivalent to a theoretical plate (HETP) respectively. From the van Deemter plot, the optimum flow rate was obtained at the lowest HETP.

2.6.2 Column temperature

Initially the column was operated under isothermal temperature (120°C) but separation of DMA and TMA took a long time. Therefore, a column temperature program was investigated to obtain a high response with a short analysis time. The column temperature programming consists of five steps as shown in Table 2.1. The selected optimum of each parameter was the one providing the highest response, the best resolution and short analysis time.

Table 2.1 Optimization of column temperature programming

Step	Parameters	Studied value
1	Initial temperature	90, 100, 110, 120 and 130°C
2	Holding time at initial temperature	0, 1, 2 and 3 min
3	Temperature ramp rate	10, 20, 30 and 40°C min^{-1}
4	Final temperature	160, 170, 180, 190 and 200°C
5	Holding time at final temperature	1, 2, 3 and 4 min

2.6.3 Other parameters

Other parameters affecting the operation of the GC-NPD system were also optimized and they are summarized in Table 2.2.

Table 2.2 Optimization of other parameters for the GC-NPD system

Step	Parameters	Studied value
1	Injector temperature	140, 150, 160, 170 and 180°C
2	Detector temperature	180, 200, 210, 220, 230 and 240 °C
3	Fuel (Hydrogen) flow rate	1, 2, 3 and 4 mL min^{-1}
4	Oxidant gas (air) flow rate	90, 100, 110 and 120 mL min^{-1}
5	Split ratio	10:1, 20:1, 30:1 and 40:1

2.7 Optimization of headspace conditions

The responses of DMA and TMA for headspace technique were based on various parameters *i.e.,* equilibration time, equilibration temperature, sample volume (phase ratio), size of the vial, salt and amount of salt. In this study, a laboratory-built thermal bath was used as shown in Figure 2.2. The analysis was done by using the optimum conditions of gas chromatograph with nitrogen phosphorus detector (GC-NPD) found earlier (see results in chapter 3).

2.7.1 Equilibration time

Mixture of DMA and TMA working standard solutions, 20 mL containing 100 and 1 μg mL^{-1}, were pipetted into 60 mL headspace vials. They were placed in a laboratory-built water bath at 70°C for 10, 20, 30, 40 and 50 minutes. 0.5 mL of the gas phase was then taken from the headspace by a gas tight syringe and injected to the GC-NPD system. The peak heights of the responses were plotted against the equilibration time.

2.7.2 Equilibration temperature

20 mL mixture of DMA and TMA working standard solution, 100 and 1 μg mL^{-1} were pipetted into 60 mL headspace vials. They were placed in a laboratory-built water bath at 40, 50, 60, 70 and 80°C. 0.5 mL of the gas phase was then taken from the headspace by a gas tight syringe and injected to the GC-NPD system. The peak heights of the responses were plotted against the equilibration temperature.

2.7.3 Sample volume (phase ratio)

Phase ratio is the rate constant of the sample phase and gas phase that consist above the sample. It is the equilibrated partition of two heterogeneous phases between sample and vapour volumes above sample (Kolb and Ettre, 1997). The best phase ratio will provide a high response and good precision. The volume of 100 μg mL^{-1} DMA and 1 μg mL^{-1} TMA standard solutions, were varied at 10, 15, 20, 25, 30 and 40 mL (phase ratio at 0.2, 0.5, 1.0, 1.4, 2 and 5) for 60 mL vial. The GC-NPD conditions, equilibration time and equilibration temperature were set at their optimum values.

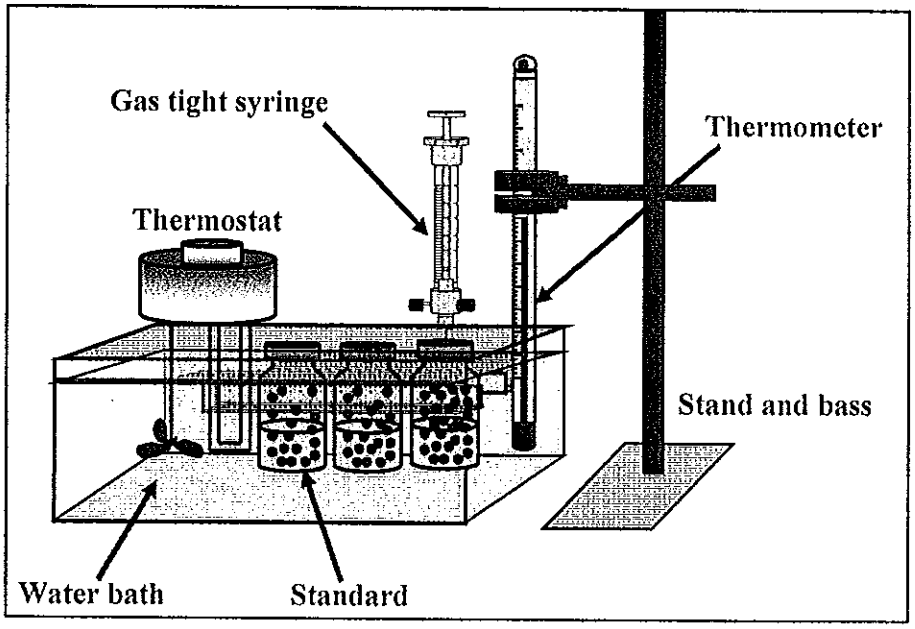

Figure 2.2 Laboratory-built water bath systems

2.7.4 Size of vial (volume)

Size of vials was studied at 10, 30 and 60 mL. Mixture of DMA and TMA working standard solution, 100 and 1 μg mL^{-1} were used. The main focus is to reduce the amount of sample, save money and chemical. 3.33, 10 and 20 mL mixture of DMA and TMA working standard solution were pipetted into 10, 30 and 60 mL vial, respectively (the phase ratio, β, in all cases was 2). A 0.5 mL gas phase mixture of DMA and TMA standard solutions was injected into gas chromatograph set at the optimum conditions for headspace and GC-NPD system.

2.7.5 Salt and amount of salt

Salt is another important parameter needs to be considered in the salting out effect to increase the response. Sodium chloride (NaCl), sodium carbonate (Na_2CO_3), sodium sulfate (Na_2SO_4) and potassium chloride (KCl) were tested at 0.5, 1.0, 1.5 and 2.0 g. 3.33 mL mixture of DMA and TMA working standard solution, 100 and 1 μg mL^{-1}, were pipetted into 10 mL vial. A 0.5 mL gas phase mixture of DMA and TMA standard solution were injected into gas chromatograph set at the optimum conditions for headspace and GC-NPD system.

2.8 Linear dynamic range (LDR, Linearity)

The standard stock solutions of DMA and TMA were diluted with ultra pure water to various concentrations in the range of 0.5-250 μg mL^{-1} for DMA and 0.01-50 μg mL^{-1} for TMA in the headspace vials with sealed cap. They were placed in a laboratory-built water bath. A 0.5 mL of the gas phase of standard mixture was taken from the headspace by a gas tight syringe and injected to the GC-NPD system. Five replications were done for all analysis. Linear dynamic range was determined by plotting the peak heights *versus* the concentrations of DMA and TMA. The linearity of the response was determined by considering the coefficient of determination.

2.9 Limit of detection (LOD)

The limit of detection (LOD), C_L (or amount, q_L), was the lowest concentration of an anlyte in a sample that can be detected. In this study, the determination of detection was based on IUPAC definition. It is derived from the smallest, X_L, that can be detected with reasonable certainly for a given analytical procedure (Long and Wineforder, 1983). The limit of detection was determined by measuring blank response (X_B). Blank was prepared by adding sodium carbonate in ultra pure water in the 10 mL headspace vials with sealed cap. They were placed in a laboratory-built water bath at 70°C. After equilibration time was reached, 0.5 mL of the gas phase was taken from the headspace by a gas tight syringe and injected to the GC-NPD system operated at optimum conditions. Blank analysis (n_B) was done more than 20 times. A mean value of the blank response, $\overline{X}_B$ can be calculated as

$$\overline{X}_B = \frac{\sum_{j=1}^{n_B} X_{Bj}}{n_B} \tag{2.1}$$

And standard deviation as

$$S_B = \sqrt{\frac{\sum_{j=1}^{nB} (X_{Bj} - \overline{X}_B)^2}{nB-1}} \tag{2.2}$$

In defining X_L, IUPAC state that

$$X_L = \overline{X}_B + kS_B \tag{2.3}$$

Where k is a number factor chosen in accordance with the confidence limit desired and the accepted value is 3 at a confidence level of 99.7%, S_B is the standard deviation for 20 times of injection. The C_L was obtained as a function of X_L and can be calculated as

$$C_L = \frac{(X_L - \bar{X}_B)}{m} \quad (2.4)$$

Where m is the analytical sensitivity (slope of the calibration curves) and C_L is the smallest concentration that can be detected with reasonable certainty for a given analytical procedure. Because the mean blank reading, $\bar{X}_B$ is not always 0 the signal must be background corrected. Equation 2.5 was obtained after substitution Equation 2.3 into 2.4.

$$C_L = \frac{kS_B}{m} \quad (2.5)$$

The limit of detection is found by relating kS_B to a concentration value by dividing by slope of calibration curve obtained from the linear regression analysis (Long and Winefordner, 1983).

2.10 Sample Analysis

Analytical procedures of DMA and TMA for fish and shrimp samples are shown in Figure 2.3. A static headspace technique combined with gas chromatography with nitrogen phosphorus detector was used for qualitative and quantitative analysis of DMA and TMA in fish and shrimp samples. The conditions of headspace and GC-NPD system were set at optimum conditions

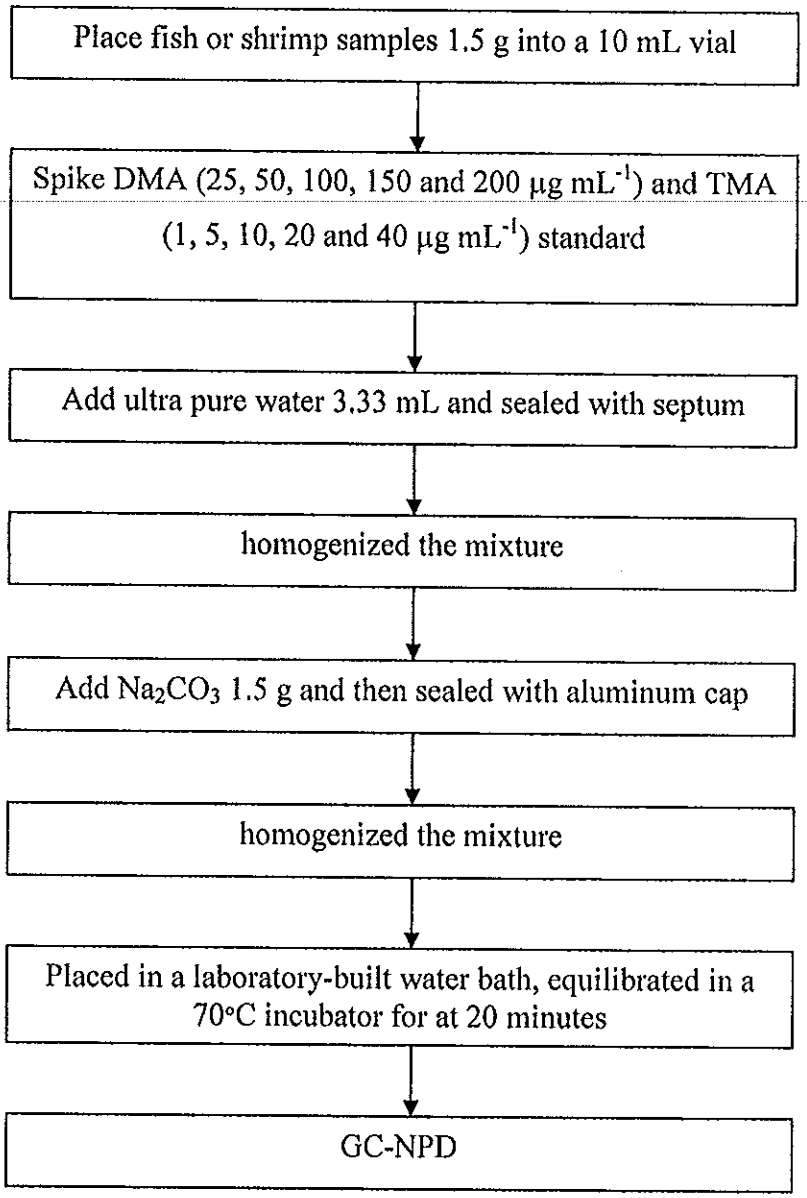

Figure 2.3 Analytical procedures of DMA and TMA for fish and shrimp samples

2.10.1 Sampling

Fish and shrimp samples were sampling from local fresh markets and supermarkets in Hat Yai district as in Table 3. All fish and shrimp samples were stored at 4°C until being analyzed.

Table 2.3 The information of samples

Type of samples	Number of sources	Number of samples
Indian mackerel	5	5
Sea bass	4	4
Giant tiger shrimp	2	2
White shrimp	6	6
Total samples	17	

2.10.2 Sample preparation

Fish and shrimp edible tissue samples were cut and minced. Samples were brought to room temperature before analysis. Amount of sample was tested at 0.5, 1.0, 1.5 and 2.0 gram followed the steps in Figure 2.3.

2.11 Matrix Interference

In headspace analysis matrix is used to express the bulk of the sample that contains the volatile compounds to be measured. The matrix effect represents an important consideration in sample reproducibility and particularly in the preparation of standard mixtures used for calibration (Kolb and Ettre, 1997). Matrix spiking is done by adding a known quantity of a component that is similar to the analyte.

In this work, the effect of matrix were studied by spiking known amount of DMA standard solution at 25, 50, 100, 150 and 200 μg mL^{-1} and amount of TMA standard solution at 1, 5, 10, 20 and 40 μg mL^{-1} into 1.5 g of fish and shrimp samples. Five replications were done for all analysis.

A sample blank was also analyzed with each set of fish and shrimp sample. A 0.5 mL of the gas phase was taken from the headspace by a gas tight

syringe and injected into GC-NPD system at optimum condition. Each experiment was done in five replicates. The responses, peak height, were plotted against the known concentrations: The slope of the standard curve and the spiked sample curve were compared for matrix interference.

2.12 Method validation

The method validation is used to demonstrate that known levels can be accurately and precisely determined in a real sample

2.12.1 Recovery

The recovery of the method was determined for each analyte by HS-GC-NPD system. The samples were spiked with the two analytes at two levels in the linear range and in the range that is needed to be determined. The recovery of the method was performed by spiking fish and shrimp sample with DMA standard solution 100 and 200 μg mL^{-1} and TMA standard solution 0.5 and 1.0 μg mL^{-1}. A blank fish and shrimp were also analyzed with each set of fish and shrimp samples. A 0.5 mL of the gas phase was taken from the headspace by a gas tight syringe and injected to the GC-NPD system operated at optimum conditions. Each experiment was done in five replicates.

2.12.2 Accuracy

Accuracy of the method was studied by analyzing two fortified fish and shrimp samples with DMA at 100 and 200 μg mL^{-1} and TMA at 0.5 and 1.0 μg mL^{-1}.

The accuracy was evaluated by the percentage of recovery for fortified samples with DMA at 100 and 200 μg mL^{-1} and TMA at 0.5 and 1.0 μg mL^{-1}.

2.12.3 Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation for statistically significant number of samples (Swartz *et al.*, 1997). The precision of the method for the determination of DMA and TMA in fish and shrimp samples were evaluated at two spiking levels of DMA standard solution 100 and 200 μg mL^{-1} and TMA standard solution 0.5 and 1.0 μg mL^{-1} in 1.5 g of fish and shrimp samples in Figure 3. A sample blank was also analyzed in parallel. A 0.5 mL of the gas phase was taken from the headspace by a gas tight syringe and injected to the GC-NPD system operated at optimum conditions. Each concentration was done in five replicates. The relative standard deviation (RSD) was then calculated for each type of samples by the following equations (Miller and Miller, 2000).

$$\%RSD = \frac{S}{\overline{X}} \times 100$$

$$S = \sqrt{\frac{\sum (Xi - \overline{X})^2}{n-1}}$$

$$\overline{X} = \sum_{i=1}^{n} Xi$$

Where S is the standard deviation

n is the total number of measurement

$\overline{X}$ is the mean of n measurement

2.13 Qualitative and quantitative analysis of DMA and TMA in fish and shrimp samples

2.13.1 Qualitative Analysis

Qualitative analysis was determined by the retention time and confirmed with the DMA and TMA standards spiked technique. The retention time, t_R, is the thresholding time from the injection of sample to recording of the peak maximum.

2.13.2 Quantitative Analysis

The quantitative analysis was based on the response from GC-NPD, *i.e.*, peak height of chromatogram which was proportional to the amount of DMA and TMA. Analytical technique was carried out by matrix math calibration curve.

2.13.2.1 Matrix match calibration curve

Matrix match calibration curve were studied by spiking known amount of DMA standard solutions at 25, 50, 100, 150 and 200 μg mL^{-1} and amount of TMA standard solutions at 1, 5, 10, 20 and 40 μg mL^{-1} into 1.5 g of fish or shrimp sample. Five replications were done for all analysis. The matrix match calibration curves were obtained by plotting the peak height obtained from the spiked samples *versus* concentration of DMA and TMA. The response from the chromatogram from the unknown samples was used to calculate the concentration from the matrix match calibration curve.

2.14 Quality assurance and quality control

In the laboratory, at any stage of headspace technique and analysis, contamination can occur. Therefore, it must be demonstrated that during the sample preparation procedure it is essentially free of DMA and TMA residue. This was assessed by preparing reagent blanks and method blank.

Reagent blanks are reagents used during the analytical process. These are analyzed in isolation in order to see whether they contribute to the measurement signal. The measurement signal from the analyte can be corrected accordingly. Reagent blanks were prepared and analyzed for DMA and TMA prior to the sample preparation steps.

Method blank is performed by carried through all the steps of headspace technique and analysis as if was an actual sample. It is used to evaluate the laboratory contamination for the targeted analytes and corrected systematic errors due to impurities that could arise from the reagents, the glassware, or the laboratory environment (Harvey, 2000).

CHAPTER 3

Results and Discussion

A gas chromatograph with nitrogen phosphorous detector (GC-NPD) was used for the determination of dimethylamine (DMA) and trimethylamine (TMA) in fish and shrimp samples using a 25 m × 0.32 mm i.d. × 0.52 μm film thickness fused silica HP-FFAP capillary column consists of nitroterephthalic acid modified polyethylene glycol. A laboratory-built stainless steel water bath with a control heating coil and stainless steel adapter for vial holder was used for the headspace system.

3.1 Optimization of the GC-NPD conditions for DMA and TMA analysis

3.1.1 Carrier gas flow rate

Carrier gas (helium, He) flow rate was optimized in experiment 2.6.1 by considering the relationship between the height equivalent to a theoretical plate (HETP) and the carrier gas flow rate. HETP can be calculated by the van Deemter equation (equation 3.1) (Grob, 2004).

$$\text{HETP} = A + \frac{B}{u} + Cu \qquad (3.1)$$

Where A is eddy diffusion term that the mobile phase diffused through the particles of packing in the column. The result is shown in the velocity of mobile phase.

B is longitudinal or ordinary diffusion term resulted from the movement of molecules after collision in the column.

C is resistant to mass transfer term that a constant amount of the mass transfer.

The van Deemter equation shows the effect of the change in linear gas velocity on HETP. This equation represents a hyperbola that has a minimum velocity, at $u = (B/C)^{1/2}$ and a minimum HETP value ($HETP_{min}$) at $A+2(BC)^{1/2}$. The constants

can be calculated from an experimental plot of HETP *versus* linear gas velocity (Grob, 2004) as shown in Figure 3.1.

The first term, eddy diffusion (A term) accounts for the geometry of the packing. This term describes the change in pathway and velocity of solute molecules in reference to the zone center. Therefore, when a sample migrates down the column, each molecule has different paths and each path is of a different length. Some molecules take the longer paths and other take the shorter paths. There are also variations in the velocities of the mobile phase within these pathways. The overall result is that some molecules lag behind the center of the zone, whereas others move ahead of the zone. Therefore, the eddy diffusion process results in flow along randomly space variable-size particles in the column. Eddy diffusion term was quantified from the equation $A = 2\lambda d_p$, λ is a constant characteristic of packing and d_p is a diameter particle of packing. The contribution of the $2\lambda d_p$ term can be decreased by reducing the particle size (Grob, 2004).

The second term, B/u, describes the longitudinal diffusion term, $B = 2\lambda d_g / u$, d_g is the diffusion coefficients of the component in gas phase. This term is a measure of effect of molecular diffusion on zone spreading. If the velocity of the mobile phase is high then the analyte spends less time on the column, with decreases the effects of longitudinal diffusion. The optimum velocity is greatest for column packed with small particles and for mobile phase in which the solutes have a high diffusivity gas of low density, *e.g.*, hydrogen or helium (Grob, 2004).

The final term, C is the resistant to mass transfer term that a constant amount of the mass transfer. $C = (8/\pi^2)[k + (1+k)^2](d_f^2 / D_l)$ where k is retention factor (capacity factor), d_f is effective film thickness of liquid phase and D_l is diffusivity of solute in liquid phase (Grob, 2004). An obvious way of reducing this term is to reduce the liquid film thickness. This causes a reduction in k and an increase in the term $k/(1+k)^2$ the k term is temperature dependent, so we can increase k and decrease $k/(1+k)^2$ by lowering the temperature. Lowering the temperature increase viscosity and thus decrease D_l. Therefore, the effect of factor $k/(1+k)^2$ and $1/D_l$ counteract each other. This term can be represented as the composite of the resistance to mass transfer in the mobile phase C_g and that in

the stationary phase C_l: $C = C_g + C_l$ the C term accounts for resistance to mass transfer in the liquid phase (Grob, 2004).

From equation (3.1), at low u, the B term is large, but quickly diminishes with increasing u and C_g to a lesser extent, C_l, then dominates. The smallest value of HETP is HETP $_{min}$, at with u is optimum, u_{opt}. The greater u_{opt}, the faster a sample can be analysed (Baugh, 1993).

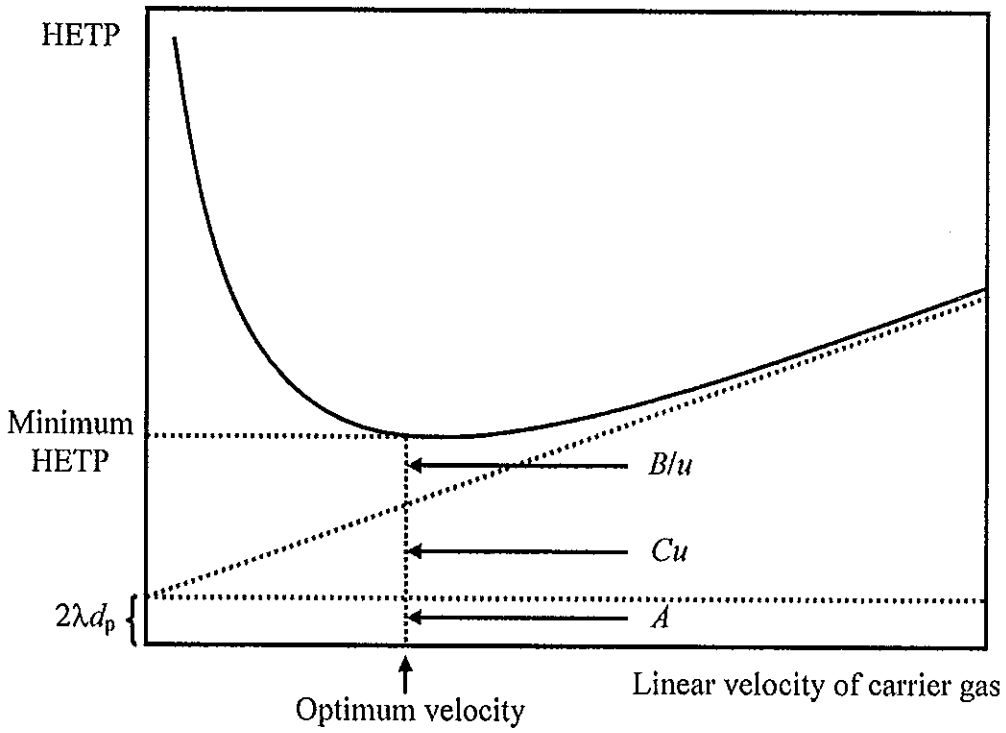

Figure 3.1 The van Deemter plot

In this research, 25 m × 0.32 mm i.d. narrow bore wall-coated open tubular (WCOT) capillary column was used for the analysis. A capillary column is a fused-silica tube of very small internal diameter (generally between 0.20-0.53 mm). The inner surface of a capillary column is coated with a thin layer of stationary phase so it is still possible for the solute molecules to come in contact with the inner walls of the tubing. In this column a liquid phase is coated on fused-silica wall with no packing material, therefore, the A term (eddy diffusion) is nonexistent because there is only one flow path and no packing material. The resistance to mass transfer term C

has the greatest effect on band broadening, and its effect in capillary columns is controlled by the mass transfer in the gas phase C_g. Thus, equation (3.1) takes a different form for capillary column, and this known as the Golay equation (Grob, 2004) in equation (3.2).

$$\text{HETP} = B/u + C_g u \tag{3.2}$$

The above equation showed that HETP is proportional to the flow rate of carrier gas (u). It is also known that an optimum carrier gas flow rate will give an optimum column resolution with the narrowest HETP (Grob, 2004).

In practical, the terms of A, B and C in the equation (3.1), are difficult to obtain. However, the plate theory assumes that the column is divided into a number of zones called *theoretical plate* (N). The zone thickness or height equivalent to a theoretical plate (HETP) is determined by assuming that there is perfect equilibrium between the gas and liquid phases within each plate. The indication of column efficiency in the term of HETP is determined by equation (3.3)

$$\text{HETP} = \frac{L}{N} \tag{3.3}$$

where L is length of column in centimeters

N is the number of theoretical plates

The plate number, N, of a column can be calculate from equation (3.4)

$$N = 16\left(\frac{t_R}{w}\right)^2 \tag{3.4}$$

where t_R is the retention time of the peak, w is the base peak width. If a width at half height ($w_{1/2}$) was used instead of a width (w) at the base, the plate number could be calculated by equation (3.5) (Tibor and Esther, 1999) (Figure 3.2)

$$N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2 \qquad (3.5).$$

Since a capillary column was used in this work, the plate number N could be calculated directly from the value obtained from a chromatogram as shown in the Figure 3.2 and equation 3.6 (Grob, 2004)

$$N = 2\pi\left(t_R h / A\right)^2 \qquad (3.6)$$

Where A is integrated peak area

h is integrated peak height

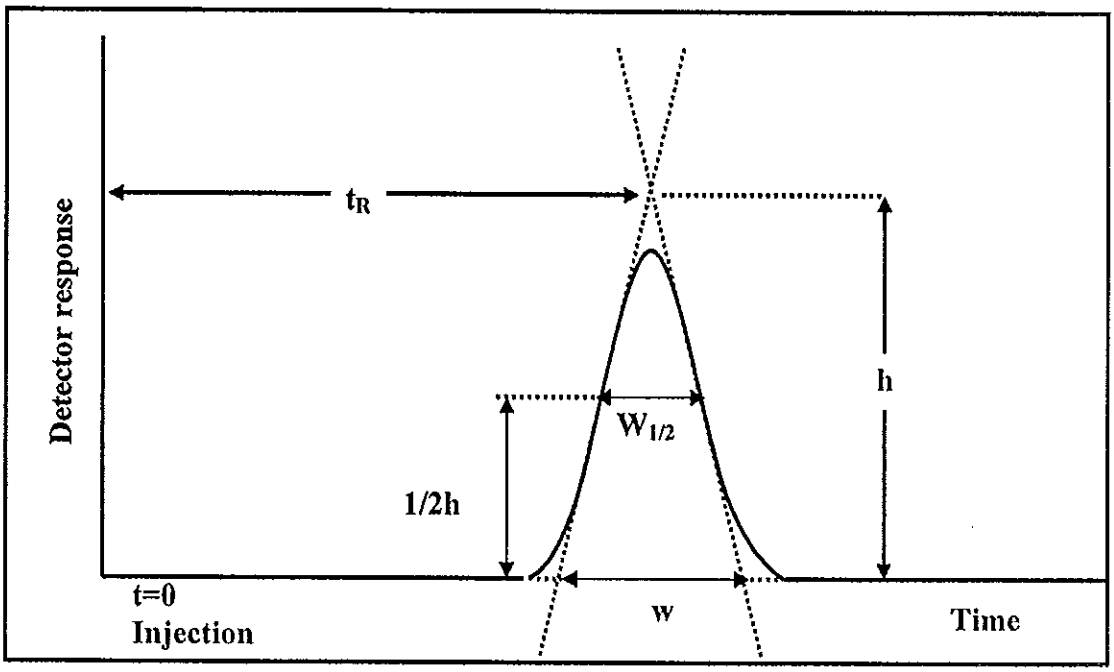

Figure 3.2 Measurement used in calculating total theoretical plates

N was calculated by equation 3.6 and substituted in equation 3.3 with a known L (column length) to obtain HETP. The values of HETP obtained at different carrier gas flow rate are shown in Table 3.1 and Figure 3.3. From the van Deemter

plot, the optimum flow rate of 3.0 mL min^{-1} for both DMA and TMA was obtained at the narrowest HETP and this provided the highest column efficiency.

Table 3.1 HETP of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various carrier gas flow rates

Flow rate (mL min^{-1})	HETP (cm)*	
	DMA	TMA
2.6	6.933±0.195	0.401±0.012
2.8	6.473±0.140	0.387±0.004
3.0	5.529±0.099	0.383±0.010
3.2	7.699±0.092	0.401±0.015
3.4	7.908±0.132	0.410±0.008
3.6	8.134±0.093	0.433±0.006

*5 replications, RSD < 4 %

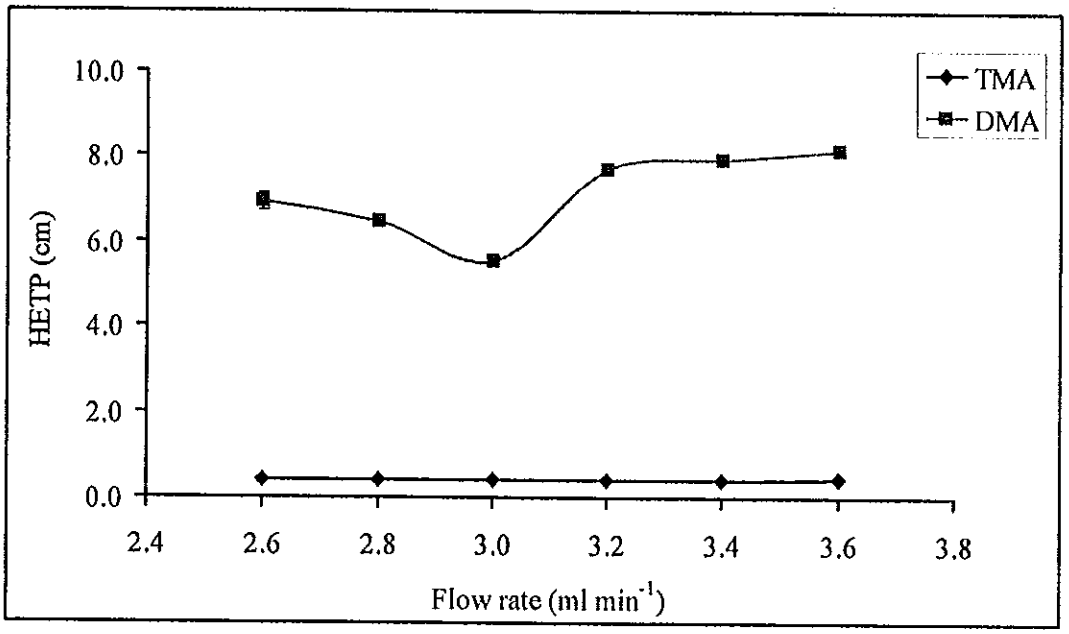

Figure 3.3 The van Deemter plot of DMA and TMA

3.1.2 Column temperature programming

Column temperature is one of the most important parameter in gas chromatography. The column temperature was first operated under isothermal temperature, 120°C but it took a long time to separate DMA and TMA. Therefore, a column temperature program was investigated.

The optimization of column temperature programming started with initial temperature and the results are shown in Table 3.2 and Figure 3.4. The analysis time also decreased when the temperature increased. The initial temperature at 120°C provides the highest response and a good separation and this was chosen.

Table 3.2 Effect of initial temperature on the responses of DMA (100 μg mL^{-1}) and was TMA (1 μg mL^{-1}) standard solution

Temperature (°C)	Responses×10^4 (μV)*	
	DMA	TMA
90	4.04±0.04	14.35±0.12
100	5.13±0.11	15.26±0.18
110	6.36±0.05	16.98±0.24
120	9.98±0.29	18.30±0.43
130	7.96±0.19	14.19±0.07

*5 replications, RSD < 4 %

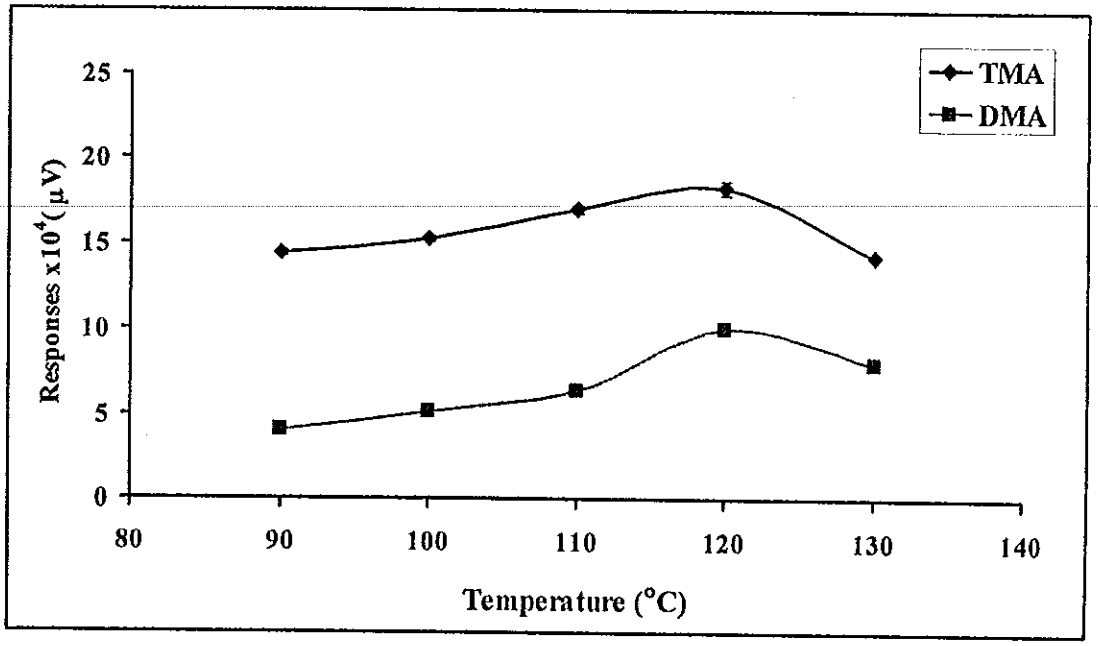

Figure 3.4 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various initial temperatures

Holding time at initial temperature of 120°C was investigated and the results are shown in Table 3.3 and Figure 3.5. The responses decrease as the holding time increased for DMA because the responses of DMA had a tailing peak but the responses for TMA between 0 and 3 minutes were similar. Then 0 minute was chosen as the optimum.

Table 3.3 Effect of holding time at initial temperature on the responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution

Time (min)	Responses×10^4 (μV)*	
	DMA	TMA
0	9.24±0.12	14.54±0.02
1	3.79±0.07	14.06±0.13
2	2.13±0.07	13.99±0.36
3	1.99±0.05	14.30±0.17

*5 replications, RSD < 4 %

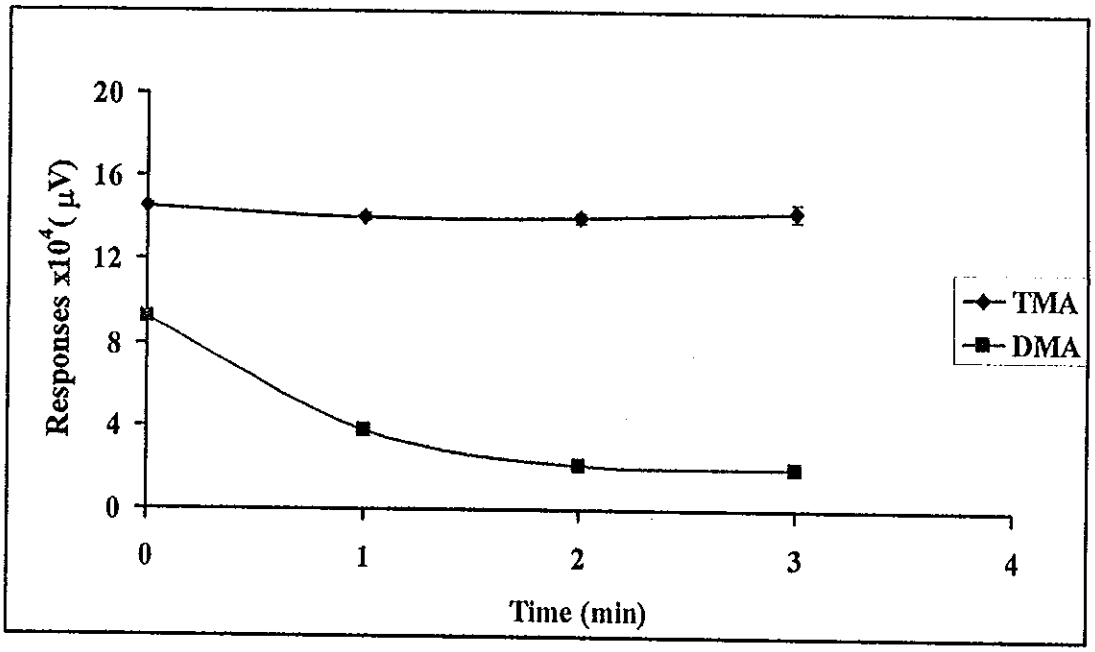

Figure 3.5 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various holding time

The temperature ramp rate of temperature was investigated between 10-40°C min^{-1} as shown in Table 3.4 and Figure 3.6. After 30°C min^{-1} the responses differed less than 10% for DMA and TMA. For this reason, the temperature ramp rate

of 30°C min^{-1} was selected as the optimum since it provided good separation and short analysis time.

Table 3.4 Effect of temperature ramp rate on the responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution

Temperature ramp rate (°C min^{-1})	Responses×10^4 (μV)*	
	DMA	TMA
10	2.92±0.10	14.21±0.23
20	3.53±0.11	12.86±0.08
25	4.81±0.09	13.07±0.16
30	7.98±0.13	14.91±0.39
35	7.71±0.12	14.29±0.39
40	7.63±0.05	13.73±0.29

*5 replications, RSD < 4 %

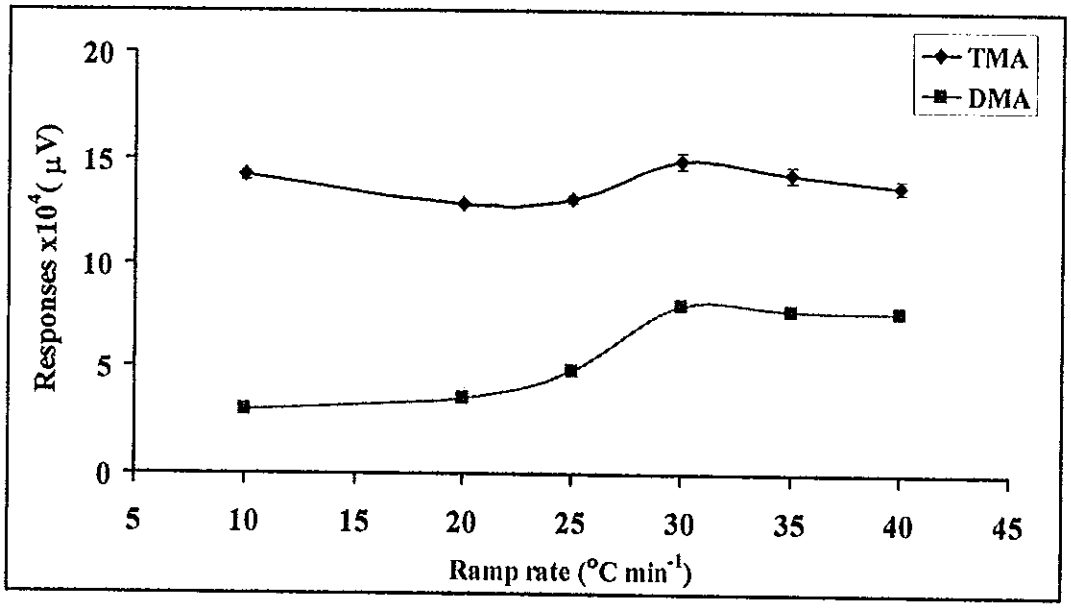

Figure 3.6 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various temperature ramp rates

The optimum final temperature was investigated between 140°C and 180°C (Table 3.5 and Figure 3.7). The highest response was found at 160°C and this was select for the optimum final temperature.

Table 3.5 Effect of final temperature on the responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution

Final temperature (°C)	Responses×10^4 (μV)*	
	DMA	TMA
140	5.95± 0.18	12.49 ± 0.41
150	4.71± 0.05	12.23±0.45
160	7.24± 0.04	13.25±0.18
170	3.69± 0.06	12.51±0.32
180	2.96± 0.11	12.29±0.33

*5 replications, RSD < 4 %

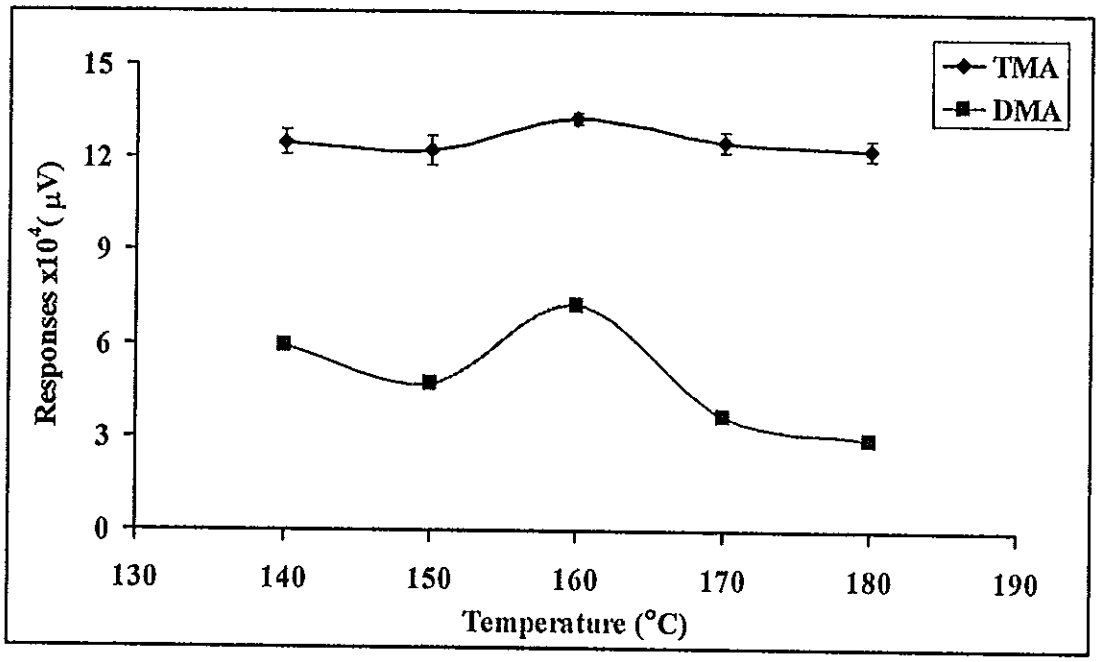

Figure 3.7 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various final temperatures

After final temperature was investigated its holding time was obtained by considering the time that DMA and TMA could be completely eluted from the column. The holding time of final temperature at zero minute was not tested because it gave a DMA response with a tailing peak and did not allow the signal to go back to baseline. The optimum holding time at final temperature was investigated from 1-4 minutes. The results showed that the responses at holding time between 1 and 4 minutes differed less than 10% (Table 3.6 and Figure 3.8). Therefore, the holding time at final temperature of 1 minute was chosen to decrease analysis time at final temperature.

Table 3.6 Effect of holding time at final temperature on the responses of DMA (100 $\mu g\ mL^{-1}$) and TMA (1 $\mu g\ mL^{-1}$) standard solution

Holding time at final temperature (°C)	**Responses×10^4 (μV)***	
	DMA	**TMA**
1	6.02± 0.06	13.93 ± 0.21
2	6.43± 0.15	14.28± 0.35
3	5.78± 0.14	13.80± 0.13
4	5.21± 0.10	13.31± 0.30

*5 replications, RSD < 4 %

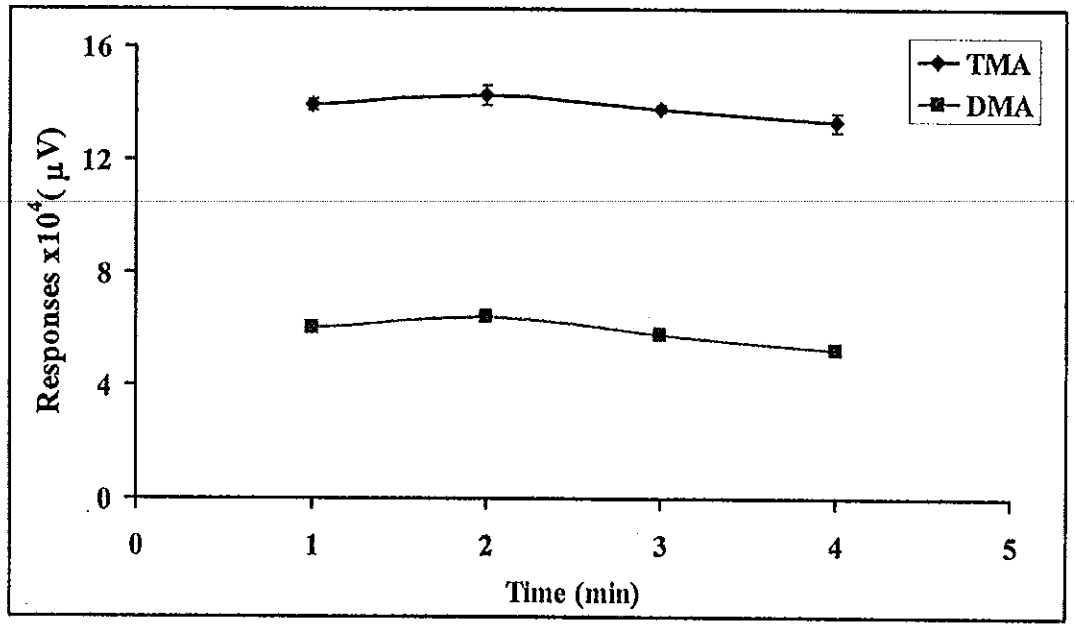

Figure 3.8 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various holding time at final temperatures

The optimum conditions of column temperature programming for GC-NPD were summarized in Figure 3.9.

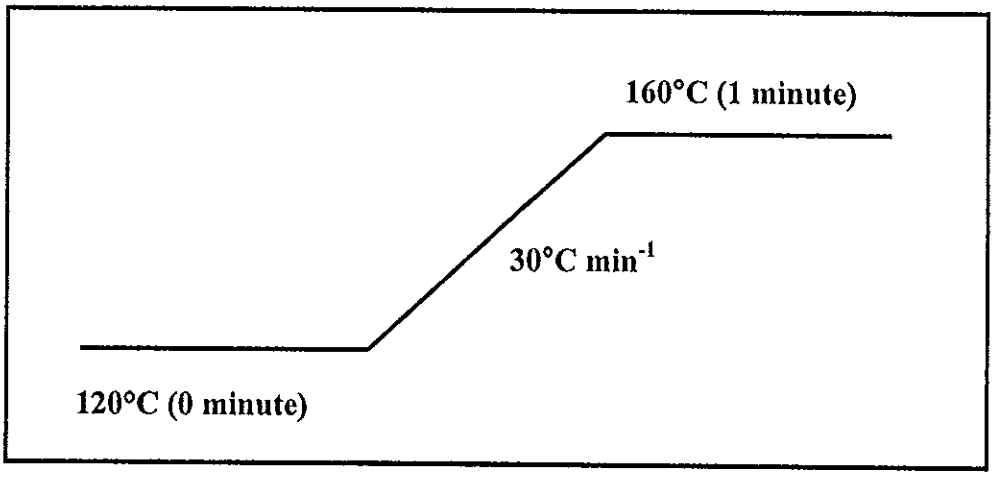

Figure 3.9 The optimum column temperature programming for DMA and TMA analysis

3.1.3 Injector temperature

Injector is an important part of the system that performs the physical task of transferring the sample into the gas chromatograph. The injector temperature must be high enough to vaporize the injected sample into the column but not too high that cause the sample components to decompose. The inlet injection of this study used a split mode where the split inlet allows the introduction of a user-selectable fraction of the injected sample into the capillary column by adjusting the relative flows of carrier gas into the column and to waste through a purge valve. Split inlet is heated, with a high thermal mass, to ensure that the entire injected sample evaporates quickly and mixes homogeneously with the carrier gas (Grob, 2004).

Table 3.7 and Figure 3.10 show the responses of DMA and TMA at injector temperature from 140-180°C where the responses differed less than 10%. By considering high resolution, short analysis time, and decrease tailing peak for DMA, 160°C was chosen as the optimum injector temperature.

Table 3.7 Effect of injector temperature on the responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution

Injector temperature (°C)	**Responses×10^4 (μV)***	
	DMA	**TMA**
140	4.00±0.06	11.54 ± 0.38
150	4.28±0.12	11.57± 0.33
160	4.46±0.09	12.10± 0.45
170	4.25±0.15	11.96± 0.23
180	4.27±0.07	11.95± 0.39

*5 replications, RSD < 4 %

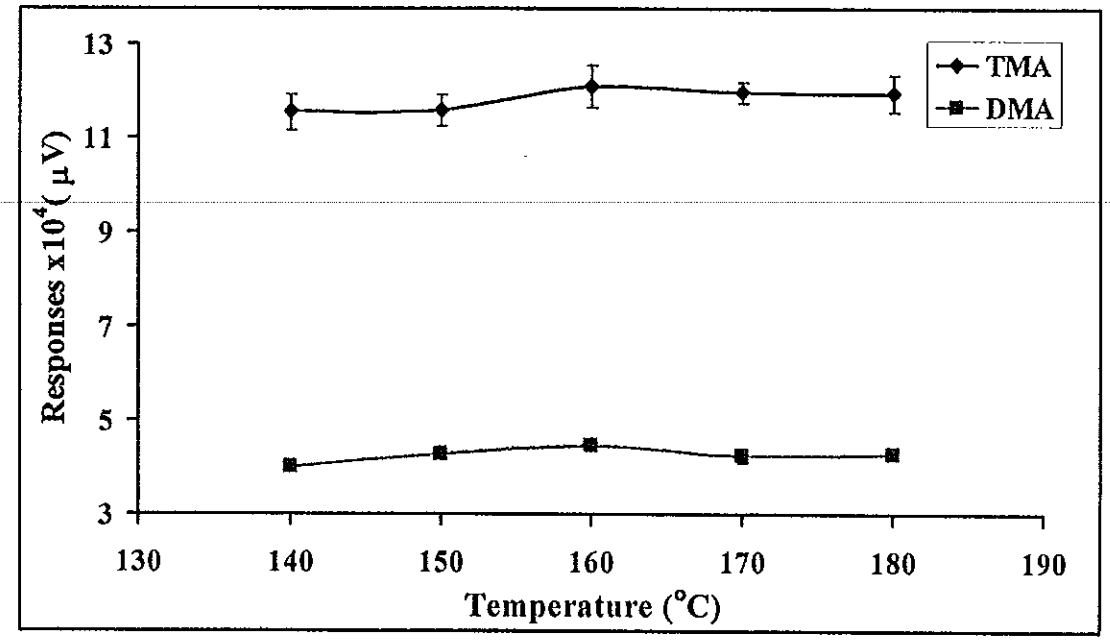

Figure 3.10 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various injector temperatures

3.1.4 Detector temperature

The detection system in gas chromatography provides the responses signal for the chemical compounds separated by the chromatographic column. The type of detector used depended on the application. In this system the analytes eluted from the column was detected by the nitrogen phosphorous detector (NPD). The NPD is based on the phenomenon that a metal anode emits positive ions when heated in a gas. It is a commonly used gas chromatographic detector for the selective determination of organic compounds containing nitrogen (N) and phosphorous (P) atom.

The detectability and the specificity of the detector are both affected by the magnitude of the heating current. The hydrogen flow affects the concentration of the hydrogen atoms in the reactive gaseous layer around the thermionic source that in turn determines the responses. The effect of the flow rate and the temperature increased the source heating current beyond the base value required to initiate the H_2/air chemistry can increase the detectability of the detector (Grob, 2004).

The hydrogen and make up gases mix with the column effluent inside the jet. The gases leave the jet and mix with air; then, they are heated at the hot thermionic source, where decomposition and ionization occur. Ions are then collected at the collector electrode, which is maintained at a potential of a few hundred volts (Grob, 2004).

The responses from various detector temperatures are shown in Table 3.8 and Figure 3.11. Although 240°C gave the highest response but the limit of the column temperature was at 250°C therefore 220°C was chosen as the optimum detector to avoid damaging the column.

Table 3.8 Effect of detector temperature on the responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution

Detector temperature (°C)	**Responses×10^4 (μV)***	
	DMA	**TMA**
180	2.27±0.07	7.23 ± 0.22
200	2.40±0.06	8.76± 0.11
210	2.86±0.07	9.04± 0.20
220	2.93±0.10	9.56± 0.35
230	2.86±0.12	10.11±0.49
240	3.82±0.09	11.27± 0.20

*5 replications, RSD < 4 %

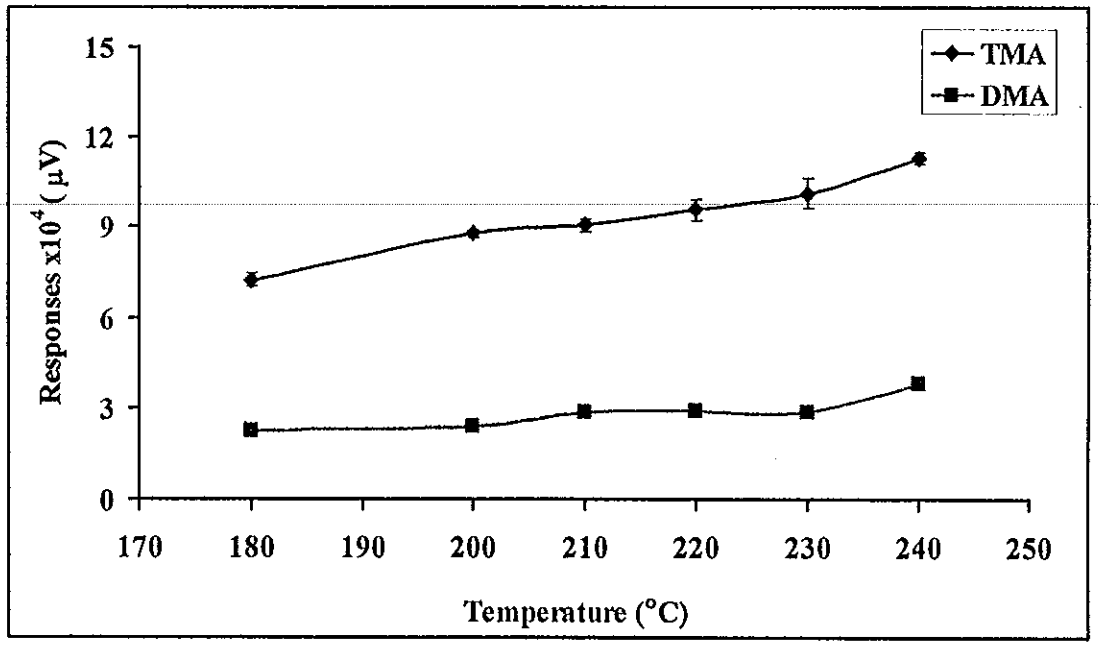

Figure 3.11 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various detector temperatures

3.1.5 The fuel gas flow rate (hydrogen gas)

The design of the nitrogen phosphorous detector used in this work is similar to a flame ionization detector (FID) but with an important difference *i.e.,* an electrically heated silicate bead doped with an alkali salt was mounted between the jet and the collector. A very low hydrogen gas flow rate is mixed with the carrier gas and burns as plasma flame when contact with the heated bead and jet. The exact mechanism for producing a responses has been subject of some discussion, but Kolb's theory seems to be widely accepted (Tripler, 1993). At the operating temperature, the bead substrate is electrically conductive and some of the alkali ions are able to acquire an electron and are thus converted to the atomic form. These atoms are relatively volatile and are emitted into the plasma, where they quickly react with combustion products, and are ionized again and recollected on the negatively polarized bead. This repeated process gives rise to the background signal and explains why the bead continues to function over an extended time. When a compound containing nitrogen or phosphorous eluted from the column into the plasma, these molecules will burn and

react with the exited atomic alkali to form phosphorous oxide anions. These reactions disturb the alkali equilibrium in the plasma and additional alkali released into the plasma, thus promoting further ion formation and in creasing the signal (Baugh, 1993).

The main disadvantage of this detector is that its performance deteriorates with time. The alkali salt employed as the bead is usually an alkali silicate that the reduced responses was due to water vapour from the burning hydrogen, converting the alkali silicate to the hydroxide. At the operating temperature of the bead, the alkali hydroxide has significant vapour pressure and consequently, the rubidium or cesium is continually lost during the operating of the detector. Eventually all the alkali is evaporated, leaving a bead of inactive silica. This an inherent problem with all NPD detectors and as a result, the bead needs to place regularly if the detector is in continuous use (Raymond, 1998).

In this system the analyte eluted from the column was detected by a nitrogen phosphorous detector (NPD). The detector consists of small hydrogen and air diffusion flame burning produced from oxidant and fuel gas. The oxidant gas was air and fuel gas was hydrogen. Thus, the flow rate of air and hydrogen gases significant influences the noise level and the sensitivity of the detector.

The effect of hydrogen gas flow rate to the responses is shown in Table 3.9 and Figure 3.12. The highest response was obtained at 2.0 mL min^{-1}, the optimum flow rate. This is the same as the recommended value of GC-NPD manual, 2.0 mL min^{-1} (The Perkin-Elmer Corporation, 1995).

Table 3.9 Effect of hydrogen gas flow rate on the responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution

Hydrogen gas flow rate ($mL\ min^{-1}$)	Responses×10^4 (μV)*	
	DMA	TMA
1.0	3.57 ± 0.13	9.71 ± 0.20
2.0	7.06 ± 0.12	11.51 ± 0.20
3.0	4.90 ± 0.12	7.99 ± 0.17
4.0	4.48 ± 0.05	6.21 ± 0.06

*5 replications, RSD < 4 %

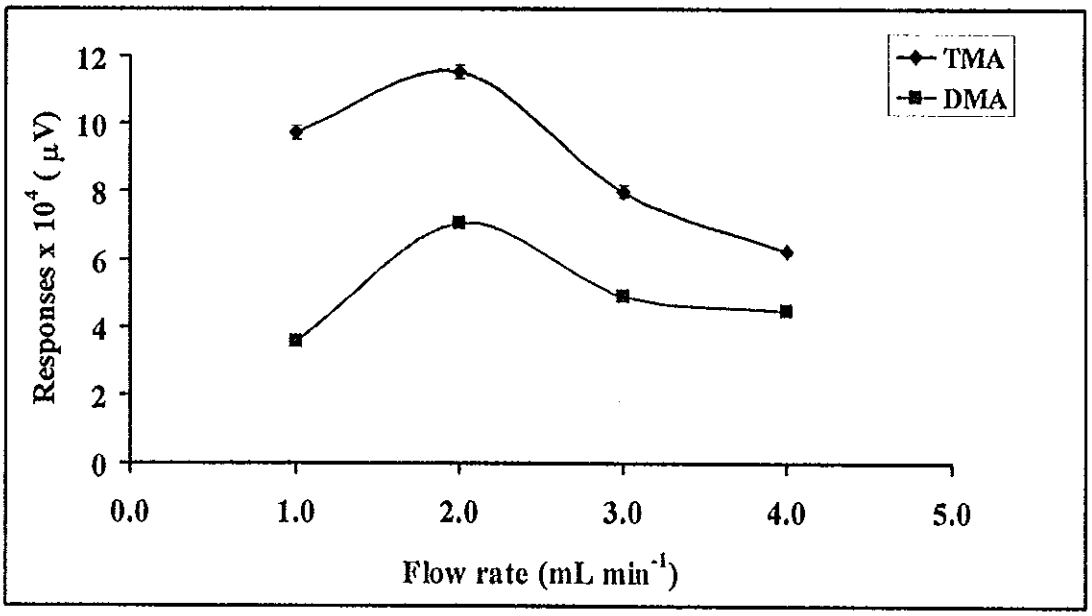

Figure 3.12 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various hydrogen gas flow rates

3.1.6 Oxidant gas flow rate (air)

Air was used as an oxidant for NPD. Typical flows of air are 100-200 $mL\ min^{-1}$ (Grob, 2004). The effect of air flow rate on the responses (section 2.6.3) is

shown in Table 3.10 and Figure 3.13. The highest response was obtained at 100 mL min^{-1}, the same as the recommend value of GC-NPD manual (The Perkin-Elmer Corporation, 1995).

Table 3.10 Effect of air flow rate on the responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution

Air flow rate (mL min^{-1})	Responses×10^4 (μV)*	
	DMA	TMA
90	2.88 ± 0.09	10.37 ± 0.34
100	4.82 ± 0.18	16.62 ± 0.39
110	4.55 ± 0.11	14.43 ± 0.28
120	4.29 ± 0.15	12.46 ± 0.32

*5 replications, RSD < 4 %

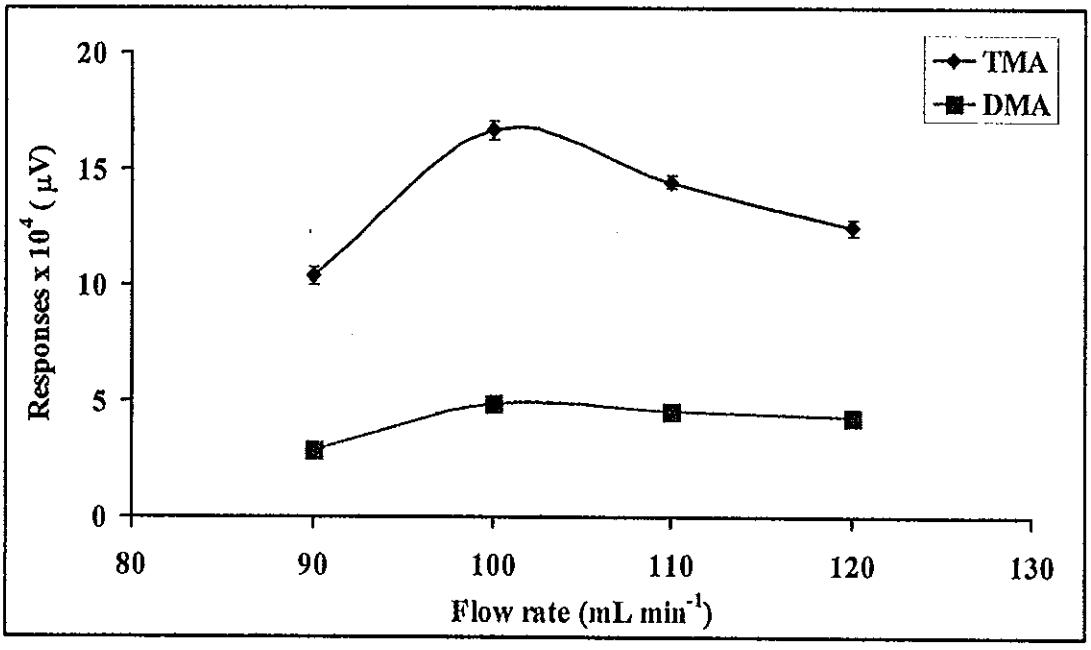

Figure 3.13 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various air flow rates

3.1.7 The split ratio

The split ratio is the ratio of the volumetric flow rate at the split purge vent to the volumetric flow rate in the GC column.

Preliminary study showed that at the split ratio less than 10:1 although a high response was obtained, it provided broad peaks and the resolution was less than one because the analytes overload the capillary column. When increase the split ratio from 10:1 up to 40:1 the responses of DMA and TMA decreased (Table 3.11, Figure 3.14) because less analytes flowed through the capillary column. The optimum split ratio was then selected at 10:1 because it gave the highest response and the resolution was higher than others.

Table 3.11 Effect of the split ratio on the responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution

The split ratio	Responses×10^4 (μV)*	
	DMA	TMA
10	8.87±0.14	12.09±0.06
20	3.81±0.13	7.26±0.16
30	2.16±0.03	4.71±0.14
40	2.07±0.03	4.34±0.11

*5 replications, RSD < 4 %

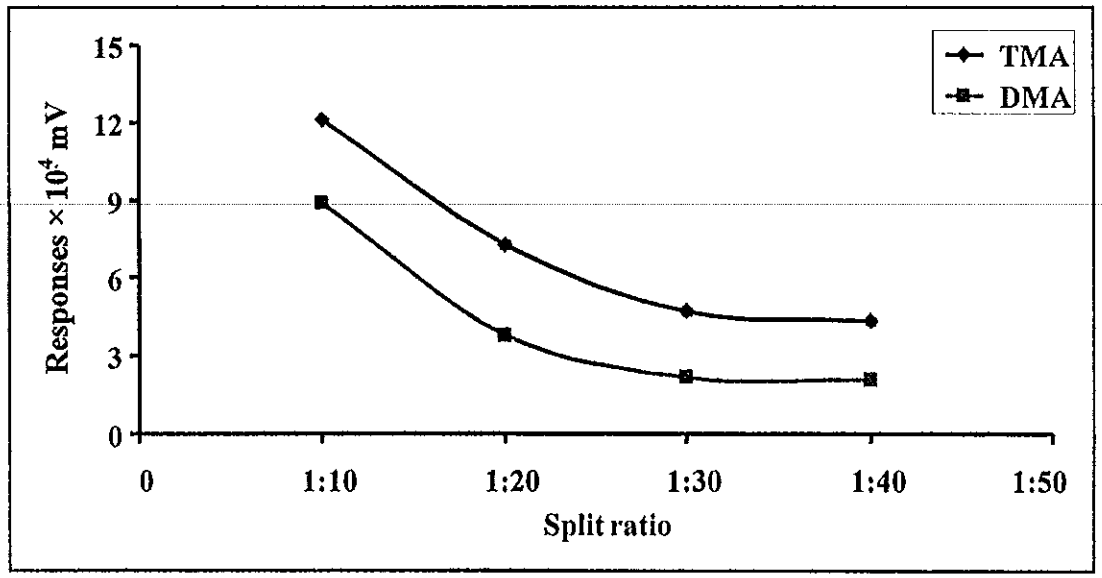

Figure 3.14 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various the split ratios

3.1.8 Summary of GC-NPD conditions

The optimum conditions for DMA and TMA analysis on capillary column (HP-FFAP, 25 m × 0.32 mm i.d., 0. 52 μm film thicknesses) with nitrogen phosphorous detector are summarized in Table 3.12. The chromatogram obtained from these optimum conditions is shown in Figure 3.15.

Table 3.12 The optimum conditions of GC-NPD for DMA and TMA

Conditions	Optimum conditions
Flow rate	
- Carrier gas (helium gas)	3 mL min^{-1}
- Fuel flow rate (hydrogen gas)	2 mL min^{-1}
- Oxidant gas flow rate (air)	100 mL min^{-1}
Column temperature programming	
- Initial temperature	120°C
- Holding time at initial temperature	0
- Temperature ramp rate	30°C min^{-1}
- Final temperature	160°C
- Holding time at final temperature	1 min
Injector temperature	160°C
Detector temperature	220°C
Split ratio	1:10

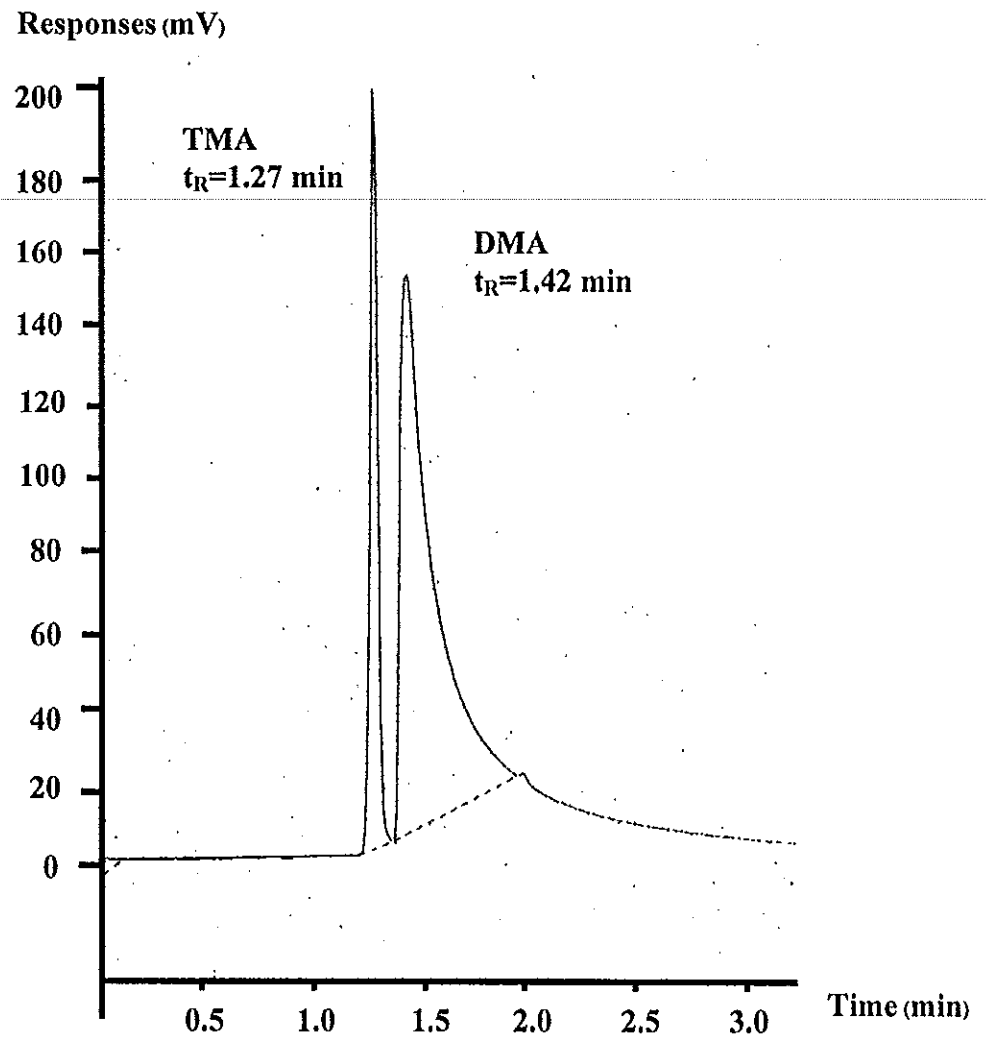

Figure 3.15 The chromatogram of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) at optimum GC-NPD conditions

3.1.9 Headspace analysis conditions

Headspace gas chromatography (GC-NPD) has been available since the late 1960s (Hachenberg *et al.*, 1977) and is a rugged, robust and popular method of sample preparation used for the introduction of volatile analytes to gas chromatograph. There are two types of headspace analysis: static and dynamic. In static analysis, the analytes are samples under conditions of equilibration and in dynamic; the analytes are exhaustively extracted from the sample (purge and trap).

For static headspace, the sample is sealed in a vial where the analytes reach equilibrium between the gas phase (headspace) and sample (liquid and solid). Once reach to equilibrium the analytes are transferred to the chromatograph for analysis. In dynamic headspace, equilibrium is never reached from the vial. The volatile analytes are swept to a trap where they held until analyses (Grob, 2004).

Static headspace was employed and the volatile DMA and TMA in the solution are present in the gas phase that is in contact with the solution of the headspace vial as shown in Figure 3.16. It relative concentrations in the gas phase depend on the partition pressure which in turn could be influenced by temperature and time (Kolb and Ettre, 1997).

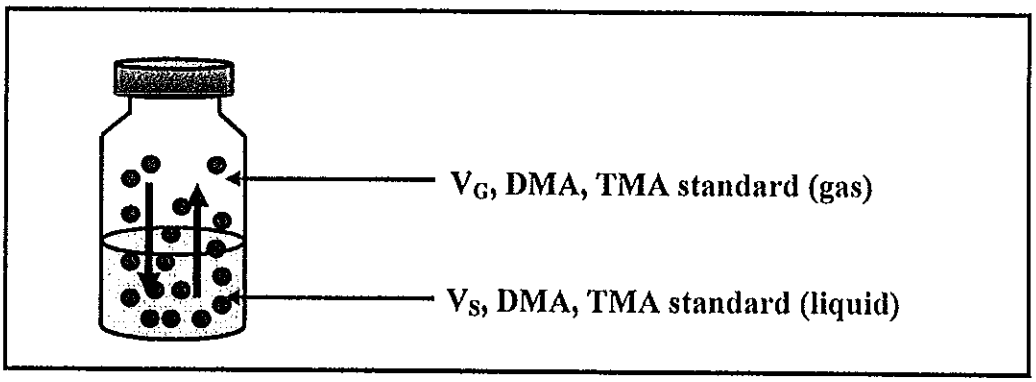

Figure 3.16 A headspace vial containing the volatile DMA and TMA in standard solution: V_G = volume of the gas phase, V_S = volume of the liquid sample

3.1.10 Equilibration temperature

The influence of equilibration temperature on the volatility and the headspace sensitivity were studied. In static headspace analysis increasing temperature to enhance sensitivity could cause a condensation problem from the evaporation of the analyte into gas phase. Therefore, the equilibration temperature should be optimized to avoid the condensation. For the laboratory-built thermal system at temperature higher than 70°C, condensation of analyte in the syringe occurred during the transfer of the gas phase headspace to GC-NPD system. To avoid

this condensation, ASTM standard practice recommended the syringe should be heated it in the oven at 90°C before sampling (Kolb and Ettre, 1997). In this work the syringe was cleaned by a syringe cleaner and heated at 370°C.

The effects of the equilibration temperature on the responses are shown in Table 3.13 and Figure 3.17. The responses of DMA and TMA increased when the equilibration temperature increased. The equilibration temperature at 70°C that gave the highest response and sensitivity without condensation.

Table 3.13 Effect of equilibration temperature on the responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution

Equilibration temperature (°C)	**Responses×10^4 (μV)***	
	DMA	**TMA**
40	10.91±0.10	4.27±0.03
50	16.23±0.15	10.51±0.29
60	16.69±0.46	18.67±0.10
70	16.78±0.15	26.83±0.81
80	17.20±0.01	44.45±1.56

*5 replications, RSD < 4 %

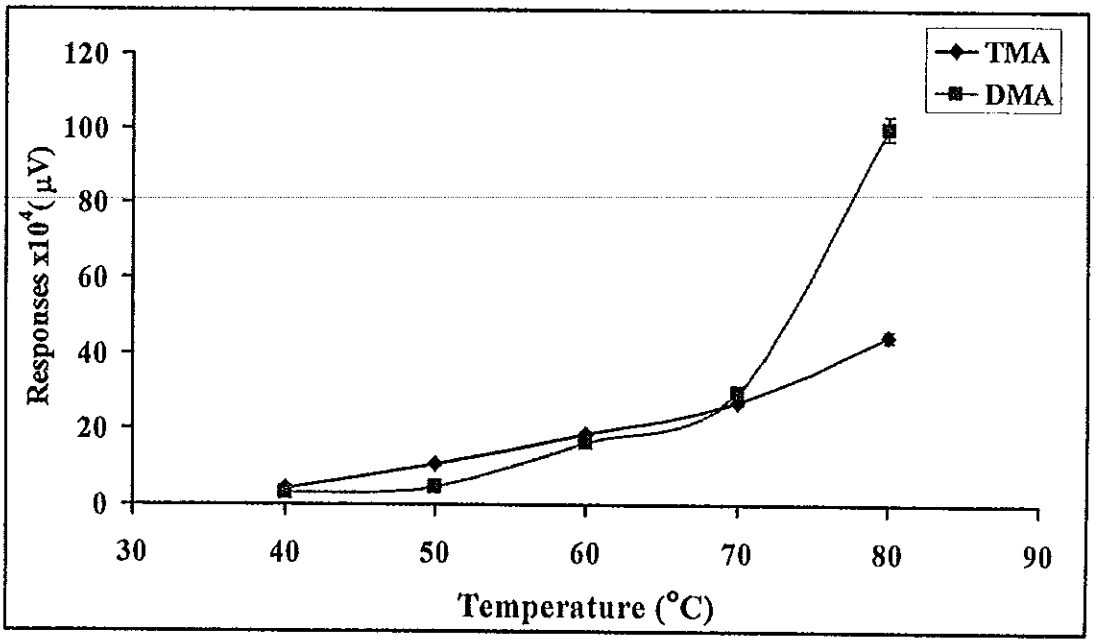

Figure 3.17 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various equilibration temperatures

3.1.11 Equilibration time

Parameters influencing the headspace sensitivity are equilibration temperature, equilibration time and sample volume (phase ratio). The equilibration time is depending on the distribution of volatile components. For this work, headspace technique for the standard DMA and TMA solutions is performed by placing the sample in a closed headspace vial to establish an equilibrium condition between the sample and gas phases in a laboratory-built thermal bath system. DMA and TMA were continuously extracted from the liquid phase (sample phase) into gas phase until an equilibration was reached (DMA and TMA in the gas phase equal to the DMA and TMA in the liquid phase). Table 3.14 and Figure 3.18 showed that the responses increased with equilibration time up to 20 minutes and became constant. Therefore, 20 minutes was selected as the optimum equilibration time.

Table 3.14 Effect of equilibration time on the responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solutions

Equilibration time (min)	Responses×10^4 (μV)*	
	DMA	TMA
10	10.91±0.10	20.80±0.43
20	16.23±0.15	22.33±0.21
30	16.69±0.46	22.57±0.49
40	16.78±0.15	23.00±0.34
50	17.20±0.01	23.12±0.63

*5 replications, RSD < 4 %

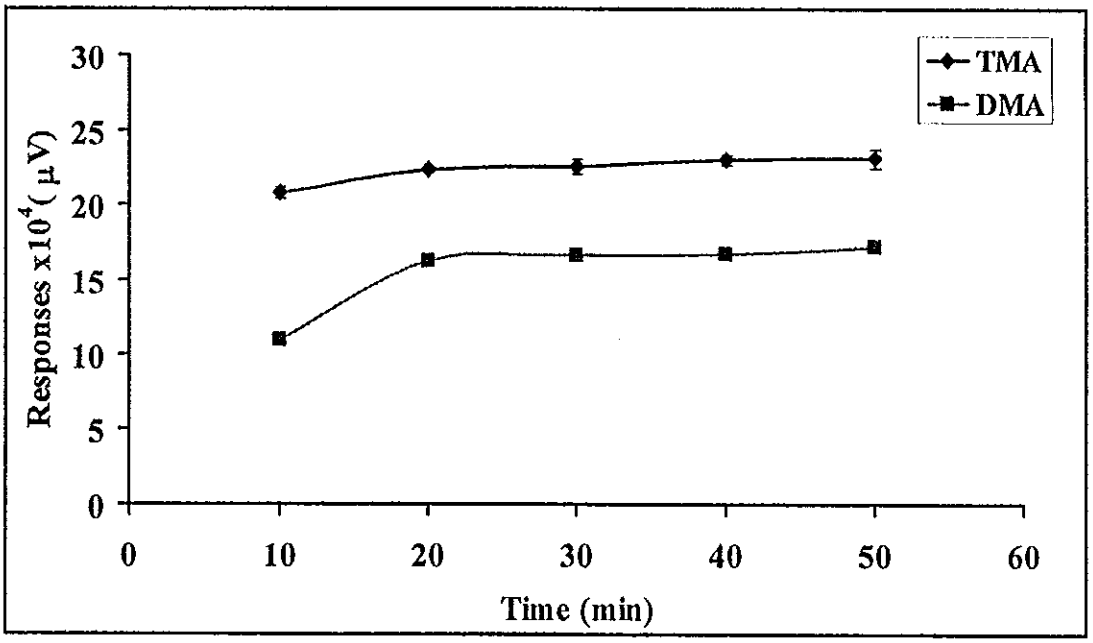

Figure 3.18 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various equilibration times

3.1.12 Sample volume (phase ratio)

The headspace sensitivity also depends on the phase ratio. The phase ratio (β) is the ratio of the volume of the gas phase to the volume of the sample phase in the vial (Figure 3.16) and is presented as equation 3.7.

$$\beta = \frac{V_G}{V_S} \tag{3.7}$$

Where V_G = volume of the gas phase

V_S = volume of the sample phase

V_V = volume of the total

When

$$V_V = V_G + V_S \tag{3.8}$$

equation (3.7) can be expressed as

$$\beta = \frac{V_V - V_S}{V_S} \tag{3.9}$$

and

$$V_S = \frac{V_V}{1+\beta}. \tag{3.10}$$

Therefore

$$\beta = \frac{V_G}{V_S} = \frac{V_V - V_S}{V_S} = \frac{V_G}{V_V - V_G} \tag{3.11}$$

The phase ratio will increase when the sample volume decrease or gas phase increase.

Headspace analysis is also related to the distribution of analyte between the two phases upon the equilibrium and is expressed by the

thermodynamically controlled equilibrium constant. In analogy to the common practice in gas chromatography, the synonymous term partition (distribution) coefficient, K, and is express as

$$K = \frac{C_S}{C_G} \tag{3.12}$$

Where K is the partition coefficient related to the mass distribution in the phase system. It depends on the solubility of the analyte in the condensed phase: compounds with high solubility will have a high concentration in the condensed phase relative to the gas phase. C_S is the concentration of analyte in sample phase (condensed phase). C_G is the concentration of analyte in gas phase (headspace).

Headspace analysis technique was used in this work and the responses of DMA and TMA using GC-NPD were measured as the peak height. The relation of the concentration of sample and peak height is expressed as equation 3.13

$$A \propto C_G = \frac{C_O}{K + \beta} \tag{3.13}$$

Where A = peak area (in this work was used peak height)

C_G = the concentration of the analyte in the headspace (gas phase)

C_O = the original sample concentration of the analyte

K = the partition coefficient

β = phase ratio of the vial

From equation (3.13) the headspace sensitivity (the peak height) is depended on the distribution coefficient (K) and phase ratio (β). The sensitivity of the headspace will increase as the distribution coefficient and phase ratio decrease.

The phase ratio for the headspace laboratory-built thermal bath system was investigated in section 2.7.3. The results are shown in Table 3.15 and Figure 3.19. The phase ratio at 1.0, 1.5 and 2.0 gave high responses and were nearly the same. The optimum phase ratio of headspace technique was chosen at 2.0 (20 mL sample volume in a 60 mL vial) because it used less sample volume. The phase ratio lower than 1.0 (0.2 and 0.5) gave less sensitivity because it did not reach thire equilibrium.

Table 3.15 Effect of phase ratio on the responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solutions

Phase ratio	Responses×10^4 (μV)*	
	DMA	TMA
0.2	12.42±0.15	14.97±0.50
0.5	16.56±0.37	16.32±0.46
1.0	19.77±0.72	19.12±0.52
1.5	19.29±0.48	18.91±0.22
2.0	18.53±0.21	18.79±0.54
5.0	15.84±0.39	16.76±0.23

*5 replications, RSD < 4 %

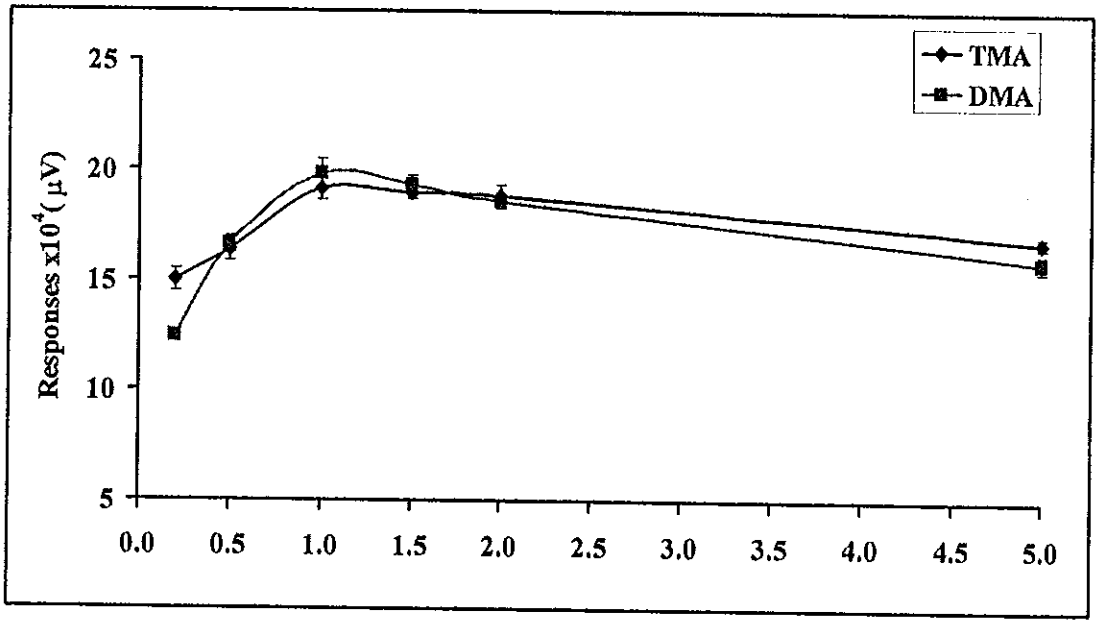

Figure 3.19 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various phase ratios

3.1.13 Vial volume size

Three size of vial *i.e.*, 10, 30 and 60 mL were used for the investigation. The analyses of DMA and TMA in fish and shrimp samples with GC-NPD system are shown in Table 3.16 and Figure 3.20. The headspace sensitivity primarily depends on the concentration in the gas phase but not on the sample vial size: the phase ratio (β) is the one that determines the final sensitivity. The result showed that a 3.33 mL liquid sample in a 10 mL vial gave the highest response because a smaller sample volume allows shorter equilibrium vial (Kolb and Ettre, 1997) (The phase ratio, β in all cases was 2). Consequently, a 10 mL of vial volume was chosen to prepare sample.

Table 3.16 Effect of size of vial volume on the responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution

Size of vial volume (mL)	Responses×10^4 (μV)*	
	DMA	TMA
10	2.37±0.13	0.93±0.04
30	1.14±0.12	0.59±0.02
60	0.75±0.06	0.69±0.08

*5 replications, RSD < 13 %

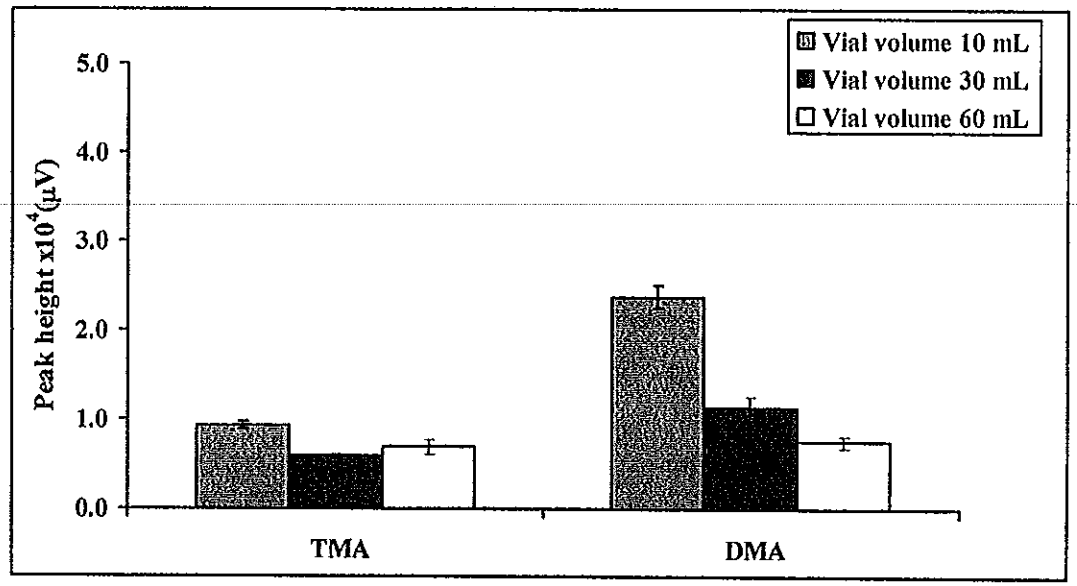

Figure 3.20 Responses of DMA (100 μg mL^{-1}) and TMA (1 μg mL^{-1}) standard solution at various size of vial volume

3.1.14 Salts and amount of salts

In headspace technique the activity coefficient can be increased by adding salt. A small partition coefficient indicates reduced the solubility of the analyte in the matrix and thus an increased concentration in the headspace in equation 3.12. This can be achieved by adding an electrolyte (salt) to the sample and the technique, commonly called salting out. However, the sensitivity is not influenced by salting out effect alone; there is an additional volume effect involved which is widely ignored (Kolb and Ettre, 1997). The addition of a high amount of salt increases the volume of liquid sample thus decreases the phase ratio (β).

In this work, the adding of NaCl, Na_2CO_3, Na_2SO_4 and KCl were tested where the amounts were varied at 0.5, 1.0, 1.5 and 2.0 g. The results are shown in Tables 3.17-3.18 and Figures 3.21-3.22. The salt and amount of salt that gave the highest response for both DMA and TMA was Na_2CO_3 at 1.5 g because it has the highest ionic strength which can be obtained from the Debye Hückel theory as equation 3.14 resuting in the decreasing of analytes solubility in the matrix. The other

reason is that sample under the addition of Na_2CO_3 is in the basic condition which kept the analyte in the original form.

$$I = \frac{1}{2}\sum_i C_i Z_i^2 \qquad (3.14)$$

Where I is ionic strength

C_i is the concentration of every ion, mol L^{-1}

Z_i are the charges on the two ions (Butler and Cogley, 1998).

The higher ionic strength can decrease the solubility of analyte in matrix resulting in higher concentration of the analyte in the gas phase (Kolb and Ettre, 1997).

So, the best salt and amount was Na_2CO_3 at 1.5 g that is the salting out to reduce the solubility of the analyte in the matrix.

Table 3.17 Effect of salt and amount of salt on the responses of DMA (150 μg mL^{-1}) standard solution

Amount of salt (g)	Responses×10^4 (μV)* for DMA			
	NaCl	Na_2CO_3	Na_2SO_4	KCl
0.5	48.4±1.3	345.4±4.7	42.7±1.6	37.3±1.0
1.0	39.4±1.3	501.8±10.8	70.2±2.4	48.8±1.2
1.5	96.9±2.2	1234.8±18.2	554.7±6.1	184.1±2.5
2.0	76.5±1.5	763.5±12.3	232.5±4.8	84.8±1.0

*5 replications, RSD < 4 %

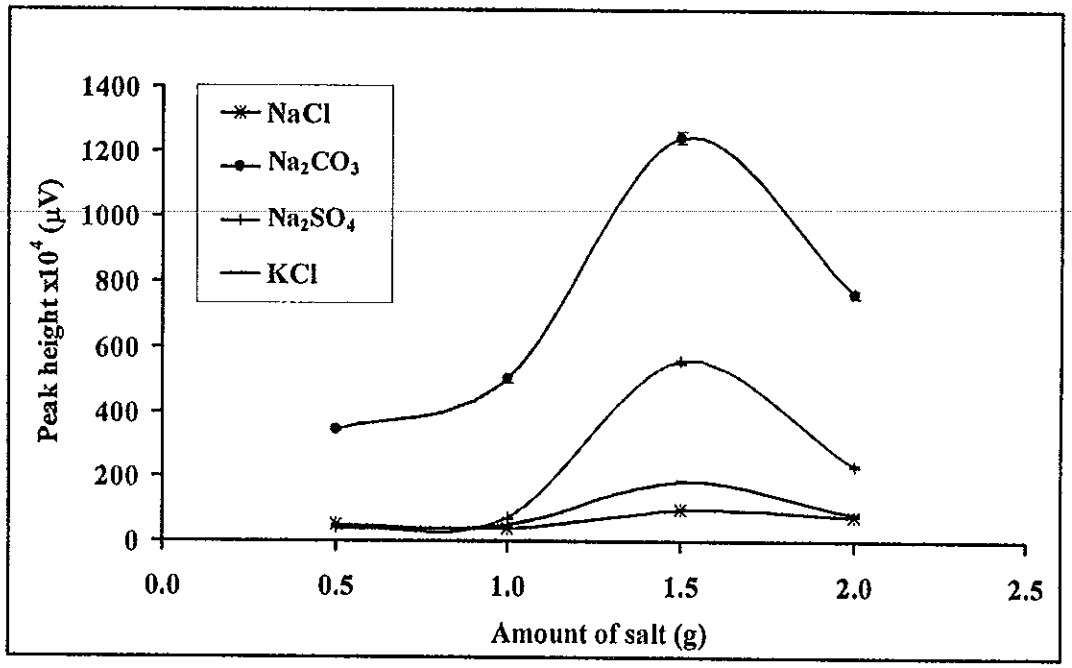

Figure 3.21 Responses of DMA (150 μg mL^{-1}) standard solution at various salts and amount of salt

Table 3.18 Effect of salt and amount of salt on the responses of TMA (1 μg mL^{-1}) standard solution

Amount of salt (g)	Responses×10^4 (μV)* for TMA			
	NaCl	Na_2CO_3	Na_2SO_4	KCl
0.5	17.7±0.5	36.7±1.4	19.5±0.8	15.7±0.2
1.0	35.3±1.3	51.2±2.0	41.5±1.4	33.4±0.8
1.5	29.8±0.9	112.8±0.6	70.8±1.7	40.4±0.6
2.0	19.8±0.6	67.7±1.1	43.4±1.4	30.2±0.7

*5 replications, RSD < 4 %

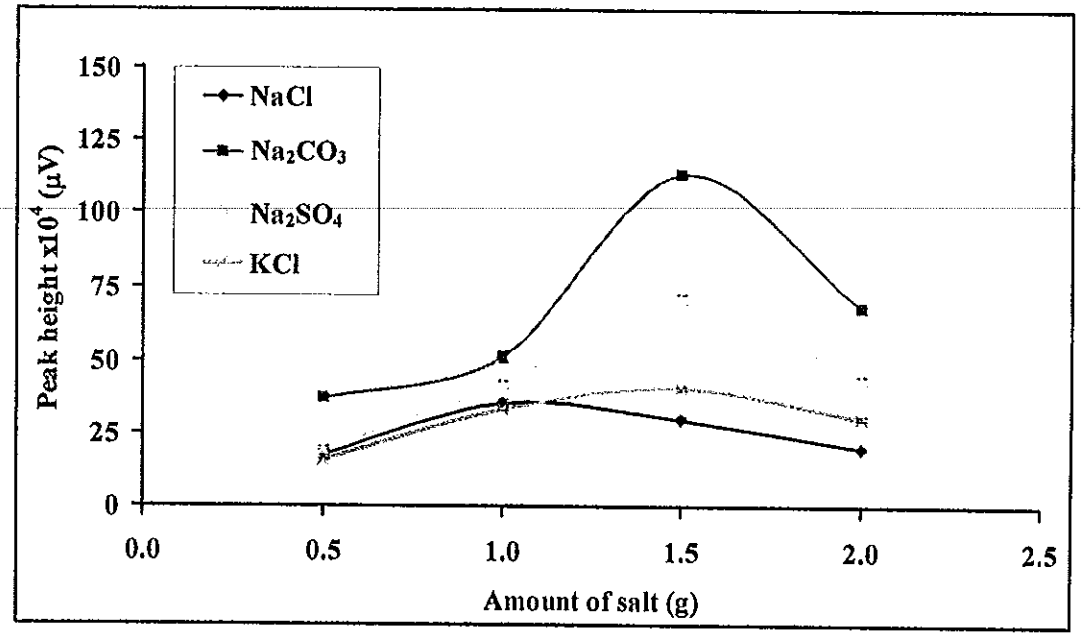

Figure 3.22 Responses of TMA (1 μg mL^{-1}) standard solution at various salts and amount of salt

3.1.15 Summary of headspace conditions

The optimum headspace conditions for the analysis of DMA and TMA in fish and shrimp samples with GC-NPD using HP-FFAP capillary column are summarized in Table 3.19. The chromatogram obtained by these conditions are shown in Figure 3.23 where the resolution more than one were obtained.

Table 3.19 The optimum conditions of headspace system for DMA and TMA analysis

Parameter	Optimum values
Headspace system	
• Equilibration time	20 minute
• Equilibration temperature	70°C
• Phase ratio	2.0
• Vial volume	10 mL
• Salt and amount of salt	Na_2CO_3 1.5 g

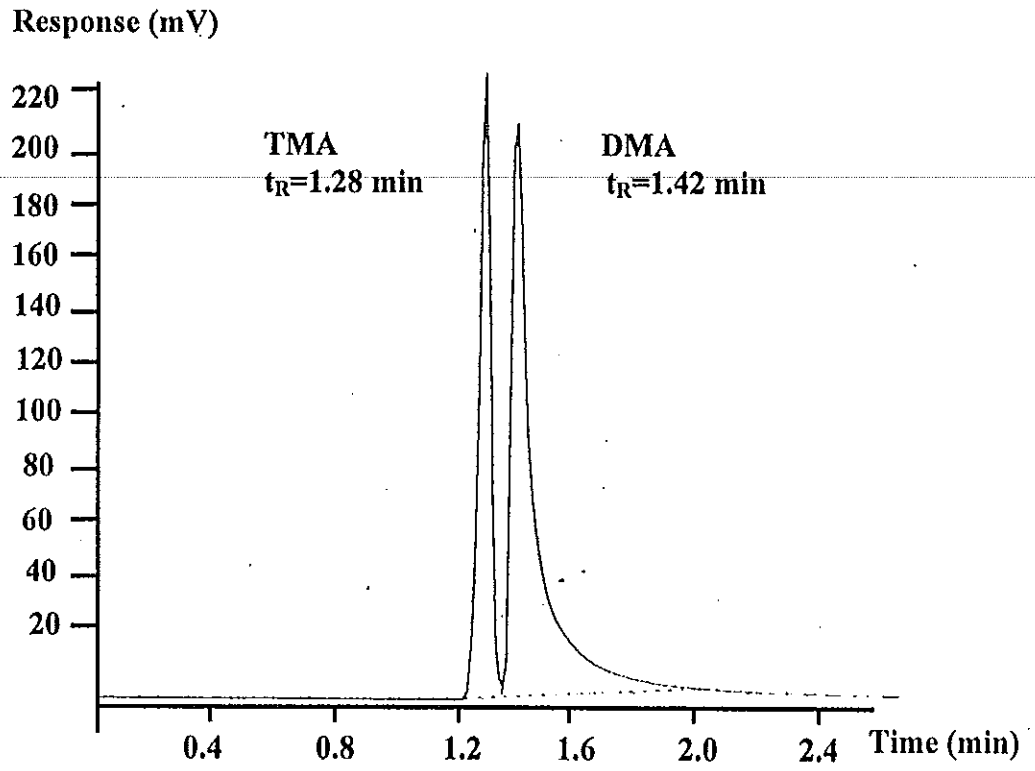

Figure 3.23 Chromatogram of TMA (1 μg mL^{-1}) and DMA (100 μg mL^{-1}) standard solution by HS-GC-NPD system

3.2 Linear dynamic range (LDR, Linearity)

The linear dynamic range or linearity of the headspace is the relationship between the original concentrations (C_O) of analyte in the sample and its concentration in the headspace. The data variables used for quantitation of analytes are peak height. In section 2.5.4, the dynamic range was investigated by serial dilutions of a stock standard solution. Linear dynamic range is achieved when the coefficient of determination (R^2) is equal or greater than 0.99 (FDA, 2000). When the value of R^2 get closer to 1 (Miller and Miller, 2000) the slope of the regression line will provide the sensitivity of the regression and method to be validated. The linearity of DMA and TMA were determined in 2.8 for the laboratory-built thermal bath with GC-NPD system. The responses of DMA and TMA at various concentrations are shown in Tables 3.20-3.21 and Figures 3.24-3.25. For each concentration, five

replications were done and good precision was obtained since the relative standard deviations (RSD) were all lower than 4%. The HS-GC-NPD system showed a wide linear dynamic range, 2.7×10^{-3}-250 μg mL^{-1} for DMA and 0.30×10^{-3}-50 μg mL^{-1} for TMA, $R^2>0.99$.

Table 3.20 The responses of DMA at various concentrations

Concentration (μg mL^{-1})	**Responses$\times10^6$ (μV)***
	DMA
0.5	0.007±0.001
1.0	0.018±0.001
5.0	0.236±0.004
7.5	0.240±0.002
10	0.29±0.01
25	2.2±0.02
50	6.0±0.1
75	8.2±0.3
100	11.0±0.3
150	17.2±0.2
175	20.1±0.6
200	21.9±0.3
225	24.4±0.5
250	27.5±0.4
275	27.6±1.0
300	28.5±0.7

*5 replications, RSD < 4 %

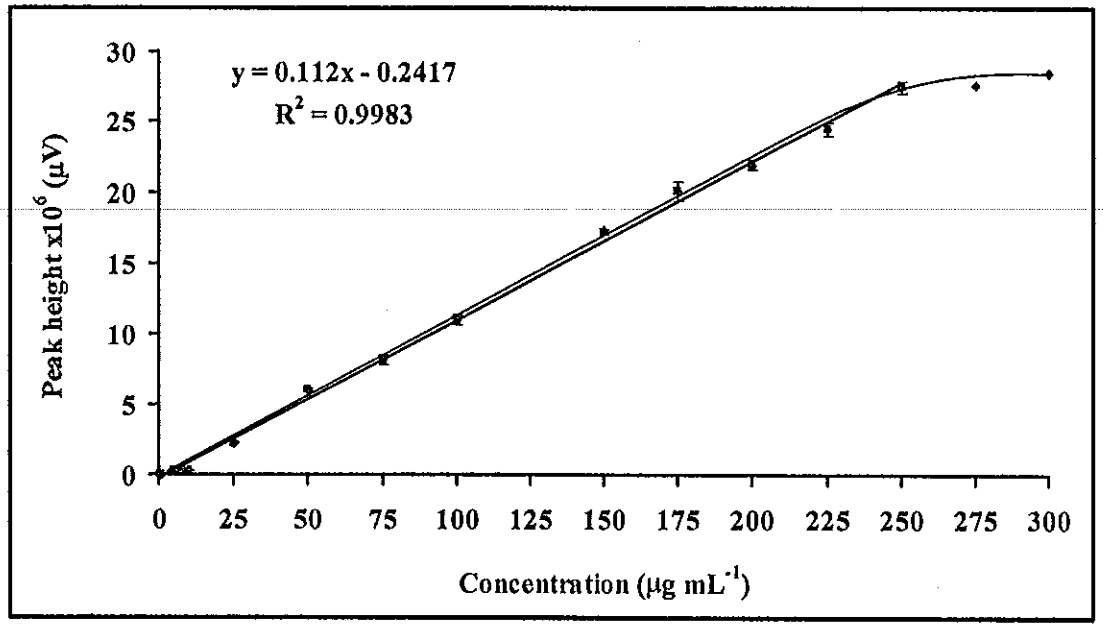

Figure 3.24 Linear dynamic range of DMA

Table 3.21 The responses of TMA at various concentrations

Concentration ($\mu g\ mL^{-1}$)	Responses$\times 10^6$ (μV)* for TMA
0.001	0.0040±0.0001
0.005	0.0029±0.0001
0.01	0.0089±0.0002
0.05	0.059±0.001
0.10	0.140±0.005
0.25	0.34±0.01
0.50	0.68±0.02
1.0	1.24±0.04
5.0	6.1±0.1
10	11.8±0.3
15	16.3±0.4
20	22.1±0.2
30	33.3±0.8
40	43.6±0.7
50	55.5±1.7
60	59.3±0.7
70	62.6±0.4
80	63.0±0.1

*5 replications, RSD < 4 %

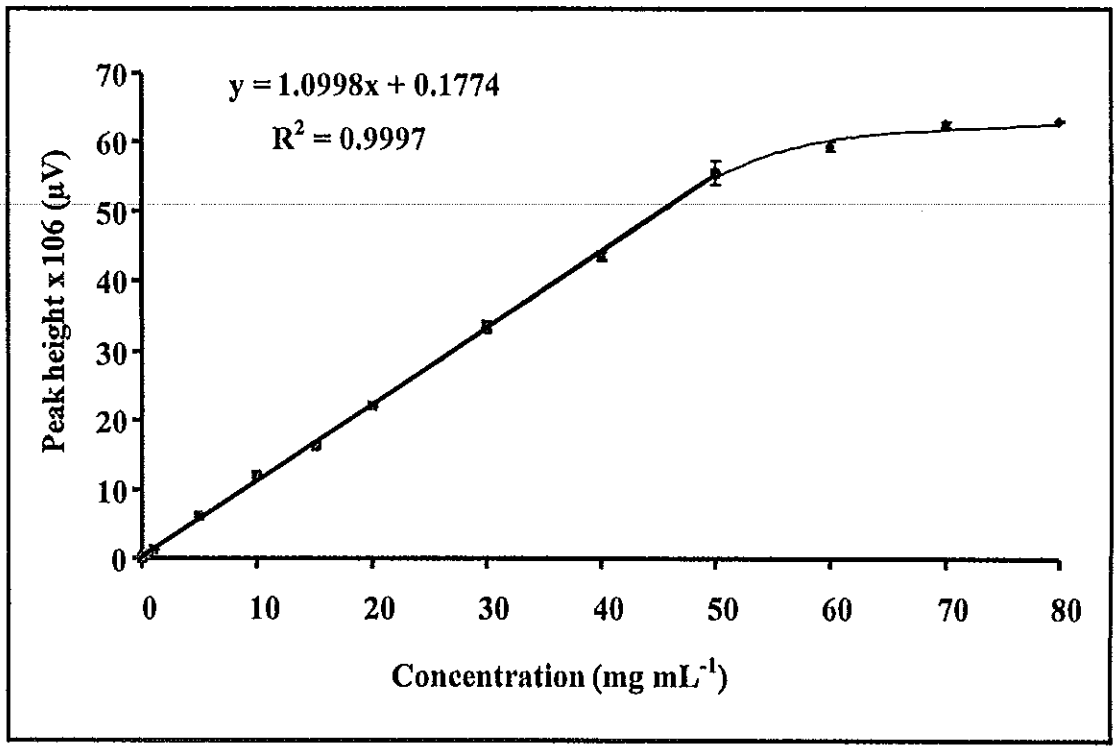

Figure 3.25 Linear dynamic range of TMA

3.3 Limit of detection (LOD)

The limit of detection (LOD) is the lowest concentration of an analyte in a sample that can be detected (Long and Winefordner, 1983). The limit of detection of DMA and TMA were investigated based on the IUPAC recommendation. The responses of blanks which appeared at retention times were measured. The maximum detectable blank signals are shown in Table 22 for headspace system. The limits of detection were calculated from equation 3.15, the standard calibration curves in Tables 3.20-3.21 and Figures 3.24-3.25.

$$C_L = \frac{kS_B}{m} \qquad (3.15)$$

Where C_L is the detectable concentration, k is the constant value at the confidence limit (k=3), S_B as the standard deviation of blank, for twenty repeatability and m is the slope of the calibration curve. The limits of detection of laboratory-built

system (HS-GC-NPD) were obtained for DMA and TMA 2.7 ng mL^{-1} and 0.30 ng mL^{-1} respectively as shown in Table 3.23.

Table 3.22 The data of the blank measurements by headspace technique, $n_B = 20$

t_R (min)	Maximum responses$\times 10^6$ (μV)*
1.28	0.21
1.28	0.20
1.28	0.21
1.29	0.20
1.28	0.20
1.29	0.22
1.29	0.20
1.29	0.19
1.30	0.21
1.29	0.20
1.29	0.21
1.29	0.20
1.29	0.22
1.29	0.21
1.29	0.21
1.29	0.22
1.29	0.19
1.29	0.21
1.28	0.21
1.29	0.19
$\overline{X}$	0.21
S_B	0.01

Table 3.23 The limit of detection for DMA and TMA standard solution with optimum conditions of HS-GC-NPD

Analytes	Limit of detection ($ng\ mL^{-1}$)
DMA	2.7
TMA	0.30

3.4 Sample Analysis

3.4.1 Sampling

Fish and shrimps were collected and analyzed by optimum conditions of HS-GC-NPD system. A total of 17 samples of fish and shrimp samples were sampling from local supermarkets and local fresh market in Hat Yai Songkhla as shown in Table 3.24.

Table 3.24 The information of samples

Type of samples	Variety of source	Number of sample
Indian mackerel	5	5
Sea bass	4	4
Giant tiger shrimp	2	2
White shrimp	6	6
Total samples	17	

3.4.2 Sample preparation

The ease of initial sample preparation is one of the advantages of static headspace extraction. Often, for qualitative analysis, the sample can be placed directly into the headspace vial and analyzed with no additional preparation. However for quantitation, it needs to use equilibrium system to obtain good sensitivity and accuracy for DMA and TMA, volatile organic compounds from solid samples. In this work, the matrices were fish and shrimp which were solid samples. Therefore, the physical state of the sample matrix needed to be changed. Two common approaches are crushing or grinding the sample and dissolving or dispersing the solid into a liquid. The first approach increases the surface area available for the volatile analyte to partition into the headspace. However, the analyte is still partitioning between a solid and the headspace. The second approach is preferred since liquid or solution sample matrices are generally easier to work than solid since the analyte partitioning process into the headspace usually reaches equilibrium faster. Also, analyte diffusion in liquids eliminates unusual diffusion path problems which often occur with solids and can unpredictably affect equilibration time (Mitra, 2003). For this work, fish and shrimp samples were ground and mixed with 3.33 mL ultra pure water into a 10 mL headspace vial and closed by septum and aluminium cap with climper and put the laboratory-built thermal bath before analysis at optimum conditions HS-GC-NPD system.

3.4.3 Sample size

For solid samples, diffusion takes much longer. This can be seen by comparing the order of magnitude of the diffusion coefficient, D: it order of magnitude is 10^{-6} in liquid and 10^{-8} to 10^{-11} in solids, while it is 10^{-1} in gases (Kolb and Ettre, 1997). Therefore, one characteristic of solid samples are the long equilibration times to increase the diffusion coefficient of DMA and TMA and enhance the analyte from liquid phase into gas phase (headspace), fish and shrimp samples were mixed in ultra pure water by adjustment the total sample volume at 3.33 mL (β=2). The results are shown in Table 3.25 and Figure 3.26. The sample size (fish

and shrimp tissue) at 1.5, 2.0 and 2.5 g gave nearly the same responses of DMA. When the sample size was lower than 1.5 g, it gave a lower response because of less analyte. For TMA, the sample size at 1.5 and 2.0 g gave nearly the same responses. The lower response at sample size 2.5 g could be because large amount of sample may reduce the diffusion coefficient in liquid phase. The sample size at 1.5 g was then selected as the optimum for sample analysis and validation method because it requires less sample.

Table 3.25 Effect of the sample size on the responses of DMA (25 μg mL^{-1}) and TMA (0.5 μg mL^{-1}) standard solutions

The amount of sample	**Responses×10^4 (μV)***	
	DMA	**TMA**
0.5	0.151±0.022	0.015±0.002
1.0	0.148±0.016	0.046±0.005
1.5	0.258±0.044	0.204±0.017
2.0	0.255±0.038	0.206±0.022
2.5	0.259±0.044	0.082±0.009

*5 replications, RSD < 17 %

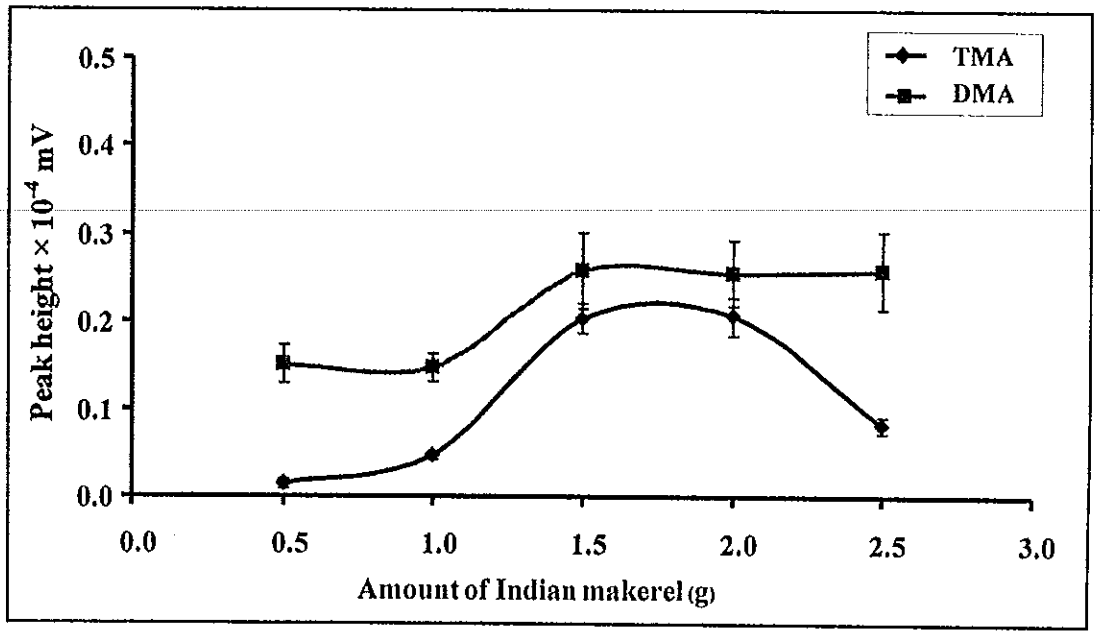

Figure 3.26 Responses of DMA (25 μg mL^{-1}) and TMA (0.5 μg mL^{-1}) standard solutions at various sample sizes

3.5 Matrix Interference

The various compositions in food could be interfered with the interest analyte in food analysis. If the analysts are not aware of this interference, it can lead to a number of different effects. One of the effects is enhancing the concentration of the analyte. Interference usually can affect the slope of the calibration curve and it could lead to a difference slope from the analyte of interest, so the slope of the calibration curve in the method of the standard additions may affect the linearity of the curve. This effect has the potential to indicate the possible present of hidden interferences (Eurachem guide, 1998). The matrix interference from two types of fish and two types of shrimp samples *i.e.*, Indian mackerel, Sea bass, Giant tiger shrimp and White shrimp were investigated. The matrix match calibration curve was used for this study by spiking various amounts of known standard of DMA and TMA into the sample as described in section 2.11. The optimum conditions were set for determining the spiking sample and standard DMA and TMA. If there is no matrix effect, the slope of standard curve and the spiked sample calibration should be equal (Eurachem,

1998). The results are shown in Tables 3.26-3.27 and Figures 3.27-3.28. When the standard and the spiked sample calibration curves were compared, both the DMA and TMA data did not give parallel regression lines and the slope of regression line in each group of standard curve and matrix match calibration curve were different.

Table 3.26 Effect of matrix on the responses of DMA in fish and shrimp samples

[DMA] ($\mu g\ mL^{-1}$)	Responses$\times 10^4$ (μV)* ± SD				
	Standard DMA	**Indian mackerel**	**Sea bass**	**Giant tiger shrimp**	**White shrimp**
25	2.28±0.06	0.48±0.01	0.17±0.01	0.37±0.04	0.19±0.01
50	4.65±0.16	1.42±0.04	1.02±0.18	1.12±0.11	1.20±0.03
100	11.63±0.33	3.16±0.02	3.24±0.46	3.92±0.50	2.97±0.05
150	17.10±0.59	5.0±0.2	6.02±0.63	5.61±0.63	5.60±0.03
200	22.38±0.73	6.97±0.08	8.93±1.33	8.71±0.97	7.47±0.09

*5 replications, RSD < 20 %

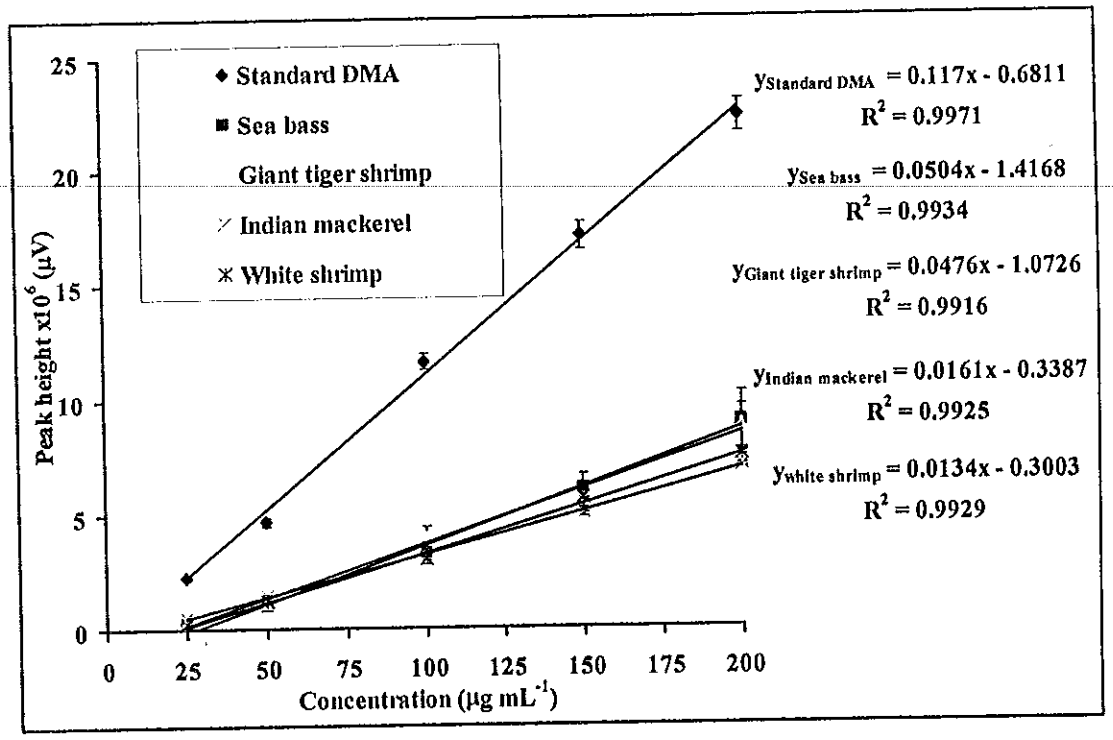

Figure 3.27 Matrix match calibration curves of DMA of fish and shrimp samples

Table 3.27 Effect of matrix on the responses of TMA in fish and shrimp samples

[TMA] (μg mL^{-1})	Responses×10^4 (μV)* ± SD				
	Standard TMA	Indian mackerel	Sea bass	Giant tiger shrimp	White shrimp
1	1.24±0.04	1.02±0.01	1.09 ±0.26	1.01±0.09	1.05±0.03
5	6.05±0.09	2.73±0.04	3.03±0.45	3.42±0.48	4.09±0.04
10	11.84±0.33	4.86±0.02	6.30±0.44	6.43±0.49	6.30±0.25
20	22.14±0.22	10.54±0.18	11.91±0.68	10.99±0.78	13.06±0.50
40	43.63±0.66	21.96±0.08	22.62±0.83	22.04±1.78	26.82±0.90

*5 replications, RSD < 20 %

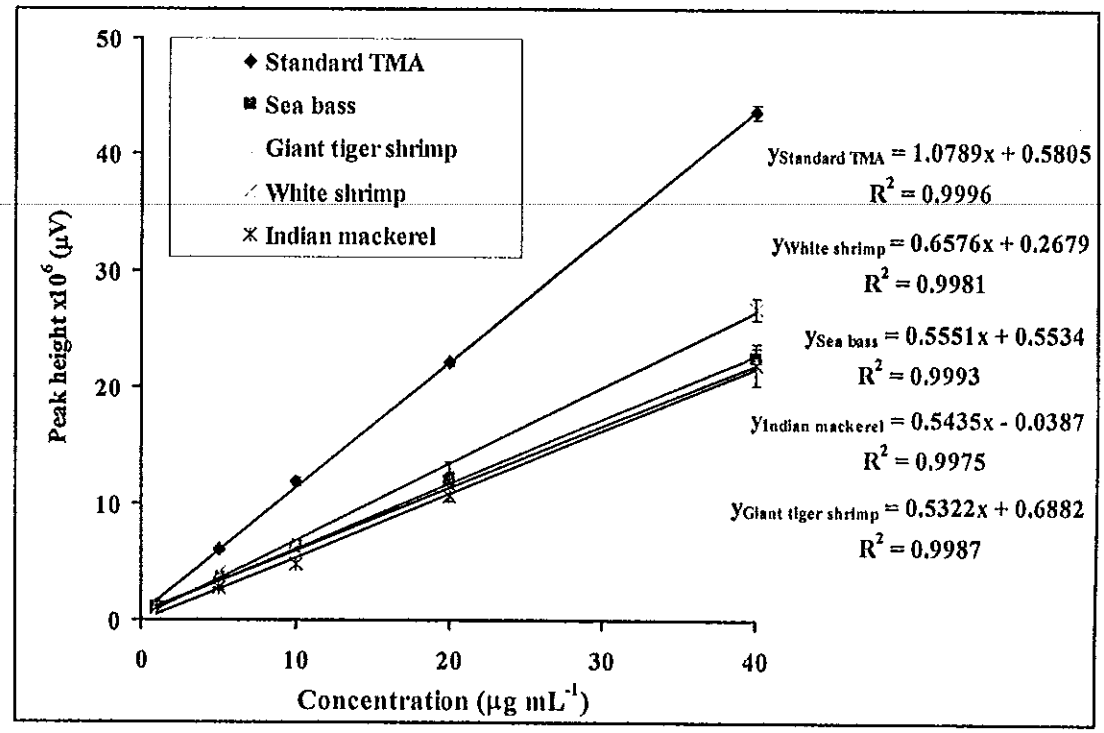

Figure 3.28 Matrix match calibration curves of TMA of fish and shrimp samples

The slopes of standard curve and matrix match calibration curves were tested using two-way analysis of variance (two-way ANOVA) by taking the null hypothesis (H_0) that the interaction of both slope is not significant and alternative hypothesis (H_1) that the interaction of slope is significant.

The results from significant test for comparison of the slopes of standard curve and matrix match calibration curves are shown in Tables 3.28-3.29. If *P* value is less than alpha (α, level of significance) then the null hypothesis was rejected at that significant level. From the results, it can be concluded that the slopes of regression line in standard curve and matrix match calibration curve were significant different as shown in Table 3.30. Therefore quantitative analysis of fish and shrimp samples must be determined from matrix match calibration curve (US-FDA, 2001).

Table 3.28 Statistic values for the comparison between the slope for DMA standard curve and matrix match calibration curve of various seafood samples

Matrix (type of seafood)	*Df*	Sum Sq	Mean Sq	*F*	*P*
Indian mackerel	4	332	83	1032	2.2×10^{-16}***
Sea bass	4	232	58	176	2.2×10^{-16}***
Giant tiger shrimp	4	252	63	290	2.2×10^{-16}***
White shrimp	4	290	72	937	2.2×10^{-16}***

Table 3.29 Statistic values for the comparison between the slope for TMA standard curve and matrix match calibration curve of various seafood samples

Matrix (type of seafood)	*Df*	Sum Sq	Mean Sq	*F*	*P*
Indian mackerel	4	701	175	2735	2.2×10^{-16}***
Sea bass	4	667	167	751	2.2×10^{-16}***
Giant tiger shrimp	4	726	182	374	2.2×10^{-16}***
White shrimp	4	435	109	632	2.2×10^{-16}***

Significant codes: '***' ($\alpha = 0.001$)

Where Df: Degree of freedom

Sum Sq: Sum square

Mean Sq: Mean square

F: ratio of two variances

P: Probability

Table 3.30 Level of significance (*P* value) from ANOVA for the comparison between the slope the standard curve and matrix match calibration curve of various seafood samples

Matrix	*P*	
	DMA	TMA
Indian mackerel	0.001	0.001
Sea bass	0.001	0.001
Giant tiger shrimp	0.001	0.001
White shrimp	0.001	0.001

3.6 Method validation

Method validation was performed to create the confidence in the analysis procedures. It provides an assurance of reliability during normal use (Swartz and Krull, 1997). To ensure the reliability of the results, method validation was evaluated by using standards, spiked samples, reagent and method blanks.

3.6.1 Accuracy and Recovery

Accuracy in HS-GC-NPD system was studied in term of recovery and precision (RSD). Recoveries of DMA and TMA in fish and shrimp samples were investigated by spiking known amount of DMA and TMA standard solution into fish and shrimp samples at concentration level of 100 and 200 μg mL^{-1} of DMA and 0.5 and 1 μg mL^{-1} of TMA (section 2.12.1). The responses obtained from spiked fish and shrimp samples for the mixture of DMA and TMA standard solution were compared. Recoveries were 54-69% for DMA (0.5 and 1 μg mL^{-1}) and 53-70% for TMA (0.5 and 1 μg mL^{-1}) (Tables 3.31 and 3.32). For DMA and TMA there are no regulation for recovery but these values of recovery are acceptable by EPA method 8070A in 1996 (13-109% recovery) for the analysis of nitrosamines by gas chromatographic

method in solid matrices which are similar to DMA and TMA. The recovery in this study is better than obtained by other works of HS-GC technique *i.e.*, the determination of volatile organochlorine compounds in landfill leachate and treated leachate which reported the recoveries in range of 24.7 to 26.9% for landfill leachate and 25.3 to 26.0% of treated leachate (Flórez Menéndez *et al.*, 2004) and volatile organic compounds in sediment samples which reported the recoveries in range of 6.5-97.6% (Kawata *et al.*, 1997). The recovery, SD and %RSD for the fortified fish and shrimp samples indicated in Tables 3.31 and 3.32. The RSDs ranged from 3.8-11.8% (%RSD, recommend method ±20).

Table 3.31 Recovery of DMA of various fish and shrimp samples at spiked concentrations of 100 and 200 μg mL^{-1}

Type of samples	**Recovery* (%) ± SD**	
	100 μg mL^{-1}	**200 μg mL^{-1}**
Indian mackerel	65±6	68±3
Sea bass	60±5	59±5
Giant tiger shrimp	61±4	54±6
White shrimp	60±2	69±4

*5 replications, RSD < 11.5 %

Table 3.32 Recovery of TMA of various fish and shrimp samples at spiked concentrations of 0.5 and 1 μg mL^{-1}

Type of samples	Recovery* (%) ± SD	
	0.5 μg mL^{-1}	**1 μg mL^{-1}**
Indian mackerel	63±6	53±6
Sea bass	67±6	59±4
Giant tiger shrimp	68±4	62±5
White shrimp	70±8	56±4

*5 replications, RSD < 11.8 %

3.6.2 Precision

The precision is the measure of the degree of repeatability of an analytical method as the percent relative standard deviation for a statistically significant number of samples (Swartz and Krull, 1997). Repeatability is a type of precision relating to measurements made under repeatable conditions *i.e.*, same method, same material, same operator and same laboratory (Eurachem, 2002). In this study, two types of fish and two types of shrimp samples were evaluated at two spiking level of 100 and 200 μg mL^{-1} for DMA, and 0.5 and 1 μg mL^{-1} for TMA, respectively, in each fish and shrimp samples followed by headspace technique before analysis in section 2.5.4. Five replicate analyses were performed at each concentration. The results are shown in Tables 3.33 and 3.34 their relative standard deviations (RSD) were 3.8-11.8%, lower than 20% (EPA, 8021B), the maximum %RSD that is allowed at spiked concentration 100 and 200 μg mL^{-1} for DMA and 0.5 and 1 μg mL^{-1} for TMA.

Table 3.33 Precision of DMA of various fish and shrimp samples at spiked concentrations of 100 and 200 μg mL^{-1}

Type of samples	Precision*, RSD (%)	
	100 μg mL^{-1}	200 μg mL^{-1}
Indian mackerel	9.6	4.7
Sea bass	8.6	8.5
Giant tiger shrimp	6.3	11.5
White shrimp	3.8	6.4

*5 replications, RSD < 11.5 %

Table 3.34 Precision of TMA of various fish and shrimp samples at spiked concentrations of 0.5 and 1 μg mL^{-1}

Type of samples	Precision*, RSD (%)	
	0.5 μg mL^{-1}	1 μg mL^{-1}
Indian mackerel	9.7	11.8
Sea bass	9.5	8.3
Giant tiger shrimp	6.3	7.3
White shrimp	10.9	8.0

*5 replications, RSD < 11.8 %

3.7 Qualitative and quantitative analysis of DMA and TMA in fish and shrimp samples

3.7.1 Qualitative Analysis

The optimum conditions of HS-GC-NPD were used to analyze DMA and TMA in fish and shrimp samples. For qualitative analysis, the retention time, t_R, of the sample chromatogram was compared with retention time of the standard

chromatogram to identify the DMA and TMA peak. The average t_R of DMA was 1.43±0.01 minutes and TMA was 1.28±0.01 minutes.

3.7.2 Quantitative Analysis

Quantitative analysis of DMA and TMA were done by considering the responses *i.e.*, peak height that was related to concentration of the analytes. Two types of fish and two types of shrimp samples *i.e.*, Indian mackerel, Sea bass, Giant tiger shrimp and White shrimp were sampling from local fresh markets and local supermarkets. All samples were prepared and analyzed at the optimum conditions in 2.5.4. The samples were analyzed by HS-GC-NPD using optimum conditions and representative chromatogram of the two types of fish and two types of shrimp samples are shown in Figure 3.29. The DMA in fish and shrimp samples were obtained in the range 2.17-5.15 and 2.35-4.55 mg/100g, respectively as shown in Table 3.35. TMA in fish and shrimp samples were obtained in the range 0.11-7.69 and ND-5.22 mg/100g, respectively (Table 3.36).

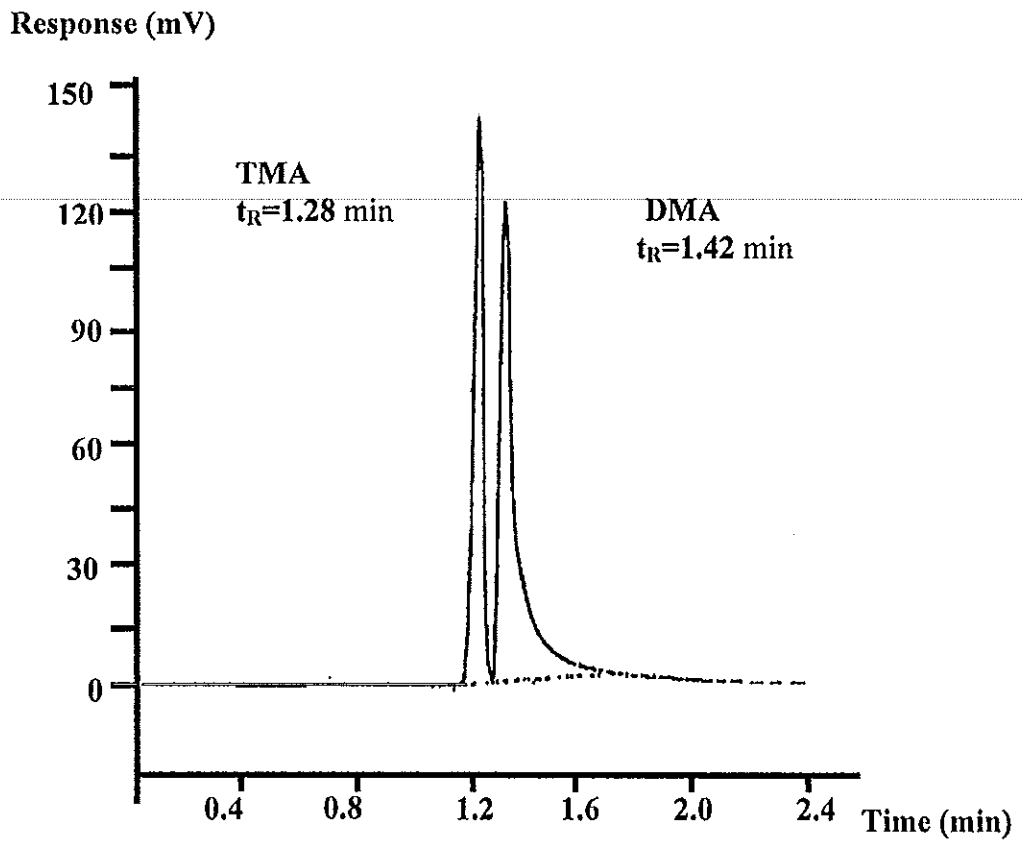

Figure 3.29 HS-GC-NPD Chromatogram of DMA and TMA from spiked Indian mackerel fish sample

Table 3.35 Amount of DMA in fish and shrimp samples by matrix match calibration curve

Sources	Amount of DMA (mg/100g)			
	Indian mackerel	**Sea bass**	**Giant tiger shrimp**	**White shrimp**
S1	5.15±0.08	4.63±0.13	2.67±0.05	4.55±0.11
S2	4.65±0.29	3.07±0.02	NS	2.51±0.04
S3	2.48±0.02	3.01±0.01	2.36±0.01	3.12±0.01
S4	2.28±0.02	NS	NS	2.54±0.03
S5	2.17±0.01	NS	NS	2.35±0.01
S6	NS	3.01±0.01	NS	3.12±0.01

ND= Not Detectable

NS= Not Sampling

S1= Klongrean fresh market

S2= Carrefour

S3= Natural fresh market

S4= Plaza market

S5= Samchai fresh market

S6= Tesco Lotus

Table 3.36 Amount of TMA in fish and shrimp samples by matrix match calibration curve

Sources	Amount of TMA (mg/100g)			
	Indian mackerel	**Sea bass**	**Giant tiger shrimp**	**White shrimp**
S1	4.65±0.11	3.27±0.10	1.12±0.09	1.85±0.14
S2	7.69±0.13	1.96±0.04	NS	ND
S3	1.05±0.27	0.11±0.02	0.69±0.02	5.22±0.22
S4	0.72±0.11	NS	NS	3.10±0.30
S5	1.77±0.17	NS	NS	ND
S6	NS	4.94±0.02	NS	ND

ND= Not Detectable

NS= Not Sampling

S1= Klongrean Fresh market

S2= Carrefour

S3= Natural Fresh market

S4= Plaza market

S5= Samchai Fresh market

S6= Tesco Lotus

3.7.3 The criteria for quality of fish product

Freshness is one of the most important criteria for quality of fish product. However, fish undergoes irreversible change (colour, texture and smell) after harvest, resulting in quality loss (Timm and Jørgensen, 2002). DMA and TMA have often been used to be an index of spoilage (Krzymien and Elias, 1990). For TMA, the EU acceptable quality and safety limits are set at 12 mg per 100 g (The European Commission Council Regulation No. 91/493/EEC, 1991). For DMA, although there is no specific limit but it can react with nitrile compounds to form a nitroso-dimethylamine (carcinogen compound). From the real sample analysis of both fishes and shrimps in the local market the values obtained are quite low *i.e.,* they are fresh and safe.

CHAPTER 4

Conclusions

An analysis method for two aliphatic amines, dimethylamine (DMA) and trimethylamine (TMA) used for seafood, fish and shrimp, freshness indication was investigated using headspace gas chromatography with nitrogen phosphorous detector (HS-GC-NPD). Chromatographic separation was carrier out by a fuse silica HP-FFAP capillary column, a 25 m × 0.32 mm i.d. × 0.52 μm film thickness consists of nitroterephthalic acid modified polyethylene glycol. Optimum conditions for GC-NPD technique were; flow rate *i.e.*, carrier gas (helium gas) 3 mL min^{-1}, fuel (hydrogen gas) 2 mL min^{-1}, oxidant gas (air) 100 mL min^{-1}; injector and detector temperature 120°C and 220°C, respectively; split ratio 10:1; column temperature programming 120°C followed by an increasing to 160°C with the temperature ramp rate of 30°C min^{-1} and kept at final temperature for 1 min.

In headspace system, a laboratory-built stainless steel water bath with a control heating coil and stainless steel adapter for vial holder was optimized and the results obtained are equilibration times 20 min, equilibration temperature 70°C, phase ratio 2.0. Vial volume at 10 mL was used to prepare sample to decrease the amount of sample. In addition, 1.5 g of Na_2CO_3 was added to increase the sensitivity of both analytes and decrease the solubility of analytes in matrix (Kolb and Ettre, 1997).

At optimum conditions good resolution ($R \geq 1$) and short analysis time (<5 min) were obtained. The HS-GC-NPD system showed a wide linear dynamic range, 2.7×10^{-3}-250 μg mL^{-1} for DMA and 0.30×10^{-3}-50 μg mL^{-1} for TMA with a coefficient of determination (R^2) greater than 0.99. The limit of detection was 2.7 ng mL^{-1} for DMA and 0.30 ng mL^{-1} for TMA. Excellent precision was obtained since the relative standard deviations (RSD) were all lower than 4%.

The matrix interference was also studied. Statistically fish and shrimp samples interfered with the responses and matrix match calibration curve was used for determination of DMA and TMA in the sample. The method was validated to ensure the reliability of the results by using standard, spiked samples, reagent and method

blanks. Validation parameters include accuracy, recovery and precision were studied. Recoveries were obtained at 54-69% for DMA (0.5 and 1 μg mL^{-1}) and 53-70% for TMA (0.5 and 1 μg mL^{-1}). For DMA and TMA there are no regulation for recovery but a recovery between 13 and 109% are acceptable by EPA method 8070A in 1996 for the analysis of nitrosamines by gas chromatographic method in solid matrices which are similar to DMA and TMA. Relative standard deviations (RSD) were 3.8-11.8% (n=5), lower than 20% (EPA, 8021B), the maximum %RSD that is allowed at spiked concentration 100 and 200 μg mL^{-1} for DMA and 0.5 and 1 μg mL^{-1} for TMA. When comparing between HS-GC-NPD method of this work with other reports (Table 4.1) it can be seen that the sample size, 1.5 g is smaller than almost all other methods other advantages of this HS technique includes being solvent free, simple to operate with less procedure steps and donot required derivatization. In term of system performance, the detection limit of this work (3.0×10^{-4} mg 100 g^{-1}) was better than others (Sukpeng, 2001; Vciana-Nogues *et al.,* 1996; Li *et al.*1997; Namieśnik *et al.*, 2003; Kaykhaii *et al.*, 2005). The recovery in this study (53-70%) is lower than other work, however, they all has the preconcentration steps, *i.e.*, by LLE (Vciana-Nogues *et al.*, 1996), SPME (Li *et al.*, 1997). Aliphatic amines with SIBA were also used for derivatization coupled to HS-SPME (Zhao *et al.*, 2003) and HS-SDME (Kaykhaii *et al.*, 2005) for liquid samples, making it easier to extract than solid samples, hence, higher recovery.

Table 4.1 Comparison between proposed method and another sample preparation method for analysis aliphatic amines in seafood

Samples	Amount of sample	Pre-treatment	Recovery (%)	Detection limits (mg 100 g^{-1})	References
Fish and shrimp	1.5 g	HS	53-70	3.0×10^{-4}	This work
Fish	50 g	HS (based on air sampling with Cabotrab or Tenax sorbent tubes) , LLE	No reported	No reported	Krzymien and Elias, 1990
Fish	10 g	LLE	97-99	0.46	Vciana-Nogues *et al.*, 1996
Fish	1 g	SPME	93	7.5×10^{-3}	Li *et al.*, 1997
Frozen seafood	20 g	HS (based on air sampling with silica gel sorbent tubes)	No reported	0.48	Sukpeng, 2001
Lake water	100 mL	Derivatization with SIBA and HS-SPME	94-102	6.4×10^{-5}	Zhao *et al.*, 2003
Air	3 g	SPME	No reported	0.67×10^{-4}	Namieśnik *et al.*, 2003
Tap and river water	2 mL	HS-SDME	96-103	2.5×10^{-4}	Kaykhaii *et al.*, 2005

For qualitative and quantitative analysis of DMA and TMA in fish and shrimp, these samples were analysed and evaluated using the matrix match calibration curve. The concentrations of DMA in all samples were obtained in the range of 2.17-5.15 mg per100g, TMA in fish and shrimp samples were obtained in the range ND-7.69 mg per 100g. These concentrations of DMA and TMA in fish and shrimp samples were lower than the EU acceptable quality and safety limit (TMA, 12 mg per 100 g) (The European Commission Council Regulation No. 91/493/EEC, 1991). For DMA, although there is no specific limit but it can react with nitrile compounds to form a nitroso-dimethylamine (carcinogen compound).

In conclusion, the proposed analysis method can be used for qualitative and quantitative analysis of DMA and TMA in fish and shrimp samples with good recovery and precision. This method has many advantages such as simplicity, less procedure *versus* LLE (Vciana-Nogues *et al.*, 1996) and derivatization technique (Zhao *et al.*, 2003); rapid, the throughput of the analysis is 12 samples per hour *versus* 4 sample per hour (Sukpeng, 2001)); small sample size (1.5 g *versus* 20 g for HS technique (Sukpeng, 2001); solvent free, no solvent *versus* 1 mL toluene per tube (Vciana-Nogues *et al.*, 1996); low detection limit in HS technique, 2.7×10^{-4} mg per 100 g *versus* 0.48 mg per 100 g (Sukpeng, 2001); cost effective, 1 bath (1.5 g sample)/vial and can be tested freshness of seafood.

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List of Publications and Proceedings

Poster presentations

1. Boonchai, P, Kanatharana, P. and Thavarungkul, P. "Development of Gas Chromatographic Technique for Seafood Freshness Determination (Dimethylamine and Trimethylamine)" The 32nd Congress on Science and Technology of Thailand (STT2006), Queen Sirikit National Convention Center, Thailand. October 8-12, 2006.
2. Boonchai, P, Kanatharana, P. and Thavarungkul, P. "Simple Technique for Seafood Freshness Determination" The 5th PERCH-CIC Annual Scientific Congress (PERCH-CIC Congress V), Pattaya, Thailand, May 6-9, 2007.