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.

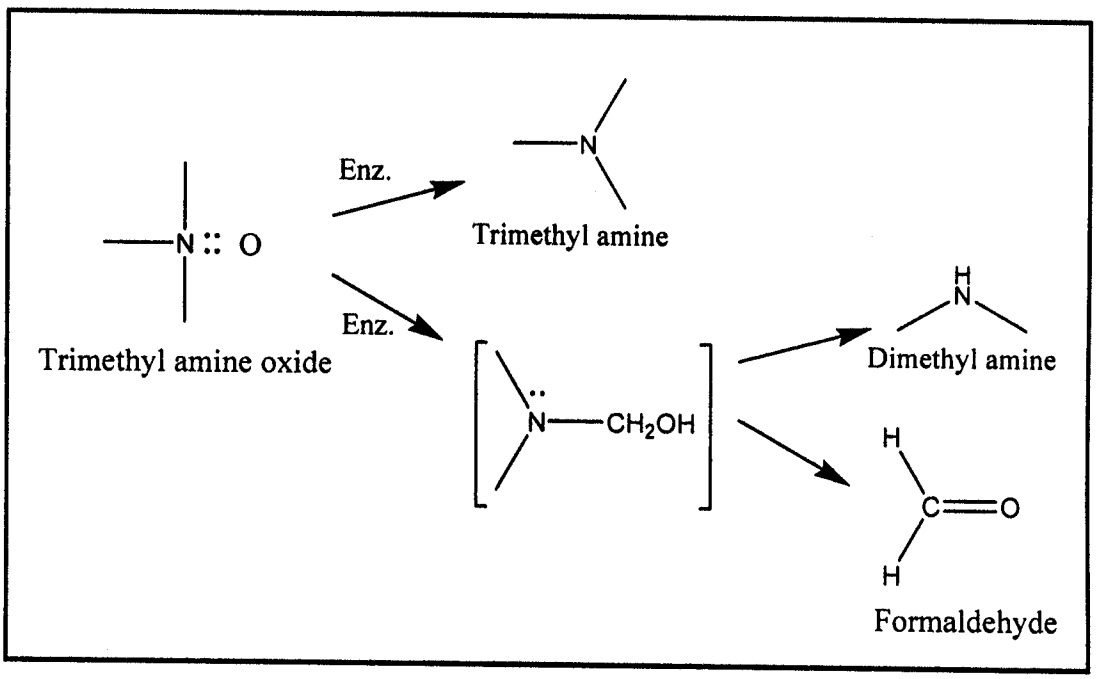


Figure 1.1 Mechanisms of trimethylamine and dimethylamine (Lindsay, 1996)

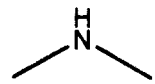
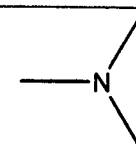
1.2 Background

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

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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.