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).

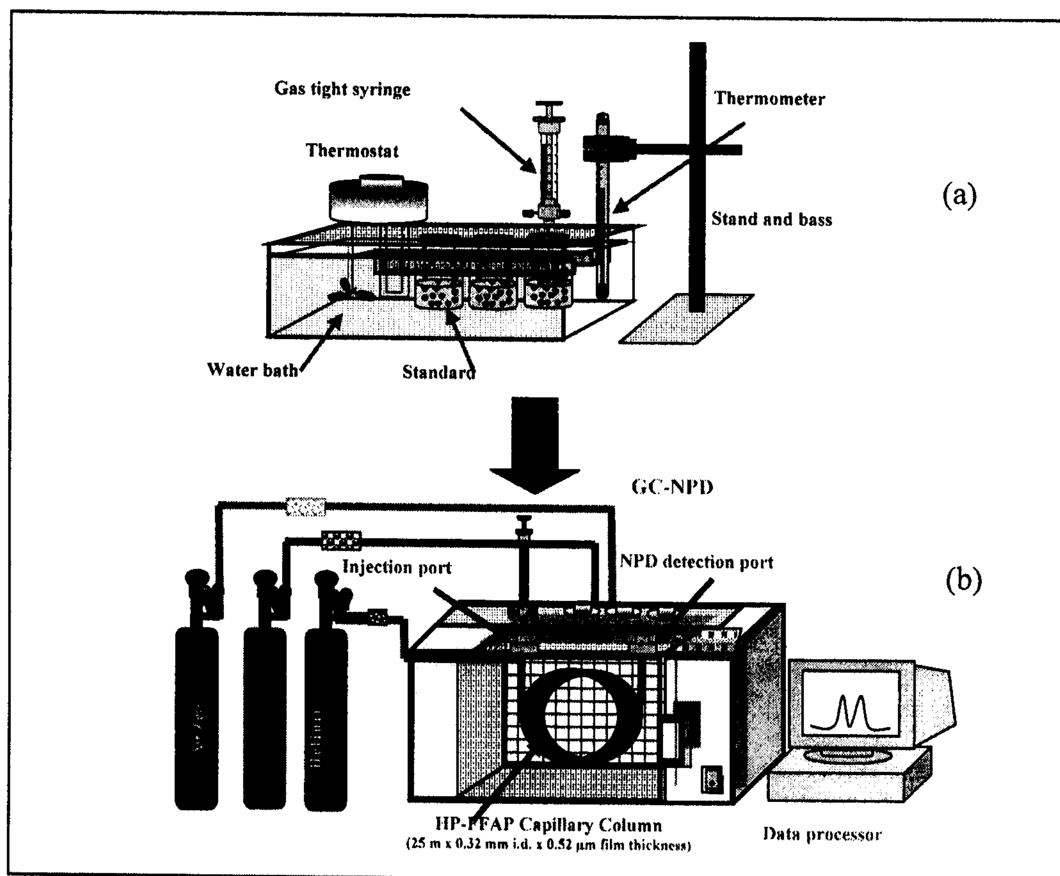

Figure 2.1 Analysis system for dimethylamine and trimethylamine, (a) Headspace (b) Gas Chromatography with Nitrogen Phosphorus Detector, (HS-GC-NPD)

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.

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.

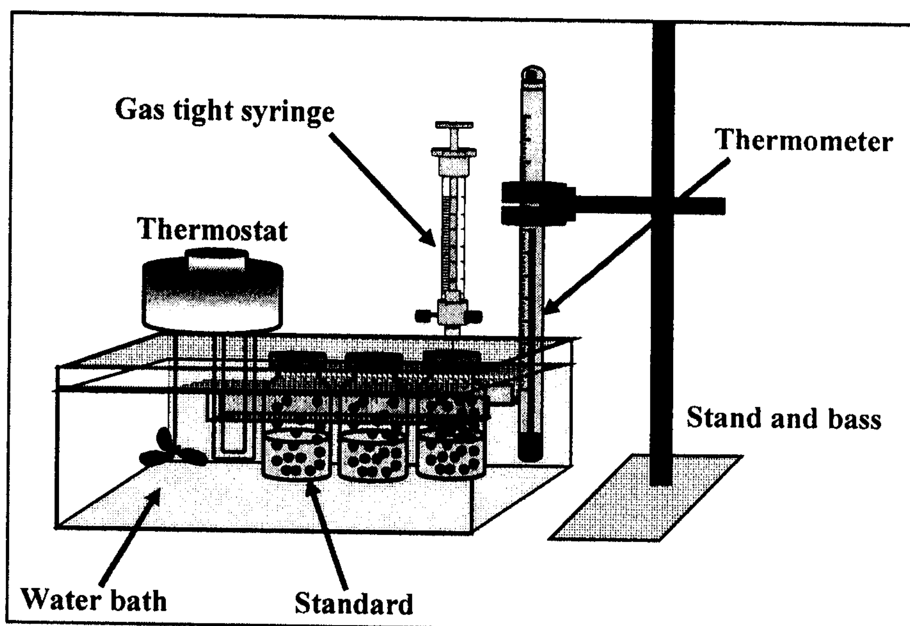

Figure 2.2 Laboratory-built water bath systems

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

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

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

$$C_L = \frac{(x_L - \overline{X}_B)}{m} \tag{2.4}$$

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, $\overline{X}_B$ is not always 0 the signal must be background corrected. Equation 2.5 was obtained after substitution Equation 2.3 into 2.4.

$$C_L = \frac{kS_B}{m} \tag{2.5}$$

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

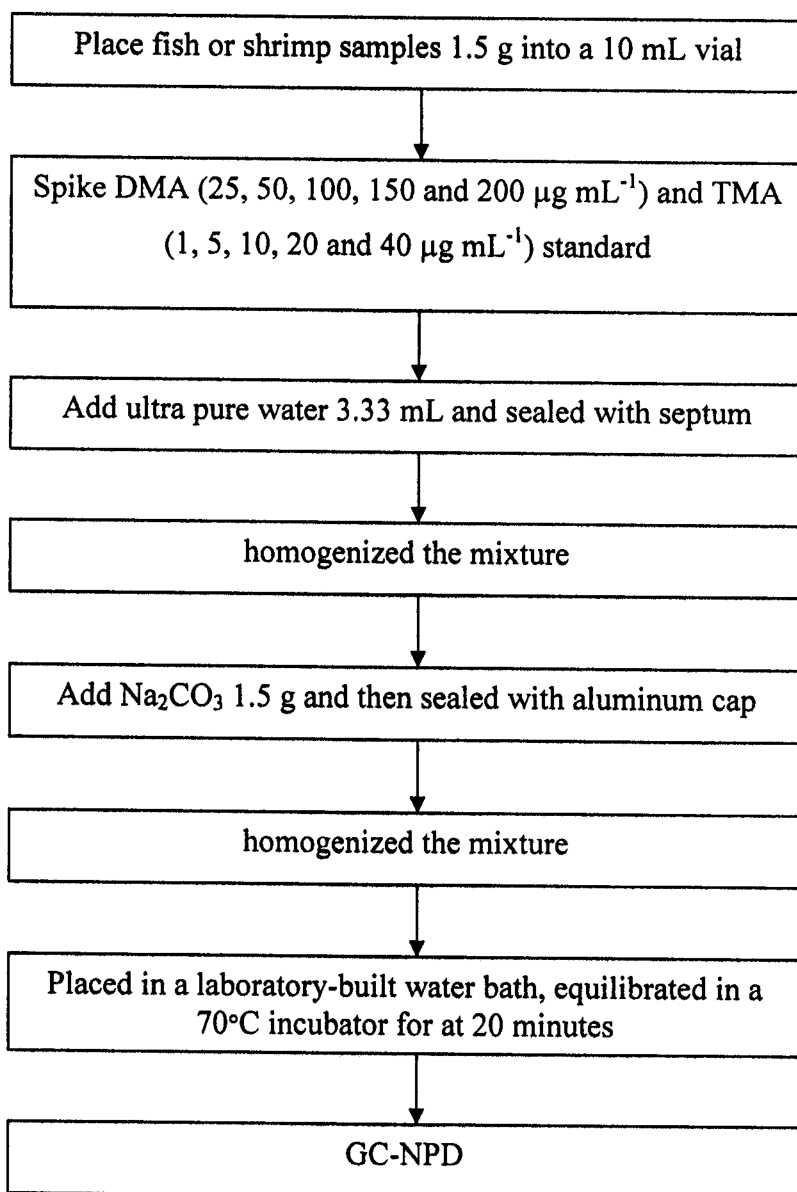

Figure 2.3 Analytical procedures of DMA and TMA for fish and shrimp samples

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 $\mu g\ mL^{-1}$ and amount of TMA standard solution at 1, 5, 10, 20 and 40 $\mu g\ mL^{-1}$ 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 $\mu g\ mL^{-1}$ and TMA standard solution 0.5 and 1.0 $\mu g\ mL^{-1}$. 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 $\mu g\ mL^{-1}$ and TMA at 0.5 and 1.0 $\mu g\ mL^{-1}$.

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

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).