# 2 MATERIALS AND METHODS

## 2.1 Safety

Heterocyclic amines are mutagenic/carcinogenic and must be handled with appropriate safety precaution including the use of chemical safety goggles, chemical resistant gloves, lab coat and efficient fume hoods (Balogh *et al.*, 2000).

## 2.2 Materials

### 2.2.1 Standard chemicals

- 2-Amino-3-methyl-3H-imidazo [4, 5-f] quinoline (IQ) (Purity: > 98%)
- 2-Amino-3, 8-dimethylimidazo [4, 5-f] quinoxaline (MeIQx) (Purity: > 99%)
- 2-Amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP) (Purity: > 98%)

All standard were purchased from Toronto Research Chemicals (Toronto, Canada)

### 2.2.2 Derivatization reagent

- N, N- dimethylformamide dimethylacetal (DMF-DMA: Sigma -Aldrich, USA) Chemical and physical properties of DMF-DMA illustrated below and chemical structure is shown in Figure 4.

Synonyms: N, N –dimethyldimethoxymethylamine

CAS registry number: 4637-24-5

Molecular formula: $C_5H_{13}NO_2$

Molecular weight: 119.16 g $mol^{-1}$

Boiling point: ~107-108 °C

Physical state: liquid

Color: yellow

Density: 0.90 g $cm^{-3}$

Chemical structure:

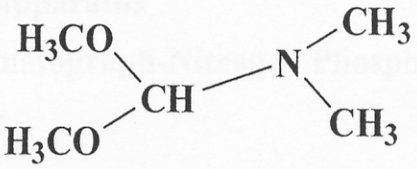


**Figure 4** Structure of DMF-DMA

### 2.2.3 Other chemicals

- Ammonium acetate ($CH_3COONH_4$, AR grade: BDH, England)
- Ammonia solution ($NH_3OH$, AR grade: BDH, England)
- Diatomaceous earth (Sciencetific, USA)
- Dichloromethane ($CH_2Cl_2$ AR grade: Merck, USA)
- Ethyl acetate ($CH_3COOC_2H_5$, AR grade: LAB-SCAN,Thailand)
- Glass wool
- Hydrochloric acid (HCl, AR grade: LAB-SCAN, Thailand)
- Methanol ($CH_3OH$, AR grade: LAB-SCAN, Thailand)
- Sodium hydroxide (NaOH, AR grade: LAB-SCAN, Thailand)
- Toluene ($C_6H_5CH_3$, AR grade: LAB-SCAN, Thailand)

- Ultra pure water ($H_2O$, Synthesis in laboratory by Maxima, ELGA, England)

### 2.2.4 Solid Phase Extraction (SPE)

- Bond Elut propylsulfonic acid cartridges (PRS, 500 mg, 3 mL: Varian, USA.)
- Octadecylsilane cartridges ($C_{18}$, 100 mg, 3 mL: Restek, USA)
- SPE vacuum manifold (Supelco, USA.)
- Vacuum pump (Gast, USA)

## 2.3 Instruments and apparatus

### 2.3.1 Gas Chromatograph-Nitrogen Phosphorus Detector (GC-NPD)

- Gas Chromatograph Auto system XL equipped with Nitrogen Phosphorus Detector (Perkin Elmer, USA)
- Computer system model intel inside pentium digital venturis

### 2.3.2 Gas Chromatograph-Mass Spectrometer (GC-MS)

- Gas chromatograph model 6890 Series (Agilent Technologies, USA)
- An auto-sampler injection model 7683 Series (Agilent Technologies, USA)
- Mass Selective Detector (MSD) model 5973 (Agilent Technologies, USA)
- Computer system model KAYAK (Hewlette Packard, USA)
- Capillary column: PE-17 ht, 30 m × 0.25 mm I.D., 0.25 μm film thickness of 50%phenyl -50%methylpolysiloxane (PerkinElmer, USA)

- Capillary column: HP-5, 30 m × 0.32 I.D., 0.25 μm film thickness of 5%phenyl-95%methylpolysiloxane (Agilent, 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)

### 2.3.3 Apparatus for derivatization

- Lab-built heating block unit (Figure 5)
- Thermocouple Module 80 TK with sensor (Fluke, USA)

### 2.3.4 Apparatus

- Amber vial 2 mL with septum and silver aluminum cap (Agilent Technologies, USA)
- Amber vial 2 mL with polypropylene screw cap and red rubber septa (Agilent Technologies, USA)
- Glass inserts (Agilent Technologies, USA)
- Thermocouple DL 297 T digital multitester (Korea)
- 11-mm crimper and 11-mm decrimper (Agilent Technologies, USA)
- Microliter pipette 10 μL, 20 μL, 200 μL, 1000 μL (Gilson, France)
- General glassware such as volumetric flask, test tube
- Evaporating rotator (EYELA, Japan)
- pH meter (millimeters, WTW Germany)

## 2.4 Methods

### 2.4.1 Lab-built heating block unit and temperature calibration

A heating block unit was designed to be used for the derivatization reaction of HCAs. The lab-built heating block unit is shown in Figure 5. It consists of an aluminum block with the dimension of 20 cm wide, × 20 cm length, × 4 cm height. Forty-five holes were drilled into the block, each with a diameter of 13 mm and 2.5 cm deep. The inner diameter of the each hole was a close fitted to a 2 mL reaction vial. The aluminum block was placed on a hot plate that was connected to a heat sensor and a temperature control unit.

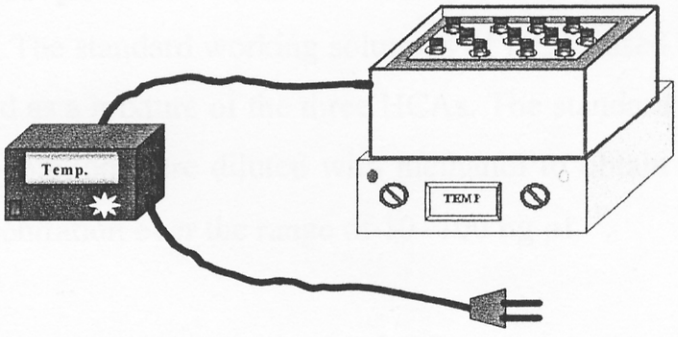


**Figure 5** A Lab built heating block unit

The temperature of the lab-built heating block unit was calibrated by using a thermocouple. The 2 mL amber vial was filled with solution blank and placed in the hole of the aluminum block. A thermocouple was then placed inside the solution blank and held with a clamp. The heater of the heating block unit was set at the temperature control unit. For each heater temperature setting the reading was done every 5 minutes for 30 minutes. From this the temperatures inside the vial corresponding to a set heater temperature was obtained and used for further studies.

### 2.4.2 Preparation of HCAs standard stock solution

Standards of 1000 ng $\mu L^{-1}$ was prepared by placing 10.00 mg of each of HCAs, *i.e.* IQ, MeIQx and PhIP in to a 10 mL volumetric flask and made up the volume with methanol. The intermediate solution was prepared by diluting the standard stock solution, 1000 ng $\mu L^{-1}$, to the concentration of 100 ng $\mu L^{-1}$ in methanol. Each standard solution was then transferred into a glass bottle with PTFE-lined screw cap. All bottles were wrapped with aluminum foil to protect them from light and then stored at 4°C as recommended by the manufacturer.

### 2.4.3 Preparation of HCAs standard working solutions

The standard working solutions of HCAs used in the experiments were prepared as a mixture of the three HCAs. The standard stock solution and intermediate solution were diluted with methanol to obtain the desired HCAs mixture concentration over the range of 10 -100 ng $\mu L^{-1}$.

### 2.4.4 Derivatization reaction of HCAs

Standard mixture solution, 200 µL, at a concentration of 100 ng $\mu L^{-1}$ was derivatized by adding 50 µL of N,N-dimethylformamide dimethylacetal (DMF-DMA) in standard mixture solution vial. This vial, without capping, was then heated at 100°C for 10 minutes (Kataoka *et al.*, 1997) in the lab-built heating block. After evaporation to dryness, the residue was dissolved in 500 µL of ethyl acetate to obtain a final concentration of the mixture solution derivatives of 50 ng $\mu L^{-1}$. Then 1 µL of this solution was injected into GC-NPD at the optimum conditions.

### 2.4.5 Optimization of derivatization reaction for HCAs

The optimization was focusing on the effect of the reaction temperature and time required to convert the heterocyclic amines into their N-dimethylaminomethylene derivatives to obtain the high efficiency.

#### 2.4.5.1 Optimization of the reaction temperature

The optimum reaction temperature was investigated by varying the temperature at 70, 80, 90, 100 and 110°C. Then, 1-μL of N-dimethylaminomethylene derivatives solution was injected to GC-NPD with splitless mode and equipped with a PE-17 ht fused-silica capillary column, 30 m × 0.25 mm ID., 0.25 μm film thickness of 50%phenyl-methylpolysiloxane. The GC-NPD operating conditions and reaction time were operated as. Column temperature program; 150°C initial temperature, (hold 5 minutes) ramped at 25°C/min to 280°C (hold 3 minutes) and ramped at 5°C/min to a final temperature of 330°C (hold 5 minutes).
Injector and detector temperature 340°C. Reaction time was 10 minutes. These parameters were performed following the application note of Kataoka *et al.,* (1997). The flow rate of the helium carrier gas was 1.5 mL $min^{-1}$. Hydrogen and air were used as the fuel and oxidant gases at the flow rates of 2 mL $min^{-1}$ and 100 mL $min^{-1}$ respectively (Perkin Elmer Gas Chromatograph Autosystem XL manual, 1995). The responses (peak heights) obtained from the chromatograms were then compared the optimum reaction temperature was the one that provided the highest response.

#### 2.4.5.2 Optimization of the reaction time

The reaction temperature was set at 90°C (the optimum condition obtained from experiment 2.4.5.1). The GC-NPD operating conditions were described in 2.4.5.1. The reaction time was determined by varying the time from 5 minutes to 30 minutes with an increment of 5 minutes.

The peak heights obtained from the different reaction time were compared and the optimum reaction time was selected by the highest response.

### 2.4.6 Optimization of the GC-NPD conditions for HCAs analysis

To optimize the GC-NPD system for analysis of HCAs, the derivative standard solution was analyzed using the optimum reaction temperature and time at 90°C for 10 min (the optimum condition obtained from 2.4.5.1-2.4.5.2). These were used throughout this work. The following parameters were studied: the carrier gas flow rate, the column temperature program, injector temperature, detector temperature, the fuel and oxidant gases flow rates, relative response factor, limit of detection, linear dynamic range, sample preparation and recovery.

#### 2.4.6.1 Optimization of the carrier gas (He) flow rate

To optimize the carrier gas flow rate, 1μL of 50 ng $\mu L^{-1}$, HCAS derivatives solution was injected to GC-NPD system. The gas chromatographic conditions were set as in 2.4.5.1.

The optimization of carrier gas flow rate was determined by varying the flow rate of helium carrier gas at 0.8, 1.0, 1.2, 1.5 and 1.8 mL $min^{1}$. Five replications of each flow rate were performed. The retention time, peak height and peak area from the chromatogram 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.

#### 2.4.6.2 Optimization of the column temperature

Initially only the column temperature that was operated under isothermal temperature was optimized, but this procedure could not adequately separated the HCA derivatives. Therefore, the column temperature

program was optimized to obtain a high resolution. To optimize the column temperature programming parameter, 1 μL of the standard working solution of HCA derivatives was injected into the GC-NPD system by varying one parameter at a time, as shown in Table 5. The selected optimum of each parameter was the one that provided the highest response and the best resolution. The values obtained were used in the optimization of next parameters.

**Table 5** Optimization of column temperature programming

| Step | Parameters | Optimized value |
|---|---|---|
| I | • Initial temperature | 150,160,170,180,190,200 and 210°C |
| II | • First hold time | 0, 1, 2, 3, 4 and 5 min |
| III | • First stage of ramp rate | 10, 20, 30, 35 and 40°C/min |
| IV | • Second stage of temperature | 250, 260, 270, 280 and 290°C |
| V | • Second hold time | 0, 1, 2, 3 and 4 min |
| VI | • Second stage of ramp rate | 5, 10, 15 and 20°C/min |
| VII | • Final temperature | 315, 320, 325, 330, 335 and 340°C |
| VIII | • Final hold time | 5, 6, 7, 8, 9 and 10 min |

2.4.6.3 Optimization of the injector temperature

In this studied, 1 μL of the standard solution derivative was injected to GC -NPD. The GC conditions were set using the results from experiments 2.4.6.1-2.4.6.2.

The optimization of injector temperature was determined by varying the temperature from 290, 300, 310, 320, 330 and 340°C respectively. Five replicates for each temperature were performed and obtained the temperature at the highest response as the optimum injector temperature.

2.4.6.4 Optimization of the detector temperature

The detector temperature was investigated by varying the temperature from 300°C to 360°C with an increment of 10°C. All responses obtained from the different temperatures were compared and the selected optimum was the temperature that provided the highest response.

2.4.6.5 Optimization of fuel ($H_2$) flow rate

The gas chromatographic conditions: carrier gas flow rate, the column temperature program, injector temperature, detector temperature were set at the optimum conditions obtained earlier except the flow rate of oxidant gas was fixed at the recommended flow rate by the Perkin Elmer Gas Chromatograph Autosystem XL manual (1995).

To optimize the flow rate of hydrogen that was used as the fuel gas, its flow rate was varied from 1 mL $min^{-1}$ to 4 mL $min^{-1}$ with an increment of 1 mL $min^{-1}$. The responses at different hydrogen flow rate were compared and the optimum hydrogen flow rate was selected at flow rate that gave the highest response.

2.4.6.6 Optimization of oxidant (air) flow rate

The GC conditions were set at the optimum conditions obtained from 2.4.6.1-2.4.6.5. The optimum oxidant gas flow rate was investigated by varying the flow rate of the oxidant at 60, 70, 80, 90, 100 and 110 mL $min^{-1}$. The optimum oxidant flow rate was obtained at the flow rate that provided the highest response.

**2.4.7 Linear dynamic range (LDR)**

The standard mixture HCA derivatives were prepared at concentration 100 ng $\mu L^{-1}$ was diluted with ethyl acetate to obtain the concentration in the range 0.80-100 ng $\mu L^{-1}$. A 1µL aliquot of standard mixture

was injected in to the GC-NPD system that was set at the optimum conditions from 2.4.6.1-2.4.6.6 Summarized in Table 6. Linear dynamic range was determined by plotting the calibration curve. The linearity of the response was determined by considering the correlation efficient.

**Table 6** The optimum derivatization reaction and GC-NPD conditions for HCA derivatives analysis

| Parameters | Optimum values |
|---|---|
| **1. Derivatization reaction** | |
| Reaction temperature | 90°C |
| Reaction time | 10 minutes |
| **2. GC-NPD** | |
| Carrier gas flow rate | 1.5 mL $min^{-1}$ |
| PE-17ht, 30 m × 0.25 mm I.D., 0.25 μm film thickness of 50% phenyl-50% methylpolysiloxane | |
| Column temperature programmed | 330°C, 5 min<br>10°C/ min<br>280°C, 0 min<br>35°C/ min<br>190°C, 3 min |
| Injector temperature | 300°C |
| Detector temperature | 340°C |
| $H_2$ gas flow rate | 2 mL $min^{-1}$ |
| $O_2$ gas flow rate | 100 mL $min^{-1}$ |

### 2.4.8 Limit of detection (LOD)

The standard mixture of HCA derivative at the concentration of 50 ng $\mu L^{-1}$ was diluted with ethyl acetate to obtain the concentration in the range 0.5-5.0 ng $\mu L^{-1}$. A 1- μL aliquot of each standard solution was injected into the GC-NPD system at optimum conditions.

Limit of detection was considered as the lowest concentration that the detector could provide a signal on the chromatogram, and the signal to noise ratio (S/N) calculated from the chromatogram was more than 3.

### 2.4.9 Response factor

The mixed HCA standard solution was prepared at five working HCA derivative concentrations *i.e.* 1.0, 5.0, 10.0, 25.0, and 50.0 ng $\mu L^{-1}$. A 1-μL aliquot of mixture solution at various concentrations was injected into GC-NPD system that operated at optimum conditions.

The response factor (RF) was determined by comparing between the responses of integrated peak height of each analyte at various concentrations and the integrated peak response of IQ derivative. Where the IQ derivative was assigned with a response factor of 1.

### 2.4.10 Confirmation of the HCA derivative structures and comparison of GC-MS and GC-NPD for HCAs analysis

The structure of HCA derivatives from derivatization reaction (obtained from experiment 2.4.5.1-2.4.5.2) were confirmed by GC-MS.

Agilent model 5973 mass-selective detector was combined with Agilent 6890 gas chromatograph, 30 m × 0.25 mm I.D., 0.25 μm film thickness PE-17ht (50% phenyl-50% methylpolysiloxane) capillary column. In the part of GC, conditions were set at optima obtained from experiments 2.4.6.1-2.4.6.3, the column temperature programming applied in GC-MS was; 180°C, hold for 3 min, then ramped at 35°C/min to 280 °C, immediately

ramped at 10°C/min to 300°C, and held for 5 min to let signal went back to the baseline. Injection port and transfer line temperature were set at 300 °C, helium carrier gas flow rate, 1 mL $min^{-1}$.

MS measurement was performed with electron impact (EI) ionization at 70 eV. MS source and MS quadrupole temperature were set at 230°C and 150°C respectively, the recommended set point for operating in HP 5973 Mass Selective detector Hardware Manual (Hewlett-Packard, 1998). For identification of HCA, derivatives were scanned m/z from 45 to 550 amu by the scan mode to obtain the qualitative information and selected ion monitoring mode (SIM) was used for quantitative analysis.

2.4.10.1 Linear dynamic range in GC -MS (PE-17 ht column)

The HCA derivatives standard solution at a concentration of 100 ng $\mu L^{-1}$ was diluted with ethyl acetate to obtain concentration in the range of 0.2-100 ng $\mu L^{-1}$. A 1-µL aliquot of standard mixture was injected into GC-MS. Linear dynamic range was determined by plotting a calibration curve.

The linearity of the response was determined by considering the correlation coefficient.

2.4.10.2 Limit of detection in GC–MS (PE-17 ht column)

The 100 ng $\mu L^{-1}$ HCA derivatives standard was diluted with ethyl acetate to obtain concentration in the range of 0.1-1.0 ng $\mu L^{-1}$. A 1-µL aliquot of standard mixture solution was injected into GC-MS at the optimum conditions.

Limit of detection was considered as the lowest concentration that mass selective detector could provide a signal on the chromatogram, The signal to noise ratio (S/N) was calculated automatically by the Chemstation operating software.

### 2.4.11 Comparison of two different capillary columns, PE-17ht and HP-5

In this study, HCAs were converted into N-dimethyl - aminomethylene derivatives and measured with nitrogen phosphorus selective detector. Initially only the capillary column PE-17 ht (50%phenyl-50% methylpolysiloxane) (Kataoka *et al.*,1997) was used for the separation of derivatives. The stationary phase (50%phenyl-50% methylpolysiloxane) was the one used by Skog *et al.*, (1998) who the introduced a GC-MS method for the determination of HCAs.

In this thesis, an HP-5, a common capillary column (5%phenyl-95% methylpolysiloxane) 30 m × 0.32 mm I.D., 0.25 μm film thickness was used and compared with PE-17 ht capillary column (50%phenyl-50%methylpolysiloxane) for analysis of HCAs (IQ, MeIQx and PhIP).

The comparison of the two columns was done by setting the GC conditions at optima obtained from 2.4.6.1-2.4.6.6 except the detector and final temperatures which were set at 300°C. This was because the temperature of HP-5 column should not be more than 325°C, therefore, column must be kept at a temperature lower than 325°C. To evaluate this parameter, 1 μL of standard working HCA derivatives was injected into the GC-NPD system. The response and sensitivity obtained from the different capillary columns were compared and selected for the analysis of heterocyclic amines in food samples throughout the root of experiments.

#### 2.4.11.1 Linear dynamic range in GC-NPD (HP-5 column)

The 100 ng $\mu L^{-1}$ standard HCA derivatives solution was diluted with ethyl acetate to obtained concentrations in the range of 0.3 to 80.0 ng $\mu L^{-1}$. A 1-μL aliquot of standard mixture was injected into GC- NPD at the optimum conditions. Linear dynamic range was determined by plotting

a calibration curve. The linearity of the response was determined by considering the correlation coefficient.

2.4.11.2 Limit of detection in GC -NPD (HP-5 column)

The 100 ng $\mu L^{-1}$ standard HCA derivatives solution was diluted with ethyl acetate to obtained concentrations in the range of 0.3 to 5.0 ng $\mu L^{-1}$. A 1-μL aliquot of standard mixture was injected into GC- NPD.

### 2.4.12 Optimization of sample preparation

The multistep procedures for isolation of the heterocyclic amines from meat were investigation. Until now, the authors of many articles have usually used only one method for HCAs separation either liquid-solid extraction (LSE) or liquid-liquid extraction (LLE) or solid phase extraction (SPE). This thesis proposed the combination of ultrasonic liquid-solid extraction (LSE) and solid phase extraction (SPE). After sample preparation the analysis was performed as previously described by Gross and Grüter (1992) with some modifications. Grilled chicken was used as a representation to optimize the extraction conditions throughout this experiment. To determine heterocyclic amines in cooked meat samples, each sample was equally divided in random fashion without bias to heavily browned areas. The meat samples were grounded by the blender. A 10.00 g aliquot of sample was placed in the mortar and spiked with 200-μL of 10 ng $\mu L^{-1}$ IQ, 25 ng $\mu L^{-1}$ MeIQx, 50 ng $\mu L^{-1}$ PhIP standard mixture solution. The solvent was allowed to evaporate for 5 minutes before processing the sample extraction step. At this point of analyses, the preparation into was divided into five equal portions (2.00 g) and placed in a 25 mL beaker. The samples were homogenized in 12 mL, 1.0 *M* sodium hydroxide by ultrasonic extraction at a frequency of 100 Hertz for 3 hours. The alkaline solution was mixed with 15 g

of diatomaceous earth. The material was then loaded to 2 cm I.D., × 45 cm in length glass column is shown in Figure 6.

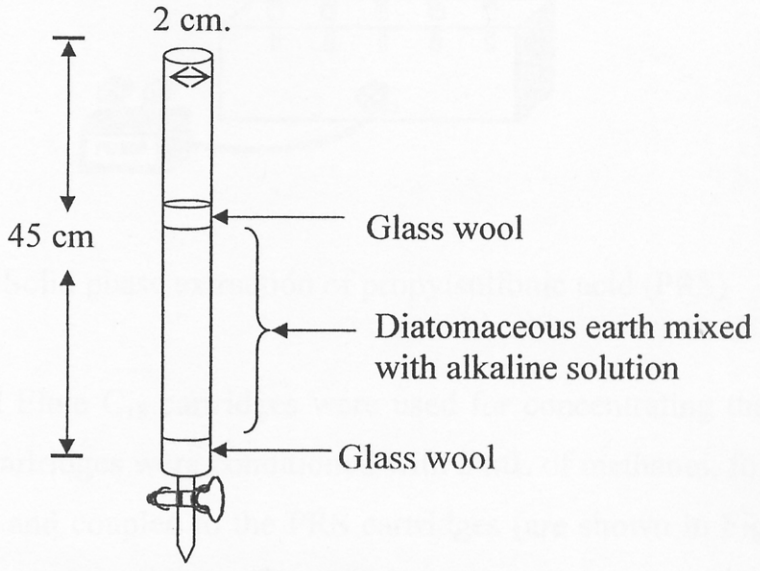


**Figure 6** The glass column packed with diatomaceous earth

In the SPE method, Bond Elut PRS cartridges (shown in Figure 7) were fitted with coupling adapters and preconditioned by 5 mL, 0.1 *M* HCl, 10 mL water and 5 mL methanol. After drying the cartridges under vacuum, 5 mL of dichloromethane was passed, and then the PRS cartridges were coupled with diatomaceous earth column. A Supelco Visiprep SPE vacuum manifold was used for manipulating of eluent flow rate with the sample solid–phase extraction cartridges. The dichloromethane was used for eluting the HCAs from diatomaceous earth. The PRS cartridges were dried under maximum vacuum (15 in. of Hg) then rinsed successively with 15 mL methanol-water (4:6, v/v) and 2 mL water.

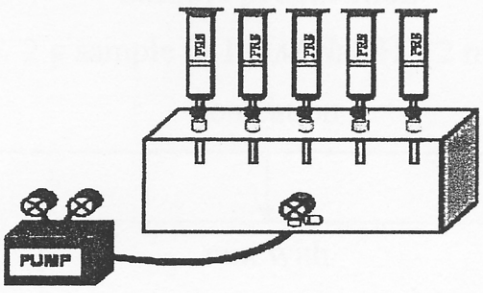


**Figure 7** Solid phase extraction of propylsulfonic acid (PRS)

Bond Elute $C_{18}$ cartridges were used for concentrating the polar amines. First, the cartridges were conditioned with 5 mL of methanol, followed by 5 mL of water, and coupled to the PRS cartridges (are shown in Figure 8) that contained the polar amines. The HCAs were eluted from the PRS cartridges to the $C_{18}$ cartridges with 0.5 *M* ammonium acetate (adjusted to pH 8.0 with concentrated ammonium hydroxide). The $C_{18}$ cartridges were rinsed with 5 mL of water and dried under vacuum. The adsorbed HCAs were eluted from $C_{18}$ into test tubes using methanol-ammonia (9:1, v/v). The extractants were concentrated under maximum vacuum and re-dissolved in 500 µL methanol and derivatized as described in 2.4.6.1-2.4.6.2. The summary of the sample preparation procedure is shown in Figure 9

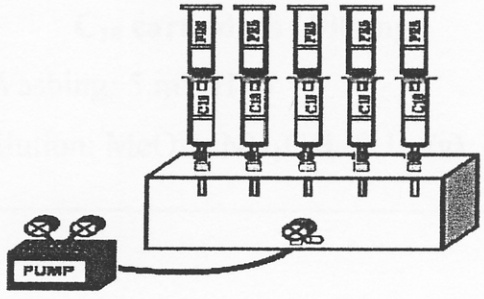


**Figure 8** Solid phase extraction of propylsulfonic acid (PRS) cartridges coupled with $C_{18}$ cartridges

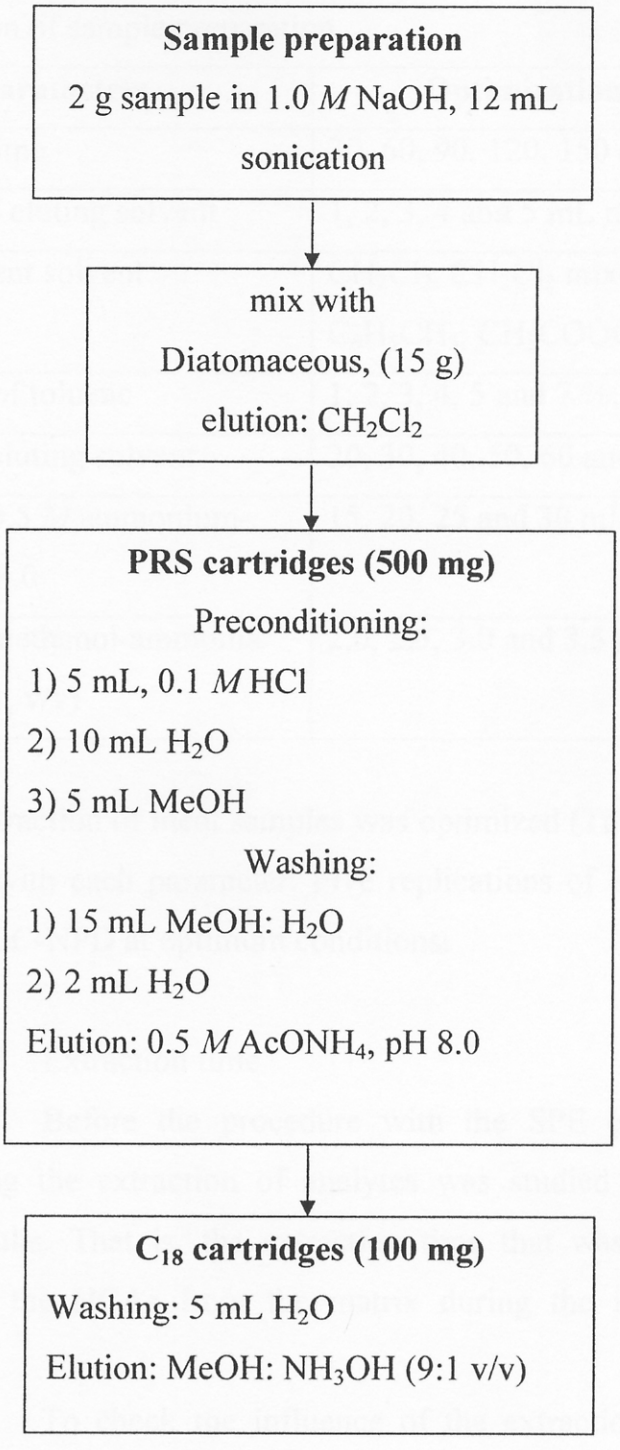


**Figure 9** Summary of the sample preparation procedure

**Table 7** Optimization of sample preparation

| Steps | Parameters | Optimization value |
|---|---|---|
| I | Extraction time | 30, 60, 90, 120, 150 and 180 min |
| II | Flow rate of eluting solvent | 1, 2, 3, 4 and 5 mL $min^{-1}$ |
| III | Type of eluent solvent | $CH_2Cl_2$, $CH_2Cl_2$ mixed with $C_6H_5CH_3$, $CH_3COOC_2H_5$ |
| IV | Percentage of toluene | 1, 2, 3, 4, 5 and 7 %. |
| V | Volume of eluting solvent | 20, 30, 40, 50, 60 and 70 mL. |
| VI | Volume of 0.5 *M* ammonium-acetate, pH 8.0 | 15, 20, 25 and 30 mL. |
| VII | Volume of methanol-ammonia solution (9:1, v/v) | 2.0, 2.5, 3.0 and 3.5 mL |

The extraction of meat samples was optimized (Table 7) and was repeated five times with each parameter. Five replications of each extractant were analyzed with GC-NPD at optimum conditions.

2.4.12.1 Extraction time

Before the procedure with the SPE cartridges, one parameter influencing the extraction of analytes was studied to ensure the accuracy of the results. That is, the extraction time that was necessary to completely released the HCAs from the matrix during the initial alkaline treatment.

To check the influence of the extraction time in the initial alkaline treatment, once the sample was homogenized in 12 mL of 1.0 *M* sodium hydroxide solution with ultrasonic, extraction time of 30, 60, 90, 120, 150 and 180 minutes were studied while other parameters were kept constant. Each experiment was done in five replicates. The extractants were analyzed by

GC-NPD. The extraction time that provided the highest response was then selected.

2.4.12.2 Flow rate of eluting solvent

The flow rate of the eluting solvent is an important parameter in the removing of the analytes from diatomaceous earth. The flow rate was varied at 1, 2, 3, 4 and 5 mL $min^{-1}$ and each flow was done in 5 replications. The extractants were derivatized and analyzed at optimum conditions. The flow rate that gave the highest response was then chosen.

2.4.12.3 Type of eluent solvent

The elution solvent is another important parameter to consideration in the eluting of the analytes from diatomaceous earth. The appropriate elution solvent for extracting HCAs from diatomaceous earth were studied. Three solvents were tested, dichloromethane, toluene mixed with dichloromethane and ethyl acetate. Each experiment was done in five replicates. The extractants were analyzed by GC-NPD. The solvent that provided the highest response was then selected.

2.4.12.4 Percentage of toluene

The percentage of toluene was tested at 1, 2, 3, 4, 5 and 7%. The resulting extractants were analyzed by GC-NPD at the optimum conditions. The least percentage of toluene that provided the highest response was selected.

2.4.12.5 Volume of eluting solvent

From the result in experiment 2.4.12.4 dichloromethane mixed with 3% of toluene was selected as eluent to elute analytes from diatomaceous earth. In order to minimize solvent used and at the

same time maintained the high response, the volume of the 3% toluene mixed with dichloromethane was studied at 20, 30, 40, 50, 60 and 70 mL. Each experiment was done in five replicates. The extractants were analyzed by GC-NPD.

2.4.12.6 Volume of 0.5 *M* ammonium acetate, pH 8.0

The PRS cartridge, which contained HCAs, was then connected with $C_{18}$ cartridge. HCAs were eluted from the PRS cartridge using 0.5 *M* ammonium acetate, pH 8.0 directly into $C_{18}$ cartridge. The volume of the solution used in this processes was an important parameter because it would affect the extraction efficiency. If the volume is too small it may not be sufficient to elute the analytes on the SPE column. Therefore, it was necessary to optimize the volume of 0.5 *M* ammonium acetate, pH 8.0. The volume of this solvent was varied at 15, 20, 25 and 30 mL. Each experiment was done in five replicates. The extractants were analyzed by GC-NPD at the optimum conditions. The least solvent volume that provided the highest response was selected.

2.4.12.7 Volume of methanol-ammonia solution (9:1, v/v)

The adsorbed aminoazaarenes were eluted from $C_{18}$, using mixture of methanol: ammonia (9:1, v/v). In order to minimize solvent used and at the same time maintained the high response, the last eluting solvent, methanol: ammonia was varied at 2.0, 2.5, 3.0 and 3.5 mL. Each experiment was done in five replicates. The extractants were analyzed by GC-NPD at the optimum condition. The least solvent volume that provided the highest response was selected.

### 2.4.13 Matrix Interference

The difficulty in extracting heterocyclic amines from a complex food matrix and the low quantity of heterocyclic amines present made accurate identification and quantification difficult. The major problems associated with the analysis of aminoazaarenes in food is that many organic compounds can be co-extracted which aminoazaarenes from foods with interference matrix. The aim of this section was to study the effect of matrix in food by spiking known amount of HCAs standard in range of 0.5 - 50.0 ng $\mu L^{-1}$ in 10.00 g of sample. The responses, peak heights were plotted against the known concentration. The slope of the standard and the spiked sample were compared for matrix interference.

### 2.4.14 Qualitative and quantitative analysis of heterocyclic amines in food

#### 2.4.14.1 Meat sampling

Some common and popular types of meat were selected and investigated. These included chicken, pork, beef and fish. The raw meat products were purchased from local supermarkets and stored at 4°C until being analyzed.

#### 2.4.14.2 Qualitative analysis

Qualitative analysis was carried out by using the retention time from the chromatogram compared with the standard HCA derivatives.

#### 2.4.14.3 Quantification and recovery

Quantitative analysis was carried out by considering the response from GC-NPD, *i.e.* peak height of the chromatogram which was proportional to the amount of HCA derivatives, by considering the response

factor. Quantification and recovery calculation of the heterocyclic amines in food samples were carried out by standard addition method. The meat samples were spiked with all the analyzed compound at different levels (0.50-50.0 ng $\mu L^{-1}$). These were added directly to the samples before the processing extraction steps. The results were plotted, and the response from the chromatogram per unit concentration of HCAs in the original sample is then determined.