CHAPTER III
EXPERIMENTAL

This chapter contains information of chemicals, instruments and preparation method of reagents and samples. The sample preparation, spectrophotometric method and HPLC technique for determination of carbaryl and carbofuran in samples are also given.

3.1 Chemicals and reagents

All reagents were at least of analytical reagent grade. Six carbamate pesticides and two organophosphate pesticides including, carbaryl (CBR), carbofuran (CBF), bendiocarb (BDC), promecarb (PMC), isoprocarb (IPC), propoxur (PPX) and parathion (PTO), monocrotophos (MCP), respectively, were purchased from Riedel-deHaen, Germany. Triton X-114 (TX-114) was purchased from Acros (USA). 2,4-dimethoxyaniline; DMA ($C_8H_{11}NO_2$) from Fluka (Japan). Sodium nitrite ($NaNO_2$) from Riedel-deHaen (Germany). Concentrated HCl was obtained from Carlo Erba (France). 4-aminoantipyrine; AP ($C_{11}H_{13}N_3O$) was purchased from Acros (USA), potassium ferricyanide ($K_3Fe(CN)_6$) and sodium tetraborate decahydrate ($B_4Na_2O_7.10H_2O$) from Fluka (Japan). Sodium hydroxide (NaOH) was purchased from Carlo Erba (France). Sodium sulphate anhydrous (Na_2SO_4) was purchased from Fluka (Japan), sodium acetate (CH_3COONa) and magnesium sulphate ($MgSO_4$) were purchased from Carlo Erba (France) and sodium chloride (NaCl) was purchased from Ajex Finechem (Australia). Carbograph and primary secondary amine (PSA) were purchased from Vertical Chromatography Co., Ltd. (Thailand). Acetonitrile (CH_3CN) and methanol (CH_3OH) were purchased from Lab scan asia (Thailand) and Acetic acid (CH_3COOH) was purchased from Carlo Erba (France).

3.2 Instruments

3.2.1 Spectrophotometry

Absorbance measurements and spectra recording were performed on a Spectrophotometer (Agilent 8453 UV-Vis spectroscopy system, Germany). A 1-cm thick quartz cell was used throughout the experiments.

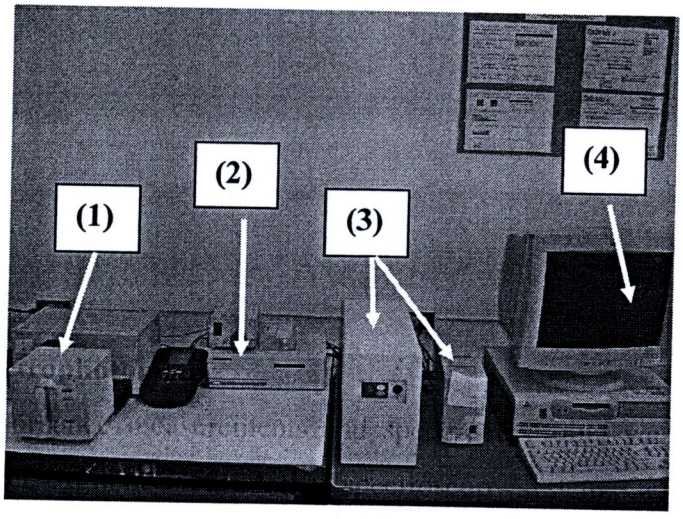

Figure 3.1 Agilent 8453 Spectrophotometer.

(1) Photodiode array detector

(2) Temperature control

(3) Stabilizers

(4) Monitor

3.2.2 Chromatographic analysis system

A chromatographic instrument consisted of a water 600 controller dual pump (Waters, USA), a Rheodyne injector with 20 μL sample loop and Waters 2996 Absorbance detector. The Empower software was used for data acquisition. A Chromolith Speed ROD (C_{18} monolithic 50 mm x 4.6 mm) column was used.

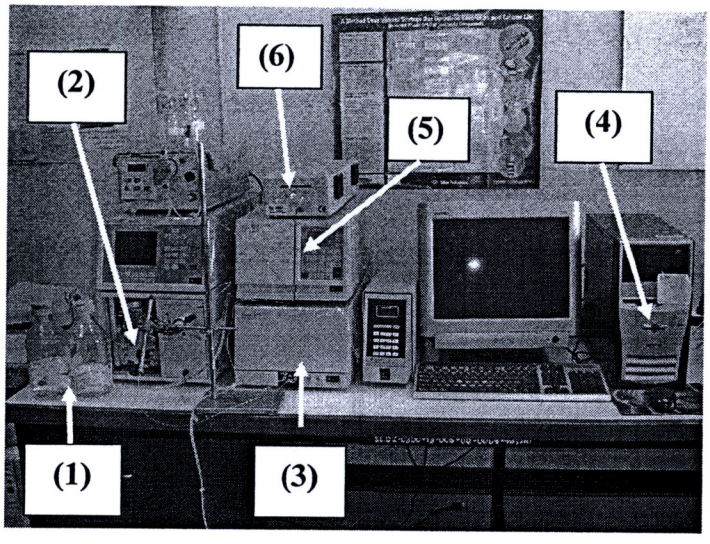

Figure 3.2 Instrumentation of RP-HPLC.

(1) Mobile phase reservoir

(2) Pump

(3) A Waters 2669 photodiode array detector (PDA)

(4) Empower software

(5) A Water 2475 Multi wavelength fluorescence detector

(6) A Water temperature control system

3.2.3 Sample preparation procedure

Reciprocal shaker (Vision Scientific Co.,Ltd., Korea) was used for extraction of vegetables. Two centrifuge instruments (Biomed group Co., Ltd., Thailand, and Kokusan type H-11N, Biomed group, Co., Ltd., Japan) were used for phase separation. Vortex (Scientific Industries, INC., USA) was used for mixing the sample or analyte in solvent. The rotary evaporator R-200 (Buchi, Switzerland) was used for elimination of solvents.

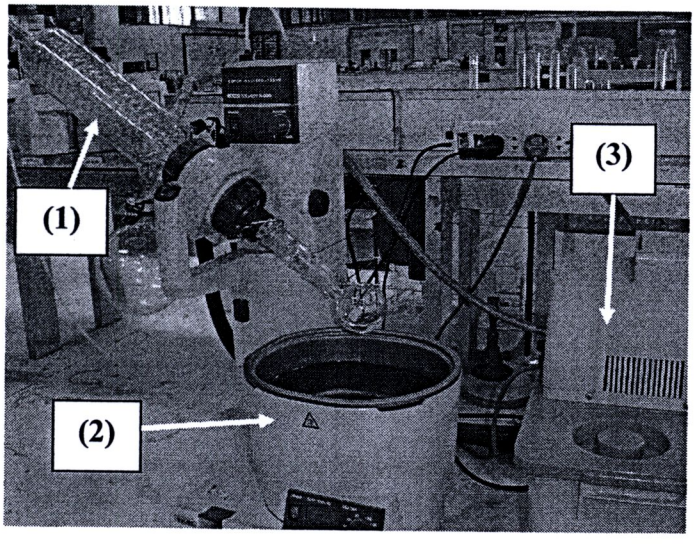

Figure 3.3 Rotary evaporator R-200.
(1) Condenser
(2) Heating bath B-490
(3) Pump

3.3 Procedures

3.3.1 Preparation of standard pesticides

The 1,000 mg L^{-1} of stock standard solutions of pesticides were prepared by dissolving 0.0250 g of each pesticide in methanol (25.00 mL) and stored at 4 °C. These standards were prepared every six months. Working solutions were prepared by appropriate dilution of the stock solutions in water.

3.3.2 Preparation of the derivatizing agents

3.3.2.1 Reagents for the derivatization of CBR

DMA (0.1 mol L^{-1}) was used as the derivatizing agent for CBR, prepared by dissolving an accurate amount of DMA (0.3830 g) in 25.00 mL of methanol. Sodium nitrite (0.1 mol L^{-1}) was prepared by dissolving 0.1725 g in 25.00 mL water and hydrochloric acid (1.0 mol L^{-1}) was prepared in water.

3.3.2.2 Reagents for the derivatization of CBR and CBF

AP (2% w/v) was used as the derivatizing agent for both CBR and CBF, prepared by dissolving 2 g AP in 100.00 mL water. Sodium hydroxide (1.0 mol $^{-1}$L) was prepared by dissolving the 4 g of NaOH pellets in 100.00 mL water. Borate buffer (0.1 mol L^{-1}) was prepared by dissolving sodium tetraborate decahydrate 3.8137 g in water and adjusted pH to 9.5 with 1.0 mol L^{-1} NaOH and then water was added to final volume of 100.00 mL. Potassium ferricyanide (8% w/v) was prepared by dissolving $K_3Fe(CN)_6$ 8 g in 100.00 mL water.

3.3.3 QuEChERS

3.3.3.1 Chemicals for QuEChERS

The chemicals used for QuEChERS including, acetonitrile, sodium sulphate anhydrous, sodium acetate, magnesium sulphate, Carbograph and primary secondary amine (PSA).

3.3.3.2 Extraction by QuEChERS

The edible part of the sample (~500 g) was cut into 1-cm pieces and blended using a commercial food mixer. The mince sample was accurately weighed (15 g) and placed in a 50 mL centrifuge tube. Aliquot of organic solvent was added and shaken at 250 rpm for 1 hour. After shaking, the extract was added with Na_2SO_4 anhydrous, $MgSO_4$ and CH_3COONa, vortexed immediately and then centrifuged for 10 min at 2,500 rpm and transferred aliquot of upper layer into a 50 mL tube containing Carbograph and PSA. The tube was shaken immediately and solution was filtered. The filtrate was then evaporated using a rotary evaporator (at 50 ^{o}C water bath) to eliminate acetonitrile. The solution was diluted with water to 10.00 mL prior to the extraction by CPE. For spiked samples, CBR and CBF were added to the represent samples and equilibrated at room temperature for 30 min before extraction.

Parameters affect on the extraction efficiency were studied including, type and amount of organic solvent (1% acetic acid in 99% methanol and 1% acetic acid in 99% acetonitrile), amount of salts (Na_2SO_4, $MgSO_4$ and

CH_3COONa) at the ranges of 5.0-15.0 g, 1.0-10.0 g and 0.5-5.0 g, respectively. Amount of Carbograph and PSA were studied for removing chlorophyll.

3.3.4 Cloud-point extraction (CPE)

3.3.4.1 Reagents for CPE

TX-114 (25% w/v) was prepared by dissolving TX-114 25 g and adjusted to 100.00 mL with water. Sodium chloride, sodium carbonate, and sodium sulphate were prepared in water.

3.3.4.2 Preconcentration by CPE

Standard or sample solutions (10.00 mL) were mixed with salts and TX-114 solution in a 15 mL centrifuge tube. The solution was shaken and kept in a thermostat water bath and then centrifuged at 3,500 rpm to complete the phase separation. The solution was kept in an ice bath for 10 min. The aqueous phase (upper phase) was withdrawn, while the SRP (lower phase) was measured the volume using a 10 mL syringe. SRP (~400 μL) was then diluted with methanol to a final volume of 1 mL before analysis.

Parameters affect on the extraction efficiency were studied including concentration of TX-114 was studied in the range of 0.5-2.5 % w/v, type and concentration of salts (NaCl, Na_2CO_3 and Na_2SO_4) were studied in the range of 0.2-5.0 % w/v. The equilibration temperature, equilibration time and centrifugation time were studied in the range of 25-55 °C, 5-30 min and 5-30 min, respectively.

3.3.5 Spectrophotometry of CBR and CBF

3.3.5.1 Procedure for derivatization

(1) Derivatization with DMA

The standard solution or sample solution (obtained from CPE) was added with 1.0 mol L^{-1} NaOH and kept at room temperature for 10 min. The diazonium ion was prepared by mixing 0.1 mol L^{-1} DMA and 0.1 mol L^{-1} $NaNO_2$ and 1.0 mol L^{-1} HCl. The naphtholate was mixed with diazonium ion to form azo dye. A blank reagent solution was performed in the same manner without CBR

spiking. Absorbance was evaluated at the maximum absorption wavelength of 510 nm. All experiments were consecutively studied in triplicate.

(2) Derivatization with AP

For the simultaneous determination, CBR and CBF were derivatized with AP. Standard solution or sample solution from CPE was added with 1.0 mol L^{-1} NaOH and allowed to complete hydrolysis for 10 min, the pH of the solution was adjusted to 9.5 by adding 0.1 mol L^{-1} borate buffer solution. 2% w/v AP and 8% w/v potassium ferricyanide was added, respectively, and mixed well. Absorbance was evaluated at the maximum absorption wavelengths of 480 nm and 510 nm for CBR and CBF derivatives, respectively. All experiments were consecutively studied in triplicate.

3.3.5.2 Optimization of the conditions for spectrophotometry

The optimum conditions for the spectrophotometric method were studied using CBR and CBF (3.0 mg L^{-1}) and the studied parameters are: concentration of reagents, pH, and stability of the derivatives.

(1) Concentration of reagents

The concentrations of reagents for derivatization of CBR were studied including; DMA, $NaNO_2$, HCl, and NaOH. The concentration ranges studied were DMA (0.2-1.0 mmol L^{-1}), $NaNO_2$ (0.1-1.0 mmol L^{-1}) and HCl (2.0-10.0 mmol L^{-1}). While, concentration of NaOH was studied according to pHs ranged from 2.0-14.0.

The concentrations of reagents for simultaneous determination of CBR and CBF were studied including; AP, borate buffer solution, $K_3Fe(CN)_6$ and NaOH. The studied concentrations of AP, borate buffer solution and $K_3Fe(CN)_6$ were 1.0-7.0 mmol L^{-1}, 0.0-7.0 mmol L^{-1} and 2.4-16.8 mmol L^{-1}, respectively. While, concentration of NaOH was studied according to pHs ranged from 2.0-14.0.

(2) pH for hydrolysis

For hydrolysis of CBR and CBF, pHs were studied in the range of 0-14.

(3) Stability of the derivatives

The stability of the derivatives was studied by leaving the derivatives to stand for over 2 hours and measured the absorbance at the maximum absorption wavelength every 20 min.

3.3.6 Method validation

The method validation was investigated using optimum condition obtained from (3.3.5.2). The parameters were: linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy and interference study.

3.3.6.1 Linearity

Linearity was investigated in triplicate analysis of standard of CBR and CBF ranged from 0.5 to 7.0 mg L^{-1}. All standards were daily prepared by dilution of the stock standard solutions containing 1,000 mg L^{-1} of each standard.

Moreover, in order to eliminate matrix effect in vegetable samples. The linearity was investigated using matrix match calibration technique, five concentration levels of analytes ranged from 0.5 to 7.0 mg L^{-1} were prepared in the sample matrices.

3.3.6.2 Limit of detection and limit of quantitation

Limit of detection (LOD) and limit of quantitation (LOQ) were obtained with blank solutions containing all the reagents, except CBR or CBF. The 3s criterion was adopted to evaluated LOD (Alvarez-Rodriguez et al., 1997). In this study, LOD and LOQ were calculated as CBR or CBF concentration giving a signal equal to 3SD and 10SD of blank (n=5), respectively.

3.3.6.3 Accuracy and precision

An accuracy was studied in term of recovery by spiking the sample with known concentrations of each analyte (0.5, 1.0, and 3.0 mg kg^{-1}) and was analyzed in five replicates. While, precision was studied in term of relative standard deviation of recovery.

3.3.6.4 Interference study

The interference was studied by adding different amounts of potentially interfering species to standard solutions of CBR and CBF. Interfering species studied were carbamate pesticides (PPX, IPC, PMC, BDC, MTM, MTC) and organophosphate pesticides (PTO, MCP). The solutions were hydrolyzed, derivatized and measured the absorbance in triplicate.

3.3.7 Sample preparation

Vegetable samples are cucumber, cabbage, yard long bean, kale and mustard (see Figure 3.4). These vegetables were purchased from local markets in Khon Kaen province. QuEChERS and CPE were used as the sample preparation steps (see 3.3.3 and 3.3.4).

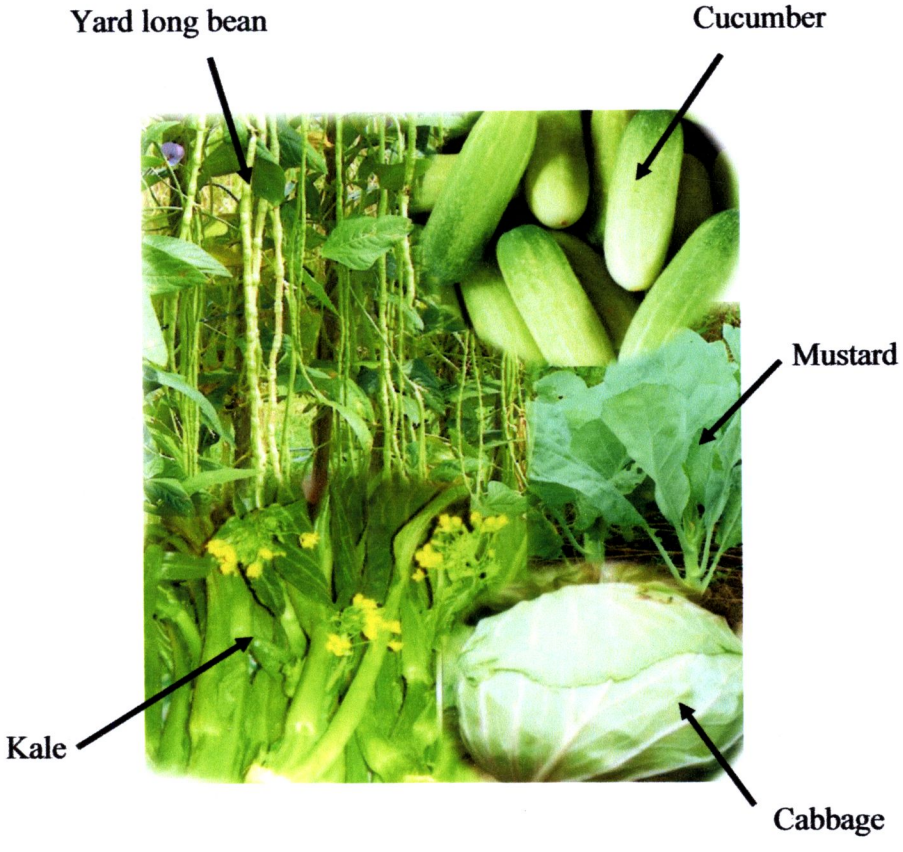

Figure 3.4 Vegetable samples

3.3.8 Simultaneous determination of CBR and CBF

Using the optimum conditions (obtained from 3.3.5.2), the absorbance of individual CBR and CBF derivative and mixture of CBR and CBF derivative were recorded using both normal mode (for simultaneous determination of CBR and CBR by simultaneous equations technique) and first-derivative mode (for simultaneous determination of CBR and CBF by zero-crossing technique).

3.3.9 Analysis of CBR and CBF by HPLC

Methanol and water were used as the mobile phase in the study. They were filtered through 0.45 μm nylon and degassed by ultrasonic bath before use. The ratios of methanol and water were studied in order to completely separate of CBR and CBF. The detection wavelengths were investigated to obtain highest sensitivity.