CHAPTER 2

EXPERIMENTAL

2.1 Apparatus and Chemicals

2.1.1 Apparatus

1. High Performance Liquid Chromatography (HPLC) HP 1100 manufactured by Agilent, Germany Aglient 1100, consisting of
    a) Agilent 1100 series Isocratic pump
    b) Agilent 1100 series Variable wavelength detector

    shown in Figure 2.1
2. Analytical column, Hypersil ODS 4.0 x 125 mm 5 microns, Bondapak C_{18}.
3. Analytical balance, Sartorius MC 1 Analytical AC 210 S, made in Germany.
5. Vacuum pump, Milipore, USA
6. UV-VIS spectrophotometer (Model UV Lamda 25, Perkin Elmer Instruments, USA)
7. Ultrasonicator (Model 8891 Cole Parmer Instrument, USA)
8. Rotary evaporator, Buchi Labortechnik AG, Switzerland
9. Kokusan H 31 series Centrifuge, Kokusan Ensinki Co.Ltd. Tokyo, Japan
10. SPE Manifold, Alletech, Associated Applied Science Ltd. UK.

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11. Whatman Filter paper No.1, England
12. Nylon filter, porous, 0.45 Sartorius Germany and filtration equipment
13. Solid phase extraction column, Florisil (3 ml, 500 mg), Waters, Supelco, U.S.A

2.1.2 Chemicals

The chemicals used in this works are listed in the Table 2.1.

Table 2.1 Chemicals

Chemical	Purity Grade	Supplier
Acetonitrile CH_3COCH_3	HPLC 99.8%	VWR Prolabo. International Ltd.
Ethyl acetate	Analytical Grade 99.97%	VWR Prolabo. International Ltd
Methanol , CH_3OH	HPLC 99.9%	Lab Scan, Ireland
Milli Q water	Deionized water more purified with Milli pore Q system	Chemistry Department, Chiang Mai University
Sodium Sulphate	99%	Carloerba
Carbofuran	99%	ChemService, USA
Carboxin	99%	ChemService, USA
Methomyl	Analytical Standard Assay (HPLC) 99.9%	Sigma – Aldrich Laborchemikalien GMBH. Germany

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Figure 2.1 HPLC (HP 1100) system used in this research works

2.2 Preparation of Solutions

2.2.1 Working standard solutions (100 μg/ml)

1 ml of each 1000 ppm stock standard solutions was pipetted into a 10 ml volumetric flask and diluted to the mark with HPLC grade methanol to yield the standard solutions of 100 μg/ml.

2.2.2 Mixed standard solutions (10 μg/ml)

Mixed standards 10 μg/ml was prepared by mixing 2.5 ml volumes of each 100 μg/ml working standard solutions into a 25 ml volumetric flask respectively and HPLC grade methanol was added to the mark. The working standard solutions were prepared by further dilution and stored at 4 °C in refrigerator.

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2.2.3 Mobile phase solutions

The HPLC grade solvents were chosen for mobile phase solutions. HPLC solvents guarantee the best possible UV transparency and the absence of contaminants which alter the elution strength or give rise to extra peaks in separation. The mobile phase used in this investigation was a mixture of acetonitrile (ACN) and water in the ratio of (40:60). Prior to use, the solvents are filtered by filter apparatus and vacuum pump. Even though, the HPLC system have filters routinely in line, the simple preventive maintenance practice of filtration can minimize clogging, filter changes and associate expense. The vacuum pump is used to supply the suction to pull the mobile phase through the filter into the filtering flask. After filtration, the solvents were degassed for 20 minutes by putting the mobile phase bottle in to an in ultrasonicator to the vacuum pump. If the gas bubbles dissolve in the solvents, some problems may occur showing the base line will be noisy or spiked with the extra peaks and analytical precision may be affected. When mobile phase are mixed, during the development of a method, it is important to note the manufacturer, lot number, grade, types and any other attributes of the solvents used. Physical properties of solvents used in this research works are shown in Table 2.2.

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Table 2.2 Physical properties of solvents used in this research works [18]

Solvent	Polarity Index	Viscosity (CP) at 20 °C	Boiling Point (°C) at 760 Torr
Acetonitrile	6.2	0.37	81.6
Ethyl Acetate	4.3	0.47	77.1
Methanol	6.6	0.60	64.7
Water	9.0	1.0	100.0

2.3 Detection Wavelength

The single standard solutions of 1 ppm of methomyl, 5 ppm of carbofuran, and carboxin dissolved in methanol were measured for the maximum absorbance by UV-VIS spectrometer. The detection wavelength in the range of 190-300 nm was studied. After finding out the maximum wavelength of the single analytes, the standards solutions were mixed and run onto the HPLC according to the different maximum wavelengths of the single standards detected by the UV- VIS spectrometer.

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2.4 Optimization of the HPLC System

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2.4.1 Choice of column

Reverse phase liquid chromatography is the most popular mode of chromatography in the HPLC in which the mobile phase is more polar than the stationary phase which is the opposite of normal phase chromatography. C_{18} is the most popular column and for a general purpose, the first choice of a bonded phase column used in the reverse phase mode is the C_{18}. It is the most appropriate because

of its wide versality for retention of most compounds and has a history of documented application usage. Furthermore, HPLC methods for the determination of pesticides residues in fruits and vegetables could employ reversed-phase chromatography with C_{18} or C_8 columns and aqueous mobile phase, followed by UV absorption or UV diode array detection [2]. In this work, C_{18} column was used because of its versality and well documentation.

2.4.2 Optimization of mobile phase composition

After selecting the C_{18} bonded phase column in the reverse phase mode, the best mobile phase composition for an adequate separation was approached by varying different ratios of solvents of Acetonitrile in water such as 20%, 30%, 40%, 50% whereas the flow rate was fixed at 1 ml/min. The retention and separation behavior were noted to achieve the desired resolution.

2.4.3 Optimization of flow rate

Chromatography is a separation processes and any mixing of sample molecules, and a reflection of the mixing in a column is dependent upon the velocity at which the chromatography takes place. To develop a seperation and to minimize the time involved in developing a method, different flow rates such as 0.6 ml/min, 0.8 ml/min, 1 ml/min, and 1.2 ml/ min were varied whereas the optimum composition of mobile phase was 40/60% v/v ACN in water. The efficiency performance and the time involving in the developing method were noted.

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2.5 Validation of the Method

A validation of the method was performed in terms of precision, linearity range, limit of detection and limit of quantification.

2.5.1 Precision

Precision of a method is the degree of scatter of the results and usually expressed as standard deviation (SD), and percent relative standard deviation of retention time and peak area. Precision is often subdivided into repeatability and reproducibility.

2.5.1.1 Repeatability test of HPLC system

Repeatability indicates the closeness of the individual results obtained by the same analyst on the same instrument with identical reagents within a short period of time (<1day) [100]. Repeatability is assessed by making replicate injections of the same solutions and gives a measure of the error in measuring peak area and peak height. For testing the repeatability, a10 µl of the 1 µg/ml of methomyl, 5 µg/ml of carbofuran and carboxin mix standards was injected 10 times within intraday onto the HPLC column. Detector response in terms of retention times and peak area were recorded to observe error from such chromatograms.

2.5.1.2 Reproducibility test of HPLC system

Reproducibility indicates the closeness of the individual results obtained by the different days after changes of experimental conditions such as, reagent sources [31]. In order to find the reproducibility of HPLC system, the same amount of

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standard solution was injected inter day (3 days) to observe the reproducibility of retention times and peak area from chromatograms.

2.5.2 Determination of detector linearity

If there is a linear relationship, a method is linear between the analytical response and concentration of analyte in the sample solution over a specified range of concentrations of the analyte. Linearity is generally assessed by calculating a linear regression coefficient (r^2). The linearity of the calibration curves was performed with 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, and 8 µl/mg of mixed pesticide standards on to HPLC operated at optimum condition. The calibration curve of each standard was constructed by plotting the detector response in terms of peak area against the concentration of each standard injected.

2.5.3 Determination of Limit of Detection and Limit of Quantification

The limit of detection is the amount of analyte which can be reliably detected under the stated experimental conditions. The limit of detection is the analyte concentration giving a signal equal to the blank signal, plus three standard deviations of the blank. The limit of detection was calculated from the calibration curve by means of the blank signal, which can be used as an estimate of the calculated intercept, plus three standard deviations of the blank. It can be used as an estimation of the calculated value from the regression line. The values lower than the limit of detection was called non detected (ND) whereas the values which were higher than the limit of quantification were acceptable values [32, 33]. A mixture of standards

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containing 0.1, 0.2, 0.4, 0.6, 0.8 μl/mg was injected into HPLC system to investigate the limit of detection and limit of quantification.

2.6 Optimization of Solid Phase Extraction

The efficiency of SPE depends on selection of the sorbent, elutrophic strength and volume of the elution solvent and the amount of sample loaded onto SPE. Firstly, the optimization was started by using Sep-Pak C_{18} SPE column conditioned by 10 ml of methanol followed by 10 ml of Milli Q water.

2.6.1 The composition of elution solvent

The composition of the elution solvent was assessed by varying different concentration of acetonitrile with water (40, 50, 60, and 70 %.) A Sep-Pak C_{18} SPE column was conditioned as described in section 2.6 and 2 μl/mg fortication level mix standard solutions was spiked onto the SPE column. In the elution step, the series of 1st fraction (3 ml), 2nd fraction (2 ml) and 3rd fraction (2 ml) of elutes were collected respectively. After this, each fraction was injected onto HPLC system.

2.6.2 Study the volume of the elution solvent

Due to the different polarities of analytes, the elution pattern of analytes present in the standard solutions, was studied and optimized. The minimum eluent volume after ACN/ H_2O (70:30) was chosen as the eluent for analytes. The volume of eluent was collected as each 1 ml for seven fractions after the solutions was passed through the SPE cartridges. Peak areas of analytes at different fractions of eluent were used for elution profile of analytes.

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2.6.3 Sorbent selection

Sorbent selection is influenced by the analyte characteristics and sample matrix. To optimitize the efficiency of the SPE, reverse phase C_{18} and normal phase florisil sorbents were tested. The cartridges packed with different sorbents were loaded with three different concentrations levels of mix standard solutions after conditioning the bonded phase. The recovery rates of each pesticide were evaluated from the chromatograms of the standard solutions before and after use of the SPE cartridges in order to access the extraction efficiency of the proposed method.

2.7 Sampling Method

The popular and commonly consumed vegetables in Thai food namely cabbage (*Brassica olecera var. capitata*) representing the brassica families were selected for this study. Vegetable samples with and without safety labeled samples were collected from different markets such as major supermarket, minimart, farm shop and local fresh market of Chiang Mai, Thailand as shown in Table 2.3 by using market oriented supply study. This kind of study monitors the marketed produce available to consumers by collecting samples from different shops [34]. Type of sample cabbage (*Brassica oleracea* L. cv. white headed cabbage cruciferae) and sample cabbages from the supermarket are shown in Figures 2.2 and 2.3.

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Figure 2.2 Type of sample cabbage

(*Brassica oleracea* L. cv. white headed cabbage cruciferae)

Figure 2.3 Sample cabbages from the super market

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Table 2.3 Criteria of samples collected from different markets

Sample No.	Sample code	Date of Collection	Description
1	LF1	20-2-06	Local fresh market (without safety label)
2	SM1	20-2-06	Super market (without safety label)
3	SM3	20-2-06	Super market (with certified safety label)
4	MM1	24-2-06	Minimart (without safety label)
5	FS1	24-2-06	Farm shop (with safety label)
6	LF2	27-2-06	Local fresh market (without safety label)
7	SM2	28-2-06	Super market (without safety label)
8	SM4	28-2-06	Super market (with certified safety label)
9	MM2	4-3-06	Minimart (without safety label)
10	MM3	4-3-06	Minimart (with certified safety label)
11	FS2	4-3-06	Farm shop (with safety label)

2.8 Sample Extraction

Ethyl acetate was selected as the solvent for the extraction of pesticides because of its effectiveness for polar and non polar pesticides from a diverse range of matrix. The other advantages include ease of evaporation. Ten grams of freshly chopped vegetable was homogenized and extracted with 50 ml of ethyl acetate and sonicated in the ultrasonic bath for 5 minutes. Thirty grams of anhydrous sodium sulphate were added and homogenized well for 10 minutes to remove water. Then, the extract was filtered through the Buchner Funnel containing Whatman filter paper No1. The 25 ml of ethyl acetate was added to the pellet to rinse for two times and then

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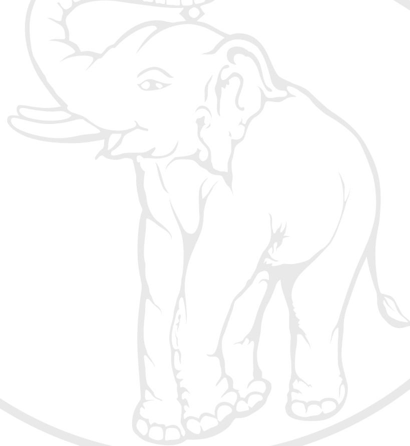
the rinsing liquid was added to the combined extraction fractions. The filtrate solution was evaporated in the rotary evaporator at 30 ºC until dryness and redissolved in 5 ml of 70 % ACN. Only 2 ml of the redissolved extract was centrifuged. After that 0.5 ml of the supernatant was loaded onto florisil SPE cartridge which had already been conditioned with 10 ml of methanol and 10 ml of Milli Q water. The column cartridge was dried with vacuum manifold and 3 ml of 70% acetonitrile in water were used to elute the analytes retained on the florisil sorbent before injection onto the HPLC. Schematic diagram of sample extraction and clean up procedure is shown in Figure 2.4.



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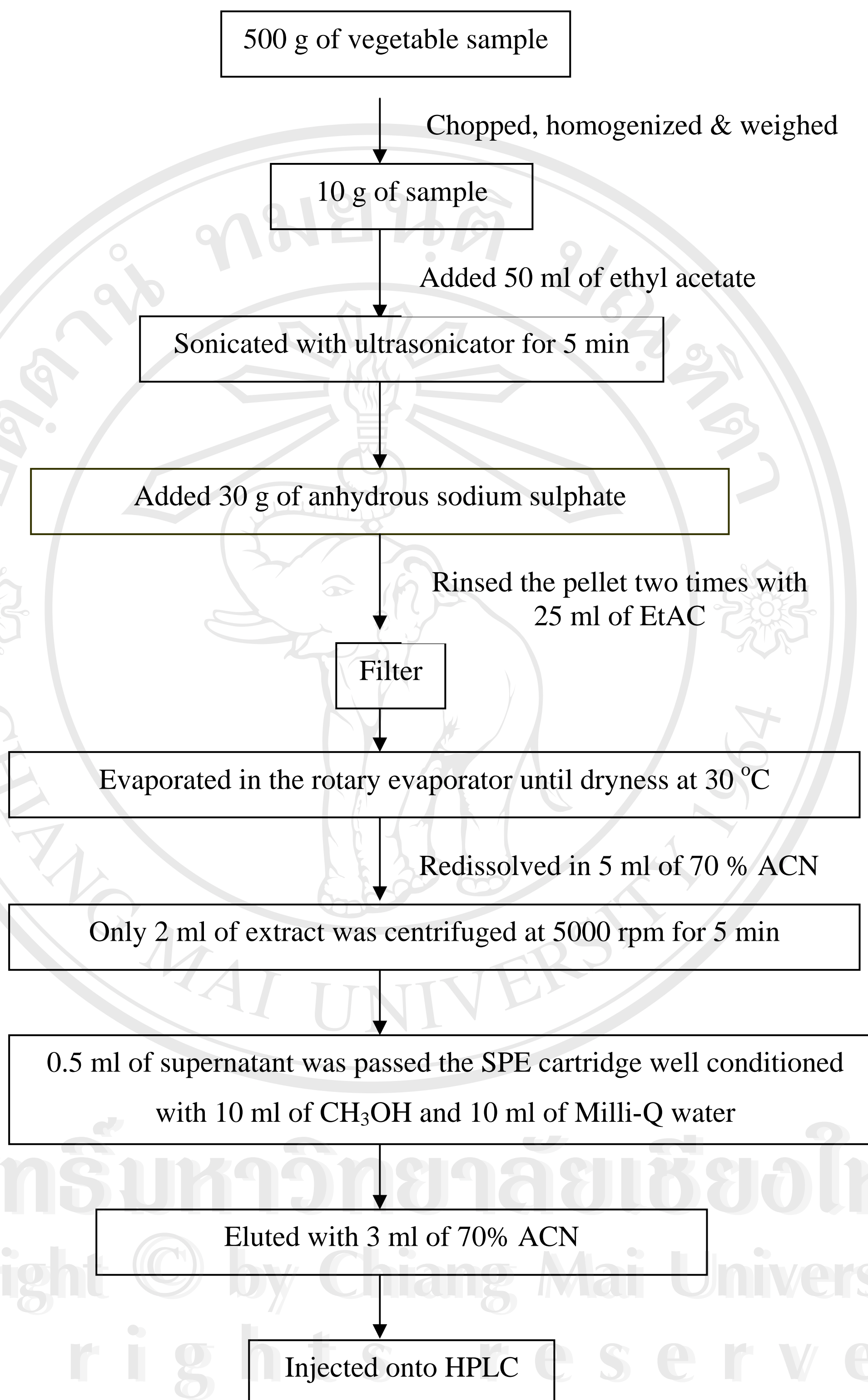

Figure 2.4 Schematic diagram of sample extraction and clean up procedures

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2.9 Accuracy

In analytical chemistry, the reliability of results could be expressed in terms of accuracy. Generally, accuracy is a measurement of the difference between the true values and the determined values as the error or relative error. In this work, the accuracy of the method was calculated through the recovery of each pesticide. The samples were spiked in order to get 0.5 μg/ml of mixed standard solutions and it was extracted by using developed extraction procedures as shown in Figure 2.3. The percent recovery of extraction method was calculated as below.

$$\%\ \text{Recovery} = \frac{\text{Sample peak response x Sample final volume}}{\text{Std peak response x Std final volume}} \times 100$$

In this experiment, the concentrated sample extracts contain a high content of co-extractives which interfere with the component of interest during the extraction step and diminish the recovery percentage. The background obtained from chromatograms of real samples was high and matrix interferences resulted in the occurrence of false positive results and inaccurate quantitation. Consequently, further optimization was required and HPLC parameters such as wavelength, flow rates and mobile phase composition were further optimized as follows.

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2.9.1 Further optimization of wavelength

In this experiment, the plant pigments co-exist in the sample matrix. Relatively high background peaks emerged together with the component of interest and interfered the peak identification and subsequented quantification especially

methomyl. Therefore, proper optimum wavelength was selected instead of former optimum wavelength (233nm).

2.9.2 Further optimization of mobile phase composition

The mobile phase composition was varied with 20%, 25%, 30 % acetonitrile in water whereas the flow rate was fixed at the former optimum flow rate at 0.8 ml/ min. The sample extract spiked with 0.5 µg/ml fortification level of standards was injected into the chromatographic systems. The background peak, resolution and retention times were evaluated.

2.9.3 Further optimization of flow rate

The further optimization of flow rate was varied from 0.6 to 1.2 ml/min whereas the optimum composition of the mobile phase was 25/75% v/v acetonitrile/water. The extracted sample spiked with 0.5 µg/ml fortification level of mix standards was injected into the chromatographic systems. The recovery rates of each pesticide at different flow rate levels were evaluated.

2.10 Confirmation Method

It is important to confirm the identity of analytes and standards by comparing their retention time. Some of the samples were spiked with known mix standards and injected on the HPLC system under the same optimum condition. The chromatograms of with and without spiking of the standards in some extracted samples were compared. In addition, some of the extracted samples were analyzed by Laboratory Center for Food and Agricultural Products Co., Ltd (LCFA) by using HPLC

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fluorescence detector for carbofuran and Gas Chromatography-Mass Spectrometry (GC-MS) for carboxin respectively.

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