2. EXPERIMENTAL

2.1 Apparatus

(i) Multisolvent Delivery System, Waters 600E, manufactured by Waters Chromatography Div., Milford, U.S.A.

(ii) Autosampler, Waters 717, manufactured by Waters Chromatography Div., Milford, U.S.A.

(iii) Tunable UV-Visible Absorbance Detector, Waters 486, manufactured by Waters Chromatography Div., Milford, U.S.A.

(iv) Chromatography Data Workstation Maxima 820, NEC PowerMate SX/16 and NEC pinwriter P6200, manufactured by NEC Technologies Inc., Boxborough, U.S.A.

(v) Guard column, Guard PAK guard column holder (Part No.88141, Waters Chromatography Div., Milford, U.S.A.), fitted with Guard PAK C_{18} μ Bondapak precolumn inserts (Part No.88070, Waters Chromatography Div., Milford, U.S.A.)

(vi) Analytical column, 3.9mm.×300mm., containing 10 μm μ Bondapak C_{18} spherical particles (Part No. 27324, manufactured by Waters Chromatography Div., Milford, U.S.A.)

(vii) Power line stabilizer/ conditioner, model AVS-400 2C, Quasar, Thailand.

(viii) Ultrasonic cleaner, Branson B-2200, Connecticut, U.S.A.

(ix) Air cylinder with a pressure regulator.

(x) Helium cylinder with a pressure regulator.

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(xi) Filter apparatus, Waters Associates, equipped with 1 liter of ground joint flask, 300 cm^3 funnel and tabulated base.

(xii) Air motor compressor vacuum pump, Waters Associates.

(xiii) Filter unit, Nalgene, U.S.A., 0.20 μ

(xiv) Analytical Balance, Sartorius.

(xv) Glass column for sample preparation, manufactured by J.T. Baker, Philipsburg, U.S.A. and Merck, Darmstadt, F.R.Germany.

(xvi) Frits (PTFE) for 8 ml glass columns (catalog No. 19828) manufactured by Merck, Darmstadt, F.R.Germany.

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2.2 Chemicals

Chemical	Grade	Supplier
Acetone, CH_3COCH_3	AR	BDH Chemical Ltd., Poole, England
Acetonitrile, CH_3CN	HPLC	Riedel-de Haën, Seelze, F.R.Germany May & Baker Ltd, Dagenham, England
Methanol, CH_3OH	HPLC	J.T. Baker, Philipsburg, U.S.A.
Octadecyl (C_{18}) 40 µm Bulk packing for chromatography		J.T. Baker, Philipsburg, U.S.A. E.Merck,Darmstadt, F.R.Germany
Ammonium acetate, CH_3COONH_4	98%	E.Merck,Darmstadt, F.R.Germany
Carbaryl standard	99.9%	Regional Medical Science Center, Chiang Mai
Distilled water		Chemistry Department, Chiang Mai University

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2.3 Preparation of Solutions

2.3.1. LC Standard Solutions

a. Stock solution of carbaryl standard (1000 μg/ml)

A 0.050 g amount of the carbaryl standard was transferred into a 50 ml volumetric flask after weighing. The standard was dissolved in acetonitrile which was also added to the 50 ml mark. The stock standard solution was stored at 4 °C in a refrigerator.

b. Working solutions (50 μg/ml, 40 μg/ml, 10 μg/ml)

0.5 ml and 0.4 ml of the stock carbaryl standard solution were pipetted into two 10-ml volumetric flasks and diluted with HPLC grade acetonitrile to the mark of the 10 ml volumetric flasks to yield the carbaryl working solutions of 50 and 40 μg/ml, respectively. Two ml volume of the 50 μg/ml working standard solution was pipetted into another 10 ml volumetric flask and HPLC grade acetonitrile was added to the 10 ml mark to make 10 μg/ml carbaryl standard. The working standard solutions were stored at 4 °C and replaced as new stock solutions were made.

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2.4 Construction of Calibration Curves

2.4.1. Calibration Curve Range 0.04 μg/ml to 2 μg/ml

The preparation of the carbaryl standard solutions in this range was carried out by pipetting 0.10, 0.20, 0.20, 0.40, 0.80 and 2.00 cm^3 of the working standard

solution with 10 μg/ml carbaryl concentration into two 25 ml volumetric flasks for the concentrations 0.04 and 0.08 μg/ml and into three 10 ml volumetric flasks for the concentrations 0.2, 0.4, 0.8 and 2 μg/ml, respectively. Each volumetric flask was filled up to the level with HPLC grade of acetonitrile, stored at 4 °C and replaced as new standard solutions were required.

2.4.2. Calibration Curve Range from 1 μg/ml to 5 μg/ml

The preparation of the carbaryl standard solutions for the concentration range 1 μg/ml to 5 μg/ml was carried out by pipetting 1.00, 2.00, 3.00, 4.00 and 5.00 cm^3 of the working standard solution with 10 μg/ml carbaryl concentration into five 10 ml volumetric flasks and adding HPLC grade acetonitrile into each of the flask for making the concentrations of 1, 2, 3, 4, and 5 μg/ml, respectively. All of these solutions were stored at 4 °C and replaced as new standard solutions were made again as necessary.

2.4.3. Calibration Curve Range from 5 μg/ml to 30 μg/ml

The preparation of the carbaryl standard solutions for the concentration range 5 μg/ml to 30 μg/ml was carried out by pipetting 3.00, 4.00, 5.00 and 6.00 cm^3 of the working standard solution with 50 μg/ml carbaryl concentration into four 10 ml volumetric flasks for making the concentrations of 15, 20, 25 and 30 μg/ml, respectively. The HPLC grade of acetonitrile was filled up to the level in each flask and all solutions were stored at 4 °C and replaced as new standard solutions were made again as necessary.

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The response peak heights and peak areas of carbaryl in these three ranges

of concentration are shown in Tables 3.1, 3.2 and 3.3. The 3 concentration ranges of carbaryl calibration curves were constructed by plotting concentration against peak area response as illustrated in Figs. 3.1-3.3 and some of the chromatograms obtained are shown in Figs. 3.4-3.6.

2.5 Preparation of the Mobile Phase

The use of clean solvents is important not only for reproducible results but also for operation with minimal maintenance. Poor solvent condition can cause baseline noise and drift, and particulate matter can block the solvent reservoir and inlet filters .

Mobile phase difficulties account for 70% or more of all liquid chromatographic problems. Degassing solvents used in the mobile phase is one of the most effective measures to eliminate these problems [24].

The preparation of the mobile phase in this work was carried out by using a binary solvent system, i.e. acetonitrile/water mixture. The composition of the mobile phase prepared in 1000 cm^3 volume was 50/50 (v/v) CH_3CN/H_2O system. This was achieved by mixing 500 cm^3 of pure CH_3CN and 500 cm^3 of distilled water together with 0.5 mmol of ammonium acetate buffer. As an unclean solvent can cause baseline noise and drift, and particulate matter can block the solvent reservoir and inlet filters, the solvent in this work had to be filtered by using a filter apparatus (filter pore size 0.20 μ) and vacuum pump. After that, the solvent was degassed for 30 minutes in an ultrasonicator by pouring the solvent into a 1000

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cm^3 suction flask, of which the side arm was connected to the vacuum pump. The flask was sealed with parafilm to prevent air from the atmosphere. This flask was allowed to stand at the room temperature before use.

2.6 Investigation for the Optimum Wavelength

In order to obtain good and reliable results, it is necessary to investigate for the optimum wavelength of the detector used because the chromophore substances can absorb UV light at different wavelengths. This investigation was attempted using 50/50 (v/v) CH_3CN/H_2O as mobile phase. The test solutions were two carbaryl standard solutions with concentrations 0.2 μg/ml and 5 μg/ml. A 20 μl volume of these 2 standards was injected onto the HPLC column at the wavelengths between 200 nm and 300 nm at a constant flow rate of mobile phase (1.5 cm^3/min). The detector responses expressed in terms of peak areas are shown in Figs. 3.7 and 3.8 and tabulated in Tables 3.4 and 3.5. The highest UV absorption for this kind of pesticide was found at 220 nm while the second absorption was at 280 nm.

2.7 Determination of the Detector Linearity

Detector linearity is the linear range of the detector response which is directly proportional to the amount of a given sample. Determination of the UV detector linearity in this work was made by injecting an exact amount of the working standard solution onto the μ Bondapak C_{18} column, operated at a constant

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flow rate at 1.5 cm^3/min for 50/50 (v/v) CH_3CN/H_2O. These chromatographic runs were made for the purpose of quantitation. The response peak area was then measured and calculated automatically after the chromatograms had been obtained by the chromatography workstation. The results are shown in Table 3.6. The concentration of standard carbaryl plotted against the response peak area resulted in a linearity curve shown in Figure 3.9. It can be seen that the linearity cut-off of the detector for carbaryl was found to be at 120 µg/ml.

2.8 Determination of the Detection Limit

Detection limit is defined as the minimum concentration of the substance that will produce a signal two times of short-term noise of background signal [25]. In this work, the investigation was carried out by injecting 20 µl volume of each concentration of carbaryl standard starting from 0.008 µg/ml downwards onto the column employing 50/50 (v/v) CH_3CN/H_2O as mobile phase at the highest sensitivity of UV the detector setting in order to see the carbaryl concentration which would give the two times noise level. The results of these are shown in Table 3.7 and Fig. 3.10.

2.9 Determination of Column Efficiency

The 2 µg/ml and 5 µg/ml of carbaryl standard solutions were injected several times onto the HPLC column to obtain suitable chromatograms to be used in determination of column efficiency. After obtaining the retention time and peak width, column efficiency was determined in terms of the number of theoretical

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plates via the following equations [26,27].

$$N = 16(t_R/W)^2 \quad \text{---------- (2.1)}$$

or $$N = 5.54\,(t_R/W_{1/2})^2 \quad \text{---------- (2.2)}$$

where N = the plate number (N) for a particular column, which is a measure of column performance or column efficiency

W = base-line peak width

$W_{1/2}$ = peak width at half height.

Additionally, N is also related to the height equivalent to a theoretical plate, H , as shown below.

$$H = L/N \quad \text{---------- (2.3)}$$

where L = column length.

In brief, an efficient column should yield a high value of N and a small value of H. Results on this part are shown in Tables 3.8 and 3.9 and Fig. 3.11.

2.10 Operation of HPLC

The entire HPLC system was switched on and warmed up for at least 10 minutes. The helium tank pressure regulator was adjusted to a setting between 50 and 90 psi. The helium was allowed to flow at 100 ml/min for 15 minutes and the flow was reduced to 20 ml/min for 50 % aqueous and acetonitrile which was used as mobile phase in this work. The helium sparge was maintained at this flow during the HPLC run. The parameters used on the chromatography workstation Maxima

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820 included run time of 7.00 minutes and the data acquisition rate of 2.00 points/second while the preacquisition delay was 0.00. For the initial peak integration parameters, the base line point was 10 and the filter window was 13 (in points) while the coarse integration sensitivity and the fine integration sensitivity were 500.0 and 500.0, respectively. The minimum peak height, minimum peak area and the minimum peak width were 100, 100, 1.00 microvolt × second, respectively. These parameters were adjusted as necessary according to verification of the peaks in chromatograms. Details of the HPLC operating conditions for carbaryl determination in this study are given in Table 3.10.

2.11 Sampling Method

Pesticide residue data is meaningless unless the sample is taken, handled, and prepared properly. Taking samples is a very complex step and it should be performed by a trained person. The main objective is to obtain an exact miniature replica of the authentic product. To maintain this homogeneity, a certain protocol must be followed. In this work, on each day during the vegetable collection period, the vegetable samples were collected from the study sites, namely Ban Sop Pao in Lamphun Province and Ban Pa Sao in Chiang Mai Province, as shown in Figs. 2.2-2.4 in 10 spots randomly. The first 5 spots were collected along one of the diagonals of the square shaped area while the others were collected on the other diagonal. The vegetables within 6 cm. of diameter in each spot were cut as shown in Fig. 2.1 and mixed together in a total weight about 2 kg in each collection. After that, the vegetable samples were divided into 10 subsamples before analysis. It should be noted also that during sample collection in Ban Sop Pao, Lamphun

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Province, and in Ban Pa Sao, Chiang Mai Province, a thermometer was used to measure the air temperature. The temperatures obtained from the two sampling sites together with the time of collection are tabulated in Tables 3.11 and 3.12, respectively.

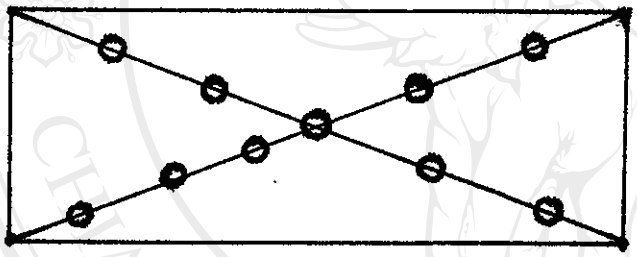

Figure 2.1 Diagram of the study sites showing points of sampling.

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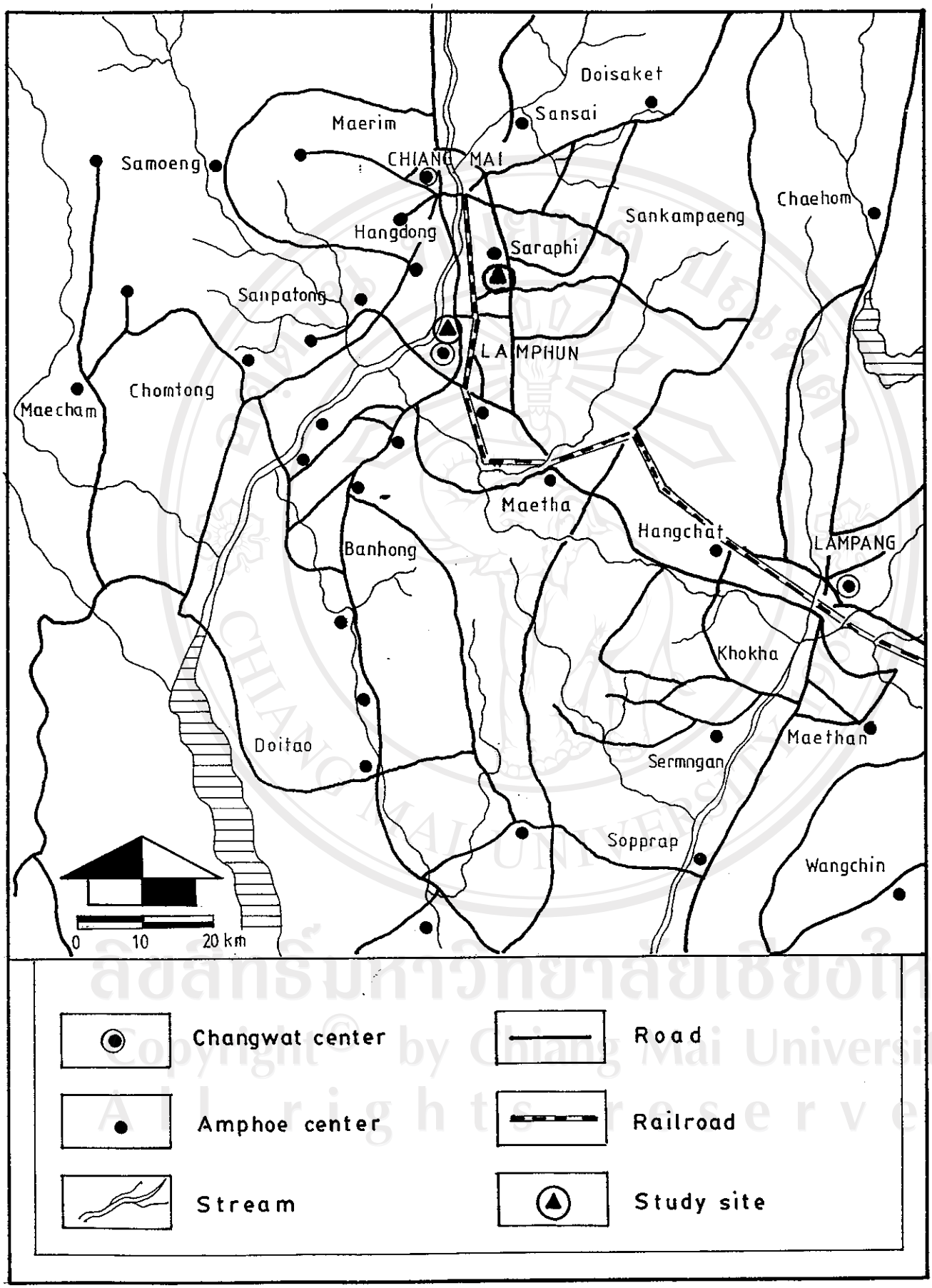

Fig.2.2 Map showing the Study Sites.

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Figure 2.3 First study site of kale in Ban Sop Pao, Lamphun Province.

Figure 2.4 Second study site of edible rape in Ban Pa Sao, Chiang Mai Province.

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2.12 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 value and the determined values as the error or relative error [28]. In this research project accuracy of results was investigated by means of percent recovery. The procedure was performed as follows.

The vegetable samples without contamination were spiked with a known volume of 40 µg/ml carbaryl standard concentration and then it was analysed in accordance with the method for sample preparation as shown in Fig. 2.5. The calculation for determination of the percent recovery could be made via the equation below.

$$\%\text{Recovery} = (C_S/A) \times 100 \quad \text{-------- (2.4)}$$

where C_S = the amount of carbaryl found in the last step for spiked sample

A = known amount of spiked carbaryl standard

The percent recovery range at concentration 1 µg/g in kale (Brassica olemacea L.var.alboglabra Bail) was found to be 90.0-94.7% and that for 5 µg/g was found to be 80.3-85.2%. The average percent recovery and coefficient of variation (C_V%) for 1 µg/g concentration were 93.3% and 1.7% and those for 5 µg/g concentration were 82.3% and 2.4%, respectively. The percent recovery range at concentration 1 µg/g in edible rape (Brassica chinensis L. var oleifera Tsen et Lee) was found to be 91.4-97.5% and that for 5 µg/g was found to be 83.2-89.2%.

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The average percent recovery and C_V% for 1 µg/g concentration were 93.8% and 1.9% and those for 5 µg/g concentration were 86.0% and 2.6% ,respectively. More results are shown in Tables 3.13 and 3.14.

2.13 Precision

The term precision is used to describe the reproducibility of results. It can be defined as the agreement between the numerical value of two or more measurements that have been made under an identical condition with the same sample [29]. The precision is commonly stated in terms of standard deviation (SD) or relative standard deviation (RSD) or C_V% which can be obtained from the following equations:

$$SD = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}} \qquad \text{--------(2.5)}$$

$$C_v\% = \frac{SD}{\bar{x}} \times 100\% \qquad \text{--------(2.6)}$$

where X is the arithmetic mean for a set of n results of $X_i = X_1, X_2, ..., X_n$. The replicate experiments were performed for determination of the precision as follows.

In order to find the reproducibility of the HPLC determination of carbaryl, the same amount of carbaryl standard was injected several times onto the HPLC column to observe reproducibility of the peak height and peak area from such

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chromatograms. The results are shown in Tables 3.15 and 3.16 and Fig.3.12.

2.14 Sample Preparation

The 40 g amount of freshly chopped vegetable was extracted with 100 ml of methanol before being sonicated in the " Branson B-2200" ultrasonic bath for 2 minutes [30]. Then, it was filtered through the glass filter funnel containing filter paper. 5.00 ml of the filtrate were combined with 245 ml of distilled water in an Erlenmeyer flask in order to dilute the amount of methanol into a ratio of 1:50. After that, the solution was sucked through the column cartridge containing octadecyl (C_{18}) sorbent which had already been conditioned with 10 ml of methanol and 10 ml of distilled water. The column cartridge was dried with gentle air stream later and 3.50 ml of 50% acetonitrile/ water (v/v) were used to elute carbaryl retained on the C_{18} sorbent before making an injection of 20 µl onto the HPLC column.

The results of these are shown in Tables 3.17 and 3.18 and Figs. 3.13 and 3.14 while the variation of carbaryl amount found in edible rape on the day directly after spraying is shown in Fig. 3.15.

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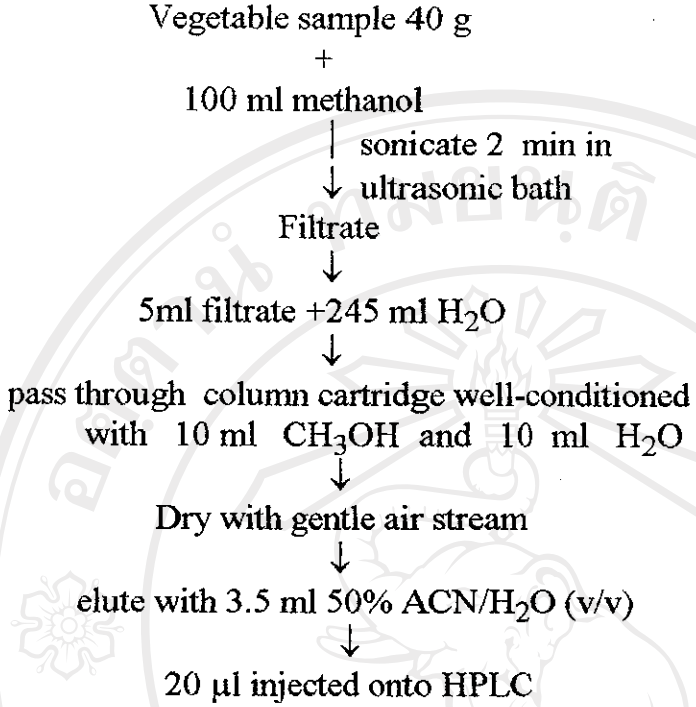

Figure 2.5 Sample preparation steps for carbaryl determination in this work.

2.15 Confirmation Method

Some of the carbaryl standards and samples were injected onto the HPLC column at 2 different wavelengths. In this work, the two wavelengths chosen were 220 nm and 270 nm. A chromatogram showing carbaryl peaks at 2 wavelengths is shown in Fig. 3.16. Each ratio value was obtained by dividing the peak area obtained at 270 nm by that obtained at 220 nm. The ratios obtained are tabulated in Table 3.19.

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