# CHAPTER 2

# EXPERIMENTAL

## 2.1 Apparatus and chemicals

### 2.1.1 Apparatus

1) Aluminium foil, Diamond (7.62 m x 45.7 cm), Reynolds Consumer Product Company, Richmond, U.S.A.
2) Analytical balance, AB304-S, Mettler Toledo, Switzerland
3) Analytical balance, PB1502-S, Mettler Toledo, Switzerland
4) Analytical column, Envirosep-CM (175 mm x 3.2 mm i.d., 5 µm), Phenomenex, U.S.A.
5) Capillary column, 007-5MS, 30 m x 0.32 mm I.D., 0.25 µm film thickness, QUADREX Corporation, Technology, U.S.A.
6) Cellulose filter membranes, 47 mm, 0.45 µm, Agilent, U.S.A.
7) Disposable syringe, 3 mL, NIPRO (Thailand) Corp., Ltd., Ayutthaya, Thailand
8) Gas chromatograph, HP 6890, G 1530A, Hewlett Packard, U.S.A.
9) Guard column, Envirosep-CM (30 mm x 3.2 mm i.d., 5 µm), Phenomenex, U.S.A.
10) Hypodermic needle, 23GX1" (0.6 x 25 mm) Thin Wall, NIPRO (Thailand) Corp., Ltd., Ayutthaya, Thailand

11) Liquid chromatography, Agilent 1100 Series LC system, Hewlett Packard, U.S.A.

12) Mass selective detector (MSD), orthogonal spray, MS-G 1946-90026, Hewlett Packard, U.S.A.

13) Oven, Model 400, Memmert, Germany

14) Paper thimble, Whatman, England

15) Parafilm®, 4 in. x 125 FT. Roll, Pechiney Plastic Packaging, Chicago, U.S.A.

16) Polypropylene screw caps, Blue, Open top, Agilent, U.S.A.

17) PTFE filter membranes, 47 mm, 0.45 µm, Agilent, U.S.A.

18) PTFE syringe filters, 13 mm, 0.45 µm, Filtrec, U.S.A.

19) Protector laboratory hood, Science Technology, Thailand

20) Red PTFE/white silicone Septa, Agilent, U.S.A.

21) Rotary evaporator, Buchi Rotavapor, R-124, Switzerland

22) SPE-24G glass manifold, J.T Baker, Philipsburg, U.S.A.

23) Ultrasonic bath, Cole-Parmer 8891, U.S.A. (150 W, 33 kHz)

24) VerticalPak™ $C_{18}$ tubes or octadecylsilica cartridges, 500 mg/10 mL, Vertical Chromatography Co., Ltd., Bangkok, Thailand

25) Wide opening screw top glass vials, Amber, write-on spot, 1.5 mL, Agilent, U.S.A.

### 2.1.2 Chemicals

**Table 2.1** List of chemicals used, their purity grade and suppliers.

| Chemical | Purity grade | Supplier |
|---|---|---|
| Acetone | A.R. | Merck, Germany |
| Acetonitrile | HPLC | BDH, England |
| Ammonium acetate | A.R. | Ajax Finechem, Auckland |
| Carbaryl (%assay 99.5 ) | A.R. | Dr. Ehrenstorfer, Germany |
| Dimethoate (%assay 99.0 ) | A.R. | Dr. Ehrenstorfer, Germany |
| Ethanol | A.R. | Lab Scan, Thailand |
| Ethyl acetate | A.R. | Lab Scan, Thailand |
| Fenvalerate (%assay 98.5 ) | A.R. | Dr. Ehrenstorfer, Germany |
| Methanol | HPLC | Merck, Germany |

## 2.2 Preparation of solutions

### 2.2.1 Preparation of stock standard solutions

Each of dimethoate, carbaryl and fenvalerate stock standard solution with concentration of 1000 mg $L^{-1}$ was prepared. 0.1010, 0.1005 and 0.1015 g of dimethoate, carbaryl and fenvalerate were transferred into a 100 mL of volumetric flask after weighing, respectively. Each standard was dissolved in methanol which was also adjusted to the 100 mL mark. The stock standard solutions were stored at 4 °C in a refrigerator.

### 2.2.2 Preparation of working standard solutions

#### 2.2.2.1 Preparation of working standard solution A

The standard solution of dimethoate, carbaryl and fenvalerate at concentrations of 10, 3 and 4 mg $L^{-1}$, respectively, was prepared by pipette 1.00. 0.30 and 0.40 mL, respectively, of stock standard solutions into 100 mL of volumetric flask and adjusted volume with methanol.

#### 2.2.2.2 Preparation of working standard solution B

The standard solution of dimethoate, carbaryl and fenvalerate at concentrations of 5.00, 1.50 and 2.00 mg $L^{-1}$, respectively, was prepared by pipette 0.50. 0.15 and 0.20 mL, respectively, of stock standard solutions into 100 mL of volumetric flask and adjusted volume with methanol.

**2.2.2.3 Preparation of working standard solution C**

The standard solution of dimethoate, carbaryl and fenvalerate at concentrations of 0.50, 0.15 and 0.20 mg $L^{-1}$, respectively, was prepared by pipette 5.00 mL of working standard solution A into 100 mL of volumetric flask and adjusted volume with methanol.

**2.2.3 Preparation of standard solution for sample fortification**

The standard solution of dimethoate, carbaryl and fenvalerate at concentrations of 50, 15 and 20 mg $L^{-1}$, respectively, was prepared by pipette 5.00, 1.50 and 2.00 mL of stock standard solutions of dimethoate, carbaryl and fenvalerate, respectively, into 100 mL of volumetric flask and adjusted volume with methanol.

**2.2.4 Preparation of standard solutions for calibration in HPLC**

The standard solutions of dimethoate, carbaryl and fenvalerate at concentrations of 0.13: 0.039: 0.052, 0.27: 0.081: 0.11, 0.50: 0.15: 0.20, 0.80: 0.24: 0.32, 1.00: 0.30: 0.40, 2.00: 0.60: 0.80, 3.00: 0.90: 1.20 and 4.00: 1.20: 1.60 mg $L^{-1}$ were prepared by pipette 0.26, 0.54, 1.00, 1.60, 2.00, 4.00, 6.00 and 8.00 mL, respectively, of working standard solution B into 10 mL of volumetric flasks and adjusted volume with methanol.

**2.2.5 Preparation of standard solutions for limit of detection in HPLC**

The standard solutions of dimethoate, carbaryl and fenvalerate at concentrations of 0.00050: 0.00015: 0.00020, 0.010: 0.0030: 0.0040, 0.017:

0.0051: 0.0068, 0.10: 0.030: 0.040 and 0.20: 0.060: 0.080 mg $L^{-1}$ were prepared by pipette 0.01, 0.20, 0.34, 2.00 and 4.00 mL, respectively, of working standard solution C into 10 mL of volumetric flasks and adjusted volume with methanol.

**2.2.6 Preparation of standard solution for precision in HPLC**

The standard solution of dimethoate, carbaryl and fenvalerate at concentrations of 6.00, 1.80 and 2.40 mg $L^{-1}$ was prepared by pipette 6.00 mL of working standard solution A into 10 mL of volumetric flask and adjusted volume with methanol.

**2.2.7 Preparation of standard solutions for calibration curve in LC/MS**

The standard solutions of dimethoate, carbaryl and fenvalerate at concentrations of 1.00: 0.30: 0.40, 2.00: 0.60: 0.80, 3.00: 0.90: 1.20, 4.00: 1.20: 1.60, 5.00: 1.50: 2.00, 6.00: 1.80: 2.40 and 7.00: 2.10: 2.80 mg $L^{-1}$ were prepared by pipette 1.00, 2.00, 3.00, 4.00, 5.00, 6.00 and 7.00 mL, respectively, of working standard solution A into 10 mL of volumetric flasks and adjusted volume with methanol.



**2.2.8 Preparation of standard solutions for limit of detection in LC/MS**

The standard solutions of dimethoate, carbaryl and fenvalerate at concentrations of 0.010: 0.0030: 0.0040, 0.10: 0.030: 0.040, 0.20: 0.060: 0.080 and 0.50: 0.15: 0.20 mg $L^{-1}$ were prepared by pipette 0.02, 0.20, 0.40 and 1.00 mL, respectively, of working standard solution B into 10 mL of volumetric flasks and adjusted volume with methanol.

**2.2.9 Preparation of standard solution for precision in LC/MS**

The standard solution of dimethoate, carbaryl and fenvalerate at concentrations of 6.00, 1.80 and 2.40 mg $L^{-1}$ was prepared by pipette 6.00 mL of working standard solution A into 10 mL of volumetric flask and adjusted volume with methanol.

**2.2.10 Preparation of spiked sample solution by extraction**

Ten grams of chopped orange peels were spiked with 1.00 mL of standard solution of dimethoate, carbaryl and fenvalerate at concentrations of 50, 15 and 20 mg $L^{-1}$, respectively. After equilibration for 1 hour at room temperature to allow adsorption of pesticides, the sample was extracted with 50 mL of ethyl acetate-acetone-ethanol (1:1:1, v/v) in ultrasonic bath for 15 minutes. The extract was filtered through a Whatman paper and collected in a 100 mL of round bottom flask. After that the extract was evaporated to near dryness on a vacuum rotary evaporator (40±1°C).

## 2.3 Chromatographic Conditions

### 2.3.1 Gas Chromatographic Conditions

The GC conditions are shown in Table 2.2. A sample was injected in the splitless mode and detected with flame phosphorus detector (FPD). Helium and nitrogen gases were used as the carrier gas and make-up gas, respectively.

**Table 2.2** The GC conditions.

| Parameter | Condition |
|---|---|
| Injection volume | 1.00 µL |
| Injector temperature | 250 °C |
| Detector temperature | 250 °C |
| Initial temperature | 230 °C |
| Temperature Rate | 5 °C $min^{-1}$ |
| Hold-up time | 4 min |
| Final temperature | 240 °C |
| Flow rate of carrier gas (He) | 1.0 mL $min^{-1}$ |
| Flow rate of make-up gas ($N_2$) | 60 mL $min^{-1}$ |
| Flow rate of oxidant ($O_2$) | 100 mL $min^{-1}$ |
| Analysis time | 4 min |
| Post time | 8 min |
| Run time | 12 min |

### 2.3.2 Liquid Chromatographic Conditions

The optimum HPLC conditions on the Envirosep-CM column are shown in Table 2.3. The optimization was carried out to separate peaks on the chromatogram with suitable resolution as well as possible. At the beginning of separation, the column was conditioned by flushing distilled water (30 min) followed by methanol (30 min), acetonitrile (30 min) and mobile phase at least 30 min to check constantly detector response before using.

**Table 2.3** The optimum HPLC conditions with the Envirosep-CM column.

| **Parameter** | **Condition** |
|---|---|
| Mobile phase | Solvent A: MeOH:$H_2O$ (1:9, v/v)<br>Solvent B: MeOH:$H_2O$ (9:1, v/v)<br>Both of them containing 5 mM, $CH_3COO\ NH_4$ |
| Injection volume | 20 µL |
| Flow rate | 0.30 mL $min^{-1}$ |
| Separation mode | Gradient: Time %A %B<br>0 80 20<br>5 80 20<br>20 60 40<br>40 20 80<br>60 0 100<br>80 0 100 |
| Wavelength detection | 220 nm |
| Analysis time | 80 min |
| Post time | 10 min |
| Run time | 90 min |

### 2.3.3 Liquid Chromatographic/Mass Spectrometry Conditions

The confirmation of each analyte was identified by LC/MS on the Envirosep-CM column. The optimum conditions are shown in Table 2.4.

**Table 2.4** The optimum LC/MS conditions with the Envirosep-CM column.

| Parameter | Optimum condition |
|---|---|
| Gas flow | 10 L $min^{-1}$ |
| Nebulizer pressure | 40 psi |
| Gas temperature | 300 °C |
| Capillary voltage | 3.5 kV |
| Fragmentor voltage | 50 V |

## 2.4. Investigation of extraction procedure for HPLC method

### 2.4.1 Investigation of extracting solvent as diluent for standard solution

1 mL of standard solution of dimethoate, carbaryl and fenvalerate at concentrations of 50, 15 and 20 mg $L^{-1}$, respectively, was pipetted into 10 mL of volumetric flask and adjusted to the 10 mL mark with ethyl acetate. Then 1 µL and 20 µL were injected in GC and HPLC system, respectively, under the optimum conditions (Table 2.2 and Table 2.3). To investigate organic solvent as diluent, the standard solution was prepared following above procedure but the organic solvent was changed one at a time from ethyl acetate to acetone, ethanol and a mixture of ethyl acetate-acetone-ethanol (1:1:1, v/v), respectively.

### 2.4.2 Investigation of extracting solvent using acetone as diluent for sample

To investigate the type of extracting solvent, the sample was extracted following the procedure in 2.2.10 but the type of extracting solvent was changed one at a time from ethyl acetate to acetone, ethanol, or a mixture of ethyl acetate, acetone and ethanol (1:1:1, v/v), respectively. The remaining residue was dissolved in 10 mL of acetone and then filtered through a disposable syringe. Finally 1 µL and 20 µL were injected in GC and HPLC system, respectively, under the optimum conditions. The purified and non-purified extracts from the same sample were processed in parallel. All procedures were carried out in triplicates.

### 2.4.3 Investigation of sonication time using acetone as diluent for sample

To investigate sonication time, the sample was extracted following the procedure in 2.2.10 but sonication time was changed one at a time from 0 (without sonication), 5, 10, 15 or 20 minutes, respectively. After extraction and evaporation, the remaining residue was dissolved in 10 mL of acetone and then filtered through a disposable syringe. Finally 1 µL and 20 µL were injected in GC and HPLC system, respectively, under the optimum conditions. The purified and non-purified extracts from the same sample were processed in parallel. All procedures were carried out in triplicates.

### 2.4.4 Investigation of solid phase extraction

#### 2.4.4.1 Investigation of eluting solvent for standard solution

1.00 mL of standard solution of dimethoate, carbaryl and fenvalerate at concentrations of 50, 15 and 20 mg $L^{-1}$, respectively, was pipetted into 10 mL of volumetric flask and diluted to 10 mL with acetone or deionized water. After that 2.00 mL of standard solution was passed through $C_{18}$-SPE cartridges which were previously conditioned with 3.00 mL of methanol following 3.00 mL of deionized water. The filtrate was collected immediately after passing the standard solution through the cartridge. The elution of analytes from the cartridge was carried out with 3.00 mL of MeOH-$H_2O$ (7:3, v/v) and the eluate was filtered through a disposable syringe before analysis. Finally 20 µL of both the un-adsorbed standard solution and the eluent were injected in the HPLC system under the optimum conditions. In the case of using acetone as diluent, another 1 µL of

each fraction was injected in the GC system. To investigate type of eluting solvent, the standard solution was extracted following above procedure but the eluting solvent was changed one at a time from a mixture of MeOH-$H_2O$ (7:3, v/v) to a mixture of ACN-$H_2O$ (7:3, v/v), a mixture of acetone-water (7:3, v/v) and pure ACN, respectively. The purified and non-purified extracts from the same sample were processed in parallel. All procedures were carried out in triplicates.

**2.4.4.2 Investigation of eluting solvent for sample**

The sample was extracted following the procedure in 2.2.10. The remaining residue was dissolved in 10 mL of acetone or deionized water. After that 2.00 mL of the extract solution was passed through $C_{18}$-SPE cartridge which was previously conditioned with 3.00 mL of methanol following 3.00 mL of deionized water. The filtrate was collected immediately after passing through the cartridge. The elution of analytes from the cartridge was carried out with 3.00 mL of MeOH-$H_2O$ (7:3, v/v) and the eluate was filtered through a disposable syringe before analysis. Finally 20 µL of both the un-adsorbed extract solution and the eluent were injected in the HPLC system under the optimum conditions. In the case of using acetone as diluent, another 1 µL of both the un-adsorbed extract solution and the eluent were injected in the GC system. To investigate type of eluting solvent, the sample was extracted following above procedure but the eluting solvent was changed one at a time from a mixture of MeOH-$H_2O$ (7:3, v/v) to a mixture of ACN-$H_2O$ (7:3, v/v), a mixture of acetone-$H_2O$ (7:3, v/v) and ACN, respectively. The purified and non-purified extracts

from the same sample were processed in parallel. All procedures were carried out in triplicates.

**2.5 Validation of HPLC method** [100-101]

**2.5.1 Calibration curve**

A 20 µL of standard solutions (Section 2.2.4) were injected in HPLC system under the optimum conditions (Table 2.2). All injection was carried out in triplicates. The linear range studied of standard solution was over the concentration ranges of 0.13 – 1.0 mg $L^{-1}$, 0.24 – 1.2 mg $L^{-1}$ and 0.2 – 1.2 mg $L^{-1}$, respectively, for dimethoate, carbaryl and fenvalerate. The calibration curve was constructed from peak area proportional to the concentration of standard solution and should be prepare cover the range of pesticide levels likely to be found in sample.

**2.5.2 Limit of detection**

A 20 µL of standard solutions (Section 2.2.5) were injected in HPLC system under the optimum conditions. All injection was carried out in triplicates. The detection limit was determined by consideration the lowest concentration of standard solution which could be detectable. Besides mentioned method, LOD was also determined by Miller-Miller method [102]. The results obtained were calculated from the equations (2.1) and (2.2).

$$LOD = 3x \frac{(S_y/x)}{b} \qquad (2.1)$$

$$S_y = \left\{ \frac{\sum(Y_1 - Y_2)}{n - 2} \right\}^{1/2} \qquad (2.2)$$

where

$Y_1$ = response value from instrument corresponding to individual x-values

$Y_2$ = value of y on calculated regression line corresponding to individual x-values

n = number of points on calibration curve

b = slope of calibration curve

**2.5.3 Accuracy**

The accuracy was presented in term of percentage of recovery. The percentage of recovery was verified by spiking the orange sample peels of standard solution of dimethoate, carbaryl and fenvalerate at concentrations of 50, 15 and 20 mg $L^{-1}$, respectively. After equilibration for 1 hour at room temperature to allow adsorption of pesticides, the sample was extracted with 50 mL, a mixture of ethyl acetate-acetone-ethanol (1:1:1, v/v) in ultrasonic bath for 15 minutes. The extract was filtered through a Whatman paper with filtering glass and collected in a 100 mL of round bottom flask. After that the extract was evaporated to near dryness on a vacuum rotary evaporator (40±1°C). The remaining residue was dissolved in 10 mL of deionized water. Then 2.00 mL of the extract was passed through $C_{18}$-SPE cartridges which were previously conditioned with 3.00 mL of

methanol following 3.00 mL of deionized water. The filtrate was collected immediately after passing the extractant through the cartridge (namely, unadsorbed solution) for combination with other fraction. After that the elution of carbaryl was carried out with 1.00 mL of acetone-$H_2O$ (7:3, v/v) and the elution of fenvalerate was carried out with 1.00 mL of ACN. Therefore a ratio of unadsorbed solution: a mixture of acetone-$H_2O$ (7:3, v/v): ACN or a ratio of eluting solvent was 2:1:1 (mL) leading the total volume obtained was approx. 4.00 mL. Finally 20 µL of the combined solution was injected in HPLC system under the optimum conditions. The purified and non-purified extracts came from the same sample and both were processed in parallel. All procedures were carried out in triplicates. The concentrations of dimethoate, carbaryl and fenvalerate were determined from linear regression equation obtained from the calibration curve and the percentage of recovery was calculated from the equation (2.3).

$$\%\text{Recovery} = \frac{\text{Spiked sample response} - \text{Unspiked sample response}}{\text{Standard added response}} \times 100 \qquad (2.3)$$

### 2.5.4 Precision

The precision was expressed as repeatability and reproducibility. The repeatability (intra-day) was determined by injection of 20 µL of standard solutions (Section 2.2.6) in HPLC system under the optimum conditions for eight times (n = 8) in the same day. For reproducibility (inter-day) was determined by injection of 20 µL of the same standard solution into HPLC under the optimum conditions for eight times with six successive days (n = 8, 6 days). All procedures were carried out in triplicates. The precision is usually reported as a percent of relative standard deviation (%R.S.D.), which can be calculated from the equation (2.4).

$$\%\,\text{R.S.D.} = \left(\frac{\text{SD}}{\overline{\text{X}}}\right) \times 100 \qquad (2.4)$$

where % R.S.D. = percent of relative standard deviation

SD = standard deviation

$\overline{\text{X}}$ = mean measured value

SD can be calculated from the equation (2.5).

$$\text{SD} = \left[\sum_{i=1}^{n} \frac{(\text{X}_i - \overline{\text{X}})}{n-1}\right]^{1/2} \qquad (2.5)$$

where $\text{X}_i$ = individual measured value

n = number of measurements

## 2.6 Investigation of extraction procedure for LC/MS method

### 2.6.1 Investigation of extracting solvent for sample

Ten grams of chopped orange peels were spiked with 1.00 mL of standard solution of dimethoate, carbaryl and fenvalerate at concentration level of 50, 15 and 20 mg $L^{-1}$, respectively. After equilibration for 1 hour at room temperature to allow adsorption of pesticides, the sample was extracted with 50 mL of ethyl acetate in ultrasonic bath for 15 minutes. The extract was filtered through a Whatman paper with filtering glass and collected in a 100 mL of round bottom flask. After that the extract was evaporated to near dryness on a vacuum rotary evaporator (40±1°C). The remaining residue was dissolved in 10 mL of deionized water. The extract was then filtered through a disposable syringe and finally 20 µL was injected in LC/MS system under the optimum conditions (Table 2.4). To investigate extracting solvent, the sample was extracted following above procedure but the extracting solvent was changed one at a time from ethyl acetate to acetone, ethanol and a mixture of ethyl acetate-acetone-ethanol (1:1:1, v/v), respectively. The purified and non-purified extracts from the same sample and both were processed in parallel. All procedures were carried out in triplicates.

### 2.6.2 Investigation of sonication time for sample

To investigate sonication time, the sample was extracted and injected in LC/MS system following the procedure in 2.6.1 using a mixture of ethyl acetate-acetone-ethanol (1:1:1, v/v) but the sonication time was changed one at a time from 0, 5, 10, 15 and 20 minutes, respectively. The purified and non-purified extracts from the same sample and both were processed in parallel. All procedures were carried out in triplicates.

## 2.7 Validation of LC/MS method

### 2.7.1 Calibration curve

A 20 µL of standard solutions (Section 2.2.7) were injected in LC/MS system under the optimum conditions (Table 2.4). All injection was carried out in triplicates. The linear ranges studied of standard solutions were over the concentration ranges of 1.0 – 2.0 mg $L^{-1}$, 0.30 – 1.5 mg $L^{-1}$ and 1.2 – 2.8 mg $L^{-1}$ for dimethoate, carbaryl and fenvalerate, respectively. The calibration curve was constructed from peak area proportional to the concentration of standard solution and should be prepared cover the range of pesticide levels likely to be found in sample.



### 2.7.2 Limit of detection

A 20 µL of standard solutions (Section 2.2.8) were injected in LC/MS system under the optimum conditions. All injection was carried out in triplicates. The limit of detection (LOD) was determined by consideration the concentration

of standard solution which gave a signal-to-noise of 3. Besides mentioned method, LOD was also determined by Miller-Miller method.

### 2.7.3 Accuracy

The accuracy was presented in term of percentage of recovery. The percentage of recovery is verified by spiking the orange sample peels of standard solution of dimethoate, carbaryl and fenvalerate at concentration level of 50, 15 and 20 mg $L^{-1}$, respectively. The sample was extracted and injected in LC/MS system following the procedure in 2.6.1 using a mixture of ethyl acetate-acetone-ethanol (1:1:1, v/v). The purified and non-purified extracts from the same sample and both were processed in parallel. All procedures were carried out in triplicates. The concentrations of dimethoate, carbaryl and fenvalerate were determined from linear regression equation obtained from the calibration curve and the percentage of recovery was calculated from the equation (2.3) as mentioned above.

### 2.7.4 Precision

The precision was expressed as repeatability and reproducibility. The repeatability (intra-day) was determined by injection of 20 µL of standard solutions (Section 2.2.9) in LC/MS system under the optimum conditions for eight times (n = 8) in the same day. For reproducibility (inter-day) was determined by injection of 20 µL of the same standard solution in LC/MS system under the optimum conditions for eight times with six successive days (n = 8, 2 days). All procedures were carried out in triplicates. The precision is usually

reported as a percent of relative standard deviation (%R.S.D.), which can be calculated from the equations (2.4) and (2.5) as mentioned above.