CHAPTER 3

MATERIALS AND METHODS

3.1 The suitable GC-PFPD conditions for analysis of organophosphorus insecticide

3.1.1 Instruments used in this study:

1) Gas chromatograph 3600 cx GC system (Varian, U.S.A.) consists of ;
   a) Pulsed flame photometric detector (PFPD)
   b) 8200 cx AutoSampler
   c) Data processing system, Star chromatography workstation version 5.0
   d) Capillary columns, DB–1, 30m x 0.32 mm I.D.,1μm film thicknes (Alltech, U.S.A.)
2) Gas chromatograph (Agilent Technologies, 6890 N. Networt) consistingof ;
   a) Flame photometric detector (FPD)
   b) AutoSampler Agilent Technologies, 7683 series injector
   c) Capillary columns with HP – 1701, 30 m x 0.32 mm I.D., 1.5 μm film thickness
3) Solid phase microextraction fiber holder and Auto sampling (Supelco, U.S.A.)
4) Solid phase microextraction fiber (Supelco, U.S.A.) for AutoSampler, in the sizes listed below:

- 100 μm Polydimethylsiloxane (PDMS)
- 7 μm Polydimethylsiloxane (PDMS)
- 85 μm Polyacrylate
- 65 μm Polydimethylsiloxane/Divinylbenzene (PDMS/DVB)
- 75 μm Carboxen/Polydimethylsiloxane (CAR/PDMS)
- 65 μm Carbowax ®/ Divinylbenzene (CW/DVB)

5) Analytical balance:
   a) Satorious Basic BP 210s, manufactured by Sartorious AG, Germany
   b) Mettler AB 204
6) Blender, Thomas Scientific, U.S.A.
7) Water distilling apparatus
8) Ultrapure water apparatus with Milli – Q system (Millipore, Beldford, MA, U.S.A.)
9) Clear vials at12 x 32 mm, 2.0 ml with screw top and septa inserted cap
10) Autopipette (Brand Tech Scientific Inc., U.S.A.)
11) Septa (PTFE faced silicone) 11 mm, Varian, U.S.A.
12) Homogenizer (T25, KIKA Labortechnik)
13) Rotary Evaporator (EYELA SB 650, Tokyo Rikakikai)
14) Micro pipette (P1000, Gilson)
15) Centrifuge (Cole- Parmer, U.S.A.)
16) Scanning electron microscopy (SEM) (Leica Stereoscan 440)
17) Steromicroscope (Olypus, Japane)

3.1.2 Chemicals used in this study:

1) Prothiophos (Rm, Riedel – deHaen,Germany)
2) Methylparathion (Rm, Riedel – deHaen,Germany)
3) Chlorpyriphos (Rm, Riedel – deHaen,Germany)
4) Methamidophos (Rm, Riedel – deHaen,Germany)
5) Mevinphos (Rm, Riedel – deHaen,Germany)
6) Profenofos (Rm, Riedel – deHaen, Germany)
7) Dicrotophos (Rm, Riedel – deHaen, Germany)
8) Acetone (PR gradeJ.J. Baker)
9) Sodium chloride (AR grade Merck, Germany)
10) Ultrapure water (Milli-Q system Millipore, Beldford, MA, U.S.A.)
11) Helium gas, 99.99% (HP grade TIG, Thailand)
12) Nitrogen gas, 99.99% (UHP grade, TIG, Thailand
13) Air, Air-Zero grade (TIG, Thailand)
14) Hydrogen, 99.99%(HP grade TIG, Thailand)
15) Sodium sulphate (PR grade, J.J. Baker)
16) Dichloromethane (AP grade, J.J. Baker
17) Octadecyltrichlorosilian (Merck, Germany)
18) Silica gel 60 (Merck, Germany)

3.1.3 Preparation of standard solutions

3.1.3.1 Preparating stock of Organophosphorus pesticides solutions (OPP)

The stock solutions of each organophosphorus pedticides i.e. prothiofos, methylparathion, chlorpyriphos, mevinphos, profenofos,dicrotophos and methamidophos were prepared by dissolving 10.00 mg of each insecticide in 10 ml of acetone in a 10 ml volumetric flask, this made the stock solution have 1000.00 mg/l concentration. The stock solution of each OPP was diluted to 50.00 mg/l with ultrapure water and then diluted to a series of dilutions to 0.005 mg/l, 0.01 mg/l, 0.10 mg/l, 0.20 mg/l , 0.30 mg/l , 0.40 mg/l ,0.50 mg/l and 1.00 mg/l with ultrapure water as shown in Table 3.1.

Table 3.1 Composition of the standard solutions

Concentration of OPP (mg/l)	Volume of 50 mg/l OPP solution
0.005	1 μl
0.01	2 μl
0.10	20 μl
0.20	40 μl
0.30	60 μl
0.40	80 μl
0.50	100 μl
0.60	120 μl

3.1.3.2 Preparation of primary mixed OPP standard solutions

The primary mixed OPP standard solutions containing 50 mg/l prothiofos, methylparathion, chlorpyriphos, mevinphos, profenofos and methamidophos in

ultrapure water were prepared by pipeting 0.5 ml of the stock standard solution of each insecticide of ultrapure water in to a 10 ml volumetric flask.

3.1.3.3 Preparation of mixed OPP working standard solutions

The OPP mixed working standard solutions containing 0.005 mg/l- 1 mg/l were prepared from the primary mixed OPP standard solutions of 50 mg/l by dilution with ultrapure water. The content of acetone in the aqueous solutions was always less than 1% in volume. Aqueous solutions were prepared daily.

3.1.3.4 Optimization of experimental parameters for GC

All the experimental parameters were optimized as follows :

The two- multiramp temperature programming and splittess injection mode were performed for investigatation on DB – 1 column (polydimethylsiloxane). The temperature of injector port was 250°C while the temperature of PFPD detector was maintained 300°C . On microliter (1µl) of 0.1 mg/l of mixed OPP standard solution was injected into the GC column. The optimized conditions of GC for these experiments were studied, using parameter shown in Table 3.2 which obtained from primary testing of GC.

Table 3.2 Parameter from experiments on the GC optimization of GC conditions using DB-1 column

Factor	Experiment No.				
	1	2	3	4	5
Initial column temperature (°C)	60	70	80	90	80
Initial holding time (min)	0.5	1	2	0.5	0.5
Heating rate at the first ramp (°C/ min)	20	20	20	15	20
Heating rate at the second ramp (°C /min)	10	20	20	20	20
Mid -point column temperature(°C)	250	200	200	200	250
Last -point column temperature(°C)	300	300	300	300	300

3.2 Coating SPME fibers with octadecyltrichlorosilane

3.2.1 Preliminary test

A study of technique on coating fiber with silica gel

The silica gel of 60 G for preparing TLC was used for coating. The gel was mixed octadecyltrichlorosilane(ODS) in water at concentration of 99% (1% water). The mixture was left at room temperature for 10 mins and then rinsed with water. The coated silica gel left air dried overnight and kept in the oven at 250 ºC for 2 hrs before use.

A test on absorption and desorption of the coated silica gel

One gram of the coated silica gel was added in a mixed OPP standard standard solution which has 1 mg/l of dicrotophos, prothiofos, methylparathion, chlorpyriphos, mevinphos, profenofos and methamidophos and left at room temperature for 10 mins. The coated silica gel with insecticides was transferred to the screw top vial then it was heated on the hotplate at 250 ºC for 5 mins before using a syringe to take 2 µl of the gas in the vial. The gas contained insecticides was then injected to the GC at injection port. After the separated peaks appeared on the chromatogram it was considered to be suitable for using the coated fiber for the next experiments.

3.2.2 Preparation of octadecylsiloxane coated fiber

. The fibers used for preparation was the 100 µ m in thickness fiber coated with polydimethylsiloxane. The fibers had been treated with 98% sulfuric acid then washed thoroughly with methanol and the bare fused-silica fiber was left dried (40:123) overnight. At room temperature, at this time the fibers were free of polydimethylsiloxane and ready to be coated with ODS. The bare fused-silica fiber, obtained by chemical polymerization method, were dipped into the ODS in water at concentration of 99% (1% water) took them out and left for 1 min then dipped them in ultrapure water once again, took them out and left air dried overnight. This process was repeated 3 times to increase the flim thickness of the fiber. The ODS fibers were conditionin the GC injection port at 250 ºC for 60 mins.

3.3 The suitable conditions of SPME/GC PFPD for analysis of organophosphorus insecticides

3.3.1 Trying condition of SPME

A AutoSampler SPME fiber was used to perform the experiments with fused silica fiber of 10 mm in length, 100 μm in diameter coated with different kinds of polymer e.g. polydimethylsiloxane (100 μm), polyaerylate (85 μm), polydimethylsiloxane/divinylbenxene (65 μm), carboxen ™/polydimethylsiloxane and carbowax®/divinylbenzene (65 μm) to compare with fiber coated with ODS developed in this study. All SPME with different coated fused silica fiber were used to extract the pesticide residues from the blended vegetable spiked with standard of OPP. The conditions of each commercial coated fiber were tried, using the recommended conditions listed in Table 3.3 and the condition of ODS coated fiber mentioned in 3.2.2 was 250°C for 1 hr.

Table 3.3 Recommendations for conditioning fused silica fiber of different kinds

Stationary Phase	Conditioning temperature	Time (hrs.)
100 μm Polydimethylsiloxane	250 ºC	1
30 μm Polydimethylsiloxane	250 ºC	1
7 μm Bonder Polydimethylsiloxane	320 ºC	2-4
85 μm Polyacrylate	300 ºC	2
65 μm Carbowax / divinylbenzene	250 ºC	0.5
65 μm PDMS / divinylbenzene	260 ºC	0.5

3.3.2 Selection of SPME coating

Each compound has different polarity so the " like dissolves like ″ or the compounds with similar polarity can absorp each other. Fiber coating should be selected as the simple rule holds for liquid polymer. SPME consists of two separate stages, absorption (retention of analytes on the stationary phase) and desorption. Development of a particular procedure for determination of pesticides using the SPME technique usually requires the optimization of the variables related to both extraction and desorption steps.

3.3.3 Selection of the sampling mode

Three sampling modes of SPME were compared; direct immersion, headspace and agitation. The most suitable sampling mode was used in the next experiments.

3.3.4 Determination of a absorption time

A time profile of some organophosphorus absorption into the ODS and PDMS fiber was determined in order to assess the optimum SPME sampling period. OPP spiked in vegetable sample and samples of 1 ml of the 1 mg/l working standard solutions of prothiophos, methylparathion, chlorpyriphos, mevinphos, methamidophos, profenofos and dicrotophos were analyzed by SPME using PFPD detection with sampling periods of 1, 2, 3, 4, 5, 10, 15, 20, 25, 30 and 60 mins. Desorption time was set at 250 ºC for 5 mins.

3.3.5 Determination of desorption temperature

The optimum desorption temperature should be determined for finding a minimum of time for desorption of the analyte. The maximum desorption temperature is limited by the stability of the polymeric coating (Table 3.3)

3.3.6 Determination of desorption time

Desorption time profiles of prothiophos, methylparathion, chlorpyriphos , mevinphos, methamidophos, profenofos and dicrotophos were established to prevent carry over of the analyte from one analysis to the next ones. OPP spiked in vegetable sample and samples of 1 ml of the 1 mg/l OPP working standard were analyzed by ODS and PDMS using PFPD detection with sampling periods of 30 mins. Desorption times at 250 ºC for 1, 2, 3, 4, 5, 10, 15, 20 mins were investigated. Subsequent to each sample injection, a blank injection of the fiber was made in order to determine if any carry-over would occur.

3.3.7 Determination of DOS fiber capacity on absorption of the OPP at different concentration

ODS fiber was dipped in the OPP mixed standard solution at concentrations of 0.1, 0.2, 0.5 and 1.0 mg/l. The test was on its absorption capacity by finding out the concentration that could give high peak area in the chromatograms.

3.3.8 Analysis of organophosphorus insecticides spiked in vegetable, using ODS SPME /GC-PFPD analytical method.

Analysis of OPPs started with preparation of vegetable by using 1 kg of vegetable, cut in halves and 500g of the sample were cut into small pices and then blended to fine particles. One gram of the blended vegetable was put in a 500 ml Erlenmeyer flask, 200ml of ultrapure water was poured into the flask. The mixture was shaken for 2 hrs then centrifuged for 2 mins. The aqueous extract solution was collected and 1 ml of the extract was pipetted into a 2 mls vial and capped with silicone septum. The extract was analyzed for OPPs using SPME/GC-PFPD. The scheme of OPPs analysis is shown in Fig. 3.1

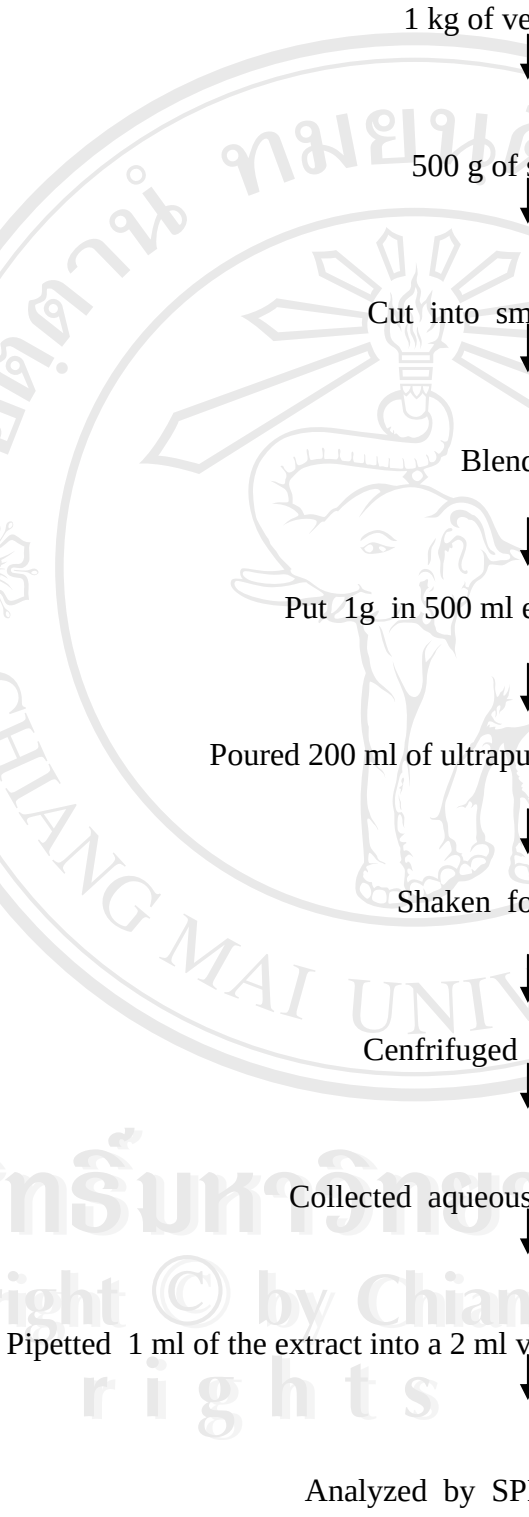

Figure 3.1 Scheme for analysis of OPPs by SPME /GC-PFPD

3.3.9 Identification of prothiophos, methyl parathion, chlorpyriphos, mevinphos, methamidophos, profenofos and dicrotophos residues in vegetable sample

Chromatograms obtained by SPME/GC-PFPD were used for identification of OPPs in vegetable. The identification was made by comparison of retention time of each insecticide residue with retention time of the working standard of the same insecticide.

3.4 Validation of the analytical method using SPME/GC-PFPD.

After having the optimum condition for analysis of prothiophos, methylparathion, chlorpyriphos , mevinphos, methamidophos, profenofos and dicrotophos, using SPME/GC-PFPD (Table 4.1 and Table 4.15), the same condition was repeated 6 times. Data were calculated and statistically compared. Validation of the method consists of determination of linear range, detection limit, precision, recovery and accuracy as described below:

3.4.1 Determination of linear range or linearity

After injection of each working insecticide standard into the GC. The peak area appeared, it was graph plotted with concentrations of the insecticide standards (10-50 μg/l, 0-1000 μg/l, 0-0.40 mg/l). The graph that has R^2 close to 1 was chosen to be linearity.

3.4.2 Detection limites

Each working insecticide standard of the OPP was diluted and injected into PDMS/GC-PFPD and ODS/GC-PFPD. Dilutions were made in a series until no peak appeared. Detection limits (LOD) were determined by comparing the signal-to - noise (S/N) ratio of a minimum 3:1 to the lowest detectable concentration.

3.4.3 Precision

Standard solutions containing 5.00 μg/l, 0.01 mg/l and 1.00 mg/l of prothiophos, methylparathion, chlorpyriphos, mevinphos, methamidophos, profenofos and dicrotophos were added to sample solution. The solutions were analyzed (six replicates) by PDMS/GC-PFPD and ODS/GC-PFPD using the same procedure and conditions. Results were then calculated to find percentage of relative standard deviation (%RSD).

Fifty grams of the sample was added with 0.7 ml of the 10 mg/l each of standard solutions of prothiophos, methylparathion, chlorpyriphos, mevinphos, methamidophos and profenofos. The samples mixed with OPP solution were analyzed (six replicates) by extraction method described by Steinwandter (52) and GC-FPD used by the chemist at Royal Project Foundation (personal contact) under the same procedure and the same condition. Data were then calculated to find percentage of relative standard deviation (%RSD).

3.4.4 Recoveries

Standard solutions containing 5.00 μg/l, 10.00 μg/l, 0.01 mg/l 0. 10 mg/l, 0.20 mg/l, 0.30 mg/l, 0.40 mg/l and 0.50 mg/l of prothiophos, methylparathion, chlorpyriphos, mevinphos, methamidophos, profenofos and dicrotophos were added to 1 g of the sample concentration for each sample and then followed the process of extraction as described in Fig. 3.1. Results were calculated based on the external calibration curve and the peak area.

3.4.5 Analysis pesticide residues from vegetables bought from the market

The optimum conditions for analysis of pesticide residues using ODS SPME/GC-PFPD developed in this study were used for analysis of OPPs from 11 vegetables i. e. sweet pepper, yard long beans (green type), Chinese kale, cauliflower, garden pea (young pod), cucumber, carrot, spring cucumber (young leaves), broccoli, tomato and cabbage.

3.4.6 Method to compare ODS, PDMS GC-PFPD and GC-FPD

Comparing percentage of recovery and percentage of standard deviation (%RSD) on analysis of OPPs using ODS , PDMS SPME/GC-PFPD with GC-FPD the method used at Royal Project's laboratory. Results obtained from the comparison were calculated using t-test as indicated below:

$$t_{calculated} = \bar{d}\,[n]^{0.5} \div s_d \qquad \text{------- 1}$$

$s_d = [\sum (d_i - \bar{d})^2 \div (n-1)]^{0.5}$ -------2

$\bar{d}$ = the average difference between method A and B

n = the number of pair of data

$t_{calculated}$ from Eq. 1 is compare with t_{table} for n - 1 degree of freedom. If $t_{calculated} > t_{table}$ at the 95% confidence level, the two results are considered to be different (56).