CHAPTER III

EXPERIMENTAL

3.1 Chemical Reagents

3.1.1 Solvent

Hexane, methylene chloride, acetonitrile and acetone that were ultra-residues of analytical grade, were purchased from J. T. Baker Chemical Company (Deverter, Holland). Ethyl ether that was analytical grade was purchased from Merck (Darmstadt, Germany).

3.1.2 Other chemicals

Extra pure silica gel (60 – 230 mesh), anhydrous sodium sulfate and florisil (60 – 100 mesh), which were analytical grade were obtained from J. T. Baker Chemical Company (Deverter, Holland). Silica gel was washed with hexane. Sodium chloride (AR Grade) was obtained from Merck (Darmstadt, Germany).

3.1.3 Standard chemicals

Analytical-grade alpha-hexachlorocyclohexane (HCH) (purity, 97.8%), beta-hexachlorocyclohexane (HCH) (purity, 98.0%), gamma-hexachlorocyclohexane (HCH) (purity, 99.5%), O,P′-DDT(purity, 97.0%), P,P′-DDT, O,P′-DDE, P,P′-DDE (purity, 99.0%), O,P′-DDD, P,P′-DDD, alpha-endosulfan (purity, 97.0%) , heptachlor epoxide isomer A (purity, 96.0%), heptachlor epoxide isomer B (purity, 98.8%), carbophenothion (purity, 92.0%) , alachlor (purity, 99%) , aldrin, dieldrin, and endrin were obtained from Dr. Ehrenstorfer (Augsburg, Germany).

3.2 Instruments and Equipments

3.2.1 GC 6890N was equipped with micro-Electron Capture Detector (μ-ECD) from Agilent Technologies

3.2.2 7693 Autosampler was from Agilent Technologies, USA

3.2.3 HP-5 (5% phenyl methyl siloxane, 30 m. x 320 mm, 0.25 μm film thickness)

3.2.4 Amber vial 2 mL was from Agilent Technologies, USA

3.2.5 Micro syringe 10.00 μL, Hamilton Company, Switzerland

3.2.6 Centrifuge, CENTAURA 2, Sanyo

3.2.7 Isotemp® vacuum oven. Model 280 A, Fisher Scientific

3.2.8 Ultrasonic cleaner (CREST model 575 D), Scientific Promotion Ltd.

3.2.8 Vortex mixer, Scientific Industries

3.2.9 6 mL Glass Reaction Tube with 1 Teflon Frit, 504394, Supelco

3.2.10 Sep-Pak® Vac NH_2 6 cc, W34199B2, Waters

3.2.11 Sep-Pak® cartridges Vac C18 6cc, W3170B1, Waters

3.2.12 Syringe adaptor for 1.5 mL, 4.0 mL and 8.0 mL SPE, 181794, Alltech

3.2.13 Glass syringe 10 mL

3.2.14 Supelclean ENVI-CARB 6 mL tubes SP1018B, Supelco

3.2.15 Carbograph 300 mg 6.0 mL, 210101, Alltech

3.2.16 Silica Gel Desiccators Cabinet Model AD 48 (KATT®)

3.2.17 Nitrogen gas 99.99% purity, TIG, Thailand

3.2.18 Volumetric pipettes 5 and 10 mL

3.2.19 Round bottom flasks 50 mL

3.2.20 Amber glass container with Teflon screw cap 50 mL

3.2.21 Erlenmeyer flasks 50, 100 and 250 mL

3.2.22 Separatory funnels 250 mL

3.2.23 Beakers 50 mL, 100 mL and 500 mL

3.2.17 Spatula

3.2.25 Dropper

3.2.26 Stirrer rod

All glasses were washed with detergent, dried in an oven and rinsed with dichloromethane before use.

3.3 Preparation of the Standard Solutions

3.3.1 The Single Standard Stock Solutions in Hexane

Each standard solution containing 1000 μg/mL in hexane was prepared by weighing 50 mg of each single standard in an amber glass container with a Teflon screw cap to 50 mL.

3.3.2 The Mixture of 17 Standard Organochlorine Pesticide Stock Solutions in hexane

The mixture of 17 standard organochlorine pesticide stock solutions containing 10 μg/mL was prepared by pipetting 0.5 mL of each single standard solution containing 1000 μg/mL into a 50 mL amber-glass container with a Teflon screw cap and the volume was adjusted to 50 mL with hexane.

3.3.3 The Mixture of 17 Standard Organochlorine Pesticides Solutions for Calibration Curve

The mixture of 17 standard organochlorine pesticide solutions of 1, 3, 5, 10, 20, 30, 50, 100, 300 and 500 ng/mL was prepared by pipetting standard mixture solutions 1mg/L and diluting them to 1.0 mL with hexane in 2.0 mL amber vial. The volume of standard mixture solutions had to be put into pipettes of each concentration as shown in Table 3.1.

Table 3.1 The measuring volume of mixed 17 standard organochlorine pesticide solutions, hexane and final concentration

Concentration of mixed 17 standard organochlorine pesticide solutions (ng/mL)	The volume of standard mixture solution (μL)	The volume of hexane (μL)
1	1.00	999
3	3.00	997
5	5.00	995
10	10.0	990
20	20.0	980
30	30.0	970
50	50.0	950
100	100	900
300	300	700
500	500	500

3.4 GC Optimisation

In this research, the analysis was performed by GC chromatographic with a PAS-1701 column at Agilent Technologies (Thailand) Ltd. and one GC chromatographic with a HP-5 column at Department of Biology (Chulalongkorn University). At the first time, we used column PAS – 1701 at Agilent Technologies (Thailand) Ltd. The GC conditions were shown in Table 4.2. It was inconvenient to extract and run with GC. So, when the Department of Biology, Chulalongkorn University, allowed the experiment to be conducted on the GC μECD at the department, all the subsequent runs were performed with column HP-5 at the department. The GC conditions were shown in Table 4.1.

To optimise GC conditions for column PAS-1701, the mixed 17 standard organochlorine pesticides solutions 10 ng/mL was used for investigation on injection

To optimise GC conditions for column PAS-1701, the mixed 17 standard organochlorine pesticides solutions 10 ng/mL was used for investigation on injection mode, flow rate and temperature of inlet, oven and detector that was within the suitable GC conditions for separation of each compound. The selectivity and resolution on separation of mixed 17 standard organochlorine pesticide solutions were studied as described in Table 4.4. The chromatogram of mixed 17 standard organochlorine pesticides in hexane 10 ng/mL under the GC conditions in Table 4.2 is shown in Figure 4.2.

For column HP -5, the mixed 17 standard organochlorine pesticide solutions 10 ng/mL was used for investigation on injection mode, flow rate and temperature of inlet, oven and detector that was within the suitable GC conditions for separation of each compound. The selectivity and resolution on separation of mixed 17 standard organochlorine pesticide solutions were studied as described in Table 4.3. The chromatogram of mixed 17 standard organochlorine pesticides in hexane 10 ng/mL under the GC conditions in Table 4.1 is shown in Figure 4.1.

3.5 The Study of Selectivity of GC

The selectivity of two columns determined by retention time of each peak under the suitable GC conditions in Section 3.4. Resolution could be determined by observing the baseline separation that was the best separation and would give resolution more than 1.5 as described in Table 4.3 (HP-5) and Table 4.4 (PAS-1701).

$$\text{Resolution} = \frac{t_{R_2} - t_{R_1}}{W_{h_{1/2_1}} + W_{h_{1/2_2}}}$$

3.6 The Study of Calibration Curve

Each concentration of mixed 17 standard organochlorine pesticide solutions in Table 3.1 was run 10 points and each point run in duplicate. The peak heights of a function of concentration were plotted. Each point was the average of duplicate runs. Summary

of Value of Slope, Retention Time, Intercept and Correlation Coefficient (R^2) of each pesticide in mixed 17 standard organochlorine pesticides by the condition in Table 4.1, was shown in Table 4.5 and calibration curve was shown in APPENDIX.

3.7 The Study of Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The Limit of Detection (LOD) and the Limit of Quantitation (LOQ) were determined by injection of matrix standard solution. The matrix standard solution was prepared by extraction sample according to the developed method in Section 3.10.4 (sample blank solutions) and the concentration of matrix standard solutions were prepared using the sample blank solutions as a dilutent the solution instead of hexane. The matrix standard concentration of 1 ng/mL and 10 ng/mL were injected into the GC system under optimum conditions in Table 4.1 until the signal-to-noise ratios of 3 for LOD and 10 for LOQ were reached, as described in Table 4.6.

3.8 The Study of Matrix Calibration Curve

The matrix standards were prepared by adding the mixed 17 standard organochlorine pesticide solutions before creating the volume process with sample blank solutions obtained prepared by the developed method. The data for the matrix calibration curve were gotten from 10-matrix standards concentration with twice injection according to GC conditions in Table 4.1. The matrix calibration curves were plotted by peak heights of a function of concentration. The volume of the mixed 17 standard organochlorine pesticide solutions were added and shown in Table 3.2. The summary of Value of Slope, Intercept and Correlation Coefficient (R^2) of each pesticide in 17 standard organochlorine pesticides in matrix under the GC conditions in Table 4.1 was described in Table 4.7.

Table 3.2 The desired concentration of matrix standards and the pipetted volume of the mixed 17 standard organochlorine pesticide solutions

Concentration of mixed 17 standard organochlorine pesticide solutions (ng/mL)	The volume of standard mixture solution (μL)	The volume of hexane (μL)
1	1.00	999
3	3.00	997
5	5.00	995
10	10.0	990
20	20.0	980
30	30.0	970
50	50.0	950
100	100	900
300	300	700
500	500	500

3.9 The Study of Matrix Effect

The influence of matrix standards on GC conditions in the quantitative analysis of organochlorine pesticides was carried out for statistic analysis using paired *t*-test: between the peak heights from the calibration curve and the matrix calibration curve at the equivalent concentrations as described in Table 4.8.

3.10 The Developing of the Sample Preparation Technique

In 2000, levels of 14 organochlorine pesticides in spices powder that not Curcumin (nutmeg, pepper and maze) were extracted with n-hexane-dichloromethane (4:1) and the extracts were cleaned in a single step on a cartridge packed with silica and florisil, quantified by GC-MS in SIM mode. Recoveries were measured from 60% for dieldrin and endrin to 97% for other pesticides. [34]Thus, we would use this method as a basis

for sample preparation and quantitative analysis of organochlorine pesticides in Turmeric sample the procedure of the basis method. [34] is as follows (Figure 3.1).

In this work, some preparation processes of the basis method were adapted for a suitable preparation of Turmeric sample. The developed methods were investigated in the background noise, interference peaks and percentage recovery (%recovery).

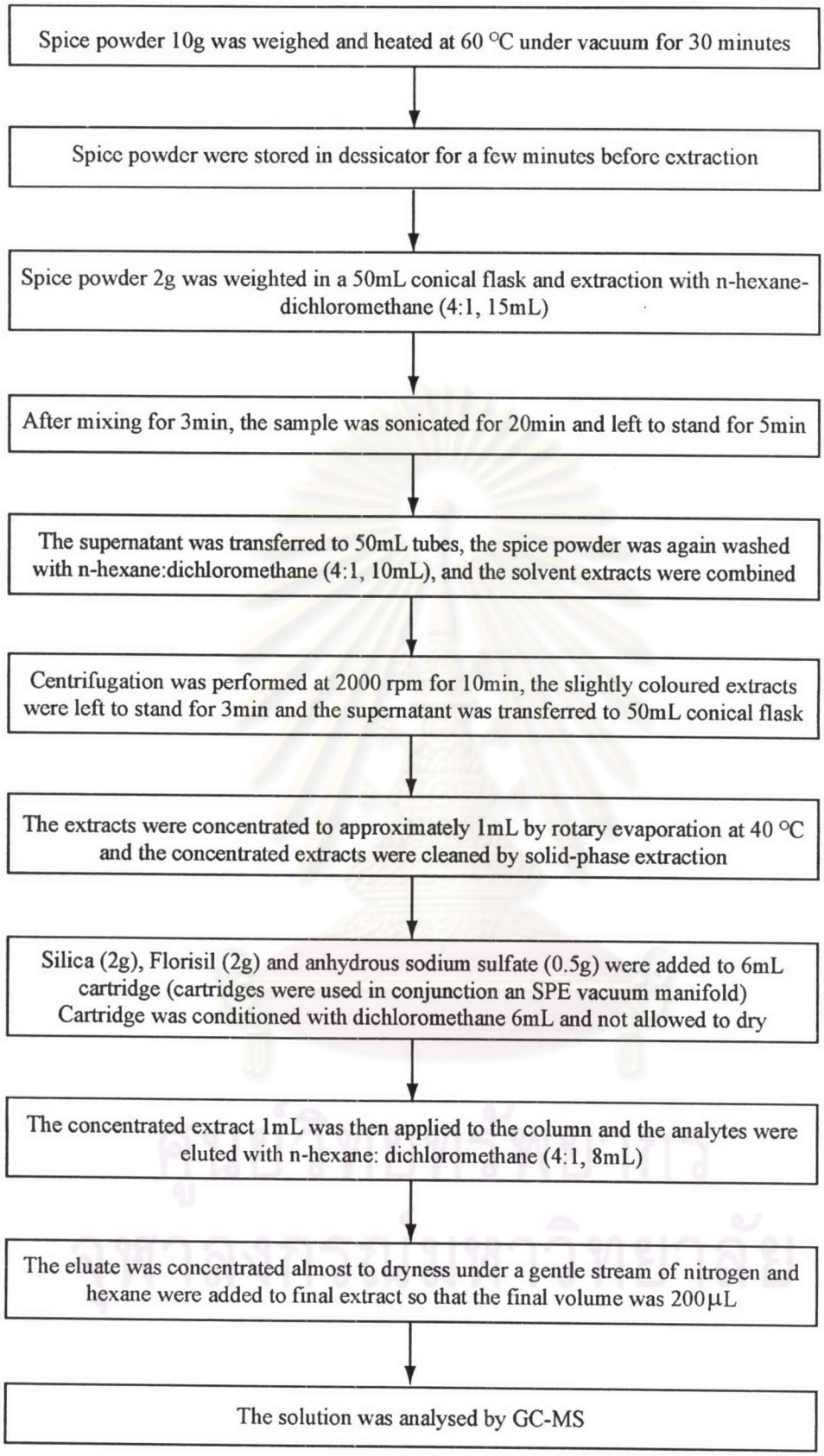

Figure 3.1 Schematic of procedure in basis method

3.10.1 The Study of the Pre-treatment with Vacuum Oven

From the basis method, it was further developed so that it could be most suitable to use with Turmeric samples, and could effectively eliminate matrices. The use of vacuum oven was adjusted so that it created least volatile interferences, and most parameters were studied so that an effective extracting method could be developed. The process was changed from sonicate to vortex because when we used sonicate, solvent that containing organochlorine dissolution will be volatilized. Furthermore, we studied by comparing sample with vacuum oven before extraction and raw sample. Before the extraction process, the mixed 17 standard organochlorine pesticide solutions were added with spiking level of 50 ng/g, concentration factor equal to 0.4 and the final concentration of 20 ng/g The comparison of the result gained through the use of vacuum oven with that without the use of vacuum oven was investigated for interfering peaks, background noises in chromatogram and in the %recovery with GC condition in Table 4.2. The results were shown in Table 4.9 and Figures 4.5 - 4.6.

3.10.2 The Study of SPE Type

The SPE packing component with 3 types of mixed mode such as silica, florisil and sodium anhydrous sulfate was used in the clean-up process of the basis method. In this section, the clean-up process was developed by connect ENVI-CARB SPE after the 3 types mixed mode SPE. As for ENVI-CARB SPE, mostly it is used to eliminate colouring substances and chlorophyll in vegetables. Therefore, it was used to extract colouring substances in Turmeric samples. The effect of ENVI-CARB SPE in sample at spiking level of 50 ng/g, concentration factor equal to 0.4 and the final concentration of 20 ng/g was investigated for the colour of solution after the elution, the interfering peaks, and background noises in chromatogram and the % recovery with GC conditions in Table 4.2. The results were shown in Table 4.10 and Figures 4.7 - 4.8.

3.10.3 The Study of the Influence of Mixed Solution Ratio for Extraction

From the result in Section 3.10.1, one could either choose the procedure with or without the vacuum oven. In this section, the mixed solvent ratio was studied. It was believed that when the value of hexane : dichloromethane increased it could help elute more interested substances and yield better %recovery. The mixed solvent of hexane: dichloromethane 4:1 (v/v) was used to extract sample spiked at spiking level of 100 ng/g, concentration factor equal to 0.4 and the final concentration of 40 ng/g compared with the mixed solvent of hexane: dichloromethane 5:2 (v/v), the fractions were collected since loaded sample at the same spiking level the results of the interfering peaks, background noises in chromatogram and the %recovery as used GC condition in Table 4.1 were shown in Table 4.11 and Figures 4.9 - 4.10.

3.10.4 The Study of the Influence of Elution Volumes of SPE

From the results in Section 3.10.3, one can achieve the best mix solvent ratio for the method. However, there are certain organochlorine pesticides cannot yield a satisfactory %recovery. This led to a change in the parameters of SPE so that better %recoveries could be expected. In this section, the influence of elution volumes of SPE between 5 mL and 8 mL was studied for elution of extracted samples using to spike sample extraction at spiking level of 25 ng/g, concentration factor equal to 0.8 and the final concentration of 20 ng/g. As I want the pre-concentration in the basis method, the concentration factor was changed from 0.4 to 0.8. The sample volumes were increased for SPE from 1 mL to 10 mL and the final volume to 1 mL from 200 μL. The results of the interfering peaks, background noises in chromatogram and the %recovery according to the GC conditions in Table 4.1 were shown in Table 4.12 and Figures 4.11 - 4.12

From this section, I shall conclude and describe the developed method of sample preparation for determination of organochlorine pesticides in Turmeric sample.

3.10.5 The Study of the Comparing of the Developed Organochlorine Pesticides Quantitative Analysis Method with the Standard Method for Tobacco

From the developed method in 3.10.4, it was used to compare with standard method for a Tobacco. [41 – 45] A study by way of comparison of this method with the standard method that was used with tobacco was in order. The purpose was to find out if the method could be used with Turmeric samples and how much in terms of %recovery the method could render. The extraction process of standard method of Tobacco used the spiked samples at spiking level of 100 ng/g, concentration factor equal to 0.06 and the final concentration of 6 ng/g. But we used the developed method from Section 3.10.4 at spiking level of 5 ng/g, concentration factor equal to 0.8 and the final concentration of 4 ng/g. The comparison of results was considered on the %recovery, the interference peaks and background noises in the chromatogram according to the GC conditions in Table 4.1 and described in Figures 4.14 - 4.15 and Table 4.13.

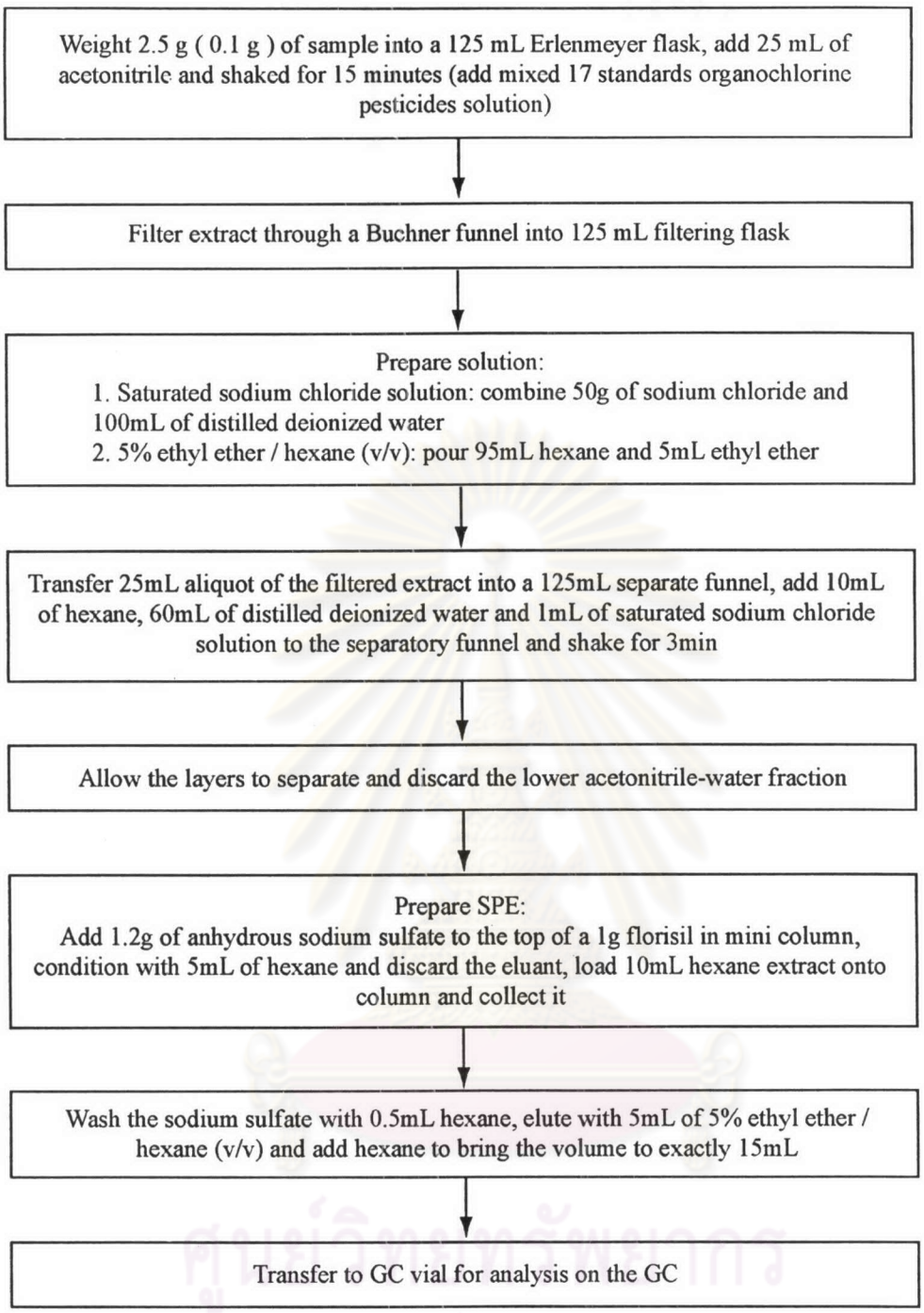

Figure 3.2 Schematic of procedure in standard method for Tobacco.

3.11 The Study of Method Detection Limit (MDL) and Method Quantification Limit (MQL)

An examination was made on the method to find out what was the lowest level of substances it could detect in terms of LOD and LOQ. With these values, MDL and MQL could be arrived at. The Method Detection Limit (MDL) and the Method Quantitation Limit (MQL) were determined by the concentration factor of the developed method. This method has the concentration factor equal to 0.8. As such, the MDL and MQL must divide 0.8 from LOD and LOQ respectively. The value of MDL, MQL and MRLs from USP regulations was according to the GC conditions in Table 4.1 and described in Table 4.14.

3.12 The Study of Precision

As for precision, there are 2 types of precision. First, intra-assay precision that is obtained by repeat analysis of spiked sample in one day and the second is intermediate precision, which is obtained by analysis on different days. The study used spiked sample at spiking level of 5, 25 and 125 ng/g. In one day, I could extract 6 times for each level (intra-assay precision and at each level it was repeated on 3 different days for intermediate precision according to the GC conditions in Table 4.1 and described in Tables 4.15 - 4.26.

3.13 The Study of Accuracy

To determine the accuracy of developed method, it was accomplished by calculating the average %recovery, SD and %RSD that were obtained from each spiking level on 3 different days, according to the GC conditions in Table 4.1 and described in Table 4.27.

3.14 The Study of Stability

The concentration of extraction in the spiked sample at spiking level of 125 ng/g was divided into 11 vials and was kept under the same conditions in refrigerator. Then, it

was compared with matrix standard of 17 standard organochlorine pesticide solutions containing 125 ng/g for the several days. The first injection was made right after the extraction process. Observe %recovery that could be compared with the means of %recovery of the precision at the spiking level of 125 ng/g ± 2SD was made. Detailed descriptions are shown in Table 4.1, Tables 4.28, 4.29 and Figures 4.16 – 4.35.

3.15 The Determination of Organochlorine Pesticides in Turmeric Products at 3 Thai Markets and 3 Commercially-packed Turmeric Powder

At 3 Thai Markets and 3 commercial areas of Turmeric powder, Turmeric was purchased and was determined to find out the amount of organochlorine pesticides according to the GC conditions in Table 4.1 and described in Table 4.30.

1. Turmeric powder from market A
2. Turmeric powder from market B
3. Turmeric powder from market C
4. Turmeric powder from commercial A
5. Turmeric powder from commercial B
6. Turmeric powder from commercial C