CHAPTER 2

2. *Experimental*

2.1 Chemicals and Materials

All the solvents used were HPLC grade unless otherwise stated. Acetonitrile, methanol, petroleum ether, diethyl ether, ethyl acetate and ethanol were purchased from J. T. Baker, a division of Mallinckrodt Baker, Inc. Phillipsburg, NJ 08865 USA and anhydrous sodium sulphate was purchased from Fluka. Ultra-pure water and methanol were filtered through a 0.45 μm filter purchased from Millipore. All the pesticide standards of purity ranging 98.9% to 100% in methanol solution (1.0 mg/ml) were purchased from AccuStandard Inc., New Haven CT, USA except the N-methylcarbamates (carbaryl, carbofuran, methiocarb) and diuron which were purchased from PolyScience, Niles, IL, USA. The use of high purity reagents and solvents help to minimise interference problems. Table (6) provides the empirical formulas and the molecular weights of the investigated pesticides.

	Empirical Formula	Molecular wt.
Carbaryl	$C_{12}H_{11}NO_2$	201.2
Carbofuran	$C_{12}H_{15}NO_3$	221.3
Methiocarb	$C_{11}H_{15}NO_2S$	225.0
Chlorothalonil	$C_8Cl_4N_2$	266.0
Endosulfan	$C_9H_6Cl_6O_3S$	406.2
Diazinon	$C_{12}H_{21}N_2O_3$	304.3
Chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$	349.0
Malathion	$C_{10}H_{19}O_6PS_2$	330.3
Methamidophos	$C_2H_8NO_2PS$	141.1
Dimethoate	$C_5H_{12}NO_3PS_2$	229.0
Profenofos	$C_{11}H_{15}O_3PSBrCl$	373.6
Cthyl- parathion	$C_{10}H_{14}NO_5PS$	291.3
Diuron	$C_9H_{10}N_2OCl_2$	232.1

Table 6. Names and molecular weights of investigated pesticides

2.2 Standard Stock Solution

The mixed standard stock solution containing all the 11 pesticides was prepared by pooling aliquots of the individual pure pesticide standard solutions and then diluting with methanol. Table (7) shows the concentrations of individual pesticide in the mixed standard stock solution. The calibration standard solutions over the concentration range of interest were then subsequently prepared by serial dilution of the mixed standard stock solution with methanol. Mass spectrometer detector

response linearity was examined over various concentration ranges. The analyte peaks obtained in the LC-API-MS (SIM) was integrated and plotted as functions of concentration.

COMPOUND	CONCENTRATION / ppm
Methamidophos	10
Dimethoate	30
Carbofuran	20
Carbaryl	30
Diuron	20
Methiocarb	50
Chlorothalonil	30
Ethyl-parathion	50
Diazinon	5
Profenofos	30
Chlorpyrifos	50

Table 7-The concentrations of 11 pesticides in the mixed standard stock solution.

The standard stock solutions used for determining the pesticide formulations were prepared by diluting the pure pesticide standard solution individually to 50 mg/l (50 ppm). Subsequently, the individual pesticide standard stock solution was diluted serially to obtain calibration standard solutions over the concentration range of interest.

2.3 Glassware

All glassware was scrupulously cleaned to minimise interference problem. A chromic acid bath was prepared by adding potassium dichromate ($K_2Cr_2O_7$) to concentrated sulphuric acid (H_2SO_4) until saturation was reached. All glassware

was soaked in chromic acid overnight and rinsed with distilled water to ensure that all acid residue was wash away. The glassware was then drained dried and stored inverted or capped with aluminium foil in a cupboard to prevent any accumulation of dust or other contaminants.

2.4 Optimising the Heated Nebuliser Parameters

The possible parameters affecting the performance of the heated nebuliser set-up are the eluent flow rate, heated nebuliser probe temperature, orifice and focusing ring voltages. No fixed relation between the pesticides and the parameters can be observed. To optimise the performance, the pesticides were injected individually by FIA-API-MS and a signal-to-noise (S/N) ratio for each compound was calculated from the individual SIM chromatograms. The influence of variations in each parameter setting on the S/N ratio of individual compounds was determined by repeated injections of the solution, keeping the other three parameters constant. For each compound, the parameter settings giving rise to the highest signal-to noise ratio were selected.

2.5 Samples

In the multiclass and multiresidue analysis of pesticides in vegetables, pesticide recovery studies were performed on one type of leafy vegetable ; water spinach (*Ipomoea aquatica)* which was obtained from a pesticide-free farm in Malaysian

Agricultural Research and Development Institute (MARDI). The known volume of standard stock solution was added to the blank control samples to obtained spiked control samples. Recoveries of pesticides were determined by comparison of the amount of each analyte extracted from the spiked samples with that of the standard calibration solutions.

For pesticide formulations, the crude samples of pesticides were obtained from the Department of Agriculture (DOA), Hextar (M) Sdn. Bhd. and some farmers from Cameron Highlands (field). Table (8) is the list of crude pesticides used in this experiment.

No	Source	Type of Formulation	Active Ingredient (AI)	Physical Form	Labelled Value
1	DOA	Carbaryl	Carbaryl	solid	5 %
2	DOA	Carbofuran	Carbofuran	solid	3 %
3	DOA	Methiocarb	Methiocarb	solid	50 %
4	DOA	Chlorothalonil	Chlorothalonil	emulsion	40 %
5	DOA	Endosulfan	Endosulfan	liquid	33 %
6	Hextar	Endosulfan	Endosulfan	liquid	33 %
7	Field	Endosulfan	Endosulfan	liquid	32 %
8	DOA	Diazinon	Diazinon	emulsion	53 %
9	DOA	Malathion	Malathion	liquid	80 %
10	DOA	Dursban	Chlorpyrifos	liquid	21.2 %
11	Hextar	Hextar 38.7 %	Chlorpyrifos	liquid	38.7 %
12	Hextar	Hextar 21.2 %	Chlorpyrifos	liquid	21.2 %
13	Field	Dursban	Chlorpyrifos	liquid	20 %
14	Field	Zesban 45 %	Chlorpyrifos	liquid	45 %
15	Field	Nurelle	Chlorpyrifos	liquid	45.9 %
16	DOA	Methamidophos	Methamidophos	liquid	50 %
17	Hextar	Hextar 50 %	Methamidophos	liquid	50 %
18	Field	Multiphos	Methamidophos	liquid	50 %

Table (8) - The crude pesticides used in pesticide formulation experiments

2.6 Sample Preparation

The sample preparation method is adopted from that of the Queensland Health Scientific Services, Australia with some slight modifications (179). The spiked samples were extracted and cleaned up as follows:

The vegetable was finely chopped and homogenised with a blender. 30 g of the homogenised sample was accurately weighed and placed in a 500ml conical flask. Then 100 μL of the standard stock solution was spiked into the sample to provide the spiked control sample. The mixture was thoroughly mixed and then 150 ml of 5% v/v ethanol in ethyl acetate was added. After ultrasonicating for 10 minutes, the supernatant liquid was filtered into a flask containing about 60 g sodium sulphate. After adding the plant tissue to the funnel, the extraction and filtration steps were repeated once more with 100 ml of the 5% v/v ethanol in ethyl acetate. The combined extract was again filtered into a round bottomed flask and was evaporated to just dryness with a vacuum rotary evaporator (40^0C water bath). The concentrated extract was dissolved in 1 ml of acetonitrile and further cleaned up with Florisil.

Florisil column chromatography clean-up was used for separation of analytes from coextractive matrices by elution with 100 ml acetonitrile. The chromatographic column (30cm x 1 cm I.D.) was slurry packed with 4 g Florisil activated at 450 ^{0}C overnight, made into a 3% v/w water/Florisil with distilled water and stirred for 1 hour before used. Approximately 0.5 cm anhydrous sodium

sulfate was placed at the top of the column to absorb any water in the sample or the solvent. The column was pre-eluted with 50 ml of acetonitrile. Just prior to the exposure of the sodium sulfate layer to the air, the washings from the flask containing the distilled off solvent was placed into the column and allowed to sink below the sodium sulfate layer. The sample in the Florisil column was eluted with 100 ml acetonitrile and the eluate was collected in a round bottom flask. It was evaporated to just dryness by rotary evaporator and then dissolved in 2 ml of methanol before it was injected into LC-API-MS system. All samples were prepared in duplicates. Fig.(7) shows the flow chart of the multiresidue analysis of pesticides in vegetables.

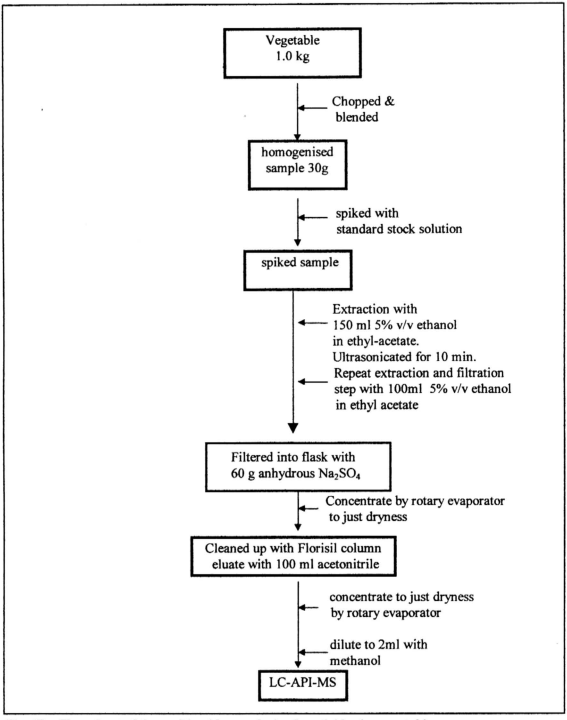

Fig. (7) - Flow chart of the multiresidue analysis of pesticides in vegetables

In the pesticide formulation analysis, different procedures for the sample preparation were used according to the type of sample. For crude pesticides which

are in solid or emulsion forms, the sample solution was prepared by weighing 1 g of the sample in a 100 ml volumetric flask and then made up to the volume with methanol. After ultrasonicating for 10 minutes, it was filtered, and an aliquot (1 ml) of the filtrate was taken and serially diluted to the concentration range of interest before it was used for quantitative analysis. For samples which are in liquid form, the sample solution was prepared directly by serial dilution of the sample to the concentration of interest before it was injected onto FIA-API-MS system for quantitative analysis.

2.7 Liquid Chromatography-Atmospheric Pressure Ionisation-Mass Spectrometry (LC-API-MS) and a Heated Nebuliser

The analyses were performed on a Perkin Elmer LC-200 pump fitted with a Rheodyne 8125 20-μL loop injector. The LC column was connected to a single quadrupole Perkin Elmer/Sciex API 100 LC-MS system equipped with a heated nebuliser interface. The separations were performed on a Techsphere 5 ODS 25 cm x 4.6 mm ID column, purchased from HPLC Technology Ltd., Wellington House, Waterloo Street West, Macclesfield Cheshire, UK. The separation was carried out starting with methanol-water at 60:40 (v/v) with isocratic elution for 5 minutes and then followed by a linear gradient elution to a final methanol-water at 100:0 (v/v) in 35 minutes. The flow rate was set at 0.8 ml/min and 1μL of the sample/calibration solution was injected onto the LC-MS system.. After each run

the column was equilibrated for 5 minutes with the LC initial conditions. The schematic diagram of the LC-API-MS is shown in Fig (8).

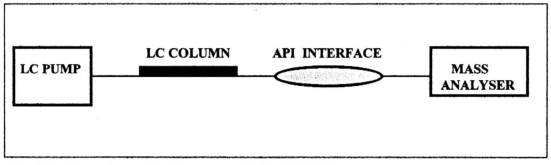

Fig (8) -The schematic diagram of LC-API-MS

The chromatograms of the blank control sample and spiked control sample as shown in Fig. (9) were recorded under time-scheduled selected ion monitoring (SIM) conditions using an acquisition window for each pesticide. The elution order of the pesticides is as shown in Table (14). In this method, the API-MS detection which was performed in the SIM mode can combine both positive and negative modes of operation (PCI/NCI) in a single run. It can be switched to and fro from PCI to NCI mode. Determination of positive ions were achieved by monitoring the $[M+H]^+$ pseudo-molecular ions or fragment ions $[M+H\text{-}CONCH_3]^+$. For the negative ions, determination of chlorothalonil and chlorpyriphos were based on $[M\text{-}Cl+O]^-$ ions, whereas $[M\text{-}C_2H_5]^-$ ion was monitored for ethyl-parathion. Table (10) shows the various ionisation patterns for the pesticides. Table (9) below shows the periods and the acquisition windows of the pesticides.

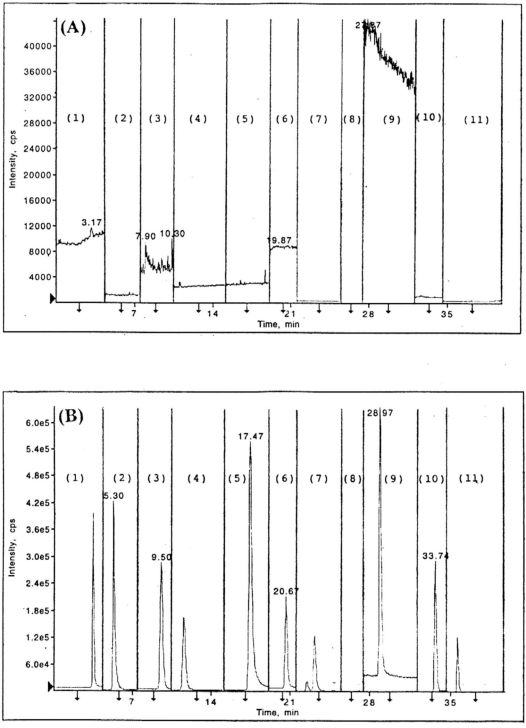

Fig. (9).- The total ion chromatograms of the blank control sample (A) and spiked control sample (B) recorded under time - scheduled selected ion monitoring (SIM) conditions using an acquisition window for each pesticide. (1) methamidophos, (2) dimethoate, (3) carbofuran, (4) carbaryl, (5) diuron, (6) methiocarb, (7) chlorothalonil, (8) ethyl-parathion, (9) diazinon, (10) profenofos, (11) chlorpyrifos

PERIOD	COMPOUND	MOL.WT	MONITORING ION M/Z	PCI/NCI MODE	ACQUISITION WINDOW (min)
1	Methamidophos	141.1	142.1$[M+H]^+$	PCI	0 - 4.0
2	Dimethoate	229.0	230.1$[M+H]^+$	PCI	4.0 - 7.4
3	Carbofuran	221.3	165.4$[M+H\text{-}CONCH_3]^+$	PCI	7.4 - 10.4
4	Carbaryl	201.2	145.0$[M+H\text{-}CONCH_3]^+$	PCI	10.4 - 15.1
5	Diuron	232.1	233.1$[M+H]^+$	PCI	15.1 - 19.3
6	Methiocarb	225.0	169.3$[M+H\text{-}CONCH_3]^+$	PCI	19.3 - 21.9
7	Chlorothalonil	264.0	245.0$[M\text{-}Cl+O]^-$	NCI	21.9 - 25.2
8	Ethyl-Parathion	291.3	261.1$[M\text{-}C_2H_5]^-$	NCI	25.2 - 28.3.
9	Diazinon	304.3	305.3$[M+H]^+$	PCI	28.3 - 32.2
10	Profenofos	373.6	375.0$[M+H]^+$	PCI	32.2 - 34.9
11	Chlorpyrifos	349.0	330.0$[M\text{-}Cl+O]^-$	NCI	34.9 - 40.0

Table 9 - The periods and acquisition windows of the pesticides

2.8 Flow Injection Analysis - Atmospheric Pressure Ionisation Mass Spectrometry (FIA-API-MS) and a Heated Nebuliser

The eluent (methanol 100 %) was delivered by a Perkin Elmer LC-200 pump fitted with a Rheodyne 8125 20-μL loop injector. The injector was connected directly without a column to a single quadrupole LC-MS system-model Perkin Elmer/Sciex API 100 ((Perkin Elmer/ Sciex, Thornhill, Canada) equipped with a heated nebuliser. Fig. (10) shows the schematic diagram of the FIA-API-MS used for the pesticide formulation experiments.

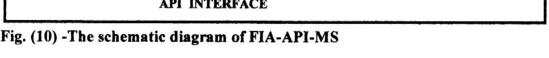

Fig. (10) -The schematic diagram of FIA-API-MS

2.9 Quantification

The instrument control and data processing utilities is based on the Macquan Software Version 1.5. The calibration graph of each pesticide was constructed using the calibration standard solutions. The above standard solutions were prepared by appropriate dilution of the stock solution. In the multiclass and multiresidue analysis of pesticides in vegetables, all pesticides were detected by the selected ion monitoring(SIM) mode on their respective $[M+H]^+$ or their characteristic fragment ions using the time-scheduled procedure and acquisition windows as shown in Table (9). The recoveries, linearity of the method was examined on pesticide free water spinach *(Ipomoea aquatica)* samples. The relationship between peak area and concentration of the respective pesticide (correlation coefficient) was obtained for each pesticide. Recoveries of each pesticide were evaluated by the external calibration method. The potential of this multiresidue method was also demonstrated on 3 types of leafy vegetables; water spinach, choysam (Chinese Mustard)*(Brassica chinensis var. para chinensis)* and kailan (Chinese Kale)*(Brassica alboglabra)* which were obtained from the local market.

In the determination of pesticide formulations, the single residue method (SRM) was used in the study. The relationship between the peak area and the concentration of the respective pesticide (correlation coefficient) was obtained. The measurement of 'AI' was evaluated by the external calibration method.