CHAPTER 3 METHOD DEVELOPMENT FOR DETERMINATION OF PESTICIDES IN SOIL SAMPLES

Soil sampling

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3.1 INTRODUCTION

Pesticides are applied widely to protect plants from disease, weeds, and insect damage, and in doing so, they usually come into contact with soil [117]. This is because pesticides can be displaced from their site of application via spray drift, volatilization, and natural rainfall, and finally would result in one part of the amount used reaches the target while other part is deposited on the soil, where it is subjected to different processes that will determine the fate of these agrochemicals [118]. Slow degradation of pesticides in the environment and extensive or inappropriate usage by farmers can lead to environmental contamination of the water, soil, air, several types of crops and indirectly affect the well-being of humans or other living things [119]. The reason for this event to occur is because soil is the principle reservoir of environmental pesticides, therefore representing a source from which residues can be released to the atmosphere, ground water and living organisms as previously mentioned.

The target compounds for analysis in soil and sediment samples have traditionally been highly hydrophobic in nature [119]. In this thesis, both λ-cyhalothrin and cypermethrin studied are highly hydrophobic with high $K_{o/w}$ value (4×10^6 for cypermethrin, 1×10^7 for λ-cyhalothrin) and practically insoluble in water (0.01 mg/L for cypermethrin, 0.005 mg/L for λ-cyhalothrin) [61]. The hydrophobicity of these compounds cause strong sorption to soil and sediment particles, which make the compounds less bioavailable [120]. Furthermore, Fernandez-Alvarez *et al*. [121] stated that these compounds are so strongly adsorbed on soil particles that they do not easily

leach from the application point due to their low solubility in water and high lipophilicity.

Although most synthetic pyrethroids have lower mammalian toxicity compared to other classes of insecticides (e.g. organochlorines and organophosphates), they can still be very harmful to certain vertebrate and mammal species including bees, chicks, fish, and shellfish [122]. Besides vertebrate and mammal species, there also has been increasing concern regarding the health risk from pesticide residues present in crop soil, as their intake or translocation by plants can easily lead to food or crop contamination. An example is fenvalerate residues that were frequently detected in Chinese tea at levels that consequently often reduce its export potential [123]. According to the Japanese ministry more recently, Japan lodged a complaint on Indonesia over the country's cocoa exported to Japan from Singapore, saying that it contained 2,4-dichlorophenoxyacetic acid (2,4-D) residues at the level of more than 0.01 ppm, which is dangerous to health [124]. Additionally, strong sorption and the resulting of slow degradation of λ-cyhalothrin may cause long term effects on beneficial soil microorganisms [125]. Other study proved that pyrethroid residues could be widespread in sediments from regions of intensive agriculture, and in some locations the high levels of these residues were likely to cause toxicity to sensitive species [126]. From all these noteworthy observations, an understanding of the status of pesticide concentration in soil and its effects is very important to ecological and human health, considering that the potential for environment or food contamination would increase with increasing pesticides residues in soil.

Prior to chemical analysis, a good sample preparation technique is needed in order to determine the analytes of interest using gas chromatography with different detector systems. However it is not as easy as thought, since the interaction between the analytes and matrix is much stronger in soil compared with food and water samples. The reason was because bound residues could be formed in soils, which result in different extraction behavior compared to the non-bound fraction in food and water. Thus, a more exhaustive extraction procedure is required to liberate the bound residues from the soil matrix [127]. Nowadays, various extraction and clean-up procedures have been proposed for the removal of pyrethroid insecticides from soil matrix. These procedures include ultrasonic extraction (USE) with various types of solvents [118, 119, 122, 127, 113, 128-130], differential pulse voltammetry (DPV) [120], accelerated solvent extraction (ASE) [131], homogenous liquid-liquid extraction (HLLE) [132], and headspace solid-phase microextraction (HS-SPME) [121].

As of today, an extraction procedure based on sonication technique is vastly applied for pyrethroid extraction in soil and sediment samples [118, 119, 122, 127, 113, 128-130]. This technique was first introduced for pesticides extraction in soil by Johnsen *et al*. in 1972 [133]. The reason why this technique is so popular is because when compared with other types of extraction, sonication provides a more efficient contact between the solid and the extraction solvent and usually resulting in a greater recovery of the analyte [134]. Furthermore, its versatility is shown in pesticides method development for soil samples with the possibility of selecting and optimizing the solvent type or solvent mixture that allows the maximum extraction efficiency and selectivity. Economically, this technique is much more cost saving since no specialized laboratory equipment is required compared to other extraction techniques such as DPV [120], SPME [121], and ASE [131] which required specialized and expensive

equipments. On the other hand, the downside of this technique is that it is not easily automated and involved manual steps of filtration using either filter paper or membrane filter.

You *et al*. [127] reported a sonication extraction method for the analysis of pyrethroid, organophosphate, and organochlorine pesticides from sediment by gas chromatography with electron capture detection. In their research, pesticide residues were extracted using sonication with acetone-methylene chloride (1:1 v/v) and the extracts were subsequently cleaned with deactivated florisil. Recoveries for λ-cyhalothrin spiked soil samples at four concentrations ranged from 102.1 % to 129.8 % with RSD values of 9.1-10.7 %. Later on, the same team of researchers studied a solution for isomerisation of pyrethroid insecticides in gas chromatography using the same extraction technique developed earlier [113]. They investigated the stability of pyrethroids using different solvents and analyte additives while GC injection conditions were optimized. In this study, the authors concluded that polar solvents enhanced pyrethroid isomerisation and acetic acid was used successfully as an isomer-stabilizing agent for GC analysis. Hence, they suggested that hexane was the best choice as an analytical solvent and pulsed splitless injection at 30 psi and 260 °C was chosen for injection. From their research, only three peaks (instead of four) were observed for cypermethrin using the DB-608 column and additional peak found for λ-cyhalothrin indicated that isomerisation had occurred.

Gu *et al*. [122] conducted laboratory incubation trials to investigate the effects of several factors on the persistence as well as the dissipation of three synthetic pyrethroid pesticides in red soils obtained from the Yangtze River Delta region in China. In that study, pyrethroid residues in soil samples were extracted using ultrasonic extraction

with the combination of petroleum-ether/acetone (2:1 v/v). Then the extracts were cleaned with florisil column before analysed with gas chromatography with electron capture detection. The method applied for pyrethroid extraction was almost similar to the work of You *et al*. [113, 127] except for the different extraction solvent used.The combination of petroleum-ether/acetone has been chosen instead of acetone-methylene chloride as proposed by You *et al*. [113, 127]. The results for the recovery studies of 3 types of pyrethroids (cypermethrin, fenvalerate, and deltamethrin) ranged from 89.7 % to 93.0 %.

Despite the importance of clean-up step for matrix co-extractants removal following the ultrasonic extraction, some researchers excluded the clean-up step and applied only the ultrasonic extraction for pyrethroid extraction in soil samples [118, 119, 128-130]. The reason for the exclusion of clean-up step is because of the high selectivity of the method developed. An example is the multiresidue analysis of insecticides in soil by Castro *et al*. [118]. They developed a rapid multiresidue method for the analysis of nine insecticides (organochlorine, organophosphorus, and pyrethroid) based on the sonication extraction of residues from a certain amount of soil placed in a small column, using ethyl acetate and determined by gas chromatography with electron capture detection. In that study, the average recovery through the method obtained for these compounds varied from 90 to 108 % with a RSD values between 1 and 11 %. According to the authors, the results of the study pointed out that the proposed method of extraction by sonication of soil samples placed in small columns using ethyl acetate as extracting solvent provides a rapid and sensitive procedure for the simultaneous determination of the selected pesticides without extraneous clean-up step.

Sánchez-Brunete *et al*. [128] applied the same extraction strategy for the simultaneous determination of 52 pesticides of various classes in soil. With the proposed analytical method, the extraction of samples was performed with a low volume of ethyl acetate and a subsequent clean up was not required since good resolution of the pesticide mixture was achieved in approximately 41 min. The authors also claimed that the good reproducibility and the low detection and quantification limits achieved with this method would allow its application to monitoring of pesticide residues in soil samples collected from various agricultural areas of Spain. From the monitoring results, several herbicides and insecticides were found. Meanwhile, cypermethrin and λ-cyhalothrin recoveries were in the acceptable ranges, 87-103 % and 97-100 % respectively.

Gonçalves *et al*. [119] established a suitable methodology for different classes of pesticides namely organochlorine, organophosphorus, pyrethroid, triazine, and acetanilide based on ultrasonic extraction and gas chromatography mass spectrometry. In this study, the authors tested several solvents (*n*-hexane, ethyl acetate, acetonitrile, and dichloromethane) in order to minimize the effect of soil co-extractives on the determination of analytes as well as to improve recoveries. Their results indicated that ethyl acetate gave the best recoveries and also best precision for all analytes and hence this solvent was chosen as the extraction solvent in ultrasonic extraction. Furthermore, from the evaluation of method performance using 5 mL of ethyl acetate as extractant in ultrasonic extraction during 15 min repeated three times, they concluded that these conditions exhibited excellent extraction capabilities. Therefore, no further optimisation would be needed. Recoveries for cypermethrin and λ-cyhalothrin spiked at 0.01 µg/g were both 103% with RSD values of 7 and 10 % respectively.

Besides ethyl acetate, some authors preferred the combination of acetonitrile/water as the extractant in the ultrasonic extraction [129, 130]. Fenoll *et al*. [129] proposed a rapid multiresidue method for the simultaneous determination of 25 fungicides and insecticides in soil based on ultrasonic extraction using acetonitrile/water as extractant and subsequent partitioning with dichloromethane. Final determination was made by gas chromatography with nitrogen-phosphorus detection. In this study, λ-cyhalothrin recoveries varied from 99% to 104 % while for cypermethrin the recoveries were ranged from 90% to 103%. The authors claimed that the proposed method is rapid, simple, and sensitive. Additionally, they also stated that the method has advantages when compared to other conventional methods due to the use of low volume of organic solvent in the sample extraction. Therefore, clean-up of soil samples was not required.

Lesueur *et al*. [130] carried out a comprehensive study on different extraction approaches for the analysis of 24 pesticides in soil samples with gas chromatography mass spectrometry and liquid chromatography ion trap mass spectrometry. In that study, a new ultrasonic solvent extraction (USE) was compared to the European Norm DIN 12393 for foodstuff (extraction with acetone, partitioning with ethyl acetate/cyclohexane and clean-up with gel permeation chromatography), the QuEChERS and a pressurized liquid extraction (PLE) method. In this work, the combination of acetonitrile/water was used as extractant and no further clean-up step is required. As reported by the authors, the newly developed USE method is accurate as a monitoring method for the extraction of the selected pesticides from soil but cannot be implemented as currently applied as quantification method due to its low recovery for certain pesticides. On the other hand, the QuEChERS method seems so far to be the most adapted method for soil sample analysis. Nevertheless, the authors also proposed

that other solvent such as acetone for instances should be investigated with USE technique to increase the recoveries of certain pesticides.

In recent years, many other techniques besides ultrasonic extraction have emerged and reported as alternatives to ultrasonic extraction, such as headspace solid-phase extraction (HS-SPME), accelerated solvent extraction (ASE), and homogenous liquid-liquid extraction (HLLE) [121, 131, 132]. SPME technique would be beneficial in terms of simplification in sample handling, reduction in sample size and solvent volume, and absence of additional clean-up procedures. However, this microextraction method is primarily used in liquid samples. Few literatures report this microextraction method to be used in solid samples, which possibly result from more limitation for the determination of pesticide residues in solid samples [132]. Additionally, this could also be attributed to some limitations in terms of fiber stability or analyte release/volatility [119]. Meanwhile, as for ASE, it requires expensive instrumentation and laborious optimization process [119]. Nowadays, an ideal sample preparation technique for pesticide residues in soil samples usually requires it to be rapid, simple, cheap, environmentally friendly, clean final extracts and minimum or without clean-up steps involved in the chemical analysis.

3.2 OBJECTIVES

The aim of this work was to optimize the ultrasonic extraction procedure for the determination of cypermethrin and λ-cyhalothrin in soil sample. The optimization was carried out with regard to the solvent type, amount of solvent, and duration of sonication. The extracted pesticides were then identified and quantified using gas chromatography with electron capture detection.

3.3 EXPERIMENTAL

3.3.1 REAGENTS AND MATERIALS

HPLC grade acetone, acetonitrile, and ethyl acetate were obtained from Merck (Darmstadt, Germany), while both pesticide standards of cypermethrin and λ-cyhalothrin with the purity of >97%, were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Reagent grade of anhydrous $MgSO_4$ was obtained from Supelco Inc. (Bellefonte, PA, USA). All extracts were filtered using filter paper (No. 4, flow rate: fast, particle retention: 20-25 µm) and syringe filter (nylon, 0.45 µm) which both were obtained from Whatman (Maidstone, Kent, UK).

3.3.2 APPARATUS AND GLASSWARE

Microliter pipettes, adjustable between 100 and 1000 µL, and pipette tips were obtained from Eppendorf (Hamburg, Germany). Microvials (2 mL) for GC injection were purchased from Agilent (Palo Alto, CA, USA) and vortex mix used in the sample extraction and partition step, was obtained from Barnstead/Thermolyne Inc. (Dubuque, IA, USA). Ultrasonicator used in samples extraction step was obtained from Branson 5510 (Danbury, CT, USA), while N-Evap nitrogen evaporator for sample concentration was obtained from Organomation Associates Inc. (South Berlin, MA, USA). All glassware were cleaned thoroughly using cleaning detergent and rinsed with tap water before dried in a drying oven at 60 ºC. Prior to use, the glassware were again rinsed with acetone and dried in an oven to remove any impurities that cannot be remove by water.

3.3.3 INSTRUMENTATION

Sample extracts were analyzed using an Agilent Model 6890 series gas chromatograph equipped with a 7683 auto-sampler, split/splitless injector, and an ECD operated at 280 ºC (Agilent Technologies). The injection mode was splitless operated at 250 ºC and the injection volume was 2.0 µL. The inlet pressure was 18.22 psi while the purge flow was 20.0 mL/min with purge time of 2 min. A DB-608 column (30 m x 0.25 mm i.d. x 0.25 µm film thickness, Agilent Technologies) was used to separate the analytes. Nitrogen was used as a carrier and makeup gas, with flow rate for carrier gas and makeup gas were at 1.2 mL/min and 60 mL/min respectively. The equilibration time for the oven was set at 1 min. The initial temperature was 150 ºC, with initial time of 2 min. The oven was heated to 250 ºC at 20 ºC/min and then held at that temperature for 25 min. The post-run temperature was 250 ºC (held for 5 min) and the total runtime was 32 min. Chemstation software was used for instrument control and data analysis. A calibration curve was constructed using seven external standards at concentrations of 0.01, 0.02, 0.05, 0.08, 0.10, 0.50, and 1.00 µg/mL.

3.3.4 PREPARATION OF STOCK STANDARD SOLUTION

Individual stock standard solutions of each pesticide were prepared in acetone at concentration of 2000 µg/mL by dissolving 0.1 g of cypermethrin and λ-cyhalothrin in 50 mL acetone and were kept refrigerated at -20 ºC in amber glass-stopped bottles in the dark. Then, intermediate working standard solutions were prepared by dilution of the stock solutions in acetone to give mixed pesticide standards of 100 µg/mL and 10 µg/mL. Finally, serial dilutions of the mixed working standard solutions were performed to give seven calibration solutions (0.01, 0.02, 0.05, 0.08, 0.1, 0.5, 1 µg/mL) in acetone. All the standard solutions were stored in scintillation vials at 4 ºC in the refrigerator. Furthermore, the standard mixture solutions were prepared freshly daily in

order to prevent any errors that can affect the results obtain raised from the possible degradation of the pesticides.

3.3.5 SOIL SAMPLES FOR FORTIFICATION

In the method development and validation studies, soil sample investigated should be free from cypermethrin and λ-cyhalothrin residues. Blank soil samples which were used as a control sample were obtained from MPOB-UKM Research Station. Top soil samples (0-10 cm), collected at various locations where no oil palm trees were planted were homogenized by hand mixing, where large debris (e.g., gravel, sticks) was removed. Then, they were sieved to < 2mm, air dried, perfectly well homogenized again by hand mixing, and stored in glass vials at -18 ºC until analysis was performed. Recoveries of cypermethrin and λ-cyhalothrin were determined using soil samples at fortification levels of 0.01, 0.02, 0.05, and 0.1 µg/g. Each solution used to provide fortification was prepared by measuring an appropriate amount of pyrethroid reference standard into a known quantity of acetone solution. Then, an appropriate amount (1.0 mL) of the fortification solution was evenly pipetted into a 100-mL beaker containing 20.0 g of the soil sample. After homogenization using vortex mixer for 2 minutes and hand shaking, the fortified samples were allowed to stand for 30 minutes prior to analysis.

3.3.6 FIELD SOIL SAMPLES FOR MONITORING STUDY

Real samples were collected from the top layer (0-20 cm) from New Labu Plantation (Sime Darby Plantation) in Labu, Negeri Sembilan. In this case, soil was sampled at approximately 1 meter around the trees. The samples were then sieved (2 mm), homogenized using cone and quartering technique, and finally stored at -18 ºC before analysing using the optimized USE method in seven replicates.

3.3.7 ANALYTICAL PROCEDURES

In the method development, an ultrasonic extraction (USE) was chosen as the extraction technique for both cypermethrin and λ-cyhalothrin in soil. Initial tests were carried out to optimize the extraction procedure. In this study, optimization of extraction parameters was divided into three experiments according to the parameters, which were extraction solvent (acetonitrile, ethyl acetate, and acetone), solvent volume (15, 20, and 25 mL), and time consumption of USE (5, 10, 15, and 20 min). In general, a homogenized soil sample (20.0 g) was weighed in a 100-mL beaker. Then, a suitable amount of solvent was added and the sample was extracted by ultrasonic extraction. After the extraction period, the extract was decanted and filtered through a piece of Whatman filter paper (filled with approximately 1 g of anhydrous $MgSO_4$) into a 20 mL scintillation vial. Then, the extract was again filtered through nylon syringe filter to remove additional fine soil particles and obtain a clear solution. An aliquot (equal to 20 % of the original volume) was taken out of the scintillation vial and evaporated to dryness using N-evaporator. Finally, the extract was reconstituted with 1 mL of acetone and ready for GC analysis.

3.3.7 (A) SELECTION OF SOLVENT

In the first set of experiments, the extraction efficiencies of various organic solvents were compared: acetone, acetonitrile, and ethyl acetate. In this experiment, 20.0 g of a homogenous soil sample was spiked as described in section 3.3.5 with a mixture of working standard solutions of cypermethrin and λ-cyhalothrin to achieve final concentrations of approximately 0.02 and 0.1 µg/g. Then, each spiking level was extracted in triplicates (n = 3) with a 20 mL of extraction solvents (acetonitrile, acetone, and ethyl acetate) by ultrasonic extraction for 20 min.

3.3.7 (B) SELECTION OF SOLVENT VOLUME

In the second set of experiments, the optimum volume of solvent was determined. The optimization experiment was carried out using acetonitrile, which gave the highest recoveries of the pesticides studied. For that, a 20.0 g homogenous soil sample was spiked as described in section 3.3.5 with a mixture of working standard solutions of cypermethrin and λ-cyhalothrin to achieve final concentration of approximately 0.05 µg/g. Then, each of the spiked soil was extracted in four replicates (n = 4) with 15, 20, and 25 mL of acetonitrile by ultrasonic extraction for 20 min.

3.3.7 (C) SELECTION OF OPTIMUM SONICATION TIME

In the final set of experiments, an optimum sonication time was investigated using 15 mL of acetonitrile which gave the highest recoveries of the pesticides studied. In order to achieve this, a 20.0 g homogenous soil sample was spiked as described in section 3.3.5 with a mixture of working standard solutions of cypermethrin and λ-cyhalothrin to achieve final concentrations of approximately 0.05 µg/g. Then, the spiked soil was extracted in four replicates (n = 4) with 15 mL of acetonitrile by ultrasonic extraction for 5, 10, 15 and 20 min.

3.3.8 QUANTIFICATION AND METHOD VALIDATION

In order to construct the calibration curve, seven working standard solutions (0.01, 0.02, 0.05, 0.08, 0.1, 0.5, 1 µg/mL) were analyzed by GC-ECD. The signal for each pesticide was measured for its peak area and individual calibration plot for cypermethrin and λ-cyhalothrin were constructed. The linearity of the signals from the instrument was studied during the construction of the calibration curve. The percent recovery was determined in four replicate experiments (n = 4) at four concentration levels (0.01, 0.02, 0.05, and 0.1 µg/g) of each pesticide by comparing the analyte peak

area from the fortified samples with that of the standard calibration solutions. Since the gas chromatographic response for cypermethrin is known to be matrix dependent [140], quantification was also carried out by using standards in non-spiked residue free soil extracts obtained by the same sample preparation each time. For each standard (standards in pure solvent and matrix-matched standards), the recovery was calculated using the following equation:

$$\% \text{ recovery} = A_{fortified}/A_{standard}$$

where,

$A_{fortified}$ = peak area of fortified sample

$A_{standard}$ = peak area of pyrethroid standard

The pyrethroid content (µg/g) in the samples for the monitoring study was calculated using the following equation:

$$\text{Pyrethroid concentration (µg/g)} = \frac{V_{extraction} \times V_{fv} \times A_{sample}}{V_{aliquot} \times W \times A_{standard}} \times \text{concentration of standard (µg/mL)}$$

$V_{extraction}$ = volume of extraction solution (mL)

V_{fv} = volume of final solution (mL)

$V_{aliquot}$ = volume of aliquot taken (mL)

W = sample weight (g)

A_{sample} = peak area of sample solution

$A_{standard}$ = peak area of standard solution

In this experiment, no internal standard was applied for quantification in the GC-ECD method since GC auto-sampler was used during the injection of samples. Repeatability of the chromatographic method for the electron capture detector was determined by injecting 0.2 µg/mL standard solution and fortified soil (0.2 µg/g) ten times *via* an auto-sampler. The accuracy and precision of the method were expressed in terms of recovery and RSD respectively in four replicate experiments. The specificity of the proposed method was assessed by analyzing blank soil samples, while the limit of detection (LOD) and limit of quantification (LOQ) of the proposed method were determined by considering a value of 3 and 10 times of the background noise obtained from blank samples.

3.4 RESULTS AND DISCUSSION

3.4.1 OPTIMIZATION OF ULTRASONIC EXTRACTION (USE)

As mentioned earlier, the reason why USE technique is so popular is because when compared with other types of extraction, sonication provides a more efficient contact between a solid sample matrix and an extraction solvent (exhaustive extraction) and usually resulting in a greater recovery of the analyte [134]. Its versatility is shown in pesticides method development for soil samples with the possibility of selecting and optimizing the solvent type or solvent mixture that allows the maximum extraction efficiency and selectivity. Additionally, ultrasonication can be done at room temperature, which allows analysis of thermolabile pesticides, especially when dealing with insecticides using gas chromatography. From an economical point of view, this technique is much more cost saving since no specialized laboratory equipment is required. Currently, there are many techniques in the literature ranging from those that are extremely long, laborious and complicated to the simplest that shake or sonicate an aqueous solution [117].

In USE method development, one of the important aspects of concerned is the extraction step. Extraction aims to remove as much as possible the analyte from the matrix, so it is crucial to optimize the extraction parameters in order to save the time and solvent used without compromising the extraction efficiency and selectivity. For this, the best extraction condition for pesticides in soil is established. This work discusses three extraction parameters for cypermethrin and λ-cyhalothrin in soil. They were types of solvent, solvent volume, and sonication time. These three parameters act as the main extraction parameters for USE technique. Cleanliness of the extracts presented in the chromatograms and good recoveries were the main criteria for the method selection. Tables 3.1-3.3 show the effects on the recovery of analytes based on (i) types of solvent, (ii) solvent volume, and (iii) sonication time.

3.4.1 (A) SELECTION OF SOLVENT FOR THE ULTRASONIC EXTRACTION

The nature of the extraction solvent represents the most important parameter that influences extraction efficiency and selectivity in USE technique. Usually, solvent selection is the first step that analysts have to engage before moving to the next step of optimization. Generally, a selection of solvent follows the polarity properties of both the solvent and analyte studied. In this case, the extraction solvent should have polarity properties compatible with both cypermethrin and λ-cyhalothrin. As of today, various solvents or combination of solvents have been tested and applied for pyrethroids extraction in soil matrix using USE technique. They are ethyl acetate [118, 119, 128], petroleum ether:acetone [122], acetone:dichloromethane [113, 127], and acetonitrile:water [129, 130].

However, only acetonitrile, acetone, and ethyl acetate were considered for optimization in this study. Water, petroleum ether, and n-hexane were not considered because they represent the most extreme polar and non-polar solvents. Hence, the possibility of matrix co-extractives of very polar and very non-polar interferences from the soil may increase. According to Vagi *et al*. [135], dichloromethane minimized the influence of matrix co-extractives on the response of analytes. In their research, they discovered that the extracts obtained with dichloromethane were cleaner, in comparison with ethyl acetate and for that reason they choose it for USE of OCPs from marine sediments, providing a rapid extraction procedure without a clean-up step. Nevertheless, dichloromethane was not considered in this work due to its toxicity [135]. Furthermore, its high volatility makes it an acute inhalation hazard [136]. Hence, it was not included in the method development step.

Mid-polar solvents such as acetonitrile, acetone, and ethyl acetate were chosen in the solvent selection step since currently they are by far the most applied extraction solvents for pyrethroid residues in soil [118, 119, 122, 127, 113, 128-130]. In addition, their mid-polarity properties would minimize the co-extractives interferences in the chromatograms and balance the extraction between the analytes and interferences. In this study, the recoveries of both analytes used for each solvent were calculated and evaluated to obtain the optimum extraction solvent efficiency. To achieve this, fortified soil samples at two levels of concentration, 0.02 and 0.1 µg/g, were extracted in triplicates for each analyte by USE for 20 min using 20 mL of solvents (acetonitrile, acetone, and ethyl acetate) as described in section 3.3.7. No clean-up step was applied. Table 3.1 summarizes the recoveries of cypermethrin and λ-cyhalothrin obtained using acetonitrile, acetone, and ethyl acetate as an extraction solvent.

Finally, the results obtained were analysed using analysis of variance (ANOVA) test to check whether there is a potential difference among the three extraction solvents tested at 0.02 µg/g fortification level. This method uses a single test to determine whether there is or is not a significant difference among the population means rather than pair-wise comparisons, as are done with the *t*-test [106]. In this single-factor ANOVA procedure for various extraction solvents, the null hypothesis H_0 was of the form

$$H_0: \mu_{\text{acetonitrile}} = \mu_{\text{acetone}} = \mu_{\text{ethyl acetate}}$$

$\mu_{\text{acetonitrile}}$ = mean recovery for acetonitrile extraction

μ_{acetone} = mean recovery for acetone extraction

$\mu_{\text{ethyl acetate}}$ = mean recovery for ethyl acetate extraction

and the alternative hypothesis H_a was

H_a: at least two of the mean recoveries are different.

To complete the hypothesis test, the calculated *F* values for both pesticides were compared with the critical value obtained from the *F*-value table (Appendix 2) at the 95% confidence level. The results of ANOVA test were summarized in Table 3.4 and Table 3.5 for λ-cyhalothrin and cypermethrin respectively. From the ANOVA tests, the calculated F values were 6.05 for λ-cyhalothrin and 1.39 for cypermethrin. Since the critical *F*-value for this ANOVA test was 5.14 at the 95% confidence level, it can be concluded that for λ-cyhalothrin, the alternative hypothesis (H_a) was accepted and there was a significant difference among the mean recoveries. Therefore, we can say that at 95% confidence level, variation of the solvents gave different recovery values for λ-

cyhalothrin. On the other hand, the null hypothesis (H_0) was accepted for cypermethrin and concluded that there was no significant difference among the mean recoveries. Hence, all extraction solvents gave equivalent results.

The results showed that all extraction solvents gave satisfactory recoveries (70-120%) except for acetone which gave recoveries more than 120% as shown in Table 3.1. In this analysis, USE achieved with acetone showed that this solvent could be acceptable for extraction of λ-cyhalothrin and cypermethrin in soil without the need to combine with other solvents. Additionally, acetone also gave the highest recoveries compared with acetonitrile and ethyl acetate. The results were in accordance with the study done by Babic *et al*. [134], Vagi *et al*. [135], and Tor *et al*. [137]. A research done by Banjoo *et al*. [138] employed an ultrasonication at room temperature using acetone and compared to methanolic KOH solvent to extract polyaromatic hydrocarbons (PAHs) in soil. Their results indicated that the analytes could penetrate the pores of a sediment matrix to a greater extent and provide a more efficient contact between the sediment particles and itself as the extracting solvent. Thus, resulting in higher quantities of PAHs being extracted. This was confirmed by the study by Tor *et al*. [137] where in their research, they stated that acetone, in combination with some mechanical forces, would disintegrate the aggregates in soil and improve the extraction.

Nevertheless, in this work acetone was discarded from the method development because of the high amount of matrix co-extractives presence in the final extracts. The final extract solutions were in an agreement with other study using acetone as an extraction solvent [135]. The organic extracts obtained by USE method with acetone were light yellow coloured even after filtration steps. Dirty extracts with even a small amount of co-extractives may decrease the column and harm the detector, hence,

upsetting the determination of the analyte of interest. To overcome this problem, an extraneous sample clean-up step prior to GC analysis is required and would result in an increase of solvents and materials consumption. Furthermore, acetone extracts also showed the highest matrix-induced response enhancement effect compared to acetonitrile and ethyl acetate. This effect can be seen from the high recovery values (> 100%) observed for acetone compared to acetonitrile and ethyl acetate as shown in Figure 3.1 and Figure 3.2. The representative bar charts clearly showed that acetone extracts gave higher recoveries and deviated from the 100% value with a bigger margin compared to other solvents.

Both acetonitrile and ethyl acetate performed acceptable extraction efficiencies for USE of λ-cyhalothrin and cypermethrin in soil matrix. The results obtained for both solvents were compatible with earlier study by Gonçalves *et al*. [119]. Their results indicated that ethyl acetate obtained the best recoveries while acetonitrile showed good properties as an extraction solvent. In this study, both solvents gave almost similar recoveries for λ-cyhalothrin and cypermethrin as shown in Table 3.1. In addition, both solvents also obtained clear final extract solutions after filtration without clean-up step required. GC-ECD chromatograms for soil extracts fortified with 0.1 µg/g pesticides were obtained as shown in Figure 3.3. Nevertheless, in this study acetonitrile showed better precision than ethyl acetate for both pesticides when assessed from their RSD values. In conclusion, the comparison of different extraction solvents for USE method showed that acetonitrile obtained the best precision, the least matrix-enhancement effect, acceptable recoveries, and clear extracts. For these reasons, acetonitrile was selected as an extraction solvent in further optimization experiments.

Table 3.1: Recoveries of pyrethroids obtained by USE method (v = 20 mL, t = 20 min) with various solvents (n = 3) at two different concentrations (0.02µg/g and 0.1 µg/g)

Analyte	**0.02µg/g**						**0.1µg/g**					
	Acetonitrile		**Acetone**		**Ethyl acetate**		**Acetonitrile**		**Acetone**		**Ethyl acetate**	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
λ-cyhalothrin	103.15	2.79	125.65	10.19	102.51	9.05	95.72	2.01	109.60	4.77	96.94	4.18
cypermethrin	104.19	6.04	122.52	14.39	107.11	15.51	104.71	3.84	118.37	6.26	107.81	6.25

Table 3.2: Recoveries of pyrethroids (0.05 µg/g) obtained by USE method (t = 20 min) with various volumes of acetonitrile (n = 4)

Analyte	15 mL		20 mL		25 mL	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
λ-cyhalothrin	103.13	3.59	106.7	3.39	106.03	5.22
cypermethrin	109.37	7.99	111.83	7.19	111.67	8.63

Table 3.3: Recoveries of pyrethroids (0.05 μg/g) obtained by USE method (15 mL of acetonitrile) with various durations of extraction (n = 4)

Analyte	5 min		10 min		15 min		20 min	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
λ-cyhalothrin	104.14	3.11	105.57	1.93	104.21	2.59	105.56	1.6
cypermethrin	116.23	2.62	116.98	3.34	112.43	6.17	117.14	1.26

Table 3.4: ANOVA test for various extraction solvents (acetonitrile, acetone, and ethyl acetate) for λ-cyhalothrin, n = 3

SUMMARY

Solvent	No. of measurement	Sum	Average	Variance
Acetonitrile	3	309.46	103.15	8.26
Acetone	3	376.96	125.65	163.94
Ethyl acetate	3	307.53	102.51	86.12

ANOVA

Source of Variation	Source of Square (SS)	Degrees of Freedom (df)	Mean Square (MS)	F (calculated)	P-value	F (critical)
Between Groups	1042.28	2	521.14	6.05	0.04	5.14
Within Groups	516.64	6	86.11			
Total	1558.92	8				

Table 3.5: ANOVA test for various extraction solvents (acetonitrile, acetone, and ethyl acetate) for cypermethrin, n = 3

SUMMARY

Solvent	No. of measurement	Sum	Average	Variance
Acetonitrile	3	312.56	104.19	39.57
Acetone	3	367.55	122.52	310.70
Ethyl acetate	3	321.33	107.11	275.82

ANOVA

Source of Variation	Sum of Square (SS)	Degrees of Freedom (df)	Mean Square (MS)	F (calculated)	P-value	F (critical)
Between Groups	581.90	2	290.95	1.39	0.318	5.14
Within Groups	1252.18	6	208.70			
Total	1834.08	8				

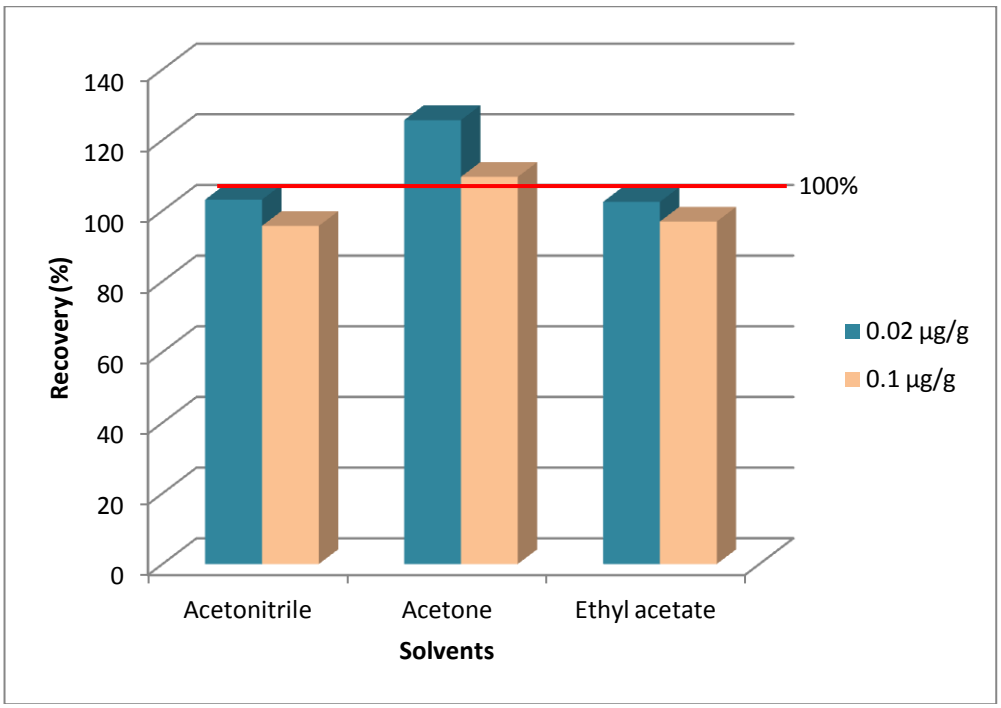

Figure 3.1: Recoveries of λ-cyhalothrin obtained by USE (20 mL of solvent for 20 min) with various solvents (n = 3)

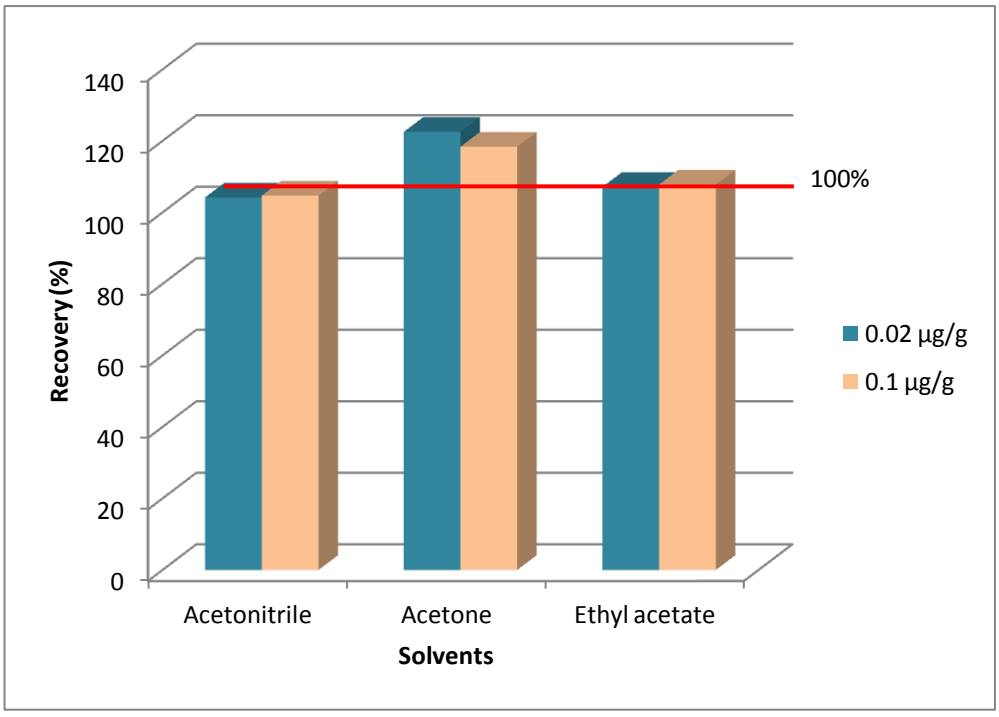

Figure 3.2: Recoveries of cypermethrin obtained by USE (20 mL of solvent for 20 min) with various solvents (n = 3)

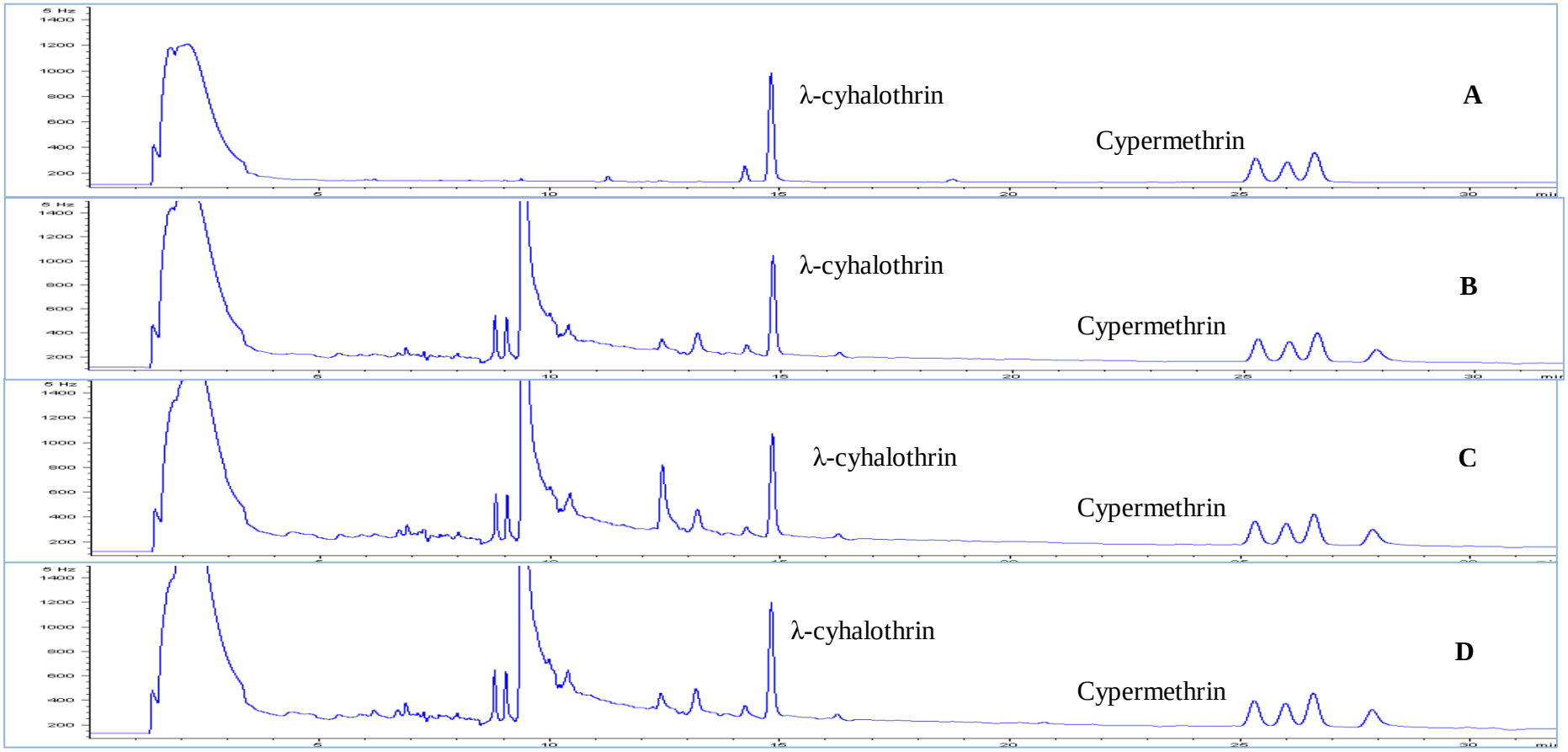

Figure 3.3: GC-ECD chromatograms of (A) pyrethroid standards and fortified (0.1 µg/g) soil extracts after sonication with (B) acetonitrile, (C) ethyl acetate, and (D) acetone.

3.4.1 (B) SELECTION OF AN OPTIMUM VOLUME OF SOLVENT FOR THE ULTRASONIC EXTRACTION

In the second experiment, different volumes of acetonitrile were investigated in order to optimize the extraction efficiency with minimum solvent consumption for USE method. For this, one critical point that must be taken into consideration is the sample size. Previous studies for pyrethroid analysis in soil sample used either 5 g [118, 119, 128, 129] or 20 g [127, 113, 130] as the initial soil sample size. Increasing the amount of sample size would result in the larger extraction volume needed in USE method. Nevertheless, in this work, 20 g was selected as an initial sample size so that it can act as a more representative size of the bulk samples compared to 5 g of sample size.

In this study, fortified soil samples (0.05 µg/g) were extracted in four replicates by USE for 20 minutes with 15, 20, and 25 mL of acetonitrile as described in section 3.3.7. Clean-up step was not applied in this experiment. Then, the recoveries for each volume were calculated and evaluated to obtain the optimum volume of acetonitrile needed. Table 3.2 summarizes the recovery results of both cypermethrin and λ-cyhalothrin obtained with 15, 20, and 25 mL of acetonitrile extraction. Finally, the results obtained were analysed using analysis of variance (ANOVA) test to check whether there is a potential difference among the three extraction volumes tested. In this single-factor ANOVA procedure for various volumes of acetonitrile, the null hypothesis, H_0 was of the form

$$H_0: \mu_{15mL} = \mu_{20mL} = \mu_{25mL}$$

μ_{15mL} = mean recovery for 15 mL acetonitrile extraction

μ_{20mL} = mean recovery for 20 mL acetonitrile extraction

μ_{25mL} = mean recovery for 25 mL acetonitrile extraction

and the alternative hypothesis H_a was

H_a: at least two of the mean recoveries are different.

To complete the hypothesis test, the calculated *F* value was compared with the critical value obtained from the *F*-value table (Appendix 2) at the 95% confidence level. The results of ANOVA test were summarized in Table 3.6 and Table 3.7 for λ-cyhalothrin and cypermethrin respectively. From the ANOVA tests, the calculated F value was 0.75 for λ-cyhalothrin and 0.09 for cypermethrin. Since these values were smaller than the critical *F* -value (4.26) at the 95% confidence level, the null hypothesis H_0 was accepted and concluded that there was no significant difference among the mean recoveries for both insecticides. Hence, all three extraction volumes gave equivalent results, indicating that the increase of the solvent volume had no significant effect on the extraction efficiency. Thus, increasing the volume of acetonitrile from 15 mL to 20 mL and finally to 25 mL gave no significant difference on the quantities of pesticides extracted in ultrasonic procedures. In addition, all extraction volumes gave satisfactory recoveries (70 - 120%), as shown in Table 3.2.

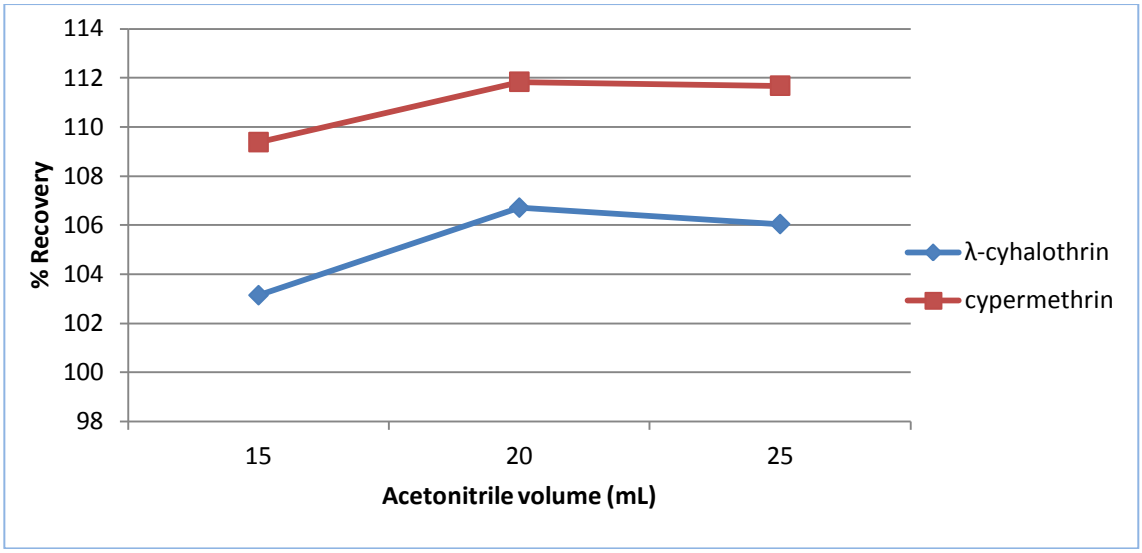

Figure 3.4: Effect of acetonitrile volume on the recoveries of λ-cyhalothrin and cypermethrin that fortified at 0.05 µg/g (n = 4)

The representative line chart for the effect of acetonitrile volume on the analyte recoveries was depicted in Figure 3.4. From the figure, it was found that 15 mL of acetonitrile was the most suitable amount to extract the pesticides with the recovery value of just slightly above 100%. In contrast, the more extensive the extraction procedure used, the more co-extracted interference can be expected and at the same time increasing the probability of matrix-enhancement effect, and consequently may harm the ECD detector, without considering the additional waste of the solvents discharged to the environment. An amount of solvent less than 15 mL was not always sufficient to allow acceptable removal of the required aliquot from the mixtures since some of the solvents will be absorbed by the soil matrices. Therefore, in order to reduce the solvent consumption and the cost of the overall procedure, 15 mL acetonitrile was selected as an optimum volume in further optimization experiment.

Table 3.6: ANOVA test for various volume of acetonitrile for λ-cyhalothrin, n = 4

SUMMARY

Volume of acetonitrile (mL)	No. of measurement	Sum	Average	Variance
15 mL	4	412.50	103.13	13.70
20 mL	4	426.80	106.70	13.12
25 mL	4	424.11	106.03	30.69

ANOVA

Source of Variation	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F (calculated)	P-value	F (critical)
Between Groups	28.88	2	14.44	0.75	0.49	4.26
Within Groups	172.50	9	19.17			
Total	201.38	11				

Table 3.7: ANOVA test for various volume of acetonitrile for cypermethrin, n = 4

SUMMARY

Volume of acetonitrile (mL)	No. of measurement	Sum	Average	Variance
15 mL	4	437.47	109.37	76.32
20 mL	4	447.30	111.83	64.58
25 mL	4	446.62	111.66	92.93

ANOVA

Source of Variation	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F (calculated)	P-value	F (critical)
Between Groups	15.07	2	7.53	0.09	0.91	4.26
Within Groups	701.50	9	77.94			
Total	716.56	11				

3.4.1 (C) SELECTION OF OPTIMUM TIME FOR THE ULTRASONIC EXTRACTION

In the third and final optimization experiment, different durations of time for USE were investigated in order to optimize the extraction efficiency with minimum time consumption. Previously, conventional methods such as Soxhlet and shake-flask required approximately 6 to 8 hours in order to extract the pesticides from the soil matrices. By using these methods, bulky glassware or orbital shaker were employed and operated at certain duration of time (minimum of 6 hours). As a result, the amount of analysis per day was no way compared to today's standard. Nowadays, modern methods have been proposed to solve time and solvent consuming problems as an alternative to conventional methods [139]. The extraction technique will take rarely more than 2 hours per analysis as more than 2 hours of time consumption is considered inefficient.

Nowadays, pyrethroid extraction from soil sample using USE ranged from as fast as 2 minutes to as long as 15 minutes [118, 119, 127, 113, 128-130]. Nevertheless, it still depends on the sample size and solvent volume used. Faster sonication time usually required two or more repetition of the same procedure to the same sample to achieve satisfactory analyte extraction. On the other hand, longer extraction time allows a more thorough contact between the soil particles and the extraction solvent and thus involves only one step extraction without repetition. But sometimes single extraction is not sufficient especially when dealing with multiresidue analysis.

In this study, an optimization of USE time was carried out by ultrasonication of fortified soil samples (0.05 µg/g) in four replicates with 15 mL of acetonitrile for 5, 10, 15, and 20 minutes as described in section 3.3.7. Clean-up step was not applied in this experiment. Table 3.3 summarizes recovery results of cypermethrin and λ-cyhalothrin

obtained with 5, 10, 15 and 20 minutes of sonication time. Finally, the results obtained were analysed using analysis of variance (ANOVA) test to check whether there is a potential difference among the four sonication times tested. In this single-factor ANOVA procedure for various sonication times, the null hypothesis H_0 was of the form

$$H_0: \mu_{5min} = \mu_{10min} = \mu_{15min} = \mu_{20min}$$

μ_{5min} = mean recovery for 5 min sonication

μ_{10min} = mean recovery for 10 min sonication

μ_{15min} = mean recovery for 15 min sonication

μ_{20min} = mean recovery for 20 min sonication

and the alternative hypothesis H_a was

H_a: at least two of the mean recoveries are different.

To complete the hypothesis test, the calculated *F* value was compared with the critical value obtained from the *F*-value table (Appendix 2) at the 95% confidence level. The results of ANOVA test were summarized in Table 3.8 and Table 3.9 for λ-cyhalothrin and cypermethrin respectively. From the ANOVA tests, the calculated F value was 0.41 for λ-cyhalothrin and was 1.05 for cypermethrin. Since these values were smaller than the critical *F* value (3.49) at the 95% confidence level, the null hypothesis H_0 was accepted and concluded that there was no significant difference among the mean recoveries for both insecticides. Hence, the four sonication time gave equivalent results and the increase of the sonication time from 5 minutes to 20 minutes exhibited no substantial impact on the extraction efficiency.

Table 3.8: ANOVA test for various sonication time for λ-cyhalothrin, n = 4

SUMMARY

Sonication time (minute)	No. of measurement	Sum	Average	Variance
5 min	4	416.57	104.14	10.52
10 min	4	422.27	105.57	4.16
15 min	4	416.84	104.21	7.27
20 min	4	422.24	105.56	2.86

ANOVA

Source of Variation	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F (calculated)	P-value	F (critical)
Between Groups	7.71	3	2.57	0.41	0.75	3.49
Within Groups	74.44	12	6.20			
Total	82.15	15				

Table 3.9: ANOVA test for various sonication time for cypermethrin, n = 4

SUMMARY

Sonication time (minute)	No. of measurement	Sum	Average	Variance
5 min	4	464.92	116.23	9.29
10 min	4	467.92	116.98	15.29
15 min	4	449.71	112.43	48.09
20 min	4	468.54	117.14	2.18

ANOVA

Source of Variation	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F (calculated)	P-value	F (critical)
Between Groups	58.75	3	19.58	1.05	0.41	3.49
Within Groups	224.54	12	18.71			
Total	283.29	15				

The effect of sonication time on the extraction of λ-cyhalothrin and cypermethrin can be seen in Figure 3.5. From the line chart, it was found that the recoveries for both pesticides were quite consistent when varying the sonication time from 5 to 20 minutes. On top of that, they all gave acceptable range of recoveries (70-120 %). However, longer extraction time could result in the possibility of degradation of some analytes when using USE [69]. Taking into consideration the above aspects, USE time was not evaluated more than 20 minutes and the optimum sonication time that gave efficient extraction and high recoveries was selected as 5 minutes. In this study, considering the basis that the recoveries of both pesticides obtained from one step of extraction (15 mL of acetonitrile and 5 minutes of sonication) ranged from 104 % to 117 %, no repetition of extraction is needed.

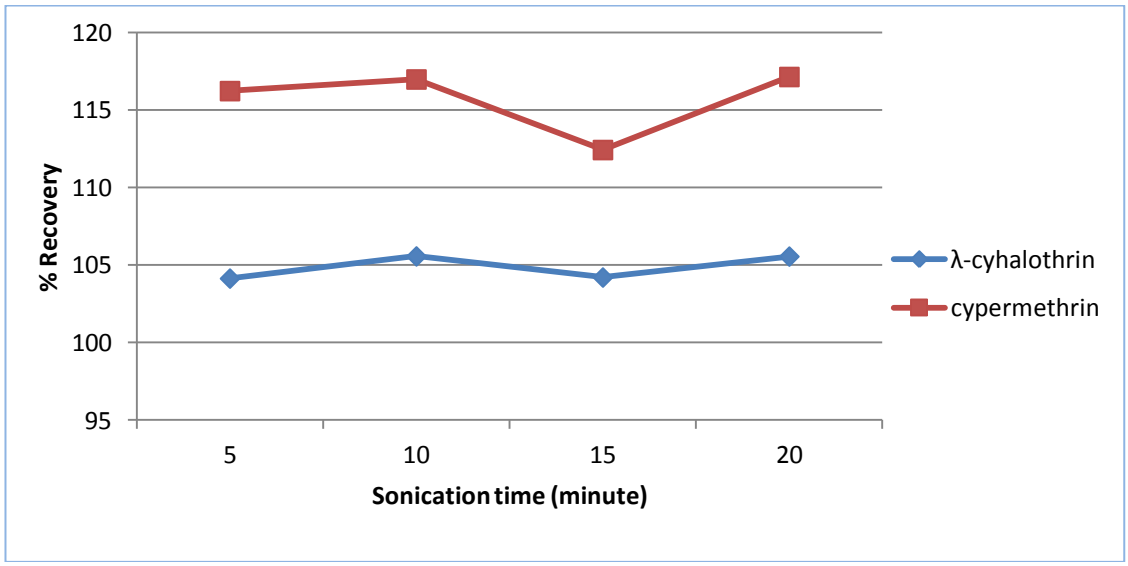

Figure 3.5: Effect of sonication time on recoveries of λ-cyhalothrin and cypermethrin fortified at 0.05 µg/g (n = 4)

Table 3.10 gives a summary of analytical methods used for quantification of λ-cyhalothrin and cypermethrin in soil. From the table, more than half of the current analytical methods used to extract pyrethroid insecticides involved ultrasonic extraction, either with clean-up step [122, 127, 113] or without clean-up step [118, 119, 128-130]. From the preliminary tests, it was found that the newly proposed USE method represents among the fastest extraction method, with 5 minutes extraction time when compared to other techniques, thus facilitating a higher throughput of batches of extracted samples per day. Only the work done by Lesueur *et al.* [130] was faster with 2 minutes extraction time. Nevertheless, the parameters for sample pre-treatment is also related to the detection method since the more sensitive and specific detection method is used, the less stages of sample treatment will be required. In this case, the more expensive and sensitive high-end mass spectrometric techniques equipped with the Ion Trap system may require less vigorous sample pre-treatment compared to the less selective detector such as ECD. The other reason was the difference in a volume of extraction solvent between the two techniques. Higher volume of solvent may require less time compared to lower volume of solvent. Secondly, an improvement can be seen in terms of solvent consumption for the sample size of more than 20 g. The proposed method required less solvent when compared to other methods with the same or larger sample size. Thirdly, the extraction took place only once with no repetition of USE as compared to other methods. This work utilized a single extraction cycle whereas in other studies [118, 119, 122, 127, 113, 128], repeated extraction was employed for the soil matrix using fresh solvent. A repeated extraction was avoided in this research since it could contribute to errors in the analysis due to the increase number of sample preparation steps. Hence, it reduces sample throughput and may adversely affect the accuracy of the method. Last but not least, from the evaluation of method performance using 15 mL of acetonitrile as an extractant in USE for 5 minutes, it was concluded that

these conditions exhibited excellent extraction capabilities with just minor defect. Therefore, no further optimization would be needed especially the clean-up step.

During the course of the optimization tests, sometimes high recovery values (>110 %) were obtained especially for cypermethrin. This is due to the matrix-induced response enhancement effect. This effect or phenomena is popular in the pesticide residue analysis. In general, the effect will either decrease the detection response or increase the analytical signal, as observed in the current research. Many compounds are not affected by matrix-induced enhancement, either because these compounds are thermally stable or have limited potential for adsorption interactions in hot vaporizing injectors, or because the matrix is unable to provide a significant protecting effect. On the other hand, the high recoveries observed for analytes susceptible to matrix-induced enhancement were explained by the protecting effect of the matrix compared with calibration standards prepared in matrix-free solvent [140]. The most common practical solution to the matrix-induced response enhancement problem, often practiced in silence at the time, was to use residue-free matrix-matched calibration standards to equalize the analyte response in calibration and sample solutions [141, 142, 143]. To overcome the problem, a method validation of the optimized method was performed based on the analysis of matrix-matched standards as discussed in the next section.

Table 3.10: Several methods commonly used for the analysis of pyrethroids in soil

Sample size (g)	Extraction				Clean-up	Determination	Reference
	Technique	Time (minute)	Solvent	Solvent volume (mL)			
10	Soxhlet extraction	480	*n*-hexane	125	-	Differential pulse voltammetry	[73]
4	Homogenous liquid-liquid extraction	30	Acetone	10	-	GC-ECD	[132]
5	Accelerated solvent extraction	10	Acetonitrile	40	SPE (Florisil)	GC-ECD	[131]
0.5	Headspace solid-phase microextraction	30	Water	0.5	-	GC-µECD	[121]
5	Ultrasonic extraction	15 (x2)	Ethyl acetate	4 (x2)	-	GC-ECD	[118]
5	Ultrasonic extraction	15 (x2)	Ethyl acetate	4 (x2)	-	GC-MS	[128]
20	Ultrasonic extraction	5 (x3)	Acetone : ethylene chloride	50 (x3)	SPE (Florisil)	GC-ECD	[127]
20	Ultrasonic extraction	5 (x3)	Acetone : ethylene chloride	50 (x3)	SPE (Florisil)	GC-ECD	[113]
50	Ultrasonic extraction	30 (x3)	Petroleum ether : acetone	50 (x3)	SPE (Florisil)	GC-ECD	[122]
5	Ultrasonic extraction	15 (x3)	Ethyl acetate	5 (x3)	-	GC-MS	[119]
5	Ultrasonic extraction	15	Acetonitrile : water	30	-	GC-NPD	[129]
20	Ultrasonic extraction	2	Acetonitrile : water	60	-	GC-MS and LC-IT-MS	[130]
20	New proposed ultrasonic method	5	Acetonitrile	15	-	GC-ECD	-

Non-ultrasonic extraction Ultrasonic extraction Proposed method

3.4.2 METHOD VALIDATION

In any method development study, a basic requirement is to assess how much analyte has been removed from soil by the selected or proposed extraction technique. On this basis, the USE method as described above was extensively tested in order to assess its linearity, selectivity/specificity, sensitivity, mean recovery (as measure of trueness or bias), and precision. The optimized method conditions are as follows: a homogenized soil sample (20.0 g) was weighed onto a 100-mL beaker. Then, 15 mL of acetonitrile was added and the mixture was mixed and extracted by ultrasonic extraction for 5 minutes. After the extraction period, the extract was decanted and filtered through a piece of Whatman filter paper (filled with approximately 1 g of anhydrous $MgSO_4$) into a 20-mL scintillation vial. The purpose of using anhydrous $MgSO_4$ was to absorb micro quantities of water. Then, the extract was filtered again through a nylon syringe filter to remove additional fine soil particles and to obtain a clear solution. An aliquot of 3 mL was taken out from the clear extract into a scintillation vial and then it was evaporated to dryness using N-evaporator. Finally, the extract was reconstituted with 1 mL of acetone and it was ready for GC analysis. The simplified method is shown in Figure 3.6.

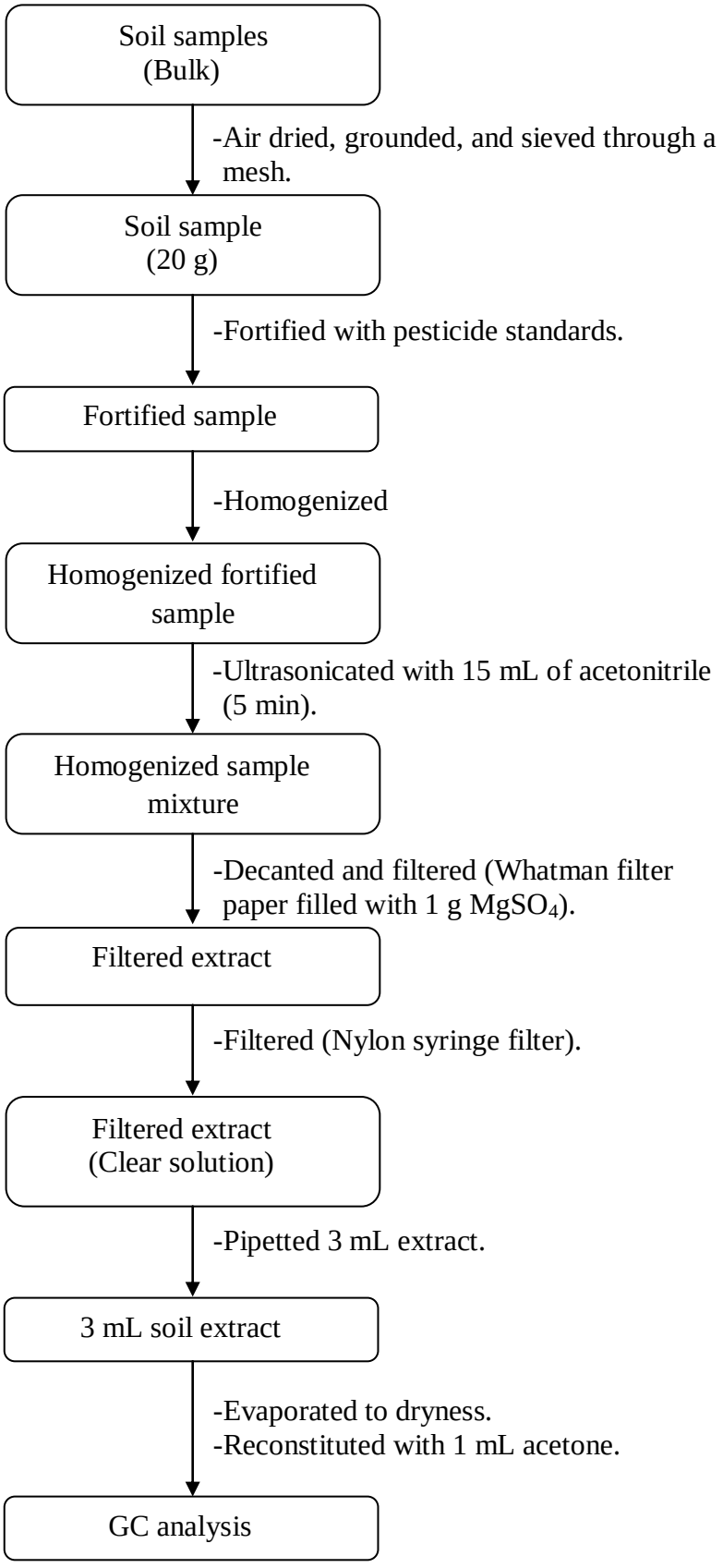

Figure 3.6: Flow chart of an optimized ultrasonic extraction method

3.4.2.1 LINEARITY AND REPEATABILITY

For a quantitative method, it is necessary to determine the range of analyte concentrations or property values over which the method may be applied [111]. Then, a calibration curve was constructed to obtain the linearity of the analytical method. The calibration curve is the relationship between instrument response and known concentration of the analyte [112]. A sufficient number of standards should be used to adequately define the relationship between concentration and response. In this case, seven working standard solutions (0.01, 0.02, 0.05, 0.08, 0.1, 0.5, 1 μg/mL) were analysed by GC-ECD and the signal for each pesticide was measured for its peak area and finally individual calibration plot for cypermethrin and λ-cyhalothrin were constructed. Figures 3.7 and 3.8 display the calibration curves for λ-cyhalothrin and cypermethrin. The figures show that both calibration curves were acceptable with regression coefficients of 0.9988 and 0.9991 respectively for λ-cyhalothrin and cypermethrin, indicating that the technique is quantitative for both pesticides.

As previously mentioned in section 3.3.8, no internal standard was used for quantification in the GC-ECD method since GC auto-sampler was used to inject the samples. Table 3.11 shows the summarized repeatability data for the retention times and peak areas. Overall, the results show that the repeatability of the chromatographic method obtained by the automatic injection was acceptable with the RSD values for peak area and retention time ranged from 1.12 to 1.67 % and 0.0039 to 0.0071 % respectively. Hence, it can be concluded that the injection technique gave small error in the analytical method.

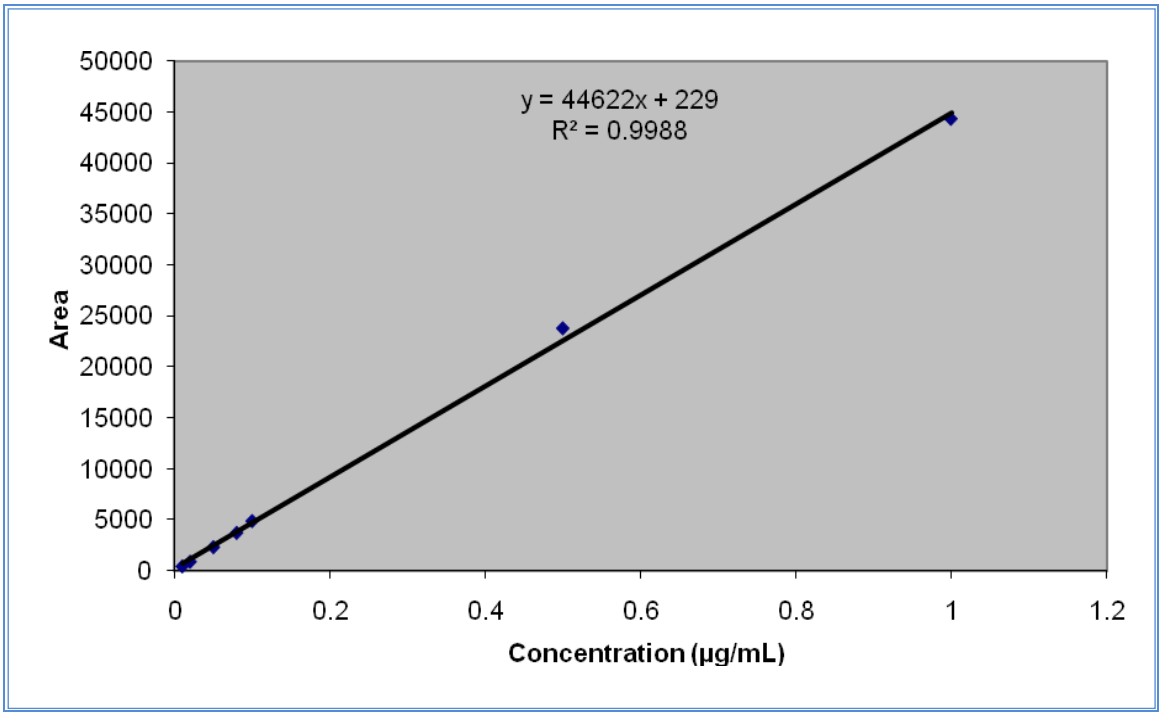

Figure 3.7: Calibration curve of λ-cyhalothrin (0.01 – 1 µg/mL)

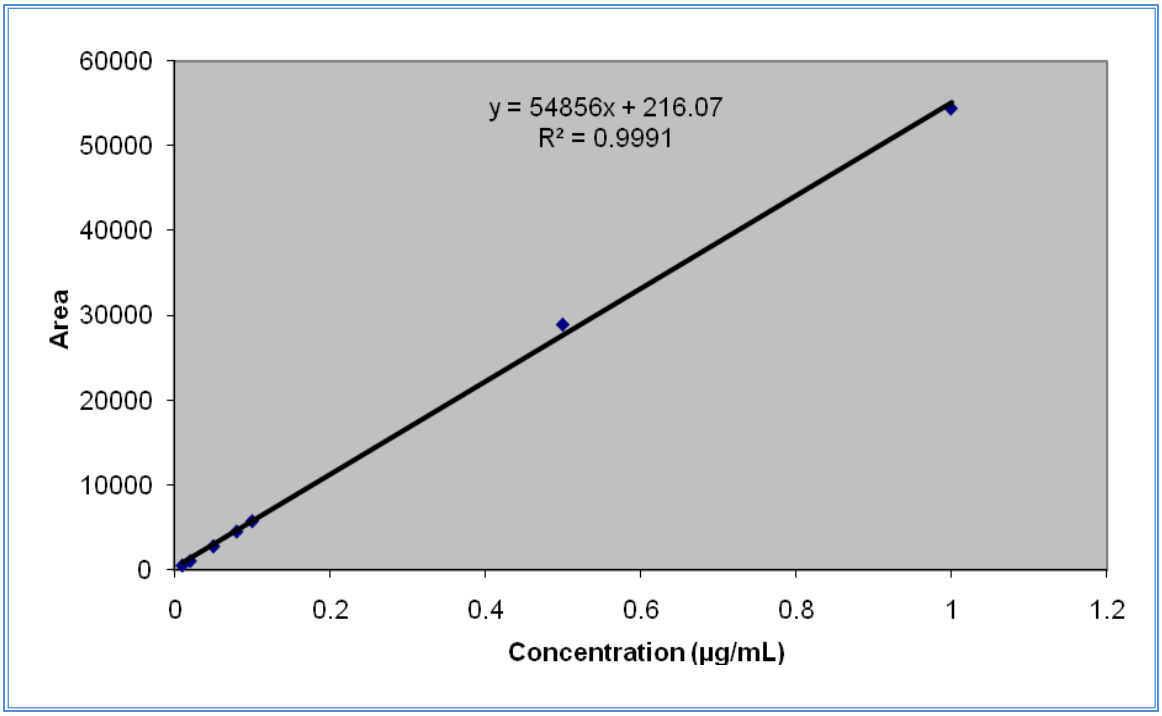

Figure 3.8: Calibration curve of cypermethrin (0.01 – 1 µg/mL)

Table 3.11: Repeatability data (retention time and peak area) of pesticides analyzed in soil and pure solvent (fortified at 0.2 µg/g, n = 10)

Compound	Repeatability (%RSD)			
	t_R		Peak area	
	Soil	Acetone	Soil	Acetone
λ-cyhalothrin	0.0047	0.0043	1.26	1.12
cypermethrin	0.0071	0.0039	1.67	1.25

3.4.2.2 SELECTIVITY/SPECIFICITY

The selectivity of the analytical method in this work was determined by comparing the chromatograms of a blank matrix solution with the fortified matrix solution. Figure 3.9 shows the pesticide standard solutions, blank soil samples, and fortified soil samples by GC-ECD. In the blank samples of soil, few interferences present at the analytes retention times, 14.8 min for λ-cyhalothrin and 26.7 min for cypermethrin. As a result, in the fortified samples, we can see that the analytes of interest were well separated from the other components present in the soil matrix and hence allowed the differentiation and quantification of the analytes. These chromatograms depict that the method developed removes much of the interferences in soil matrices and thus exhibited its selectivity.

Additionally, from the chromatographic point of view, the method presented herein does not require a clean-up step of the soil extracts which was evident from the absence of interfering peaks in the blank (uncontaminated samples). Nevertheless, there are few impurity peaks but they do not hinder the identification and quantification activities. In the meantime, as previously discussed in Chapter 2, the analytical signal

for mixture of isomers (cypermethrin) was obtained by summing the peak areas of all isomers.

3.4.2.3 LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

In this study, blank soil samples were used to establish the detection and quantification limits for each pesticide. The LOD values of the proposed method were determined by analysing the decreasing concentrations of analytes spiked on soil until obtaining a signal/noise (S/N) ratio of 3 while LOQ were derived from LOD values to give a S/N of 10. In this study, the sensitivity of the method proposed was important in order to determine its appropriateness for environmental behaviour and pollution monitoring studies. LOD for the proposed method was found to be 0.0025 μg/g for λ-cyhalothrin and 0.01 μg/g for cypermethrin. Meanwhile, their LOQ values were 0.0075 μg/g and 0.03 μg/g for λ-cyhalothrin and cypermethrin respectively.

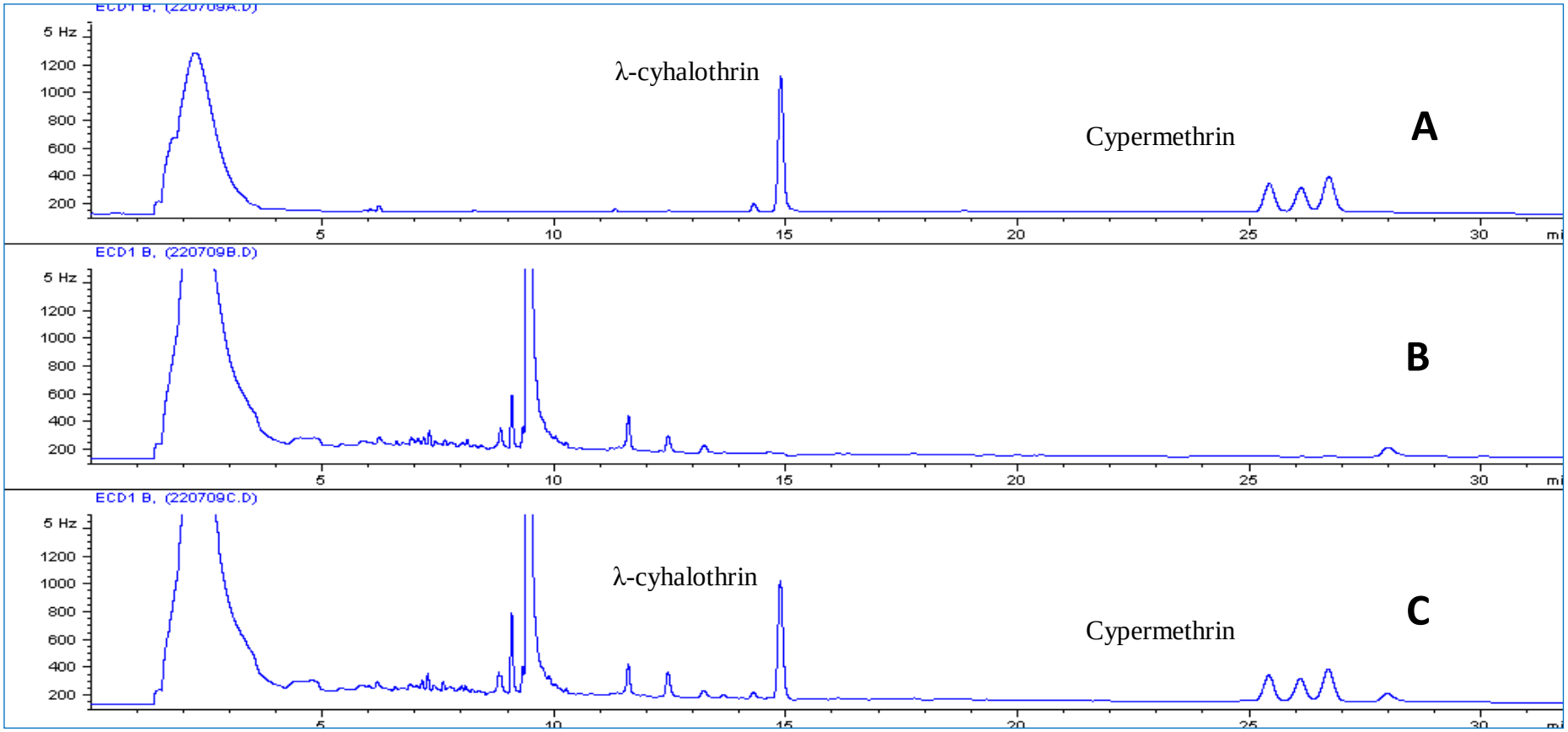

Figure 3.9: Selectivity chromatograms: (A) Pesticide standards in pure acetone; (B) Blank soil sample; (C) Spiked soil sample

3.4.2.4 RECOVERY AND PRECISION

As mentioned earlier, high recovery values were observed during the method optimization steps and were described by the occurrence known as matrix-induced response enhancement effect. This effect could occur for particular pesticides and matrix types, depending on the status of the capillary column [143, 144]. Afterwards, the cause of these effects was found to be related with the extract matrix. This is due to the blank extracts were free of interferences at the pesticides retention time and the same concentration injected in pure solvent gave lower peak areas compared to the recovery standards. Therefore, matrix-matched standards in free residue soil extracts were applied in order to avoid quantitative errors. The homogenized samples were subsequently used as blanks and in the preparation of matrix-matched standards for calibration. To do this, matrix-matched calibration standards were prepared by adding known quantities of standard mixture solutions to the corresponding blank sample extracts at concentrations of 0.01, 0.02, 0.05, and 0.1 µg/g. Then, calculations were performed based on the analysis of matrix-matched standards. The results were compared with the recoveries obtained using standards in pure solvent as shown in Table 3.12.

Table 3.12: Recoveries and relative standard deviation (RSD) of pyrethroids in soil samples using calibration standard in pure solvent and matrix-matched standard

Pesticides	Spike level (mg/kg)	Standard in acetone		Matrix-matched standard	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
λ-cyhalothrin	0.01	107.51	3.59	101.49	3.95
	0.02	109.16	2.18	93.99	2.19
	0.05	98.37	0.66	100.92	0.67
	0.1	108.17	2.57	100.15	2.57
Cypermethin	0.01	104.01	2.86	99.45	2.86
	0.02	117.83	2.50	90.59	2.50
	0.05	101.94	0.46	98.36	0.45
	0.1	134.63	1.70	99.50	1.70

n = 4

In the meantime, calibration curves for standards in solvent were plotted versus calibration curves for matrix-matched standards, and the difference in slope was calculated. Figure 3.10 and Figure 3.11 illustrate the calibration curves for λ-cyhalothrin and cypermethrin. Good linearity was obtained for the pesticides with regression coefficients of 0.9906 and 0.9911 respectively for λ-cyhalothrin and cypermethrin, indicating that the calculation using matrix-matched standards is quantitative for both pesticides.

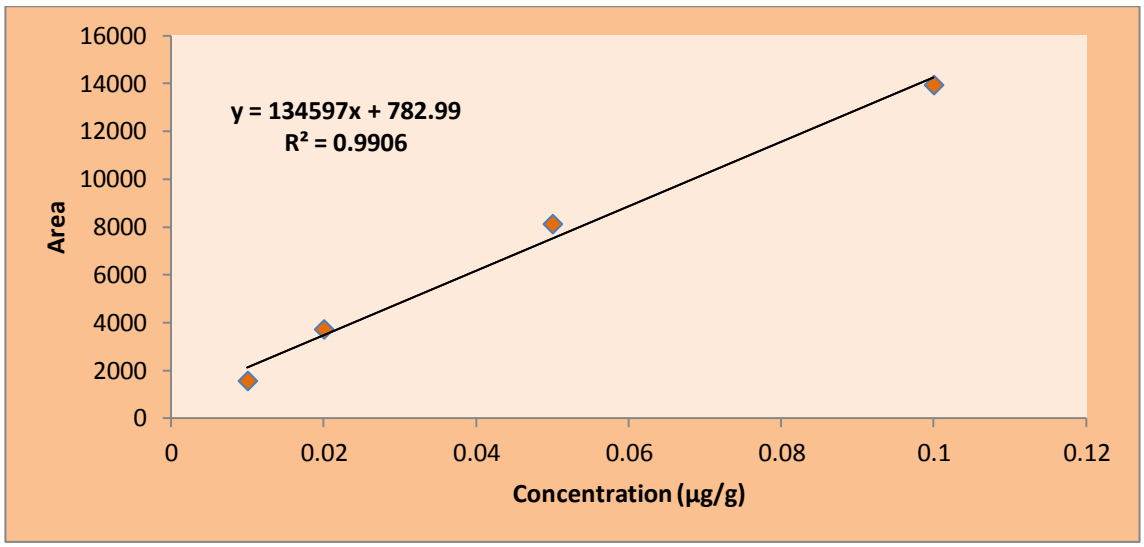

Figure 3.10: Matrix-matched calibration curve of λ-cyhalothrin (0.01-0.1 µg/g)

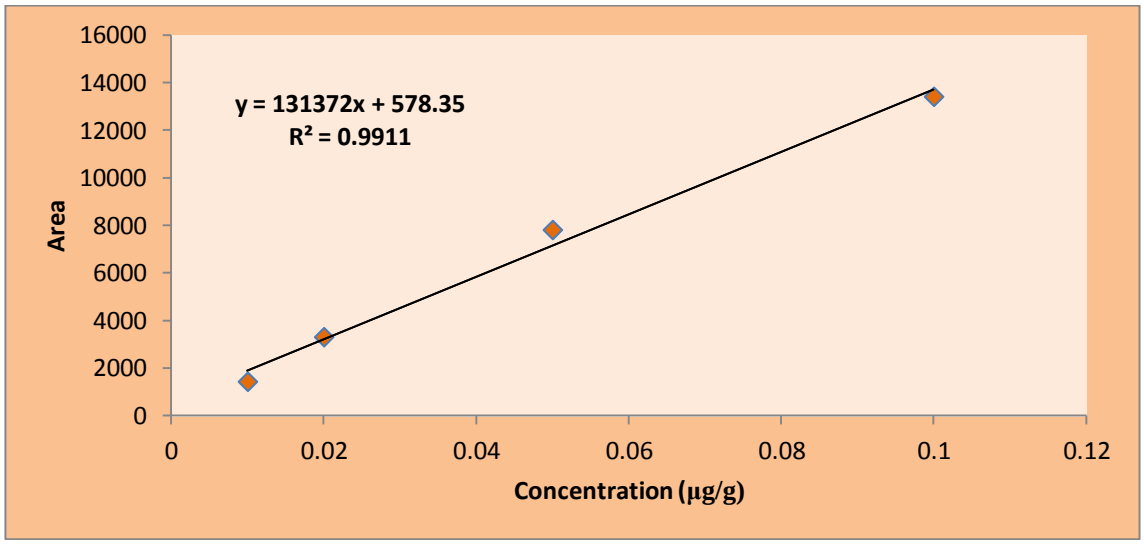

Figure 3.11: Matrix-matched calibration curve of cypermethrin (0.01-0.1 µg/g)

Table 3.13 shows the comparison between matrix-matched standard calibration curve and standard in pure solvent calibration curve. The differences in slope between the two curves were found to be 1.07 and 1.04 for λ-cyhalothrin and cypermethrin respectively. The values indicate that for both pesticides, the calibration curves made using matrix-matched standards gave higher analyte signals compared to calibration curves using standards in solvent. On that account, matrix-matched standards were selected in this study in order to avoid quantitative errors.

Table 3.13: Comparison of matrix-matched standard calibration with standard in solvent calibration

Pesticide	Matrix calibration			Solvent calibration			Slope matrix/slope standard
	Slope	y-Intercept	R^2	Slope	y-Intercept	R^2	
λ-cyhalothrin	134597	782.99	0.9906	125321	546.12	0.99	1.07
Cypermethrin	131372	578.35	0.9911	126030	277.83	0.99	1.04

According to Lentza-Rizos *et al*. [75] the matrix effect was found to be variable from system to system and over time, with the response to standards in solvent alone sometimes being greater (negative matrix effect) and sometimes less (positive matrix effect) than the response to matrix-matched standards. This was in accordance with what was mentioned by Poole *et al*. [140] in which they stated that the variability of results from different laboratories arises because the activity of different injection devices (largely associated with liners) is not constant and is affected by the injector use history. In a sequence of injections each prior injection has the potential to modify the activity of the injector by depositing active matrix components in the liner. Thus, matrix-induced enhancement cannot be considered as solely an analyte effect with an

enhancement value assigned to each analyte but rather it is an analyte property subject to system modification. As system properties are not easy to control or generally easily varied, matrix-induced enhancement values can and do vary widely in absolute terms. In other words, when different instrument is used matrix-induced response enhancement values may not be comparable to those observed for the same samples on another instrument.

From the results obtained in Table 3.12, it was found that the pesticides response from standard solutions in solvent were lower than those obtained from standards in soil extracts (positive matrix effect) and resulted in higher recoveries when calculations were made using standards in pure solvent. In the meantime, when calculations were made using matrix-matched standards, it was found that most pesticides recoveries were all decreased as can be seen in Figures 3.12 and 3.13. In general, the recovery values obtained using this method for both pesticides were lower than the ones previously obtained and were acceptable ranging from 91 to 102 %. The overall RSD values ranged from 0.5 to 3.9 %. Since both the recovery and RSD values meet the method performance criteria, this indicates the good precision and accuracy of the proposed method. In addition, the application of matrix-matched standards in the recovery calculations could in essence compensate for the response enhancement.

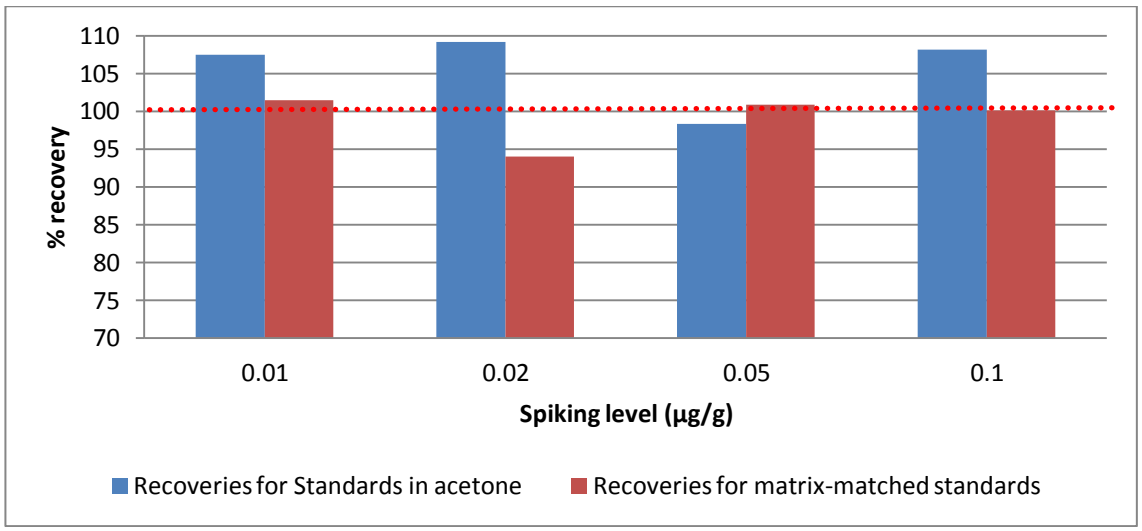

Figure 3.12: Recoveries and relative standard deviation (RSD) of λ-cyhalothrin from soil after quantification using standards in acetone and matrix-matched standards (n = 4)

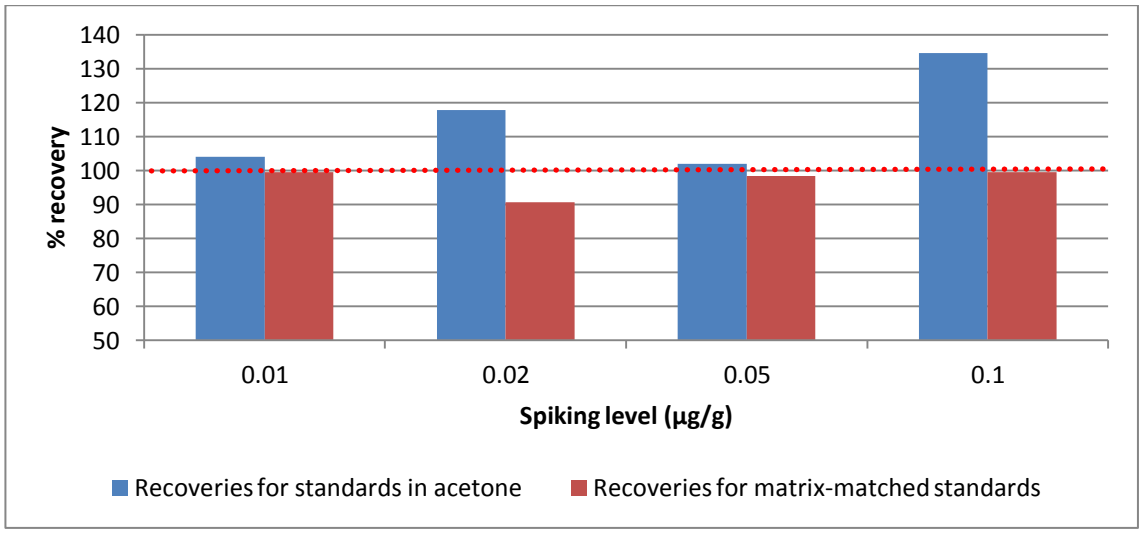

Figure 3.13: Recoveries and relative standard deviation (RSD) of cypermethrin from soil after quantification using standards in acetone and matrix-matched standards (n = 4)

3.4.2.5 RUGGEDNESS/ROBUSTNESS

In the initial tests, variations in the extraction techniques and parameters previously mentioned (extraction solvent, extraction volume, and extraction time) generally had little effect on the mean recovery of both cypermethrin and λ-cyhalothrin. This argument was further backed up by the statistical analysis data. The outcomes showed that when the aforementioned parameters varied, no significant difference was observed among the various parameters studied. This showed that the method was adequately robust to be successfully applied by inexperienced analysts.

3.4.3 REAL SAMPLES MONITORING

The developed method was applied to real soil samples from an oil palm plantation in Labu, Negeri Sembilan, Malaysia. The main objective was to identify the quantities of λ-cyhalothrin and cypermethrin that were contaminating the soil. Each sample was analysed in triplicate following the optimized procedure described previously. At the beginning of each set of samples, analytical grade acetone, standard prepared in pure solvent, blank sample, and fortified sample were analyzed to check whether the system is under the correct conditions. These routine procedures were done in order to:

a) Check any possibility of contamination in the chromatograph that could cause false positive.
b) Check the performance of the ultrasonic extraction procedures (acceptable recoveries at 70-120 %).
c) Check the response of the detector to avoid errors in quantification caused by instrument fluctuation.

From the monitoring study, it was found that the sampling area was a very low contaminated site since neither λ-cyhalothrin nor cypermethrin residues was detected using this method, indicating that these soil samples did not contain any of the pesticide residues studied. As mentioned in the previous chapter, the results also confirmed that the application of agrochemicals on oil palm in plantations, especially cypermethrin and λ-cyhalothrin is according to the label instructions and the harvesting according to GAP.

3.5 CONCLUSION

Sample extraction based on ultrasonic extraction (USE) was successfully developed to determine λ-cyhalothrin and cypermethrin in soil. In the preliminary studies, three extraction parameters, namely extraction solvent, extraction volume, and extraction time were earlier optimized for the USE procedure. Statistical evaluation utilizing the single factor ANOVA test indicated that the differences between the methods of extraction in most cases were not significant. Acetonitrile was chosen as the solvent of choice to extract the pesticides from the soil matrix. The comparison of different extraction solvents for USE showed that acetonitrile gave the best precision, the least matrix-enhancement effect, acceptable recoveries, and clear extracts. For these reasons, acetonitrile was selected as an extraction solvent in further optimization experiments. Then, increasing the volume of acetonitrile from 15 mL to 20 mL and finally to 25 mL gave no significant differences on the quantities of pesticides extracted in ultrasonic procedures. Therefore, in order to reduce the solvent consumption and the cost of the overall procedure, 15 mL acetonitrile was selected as an optimum volume in further optimization experiment. Last but not least, it was found that since longer extraction time could result in the possibility of degradation of some analytes when using USE, the optimum sonication time that gave efficient extraction and high recoveries was selected as 5 minutes.

The optimized method also went through the validation studies where validation parameters, namely linearity (calibration curve), selectivity/specificity, sensitivity (LOD, LOQ), recovery (accuracy), and precision (relative standard deviation) were applied. Recoveries obtained for both pesticides ranged from 91-102 % with RSD values of 0.5-4 %. The sensitivities of the method were acceptable with LOD of 0.0025 µg/g and 0.01 µg/g for λ-cyhalothrin and cypermethrin respectively. Matrix effects commonly encountered in the determination of pesticide residues were avoided in this study by constructing calibration graphs with soil as matrix (matrix-matched standards). In this study, matrix-matching of standards is considered to be necessary for the reliable quantification of both λ-cyhalothrin and cypermethrin in soil.

The major advantage of ultrasonication is the much lower extraction time and the elimination of an additional clean-up stage involving additional glassware and apparatus [91]. This method gave good extraction efficiency, precision, and recovery of both λ-cyhalothrin and cypermethrin combined with little, fast, and simple sample extraction procedures. Additionally, the method proposed does not require any clean-up step since there was no interfering peaks in the blanks at the analytes retention time in the chromatogram. The method also introduces low solvent consumption and therefore reduces the risk for human health and the environment. It also produces an improvement in comparison with other USE methods [118, 119, 122, 127, 113, 128-130]. Last but not least, although not automated, simultaneous extraction of up to eight samples can be easily handled thus makes it an ideal technique for laboratories involved in analyzing a large number of soil samples.