CHAPTER 2 METHOD DEVELOPMENT FOR DETERMINATION OF PESTICIDES IN PALM OIL MATRICES

Oil palm fruitlets

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2.1 INTRODUCTION

Nowadays, one of the most economical and efficient pest control method in oil palm plantations is the use of selective pesticide which kills or retards the growth of certain plants, weeds and insects, without causing particular harm to other organisms. In that sense, the use of insecticides in oil palm plantations is minimal compared to other pesticides such as herbicides and fungicides. This is because insecticides are only applied when there is an insect attack which numbers exceeds certain threshold level.

One of the important impacts of pesticide application for crop protection is on food safety. Residues of the pesticide could persist for a long time in the crop and finally in the food products. In the case of palm oil, although one of the steps in the processing of the crude palm oil is to obtain refined bleached deodorized palm oil (RBDPO) involves water washing, which removes most of the water soluble pesticides such as herbicides, some of the insecticides used in oil palm plantations are lipid soluble. These compounds are lipophilic, with high n-octanol-water partition coefficients ($K_{o/w}$). Most of them are less soluble in water, suggesting the residues may concentrate in the oil during the extraction from the fruit of oil palm.

Some of the lipophilic insecticides used in oil palm plantations are cypermethrin and λ-cyhalothrin. They are both non-polar pesticides with high $K_{o/w}$ values which mean that they are not easily washed off by water during the processing steps. Consequently, pesticide residues breakthrough could occur during the pressing of the oil out from the fruits. Hence, pesticide residues in palm oil and palm kernel oil constitute an important parameter of the quality of these edible oils. Monitoring the residue level of pesticides in palm oil is one of the aims of developing methods. This is important to ensure that palm oil is free from chemical residues, safe for human consumption and particularly meet the pesticide residue regulatory requirements of importing countries [68].

The identification of pesticide residues in food with high fat content such as vegetable oils is a difficult and challenging task since the inherent complexity of the matrix could interfere in the determination and quantification of the target analytes. Taken into account that some of the pesticides used are lipid soluble non-polar compounds, they tend to concentrate and remain in the oil throughout the processing steps. Furthermore, the matrix also stabilizes and protects the compounds of interest from degradation or oxidation phenomena, thus making possible the persistence of these compounds even at low concentration levels for long periods [69]. This urges the need to develop more rigorous extraction and clean-up steps in order to minimize or if possible, complete removal of the co-extraction of fatty materials from the sample.

It is well known that the main problem associated when dealing with these kinds of matrices is that dirty extracts with even a small amount of fats may disrupt the columns and harm the detectors, hence, upsetting the right analyte determination through signal suppression. The presence of high concentration of fatty acids in the samples may complicate the GC detection system of low pesticide concentrations. This

is due to the presence of high matrix peaks in the chromatograms that mask the analytes of interest. Although it has been a practice for some laboratories to change the column at intervals, it is very costly to change the detector. Consequently, further purification of the extract is required before the analytes determination. This step is called a clean-up step and aims at the isolation of the target analytes from potentially interfering co-extractives as well as discarding the extraction solvent and preparing the target analytes in an appropriate chemical form for its characterization and quantification [70]. Therefore, sample pre-treatment and extraction procedures are the key steps and bottleneck where most of errors occur in this kind of analysis.

In principal, method development of pesticide residues involves the development of strategies to isolate or extract the pesticide fraction from the whole fatty matrix. This procedure comprises extractions of analytes from their bulk matrix into an appropriate solvent, followed by removal of potentially interfering substances from the solvent extracts to small volumes prior to analysis. This choice of sample pre-treatment is related to the detection method since the more sensitive and specific detection method is used, the less stages of sample treatment will be required [70]. For instance, the non-selective detectors such as NPD, FID or ECD in gas chromatography (GC) or UV in liquid chromatography (LC) require more rigorous and thorough sample pre-treatment compared to more sensitive high-end mass spectrometric techniques such as tandem mass spectrometry with triple quadrupole (QQQ) or quadrupole time-of-flight (Q-TOF) instruments attached to GC or LC. Finally, it should be noted that it is desirable that the chosen method involves low solvent consumption, to be environmentally friendly and also safer to apply by the analyst [71].

The technique of choice for determination and quantification of pesticide residues currently revolves around the use of chromatographic methods, especially gas chromatography (GC), high-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC). These chromatographic methods coupled to the development of various detectors such as flame ionization (FID), nitrogen-phosphorus (NPD), flame photometric (FPD), electron capture (ECD), and mass spectrometric (MS) for GC and ultraviolet (UV), diode array (DAD), fluorescence (FD), and mass spectrometric (MS) for HPLC, have turned these methods into highly successful analytical tools for pesticide residues analysis.

Recently, two reviews of analytical techniques for sample pre-treatment in fatty vegetable matrices have been documented for various pesticide residues [70, 72]. The reviews address the main sample treatment methodologies for pesticide residue analysis in fatty vegetable matrices and numerous vegetable oils. From the reviews, olive and olive oil represent the most frequently analysed samples for pesticide residues, followed by soybean and soybean oil, avocado, sunflower oil, corn oil, and other vegetable oils. What's more, currently the most popular methods involve the use of one or the combination of some of the following techniques for both the sample extraction and clean-up steps: liquid-liquid partitioning, adsorption chromatography, gel permeation chromatography (GPC), solid-phase extraction (SPE), and matrix solid-phase dispersion (MSPD) [70]. Interestingly, according to the reviewers, more than 70 % of the methods discussed were based on liquid partitioning with organic solvents followed by a clean-up with either SPE or GPC.

Determination of halogenated pesticides such as pyrethroids is usually carried out by gas chromatography with electron capture or mass spectrometry detection. Many methods have been reported in the literature for the determination of pyrethroid residues in fatty matrices employing various extraction and clean-up procedures using analytical techniques such as gas chromatography with ECD, MS or tandem MS detection [73-84]. However, a further clean-up is generally required for the determination using electron capture detection, because of excessive interferences in the chromatogram from matrix components and the need to protect the chromatographic system from the losses of efficiency and sensitivity resulting from the presence of traces of fatty materials. It has been estimated that a fat residue of less than 0.25 mg/mL is required for ECD analysis [85].

In the late 1990's, two types of methodologies with different approaches (SPE and GPC) were reported for pyrethroids in vegetable oils and butter fat [73, 74]. Ramesh *et al*. studied the efficiency of purification of pyrethroids by SPE using graphitized carbon black (GCB) as the sorbent in vegetable oils (groundnut oil, soybean oil, sunflower oil, olive oil) and butter fat [73]. The authors proposed a straightforward approach in sample clean-up using SPE without a preliminary acetonitrile-hexane partitioning. In this work, sample purification was achieved by direct introduction of the oil samples to the SPE column packed with GCB. Analytes were eluted from the column with acetonitrile, followed by concentration and reconstitution with acetone. Finally, quantification was achieved by GC with electron capture detection. The method was studied using seven highly persistent pyrethroid insecticides (cypermethrin, deltamethrin, fenvalerate, cyfluthrin, allethrin, cyhalothrin, and permethrin) with the recoveries in the range between 86% and 105%. An interesting point in their research is that the addition of methyltrioctylammonium chloride (MTOAC) to the oil samples,

prior to sample extraction. The authors reported that MTOAC plays an important role in the pre-concentration of pyrethroid molecules from oil/high fat content samples. Nevertheless, the actual role of MTOAC in the separation technique has yet to be established.

Based on gas permeation chromatography technique, Di Muccio *et al*. [74] proposed a method for pyrethroid insecticides in soya oil using a combination of a solid-matrix dispersion partition followed by high-performance size-exclusion chromatography on a mini-column of 7.8 mm I.D. They reported 99.8% of fatty matrix removal with acceptable quantification for 9 out of 14 pyrethroids tested. The chromatograms obtained via GC-ECD for soya oil extract were quite nasty with several interferences still remained, preventing quantification (fluvalinate, permethrin) and low recoveries (λ-cyhalothrin, esfenvalerate, tralomethrin) for some pyrethroids. This is due to insufficient sample pre-treatment which allowed co-extract of the contaminants and hence masking the analyte signals. On the other hand, the recovery of cypermethrin was 72.6%.

Both of these methods (SPE and GPC) are the most commonly applied analytical techniques for pyrethroid extraction in vegetable oils [73, 75, 76, 78- 80, 83, 84, 86]. These clean-up steps were usually combined with preliminary liquid-liquid partition with either acetonitrile-hexane or acetonitrile alone. Both methods have their advantages and disadvantages. SPE is vastly used in sample purification technique in various matrices for different types of analytes, mainly for clean-up purposes after the extraction steps of the methodology. Amongst the advantages of SPE over liquid partitioning procedures are higher precision and throughput, lower solvent consumption, and avoiding the formation of emulsions [70]. Additionally, SPE can be easily

incorporated into automated analytical procedures with relatively simple and inexpensive equipment, which can lead to greater accuracy and precision and higher laboratory throughput [87]. On the contrary, this method is time consuming and laborious, since some SPE techniques involve many steps (sorbent conditioning, sample addition, washing, and analyte elution) with some manual stages that need continual analyst attention.

On the other hand, GPC is a technique equipped with polymeric porous microspheres column which enables the separation of compounds according to their molecular weights. Thus, base on this principle, low molecular weight pesticide fraction is separated from the high molecular weight triglycerides fractions. According to Gilbert-López *et al*. [70], clean-up using GPC after a preliminary liquid-liquid partition with acetonitrile is currently one of the more regularly applied technique in routine laboratories for the analysis of pesticide residues in vegetable oils by GC with different detectors (ECD, NPD, MS). Nevertheless, direct extraction and clean-up of pesticides from vegetable oils without preliminary liquid-liquid partition step is not uncommon. The employment of GPC alone without initial partitioning step is highly not recommended since it may cause adverse effects to the columns and chromatographic systems. Therefore, liquid-liquid partitioning is the step to be considered when developing method based on GPC techniques to achieve cleaner extracts. One of the reasons why GPC technique is favoured compared to other techniques is because of its high degree of automation using auto-sampler injection and on-line GPC-GC coupling. This GPC-GC coupling concept is achieved by on-line transfer of pesticide-containing fraction from the GPC fractionation step to the gas chromatograph using a loop-type interface. In contrast, the major disadvantage of this technique is the partial overlapping between the pesticide fraction and the components from the matrix since thorough

optimization of the GPC condition is needed and sometimes it is difficult to fully separate the pesticide fraction from the matrix. Furthermore, GPC instrument is more expensive with high maintenance cost, while the large amount of toxic solvents consumed per analysis makes this technique not very environmental friendly.

Some authors chose GPC extraction and clean-up technique for pyrethroid analysis in vegetable oil since it was automated and many samples could be analysed per day [74, 78, 80, 84]. Others adopted the more economical and environmental friendly SPE technique which required less solvent [73, 75, 76, 79, 83]. Barrek *et al*. [84] described the development of a method for analyzing pesticide residues in olive oil by GC-MS and HPLC-MS, using GPC technique. Twenty pesticides were separated and analyzed by GC-MS, while the other 11 were analyzed by HPLC-MS in electrospray ionization mode. In this work no liquid-liquid extraction was adopted prior to GPC clean-up technique. Fortified oil sample was dissolved with tetrahydrofuran and homogenized in an ultrasonic bath. Then, the fraction of the diluted oil sample was injected into the GPC instrument equipped with two sets of column in series and eluted in tetrahydrofuran (THF) at a flow rate of 1 mL min^{-1}. The recoveries achieved for cypermethrin and λ-cyhalothrin from olive oil were 91.5% and 99.1% respectively. Nevertheless, some of the pesticides (EPTC, fenthion, methidathion and acrinathrine) studied gave low recoveries. In the case of acrinathrine, it has a larger steric volume close to that of triglycerides, making its separation from the matrix difficult and explaining its low recovery yield. This is the perfect case which revealed one of the primary disadvantages of this technique.

García Sánchez *et al*. [80] reported a method for the multiresidue analysis of 26 pesticides in olive oil using a combination of liquid-liquid extraction (acetonitrile-hexane) and gel-permeation chromatography clean-up. In this study, the efficiency of the mobile phase for the GPC was studied. According to the authors, dichloromethane was found to provide the most efficient mobile phase among the seven organic solvents studied (*n*-hexane, cyclohexane, petroleum ether, dichloromethane, diethyl ether, acetonitrile and methanol), with pesticide collection window between 14 and 23 min. The recoveries obtained for all 26 pesticides were satisfactory and ranged between 84% and 110% while the recoveries for λ-cyhalothrin and cypermethrin were 86-103% and 89-105% respectively. Additionally, the application of the optimized method to the real olive oil samples revealed that only endosulfan sulphate was detected in the refined olive oil, while diuron, terbuthylazine, endosulfan sulphate and diflufenican were all detected in the virgin olive oil.

A similar extraction procedure was adopted by Ballesteros *et al*. [78] for the determination of pesticide and polycyclic aromatic hydrocarbon residues in olive and olive-pomace oils in a single injection by GC-MS^2. Based on the earlier work of Sánchez *et al*. [80], oil samples were previously extracted with an acetonitrile/*n*-hexane mixture and cleaned up by GPC. The application of electronspray ionization and chemical ionization allow pesticides and polyaromatic hydrocarbons (PAHs) to be determined in a single analysis. Furthermore, using the optimized operating conditions, pesticide and PAH residues would be present in the same fraction following clean-up and gel permeation chromatography. All the recoveries exceeded 84% and mostly around 90% for the analytes studied. The recoveries for λ-cyhalothrin and cypermethrin were 86-108% and 94-109% respectively.

Besides GPC, SPE is one of the most popular techniques in sample pre-treatment of pesticide residues in food matrices. Amvrazi *et al*. [79] evaluated a comprehensive study on different extraction and clean-up procedures based on the classical liquid partitioning and SPE technique for the multiresidue determination of 35 pesticides in olive oil by GC with nitrogen phosphorus detection (NPD) and electron capture detection (ECD). In this study, three different liquid-liquid extraction procedures based on (i) partition of pesticides between acetonitrile (ACN) and oil solution in n-hexane, (ii) partition of pesticides between saturated ACN with n-hexane and oil solution in n-hexane saturated with ACN, and (iii) partition of pesticides between ACN and oil, were tested and evaluated for the optimization of the highest pesticide recoveries with the lowest oil residue in the final extracts. Then, different types of SPE sorbents (N-Alumina, Florisil, C18, Envi-Carb, Diol, CN, Ph, and NH_2) were tested for their efficiency in the clean-up steps. The authors proposed the liquid-liquid extraction of the oil solution in n-hexane with acetonitrile followed by a SPE clean-up of the extract using GCB for the organophosphorus and triazine compounds gave the highest recoveries of all of the pesticides studied with less oil residues in the sample, while for pyrethroids and organochlorine compounds, the acetonitrile extract (from the GCB) was additionally cleaned through a Diol-SPE cartridge. The recoveries for λ-cyhalothrin and cypermethrin were 86-91% and 105-108% respectively. The developed method was then applied in the study of pesticide residues assessment in different types of olive oil and preliminary exposure assessment of Greek consumers to the pesticide residues detected [83].

Recent advances focus on the use of a combination of two or more commercially available SPE sorbents for clean-up [88]. Recently, the use of primary secondary amine (PSA) and graphitized carbon black (GCB) for the SPE clean-up of fatty acid matrix

components from food extracts in multiresidue pesticide analysis was evaluated [88, 89]. Shimelis *et al*. [88] underlined the use of dual layer SPE, a primary-secondary amine in combination with graphitized carbon black for sample clean-up during multiresidue pesticide screening of agricultural and food products. The retention of fatty acids (palmitic acid, linoleic acid, oleic acid, and stearic acid) by the PSA sorbent was quantified and the effect of the elution solvent on the retention of fatty acid on the SPE cartridge was evaluated. According to the authors, the use of stronger elution solvents to elute certain pesticides from GCB was shown to interfere with the capacity of PSA to bind fatty acids. Since GCB has a strong affinity for planar molecules, this sorbent usually applied in sample clean-up step to effectively removes pigments such as chlorophyll, carotenoids, as well as sterols in foods especially vegetable oils. The authors suggested that carbon may contribute to the retention of fatty acids by the dual layer SPE when a mixture of acetonitrile:toluene (3:1) solvent is used, and therefore, be beneficial to overall sample clean-up. Hence, practical applications of dual-layer GCB/PSA cartridges with acetonitrile:toluene (3:1) elution solvent should be limited to food samples with low levels of fatty acids since only the use of 100% acetonitrile as an elution solvent maximizes PSA capacity for retention of fatty acids. The addition of toluene can significantly weaken this binding ability. With a few exceptions, pesticide recoveries were between 85% and 110% including cypermethrin, and sample-to-sample differences of less than 5% were achieved, demonstrating the versatile suitability of the dual-layer SPE to sample clean-up. λ-cyhalothrin was not the compound of interest in this study.

An almost identical study was documented by He *et al*. [89] on the use of PSA and GCB for the SPE clean-up of food extracts in pesticide residues analysis. They investigated the influence of elution protocols on the capacity of PSA for removal of

fatty acids. In this study, the authors claimed that when PSA is combined with GCB to remove pigments, the capacity of PSA for removal of fatty acids is dramatically reduced and the degree of the decrease is highly dependent on the conditioning and elution protocols. The use of toluene, hexane and /or acetone in the elution steps severely reduces the capacity of PSA for removal of fatty acids. This finding was in the accordance with what was reported by Shimelis *et al*. [88]. The difference between these two literatures was the used of GCB/PSA sorbents, whether two cartridges in serial [89] or in dual layer form [88]. He *et al*. also claimed that the applications of GCB/PSA dual layer should be limited to non-fatty foods and/or food with a low amount of fat, but if GCB has to be used for removal of colours and sterols, they suggested that PSA cartridge should be used with carbon cartridge in serial.

Esteve-Turrillas *et al*. [76] proposed a method using combined solid-phase extraction and tandem mass spectrometry detection for the determination of 11 pyrethroid insecticide residues in vegetable oils (olive oil, sunflower oil, corn oil, and soybean oil). The authors tested several types of sorbents such as Florisil, alumina, C_{18}, and GCB in order to minimize fat residues. From the study, they suggested that the combination of basic alumina and C_{18} solid-phase extraction clean-up with preliminary acetonitrile:hexane liquid-liquid extraction gave the most effective clean-up which provided an oil residue of 2.2 mg (0.04%,w/w), allowing the gas chromatographic determination of pyrethroid insecticides without interference peaks. Furthermore, the authors also recommended the use of the whole partition acetonitrile extract from the liquid-liquid partitioning step as an SPE elution solvent, since this technique would avoid the solvent change or evaporation step; reducing analysis time, solvent consumption, and possible evaporation losses of analyte. Their results revealed that the

pyrethroid recoveries varied from 91% to 104% with the recoveries for λ-cyhalothrin and cypermethrin were 92%-104% and 95%-104% respectively.

Most of the techniques discussed earlier involve the use of liquid-liquid partitioning extraction using acetonitrile and hexane as solvents prior to cleanup with either SPE or GPC. But recently, classic liquid-liquid partitioning extraction has been replaced by a more cost-effective extraction technique, low-temperature precipitation. Lentza-Rizos *et al.* [86] were the first to introduce the low-temperature extraction in olive oil. This extraction technique was later applied to determine organophosphorus pesticides in soybean oil, peanut oil, and sesame oil [90]. Afterward, modification was made to the low-temperature technique by adding the extra clean-up steps via solid-phase extraction (SPE) [75] and dispersive solid-phase extraction (d-SPE) [82]. The clean-up steps were necessary in order to obtain cleaner sample extracts by removing of excessive interferences in the chromatogram from matrix components for the determination using electron capture detection.

In the earlier work by Lentza-Rizos *et al.* [86], they developed a simple, extremely low-cost method using low-temperature lipid precipitation for the rapid analysis of virgin olive oil for organophosphorus insecticides and triazine herbicides commonly used in olive groves. The method gives a good clean-up for GC analysis with nitrogen-phosphorus detection and the recoveries were between 77% and 104% with RSD values of 7-16%. Later, the same extraction strategy was used in addition with SPE clean-up step for the determination of endosulfan and pyrethroid (cypermethrin, deltamethrin, fenvalerate, λ-cyhalothrin, and permethrin) insecticides in virgin olive oil using gas chromatography with electron-capture detection [75]. In this study, two initial extraction techniques were evaluated with both gave an extract in acetone equivalent to

1 g oil/mL. The first technique involved the classical partitioning of virgin olive oil between hexane and acetonitrile phases, while the second technique involved the partitioning of oil and acetonitrile followed by removal of the oil by precipitation at -20ºC. Furthermore, they also studied the ability of different sorbent materials (Isolute Florisil, Silica gel, alumina-N and Sep-Pak alumina-N) to remove traces of oil remaining in extracts of olive oil after initial extraction step. According to the authors, the low-temperature method was chosen as the method of choice since this method proved to be considerably faster and more cost-effective. Although both methods gave acceptable results in terms of recoveries, Sep-Pak alumina-N cartridge with the acetonitrile solvent system provided a simple and satisfactory clean-up procedure. The recoveries obtained varied from 71% to 91% while the recoveries for cypermethrin and λ-cyhalothrin were 80% and 84% respectively. The authors claimed that the method developed was simple, inexpensive, efficient, and particularly it consumed only small amount of solvent.

Li *et al.* [90] proposed an almost similar method of extraction for the multi-residue determination of 14 organophosphorus pesticides in soybean oil, peanut oil, and sesame oil by gas chromatography with flame photometric detector (FPD). The finding indicated that different matrix influenced the response and retention time of pesticides studied, and matrix-matched calibration standards were recommended to be used in order to counteract the matrix effect. The study showed that the analytical signals of 14 pesticides, retention time and peak shape of fenamiphos influenced by the matrix effect. The recoveries obtained were in the range from 51.3% to 112.4% with RSDs less than 14.9%. The same authors proposed the same approach for the determination of 28 analytes from various types of pesticide in soybean oil by gas chromatography mass spectrometry (GC-MS) [82]. Pesticides of low molecular mass were separated from the

fatty matrix which has a high molecular mass, by using low-temperature fat precipitation, followed by a clean-up process based on dispersive solid-phase extraction with primary secondary amine and C_{18} as sorbents, and magnesium sulphate for the removal of residual water. The authors also compared the efficiency of extraction step between liquid-liquid partitioning and low-temperature precipitation. In this study, the two procedures gave similar recoveries but low-temperature extraction proved to be much faster and easier. Additionally, this technique also avoids the use of hexane, which is very expensive and toxic to humans [82]. Meanwhile, the use of dispersive-SPE could effectively reduced time, expense, and hazardous waste. The recoveries of most pesticides were acceptable with the recoveries for cypermethrin and λ-cyhalothrin was 55-71% and 64-82% respectively.

Besides SPE and GPC based technique, other pesticide extraction technique that has been applied for pyrethroid analysis in fatty matrices is matrix solid-phase dispersion (MSPD). MSPD is an SPE based strategy in which a fine dispersion of the matrix is mixed with a sorbent material (C_{18}, alumina, Silica, etc.) with a mortar and a pestle [70]. Some of its advantages compared to other techniques are possible elimination of emulsion formation, solvent consumption is substantially reduced, enhanced extraction efficiency of the analytes since the entire sample is exposed to the extractants, and finally it can be used to extract analytes from both solid and liquid samples. On the contrary, the main disadvantage of this extraction technique is the lack of automation of the procedure. Ferrrer *et al.* [77] developed and evaluated a novel analytical approach based on MSPD for the quantitative analysis of a selected group of widely used pesticides which could be found at trace levels in olive oil and olives. MSPD was used as a clean-up technique with a preliminary liquid-liquid extraction of petroleum ether saturated with acetonitrile and acetonitrile saturated with petroleum

ether. Aminopropyl was used as sorbent material with a clean-up performed in the elution step with Florisil, followed by mass spectrometric identification and quantification of the selected pesticides using both GC-MS and LC-MS^2. The recoveries obtained in this research were between 85% and 115% with the recoveries for cypermethrin were in the range between 103 and 126%.

Kodba *et al.* [81] proposed a new, single-step extraction and purification method developed for the separation of 26 organochlorine pesticides, three pyrethroid pesticides and six polychlorinated biphenyls (PCBs) from fatty foods of either animal or vegetable origin. The method included homogenisation of extracted fat and diatomaceous earth. Separation was achieved using a mini Pasteur pipette where MSPD technique was carried out with only 5 mL of dimethylsulfoxide as an eluting solvent. A Pasteur pipette was joined to a pre-packed slurry filled Florisil column, water deactivated to 15% where a liquid-liquid extraction and adsorption chromatography successively took place. The elution of the analytes was performed with n-hexane/diethyl ether. Excellent recoveries were obtained for pyrethroid pesticides, mostly above 80% with recovery for cypermethrin was 91% with RSD value of 9%. The authors claimed that the developed method was more advantageous than the conventional extraction and purification methods currently used for the sample preparation due to its greater sample throughput, simplified sample preparation, and shorter sample preparation time.

Although many references are devoted to the development of analytical methods to determine pesticide residues in vegetable oils especially olive oil, very few reports documented the method for analyzing pesticide residues in palm oil [91-102]. Previous studies of pesticides method development in palm oil matrices dealt with OC, OP, paraquat, glyphosate, deltamethrin, glufosinate ammonium, and fluroxypyr. Currently,

cypermethrin and λ-cyhalothrin were not considered in the same matrices. Ainie *et al.* [91, 97] evaluated the feasibility of the method developed by Imperial Chemical Industry (ICI) in determining paraquat residue in palm oil and palm oil products using ion exchange column chromatography and determination by spectrophotometer measured at a wavelength of 396 nm. In this study, the method used for paraquat analysis was an adaptation of the ICI United Kingdom method for oil containing crops such as rapeseed, sunflower seed, olives and grain, vegetables, fruits and others. Two different types of cationic exchange resins, Duolite and Amberlite, were studied for paraquat recoveries from the oil matrix. From the study, the percentage recoveries ranged from 50% to 83% using Duolite resin and greater than 90% when Amberlite resin was used. The estimated limit of detection based on recovery data of this experiment was 0.01 µg/g. According to the authors, the ICI method could be applied for determination of paraquat residue in palm oil and palm oil products with Amberlite resin that gave better recoveries compared to Duolite resin.

The same authors studied the application of gel permeation chromatography to separate monocrotophos from RBD palm olein without preliminary liquid-liquid extraction [93]. They reported a straightforward approach in GPC extraction technique by direct introduction of the diluted oil into the GPC system. Fortified oil sample was diluted with the elution solvent (cyclohexane:ethyl acetate [1:1]) and fraction of the diluted oil sample was injected to the GPC instrument equipped with Bio-Beads SX-3. Elution solvent was pumped through the column at a constant flow rate of 2.0 mL/min. Determination of monocrotophos was done by gas chromatography with flame photometric detector. The recoveries obtained in this research for monocrotophos ranged between 74% and 102% with RSD of 3.5% - 13.5%. The limit of detection was 0.01 µg/mL. Their finding showed that the GPC approach without an extra Florisil or

Alumina column clean-up was suitable for the extraction of monocrotophos residue in oil matrix. They also suggested that the developed method could be used as a multi-residue clean-up technique for all organophosphorus pesticides in oil matrix. Other studies by the same group of researchers dealt with glyphosate and deltamethrin residues in palm oil matrix using cation exchange chromatography and GPC technique respectively [98, 99].

Norizah [102] employed the QuEChERS technique for the determination of glufosinate ammonium in palm oil matrix. This technique is also known as a dispersive solid-phase extraction (d-SPE). It is one of the well known and popular techniques at the moment employed in pesticide residues and other contaminants in food analysis. It was first introduced in 2003 by Anastassiades *et al.* [103]. This technique is based on a liquid partitioning of the oil sample with acetonitrile followed by a dispersive SPE clean-up with mixture of different sorbents (C_{18}, PSA, GCB, etc.). These steps constitute a clean-up procedure that uses shaking, centrifugation, and dispersive SPE. Amongst main advantages of this approach are its simplicity, cheap disposable reagents and materials, small volume of organic solvent consumed, and high throughput. At the moment, Norizah [102] was the first to apply this technique in palm oil matrix using HPLC-MS^2. In this study, the recoveries of glufosinate ammonium from fortified samples were in the range of 77% to 109% with RSD values of less than 10%. Additionally, the limit of detection of the method was 0.002 µg/g.

In another study, Yeoh *et al.* [100] proposed a method for the determination of acephate, methamidophos, and monocrotophos in crude palm oil using low-temperature precipitation and SPE clean-up. In this study, pesticide residues in crude palm oil were extracted with acetonitrile, and a clean-up process was performed by cooling the entire

extract below 10 ºC, followed by a discolouring process using a carbon black SPE cartridge. The extract was then analysed using gas chromatography coupled with a pulsed flame photometry detector. In their early work to optimize the GC analysis, they encountered the matrix effect, raised by the interaction between the active sites in the GC system with the analytes of interest, causing the loss of analyte's response and peak tailing in the chromatogram. Although the use of matrix-matched standard proves to reduce some of the effects caused by these active sites in other matrices, they opted not to apply it in the analysis of palm oil samples since it could shorten the column life. Hence, they used an analyte protectant instead, using d-xylose. The recoveries obtained for all pesticides were acceptable in the range of 85-109% with RSD values less than 15%. The method was reported to be simple, fast, and cost effective with LOD of 0.01 µg/g.

Halimah *et al.* [92] worked with chlorpyrifos in refined palm olein using the method adopted from Cloborn *et al.* [104] for determination of chlorpyrifos in milk and body tissue of cattle. They investigated the suitability of the GC method for determination of chlorpyrifos in oil samples using both ECD and FPD detectors. The approach taken was liquid-liquid extraction using n-hexane and acetonitrile and clean-up with self prepared silicic acid column chromatography. In the first experiment, the FPD detector used gave the recoveries ranging from 89% to 100% with RSD values from 3% to 11%. In the second experiment using ECD detector, the recoveries obtained were greater than 97% with RSD values from 0.5% to 2%. As reported by the authors, one of the advantages of using ECD is its ability to detect halogen atoms, in this case chlorine. This enables the detection of both chlorpyrifos and its major metabolite (3, 5, 6-trichloro-2-pyridinol). As a detector, FPD only allows the detection of the phosphorus atom. It won't be able to detect the metabolite of chlorpyrifos since there is no

phosphorus atom in its metabolite. Hence, the GC methods using ECD and FPD described in this study are sensitive enough for the determination of chlorpyrifos in refined palm olein. Nevertheless, the GC with ECD detector is the preferred method compared to FPD.

Later, the same authors proposed a comprehensive study on the optimization of extraction and clean-up procedures for chlorpyrifos residue in RBD palm olein and analyzed by gas chromatography with electron capture detector [94]. An improved method for extraction and clean-up techniques of chlorpyrifos residue from oil matrix was established after a series of trials. The authors optimized the clean-up and recovery of the analyte by using commercial SPE cartridges packed with silica and eluents of different composition and polarity. From the results obtained, optimization of all these factors has resulted in greater than 90% recovery of chlorpyrifos from fortified oil samples. According to the authors, the optimized polarity of the eluting solvent has resulted in significant reduction of the eluent volume, without sacrificing the extraction efficiency of chlorpyrifos residue. They also suggested that the proposed method to be applicable for the analyses of chlorpyrifos residue in other vegetable oils.

Apart from organophosphorus pesticides (acephate, methamidophos, monocrotophos, and chlorpyrifos), there are also literatures discussed on the determination of organochlorine pesticide residues in palm oil and its products [95, 96]. Md. Pauzi *et al.* [95] studied and compared different types of extraction techniques, SPE and sweep co-distillation (SCD), for the determination of 15 organochlorine pesticide residues in refined palm oil by gas chromatography with electron capture detector. From the study, it was found that the 15 OCPs tested were recovered well above 80% when determined by both clean-up methods, except for endrin ketone with recoveries below

80%, which has gone through the SCD clean-up. According to the authors, the conditioning step that consisted of two sub steps for SPE clean-up was vital in order to avoid any extraneous peaks found in procedural blanks from commercial SPE cartridge. These peaks have been attributed to phthalate plasticizers in the polypropylene housing material of these cartridges. Apart from that, the slightly high mean recoveries were also observed for p, p'-DDE (101.2%-105.6%) and p, p'-DDD (100.3%-15.9%) coupled with a correspondingly low mean recovery of p, p'-DDT (84.6-89.4%) for SCD clean-up. They suggested that this was due to the consistent minor breakdown of p, p'-DDT to p, p'-DDE and p, p'-DDD in the hot distillation tubes. The authors also suspected that degradation of structural properties might be responsible for these consistently low recoveries of endrin ketone. In conclusion, they suggested that sweep co-distillation clean-up were equivalent to SPE, except for endrin ketone compound.

Later, Halimah *et al.* [96] developed an optimum condition for the quantitative recovery of organochlorine pesticide residues in palm oil using a commercial sweep co-distillation apparatus. They studied the parameters affecting the efficiency of this technique such as distillation fractionation tube temperature, nitrogen carrier flow, sweep time, and eluting solvent mixture. They discovered that under the optimized condition (245 ºC distillation temperature, 250 mL/min nitrogen flow rate, and 45 min sweep time) and using a trap packed with sodium sulphate and partially deactivated Florisil, the recoveries of 14 organochlorine pesticide residues at ppm and ppb levels in fortified oil matrix were more than 80%, with RSDs ranged from 5.6% to 9.9%. However, they were unable to get a good recovery for endrin ketone (below 80%). These results were comparable with the previous research by Md. Pauzi *et al.* [95].

A recently published work dealt with the determination of the herbicide fluroxypyr in CPO and CPKO by high performance liquid chromatography with diode array detector [101]. In this study, herbicide residue was extracted from the palm oil matrices by liquid-liquid extraction, followed by low-temperature precipitation to separate the analyte from the bulk oil matrices. The extraction method used in this experiment was a modification of the multi-residues method outlined by Gillespie *et al.* [105]. The authors replaced commercial C_{18} SPE cartridges by low-temperature precipitation clean-up in order to separate the analyte from matrix interferences. This technique proved to be a cheaper approach compared to the SPE clean-up since no special apparatus and glassware were needed for the low-temperature step. The recovery of fluroxypyr obtained from CPO and CPKO were 78-111% and 91-107% respectively, with a minimum detection limit of 0.05 µg/g for both CPO and CPKO. According to the authors, when fluroxypyr was used for weed control in oil palm plantations, no residue was detected in CPO and CPKO, irrespective of the sampling interval and the dosage applied at the recommended or doubles the manufacturer's recommended dosage.

2.2 OBJECTIVES

The purpose of this work was to develop a simple, cheap, and efficient method of extraction and analysis of cypermethrin and λ-cyhalothrin residues in crude palm oil (CPO) and crude palm kernel oil (CPKO) based on low-temperature extraction using acetonitrile. Then, solid-phase extraction (SPE) and dispersive solid-phase extraction (d-SPE) were tested as clean-up procedures to obtain the best overall recoveries for cypermethrin and λ-cyhalothrin.

2.3 EXPERIMENTAL

2.3.1 REAGENTS AND MATERIALS

HPLC grade acetone and acetonitrile were obtained from Merck (Darmstadt, Germany) while reagent grade anhydrous $MgSO_4$ and NaCl were obtained from Supelco Inc. (Bellefonte, PA, USA). Both pesticide standards of cypermethrin and λ-cyhalothrin with the purity of >97%, were purchased from Dr. Ehrenstorfer (Augsburg, Germany). For the dispersive-SPE method, bulk primary secondary amine (PSA, 100 g) and Supelclean ENVI-Carb graphite carbon black (GCB, 50 g) were used as the sorbents and they were both purchased from Supelco Inc. (Bellefonte, PA, USA).

SPE cartridges used for the clean-up experiments were graphitized carbon black (Carbograph, 500 mg/6 mL) obtained from Alltech Inc. (Deerfield, IL, USA) and C_{18} (LiChrolut RP-18, 500 mg/6 mL) purchased from Merck KGaA (Darmstadt, Germany), while primary secondary amine (PSA, 500 mg/2 mL), Florisil (500 mg/6 mL), and Silica (500 mg/6 mL) were all supplied by International Sorbent Technology (Hengoed, Mid-Glamorgan, UK).

2.3.2 APPARATUS AND GLASSWARE

Microliter pipettes, adjustable between 100 and 1000 µL, and pipette tips were obtained from Eppendorf (Hamburg, Germany), while SPE vacuum manifold was from Supelco Inc. (Bellefonte, PA, USA). 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). Ten mL graduated vials used to collect the analytes eluted from the SPE cartridges were obtained from Alltech Inc. (Deerfield, IL, USA). N-Evap nitrogen evaporator for sample concentration was obtained from Organomation Associates Inc.

(South Berlin, MA, USA). Finally, screw cap test tubes with various sizes (50 mL and 15 mL) were purchased from Favorit. All glassware were cleaned thoroughly using cleaning detergent and rinsed with tap water before drying in an oven at 60 ºC. Prior to use, the glassware were again rinsed with acetone and dried in an oven to get rid of any impurities that could not be removed by water.

2.3.3 INSTRUMENTATION

Sample extracts were analyzed on 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. This is the most that could be injected without overfilling the liner, since with these conditions the approximate vapour volume is 794 µL. The inlet pressure was 15.56 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 the 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 100 ºC, with an initial time of 1 min. The oven was heated to 250 ºC at 10 ºC/min, then to 280 ºC at 3 ºC/min, and finally held at 280 ºC for 15 min. The post-run temperature was 280 ºC (held for 5 min) and the total runtime was 41 min. Chemstation software was used for instrument control and data analysis. Calibration curve was carried out using seven external standards at concentration of 0.01, 0.02, 0.05, 0.08, 0.10, 0.50, and 1.00 µg/mL.

2.3.4 PREPARATION OF STOCK STANDARD SOLUTIONS

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

2.3.5 CPO AND CPKO SAMPLES FOR FORTIFICATION

In the method development and validation studies, the crude oil investigated should be free from cypermethrin and λ-cyhalothrin residues. A blank crude palm oil (CPO) that is used as a control was obtained from MPOB Labu refinery, while blank crude palm kernel oil (CPKO) was obtained from Felda Pandamaran refinery. Samples of CPO and CPKO were melted at 60 ºC in an oven and then homogenized by shaking the samples. After homogenization, recoveries of cypermethrin and λ-cyhalothrin were determined using oil samples at fortification levels of 0.05, 0.08, 0.1, 0.5, and 1.0 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 screw cap test tube containing 5.0 g of the oil sample. After homogenization for 5

minutes using vortex mixer, the fortified samples were allowed to stand for 30 minutes prior to analysis.

2.3.6 REAL SAMPLES FOR MONITORING STUDY

A total number of 30 crude palm oil samples were used in the monitoring study. The CPO samples were collected from different producers, refineries, and regions of Malaysia, which obtained from Registration and Licensing Department, Wisma Sawit, MPOB Kelana Jaya. Each sample was analysed for the pesticide residues using the developed method in triplicate.

2.3.7 ANALYTICAL PROCEDURES

In the method development, two types of extraction techniques were tested for the best overall extraction efficiency of the pyrethroid insecticides in CPO and CPKO. The first technique was based on low-temperature precipitation of the oil matrices from the acetonitrile layer, followed by solid-phase extraction clean-up using commercial SPE cartridges. Initial tests were carried out to optimize the extraction and clean-up procedure. In this study, extraction volume and freezing time were investigated and optimized. Furthermore, 6 types of SPE sorbents (GCB, PSA, GCB/PSA, C18, Florisil, and Silica) were tested in the clean-up step.

In the second approach, dispersive-SPE technique was studied as the clean-up step after extraction by low-temperature precipitation with acetonitrile. The combination of magnesium sulphate with PSA, and with both PSA and GCB were evaluated for their clean-up efficiency of the samples.

2.3.7.1 SOLVENT EXTRACTION AND LOW-TEMPERATURE PRECIPITATION

Samples of 5.0 g homogenous oil (CPO or CPKO) were transferred into 50-mL screw cap test tubes. Each sample was fortified with a suitable volume of working standard solution for the recovery experiment. The fortified samples were mixed well using a vortex mixer and allowed to stand for 30 minutes for equilibration. Acetonitrile (10, 15, and 20 mL) was added to the fortified samples in each tube and the mixtures were shaken for 5 minutes using a vortex mixer. The oil precipitated to the bottom of the test tubes, and the acetonitrile extract rose to the top. Each tube was kept either horizontally or vertically in a freezer (-20 ºC) for 1, 2, or 24 hours for oil precipitation before undergoing clean-up procedure.

2.3.7.1 (A) SOLID PHASE-EXTRACTION CLEAN-UP

SPE cartridges were first conditioned with 5 mL acetonitrile. An aliquot (equal to 20% of the original volume) of the upper layer of the acetonitrile extract from the low-temperature extraction step was transferred into the cartridge. The extract was initially allowed to flow under gravity, and then a gentle pressure was applied to achieve a flow of approximately one drop per second. Collection of the eluate was begun at this point into a 10-mL graduated vial. The column was then eluted with an additional acetonitrile and the volume collected was adjusted to 5 mL. Finally the eluate was mixed and ready for GC analysis.

2.3.7.1 (B) DISPERSIVE SOLID-PHASE EXTRACTION CLEAN-UP

An aliquot (1 mL) of the upper layer of the acetonitrile extract from the low-temperature extraction step was transferred to a dispersive-SPE tube containing one of the following for testing: (1) 0.3 g of anhydrous $MgSO_4$, (2) 0.3 g of anhydrous $MgSO_4$ and 0.1 g of PSA, (3) 0.3 g of anhydrous $MgSO_4$, 0.1 g of PSA and 0.025 g of GCB, (4) 0.3 g of anhydrous $MgSO_4$, 0.2 g of PSA, and (5) 0.3 g of anhydrous $MgSO_4$ and 0.3 g of PSA. The tubes were then capped tightly, and shaken for 1 min before centrifuged at 3000 rpm for 1 minute. Finally, 0.5 mL aliquot of extract solution from each tube was transferred to vials for GC analysis.

2.3.8 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 analysed by GC-ECD for each concentration level. The signal for each pesticide was measured for its peak area and an individual calibration plot for cypermethrin and λ-cyhalothrin was constructed. The linearity of the signals from the instrument was studied during the construction of the calibration curve. The percent recovery was determined in six replicate experiments at 5 concentration levels (0.05, 0.08, 0.1, 0.5, and 1.0 µg/g) by comparing the analyte peak area from the fortified samples with that of the standard calibration solutions. 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 sample for the monitoring study was calculated using the following equation:

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

where,

$V_{extraction}$ = 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 injection of 0.2 µg/mL standard solution and oil fortified at 0.2 µg/g. Both the standard solution and fortified oil were injected ten times *via* an auto-sampler. The accuracy and precision of the method were expressed in terms of recovery and RSD respectively in six replicate measurements. The specificity of the proposed method was assessed by analyzing blank oil 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.

2.4 RESULTS AND DISCUSSION

2.4.1 OPTIMIZATION AND SELECTION OF SAMPLES PRE-TREATMENT

In this thesis, both types of clean-up techniques were optimized and applied to crude palm oil (CPO) and crude palm kernel oil (CPKO) in order to obtain the best extraction and clean-up efficiency of cypermethrin and λ-cyhalothrin residues. Different sorbents for SPE were studied for their clean-up efficiency to remove matrix interferences in the extracts. While for the d-SPE technique, different combinations of sorbent materials were tested. Cleanliness of the extracts presented in the chromatograms and good recoveries were the main criteria for the method selection.

2.4.2 SOLVENT EXTRACTION AND LOW-TEMPERATURE PRECIPITATION EXTRACTION

As mentioned earlier, the extraction method applied in this study was a modification of the method for multiresidue analysis as outlined by Lentza-Rizos *et al*. [75]. This basic procedure is based on a liquid partitioning of the oil with acetonitrile without the involvement of hexane. In the extraction step, acetonitrile was chosen as the extraction solvent since it is the only few solvents that immiscible with oil, including methanol. To date, acetonitrile is probably the most extensively used solvent for the sample extraction of pyrethroids in vegetable oils [73, 75-80, 82, 100].

In the initial tests, variations in the extraction techniques and parameters previously mentioned (extraction volume, position of the test tube in the freezer, decanting the liquid phase immediately after freezing, and freezing time) generally had little effect on the mean recovery of both cypermethrin and λ-cyhalothrin. This showed that the method was adequately robust to be successfully applied by inexperienced technicians. Nonetheless, it is very important to optimize the extraction procedure in

order to save the time and solvent used. The effects on the recovery of (i) the acetonitrile volume, (ii) decanting the liquid phase from the precipitated oil after low-temperature precipitation, and (iii) the freezing time were studied. In the first step, different volumes of acetonitrile were investigated in this work to optimize the extraction procedure. Volume ratios of sample to solvent of 1:2, 1:3, and 1:4 were employed, which gave 10, 15, and 20 mL of acetonitrile to each of 5 g oil sample. Then, recoveries for each volume were calculated and evaluated to obtain the optimum volume of acetonitrile needed.

In this study, fortified CPO samples (0.1 µg/g) were extracted in four replicates by solvent extraction for 5 minutes with 10, 15, and 20 mL of acetonitrile, followed by low-temperature precipitation at -20 °C for 24 hours. SPE (GCB/PSA) was applied as the clean-up step. Table 2.1 shows the effect on the recovery of the acetonitrile volume. Finally, the results obtained were analysed using analysis of variance (ANOVA) test to check whether there is potential difference among the three extraction volumes tested. This method uses a single test to determine whether there is or is not a difference among the population means rather than pair-wise comparisons, as is done with the *t*-test [106]. In this single-factor ANOVA procedure for various ACN volumes, the null hypothesis H_0 was of the form

$$H_0: \mu_{10mL} = \mu_{15mL} = \mu_{20mL}$$

μ_{10mL} = mean recovery for 10 mL acetonitrile extraction

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

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

and the alternative hypothesis H_a was

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

Table 2.1: Recoveries and relative standard deviation (RSD) of pyrethroids from CPO obtained by low-temperature precipitation (24 hours, -20 °C) and SPE (GCB/PSA) clean-up with various volume of acetonitrile (n=4)

Compound	**0.1 µg/g**					
	10 mL		15 mL		20 mL	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
λ-cyhalothrin	92.49	1.62	89.98	4.68	94.63	3.34
cypermethrin	86.93	1.44	89.77	2.14	85.61	5.4

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 2.2 and Table 2.3 for λ-cyhalothrin and cypermethrin respectively. From the ANOVA tests, the calculated *F* value was 2.17 for λ-cyhalothrin and 2.04 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 and hence the three extraction volumes gave equivalent results. Thus, increasing the volume of acetonitrile from 10 mL to 15 mL and finally to 20 mL gave no significant difference. In addition, all extraction volumes gave satisfactory recoveries (70 - 120%), as shown in Table 2.1. Although the recoveries were higher for 15 mL (cypermethrin) and 20 mL (λ-cyhalothrin), 10 mL of ACN was selected since higher volume of extraction solvent would not only extracts the analyte of

interest, but would also bring along other impurities from oil matrix, and consequently harm the ECD detector, not to mention the additional waste of the solvents discharged to the environment.

In the meantime, 5 mL of ACN was not tested since other volumes lower than 10 mL was not always sufficient to allow acceptable removal of the required aliquot from the mixtures without taking unwanted frozen material. So, the minimum volume of the extraction solvent needed for the analyte extraction from the oil sample was opted to be twice the sample weight (5 g), which is 10 mL.

Table 2.2: ANOVA test for various volume of ACN, n = 4 (λ-cyhalothrin)

SUMMARY					
Acetonitrile volume (mL)	Count	Sum	Average	Variance	
10 mL	4	369.97	92.49	2.26	
15 mL	4	359.90	89.98	17.77	
20 mL	4	378.50	94.63	9.99	
ANOVA					
Source of Variation	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F (calculated)	F (critical)
Between Groups	43.34	2	21.67		
Within Groups	90.06	9	10.01	2.17	4.26
Total	133.40	11			

Table 2.3: ANOVA test for various volume of ACN, n=4 (cypermethrin)

SUMMARY				
Groups	Count	Sum	Average	Variance
10 mL	4	347.73	86.93	1.56
15 mL	4	359.08	89.77	3.70
20 mL	4	342.44	85.61	21.35

ANOVA					
Source of Variation	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F (calculated)	F (critical)
Between Groups	36.14	2	18.07		
Within Groups	79.84	9	8.87	2.04	4.26
Total	115.98	11			

Secondly, the minimum time for which the sample needed to be left in the freezer at -20 ºC for fat precipitation was studied. From the recovery table shown in Table 2.4, all three freezing durations studied gave acceptable recoveries (70 – 120%) for both λ-cyhalothrin and cypermethrin from fortified CPO samples (0.1 µg/g). In this study, an hour was picked as the minimum freezing time whereas 24 hours was selected as the maximum freezing time to freeze and finally precipitate the oil sample in the mixture. The results obtained in this study showed that there was no significant difference in both cypermethrin and λ-cyhalothrin recovery when the freezing time was varied between 1, 2, and 24 hours as shown in Table 2.4. To confirm this assumption, a statistical analysis was used. An ANOVA test applied to these data showed no significant difference on the mean recoveries among the different freezing duration at 95% confidence level. Hence, null hypothesis H_0 was accepted,

$$H_0: \mu_{1hr} = \mu_{2hrs} = \mu_{24hrs}$$

μ_{1hr} = mean recovery for 1 hour freezing time

μ_{2hrs} = mean recovery for 2 hours freezing time

μ_{24hrs} = mean recovery for 24 hours freezing time

while the alternative hypothesis H_a (at least two of the mean recoveries are different) was rejected.

Table 2.4: Recoveries and relative standard deviation (RSD) of pyrethroids from CPO obtained by low-temperature precipitation (10 mL ACN, 24 hours, -20 °C) and SPE (GCB/PSA) clean-up with various freezing time (n=4)

Compound	**0.1 µg/g**					
	1 hr		2 hr		24 hr	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
λ-cyhalothrin	95.39	3.59	96.68	1.04	95.32	2.61
cypermethrin	91.33	1.17	90.16	2.55	93.00	0.37

The results of ANOVA tests were summarized in Table 2.5 and Table 2.5 for λ-cyhalothrin and cypermethrin respectively. For both pesticides, the calculated *F* value was smaller than the *F*-critical value at 95% confidence level. Thus, increasing the time in the freezer would not make any different when recoveries are concern and the minimum time for satisfactory fat removal during low-temperature precipitation was found to be 2 hours. Meanwhile, one hour was not sufficient to freeze the oil completely, with small pieces of dispersed frozen oil still present in the acetonitrile layer. In this case, it could hinder the possibility of removing sufficient extract without

also taking any solid frozen material. In the meantime, 24 hours was too time consuming and not very efficient in method development.

Table 2.5: ANOVA test for various freezing time, n=4 (λ-cyhalothrin)

SUMMARY				
Groups	Count	Sum	Average	Variance
1 hour	4	381.55	95.39	11.75
2 hours	4	386.73	96.68	1.01
24 hours	4	381.27	95.32	6.17

ANOVA					
Source of Variation	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F (calculated)	F (critical)
Between Groups	4.73	2	2.363		
Within Groups	56.81	9	6.313	0.37	4.26
Total	61.54	11			

Table 2.6: ANOVA test for various freezing time, n=4 (cypermethrin)

SUMMARY				
Groups	Count	Sum	Average	Variance
1 hour	4	365.32	91.33	1.14
2 hours	4	360.63	90.16	5.27
24 hours	4	372.00	93.00	0.12

ANOVA					
Source of Variation	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F (calculated)	F (critical)
Between Groups	16.32	2	8.16	3.75	4.26
Within Groups	19.60	9	2.18		
Total	35.92	11			

The third preliminary study referred to the positioning of the test tubes in the freezer. Initially, test tubes of sample mixtures were placed vertically in a separate beaker during the freezing step, leaving the frozen oil precipitated on the bottom of test tubes. However, it was then found that it was better and easier to remove an aliquot of extract from the frozen oil if the test tubes were kept horizontally in the freezer. On the removal from the freezer, the test tubes were instantaneously stood vertically, leaving the frozen oil adhered to the test tube wall. Finally, the effect of decanting the liquid phase after freezing step was investigated.

In this test, two techniques were tested; the first was decanting the liquid phase immediately to a small beaker after the freezing step, while the second involved directly pipetting aliquot from sample mixtures. Fortified CPO samples (0.1 µg/g) were extracted with 10 mL of ACN and frozen for 2 hours for oil precipitation before clean-up with GCB/PSA SPE cartridges. Table 2.7 exhibited the effects on recoveries of these techniques where both of them gave acceptable recoveries (70-120%). A *t*-test analysis of these data showed no significant difference between the mean recoveries at 95% confidence level, indicating that whichever techniques used, it had no effects on the recoveries. The critical value of *t* at the 95% confidence level for 6 degrees of freedom was 2.45 obtained from *t*-value table (Appendix 1). Since this value was greater than the calculated *t*-value for both λ-cyhalothrin (0.59) and cypermethrin (0.42), null hypothesis H_0 was accepted,

$$H_0: \mu_{dec} = \mu_{notdec}$$

μ_{dec} = mean recovery for decanted aliquot

μ_{notdec} = mean recovery for not decanted aliquot

while the alternative hypothesis H_a ($\mu_{dec} \neq \mu_{notdec}$) was rejected and hence it can be deducted that the way the aliquot was taken from the mixtures was not a factor that can influence the recovery of pesticides from the oil.

Table 2.7: Effect on recoveries and relative standard deviation (RSD) of decanting the liquid phase after freezing of pyrethroids from CPO (2 hours, -20 °C, 10 mL ACN), n=4

Compound	**0.1 µg/g**			
	Decanted		In contact with solid	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
λ-cyhalothrin	92.36	1.33	93.2	2.76
cypermethrin	98.03	1.09	98.28	0.56

From the statistical data analysis and on the basis of the preliminary tests described above, the optimized solvent extraction and low-temperature precipitation was as follows: a 5 g of oil was weighed out into a 50-mL screw cap test tube. Then, 10 mL of acetonitrile was added and the mixture was mixed and homogenized using a vortex mixer for 5 minutes. After homogenization, the mixture was left to stand for a while to allow phase separation between the oil and acetonitrile layer. Normally good separation took about 1-2 min for CPO and a little bit quicker for CPKO. The test tube was then transferred into the freezer and kept horizontally for a minimum of 2 hours for oil precipitation. After 2 hours, the test tube was removed from the freezer slowly and carefully, taking care not to disturb the precipitated solids. Then, it was kept vertically leaving the frozen oil adhered to the test tube wall. The acetonitrile extract was transferred immediately using a Pasteur pipette into a small beaker, leaving the frozen oil in the test tube. It was left for a while before an aliquot of the extract underwent the clean-up step. Two different clean-up methodologies were tested for co-extractives elimination: (i) solid-phase extraction (SPE); and (ii) dispersive solid-phase extraction (d-SPE).

2.4.3 SOLID-PHASE EXTRACTION CLEAN-UP

One highly important aspect in SPE is the selection of sorbent [107]. This would depend on both the analyte and the matrices studied. In this work, initial tests were made to evaluate the capacity of different sorbent materials to remove traces of oil and other co-extractants remaining in oil extracts after an initial extraction step. Then, the chromatograms were assessed for baseline noise and presence of interfering peaks. A brief description and characteristics of these materials are shown in Table 2.8.

Table 2.8: Characteristics of SPE cartridges studied

SPE Cartridge	Symbol in Study	Sorbent Material	Retention Mechanism
Carbograph Extract-Clean	GCB	Graphitized non-Porous carbon	Reversed phase or adsorption
Isolute-PSA	PSA	Polymerically bonded Primary secondary amine	Weak anion exchange
LiChrolut RP-18	C_{18}	Polymerically bonded octadecyl silane	Reversed phase
Isolute-Silica	Silica	Silica gel	Adsorption
Isolute-Florisil	Florisil	Magnesium silicate	Adsorption

The extract of the optimized solvent extraction and low-temperature precipitation was subjected to clean-up procedures via different SPE cartridges based on different sorbent materials in order to find the most efficient clean-up that would allow the determination and quantification of both cypermethrin and λ-cyhalothrin by GC-ECD. CPO samples which fortified at three different fortification levels (0.05, 0.08, and 0.2 µg/g) were extracted with the optimized acetonitrile extraction and low-temperature precipitation described previously. Then, the extracts were cleaned using six SPE cartridges according to the procedure discussed in section 2.3.7.1 (A). In this procedure,

the SPE sorbents acted as chemical filters and retained the matrix co-extractants while allowing the insecticides to be eluted.

The mean recoveries (n = 3) of cypermethrin and λ-cyhalothrin determined by GC-ECD are shown in Table 2.9. For cypermethrin, the recoveries ranged from 94.7% to 97.8% for GCB/PSA, from 91.6% to 96.2% for GCB, from 86.0% to 93.4% for PSA, from 84.1% to 95.7% for C-18, from 89.8% to 92.4% for Florisil, and from 90.7% to 93.7% for Silica. On the other hand, the recoveries for λ-cyhalothrin ranged from 90.3% to 98.6% for GCB/PSA, from 91.0% to 95.6% for GCB, from 67.6% to 82.9% for PSA, from 81.5% to 102.1% for C-18, from 63.6% to 71.8% for Florisil, and from 86.5% to 93.5% for Silica. Representative bar charts for the recoveries of both cypermethrin and λ-cyhalothrin cleaned with various SPE cartridges were plotted and showed in Figure 2.1 and Figure 2.2, respectively. The results showed that all SPE sorbents studied gave acceptable recovery values for cypermethrin, while for λ-cyhalothrin all sorbents except for Florisil gave acceptable recoveries. The highest mean recovery for both insecticides among the studied sorbents was exhibited by the combination of GCB/PSA. On the other hand, the highest precision in term of RSD was shown by Florisil for cypermethrin and GCB for λ-cyhalothrin.

Table 2.9: Recoveries of pyrethroids from CPO spiked at 3 concentration levels with various SPE sorbents clean-up

Pesticides	Spiking levels (μg/g)	GCB/PSA		GCB		PSA		C-18		Florisil		Silica	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Cypermethrin	0.05	97.76	6.03	94.21	3.71	92.41	7.44	95.67	5.96	89.75	4.3	93.66	6.22
	0.08	94.70	3.60	96.18	3.62	93.39	1.3	93.43	4.00	92.41	3.99	93.09	2.44
	0.20	95.81	4.26	91.58	6.86	85.96	5.08	84.10	20.70	90.49	8.16	90.69	8.42
	mean	96.09		93.99		90.59		91.07		90.88		92.48	
	RSD	1.61		2.46		4.46		6.74		1.51		1.70	
λ-cyhalothrin	0.05	98.59	4.61	95.58	2.94	82.91	13.2	102.07	11.11	71.63	18.8	93.53	8.01
	0.08	90.27	3.40	91.45	5.79	78.54	7.22	90.04	3.03	71.79	8.58	89.35	7.06
	0.20	91.77	4.63	90.98	5.59	67.59	6.71	81.54	14.04	63.63	12.26	86.45	7.68
	mean	93.54		92.67		76.35		91.22		69.02		89.78	
	RSD	4.74		2.73		10.34		11.31		6.76		3.96	

n = 3

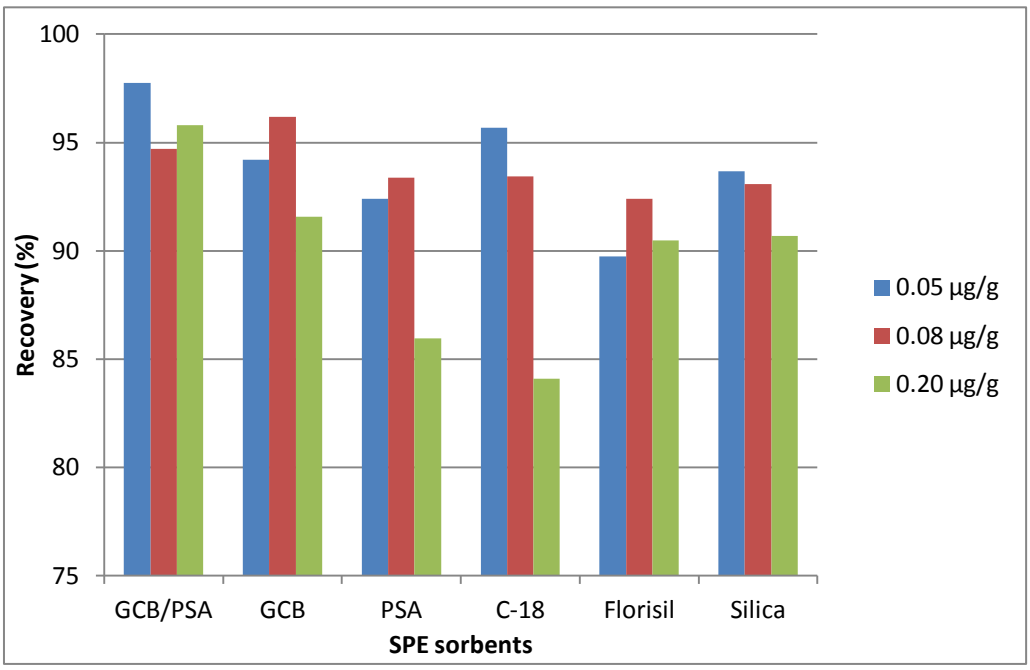

Figure 2.1: Recoveries (n = 3) of cypermethrin from CPO analyzed by GC-ECD and fortified at 0.05, 0.08, and 0.2 µg/g with various SPE sorbents clean-up

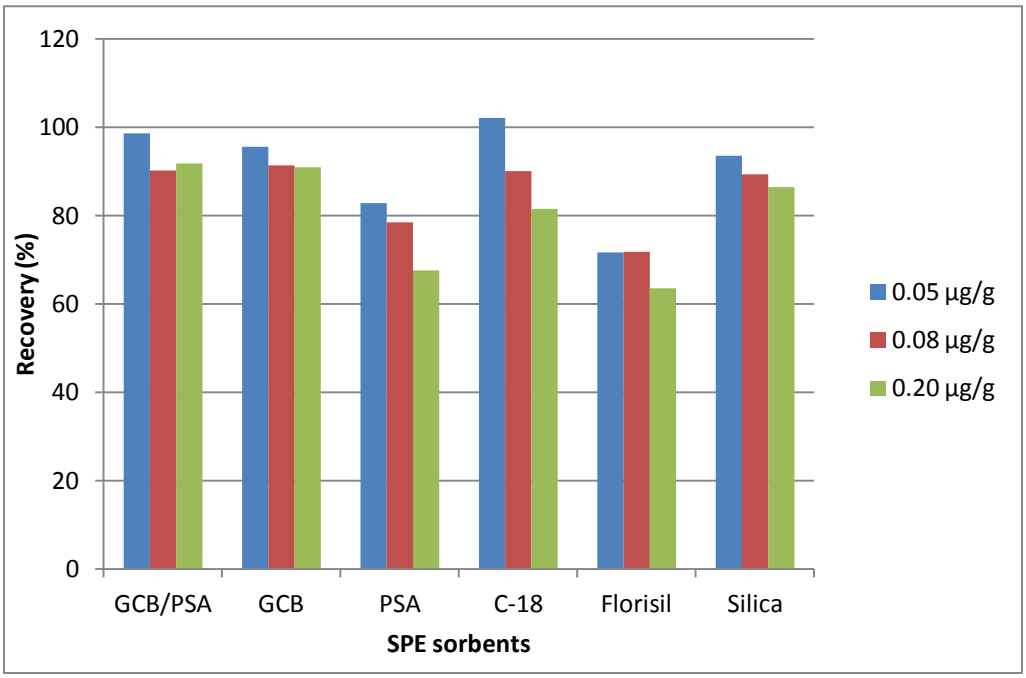

Figure 2.2: Recoveries (n = 3) of λ-cyhalothrin from CPO analyzed by GC-ECD and fortified at 0.05, 0.08, and 0.2 µg/g with various SPE sorbents clean-up

GC-ECD chromatograms for oil extracts fortified with 0.2 µg/g pesticides were obtained after using each of the previously clean-up SPE sorbents in order to know the peak outline and differences between the compared sorbents (Figure 2.3 A-F). In the meantime, for easy comparison, Figure 2.4 showed the pyrethroid standards in acetone. From the aforementioned chromatograms, we can see that high interference peaks were eluted between 4 and 17 minutes, and several minor peaks were observed between 29 and 36 minutes, both of which were before and after the pyrethroid compounds retention time respectively.

Silica and C-18 cartridges were not considered further in the clean-up method development since their chromatograms showed the early (4-17 min) and late (29-31, 33, and 36 min) interference peaks. Consequently, there were three sorbents left for consideration in the clean-up step in this work; GCB, PSA, and Florisil. The similar effect of PSA and Florisil can be seen in the chromatograms, since both sorbents gave cleaner early interference peaks in the range 4-17 min. While for the late interferences, both sorbents significantly decreased the peaks in the range 29-31 min, but the peaks at 33 min and 36 min were still present. The only difference between these two sorbents was the elimination of sharp peak at 9 min retention time by PSA, which Florisil was unable to remove it, and as a consequence rejected from the method development. In the case of GCB, the early eluted interferences were quite nasty from 4-17 min, but lower interference peaks were observed at 33 min and 36 min. The main purpose of using GCB was to remove the carotenoids in the oil as decolorizing effect, resulting in a clear and colourless final solution of the sample as shown in Figure 2.5 (A) and Figure 2.5 (B). This is because GCB has a strong affinity towards planar molecules and thus effectively removes pigments (carotenoids) as well as sterols that are commonly present in foods [85]. Nevertheless, sometimes clear and colourless extracts are not necessarily

indicated that the extracts are cleaner than coloured counterparts in residue analysis, because some nasty matrix components are clear and colourless in solution. On the other hand, some benign components are strongly coloured even at low concentration, such as chlorophyll. Summary of chromatograms profile obtained for SPE sorbents comparison study is shown in Table 2.10.

Table 2.10: Summary of SPE sorbents comparison study

SPE sorbents	GC-ECD chromatogram			
	4-17 min	29-31 min	33 min	36 min
GCB/PSA	Cleaner	Cleaner	Cleaner	Cleaner
GCB	Dirtier	Dirtier	Cleaner	Cleaner
PSA	Cleaner	Cleaner	Dirtier	Dirtier
C-18	Dirtier	Dirtier	Dirtier	Dirtier
Florisil	Cleaner	Cleaner	Dirtier	Dirtier
Silica	Dirtier	Dirtier	Dirtier	Dirtier

Higher interference peaks Lower interference peaks

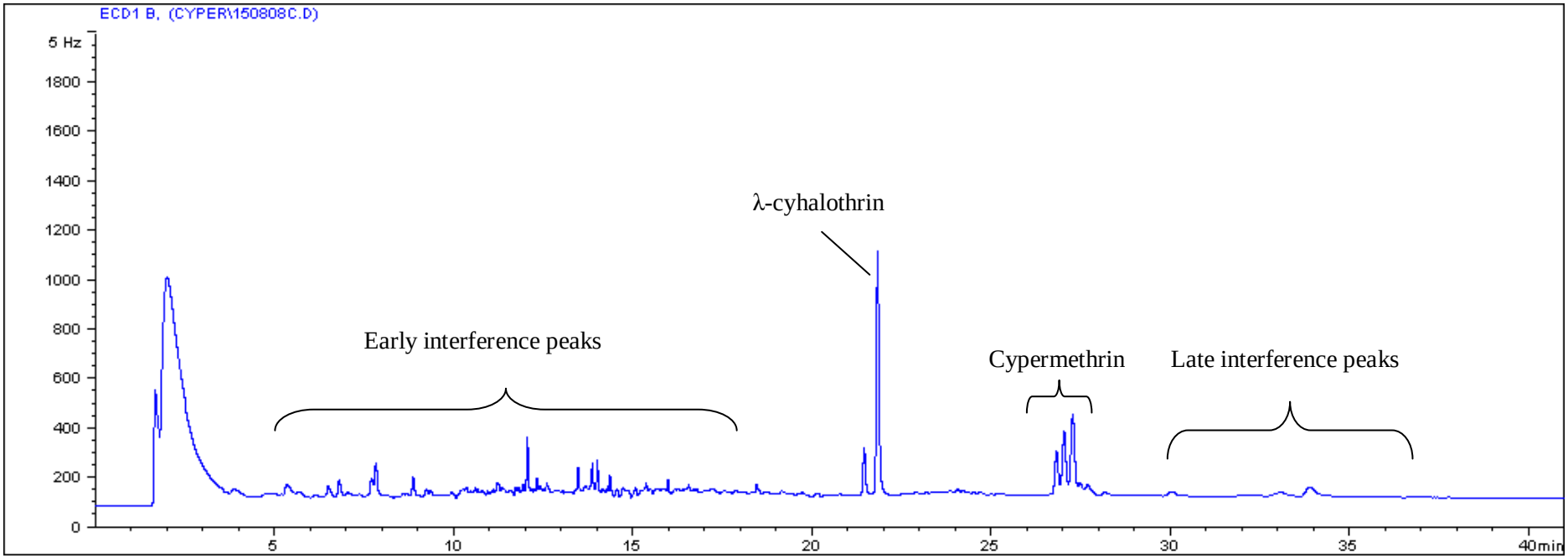

Figure 2.3 (A): GC-ECD chromatogram of fortified (0.2 µg/g) crude palm oil extracts after clean-up with GCB/PSA SPE cartridge

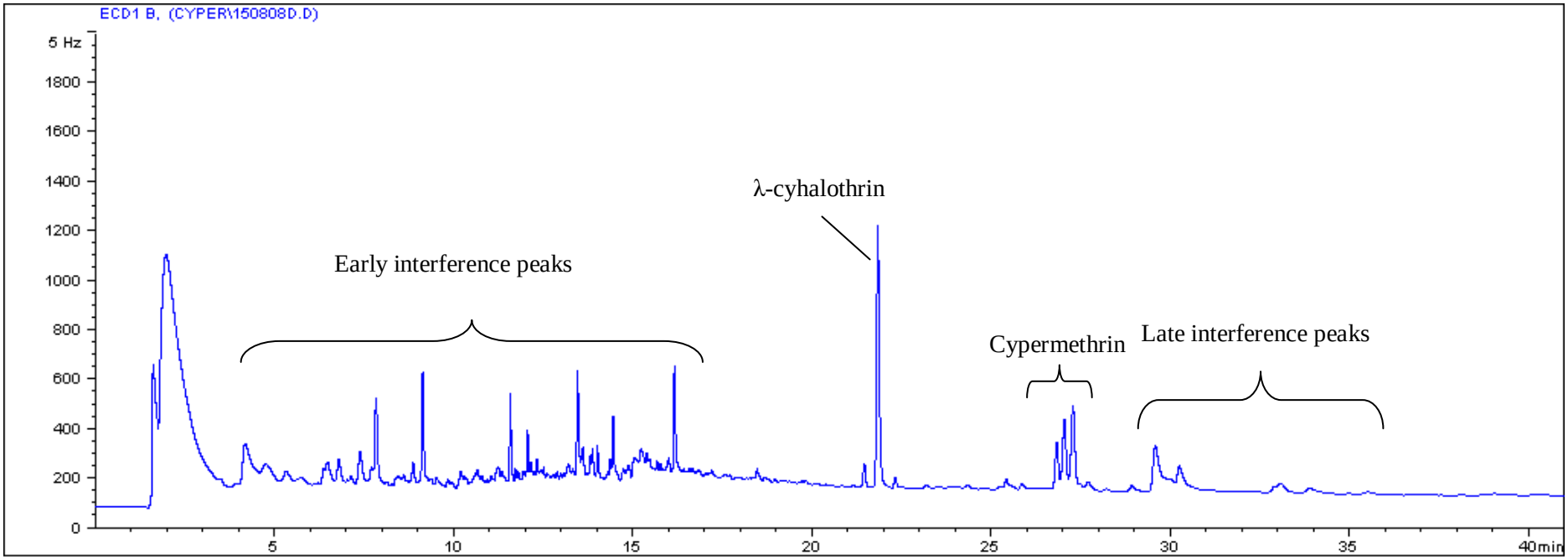

Figure 2.3 (B): GC-ECD chromatogram of fortified (0.2 µg/g) crude palm oil extracts after clean-up with GCB SPE cartridge

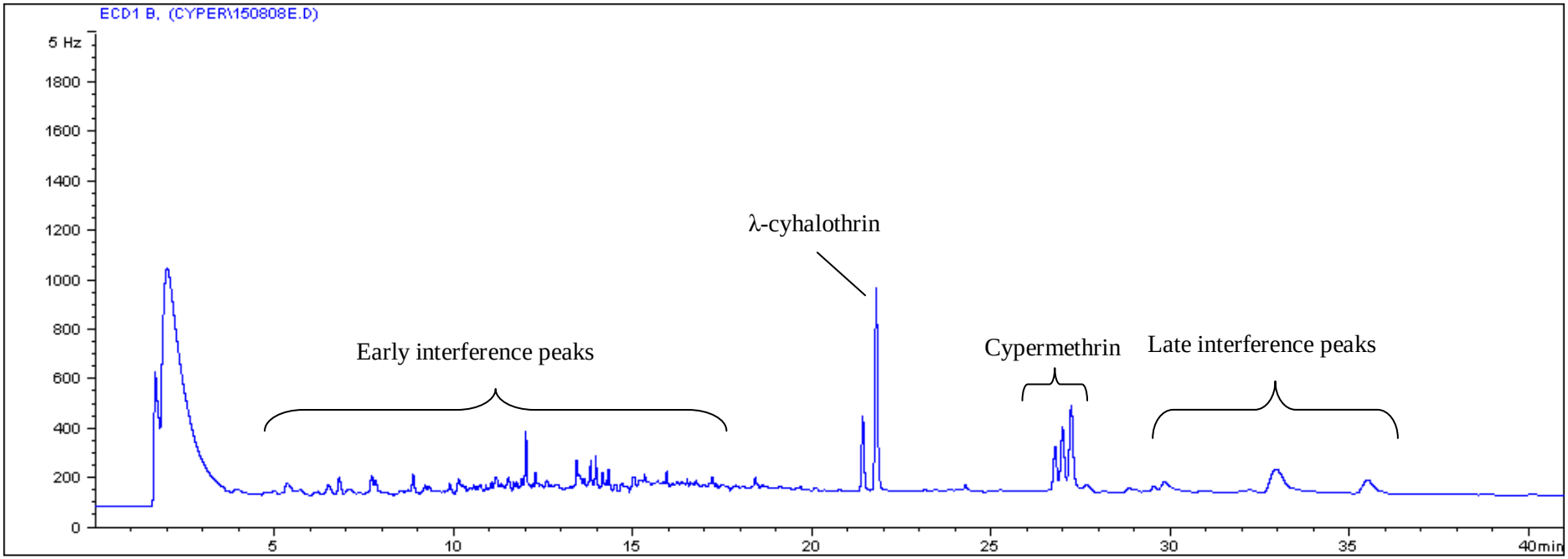

Figure 2.3 (C): GC-ECD chromatogram of fortified (0.2 µg/g) crude palm oil extracts after clean-up with PSA SPE cartridge

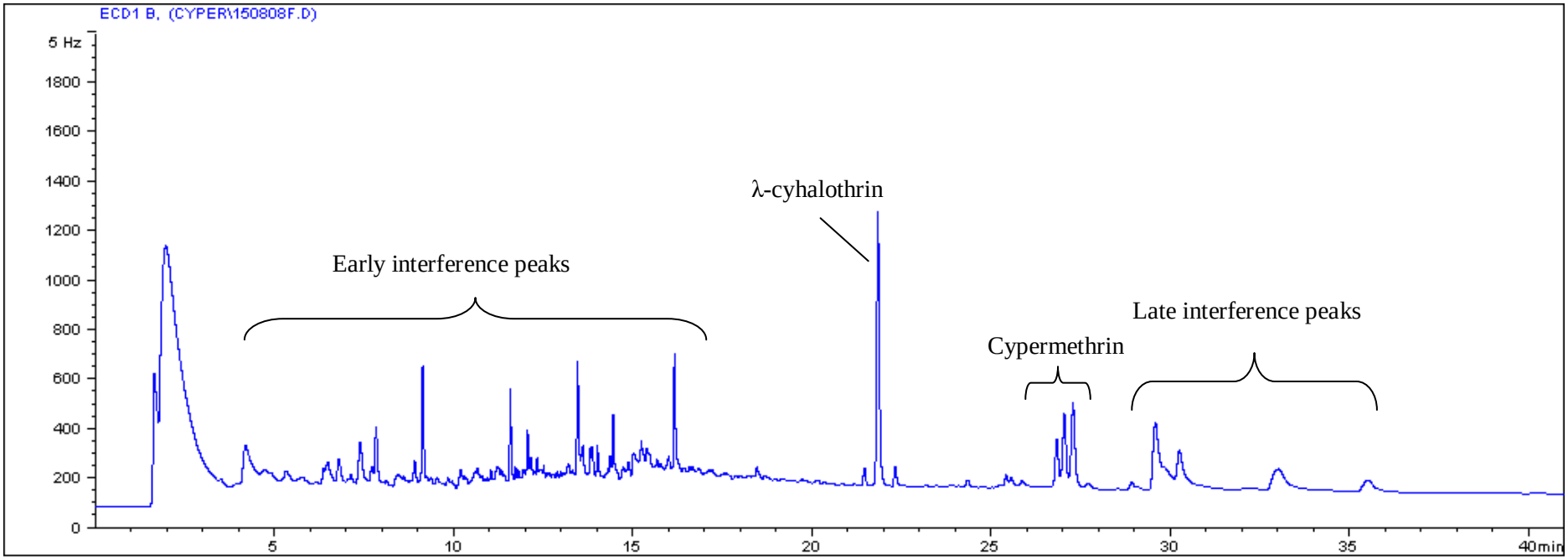

Figure 2.3 (D): GC-ECD chromatogram of fortified (0.2 µg/g) crude palm oil extracts after clean-up with C-18 SPE cartridge

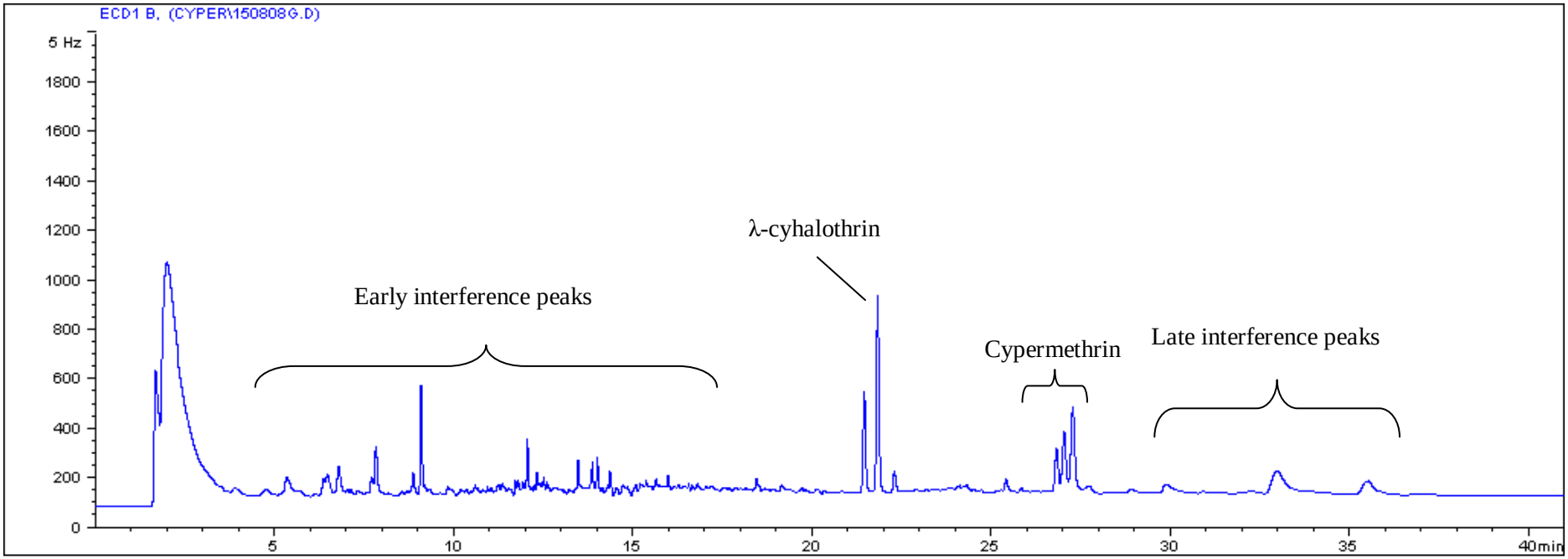

Figure 2.3 (E): GC-ECD chromatogram of fortified (0.2 µg/g) crude palm oil extracts after clean-up with Florisil SPE cartridge

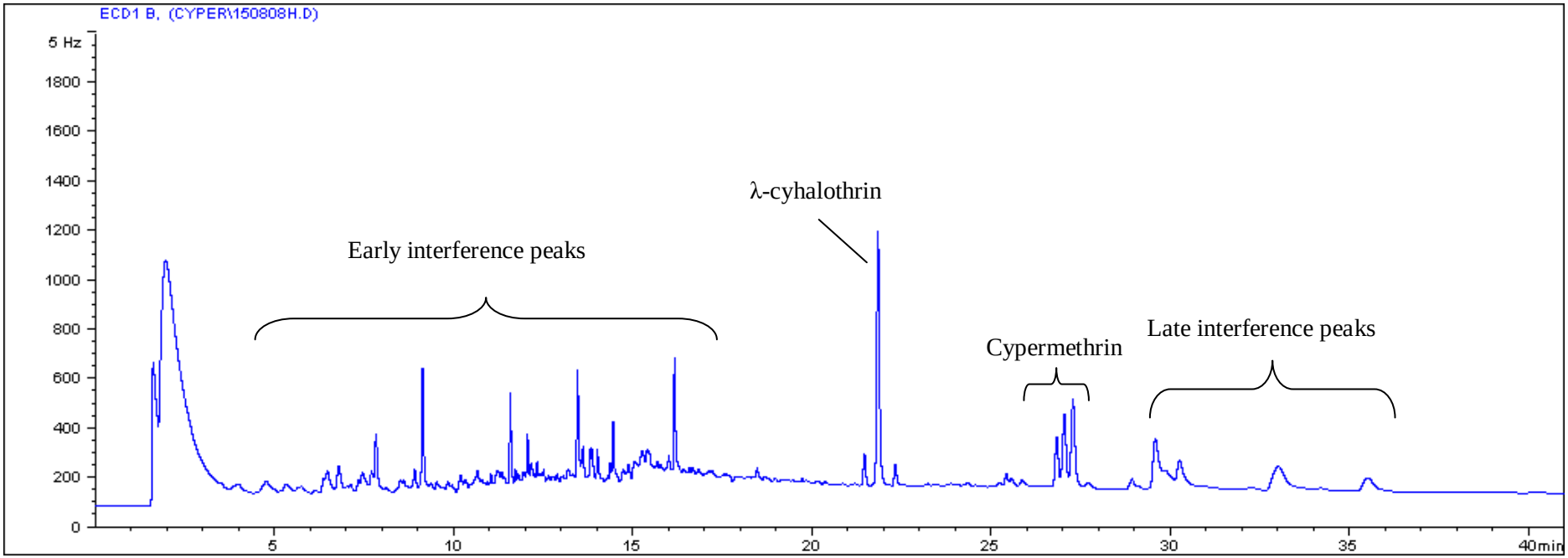

Figure 2.3 (F): GC-ECD chromatogram of fortified (0.2 µg/g) crude palm oil extracts after clean-up with Silica SPE cartridge

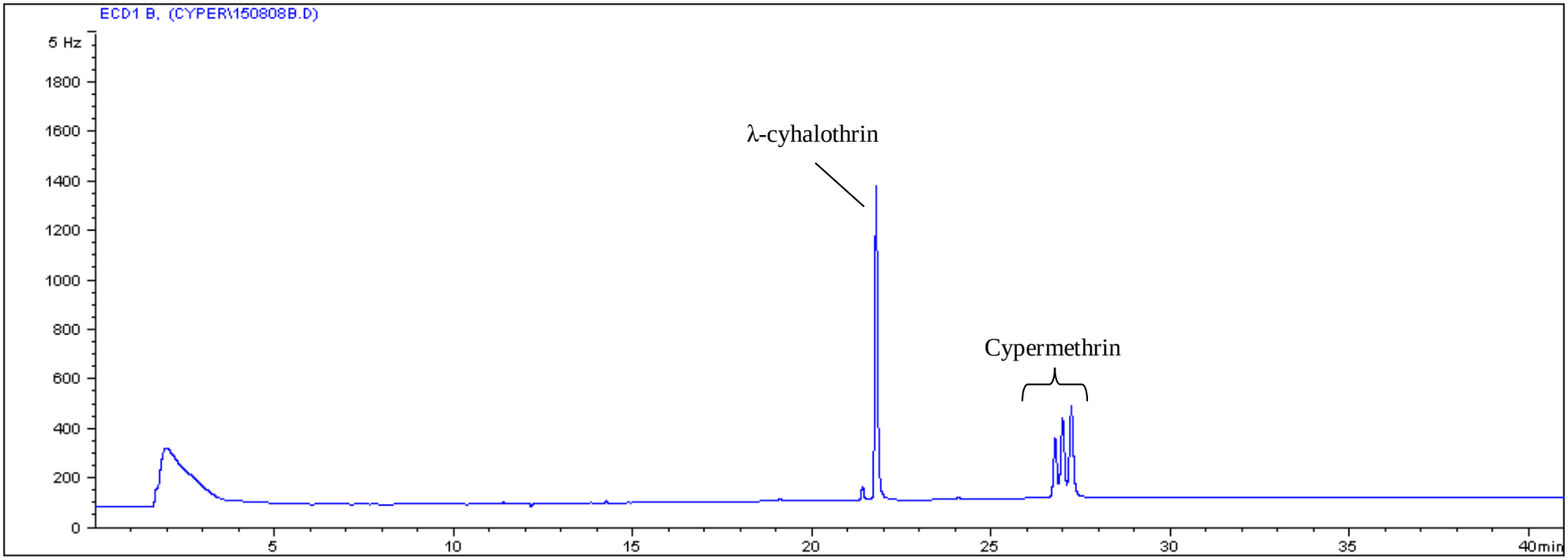

Figure 2.4: GC-ECD chromatograms of mixed pyrethroid standard solution in acetone

Combination of sorbents was studied in this work to improve the clean-up procedure since the use of single sorbent as discussed previously could not eliminate completely the interference peaks present in the chromatograms. Thus, sorbent combination was explored to overcome this problem and successively increase the efficiency of the overall clean-up procedures. To do this, PSA was chosen instead of other sorbents to combine with GCB for removal of interferences because PSA has a much higher ion-exchange capacity for removal of fatty acids in foods and vegetables [103, 108]. Although GCB removes most of the visible pigment in the extracts, it could not eliminate the fatty acid matrix interferences present in the oil. In addition, according to Anastassiades *et al*. [103], pigments give no visible peaks and this was in accordance with the results obtained, since no correlation was observed between extracts colour and cleanliness of the chromatogram, as depicted in Figure 2.3 and 2.5 respectively. Figure 2.3 (B) showed that GCB failed to remove interference peaks that crowded in the chromatogram between 4 and 17 min although the final solution was clear as in Figure 2.5 (A), compared to Figure 2.5 (B) when Florisil, PSA and Silica were used as the sorbents in the clean-up step.

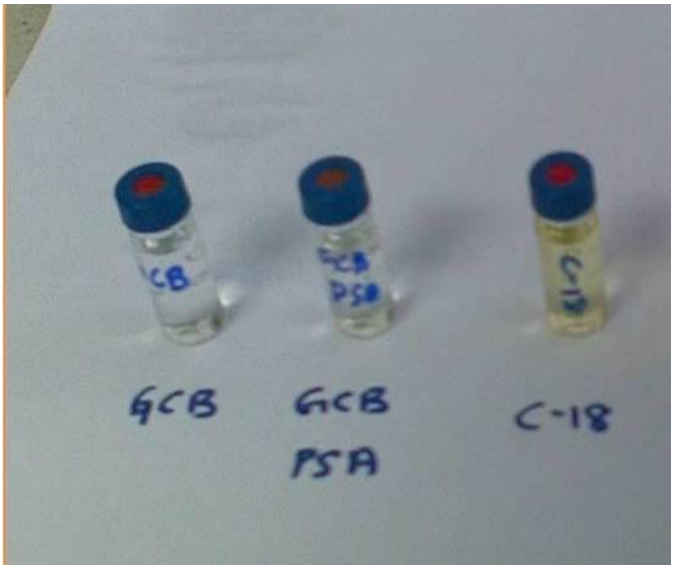

Figure 2.5 (A): Final solution of GCB, GCB/PSA, and C-18 clean-up

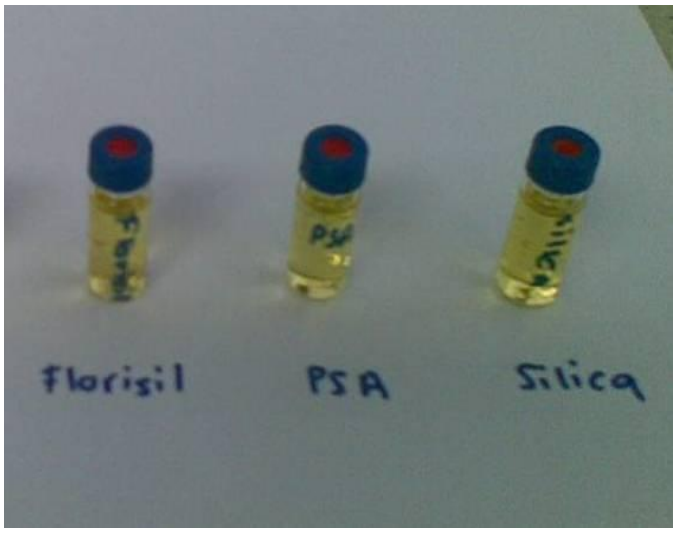

Figure 2.5 (B): Final solution of Florisil, PSA, and Silica clean-up

Solid-phase extraction clean-up with a combination of GCB and PSA sorbents improved the matrix interferences removal of the oil extract as shown in Figure 2.3 (A). Marked improvements of the chromatogram were observed especially at 4-17, 29-31, 33, and 36 min retention time, where most of the peak interferences were significantly decreased. We have discussed previously the work done by Shimelis *et al*. [88] and He *et al*. [89] on which both studies agreed that practical applications of dual-layer GCB/PSA cartridges should be limited to food samples with low levels of fatty acids since only the use of 100 % acetonitrile as an elution solvent maximizes PSA capacity for retention of fatty acids. At the same time, He *et al*. [89] also proposed that if GCB has to be used for removal of colour and sterol, they suggested that PSA cartridge should be used with carbon cartridge in serial (Figure 2.6), not in dual-layer (Figure 2.7) as proposed by Shimelis *et al*. [88].

After taking both points into consideration, serial combination of SPE sorbents was chosen since the only purpose of GCB was to remove the pigments in the oil sample. Furthermore, in this situation, 100 % acetonitrile was used as an elution solvent. The serial design of PSA and GCB cartridges was illustrated in Figure 2.6. Each sorbent was conditioned separately with acetonitrile. Sorbents were not allowed to dry during the conditioning step. Then, oil extract was loaded into GCB cartridge and concurrently an adaptor was attached into the top of the PSA cartridge reservoir.

This type of SPE design would allow the filtration of matrix co-extractants separately by GCB and PSA. The first cartridge will retain pigments and other colourised matrices, while the second cartridge will retain the fatty acids composition of the extracts. Other benefit of this serial design is that when compared to dual-layer sorbents in one cartridge, analysts could use the cartridges separately for other specific

purposes, since the sorbents were packed in separate cartridge tubes. Hence, it avoids the interaction between the sorbents. This would save costs and increase the flexibility of the materials in the laboratory. From the baseline noise of the chromatogram, the presence of interfering peaks and the recovery values, it was concluded that the most effective clean-up procedure of those tested was the application of GCB/PSA SPE cartridges in serial design, and acetonitrile as the conditioning and eluting solvent.

The clean-up efficiency of the optimized method was assessed by determining the amount of oil co-extracted from the samples into the extract. This was done gravimetrically after clean-up using GCB/PSA SPE cartridges. The optimized acetonitrile extraction together with low-temperature precipitation and GCB/PSA SPE clean-up was applied to blank CPO and CPKO samples without fortification. The vial of the final solution was weighed before the addition of extract. Then, sample extract obtained after the clean-up step was dried via N-evaporator to dryness to obtain the oil residue. The oil residue together with the vial was re-weighed for oil residues determination. From the results obtained, the amount of oil co-extracted for CPO and CPKO samples after the clean-up procedure was 1.7 ± 0.6 mg/g (n=6) and 2.7 ± 1.2 mg/g (n=6), respectively. These values represented 0.2 and 0.3 % of the sample mass and the results showed that the clean-up step was able to eliminate 99.75 % of the lipid using this method, indicating sufficient for the chromatographic system to maintain its separation efficiency for more than 100 sample injections.

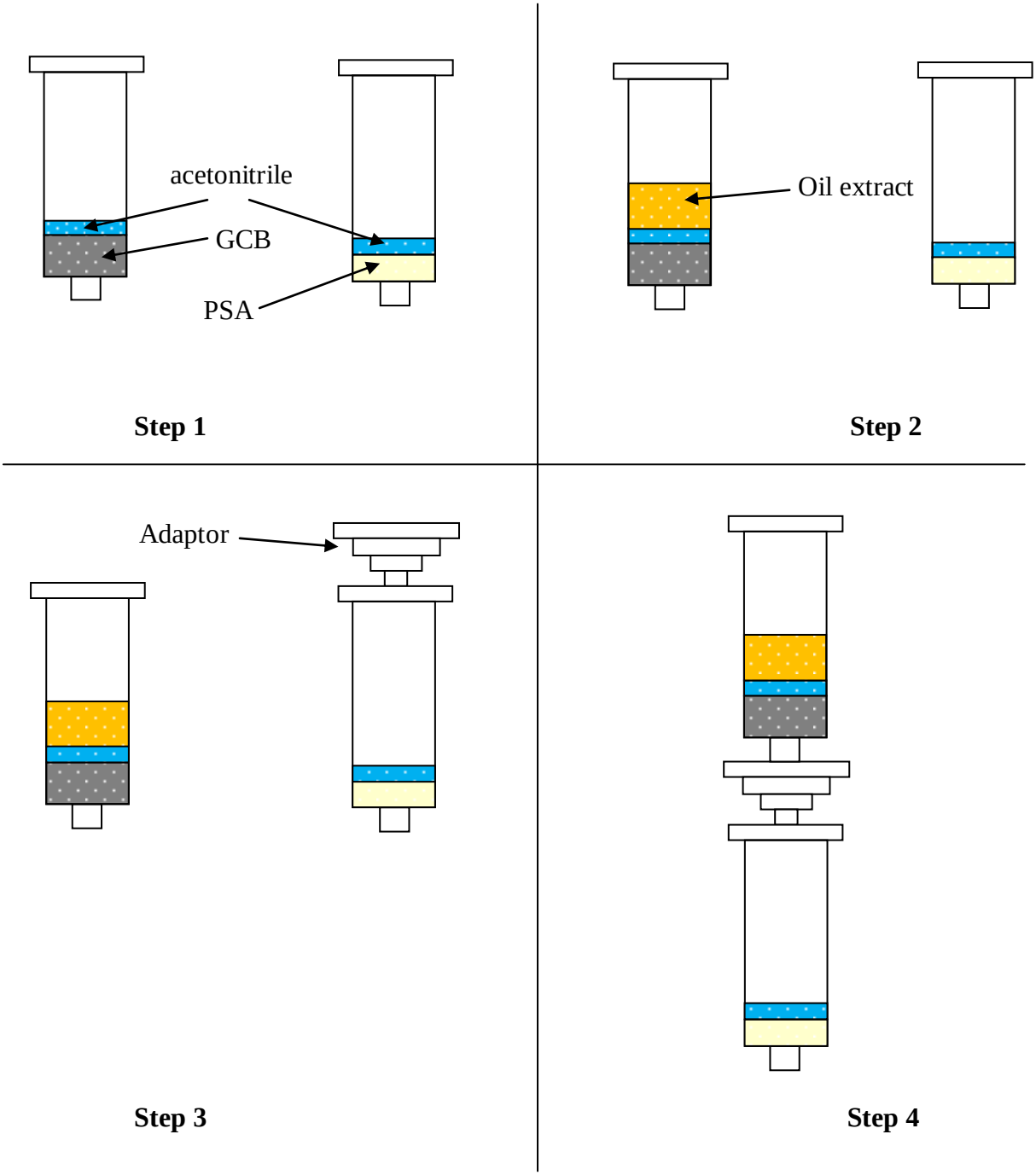

Figure 2.6: A serial design of PSA and GCB cartridges for clean-up of palm oil samples

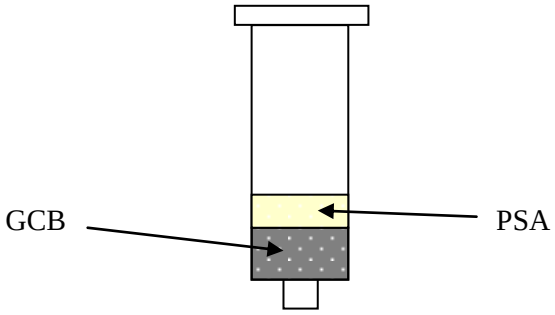

Figure 2.7: Dual-layer design of PSA and GCB cartridges

2.4.4 DISPERSIVE SOLID-PHASE EXTRACTION CLEAN-UP

In 2003, Anastassiades *et al*. published an extraction technique that provided high quality results with minimum number of steps and low solvent and glassware consumption called QuEChERS [103]. The original procedure of this technique involves two parts. The first part consists of extracting the homogenised sample by hand-shake or Vortex with the same amount of acetonitrile in order to have a final extract, concentrated enough without the need of a solvent evaporation step [109]. The advantage of extracting with acetonitrile rather than acetone is that acetonitrile separates more easily from water with the addition of a proper combination of salts (anhydrous magnesium sulphate, $MgSO_4$, and sodium chloride, NaCl) which provides a well defined phase separation without dilution with hazardous non-polar organic solvents. After centrifugation, which provides a perfect physical separation of phases, clean-up and removal of residual water is performed simultaneously by using a rapid procedure called dispersive solid-phase extraction (d-SPE), in which PSA sorbent and more anhydrous $MgSO_4$ are mixed with the sample extract.

As mentioned earlier, dispersive SPE is based on the SPE methodology, but the sorbent is directly added to the extract without conditioning and the clean-up is being easily carried out by shaking and centrifugation, rather than passing it through an SPE column. Initially, this method was designed for samples with more than 75 % moisture, especially for vegetables and fruits. But lately, it has been modified in order to apply the method to high fat samples including vegetable oils [82, 110]. To do this, freezing step is introduced prior to d-SPE, to separate the acetonitrile layer from the oil matrix.

In this work, the extract of the optimized solvent extraction and low-temperature precipitation was subjected to d-SPE clean-up step with various sorbent materials. The sorbents (0.3 g anhydrous $MgSO_4$ (d-SPE1), 0.3 g anhydrous $MgSO_4$ + 0.1 g PSA (d-SPE2), 0.3 g anhydrous $MgSO_4$ + 0.1 g PSA + 0.025 g GCB (d-SPE3), 0.3 g anhydrous $MgSO_4$ + 0.2 g PSA (d-SPE4), and 0.3 g anhydrous $MgSO_4$ + 0.3 g PSA (d-SPE5)) were compared in dispersive SPE study in order to find the best sorbent materials available for the performance of the pesticide residues determination with higher recoveries and lower matrix interferences. This was assessed from the chromatograms. The summary of sorbent combination is shown in Table 2.11. In this study, anhydrous $MgSO_4$ was used to absorb micro quantities of water in the solvent, while the applications of PSA and GCB were the same as previously discussed in section 2.4.3.

Table 2.11: Combination of sorbents used for d-SPE

Dispersive-SPE	Sorbents (g)		
	$MgSO_4$	PSA	GCB
d-SPE1	0.3	0	0
d-SPE2	0.3	0.1	0
d-SPE3	0.3	0.1	0.025
d-SPE4	0.3	0.2	0
d-SPE5	0.3	0.3	0

The CPO sample fortified at 0.1 µg/g insecticides was extracted with the optimized acetonitrile extraction and low-temperature precipitation described previously, and the extracts were cleaned using d-SPE by the procedure discussed in section 2.3.7.1 (B). The respective mean recoveries (n=3) of cypermethrin and λ-cyhalothrin determined by GC-ECD are shown in Table 2.12 and ranged from 88.2 to 99.1 % for d-SPE1, 72.9 to 90.5 % for d-SPE2, 71.1 to 90.4 % for d-SPE3, 66.6 to 80.0 % for d-SPE4, and 61.7 to 72.8 % for d-SPE5. The recovery profiles of the d-SPE sorbents were summarised in Figure 2.8. From the chart, it showed that increasing the PSA sorbent content would result in proportional decreased of the recoveries of both cypermethrin and λ-cyhalothrin.

The representative chromatograms of the different sorbents studied are shown in Figure 2.9 (A-E). Although high recoveries were reported when only $MgSO_4$ was used as the sorbent, large impurity peaks were also observed at 3-10 min of the retention time while some minor impurity peaks were present at 16 and 22 min of the retention time (Figure 2.9 [A]). In the meantime, when combination of 0.3 g $MgSO_4$ and 0.1 g PSA were used as the sorbent, major impurity peaks obtained at 3-10 min and minor peaks found at 16 min were both tremendously decreased, whereas peaks at 22 min were removed completely (Figure 2.9 [B]). Preliminary studies showed that when 0.025 g of GCB was included in the sorbent combination, GCB gave only little effect to the cleanliness of the chromatograms and the recoveries of both cypermethrin and λ-cyhalothrin as shown in Figure 2.9 (C). This explained why the almost identical chromatograms obtained for d-SPE2 and d-SPE3 (Figure 2.9 B and Figure 2.9 C), while their recovery values were also quite similar, but lower than d-SPE1 (Table 2.12). In the following study, it showed that the increased of PSA content in the d-SPE to 0.2 g and later 0.3 g with the absence of GCB, resulted in the drop of recoveries for both pesticides (Table 2.12) with no improvement of the cleanliness of the chromatograms, except for some minor peaks at 16 min of the retention time (Figure 2.9 D and Figure 2.9 E).

Table 2.12: Recoveries (0.1 µg/g) and relative standard deviation (RSD) of pyrethroids in CPO samples with various d-SPE sorbents (d-SPE1, d-SPE2, d-SPE3, d-SPE4, and d-SPE5)

Sorbent material	Cypermethrin		λ-cyhalothrin	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
d-SPE1	99.09	2.31	88.15	1.65
d-SPE2	90.49	5.91	72.89	2.68
d-SPE3	90.41	4.29	71.09	3.77
d-SPE4	79.97	5.57	66.64	5.46
d-SPE5	72.77	3.34	61.68	4.63

n = 3

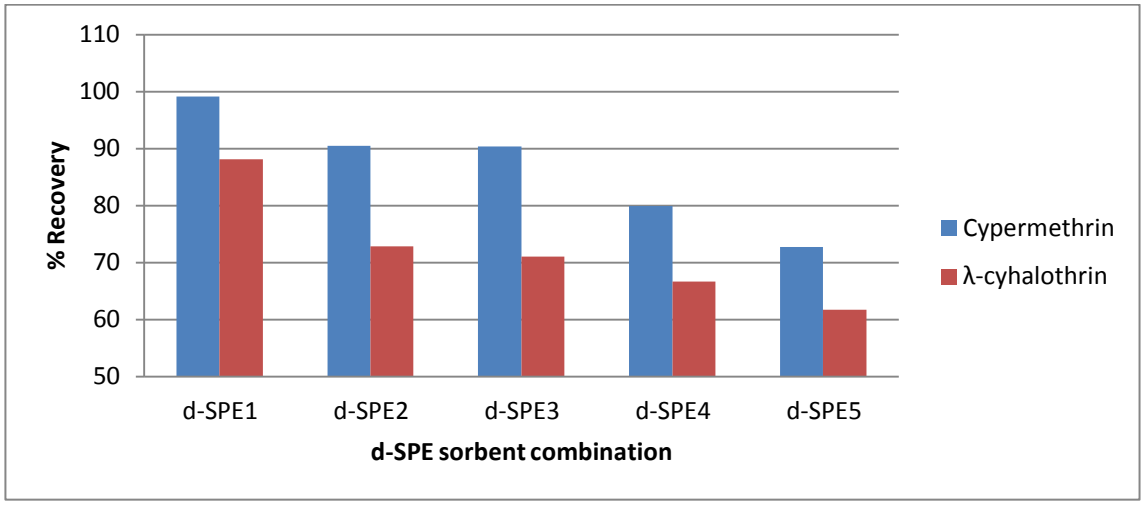

Figure 2.8: Recoveries (n = 3) of pyrethroids from CPO analyzed by GC-ECD and fortified at 0.1 µg/g with various d-SPE sorbents used for clean-up

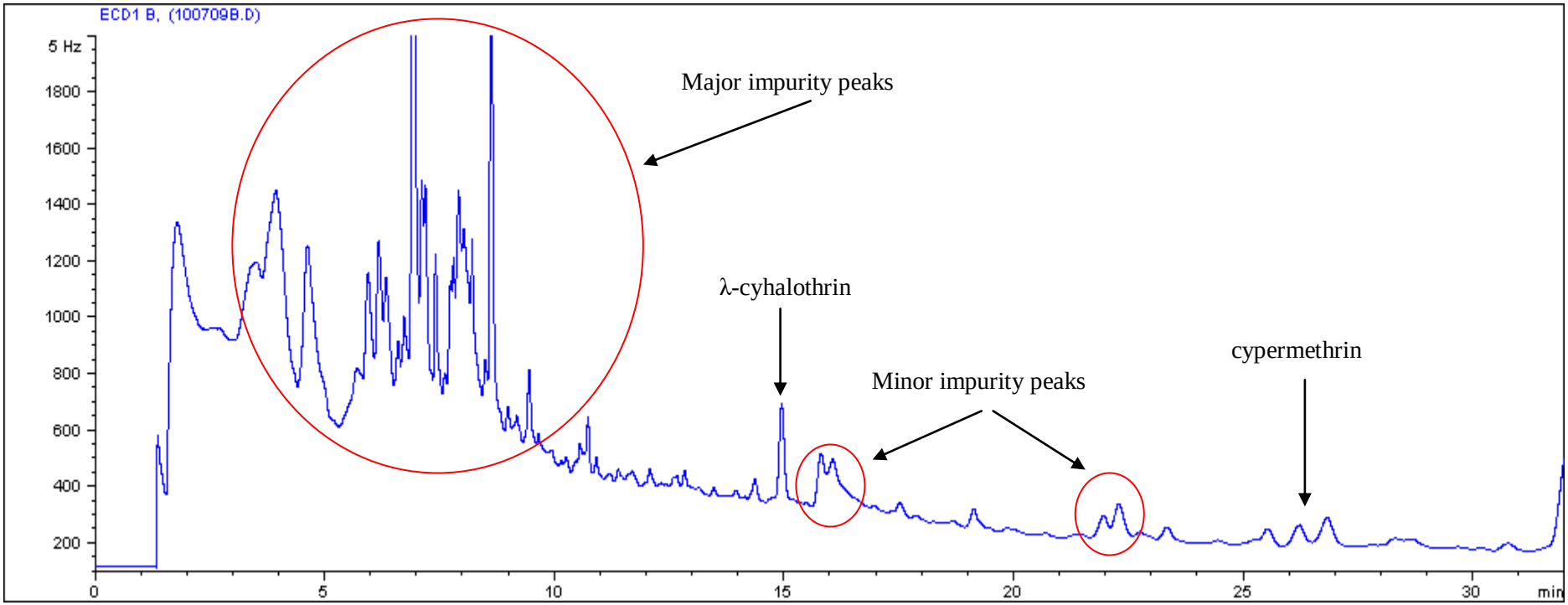

Figure 2.9 (A): GC-ECD chromatogram of fortified (0.1 µg/g) CPO extracts after clean-up with d-SPE1 (0.3 g $MgSO_4$)

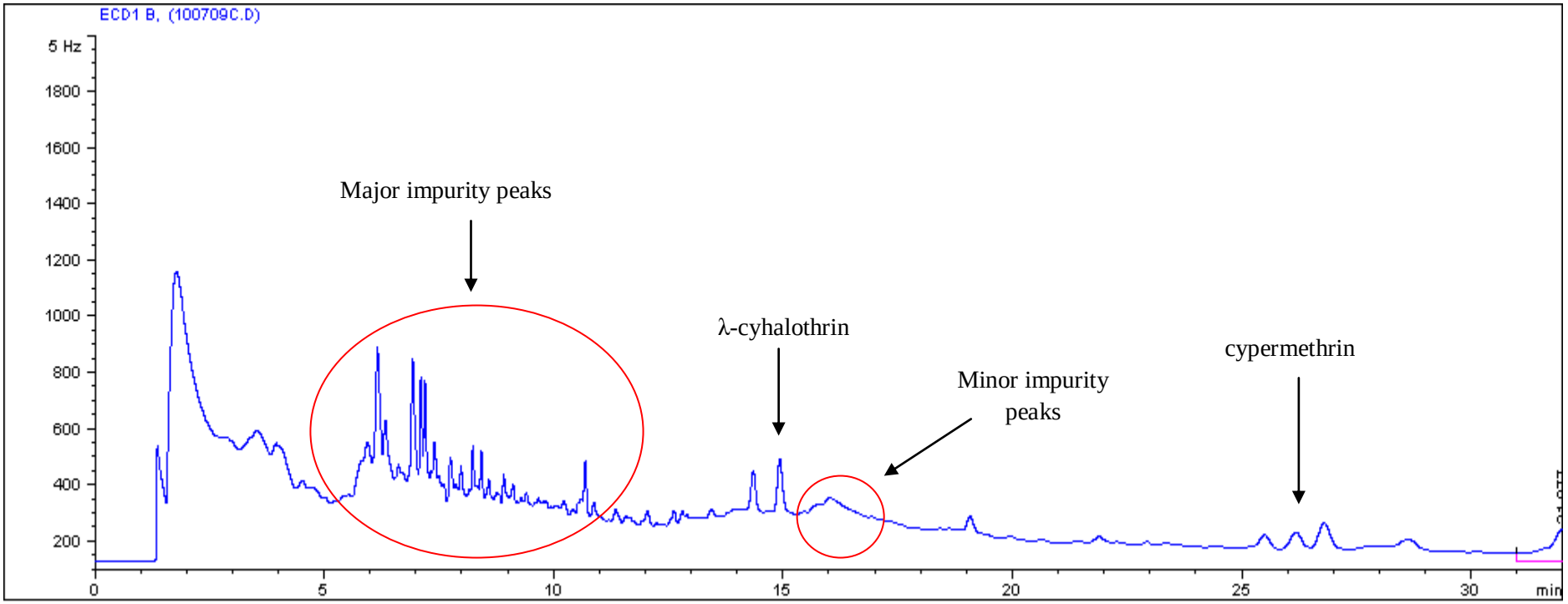

Figure 2.9 (B): GC-ECD chromatogram of fortified (0.1 µg/g) CPO extracts after clean-up with d-SPE2 (0.3 g anhydrous $MgSO_4$ + 0.1 g PSA)

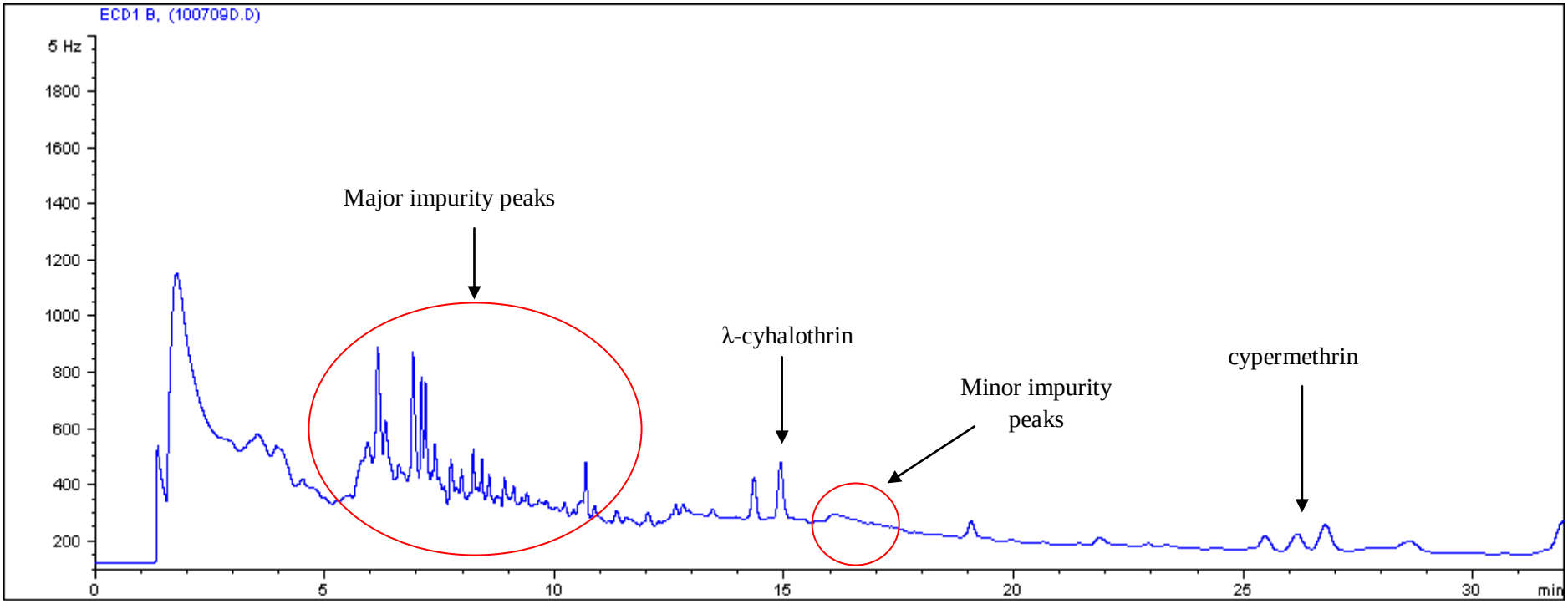

Figure 2.9 (C): GC-ECD chromatogram of fortified (0.1 µg/g) CPO extracts after clean-up with d-SPE3 (0.3 g anhydrous $MgSO_4$ + 0.1 g PSA + 0.025 g GCB)

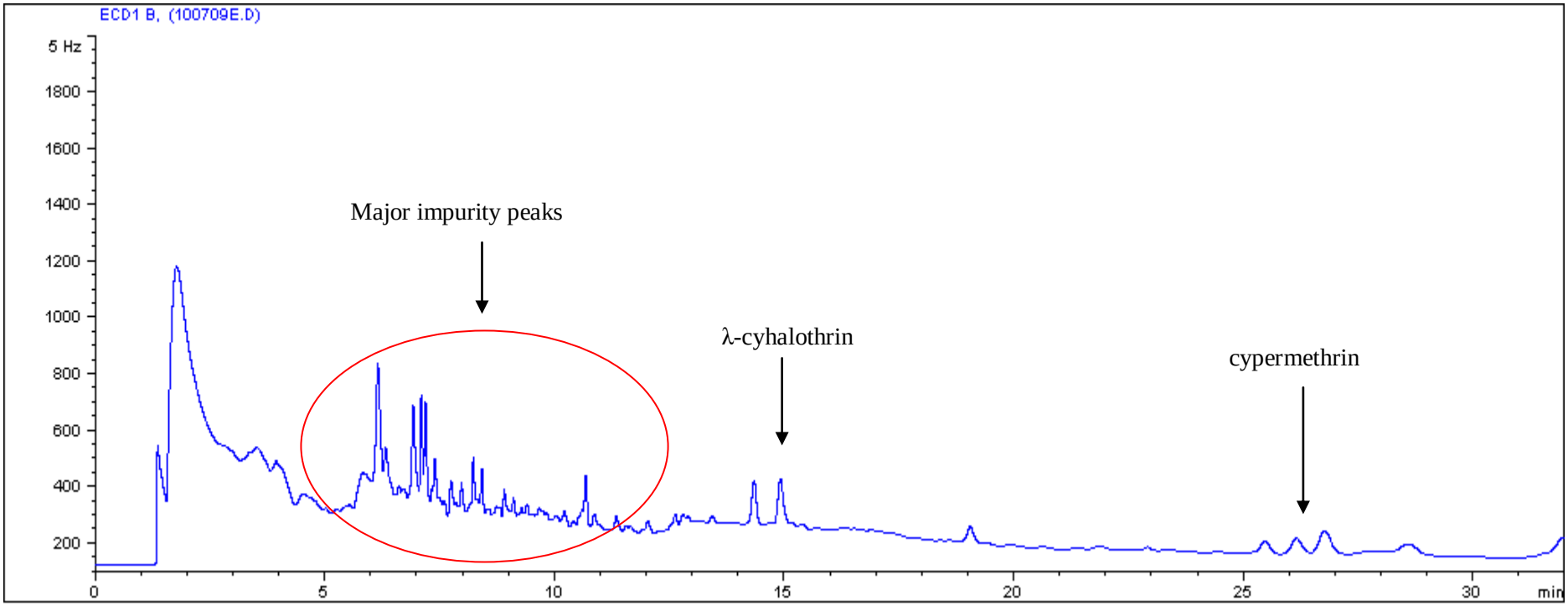

Figure 2.9 (D): GC-ECD chromatogram of fortified (0.1 µg/g) CPO extracts after clean-up with d-SPE4 (0.3 g anhydrous $MgSO_4$ + 0.2 g PSA)

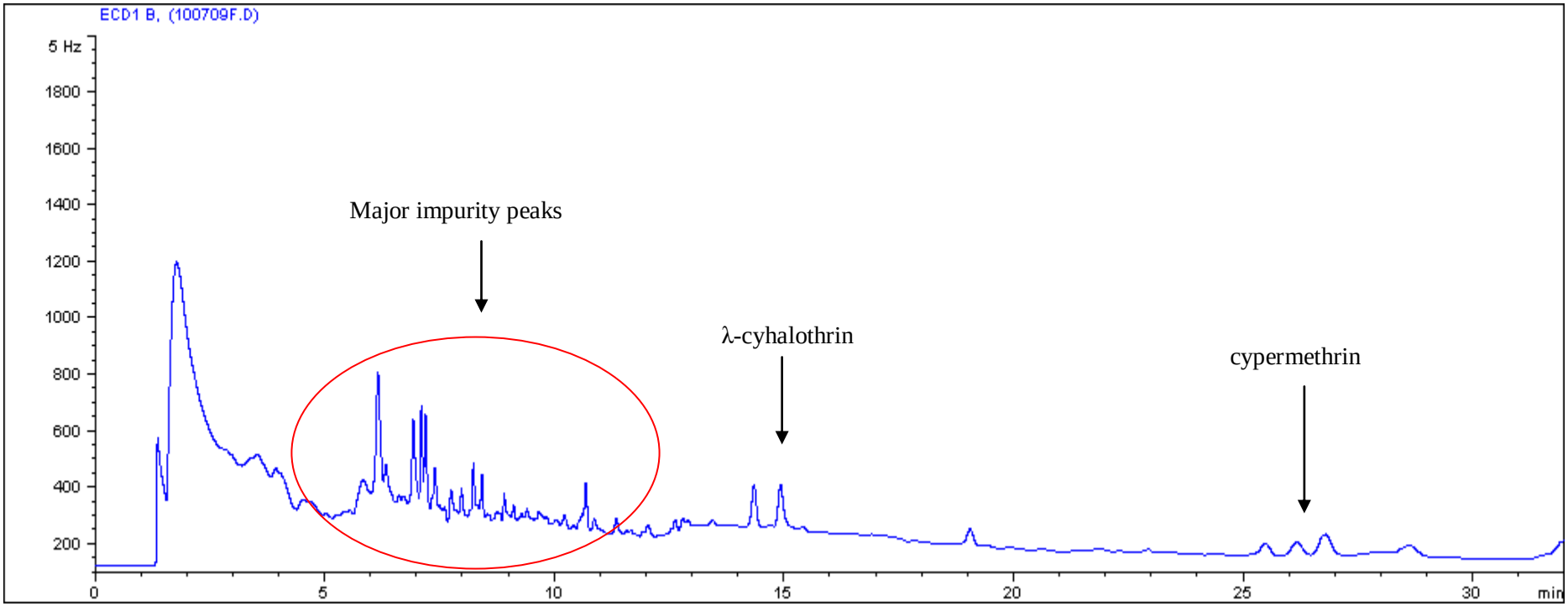

Figure 2.9 (E): GC-ECD chromatogram of fortified (0.1 µg/g) CPO extracts after clean-up with d-SPE5 (0.3 g anhydrous $MgSO_4$ + 0.3 g PSA)

To further confirm the finding, another set of analysis was carried out where anhydrous $MgSO_4$ was kept constant at 0.3 g while PSA content was increased gradually from 0 to 0.1 g. No GCB was considered in this test. Results as shown in Table 2.13 revealed that the increased of PSA content would significantly decrease the recovery values of both cypermethrin and λ-cyhalothrin. The effect of various PSA contents in the decreasing profile of the recoveries is represented in the line chart in Figure 2.10. The representative chromatograms are shown in Figure 2.11 (A-D). In this test, the results obtained were the same as the previous study, indicating that PSA has the ability to remove matrix co-extractants in the extracts. But in doing so, it will also decrease the recoveries of the studied pesticides. Figure 2.11 showed that the interference peaks (major and minor) at 3-10, 16, and 22 min of the retention time were all decreased in magnitude when PSA was used in d-SPE. The degree of impurity peaks reduction would depend on the quantity of PSA used. The higher the sorbent contents, the cleaner the chromatograms, but the recoveries were lower, as shown in the line chart (Figure 2.10).

Table 2.13: Recoveries (0.1 µg/g) of pyrethroids in CPO samples clean-up with 0.3 g $MgSO_4$ and various PSA sorbent contents

PSA content (µg/g)	Recovery, % (RSD,%)	
	cypermethrin	λ-cyhalothrin
0	86.68 (2.18)	80.56 (4.41)
0.025	85.42 (5.44)	80.26 (6.13)
0.05	80.43 (2.56)	79.23 (3.71)
0.1	79.79 (2.67)	74.56 (3.47)

n = 3

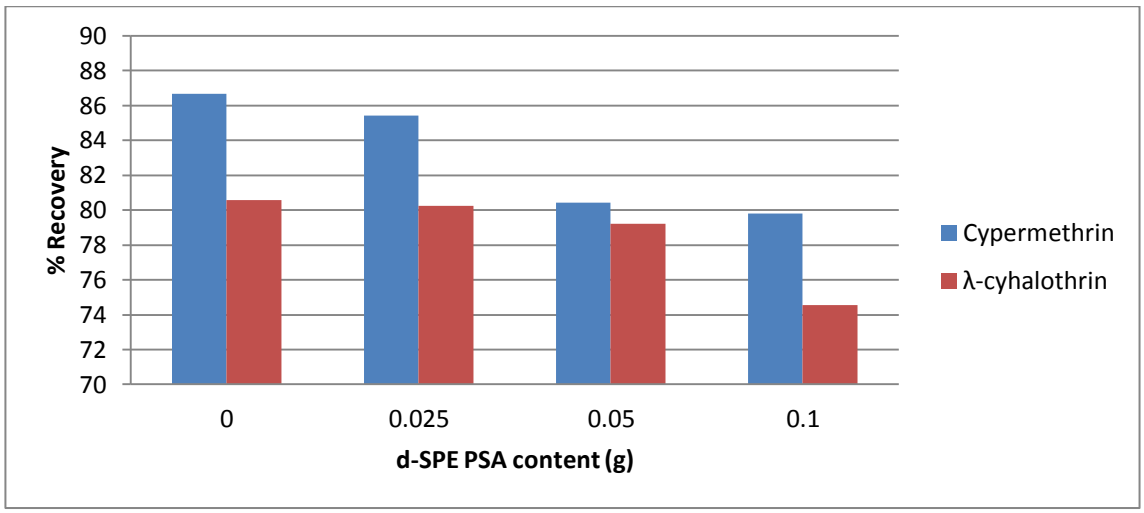

Figure 2.10: Recoveries (n = 3) of pyrethroids from CPO fortified at 0.1 µg/g and analyzed by GC-ECD with different amount of PSA sorbent used for clean-up

From the preliminary study of the d-SPE clean-up, three points can be highlighted as a summary for this clean-up technique. First, the use of PSA as sorbent in d-SPE reduced the nasty interference peaks in the chromatograms and gave cleaner extracts with less matrix co-extractants in the final solution. From the results, higher PSA content gave cleaner chromatogram profile especially in the early elution peak interferences. When PSA content reached 0.1 g, the effect of the sorbent reaches a maximum, and further increased of PSA content would not give any big difference in terms of chromatogram cleanliness.

Secondly, higher PSA content comes with a great reduction of recoveries for both cypermethrin and λ-cyhalothrin. A proportional decreased of recovery was observed when PSA content was increased from 0 to 0.1 g. This finding can be some kind of hindrance for the application of d-SPE clean-up to palm oil matrices because it is very important to obtain a clean final extracts and at the same time maintaining good recoveries. Dirty extracts with even a small amount of fats may disrupt the columns and harm the detectors and hence upset the right analyte determination through signal suppression. Furthermore, the presence of high concentrations of fatty acids in the samples can complicate the GC detection system of low pesticide concentrations. This is due to the presence of high matrix peaks in the chromatograms that mask the analytes of interest and finally decrease the detection limit of the method. Last but not least, GCB gave no effect in the chromatogram. Its only purpose was to decolorize the extracts from carotenoids. After taking all the findings into consideration, it was concluded that d-SPE clean-up technique was not very efficient for cypermethrin and λ-cyhalothrin in palm oil matrices and hence rejected from the method development.

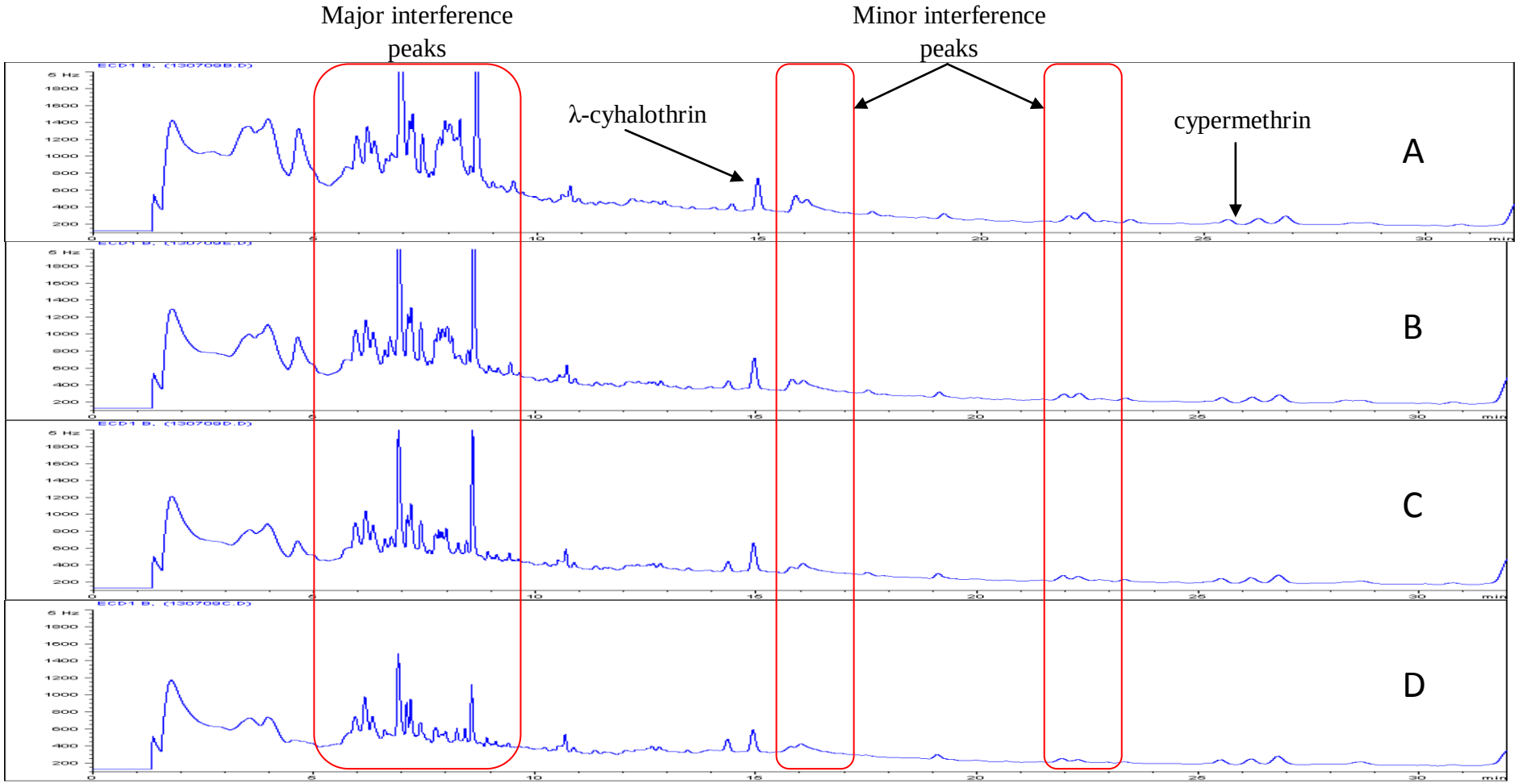

Figure 2.11: GC-ECD chromatogram of fortified (0.1 µg/g) CPO extracts after clean-up with (A) 0.3 g anhydrous $MgSO_4$; (B) 0.3 g anhydrous $MgSO_4$ + 0.025 g PSA; (C) 0.3 g anhydrous $MgSO_4$ + 0.05 g PSA; (D) 0.3 g anhydrous $MgSO_4$ + 0.1 g PS

2.4.5 METHOD VALIDATION

Method validation can be interpreted as being the process of defining an analytical requirement, and confirming that the method under consideration has performance capabilities consistent with what the application requires [111]. Method validation is usually considered to be very closely tied to method development, although it is often not possible to determine exactly where method development finishes and validation begins. Many parameters of the method performance that are associated with method validation are in fact usually evaluated, at least approximately, as part of method development. Hence, it is implicit in the method validation process that the studies to determine method performance parameters are carried out using equipment that is within specification, working correctly, and adequately calibrated. Likewise the operator carrying out the studies must be competent in the field of work under study and have sufficient knowledge related to the work to be able to make appropriate decisions from the observations made as the study progresses.

In this study, the method must be tested to assess for linearity, selectivity/specificity, sensitivity, mean recovery (as measure of accuracy), and precision. The optimized method conditions are as follows: a 5 g of oil (CPO, CPKO) was weighed out into a 50-mL screw cap test tube. Then, 10 mL of acetonitrile was added and the mixture was mixed and homogenized using a vortex mixer for 5 minutes. After homogenization, the mixture was left to stand for a while to allow a phase separation between the oil and acetonitrile layer. Normally a good separation took about 1-2 min for CPO and a little bit faster for CPKO. The test tube was then transferred into the freezer and kept horizontally for a minimum of 2 hours for oil precipitation. After 2 hours, the test tube was removed from the freezer gently without disturbing the precipitated solids. Then, it was stood vertically leaving the frozen oil adhered to the

test tube wall. The acetonitrile extract was transferred immediately using a Pasteur pipette into a small beaker, leaving the frozen oil in the test tube. It was left for a while before 2 mL of acetonitrile extract was loaded into pre-conditioned GCB cartridge and concurrently an adaptor was attached at the top of the pre-conditioned PSA cartridge reservoir. The extract was initially allowed to flow under gravity, and then a gentle pressure was applied to achieve a flow of approximately one drop per second. Collection of the eluate was started at this point into a 10-mL graduated vial. The column was then eluted with 2 mL of acetonitrile and the volume collected was adjusted to 5 mL. Eluate was then mixed and ready for GC analysis. The simplified method is shown in Figure 2.12.

2.4.5.1 LINEARITY AND REPEATABILITY

For any quantitative method, it is necessary to determine the range of analyte concentrations or property values over which the method may be applied [111]. Then, calibration curve was constructed to obtain the linearity of the analytical method. A 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. Figure 2.13 and 2.14 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 2.3.8, no internal standard was used for quantification in the GC-ECD method since GC auto-sampler was used in the injection of samples. Repeatability of the chromatographic method for the electron capture detector was determined by injection of 0.2 µg/mL standard solution and oil fortified at 0.2 µg/g. Both the standard solution and fortified oil were injected ten times *via* an auto-sampler. Table 2.14 shows the summarized repeatability data for retention times and peak areas. Overall, the results showed that the repeatability of the chromatographic method obtained by automatic injection was acceptable with the RSD values for peak area and retention time ranged from 0.47 to 1.61 % and 0.0017 to 0.0086 % respectively. Hence, it can be concluded that the injection technique gave small error in the analytical method.

Table 2.14: Repeatability data (retention time and peak area) of pesticides analyzed in CPO, CPKO, and pure solvent (fortified at 0.2 µg/g, n = 10)

Compound	Repeatability (% RSD)					
	t_R			Peak area		
	CPO	CPKO	Acetone	CPO	CPKO	Acetone
λ-cyhalothrin	0.0069	0.005	0.0086	1.27	0.47	0.65
cypermethrin	0.0017	0.0085	0.0056	1.35	1.61	1.48

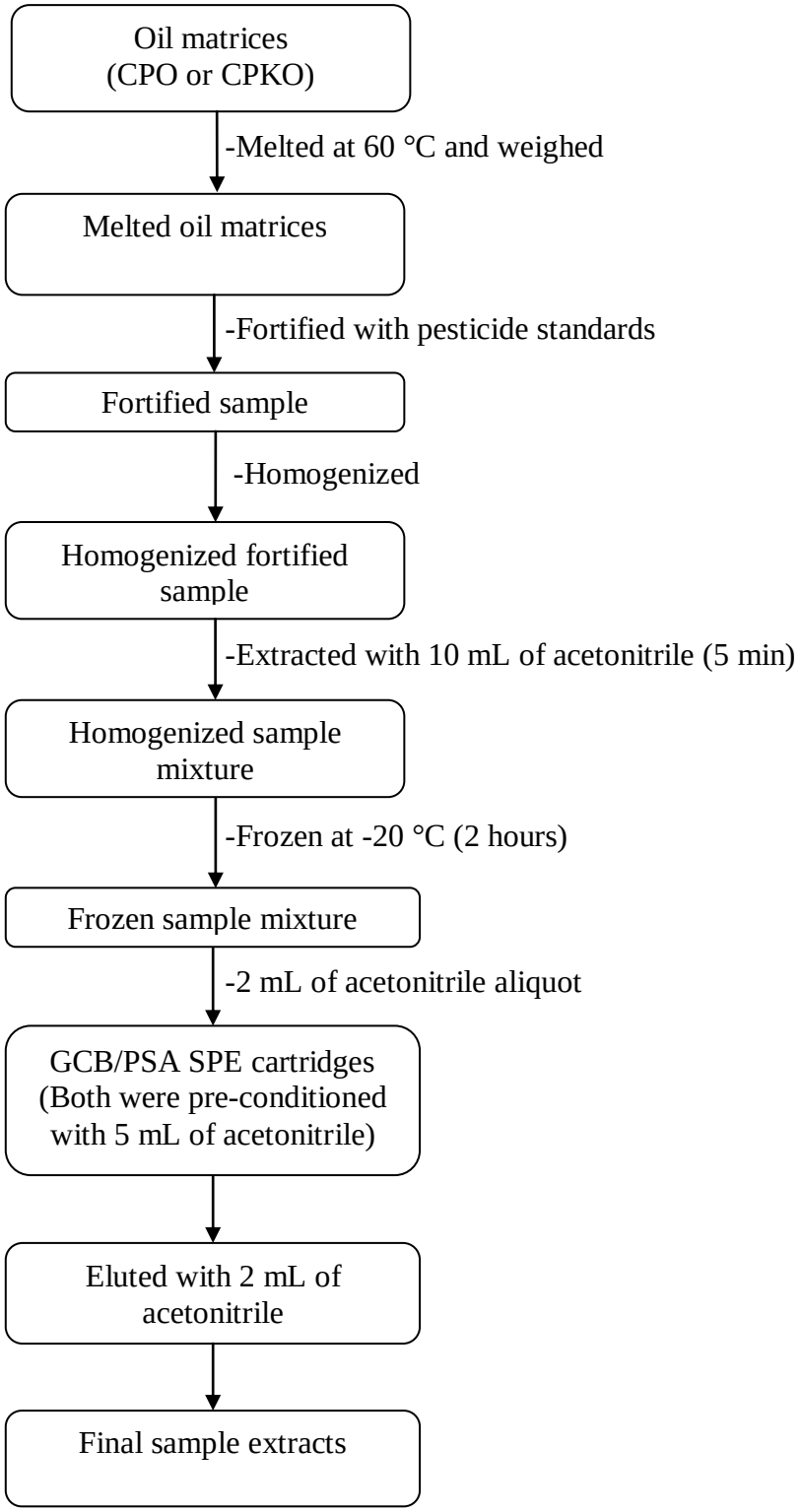

Figure 2.12: Flow chart of an optimized extraction and clean-up of pesticides using low-temperature precipitation and SPE method

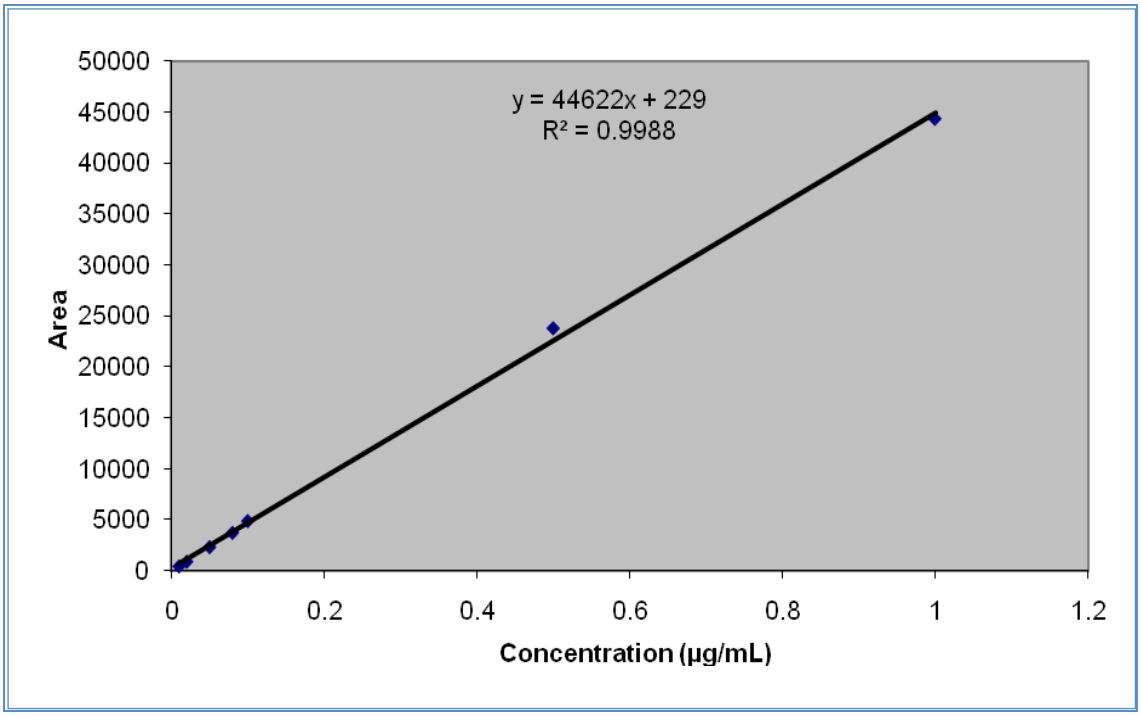

Figure 2.13: Calibration curve of λ-cyhalothrin (0.01 – 1 µg/mL)

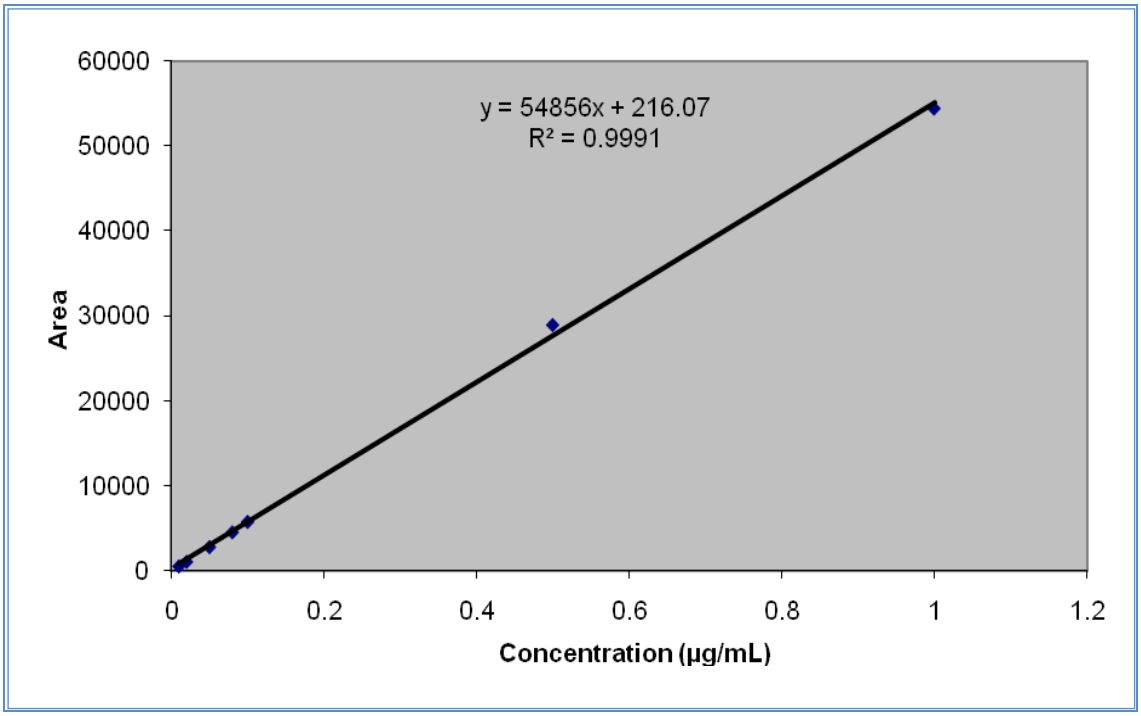

Figure 2.14: Calibration curve of cypermethrin (0.01 – 1 µg/mL)

2.4.5.2 SELECTIVITY/SPECIFICITY

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components (impurities, degradants, and matrix components) in the sample matrix under the stated conditions of the test [112]. In method development, it is necessary to establish that the signal produced at the measurement stage, or other measured property, which has been attributed to the analyte, is only due to the analyte and not from the presence of something chemically or physically similar or arising as a coincidence [111]. Whether or not other compounds interfere with the measurement of the analyte will depend on the effectiveness of the isolation stage (extraction/clean-up) and selectivity/specificity of the measurement stage (gas chromatography). Selectivity and specificity are measures which assess the reliability of measurements in the presence of interferences. In this case, it is far more difficult to state that nothing interferes than pointing out the interferences, since there is always the possibility of encountering some interference in the real samples in the real world. For that reason, the analyst always plays an important role to decide at what point it is reasonable to stop looking for interferences.

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 2.15 and 2.16 show the pesticide standard solutions, blank oil samples, and fortified oil samples by GC-ECD. In the blank samples of CPO and CPKO, few interferences were present at the analytes retention times, 21.9 min for λ-cyhalothrin and 27.5 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 oil matrix and hence allowed the differentiation and quantification of the analytes. This shows that the method developed could remove much of the interferences in oil

matrices and thus exhibited its selectivity. In the meantime, multiple peaks were observed in the chromatograms for cypermethrin due to the separation of diasteroisomers (Figure 2.15 and 2.16) [113]. Normally, there are four peaks for cypermethrin (cypermethrin I, II, III, IV) when analyzed using HP-5 column, but only three peaks were observed since cypermethrin II and IV could not be separated using DB-608 column [113]. However, DB-608 column was preferred since it gave higher total peak areas compared to HP-5 column. From the analytical point of view, the problems of analyzing pyrethroid pesticides lie in difficult separation of enantiomers and diasteroisomers [81]. In the case of a mixture of isomers, the analytical signal was obtained by summing the peak areas of all three peaks.

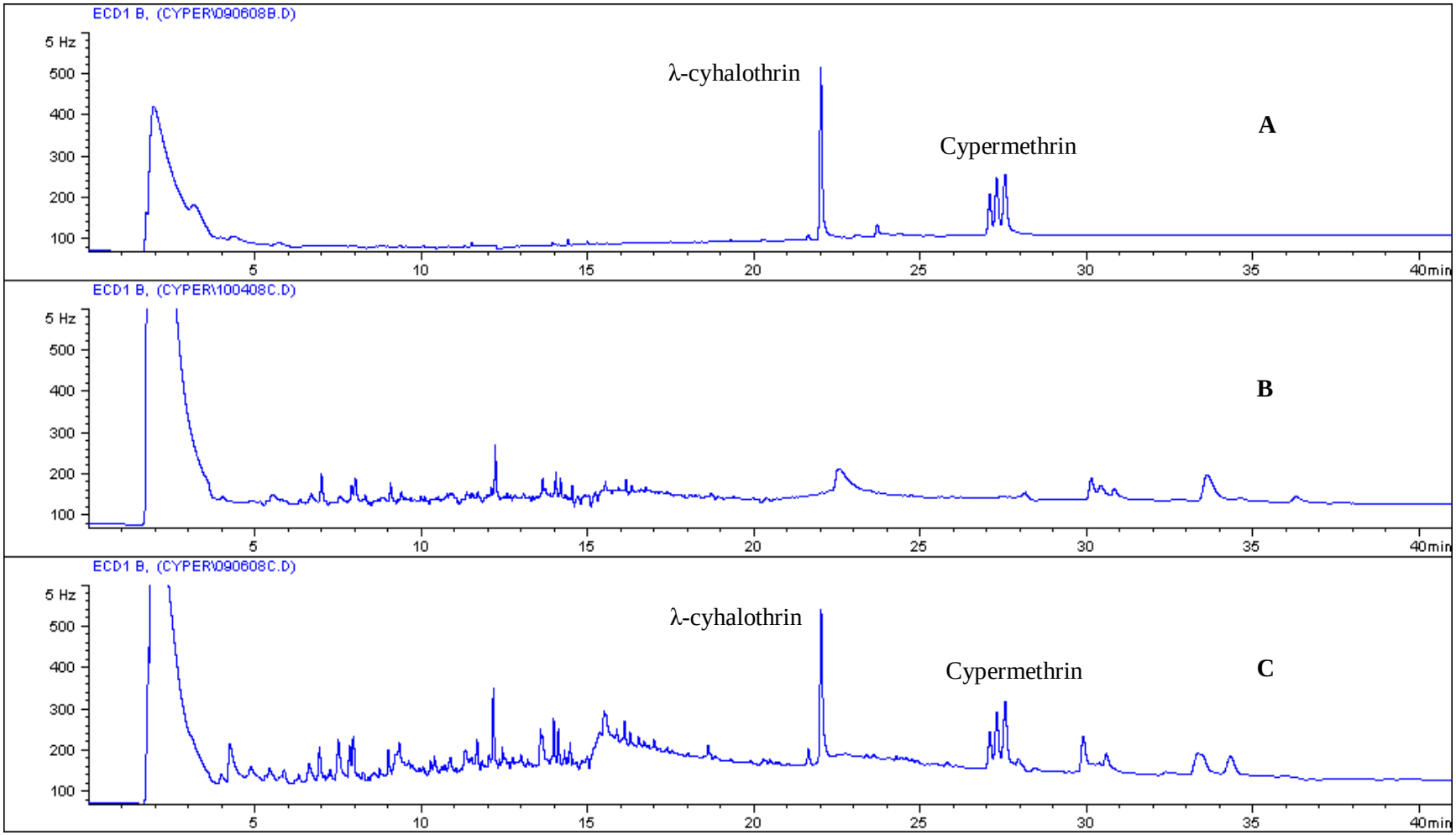

Figure 2.15: Selectivity chromatograms (A) Pesticide standards in pure acetone; (B) Blank CPO sample; (C) Spiked CPO sample

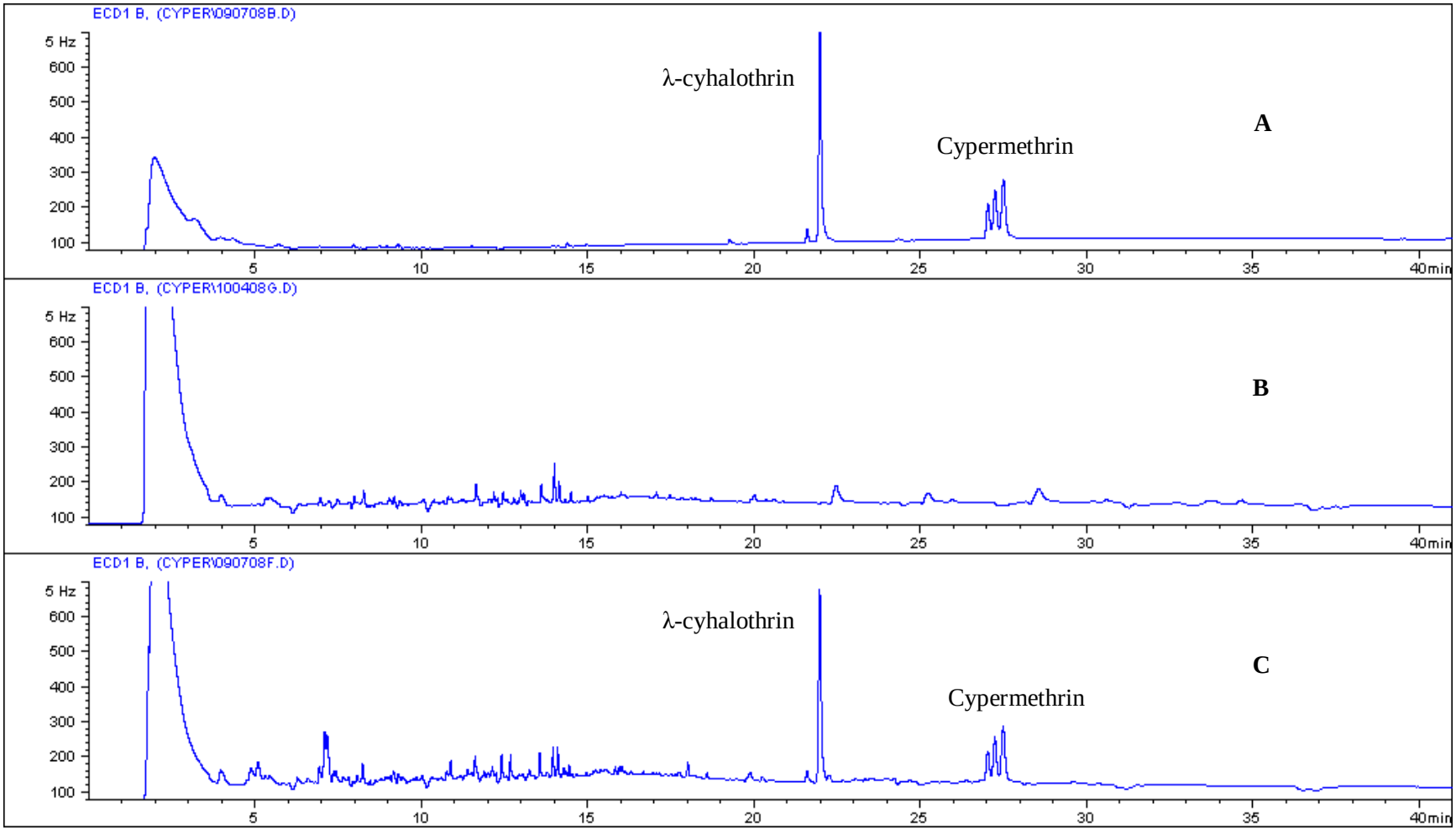

Figure 2.16: Selectivity chromatograms (A) Pesticide standards in pure acetone; (B) Blank CPKO sample; (C) Spiked CPKO sample

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

In trace analysis, it is important to know the lowest concentration of the analyte that can be confidently detected by the method [111]. The LOD is defined as the concentration of analyte that results in a peak height three times the noise level when injected into the chromatographic system. The LOD is the lowest concentration of the analyte in a sample that can be detected but not necessarily quantifiable [114]. As for LOQ, it is strictly the lowest concentration of analyte that can be determined with an acceptable level of accuracy (70-120 %) and precision (< 20 %). It could be variously defined but must be a value greater than the LOD.

In this study, blank oil samples were used to establish the detection and quantification limits for each pesticide. The LOD values of the proposed method were determined at a signal-to-noise ratio (S/N) of 3:1 for the individual pesticides in oils by GC-ECD, whereas the LOQ values were obtained at an S/N of 10:1. Table 2.15 shows the LOD and LOQ values obtained for each pesticide by GC-ECD. The values obtained are lower than the national MRLs. In Malaysia, national MRLs are documented in the Food Act 1983 (Act 281) and Regulations. In this Act, all the agrochemicals registered for usage in the agricultural sector can be found under Regulation 41 of Schedule Sixteenth [115]. Since the LOD and LOQ values obtained for the proposed method are lower than the national MRLs for CPO (λ-cyhalothrin: 0.1 µg/g; cypermethrin: 0.5 µg/g), it can be concluded that the method is sensitive enough to quantify both pesticides in CPO and CPKO samples.

Table 2.15: Limit of detection (LOD), Limit of quantification (LOQ), and Maximum residue limits (MRLs)

Compound		Pesticide level (µg/g)	
		CPO	CPKO
λ-cyhalothrin	LOD	0.025	0.025
	LOQ	0.075	0.075
	MRL*	0.1	0.1
Cypermethrin	LOD	0.05	0.05
	LOQ	0.15	0.15
	MRL*	0.5	0.5

*Food Act 1983 (Act 281) and Regulations Schedule sixteenth [115]

2.4.5.4 RECOVERY AND PRECISION

It is not usually known how much of a particular analyte is present in a test portion. Therefore, it is difficult to be certain how successful the method has been at extracting it from the matrix. One way to determine the efficiency of extraction is to spike test portions with the analyte at various concentrations, then extract the fortified test portions and measure the analyte concentration. The inherent problem with this is that the analyte introduced in such a way will probably not be held as strongly as that which is naturally present in the test portion matrix and so the technique will give an unrealistically high impression of the extraction efficiency [111]. However, having said that, recovery study via fortification is the most common way of determining extraction efficiency, and it is recognised worldwide as an acceptable way of doing so in pesticide residues analysis. However, the drawback of the technique should be borne in mind. Hence, recovery of the analyte need not be 100 %, but the values should be consistent, precise and reproducible. Furthermore, it should be performed by comparing the

analytical results for extracted samples at least three concentrations (low, medium, and high) with pesticide standards that represent 100 % recovery.

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogenous of samples. The precision determined at each fortification level should not exceed 20 % of the coefficient of variation (CV) [112]. In this study, oil samples were fortified at five levels (0.05, 0.08, 0.1, 0.5, and 1.0 µg/g) and six replicates were analyzed for each level. The method performance should meet the acceptable criteria of 70-120 % mean recoveries and coefficient of variation of not more than 20 % [116]. Table 2.16 shows the pesticide recovery results in CPO and CPKO. Recoveries obtained for both pesticides ranged from 81-102 % for CPO and 84-105 % for CPKO. The overall RSD values ranged from 1.3-3.4 % for CPO and 0.1-7.1 % for CPKO. Since both the recovery and RSD values meet the method performance criteria, this indicates the good precision and accuracy of the proposed method.

Table 2.16: Recoveries and relative standard deviation (RSD) of pyrethroids from CPO and CPKO

Pesticides	Spike level (mg/kg)	CPO		CPKO	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
λ-cyhalothrin	0.05	100.49	1.74	100.07	0.08
	0.08	96.34	2.80	90.58	3.39
	0.1	100.20	2.35	88.45	3.81
	0.5	91.52	1.37	102.70	3.89
	1	90.39	3.41	99.17	3.08
Cypermethin	0.05	98.98	3.46	93.00	7.06
	0.08	100.38	2.10	84.20	2.37
	0.1	102.47	2.72	88.82	5.37
	0.5	80.99	3.02	113.50	2.98
	1	98.90	2.03	105.05	0.67

n = 6

2.4.5.5 RUGGEDNESS/ROBUSTNESS

A measure of an effective analytical method is how well its performance stands up to less than perfect implementation [111]. This is because, in any method, there will be certain stages which, if not carried out sufficiently and carefully, will have a severe effect on method performance and may even result in the method not working at all. In this study, these stages were identified during the method development part in section 2.4.2. It involves making deliberate variations to the method, and investigating the subsequent effect on method performance, namely precision and accuracy.

In the initial tests, variations in the extraction techniques and parameters previously mentioned (extraction volume, position of the test tube in the freezer, decanting the liquid phase immediately after freezing, and freezing 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 indicated that the method was adequately robust to be successfully applied by inexperienced analysts.

2.4.6 REAL SAMPLES MONITORING

The developed method was applied in the analyses of 30 commercial CPO samples from various refineries throughout Malaysia. 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 extraction and clean-up 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 none of these CPO samples contained cypermethrin and λ-cyhalothrin residues. This was expected since according to Ainie *et al*. [68], no pesticide residues were detected in oil palm plantations so far based on more than 100 samples of palm oil analysed. 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.

2.5 CONCLUSION

Sample extraction and clean-up technique based on acetonitrile extraction, low-temperature precipitation and solid-phase extraction was successfully developed to determine cypermethrin and λ-cyhalothrin in crude palm oil and crude palm kernel oil. This is the first time that this type of extraction and clean-up technique is used and applied to cypermethrin and λ-cyhalothrin in palm oil matrices. In this study, acetonitrile was chosen as the solvent of choice to extract the pesticides from the oil matrix. Then, the whole mixture was frozen at -20 ºC to precipitate the bulky oil matrix and separate it from the acetonitrile layer. Sample extract was then underwent the clean-up step using SPE cartridges contained graphitized carbon black and primary secondary amine sorbents.

During the optimization process, the effects of some experimental parameters such as extraction volume, freezing time, and SPE sorbents were evaluated to obtain the most efficient extraction and clean-up technique that give the best results. Furthermore, variation in the positioning of the test tube in the freezer and decantation of the liquid phase immediately after freezing gave no significant difference on the results obtained. This indicated that the method was adequately robust to be successfully applied by inexperienced technicians.

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. The recoveries obtained for both pesticides ranged from 81-102 % for CPO and 84-105 % for CPKO. The overall RSD values ranged from 1.3-3.4 % for CPO and 0.1-7.1 % for CPKO. The developed method can be used to determine both pesticide residues in CPO from local refineries throughout Malaysia since the LOD and LOQ obtained are lower than the MRLs as specified in Food Act 1983 (Act 281) and Regulations [115]. From the monitoring study, no pesticide residues were detected in any of the 30 samples analysed. As a conclusion, the results demonstrated that the proposed optimized method is specific, sensitive, accurate, and precise within the established linearity range.