CHAPTER 3

ANALYTICAL METHODOLOGY FOR ENDOSULFAN AND ITS METABOLITES

3.1 INTRODUCTION

Analytical methods play an important role in the detection and quantification of residue levels of the test substances or its metabolites in biological samples. In the past, gas chromatograph-electron capture detector (GC-ECD) has become the main analytical instrument in the determination of OC pesticides in both the biotic and abiotic environment. This chapter reviews the various aspects related in analytical methodology including the published methods and the method development for the present study.

3.2 METHOD DEVELOPMENT FOR RESIDUE ANALYSIS OF ENDOSULFAN AND ITS METABOLITES FOR THE PRESENT STUDY

Siyali (1972) reported that analysis of OC compounds was improved when electron capture detector was introduced in 1960 with detection limit in the range of 10^{-12} gram. Many surveys of the levels of OC pesticides in blood and tissues have been conducted since this invention (Siyali, 1972; Gupta, 1978; Nath *et al.*, 1978).

However, the analysis by using GC-ECD often leads to misidentification or high uncertainties in the identification of certain compounds although comparison of the retention times of standards can give confirmatory data.

3.2.1 Plasma

At present, there has been little reported extraction procedure for extraction of endosulfan and its metabolites from biological fluid. The only reported procedure was by Siyali (1972) using liquid-liquid extraction (LLE) procedure for extraction of OC pesticides from whole blood. In the present method, extraction was initially carried out by LLE using plasma but subsequently, solid phase extraction (SPE) was chosen as the better technique.

LLE was replaced by SPE because of the low recoveries for the parent compounds (α- and β-isomers) and endosulfan sulfate. Endosulfan diol was not extracted when LLE was applied. Hence, the extraction procedure for plasma was performed using SPE with C18 end-capped column. The C18 column was manufactured using a tri-functional silane and end-capped to reduce the polar secondary interactions associated with surface silanol groups.

A pH2 buffer was added into the plasma in a ratio of 1:1 to enhance the recovery of endosulfan diol.

3.2.2 Tissue sample

The extraction procedure of endosulfan (α- and β-isomers) and endosulfan sulfate was based on Soxhlet extraction (IAEA, 1998). Sample clean-up was initially carried out using sulfuric acid to remove lipids from the tissue prior to the introduction of the sample to the Florisil packed chromatographic column. However, SPE was found to be the better clean-up technique as it was able to yield high recoveries of the analytes and was efficient in removing the lipid contents.

The SPE clean-up procedure was performed using Florisil column, which is packed with powdered magnesia-silica gel. This technique has the advantage of combining filtration, extraction and adsorption into one single step resulting in faster and in some case, a more efficient clean-up method.

The extraction procedures of plasma and tissues are explained in Section 3.3 and 3.4.

3.2.3 Analysis of endosulfan and its metabolites in biological samples

Mass spectrometry is very powerful in identification of organic compounds, because only molecules belonging to the same substances should be present in the ion source while generating the mass spectrum. The specificity and sensitivity of mass spectrometry has also rapidly superseded conventional detectors for complex analytical problems, thus making it an unrivalled technique in providing qualitative and quantitative results in environmental analysis (Bruner, 1993).

In the present study, gas chromatographic-mass spectrometry (GC-MS) method was chosen instead of GC-ECD for the separation and detection of endosulfan and its metabolites in plasma and tissues to avoid false positive results given by the electron capture detector. In addition, its linearity is poor, and a lot of attention has to be paid to cleanliness and the purity of gases and solvents (Willett, 1991). The advantage of mass spectrometer (MS) over other methods of identification is that the response is given by integer mass units. Since the MS when combined with a GC will serve as a multi-detector, the computerized data acquisition system in the market today improves our ability to identify most compounds in a reasonably pure form by GC.

The J & W DB1-MS column was used in the present study. This column was chosen as it was able to give better resolution to β-endosulfan and endosulfan diol compared to other columns such as DB1 and DB5.

The total ion chromatogram is shown in Figure 3.1.

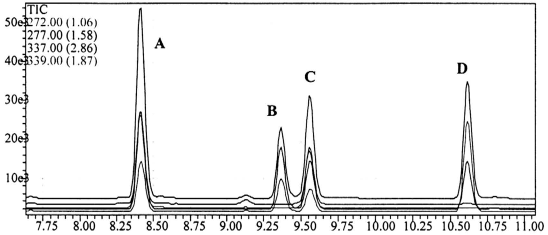


Figure 3.1. Total ion chromatogram of α-endosulfan (A), endosulfan diol (B), β-endosulfan (C) and endosulfan sulfate (D)

The mass spectra of endosulfan and its metabolites are shown in Figures 2.9, 2.10, 2.11 and 3.2.

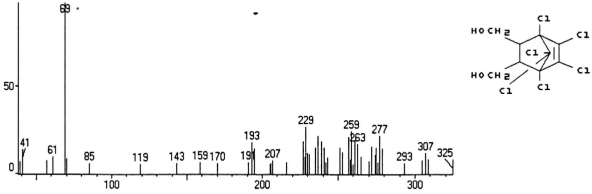


Figure 3.2. Mass spectrum of endosulfan diol

3.2.4 Solid phase extraction (SPE)

Solid phase extraction (SPE) is a technique based on the principle of liquid-solid chromatography and it is analogous to liquid-liquid extraction (Bruner, 1993). As the liquid sample is passed through the SPE column, compounds are extracted from the sample onto the sorbent material in the column. Interferences can then be selectively removed from the column through the correct choice of wash solvents. Finally, the desired analytes may be selectively recovered from the column by an elution solvent, resulting in a highly purified extract. This extract is often significantly more concentrated than the original sample. Alternatively, an extraction column may be selected which retains the interferences in the sample, but allows the analytes to pass through unretained.

SPE offers several advantages, namely reduction in solvent consumption, high recoveries of the analytes and a faster operation in which a larger number of extraction processes may be carried out within a given time (Bruner, 1993).

3.3 EXTRACTION PROCEDURE FOR ENDOSULFAN AND ITS METABOLITES (ENDOSULFAN SULFATE AND ENDOSULFAN DIOL) IN PLASMA

A schematic diagram of the extraction procedure is shown in Figure 3.3. A 1000 μl volume of the mixture of plasma sample and KH_2PO_4 (0.04M, pH2) buffer (1:1) was transferred using a plastic disposable transfer pipette to the C18 end-capped column placed at a solid phase extraction manifold after the column was pre-conditioned with 1 ml of methanol and 1ml of distilled water. The plasma was allowed to flow under vacuum condition until finished. 6 ml of distilled water was added to the column under the same vacuum condition until finished. This step, which is called the interference elution is to clean any endogenous compounds, which are trapped at the packing material. Following that, the column was vacuumed at 10 inHg for 15 minutes. To elute endosulfan and its metabolites out of the column, 2 ml of methanol was added to the column and collected in a labeled glass tube. The content of the glass tube was then evaporated to dryness under a stream of nitrogen and reconstituted to 100 μl of methanol. 2 μl of the concentrated extract was injected into the gas chromatograph-mass spectrometer (GC-MS). The detailed GC-MS procedure is explained in Section 3.5.8.

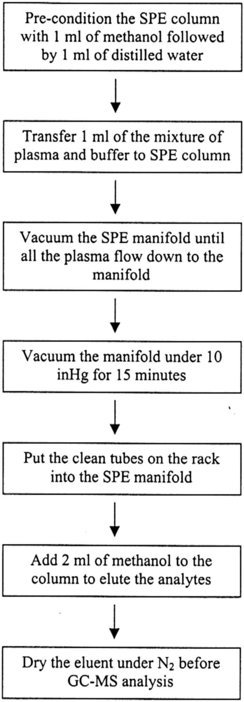


Figure 3.3. Schematic diagram for the extraction of endosulfan and its metabolites in plasma

3.4 EXTRACTION PROCEDURE FOR ENDOSULFAN AND ITS METABOLITE (ENDOSULFAN SULFATE) IN TISSUE WITHOUT FREEZE-DRYING

Tissue was dried on filter paper to remove any superficial fluid. Anhydrous sodium sulfate was added to the sample and manually homogenized to determine whether the sample was adequately dried. If not, more anhydrous sodium sulfate should be added until a dry mixture was obtained. Normally, three times the sample weight used should be adequate. Once this has been achieved, the mixture was ground with the mortar and pestle until it was well homogenized and the sample appeared dry. The mixture was transferred to a clean extraction thimble and the dehydrated tissue was extracted with about 200 ml of dichloromethane (DCM) for 8 hours in a Soxhlet apparatus, cycling 4 to 5 times per hour. The extract was then concentrated to about 1 ml and transferred to a clean vial. The concentrated extract was further dried under a stream of nitrogen before reconstituted to 1 ml of DCM prior sample clean-up.

3.4.1 Sample clean-up utilizing SPE

A schematic diagram of the extraction procedure is shown in Figure 3.4. A mixture of 500 μl of the above concentrated extract and 500 μl of DCM was transferred using a disposable plastic pipette to the Florisil column placed at a solid phase extraction manifold after the column was pre-conditioned with 6ml of dichloromethane, 6 ml of n-hexane and 6 ml of DCM again. The sample was collected and allowed to flow without vacuum. To elute endosulfan and its metabolite out of the column, 6 ml of dichloromethane was added to the column and collected in a labeled glass tube. The content was evaporated to dryness under a stream of nitrogen and reconstituted to 100 μl of DCM. 2 μl of the concentrated extract was injected into the gas chromatograph. The detailed GC-MS procedure and conditions were explained in Section 3.5.8.

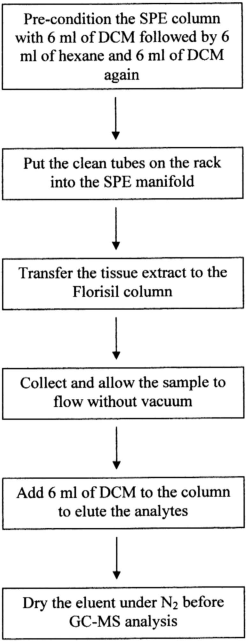


Figure 3.4. Schematic diagram for the clean-up procedure for tissue after extraction

3.4.2 Determination of extractable organic matter (EOM)

The extractable organic matter (EOM) weight of a sample is operationally defined as the weight of material extracted with the solvent employed. An aliquot of the sample extract is taken, the solvent is evaporated and the residue is weighed to determine the quantity of lipids extracted in the aliquot and from it to the total sample. The results are reported in μg lipids per gram dry weight extracted. Hence, the quantity of EOM is:

$$\text{EOM } (\mu g/g) = \frac{\text{Weight of residue (mg) X Volume of the extract (ml) X 100}}{\text{Volume evaporated (ml) X Quantity of sample extracted (g)}}$$

EOM will only be determined if the sample shows the presence of endosulfan or its metabolite in this study.

100 μl was removed from a 1 ml sample extract, without clean-up and placed in a clean glass tube. The 100 μl of the aliquot was evaporated under a stream of nitrogen and the residue was weighed. Three determinations were made and the average was taken.

3.5 EXPERIMENTAL

3.5.1 Chemicals

1.	Standards of α-endosulfan, β-endosulfan, endosulfan sulfate and endosulfan diol	Dr. Ehrenstorfer, Germany, analytical standard (99.0% purity)
2.	Potassium dihydrogen orthophosphate	BDH Chemicals, England; analytical grade
3.	Anhydrous sodium sulfate	BDH Chemicals, England; analytical grade
4.	Methanol	Fisher Scientific; 99.5% purity
5.	Dichloromethane (Analytical grade)	Fisher Scientific; 99.5% purity
6.	n-hexane (Analytical grade)	Fisher Scientific; 99.5% purity
7.	Phosphoric acid	Fisher Scientific
8.	C18 end-capped cartridge	Chromabond
9.	Florisil cartridge	International Sorbent Technology (IST)

3.5.2 Apparatus

1.	Rotary evaporator	EYELA
2.	Analytical balance	ER-180A
3.	Solid phase extraction manifold	IST
4.	Gas chromatograph-Mass spectrometer	GC-MS (QP5050A) Shimadzu

3.5.3 Stock solutions

Stock solutions (100 μg/ml) of α-endosulfan, β-endosulfan, endosulfan sulfate and endosulfan diol were prepared in methanol and stored at 4°C.

3.5.4 Standard solutions

Standard solutions of α-endosulfan, β-endosulfan, endosulfan sulfate and endosulfan diol were prepared as described in Appendix 2.

3.5.5 Preparation of buffer

KH_2PO_4 buffer solution was prepared as described in Appendix 2.

3.5.6 Sample preparation for plasma

The samples to be analysed were taken out from the freezer (-20°C) and thawed at room temperature. After complete thawing, a 500 µl samples were transferred to a clean glass tube. The samples then underwent an extraction procedure as described in Section 3.3 and then analysed under the chromatographic conditions as described in Section 3.5.8.

3.5.7 Sample preparation for tissue

The samples to be analysed were taken out from the freezer (-20°C) and thawed at room temperature. After complete thawing, the samples underwent an extraction procedure as described in Section 3.3 and then analysed under the chromatographic conditions as described in Section 3.5.8.

3.5.8 Gas Chromatograph-Mass Spectrometer

A Shimadzu QP5050A gas chromatograph coupled with mass spectrometer was used for the quantitative analysis of residue of endosulfan and metabolites in plasma and tissues. The J&W DB1-MS fused silica capillary column (30m x 0.25mm i.d.) was used. The operating conditions for the Gas Chromatograph are as follows:

Oven temperature: Initial temperature 120°C

Ramp 30°C/min to 200°C followed by 5°C/min to 270°C and hold for 2 minutes

Injector temperature: 280°C

Interface temperature: 300°C

Carrier gas: Helium (Highly purified)

Carrier gas pressure: 120.7 kPa

Carrier flow rate: 75.9 ml/min

Column flow rate: 1.5 ml/min

Injection mode: Splitless

Injection volume: 2 μl

Mass spectrometer:

The detection was done using quadruple detector with electron ionization mode detection.

Data acquisition mode: Selected Ion Monitoring (SIM)

Ion monitored: α-endosulfan (272, 277, 337, 339); β-endosulfan (272, 277, 337, 339); endosulfan sulfate (272, 277) and endosulfan diol (272, 277)

3.5.9 Calibration curve

A calibration curve is a relationship between known concentrations of the analyte and the response of the instrumentation. The standard samples used consist of known concentrations of α-endosuflan, β-endosuflan, endosulfan sulfate and endosulfan diol. The calibration curve was constructed with a minimum of four values from the expected range of concentrations. In this study, calibration was done using external standards and the expected range was from 30 ng/ml to 1000 ng/ml covering at least four concentration points. Standard solutions of the mixture of endosulfan and its metabolites were prepared: 30, 50, 70, 100, 300, 500, and 1000 ng/ml as described in Appendix 2. The calibration curve of each standard was plotted as the area of the analyte against the concentration of the standard solutions. The calibration curve must be linear with R^2 value greater than 0.99.

3.5.10 Quantification

The direct comparison technique using external standards was chosen for the present study because the test substances identified in the sample clearly matched with known standards based on their retention times.

3.6 PRECISION, ACCURACY AND RECOVERY

3.6.1 Precision and efficiency of the analytical methods

The precision and the efficiency of the analytical method should be established by the replicate analysis of samples of the appropriate matrix. Precision describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogenous volume of biological matrix, usually expressed as the standard deviation of the coefficient variation. Efficiency is defined as a capability of producing a desired value, while accuracy is the combination of bias and precision of an analytical procedure which reflects the closeness of a measured value to a true value. Hence, precision and accuracy should be evaluated as a matter of course during initial implementation procedure just before initiation of sample analysis.

In this study, precision was measured using a minimum of five replicates per concentration. Three concentrations were chosen, being within the range of the calibration curve. The three concentrations of endosulfan and its metabolites identified as being low, medium and high were 10, 50 and 100 ng/ml. The coefficient variation (C.V.) was measured for every concentration and is measured as standard deviation (S.D.) / Mean.

3.6.2 Blank

Blanks represent an opportunity to evaluate and monitor the potential introduction of contaminants into samples during processing. Contributions to the analyte signal can arise from contaminats in the reagents, those arising from passive contact between the sample and the environment (i.e. atmosphere) and those introduced during sample handling by hands, implements or glassware. It is essential to establish a consistently low (i.e. with respect to analytes) blank prior to initiating analysis or even the determination of the method detection limit. In addition, it is necessary to perform blank determinations on a regular basis (i.e. every batch of samples).

3.6.3 Recovery

Recovery reflects the ability of the analyst to fully recover surrogate compounds introduced to the sample matrix or blank at the beginning of the procedure. The primary criteria for selection of compounds to be used for testing recovery are that they: 1) have physical (i.e. chromatographic/partitioning) properties similar to and if necessary spanning those of the analytes of interest, 2) do not suffer from interferences during gas chromatographic analysis, and 3) be baseline resolved from the analytes of interest. In other words, the recovery of an analyte is the result obtained from an amount of the analyte fortified to and extracted out from the biological matrix, compared to the result obtained from the pure standard.

For the analysis of recovery in plasma, five extracted samples of endosulfan and its metabolites were prepared for low (10 ng/ml), medium (50 ng/ml) and high concentrations (100 ng/ml).

As for the analysis of recovery in tissue samples, five extracted samples of endosulfan and its metabolite except endosulfan diol were prepared for low (100 ng/ml), medium (500 ng/ml) and high concentrations (1000 ng/ml). Endosulfan diol was not spiked into the tissue samples since it was not present in the tissues and would be excluded in tissue residual analysis. From the chromatogram, the peak area of the extracted samples was divided by the peak area of the pure standards.

$$\text{Recovery} = \frac{\text{Area of extracted samples}}{\text{Area of pure standards}} \times 100\%$$

3.7 RESULTS AND DISCUSSION

3.7.1 Calibration curve

Linear model calibration was used, in which the independent variable (X) is the concentration of endosulfan and its metabolites and the dependent variable (Y) is the detector response (peak area). The standard curve based on the peak area of each of endosulfan and its metabolites was linear from 30 ng/ml to 1000 ng/ml giving a linear regression (R^2) value greater than 0.99 with intercept set at zero.

Concentration, X (ng/ml)	Peak area, Y			
	α-endosulfan	β-endosulfan	Endosulfan sulfate	Endosulfan diol
30	*na	na	375	na
50	1394	375	1397	na
70	1981	679	1729	na
100	3518	1105	3491	472
300	na	na	na	1661
500	13777	5401	17752	2549
1000	26692	9841	35857	4610

*na = Not available

Detection limit for the chemicals were as follows: 3 ng/ml (α-endosulfan), 10 ng/ml (β-endosulfan), 30 ng/ml (endosulfan sulfate) and 100 ng/ml (endosulfan diol).

The calibration curve of α-endosulfan, β-endosulfan, endosulfan sulfate and endosulfan diol are shown in Figure 3.5 - 3.8.

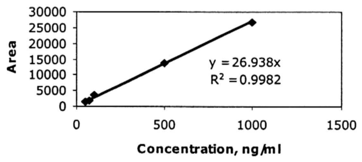


Figure 3.5. Standard Curve of α-Endosulfan

Figure 3.6. Standard Curve of β-Endosulfan

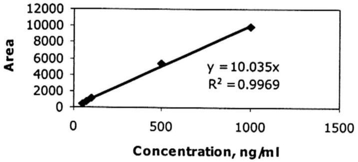


Figure 3.7. Standard Curve of Endosulfan Sulfate

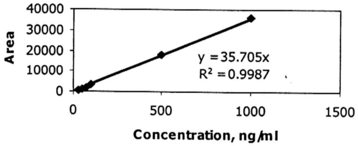
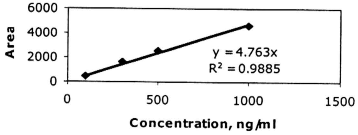


Figure 3.8. Standard Curve of Endosulfan Diol



3.7.2 Efficiency of the analytical methods

The results obtained for the recovery and precision of the analysis are tabulated in Table 3.1. For plasma, the high percentage of mean recoveries for low, medium and high concentrations ranging from 87.6 to 102.3% (α-endosulfan); 82.2 to 92.1% (β-endosulfan); 85.7 to 91.0% (endosulfan sulfate) and 101.5 to 132.1% (endosulfan diol) with coefficient variations ranging from 4.6 to 25.3% indicates that the methodology used for the extraction procedure was acceptable for the present study. In addition, high recovery of 132.1%, which corresponded to endosulfan diol was also observed in the medium concentration. This was considered quite high and unusual compared to the others and could be attributed to baseline disturbances. In addition, this may also be attributed to exogenous contamination from SPE cartridge or from glassware used. Solvent blank (hexane) was injected into the GC-MS after every 10 samples to identify the level of contamination at the baseline.

Table 3.1. Percentage recovery (C.V.) of α-endosulfan, β-endosulfan, endosulfan sulfate and endosulfan diol fortified in plasma

	Spiked concentration (ng/ml)		
Compound	100	50	10
α-Endosulfan	80.0 (5.8)	102.3 (10.7)	87.6 (8.2)
β-Endosulfan	87.4 (7.1)	92.1 (6.5)	82.2 (25.3)
Endosulfan sulfate	91.0 (10.0)	84.7 (8.7)	85.7 (6.1)
Endosulfan diol	111.7 (4.6)	132.1 (7.4)	101.5 (21.6)

For the liver samples, the mean recoveries for low, medium and high concentrations were ranges from 73.0 to 109.5% (α-endosulfan); 73.1 to 116.0% (β-endosulfan) and 75.0 to 112.9% (23.7 (endosulfan sulfate) with coefficient variations from 8.6 to 23.7% (Table 3.2).

For the kidneys samples, the mean recoveries for low, medium and high concentrations were ranges from 81.8 to 92.0% (α-endosulfan); 74.5 to 101.6% (β-endosulfan) and 90.8 to 105.4% (endosulfan sulfate) with coefficient variations from 6.0 to 17.3% (Table 3.3).

Table 3.2. Percentage recovery (C.V.) of α-endosulfan, β-endosulfan and endosulfan sulfate fortified in liver samples

	Spiked concentration (ng/ml)		
Compound	100	500	1000
α-Endosulfan	94.7 (12.4)	73.0 (18.8)	109.5 (22.2)
β-Endosulfan	116.0 (8.6)	96.7 (22.2)	73.1 (8.9)
Endosulfan sulfate	75.0 (10.5)	112.9 (23.7)	96.1 (20.6)

Table 3.3. Percentage recovery (C.V.) of α-endosulfan, β-endosulfan and endosulfan sulfate fortified in kidneys samples

	Spiked concentration (ng/ml)		
Compound	100	500	1000
α-Endosulfan	81.8 (16.8)	91.8 (13.3)	92.0 (13.6)
β-Endosulfan	101.6 (17.3)	125.5 (13.6)	74.5 (12.1)
Endosulfan sulfate	90.8 (6.0)	91.8 (10.9)	105.4 (11.6)

3.7.3 Precision of repeated GC injections

In determining the error factor contributed by the GC, three different concentrations of the standards solution mixture of α-endosulfan, β-endosulfan, endosulfan sulfate and endosulfan diol were repeatedly injected into the GC. The results obtained by repeated injections are tabulated in Table 3.4-3.7.

Table 3.4. Precision of the GC analysis for α-endosulfan

Concentration (ng/ml)	Area recovered 1	2	3	4	5	Coefficient Variation (%)
1000	8378	9324	9703	9309	8608	6.1
500	2961	2991	2882	2667	2570	12.7
100	759	725	764	837	885	8.2

Table 3.5. Precision of the GC analysis for β-endosulfan

Concentration (ng/ml)	Area recovered 1	2	3	4	5	Coefficient Variation (%)
1000	6581	7121	7432	7103	6650	5.1
500	2093	2131	2074	1893	1911	5.5
100	543	383	640	775	526	25.3

Table 3.6. Precision of the GC analysis for endosulfan sulfate

Concentration (ng/ml)	Area recovered 1	2	3	4	5	Coefficient Variation (%)
1000	5238	6719	6720	6548	5844	10.5
500	889	813	941	649	732	14.6
100	356	336	314	352	369	6.1

Table 3.7. Precision of the GC analysis for endosulfan diol

Concentration (ng/ml)	Area recovered 1	2	3	4	5	Coefficient Variation (%)
1000	3446	4014	4461	4419	4077	10.0
500	1611	1386	1462	1297	1143	12.7
100	384	455	394	540	299	21.6

The coefficient variations of repeated GC injections ranged from 6.1 to 12.7% (α-endosulfan), 5.1 to 25.3% (β-endosulfan), 6.1 to 14.6% (endosulfan sulfate) and 10.0 to 21.6% (endosulfan diol). These do not appear to be a correlation between coefficient variation and the concentration of the injected samples.

3.8 CONCLUSION

In the present study, the SPE procedure that was developed for the extraction of plasma provided consistent results and yielded high recoveries for all the analytes. The procedure was relatively rapid compared to the published methods utilizing LLE. Sample clean-up for the tissues samples was found to be efficient in removing lipid contents when SPE was applied and was more effective and faster compared to the classical method using chromatographic columns. Hence, this method of sample clean-up gives faster operation in which a large number of samples may be processed within a given time.