APPENDICES

APPENDIX 1
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DATA SOSIO DEMOGRAFI
SOCIO DEMOGRAPHIC DATA

Jantina **1. Lelaki** **2. Perempuan** **9. Tidak perolehi** [ ] **A1**
Sex Male Female Not available

Tarikh Lahir
Date of Birth (**hh**/dd) (**bb**/mm) (**tt**/yy)

Umur (tahun) _____ **99. Tidak perolehi** [ ][ ] **A3**
Age (in completed years) (**Tahun**/Year) Not available

Bangsa **1. Melayu** **2. Cina** **3. India** [ ] **A4**
Race Malay Chinese Indian
4. Lain-lain _____ **9. Tidak perolehi**
Others (**Nyatakan**/Specify) Not available

Ugama **1. Islam** **2. Buddha** **3. Hindu** [ ] **A5**
Religion Muslim Buddist Hindu
4. Lain-lain _____ **9. Tidak perolehi**
Others (Nyatakan\Specify) Not available

Apakah pekerjaan bapa anda? [ ][ ] **A6**
What is the occupation of your father? (**Nyatakan pekerjaan & tempat kerja**\Specify Occupation & Industry)

Apakah pekerjaan ibu anda?
What is the occupation of your Mother? (**Nyatakan pekerjaan & tempat kerja**\Specify Occupation & Industry) [ ][ ]**A7**

1. Pekerja iktisas/professional, teknik & yang berkaitan
Professional, technical & related workers
2. Pekerja pentadbiran & pengurusan
Administrative & managerial workers
3. Perkeranian & yang berkaitan
Clerical & related workers
4. Penjual
Sales workers
5. Pekerja perkhidmatan
Service workers
6. Pekerja pertanian,ternakan & perhutanan, nelayan & pemburu
Agricultural, animal husbandry and forestry workers, fisherman & hunters
7. Pekerja pengeluaran & yang berkaitan, operator alat pengangkutan & buruh
Production and related workers, transport equipment operators and labourers
8. Pesara
Pensioners
9. Penggangur/Suri rumah tangga
Unemployed/Housewife
10. Tidak perolehi
Not available

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DATA TEMPAT TINGGAL/ALAM SEKITAR [ ][ ][ ] **B**
RESIDENTIAL/ENVIRONMENTAL DATA

Berapa lamakah anda tinggal di tempat kediaman sekarang? _____
How long have you been living at the current place of residence? (**Bulan**/Months)
999. Tidak perolehi
Not available

Sudah berapa lamakah rumah anda di cat pada kali yang terakhir? [ ][ ][ ] **B**
How long has it been since your house was last painted? _____ (**Bulan**/Months)
000. Tidak berkenaan **999. Tidak perolehi**
Not applicable Not available

Dimanakah anda tinggal sebelum ini (Nyatakan Kampung/Taman, Jalan, Pekan & Negeri)?
Where were you staying before this (State Village,Street, Town & State) ?

.....Kg/Village:Taman/Garden
.....Jalan/Road
.....Pekan/Town
..... Negeri/State

Berapa lamakah anda tinggal di tempat ini? _____ [ ][ ][ ] **B**
How long were you staying in this place (**Bulan**/Months)

000. Tidak berkenaan **999. Tidak perolehi**
Not applicable Not available

Apakah sumber utama air <u>minuman</u> untuk isirumah anda sekarang? [ ] **B4**
What is the main source of drinking water in your household?

1. Air paip dari rumah sendiri
Own water pipe (treated water)
2. Air paip awam
Public water pipe (treated water)
3. Lain-lain _____
Others (**Nyatakan**/Specify)
9. Tidak perolehi
Not available

Apakah yang di lakukan sebelum air itu dijadikan air minuman? [ ] **B5**
What is done to the water before drinking?

1. Memasaknya terlebih dahulu
Boil the water
2. Air Mineral
Mineral Pot/
3. Penapis Air
Water Filter
4. Mencampurkan ubat (melakukan pengklorinan)
Chlorinate (add chemical)
5. Tidak buat apa apa(minum terus)
Nothing (drink direct from source)
6. Lain-lain _____
Others (**Nyatakan**/Specify)
9. Tidak perolehi
Not available

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Bagaimanakah air minuman disimpan? [ ] **B6**

How do you store your drinking water?

1. Dalam bekas logam besi
In a metal container
2. Dalam bekas plastik
In a plastic container
3. Dalam bekas simen
In a cemented container
4. Dalam tempayan/tembikar
In a ceramic urn
5. Tidak perlu di simpan(minum terus dari sumber)
No need to store
6. Lain-lain _____
(Nyatakan/Specify)
9. Tidak perolehi
Not available

DATA SEKOLAH
SCHOOL DATA

Berapa lamakah anda belajar di sekolah ini _____ [ ][ ][ ]C
How long have you been in this school? **(Bulan**/Months)
999. Tidak perolehi
Not available

Bagaimanakah anda ulang alih ke sekolah? _____ [ ]C2
How do you comute between home and school? **(Nyatakan/Specify)**

1. Kenderaan bertutup seperti bas, kereta, dll berhawa dingin (Lanjut ke soalan 3)
Covered vehicle eg air conditiones buses, cars, etc (Go to Q3)
2. Kenderaan terdedah/Berjalan kaki
Exposed vehicles/Walk
3. Kedua duanya (1 & 2)
Both (1 & 2)

Pada purata berapa lamakah perjalanan dari rumah anda ke sekolah (ulang alih)? [ ][ ][ ]C
On an average day how much time do you spend travelling _____ **(Min**/Min)
from home to school & vice-versa
000 Tidak Berkenaan **999. Tidak perolehi**
Not applicable Not available

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Pernahkah anda belajar di sekolah lain sebelum ini? [ ]C3
Have you studied in other schools prior to this school?
1.Ya **2. Tidak (lanjut ke bahagian D)** **9. Tidak perolehi**
Yes No (go to section D) Not available

Jika pernah belajar di sekolah sekolah lain nyatakan nama dan alamat sekolah yang terakhir _____
_____
If ever been to other schools, state the name and address of your last school.

Berapa lama anda belajar di sekolah tersebut? _____ [ ][ ][ ]
How long were you in this school? **(Bulan/Months)**
000. Tidak Berkenaan **999. Tidak perolehi**
Not applicable Not available

PEMAKANAN & RIADAH DI LUAR
DIET & OUTDOOR RECREATION
Secara purata dimana dan berapa kerap anda meluangkan masa di kawasan luar dalam seminggu?
On an average where and how frequently do you spend your time out of doors in a Week?

Tempat Place	(i) **Kali dalam seminggu** No of times in one week	(ii) **Berapa minit dalam satu kali** No of minutes per visit
	0. Tidak ada/Jarang None/Rarely **9. Tidak perolehi** Not available	**0. Tidak berkenaan** Not applicable **9. Tidak perolehi** Not available
kolah /School **mpat Tinggal** / Residence **n-lain (Nyatakan tempat lamat)**/Others (State place & ress)	_____ [ ] **D1a(i)** _____ [ ] **D1b(i)**	_____[ ][ ][ ] **D1a(ii)** _____[ ][ ][ ] **D1b(ii)**
	_____ [ ] **D1b(i)**	_____[ ][ ][ ] **D1c(ii)**
	_____[ ] **D1c(i)**	_____[ ][ ][ ]**D1c(ii)**

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Secara purata, berapa kalikah anda memakan makanan-makanan tersebut dalam seminggu?
On an average week, how often do you eat the following foods?

Jenis-jenis makanan: Types of food:	**Bilangan hari dalam seminggu**/Number of days in a week	**Kekerapan dalam sehari**/No of times per day
	0.Tidak makan/Jarang Don't eat/Rarely **9. Tidak perolehi** Not available	**0.Tidak berkenaan** Not applicable **9.Tidak perolehi** Not available
yur-sayuran (contoh: bayam, kuchai ....) afy vegetables (eg: spinach. Kuchai ....)	[ ] **2a_week**	[ ] **2a_day**

yur Jenis berbuah(contoh: tomato, terung..) it vegetable (eg: tomato, brinjal ...)	[ ] 2b_week	[ ] 2b_day
i-ubi (contoh: kentang, keladi ...) ot vegetable (eg: potato, yam ...)	[ ] 2c_week	[ ] 2c_day
ah-buahan (contoh: betik, oren ...) its (eg: papaya, orange ....)	[ ] 2d_week	[ ] 2d_day
kanan laut food **kan** ish **Lain-lain (Contoh: udang, kerang ...)** Others (eg: prawns, shell fish ...)	[ ] 2ei_week [ ] 2eii_week	[ ] 2ei_day [ ]2eii_day
Makanan dalam tin Canned food **Minuman dalam tin** Canned drinks	[ ] 2fi_week [ ] 2fii_week	[ ] 2fi_day [ ] 2fii_day

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SEJARAH KESIHATAN:
HEALTH HISTORY

Adakah anda atau sesiapa lagi di rumah anda merokok? [ ] **E1a**
Do you or any other person in your house smoke cigarettes?

1. Ya Yes — **2. Tidak (lanjut ke soalan 2)** No (Go to Q2) — **9. Tidak perolehi** Not available

Jika ya, siapa yang merokok, anda sendiri atau lain-lain, ataupun anda serta lain - lain yang berada di rumah anda?
If yes, who smokes, just you, only others or you and others living in your house?

	Sejarah Merokok/History of Smoking					
	Masih Currently	**Sudah berhenti** Stopped since	Tidak pernah Never	**Tidak Berkenaan** Not applicable	**Tidak perolehi** Not available	
Ya	**1 ***	**2**	**3 ***	**0**	**9**	[ ] **E1bi**
Lain-lain Others	**1 ***	**2 ***	**3 ***	**0**	**9**	[ ] **E1bii**

* **Lanjut ke soalan 2**/Go to Q2

Jika anda sudah berhenti merokok, berapa lamakah anda telah berhenti? [ ][ ] **E1c**
If you have stopped smoking, how long has it been since you last smoked? _____ (**Bulan**\Months)

0. Tidak berkenaan
Not applicable

9. Tidak perolehi
Not available

Pernahkah doktor memberitahu anda dan/atau pernahkah anda mendapat rawatan bagi penyakit-penyakit berikut?
Has the medical doctor ever told you that you have the following illness and/or have you had treatment for any of them?

a) Kurang Darah >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>> [ ] **E2a**
Anaemia

b) Sakit buah pinggang >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>> [ ] **E2b**
Kidney problems

c) Lela >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>> [ ] **E2c**
Asthma

d) Sakit paru-paru >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>> [ ] **E2d**
Lung (pulmonary) problem

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e) Alahan kulit/gatal-gata >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>> [ ] **E2e**
Skin allergy/rashes,itchiness

f) Demam kuning >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>> [ ] **E2f**
Jaundice

g) Bersin, mata merah, selsema >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>> [ ] **E2g**
Hay fever

h) Lain-lain _____ >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>> [ ] **E2h**
Others (**Nyatakan**/Specify)

1. Ya
Yes

2. Tidak
No

9. Tidak perolehi.
Not available

Berapa kalikah anda mengalami masalah-masalah kesihatan berikut dalam tahun yang lalu?
How many episodes of the following health problems did you experience in the past year

Kekerapan/No of times

a) Batuk-batuk yang berpanjangan _____ [ ] **E3a**
Prolonged cough

b) Cirit-birit _____ [ ] **E3b**
Diarrhoea

c) Loya dan muntah-muntah _____ [ ] **E3c**
Nausea and vomiting

d) Selsema atau berhingus _____ [ ][ ] **E3d**
Colds and runny nose

e) Penyakit-penyakit kulit/ruam-ruam
Skin infection/ rash _____ [ ] E3e

f) Mata merah atau pedih
Red or smarting eyes _____ [ ] E3f

g) Hidung berdarah
Nose bleed _____ [ ] E3g

h) Terkokol-kokol (sawan babi)
Convulsions _____ [ ] E3h

i) Lain-lain _____
Others **(Nyatakan**/Specify) _____ [ ] E3I

Tidak pernah **9.Tidak perolehi**
No, never Not available

PHYSICAL EXAMINATION

[ ][ ].[ ]F
[ ][ ][ ].[

Weight of respondent ___.__ kg
Height of respondent ___.__ cm

ional status by appearance:	Codes: 1	2	3	0	
colour	Changes	Normal (go to Q4)			[ ] F3
allor	Present	Absent		Not applicable	[ ] F3a
Cynosis	Present	Absent		Not applicable	[ ] F3b
aundice	Present	Absent		Not applicable	[ ] F3c
morrhage in the skin	Present	Absent (go to Q5)			[ ] F4
urpura	Yes	No		Not applicable	[ ] F4a
cchymosis	Yes	No		Not applicable	[ ] F4b
aematoma	Yes	No		Not applicable	[ ] F4c
Lesions	Present	Absent(go to Q6)			[ ] F5
czema	Yes	No		Not applicable	[ ] F5a
ash	Yes	No		Not applicable	[ ] F5b
ermatitis	Yes	No		Not applicable	[ ] F5c
Others (Specify)	Yes	No		Not applicable	[ ] F5d
ema	Present	Absent(go to Q7)			[ ] F6
ce	Present	Absent		Not applicable	[ ] F6a
nkle	Present	Absent		Not applicable	[ ] F6b
scites	Present	Absent		Not applicable	[ ] F6c
Discolouration	Present	Absent(Go to Q8)			[ ] F7
ed	Present	Absent		Not applicable	[ ] F7a
y & pale	Present	Absent		Not applicable	[ ] F7b
hers (Specify)	Present	Absent		Not applicable	[ ] F7c
s	Changes	Normal (go to Q9)			[ ] F8
x-opthalmos	Present	Absent		Not applicable	[ ] F8a
tosis	Present	Absent		Not applicable	[ ] F8b
te of Prosis	Right	Left		Not applicable	[ ] F8c
everity of Prosis	Mild	Severe		Not applicable	[ ] F8d
naemia	Present	Absent		Not applicable	[ ] F8e
everity of Anaemia	Mild	Severe		Not applicable	[ ] F8f
undice	Present	Absent		Not applicable	[ ] F8g
onjunctivitis	Present	Absent		Not applicable	[ ] F8h
te of Conjuntivitis	Right	Left		Not applicable	[ ] F8 I

nflammation of the cornea…..	Present……	Absent ……		Not applicable…….	[ ] F8j
Pterygium……	Present……	Absent ……		Not applicable…….	[ ] F8k
Site of Pterygium……	Right ……	Left ……		Not applicable…….	[ ] F8l
uth & Pharynx Abnormalities	Present	Absent (go to Q10)			[ ] F9
Lips - cracks & fissure……	Present……	Absent ……			[ ] F9a
Teeth……	Unhealthy……..	Healthy ……			[ ] F9b
Gums - blue line or lead …….	Present……	Absent ……			[ ] F9c
Tongue – tremor……	Present……	Absent ……			[ ] F9d
ape of the Chest	Abnormal	Normal (go to Q11)			[ ] F10
Barrel-shaped……	Present……	Absent ……		Not applicable…….	[ ] F10a
Flattening……	Present……	Absent ……		Not applicable…….	[ ] F10b
Kyphosis……	Present……	Absent ……		Not applicable…….	[ ] F10c
Scoliosis……	Present……	Absent ……		Not applicable…….	[ ] F10d
rpe of Respiration	Abnormal	Normal			[ ] F11
achea Position	Changes	Normal (go to Q13)			[ ] F12
Shift to ……	Left ……	Right ……		Not applicable…….	[ ] F12a
ngs:					
Breath Sounds……	Abnormal…… …	Normal…….. (go to Q14)			[ ] F13a
Crepitations……	Present……	Absent ……		Not applicable…….	[ ] F13b
Site of Crepitations……	Right ……	Left ……	Both	Not applicable…….	[ ] F13c
Rhonchi……	Present……	Absent ……		Not applicable…….	[ ] F13d
Site of Rhonchi……	Right ……	Lef t ……	Both	Not applicable…….	[ ] F13e
Diminished breath sounds……	Present……	Absent ……		Not applicable…….	[ ] F13f
eart:					
ate: _____ beats/min					[ ][ ][ ] F1
ounds……	Abnormal……	Normal (go to Q15)			[ ] F14b
ystolic murmurs……	Present……	Absent ……		Not applicable…….	[ ] F14c
Diastolic murmurs……	Present……	Absent ……		Not applicable…….	[ ] F15d
ericardial rub……	Present……	Absent ……		Not applicable…….	[ ] F14e
Others: (Specify……)	Present……	Absent ……		Not applicable…….	[ ] F14f
ood Pressure (mmHg)					
rd the average of two readings]					
SBP _____.					[ ][ ][ ] F1
DBP _____.					[ ][ ][ ] F1
ver	Palpable	Not palpable (go to 17)			[ ] F16
ize _____ cm (from costal					
argin) ……				Not applicable…….	[ ][ ] F16a
Consistency……	Soft ……	Firm	Hard	Not applicable…….	[ ] F16b
Surface……	Smooth……	Nodular		Not applicable…….	[ ] F16c
leen	Palpable	Not palpable			[ ] F17
dney	Ballotable	Not Ballotable (go to Q19)			[ ] F18
if ballotable……	Right ……	Left ……	Both	Not applicable…….	[ ] F18a
rvous System:					
	Abnormal	Normal(go to 20)			[ ] F19a
rdination of movement:	Impaired……	Normal ……		Not applicable…….	[ ] F19b
pper Right Limb……	Impaired……..	Normal ……		Not applicable…….	[ ] F19bi
pper Left Limb……	Impaired……..	Normal ……		Not applicable…….	[ ] F19bii
ower Right Limb……	Impaired……..	Normal ……		Not applicable…….	[ ] F19biii
ower Left Limb……	Impaired……..	Normal ……		Not applicable…….	[ ] F19biv
emors of Fingers	Present	Absent			[ ] F20
nsation	Abnormal	Normal (End)			[ ] F21
ibration sense test in feet:					
Right……	Impaired……..	Normal ……		Not applicable…….	[ ] F21ai
Left ……	Impaired……..	Normal ……		Not applicable…….	[ ] F21aii

Pin prick test.....					
. Upper right limb.....	Impaired.....	Normal		Not applicable.....	[ ] **F21bi**
. Upper left limb.....	Impaired.....	Normal		Not applicable.....	[ ] **F21bii**
. Lower limb right.....	Impaired.....	Normal			[ ] **F21biii**
. Lower limb left.....	Impaired.....	Normal		Not applicable.....	[ ] **F21biv**

APPENDIX 2

ANALYTICAL PROCEDURE FOR α-, β-ENDOSULFAN, ENDOSULFAN SULFATE AND ENDOSULFAN DIOL

1 IDENTIFICATION OF ANALYTE

Compare the peaks for four ion sets in Selected Ion Monitoring (SIM) of α-endosulfan (272, 277, 337, 339); β-endosulfan (272, 277, 337, 339); endosulfan sulfate (272, 277) and endosulfan diol (272, 277) from plasma and tissues with the working standard solutions.

The identity is considered positive if the respective ion sets appear at the same retention time (± 0.5 minute).

2 QUANTIFICATION

The sample concentration will be determined using the calibration curve plotted as the peak area of the standard against the concentration of the standard. Calibration using external standards will be used in the present study.

3 PREPARATION OF STANDARD SOLUTIONS

Prepare the stock solutions (100 μg/ml) in methanol and store in amble glass bottles at 4°C for further dilution.

3.1. Accurately weigh 10 mg of each endosulfan and its metabolites into a 100 ml volumetric flask. Dissolve endosulfan and its metabolites in methanol and make

up the volume. The concentration is about 100 μg/ml for each standard. Transfer to amble bottles for storage.

3.2. Transfer 1000 μl of each stock solution to a 10 ml volumetric and make up the volume to obtain a final concentration of 10 000 ng/ml solution mixture.

3.3. Transfer 1000 μl of the 10 000 ng/ml mixture to a 10 ml volumetric flask and make up the volume to obtain a final concentration of 1000 ng/ml solution mixture.

3.4. Prepare the following working standard solutions from the 1000 ng/ml solution mixture and inject them into the gas chromatograph-mass spectrometer (GC-MS).

Amount of stock solution (μl)	Amount of methanol (μl)	Final concentration of the mixture (ng/ml)
500	500	500
300	700	300
100	900	100
70	930	70
50	950	50
30	970	30

4 CALIBRATION CURVE

4.1. From the standard chromatogram printed out, plot the peak area of endosulfan and its metabolites against the concentration of the standards.

4.2. The calibration curves must be linear with R^2 value greater than 0.99.

5 PREPARATION OF QC SAMPLES FOR PLASMA

5.1. For the preparation of KH_2PO_4 buffer solution, accurately weigh 5.4436 g into a 1000 ml volumetric flask. Dissolve KH_2PO_4 in distilled water and make up the volume. The concentration is 0.04M. Add drops of phosphoric acid to obtain a pH2 solution.

5.2. For the QC samples, three concentrations were chosen, these concentrations being within the range of the calibration curve. The three concentrations of endosulfan and its metabolites identified as being low, medium and high are 10, 50 and 100 ng/ml.

5.3. From a stock solution of 100 000 ng/ml endosulfan and metabolites, transfer 1000 µl of each stock solution to a 10 ml volumetric and make up the volume to obtain a final concentration of 10 000 ng/ml solution mixture.

5.4. From 5.3, further dilute to three concentration of solution mixture by following the serial dilution below:

Amount of stock solution (µl)	Amount of methanol (µl)	Final concentration of the mixture (ng/ml)
100	9900	100
500	9500	500
1000	9000	1000

5.5. The volume spiked and the final concentration of endosulfan and its metabolites in plasma are shown below:

Concentration of the solution mixture from 5.4. (ng/ml)	Volume of mixture of plasma and KH_2PO_4 buffer (µl)	Volume of solution mixture spiked (µl)	Final concentration of the mixture in plasma (ng/ml)
100	900	100	10
500	900	100	50
1000	900	100	100

6 SAMPLE PREPARATION/EXTRACTION FOR PLASMA

6.1. Pre-condition the C18 end-capped column at the solid phase extraction (SPE) manifold with 1ml of methanol followed by 1 ml of distilled water.

6.2. Using a plastic disposable transfer pipette, transfer 1000 µl of sample (plasma and buffer [1:1], standards) containing mixture of endosulfan and its metabolites to the C18 end-capped column.

6.3. Let the plasma flow under vacuum condition at 1 inHg until finished.

6.4. Add in 6 ml of distilled water under the same vacuum condition until finished. This interference elution step is to clean out endogenous compounds which are trapped at the packing material.

6.5. Apply vacuum to the column at 10 inHg for 15 minutes.

6.6. Stop the vacuum and put in a labeled glass tube inside the vacuum manifold.

6.7. Add in 2 ml of methanol to elute out endosulfan and its metabolites from the column and collect the eluent in the labeled glass tube.

6.8. Evaporate the contents of the glass tube to dryness under a gentle stream of nitrogen.

6.9. Reconstitute the extract in 100 µl of methanol. Then, inject 2 µl of the concentrated extract into the gas chromatograph-mass spectrometer (GC-MS). The conditions of the GC-MS are described in Section 9.

7 PREPARATION OF QC SAMPLES FOR TISSUES

7.1. For the QC samples, three concentrations were chosen, these concentrations being within the range of the calibration curve. The three concentrations of endosulfan and its metabolites identified as being low, medium and high are 100, 500 and 1000 ng/g.

7.2. From a stock solution of 100 000 ng/ml endosulfan and metabolites, transfer 1000 µl of each stock solution to a 10 ml volumetric and make up the volume to obtain a final concentration of 10 000 ng/ml solution mixture.

7.3. From 7.2, further dilute to two concentration of solution mixture by following the serial dilution below:

Amount of stock solution (µl)	Amount of methanol (µl)	Final concentration of the mixture (ng/ml)
100	900	1000
500	500	5000

7.4. Weigh approximately 1 g of the selected tissues (liver and kidneys) before spiking in any solution mixture of endosulfan and metabolites. The volume spiked and the final concentration of endosulfan and metabolites in tissues are shown in the table below:

Concentration of the solution mixture from 7.2 and 7.3 (ng/ml)	Volume of solution mixture spiked (µl)	Final concentration of the mixture in tissues (ng/g)
1000	100	100
5000	100	500
10 000	100	1000

7.5. The samples in 7.4 undergo Soxhlet extraction for 8 hours before clean-up process with Florisil column utilizing SPE.

7.6. Concentrate the extract to approximately 1ml using a rotary evaporator and transfer it to a clean vial.

7.7. Dry the concentrated extract in 7.6 under a gentle stream of nitrogen.

7.8. Reconstitute the extract in 1 ml of dichloromethane (DCM).

7.9. Dilute 500 µl of the extract from 7.8 with 500 µl of DCM prior to sample clean-up in Section 8.

8 SAMPLE CLEAN-UP FOR TISSUES

8.1. Pre-condition the Florisil column at the SPE manifold with 6 ml of DCM followed by 6 ml of hexane and 6 ml of DCM again. Do not apply vacuum throughout the clean-up process.

8.2. Using a plastic disposable pipette, transfer 1000 µl of sample (tissues and standards) to the Florisil column.

8.3. Let the sample flow and collect the eluent in a labeled glass tube.

8.4. Add in 6 ml of DCM to further elute out endosulfan and its metabolites from the column.

8.5. Evaporate the contents of the glass tube to dryness under a gentle stream of nitrogen.

8.6. Reconstitute the extract in 100 µl of DCM. Then, inject 2 ml of the concentrated extract into the GC-MS. The conditions of the GC-MS are described in Section 9.

9 CHROMATOGRAPHIC SYSTEM

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 minute

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.

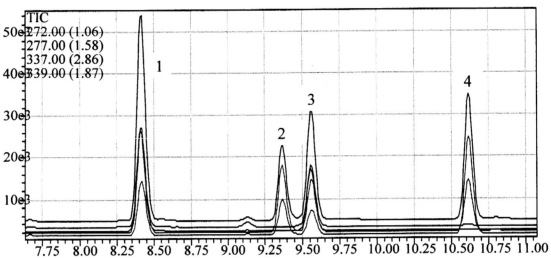
Data acquisition mode: Selected Ion Monitoring (SIM)

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

10 CALCULATIONS

Calculate the concentration of the samples by using the equation obtained from the calibration curves; $y = mx$ with intercept set to zero.

SELECTED CHROMATOGRAM OF ENDOSULFAN AND ITS METABOLITES FROM STANDARDS SOLUTION MIXTURE



Representative chromatogram of 1000 ng/ml standards solution mixture.

Peak identity: 1 = α-endosulfan, 2 = endosulfan diol, 3 = β-endosulfan and 4 = endosulfan sulfate

APPENDIX 3

DETERMINATION OF EXTRACTABLE ORGANIC MATTER (EOM)

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

1.1. Remove 100 µl from a 1 ml sample extract, without clean-up and place it in a clean glass tube.

1.2. Evaporate the extract under a gentle stream of nitrogen.

1.3. Weigh the residue on a weighing balance.

1.4. Repeat the above procedure thrice and take the average from the three determinations.

1.5. Calculate the quantity of EOM with the formula below:

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

APPENDIX 4

RADIOIMMUNOASSAY PROCEDURES: TESTOSTERONE, T3 AND INSULIN

A) TOTAL TESTOSTERONE

1. Before assay, allow the stored sera to come to room temperature (15 - 28°C) and mix by gentle swirling and inversion.
2. Label four plain uncoated (12 x 75 mm) polypropylene tubes T for "total counts" and NSB for "nonspecific binding" in duplicate.
3. Label twelve Total Testosterone Ab-Coated Tubes A (maximum binding) and B through F in duplicate.

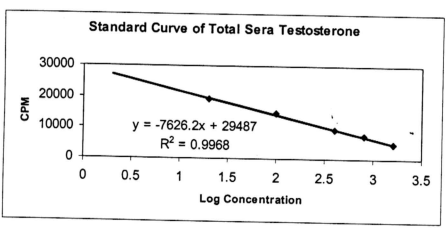
Calibrators	ng/dL	nmol/L
A (MB)	0	0
B	20	0.7
C	100	3.5
D	400	14
E	800	28
F	1600	55

4. Pipette 50 µl of the zero calibrator A into the NSB and A tubes, and 50 µl of each remaining calibrator, control and test sample (sera) into the tubes prepared. Pipette directly to the bottom.
5. Add 1.0 ml of ^{125}I Total Testosterone to every tube and vortex.
6. Incubate for 3 hours at 37°C.

7. Decant thoroughly. Removing all visible moisture will greatly enhance precision. Using a foam decanting rack, decant or aspirate the contents of all tubes (except the T tubes) and allow them to drain for 2 or 3 minutes. Then, strike the tubes sharply on absorbent paper to shake off all residual droplets.
8. Count for 1 minute in a gamma counter.

CALIBRATION CURVE OF TOTAL SERA TESTOSTERONE

Plot the calibration curve of total sera testosterone as count per minute (CPM) against the log concentration of the calibrators ranges.



CALCULATION

Calculate the concentration of testosterone in the samples by using the equation obtained from the calibration curves.

B) TOTAL T3

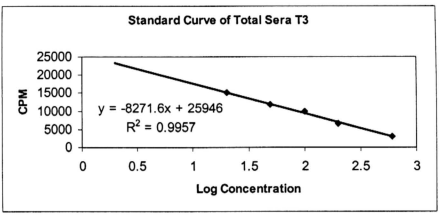
1. Before assay, allow the stored sera to come to room temperature (15 - 28°C) and mix by gentle swirling and inversion.
2. Label four plain uncoated (12 x 75 mm) polypropylene tubes T for "total counts" and NSB for "nonspecific binding" in duplicate.
3. Label twelve Total T3 Ab-Coated Tubes A (maximum binding) and B through F in duplicate.

Calibrators	ng/dL	nmol/L
A (MB)	0	0
B	20	0.31
C	50	0.77
D	100	1.54
E	200	3.07
F	600	9.22

4. Pipette 100 µl of the zero calibrator A into the NSB and A tubes, and 100 µl of each remaining calibrator, control and test sample (sera) into the tubes prepared. Pipette directly to the bottom.
5. Add 1.0 ml of ^{125}I Total T3 to every tube and vortex briefly and gently.
6. Incubate for 2 hours at 37°C.
7. Decant thoroughly. Removing all visible moisture will greatly enhance precision. Using a foam decanting rack, decant or aspirate the contents of all tubes (except the T tubes) and allow them to drain for 2 or 3 minutes. Then, strike the tubes sharply on absorbent paper to shake off all residual droplets.
8. Count for 1 minute in a gamma counter.

CALIBRATION CURVE OF TOTAL SERA T3

Plot the calibration curve of total sera testosterone as count per minute (CPM) against the log concentration of the calibrators ranges.



CALCULATION

Calculate the concentration of testosterone in the samples by using the equation obtained from the calibration curves.

C) INSULIN

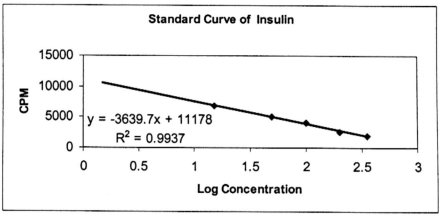
1. Before assay, allow the stored sera to come to room temperature (15 - 28°C) and mix by gentle swirling and inversion.
2. Label four plain uncoated (12 x 75 mm) polypropylene tubes T for "total counts" and NSB for "nonspecific binding" in duplicate.
3. Label twelve Insulin Ab-Coated Tubes A (maximum binding) and C through G in duplicate.

Calibrators	µIU/ml
A (MB)	0
C	15
D	50
E	100
F	200
G	350

4. Pipette 200 µl of the zero calibrator A into the NSB and A tubes, and 200 µl of each remaining calibrator, control and test sample (sera) into the tubes prepared. Pipette directly to the bottom.
5. Add 1.0 ml of ^{125}I Insulin to every tube and vortex.
6. Incubate for 3 hours at room temperature (15 - 28°C).
7. Decant thoroughly. Removing all visible moisture will greatly enhance precision. Using a foam decanting rack, decant or aspirate the contents of all tubes (except the T tubes) and allow them to drain for 2 or 3 minutes. Then, strike the tubes sharply on absorbent paper to shake off all residual droplets.
8. Count for 1 minute in a gamma counter.

CALIBRATION CURVE OF INSULIN

Plot the calibration curve of total sera testosterone as count per minute (CPM) against the log concentration of the calibrators ranges.



CALCULATION

Calculate the concentration of testosterone in the samples by using the equation obtained from the calibration curves.

APPENDIX 5

HAEMATOXYLIN AND EOSIN STAINING PROCEDURE

Slides should always be left overnight in an oven at 37°C to prevent sections from floating off.

1) Place slide in xylene to dewax	2 min
Hydrate:	
2) 1st Absolute Alcohol	2 min
3) 2nd Absolute Alcohol	2 min
4) 95% Alcohol	2 min
5) 80% Alcohol	2 min
6) 70% Alcohol	2 min
7) 50% Alcohol	2 min
8) Wash in water	2 min
9) Haematoxylin (Mayer's or Harris's)	20 min
10) Wash in water	2 min
11) Differentiate in 0.5% acid alcohol	a dip
12) Wash in water	2 min
13) Blue in water. Microscopic check. If haematoxylin is too heavy further differentiation is acid alcohol in required	
14) Wash in water	30 sec - 1 min (X2)
15) Eosin	1 - 2 min
16) Wash in water	30 sec - 1 min (X2)
17) Dehydrate to alcohols	
18) Mount in DPX	

Staining times are a guide. Microscopic examination is essential

RESULTS

Nuclei : Blue

Other tissues : Varying shades of pink and red

APPENDIX 6

PREPARATION OF MAYER'S HAEMATOXYLIN

Prepare the stain as follows:

Haematoxylin	1 g
Distilled water	1000 ml
Potassium or ammonium alum	50 g
Citric acid	1 g
Chloral hydrate	30 g
Sodium iodate	0.2 g

1. Dissolve haematoxylin, potassium alum and sodium iodate in the distilled water by warming and stirring, or by allowing to stand at room temperature overnight.
2. Add chloral hydrate and citric acid.
3. Boil the mixture for 5 minutes, then cool and filter.
4. The stain is ready for use immediately.

Appendix 7

Chi-Square Table

df\area	.995	.990	.975	.950	.900	.750	.500	.250	.100	.050	.025	.010	.005
1	0.00004	0.00016	0.00098	0.00393	0.01579	0.10153	0.45494	1.32330	2.70554	3.84146	5.02389	6.63490	7.87944
2	0.01003	0.02010	0.05064	0.10259	0.21072	0.57536	1.38629	2.77259	4.60517	5.9946	7.37776	9.21034	10.59663
3	0.07172	0.11483	0.21580	0.35185	0.58437	1.21253	2.36597	4.10834	6.25139	7.81473	9.34840	11.34487	12.83816
4	0.20699	0.29711	0.48442	0.71072	1.06362	1.92256	3.35669	5.38527	7.77944	9.4873	11.14329	13.27670	14.86026
5	0.41174	0.55430	0.83121	1.14548	1.61031	2.67460	4.35146	6.62568	9.23636	11.07050	12.83250	15.08627	16.74960