Procedure for the Analysis of Persistent Organic Pollutants in Environmental and Human Matrices to Implement the Global Monitoring Plan under the Stockholm Convention

Protocol 2:
Protocol for the Analysis of Polychlorinated Biphenyls (PCB) and Organochlorine Pesticides (OCP) in Human Milk, Air and Human Serum

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United Nations Environment Programme (UNEP)
Division of Technology, Industry and Economics

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For:

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1 SCOPE

The Global Monitoring Plan of the Stockholm Convention sets a framework for the analysis of persistent organic pollutants (POPs); therein, the congeners recommended for analysis in the core matrices are listed (see chapter 2 of the "Guidance on the global monitoring plan for persistent organic pollutants", UNEP 2013). A protocol is needed to ensure that these compounds are always analysed correctly in various laboratories and in the same way. In order to assist POPs laboratories in the analysis of POPs, Chemicals Branch of the Division of Technology, Industry and Economics (DTIE) of the United Nations Environment Programme is developing generic procedures for the analysis of initial and new POPs.

This procedure covers polychlorinated biphenyls (PCB) and organochlorine pesticides (OCP). The present protocol describes the method for sample preparation, extraction, purification and analysis of six indicator PCB and OCPs (see Table 1) in human milk, human serum and air.

Table 1: PCB and OCPs to be analysed with the underlying protocol

PCB congener number	Structure
28	2,2',4-trichlorobiphenyl
52	2,2',5,5'-tetrachlorobiphenyl
101	2,2',4,5,5'-pentachlrobiphenyl
138	2,2',3,4',5,5'-hexachlorobiphenyl
153	2,2',4,4',5,5'-hexachlorobiphenyl
180	2,2',3,4,4',5,5'-heptachlorobiphenyl

Pesticides (initial POPs)	Pesticides (new POPs)
Aldrin	α-HCH
Chlordanes	β-HCH
cis-chlordane	Lindane (γ-HCH)
trans-chlordane	Endosulfan
trans-nonachlor	α-Endosulfan
Oxychlordane	β-Endosulfan
DDT	Endosulfan sulfate
o,p'-DDD	Pentachlorobenzene
p,p'-DDD	
p,p'-DDE	
o,p'-DDE	
o,p'-DDT	
p,p'-DDT	
Dieldrin	
Endrin	
Heptachlor	
cis-Heptachloro epoxide	
trans-Heptachloro epoxide	
Hexachlorobenzene (HCB)	
Mirex	

The following POPs pesticides are not addressed in this protocol since the analytical procedure requires higher sophistication: chlordecone and toxaphene.

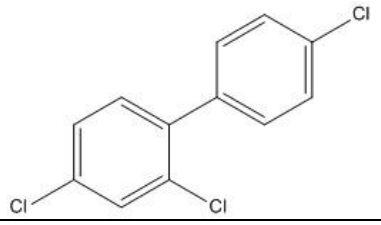
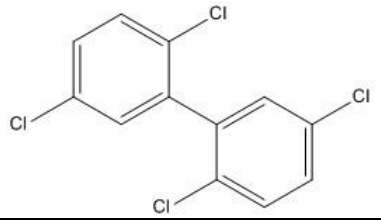
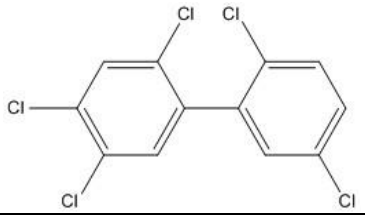
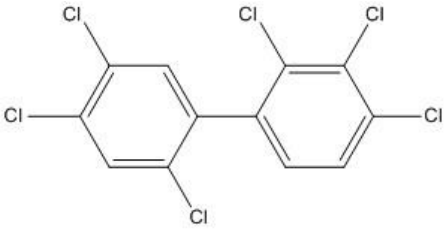
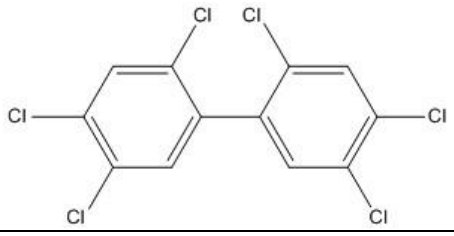
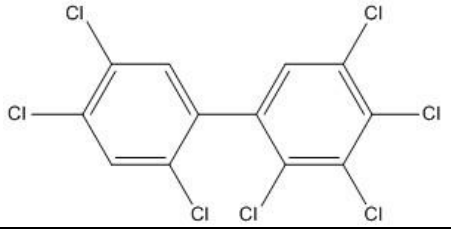
PCB 28	
PCB 52	
PCB 101	
PCB 138	
PCB 153	
PCB 180	

Figure 1: Structures of the six indicator PCB congeners

2 PRINCIPLE

All analytes of interest, *i.e.,* six indicator PCB and OCP need to be released from their matrices because most matrix components interfere in the final determination. PCB and OCP can be extracted from human milk samples by liquid-liquid extraction (LLE), from air samples (on polyurethane foams (PUFs)) with Soxhlet extraction and from human serum samples with solid phase extraction (SPE). Purification from all the extracts can be performed over an Al_2O_3 column, followed by fractionation on an 1.5% (w/w) deactivated silica column and purification and/or an acidic silica column. The instrumental analysis of the cleaned extracts of all samples is carried out by gas chromatography coupled to an electron capture detector (GC-ECD) with a double column system, after which all target compounds can be identified and quantified.

3 PRECAUTIONS

Before starting with the analysis and the preparation of the necessary materials it is essential to take two precautions.

1. The blank contamination of solvents and materials used during the analysis must be tested to prove they do not contain any PCB and OCP of interest.
2. The present protocol describes the analysis of PCB and OCP. However, it is possible to change certain parameters and analytical conditions described in this protocol, while still obtaining the same results. In case of such changes, the entire method should be optimised and validated to ensure the comparability of data.

4 MATERIALS AND REAGENTS

4.1 Materials

Balance (precision 0.01 g)

Round-bottom flask (1 L)

Shaking machine

Dessiccator

Oven (140 °C)

Glass centrifuge tubes (10 mL and 25 mL)

Pipettes (100 µL and 2 mL)

Soxhlet glassware and electrical heating device or water bath

Passive sampler for PUF disks

Whirl mixer

Ultrasonic bath

Refrigerator (4 °C)

Centrifuge (3,000 rpm)

Kuderna-Danish (KD) glassware

Glass collection tubes (10 mL, 20 mL and 30 mL)

SPE device

Glass columns with glass frit 22 cm x 20 mm internal diameter (id)

Glass columns for silicagel 15 cm length x 11 mm id

Glass GC-vial (2 mL)

Seal, silver aluminium 11 mm, PTFE/ Rubber Liner (seals should not contain sulphur) Chromacol 9102013285

Capper/Decapper

Glass Pasteur Capillary Pipettes

Capillary column CP-SIL 8 CB (Agilent Chrompack CP8753), length 60 m, id 0.25 mm, film thickness 0.25 µm

Capillary column CP-SIL 19 CB (Agilent Chrompack CP8722), length 60 m, id 0.25 mm, film thickness 0.25 µm

4.2 Reagents and chemicals

Water, demineralised

Al_2O_3, MP Alumina B-Super I (EcoChrom), MP Biochemicals 04571, Eschwege, Germany,

Silica gel 60 (0.063 mm-0.200 mm), Merck 1.07734, Darmstadt Germany

H_2SO_4, 95%-97%, pro analysis, Sigma Aldrich 30743, Steinheim, Germany

n-Hexane, Ultra Resi-Analyzed, J.T. Baker 9262, via Avantor Performance Materials B.V., Deventer, The Netherlands

n-Pentane, Picograde, Promochem, SO-1282, via LGC Standards, Wesel, Germany

Iso-octane, suprasolve, Merck product No 1.15440, Darmstadt, Germany

Stock PCB 103, Ultra Scientific RPC-040AS, 100 µg/ml in isooctane, North Kingstown, Rhode Island, United States

Stock PCB 198, Ultra Scientific RPC-075AS, 100 µg/ml in isooctane, North Kingstown, Rhode Island, United States

Internal standard (I.S.) (PCB 103 + PCB 193) (125 ng/mL in iso-octane)

Acetone, Ultra Resi-Analyzed, no. 9254, J.T.Baker, Deventer, the Netherlands

I.S. (PCB 103 + PCB 193) (125 ng/mL in acetone)

Formic Acid. 98-100%, Sigma Aldrich 27001, Steinheim, Germany

PUF disk, 14 cm x 1.35 cm, surface area 365 cm^2, mass 4.40 g, volume 207 cm^3, Tisch Environmental, Cleves, OH, USA

Dichloromethane (DCM), Picograde, , Promochem SO-1185, via LGC Standards, Wesel, Germany

Hexane/ DCM (5:1, v/v)

NaCl, Sigma Aldrich, S9625, Steinheim, Germany

Nitrogen gas

Toluene, pro analysis, analytical reagent grade, Fisher Chemical, Loughborough, Leicestershire, UK

Boiling granules, washed with acetone and toluene

SPE cartridges: Oasis™ HLB (500 mg/6 ml), Waters 186000115, Milford Massachusetts USA

Methanol, HPLC gradient grade, J.T.Baker, Deventer, the Netherlands

Na_2SO_4 anhydrous, pro analysis; heated for 16 hours at 400 °C, Sigma Aldrich 71960, Steinheim, Germany

Diethyl ether (DEE), pro analysis, Merck Emsure ® 1.00921, Darmstadt, Germany

DEE in hexane (15%, v/v)

Silanized glass wool, J.T. Baker, Deventer, the Netherlands

Hexane/ DCM (4:1, v/v)

Individueel PCB stock solutions, Ultra Scientific

PCB calibration solution (0-250 ng/mL)

OCP calibration solution (0-250 ng/mL), Custom made by Accu Standard

4.3 Instrumentation

GC-ECD, Agilent, Santa Clara, CA, United States GC: 6890.

Injector: 7683

ECD: G2397A (µ-ECD)

Software for integration, datahandling and storage, Chemstation Software: D.03.00.611,

Agilent, Santa Clara, CA, United States

5 PREPARING AL_2O_3, DEACTIVATED SILICA AND ACIDIC SILICA

5.1 Preparation of Al_2O_3 with 8% water

- Add 8 g demineralised water to 92 g Al_2O_3 in a round-bottom flask. Use a precision balance;
- Homogenize the mixture by shaking manually until no lumps larger than ca. 1 cm^3 are visible;
- Subsequently, shake the Al_2O_3 on a shaking machine for a few hours; and
- Store in a desiccator overnight (not shorter!).

5.2 Preparation of deactivated silica with 1.5% water

- Add approximately 0.5 kg of silicagel to a round-bottom flask;
- Heat the silicagel in an oven at 140 °C for 1 night;
- Let the silicagel cool down to room temperature in a dessiccator;
- Add 1.5 g demineralized water to 98.5 g silicagel;
- Homogenize the mixture by shaking manually until no lumps larger than ca. 1 cm^3 are visible;
- Subsequently, shake the silicagel on a shaking machine for a few hours; and
- Store in a desiccator overnight.

5.3 Preparation of acidic silica (40% H_2SO_4 (w/w))

- Use a precision balance to add in a round-bottom flask of 1 L, 200 g H_2SO_4 to 300 g of silicagel;
- Homogenize the mixture by shaking manually until no more lumps larger than ca. 1 cm^3 are visible;
- Subsequently, shake the silicagel on a shaker for a few hours; and
- Store in a desiccator overnight (not shorter).

6 METHOD

6.1 Sample preparation

Note: All the glassware should be rinsed with hexane or pentane prior to use!

6.1.1 Human milk

- Homogenise the samples manually by shaking for 1 min;
- Weigh 5 mL milk in an glass centrifuge tube (25 mL);
- Add 100 µL I.S. (125 ng/mL in iso-octane);
- Add 2 mL of formic acid.

6.1.2 Air

For air sampling PUF disks are used

6.1.2.1 Preparation of the PUF

- Pre-cleaning of a PUF:
  - Perform a Soxhlet extraction on the PUF with acetone (24 h), followed by hexane (24 h); and
  - Dry the PUF and store in a desiccator (24 h).

6.1.2.2 Air sampling:

Place a PUF in a passive sampler for three months at an outdoor sampling location. Avoid any possible contamination from hands (wear gloves), etc.

6.1.2.3 Air sample preparation:

- Take the PUF out of the sampler; and
- Add 100 µL I.S. (125 ng/mL in iso-octane) to the PUF

6.1.3 Human serum

- Homogenise the human serum sample (10 mL) manually by shaking for 1 min;
- Weigh 5 mL of human serum in a glass centrifuge tube (10 mL);

- Add 100 µL I.S. (125 ng/mL in iso-octane);
- Vortex and sonicate the samples for 20 min;
- Store the samples overnight in a refrigerator;
- Add 1.5 mL formic acid and 2 mL demineralized water to the sample; and
- Vortex and sonicated for 20 min.

6.2 Sample extraction

Note: All the glassware should be rinsed with hexane or pentane prior to use!

6.2.1 Human milk

LLE is used for the extraction of human milk

- Add 12 mL hexane/ DCM (5:1, v/v) to the sample;
- Shake manually and centrifuge for 10 min at 3000 rpm;
- In case emulsions are formed between the two phases, add small amounts of NaCl until the emulsions are disrupted; centrifuge when needed;
- Transfer the upper, organic layer to an empty glass collection tube (30 mL);
- Repeat the extraction with 12 mL hexane/ DCM (5:1, v/v);
- Transfer the upper, organic layer to the same collection tube;
- Add 1mL iso-octane to the extract; and
- Concentrate the extract to 1 mL in a water bath (max 40 °C) under a nitrogen flow.

6.2.2 Air

- Cut the PUF into pieces with a clean scissors;
- Perform a Soxhlet extraction on the PUF with DCM (12 h);
- Add 1 mL *iso*-octane, and 2 boiling granules to the KD-flask; and
- Concentrate the extract to 1 mL by using either a rotary evaporator or KD. In case a rotary evaporator is used, ensure it is kept clean by thoroughly rinsing the cooler between the samples.

6.2.3 Human serum

SPE is used for the extraction of serum

- Install an SPE cartridge on the SPE device;
- Wash the SPE cartridge with 5 mL DCM with a flow of 1.5 mL/min;
- Condition the SPE cartridge with 5 mL methanol followed by 5 mL water with a flow of 1.5 mL/min;
- Add the sample extract to the SPE cartridge with a flow of 0.4 mL/min;
- Wash the SPE cartridge with 1 mL water;

- Dry the cartridge under a nitrogen stream at 20 psi for 10 min, followed by centrifugation (15 min, 4000 rpm);
- Elute the PCB and OCPs from the cartridge in a clean collection tube (20 mL) with 5 mL hexane followed by 3 mL DCM; and
- Concentrate the extract to approximately 1 mL in a water bath (max 40 °C) under a nitrogen flow.

6.3 Sample purification

6.3.1 Human milk, air, and human serum

Sample purification of extracts of human milk, air and human serum consists of 3 steps. First, the extracts need to be purified over an Al_2O_3 column. The second step involves the fractionation over an 1.5% (w/w) deactivated silica column. After this second step the extracts need to be analysed with GC-ECD for OCPs vulnerable to oxidation (aldrin, dieldrin, endrin, α and β–endosulfan) and α and β-heptachloroepoxide. After analysis of the extracts, an additional purification step can be performed over an acidic silica column, to obtain cleaner chromatograms for the OCP fraction.

6.3.1.1 Purification over an Al_2O_3 column

- Prepare an Al_2O_3 column by filling a glass column (with a glass frit) with 15 g of 8% deactivated Al_2O_3 (see paragraph 5.1) followed by 1 cm of Na_2SO_4;
- Vibrate the column until the height of the column does not drop anymore;
- Rinse the column wit 20 mL pentane;
- Bring the sample extract on the column;
- Rinse the sample tube 3x with 1 mL pentane;
- Place a KD-flask or other flask under the column;
- As soon as the solution has sunk into the column, elute the PCB and OCPs from the column with 210 mL pentane;
- Add 1 mL iso-octane, and 2 boiling granules to the KD-flask; and
- Concentrate the extract to 1 mL by using either a rotary evaporator or KD.

6.3.1.2 Fractionation over an 1.5 % (w/w) deactivated silica column

- Prepare an 1.5% (w/w) deactivated silica column by filling a glass column with 1.8 g of deactivate silica with 1.5% water (see paragraph 5.2) followed by 1 cm of Na_2SO_4.
- Vibrate the column until the height of the column does not drop anymore;
- Add 6 mL hexane to condition the column;
- Add the sample extract to the column as soon as the meniscus reaches the Na_2SO_4 layer;
- Place a collection tube (20 mL) under the column to collect fraction 1:
  - Rinse the sample tube three times with 1 mL hexane and add this to the column;
  - Elute with the 11 mL hexane;

- Place a new tube under the column as soon as the hexane has sunk into the column to collect fraction 2:
  - Elute with 10 mL DEE in hexane (15%, v/v) (followed by 25 mL DEE when β-endosulfan has to be analysed);
- Add 1 mL iso-octane to both tubes;
- Concentrate the extracts to 500 µL in a water bath (max 40 °C) under a nitrogen flow; and
- Transfer the extracts into a GC vial and analyse by GC-ECD or GC/MS..
  - Fraction 1 contains all PCB and the non polar pesticides (see Apendix 1).
  - Fraction 2 contains the polar pesticides (see Apendix 1).

6.3.1.3 Purification over an acidic silica column

Note: Prior to purification over an acidic silica column the extracts should be analysed for OCPs vulnerable to oxidation (see paragraph 6.3).

- Prepare an acidic silica column by filling a glass pasteur pipette consecutively with glass wool, and 1 g of 40% H_2SO_4-silica (see paragraph 5.1).
- Rinse the column with 6 times 1 mL Hexane/ DCM (4:1, v/v);
- Bring the sample extract on top of the column;
- Place a collection tube (10 mL) under the column;
- Rinse the sample tube 2 times with 0.5 mL hexane/ DCM (4:1, v/v);
- Elute the PCB and OCPs from the column with 2 mL hexane/ DCM (4:1, v/v);
- Add 1 mL iso-ocatane to the collection tube.
- Concentrate the extracts to 500 µL in a water bath (max 40 °C) under a nitrogen flow; and
- Transfer the extracts into a glass GC vial and analyze with GC-ECD (see paragraph 7).

7 INSTRUMENTAL ANALYSES

Please note that the gradient and ECD settings are dependent of the GC-ECD system and on the type of columns used. Those settings should be optimized for the in-house instruments and columns.

- Install the analytical columns in the GC;
- Check the system by performing a signal-to-noise check by injecting the calibration solution with the lowest concentration; normally a signal-to-noise (S/N) level of 3 is acceptable;
- Make a method in the software for the analyses of PCB and OCPs. Settings for separation and detection on a GC-ECD are given in Table 2;
- Place all the vials with extracts, blanks, and calibration solutions in the tray of the autosampler;
- Make a sequence in the computer, the calibration solutions, the samples, blank and the reference material in random order; and
- Start the sequence.

Table 2: Settings for PCB and OCP analyses on a GC-ECD double column system

Column 1:	Capillary column CP-SIL 8 CB (Agilent Chromoack CP8753), length 60 m, id 0.25 mm, film thickness 0.25 µm
Column 2:	Capillary column CP-SIL 19 CB (Agilent Chrompack CP8722), length 60m, id 0.25 mm, film thickness 0.25 µm
Temperature program:	90 °C (3 min), 30 °C/min to 200 °C (15 min), 5 °C/min to 265 °C (5 min), 3 °C/min to 275 °C (15 min). Total time = 58.00 min.
Carrier Gas type:	Helium gas
Gas flow:	1 mL/min
Injection volume:	1 µL
Injection temperature:*	80 °C (0.7 min), 12 °C/min to 250 °C (4 min), 12 °C/min to 350 °C (44 min) Stabilization time: 0.1 min
Injection mode:	Pulsed splitless
Pulse pressure:	170 kPa
Pulse time:	1.5 min
Purge flow:	50 mL/min
Purge time:	1.4 min
Detector:	ECD (2x)
Makeup flow:	30 mL/min
Make-up gas:	N_2

Not all injectors are capable to perform this cold-splitless technique. In that case use a fixed injection temperature of 270 °C.

8 QUANTIFICATION

For identification and quantification software programs are available like Chemstation, Totalchrom etc. Use the software to identify the target peaks (on both columns) in the GC-ECD chromatograms based on retention time (RT) (see Table 3 for the retention times of PCB

Table 3: GC-ECD-settings for PCB and OCP separation on a double column system with a CP-SIL 8 CB column and a CP-SIL 19 column

Compound	RT (min) on CP-SIL 8	RT (min) on CP-SIL 19
PCB 28	20.267	21.624
PCB 52	22.003	23.327
PCB 103 (I.S.)	24.108	24.988
PCB 101	26.306	27.442
PCB 153	30.214	31.416
PCB 138	31.641	33.365
PCB 180	35.709	37.771
PCB 198 (I.S.)	38.250	39.572
Pentachlorobenzene	12.569	12.98
α–HCH	15.788	18.887
Hexachlorobenzene	16.297	16.871
β -HCH	16.938	24.571
γ-HCH	17.403	21.054
Heptachlor	21.235	22.278
Aldrin	23.047	23.712
PCB 103 I.S.	24.111	24.988
cis-Heptachlorepoxide	24.939	26.995
Oxychlordane	25.013	26.312
trans-Chlordane	26.072	28.423
o,p'-DDE	26.18	27.542
α-Endosulfan	26.697	28.299
cis-Chlordane	26.785	28.763
trans-Nonachlor	27.025	28.845
p,p'-DDE	27.609	28.993
Dieldrin	27.883	29.947
o,p'-DDD	28.014	30.439
Endrin	28.876	31.025
p,p'-DDD	29.491	32.885
o,p'-DDT	29.717	31.258
cis-Nonachlor	29.862	33.471
Endosulfan sulfate	31.303	38.354
p,p'-DDT	31.371	33.968
Mirex	38.055	37.945
PCB 198 I.S.	38.255	39.572

I.S.: Internal standard

and OCPs on a CP-SIL 8 CB column and a CP-SIL 19 CB column). p,p'-DDT can sometimes be instable at more polar columns such as CPSil 19. In that case the less polar column should be used for the determination.

Use the peak heights of the peaks in the calibration solutions to draw a calibration curve of each of the target compounds. Compare the peak heights and retention times of the peaks in the calibration solution with those of the peaks in the samples and calculate the PCB and OCP concentrations on both columns. Of the results of both columns, the lowest concentration per compound is used for further calculation, since it is assumes that the higher value is caused by co-elution and would result in an overestimation of the concentration.

9 QA/QC

For quality control purposes, include a blank, a duplicate sample and an internal reference material in each series of maximum 12 samples. Participating interlaboratory studies (ILS) and analysing certified reference materials (CRMs) on a regular base is strongly recommended to assure the quality of the analyses.

10 References

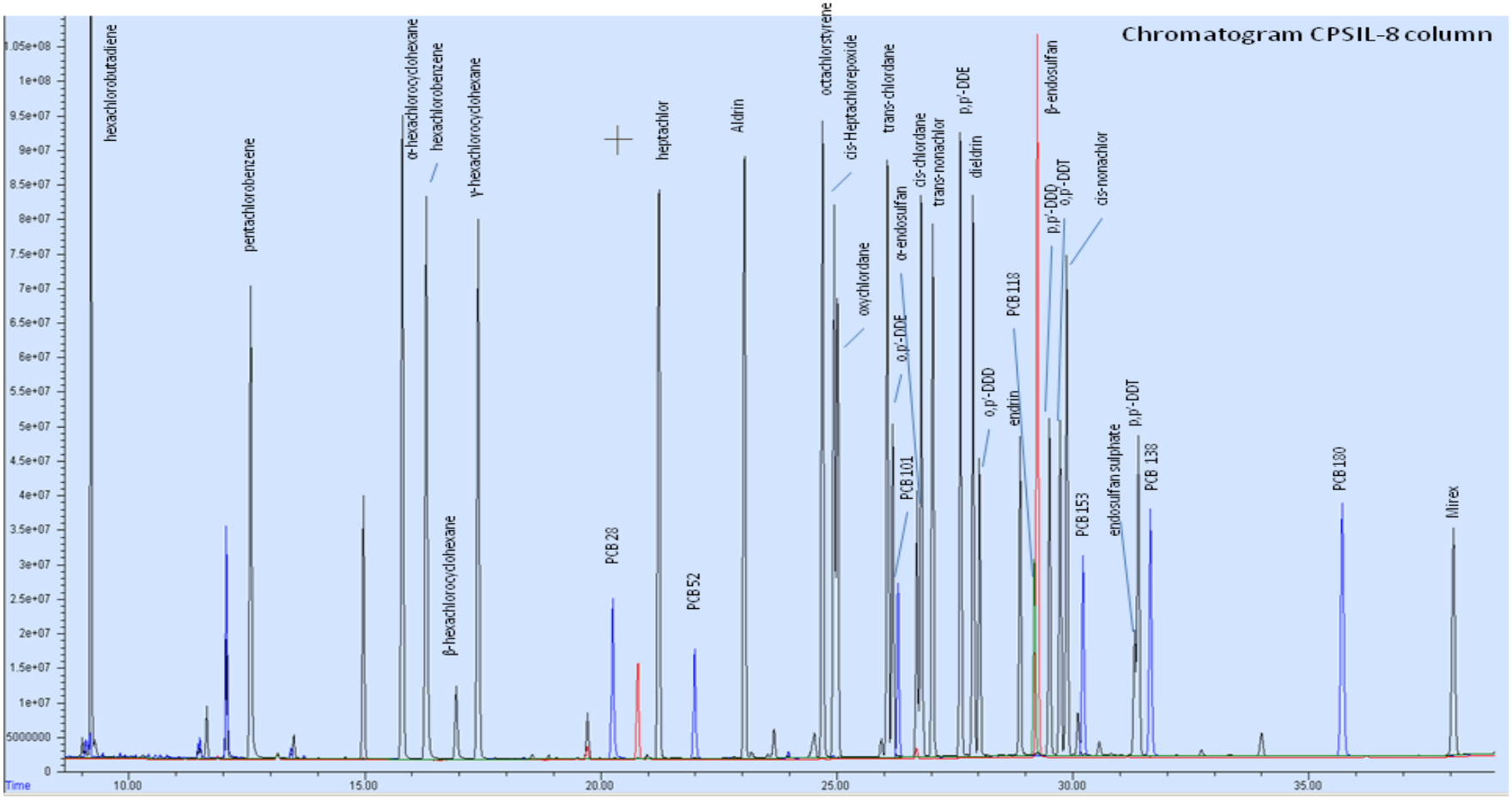
UNEP (2013): Guidance on the global monitoring plan for persistent organic pollutants. UNEP/POPS/COP.6/INF/31, 4 February, accessible from www.pops.int

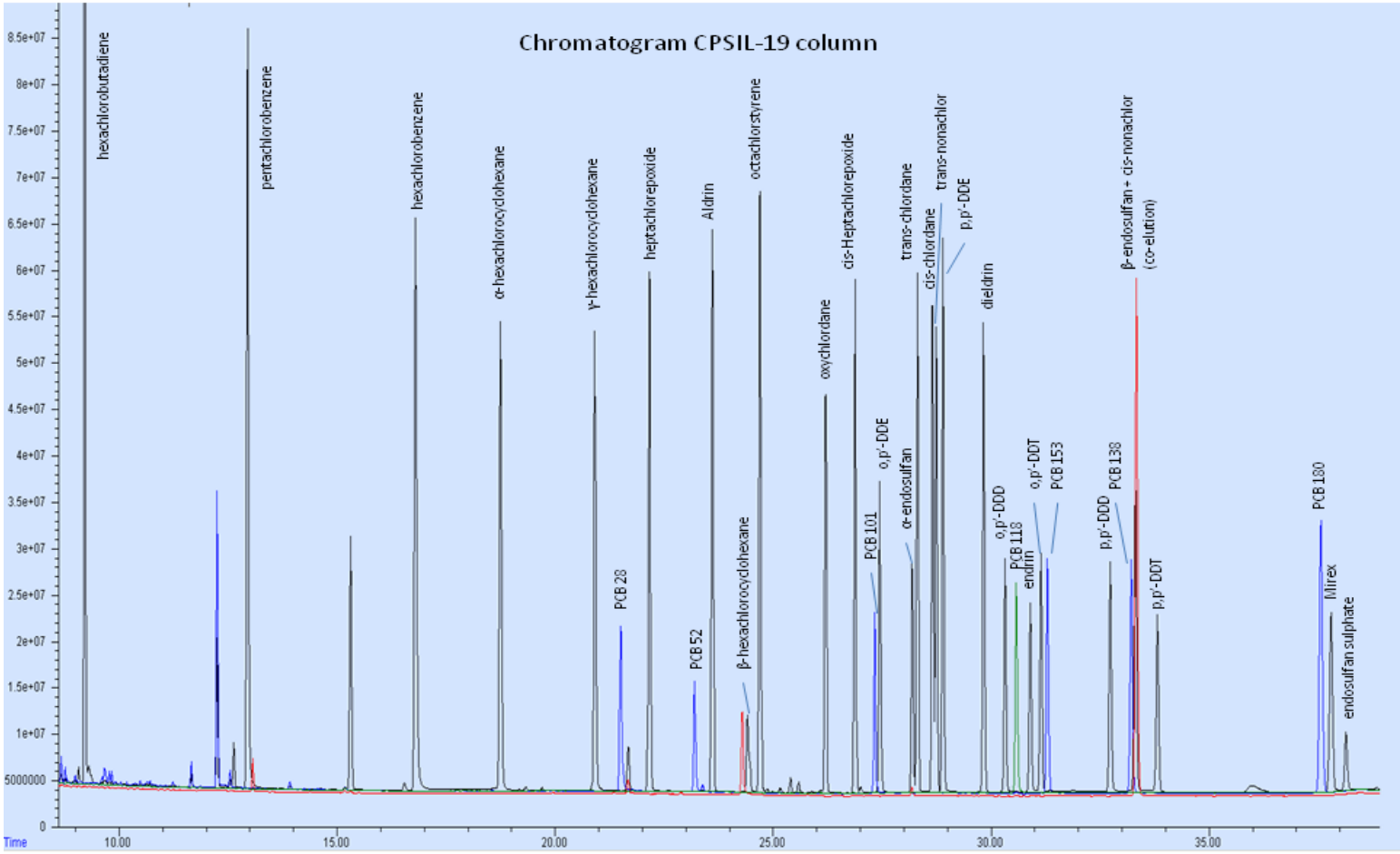
11 APPENDIX 1

Table 4: Fractionation of OCPs on an 1.5% (w/w) deactivated silica column

Fraction 1 (non-polar pesticides)	Fraction 2 (polar pesicides)
o,p'-DDT (ca. 50%)	cis and trans-chlordane
HCB	Trans-nonachlor
Heptachlor (ca. 50%)	Oxychlordane
Mirex	o,p'-DDD
Pentachlorobenzene	p,p'-DDD
	o,p'-DDT (ca. 50%)
	p,p'-DDT
	o,p'-DDE
	p,p'-DDE
	Dieldrin
	Endrin
	α- and β-endosulfan
	α-HCH
	β-HCH
	γ-HCH
	α and β-heptachlor epoxide
	Heptachlor (ca. 50%)
	Endosulfan sulfate

12 APPENDIX 2. CHROMATOGRAMS FROM CPSIL8 AND CPSIL19 COLUMNS




Chromatogram CPSIL-19 column
hexachlorobutadiene
pentachlorobenzene
hexachlorobenzene
α-hexachlorocyclohexane
γ-hexachlorocyclohexane
heptachlorepoxide
Aldrin
octachlorstyrene
oxychlordane
cis-Heptachlorepoxide
trans-chlordane
cis-chlordane
trans-nonachlor
p,p'-DDE
dieldrin
β-endosulfan + cis-nonachlor (co-elution)
PCB 28
PCB 52
β-hexachlorocyclohexane
PCB 101
o,p'-DDE
α-endosulfan
o,p'-DDD
PCB 118
endrin
o,p'-DDT
PCB 153
p,p'-DDD
PCB 138
p,p'-DDT
PCB 180
Mirex
endosulfan sulphate
Time
10.00
15.00
20.00
25.00
30.00
35.00