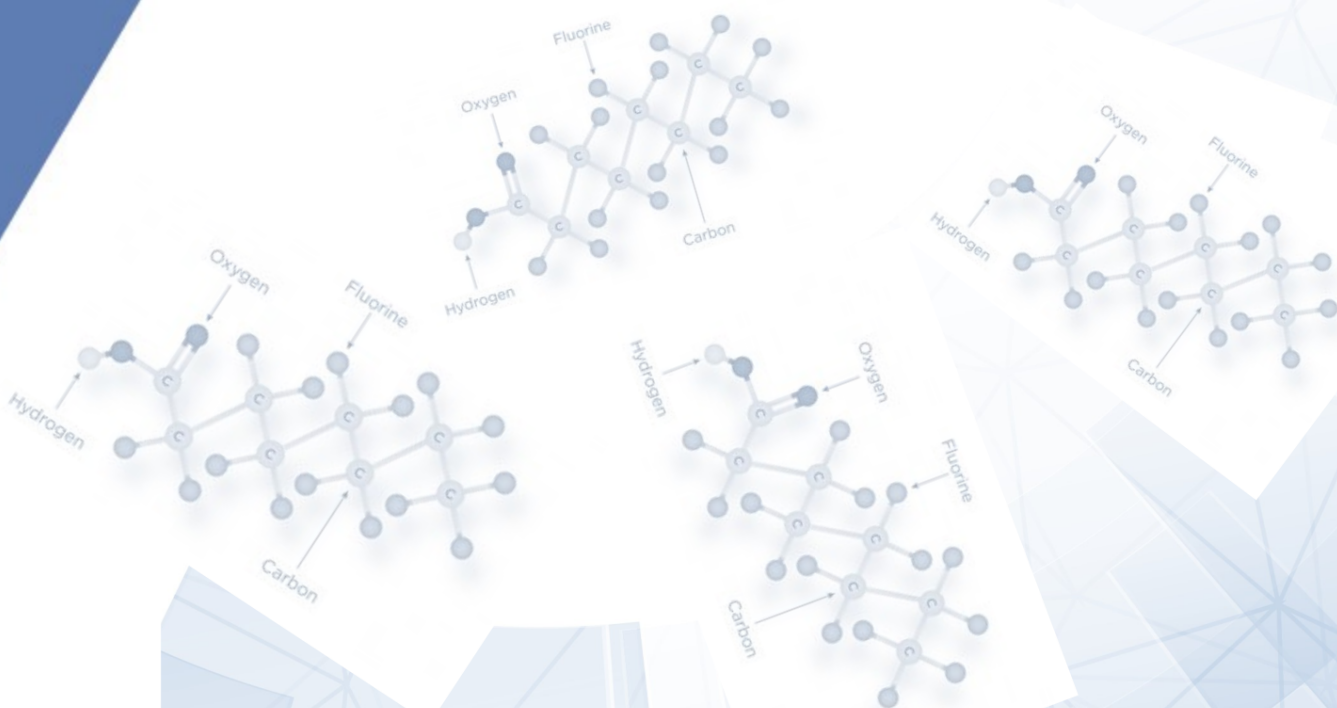




Global Monitoring Plan on Persistent Organic Pollutants

Protocol 3: Protocol for the Analysis of Polybrominated Diphenyl Ethers (PBDE) in Human Milk, Air and Human Serum

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

**Protocol 3:
Protocol for the Analysis of Polybrominated Diphenyl Ethers (PBDE) 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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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.

The Global Monitoring Plan (GMP) established through article 16 of the Stockholm Convention (<http://chm.pops.int/Convention/ConferenceofthePartiesCOP/Meetings/COP5/COP5Documents/tabid/1268/Default.aspx>) requires the analysis of the persistent organic pollutants (POPs) in relevant matrices. A protocol is needed to ensure that these compounds are always analysed correctly and in the same way.

This procedure covers polybrominated diphenyl ethers (PBDE). The present protocol describes the method for sample preparation, extraction, purification and analysis of eight PBDE that are recommended for analysis (see Table 1) in human milk, human serum and air according to the GMP.

Table 1: PBDE congeners to be analysed with the underlying protocol

PBDE congener number	Structure	Relevant core matrix
17	2,2',4-Tribromodiphenyl ether	Optional: Air
28	2,4,4'-Tribromodiphenyl ether	Optional: Air
47	2,2',4,4'-Tetrabromodiphenyl ether	Air, human milk/blood
49	2,2',4,5'-Tetrabromodiphenyl ether	
99	2,2',4,4',5-Pentabromodiphenyl ether	Air, human milk/blood
100	2,2',4,4',6-Pentabromodiphenyl ether	Optional: Air, human milk/blood
153	2,2',4,4',5,5'-Hexabromodiphenyl ether	Air, human milk/blood
154	2,2',4,4',5,6'-Hexabromodiphenyl ether	Air, human milk/blood
183	2,2',3,4,4',5',6-Heptabromodiphenyl ether	Air, human milk/blood

2 PRINCIPLE

All PBDE need to be released from their matrices because most matrix components interfere in the final determination. PBDE 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 acidic silica column followed by purification over an 1.5% (w/w) deactivated silica column. The instrumental analysis of the cleaned extracts of all samples is carried out by gas chromatography coupled to (low resolution) mass spectrometry (GC-MS), 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 PBDE of interest.
2. The present protocol describes the analysis of PBDE. 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

Vacuum dessiccator

Ovens (37 °C and 140 °C)

Whirl mixer

Glass jar (15 mL)

Glass tube (20 mL)

Pipette (100 µL)

Refrigerator (4 °C)

Kuderna-Danish (KD) glassware

Passive sampler for polyurethane foam (PUF) disks

Boiling granules, washed with acetone and toluene

Soxhlet glassware and electrical heating device or water bath

Ultrasonic bath

SPE device

Collection tubes (20 mL)

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

Glass columns for silicagel 15 cm length x 11 mm id

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

Pasteur pipettes

Glass GC-vial (2 mL)

Capper/ decapper

Seal, silver aluminum 11 mm, PTFE/ Rubber Liner

Capillary column, CP-sil-8CB, WCOT fused silica, 50 m x internal diameter (I.D) 0.25 mm x Film 0.25 µm, Varian

4.2 Reagents

H₂SO₄, 95-97%, pro analysis, Sigma Aldrich 30743, Steinheim, Germany

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

Water, demineralized

Acetone, Ultraresi, J.T.Baker, Deventer, the Netherlands

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

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

BDE 58 stock solution, Wellington, BDE-58, 50 µg/ml, Ontario Canada

Internal standard (I.S.) (BDE 58) (100 ng/mL in iso-octane)

I.S. (BDE 58) (100 ng/mL in acetone)

Polyurethane foam (PUF) disk, 14 cm x 1.35 cm, surface area 365 cm², mass 4.40 g, volume 207 cm³, Tisch Environmental, Cleves, OH, USA

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

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

2-propanol, LC-MS Chromasolv[®], Fluka 34965, Steinheim, Germany

Formic acid/ 2-propanol (4:1, v/v)

Water/2-propanol (4:1, v/v)

Sodium oxalate, Sigma-Aldrich, Steinheim, Germany

0.15 M Sodium oxalate solution

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

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

Heptane, J.T.Baker, Deventer, the Netherlands

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

SPE cartridges: Oasis[®] HLB custom made SPE extraction cartridges (540 mg/3 ml), Waters product No 186003852, Milford Massachusetts, USA

DCM/ methanol (7:3, v/v)

Water/ methanol (19:1, v/v)

Water/ 2-propanol (7:3, v/v)

Water/ methanol (3:7, v/v)

Nitrogen gas

Hexane/ DCM (3:1, v/v)

Na₂SO₄ anhydrous, pro analysis; heated for 16 hours at 250 °C

Hexane/ DCM (7:3, v/v)

DEE in hexane (15%, v/v)

PBDE solution (Wellington PBDE solution, concentration 1000-5000 ng/mL), BDE-MXE, Wellington, Ontario, Canada

Calibration solution: PBDE solution diluted 5, 10, 20, 50, 225, 550, 1000 and 2500-fold

4.3 Instrumentation

GC-MS with electron capture negative ionization (ECNI), Agilent, Santa Clara, CA, United States:

GC: 6890N

Injector: 7683B

MSD: 5975 inertXL MSD

CI bron: G3170-65403 (kit)

EI bron: G2591-64710 (kit)

Software for integration, data handling and storage, Agilent Chemstation, Version E.02.00.493, Agilent, Santa Clara, CA, United States

5 PREPARING ACIDIC SILICA AND DEACTIVATED SILICA

5.1 Preparation of acidic silica (40% H₂SO₄ (w/w))

- Use a precision balance to add, in a round-bottom flask of 1 L, 200 g H₂SO₄ to 300 g of silicagel;
- Homogenize the mixture by shaking manually until no more lumps larger than ca. 1 cm³ are visible;
- Subsequently, shake the silicagel on a shaker for a few hours; and
- Store in a dessiccator overnight.

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 desiccator;
- Add 1.5 g demineralised water to 98.5 g silicagel;
- Homogenize the mixture by shaking manually until no lumps larger than ca. 1 cm³ are visible;
- Subsequently, shake the silicagel on a shaker for a few hours; and
- Store in a dessiccator overnight.

6 METHOD

6.1 Sample preparation

Note: All the glassware should be rinsed with acetone prior to use!

6.1.1 Human milk

- Condition the human milk samples (25 mL) in an oven of 37 °C for 2 h;
- Vortex the samples for 1 min;
- Weigh 5 mL milk in an empty glass tube (20 mL);
- Add 100 µL I.S. (100 ng/mL in iso-octane); and
- Store the samples overnight in a refrigerator.

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);
 - Dry the PUF and store in a dessiccator (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. (100 ng/mL in iso-octane) to the PUF.

6.1.3 Human serum

- Homogenise the human serum samples (10 mL) manually by shaking for 1 min;
- Weigh 5 mL of human serum sample in a glass jar (15 mL);
- Add 100 µL I.S. (100 ng/mL in acetone);
- Vortex the samples for 1 min;
- Store the serum sample for one night at 4 °C;
- Condition the samples at room temperature;
- Add 5 mL formic acid/ 2-propanol (4:1, v/v);

- Vortex and sonicate for 5 min;
- Place the samples in the dark for 50 min to allow the proteins to denature;
- Add 5 mL water/2-propanol (4:1, v/v); and
- Vortex and sonicate for 5 min;

6.2 Sample extraction

Note: All the glassware should be rinsed with acetone prior to use!

6.2.1 Human milk

- Add subsequently to the sample:
 - 2.5 mL of 0.15 M sodium oxalate solution
 - 5 mL of methanol
 - 5 mL of DEE
 - 5 mL of n-heptane.
- Shake the sample tube carefully between additions of solvents;
- In case emulsions are formed between the two phases, add small amounts of methanol until the emulsions are disrupted;
- Transfer the upper, organic layer to a KD flask;
- Repeat the extraction two more times. First by subsequently adding 5 mL of DEE and 5 mL of n-heptane, and secondly by adding 3 mL of each;
- Transfer the organic phase to the same KD flask between each extraction;
- Add 1 mL of *iso*-octane and 2 boiling granules;
- 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.

6.2.2 Air

- Perform a Soxhlet extraction of the PUF with DCM (12 h);
- Add 1 mL of *iso*-octane and concentrate the extract to 1 mL by using either a rotary evaporator or KD.

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 3 mL methanol followed by 3 mL DCM/ methanol (7:3, v/v) with a flow of 1.5 mL/min;
- Condition the SPE cartridge with 5 mL methanol followed by 5 mL water/ methanol (19:1, v/v) with a flow of 1.5 mL/min;
- Add the sample extract (15 mL) to the SPE cartridge with a flow of 0.4 mL/min;

- Add 5 mL water/ methanol (19:1, v/v) to the SPE cartridge with a flow of 0.4 mL/min;
- Wash the SPE cartridge with 10 mL water/ 2-propanol (7:3, v/v) with a flow of 1.5 mL/min;
- Dry the cartridge for 45 min under a vacuum of 38 kPa;
- Clean the cartridge with 3 times 20 µL water/methanol (3:7, v/v);
- Elute the PBDE from the cartridge in a clean collection tube (20 mL) with 12 mL DCM/ methanol (7:3, v/v) with a vacuum of 8 kPa;
- After the 12 mL DCM/methanol had disappeared in the cartridge, let the pressure increase up to 20 kPa for 10 seconds to elute the remaining solvent;
- Add 1 mL iso-octane to the extract;
- Concentrate the extract to approximately 500 µL in a water bath (max 40 °C) under a nitrogen flow; and
- Add hexane/ DCM (3:1, v/v) to the extract up to 1 mL.

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 two steps. The first step involves the clean-up over an acidic silica column. The second step involves the cleaning over a 1.5% (w/w) deactivated silica column.

6.3.1.1 *Purification over an acid silica column*

- Prepare an acidic silica column by filling a glass column (with a glass frit) with 20 g 40% H₂SO₄-silica (see section 5.1) followed by 1 cm of Na₂SO₄. Vibrate the column between the different additions until no air bubbles are visible;
- Rinse the column with 25 mL hexane/ DCM (7:3, v/v);
- Bring the sample extract on top of the column;
- Rinse the sample tube 2 times with 1 mL hexane/ DCM (7:3, v/v);
- Place a KD-flask under the column;
- Elute the PBDE from the column with 75 mL hexane/ DCM (7:3, v/v);
- 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 *Purification over an 1.5 % (m/m) 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 5.2) followed by 1 cm of Na₂SO₄. Vibrate the column between the different additions until no air bubbles are visible;
- Add 6 mL hexane to condition the column;
- Add the sample extract to the column as soon as the meniscus reaches the Na₂SO₄ layer;
- Place a KD-flask under the column as soon as all off the extract is sunk into the column;

- Rinse the extract tube three times with 1 mL hexane and add it to the column;
- Elute with 11 mL hexane followed by 12 mL DEE in hexane (15%, v/v);
- After collecting all eluates, add 1 mL *iso*-octane and 2 boiling granules to the KD-flask;
- Concentrate the extract to 500 µL by using either a rotary evaporator or KD; and
- Transfer the extract into a glass GC vial and analyze with GC-MSD (ECNI mode) (see Section 7).

7 INSTRUMENTAL ANALYSES

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

- Install the analytical column;
- Tune the MS. If the tuning is not working, the source needs to be cleaned;
- Check the system by performing a signal-to-noise check for every PBDE by injecting the calibration solution with the lowest concentration;
- Make a method in the software for the analyses of PBDE. Settings for separation and detection on a GC-ECNI-MS 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 blank and the reference material in random order; and
- Start the sequence.

Table 2: Settings for PBDE analyses on a GC-ECNI-MS

Column:	Capillary column, CP-sil-8CB, WCOT fused silica, 50 m x internal diameter (I.D) 0.25 mm x Film 0.25 µm, Varian
Injection volume:	1 µL
Injection mode:	Pulsed splitless
Carrier Gas:	Helium
Nominal initial flow:	2.6 mL/min
Tune gas:	Methane

8 QUANTIFICATION

For identification and quantification software programs are available like Chemstation, Masshunter, etc.

Use the software to identify the target peaks in the GC/MS chromatograms based on retention time (RT) and m/z transition (see Table 3 for the settings for PBDE detection and quantification after separation on a CP-Sil-8CB column).

Use the peak areas of these peaks in the calibration solutions to draw a calibration curve of each of the target compounds. Compare the peak areas and retention times of the peaks in the calibration solution with those of the peaks in the samples and calculate the PBDE concentrations.

Table 3: GC/MS-settings for PBDE separation on CP Sil-8CB column

Compound	Description	m/z	RT (min)
BDE 17	Target compound	79	18.7
BDE 28	Target compound	79	19.2
BDE 47	Target compound	79	30.8
BDE 49	Target compound	79	29.0
BDE 99	Target compound	79	38.0
BDE 100	Target compound	79	36.7
BDE 153	Target compound	79	42.7
BDE 154	Target compound	79	41.3
BDE 183	Target compound	79	47.5
BDE 58	I.S.	79	31.5

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 in 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