Standard operation procedure (SOP) for the extraction and clean-up of total PCBs and select OH-PCBs in human serum

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1.0 Scope and Application

This method covers the extraction and cleanup for analysis of PCBs and OH-PCBs in approximately 4 g of blood plasma or serum. The serum is acidified and deactivated by hydrochloric acid (HCl) and 2-propanol, and the lipids are then extracted by liquid-liquid partitioning with a mixture of hexane and methyl-*tert* butyl ether. The samples are cleaned up by applying sulfuric acid and different sulfuric acid impregnated silica gel columns. The instrumental analyses are performed by gas chromatography (GC) with tandem mass spectrometry (MS/MS) and are described elsewhere.

The method is originally described by Hovander, L.; Athanasiadou, M.; Asplund, L.; Jensen, S.; Wehler, E. K. Extraction and cleanup methods for analysis of phenolic and neutral organohalogens in plasma. *J Anal Toxicol* **2000**, *24*, 696-703.

The adaptation described in this SOP is published by Marek, R.F.; Thorne, P.S.; Wang, K.; DeWall, J.; Hornbuckle, K.C. PCBs and OH-PCBs in serum from children and mothers in urban and rural U.S. communities. *Environ Sci Technol* **2013**, *47*, 3353-3361.

2.0 Interferences and QA/QC

2.1 Method blanks

Interferences from glassware, solvent and chemicals are monitored by running method blanks. The method blank is spiked with surrogate standards and run through the entire extraction process and clean up steps in parallel with the samples. In every series of 10 human serum samples one method blank should also be run. The method blank is prepared by adding 1% KCl in the same amount as the serum samples, i.e. about 4 mL.

2.2 Control of GC Instruments

Pure hexane is run as the first sample for all instruments to check for instrumental interferences. Hexane should also be run between calibration standards and samples for control of memory effects. Also the last sample should be pure hexane to check the instrument.

A calibration standard with concentrations close to the limit of detection should be included to check the response of the instrument.

2.3 Cleaning of glassware

All glassware is rinsed with hot water as soon as possible after use. The glassware is left in soapy water no longer than 24 hrs and rinsed with tap water

until soap bubbles are gone followed by 2 rinses with DI water. All glassware is heated in a furnace at 450 °C overnight. After cooling, the glassware is capped with aluminum foil and stored in a clean environment to be protected from dust and other contaminants. Plastic caps are washed in the same method as the glassware, followed by a triple-rinsing of methanol, hexane, and acetone. Caps are also stored in a clean environment protected from dust and other contaminants.

2.4 Surrogate Recovery

Before starting the extraction procedure all samples are spiked with surrogate standards (SS). At the same time, one empty GC vial is also spiked with the same surrogate standard for each class of compounds. After the serum samples have gone through the extraction and clean up steps the samples are evaporated to an appropriate volume. Then the samples AND the GC vial that contains the SS are spiked with the internal standard. These GC vials represent 100% of what has been added to the samples and will be used for the calculation of the recovery of the SS in the samples.

2.5 Laboratory Reference Material (LRM)

LRM consists of human serum purchased from a blood bank. This serum is used to check the reproducibility of the method and instrument over time since each LRM sample is from the same serum batch.

2.6 Standard Reference Material (SRM)

Standard Reference Material 1957: Organic Contaminants in Non-Fortified Human Serum was purchased from the National Institute of Standards and Technology (NIST). SRM is analyzed to check the reproducibility of the method and instrument over time (internal consistency) and serves as an external method control. One SRM sample is analyzed for each batch of 10 human serum samples.

3.0 Safety

3.1 Blood

Working with blood involves risk since it may contain viruses. Even if the risk is very low the work with serum should include precautions that minimize the risk of contagion.
In this method, viruses are de-activated by alcohols and 2-propanol.
Gloves and eye protection (goggles) should always be worn when handling serum and in the extraction procedure until the 2-propanol has been added. All glassware that has been in contact with blood/plasma/serum, for example Pasteur pipettes, should be disposed of in the biohazard sharps container. Gloves should be disposed of in the regular (non-sharps) biohazard container.

Disinfecting wipes are located on the bench top and should be used to wipe down all surfaces after serum has been used, including the bench top and balance used to weigh the serum, and disposed of in the biohazard container. All employees working with serum will have undertaken the university biohazard safety training course.

3.2 Diazomethane

Uses, Safety Hazards, and Exposure Symptoms. Diazomethane (CH_2N_2) occurs as a very toxic, explosive yellow gas and it is prepared as a solution in diethylether. It is used for converting the OH-PCBs into their methyl esters. Diazomethane is toxic by inhalation or by contact with the skin or eyes. Exposure symptoms include chest discomfort, headache, weakness and, in severe cases, collapse. CH_2N_2 may explode when in contact with ground-glass joints or when heated to about 100.0 °C. Consequently specialized, scratch-free and rounded-edge glassware and a blast shield should be employed for its use. Glass pipettes, not plastic pipettes, will be used to aliquot the diazomethane to samples. Transport of diazomethane (or other forms of shock) should be minimized. Diazomethane vials with crystals present should NOT be used and should be reported to the Synthesis Core Director immediately.

Usage Overview. The diazomethane is prepared in the laboratory of MTF by the Synthesis Core. When diazomethane is needed the samples will be transported to IREH and the derivitization procedure will take place there. The evaporation of excess diazomethane from samples will also be performed at IREH before the samples can be transported back to ECE.

Protective Equipment. All diazomethane usage will take place in a well-ventilated fume hood with the sash in the down, protective position. Protective clothing such as long pants and closed-toed, impervious shoes need to be worn. Doubled Nitrile gloves, protective glasses, face shield, and a lab coat need to be worn at all times when handling the diazomethane. The addition of diazomethane to samples will take place behind a transportable explosion shield placed in the fume hood.

Used equipment like gloves and pipettes will sit in the hood to allow the diazomethane to completely evaporate before being discarded.

Additional Information. Additional information on diazomethane safety can be found in the Standard Operating Procedure for Diazomethane Generation available through the Synthesis Core.

4.0 Apparatus and Glassware

4.1 Apparatus

Barnstead|Thermolyne Rotisserie (custom)

Fisher Scientific Mini-Vortexer

The Meyer N-Evap Analytical Evaporator equipped with Popper non-sterile pipetting needles with blunt end and standard hub, ref 7936,18x4”

Mettler Toledo AG245 analytical balance
Thermolyne 30400 Furnace
ThermoScientific Sorvall Legend XTR centrifuge equipped with a FiberLite 6x250LE rotor and Sorvall adapters, cat. No. 00456
Silica column holder (custom)

4.2 Glassware and Supplies

Pasture pipettes, borosilicate & non-sterile, 9" Fisher Cat. No. 13-678-20D
Pasture pipettes, borosilicate & non-sterile, 5 3/4" Fisher Cat. No. 13-678-20B
16x125 mm (15 mL, No. 9826) Pyrex test tubes w/ Teflon-lined screw caps
Corning screw cap w/ PTFE liner 15mm-415 (No. 9998-15)
Glass funnel (custom)
GC sample vials (2mL capacity)
GC vial inserts, Wheaton Cat. No. 225350-631, 0.35 mL glass flat bottom limited volume insert
Teflon aluminum crimp caps with Teflon septa, SUN-Sri 200 100 TFE/RUB 11MM seal
Glass wool, Pyrex borosilicate, Fisher Cat. No. 11-388 from Corning, Inc. X3950
Solvent reservoirs (custom)
Wheaton vial file No. 228778 (holds 60 2 mL M-T vials)

5.0 Chemicals and Solvents

5.1 Raw Materials

Silica gel 70-230 Mesh, S826-1	Fisher Scientific
Potassium Chloride EP/BP/USP/FCC, P333-500	Fisher Scientific
Potassium Hydroxide NF/FCC Pellets, P251-3	Fisher Scientific
Hexanes, pesticide, H300-4	Fisher Scientific
2-Propanol, histological, A426P-4	Fisher Scientific
Methyl tert-Butyl Ether, HPLC, E127-4	Fisher Scientific
Methylene Chloride, pesticide, D142-4	Fisher Scientific
Methanol, optima, A454-4	Fisher Scientific
Ethanol, absolute, 200 proof , 99.5% A.C.S reagent	Sigma-Aldrich
Water optima, W7-4	Fisher Scientific
Sulfuric Acid certified ACS plus, A300-212	Fisher Scientific
Hydrochloric Acid Solution 6N, SA56-500	Fisher Scientific

5.2 Reagents

1:1 Hx:MTBE (v/v)
1% KCl (w/w): 5 g KCl, 495 g reag. H2O
KOH: 100 mL reagent water, 5.622 g KOH, 100 mL EtOH
9:1 Hx:MTBE (v/v): 50 mL MTBE, 450 mL Hx
2 M HCl: 1part 6 N HCl to 2 parts reagent water (50 mL acid + 100 mL H_2O)

5.3 Preparation of acidified silica gel columns

Combust the silica gel overnight at 450 °C. When it has cooled, transfer the gel to a screw-capped glass bottle. Weigh a portion of the gel in a new screw-capped glass bottle and add by weight concentrated H_2SO_4 to a relationship of 2:1 (silica gel:acid). Shake the bottle thoroughly until no lumps are seen.

The columns are prepared by stuffing a small amount of glass wool in the bottom of a 9" Pasteur pipette and then using a small glass funnel to add 0.1 g of pure activated silica gel. Then 1 g acidified silica gel is added on the top.

6.0 Standards

6.1 Surrogate Standards (SS)

6.1.1 PCB

4-monochlorobiphenyl	(^{13}C PCB 3)
4,4'-dichlorobiphenyl	(^{13}C PCB 15)
2,4,4'-trichlorobiphenyl	(^{13}C PCB 28)
2,2',5,5'-tetrachlorobiphenyl	(^{13}C PCB 52)
2,3',4,4',5-pentachlorobiphenyl	(^{13}C PCB 118)
2,2',4,4',5,5'-hexachlorobiphenyl	(^{13}C PCB 153)
2,2',3,4,4',5,5'-heptachlorobiphenyl	(^{13}C PCB 180)
2,2',3,3',4,4',5,5'-octachlorobiphenyl	(^{13}C PCB 194)
2,2',3,3',4,5,5',6,6'-nonachlorobiphenyl	(^{13}C PCB 208)
Decachlorobiphenyl	(^{13}C PCB 209)

6.1.2 OH-PCB

3',4'-dichloro-4-biphenylol	(^{13}C 4'OH-PCB 12)
2',3,4',5,5'-pentachloro-4-biphenylol	(^{13}C 4' OH-PCB 120)
2,2',3,4',5,5',6-heptachloro-4-biphenylol	(^{13}C 4 OH-PCB 187)

6.2 Internal Standards (IS)

6.2.1 PCB

2,3',4',5-tetrachlorobiphenyl	(^{13}C PCB 70)
2,3,3',5,5'-pentachlorobiphenyl	(^{13}C PCB 111)
2,2',3,4,4',5'-hexachlorobiphenyl	(^{13}C PCB 138)
2,2',3,3',4,4',5-heptachlorobiphenyl	(^{13}C PCB 170)

6.2.2 OH-PCB

2,4,6-trichlorobiphenyl (PCB 30)
2,2',3,4,4',5,6,6'-octachlorobiphenyl (PCB 204)

6.3 Calibration standards

6.3.1 PCB

PCB calibration mixture contains all 209 PCB congeners, the SS, and the IS.

6.3.2 OH-PCB

OH-PCB calibration mixture contains 65 OH-PCB congeners, the SS, and the IS

7.0 Sample handling

The serum should be kept frozen at approximately -20 °C until extraction. When it is time for extraction, let the serum thaw at room temperature or in a fridge overnight. Vortex the sample before weighing the amount for analysis.

8.0 Surrogate Spiking and Extraction Procedure

Weigh approximately 4 g thawed serum from the container to a 15 mL Pyrex screw-capped glass test tube, recording the exact serum weight to 4 decimal places.
Spiking solutions should warm to room temperature before spiking. Check weight of spikes to ensure no evaporation. Rinse syringe 3x with DCM and then 10x with Hx (for PCBs) or 10x with MeOH (for OH-PCBs). Add surrogate standard to samples and vortex thoroughly. Surrogate-spike 1 empty GC vial with insert (this is the reference) with PCB surrogate standard. Spike 1 test tube with OH-PCB surrogate standard plus 10 drops MeOH – this reference will be derivatized with samples in step 10.0. Spike 1 LRM sample. Spike the method blank (4 mL 1% KCl). Rinse syringe 3x with DCM and then 10x with Hx (for PCBs) or 10x with MeOH (for OH-PCBs), and reweigh standards. Use the appropriate amount of surrogate standard so that injected mass is ~5 ng.

Add 1 mL HCl 6 M to the serum and vortex. Add 5 mL 2-propanol and vortex. Add 5 mL hexane:MTBE (1:1, v/v), invert 5 min and centrifuge 5 min. Transfer the organic solvent phase to a new Pyrex test tube containing 4 mL aqueous KCl (1%, w/w). Re-extract the serum with 3 mL hexane/MTBE (1:1). Invert the test tube with organic solvent and the KCl for 3 min, centrifuge 5 min and transfer organic solvent phase to an additional new test tube (or turbo-vap vial). Re-extract the aqueous phase with 3 mL Hx:MTBE 1:1.

9.0 Substance class separation

9.1 Separation of neutral and hydroxylated PCBs

Evaporate the extract from previous step to dryness with N_2 and water bath heated to 30 ^oC. A small amount of 1:1 Hx:MTBE can be added when sample volume is evaporated to ~0.5 mL. Sample can dry completely under N_2. Dissolve remaining lipids in 4 mL hexane and vortex. Add 2 mL KOH-solution (0.5 M in 50% ethanol), invert 3 min and centrifuge 3 min. Transfer the organic solvent phase (neutral fraction) to a new test tube. Re-extract the alkaline solution with 3 mL hexane. Save the organic phase, which contains the PCBs, for step 11.1 (lipid removal from PCB fraction).

Acidify the alkaline solution with 0.5 mL HCl (2 M). Check the pH to ensure the acidity, and add an additional allotment if not acidic. Extract the OH-PCBs with 4 mL hexane/MTBE (9:1), invert 3 min and centrifuge 3 min. Transfer the organic solvent phase (phenolic fraction) to a new test tube. Re-extract the acidic phase with 3 mL Hx/MTBE (9:1).

10.0 Derivatization of OH-PCBs (Oakdale lab)

Materials: mark 4 mL on test tubes, 13x4 mL covered Hx, 3 combusted pipettes, 2 dropping bulbs, 13 combusted evap. Needles, samples and reference

(OH-PCB fraction is dissolved in 7 mL Hx:MTBE (9:1))
Concentrate the phenolic fraction to about 1 mL by evaporation with N_2 and 3 drops of MeOH and vortex. Mark sample level in test tube with Sharpie. Add 0.5 mL of diazomethane to samples and reference. Keep in fridge at 4-8 °C for at least 3 hours or overnight. Evaporate the excess of ether and diazomethane under a gentle flow of N_2 and concentrate to a few drops. Dissolve in 4 mL hexane. At this point, samples will be stored at -20 $^0C$for future analysis of OH-PCBs.

11.0 Lipid removal

11.1 PCB fraction

(The PCB fraction is dissolved in 7 mL hexane)
Add 2 mL concentrated H_2SO_4. Invert 2 min and centrifuge 5 min. Transfer organic solvent phase to new test tube (or turbo-vap vial). Re-extract the acidic phase with 3 mL hexane. Concentrate the organic phase to 0.5 mL by evaporation with N_2. Transfer the organic solvent to a column of silica/conc. H_2SO_4 (2:1, w,w, 1 g) with 0.1 g activated silica gel in the bottom. Elute to a volume of 10 mL hexane.

11.2 OH-PCB faction

(Samples are dissolved in 4 mL Hx.)
Add 2 mL concentrated H_2SO_4. Invert 2 min and centrifuge 5 min. Transfer the organic solvent phase to a new test tube. Re-extract the acidic phase with 3 mL hexane. Concentrate the organic solvent to 0.5 mL by evaporation with N_2. Transfer to a column of silica/conc. H_2SO_4 (2:1, w,w, 1 g) with 0.1 g activated silica gel in the bottom. Elute with 10 mL DCM . Change sample and reference solvent to Hx before GC analysis by evaporating to 0.5 mL, adding 3 mL Hx, evaporating to 0.5 mL, adding 3 mL Hx, evaporating to 0.5 mL, adding 3mL Hx.

12.0 Spiking with I.S. and transfer to GC vials

Evaporate the Hx from PCBs with a gentle stream of N_2 until a few drops are left. Transfer the sample to a GC vial with insert, rinsing test tube twice with Hx. Concentrate to the 0.5 mark on vial by letting stand open in hood for a few hours. Spike each sample fraction, method blanks, LRMs, and references with the appropriate amount of internal standard so that injected mass is ~5 ng. Alternatively, use a very gentle stream of N_2. Samples are now ready for analysis on the instrument.