Standard operation procedure (SOP) for the extraction and clean-up of all 209 PCBs in human serum

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1.0 Scope and Application

This method covers the extraction and cleanup for analysis of 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 sulfuric acid activated 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 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: bloodborne pathogens

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 acid and 2-propanol.
Gloves and eye protection (goggles) should always be worn when handling serum and in the extraction procedure. 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 Bloodborne Pathogens safety training course. A current copy of the Bloodborne Pathogens Exposure Control Plan is in SC1246.

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
FMS with custom H_2SO_4-acidified silica gel columns

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)
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
Wheaton vial file No. 228778 (holds 60 2 mL M-T vials)

5.0 Chemicals and solvents

5.1 Raw materials

Potassium Chloride EP/BP/USP/FCC, P333-500	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
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

6.0 Standards

6.1 Surrogate Standards (SS)

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)

6.2 Internal Standards (IS)

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)

6.3 Calibration standards

PCB calibration mixture contains all 209 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. Rinse syringe 3x with DCM and then 3x with Hx. Use the appropriate amount of surrogate standard so that injected mass is ~10 ng. Surrogate-spike 1 empty GC vial with insert (this is the reference) with PCB surrogate standard. Spike the method blank (test tube containing 4 mL 1% KCl). Spike 1 SRM sample. Spike surrogate standard to samples and vortex thoroughly, rinsing the syringe between each sample. When finished spiking all samples again rinse syringe 3x with DCM and then 3x with Hx. The samples, blank, and SRM will go through all

the remaining steps together. The reference is kept in the freezer until spiking the samples/SRM/blank with Internal Standard.

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 at 3000 rpm and 20 ^{o}C. 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) vortex each test tube after addition of the 3 mL hexane/MTBE, invert 5 min, centrifuge 5 min, transfer top layer to the test tube with KCl wash and first organic phase. Invert the test tube with the 8 mL organic solvent and the KCl for 3 min, centrifuge 5 min and transfer organic solvent phase to a new, empty test tube. Re-extract the aqueous phase with 3 mL Hx:MTBE 1:1 invert 3 min, centrifuge 5 min, transfer top (solvent) layer to the test tube for a total of 11 mL Hx/MTBE. Use N-Evap to concentrate samples until almost dry (1 drop left in the test tube). Add 2 mL Hx.

9.0 Lipid removal

9.1 Mixing with concentrated H_2SO_4

Add 1 mL concentrated H_2SO_4. Invert 2 min and centrifuge 5 min. Transfer organic solvent phase to small FMS tubes. Re-extract the acidic phase with 1 mL hexane: invert 2 min, centrifuge 5 min, transfer top (solvent) layer to the FMS tubes.

9.2 FMS cleanup columns

9.2.1 FMS instrument prep

Turn the main power on (power switch on top right console, **Figure 1**). The LCD control module (**Figure 2**) will read "Multiple process?". Select NO. The module will then read "Main menu?". Select NO. The module will then read "PH menu?". Select NO. The module will then read "UV menu?". Select NO. The module will then read "Service menu?". Select YES. The module will then read "PC communication?". Select YES. The module will then read "PC interface". Select YES. Turn on one or both sampling modules using switch located to the left of the pressure readers (**Figure 3**).

Figure 1. Main console power

Figure 2. LCD control module

Figure 3. Sampling module

9.2.2 Software prep

Double click on DMS6000 icon on home screen. Select Applications -> PLE-CleanUp. This will open a new window. Select File -> Open -> Log -> Cleanup. This will open a new window to choose the file. Open the Cleanup Programs file, then select k1. Press open.

9.2.3 Pre-cleaning FMS lines

Fill 2 large vials with methanol. Add sample lines, swish, remove sample lines and wrap in new aluminum foil. Add fraction lines, swish, remove fraction lines and wrap in new aluminum foil. Dump methanol into waste and fill with acetone. Repeat line cleaning with the acetone and then hexane. Refill with new hexane and add sample lines. Add fraction lines to 2 empty large vials. Run the washsystem-5 program: Select File -> Open -> Editor -> cleanup -> Wash Programs. Dump all solvent into the waste jug.

9.2.4 Sample cleanup

Use adapters to connect new cleanup columns to the FMS (**Figure 4**). Add a sample line to each sample in small capped FMS tubes and a fraction line to a large capped FMS tube (**Figure 5**), *taking great care to match sample 1 in small FMS tube with sample line 1 and fraction line 1 with sample 1 in large FMS tube, etc.* Make sure the sample line extends all the way down into the tip of the small FMS tube (**Figure 6**).

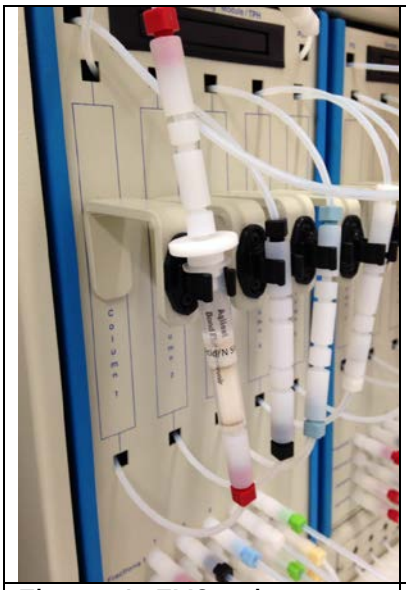

Figure 4. FMS columns with adapters on top.

Figure 5. Insert sample lines into samples in small, capped FMS tubes (left) and fraction lines into large, capped FMS tubes (right).

Figure 6. Sample line extends all the way down in sample tube.

Select File -> Open -> Editor -> Cleanup. Select samplecleanup-x, where x corresponds to the number of sample pairs you will run. Each sampling module can operate by itself or the two modules can operate in parallel. For example, if you are running two samples, you would load one sample on the left hand module in column 1, and the other sample on the right hand module in column 1. Then you would select samplecleanup-1 for the cleanup file. This will run both samples at the same time. If running four samples, you would place two on each side and select samplecleanup-2. If running three samples, you must run all on one sampling module, turn off the other module, and select samplecleanup-3.

9.2.5 Post-cleaning FMS lines

Disconnect columns and adapters. Columns should evaporate in the hood before being discarded in the blue biohaz tub. Adapters should be triple rinsed before next use. Fill 2 large vials with hexane and add sample lines. Add fraction lines to 2 empty large vials. Run the washsystem-5 program: Select File -> Open -> Editor -> cleanup -> Wash Programs. Dump all solvent into the waste jug. Refill the 2 large vials with methanol. Add sample lines, swish, remove sample lines. Add fraction lines, swish, remove fraction lines. Dump methanol into waste and fill with acetone. Repeat line cleaning with the acetone and then hexane. Wrap sample and fraction lines in new aluminum foil.

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

Evaporate the Hx from samples with a gentle stream of N_2 until a few drops are left, ~0.5 mL. Transfer the sample to a GC vial with insert, rinsing test tube twice with 15 drops of Hx. Spike each sample, method blank, SRM, and reference with 10 uL of ~1000 ng/mL internal standard so that injected mass is ~10 ng. Rinse the syringe between each injection with 3x DCM and 3x Hx. Samples are now ready for analysis on the instrument.