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Liquid-liquid extraction of 3,3'-dichlorobiphenyl (PCB11) and its hydroxylated metabolites from animal tissues V.1

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Abstract

This protocol describes a method to extract PCB11 (3,3'-dichlorobiphenyl) and its monohydoxylated metabolites from mouse tissues via a liquid-liquid extraction followed by solid-phase extraction clean-up. The level of PCB11 and OH-PCB11s in the tissue are subsequently measured by gas chromatography-tandem mass spectrometry.

Attachments

XLSX

worksheet and quanti...

38KB

Materials

Chemicals list

- 2-propanol (Fisher, BPA4164)
- acetone (Fisher, BPA18P4)
- diazomethane (CH_2N_2) (Hudlicky, 1980; Sigma Aldrich, 2003)
- dichloromethane (DCM), pesticide grade (Fisher, D12-4)
- diethyl ether (Sigma-Aldrich, 673811)
- distilled water
- ethanol (EtOH) (Fisher, A4094)
- hexane, pesticide grade (Fisher, H300-4)
- methanol (Fisher, A412-4)
- milli-Q water
- phosphoric acid (H_3PO_4, Fisher, A242-500)
- potassium hydroxide (KOH) (Fisher, P250-1)
- silica gel, 150-230 mesh (Fisher, 042727A1)
- sodium chloride (NaCl) (Fisher, BP358-212)
- hydrochloric acid (HCl) (Fisher, S25838)
- potassium chloride (KCl) (Fisher, P217-500)
- sulfuric acid (H_2SO_4), concentrated (Fisher, A300C-212)

Analytical standards

3,5-Dichlorobiphenyl (PCB14), 3,3'-dichlorinated biphenyl (PCB11), 3,3'-dichlorobiphenyl-2-ol (2-OH-PCB11), 3,3'-dichlorobiphenyl-4-ol (4-OH-PCB11), 3,3'-dichlorobiphenyl-5-ol (5-OH-PCB11),3,3'-dichlorobiphenyl-6-ol (6-OH-PCB11) were synthesized and authenticated as described previously (Li, 2018; Dhakal, 2012, Dhakal, 2020, and Zhu, 2013). 2,4,6-Trichlorobiphenyl (PCB30), 3,4,4',5,5',6'-octachlorobiphenyl (PCB204), and 2',3,3',4',5,5'-hexachlorobiphenyl-4-ol (4'-OH-PCB159) were purchased from AccuStandard Inc. (New Haven, CT, USA). Deuterated 2,3,5,6-tetrachlorobiphenyl (d-PCB65) was provided by CND Isotopes Inc., Quebec, Canda.

Analytical standard solutions

- Surrogate standard (SS)
  d-PCB65 (SS_PCB, 100 ng/mL in isooctane)
  PCB14 (SS_PCB, 100 ng/mL in isooctane)
  4'-OH-PCB159 (SS_OH-PCB, 100 ng/mL in MeOH)
- Internal standard (IS)
  PCB30/204 (100 ng/mL each in isooctane)
- Analytes
  PCB 11 (100 ng/mL in isooctane)
  2-OH-PCB11; 4-OH-PCB11; 5-OH-PCB11; 6-OH-PCB11 (100 ng/mL each in MeOH)

Accessories

- TissueRuptor II (Qiagen, 9002755)
- TissueRuptor Disposable Probes, nonsterile (Qiagen, 990890)

- Serological pipette, 10 mL, (Fisher, 13-678-11E)
- Tube racks
- Disposable Glass Pasteur pipette (short pipette), 5 3/4”, 2 mL (Fisher, 13-678-20A, box of 720)
- Disposable Glass Pasteur pipette (long pipette), 9”, 2 mL (Fisher, 13-678-20C, box of 720)
- Disposable Glass tubes, size 16 x 125 mm (Fisher, 14-959-35A)
- Disposable Glass tube caps (Fisher, 02-883-8D, black screw caps with PTFE liners)
- Repeater pipette (BrandTech, UX-24806-05)
- Syringe Tips NanoRep, 50 mL (Rainin, ENC-50ml)
- SPE Cartridges (Sigma-Aldrich, 52797-U)
- GC vials, 2ml (Fisher, 03-391-6, crimp wide opening autosampler vials)
- Crimp aluminum seal cap, 11 mm (Fisher, 200154, PTFE/silver 100 pack)
- Crimper

Instruments

- Eppendorf centrifuge 5810, (Eppendorf, 022625004)
- Vortex mixer, (Fisher, 50-728-002)
- Tube rotator, (Fisher, 88-861-122)
- Furnace (Barnstead Thermolyne, 30400)
- AquaTherm Water Bath Shaker, (Stellar, SL-SWB-17)
- N-EVAP analytical evaporator with needles, (Thomas Scientific, 1156Y21)
- SPE Vacuum Manifold, 12 ports, (Sigma-Aldrich, 57-160-U & 57-162-U)

Reagents and solvents

- 1:1 hexane:dichloromethane (v/v): The mixture of 100 mL of hexane and 100 mL of DCM were measured with graduated cylinder and mixed well.
- 9:1 Hexane: diethyl ether (v/v): 90 mL of hexane and 10 mL of diethyl ether were measured with graduated cylinder, and mixed well in amber bottle with cap.
- 1% KCl (w/w): 5 g KCl was dissolved in 500 mL of Milli-q water.
- 6 M HCl: Add 50 mL of HCl (12M) to a volumetric flask (100 mL), then add Milli-Q water to the marking.
- 0.1 M phosphoric acid in 0.9% NaCl solution: Add 0.9 g of NaCl and 1.15 g of 85% phosphoric acid to a volumetric flask (100 mL) and then add Milli-Q water to the marking.
- Acidified silica gel: The silica gel in a beaker was combusted at 450 °C for overnight. After cooling down, the sigical gel was transfer to a screw-capped glass bottle. The mixture of combusted silica gel and sulfuric acid by weight ratio of 5 : 1 (For example, 40 g of silica gel was mixed with 8 g of sulfuric acid) was shaked vigorously until no lumps were observed. The acidified silica gel was kept in another screw-capped glass bottle and ready for the SPE cartridge.
- 1 M KOH in 95% EtOH: add 2.8 g (0.1 mol) of KOH to a clean beaker (100 mL), then add 2.5 mL of Milli-Q water. Stir the mixture until KOH is dissolved completely, then add 47.5 mL EtOH and get the solution mixed well before using.

Safety warnings

The following section describes precautions for the safe handling of chemicals used in this protocol. Consult the Safety Data Sheet for additional information regarding all chemicals used in this protocol:

Acetone:
Work under hood. Do not inhale the substance/mixture. Avoid the generation of vapors/aerosols. Keep away from open flames, hot surfaces, and sources of ignition. Take precautionary measures against static discharge. Change contaminated clothing. Preventive skin protection is recommended. Wash hands after working with substance.

Diazomethane:
Work under a well-ventilated fume hood with sash down. Do not inhale the substance/mixture. Avoid contact with exposed skin. Keep away from open flames, hot surfaces, and sources of ignition. Avoid contact with irregular glass surfaces. Proper PPE is required upon handling, including a lab coat, fresh gloves, and a face shield. Change gloves immediately after handling and discard them into the hazard container.

Dichloromethane (DCM):
Wear personal protective equipment/face protection. Do not get in the eyes, on skin, or clothing. Avoid ingestion and inhalation. Vapors are heavier than air and may spread along floors. Handle product only in an enclosed system or provide appropriate exhaust ventilation. Reacts with aluminum and its alloys.

Diethyl ether:
Avoid contact with skin, eyes, and clothing. Follow good hygiene procedures when handling chemical materials. Follow proper disposal methods. Do not eat, drink, smoke, or use personal products when handling chemical substances. Ground and bond containers when transferring material. Do not get in the eyes, on the skin, or clothing. Empty containers retain product residue (liquid and/or vapor) and can be dangerous. Keep away from heat, sparks, and flame. Do not ingest or inhale. Prevent thebuild-up of vapors to explosive concentration.

Hexane:
Work under a hood. Do not inhale the substance or mixture. Avoid generating vapors or aerosols.

Hydrochloric Acid:
Do not breathe vapors or aerosols. Avoid substance contact. Ensure adequate ventilation. Evacuate the danger area, observe emergency procedures, and consult an expert.

Methanol:
Use in a chemical fume hood. Wash hands before breaks and immediately after handling the product. Avoid contact with skin, eyes, and clothing. Take precautions against static discharge.

Methyl *tert*-butyl ether (MTBE):

Do not breathe vapors or aerosols. Avoid substance contact. Ensure adequate ventilation. Keep away from heat and sources of ignition. Evacuate the danger area, observe emergency procedures, and consult an expert.

Phosphoric acid (H_3PO_4)

Wash hands after handling. Do not mix with bases. Use in a chemical fume hood. Follow good hygiene. Do not eat, drink, smoke, or use personal products when handling chemical substances. Use only in well ventilated areas. Prevent contact with eyes, skin, and clothing.

Polychlorinated biphenyls (PCBs):

May cause damage to organs through prolonged or repeated exposure. Very toxic to aquatic life with long-lasting effects. Do not breathe dust/fume/gas/mist/vapors/spray. Avoid release to the environment. This statement does not apply where this is the intended use. Get medical advice/attention if you feel unwell. Collect spillage. Dispose of contents/containers following relevant regulations.

Potassium chloride (KCl):

Minimize dust generation and accumulation. Wash hands after handling. Avoid dispersal of dust in the air (i.e., clearing dust surfaces with compressed air). Routine housekeeping should be instituted to ensure that dusts do not accumulate on surfaces. Dry powders can build static electricity charges when subjected to the friction of transfer and mixing operations. Follow good hygiene procedures when handling chemical materials. Do not eat, drink, smoke, or use personal products when handling chemical substances. Avoid generation of dust or fine particulate. Avoid contact with eyes, skin, and clothing.

Potassium hydroxide (KOH):

Do not get in eyes, on skin, or on clothing. Wear personal protective equipment/face protection. Use only under a chemical fume hood. Do not breathe dust. Do not ingest. If swallowed then seek immediate medical assistance.

2-Propanol:

Wear personal protective equipment/face protection. Keep away from open flames, hot surfaces and sources of ignition. Use spark-proof tools and explosion-proof equipment. Use only non-sparking tools. Take precautionary measures against static discharges. Do not get in eyes, on skin, or on clothing. Do not breathe mist/vapors/spray. To avoid ignition of vapors by static electricity discharge, all metal parts of the equipment must be grounded.

Sulfuric acid (H_2SO_4):

Avoid contact with skin, eyes, and clothing. Follow good hygiene procedures when handling chemical materials. Follow proper disposal methods. Do not eat, drink, smoke, or use personal products when handling chemical substances.

Ethics statement

The responsible Institutional Animal Care and Use Committee (IACUC) approved the animal protocol used to generate tissue samples. All animals were utilized in accordance with all Public Health Service (PHS) policies and the Guide for the Care and Use of Laboratory Animals, National Institutes of Health (NIH) Publication No. 85-23, revised 2010.

Before start

Always wear proper Personal Protective Equipment and work in a fume hood when working with hexanes, hydrochloric acid, MTBE, diazomethane, polychlorinated biphenyl (PCBs) and their derivatives, and strong acids and bases.

Note that this protocol involves work with experimental animals and requires prior approval by the users' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee.

Preparation

1 Combust silica gel, glass wool, all glassware (cartridges, short and long pipettes, tubes, beaker/volumetric flask, funnel), scalpel, forceps, and spatula.

2 Print the working sheet (see attachment for an example).

3 Label all sample tubes (**Tube-A**, **Tube-B**, **Tube-C**, **Tube-D**, and **Tube-E**).

Quality control samples

4 Quality control samples include:
- Method blank (MB): x2 or x3 to make sample number even
- Tissue blank (TB): tissue from control animals
- Tissue samples: tissue from exposed animals
- Ongoing Precision and Recovery (OPR): tissue blank spiked with surrogate standard (SS), internal standard (IS), and analytes
- Reference standard: includes SS, IS, and analytes
- Instrument blank or solvent: hexane only

Note: *For the spike test, use only blank tissue. For actual tests, use blank tissue plus exposed samples.*

DAY 1: TISSUE HOMOGENIZATION, EXTRACTION, AND DERIVATIZATION

5 Remove the analytical standards for the ORP and the SS from the freezer and allow to warm to room temperature (~ 30 min).

Tissue homogenization

6 Label tubes as A1, A2 etc, and **Reference standard**.

7 Place tissues (liver 100-200 mg, brain 200 mg, adipose 50-80 mg) in **tube-A**. Record weight on the working sheet.

Note: *Wash scalpel, forceps, and spatula between samples with water, acetone, and hexane subsequently.*

8 Add 3 mL of 2-propanol to all **tube-A** samples with repeater pipettor.

9 Homogenize the sample (~30-60 s).

Note: *Rinse the TissueRuptor blade between samples with water in a beaker, followed by 2-propanol (~ 5 mL in a test tube).*

10 Add 1 mL of diethyl ether with a repeater pipettor in a fume hood to each sample, recap, and vortex gently.

Addition of Analytical Standards

11 Spike all **tube-A** samples (MB, TB, tissue, OPR, and **Reference standard**) with SS_PCB (10 ng d-PCB65 and 10 ng PCB14).
- d-PCB65, 100 µL x 100 ng/mL = 10 ng each
- PCB14, 100 µL x 100 ng/mL = 10 ng each

12 Spike all **tube-A** samples (MB, TB, tissue, OPR, and **Reference standard**) with SS_OH-PCB (10 ng 4'-OH-PCB159).
- 4'-OH-PCB159, 100 µL x 100 ng/mL = 10 ng each

13 Spike the following analytes only to **tube-A** OPR samples and the tube for the **Reference standard**.
- 100 µL of PCB11 standard (100 µL x 100 ng/mL = 10 ng)
- 100 µL of OH-PCB11 standard mixture (100 µL x 100 ng/mL = 10 ng each)

14 Cap **tube-A** and the **Reference standard**.

15 Put the **Reference standard** aside for the derivatization step.

Extraction

16 ***Note:*** *For the entire method, all centrifugation steps are for 5 mins at 3000 rpm. Tubes are inverted on the tube rotator for 5 mins at 40 RPM.*

Note: For the evaporation or "blow down" step with nitrogen, a warm water bath (35 °C) can be used if needed.

17 Invert **tube-A** on the tube rotator and centrifuge.

18 Add 5 mL of phosphoric acid (H_3PO_4) in NaCl with repeater pipettor to **tube-B.**

19 Transfer the supernatant from **tube-A** to **tube-B** with a long pipet.

20 Adding 1 mL of 2-propanol and 3 mL of hexane-diethyl ether (9:1) to **tube-A** with a repeater pipettor, vortex for 10 secs, and centrifuge.

21 Transfer the supernatant from **tube-A** to **tube-B.**

22 Invert **tube-B** for 5 min and centrifuge.

23 Transfer the organic layer (top layer) to a new **tube-C** with new long pipettes

24 Re-extract **tube-B** with 3 mL of hexane-diethyl ether (9:1), vortex, and centrifuge

25 Transfer the top layer to combine with the extract in **tube-C** (~ 7 mL).

26 Evaporate solvent in **tube-C** under a gentle stream of nitrogen to ~100 µL.

Note: Do not evaporate to dryness.

Derivatization of OH-PCBs - Day 1

27 Add 5 drops of methanol to **tube-C** all the samples except **Reference standard** and vortex for 5 s.

28 In the fume hood, using a 10 mL serological pipette, add ~0.5 mL of diazomethane to each sample and 1 mL to the **Reference standard**.

29 Cap the tubes and keep them in a solvent-proof refrigerator (**NOT freezer**) at 4-8 °C for at least 3 h or overnight (approximately 16 h).

DAY 2: BASE CLEANUP, LIPID REMOVAL, AND INTERNAL STANDARD SPIKING

30 Label GC vials.

31 Make fresh KOH-95% EtOH solution and set it aside for the base cleanup step.

32 Remove the IS standards from the freezer and allow to warm to room temperature (~ 30 min).

33 Turn on the water bath with setting of 50 °C.

Derivatization of OH-PCBs - Day 2

34 Evaporate the excess of ether and diazomethane in a fume hood under a gentle N2 flow (no yellow color, ~200 µL).

35 Evaporate the **Reference standard** to near dryness (~ 50 µL) and put it aside for the IS spiking step.

Base clean-up step

36 Add 3 mL of hexane with a repeater pipettor and vortex.

37 Add 2 mL of KOH-95% EtOH solution with a repeater pipettor and vortex.

38 Heat for 1 hour in a water bath at 50 °C, vortexing the samples approximately every 15 mins.

39 Add 7 mL of Mili-Q water with repeater pipettor, invert, and centrifuge.

40 Transfer the top layer to **tube-D** with a short pipette.

41 Add 2 mL of hexane using a repeater pipettor to **tube-C** and vortex.

42 Let samples sit on the lab bench for 15 mins, centrifuge, and transfer the top layer to combine with **tube-D** (volume ~5 mL).

43 Evaporate the solvent under a gentle stream of nitrogen to ~0.5 mL

Lipid removal (tube-E, volume ~10mL)

44 Prepare the SPE cartridges.

Note: *The SPE cartridge can also be prepared using the dry reagent loading method: Glass wool is placed on the bottom of the SPE cartridges, and 0.2 g of silica gel and 2 g of acidified silica gel are added. Then, 3 mL of hexane:DCM (1:1) mixture is passed through the cartridge to rinse the filled cartridge.*

Note: *DCM is toxic, need to work in fume hood with good ventilation condition.*

44.1 Add uncombusted tubes to the bottom of the SPE manifold to collect waste eluent from the SPE cartridge.

44.2 Replace the top and place the clean SPE cartridges into each port.

44.3 Add glass wool to the SPE cartridge and pack it down with the end of a glass pipet.

44.4 Add 0.2 g of silica gel with a combusted small glass funnel to the SPE cartridges.

44.5 Add 2 mL of hexane:DCM (1:1) with a long pipette to rinse to the SPE cartridge.

44.6 In a small beaker, mix hexane:DCM (1:1) with acidified silica gel (5:1), swirl, and add the entire suspension with a short pipette on top of the silica gel in each SPE cartridge.

44.7 Mix the suspension with a short glass pipette tip to eliminate air bubbles.

44.8 Let the silica gel precipitate to the bottom before passing ~ 3 mL of hexane:DCM (1:1) into the bottom waste tube.

Note: *Always keep the solvent above the acidified silica gel.*

45 Replace the waste tubes at the bottom with freshly labeled, combusted **tube-E**. Align **tube-D** order in the tube rack with the order of **tube-E.**

46 With long glass pipettes, slowly load the extracts from **tube-D** into the SPE cartridge and pass it through the cartridge for collection.

Notes:

- *Adjust the vacuum of the manifold accordingly, and let the eluent drip drop by drop.*
- Close *off the vacuum once the gel is dry to avoid evaporating the sample liquid.*
- *Always keep the solvent level above silica gel.*

47 Rinse **tube-D** twice (2 x 0.5 mL) with hexane:DCM (1:1) and pass through the cartridge each time.

48 Repeatedly adding hexane:DCM (1:1) mixture (total volume ~ 9 mL) till the eluent in **tube-E** reaches 10 mL mark.

49 Turn off the manifold vacuum and remove **tube-E** from the SPE manifold.

50 Concentrate the samples in **tube-E** under a gentle stream of nitrogen to ~200 µL.

51 Add 3 mL of hexane to **tube-E**, then continue to concentrate the sample under a gentle stream of nitrogen to ~50 µL.

Note: Do not evaporate to dryness.

Internal Standard (IS) Spiking IS and preparation for analysis

52 Spike 100 µL of IS (PCB30+PCB204, 100 µL x 100 ng/mL = 10 ng each) to each **tube-E** and the **Reference standard** using a single channel pipette.

53 Vortex each **tube-E**.

54 Transfer extracts from **tube-E** and the **Reference standard** with a long pipette to a combusted crimp-style GC vial.

55 Rinse **tube-E** with hexane and combine with to the GC vial to give a final volume of ~0.6 mL.

56 Prepare the solvent blank by filling a GC vial with hexane.

Note: *Use the same hexane used for the extractions.*

57 Cap all vials immediately with a crimp cap and crimper to seal the cap.

58 Store samples in solvent-proof -20°C freezer until instrument analysis.

Note: *Do not store samples in a regular -20°C freezer because solvent vapors will destroy the freezer over time.*

59 Use the Excel worksheet included with this protocol to calculate the levels of PCB11 and its hydroxylated metabolites after gas chromatographic analysis.

DISPOSAL

60 Dispose of all items as follows:

- Scalpel – sharps container
- Homogenizer probe – If actual test, dispose in the PCB container. If spike test, dispose in the hazard waste container.
- SPE Cartridges—Remove the cartridges from the SPE manifold and dispose of all content in the PCB waste container. Place the cartridges in the sink to be washed. Add the original waste tubes to the manifold, open the vacuum tube on the manifold, and turn on the vacuum. Clean the cartridge with acetone and then hexane. Turn off the vacuum and remove it. Let the top dry on its end, and once fully dry, place the manifold back on the shelf.
- Evaporator – Place needles in the beaker to be combusted.
- **Tube-A**, **Tube-B**, **Tube-C**, **Tube-D**, **Tube-E**, and other aqueous solutions – Aqueous hazardous waste
- Organic solvents (waste tubes with hexane and 2-propanol) – Organic hazardous waste
- All PCB-exposed disposable glassware, foil, GC vials, and gloves – Blue PCB hazardous waste container.
- Serological pipette – glass hazard container.

ARCHIVING OF ALL SAMPLE EXTRACTS

61 Recap the samples after the GC-MS/MS or GC-ECD analysis

62 Store all samples, including the solvent blank, in a solvent-proof -20°C freezer until the data are published.

Note: *Do not store samples in a regular -20°C freezer because solvent vapors will destroy the freezer over time.*

Protocol references

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