Analytical Chemistry
Method 70-1
Job No. 16303

ANALYSIS OF BIOLOGICAL MATERIALS FOR POLYCHLORINATED BIPHENYLS

SCOPE

This methodology was developed for the determination of the amount and type of polychlorinated biphenyls (PCB) in biological materials. Absolute confirmation of PCB structures is not obtained with this method. Structure proof can be obtained using additional techniques such as mass spectrometry to further identify the GC fractions.

PRINCIPLE

The PCBs present in the biological materials are extracted into hexane and the bulk of the fat removed by partitioning with N,N-dimethyl formamide. Interfering components are then removed from the extracts by chemical treatment and column chromatography. The amount and type of PCB present is determined by electron capture gas chromatography (EC/GC).

REAGENTS

Hexane	Nanograde, Mallinckrodt Chemical Works, Catalog No. 4159.
N,N-Dimethyl Formamide	Certified, Fisher Scientific Company, Catalog No. D-119. (Saturated in a separatory funnel with hexane.)
Ethyl Ether	Anhydrous, AR grade, Mallinckrodt Chemical Works, Catalog No. 0848.
Sodium Sulfate	Anhydrous, granular: AR grade, Mallinckrodt Chemical Works, Catalog No. 8042. Heat at 400°C for one hour prior to use.
Alumina Adsorption	(for chromatographic analysis) 80/200 mesh, Fisher Scientific Company, Catalog No. A540. Heat at 400°C for a minimum period of 4 hours and deactivate with 5% (w/w) distilled water.
	Alumina column preparation: fill a chromatographic column with hexane up to the point where the reservoir joins the column and push a glass wool plug to the bottom with a glass rod. In a 50 ml beaker, measure 35 ml of deactivated alumina (~30g), and pour this slowly into the column. Tap or vibrate the column to settle the alumina and top the

	alumina with 2-3 cm of anhydrous sodium sulfate. Wash the column with 50-100 ml of hexane prior to the addition of the sample.
Distilled Water	Extracted with hexane to remove hexane-soluble electron capturing impurities.
Sulfuric Acid	Analytical Reagent Grade, SG = 1.84.
Potassium Oxalate	Analytical Reagent Grade.
Potassium Hydroxide	Analytical Reagent Grade.
Ethanol	Formula 2B.
2.5% (w/v) Alcoholic Potassium Hydroxide	Dissolve ~12.5 grams of AR grade KOH in 500 ml of ethanol.
9/1 (v/v) Sulfuric Acid - Water	Carefully add 270 ml of AR grade sulfuric acid to 30 ml of distilled water in a 500 ml iced beaker.
5% (w/v) Aqueous Potassium Oxalate	Dissolve ~5 grams of AR grade potassium oxalate in 100 ml of distilled water.
PCB Standards	Aroclor 1242, 1248, 1254 and 1260.

APPARATUS

1. Separatory funnels equipped with ground glass stoppers and Teflon stopcocks: 125, 250, 500, 1000 and 2000 ml capacities.
2. Kunderna-Danish Evaporative Concentrators, 500 ml capacity equipped with 3-ball Snyder columns and graduated 5 ml capacity vials: Ace Glassware Company, Catalog No. 6707.
3. Chromatographic columns, glass, 10" X 20 mm (OD) with a 5" X 50 mm (OD) reservoir at the top, equipped with Teflon stopcocks.
4. Sintered glass filter funnels, 350 ml capacity, 80M.
5. Flat bottomed boiling flasks, 125 ml capacity: Ace Glassware Company, Catalog No. 6896, Code - 04.
6. Liebig Condenser, 200 mm in length: Ace Glassware Company, Catalog No. 5915, Code - 12.
7. Hot plates, Corning PC-100: Fisher Scientific Company.
8. Water bath, Thelco, Precision Scientific, Model No. 84, Fisher Scientific Company.
9. VirTis "23" Macro Homogenizer, Catalog No. 6-105AF.

10. Standard VirTis Macro Flasks, 250 ml capacity, Catalog No. 16-094.

11. 10 μl Hamilton Syringes, Catalog No. 701N.

12. Grinding Mills, Catalog No. 8-450, and Grinding Unit, Catalog No. 8-455, Fisher Scientific Company.

13. Usual laboratory glassware.

PROCEDURES

Sample Preparation

Whole animal and individual tissue (muscle, liver, heart, lungs, spleen, etc.) samples:

All individual and composite samples weighing more than 50 grams should be weighed and ground by passing through a meat grinder. The samples are then re-ground, with mixing between grindings, until the tissue appears homogenous. A portion of the ground tissue (25-35 grams) is transferred to a tared VirTis flask for analysis.

Samples weighing less than 50 grams are cut into small chunks (1-2 cm^3) and the entire sample is transferred to a tared VirTis flask for analysis.

1. Add ~100g of anhydrous sodium sulfate and 100 ml of hexane to the flask. Blend the mixture for 2-3 minutes or until the sample is well homogenized.

2. Decant the hexane extracts into a 350 ml sintered glass filter funnel collecting the filtrate in a 600 ml beaker. A bell jar and glass plate assembly large enough to fit over a 600 ml beaker is employed to allow vacuum filtration.

3. Re-extract the homogenate with two more 100 ml portions of hexane decanting each in turn into the filter funnel. (If the tissue homogenate cakes on the sides of the flask at any time, the sides should be scraped to insure that adequate exposure to the hexane is obtained.)

4. After the final extraction, clean the homogenizer shaft and blades by washing with hexane. Transfer the homogenate and washings from the flask into the filter funnel with the aid of small portions of hexane and press the homogenate with a spatula to remove as much liquid as possible. Complete the filtration by washing the funnel and cake with several portions of hexane.

5. Place the combined hexane extracts on a steam bath and completely evaporate the solvent with the aid of a gentle stream of filtered air. After cooling to room temperature, re-weigh the beaker.

6. Prepare a hexane solution containing a maximum of 3.5 grams of fat per 25 ml and use 25 ml of this solution for the DMF/Hexane partition.

Fat:

1. On a glass plate, chop the entire frozen fat sample into small pieces with a sharp knife, transfer it to an appropriately sized, tared, beaker and re-weigh.

2. Add anhydrous sodium sulfate and grind with a heavy, flat end, glass rod. Continue grinding, adding sodium sulfate as needed, until a dry granular mixture is obtained.

3. Add an equal volume of hexane and warm, with careful stirring to boiling. Decant the supernate through a sintered glass filtering funnel, collecting the filtrate in a tared beaker.

4. Repeat the extraction step with 2-3 more portions of hexane and after decanting the final extract through the funnel, transfer the extracted residue to the funnel and press with a spatula to remove as much liquid as possible.

5. Complete the filtration by washing the beaker, funnel and cake with several portions of hexane.

6. Place the beaker containing the combined hexane extracts on a steam bath and with the aid of a gentle stream of air completely evaporate the solvent. After cooling to room temperature, re-weigh the beaker.

7. Prepare a hexane solution containing a maximum of 3.5 grams of fat per 25 ml and use 25 ml of this solution for the DMF/Hexane partition.

Milk:

1. Weigh ~50 grams of whole milk in a 150 ml beaker and quantitatively transfer it to a 500 ml separatory funnel with the aid of several portions of water.

2. Add 5 ml of the 5% aqueous potassium oxalate solution and swirl to mix.

3. Add 100 ml of diethyl ether, shake for several minutes, add 50 ml of hexane and shake for several more minutes.

4. Allow the mixture to stand for 10-15 minutes and discard the lower aqueous layer.

5. Filter the remaining solvent layer through a 4" funnel plugged with glass wool covered with anhydrous sodium sulfate into a tared 400 ml beaker.

6. Evaporate the solvent, allow the beaker to cool, and re-weigh.

7. Prepare a hexane solution containing a maximum of 3.5 grams of fat per 25 ml and use 25 ml of this solution for the DMF/Hexane partition.

Eggs:

1. Crack fresh eggs and transfer each to an appropriate sized, tared beaker and re-weigh the beaker. Composite egg samples are prepared for analysis by homogenizing the individual eggs and a sample of the homogenate (25-50g), equivalent to an individual egg, is used.

2. Add anhydrous sodium sulfate (3X), re-weigh the beaker, and carefully grind with a heavy, flat end glass rod until a homogenous granular mass is obtained.

3. Weigh ~150-175 grams of the granular mixture into a VirTis flask, add 100 ml of hexane, and blend for 2-3 minutes.

4. Decant the hexane extracts into a 350 ml sintered glass filtering funnel, collecting the filtrate in a 600 ml beaker.

5. Re-extract the homogenate with two more 100 ml portions of hexane decanting each in turn into the filter funnel. (If the tissue homogenate cakes on the sides of the flask at any time, the sides should be scraped to insure that adequate exposure to the hexane is obtained.)

6. After the final extraction, clean the homogenizer shaft and blades by washing with hexane. Transfer the homogenate and washings from the flask into the filter funnel with the aid of small portions of hexane, and press the homogenate with a spatula to remove as much liquid as possible. Complete the filtration by washing the funnel and cake with several portions of hexane.

7. Place the combined hexane extracts on a steam bath and completely evaporate the solvent with the aid of a gentle stream of filtered air. After cooling to room temperature, re-weigh the beaker.

8. Prepare a hexane solution containing a maximum of 1.5 grams of residue per 25 ml and use 25 ml of this solution for the DMF/Hexane partition.

Sample Clean Up

DMF/Hexane Partition, Chemical Treatment, and Column Chromatography:

1. Place an appropriately sized aliquot of the hexane extracts, not exceeding 25 ml, in a 500 ml separatory funnel. If the aliquot taken is some volume less than 25 ml, add enough additional hexane to yield a final volume of 25 ml.

2. Add 50 ml of DMF (hexane saturated) to the separatory funnel, stopper the funnel, and shake vigorously for at least 1 minute. Allow the layers to separate and transfer the lower DMF layer to a second separatory funnel.

3. Repeat the extraction of the hexane layer with two more 50 ml portions of DMF (hexane saturated) collecting each in turn in the second separatory funnel. Discard the remaining hexane layer.

4. Add 100 ml of distilled water to the combined DMF extracts, swirl to mix, without excessive agitation, allowing the hexane to layer out.

5. Transfer the lower aqueous-DMF layer to another 500 ml separatory funnel.

6. Extract the aqueous DMF sample with a 50 ml portion of hexane. After the layers have separated, add the first hexane extract to the second separatory funnel and transfer the aqueous-DMF layer to the original separatory funnel.

7. Re-extract the aqueous-DMF with two more 50 ml portions of hexane. Discard the aqueous-DMF layer and combine the hexane extracts.

8. Filter the combined extracts through a 4" funnel plugged with glass wool which is covered with sodium sulfate. Collect the filtrate in a Kunderna-Danish evaporative concentrator, add a small boiling chip, put the Snyder column in place, and reduce the hexane volume to less than 5 ml by heating the apparatus in a 80-90°C water bath. (CAUTION: SOLVENT VAPORS MUST BE VENTED TO A HOOD.)

9. After cooling, remove the 5 ml graduated tube and transfer the concentrate of extracts to a 125 ml extraction flask with the aid of several small portions of solvent.

10. Evaporate the extract just to dryness with a gentle stream of dry filtered air and add 25 ml of 2.5% alcoholic potassium hydroxide.

11. Add a boiling chip, put a water condenser in place, and allow the solution to reflux for 45 minutes.

12. After cooling, transfer the solution to a 250 ml separatory funnel with the aid of 25 ml of distilled water.

13. Rinse the extraction flask with 25 ml of hexane and add it to the separatory funnel.

14. Stopper the separatory funnel and shake vigorously for at least 1 minute. Allow the layers to separate and transfer the lower aqueous phase to a second separatory funnel.

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15. Extract the saponification solution with a second 25 ml portion of hexane. After the layers have separated, add the first hexane extract to the second separatory funnel and transfer the aqueous alcohol layer to the original separatory funnel.

16. Repeat the extraction with a third 25 ml portion of hexane. Discard the saponification solution and combine the hexane extracts.

17. Carefully add 25 ml of the sulfuric acid solution (9:1 concentrated sulfuric acid/water) to the hexane extracts.

18. Stopper the separatory funnel and shake virorously for at least one minute. Allow the layers to separate and discard the lower aqueous -acid layer. Repeat this step until the acid layer is colorless.

19. Wash the hexane with 25 ml portion of water.. Discard the water wash.

20. Filter the hexane extract through a 4" funnel plugged with glass wool which is covered with a layer of sodium sulfate into a Kunderna-Danish evaporative concentrator.

21. Add a small boiling chip, put the Snyder column in place and reduce the hexane volume to less than 5 ml by heating the apparatus in a 80-90°C water bath.

22. After cooling, remove the 5 ml graduated tube and transfer the hexane extract to an alumina adsorption column washing it in with several 5 ml portions of hexane.

23. Carefully add 100 ml of hexane to the column reservoir and collect the total eluent in either a 250 ml volumetric flask or a Kunderna-Danish evaporative concentrator.

24. If the column eluent is collected in a volumetric flask, dilute to volume with hexane and proceed with the gas chromatographic analysis.

25. If the column eluent is collected in a Kunderna-Danish evaporative concentrator, reduce solvent volume, cool, dilute to volume and proceed with gas chromatographic analysis.

Electron Capture Gas Chromatographic Procedure:

Instrument: F&M 402 Biomedical Gas Chromatograph
Detector: High Temperature Ni^{63} Electron Capture Cell
6mm X 6' Glass Column, 4% XE-60 on 80/100 Mesh Chromosorb W, HP, AW-DMCS

Column Temperature: 160-190°C
Detector Temperature: 300°C
Injection Port Temperature: 195-215°C
Pulse: 150

Flow Rates

Helium Carrier ~60 ml/min
Argon-Methane Purge ~120 ml/min

Using EC/GC as the determinative step, inject in duplicate 1-10 µl of each solution into the chromatograph. By comparison with standard solutions injected, in duplicate, under the same operating conditions, determine the amount and type of Aroclor using the individual or total peak height method.

The electron capture detector should also be used to guide the isolation procedures. Biological extracts can be checked for the presence of PCBs and/or interferences by injecting µl portions of the extracts at various points in the extraction and concentration schemes. In this manner, it can be determined if the sample needs to be concentrated or diluted and if the clean up procedures should be employed.

DISCUSSION

Extraction

The outlined procedures for the general extraction of PCBs from biological materials with hexane were found in practice to be sufficiently simple, quantitative and rapid for routine use.

Based on the assumption that fat or fatty organs generally yield the lowest recoveries a series of experiments were conducted with hexane solutions of chicken fat fortified with known amounts of Aroclor. The spiked fat samples were cleaned up (DMF/Hexane partition and alumina chromatography) and the amount of PCB determined by EC/GC. All recoveries fell in the range of 98-102%; for this reason recovery experiments were not carried out with each individual tissue. While a more realistic evaluation of the overall recoveries might produce recoveries in the range of 70-90%, it was felt that this would be more than sufficient for our purposes, especially in light of the quantitation problems which exist.

Sample Concentration

Concentration of sample extracts is necessary, prior to clean up by partition, chromatographic or chemical means, to reduce sample size and increase sensitivity. The preferred method of concentrating allows minimum loss through volatilization or chemical decomposition and requires a minimum time. The three methods of solvent volume reduction most commonly used are evaporation by exposure to a stream of air, evaporation employing a Kunderna-Danish evaporative concentrator equipped with a Snyder column, and evaporation under reduced pressure. We have used all three techniques and have not encountered any significant losses from volatilization or chemical alternation. However, the Kunderna-Danish evaporative concentrator and the stream of air were employed because of the ease of use.

Column Adsorption Chromatography and Chemical Clean Up

Silica gel, Florisil and Alumina deactivated with 0, 1.0, 1.5, 2.0 and

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5% water were investigated as adsorbants for the elimination of interferences. Alumina (5% water) was found to be more effective and reproducible than either silica gel or Florisil. The activity of alumina varies with age and lot, therefore, 5% water was added to the alumina, after heating for a minimum of 4 hours at 400°C, to insure a reproducible activity.

Saponification and subsequent extraction of the sample with sulfuric acid is an effective way to remove a number of chlorinated hydrocarbon interferences as well as other matrix interferences. PCBs are not affected.

Electron Capture Gas Chromatography

Columns

Column performance is the key to effective gas chromatographic analysis and as such the choice of column materials is particularly important. Ideally, the support employed should be inert, mechanically strong, and of high surface area. For these reasons, Chromosorb W, HP, AW-DMCS was used in all of our work.

A variety of polar and non-polar liquid phases were investigated. The following columns were found to provide adequate separation, etc., for use in PCB analysis by electron capture: 4% (w/w) DC-200, SF-96, OV-17, SE-30, SE-54, XE-60, Apiezon L, and 6% QF-1. DC-200 and XE-60 or QF-1 have been found to be the most suitable of these liquid phases.

Another important consideration when working with an extremely sensitive detector and consequently low levels of materials is column conditioning. With polar phases such as XE-60 and QF-1, we have found that operating a new column overnight at a temperature 25-50°C higher than to be used during analysis results in a more stable column. A no-flow conditioning technique is employed to condition non-polar columns. The column is purged with carrier gas, heated for 30 minutes at an elevated temperature without carrier flow and then cooled to room temperature. At the end of this cycle, the carrier flow is resumed and the conditioning is completed as in the case of the polar liquid phase. Two precautions: during conditioning, the column should not be connected to the detector and one should not exceed the maximum safe temperature of the liquid phase.

Since all liquid substrates bleed to one degree or another and columns eventually degrade, we characterize all new columns with two column performance indicators - the number of theoretical plates (N) and a tailing factor (T) p,p'-DDT is employed to check these parameters because it is known to degrade on "poor" columns. In this manner, we can determine if the performance of a new column is satisfactory and when the column performance begins to fall off. We consider a column good if the number of theoretical plates per foot is on the order of 400-500 with tailing factors of 1.0-1.3. Calculation of these parameters is shown in the Appendix. Additionally, there should be no significant

extraneous peaks upon injection of a pure p,p-DDT standard.

Other chromatographic conditions that can be adjusted are column temperature and flow rates. Although resolution of a mixture increases with decreasing temperature, a temperature should be chosen that allows the elution of all components within a convenient time period. The flow rates are optimum for our instrument, column and detector system and, of course, should be adjusted if better results can be achieved.

Two gas chromatographic systems have been used for PCB analysis - F&M Model 402 and 5750. We find that any system of instrument and column suitable for chlorinated pesticides is satisfactory for PCB analysis. The bulk of analyses in our laboratories was carried out using the system outlined. The use of the high temperature Ni^{63} electron capture cell is highly recommended. The ability to operate at higher temperatures prevents maintenance problems due to contamination from high boiling components. Glass columns should also be employed.

Detection and Measurement

Quantitative determinations employing the electron capture detector are non-stoichiometric measurements made by comparing peak heights or areas for known concentrations with those for unknown compositions. Except for sharp peaks, peak area measurements are usually more reproducible than peak height measurements are are extremely time consuming unless a recording integrator is employed. However, peak height measurements are as accurate as disc integration of triangulation and if the peak shape represents a gaussian curve, the height may be considered independent of the base. Consequently, peak height measurements were generally used. Three variations of the peak height quantification procedure were employed.

Case I — EC gas chromatogram of PCB unknown unchanged with respect to standard PCB with no evidence of interferences.

Case II — EC gas chromatogram of PCB unknown altered with respect to standard PCB with no evidence of interferences.

Case III — EC gas chromatogram of PCB unknown unchanged with respect to standard PCB with evidence of interference.

The amount of PCBs in Case I samples were determined by preparing a plot of the major peak height vs. concentration; for Case II, a plot of the total sum of all major peaks vs. concentration. With Case III samples, a peak free from interference was used. When dominant interferences were present, one or more of the chemical clean up procedures was employed.

In all cases, the response of the electron capture detector must be linear for quantitative analysis. With our instrument any response less than 50 at an attenuation of 8 X 10 fell into the linear response range at a pulse rate of 150. This corresponds to approximately 5 X 10^{-9}g of Aroclor 1242.

Contamination

In determining PCB's in biological materials by electron capture gas chromatography, laboratory sources of contamination can be a major problem. The samples and extracts should never be allowed to come in contact with materials other than glass, Teflon or metal. Laboratory glassware should be thoroughly washed with hot, soapy water, rinsed with distilled water, acetone, and then hexane. All equipment should also be rinsed again with hexane just prior to use and blanks should be frequently carried through all steps of the procedures to insure against the possibility of contamination.

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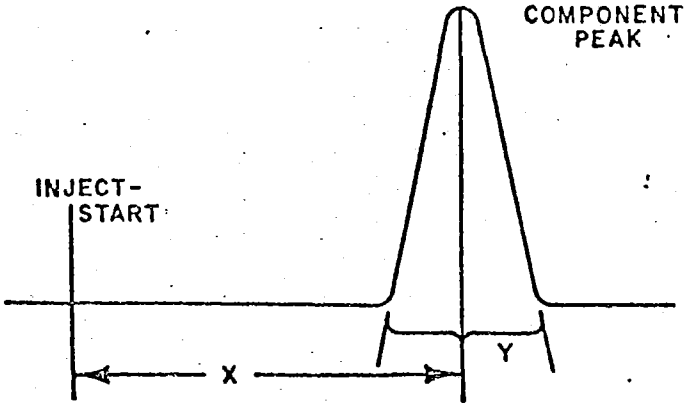
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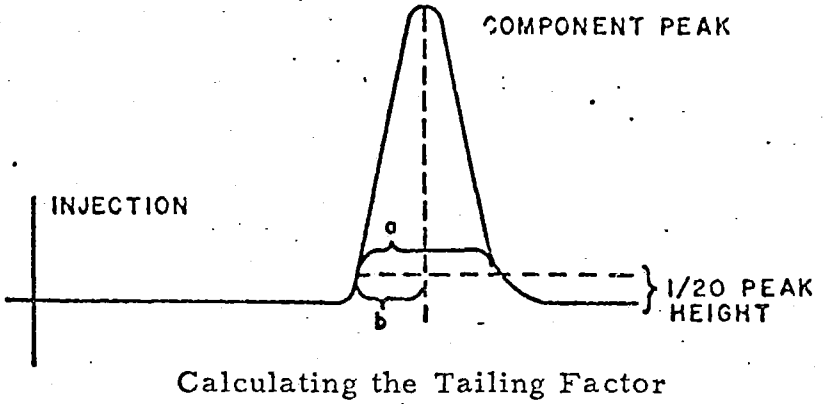
COLUMN PERFORMANCE INDICATORS



Calculating Column Efficiency

1. Theoretical Plates, N

$$N = 16(x/y)^2$$



Calculating the Tailing Factor

2. Tailing, T

$$T = a/2b$$

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