Contents

A. INTRODUCTION

1. Theory

Rendered or extracted animal fat is dissolved in cyclopentane and an aliquot is cleaned up using gel permeation chromatography (GPC), which separates target analytes from lipids. The GPC extracts are analyzed by capillary gas chromatography using an electron capture detector. Peaks detected in sample chromatograms having relative retention times (RRT) matching those determined for target analytes, and relative responses greater than those measured in a concurrently analyzed control fortified at half of each analyte's target level (TL) are identified as positive.

2. Applicability

This method will detect the analytes listed below in rendered or extracted fat at target levels specified in Table 1 (Section K.1).

Aldrin	p,p'-DDE	Linuron
Alpha BHC	o,p' - DDT	Methoxychlor
Beta BHC	p,p'-DDT	Mirex
Delta BHC	Dichlofenthion	trans-Nonachlor
Captan	Dieldrin	Oxychlordane
Carbophenothion(Trithion®)	Endosulfan I	Phosalone
cis-chlordane	Endosulfan II	Ronnel
trans-chlordane	Endosulfan sulfate	Stirophos (Gardona®)
Chlordene	Endrin	o,p' - TDE
Chlorpyrifos	Endrin Ketone	p,p'-TDE
Chlorpyrifos methyl	Heptachlor	Toxaphene
Chlorfenvinphos(Supona®)	Heptachlor epoxides	2,2',4,4',5,5'-HBB[1]
Coumaphos-O	Hexachlorobenzene	Halowaxes
Coumaphos-S	Lindane	Polybrominated biphenyls
o,p' - DDE		Polychlorinated biphenyls[2]

[1] Hexabromobiphenyl
[2] Aroclors 1254 and 1260

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

   a. Oven - Capable of maintaining a temperature of 100 ± 5 °C, Model No. MO1450SA, Lindberg/Blue.

   b. Class A volumetric flasks, volumes as needed to prepare standards.

   c. Acrodisc Filters - 0.45 µm pore size, Cat. No. CR PTFE, Gelman.

   d. Syringe - 10 mL, Luer lock.

   e. Disposable glass culture tubes - 16 x 125 mm, Cat. No. RP265BK, E & K Scientific.

   f. GPC sample input tubes – 16 x 100 mm, Cat. No. 73500 16100, Kimble.

   g. Adjustable volume pipettors, 10 -100 µL and 100 -1000 µL, Cat. Nos. 022472003, 022472101, Eppendorf.

   h. Balance - Readable to 0.01g, Cat. No. PM2000, Mettler.

   i. Bottle-top dispensers - 2 -10 mL, Cat. No. 22-22-020-9, Brinkmann.

2. Instrumentation

   a. GPC system - Cat. No. AS2000, equipped with 2.5 mL sample loop, O.I. Analytical Sample Preparation Products Division.

   b. Optima GPC column - Cat. No. 624-123, O.I. Analytical Sample Preparation Product Division.

   c. Gas chromatograph (GC) - Hewlett Packard 6890 equipped with a split/splitless injection port and an electron capture detector (^{63}Ni).

   d. GC column - Fused silica, 30 m x 0.45 mm ID, 0.7 µm film, Cat. No. DB 608, J&W Scientific, Inc.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted.

1. Reagents

   a. Ethyl acetate - pesticide quality, Cat. No. 100-4, Burdick & Jackson.

   b. Cyclopentane - pesticide quality, Cat. No. 057-4, Burdick & Jackson.

   c. Iso-Octane - pesticide quality, Cat. No. 362-4, Burdick & Jackson.

2. Solutions

   a. GPC Mobile Phase: Ethyl acetate/cyclopentane, 70:30 v/v:

      Mix together 70 volumes ethyl acetate and 30 volumes cyclopentane, measured independently.

   b. GPC Rinse solvent:

      Mobile phase, redistilled mobile phase, ethyl acetate or cyclopentane may be used for this purpose

   c. GPC diluent: iso-octane with dibutyl chlorendate:

      See D.2.b.v.

D. STANDARDS

Note: Individual standards may be necessary to establish the retention times of most analytes. Relative retention time data from this method cannot be used for analyte identification in complex mixtures where separation is marginal. Relative retention times (vs. Aldrin) must be unequivocally established for all analytes for which this method is applicable.

1. Source

   Standards may be obtained from:

Ultra Scientific	Chem Service Inc.	Division of Pesticide and Industrial Chemicals
250 Smith Street	P.O. Box 599	Center for Food Safety and Applied Nutrition
North Kingstown, RI	West Chester, PA	5100 Paint Branch Parkway
		College Park, MD 20740-3835

2. Preparation

   a. Stock standards

      Stock standards for compounds listed in Table 1 (see Section K.1) may be obtained neat or in solutions as individual or mixed standards. Purchased solutions should be in solvents that are miscible with iso-octane. Recommended concentration range for most stock solutions is 0.1 to 1.0 mg/mL.

b. Working Standards

Note: Verify that all compounds in any given mixed standard can be chromatographically resolved before preparing the standard. If two analytes cannot be resolved, remove the one showing the greater response at its specified concentration. Different combinations and/or concentrations of standards may be prepared to meet specific needs dependent on chromatographic resolution attainable.

i. Primary Mixed Standard:

Dilute stock standard solutions in iso-octane to prepare a mixed standard containing analytes at concentrations (or some consistent multiple of those concentrations) listed in Table 1, column 4.

Note: Individual analytes at concentrations between 0.8X and 1.25X the recommended relative levels may be included in the primary mixed standard if necessary, as long as appropriate corrections are made when screening (see F.4.d.iii).

ii. Secondary mixed standards [Containing analyte(s) not present in primary mixed standard in iso-octane]:

Prepare standards containing remaining analytes. Mixed standards should contain analytes at concentrations proportional to their relative target levels (Table 1, column 3).

iii. Control Fortification Standard:

Dilute appropriate Primary/secondary mixed standard(s) with iso-octane to yield an analyte concentration, in µg/mL, equivalent to 2.5 times the TL specified in Table 1, column 3. A mixed standard prepared at concentrations specified in Table 1, column 4 requires adding 25 mL standard to a 100 mL volumetric flask and diluting to volume with iso-octane.

iv. Aldrin Fortification Standard (1.0 µg/mL):

Prepare by making appropriate dilutions of an aldrin stock standard using iso-octane. (Other concentrations may be used, if needed).

v. GPC Diluent (ISTD): 0.0125 µg/mL DBC in iso-octane:

Prepare by making appropriate dilutions of a DBC stock standard using iso-octane.

vi. GC Standard:

Fill a 5 mL volumetric flask with GPC diluent and concentrate under nitrogen until approximately 1 mL has evaporated. Pipet 125 µL Control Fortification Standard and 62.5 µL Aldrin Fortification Standard, dilute to volume with iso-octane, and mix.

3. Storage and Stability

   a. Store stock standards in tightly sealed glass containers at < -10 °C. Injection and fortification standards may be stored at room temperature.

   b. Stock standard solutions are stable for three years when tightly sealed and stored in a freezer.

   c. Fortification standards are stable for one year when tightly sealed and stored in a freezer and three months when stored at room temperature.

   d. Prepare a new GC Standard at least every three months or whenever new fortification standards or GPC diluents are prepared.

      Note: Expired stock and fortification standards can be used if it can be demonstrated that they have not deteriorated by comparison to new solutions of approximately equal concentration prepared from certified reference standards that have not expired.

E. SAMPLE PREPARATION

Place animal fat sample into a glass bottle or beaker and render in an oven at 100 ± 5 ºC until an adequate amount of fat has been rendered. Fat from low fat samples may be alternatively extracted using an appropriate pesticide grade solvent, followed by removal of all solvent by evaporation/distillation.

F. ANALYTICAL PROCEDURE

1. Test Sample Preparation

   a. Weigh 0.5 ± 0.03 g liquid fat samples into disposable graduated glass culture tubes.

   b. Prepare negative and positive controls to accompany samples.

      i. Weigh out 3 portions of blank fat, i.e. fat shown to have no interfering peaks greater than one-third the peak height of any affected (co-eluting) analytes as measured in the GC Standard.

      ii. Prepare duplicate screening controls by fortifying two of the fat blanks with 100 µL Control Fortification Standard. Separate the two fortified controls in the GPC analysis sequence by at least half the total number of samples to be analyzed.

   c. Fortify all tubes with 50 µL of Aldrin Fortification Standard, then dilute to 5 mL with cyclopentane.

   d. Filter tube contents through a 0.45 µm filter into GPC input tubes. Wash filter and syringe with cyclopentane or ethyl acetate between samples.

2. GPC Cleanup

   a. Load samples onto the GPC System.

   b. Proceed with sample cleanup according to the parameters determined as described in section K.2 and the instructions in the GPC operator's manual.

   Examples of typical operating parameters are listed below.

Mobile phase flow rate:	4.5 - 5.0 mL/min
Dump time:	10 min
Evaporation time:	12 min
Evaporation temperature:	56 - 65 °C
High pressure sensor set to:	1
Ultra Sonic Sensor preload time:	0.03 sec
USS debounce time:	0.05 sec
USS maximum load time:	15 sec
Keep time:	0 sec
Diluent addition:	1 addition, 2 sec at 1 mL/sec
Mixing time:	10 sec
Chamber transfer time:	5 sec
Chamber rinse time:	20 sec
Chamber wash temperature:	55 °C
Vacuum pressure:	250 Torr
Final reconstituted volume:	2 mL in iso-octane

3. Gas Chromatographic Analysis

   a. Inject standards, followed by GPC extracts, into the GC. Re-inject the standard or positive control at the end of the run to verify instrument stability.

   b. GC/ECD Operating Conditions[1]

   Note: Initial settings to be used are listed below. These may be optimized, if necessary, to achieve best sensitivity and analyte resolution.

Injector temperature:	250 °C
Detector temperature:	325 °C
Carrier gas:	Ultra High Purity Helium
Carrier gas flow rate:	18 mL/min, constant flow
Make up gas:	Nitrogen
Make up gas flow rate:	30 mL/min
Injection mode:	Splitless with 1 minute purge time

Oven temperature program:	
Initial temperature:	100 °C
Initial time:	1 minute
Ramp #1:	30 °C/min to 170 °C and hold 10 minutes
Ramp #2:	3.5 °C /min to 280 °C and hold for 10 minutes
Total run time[2]:	55 minutes

---

[1] The GC run time should be at least 5 minutes greater than the retention time of the last eluter to prevent carry-over of the sample contaminants into the subsequent run.

[2] Maintain GC oven temperature at 100 °C to 200 °C when not in use.

c. Initial Instrument setup and suitability testing.

Perform the following whenever a new instrument, detector, or analytical column is to be used, or whenever changes in system performance (elution order or relative response) are suspected.

i. Verify that the GC parameters chosen result in adequate separation of all components in the mixed standard to be used.

ii. Verify that all analytes in the scope of this method (Table 1) can be detected at one-half the level found in the GC Standard by injecting 1:1 iso-octane dilutions of that standard.

iii. Determine RRT windows (vs. Aldrin) for all analytes in the scope of this method.

d. Sample Chromatograms

See Section K.4

4. Screening Procedure

a. Verify that response of the two screening controls is acceptable. Samples must be reanalyzed if either control fails to meet the following requirements:

i. Measurable peaks corresponding to all analytes in the control are present.

ii. Aldrin recovery exceeds 60%.

b. Determine retention times for all analyte peaks in the standard.

c. Identify any residue peaks in samples based on retention time matching

   i. Compare RRT of the sample peak with the RRT window calculated for peaks found in the GC standard.

   ii. Compare RRT of any sample peaks that remain unidentified after step I (i.e. analyte not in screen control) with RRT values previously determined for all remaining analytes for which this method is applicable. For multi-peak analytes, RRT matching is adequate for positive identification. For single peak analytes, re-inject the sample along with a standard containing the tentative match to effect positive identification.

   iii. Interferences: The following pairs of compounds were observed to co-elute using chromatographic conditions specified in this method:

oxychlordane/chlorpyrifos	captan/stirofos
trans-nonachlor/trans-chlordane	endosulfan II/o,p'-DDT
heptachlor/dichlofenthion	mirex/phenylbutazone
ronnel/chlorpyrifos methyl	endosulfan I/alpha-chlordane

Positive identification of any analyte eluting at retention times characteristic of these co-eluters will require re-analysis using chromatographic parameters known to resolve the components or use of a specific detector, such as a mass spectrometer.

d. Determine if analyte concentration exceeds screen threshold.

   i. Determine an ISTD corrected response for all analytes detected in all extracts by dividing analyte peak height by ISTD peak height.

   ii. Correct responses recorded for positive controls for any interferences found in the negative control by subtracting the negative control responses.

   iii. If the positive control was fortified with a mixed standard that contains analytes present at relative concentrations higher or lower than specified in Table 1, normalize the responses observed for those analytes by multiplying by the ratio (specified concentration/actual concentration).

   iv. Determine a reference response based on the two positive controls. If the analyte response in the two controls differs by less than 40% ($0.71<R_1/R_2<1.4$) use either the lower response or an average of the two responses as the basis for screening decisions. Otherwise, use the lower response.

e. Apply screen criteria to determine status.

i. If the response of any sample peak matching the retention time of one of the peaks in the control exceeds the reference response as determined in step F.4.d.iv, the sample is screen positive for that analyte. This applies even if the identity of the compound is not known with certainty (see 4.c.iii).

If an analyte not present in the control is tentatively identified on the basis of RRT data, it must be reanalyzed along with an appropriate positive control.

ii. A sample is screen negative for all analytes for which there is no screen positive response.

G. CALCULATIONS

1. Aldrin Recovery.

Under conditions specified in this method, the GC standard reflects the same analyte concentrations found in the fortified control. If injection volumes are constant, recovery can be determined as follows:

a. Calculate the ratio of analyte /DBC peak heights in both the GC standard (R_{Std}) and control extract ($R_{Control}$).

b. Calculate a percent recovery (%R) for the analyte using:

$$\% R = \frac{(R_{Control})}{(R_{Std})} \times 100\%$$

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Safety glasses, laboratory coat, and nitrile gloves.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Reagents:		
Ethyl acetate/ cyclopentane mixture	Highly flammable. Irritating to skin and mucous tissues.	Prepare mixture in fume hood. Vent GPC vapors to a fume hood.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Ethyl acetate/ cyclopentane mixture	See Above	Store with the flammable waste solvents until disposal by the waste disposal contractor or in house specialist. Observe all Federal, state and local environmental laws

I. QUALITY ASSURANCE PLAN

1. Performance Standards for Screening Analysis (FSIS requirements)
   a. No false positives detected when known blank samples are analyzed.
   b. No false negatives result when samples containing analytes at levels ≥ TL are analyzed.

2. Critical Control Points and Specifications

	Record	Acceptable Control
a	Chromatography	DB-608 or equivalent column that separates most target compounds. The run time should be at least 5 minutes greater than the retention time for the last eluting compound
b	Acceptable aldrin recovery	> 60%
c	Blank fat used to prepare positive and negative controls	Within the retention time windows of the target compounds, no peaks showing responses greater than one third that seen in the GC standard should be observed in negative control..
d	Positive Screening controls	All compounds in the screening recovery are detected.
e	Rendering oven temperature	100 ± 5 °C
f	Sample weight	0.5 ± 0.03 g fat

3. Readiness To Perform (FSIS Training Plan)

   a. Familiarization

      Note: Analyst only need familiarize using analytes contained in Primary mixed standard (D.2.b.i).

      i. Phase I: Standards.

         A reagent blank and 3 GC standards containing all compounds specified in D.2.b.i., prepared at concentrations ½, 1, and 2X those specfied in D.2.b.vi. Inject over 3 different days.

      ii. Phase II: Fortified samples.

         3 recovery sets, each run on a separate day, consisting of 5 blank fats, analyzed unfortified and fortified at one-half the TL (10 analyses per set).

         Note: Phase I and Phase II may be performed concurrently.

      iii. Phase III: Check samples for analyst accreditation.

         (a) A total of 6 blind check samples, consisting of one or two unfortified blanks, with the remainder fortified at one-half TL.

         (b) Report analytical findings to Quality Manager (QM).

         (c) Notification from QM is required to commence official analysis.

   b. Acceptability criteria.

      Refer to section I.1 above.

4. Intralaboratory Check Samples

   Refer to section I.1 above.

   a. System, minimum contents.

      i. Frequency: One per week per analyst when analyses are run. See 3.a.iii for general requirements for check samples.

      ii. Records are to be maintained for review.

   b. Acceptability criteria.

      If unacceptable values are obtained, then:

      i. Stop all official analyses by that analyst.

      ii. Take corrective action.

5. Sample Acceptability and Stability
   a. Matrix: fat, adipose tissue.
   b. Sample receipt size: sufficient sample to obtain 0.5 g of rendered or extracted fat.
   c. Condition upon receipt: not spoiled or rancid.
   d. Sample storage:
      i. Time: Organophosphates, approximately 1 month.
         CHCs, PCBs, and PBBs, indefinite.
      ii. Condition: Refrigerated or frozen.
6. Sample Set

   The GPC instrument specified allows processing of up to 23 samples
   a. Each sample set must include:
      i. One negative control (blank fat).
      ii. Two Positive controls.
      iii. Up to 20 samples.
7. Sensitivity

   This screen tests for likely presence of analytes at or above target levels specified in table 1.

J. WORKSHEET

See Section K.3 for example worksheet.

K. APPENDIX

1. Table 1. Analyte Specifications

Analyte	Typical RRT[1]	TL[2] (ppm)	Std. Conc., µg/mL[3]
HCB	0.48	0.1	1.0
Alpha-BHC	0.54	0.1	1.0
Chlordene	0.62	0.1	1.0
Lindane	0.69	0.1	1.0
Beta-BHC	0.73	0.1	1.0
Heptachlor	0.83	0.03	0.3
Dichlofenthion	0.83	0.1	NA
Delta-BHC	0.92	0.1	1.0
Aldrin	1.00	0.1	NA
Ronnel	1.04	0.03	0.3
Chlorpyrifos methyl	1.05	0.1	NA
Linuron	1.18	0.5	5.0
Oxychlordane	1.24	0.04	0.4
Chlorpyrifos	1.24	0.1	NA
Heptachlor Epoxide B	1.31	0.1	1.0
Heptachlor Epoxide A	1.36	0.1	1.0
Trans-Nonachlor	1.41	0.15	1.5
Trans-Chlordane	1.41	0.04	NA
Alpha-Chlordane	1.47	0.02	0.2
Endosulfan I	1.47	0.02	NA
Chlorfenvinphos	1.51	0.05	0.5
2,4'-DDE	1.51	0.1	NA
Dieldrin	1.55	0.1	1.0
p,p'-DDE	1.63	0.1	1.0
Stirofos	1.68	0.04	0.4
Captan	1.68	0.04	NA
2,4'-DDD (o,p'-TDE)	1.75	0.15	1.5
Endrin	1.77	0.1	1.0
Endosulfan II	1.86	0.04	0.4
o,p'-DDT	1.86	0.15	NA
p,p'-TDE	1.89	0.1	1.0
p,p'-DDT	2.01	0.1	1.0
Carbophenothion	2.03	0.06	0.6
Endosulfan Sulfate	2.07	0.1	1.0
DBC (method ISTD)	2.25	NA	NA
Mirex	2.32	0.1	1.0

Endrin Ketone	2.36	0.1	1.0
Methoxychlor	2.40	0.5	5.0
Phosalone	2.47	0.02	0.2
Coumaphos-O	2.71	0.4	4.0
Coumaphos-S	2.79	0.2	2.0
Hexabromo Biphenyl	3.16	0.1	1.0
Multi-Peak Analytes (require separate standards)			
Toxaphene	NA		10
Polybrominated Biphenyls	NA	0.1	1.0
Polychlorinated Biphenyls (Aroclors 1254, 1260)	NA	0.5	5.0

[1.] Vs. Aldrin

[2] Target level for analyte in a sample. This is the lowest analyte concentration that must be detected using this method.
[3] Suggested Concentrations for Primary Mixed Standard (see D.2.b.i.)

2. GPC column calibration procedures

UV column calibration procedures

These instructions are for use with the UVD-1 detector sold by O.I. Analytical. References: O.I. Analytical GPC Maintenance Manual, EPA Method 3640A.

a. Prepare Calibration solution consisting of:

   i. 25 mg/mL corn or vegetable oil.
   ii. 1.0 mg/mL Bis(2-ethylhexyl)phthalate.
   iii. 0.08 mg/mL sulfur.

b. The calibration solution used must include corn or vegetable oil and sulfur. Other components (methoxychlor, phthalate, perylene) are optional for determination of dump and collect times. The calibration solution should be prepared in cyclopentane.

c. Pump solvent through the GPC column for at least 20 minutes with the UV detector and chart recorder attached and turned on before calibration is attempted. UV wavelength should be 254 nm, rise time 1.0, recorder input = 10 mV. These parameters can be changed at the operator's discretion. If available, a start pulse cable can be connected from the rear of the GPC module to the recorder.

d. Filter 5 mL of calibration solution into a sample input tube and load on the GPC. Set dump time to 30 minutes or longer and start the run. If start pulse cable is not attached, press the EVENT switch on the detector to signify the start of elution time. After sulfur peak has eluted, the GPC operation may be stopped.

e. The elapsed time between the beginning of the run and the end of the elution of the corn or vegetable oil peak will be the dump time.

f. The elapsed time between the end of the elution of the corn or vegetable oil peak and the beginning of the sulfur peak will be the collect time.

g. After dump and collect times are determined, program the GPC using these times and process a positive control using this new program. Minor adjustments in dump and collect times (up to 2 minutes) may be necessary to achieve optimum recoveries.

Calibration by fractionation

Note: This procedure may be modified as needed (example: to calibrate GPC parameters for specific compound).

a. Run GPC pump for at least 20 minutes to stabilize the system.

b. Add 2 mL of fortification standard to a sample input tube and dilute to approximately 5 mL with cyclopentane.

c. Load sample and discard first six minutes of elution from column.

d. Collect the next ten two-minute fractions from the column. If GPC ONLY mode is being used, place collection vials in appropriate positions. If GPC WITH EVAPORATION mode is being used, collect the fractions from the column output tube (disconnect from GPC).

e. Analyze each fraction by GC to determine dump and collect times.

3. Example Worksheet.

See following page.

GPC WORKSHEET

Matrix: FAT
Method Used: HEC1.____

Sample size = 0.5 g weighed, 0.25 g processed
Final volume of GPC extract = 2.0 mL
Aldrin and DBC at 200 ppb

Date Processed by GPC: ____________________

Seq.	SAMPLE ID	Aldrin Recovery	Status: +/-	Results and Comments
1	Neg. Control			
2	Pos. Control			
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				

Samples Weighed by ____________
Balance: ____________
Refrigerator: ____________
Eppendorf(s): ____________
Repipettor: ____________
Oven: ____________

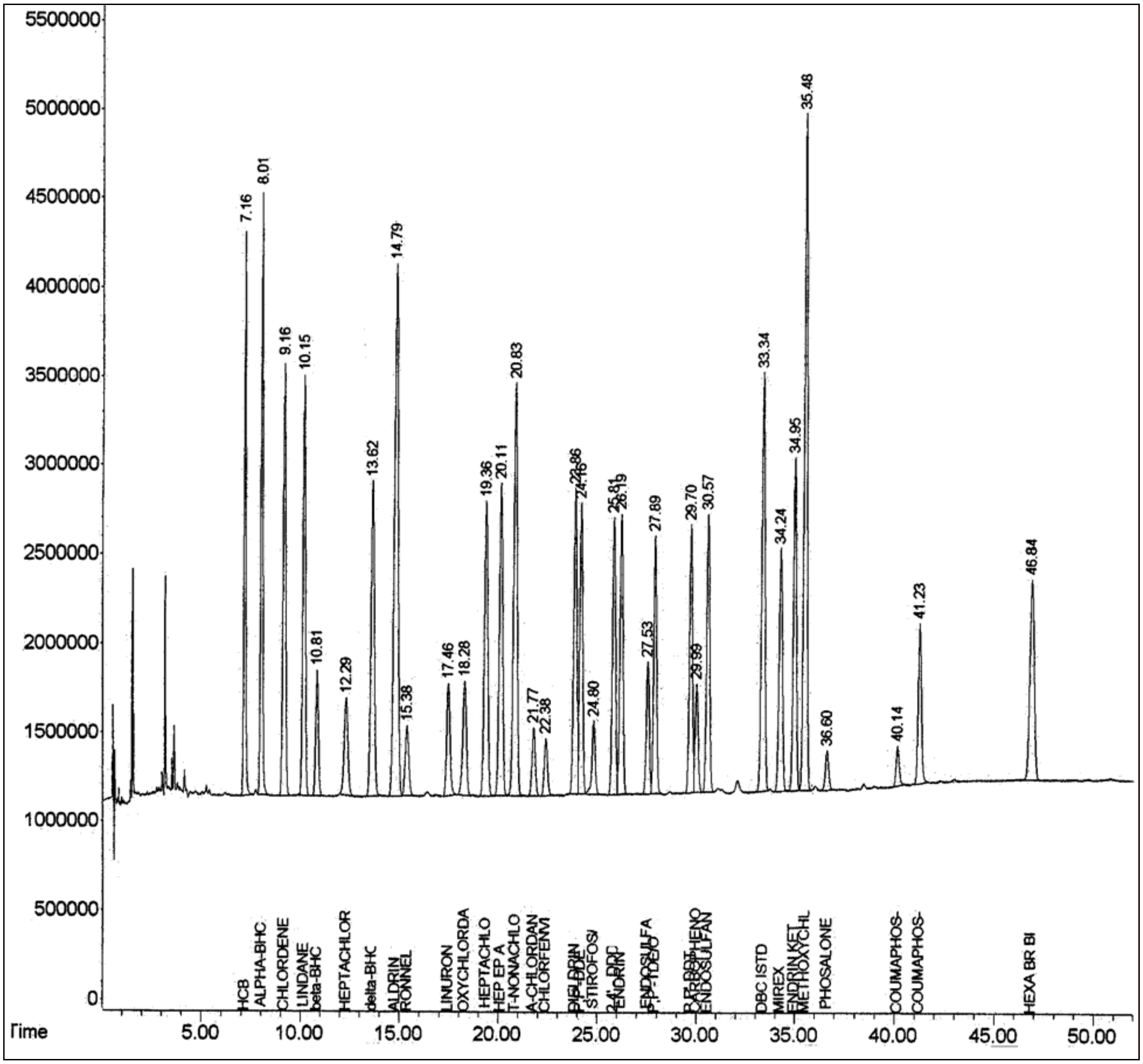
GPC: ___________ GC:___________
Aldrin Fortification Standard: ___________
Control Fortification Standard: ___________
GC Standard: ___________
Diluent: ___________
Cyclopentane: ___________

Comments: ____

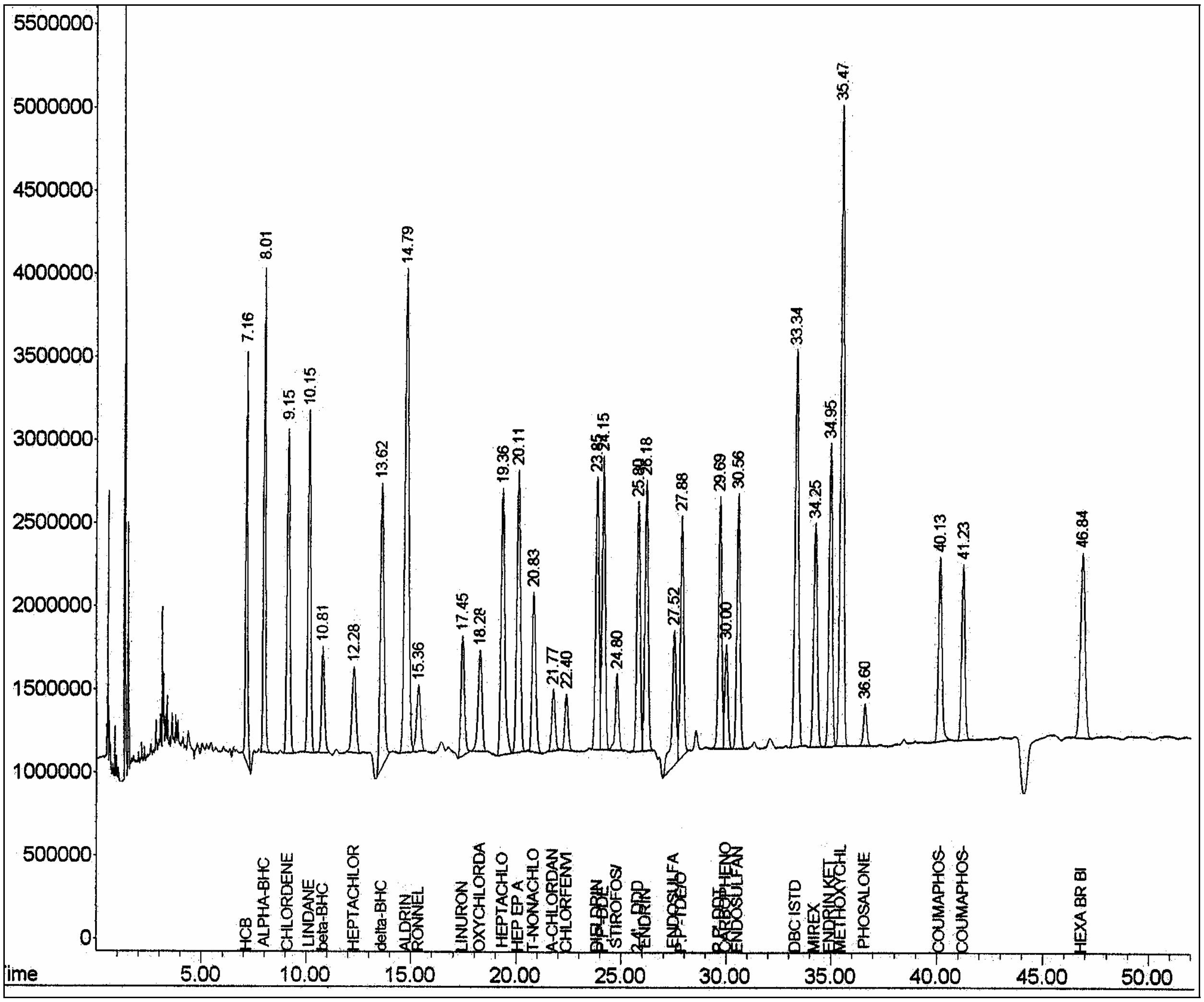
Analyst Signature and Code: ____________________ Date: ____________
Peer Reviewer Initials: ____ Date: ____________
Supervisor/Acting Supervisor Initials: ____ Date: ____________

4. Chromatograms

A. GC Standard (0.5 X TL)



B. Positive Control Made From Poultry Fat (0.5 X TL)



Approvals

Approved by:

Stephen Powell

David Martin

Jess Rajan

Charles Pixley

Phyllis Sparling

Approvals on file.