EXPERIMENTAL AND METHODS

Samples

Herring Oil	Supplied by van den Berghs and Jurgens Ltd., Purfleet, Essex.
Heron Eggs	Collected from Heronry at Tumby, Woodstide, Lincolnshire, March 1967, by Toxic Chemicals Section, Nature Conservancy, Monks Wood.
Arochlor 1254	Supplied by Monsanto Ltd.

Reagents

Dimethylformamide (DMF)	Redistilled under vacuum at 50°C.
Hexane	Petroleum fraction SBP 60-70. Redistilled and fraction boiling between collected.
Florisil	Synthetic silicic acid - magnesia adsorbent manufactured by Floridin Co. Activated. Heat to 120°C overnight. Deactivated. Activate as above, cool and add 3% w/v distilled water. (See Appendix)
Acetic acid, glacial	Analytical Reagent Grade.
Nitric acid	Analytical Reagent Grade, SG = 1.42
Sulphuric acid	Analytical Reagent Grade, SG = 1.84
Acetone	Redistilled.
Sodium sulphate	General Purpose Reagent, anhydrous, granular: washed with hexane prior to use.

Extraction and "clean-up" of samples for gas-liquid chromatography - mass spectral analysis

Fish Oil

The crude herring oil (220 g) was dissolved in 500 ml of hexane and dried over anhydrous sodium sulphate.

Eggs

The total egg weight was 67 g and the final hexane extract volume prior to the DMF partition was 100 ml.

Chromatographic "clean-up"

Fish Oil

The solution in hexane was concentrated to 50 ml and percolated through a column 1 cm wide containing 20 g of activated Florisil. The column was eluted with hexane until there was no further elution of peaks corrasponding to those occurring in the first fraction of routinely analysed samples (see Appendix A). The resulting eluate was concentrated to 10 ml and passed down an activated Florisil column (6 g) eluted with hexane and fractions collected until no further peaks were eluted, as shown by GLC electron capture. This process was repeated three times.

The hexane eluate obtained as above contains pp'-DDT and its congeners polychlorinated biphenyls, non-oxygenated "drin" insecticides. The more polar compounds such as HEOD, endrin and heptachlor epoxide are not eluted under these conditions. The solvent was evaporated from the eluate and the residue was nitrated with a mixture of nitric and sulphuric acid 4 : 1 v/v. The acid mixture was poured into water and extracted with hexane. The resulting hexane solution was again chromatographed through activated Florisil and the eluate was evaporated to 1 ml.

Heron Egg

Purification of the heron egg extract, following DMF partition, was achieved by chromatography down a deactivated Florisil column (25 g) using hexane (150 ml) as eluent. The concentrated eluate (10 ml) was further purified by chromatography down activated Florisil (6 g) using hexane as eluent.

Portions of the above samples were examined by gas-liquid chromatography with electron capture detection, micro-coulometric detection and mass spectrometric and flame ionisation detection.

Standards of Arochlor 1254 were also examined by GLC with the same column conditions and detection procedures.

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Gas chromatographic operating conditions for electron capture detection and micro-coulometric detection were as follows:-

Instrument:	Pye 104
Column:	152 cm x 0.3 cm glass tube packed with 3.8% SE30 on Diatoport S.
Inlet pressure:	18 psi.
Temperature:	185°C.
Carrier Gas:	Oxygen free Nitrogen.
Electron capture detector:	63 Ni source with pulsed DC supply and 150 microsecond pulse interval.
Microcoulometric detector:	Inlet temperature 200°C Furnace temperature 900°C
Electrolyte	70% glacial acetic acid : 3% water.
Oxygen flow rate:	60 ml/minute

The GLC/mass spectrometer operating conditions were:-

Instrument:	Pye 104 chromatograph with flame ionisation detector and 1 : 1 split between detector and mass spectrometer, an AEI MS 12.
Chromatographic column:	152 cm x 0.3 cm glass column packed with 2% Silicone SE30 and 0.2% Epikote 1001 on 85-100 mesh Diatoport S.
Carrier gas:	Helium
Flow rate	40 ml/minute
Temperature	184°C
Ionisation voltage	70 volts.

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RESULTS AND DISCUSSION

Chromatograms of extracts of heron egg and herring oil and Arochlor 1254 obtained by gas-liquid chromatography and electron capture detection are given in Figures 2-4 respectively. The chromatograms obtained by microcoulometric detection are given in Figures 5-7. These show that many of the components of the live samples respond both to electron capture and microcoulometric detection and are probably halogenated compounds.

Mass spectral - GLC examination was carried out using a gas chromatograph with a SE30 column identical to that used for electron capture, the gas chromatograph was coupled to a MS12 mas spectrometer with a Watson-Riemann separator. The chromatogram was recorded with a flame ionisation detector such that there was a 1:1 gas flow split between the detector and the separator. The chromatograms obtained are given in Figures 8-10 and portions of the mass spectra in Figures 11-13.

The extracts were also examined by thin layer chromatography and the R_f values obtained together with those of various chlorinated insecticides are given in Table 2.

The isotopes of chlorine, Cl^{35} and Cl^{37}, occur in a ratio of 3 to 1 (precisely 75.4 : 24.6) and a compound containing one Cl atom per molecule will exhibit a doublet parent peak one doublet corresponding to an ion with a Cl chlorine atom of mass 35 and the other to an ion with a chlorine atom mass 37. Further, the intensity of these peaks will be in the ratio of 3:1. Generally the expression for the probability of ions of the type $R\,Cl^{35}_{n-a}\,Cl^{37}_{a}$ is:-

$$\frac{n!}{(n-a)!a!}\,(0.75)^{n-a}\,(0.25)^{a}$$

(where n = number of chlorine atoms per ion). Using this expression it is possible to calculate the number and relative intensity of the peaks in the group due to a chlorinated ion existing in these several isotopic forms. This is illustrated in Figure 13 and illustrates

how the number of chlorine atoms per ion may be determined from a characteristic peak pattern. The assignment of the chlorine atoms per molecule and the molecular weight for compounds in each peak is given in Table 3 and the number of Cl atoms associated with each peak is illustrated in Figures 7-9. From the results obtained there can be no doubt that the interference encountered in the determination of organochlorine insecticides was due to polychlorinated biphenyls.

A comparison of the relative retention values of Arochlor 1254, heron egg and herring oil extract and some organochlorine insecticides is given in Table 4 and this illustrates that with SE 30 it is virtually impossible to separate the insecticides of the DDT group from some of the components of Arochlor 1254.

The separation of Arochlor 1254 from the less polar insecticides such as aldrin and pp'-DDE is also not possible by the technique of thin layer adsorption chromatography, and column chromatography employing adsorption is not likely to prove any more helpful. However, the determination of organochlorine residues in environmental samples requires that some separation procedure is employed prior to gas-liquid chromatography. The routine analysis of samples in Tunstall Laboratory makes use of the difference in polarity between compounds containing the oxirane group, heptachlor epoxide, HEOD and endrin and the non-oxygenated chlorine compounds to separate the two groups of compounds (See Appendix). Since the non-oxygenated compounds are eluted in the first fraction by hexane there is no interference in the determination of oxygenated chlorine insecticides by the polychlorinated biphenyls. This is not the case in all analytical techniques for the determination of organochlorine insecticides, for example, that of tha Government Chemists[13] where all the insecticides are eluted in one fraction. Although different stationery phases are then used to overcome this difficulty they are not completely successful[14].

The occurrence of chlorinated biphenyls in the environment raises questions as to the source of such pollution, how long this pollution

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has existed, its relationship to changes in particular ecosystems and the possibility of pollution by other chlorinated compounds.

The causes of pollution are less obvious but it is possible that direct discharge into the environment occurs directly into the atmosphere as in jet turbine lubrication, or directly into rivers by industrial waste disposal. Once in the environment the polychlorinated biphenyls are biologically very hard and will persist for some years.

Polychlorinated biphenyl pollution has not been detected or inferred prior to 1963 but it would appear likely that it has occurred previously. If this pollution had been recognised earlier it is likely that there would not have been the pressure against the DDT group of insecticides. It seems very likely that with the analytical procedures employed for organochlorine insecticide determination at that time any interference in the region of DDT would be reported as DDT and hence in some of the predatory birds particularly fish eating birds they appeared to have high organochlorine insecticide levels.

The toxicity of polychlorinated biphenyls has been studied with respect to rat and guinea-pigs[15]. The compounds studied would appear to be at least as toxic as DDT. Also Risebrough, et al[10] state that these compounds are more powerful inducers of hepatic microsomal enzymes than is DDT.

Since polychlorinated biphenyls occur in the environment it is to be expected that compounds with similar industrial usage will also occur. These include the chlorinated paraffins and the chlorinated naphthalenes. The chlorinated naphthalenes also interfere with the determination of organochlorine insecticides.

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APPENDIX 1

The determination of organochlorine insecticides in animal products

The method described is suitable for the determination of mixed insecticide residues in animal products. It is suitable for the determination of γBHC, HHDN, heptachlor, pp'-DDE, pp'-TDE, pp'-DDT, heptachlor epoxide, endrin and HEOD at concentrations of one part per hundred million in animal tissues, fats, oils and eggs. The procedure consists of extraction with hexane or hexane/acetone followed by clean-up with dimethylformamide., hexane partition, liquid solid chromatography on Florisil and determination of the chlorinated insecticides by gas liquid chromatography with electron capture detection.

Method

Apparatus

Separating funnels	100 ml and 250 ml and 1 litre capacity.
Volumetric flasks:	100 ml, 50 ml, 25 ml, 20 ml and 10 ml capacity.
Graduated stoppered cylinders	25 ml capacity.
Soxhlet extraction apparatus	
Filter funnel	4" short stem (for buffer).
Chromatographic column:	The column consists of thick wall glass tubing 55 cm long x 0.5 cm bore. A female S29 ball joint is fused at the bottom. The outlet of the column is made from a female S13 joint. The top of the column is connected to a S29 male joint which fits to an air line.
High speed homogeniser	
Centrifuge	
Beakers	

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Reagents

Sodium sulphate, anhydrous granular. General purpose reagent. Wash with hexane prior to use.

Hexane petroleum fraction SBP 60-70 Redistill and collect fraction boiling between 64° and 66°C.

Acetone. General purpose reagent, redistilled.

Sand. Horticultural Sharp sand. Hexane washed.

Diethyl ether. Analytical reagent.

Florisil 60-100 mesh. Activate by heating overnight at 120°C. Deactivate by addition of 3% v/w distilled water into conical flash stopper and shake for 30 minutes.

Distilled water.

Potassium oxalate. General purpose reagent.

Methanol. Analytical reagent grade.

All reagents and apparatus should be examined by gas liquid chromatography of a hexane extract to ensure freedom from contamination.

Extraction

Animal Fats, muscle tissue, kidney, liver and brain. Freeze sample by storing in polystyrene box with Cardice. With sharp knife chop up frozen sample, mix well and weigh 4 g into a 250 ml beaker. Add 10 g of sand and grind with a heavy glass rod with flattened end, add sufficient sodium sulphate and grind again to give a dry uniform granular mass. Warm the ground material with successively 50, 20, and then 20 ml portions of hexane on a steam bath until it is gently boiling, stir carefully, decant the solution through a Whatman No. 1 filter paper into a 100 ml volumetric flask and carefully transfer the solid to the filter paper. Wash the beaker, filter paper and its contents with a further 10 ml warm hexane, cool the flask and its contents to 20°C and make up to the mark with hexane.
Treat a 25 ml solution of this by DMF partition.

Butter. Dissolve 1 g of clarified butter in 10 ml hexane. Transfer to 100 ml separating funnel using 15 ml hexane for transfer and washing. Carry out DMF partition on this solution.

Eggs. If the eggs are fluid incorporate sufficient sodium sulphate to give a granular mass. If the contents are dehydrated and solid grind them with sand and sodium sulphate. Transfer the granular mass to a suitable extraction thimble and extract for 26 hours in a Soxhlet extractor with acetone - hexane (1 + 2). Cool the extract, filter through a filter paper containing sodium sulphate into a concentrator filter with a tube. Concentrate to a small volume and transfer to 100 ml flask. Make up to volume with hexane. Take the volume equivalent of 1 g weight for DMF partition.

Fish Oil. Dissolve 4 g of fish oil in hexane in graduated 100 ml flask and make up to volume with hexane. Take 25 ml for DMF partition.

Milk. Weigh 100 g milk into a 250 ml beaker. Transfer the sample to a 1 litre separating funnel. Rinse the beaker several times with water and add the water washings to the milk. Add 10 ml of 5% aqueous potassium oxalate solution and mix. Add 200 ml diethyl ether and shake for 2 minutes. Add 100 ml redistilled hexane and shake for one minute. Allow the mixture to stand for ten minutes discard the lower layer, stand for a further minute and discard the remaining aqueous layer. Filter the solvent layer through a 4" funnel plugged with glass wool which is covered with granular sodium sulphate into a 600 ml beaker. Wash the separating funnel with three volumes of hexane 15 ml, and filter into the 600 ml beaker. Evaporate to dryness, dissolve the residue in 10 ml hexane and transfer to 100 ml volumetric flask. Make up to volume with hexane. Add 10 ml of this solution and 15 ml hexane to a 100 ml separating funnel for the DMF partition procedure.

Liquid-liquid partition process

Place 10 ml of DMF in a 100 ml separating funnel. Allow a little of this to run through into the lower 100 ml separating funnel such that both taps are lubricated by clean DMF. This ensures that any leaking taps can be discarded without loss of sample. Place 25 ml

of the hexane extract into the upper 100 ml separating funnel, shake vigorously for 1 minute, allow the layers to separate and run off the DMF phase into the lower 100 ml separating funnel. Shake the hexane extract with two further 10 ml of DMF and add these to the first DMF extract. Discard the hexane phase. Wash the combined extracts with 10 ml of hexane saturated with DMF and run off the DMF layer into a 250 ml separating funnel. Shake the aqueous sodium sulphate/DMF/hexane mixture and allow to settle for 40 minutes. Run off the aqueous phase and wash the hexane with two further 20 ml of distilled water. Dry the hexane extract by using a small quantity of sodium sulphate before the next clean up procedure.

Liquid-solid chromatography

Using a plug of hexane washed cotton wool as a support add to the column 3 g of Florisil and top this with 1 cm of sodium sulphate.

Run on to the column the hexane solution obtained from the DMF partition and wash in with a little hexane. Using a 25 ml graduated flask as a receiver elute with hexane to a volume of 20 ml. Replace the receiver with another 25 ml flask and elute with 25 ml of 10% diethyl ether in hexane.

These two eluates are examined by gas-liquid chromatography. The first fraction will contain any γBHC, heptachlor, HHDN, pp'-DDE, pp'-DDD and pp'-DDT which may be present in the original extract. The second fraction contains any heptachlor epoxide, HEOD and endrin which may be present.

Gas-liquid chromatography

Instrument conditions for 1st fraction:

Column:	1 m x 3 mm i.d. all glass
Column packing:	3.8% SE30 on Diatoport S 80-100 mesh
Carrier gas:	N_2, oxygen free
Inlet pressure:	1.75 kg/cm^2
Detector:	Electron capture
Temperature:	184°C

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Instrument conditions for 2nd fraction:

Column:	1.5 m x 3 mm i.d. all glass
Column packing:	2% Oronite polybutene + 0.2% Epikote 1001 on Celite 85-100 mesh
Carrier gas:	N_2, oxygen free
Inlet pressure:	1.75 kg/cm^2
Detector:	Electron capture
Temperature:	184°C

Standard solutions:

Standard 1 (a)

A hexane solution containing 0.004 γ/ml γBHC, 0.004 γ/ml HHDN and 0.04 γ/ml pp'-DDT.

Standard 1 (b)

A hexane solution containing 0.004 γ/ml heptachlor 0.02 γ/ml pp'-DDE and 0.02 γ/ml pp'-DDD.

Standard 2

A hexane solution containing 0.01 γ/ml heptachlor epoxide, 0.02 γ/ml HEOD and 0.02 γ/ml endrin.

Method

Inject 40 µl of sample into the appropriate column and record the chromatogram. The sample then may be diluted or concentrated to give a peak of the same height as in appropriate standard.

Due to the decrease in sensitivity of the detector during prolonged running it is impracticable to construct a calibration curve for the estimation of insecticide present. To overcome this difficulty it is essential to inject a standard after every two samples and to mean the two standard peak heights for calculating the concentration of the insecticide in the egg samples between the standard samples.

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Calculation:

Let mass of egg	=	W g
Initial volume of hexane containing W g	=	100 ml
Volume taken for clean up	=	5 ml
Final volume after clean up (20 or 25 ml)	=	Z ml
Volume of standard = volume of sample injected	=	40 µl
Concentration of standard	=	C γ/ml
Dilution or concentration of final volume	=	$\frac{D}{Z}$
Mean peak height of standard at R_T	=	P_S cm
Peak height of sample at R_T	=	P_E cm
Then ppm of each component	=	$\frac{P_E \times 100 \times Z \times D \times C}{P_S \times W \times 4 \times Z}$ ppm

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