Rapid Determination and Confirmation of Low Levels of Hexachlorobenzene in Adipose Tissues

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Hexachlorobenzene (HCB) has attracted increased attention from pesticide chemists because of its occurrence in a wide variety of matrices including human blood (SIYALI 1972), milk (SIYALI 1973), and adipose tissue (BRADY and SIYALI 1972), in addition to animal fats, eggs and wheat (SMYTH 1972). It is used as a plasticizer in polyvinyl chloride plastics, in fireproofing textiles and is a by-product in the industrial production of perchloroethylene. It may be more common in human adipose tissue than had previously been believed (SIYALI 1973). Although prohibited for use on animal feed and foods for human consumption, it is widely used as a fungicide on seed wheat. The potential of HCB to translocate in plants has not been extensively investigated.

Recovery trials for HCB in adipose tissue were conducted using the method described in EPA's Manual of Analytical Methods. This is a modification of the partitioning and cleanup method by Mills, Onley and Gaither (1963). Low recoveries were obtained because of the unfavorable partition ratio of HCB in the acetonitrile-petroleum ether solvent system (0.33 in acetonitrile). In attempting to improve the recovery of HCB from fatty substances, other investigators have resorted to "direct elution" cleanup. This generally involved extraction with hexane or petroleum ether and proceeding immediately to a Florisil column thus bypassing the partition cleanup steps. Elution of HCB from the column was accomplished with solvent mixtures such as 400 ml methylene chloride-hexane (1:4) and 250 ml methylene chloride-pentane (1:4) (SMYTH 1972 and STIJVE 1971).

The method described herein utilizes 100 ml hexane and a Florisil column to separate HCB from adipose tissue coextractives. This has generally proved to be superior to solvent mixtures in that fewer interfering substances are eluted.

The various approaches to the confirmation of HCB have included gas chromatography on columns of different polarity (TAYLOR and KEENAN 1970), TLC (SMYTH 1972), reversed phase TLC (STIJVE 1972) and partition coefficients in selected solvent systems. Recent publications describe the preparation of ether derivatives of HCB as a confirmatory test (COLLINS et al. 1972, HOLDRINET 1974). These reports describe the formation of the monosubstituted ether using various techniques.

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Our work on HCB confirmation in adipose tissues involved the preparation of several derivatives from various alcohols. The two most useful compounds were isopropoxypentachlorobenzene (IPB) and bis-isopropoxytetrachlorobenzene (BITB) derived from 2-propanol. The latter product appeared to be less subject to further substitution than the former and the reproducibility of the reaction was more easily controlled.

This study presents a sensitive, rapid and reliable procedure both for determination and confirmation of HCB in lipid material.

EXPERIMENTAL

Apparatus*

A Tracor, MT-220 gas chromatograph, equipped with a tritium electron capture detector operated in the d.c. mode, was used. The gas chromatographic column was 1.8 m x 4 mm, i.d. pyrex glass, packed with 1.5% OV-17/1.95% QF-1 on 80-100 mesh Supelcoport.

The column was operated at 200° with a nitrogen flow rate of 60 ml/min. Other instrument temperatures were: detector, 210°; inlet, 220°; transfer line, 220°.

The following glassware was used: 300 mm x 25 mm o.d. Chromaflex columns (KONTES, K-420530); Kuderna-Danish concentrator assembly (K-570000) fitted with 25 ml graduated tube (Special Order, K-897900; micro Snyder columns (K-569250); disposable pipets.

Reagents & Materials

Hexachlorobenzene, 99+%, analytical reference standard
Pyridine, Spectrograde
Potassium Hydroxide, reagent grade
Sodium Sulfate, anhydrous, granular, Soxhlet extracted with benzene, oven dried at 130°
Sodium Sulfate, 2% aqueous solution, pre-extracted with hexane
Florisil, held at 130° for 24 hr. prior to use.
1-propanol, reagent grade
1-butanol, ethanol, 2-propanol, hexane, pesticide quality or equivalent.
Keeper solution, 1% paraffin oil in hexane.

*Use of trade names is for identification purposes only and does not constitute endorsement by the Environmental Protection Agency.

Procedure

Approximately 500 mg of chicken fat fortified with HCB was dissolved in 1-2 ml hexane and transferred with a disposable pipet to a 100 mm Florisil column topped with 12 mm Na_2SO_4. The column was prewashed with hexane. Elution was performed with 100 ml hexane at a flow rate of 5 ml/min. A Kuderna-Danish evaporator was placed under the column for collection of the eluate. The volume of solvent was reduced by steam evaporation and a nitrogen stream to variable volumes depending upon the HCB concentration. For the evaporation of standard solutions of HCB, 5 drops of 1% paraffin oil in hexane were added to act as a keeper. An aliquot was then injected into the gas chromatograph and compared with standards.

The derivatization reaction was carried out in a 25 ml concentrator tube fitted with a micro Snyder column. The reaction reagents consisting of 0.2 ml pyridine and 0.5 ml 10% KOH in 2-propanol were added to the evaporated sample extract or HCB standard in the tube. The contents were thoroughly mixed and the tube was heated in a boiling water bath. For the formation of the monosubstituted derivative, a 10 min. reaction time was used, while 30-60 minutes was required for the disubstituted product. The tube was then removed and immediately cooled under running water. Ten ml of 2% Na_2SO_4 and 5 ml hexane were added and the tube was shaken for 1 minute. After the phases separated, an appropriate volume adjustment was made and an aliquot was injected into the gas chromatograph.

RESULTS AND DISCUSSION

The recovery of HCB from fortified chicken fat by elution with 100 ml hexane through a 100 mm Florisil column was quantitative at several concentration levels (Table I). The eluates were injected into the gas chromatograph after evaporation in a K-D concentrator and without additional sample cleanup. Heptachlor, aldrin, p,p'-DDE and o,p'-DDT were also partially eluted. The various isomers of hexachlorocyclohexane, heptachlor epoxide, dieldrin, endrin, p,p'-DDT and p,p'-DDD were retained by the Florisil column.

TABLE I

Recovery of HCB from Fortified Chicken Fat by Direct Elution with 100 ml of Hexane

Fat, mg	HCB Added, ng	HCB Recovered, ng	HCB Conc. µg/g	Recovery %
403	4	4.1	0.010	102
494	8	7.0	0.016	88
477	16	14.8	0.034	92
474	25	24.8	0.053	99
583	50	51.5	0.086	103
482	48	51.2	0.100	107
530	150	143	0.283	95
497	160	160	0.322	100
446	250	254	0.561	102
555	350	301	0.631	86
528	500	477	0.947	95
525	1000	1003	1.900	100

Mean 97.4%
Range 86-107%
Std. Dev. ± 6.3%

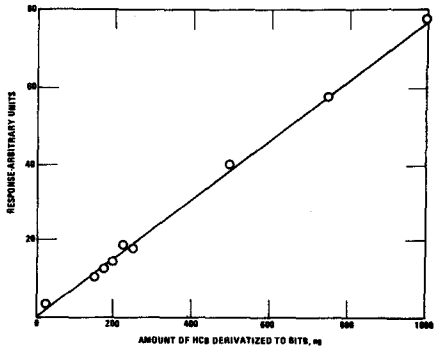

Figure 1. Response curve for BITB. Reaction conditions: 0.2 ml pyridine + 0.5 ml 10% KOH in 2-propanol heated for 1 hour at 100°C. Column: 1.5% OV - 17 + 1.95 QF-1 on 80/100 mesh Supelcoport. 1.8 m x 4 mm I.D. Pyrex glass. Oven temperature 200°C. N_2 flow rate 60 ml/min.

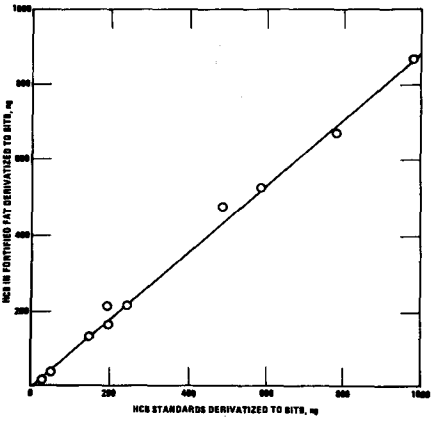

Figure 2. Standard curve of derivatized HCB standards and HCB fortified fat. Reaction conditions: See Figure 1.

A series of different amounts of HCB standard were derivatized to BITB and chromatographed to establish the linearity of the reaction. Figure 1 indicates that a straight line relationship is obtained for this derivative within analytical deviation. The points on the graph represent amounts of HCB derivatized from 25 to 1000 ng. A standard deviation of ± 4.7 mm was obtained from the injection of 10 derivatized standards at a mean peak height of 123 mm at the 25 ng level of HCB. Reproducibility and linearity of the derivative suggested that it would be a valuable adjunct to quantitation of HCB.

To assess this potential, HCB in variable amounts was added to rendered chicken fat. After extraction and elution through Florisil the sample was derivatized and compared with standards that were subjected to the derivatization step only. The results in Figure 2 indicate that the recovery of HCB through the entire procedure was consistent, ranging in most instances, from 84 to 88 percent of the derivatized standards. The HCB fortification levels in the fat ranged from 0.05 to 2.0 µg/g. Approximately 8 percent of the loss could be attributed to the solvent evaporation and Florisil elution processes. The results indicated that this procedure could be extremely useful in instances when similarly eluting compounds prevent the accurate measurement of the HCB peak in a sample. This is illustrated in Figure 3 by chromatograms of a fat sample fortified with 53 ppb HCB analyzed without derivatization and the same fat sample subjected to derivatization. It should be noted that the same instrumental conditions were used for both analyses. In addition, the derivatization allows the analyst to confirm the presence of HCB as low as 5 ppb in adipose tissue.

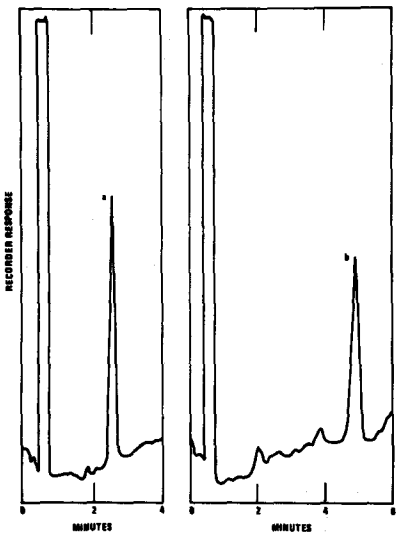

Figure 3. Chromatograms of (a) HCB by direct elution method. Injection: 5 µl from 8 ml hexane, 474 mg chicken fat fortified with 53 ppb HCB. (b) Bis-isopropoxytetrachlorobenzene derivative of sample (a). Injection: 3 µl from 3 ml hexane. Column: 1.5% OV-17 + 1.95% QF-1 on 80/100 mesh Supelcoport 1.8 m x 4 mm I.D. Pyrex glass. Oven temperature: 200°C. N_2 flow rate: 60 ml/min.

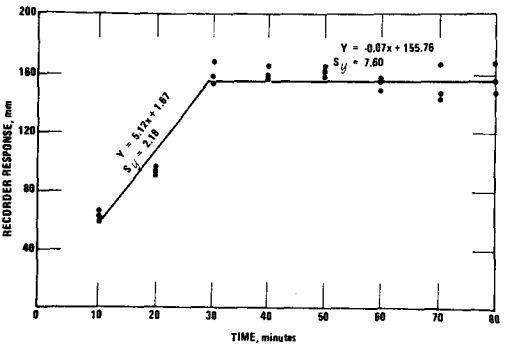

Figure 4. Production of BITB at different reaction times. Reaction conditions: See Figure 1.

Figure 4 illustrates the effect of reaction time on the yield of BITB in the presence of 0.2 ml pyridine. Three standards containing 50 ng HCB were derivatized for each 10 min. period between 10 and 80 min. It was observed during this investigation

that in the absence of pyridine at a 10 min. reaction time the derivatization produced mainly IPB and a very small amount of BITB. In the presence of pyridine (0.05 to 0.5 ml) the production of BITB at 10 min. reaction time was significant and increased to a maximum in approximately 30 min. The yield remained essentially unchanged up to 80 min. At 70 to 80 min., however, the points on the curve were more diverse. Thus, pyridine appeared to promote additional reaction to the disubstituted product. The trend lines of Figure 4 were computed by the method of least squares.

The apparent catalytic action of pyridine in the formation of HCB ethers was observed by a previous investigator who theorized that it was due to the hetero nitrogen atom and certain steric considerations (ROCKLIN 1956).

Although not the principle object of investigation in this study, the monosubstituted derivative was observed to give linear response at shorter reaction times, i.e. 10 min., when it was the predominant product formed. Where BITB yield is at a maximum (30-80 min.), IPB is a minimum, indicating the derivatives are in equilibrium depending upon the reaction time at a constant pyridine level.

TABLE II

Effect of Derivatization Reaction on Selected Pesticides*

Altered	Unaltered
isomers of HCH	aldrin
heptachlor	dieldin
heptachlor epoxide	endrin
p,p'-DDT	p,p'-DDE
o,p'-DDT	PCB's
p,p'-DDD	

*Reaction Conditions: 0.2 ml pyridine + 0.5 ml 10% KOH in 2-propanol heated for 30 min. at 100°C.

The effect of the derivatization reaction on several selected chlorinated pesticides is summarized in Table II. As would be expected, DDE and the PCB's were not affected, while the DDT's were dehydrochlorinated to the corresponding DDE's. None of the unchanged pesticides interferred gas chromatographically with either IPB or BITB.

Table III lists the relative retention times of the HCB ether derivatives obtained with four different alcohols. In all cases, pyridine, a boiling water bath and a 10% solution of KOH in the respective alcohol was used. No attempt was made to optimize reaction times except in the case of the 2-propanol derivatives. These derivatives possess potential for quantitation and confirmation by offering several options when attempting to confirm HCB in the presence of several interfering peaks.

This was demonstrated in the course of our work when it became necessary to confirm the presence of HCB in rat adipose tissue which also contained 160 ppm of Aroclor 1016. Derivatization to BITB was not feasible due to interference from a later eluting Aroclor peak. However, it was possible to confirm and quantitate HCB (0.3 ppm) based on the monosubstituted derivative (IPB) which was well separated from other components in the sample.

TABLE III

Retention Times of Ether Derivatives of Hexachlorobenzene Relative to Aldrin*

	Derivative	
Alcohol	Monosubstituted	Disubstituted
ethanol	0.57	0.68
1-propanol	0.76	1.21
2-propanol	0.65	0.86
butanol	1.02	2.19

*Column: 1.5% OV-17/1.95% QF-1 on 80/100 mesh Supelcoport. 200°C N_2 flow rate 60 ml/min. RRT of HCB = 0.48. Identity of derivatives confirmed by GC/MS.

SUMMARY

The described procedure can be utilized for rapid, simple quantitation and confirmation of hexachlorobenzene in fatty tissue at levels as low as 5 ppb without the use of sophisticated and expensive equipment. Interferences can be circumvented in many instances without additional separation by selection and preparation of the appropriate derivative.

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