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Hexachlorocyclohexanes in human blood serum

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Abstract

A simplified method allowing the determination of trace concentrations (ng/ml) of hexachlorocyclohexanes in human sera suitable for the analysis of large numbers of samples has been developed. Comparison of acid (conc. H_2SO_4,) and basic (5 *M* KOH) digestion has shown that good recoveries are obtained with the former when an internal standard, 1,2,4,5-tetrabromobenzene (TBB), is used to correct for volatilization losses. Good separation between α-, β- and γ-HCH isomers, TBB, hexachlorobenzene and some interfering compounds is obtained with DB-5 columns. The use of electron-capture detection (ECD) or negative ion chemical ionization mass spectrometry (NICI-MS) in the selected ion monitoring mode (*m/z* 71) provides sufficient sensitivity for quantitative determination. ECD is the most sensitive method for β-HCH and NICI-MS for the other isomers. GC–NICI-MS is needed for the unambiguous determination of δ-HCH due to the coelution with one interfering compound. The method has been successfully applied to the analysis of a series of 625 samples collected in a population situated near the effluents of an organochlorinated solvent factory. The results have shown that β-HCH is selectively accumulated in human sera and prompt to the preferential investigation of the toxic effects of this isomer in humans and mammals. © 1997 Elsevier Science B.V.

Keywords: Hexachlorocyclohexanes; Organochlorine compounds; Human blood serum

1. Introduction

Organochlorinated compounds are ubiquitously distributed in the environment as a consequence of their high chemical stability and widespread use in the past decades. These compounds have entered into the human diet via the food chain or respiration [1–4] and have accumulated in the adipose tissues due to their lipophilic properties. Increasing concern is growing on the possible health effects as more information on man's intake becomes available [5–9].

The hexachlorocyclohexanes (HCHs) constitute a major group of organochlorinated compounds that have widely been used as insecticides in the 1940–1970s. These compounds have been commercialized as lindane (>99% γ-HCH, the isomer with insecticide properties), fortified benzene hexachloride (FBHC; 40–48% γ-HCH) and benzene hexachloride (BHC, ca. 14% γ-HCH), the most commonly used. This last mixture is essentially composed by α-, β-, γ-, δ- and ε-isomers in a proportion of 55–70%, 7–14%, 10–18%, 6–10% and 3–4%, respectively [10].

After the seventies, the production and use of HCHs declined quickly in the developed countries, and more slowly in the developing areas. However, despite the discontinuation of their synthesis, they are still found as common contaminants in the environment [2]. In addition to input diversity, the significant isomer differences in water solubility and vapor pressure complicates further the understanding of the environmental occurrence of these compounds. Thus, the high abundance of α-HCH in the

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atmospheric samples collected in the Northern Hemisphere still remains to be explained [11,12].

In human fluids and tissues, the α- and δ-isomers are found in low concentration. In the case of lindane this may be explained by its high water solubility ($2.1 \cdot 10^{-5}$ mol/l at 20°C). In fact, the dominant HCH in human blood samples is the one having all the chlorines in equatorial position, the β-isomer [8,9,13]. The predominance of this compound may a priori be a consequence of its low dipole moment, low water solubility ($7.0 \cdot 10^{-7}$ mol/l at 20°C) and high stability [2,10].

The common occurrence of organochlorinated compounds, including HCH, in human tissues prompts to the need of combined analytical-epidemiological studies for the assessment of possible health effects related to their accumulation. Several methods have been reported for the analysis of these compounds in human tissues like blood [14–30], milk [31,32] or adipose tissue [33]. However, blood is the ideal medium for body burden estimation because it gives information on the compounds accumulated in fats and is relatively easy to obtain. Methods for the analysis of trace amounts (ng/ml) of these compounds in large series of blood samples are needed.

Gas chromatography (GC) coupled to electron-capture detection (ECD) is currently the instrumental technique of choice [34]. Its application needs clean solutions to avoid interfering compounds that limit performance of the capillary columns. However, the analysis of large series of blood samples requires the simplification of the handling procedures on order to the optimize man power and analysis time.

n-Hexane extraction and direct instrumental analysis of the extracts was the first method reported for the analysis of organochlorinated compounds in blood sera samples [14]. This method was subsequently improved by addition, before extraction, of formic acid as denaturalizing agent [15] and is still in use for its simplicity [17–19]. However, it undergoes severe problems for the life time and performance of the capillary columns due to the large amounts of compounds not amenable to GC analysis remaining in the extracts. The methods developed subsequently can be differentiated for their procedures of matrix denaturalization (methanol [20–24], formic acid [25,26] or sulphuric acid [27–29]), extraction of the organochlorinated compounds (currently with solvents but also with solid-phase [30]) and clean-up or fractionation (mainly with florisil [23,25] or silica [20,21,27]). Column chromatography fractionation has the drawback of long time consumption in the analysis of large series of samples.

The method reported in this study uses sulphuric acid for matrix denaturalization and extract clean-up. This double purpose involves the use of this reagent at two steps. It is initially added to the sera samples before extraction and then to the collected *n*-hexane extracts. The double use of sulphuric acid allows the preparation of solutions of organochlorinated compounds free from products interfering the performance of the capillary column with a reasonable man power and time. Unfortunately, the use of this reagent involves that the method is not adequate for the analysis of dieldrin or heptachlor [35].

The method presented here has been successfully used for the analysis of HCH in a series of 625 samples collected among the inhabitants living in a population situated near an organochlorinated solvent factory [5,36]. This population was highly exposed to some organochlorinated compounds and the levels of HCH had to be investigated, The analytical method used for this study was designed to work with small sample amounts. GC coupled to ECD and chemical mode mass spectrometry with negative ion recording MS (NICI) were the instrumental techniques used for the analyses.

2. Experimental

2.1. Materials

15 ml screw-capped Pyrex centrifuge tubes capped with Teflon septa (ref. Pyrex SVL 611/54; Afora, Barcelona, Catalonia, Spain), were used to keep and digest the samples. These tubes, the Pasteur pipettes and the vials for the chromatographic analysis were heated at 400°C for 12 h before use. After analysis the Pyrex tube and the Pasteur pipettes were disinfected by immersion in a commercial lye solution for 24 h. Then the tubes were re-cycled by ultrasonic cleaning in Extran AP 13 (Merck, Darmstadt, Germany) solution for 10 min and rinsed with Milli-Q

water, acetone and *n*-hexane. The Pasteur pipettes were discarded in a litter for biological residues.

Residue analysis *n*-hexane (ref.1.04371), iso-octane (ref. 1.15440), concentrated sulphuric acid 95–97% (ref. 1.00731) and acetone (ref. 1.00012) were from Merck. The potassium hydroxide pellets (Panreac, Barcelona, Catalonia, Spain) were cleaned by sonication in *n*-hexane for 10 min. The procedure was repeated three times replacing the *n*-hexane in each case. They were then dissolved in Milli-Q water to obtain a 5 *M* solution. This solution was recleaned by three successive liquid–liquid extraction steps with *n*-hexane. The purity of the solvents and reagents was checked by analysis of 2 ml of Milli-Q water following the same procedures and the dilution factors as for the samples. Not even traces of HCHs were detected.

The γ-HCH and the 1,2,4,5-tetrabromobenzene (TBB) used to prepare the solutions were from Aldrich-Chemie (Steinheim, Germany), α- and δ-HCHs were from Promochem (Wesel, Germany) and β-HCH was from Dr. Ehrenstorfer (Augsburg, Germany). The standard mixtures of HCHs and TBB were prepared in iso-octane.

2.2. Sampling

Around 40 ml of venous blood were collected into vacutainer tubes without preservatives, the samples were left to coagulate naturally for 3–7 h. Then, the samples were centrifuged to separate the serum which was transferred into screw-capped Pyrex centrifuge tubes capped with Teflon-faced silicone rubber septa. These tubes were stored at −20°C until analysis.

The serum samples used in the recovery studies were obtained from people living in Barcelona, a regular city without specific contamination by organochlorinated compounds. The case study samples correspond to the village of Flix.

2.3. Extraction and clean-up

50 μl of the surrogate solution (0.36 μg/ml of TBB) were added to 2 ml aliquots of serum in the same Pyrex centrifuge tubes where the samples were stored. The mixture was vortex stirred for 30 s at 2000 rpm. Acid digestion of the mixture was performed by addition of 3 ml of *n*-hexane and 2 ml of concentrated sulphuric acid (drop by drop). Then, the tube was locked and again vortex stirred for 30 s. The tube was allowed to cool at room temperature and then five drops of acetone were added to help phase separation. The supernatant *n*-hexane phase was removed and the remaining sulphuric acid solution was re-extracted two more times with 2 ml of *n*-hexane (acetone was added again and the mixture stirred like in the first step). All extracts were collected together and the resulting 7 ml of *n*-hexane were purified by vortex stirring (2000 rpm) with 2 ml of sulphuric acid for 3 min. Then, the *n*-hexane phase was concentrated to almost dryness under a nitrogen stream and rediluted to 500 μl with iso-octane. Some samples spiked with HCH standards were treated with 2 ml of 5 *M* KOH for comparison. After basic digestion the procedure was continued as described above.

In order to compare the recoveries obtained by both methods, 2 ml aliquots of four sera samples were spiked with α-, β-, γ- and δ-HCH at concentrations of 44, 58, 44 and 41 ng/ml, respectively. These standard solutions were added before the TBB surrogate. Three replicates were processed by acid treatment and one aliquot was digested following the basic procedure. One aliquot of each sample was analyzed without addition of the standards for substraction of the HCH background levels.

2.4. Instrumental analysis

GC analyses were performed with a Hewlett-Packard model 5890A provided with an ECD system. Two 5% phenyl–95% methylsiloxane coated capillary columns were used: a 30 m×0.25 mm I.D. DB-5 (J and W Scientific, Folsom, CA, USA) and a 50 m×0.25 mm I.D. CP-SIL 8CB (Chrompack, Middelburg, The Netherlands). Film thickness was 0.25 μm in both columns. A fused-silica precolumn of 2 m×0.32 mm I.D. was used in all cases. The DB-5 column was heated from 80°C (holding time 2 min) to 300°C at 6°C/min, keeping the final temperature for 10 min. The CP-SIL column was heated from 80°C (holding time 1 min) to 180°C (holding time 3 min) at 15°C/min and to 300°C at 4°C/min, keeping this last temperature for 10 min. The injector and detector temperatures were 270°C and 310°C, respec-

tively. Injection was performed in split/splitless mode/hot needle technique), keeping the split valve closed for 35 s. Helium (50 cm/s) and nitrogen (60 ml/min) were the carrier and the make up gases, respectively.

GC–NICI-MS analyses were performed with a Fisons MD 800. The samples were injected in split/splitless mode (48 s) at 280°C and data acquisition started after a solvent delay of 4 min. Source temperature was 150°C. Methane was used as reagent gas. The chromatographic conditions were the same as described above. Data were scanned from *m/z* 60 to 500 at 1 s per decade with dwell and interchannel delay times of 0.06 and 0.02 s, respectively. Data were also acquired in selected ion monitoring at *m/z* 71, 81 and 255 with dwell and span times of 0.06 s and 0.10 s respectively.

3. Results and discussion

3.1. Acid vs. basic digestion

The analytical method includes two steps of sulphuric acid addition. In the first step sulphuric acid oxidizes the serum and destroys many of the compounds that may diminish the extraction capacity of *n*-hexane. Sulphuric acid attack also destroys many interfering compounds that may be present in the *n*-hexane extracts together with HCHs. However, as it will be shown later, not all compounds are eliminated.

The second sulphuric acid portion is added to the collected *n*-hexane extracts. This additional oxidation step is devoted to further elimination of compounds that may interfere in the column chromatographic separation. Lack of this additional clean-up step involves the appearance of a number of interfering compounds in the GC traces and a great decrease of the live time of the capillary columns, even when a pre-column is used.

Basic digestion is an alternative method that is used for the analysis of organochlorinated compounds in tissues of many organisms. Digestion in 5 *M* KOH is a common procedure [37]. In the present study this procedure has also been tested for comparison.

The quantitative recoveries obtained by digestion with the acid and basic methods are given in Table 1. A significant decrease in the α-, γ- and δ-HCHs peaks corresponding to the chromatogram of the basic treated sample is observed. Only β-HCH gives similar recoveries with both methods. One important aspect to be mastered in the HCH analyses of serum samples is the potential volatilization of the compounds upon evaporation of the *n*-hexane solutions. In order to account for these losses, all samples were spiked with TBB. This compound has similar vapor pressure to these HCH isomers and sufficient chemical stability to overcome the basic and acid chemical digestions. TBB elutes between the HCH peaks upon capillary column GC analysis.

The recoveries reported in Table 1 have been corrected for the volatility losses of the TBB internal standard (normally in the range of 82–91%). The values corresponding to sulphuric acid digestion are generally in good agreement with recovery data reported in other studies involving the analysis of HCH in human or domestic animal serum or blood [20,21,25–28].

3.2. Retention times and overlapping

The relative retention times (RRT) of these HCH isomers were calculated in two 5% phenyl–95% phenyl polysiloxane columns, DB-5 and CP Sil 8CB.

Table 1
Comparison of the average recoveries resulting from acid and basic digestion of blood sera samples spiked with standard HCH solutions

Isomer	Spiked concentration (ng/ml)	Recoveries (%)	
		Basic treatment	Acid treatment
α-HCH	44	13	69
β-HCH	58	69	70
γ-HCH	44	15	82
δ-HCH	41	3	84

Low polarity columns coated with this stationary phase are currently used for the analysis of organochlorinated compounds. These columns have enough stability to undergo the analysis of large series of samples requiring heating at 300°C as final temperature program. Two different columns of this group were tested. In previous studies it has been observed that these columns may exhibit different selectivity effects [38,39].

Representative chromatograms of the mixtures obtained with these two columns are shown in Fig. 1. No changes in elution order between the HCH isomers, TBB or hexachlorobenzene, another common compound in the blood samples [5], are observed. The separation between the β and γ-isomers is greater for the CP-SIL 8CB column showing a difference of retention times (relative to TBB) of 1.7 whereas the difference for the DB-5 column is 0.9. However, in the CP-Sil 8CB column β-HCH and TBB almost coelute. This involves great difficulties for the use of CP-Sil 8CB columns in the analysis of HCHs when TBB is used as internal standard.

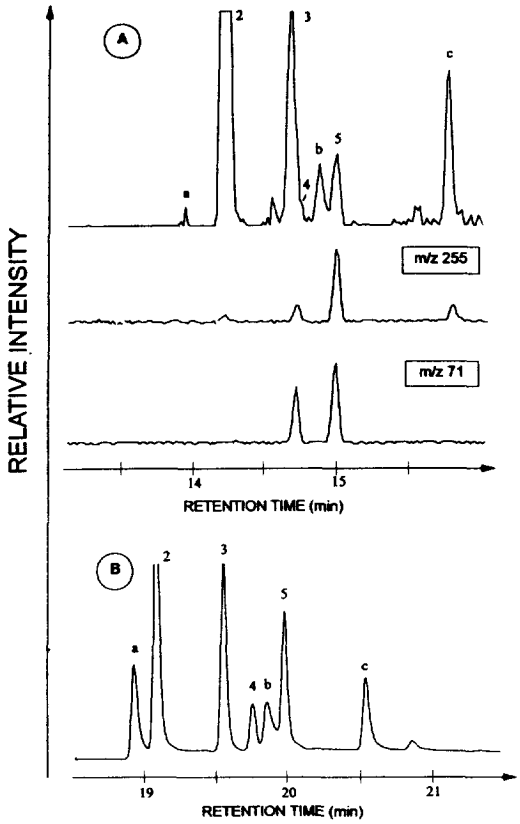

Fig. 1. Chromatograms showing the HCH composition in the serum samples analyzed in this study: (A) GC–NICI-MS with a CP-Sil 8 CB column. (B) GC–ECD with a DB-5 column. Peak assignments are as follows: 1=α-HCH; 2=hexachlorobenzene; 3=TBB; 4=β-HCH; 5=γ-HCH; a, b and c are interfering compounds.

Another aspect to be considered is the systematic occurrence of some peaks eluting between the HCH (peaks a, b and c in Fig. 1). Their mass spectra are shown in Fig. 2. Unambiguous structural identification has not been possible at the low concentrations in which these compounds are found in the samples. Two of them, a and c, do not contain chlorine atoms and may be generated during sulphuric acid attack of sera; b is a trichloronaphthalene [41] found in all samples analyzed.

Polychlorinated naphthalenes (PCN) are persistent and ubiquitous in several environmental and biological matrices [42–44]. Their origin can be related to their use in industry, combustion or chlorination processes [42,45]. In Hallowax 1014, the industrial product most commonly used, there is a predominance of the tetra-, penta- and hexachlorinated congeners. The congeners mono-, di- and tri-chloro substituted predominate in the PCN mixtures originating from combustion and chlorination. These latter compounds are those currently found in most biological matrices, maybe because of their higher environmental mobility [42]. In the samples analyzed, PCN containing two, four and six chlorine substituents were found in addition to compound b.

One problem derived from the presence of these peaks is the overlapping between compound c and δ-HCH in both columns. The presence of δ-HCH has to be investigated using GC–NICI–MS for the examination of the m/z 71 and 255 mass fragment ions. m/z 255 is characteristic of the HCHs allowing to determine which m/z 71 peaks correspond to HCH. The relative intensities of m/z 255 in the HCH isomers are 25, 17, 44 and 23% for the α, β-, γ- and δ-HCH, respectively, but different relative intensities are expected by changing instrumental parameters [41]. However, this mass fragment also records the intensity of the ^{13}C isotope of the molecular ion of compound c (Fig. 2). For this reason δ-HCH quantitation must be performed on the m/z 71 fragmentogram. Quantitation with NICI MS requires the use of

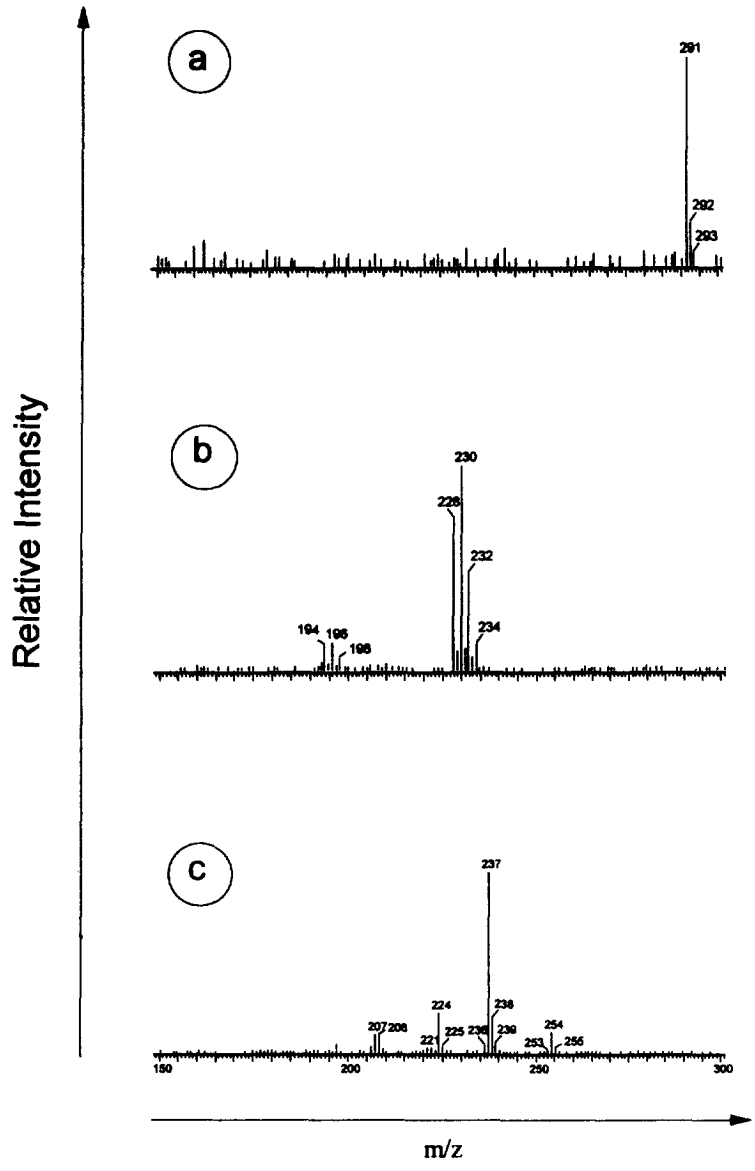

Fig. 2. Mass spectra of peaks interfering in the analysis of hexachlorocyclohexane isomers in human blood sera after sulphuric acid clean up.

this technique in selected ion monitoring (SIM) mode. Scanning does not provide enough sensitivity for the low amounts in which HCH are present in the human serum samples.

3.3. Detection and quantitation limits

Detection and quantitation limits (DL and QL, respectively) for ECD and NICI-MS in m/z 71 SIM mode were determined for the HCH isomers. Following the IUPAC recommendations [40], these limits were calculated in the range of lowest concentration levels, 0.2 to 1.0 ng/ml. The final DL and QL values also include the dilution factors of the method (Table 2).

Except in the case of β-HCH, the DL and QL

Table 2
Detection and quantitation limits (DL and QL, respectively) and operational detection limit (ODL) for the hexacyclohexane isomers in blood sera analyzed by GC in combination with ECD and NICI–MS detection

Isomer	ECD			NICI–MS (*m/z* 71)	
	DL[a]	QL[a]	ODL[b]	DL[a]	QL[a]
α-HCH	0.2	0.6	0.02	0.1	0.3
β-HCH	0.6	2.1	0.04	1.4	4.5
γ-HCH	0.2	0.8	0.02	0.1	0.3
δ-HCH	0.2	0.7	0.03	0.1	0.3

The mass spectrometer was operated in SIM to record the traces of *m/z* 71, 81 and 255 ions. Units in ng/ml.
[a]Defined by IUPAC [40].
[b]Operationally defined as an area threshold of 100 in the specific instrument used for analysis.

values are higher for ECD than for MS NICI in *m/z* 71 SIM mode. This effect points to different ionization rates of the β-isomer that probably reflects its higher stability and low dipole moment.

In the case of ECD chromatograms it was observed that peak areas down to 100 in the arbitrary units of the data system could not be quantitated. The resulting operative detection limit (ODL) was significantly lower than the DLs calculated with the IUPAC criterion (Table 2).

3.4. Quantitation

HCH were determined in the blood serum of 625 persons from a population chronically exposed to organochlorinated compounds [5,36]. Quantitation was performed by interpolation in calibration curves ranging over the same order of magnitude as the concentrations in the samples. Three calibration curves were used, 1–10 ng/ml, 10–150 ng/ml and 150–600 ng/ml. After interpolation the values were corrected by the recovery of the internal standard.

The results are summarized in Table 3. The α- and γ- isomers were only detected in few samples (ca. one tenth of the total population) and in these cases they were in low concentration (0.03–4.8 ng/ml). In contrast, β-HCH was detected in 87% of the samples at concentrations in the order of 0.08–234 ng/ml. Thus, the mean concentration of this isomer is 920 and 230 times higher than the α- and γ-isomers,

Table 3
Mean and standard deviation of the HCH isomers found in the sera samples of a population exposed to the airborne effluents of an organochlorinated solvent factory (n=625)

Isomer	Mean (ng/ml)	SD (ng/ml)
α-HCH	0.01	0.16
β-HCH	9.2	15.0
γ-HCH	0.04	0.32

respectively. As indicated above, the higher bioaccumulation of the β-isomer is probably due to its low water solubility and high chemical stability. Major attention has currently been devoted to γ-HCH because of its insecticide effect and toxicity (LD_{50}= 125 ng/kg [10]). β-HCH has lower toxicity, LD_{50}= 6000 ng/kg [10]. However, since this compound is by far the major HCH isomer present in human tissues it deserves considerably more attention that it has received in the environmental toxicology studies developed to date.

4. Conclusions

Sulphuric acid digestion and clean up combined with GC analysis and detection by ECD or NICI-MS in *m/z* 71 SIM mode allow the determination of HCH mixtures in human sera samples at concentrations of 0.1–1.4 ng/ml. This simplified method is adequate for the analysis of large series of samples. Good recoveries are obtained (70–84%) using TBB as internal standard that allows to correct for volatilization losses.

This method has been used in a study encompassing 625 samples collected in a population exposed to the airborne effluents of an organochlorinated solvent factory. In agreement with other reports, the results confirm that β-HCH largely predominates the HCH distributions vs. other isomers.

The comparison of the HCH mixtures in these human sera with those characteristic of the environmental HCH sources evidence a selective enrichment of the β-isomer. This selective accumulation may be related with its low water solubility and high chemical stability. The high abundance of β-HCH indicates that this is maybe the target compound to

address environmental toxicology studies on HCH in humans and mammals.

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