Analysis of PCBs with special emphasis on comprehensive two-dimensional gas chromatography of atropisomers

Mikael Harju
List of papers

This thesis is based on the following papers, which will be referred to by their respective roman numerals.

I. Determination of the rotational energy barriers of atropisomeric polychlorinated biphenyls
   Harju, Mikael; Haglund, Peter

II. Effects of temperature and flow-regulated carbon dioxide cooling in longitudinally modulated cryogenic systems for comprehensive two-dimensional gas chromatography
    Haglund, Peter; Harju, Mikael; Danielsson, Conny; Marriott, Philip

III. Shape selectivity: a key factor in comprehensive two-dimensional gas chromatographic analysis of toxic PCBs
     Haglund, Peter; Harju, Mikael; Ong, Ruby; Marriott, Philip

IV. Comprehensive two-dimensional gas chromatography of the 209 PCBs
    Harju, Mikael; Danielsson, Conny; and Haglund, Peter
    Submitted to Journal of Chromatography A

V. Comprehensive two-dimensional gas chromatography (GC×GC) of atropisomeric PCBs, combining a narrow bore β-cyclodextrin column and a liquid crystal column
    Harju, Mikael; Haglund, Peter

VI. Determination of levels, enantiomeric fractions and tissue distribution of atropisomeric PCBs in grey seals (Halichoreus Grypus) of varying health status using comprehensive two-dimensional gas chromatography
    Harju, Mikael, Bergman, Anders; Olsson, Mats; Anna Roos; and Haglund, Peter
    Submitted to Journal of Chromatography A

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>µECD</td>
<td>Micro electron capture detector</td>
</tr>
<tr>
<td>1D-GC</td>
<td>One-dimensional gas chromatography</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimension</td>
</tr>
<tr>
<td>AM1</td>
<td>Austin model 1</td>
</tr>
<tr>
<td>CD</td>
<td>Cyclodextrin</td>
</tr>
<tr>
<td>CO₂ (l)</td>
<td>Liquid carbon dioxide</td>
</tr>
<tr>
<td>DDD</td>
<td>Dichlorodiphenyldichloroethane</td>
</tr>
<tr>
<td>DDE</td>
<td>Dichlorodiphenyldichloroethylene</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethylene</td>
</tr>
<tr>
<td>ee</td>
<td>Enantiomer excess</td>
</tr>
<tr>
<td>EF</td>
<td>Enantiomer fraction</td>
</tr>
<tr>
<td>ER</td>
<td>Enantiomer ratio</td>
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<tr>
<td>FID</td>
<td>Flame ionization detector</td>
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<tr>
<td>GC×GC</td>
<td>Comprehensive two-dimensional gas chromatography</td>
</tr>
<tr>
<td>HCH</td>
<td>Hexachlorocyclohexane</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>LMCS</td>
<td>Longitudinally modulated cryogenic system</td>
</tr>
<tr>
<td>MDGC</td>
<td>Multidimensional gas chromatography</td>
</tr>
<tr>
<td>MSD</td>
<td>Mass spectrometric detection</td>
</tr>
<tr>
<td>N₂ (l)</td>
<td>Liquid nitrogen</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PC</td>
<td>Principal component</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated biphenyl</td>
</tr>
<tr>
<td>PCDD</td>
<td>Polychlorinated dioxins</td>
</tr>
<tr>
<td>PCDF</td>
<td>Polychlorinated furans</td>
</tr>
<tr>
<td>PCN</td>
<td>Polychlorinated naphthalene</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PMCD</td>
<td>Permethylated cyclodextrin</td>
</tr>
<tr>
<td>RI</td>
<td>Retention index</td>
</tr>
<tr>
<td>RRT</td>
<td>Relative retention time</td>
</tr>
<tr>
<td>Rs</td>
<td>Peak to peak resolution</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal to noise</td>
</tr>
<tr>
<td>TEF</td>
<td>Toxie equivalence factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
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1 Introduction

1.1 Polychlorinated biphenyls (PCBs)
Polychlorinated biphenyls (PCBs) comprise a class of chlorinated aromatic compounds, with one to ten chlorine atoms (x=1-10) attached to a biphenyl backbone, Figure 1. The empirical formula is C_{12}H_{10-x}Cl_x and there are 209 structurally different compounds, called congeners. Each congener has been assigned a systematic number by Ballschmiter et al. \(^1\) in a scheme that was later revised by Guitart et al. \(^2\), see Appendix I.

![Figure 1. Structural formula of polychlorinated biphenyls (PCB; x =1-10).](image)

PCBs are highly lipophilic compounds with low water solubility and volatility. They also have low electrical (but high thermal) conductivity, low flammability and high resistance to thermal degradation \(^3\).

1.2 Production and use
The first PCBs were synthesised in 1864, but it was not until 1929 that PCBs were produced commercially \(^3\). Their chemical and physical stability and excellent electrical insulating properties led to their commercial use as dielectric fluids in capacitors, transformers, paints, heat transfer fluids, hydraulic fluids, lubricating and cutting oils etc. Commercial polychlorinated biphenyl mixtures were synthesized by chlorination of biphenyls with chlorine gas, and the average degree of chlorination was controlled, by adjusting the reaction conditions, to yield the desired physical and chemical properties. The degree of chlorination is generally in the range 30-60 % on a weight basis (w/w), giving mostly PCBs with four to seven
chlorines. Total estimated production through 1976 amounted to $6.1 \times 10^5$ metric tons, with Monsanto Corporation in the United States as the largest producer. The trade names for the mixtures of PCBs they produced were Aroclor, followed by specific numbers designating their degree of chlorination, and other commercial PCB products were Fenclor, Clophen and Kanechlor.

In 1966 Sören Jensen detected high levels of PCBs during the chromatographic analysis of DDTs and related compounds in samples from the Swedish environment. Consequently, Sweden banned all use of PCBs in open systems in 1971. The legislation was extended in 1979 to include closed systems and a final time limit was set (January 1st 1995) to end the use of existing PCBs in transformers and capacitors.

The world-wide production of PCB mixtures was reduced in the 1970s, although some production continued until 1983. In 1973 recommendations were put forward concerning the control of PCBs and restriction of their release into the environment by the OECD (Organization for Economic Cooperation and Development) proposing that the use of PCBs should be limited to closed systems. The OECD also called for stricter control and documentation of the manufacture, import, export and disposal of these compounds. However, even though the use of PCBs was decreased in the 1970s, no decline in these persistent organic pollutants was seen in biota until the early 1990s, although levels in the environment and biota have more than halved since then.

### 1.3 Emission sources

PCB sources include emissions from landfills containing capacitors and transformers together with PCB discharges from precipitated sludge, spill and improper (illegal) disposal of PCB-containing products. Other sources of PCBs released into the environment include emissions from the combustion of industrial and municipal solid waste. Significant releases of PCBs to nearby areas may occur from explosions or overheating of transformers and capacitors.

### 1.4 Environmental levels

In the industrial synthesis of PCBs not all of the 209 possible congeners are formed, since some positions of the biphenyl are more reactive than others.
Consequently, congeners with chlorines in either the 2,5- or 2,4-positions are preferentially produced. PCBs with such substitution patterns, e.g. PCBs 138, 153 and 180, are therefore abundant in technical PCB formulations and in the environment. However, 136 and 76 congeners are found in these technical PCB mixtures at or in excess of 0.05 % or 1.0 % (w/w), respectively.

Some, but not all, of these congeners are found in biota and some congeners are found in biota that are not found at levels greater than 0.05 % (w/w) in any Aroclor. Usually only a few congeners are monitored in environmental samples. Monitoring of seven congeners (PCBs 28, 52, 101, 118, 138, 153 and 180) gives a good approximation of the total PCB content and simplifies the analytical procedure as abundant congeners are easy to measure. Those congeners can be found at very high levels in environmental samples and make up approximately 30-60 % (w/w) of the total amount of PCBs found, in e.g. Seals from Scottish and Swedish waters.

1.5 Environmental impact of PCBs

PCBs have been identified in nearly all environmental compartments of the ecosystem, such as air, water, sediments, wildlife and humans. In aquatic organisms high in the food chain, e.g. fish, seals, and whales, the levels can be exceptionally high due to the persistence and high lipophilicity of these substances. The bioaccumulation potential of PCBs in animals depends on the animals’ fat content, and certain PCBs, mainly the higher chlorinated congeners, biomagnify through the food chain. In aquatic organisms such as seals the PCB levels can reach exceptionally high concentrations.

PCBs cause a multitude of biochemical and toxicological responses in humans and wildlife, including adverse effects on reproductive, neurological and endocrine systems (such as thyroid atrophy) and porphyria. Some adverse human health effects have been observed amongst occupationally exposed workers, including chloracne, hepatic responses and reductions in birth weight. No increased mortality has been detected, but inconclusive evidence of cancer induction has been found in animal tests.

The rotational energy barrier of PCBs is an important factor affecting their potential toxicity. PCBs with low energy barriers, i.e. non- and mono-ortho
PCBs, are co-planar and exhibit dioxin-like toxicity, while certain PCBs with high energy barriers can be potent phenobarbital-type inducers.

1.6 Aims and scope

The main focus of this thesis is on the analytical aspects of polychlorinated biphenyls, in particularly the 19 atropisomeric PCBs. It covers the experimental determination of atropisomer stability as well as the development and evaluation of a relatively new multidimensional chromatographic technique, comprehensive two-dimensional gas chromatography, and its application to environmental samples.
2 Properties and effects of atropisomeric PCBs

Atropisomerism is a type of conformational isomerism referring in practical terms to conformers that can be individually isolated and have sterically hindered rotation about a chiral axis. In biphenyls the chiral axis is maintained through the hindered rotation about a single σ-bond, as shown in Figure 2. This restriction of rotation is sustained by large substituents (e.g. chlorines) in the ortho position, i.e. the 2,2'- and 6,6'-positions (Figure 1) of the phenyl rings. PCBs with restricted rotation have an energy maximum at planar conformation, which creates axially chiral enantiomers (atropisomers). In order to be chiral, a PCB must be non-planar and lack both an axis of symmetry and a center of inversion 19.

![Figure 2. The two optical isomers (enantiomers) of PCB 45](image)

Of the 209 PCB congeners, 78 display axial chirality and fulfill the basic requirements for optical activity. Kaiser predicted that PCBs with three or four ortho chlorines should have activation energies in excess of 84 kJ×mol⁻¹ and resist rotation (and enantiomerization) at most environmental conditions. These predictions were later confirmed by Oki 21, who also defined atropisomers as isomers that can be isolated since they have a half-life > 1000 s, which is equivalent to an energy barrier of > 90 kJ×mol⁻¹ at 27 °C. Nineteen PCBs fulfill this criterion and exist as stable atropisomers at ambient temperatures, see Figure 3. Twelve of these have been detected in commercial PCB mixtures at levels greater than 1% w/v 22, i.e. PCBs 45, 84, 91, 95, 132, 135, 136, 144, 149, 171, 174, and 183.
Kaiser also predicted that the so-called “buttressing effect” would increase the energy level by an additional 17 kJ×mol⁻¹. The buttressing effect occurs when chlorines are present in 3,3’,5,5’-positions adjacent to chlorines in 2,2’,6,6’-positions. Such chlorines increase the steric hindrance (preventing the outward bending of the ortho chlorine in the transition state, co-planar conformation) when the biphenyl rotates and thus the enantiomerization barrier.

2.1 Calculation of the rotational barriers

The energy barrier restricting rotation through the co-planar conformation has been calculated for tri- (2,6,2’-substituted) and tetra-ortho (2,2’,6,6’-substituted) PCBs by semi-empirical and ab initio methods. These calculations have demonstrated that the tri-ortho and tetra-ortho chlorinated biphenyls have a considerably higher rotational energy barrier than the stipulated energy barrier limit of 90 kJ×mol⁻¹ required for compounds to be classified as atropisomers.

Semi-empirical calculations by the AM1 method have predicted energy barriers for the tri-ortho chlorinated biphenyls of 110-120 kJ×mol⁻¹ and for the tetra-ortho biphenyls of 155-165 kJ×mol⁻¹. Anderson et al. used the same method, but found higher barriers, i.e. 180-220 kJ×mol⁻¹ for tri-ortho PCBs and 370-480 kJ×mol⁻¹ for tetra-ortho PCBs. Cullen et al. derived similar figures using INDO (intermediate neglect of diatomic overlap) quantum mechanical calculations (125-150 kJ×mol⁻¹ for tri-ortho and 230-270 kJ×mol⁻¹ for tetra-ortho PCBs).

The AM1-calculated barrier for PCB 132 (118 kJ×mol⁻¹ at 0 K) differs substantially from the experimentally determined rotational energy of >160 kJ×mol⁻¹ (180 °C), determined by a gas chromatographic technique, suggesting that the inter-conversion barrier is increased through interaction with the slightly polar cyclodextrin stationary phase used in this case.

Further, ab initio calculated values (B3LYP/6-31G* basis set) for the rotational barrier of PCB 132 (185 kcal×mol⁻¹) compared well with the experimentally derived results (182 kJ×mol⁻¹) and it may be concluded that this sophisticated method of calculation yields better results than the semi-empirical methods and Paper I.
Figure 3. Structural formulas of the 19 atropisomeric PCBs.
2.2 Experimental determination of the rotational barriers

The energy needed for enantiomeric inter-conversion to take place can be determined using either on-column or off-column methods. Stopped flow on-column chromatographic racemization experiments are faster and simpler than semi-preparative isolation of enantiomers and off-column racemization experiments. Other techniques are also available for the determination of enantiomerization kinetics: dynamic chromatography is the method of choice for compounds with an enantiomerization barrier lower than 130 kJ×mol\(^{-1}\), while NMR-spectroscopy may be used if the barrier is lower than 80 kJ×mol\(^{-1}\).

Single enantiomers have to be isolated, at least in a semi-preparative fashion, before their racemization (enantiomerization) kinetics can be studied with off-column techniques. Semi-preparative LC enrichment has been applied to chiral PCBs, using a variety of LC columns. PCBs 88 and 139 and PCB 197 enantiomers have been fractionated on triacetylcellulose. Permethylated β-cyclodextrin (PMCD) coated on nucleosil particles has been used to isolate fractions enriched in PCB 132 enantiomers. Haglund used the same type of column to isolate mg amounts of each enantiomer of PCBs 84, 131, 132, 135, 136, 174, 175, 176 and 196 and to partially resolve PCBs 91, 95, 139, 171 and 197. Finally, several additional PCBs have been isolated using a chiral stationary phase of (+)-poly(diphenyl-2-pyridylmethyl methacrylate) (Paper I), namely PCBs 144, 149, and 183.

The absolute conformation is difficult to assess, but it is possible to uniquely assign enantiomers through the way they rotate plane-polarized light. If a solution of a compound rotates light to the right or left the compound is denoted (+) and (−), respectively. Optical rotation measurements show that the (−)-enantiomers of PCBs 88 and 139 elute before the (+)-enantiomers from triacetylcellulose and PMCD. However, the (−)-enantiomers of PCBs 84, 131, 132, 135, 136, 174, 175, 176 and 196 elute before the (+)-enantiomers from a PMCD column. The same enantiomer elution order, (−) before (+), was observed for PCBs 144, 149 and 183 on (+)-poly(diphenyl-2-pyridylmethyl methacrylate) HPLC columns (Paper I).
2.2.1 Off-column methods
We monitored the racemization of enantiopure compounds as a function of
time at three or four temperatures (270-300°C, Figure 4) and obtained linear
Arrhenius plots (Figures 5). The energy needed to overcome the
racemization barriers was then calculated from the slope of the Arrhenius
plots (Paper I and Schurig et al. 33).

The rotational energy barriers were determined for twelve of the nineteen
environmentally stable atropisomeric polychlorinated biphenyls (PCBs),
viz. PCB 84, 131, 132, 135, 136, 144, 149, 174, 175, 176, 183, and 196,
through thermal racemization of enantiomerically pure PCBs. The rate of
racemization was primarily determined using off-line gas chromatography
(GC) or high-performance liquid chromatography (HPLC), with
permethylated cyclodextrin as the chiral selector. GC was used for PCBs 84,
132, 136, 149, 174, and 176, while PCBs 131, 175, and 196 were analyzed
using HPLC. The remaining PCBs, i.e. PCB 144 and 183, were separated by
HPLC using a Chiralpak OP(+) polymethacrylate column. The Gibbs free
energy of activation was found to be 176.6 to 184.8 kJ×mol⁻¹ for tri-ortho
PCBs. For tetra-ortho PCBs the rotational energy barrier was estimated to
be 250 kJ×mol⁻¹. Our value for PCB 132 (184.9 kJ×mol⁻¹) is in close
agreement with the value reported by Schurig 33 (183.7 kJ×mol⁻¹) and, in all
cases, the rotational energy barrier is far above that is required for
environmental stability, i.e. 90 kJ×mol⁻¹ according to Oki 21.

2.2.2 On-column method
In stopped flow on-column methods no isolation of enantiomers is required
since only the racemic substance is needed. Schurig and co-workers used
two methods to determine the racemization barriers by on-column stopped
flow techniques. The first method was simple and not as precise as the off-
column method described above. Using this method, racemic PCB 132 was
enantiomerically separated by HRGC on a column coated with Chirasil-
Dex. After some time, the flow was stopped and the temperature was
rapidly increased to a kinetic inter-conversion temperature of (320°C),
which was maintained for 3 min. The oven temperature was then rapidly
cooled to the previous temperature and the analysis commenced. This gave
rise to three peaks, the middle peak of which originated from inter-
converted enantiomers of the primarily separated enantiomers 33.
Figur 4. Racemization of (+)-PCB#149 (the second eluting enantiomer) at different times (0-128 min) at 280ºC.

The second on-column method was based on the stopped flow technique with a dual-oven MDGC system equipped with two Chirasil-Dex columns and an inter-conversion zone. The first or second eluting enantiomer was transferred from the first column through a 6-port valve to the inter-conversion zone column, where enantiomerization was initiated by rapidly heating to 300 or 320ºC while the flow was turned off. After a given time the column was rapidly cooled and the enantiomers were separated on the second Chirasil-Dex column. The results from the on-column stopped flow experiments agree well with the results obtained by off-column rotational barrier determination. The cited authors also determined the rotational barriers of two additional chiral PCBs (95 and 149). Finally, they tried to estimate the racemization barrier of PCB 136, which was shown to be in excess of 210 kJ×mol⁻¹, in agreement with the results in Paper I, where a barrier of approximately 250 kJ×mol⁻¹ was found.

Jung and Schurig suggested that interactions with the cyclodextrin stationary phase raise the interconversion barrier by 4-17 kJ×mol⁻¹ [37], but in later off-column experiments it was proved that the interactions with the stationary phase do not increase the energy barrier significantly [30,33,36;Paper I].
2.3 Buttressing effect

The buttressing effect contributes 4-31 kJ×mol\(^{-1}\) to the rotational energy barrier\(^{25}\), according to calculations by the AM1 semi-empirical method, depending on the number of ortho-chlorines and vicinal meta-chlorines. No such effect was found by INDO calculations\(^{24}\). This effect is also considered in Paper I, in which PCBs with and without buttressing chlorines were compared. The PCBs were grouped into congeners with and without a vicinal meta-chlorine, which increased the statistical power of the analysis, resulting in a statistically significant (p = 0.01) buttressing effect. PCBs with one buttressing chlorine had 6.4 kJ×mol\(^{-1}\) higher rotational energy barrier than atropisomeric PCBs lacking buttressing chlorines. This is lower than the barrier of 13.8 kJ×mol\(^{-1}\) predicted by Andersson et al.\(^{25}\).
2.4 Rotational energy barrier calculations

Racemization of an atropisomeric PCB can be seen as a reversible first-order reaction:

\[
A \overset{k_1}{\rightleftharpoons} B
\]

The reaction rate for the forward and reverse enantiomerization reactions gives a net rate of change that depends on time \( t \):

\[
\frac{d[A]}{dt} = -k_1[A] + k_2[B]
\]

At the start of the experiment only one enantiomer is present in the solution so the initial concentration of \( A \) is \([A]_0\) and the concentration of \( B \) is zero. The interchange of one enantiomer to another does not change the total amount so at all times \([A] + [B] = [A]_0\). Therefore:

\[
\frac{d[A]}{dt} = -k_1[A] + k_2([A]_0 - [A]) = -(k_1 + k_2)[A]_0 + k_2[A]_0
\]

The solution of this first order-differential equation is:

\[
[A] = \left[\frac{k_2 - k_1 \exp(-(k_1 + k_2)t)}{k_1 + k_2}\right][A]_0
\]

Enantiomerization of PCBs takes place through rotation of the C\(_1\)-C\(_1'\) carbon-carbon bond that separates the phenyl rings (Figure 1), so we can assume that the two rate constants are equal, thus \( k_1 = k_2 = k \). This simplifies the equation to:

\[
\ln \left[ \frac{[A]_0}{[A]_0 - 2[A]} \right] = 2kt
\]

One way to derive the rate constant is to plot \( \ln[A]_0/[A]_0 - 2[A] \) versus time \( t \). The slope of such a plot is \( 2k \). An increase in temperature \( T \) (Kelvin) gives a lower rate constant \( k \) (sec\(^{-1}\)) which can be used to calculate the Arrhenius energy of activation \( E_a \), Gibbs free energy of activation \( \Delta G^\dagger \) or the entropy of activation \( \Delta S^\dagger \).
2.5 Enantiomeric fraction calculations

Enantiomeric Fractions (EFs) of the atropisomeric PCBs are calculated by dividing the peak area \( E_+ \) corresponding to the respective enantiomers by the sum of peak areas \( (E_+ + E_-) \):  

\[
EF_\gamma = \frac{E_+}{(E_+ + E_-)} \quad \text{and} \quad EF_\delta = \frac{E_1}{E_1 + E_2}
\]

The optical rotation of atropisomeric PCBs specifies how the formula is to be applied: the enantiomers causing clockwise rotation of polarized light \( E_- \) should always be used as the divisor, while the first eluting enantiomer \( E_1 \) should be used as the divisor when the optical rotation properties are unknown.

More frequently is the use of the enantiomeric ratios (ER) it is not until recent years that the use of EFs is becoming more popular. ERs are calculated by dividing the first eluting with the second eluting enantiomer as specified below:

\[
ER = \frac{E_1}{E_2}
\]

\[
EF = \frac{ER}{1 + ER} = \frac{1}{1 + \frac{1}{ER}}
\]

In pharmacological sciences the enantiomeric excess is frequently used, here the difference of the areas is divided by the summed areas and \( E_1 \) is always the peak with the enantiomeric excess.

\[
%ee = \frac{E_1 - E_2}{E_1 + E_2} \times 100
\]

The optical rotation has been experimentally assigned using pure enantiomers of PCBs 84, 132, 135, 136, 149, 174 and 176. On Chirasil-Dex the (+)-enantiomer elutes before the (−)-enantiomer for all atropisomers except PCBs 135 and 174, for which the elution order is reversed (Paper I).
2.6 Enantiospecific biological activity of chiral PCBs

PCBs are potent inducers of drug-metabolizing enzymes. PCB congeners differ in activity depending on their chlorine substitution patterns. PCBs with two para and two meta chloro substituents induce certain drug-metabolizing enzymes (Figure 6, left), and exhibit the same type of effects as the polychlorinated dioxins (PCDD) because of their structural similarity in coplanar form, which allows them to interact with the Ah-receptor. These compounds are also called MC (3-methylcholanthrene)-type inducers and characteristically increase Cytochrome P450 1A (CYP1A) and P450 1B (CYP1B) enzyme levels. If a PCB has one or more ortho chlorines introduced onto the biphenyl ring its activity changes since the coplanar conformation becomes less populated. PCBs with ortho substituents, like those with two ortho and two para substituents (Figure 6, right) cause phenobarbital (PB) type induction of drug-metabolizing enzymes.

Figure 6. PCBs with meta and para substituents on the left, compared to ortho and para substituents on the right.

PCBs with three or four ortho chlorines do not exist in coplanar configurations and will be stable atropisomers at ambient temperature. This also implies that these PCBs cannot be MC-type inducers. Studies performed by Puttnam et al. showed that racemic PCB 88 is inactive as an inducer of drug-metabolizing enzymes in Sprague-Dawley rats, while racemic PCBs 88, 139, and 197 all induced BPDM (benzphetamine N-demethylase) enzymes in a chick embryo hepatocyte culture. Furthermore, racemic PCB 139 (with an additional chlorine in para position as compared to PCB 88) is a PB-type inducer. This shows that the 2,2’,4,4’-substitution present in both PCB 139 and 197 is important for their drug-metabolizing enzyme activity. PCBs with more than two ortho
chlorines are PB-type inducers \(^{41}\) and tetra-\textit{ortho} PCBs induce BPDM activity \(^{43}\).

In a study by Puttmann et al. \(^{41}\) both racemates and atropisomers (enantiomers) of PCBs 139 and 197 were given through intraperitoneal injection to immature male Sprague-Dawley rats. The rats’ livers were later analyzed for drug-metabolizing enzyme activity (aminopyrine \(N\)-demethylase and aldrin epoxidase) and total cytochrome P-450 content.

High doses of PCB 139 racemate had statistically significant effects on the enzyme systems. The (+)-atropisomer of PCB 139 was a significantly stronger inducer of drug-metabolizing and cytochrome P-450 activity than the racemate, while (−)-PCB 139 had significantly lower activity.

PCB 197 is a tetra-\textit{ortho} and PCB 139 a tri-\textit{ortho} PCB (Figure 3). This means that PCB 197 has a high rotational barrier, but also low potency as a PB-type inducer of the cytochrome P-450 system \(^{41}\). Only the (−)-enantiomer of PCB 197 induced statistically significant rat liver aminopyrine \(N\)-demethylase activity. The (−)-enantiomers of PCB 197 (and PCB 88) also induced greater BPDM activity in chick hepatocyte culture \(^{43}\) than the corresponding (+)-enantiomers, while the opposite applied to PCB 139. BPDM and aminopyrine \(N\)-demethylase is associated with the same isozyme of cytochrome P-450.

The strongest chiral selectivity was observed for EROD induction in chick hepatocytes, for which (−)-PCB 139 is a potent EROD inducer and (+)-PCB 139 is a very weak inducer. The (+)-PCB 88 and PCB 197 enantiomers also showed EROD induction, while the (−)-enantiomers were inactive. Thus, EROD activity seems to be induced by the enantiomer that does not induce BPMD or aminopyrine \(N\)-demethylase.

However, it is important to note that the observed differences in induction between the (+)- and (−)-enantiomers may depend on factors other than potency. It is possible that pharmacokinetic parameters, such as absorption, distribution, metabolism and excretion, differ and create anomalies in the relative dose of the enantiomers. This was observed by Puttmann et al. \(^{41}\) who found an enantiomeric ratio 2:1 of the (+) and (−) PCB 139 enantiomers after administration of racemate immature male Sprague-Dawley rats.

A similar discovery was made by Mössner et al. \(^{44}\). They found only the (+)-\(\alpha\)-HCH (hexachlorocyclohexane) in the brain of neonatal and stillborn
northern fur seals. Also, in female adult common dolphin, high levels of α-HCH were found, with a (+)/(-) α-HCH enantiomeric ratio (ER) of ≥ 50. α-HCH accumulated primarily in the white matter of the brain tissue. In liver and blubber of northern fur seal both α-HCH enantiomers were present at ERs of 1.5-2.0, while PCBs were found in slightly higher concentrations in the blubber than in the liver and brain. DDT’s (including DDD and DDE) accumulate primarily in liver and blubber tissue. Thus, pharmacokinetic parameters appear to be responsible for the enantioselective accumulation of (+)-α-HCH in liver and blubber, and (especially) in the white matter of the brain. It is unclear whether enantioselective hindrance by the blood-brain barrier, protein complexation with one enantiomer, or enzymatic biotransformation causes the enrichment of (+)-α-HCH.

However, events in experiments in vitro, e.g. in chick hepatocyte cultures, are less strongly influenced by pharmacokinetic parameters than they are in vivo. Thus, despite the pharmacokinetic effects it seems certain that the tested atropisomers (enantiomers) have enantioselective effects on cytochrome P-450 induction, EROD, BPMD, aldrin epoxidase and aminopyrine N-demethylase activity.
3 Analysis

Analysis of polychlorinated biphenyls (PCBs) is a complex task since more than 140 congeners have been found in commercial mixtures and are frequently found in the environment, in addition some of them coelute. Thus, confident peak assignment and quantification requires comprehensive congener-specific analysis. Further, many other xenobiotics are also found in environmental samples, e.g. chlordanes, DDTs (DDD and DDE), toxaphene, and polybrominated diphenyl ethers, although most of these compounds are generally less abundant than the major PCB congeners. Clearly, there is a significant risk of analytical bias if individual PCB enantiomers have to be quantified in the presence of all other PCBs and pesticides, especially since the maximum acceptable error in the enantiomer fraction (EF) determinations is generally only a few percent.

Sample clean-up using pre-fractionation procedures are commonly employed before analysis to separate polar from less polar substances. Alumina, silica and Florisil columns may all be used to isolate a bulk PCB fraction. Further fractionation might be required to reduce the complexity of the resulting mixtures. PCBs may be separated according to the number of ortho chlorines substitution, i.e. according to planarity, using porous graphitic carbon \(^{(45)}\) or pyrenyl silica \(^{(46)}\), on which planar compounds are retained due to \(\pi-\pi\) electron interactions between the analytes and the stationary phase. A pyrenyl silica column was used by Ramos et al. \(^{(47,48)}\) to separate the atropisomeric PCB into three fractions which could be analyzed by chiral GC with electron capture detection (ECD). This fractionation technique removes many potential analytical interferences, and has recently been used in several studies on chiral PCBs \(^{(49-51)}\).

Another approach for analyzing chiral PCBs was presented by Wong et al. in a suite of publications \(^{(52-57)}\) in which they fractionated the sample extract (after removing lipids) into two fractions using 100 % activated silica gel. The PCBs eluted in the first fraction and chlorinated pesticides in the second. The atropisomeric PCBs were then analyzed using HRGC/MSD and several different cyclodextrin columns, selected not only to resolve the chiral PCBs, but also to separate them from possible coeluting compounds, especially PCBs with the same degree of chlorination. Thus, care was taken to avoid possible analytical bias that could have affected the analytical determination of the EFs.
A comparison has been made between the GC/MSD technique described above and a multidimensional GC/MSD technique, showing good agreement in results. The use of multidimensional gas chromatography in chiral GC will be discussed further below.

### 3.1 Chiral GC analysis using Cyclodextrin columns

Cyclodextrin is a cyclic oligosaccharide with various numbers of connected \( \alpha \)-D-glucose monomers. The number of monomers defines the type of cyclodextrin: \( \alpha \)-cyclodextrin has six glucose monomers, \( \beta \)-cyclodextrin seven and \( \gamma \)-cyclodextrin eight. Cyclodextrin has hydroxyl groups on C2, C3 and C6, which have to be derivatized, usually by deactivation with methyl groups, or other alkyl and/or silyl groups to decrease its polarity and make it less rigid (crystalline). The geometry of cyclodextrins is a torus with both an outer and an inner rim (Figure 7). As they are available in different sizes (\( \alpha \), \( \beta \), \( \gamma \)) and with many different C2, C3 and C6 functionalities it is possible to find cyclodextrin columns that separate all of the chiral PCBs, as well as many chiral pesticides and other persistent chiral pollutants.

Retained analytes form a host-guest complex with the cyclodextrin (CD). For instance, non-polar compounds may interact with the non-polar interior of the CD, as illustrated in Figure 8. PCB 45 fits into the lipophilic cavity of a derivatized cyclodextrin and is enantioselectively retained to a greater degree than its enantiomeric counterpart due to selective interaction with the groups on the rim of the CD-torus.

Many different \( \beta \)- and \( \gamma \)-cyclodextrin columns have been used as chiral selective phases (CSPs) for the separation of atropisomeric PCBs (Table 1). Heptakis(2,3-di-O-methyl-6-O-tert-hexyldimethylsilyl)-\( \beta \)-CD was the first cyclodextrin CSP to be used for GC separation of atropisomeric PCBs (enantiomers of PCBs 45, 95 and 139). Later, Hardt et al. were able to separate as many as 15 of 19 atropisomeric PCBs, i.e. PCBs 45, 84, 88, 91, 95, 131, 132, 135, 136, 139, 149, 174, 175, 176 and 183, but in this case they used four different cyclodextrin stationary phases. Vetter et al. separated seven atropisomers on a 6-O-tert-butyldimethylsilylated \( \beta \)-cyclodextrin column, notably PCB 144, which had not been previously separated (but the enantiomeric resolution was low).
Figure 7. Chemical structure and geometry of Cyclodextrins with assigned points of derivatization (positions 2, 3 and 6), reproduced with permission \cite{64}.
Figure 8. Schematic representation of a host-guest complex between PCB 45 and a covalently bound PMCD molecule, reproduced from 32.

Jaus and Oehme used a partially ethylated γ-cyclodextrin column 65 to separate seven atropisomers, including, for the first time, PCB 197 (with good resolution) 62.

Wong et al. separated all 19 atropisomeric PCBs using seven cyclodextrin columns. None of these columns gave a resolution greater than 0.5 for PCBs 144 and 175. For all other atropisomers a column is available that gives a resolution close to or exceeding 1.0. The cited authors concluded that at least four cyclodextrin columns were needed to resolve all atropisomers with a resolution \( (R_s) \geq 0.5 \) 63. However, five or six different columns are needed if an acceptable \( (R_s \sim 1) \) enantiomeric resolution is needed for most atropisomeric PCBs and coelution with non-chiral PCBs is to be avoided.

The requirements depend on the analytical technique used. If GC-ECD analysis is used for detection, more pre-separation is needed than if GC-MS (single ion monitoring) or multi-dimensional GC is deployed (in which cases HPLC fractionation of samples to remove non- and mono-ortho PCBs is almost essential). Each technique limits the types of columns that can be used, and the kinds of interferences that have to be avoided to achieve acceptable quality.
Table 1. Atropisomeric PCBs and stationary phases that enable their enantiomeric separation.

<table>
<thead>
<tr>
<th>Column</th>
<th>Ref 45 84 88 91 95 131 132 135 136 139 144 149 171 174 175 176 183 196 197</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heptakis(2,3-di-O-methyl-6-O-tert-hexyl-dimethylsilyl)-β-cyclodextrin</td>
<td>60 x x x x x x x x x x</td>
</tr>
<tr>
<td>Heptakis(2,3-di-O-methyl-6-O-tert-butyl-dimethylsilyl)-β-cyclodextrin</td>
<td>40,60 x x x x x x x x x x</td>
</tr>
<tr>
<td>Heptakis(2,3,6-tri-O-tert-butyldimethylsilyl)</td>
<td>61 x x x x x x x</td>
</tr>
<tr>
<td>Heptakis(2,6-di-O-methyl-3-O-Pentyl)-β-cyclodextrin</td>
<td>60 x x x x x x x x x x x</td>
</tr>
<tr>
<td>Octakis(2,6-di-O-methyl-3-O-Pentyl)-γ-cyclodextrin</td>
<td>58,60 x x x x x x x x x x x</td>
</tr>
<tr>
<td>Octakis(2,3,6-tri-O-ethyl)-γ-cyclodextrin</td>
<td>62 x x x x x x x x x x x</td>
</tr>
<tr>
<td>Heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin</td>
<td>40,59,62 x x x x x x x x x x x</td>
</tr>
<tr>
<td>Heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin</td>
<td>63 x x x x x x x x x x x x</td>
</tr>
<tr>
<td>Heptakis(2,3-di-O-methyl)-β-cyclodextrin</td>
<td>63 x x x x x x x x x x x x</td>
</tr>
<tr>
<td>Octakis(2,6-di-O-pentyl-3-trifluoro-acetyl)-γ-cyclodextrin</td>
<td>63 x x x x x x x x x x x x</td>
</tr>
<tr>
<td>(S)-2-hydroxypropyl methyl ester-β-cyclodextrin</td>
<td>63 x x x x x x x x x x x x</td>
</tr>
<tr>
<td>Hydroxypropyl-permethy-trifluoro-acetyl-γ-cyclodextrin</td>
<td>63 x x x x x x x x x x x x</td>
</tr>
</tbody>
</table>
Fortunately, only a few of the atropisomers are abundant in biota, which decreases the need for a large set of cyclodextrin columns. Probably not more than two different columns would be required in practice, for routine analysis. Most of the analytes of environmental interest are resolved using the Chirasil-Dex columns, which were first used by Schurig et al. for the separation of PCBs 84, 91, 95, 132, 136 and 149. Later, Haglund and Wiberg separated three additional atropisomers, i.e. PCBs 135, 174 and 176. Furthermore, the elution order of the (+)- and (–)-enantiomers have also been determined for many components on Chirasil-Dex: the (+)-enantiomers of PCBs 135 and 174 elute before the (–)-enantiomers, while the opposite applies for PCBs 84, 132, 136, 149 and 176 see also Paper I. This column was therefore selected for our analysis of atropisomeric PCBs in marine mammal samples.

### 3.2 Multidimensional gas chromatography (MDGC)

When one-dimensional gas chromatography (1D-GC) fails to resolve coeluting substances, multidimensional gas chromatography (MDGC) might be the solution. This technique is much more attractive than performing extensive off-line clean-up, which is expensive, time consuming and may both introduce contaminants and lead to solute losses.

In 1D-GC the peak capacity might not be sufficient to ensure unambiguous identification and quantification of target analytes, and there is considerable risk of analytical bias. By using MDGC, the peak capacity, in selected areas, is increased and the target analytes may be resolved from coelutants on a second column with a different kind of selectivity to the first column. In MDGC some of the effluent, containing the analytes of interest, is diverted to the second column through a heart-cut switching valve, usually a Deans switch. The more sophisticated MDGC systems consist of two independently temperature programmable ovens, dual detectors and a live T-piece that transfers a small portion of the first column effluent to the second column, usually via a cold trap.

This technique is particularly useful for separation of complex samples like petroleum distillates, where the analytes of interest may be embedded amongst several hundred other components, pesticides and other man-made persistent environmental pollutants, and also for enantioselective analyze. Specifically, the MDGC technique have been useful for analyzing chlordanes and toxaphenes; groups of chemicals consisting of hundreds to
thousands of congeners, which are nearly impossible to analyse by 1D-GC even if mass selective detection is employed. MDGC have also been used in the analysis of chiral toxicants, where chromatographic interferences frequently lead to analytical bias, resulting in incorrect estimates of enantiomeric fractions.

However, this technique has the following obvious limitations. Heart-cutting is only applicable to a few regions of the chromatogram, which may make multiple analytical rounds essential. Further, the instruments involved are complex, so operators need high levels of training, and it is also quite time consuming. These systems are therefore not commonly found in most laboratories.

3.2.1 Multidimensional GC of PCBs

Extensive work has been done on MDGC separation of PCBs. Generally, the aim has been to quantify specific congeners of some of the most toxic PCBs, i.e. non- and mono-ortho PCBs. Analysis of these PCBs is especially problematic since they are present at low levels in biotic and abiotic samples, so extensive LC fractionation is usually needed to obtain a clean non- and mono-ortho fraction. The concentrations of major PCB congeners (PCBs 138, 153 and 180) and non- and mono-ortho PCBs often differ by three orders of magnitude. In 1989 Schultz et al. re-examined technical PCB formulations of Aroclor and Clophen by MDGC in order to specifically identify and determine all congeners with a fractional concentration greater than 0.05 % w/w in any of the technical PCB mixtures. For this, they combined a non-polar SE-54 first column, from which they heart-cut transferred one or two regions per run onto the second column (OV-210 or C-87) for further separation and unambiguous identification and quantification of individual congeners. In this way they determined the concentrations of 132 congeners present in these technical mixtures. The information they obtained can be used for quantitative congener-specific determination of PCBs in environmental samples. In a later study, the relative retention times for all 209 PCBs were determined for a multitude of different columns. This data set provides a wealth of information to facilitate an intelligent choice of MDGC column combinations.
3.2.2 Multidimensional GC of atropisomeric PCBs

Atropisomers of PCBs have been analyzed by MDGC with an achiral column for pre-separation, followed by heart-cut transfer of the appropriate chromatographic region to a chiral column for enantiomer separation. Sometimes, but not always, the chromatographic bands have been cryogenically refocused before the chiral separation. However, this technique is tedious since it requires careful optimization and calibration. The main problem is to divert the correct fraction to the chiral column. Ageing of the columns, slight leaks, and various other factors can introduce shifts in the retention times. A good way to solve this problem is to use relative retention indices instead of absolute retention times and to perform real-time adjustment of the column switching times or possibly retention time locking. Nevertheless, it would still be a tedious task to analyze all of the atropisomeric PCBs of interest, since only a few heart-cuts can be made in each analysis. Thus, multiple injections would be required, each using different column switching times. However, in some cases, MDGC has been used as a confirmatory technique to verify the validity of enantiomeric ratios determined by GC/MSD.

3.3 Comprehensive two-dimensional gas chromatography (GC\times GC)

The resolution achieved in MDGC is much higher than in 1D-GC, but the total peak capacity is limited since only a few heart-cuts can be made in a single run. Two or more runs are therefore needed to separate all the components of interest.

An alternative to the MDGC heart-cut approach is to use comprehensive two-dimensional gas chromatography (GC\times GC). The systems involved are related as two serially connected columns of differing selectivity are used in both cases, usually with a non-polar first dimension column that separates components based on their volatility, and a second column that separates them according to some specific type of analyte-stationary phase interaction, usually polar, but in some cases shape-selective.

In MDGC the resolution is generally higher for the heart cut fractions than is achievable by GC\times GC. Nevertheless, the GC\times GC technique has many advantages compared to MDGC. In contrast to MDGC, GC\times GC is truly
comprehensive, meaning that the entire effluent from the first column is passed through the second column, thereby conserving mass.  

The heart of the GC×GC systems is the modulator (Figure 10), which is positioned between the two columns and prevents components separated in the first column from remixing in the second column. It also refocuses (compresses in time) the material eluting from the first column before re-injecting it into the second column for further separation. The refocusing process increases the detector signal intensity and the signal-to-noise (S/N) ratio. Kinghorn et al. showed that an increase in S/N of a factor of ten is possible. Commonly, the increases in S/N is between 5 to 10 fold, depending on the temperature gradient, the peak width of the focused analyte, band broadening in the second dimension column and the number of modulations over each first dimension peak.

A schematic illustration of the action of GC×GC is shown in Figure 9. The resulting GC chromatogram is generally sliced into time slices equal the modulation period and arranged in a matrix format, which is then plotted as either a 2D (contour) or 3D (surface) plot.

GC×GC provides unprecedented total peak capacity, thereby improving the separation not only between analytes, but also between the analytes and the matrix background (which, in turn reduces background-associated bias). This allows individual substances in complex matrices to be analyzed without the extensive sample fractionation that is otherwise required. If two columns with independent modes of separation are combined and the peak capacities of the first and second dimension columns are 500 and 20, respectively, the total peak capacity is the product of the two, i.e. 10000. If the elution order of all substances in the two dimensions is random and the system properly tuned all the separation space could be utilized. Interestingly, ordered structures are frequently found since the chemical classes’ share some properties, i.e. the substance groups are clustered due to molecular similarities. Consequently, the number of single component peaks cannot (theoretically) exceed 18 % of the available peak capacity, leading to a peak capacity of 1800 peaks, which is still excellent.
Figure 9. Schematics diagrams of the function of GC×GC: a) Two analytes peaks A and B which is partially resolved in 1D-GC; b) Cryogenically focussed into a narrow band and at a set interval (2-10 sec) is released to the fast 2D column, c) The chromatogram is converted into a x/y matrix (1D in the x-axis and 2D in the y-axis) and plotted as a topographic plot where the components A and B are separated.
Figure 10. Schematics diagrams of six types of GC×GC modulator: a) Metal clad thermal modulator; b) Thermal sweeper; c) LMCS; d) KT2003 loop modulator; e) KT2001 N\textsubscript{2}(l) cooling with thermal jets or dual jet CO\textsubscript{2}; f) Semi-rotating CO\textsubscript{2} cryogenic jet.
An additional advantage of the high peak capacity provided by GC×GC is that a lot of chromatographic space is empty, making it easy to determine the baseline level. In 1D-GC the true baseline is difficult to define due to the almost continuous elution of sample constituents from the column, which inevitably affects the precision of the peak area determination.

The ordered peak distribution that is achieved by GC×GC gives a 2D chromatographic pattern based on the structural similarity of the compounds; i.e. chemical classes are easily recognized, facilitating analyte identification. The pattern is also useful in the identification of unknown substances and in pattern analysis. For instance, it may be possible to pinpoint a point release of a xenobiotic into an area, since samples close to the source will generally have a pattern more influenced by the source than samples from areas further away from it.

3.3.1 History

The technique of comprehensive two-dimensional gas chromatography was invented by John B. Phillips in the late 1980s. He originated the idea of modulating the first dimension column effluent and pioneered the development of GC×GC. The first interface consisted of a metal-clad, thick-film capillary column, schematically represented in Figure 10a. Material eluting from the first column was retained by phase-ratio focusing and then remobilized by resistive heating. This system was difficult to manufacture and was replaced by a thermal sweeper system (Figure 10b), which was unfortunately not robust enough for routine use and was too limited in the range of analyte volatility it could handle. It was therefore phased out and replaced by a number of cryogenically cooled modulator systems in which the analytes are cryogenically trapped and later thermally desorbed by means of convection currents of oven air, thermal jets, or heating wires (Figure 10c-f).

In our studies, we used the longitudinally modulated cryogenic system (LMCS) of Marriott and Kinghorn, which focuses components eluting from the primary column at the position of the cryogenically cooled (mechanically actuated) moving trap. After a pre-set time the trap is rapidly moved away (upstream) and the analytes are revolatilized and then rapidly separated in the secondary column.
3.3.2 Comparison of modulator technologies

Marriott et al. \(^{91}\) compared two modulator interfaces, the LMCS and the thermal sweeper system, for the analysis of a mixture of 24 semi-volatile aromatic compounds. The analytical method was optimized on the thermal sweeper and later the same method and columns was used on the LMCS. Application limits of the thermal sweeper demanded that the temperature programming and lengths of the columns would have to be optimized on the thermal sweeper not to get analyte breakthrough on the thick phase modulator column. Peak widths and symmetry was used as a quality measure and was shown to be comparable between the two systems. The installation and optimization on the other hand is more easily achieved on the LMCS than on the sweeper system – a thick phase modulator column had to be installed between the first and the second dimension columns on the latter.

A recent study by Kristenson et al. \(^{92}\) compared all of the above mentioned modulator systems except the metal clad thermal modulator. As a quality measure they compared the analyte peak widths at half height and the resolution of two PCBs in the second dimension, which coelute in the first dimension. In addition, they evaluated the ruggedness and ease of optimization and commented on the practical limits of some of the modulators. The parameter that was important to get an optimal focusing of analyte was the flow rate of coolant N\(_2\) and liquid CO\(_2\) in the cryogenic devices and the stroke velocity and pause time of the thermal sweeper. The thermal sweeper was found to be limited in its dynamic temperature range and unsuitable for analysis of semi-volatiles due to the 100\(^\circ\)C higher temperature difference between sweeper and the highest temperature used by the GC. They concluded that all the cryogenic modulators are suitable for GC×GC of semi-volatiles but that there are some limitations with the KT2001 which makes this device more suitable for higher volatility compounds. They also stated that the LMCS and KT3003 needed additional optimization to be useful in the entire volatility range and that the CO\(_2\) jet modulators seemed to give the narrowest peaks over the entire application range.

3.3.3 GC×GC tuning

Optimization of GC×GC instruments is more complex than optimization of 1D GC systems, due to the need to tune the second dimension separation. The most important part of the optimization is to assemble an appropriate column set, as both columns are generally placed in the same oven and tuned simultaneously.
Tuning in GC×GC has been discussed by Venkatramani et al. and Beens et al.\textsuperscript{94,95} who suggested the following guidelines:

- The first dimension column should give rise to equal activity coefficients for all compounds studied, hopefully separating components mainly through differences in their boiling-points.\textbf{Comment:} in \textit{Paper III}, we deliberately broke this “rule” by using a highly selective column that gave large differences in activity coefficients for the substances, but excellent separation of the target PCB congeners.

- Temperature programming should elute components with equal retention factors.

- The secondary column should be short enough, in principal, to separate each modulated fraction under isothermal conditions.

- The differences in activity coefficients should be large on the second column, i.e. there should be big differences in selectivity between the two columns. This is usually achieved by a combining of a non-polar first dimension and a polar second dimension column.

Beens et al.\textsuperscript{95} generalized the GC×GC tuning rules, as follows:

- Match the dimensions of the second column to the modulation time. The secondary column should process analytes rapidly enough to avoid overlap of the most and least strongly retained components under essentially isothermal conditions.

- Tune the oven ramping rate to match the dimensions of the first dimension column and the separation characteristics of compounds on this specific column.

- Determine a flow-rate that is appropriate for both columns. Here the second dimension column is both short and narrow compared to the first column, so a flow rate of 30-40 cm/s in the first column gives rise to a flow rate of 100-200 cm/s on the second dimension column, which decreases the analysis time to a few seconds without any great loss in resolution compared to using an optimal linear flow. Hydrogen is preferred as carrier due its high diffusion rate.
The new types of modulators that are based on cryogenic cooling instead of phase ratio focusing (thermal sweepers) also requires tuning.

In *Paper II* the importance of proper tuning of the cryogenic modulation system was addressed. In this work, we used the LMCS system and encountered numerous problems, including non-Gaussian first dimension peak envelopes, breakthrough of analytes in the trap leading to chair-shaped fronting of modulated peaks; inefficient release of analytes, and modulated bleed, which can increase the analytical bias. All these phenomena usually indicate modulation problems.

These problems may be related to the following factors:

- Slow temperature ramping rates, which will cause the analytes to elute from the column at a low temperature, and thus decrease the desorption rate from the trapped region.

- Long first dimension columns or fast temperature ramping rates, which will cause analytes to elute at high temperature. Breakthrough might occur if the modulation temperature is set too close to the oven temperature.

- Modulation on a thick-walled column with high thermal mass and a slow desorption rate. The column geometry is of major importance, since a column with a narrow internal diameter, a thin capillary wall and a thin stationary phase improves focusing and re-volatilization.

- Column bleed is usually not a problem on columns currently produced by the major manufacturers. However, if problems do arise, specially manufactured low-bleed columns are available that phenylene- or carborane- stabilized. Still, in trace analysis of low level compound, such as dioxins and non- and mono-ortho PCBs using polar columns and a high temperature difference between the trap and the oven, the modulated bleed from the first column might cause problems.

Generally, the revolatilization rate is dependent on the temperature difference between the trap and the oven, the carrier gas flow rate, the column dimensions, and the column’s physico-chemical properties, requiring appropriate tuning of the trap temperature. The trap works best
with a constant temperature difference between the oven and trap. In Paper II it was set 70ºC below the oven temperature, but depending on the oven temperature programme used in the analysis of low volatility compounds, such as pesticides, PCBs, PCNs, dioxins and furans, toxaphenes and bornanes the trap temperature might have to be tuned in the range of 70 to 150ºC.

The actively heated cryogenic modulators like the Zoex KT2001 and KT2003 instruments (Figure 10d, e), are not influenced as much by the trapping and oven temperature difference. The thermal jets of these systems will rapidly heat the cooled area to a temperature above the current oven temperature (50ºC or more). This may, however, cause problems if the oven temperature program ends at a temperature close to the maximum temperature of the column, but it is unlikely to lead to severe problems as the area of modulation is short and the amount of stationary phase is small. However, this effect did lead to serious problems in the thermal sweeper systems, where a positive temperature offset of 100ºC was needed, which compromised the stationary phase integrity after a few hours and led to deterioration of the phase-ratio focusing. The effect was especially pronounced when semi-volatile compounds were analyzed. The thermal sweeper systems are also prone to leaks as they contain many press-fit connections.

3.4 Analysis of PCBs by GC×GC

So far no GC column has been developed that allows unambiguous determination of even the seven regulatory PCBs. Several of these compounds coelute with one or more of the other PCB congeners in 1D-GC, and not even MS detection solve the problem. Unambiguous peak assignment and quantification of the WHO-PCBs is even more demanding as these compounds are generally present at much lower concentrations than most of the major PCBs. Usually pre-fractionation is used to decrease the possibility of coelution. While this is a common and well-validated procedure, it is labour intensive and makes the analyses costly. Naturally, a method allowing simultaneous determination of a broad set of PCB congeners would be both attractive and useful, and a few research groups have attempted to use GC×GC to analyze PCBs, Paper III and IV.

In 1996 Patterson and coworkers used one of the first thermal sweeper systems (in combination with a high-resolution MS) to separate a 24-
component mixture containing the non-ortho PCBs 81, 77, 126 and 169 and a suite of PCDDs and PCDFs. For this, they exploited a combination of a DB-5 and an OV-1701 column, which separated the compounds in seven minutes, with a sensitivity increase of two orders of magnitude, and impressive sensitivity for the most toxic dioxin congener (2,3,7,8-TCDD) – 335 attograms with an S/N of 9.

In Paper III the LMCS system was used to analyze the toxic non-ortho PCBs 77, 105, 118, 126, 156, and 169. These congeners were successfully separated from other PCB congeners present in technical PCB mixtures within a period of 8 min (the total analysis time was 17 min). This separation was achieved by a combination of a shape selective liquid crystal column in the first dimension combined with a short, narrow bore non-polar 5%-phenyl methyl column in the second dimension. In addition to the toxic PCBs, seven regulatory PCBs were resolved, i.e. PCBs 28, 52, 101, 118, 138, 153, and 180. The planar PCBs (PCBs 77, 81, 126 and 169) were strongly retained on the liquid crystal column, and so eluted at a relatively high temperature and early from the 5%-phenyl second dimension column. These results indicate that the toxic PCBs may be analyzed without the need for an extensive cleanup procedure, and within a short time period. It would be possible to analyze three samples per hour, as compared to one sample per hour with standard GC/MS analysis. In addition, the ECD is sensitive enough to allow confident quantifications of trace analytes.

Korytar et al. used a thermal sweeper system to analyze a complex mixture containing 90 PCBs. They were able to completely separate the 12 toxic WHO non- and mono-ortho PCBs using two of three tested column combinations, i.e. HP-1 – HT-8 and HP-1 – SupelcoWax-10. The carborane HT-8 column, which is slightly shape selective, presented the PCB congeners as ordered structures, showing the highest retention factors for the non-ortho PCBs. Altogether, they were able to separate 78 of the 90 PCBs congeners with the HP-1 – HT-8 column combination. The HP-1 – BPX-50 combination only separated 71 congeners and did not separate the 12 toxic PCBs, while the HP-1 – SupelcoWax-10 column combination resolved no less than 84 congeners. To monitor the analytes they used the GCxGC in combination with a micro ECD, which gave excellent detection limits of 10 fg on-column. Further, they analyzed a cod liver extract and a standard mixture containing 17 TCDD/Fs and the 90 PCBs.
In the studies reported in Paper IV we evaluated the separation potential of GC×GC technology with respect to all 209 possible PCB congeners. These experiments were performed with the LMCS cryogenic modulator, and a non-polar DB-XLB as the first dimension column, selected for its low-bleed qualities and high PCB separation capacity. As second dimension column, a liquid crystal (LC-50) or a cyanopropyl column (SP-2340 and BPX70) was used, due to their dissimilarity in selectivity with respect to the nonpolar columns. These combinations are even more selective in the second dimension than the one used by Korytar et al. The cyanopropyl column has a strong dipolar momentum, while the smectic liquid crystal has an unparalleled shape selectivity, with a strong retention factor, especially for non-ortho PCBs. The cyanopropyl stationary phase also retains the planar PCBs, since the π-electron density of a planar compound is relatively easily polarized (induced dipole), but its selectivity is not nearly as strong as in liquid crystal phases. In these studies ECD was used, but a comparison of µECD and FID detectors, that was also performed in these experiments, showed that the µECD spoils some of the GC×GC chromatographic resolution due to detector-specific band broadening (peak tailing), probably because the makeup gas is less mobile in some regions of the detector than others, especially close to or at the column interface.

The seven frequently measured CBs 28, 52, 101, 118, 138, 153, 180, and the WHO-PCBs 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169 and 189 were all separated from Aroclor PCBs on the DB-XLB/LC-50 column set, whereas CBs 118 and 131 coeluted on the DB-XLB/SP-2340 column combination. With these column combinations we were able to separate 176 and 181 of the 209 congeners (on DB-XLB/SP-2340 and DB-XLB/LC-50, respectively), and 126 of 136 Aroclor PCBs, leaving just five unresolved congener pairs. For comparison, several technical Aroclors and a grey seal blubber sample from the Baltic Sea were also analyzed. For the identification and quantification of PCBs in the seal blubber sample and technical Aroclor mixtures GC×GC software was used which improves the ease of identification and quantification by comparing the interrelated distances between PCBs in two-dimensional chromatograms derived from a standard and the samples.

Comparing one-dimensional GC and GC×GC on this DB-XLB combination, we concluded that the improvement in total congener separation is improved by using GC×GC. Furthermore, the non-ortho and most mono-ortho PCBs are well separated from the other PCBs, which is
important since some of these congeners are present at barely detectable levels, while the concentration of some of the PCBs (especially 138, 153 and 180) might be 100-1000 times higher.

Korytar et al. 99 used the thermal sweeper system (Figure 10b), so they were obliged to optimize the column types and column dimensions within the operational restrictions imposed by the thermal sweeper to avoid breakthrough or badly modulated peaks. For this technique to work there must be a phase ratio focusing of the analyte on the thick phase (~3µm) modulator column. The analyte should have an elution speed below 10 mm/sec, which makes it important to optimize the first column phase ratio and column length, as well as to choose a temperature gradient that keeps the elution speed below 10 mm/sec on the modulator column. The LMCS system (Figure 10c) and the other cryogenic cooling systems are much more flexible, since they can be optimized solely by tuning the desired chromatographic parameters in the first and second dimensions, without having to bother with the limitations of the modulation technique. At least, this is my experience using the thermal sweeper and the LMCS system.

One practical disadvantage of using the sweeper system for high resolution PCB analysis is the excessive retention times sometimes obtained. In Korytar’s work the total analysis time was more than 2.5 hours. A similar separation was obtained in half the time using the LCMS system (Paper IV).

3.5 Analysis of chiral PCBs by GC×GC

MDGC has frequently been used in the analysis of chiral organochlorines, and in a few cases (chapter 3.2.2) it has been used specifically for the analysis of chiral PCBs. In investigations described in Paper V and VI the performance of a relatively new multidimensional technique was examined. In these studies, we utilized comprehensive two-dimensional gas chromatography with a combination of chiral and polar or shape selective columns: a chiral permethylated β-cyclodextrin, Chirsil-Dex column, with an LC-50 or a new high temperature (up to 290 °C) VF-23MS cyanopropyl column. With these systems we evaluated both the enantiomeric and the congener separation in both dimensions of a mixture of 144 PCBs that occur in technical PCB mixtures at levels of 0.05 % w/w or more. As the enantiomeric fractions will be affected by changes in the concentration of either PCB enantiomer the analytical procedure is of the highest importance.
Of the nine chiral PCBs for which Chirasil-Dex shows enantiomeric selectivity, i.e. PCBs 84, 91, 95, 132, 135, 136, 149, 174 and 176, six were baseline separated from otherwise coeluting PCBs in the second dimension using the liquid crystal column. However, PCB 95 was found to coelute with PCB 93 while PCB 84 was not completely resolved from PCB 99, which is a major congener in environmental samples. Furthermore, PCB 132 was not completely resolved from PCB 141. As mentioned above, we also used a cyanopropyl column (Paper VI), which resolved PCB 95 from 93, but PCB 132 coeluted with 141, while PCB 84 was partially resolved from PCB 56 instead, which is an improvement because PCB 56 was found in tissue samples of Baltic grey seal at levels far below the levels found for PCB 99. When evaluating levels and EFs of PCBs in blubber, liver, brain and muscle of female Baltic grey seals and a fetus muscle sample, the levels of many PCBs and the EF levels could be estimated in a single run, but not PCB 95, which meant that we had to use a cyanopropyl second dimension column for this PCB. In this particular analysis mass spectrometry would not have been especially useful since many of the coeluting PCBs have the same number of chlorines and would be irresolvable by MS. MDGC with a heart-cut approach could effectively separate the chiral PCBs from possible coelutants, but probably not in a single run, as was achieved for most of the nine PCBs by GC×GC (though not PCB 95). Further, almost all of the other PCBs could be quantified in the same analysis, which is not possible with MDGC even if MS is used for detection. PCBs that are not included in the heart-cut might coelute more frequently with other PCBs. In GC×GC most of the non-ortho PCBs were either not detected at all, or they were present at concentrations below the limit of detection and thus could not be quantified.

Fractionating the PCB extract of environmental samples with pyrenyl-silica liquid chromatography gives many fractions with differing degrees of chlorination, and the 19 atropisomeric PCBs are distributed in three of these fractions. Chiral PCB analysis of a few samples that each generates many fractions would be very time-consuming, especially since the analysis of chiral PCBs requires a slow temperature gradient (0.5-1°C/min or even isothermal conditions), so analysis times exceed an hour. Thus each sample would take several hours to analyze, especially if the 12 most toxic PCBs as well as the seven most frequently measured PCBs were to be quantified.
3.6 Column characterization

As a pre-study to the practical use of GCxGC, columns with different selectivities were characterized, starting with a smectic liquid crystal, a 5% phenyl dimethyl siloxane and a permethylated β-cyclodextrin column, in order to determine the PCB retention order \(^ {101}\). This investigation was later expanded to include additional columns, some of which were used in Papers II through VI, see Appendix II, where the relative retention times of all 209 PCBs are tabulated for 12 types of column. The analysis was performed on a GC/MSD and the temperature programming, head pressure and column dimensions are listed in Table 2. The column types were as follows:

- A standard J&W DB-5 (5% phenyl) capillary column and a proprietary J&W DB-XLB column: a low polarity, low bleed column with an exceptional separation capability for PCBs.
- A narrow bore J&W DB-WAX microbore column with a high polarity polyethylene glycol bonded phase (PEG), and a J&W DB-210 microbore column, with a bonded and highly polar stationary phase consisting of (50%-Trifluoropropyl)-methylpolysiloxane.
- A SGE SolGel-1 column, which contains a bonded (100% methyl) polydimethylsiloxane phase and a SGE SolGel-WAX column, which contains a bonded polyethylene glycol phase, encapsulated in synthetic glass (Sol-Gel).
- A Macherey-nagel Optima δ-6 column, which has a seemingly “auto selective” polarity with cross-linked methyl-phenyl-polysiloxane.
- A highly polar capillary column, the Varian CP-SIL88, consisting of 100% biscyanopropyl.
- Two J&K Scientific LC-50 columns, with slightly different dimensions as shown in Table 2 and a smectic liquid crystal stationary phase of 50% liquid crystalline methylpolysiloxane.
- Two Varian CP Chirasil-Dex CB columns, of differing dimensions, with chiral selectivity, containing 10% permethylated β-cyclodextrin.
<table>
<thead>
<tr>
<th>Column set</th>
<th>Capillary column Dimensions</th>
<th>Init. Temp</th>
<th>Init. Hold</th>
<th>1st rate</th>
<th>1st level</th>
<th>2nd rate</th>
<th>Final Temp</th>
<th>Headpress.</th>
<th>Flow rate&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB5</td>
<td>30 0.25 0.25 90 1.5 30 140 2 270 72 37</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>XLB</td>
<td>60 0.18 0.18 90 1.5 30 140 2 320 480 30&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Opt6</td>
<td>50 0.20 0.20 90 1.5 30 140 2 320 410 37&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Sol1</td>
<td>30 0.25 0.25 90 1.5 30 140 2 280 56 35</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>SolW</td>
<td>30 0.25 0.25 90 1.5 30 140 2 280 64 35</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>DBW</td>
<td>10 0.10 0.10 70 1.5 30 100 2 250 350 45</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>LC.15</td>
<td>10 0.15 0.10 90 1.5 30 100 2 210&lt;sup&gt;c&lt;/sup&gt; 310 46</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC.25</td>
<td>20 0.25 0.10 80 2 3 260 - - - -</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD.1</td>
<td>10 0.10 0.10 90 2 30 140 2 260 380 46</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>CD.25</td>
<td>25 0.25 0.25 80 2 3 260 - - - -</td>
<td></td>
<td></td>
<td></td>
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</table>

<sup>a</sup> Average flow rate in the capillary column, determined by measuring the holdup time for a headspace injection of argon.
<sup>b</sup> Carrier gas flow in constant pressure mode at 90°C.
<sup>c</sup> Further temperature ramping of 30°C/min to 240°C was made even if PCB 209 and OCN eluted below 210°C.
The GC×GC column combinations used were selected by consulting an extensive relative retention time (RRT) database including RRTs for all 209 PCBs. The database includes figures obtained from an extensive inter-laboratory effort coordinated by G.M. Frame and the in-house GC/MSD evaluation of 12 additional columns with a wide spread of stationary phase properties (polar, non-polar, chiral and shape selective) as described above. The selection of columns for GC×GC is based on differences in selectivity. Commonly, only a non-polar column is needed in the first dimension since separation is based largely on differences in the inherent vapor pressures of individual PCBs and, thus, on the number of chlorines in the different PCBs, although the substitution pattern will influence the retention order to a degree, even on a 100 % methyl polysiloxane column. Three types of chromatographic selectivity can be deployed in the second dimension based on dipolarity (cyanopropyl, PEG and tri-fluoropropyl stationary phases), induced polarity (phenyl columns; especially 50 % phenyl) and shape selectivity (liquid crystal, carborane and, in part, octyl and octadecyl type of stationary phases).

Evaluating the separation capability or differences in selectivity of different column combinations can be done in several ways. The first is simple comparison by regression analysis, where RRT data of two different columns are plotted against each other and the linear regression coefficient is calculated, a low regression coefficient indicating significant differences in selectivity.

Furthermore, the spread around the regression line in a plot of a non-polar vs. a polar GC column describes the selective analyte – stationary phase interactions in the latter. The distance between an individual PCB and the regression line (orthogonal to the non-polar column RT axis), ∆RT, will therefore correlate with the second dimension retention time in GC×GC (with a non-polar first and polar second dimension column). Thus, an approximate GC×GC substance pattern can be derived by plotting the first dimension retention times vs. ∆RT. This makes it easy to evaluate the probability of separating two substances in the second dimension that would coelute on the first dimension column.

Another possibility is to use a retention index (RI) system for PCBs. In this approach, a PCB representing each chlorination degree from mono to deca is selected (in this case PCBs 1, 4, 19, 54, 104, 155, 188, 202, 208 and 209) that is among the first PCBs of its respective class to elute, and each of these
PCBs is assigned a retention index of 100 to 1000. After the calculation of each retention index for the first and second dimension columns, the RI of the first is subtracted from the retention index of the second, giving relative retention index (ΔRI) values for each PCB. Plotting the RI for the first column against the ΔRI will give a similar plot to a GC×GC two-dimensional chromatogram. This technique has been described in detail in the thesis by Jingzhen Xu.

Another way to compare capillary column phases is to use principal component analysis (PCA). Information in the RRT database of Frame and the 12-column RRT data generated in our lab was collected into a single spreadsheet, checked for skewedness of the RRT data (due to the oven temperature programmes used) and a set of RRT data was selected as representative for each column type. The data were centered and normalized, and the principal components (PCs) were extracted. Figure 11 showing the corresponding score and loading plots. The first three PCs describe 99.7 % of the variation in the data, and 98.7 % is explained by the first component, showing that the main variation in the RRT data for all the different columns is strongly inter-related, with strong dependence on the vapor pressure of the PCBs.

In Figure 11 only the second and third PCs are plotted, which explain 0.8 % and 0.2 % of the variation in the RRT data, respectively. The further the columns are from each other in the loading plot, the higher the selectivity difference between them. Here, there is clearly a considerable distance between the non-polar columns (such as the 100%Me and DB-XLB columns) and the strongly polar cyanopropyl columns (75% CyP and 100% CyP) and liquid crystal column (LC50), while the 50% phenyl column (50%Ph) has quite different locations showing that the selectivity of this type of column is distinct, due to its polarity-inducing potential. The other shape selective columns are much closer to the non-polar columns: the carborane and (especially) the octyl and octadecane columns. Even so, the potential of the carborane column as a selective stationary phase has been demonstrated by Korytar et al.

The scores plot shows clear correlations between the positioning of the non-ortho, tri- and tetra-ortho PCBs with the various column types, since the polar and liquid crystal phases are oriented in the same direction as the PCBs 77, 126 and 169 while the nonpolar and phenyl column are closer to the tetra-ortho PCBs 54 and 155.
Figure 11. Score (above) and loading (below) plot. The second and third PCs defined by loadings (different columns) and scores (PCBs) relating column selectivity to PCB congener pattern. The score and loading plots describe the correlation among PCB congeners and GC phases, respectively. Objects close to each other have similar properties and vice versa.
4 Chiral PCBs in environmental samples

Levels and chiral signatures of atropisomeric PCBs have seldom been studied, largely because environmental analysis is mainly concerned with measuring total levels of PCBs, or levels of the most toxic congeners, i.e. the non- and mono-ortho PCBs. These congeners have a similar structure in their planar conformation to the toxic polychlorinated dibenzodioxins and furans (PCDD/Fs), and have been assigned dioxin toxic equivalency (TEF) values by the WHO (TEF)\(^{106}\). PCB 126 has been assigned the largest value (TEF = 0.1) of the PCBs and is often monitored.

There are also certain requirements to consider when analyzing chiral PCBs that are not easily met. Frequently, rigorous cleanup and fractionation (by HPLC on a pyrenyl silica column\(^{47}\)) of the samples are needed to decrease bias caused by other pollutants, and more than one chiral column is required to achieve sufficient resolution. Chiral analyses also take a long time as isothermal conditions or slow temperature ramps are needed, to ensure there is sufficient chiral resolution. Furthermore, reversal of the elution order of enantiomers has also been observed from one batch of cyclodextrin columns to another\(^{107}\). Knowledge of the elution order is important to allow results from different environmental samples to be compared. Therefore, the elution order has to be checked using pure enantiomers prior to analysis.

As chiral PCBs are released into the environment as racemic mixtures and abiotic processes such as oxidation, hydrolysis, adsorption, desorption and photochemistry are non-selective, the non-racemic ratios of chiral substances found in the environment must be due to biotic enzymatic transformations\(^{108,109}\). This makes enantiomers potentially useful as tracers of biological activity\(^{108-114}\). In addition, these bioprocesses may give rise to differences in enantiomeric fractions (EFs) that are unique for specific organisms: even neighboring river streams might show variations due to differences in their microbial communities\(^56\).

Atropisomeric PCBs have been studied in many different samples, mainly from the marine environment\(^{25,50,52,55,57,74,115}\) and riparian biota\(^{47,55}\), but samples from humans\(^{51,72}\) and abiotic material (sediments) have also been examined\(^{56,57,73,75}\).
4.1 Sediments

Sediments comprise one of the major sinks for environmental pollutants, so monitoring these materials is important for our understanding of bioconcentration, bioaccumulation and biomagnification in the aquatic food chain. Such sediment studies should also provide valuable indications of temporal, spatial and regional trends in pollutant levels, and of pollutant releases from point sources, including river sediments.

Benicka et al. 73 analyzed the chiral PCBs 84, 91 and 95 in a standard reference material (SRM) of river sediment from the Hudson River, New York. While racemic levels were found for PCB 91, enantioselective degradation was observed for PCB 95. These findings were later verified by Wong et al. 57, who also analyzed sediment samples from selected sites in various parts of the United States 56. In this study PCBs 91, 95, 132, 136, 149, 174, and 176, were analyzed, and results showed that many chiral PCBs showed non-racemic profiles in the river basin sediments, indicating that enantioselective biotransformation processes may have occurred. They also found a specific non-racemic profile at highly contaminated locations that was consistent with well-known reductive chlorination patterns, suggesting that the PCBs found at these sites are biotransformed.

Furthermore, while non-racemic PCB 91 was found (ER = 0.56) close to the most heavily contaminated location, racemic levels of PCB 91 were found further downstream, where the total PCB concentrations were lower. In contrast to these findings, Wong et al. 57 found mainly racemic PCBs 91, 95, 136, 149, 174, 176, and 183 in sediment samples and a certified reference material (CRM) from Lake Ontario, Canada, suggesting that very little biotransformation had occurred at these locations. This was consistent with other reports that evidence for reductive dechlorination in this area was inconclusive. Wong et al. assumed that this site is more representative of naturally contaminated areas. These findings are consistent with the analytical results reported by Glausch et al. 75, who analyzed river sediments from a river in southern Germany and only found racemic mixtures of PCBs 95, 132 and 149.

4.2 Aquatic organisms

Enantioselective degradation of atropisomeric PCBs in marine biota was studied by Hühnerfuss et al. 116. The blue mussel (Mytilus edulis L.) was chosen for this investigations for its ability to accumulate lipophilic environmental contaminants, its low enzymatic degradation potential, and
because it is a good indicator of water pollutants in marine ecosystems. Blue mussels were collected from the German Bight, and analyzed for coplanar and atropisomeric PCBs. Five atropisomeric PCBs were found in the blue mussel samples, viz. PCBs 88, 149, 171, 174, and PCB 183 (possibly originating from commercial PCB formulations). However, only PCB 149 was present in sufficiently high concentrations to allow enantiomeric separation by GC-ECD with a capillary column coated with a modified cyclodextrin phase. Samples collected from only two of six sites in the German bight showed statistically significant evidence for enzymatic degradation, with ER values of 1.2. The cited authors concluded that only slight enzymatic degradation occurs in blue mussels.

On the other hand, Wong et al. 55 detected a strongly enantioselective distribution of atropisomeric PCBs in aquatic wildlife and riparian biota (fish, bivalves, crayfish, water snakes, barn swallows) from a highly contaminated site at Lake Hartwell, SC, United States. They also found mainly non-racemic PCBs 91, 95, 132, 136, 149, 174, 176 and 183 in river fish and bivalves nationwide. The enantiomeric profiles of sediment from the same sites were markedly different, suggesting that enantioselective bioprocesses may vary between different organisms due to differences in their ability to metabolically degrade PCBs.

Three reports have been published on chiral PCBs in cetaceans found dead and beached in the Mediterranean Sea 49,50 and native subsistent harvest in Barrow, AK 52. Generally, the cetaceans showed non-racemic ratios of the chiral PCBs. In striped dolphins 49, the PCBs 95, 132, 135, 149 and 176 were non-racemic with an enantiomeric excess of the second eluting enantiomer in almost all samples (blubber and liver). PCBs 136 and 174, in contrast, were found to be racemic. Four cetacean species were studied by Jimenez et al. (Risso’s Dolphin, bottlenose dolphin, fin whale and long-finned pilot whale) 50. The results were similar to those of Reich et al. 49, see above. In two of the investigated dolphin liver samples Reich et al reported, for the first time in biological samples, significant enantioselective degradation of PCBs 84 and 91 with enantiomeric excesses (ee) of 86 % and 32 % for PCBs 91 and 84, respectively. Usually lightly chlorinated PCBs are found at low concentrations as they are more easily metabolized which makes them harder to detect.

Blubber and liver samples of bowhead whale (Barrow, AK) and zooplankton were analyzed by Hoekstra et al. 52 for chiral PCBs and they
noted, as for cetaceans from the Mediterranean Sea (see above), an enantioselective accumulation of the chiral PCBs in blubber, but not in liver or zooplankton. As the whales feed on zooplankton, which have only racemic chiral PCBs, this suggests that the non-racemic levels found in blubber must be due to enantioselective uptake, metabolization and/or excretion. Further, their results suggest that EFs were correlated with the body length of the whales, and that the non-racemic levels found for these chiral PCBs is influenced by the total PCB concentration, age and/or metabolic changes in the whales during sexual maturity.

Klobes et al. \textsuperscript{115} studied the chiral PCB 149 in blubber from harbor and grey seals from Iceland, and found ERs > 1 in both species (1.5-2.3 and 1.7-2.3 for grey and harbour seals, respectively). Even though these species had similar ERs, a difference compared to other seal species was observed, suggesting that there are metabolic differences between the species, and that the habitat might influence the degree of enantiospecificity.

In the investigations reported in \textbf{Paper VI}, nine chiral PCBs, viz. PCBs 84, 91, 95, 132, 135, 136, 149, 174 and 176, were analyzed in six female Baltic grey seals and a fetus from one of the grey seals. Seals and other marine organisms in the Baltic Sea area, e.g. salmon, herring, and sea birds, are heavily exposed to xenobiotics from agricultural and industrial activities around the Baltic. Consequently, PCBs are found at extremely high levels (mg/kg body weight) \textsuperscript{10} in Baltic seals and they are suspected to be the causal agents of occlusions and sterility among female grey seals. The levels of PCBs are higher in affected than in healthy animals, but there is considerable natural variation. The EFs of these atropisomeric PCBs are of particular interest, since they may reflect differences in metabolization rates and specificities.

EFs of some of the atropisomeric PCBs were difficult to determine, as the levels were low and there were many interferences. The concentrations of PCBs 84 and 91 were close to or below the signal to noise level (s/n ≥ 5) and PCBs 136 and 176 were not detected in the samples. Presumably, therefore, the metabolic degradation is higher for lightly chlorinated PCBs. However, EFs were determined for the remaining five atropisomeric PCBs, i.e. PCBs 91, 95, 132, 149 and 174. All these atropisomers had EFs that deviated from racemic to a greater or lesser extent. PCBs 95 and 132 had EFs > 0.5 while PCBs 91, 149 and 174 had EFs < 0.5. The largest deviations from racemic enantiomer composition was observed for the PCBs 91, 95
and 132 (Figure 12) while PCBs 149 and 174 were closer to racemic (with EFs of 0.33-0.45 and 0.38-0.49, respectively) even though there was clear evidence of the enantioselective degradation of these xenobiotics too.

The deviations from racemic ratios might correlate with the presence of vicinal hydrogens in both ortho/meta and meta/para positions. Generally, the congeners that lack vicinal meta/para hydrogens are more persistent and readily bioaccumulated \(^{117}\). In particular, PCB congeners with a 2,4,5 or 2,3,5 chlorine substitution pattern, on both phenyl rings, have shown to be more persistent and less susceptible to metabolic degradation \(^{117}\). A clear relationship between the substitution pattern and degree of enantioselective transformation was found. The correlations between the presence of vicinal hydrogens in ortho/meta and meta/para positions and racemic status of the PCBs shows (Table 3) that the most non-racemic PCBs had vicinal hydrogens in at least two positions. For instance, PCB 132 has both ortho/meta and meta/para hydrogens while PCB 174 has vicinal hydrogens only in the meta/para position.

The deviation in EFs from racemic was larger in liver than in blubber, indicating that enantioselective metabolism occurs in the liver. However, there was no selective passage of the studied atropisomeric PCBs across the placenta, and no selective blood-brain barrier (Paper VI), although enantioselective accumulation of the chiral pesticide α-HCH has been observed in the brain \(^{44}\). Similarly, no correlation between EFs and health status was observed, which agrees with the results of Klobes et al. \(^{115}\) – although there was a correlation between the total PCB levels found in the grey seal samples from the Baltic Sea and their health status.

### 4.3 Human tissues

Human milk samples were analyzed for chiral PCBs by Glausch et al. \(^{72}\). These authors focused on PCB 132 because of its thermal stability and high metabolic transformation potential as compared to other atropisomeric PCBs (related to its vicinal hydrogens in both ortho, meta and meta, para positions). PCB 132 is also abundant in commercial PCB formulations.
Figure 12. Enantiomeric fractions of atropisomeric PCBs in different female Baltic Grey seal tissue samples.
Chiral PCBs in environmental samples

The results showed an enrichment of the second eluting enantiomer, which indicates enantioselective metabolism, or bioaccumulation, or even a combination of these processes. In a later study these authors studied PCBs 95 and 149, but only slight enantiomeric enrichment was found for PCB 95, while PCB 149 was racemic. This can be explained by the differences in substitution pattern, discussed above, since the probable relative metabolization rate is $132 > 95 > 149$.

Table 3. Substitution patterns of the atropisomeric PCBs.

<table>
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<tr>
<th>IUPAC</th>
<th>Substitution Pattern</th>
<th>No. ortho-meta</th>
<th>No. meta-para</th>
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<td>95</td>
<td>236-25</td>
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Human tissue samples (liver, muscle, brain, kidney) have been analyzed from Belgian males and females collected in an extensive investigation of the possible effects of a feed contamination episode in which 50 kg PCB (contaminated with 1 kg of dioxins) was mixed into 500 tons of feed that...
was then distributed to animal farms in January 1999\textsuperscript{118}. The chiral PCBs (95, 132 and 149) studied were found in racemic proportions in muscle, kidney and brain but in non-racemic proportions in liver samples. Correlation analysis suggested that the enantioselective metabolism of the three chiral PCBs followed the same trends (although ratios differed) and that the ER patterns depended more on the daily intake than on age or sex of the subjects.
5 Concluding remarks and future work

The GC×GC technique has to be further validated and possibly improved in order to be routinely used for the analysis of environmental samples. Analysis of PCBs and other semi-volatile persistent environmental pollutants and chiral substances should be validated by doing parallel analysis of samples by GC×GC and standard validated analytical methods, e.g. cleanup with pyrenyl silica LC and analysis by GC/MSD of PCBs including chiral PCBs. A great deal of enantioselective biotransformation of chiral PCBs is seen in many parts of the biota which would make it important to further evaluate the effects of chiral PCBs on organisms, even if no correlation between nonracemic levels of PCBs and health status have been found. The food web of the Baltic Sea and the sediments should be analysed for chiral PCBs to be able to evaluate the possible effect and the source(s) for the enantioselective bioaccumulation and degradation.

Even with current GC×GC technologies, the cryogenic modulators can be further simplified and integrated into the gas chromatographs and has to be easier to use for the common analytical population. The development of GC×GC has led to more rugged devices and will probably be further improved with new technology and software to increase the usability of this device for routine analysis. I see in the near future a GC×GC device where the entire instrument will be integrated on a single silicone chip. The first and second dimension columns will be etched into the silicon chip with a micro cooling device as modulator, preferably the cooling will be achieved by which heat is transported from one side of a silicon plate to the other side with a nanometre wide gap between the plates to take advantage of the electron tunnelling effect. Etching the modulator column on a chip which has an extremely low thermal mass will decrease the energy consumption and increase the speed of heating and cooling the device to be able to cope with the 10 000°C/second which will be needed. In addition, an integrated FID, µECD, TCD with a cell volume of 10µL and possibly a small time of flight MS would be appropriate as detectors. The electronics would be separate from the device and the silicon chip (etched first, second and modulation column) would be modular and be changed between different analytical applications or when the columns start to deteriorate or when the detectors are dirty.
6 Acknowledgement

Jag vill tacka alla trevliga människor på Miljökemi, jag tror knappast att man kan få uppleva en bättre arbetsplats än denna och den gemenskap som jag hoppas att alla känner och alla dessa gemensamma och roliga aktiviteter som ordnas. Jag vill speciellt tacka min huvudhandledare Peter Haglund som ledde in mig på denna forskarbana och som fungerat som ett bra bollplank för ideer när problem uppstått (vilka har varit många) samt alla dessa massakreringsar med rödpenna som du utfört på mina intet ont anande manuskript. Konferans resor till Italien som du och jag har åkt på har varit mycket trevliga och nyttiga vilket jag verkligen uppskattar att jag har fått möjlighet att göra. Början av min doktorering var lite kärv med tiden blev vägen lite jämnare och trevligare att färdas efter. Allt började med att jag sökte ett Kempe stipendium för att göra kunna göra några intressanta analyser av kirala PCB med en ny typ av analysinstrument (tvådimensionell gas kromatografi) och vi räknade kallt med att vi skulle kunna göra projektet på ungefär 3 månader och här är jag 6 år senare och har äntligen lyckats utföra vettiga analyser av kirala PCB. Tänk vad tiden går! Jag vill också tacka min biträdande handledare Mats Tysklind som kom som en räddande modellerande ängel med ett (flamm)skydd projekt som förbättrade min personliga ekonomi betydligt och som gjorde att jag fick tänka lite mer vari(er)at.

Men viktigaste av allt så vill jag tacka min familj och min flickvän och blivande mor till vårat barn, jag älskar dig!

Jag vill tacka Anders O som har dragit med mig på alla fester och fisketurer mm och som introducerade mig för Åsa, du hade nog planerat det där från böjan.
7 References

References


References

8 Appendix I

Systematic numbering of PCB according to IUPAC rules.

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9 Appendix II

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