Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1019 (2003) 127–142
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of atropisomeric and planar polychlorinated biphenyls, their enantiomeric fractions and tissue distribution in grey seals using comprehensive 2D gas chromatography

Mikael Harju [a,*], Anders Bergman [b], Mats Olsson [b], Anna Roos [b], Peter Haglund [a]

[a] *Department of Chemistry, Institute of Environmental Chemistry, Umeå University, SE-901 87 Umeå, Sweden*
[b] *Contaminant Research Group, Swedish Museum of Natural History, Box 50007, SE-10405 Stockholm, Sweden*

Abstract

High prevalence of uterine occlusions and sterility is found among Baltic ringed and grey seal. Polychlorinated biphenyls (CBs) are suspected to be the main cause. The CB concentrations are higher in affected than in healthy animals, but the natural variation is considerable. Thus, it might be possible to assess the health status of seals by CB analysis. The ratios of chiral compounds (enantiomeric fractions (EFs)) such as atropisomeric CBs are of particular interest, since these may reflect differences in metabolic rates. An analytical procedure was developed and used to determine the levels of atropisomeric CBs, planar-CBs (WHO-PCBs) and total CBs in seals of different health status. Comprehensive 2D gas chromatography (GC × GC) was used to separate the target analytes from other CBs and interferences and a micro electron-capture detector (μECD) was used for detection. EFs of the atropisomeric CBs were difficult to determine as the levels were low and the interferences many. Two column combinations had to be used to avoid biased results—both had a chiral column as first-dimension column. The second-dimension column was coated with either a high-polarity cyanopropyl or a liquid crystal phase. EFs were determined for five atropisomeric CBs, i.e. CBs 91, 95, 132, 149 and 174. The results were verified by GC × GC–time-of-flight mass spectrometry (TOF-MS). Some atropisomeric CBs had EFs that deviated strongly from the racemic-mixture value. The deviations were larger in liver than blubber, which indicates enantioselective metabolism. However, there was no selective passage of the studied atropisomeric CBs across placenta and no selective blood–brain barrier. Similarly, no correlation between EFs and health status was observed, although there was a correlation between the total CB levels and health status.

Keywords: Gas chromatography, comprehensive two-dimensional; Atropisomers; Enantiomeric fractions; Baltic grey seal; Polychlorinated biphenyls

1. Introduction

The Baltic Sea is a highly polluted marine environment, in which top predators such as marine mammals have high levels of persistent organic pollutants (POPs), e.g. DDTs, PCBs, PBDEs, PCDD/Fs and chlordanes. Three seal species live in the Baltic: the ringed (*Phoca hispida botnica*), grey (*Halichoerus grypus*) and harbour (*Phoca vitulina vitulina*) seals. In the mid-1970s, uterine occlusions were frequently found in ringed seals and grey seals. On the basis of

* Corresponding author. Fax: +46-90-12-81-33.
E-mail address: mikael.harju@chem.umu.se (M. Harju).

0021-9673/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2003.08.102

available data on contaminant concentrations in the Baltic, CBs are suspected to be responsible for the occurrence of sterility among Baltic seals. This hypothesis is supported by laboratory experiments in which CBs have been shown to cause reproductive failure in mammals. For instance, studies on American mink (*Mustela vison*) have shown that CBs strongly affect reproduction [1–4].

The levels of POPs, have been shown to be higher in affected animals than in healthy animals [5,6]. However, there is considerable biological variation in the POP data. It is therefore difficult to judge whether the tissue levels are high enough to cause reproductive impairments when using procedures such as biopsies. Instead, full pathological evaluation is required.

Enzyme induction is a secondary effect of the high concentrations of POPs in mammals [7]. Detoxification systems such as the P-450 enzymes may transform lipophilic compounds to more hydrophilic compounds that may be excreted. Different isozymes of P-450, varying in their substrate specificities, are induced to differing degrees by various classes of inducing agents. Thus, changes in both metabolic capacity and specificity may result from POP exposure. In this study we investigate whether such differences lead to variations in the relative ratio of CBs, including atropisomeric CBs. Such ratios often reflect the biological processes occurring in a species better than the total concentrations of pollutants. It is also easier to determine ratios than absolute concentrations. Thus, it might be easier to assess the health status of seals by such ratio measurements.

Among the 209 CB congeners, 19 tri- and tetra-*ortho*-chlorinated congeners were predicted by Kaiser [8] to exist in stable atropisomeric conformations at ambient temperatures due to a restricted rotation around the central σ-bond in the biphenyl group. This was later experimentally verified [9–11].

Chiral CBs are released into the environment as racemic mixtures. Their composition in sediments is also generally close to racemic [12–14]. However, organisms have been shown to enantiomerically enrich many of the atropisomeric CBs [15], which suggests that enantioselective biotransformation occurs. Non-racemic CB enrichment has also been found in humans [16,17]. This is of particular concern because of the health risk it may pose to infants. It has also been reported that enantioselective biological activity differs between several of the atropisomeric CBs [18,19], which makes it important to identify the individual atropisomeric CBs.

No single chiral gas chromatographic (GC) column can enantiomerically separate all 19 atropisomers. At least four columns are needed [14,20], although one research group has shown that it is possible to separate 15 atropisomeric CBs by using three different chiral columns [21]. Currently, the permethylated β-cyclodextrin Chirasil-Dex column is the most commonly used chiral column, since the stationary phase is bonded, cross-linked and has acceptable stability and bleed. On such a column, nine of the 19 atropisomeric CBs can be enantiomerically separated (R_s = 0.7–1.5), namely CBs 84, 91, 95, 132, 135, 136, 149, 174 and 176 [22]. Fortunately, this includes all but one (CB 183) of the most environmentally abundant atropisomers—the others being CBs 91, 95, 135, 149,174 and 176.

Close to 140 CB congeners have been found in the environment, some of which coelute and require comprehensive congener-specific analysis for accurate peak assignment and quantification. The study of individual atropisomeric CB enantiomers in the presence of all other congeners is, of course, even more complex. Many coelution problems occur, which makes accurate chiral analysis difficult, especially since the maximum acceptable error in chiral analysis is generally only a few per cent. Two approaches to this problem have been adopted, based on the use of either a suite of different chiral selective columns, together with a mass selective detector (MSD), or multidimensional gas chromatography (MDGC) [13,14,16,17].

An alternative technique is to use a comprehensive 2D-gas chromatographic (GC × GC) system with an enantioselective first-dimension column, for the chiral separation, and a second-dimension column of markedly different selectivity, e.g. a shape-selective or polar column [23]. Such a combination potentially allows both enantioselective separation of the atropisomeric CBs and their separation from interfering compounds. As a detection system for GC × GC the μECD has been shown to have appropriate properties to be used as a fast and selective detector for the analysis of organochlorines, as was first shown by de Geus et al. [24], while Korytar et al. [25] demonstrated that the analysis of a complex mixture of CBs could be achieved in GC × GC using a μECD.

In a previous study, most of the nine atropisomers that can be separated on Chirasil-Dex were resolved from other CBs present in a seal sample using a liquid crystalline second-dimension column [23]. However, CB 95 coeluted with CB 93, and CB 84 was only partially resolved. Therefore, in the present study we included another column set with a cyanopropyl second-dimension column. The two column sets complement each other as the second-dimension columns separate compounds based on two different molecular characteristics; shape and polarity, respectively [26]. Seal samples from age-matched individuals differing in health status were analyzed and the relative abundance of both chiral and normal CBs was compared. The samples also included various tissue types to allow comparison of the tissue distribution.

2. Experimental

2.1. Chemicals

Five mixtures (C-CS-01 through C-CS-05) with a total of 144 CBs were purchased from AccuStandard Inc. (New Haven, CT, USA) as iso-octane solutions at a concentration of 10 ng of each congener/μl. In addition, individual CB reference standards (>98% purity) of CBs 84, 91, 95, 132, 135, 136, 174 and 176 were obtained from the same source, while CB 149 was from Dr. Ehrenstorfer (Augsburg, Germany). A WHO-PCB standard containing 12 non- and mono-*ortho* CBs was obtained from LGC Promochem Inc. (Borås, Sweden), and a seven-congener CB standard with CBs 28, 52, 101, 118, 138, 153 and 180 from Larodan Fine Chemicals Inc. (Malmö, Sweden). The CB formulations (C-CS-01 through C-CS-05) contain 136 CB congeners that are abundant (i.e. present in excess of 0.05%, w/w) in the Aroclor formulations 1242, 1254, or 1260 according to Frame [27]. In addition, they contain several of the less abundant congeners. Each mixture contains congeners that are easily resolved using 5%-phenyl methylpolysiloxane types of stationary phases. A quantification standard was prepared from aliquots of these five mixtures, corresponding to 620 ng of each congener, and 600 ng each of CBs 126 and 169. The standard was diluted with iso-octane to a final concentration of 1.2 ng/μl. As an internal standard for the quantification standard and seal organ samples, native (^{12}C) CB 142, acquired from Dr. Ehrenstorfer (Augsburg, Germany), was used.

2.2. Samples

Blubber, liver, muscle, and brain tissue samples of six Baltic grey seals (*H. grypus*) of varying health status were obtained from the Swedish Museum of Natural History (Stockholm, Sweden). Data on the grey seal samples can be found in Table 1. All grey seals were female adults, 12–26 years old, collected from the east coast of Sweden in the Gulf of Bothnia. Three of the adult seals had serious pathological organ alterations and three had mild or moderate alterations. In addition, a fetus from one of the females with severe alterations was included.

Table 1
Specifics of the female grey seals (*H. grypus*) from the Baltic Sea

Sample #	Health status	Age (year)	Weight	Tissues (wet weight, g)							
				Blubber	Fat (%)[d]	Liver	Fat (%)[d]	Muscle	Fat (%)[d]	Cerebrum	Fat (%)[d]
1[c]	[b]	15	148 kg	4.1	90	22	4.1	92	1.0	52	10
2	–	Fetus	1660 g					46	4.4		
3	[b]	24–26	90 kg	2.8	86	22	3.9				
4	[b]	22	83 kg	2.7	80	34	3.9				
5	[a]	19	126 kg	4.0	92	22	3.8				
6	[a]	15	134 kg	3.4	87	31	4.0				
7	[a]	12	117 kg	3.0	89	30	4.2				

[a] Mild to moderate pathological alterations.
[b] Serious pathological alterations.
[c] Pregnant with # 2.
[d] Percentage of extractable amount of lipids.

2.3. Sample preparation

The blubber, liver, muscle and cerebral samples of the grey seals were stored at −20 °C until the time of analysis, for which 3–4 g of blubber, 20–35 g of liver, or 50 g of cerebral tissue (wet weight) was used. The tissue samples were macerated and dried with anhydrous Na_2SO_4. Lipids and lipophilic compounds were first extracted with acetone/*n*-hexane (5/2, v/v) followed by *n*-hexane/diethyl ether (9:1, v/v) and the lipid content was determined gravimetrically (see Table 1). The samples were reconstituted in *n*-hexane, spiked with CB 142, and the lipids were removed using a gravity fed multilayer column containing, from the bottom: basic silica (NaOH-treated), activated silica, 40% H_2SO_4 on silica (w/w), 20% H_2SO_4 on silica (w/w) and a thin layer of anhydrous Na_2SO_4. The sample was eluted with *n*-hexane. Two milliliters of iso-octane was added to each extract, which was then concentrated to 1 ml by rotary evaporation and nitrogen blow-down. After transfer to GC-vials the samples were ready for GC × GC analysis.

2.4. Equipment

All samples were analyzed using GC × GC/μECD. In addition, one sample was analyzed using GC × GC/TOF-MS to verify the GC × GC/μECD results. The GC × GC/μECD system consisted of two GCs connected using an interface built in-house, which was operated by the Aux 2 channel of the 6890 GC. The first GC was an Agilent HP5890 and the second an Agilent HP6890 (Agilent Technologies Inc., Palo Alto, CA, USA). The second oven was equipped with a longitudinal modulating cryogenic system (LMCS) and an electron-capture detector (μECD) unit. The GC × GC/TOF-MS validation analysis was run on a Pegasus 4D system (LECO Inc., St. Joseph, MI, USA) equipped with an Agilent 6890 gas chromatograph. The system has a two-stage cryo-jet modulator (licensed from Zoex Inc., Lincoln, NE, USA) and a secondary column oven installed inside the Agilent 6890 at the MS interface entrance.

2.5. Columns

Analyses of CB mixtures and samples were carried out using two column combinations including, in both cases, a 3 m × 0.25 mm i.d. deactivated retention gap and a 10 m × 0.10 mm, 0.1 μm Chirasil-Dex CB first-dimension column (Varian, Walnut Creek, CA, USA), which has immobilized permethy l 2,3,6-tri-*O*-methyl β-cyclodextrin as the chiral selector. The second-dimension column was a 1.4 m × 0.15 mm, 0.1 μm poly (50% liquid crystalline/50% dimethyl) siloxane column (LC-50; J&K Environmental Ltd., Sydney, Nova Scotia, Canada) or a 1.5 m × 0.1 mm, 0.1 μm > 75% biscyanopropyl polysiloxane column (VF-23MS; Varian, Walnut Creek, CA, USA), which is a bonded and cross linked experimental stationary phase with a maximum temperature of 290 °C. Standard press-fits (Agilent Technologies Inc., Palo Alto, CA, USA) were used to connect the columns. Polyimide resin was used to secure the columns to the press-fits and ensure a leak-free connection.

2.6. Gas chromatography

The GC ovens in both the GC × GC/μECD and GC × GC/TOF-MS systems were used with differential temperature ramping, in which the second-dimension GC column was kept at a higher temperature than the first. On both instruments, the Chirasil-Dex column with the retention gap was mounted in the first GC oven and threaded through the cryogenic modulating devices (LMCS or LECO cryojet) inside (GC × GC/μECD) or in close proximity to (LECO cryojet) the secondary oven. In the secondary oven the column was connected to the LC-50 (in the case of the GC × GC/μECD) or VF-23MS column, which was then connected to the detector (μECD or TOF-MS). Samples of 1 μl were injected in the split-less mode (split opening at 2 min) at a temperature of 250 °C. The first GC oven temperature program was as follows: 80 °C for 2 min, rising by 30 °C/min to 110 °C, then by 0.5 °C/min to 155 °C (180 °C for the GC × GC/TOF-MS), and finally by 10 °C/min to 250 °C, which was held isothermally for 5 min. The second GC oven was ramped in a similar way, but with a 50 or 70 °C offset for the VF-23MS and LC-50 columns, respectively. However, the final temperature of the second GC oven was limited to 275 °C for the LC-50 and 280 °C for the VF-23MS. Hydrogen (GC × GC/μECD) or helium (GC × GC/TOF-MS) was used as carrier gas in the constant flow mode, controlled by an electronic pressure control (EPC) system.

The initial head pressure in the GC × GC/μECD instrument was 290 kPa (LC-50) or 414 kPa (VF-23MS) while the initial pressure in the GC × GC/TOF-MS was 414 kPa. CBs were detected using an μECD detector (300 °C; 150 ml/min of nitrogen make up gas) operated at a data collection rate of 50 Hz or by a TOF-MS, with the conditions specified below.

2.7. Mass spectrometry

The ion source was operated in electron ionization mode (70 eV) and full scan mass spectra were collected between 50 and 500 *m/z* at a data collection rate of 50 Hz. The transfer line and ion source were kept at 250 and 200 °C, respectively, and the electron multiplier was set to 1800 V.

2.8. Cryogenic modulation

The compounds eluting from the first GC column were cryogenically modulated using the longitudinal modulating cryogenic system (LMCS), which has been described in detail elsewhere [28] or the LECO cryojet device. Modulation was applied 15 cm from the end of the first-dimension Chirasil-Dex column, leaving 4 cm after the trap for press-fit connection to the second-dimension LC-50 (only GC × GC/μECD) or VF-23MS column. In the LMCS the carbon dioxide cryogen was flow-regulated to keep the trap 120–140 °C below the oven temperature, while the LECO cryojet used pressurized nitrogen gas led via a gas transfer line through a liquid nitrogen cooled container, which kept the temperature at the cryogenic levels (exact temperature unknown) of the two serially operated cryojets. In the case of the LMCS, effluent from the first column is focused in the cryogenic trap and desorbed by convectional heat from the air in the oven when the trap moves away from the focused region. In contrast, the LECO cryojet relies, instead, on active desorbtion from two serially operated heating jets [29]. The modulation period was set to 5 s. The second-dimension retention times were determined using the apparent retention times obtained using 5 and 5.5 s modulation periods. The second-dimension retention times of compounds of interest were derived by consecutive additions of the modulation period to the apparent retention times until the same value was obtained.

2.9. Data analysis

The GC × GC and 1D-GC systems were controlled by Chemstation software (version 6.3; Agilent Technologies Inc., Palo Alto, CA, USA) run on a standard PC. The raw data were transformed to comma-separated value (CSV) files and imported, for qualitative and quantitative data evaluation, into GC-Image v1.1, a program designed for two-dimensional gas chromatographic image processing and analysis developed by the Computer Science and Engineering Department of the University of Nebraska (Lincoln, NE, USA). GC × GC peak identification, baseline correction, area and volume determinations were all done automatically within GC-Image [30]. The CBs in the seal tissue samples were identified using a template made generated from 144 CB standard, which contains CBs that are abundant (>0.05% w/w) in Aroclors. The search algorithm was set to find peaks within a modulation window of ±1 modulation period (5 s), and within 100 ms, in the second-dimension. In some rare cases miss-assignments were observed, generally resulting from coelution or near coelution with neighboring CBs. In these cases manual correction was applied to improve the description of the real peak-to-peak boundaries. Graphical contour and 3D surface presentations were generated with Transform (v3.0, Research Systems, Inc. Boulder, CO, USA) after converting the (CSV) file into a *x/y* matrix with software developed by P.J. Marriott (2D-GC Converter V2.2; RMIT University, Melbourne, Australia).

Using this software the levels of the 13 WHO-PCBs comprising the non- and mono-*ortho* CBs and the 7 CBs standard (commonly quantified in environmental monitoring programmes) as well as the concentrations and enantiomeric fractions (EFs) of nine atropisomeric CBs was determined for each sample by a single GC × GC analysis.

EFs of the atropisomeric CBs were calculated by dividing the first peak area (1D-GC) or volume (GC × GC) by the sum of the peaks of the first (E_+) and second (E_-) eluting enantiomers [31,32]:

$$\mathrm{EF}_{+/-} = \frac{E_+}{(E_+ + E_-)} \quad \text{or} \quad \mathrm{EF}_x = \frac{E_1}{(E_1 + E_2)} \qquad (1)$$

The optical rotation of atropisomeric CBs 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. The optical rotation has been experimentally assigned using pure enantiomers of CBs 84, 132, 135, 136, 149, 174 and 176. On Chirasil-Dex the (+)-enantiomer elutes before the (−)-enantiomer for all atropisomers except CBs 135 and 174, for which the elution order is reversed [11,22].

3. Results and discussion

3.1. GC × GC separation and performance

In GC × GC it important to ensure that both the first and second-dimension peak shapes are reproducible. With fast detectors, obtaining sufficient data points over a second-dimension peak is a trivial task. It is much more difficult to obtain sufficient samplings of a first-dimension peak. Murphy et al. have studied the effect of first-dimension sampling rate on the resolution in the first-dimension [33]. They stated that to trace the first-dimension peak shape, at least four modulation cycles are needed. However, the more samplings over a first-dimension peak are there, the better the peak definition. The problem is that the effective sampling rate has to be adjusted to the resolution in the second-dimension and the total analysis time. It is possible to obtain enough samplings over a first-dimension peak whilst maintaining second-dimension resolution by a slow oven temperature gradient. However, as time is an important factor for most analysts, it is usually advantageous to minimize the number of first-dimension samplings as far as possible.

For resolving chiral compounds, the chiral selectivity of the column is often a key determinant of the analysis speed in 1D-GC as well as GC × GC. On both types of systems one might improve the chiral resolution by decreasing the stationary phase thickness or capillary column bore, but in either case a slow temperature ramp is generally needed, especially to improve the resolution of recalcitrant (non-polar) analytes such as atropisomeric CBs.

It is very important in chiral analysis to be able to capture small variations in EFs. To conserve the peak resolution that is achieved in the first column throughout the modulation process, more than four modulations per peak are essential, and it is important to have at least two samplings in the region that defines the valley between peaks. This consideration led to the use of a slow ramping rate (0.5 °C/min) in the analysis of atropisomeric CBs, giving a peak width at base of 38 s (6σ). At a modulation period of 5 s, 8–9 modulations were obtained per peak. For a chiral CB with a resolution (R_s) of 1.0 (in this case CB 95) four modulations defined the valley between the peaks.

To evaluate the quality of the EF determinations, a standard mixture containing the racemic CBs 84, 91, 95, 132, 135, 136, 149, 174 and 176 was analyzed by 1D-GC and GC × GC. Peak area or volume was automatically determined using Chemstation for the 1D-GC data and GC-Image for the GC × GC data. In addition, the areas of the GC × GC peaks corresponding to a specific analyte were manually summed. All three ways to determine EFs resulted in acceptable average values, ranging from 0.47 to 0.52. The results also showed that 1D-GC gives the best precision in the EF determinations, as can be seen in Table 2, with relative standard deviations (R.S.D.s) of less than 1%. Similar R.S.D.s were obtained by GC × GC for compounds with a resolution (R_s) higher than 1, i.e. CBs 91, 132 and 149. The quality of the GC × GC data was slightly worse for the partially resolved peaks, for which R.S.D.s obtained were between 1.0 and 3.0 using GC-Image and between 1.0 and 4.0 using manual summation of peak areas. These differences in

Table 2
Arithmetic mean and relative standard deviation of atropisomeric fractions (EF)

CB	Chiral R_s	Average EF			R.S.D. (%) ($n = 5$)		
		1D-GC	Sum area	GC image	1D-GC	GC image	Sum area
84	0.92	0.49	0.52	0.47	0.8	1.6	3.1
91	1.27	0.50	0.50	0.49	0.1	0.7	0.3
95	0.97	0.49	0.51	0.47	0.3	1.9	2.4
132	2.0	0.48	0.50	0.50	0.7	0.5	0.6
135	0.62	0.49	0.49	0.48	0.5	2.1	4.0
136	0.92	0.49	0.51	0.49	0.2	1.1	1.6
149	1.22	0.50	0.50	0.50	0.1	0.3	1.0
174	0.57	0.49	0.51	0.47	0.4	3.0	3.3
176	0.75	0.50	0.48	0.48	0.5	1.0	2.3

Also included is the chiral resolution between enantiomers.

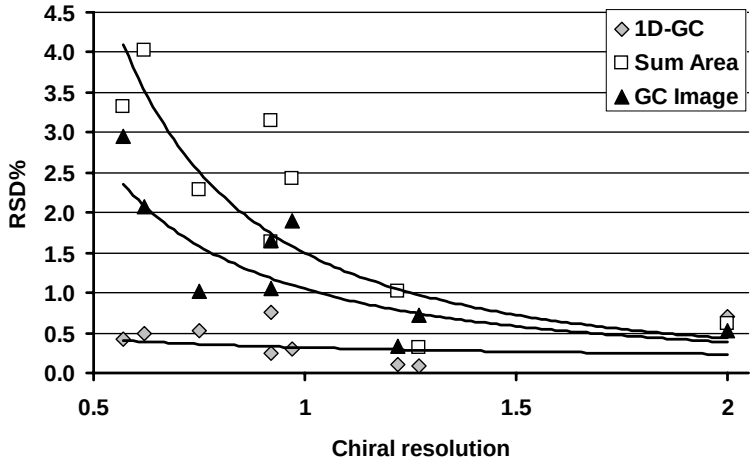

Fig. 1. Correlation between the peak to peak chiral resolution and the relative standard deviation of the determined atropisomeric fractions (EF) ($n = 5$) using three methods for EF determination.

results can be attributed to the fact that slight variations (of just a few seconds) in first-dimension retention time will cause area redistributions between second-dimension peaks that lie close to the valley region of partially resolved peaks. Here, the manual methodology employed was to sum the first seven peaks of the first enantiomer and the later seven peaks of the second enantiomer. GC-Image finds the valley by interpolation.

Clearly, the resolution of the atropisomers influences the precision of the EF determination (Table 2). While the precision was not significantly influenced by the resolution in the 1D-GC system, as shown in Fig. 1, the GC × GC system gave a significant increase in R.S.D. when the resolution decreased below 1, indicating that the GC × GC technique may be less suitable for the quantification of poorly resolved enantiomeric peaks. Nevertheless, the RMS values of the GC × GC derived EFs were low enough, at 0.3 to 3.0%, to allow its use in the seal analyses, especially since GC × GC significantly reduces the risk of coelution with other CBs which, most likely, would result in much poorer results.

The EFs derived in the analysis of the 144-CB mixture provide indications of CBs that may coelute, or partially coelute, with the atropisomeric CBs. As can be seen in Table 3 and Figs. 2 and 3, all of the atropisomers partially or completely coelute with one or more CBs in the first GC × GC-dimension (and in 1D-GC), while most of the coelutions are resolved in the second-dimension. GC × GC chromatograms obtained using the two-column combinations are shown in Fig. 2 (Chirasil-Dex/liquid crystal) and Fig. 3 (Chirasil-Dex/cyanopropyl). Clearly, some of the atropisomeric CBs coelute with Aroclor congeners as well as some of the nonachloro-bornanes that were tentatively identified using GC × GC/TOF-MS, and several other peaks that were not identified by

Table 3
Coelutions for the nine studied atropisomeric CBs in 1D-GC and GC × GC

IUPAC	Coelutants		
	1D-GC	GC × GC	
		LC-50	VF-23MS
84	**56**, *90*, **99**, **101**	99	**56**
91	**63**	–	–
95	*93*	*93*	–
132	**176**, **141**	**141** (0.7)[a]	**141**
135	**110**, **82**	–	–
136	**115**	–	–
149	**77**, **124**	–	–
174	**202**	–	–
176	**132**, **141**	–	–

CBs are marked in bold and underlined or bold or in italic are present in Aroclors 1242, 1254, or 1260 at concentrations above 1.0%, between 0.05–1.0% or below 0.05%, respectively.

[a] GC × GC resolution (R_s) in the second-dimension.

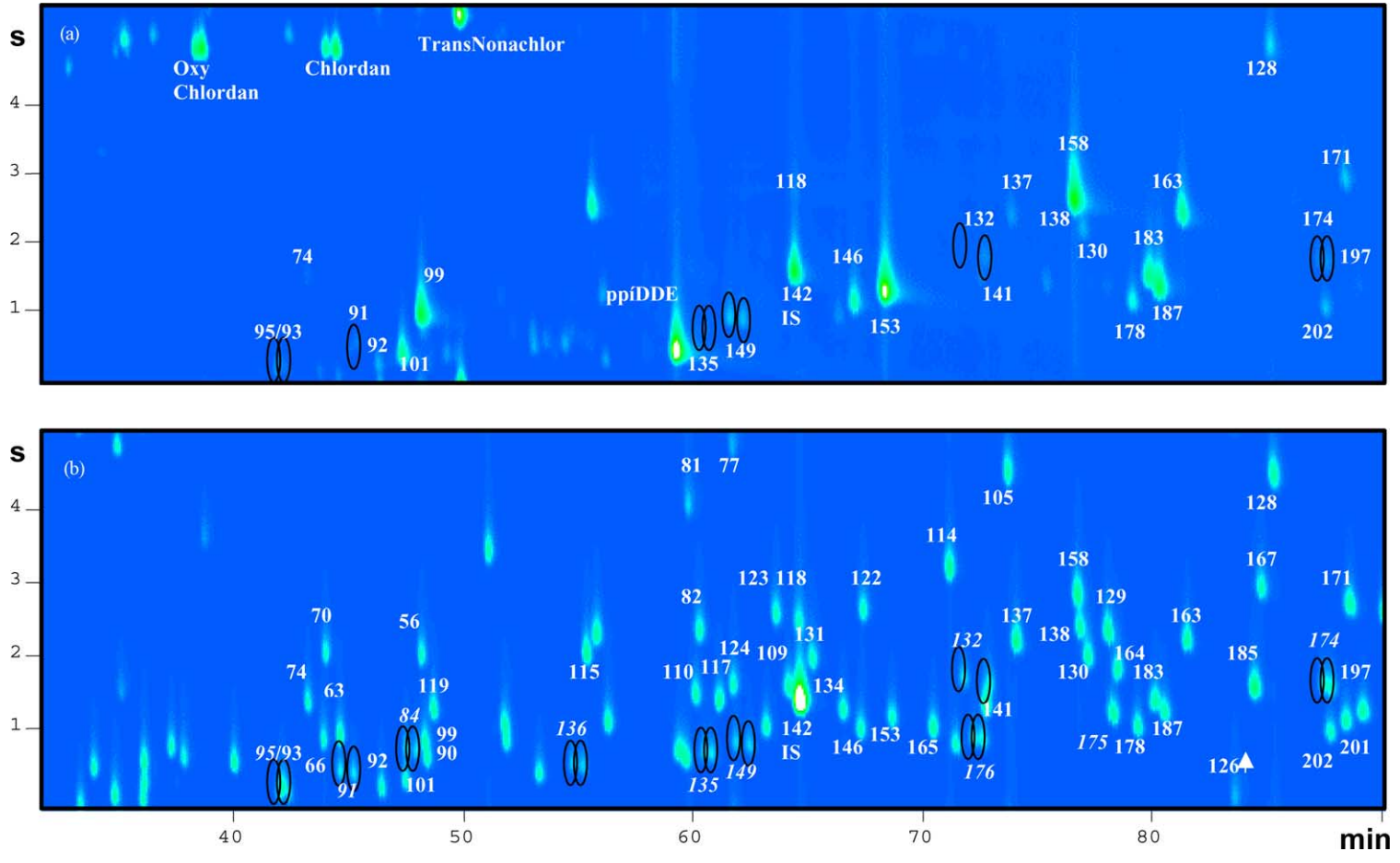

Fig. 2. GC × GC contour plot of the region 32–90 min on the column combination Chirasil-Dex and LC-50. (a) Grey seal blubber sample # 5 and (b) standard of the 144 Aroclor CBs. The CBs and a few nonachlorinated bornanes and a oxychlordane were tentatively identified by GC retention times and GC × GC/TOF-MS spectra.

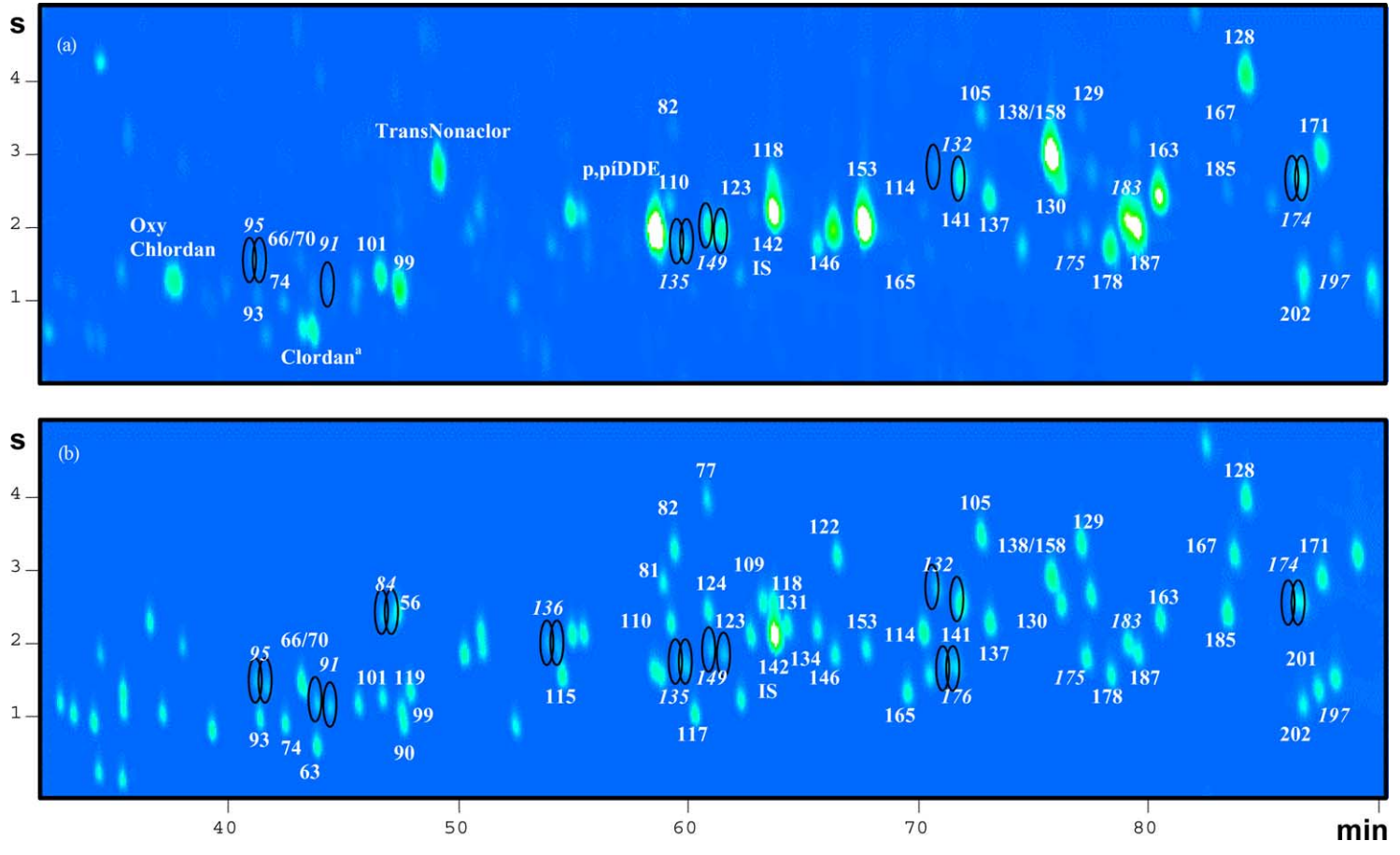

Fig. 3. GC × GC contour plot of the region 32–90 min on the column combination Chirasil-Dex and VF-23MS. (a) Grey seal blubber sample # 5 and (b) standard of the 144 Aroclor CBs.

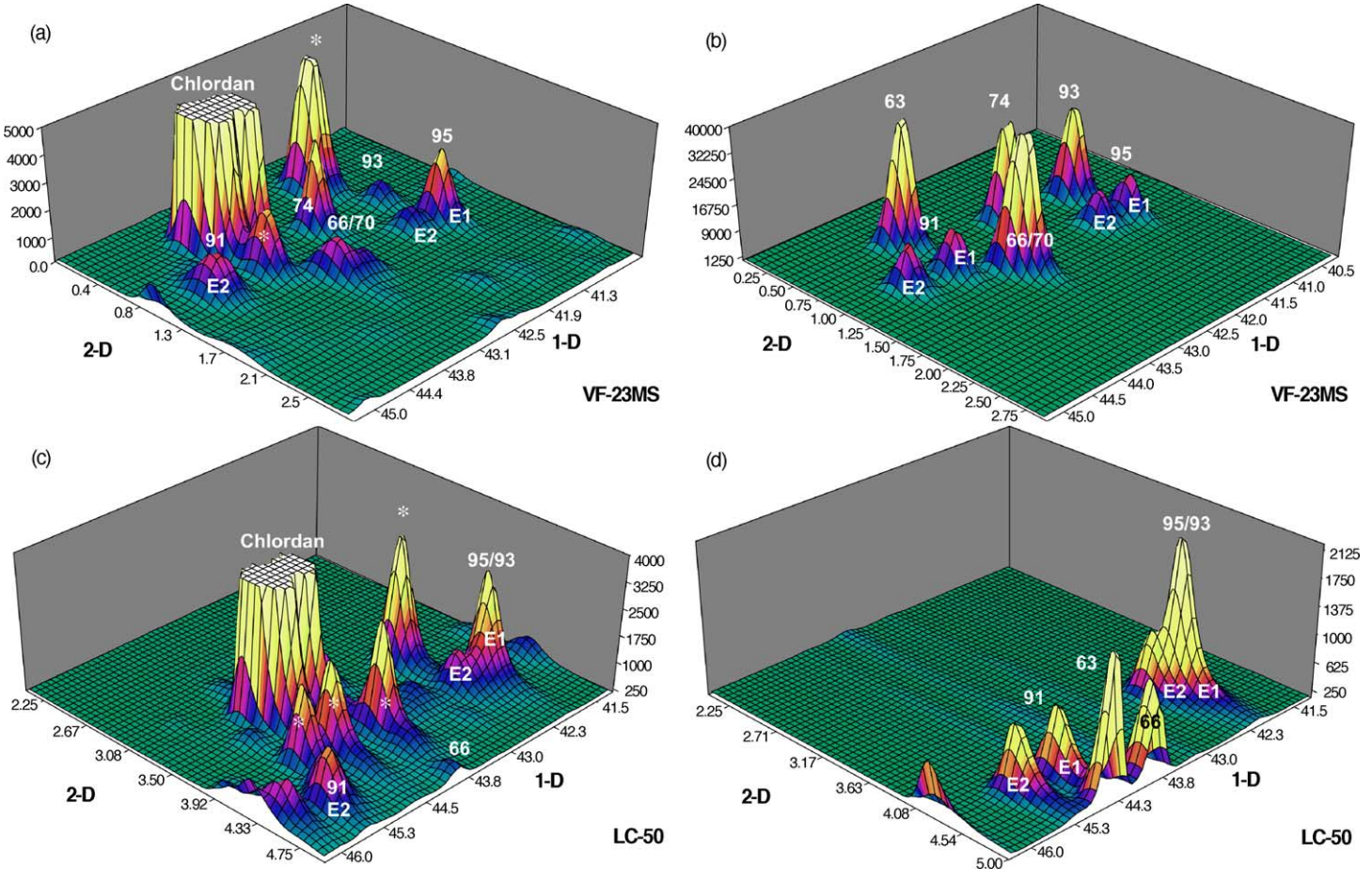

Fig. 4. 3D plot of the region 40 to 46 min in the first-dimension and 0–3 s in the second-dimension with include the atropisomeric CBs 91 and 95. The Chirasil-Dex column combined with either a VF-23MS (a and b) or a LC-50 column (c and d). Analysis of a Grey seal blubber sample # 5 (a and c) and the 144 Aroclor CB standard (b and d). Elution order of atropisomers assigned as E_1 and E_2 for the first and last eluting enantiomer, respectively.

TOF-MS since they were present at too low levels. In GC × GC, CB 84 coelutes with CB 99 on the LC-50 column combination and with CB 56 on the VF-23MS column combination. CB 132 coelutes with CB 141 on the VF-23MS column combination, but this pair is partially resolved ($R_s = 0.7$) on the LC-50 combination. On the other hand, CBs 95 and 93 coelute on the LC-50 column combination while they are separated on the VF-23MS combination. However, CB 93 is not found in Aroclors in excess of 0.05% (w/w), suggesting that determinations of CB 95, which is found at high levels in Aroclors would not be affected to any great extent [11]. This assumption proved to be incorrect, when the technique was applied to the seal samples (see below and Fig. 4). As can be clearly seen from the 3D plot, the first eluting enantiomer peak of CB 95 is higher in the LC-50 chromatogram of the seal blubber sample (Fig. 4a) and standard chromatogram (Fig. 4b) than in the VF-23MS chromatogram of the seal blubber sample (Fig. 4c) and standard (Fig. 4d). To avoid such problems, both column combinations were used to verify the accuracy of the CB congener assignments. For instance, only the second eluting CB 91 enantiomer was found, due to the low concentrations of this CB present. As the signal was of equal intensity using both column combinations, it could be concluded that the signal was from CB 91 and not from an interfering substance (Fig. 4a–d). Further, this conclusion was additionally verified by the GC × GC/TOF-MS analysis.

Liquid crystal columns retain non- and mono-*ortho* CBs strongly; while the cyanopropyl columns have less pronounced selectivity. Consequently, tri- and tetra-*ortho* CBs, which include the atropisomeric CBs, are among the first eluting congeners from the LC-50 column (Fig. 2b), while they are more evenly spread by the VF-23MS column (Fig. 3b). This selectivity difference reverses the elution order of many CBs, e.g. CBs 95 and 93, CBs 91 and 63, CBs 136 and 115, and CBs 135 and 117.

The problems caused by coelution are greater when using a detector like an μECD as compared to a mass spectrometry (MS) system. The ECD responds to all electron-capturing compounds and signals from CBs close to the baseline may therefore be subject to interference from other anthropogenic substances or background noise from the matrix. Sometimes the large differences in the relative abundance of different CBs may also cause problems. It is easy to separate the atropisomeric CB 84 from CBs 99 and 101 if they are all present in a standard with equal levels of the three components. In the seal tissue samples, however, CB 84 was found at much lower levels than either CB 99 or CB 101, which made it difficult even to identify the CB 84 peaks. Using the LC-50 column combination, the first eluting enantiomer would elute in the tailing peak of CB 101, while the later eluting enantiomer would be difficult to distinguish from the CB 99 peak. Using the VF-23MS combination, on the other hand, CB 84 is well separated from both 99 and 101, but partially coelutes with CB 56. CB 56 was found at low levels while CB 84 as well as 136 was only found at trace levels, or not at all, in the samples in these Baltic grey seals, which makes the partial coelution problem irrelevant in this study, but might affect the analysis of other biota samples. In the seal samples, CB 141 was found at similar levels to CB 132, and the partial coelution of CB 141 with the later eluting enantiomer of CB 132 will increase the quantification error for this enantiomer.

3.2. Atropisomeric CBs and health status

The atropisomeric CBs found at highest concentrations were CBs 132, 149 and 174, as shown in Table 4, but they still only contributed 0.4–1% (w/w) to the total amount of CBs found, while the concentration of CB 153 was 80–400 times higher, contributing 13–18% (w/w) to the total amount of CBs. Three other atropisomeric CBs were positively identified, i.e. CBs 91, 95 and 135. CBs 136 and 176 were not detected in the samples, while CB 84 was found at low levels. The first eluting enantiomer of CB 84 was identified using the cyanopropyl column combination, but its EF could not be determined due to coelution of the second eluting enantiomer with CB 56, which was present in the samples at slightly higher concentrations.

The EFs were also verified by GC × GC/TOF-MS. The results obtained with this technique and GC × GC-μECD with the two different column combinations are presented in Table 5. Analyses of the quantification standard of 144 CB and a Baltic grey seal blubber sample verified that the EFs of CBs 91, 95, 132, 149 and 174 had been correctly determined. A skewed ratio of CB 135 was however found using TOF-MS, indicating interference from

Table 4
Concentrations of CBs in Baltic Grey seal tissue samples of the seven indicator congeners (EC7), six detected chiral CBs and 12 non- and mono-*ortho* CBs (WHO)

	Muscle		Cerebrum	Liver						Blubber					
	# 1	# 2	# 1	# 1	# 3	# 4	# 5	# 6	# 7	# 1	# 3	# 4	# 5	# 6	# 7
EC7 ($\mu g\,g^{-1}$ lipid)															
28	–	0.002	–	–	0.002	0.004	–	–	0.003	–	–	0.002	–	0.002	–
52	0.06	0.08	0.01	0.08	0.28	0.27	0.03	0.06	0.13	0.13	0.35	0.42	0.09	0.18	0.28
101	0.23	0.24	0.03	0.26	1.0	1.2	0.12	0.32	0.48	0.48	0.98	1.4	0.41	0.47	0.96
118	–	0.18	–	–	0.52	0.46	–	0.30	0.31	0.21	0.36	0.54	0.19	0.26	0.37
138	4.5	4.6	0.49	5.1	19	31	0.83	1.9	7.0	14	14	19	3.4	3.2	18
153	6.5	6.5	0.7	6.7	25	40	0.90	2.0	8.2	20.6	22	25	5.0	3.8	25
180	7.5	5.8	0.84	8.6	49	77	1.7	4.0	9.0	27	40	35	8.9	5.8	32
Chiral ($ng\,g^{-1}$ lipid)															
91	4.7	6.8	0.59	12	32	22	7.1	25	15	27	62	37	15	18	47
95	35	26	3	46	119	66	33	50	65	36	102	85	34	51	91
132	10	13	1.2	55	190	160	31	76	130	32	120	100	28	44	120
135	18	17	2.0	12	120	64	8.5	26	43	34	120	81	40	41	120
149	140	130	15	280	1000	1000	100	260	570	380	930	830	250	290	1000
174	31	25	3.7	62	347	296	27	146	136	91	307	199	69	78	306
WHO ($ng\,g^{-1}$ lipid)															
77	–	–	–	–	28	26	4.2	3.4	–	3.9	–	5.9	1.6	–	3.2
81	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
105	25	31	2.7	63	130	170	32	75	82	57	90	210	43	56	120
114	2.9	4.5	0.5	7.7	20	38	3.8	13	12	7.1	19	11	–	8.2	15
118[a]	–	180	–	–	520	460	–	300	310	210	360	540	190	260	370
123	–	–	–	–	8.2	3.6	–	5.9	8.5	–	–	2.6	–	–	4.5
126	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
156	94	96	14	170	670	930	64	230	230	290	460	640	190	130	430
157	76	61	18	190	460	500	82	300	220	230	330	380	120	85	310
167	–	3.3	–	6.5	17	36	6.9	100	12	3.3	7.2	8.4	5.6	2.1	4.5
169	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
189	60	44	9.0	250	380	390	78	120	87	130	240	170	80	45	230

[a] Eluting in the peak tailing of the internal standard CB 142 which increase the LOD for this compound.

Table 5
Comparison of enantiomeric fractions of a Baltic grey seal blubber sample # 7 by two different detection methods and quantification by GC Image v1.1 and a manual summation of enantiomeric peak areas

Method	Enantiomeric fractions					
	91	95	132	135[a]	149	174
μECD (GC Image)	<0.01	0.75	0.84	0.4	0.39	0.43
μECD (manual)	<0.01	0.75	0.88	0.2	0.36	0.45
TOF-MS	<0.05	0.87[b]	0.91	0.1	0.37	0.45

[a] Probable coelution with an unidentified compound influencing the quantification on μECD but not TOF-MS.
[b] CB 93 not fully resolved in the second-dimension from CB 95 ($R_s = 0.8$) on TOF-MS giving a slight contribution to the E_1 CB 95 peak.

a non-CB component in GC × GC-μECD. No EFs are therefore presented for that congener. Due to the use of helium rather than hydrogen as carrier gas, the flow was slower and the retention times longer with GC × GC/TOF-MS. Consequently, CB 132 was separated from CB 141. While the first eluting enantiomer of CB 91 was below the detection limit ($S/N < 3$) on either GC×GC-ECD or TOF-MS, verifying that the second eluting enantiomer peak was indeed a pentachlorinated biphenyl and that there is a major difference in the enantiomeric ratios in these samples.

Considerable deviations from racemic ratios of the Baltic grey seal CBs were found for the six quantified atropisomers. The atropisomers found at the highest levels, CBs 135, 149 and 174, have EFs close to racemic, suggesting that they are not as easily enantioselectively metabolized as the atropisomers that have greater deviations from racemic: viz. CBs 91, 95 and 132. Of all the atropisomers, these CBs are also the most frequently found congeners in biological samples. CB 91 is also commonly found, but in this study the congener was only found at low levels (Table 4). It has an average EF below 0.1, indicating that selective metabolization of the first eluting congener takes place. CB 132 has an average EF of 0.9, suggesting that highly selective metabolization of the second eluting congener may occur. CB 95 also deviates strongly from racemic with EFs of 0.7–0.9. This component had to be determined using the cyanopropyl column combination as CB 95 partially coelutes with CB 93 on the LC-50 column combination, causing CB 93 to contribute approximately 50% of the total volume of the first eluting enantiomer of CB 95, as visualized in the 3D plot of Fig. 4a. All other components were quantified using the LC-50 column combination.

Comparison of the atropisomeric CB data derived from the six adult female grey seals (Fig. 5) shows that the EFs were generally lower for CB 149 and higher for CB 132 in liver than in blubber samples. The larger deviations from racemic in liver as compared to blubber could indicate that enantioselective metabolization takes place in the liver. CBs are more easily metabolized when there is vicinal hydrogen available, all nine atropisomers that were studied have vicinal hydrogen's *meta–para* and a few CBs have also free hydrogen in *ortho–meta* position, (CBs 84, 91, 132). Congeners with vicinal *ortho–meta* hydrogens have been shown to be metabolized by grey seal liver microsomes [34]. However, it is unclear whether this process is enantioselective. Otherwise, the EF profiles appear to be similar throughout the body of grey seal 1 (Fig. 5). Similar profiles were also found for muscle tissues of a mother seal (grey seal 1) and its fetus (grey seal 2), indicating that transfer of CBs through the placenta to the fetus occurs without any change in EF. Thus, there seems to be no selective passage of the studied atropisomeric CBs across the placenta, and no selective blood–brain barrier. No differences in EF ratios were found between the grey seals with poor health status (grey seals 1, 3 and 4) and the seals with mild or moderate pathological alterations (grey seals 5–7).

3.3. Achiral CBs and health status

Total CB contents were determined using the 144-CB mixture. All detected congeners were included and the data were expressed on a lipid weight basis. Relating the sum of CBs to the health status of the animals, there seems to be higher levels in the animals with severe pathological changes (Fig. 6). However, one exception to this general rule was found: grey seal 7 (good health status) had slightly higher levels of CBs than grey seal 1 (poor health status). This discrepancy may be due to the higher body-weight (and lipid content) of the latter, or to the fact that this pregnant seal had transferred a significant amount of CBs to its fetus. The mother and fetus had almost equal muscle tissue levels. A significant difference ($P < 0.05$) was also found between liver CB levels of animals that were seriously affected and those that were less strongly affected.

Seven congeners, i.e. CBs 28, 52, 101, 118, 138, 153, and 180, are frequently measured in environmental monitoring programs as representatives of the CB family. They are generally found at high levels in environmental samples and accounted for approximately 50% of the total CBs in our samples, as seen in Table 4. CBs 138, 153 and 180 were the most abundant, while CBs 28 and 52 were found at much lower concentrations, probably because the less highly chlorinated congeners are more easily metabolized. As these compounds contribute a major part to the total CBs they exhibit similar relationships to the seals' health status.

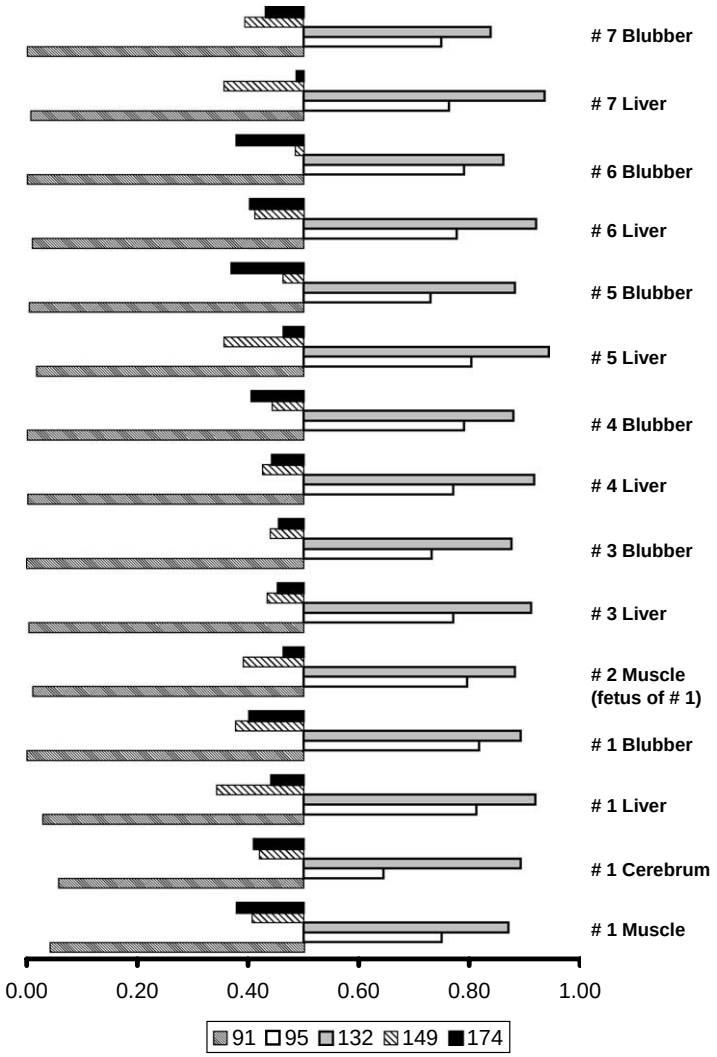

Fig. 5. Enantiomeric fractions of atropisomeric CBs in different female Baltic Grey seal tissue samples.

The WHO-PCBs are considered the most toxic of all CBs due to their structural similarity to the polychlorinated dioxins and furans (PCDD/Fs). These non- and mono-*ortho* CBs may adopt a coplanar conformation that is isosteric with the PCDD/Fs, and they have been assigned dioxin toxic equivalency factors (TEFs) by the World Health Organization (WHO) [35,36]. WHO-PCBs are usually analyzed by GC high-resolution MS as their environmental levels are low. GC-ECD cannot be used, due to coelution of many WHO-PCB congeners with more abundant CBs. Fractionation of the CBs into a planar and a non-planar fraction using activated carbon columns [37] may yield cleaner fractions that are suitable for ECD analysis. In GC × GC such a separation may be possible without pre-separation if the columns and column-dimensions are carefully chosen and optimized. An attempt was made to determine the WHO-PCBs in the same GC × GC-μECD run as the chiral analytes (Table 4). However, the non-*ortho* CBs were difficult to detect. Only CB 77 could be determined with sufficient accuracy.

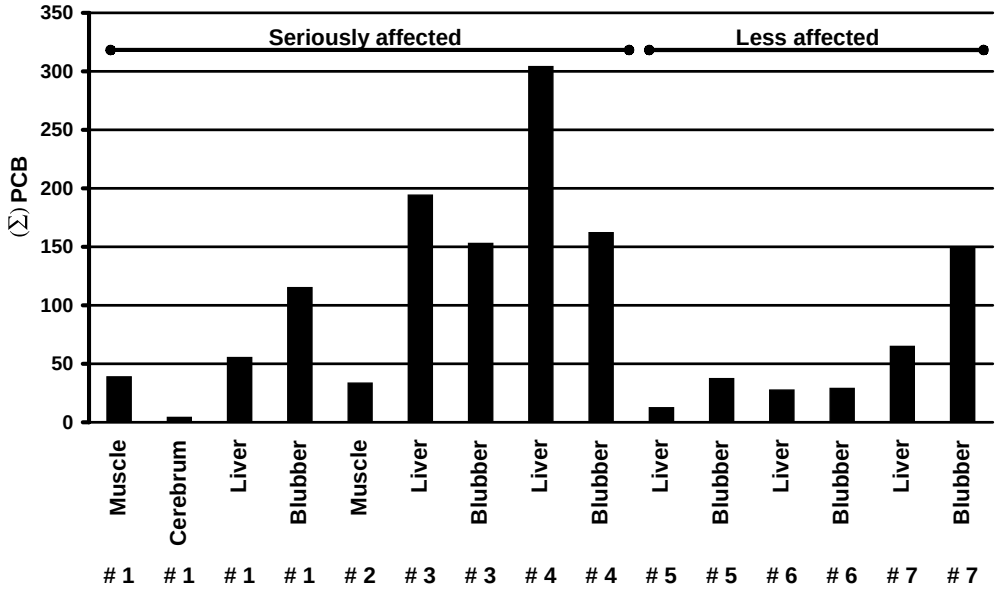

Fig. 6. Total CB levels in Grey seal tissue samples ($\mu g\,g^{-1}$ lipids).

Levels of the remaining non-*ortho* CBs (CBs 81, 126 and 169) were below the limits of detection. It was not possible to concentrate the extracts further as some of the other congeners are present at 1000-fold higher levels and the narrow bore capillary columns used would have been severely overloaded if more material was injected. However, most of the mono-*ortho* CBs could be detected as they are more abundant than the non-*ortho* CBs (Table 4). The levels of the mono-*ortho* CBs 105, 118, 156, 157 and 189 were in the 100 ng/g lipids range, while only traces of CBs 114, 123 and 167 were found.

4. Concluding remarks

The aims of this study were to optimize the analysis of chiral CBs in standard mixtures and grey seal samples by 2D-GC. Many atropisomers were successfully resolved and also separated from possible coelutants, e.g. *p,p′*-DDE and nonachloro-bornanes (Figs. 2 and 3). Conventional 1D-GC would have failed to do this, unless extensive cleanup had been performed to remove pesticide residues and other interferences. These components are generally found at similar or higher (up to 100-fold) levels than the pentachlorobiphenyl atropisomers.

The study also showed that the EFs of some CBs deviate strongly from racemic and that the ratio deviates more in liver than in blubber, indicating that enantioselective metabolism occurs. However, no correlation between EFs and health status was observed, although there was a correlation between the total CB levels and health status.

In addition, since GC × GC-μECD is very sensitive it would not be difficult to investigate the entire food chain: from sediments and sediment-living organisms to fish and seals.

References

[1] R.J. Aulerich, R.K. Ringer, Arch. Environ. Cont. Toxicol. 6 (1977) 279.
[2] M.R. Bleavins, R.J. Aulerich, R.K. Ringer, Arch. Environ. Cont. Toxicol. 9 (1980) 627.
[3] S. Jensen, J.E. Kihlstrom, M. Olsson, C. Lundberg, J. Orberg, Ambio 6 (1977) 239.
[4] J.E. Kihlstrom, M. Olsson, S. Jensen, A. Johansson, J. Ahlbom, A. Bergman, Ambio 11 (1992) 563.
[5] G. Blomkvist, A. Roos, A. Bignert, M. Olsson, S. Jensen, Ambio 21 (1992) 539.

[6] O. Andersson, A. Wartanian, Ambio 21 (1992) 550.
[7] B. Brunstrom, Ambio 21 (1992) 585.
[8] K.L.E. Kaiser, Environ. Pollut. 7 (1974) 93.
[9] V. Schurig, A. Glausch, M. Fluck, Tetrahedron Asymmetry 6 (1995) 2161.
[10] V. Schurig, S. Reich, Chirality 10 (1998) 425.
[11] M.T. Harju, P. Haglund, Fresenius J. Anal. Chem. 364 (1999) 219.
[12] C.S. Wong, A.W. Garrison, W.T. Foreman, Environ. Sci. Technol. 35 (2001) 33.
[13] C.S. Wong, P.F. Hoekstra, H. Karlsson, S.M. Backus, S.A. Mabury, D.C.G. Muir, Chemosphere 49 (2002) 1339.
[14] E. Benicka, R. Novakovsky, J. Hrouzek, J. Krupcik, P. Sandra, J. de Zeeuw, J. High Resolut. Chromatogr. 19 (1996) 95.
[15] H. Huhnerfuss, B. Pfaffenberger, B. Gehrcke, L. Karbe, W.A. Konig, O. Landgraff, Mar. Pollut. Bull. 30 (1995) 332.
[16] G.P. Blanch, A. Glausch, V. Schurig, Eur. Food Res. Technol. 209 (1999) 294.
[17] A. Glausch, J. Hahn, V. Schurig, Chemosphere 30 (1995) 2079.
[18] M. Puttmann, A. Mannschreck, F. Oesch, L. Robertson, Biochem. Pharmacol. 38 (1989) 1345.
[19] L.E. Rodman, S.I. Shedlowsky, A. Mannschreck, M. Puttmann, A.T. Swim, L.W. Robertson, Biochem. Pharmacol. 41 (1991) 915.
[20] C.S. Wong, A.W. Garrison, J. Chromatogr. A 866 (2000) 213.
[21] I.H. Hardt, C. Wolf, B. Gehrcke, D.H. Hochmuth, B. Pfaffenberger, H. Huhnerfuss, W.A. Konig, Hrc-J. High Res. Chromatogr. 17 (1994) 859.
[22] P. Haglund, K. Wiberg, Hrc-J. High Res. Chromatogr. 19 (1996) 373.
[23] M. Harju, P. Haglund, J. Microcol. Sep. 13 (2001) 300.
[24] H.J. de Geus, J. de Boer, U.A.Th. Brinkman, Chromatographia 55 (2002) 339.
[25] P. Korytar, P.E.G. Leonards, J. de Boer, U.A.Th. Brinkman, J. Chromatogr. A 958 (2002) 203.
[26] M. Harju, P. Haglund, C.J. Danielsson, Chromatogr. A, submitted for publication.
[27] G.M. Frame, Fresenius J. Anal. Chem. 357 (1997) 701.
[28] P.J. Marriott, R.M. Kinghorn, Anal. Chem. 69 (1997) 2582.
[29] E.B. Ledford Jr., C. Billesbach, J. High Resolut. Chromatogr. 23 (2000) 202.
[30] S.E. Reichenbach, M. Ni, V. Kottapalli, A. Visvanathan, E.B. Ledford, Jr., A Quick Tour of GC-Image—Software for Two-Dimensional Gas Chromatographic Image Processing and Analysis, 2002.
[31] T. Harner, K. Wiberg, R. Norstrom, Environ. Sci. Technol. 34 (2000) 218.
[32] H.J. de Geus, P.G. Wester, J. de Boer, U.A.Th. Brinkman, Chemosphere 41 (2000) 725.
[33] R.E. Murphy, M.R. Schure, J.P. Foley, Anal. Chem. 70 (1998) 1585.
[34] H. Li, J.P. Boon, W.E. Lewis, M. Van den Berg, M. Nyman, R.J. Letcher, Environ. Toxicol. Chem. 22 (2003) 636.
[35] M. Van den Berg, L. Birnbaum, A.T. Bosveld, B. Brunstrom, P. Cook, M. Feeley, J.P. Giesy, A. Hanberg, R. Hasegawa, S.W. Kennedy, T. Kubiak, J.C. Larsen, R.F.X. van Leeuwen, D.A.K. Liem, C. Nolt, R.E. Peterson, L. Poellinger, S. Safe, D. Schrenk, D. Tillitt, M. Tysklind, M. Younes, F. Wärn, T. Zacharewski, Environ. Health Persp. 106 (1998) 775.
[36] U.G. Ahlborg, G.C. Becking, L.S. Birnbaum, A. Brouwer, H.J.G.M. Derks, M. Feeley, G. Golor, A. Hanberg, J.C. Larsen, Chemosphere 28 (1994) 1049.
[37] J. de Boer, C.J.N. Stronck, F. Van der Valk, P.G. Wester, M.J.M. Daudt, Chemosphere 25 (1992) 1277.