

## Enantioselective semi-preparative HPLC separation of PCB metabolites and their absolute structures determined by electronic and vibrational circular dichroism.

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### Introduction

Atropisomeric PCBs and their metabolites can be separated on derivatised cyclodextrin (CD) based chromatographic columns <sup>i-v</sup>. The relevance of enantioselective analysis is supported by studies in which atropisomers of PCBs have been shown to exhibit a differential potency to induce several xenobiotic-metabolising enzymes or to accumulate uroporphyrin <sup>vi</sup>. In a thorough study on this subject, enantiomeric excesses of several atropisomeric methylsulfonyl PCBs have been found in different human, rat and whale tissue samples <sup>vii-viii</sup>. Some of the congeners are exclusively present in one enantiomeric form only. This is in contrast with the chiral PCB parents determined so far in biota. The results from a current study on the transformation of PCB 149 and its metabolite 3-MeSO<sub>2</sub>-CB149 by rat hepatocytes have suggested that the enantiomeric excesses of the metabolite are caused by a highly enantioselective preferential transformation of the (*S*)-3-MeSO<sub>2</sub>-CB149, while its mother compound is being transformed nearly racemically <sup>ix</sup>. Up till now, no such thorough investigation has been carried out for the atropisomeric hydroxylated, methoxylated and methylthionylated metabolites. Specific toxicological effects of each enantiomer of either methylsulfonyl, hydroxy, methoxy or methylthionyl metabolites have not been studied in detail due to the fact that pure enantiomeric components have not been available in sufficient amounts.

The only completely reliable method for determining the absolute configuration of chiral molecules is anomalous X-ray diffraction, but its inherent disadvantage is that sufficiently well-shaped single

crystals are needed. All other methods are based on comparing experimental data obtained by chiroptical methods, *i.e.*, polarimetry, optical rotation dispersion (ORD), electronic circular dichroism (UV-CD) or vibrational circular dichroism VCD, with the data of structural analogues of known configuration<sup>x</sup>. But it is important to keep in mind that the assignment of the absolute structure by means of these methods is, in principle, based on reasons by analogy, which is often unreliable or could even be misleading.

The UV-CD method is suitable for chiral compounds containing at least one chromophoric group, which, due to the presence of the biphenyl systems, is the case here. The UV-CD method was, therefore, used in this study for a first, preliminary estimation of the absolute structure. Despite the fact that a much larger amount of substance is needed for applying the VCD method, because of the relatively weak absorption signals, it has three major advantages over the UV-CD method: (i) all chiral molecules exhibit the vibrational circular dichroism effect, not only those which contain chromophores; (ii) the VCD spectra exhibit many more signals; and (iii) in case of flexible molecules the VCD signals can be also dependent on the actual conformation, thus allowing, in principle, a conformational analysis. Furthermore, in combination with theoretical *ab initio* calculations, VCD is a genuine and elegantly alternative method for structure determination in an absolute sense<sup>xi-xiv</sup>.

The present paper represents a first result of an ongoing systematic study of atropisomeric methylsulfonyl, methylthionyl, hydroxy, and methoxy metabolites of environmentally most relevant PCBs. This involves semi-preparative enantioselective HPLC separation to obtain pure atropisomers from synthesized PCB metabolite standards, their configuration estimation using the electronic circular dichroism (UV-CD) method and the determination / confirmation of these absolute configurations applying the combined vibrational circular dichroism (VCD) / *ab initio* approach. The following substances have been investigated: 4-HO-, 4-MeO-, 4-MeS-, 4-MeSO<sub>2</sub>-, 3-MeS- and 3-MeSO<sub>2</sub>-CB149.

## Materials and Methods

PCB metabolites were synthesised according to procedures which will be published elsewhere. The solvents used were Lichrosolv grade and purchased from Merck (Darmstadt, Germany). All other chemicals were p.a. grade and also purchased from Merck.

The HPLC system consists of an on-line vacuum degasser Degasys DG-1310, a Gynkotek GINA autosampler, a Gynkotek M480 HPLC gradient pump, a Gynkotek column oven, a Gynkotek UVD340S detector and an Advantec SF-2120 Super Fraction Collector. Chromatographic runs were performed under isocratic conditions. Solvent composition was optimised individually for each compound. Four normal-length chiral HPLC columns (200 mm long) and three short chiral pre-columns (30 mm long) have been evaluated in this study.

Optical rotation of separated enantiomer solutions in *n*-hexane was measured on a PE Polarimeter 341 (Perkin-Elmers Corp., Norwalk, CT, USA) with a normal aperture. The Na/Halogen lamp was used for measurements on 589 nm wavelength (Na line), while the mercury (Hg) lamp was applied for 436 nm wavelength measurements.

UV-CD spectra of separated enantiomers (solution in *n*-hexane) were obtained using the AVIV Model 215 Circular Dichroism Spectrometer (AVIV Instruments, Inc., Lakewood, NJ, USA). Quartz cuvettes with 10.00 mm path length were employed.

VCD spectra were recorded for separated enantiomers, dissolved in deuterated chloroform (for NMR spectroscopy, Merck). A Bruker IFS 66/S, PMA 37 VCD spectrometer (Bruker Optik GmbH, Ettlingen, Germany) was used with a 210  $\mu\text{m}$  pathlength KBr cell (Bruker). Each VCD spectrum was measured in 30 min with a resolution of 6  $\text{cm}^{-1}$ . VCD spectra were baseline corrected against spectrum of pure  $\text{CDCl}_3$ .

For each compound – except for 4-HO-CB149 – one single enantiomer was geometry optimised to its respective energy minimum at the B3LYP/6-31G\* level of theory using the implementation of Gaussian 98<sup>xv</sup>. A vibrational analysis followed employing the same method and basis set. The values of the frequencies were not scaled. The procedure for calculating the VCD intensities using density functional theory (DFT) is based on the gauge-invariant atomic orbitals (GIAOs)<sup>xvi,xvii</sup> approach, developed and adopted by Cheeseman *et al.*<sup>xiii</sup> as implemented in the Gaussian 98 package. In order to improve the appearance of the spectra a Lorentzian peak profile with a FWHM of 15  $\text{cm}^{-1}$  was applied to the peaks of the obtained stick spectra.

## Results and Discussion

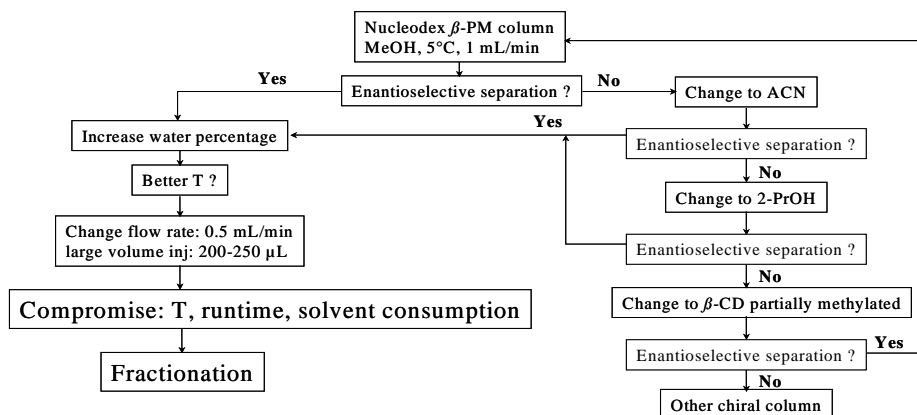
### 1. HPLC optimisation and enantiomer separation

The optimisation of enantioselective HPLC separation of atropisomeric PCB metabolites aims to achieve the best compromise between enantioselective separation quality, chromatographic runtime and costs of labour and solvents used. The enantioselective separation quality depends on a number of parameters which need to be carefully optimised. The most important are: column stationary phase, mobile phase composition, column oven temperature, solvent type, mobile phase pH, flow rate and injection volume. Based on the results obtained from a previous study<sup>xviii</sup>, a starting point with the following parameters was chosen: 250 mm  $\times$  8 mm  $\times$  5  $\mu\text{m}$  Nucleodex  $\beta$ -PM column, MeOH mobile phase at 1.0 mL/min, oven temperature 5°C and a 20  $\mu\text{L}$  injection volume. The optimisation process is then carried out according to the general procedure depicted in Figure 1. Optimised chromatographic conditions of the respective separation process of the metabolites of interest are summarised in Table 1.

**Table 1:** Chromatographic conditions for a semi-preparative separation of PCB metabolites into their respective enantiomers. Other parameters are similar for all compounds: column oven temperature 5°C, isocratic mobile phase with a flow rate of 0.5 mL/min. (PM = permethylated cyclodextrin).

Compound	Amount (mg)	Column	Mobile phase	Solvent consumption
4-MeO-CB149	49	250 × 8 mm Nucleodex $\beta$ -PM	0.5% HAc in MeOH	5 L MeOH
4-MeS-CB149	45	250 × 8 mm Nucleodex $\beta$ -PM	MeOH/H <sub>2</sub> O 95/5	8 L MeOH
4-MeSO <sub>2</sub> -CB149	50	250 × 8 mm Nucleodex $\beta$ -PM	(w/w)	12 L MeCN
3-MeS-CB149	45	250 × 8 mm Nucleodex $\beta$ -PM	MeCN/H <sub>2</sub> O 70/30(v/v)	10 L MeOH
3-MeSO <sub>2</sub> -CB149	50	250 × 8 mm Nucleodex $\beta$ -pPM*	MeOH	Test only
4-HO-CB149	40	30 × 4 mm Nucleodex $\beta$ -OH	100% <i>n</i> -hexane	

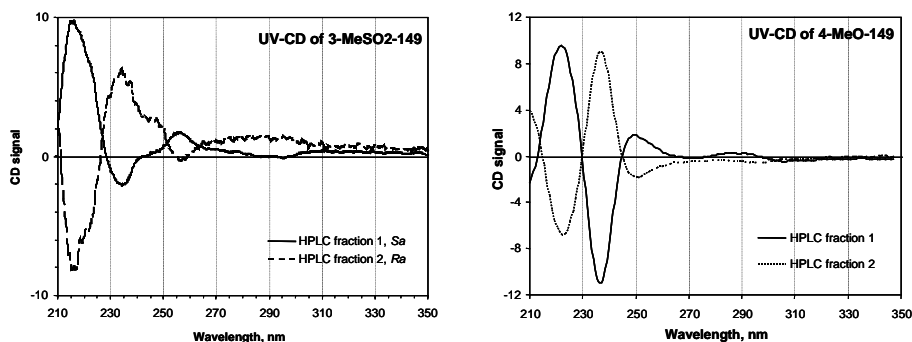
\* not fully permethylated  $\beta$ -cyclodextrin



**Figure 1:** Flow diagram of the general optimisation procedure for enantioselective HPLC separation of atropisomeric PCB metabolites (PM = permethylated cyclodextrin).

## 2. Attempt of preliminary absolute structure estimation using UV-CD

Based on the hypothesis that compounds with similar structure would exhibit similar UV-CD behaviour it is very tempting to try to estimate the configuration of newly separated atropisomers by comparing their UV-CD spectra with that obtained from 3-MeSO<sub>2</sub>-CB149 enantiomers, the absolute configuration of which was determined in a previous study<sup>xviii</sup>. These compounds belong to the same class, they even share the same PCB backbone, where their chiral centre resides. These substances differ only in type and position of the residual 'functional group'. It was, therefore, assumed that atropisomers with a similar UV-CD spectra and optical rotation behaviour would possess the same configuration. An example is given in Figure 2, where the UV-CD spectra of 4-MeO- and 3-MeSO<sub>2</sub>-CB149 enantiomers are compared. The maxima / minima on the CD spectra of both, firstly eluted 3-MeSO<sub>2</sub>-CB149 and firstly eluted 4-MeO-CB149, highly correspond to each other. Furthermore, both compounds the polarized light to the same direction, *i.e.*, to the right (+). From this observation, the absolute configuration of the first eluted 4-MeO-CB149 atropisomer was estimated as (*Sa*) (small *a* denotes the axial chirality of the biphenyl system<sup>xix</sup>). The configuration estimation for 4-MeS-, 4-MeSO<sub>2</sub>- and 3-MeS-CB149 enantiomers has been followed the same line of reasoning and is summarized in Table 2. However, this hypothesis disregards the contributions



**Figure 2:** UV-CD spectra of separated enantiomers of atropisometric PCB metabolites after the enantioselective HPLC separation into respective enantiomers. Experimental conditions: see text, Materials and Methods section.

**Table 2:** Characteristics of separated enantiomers from PCB metabolites.

compound	amount (mg)	Enant. purity (%)	Conc. (mg/mL)	OROT (°) 589 nm	OROT (°) 436 nm	Abs. struct. est. w/ UV- CD	Abs. struct. det. w/ VCD
3-MeSO <sub>2</sub> -CB149 fr.1	22.6	98.8	15.1	+ 0.03			<i>Sa</i>
3-MeSO <sub>2</sub> -CB149 fr.2	21.7	98.5	14.5	- 0.02			<i>Ra</i>
3-MeS-CB149 fr.1	16.5	98.5	11.0	+ 0.04		<i>Ra</i>	<i>Sa</i>
3-MeS-CB149 fr.2	19.1	95.0	12.7	- 0.05		<i>Sa</i>	<i>Ra</i>
4-MeO-CB149 fr.1	18.9	99.9	12.6	+ 0.07		<i>Sa</i>	<i>Ra</i>
4-MeO-CB149 fr.2	18.3	97.9	12.2	- 0.08		<i>Ra</i>	<i>Sa</i>
4-MeS-CB149 fr.1	19.0	99.9	12.7		+ 0.03	<i>Sa</i>	<i>Ra</i>
4-MeS-CB149 fr.2	19.3	98.7	12.9		- 0.03	<i>Ra</i>	<i>Sa</i>
4-MeSO <sub>2</sub> -CB149 fr.1	27.2	99.9	18.1	+ 0.05	+ 0.09	<i>Sa</i>	<i>Ra</i>
4-MeSO <sub>2</sub> -CB149 fr.2	21.2	97.0	14.1	- 0.03	- 0.09	<i>Ra</i>	<i>Sa</i>

of the type and the position of the 'functional group' to the UV behaviour of the molecule as a whole. This has been proved to be misleading as shown in the next section.

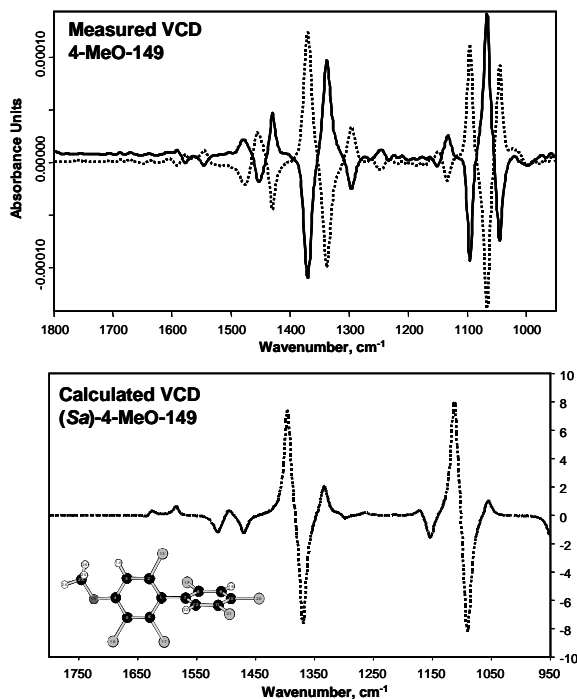
### 3. Absolute structure determination using VCD and *ab initio* DFT calculation

In Figure 3 the experimental VCD spectra of the two 4-MeO-CB149 enantiomers as well as the calculated VCD spectrum of the (*Sa*) enantiomer (see inset for the structure) are presented. All main features of the VCD spectrum of 4-MeO-CB149 fraction 2 could be reproduced in the calculation of the spectrum corresponding to the (*Sa*) enantiomer. Only the intensity of the band at 1043  $\text{cm}^{-1}$  is strongly underestimated, though it is still visible. However, the matching of the intensities and signs of at least 7 bands (at 1429, 1369, 1337, 1293, 1132, 1096, and 1067  $\text{cm}^{-1}$ ) allows the unequivocal assignment of fraction 2 to the (*Sa*) enantiomer. A similar procedure has been applied to all the metabolites of interest. The results, also summarized in Tables 2 for comparison purposes, show that there is a contradiction between the two methods for structure elucidation of PCB metabolites' enantiomers. Due to the fact that the VCD / *ab initio* DFT calculation deal with the configuration / conformation of the molecule bonds in an absolute sense and no approximate assumption has to be made, the results obtained by this method is much more reliable and are taken as the final results.

### Conclusion

A HPLC method has been optimised for semi-preparative separation of atropisomeric PCB metabolites into their pure enantiomers. The Nucleodex  $\beta$ -PM, permethylated  $\beta$ -cyclodextrin, is proven to be a workhorse for this purpose. There are, however, cases, when only a Nucleodex  $\beta$ -pPM or  $\beta$ -OH, partially permethylated or native  $\beta$ -cyclodextrin, respectively, can be used to achieve enantioselective separation. About 20 mg of each enantiomer with an enantiomeric purity exceeding 95% has been obtained from the 5 investigated PCB metabolites: 3-MeSO<sub>2</sub>-, 3-MeS-, 4-MeO-, 4-MeS- and 4-MeSO<sub>2</sub>-CB149. These pure enantiomers will be used for further research on their toxicology and metabolism.

***The assumption that enantiomers of analogues compounds with similar configuration exhibit similar UV-CD and optical rotation behaviour proves to be very misleading. The structure assignment can be determined reliably and with a very high certainty only by comparing measured VCD spectra with***



**Figure 3:** Experimental VCD spectra (upper part) of separated 4-MeO-CB149 fractions: fraction 1 (solid line) and fraction 2 (dotted line), and calculated spectrum (lower part) of the *Sa* enantiomer; the geometry of the corresponding energy minimized structure is shown in the inset.

**ab-initio DFT calculated spectra. With this method, no previously known absolute structure is needed. Apart from the need of computer, software and computing knowledge, however, much larger amounts of substances are necessary.**

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