COUNTER-CURRENT CHROMATOGRAPHIC SEPARATION OF TOXAPHENE – PART 1: POLAR CONSTITUENTS

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Introduction

High-speed counter-current chromatography (HSCCC) is a separation technique based on partitioning processes between two immiscible liquid phases[1]. In contrast to conventional column chromatography, counter-current chromatography does not involve the use of solid support materials. Therefore, HSCCC allows for the preparative separation of compound mixtures without the danger of substance losses due to irreversible adsorption on the stationary phase[2]. For this and other reasons, HSCCC is an increasingly popular technique, e.g. when natural products are to be isolated from complex plant matrices[3].

Because of the high sample capacity of HSCCC (several 100 mg), the question arises whether this technique is also capable of separating complex mixtures of structurally similar compounds. To investigate this, we fractionated technical toxaphene using HSCCC in the reversed phase mode. Toxaphene is an insecticidal multi-component mixture which has been produced by exhaustive photochlorination of camphene. It consists of more than 1000 structurally closely related components[4] (Figure 1), mainly polychlorinated bornanes, camphenes, dihydrocamphenes and bornenes bearing six to ten chlorine atoms per molecule[5,6] (Figure 2). Even today, for the vast majority of the compounds of technical toxaphene (CTTs), structures are not completely elucidated and only few CTTs are commercially available in pure form. These include some of the most abundant congeners in biota of high trophic levels.[5] However, the presence of individual congeners in toxaphene residue patterns is sample-dependent.[7] Sediment samples, for instance, often contain unknown lower chlorinated congeners.[8,9] For this reason, our study focussed on the early eluting toxaphene fractions under reversed phase HSCCC conditions, which are expected to be enriched in lower chlorinated und thus more polar congeners.

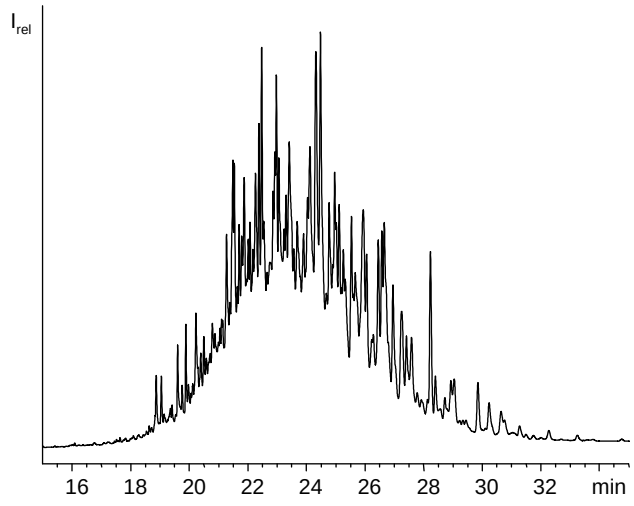

Figure 1: GC/ECD chromatogram of technical toxaphene (Melipax®). Column: CP-Sil 2, 50 m x 0.25 mm, 0.25 µm d_f.

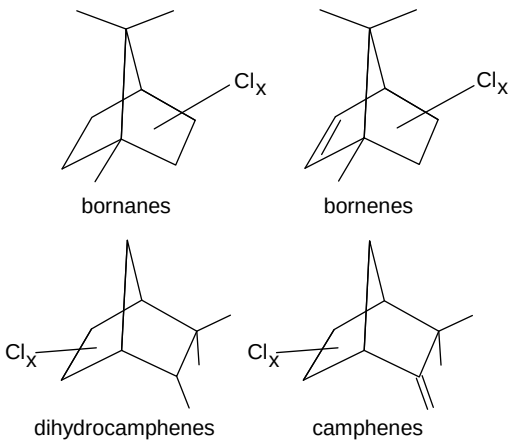

Figure 2: Skeletal structures of the most important compound classes in technical toxaphene[5].

Materials and methods

High-speed counter-current chromatography (HSCCC)

HSCCC was performed on a model CCC-1000 planetary high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD, USA), equipped with three preparative coils of PTFE tubing connected in series. The total column volume was 325 mL. For solvent delivery, a quarternary P580 HPLC pump (Dionex, Idstein, Germany) was operated at a flow rate of 1.5 mL min^{-1}. Thirty-nine fractions (351 mL) were collected every 6 min (9 mL) using an Isco Retriever 500 (Teledyne Isco, Lincoln, NE, USA).

A two-phase solvent system was developed consisting of *n*-hexane, methanol and water (HMWat) in a volume ratio of 34:24:1. The solvents were thoroughly mixed in a separatory funnel and equilibrated over night. Upper and lower phase were separated shortly before use. The resulting volume ratio between upper and lower phase was approximately 1:1. The coils were first filled with the upper stationary phase. Subsequently, rotation was started (1000 rpm) and the lower mobile phase was introduced in the head to tail mode. Injection of 500 mg of toxaphene (active ingredient, isolated from the East German formulation Melipax®), dissolved in a mixture of both phases, occurred using a 10 mL sample loop after hydrodynamic equilibrium was established.

Analysis of HSCCC fractions and data evaluation

All HSCCC fractions were blown down to dryness with a gentle stream of nitrogen, weighed and redissolved in *n*-hexane. These stock solutions were then diluted with isooctane to final concentrations of about 100 ng μL^{-1}. The diluted solutions were analyzed using GC/ECNI-MS on a CP3800 gas chromatograph coupled to a 1200 triple-quadrupole mass spectrometer (Varian, Darmstadt, Germany). GC separations were performed on a 30 m HP-5ms capillary column (0.25 mm i.d., 0.25 µm d_f) with helium 5.0 as carrier gas at a constant flow rate of 1.4 mL min^{-1}. Injector and transfer line temperatures were set at 230 °C and 280 °C, respectively. Sample introduction occurred by splitless injection (2 min splitless time). The oven temperature program started at 80 °C (1 min), was then ramped at 40 °C min^{-1} to 180 °C, followed by a 2 °C min^{-1} ramp to 270 °C (held for 4.5 min). The total run time was 45 min. The ion source of the MS was maintained at 150 °C. Methane 5.0 was used as buffer gas at a source pressure of approximately 8.5 Torr. The electron energy and emission current were set at 70 eV and 150 µA, respectively. Spectra were recorded in SIM mode (scan time 0.5 s, peak width 0.7 u), covering the $[M\text{-}Cl]^-$ and $[M]^-$ fragment ion clusters of penta- to undecachloro-CTTs.

A two-dimensional contour plot was created by plotting the HSCCC fraction number versus the GC retention time using SigmaPlot 2001 (Systat Software, Erkrath, Germany). Colour codes were assigned according to the relative signal abundances of the TIC chromatograms after baseline correction and within-run normalization. Classification of CTTs (degree of chlorination, saturated/unsaturated compounds) was based on the ratios of isotopic peaks observed in the ECNI-MS-SIM spectra.

Results and discussion

Under the above HSCCC conditions, the first toxaphene compounds eluting from the column were found in fraction 8, which contained approximately 0.2 mg of substance (Figure 3). Fractions 9-11 yielded around 1 mg of substance on average. A significant increase in yield was observed for the following fractions, which yielded about 4-8 mg (5.3 ± 1.2 mg) until the fractionation was terminated after fraction 39. The total volume of mobile phase used for the separation corresponded to about one column volume. It can be assumed that at that point all solutes that exhibit a greater affinity to the lower mobile phase (mostly methanol/water) than to the upper stationary phase (mostly *n*-hexane/methanol) had eluted from the column. The total amount of polar CTTs collected was 150 mg.

The GC/ECNI-MS chromatograms revealed that the fractions generally contained more than just one peak, which is not unexpected considering the complexity of technical toxaphene (see Figure 1). However, far less peaks were present in the individual HSCCC fractions. Usually, only a few peaks were dominating the chromatogram of a fraction. The two-dimensional contour plot visualizes the course of the separation very well (Figure 4). For convenience, the most abundant peaks were labelled according to their degrees of chlorination and saturation.

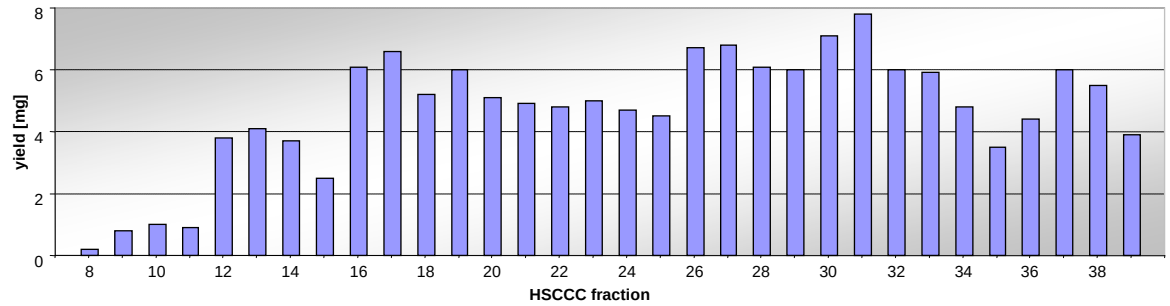

Figure 3: HSCCC fractionation of Melipax®, yields per fraction. Elution started in fraction 8 and was terminated after fraction 39. In total, approx. 150 mg of polar toxaphene constituents were collected.

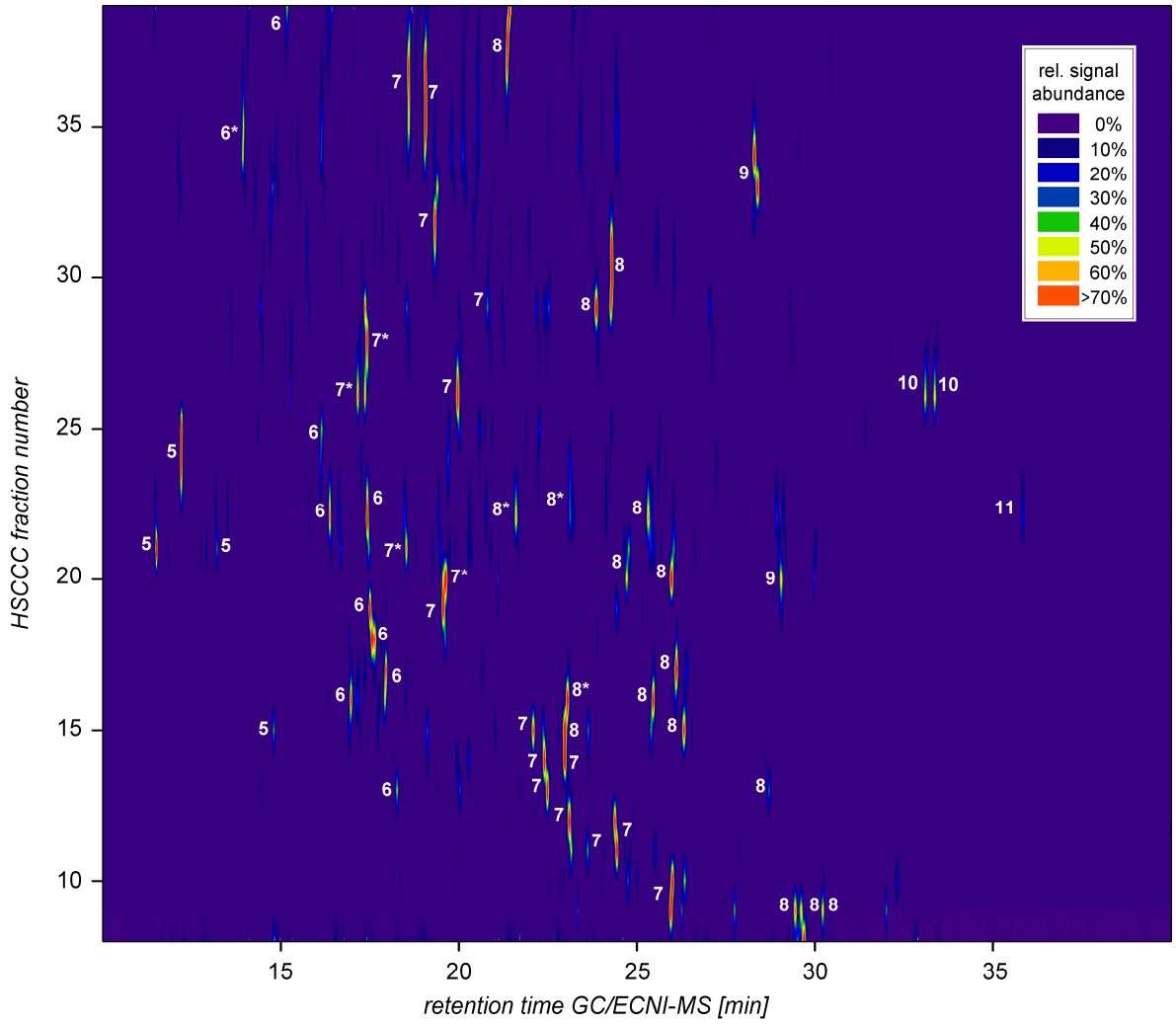

Figure 4: Overview of toxaphene fractionation by HSCCC (selection corresponds to about 30 % of the injected amount of Melipax). The shown elution range covers polar constituents of toxaphene. Numbers: degree of chlorination. *: unsaturated compounds.

Interestingly, the first eluting CTTs (fractions 8-11) were saturated compounds substituted with seven to eight chlorine atoms. This is in contrast to preliminary expectations regarding the elution order of CTTs. As the HSCCC separation was performed in reversed phase mode, compounds with the lowest degree of chlorination (penta- or hexachloro-CTTs in the case of technical toxaphene) were expected to elute first. The presence of hepta- and octachlorinated CTTs in the first HSCCC fractions indicates that these compounds may be rather untypical CTTs. The observation that these toxaphene constituents eluted at the end of the respective elution windows of hepta- and octachloro-CTTs corroborates this hypothesis.

Generally speaking, the fractions shown in Figure 4 contained more untypical CTTs than average. About 15 % of the main peaks in the contour plot originated from unsaturated compounds. Furthermore, four abundant pentachloro-CTTs could be identified, one of them showing considerable enrichment (fractions 24-25). Several pentachlorobornanes have previously been detected in sediment samples.[8,9] A similarly strong enrichment was observed for undecachlorinated homologs. In total, ten undecachloro-CTTs could be detected, albeit with minor abundances except for one congener which reached 25 % signal abundance in fraction 22. Again, it is surprising that compounds so highly chlorinated eluted so early.
The total number of components that could be differentiated using the described offline HSCCC x GC/ECNI-MS coupling added up to almost 400. Bearing in mind that only 30 % of the injected amount of toxaphene was fractionated, the total number of toxaphene constituents determined this way will probably correlate well with the currently most comprehensive estimate of >1000 CTTs, as determined by comprehensive GC (GC x GC).[4] It is obvious that a peak number that large cannot be completely separated in a single GC run. Therefore, the use of HSCCC fractionation prior to GC analysis clearly enabled the identification of coelutions. This is in total agreement with the detection of peaks having the same GC retention time, but different mass spectra and/or location in the contour plot. The different retention mechanisms of counter-current chromatography and the GC separation on the non-polar HP-5ms column evidently improved the amount of information that could be drawn from the complex toxaphene mixture.

Conclusions

Due to the pronounced enrichment of minor toxaphene components by HSCCC fractionation, some CTTs could be detected which were below the limit of detection in non-fractionated toxaphene, even when GC/ECNI-MS-SIM was employed.

The separation power of HSCCC x GC/ECNI-MS was comparable to that of GC x GC. However, using HSCCC, higher amounts of substance can be injected so that this technique is suited for the isolation and structure investigation of interesting compounds.

These results illustrate that counter-current chromatography can indeed provide remarkable opportunities for the characterisation of toxaphene in particular, and for complex mixtures in general. Future work will aim at further improvement of the separation efficiency and at the coverage of the entire range of toxaphene components.

References

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