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Sequential fractionation procedure for the identification of potentially cytochrome P4501A-inducing compounds

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Abstract

A multistep fractionation procedure for the separation of nonpolar aromatic compounds with respect to cytochrome P4501A induction is presented. Normal-phase HPLC on nitrophenylpropyl silica and cyanopropyl silica was tested for group-specific separation as a first fractionation step. Subsequent individual compound-specific PAH fractionation was done by means of reversed-phase HPLC. Electron-donor–acceptor HPLC and size-exclusion chromatography were applied to separate PAHs, PCBs, PCNs and PCDD/Fs according to their number of aromatic carbon atoms, their hydrophobicity, their degree of chlorination, their planarity and their molecular size. The method was validated for complex environmental mixtures on the basis of two sediment extracts.

Keywords: Multistep fractionation; Preparative chromatography; Cytochrome P4501A-inducing compounds

1. Introduction

Numerous aromatic environmental pollutants are known or suspected Ah-receptor mediated toxicants, which are able to induce cytochrome P4501A (CYP1A) in exposed cells. These compounds include halogenated aromatic hydrocarbons (HAHs) such as polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs), and naphthalenes (PCNs) as well as polycyclic aromatic hydrocarbons (PAHs) including alkylated and heterocyclic polyaromatic compounds [1]. The CYP1A-inducing potency is closely related to the affinity of the inducer for the cellular aryl hydrocarbon (Ah) receptor and depends on the individual aromatic structure and substitution pattern. In the environment CYP1A inducers in general occur in complex mixtures together with innumerable noninducing compounds, which may interfere with biological and chemical analysis. The identification of individual CYP1A inducers and the assessment of their contribution to overall hazards in complex environmental mixtures requires the removal of noninducing compounds which may by cytotoxic or inhibit enzyme activity and an extensive and well defined separation of CYP1A inducers prior to biotesting.

Several approaches for the separation of aromatic

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environmental pollutants have been presented [2–4]. However, an isomer specific fractionation procedure for CYP1A-inducing compounds in complex environmental mixtures is still not available.

The objective of the present study was to develop such a fractionation procedure by the transfer, adaptation and combination of available liquid chromatographic methods including normal-phase (NP-LC), reversed-phase (RP-LC), electron-donor–acceptor (EDA-LC) and size-exclusion chromatography (SE-LC). The validation for complex environmental samples was based on sediment extracts from two contaminated German creeks, Spittelwasser (Sp) and Forellenbach (Fo). The creek Spittelwasser drains the industrial region of Bitterfeld into the River Mulde, which is a tributary to the River Elbe. Spittelwasser sediment extracts proved to exhibit high CYP1A-inducing potency [5] and to be contaminated with numerous toxicants including PAHs [6], PCDD/Fs [7] and PCNs [8]. The creek Forellenbach in the catchment area of the River Neckar was contaminated by hospital effluents, which resulted in high PAH concentrations in the sediments [9].

2. Experimental

2.1. Chemicals

HPLC-grade solvents *n*-hexane (HX), dichloromethane (DCM), tetrahydrofuran (THF), and acetonitrile (ACN) for sample extraction, clean up, fractionation, and analysis were obtained from Merck (Darmstadt, Germany). PAH standards were purchased from Ultra Scientific (North Kingstown, USA) except binaphthalenes, methylchrysenes (MeCHR), methylbenz(*a*)anthracenes (MeBaA) and dinaphthofurans, which were obtained from Chiron (Trondheim, Norway). PCDD/Fs, PCNs and PCBs were purchased from Promochem (Wesel, Germany). Naphthalenylbenzothiphenes were synthesized by Jan T. Andersson (University of Münster). PCBs are signified by the IUPAC numbers (CB1 to CB209), the PCNs according to the nomenclature by Wiedmann and Ballschmitter (CN1 to CN75) [10].

2.2. Sampling and sample extraction

The sediments were sampled in slack water zones in 1998 in the creeks Spittelwasser (Sp) and Forellenbach (Fo), respectively. For both sediments several individual samples from the top layer were pooled, homogenized, and freeze dried. The sediments were Soxhlet extracted with DCM for 24 h. Elemental sulfur was removed by shaking the extract with activated copper overnight.

2.3. Clean up

Nonpolar aliphatic and polar compounds were removed applying a clean up on alumina (activity I, ICN Biomedicals, Eschwege, Germany), which was deactivated with 4.5% distilled water. An aliquot of 10 ml desulfurized sediment extract corresponding to 100 g sediment was sorbed on 18 g deactivated alumina by evaporation of the solvent in a rotation evaporator. The loaded alumina was transferred onto 60 g neat deactivated alumina in HX in a column with a diameter of 3 cm. Nonpolar aliphatic compounds were removed by eluting the column with 72 ml HX. Nonpolar aromatic compounds including PAH and HAH were subsequently eluted with 270 ml HX–DCM (95:5, v/v).

2.4. HPLC instrumentation

All separations were run on an HPLC system equipped with two high pressure pumps (Kontron HPLC-pump 422, Biotek Instruments, Neufahrn, Germany) and a dual mode UV–Vis detector (Kontron HPLC detector 430, Biotek Instruments, Neufahrn, Germany) operated at 225, 250, 280 or 310 nm depending on the analytes. Fractions were collected using an automated fraction collector (SF-2120, Advantec MFS, Pleasanton, CA, USA).

2.5. Normal-phase HPLC

Using stainless steel columns (4×250 mm), respectively, two stationary NP phases were tested for their separation capacity of PAHs with two to six aromatic rings: nitrophenylpropyl silica (NO) (5 μm Nucleosil 100-5 NO_2, Machery and Nagel, Düren, Germany) and cyanopropyl (CN) silica (5 μm Nucleosil 100-5 CN, Machery and Nagel). Standard solutions at individual concentrations of 10–50 mg/l were used for the determination of structure dependent retention behavior on both columns using 0.7

ml/min HX or HX–DCM (95:5, v/v) as mobile phases at 10 °C (Table 1).

Since the separation efficiency of the CN phase was insufficient, for fractionation a preparative NO column (21 mm diameter) was used. Appropriate run times and separation efficiency were achieved with 19 ml/min HX–DCM (95:5, v/v) as mobile phase. Seven fractions were collected. Recoveries were determined using PAH standard solutions at individual concentrations of 10 mg/l.

Table 1
Normal phase capacity factors of selected aromatic compounds on cyanopropyl silica (k_{CN}) and nitrophenylpropyl silica with *n*-hexane (k_{NO100}) and *n*-hexane–dichloromethane (95:5, v/v) ($k_{NO95/5}$) as mobile phase, fractionation course, recovery in the respective fractions and concentrations in fractions of the Forellenbach (C_{Fo}) and Spittelwasser (C_{Sp})

	k_{CN}	k_{NO100}/ $k_{NO95/5}$	Recovery (%)	C_{Fo} (μg/kg)	C_{Sp} (μg/kg)
F1 (3–6 min)					
CN 66	–	–/0.11	77	–	–
1,2,3,6,7,8-HxCDF	–	–/0.25	79	–	–
2,3,7,8-TCDD	–	–/0.28	85	–	–
Naphthalene	0.26	0.31/0.31	41	–	2300
CB 169	–	–/0.36	78	–	–
Acenaphthene	0.26	0.43/0.38	51	–	–
F2 (6–7 min)					
1-Phenylnaphthalene	0.34	0.77/–	–	–	–
Fluorene	0.36	0.79/0.62	55	211	1700
Acenaphthylene	0.38	0.82/0.63	52	2	190
F3 (7–9 min)					
Anthracene	0.46	0.88/–	86	1400	1900
4H-Cyclopenta(*def*)phenanthrene	0.44	1.37/–	–	320	1700
Phenanthrene	0.48	1.46/0.85	81	2730	12 600
2-Phenylnaphthalene	0.49	1.57/–	–	190	6000
3-(1-Naphthalenyl)benzothiophene	0.51	1.63/–	–	–	–
1,1′-Binaphthalene	0.51	1.72/–	–	–	–
Benzo(*b*)naphtho[2,1-*d*]furan	0.47	1.74/–	–	–	–
F4 (9–10.5 min)					
Benzo(*b*)naphtho[1,2-*d*]furan	0.54	2.13/–	–	–	–
Pyrene	0.56	2.24/–	80	3140	10 700
Benzo(*b*)naphtho[2,3-*d*]furan	0.59	2.28/–	–	–	–
Benzo(*b*)naphtho(2,1-*d*)thiophene	0.74	2.71/–	–	–	–
3(2-Naphthalenyl)benzothiophene	0.66	2.79/–	–	–	–
Fluoranthene	0.64	2.80/1.45	79	4600	5800
F5 (10.5–14.5 min)					
1-Methylbenz(*a*)anthracene	0.74	3.26/–	–	–	–
Benzo(*g,h,i*)fluoranthene	0.67	3.34/1.96	60	640	5000
Dinaphtho[1,2-*b*;2′,1′-*d*]furan	0.73	3.44/–	–	–	–
Dinaphtho[2,1-*b*;1′,2′-*d*]furan	0.74	3.58/–	–	–	–
Benz(*a*)anthracene	0.83	3.88/2.10	80	4700	2400
2-Phenyldibenzothiophene	0.86	4.02/–	–	–	–
Dinaphtho[1,2-*b*;2′,3′-*d*]furan	0.83	4.16/–	–	–	–
Cyclopenta(*cd*)pyrene	0.79	4.30/–	–	66	320
Chrysene/triphenylene	0.83	4.39/2.2	79	4800	3900
1-Methylchrysene	0.83	4.55/–	–	–	–
2,2′-Binaphthalene	0.82	4.89/–	–	–	–
2-Methylchrysene	0.82	5.01/–	81	–	–
Dinaphtho[2,3-*b*;2′,3′-*d*]furan	1.02	5.44/–	–	–	–

Table 1. Continued

	k_{CN}	k_{NO100}/ $k_{NO95/5}$	Recovery (%)	C_{Fo} (μg/kg)	C_{Sp} (μg/kg)
F6 (17.5–21 min)					
1-Methylbenz(*a*)pyrene	0.96	5.79/–	–	–	–
3-Methylcholanthrene	0.89	5.98/–	–	–	–
Benzo(*e*)pyrene	1.02	6.04/–	–	5500	1700
9-Methylbenz(*a*)pyrene	1.02	6.06/–	–	–	–
7-Methylbenz(*a*)pyrene	0.99	6.12/–	–	–	–
Benzo(*k*)fluoranthene/benzo(*b*)fluoranthene	1.04	6.64/3.22	72	6900	1400
Benzo(*a*)pyrene	0.97	6.70/3.38	70	4400	1800
Benzo(*a*)fluoranthene	1.04	6.91/–	–	1400	320
Benzo(*j*)fluoranthene	1.12	7.11/–	–	4200	3800
Perylene	1.08	7.30/–	82	1800	520
F7 (21–35 min)					
Anthanthrene	1.13	9.17/–	–	2700	360
Indeno(1,2,3-*cd*)fluoranthene	1.28	9.53/–	4.97	70	72
Indeno(1,2,3-*cd*)pyrene	1.24	10.62/–	55	8500	2700
Benzo(*g,h,i*)perylene	1.18	11.06/5.28	54	12 400	3400

–, not determined.

2.6. Reversed-phase HPLC

PAHs coeluting with chrysene (CHR), benz-(*a*)anthracene (BaA) and triphenylene (TRP) on the NO column were separated by RP-LC. Analytical scale separations were run at 30 °C on a stainless steel column (4×250 mm) packed with a polymeric and endcapped octadecylsilica phase with a pore diameter of 100 Å developed for optimized PAH separation (Nucleosil 100-5 C_{18} PAH, Macherey and Nagel) applying acetonitrile–water (ACN–WAT) (75:25, v/v) as mobile phase at a flow-rate of 0.5 ml/min. For preparative scale fractionation a column diameter of 21 mm and a flow-rate of 14 ml/min were applied. Ten fractions were collected.

For chemical analysis reversed-phase fractions were transferred to hexane by triple liquid–liquid extraction after adding twice the volume of half saturated NaCl solution in order to enhance extraction efficiency.

2.7. Electron-donor–acceptor HPLC

PCBs, PCNs and PCDD/Fs, which coelute from the NO column in the first fraction, were separated by EDA-LC using a stainless steel column (10×250 mm) equipped with a stainless steel guard column (10×20 mm), both packed with 2-(1-pyrenyl)ethyldimethylsilylated silica (PYE) (5 μm Cosmosil Pye, Nacalai Tesque, Kyoto, Japan) with an average pore diameter of 120 Å at a mobile phase flow of 8 ml/min (for selection of mobile phase see below). The use of two additional high-pressure valves (Valco, Schenkon, Switzerland) allowed a separate elution of the guard column and the main column according to the backflush method presented by Krahn et al. [11].

Guard column and main column were eluted isocratically with HX for 8 min. Subsequently, the guard column was isolated and the main column was eluted further for 2 min with HX followed by a gradient from 100% HX to 100% DCM within 3 min. These conditions were held for 10 min. Subsequently, the guard column was back flushed with DCM for 5 min.

Cut-off points of the fractionation procedure providing eleven fractions were selected according to the retention of standard compounds. For recovery analyzes an extended array of standards was applied including the PCB standard EC4065 (Promochem) and the PCDD/F standard EDF 949 (Promochem) as well as individual PCN standards. Individual concentrations were in the range of 0.0083–2.5 mg/l.

2.8. Size-exclusion chromatography

PCDDs, PCDFs and PCNs with a similar number of chlorine substituents coeluting in EDA-LC, supplemented by PCBs, were separated by SE-LC at 10 °C on two stainless steel columns (25×600 mm) packed with a porous polystyrene–divinylbenzene copolymer with a pore size of 50 Å and a particle size of 10 μm (PLgel, Polymer Laboratories, Waltrop, Germany) with 10 ml/min THF as mobile phase. For method development individual PCBs, PCNs and PCDD/Fs together with technical mixtures (Aroclor 1232 and Halowax 1013) were used. A fractionation procedure was established collecting three fractions characterized by (1) PCBs and PCDDs, (2) PCDFs and (3) PCNs, respectively. Recovery was determined from a THF solution of 2,3,7,8-TCDD, 1,2,3,6,7,8-HxCDF, CB 169, and CN 67 with a concentration of 10 mg/l each analyzing the fractions using size-exclusion HPLC under the same conditions as used for fractionation.

2.9. Chemical analysis

2.9.1. GC–MSD analysis

PAHs were analyzed applying a gas chromatograph (HP 6890, Agilent Technologies, Waldbronn, Germany) equipped with a split/splitless injector and a mass selective detector (5973 MS) operated at an electron impact energy of 70 eV and a source temperature of 230 °C. After splitless injection at 280 °C, the analytes were separated on a capillary column (HP-5MS, 30 m×0.25 mm I.D., 0.25 μm film thickness, Agilent Technologies) using 1 ml/min He as carrier gas. The oven temperature was programmed from 70 to 280 °C at a rate of 7 °C/min followed by isotherm conditions for 2 min and subsequent heating at 7 °C/min to a final temperature of 300 °C and a final time of 2 min. The transfer-line temperature was held at 280 °C. PAHs were quantified on the basis of the molecular ion using a mixture of deuterated PAHs (ES2528, Promochem) as internal standard.

2.9.2. High-resolution GC–MS analysis of PCDD/Fs, PCBs and PCNs

Identification and quantification of individual compounds were accomplished with a gas chromatograph (HP 5890 II) coupled with a high-resolution mass spectrometer (Finnigan MAT 95). Compounds were separated on a fused-silica capillary column (DB-5MS, 60 m×0.25 mm I.D., 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA) using a constant flow of helium of 1.5 ml/min with an initial pressure of 27 p.s.i. (1 p.s.i.=6894.76 Pa) (1.86×10^5 Pa) at 105 °C. The column oven temperature was programmed from 105 to 180 °C at a rate of 30 °C/min and then to 260 °C at 1.4 °C/min followed by a rate of 30 °C/min to a final temperature of 305 °C with a final holding time of 10 min. Injection was done on-column. The transfer line temperature was held at 290 °C. The mass spectrometer was operated at an electron impact energy of 70 eV with a mass resolution of 8000–10 000. All compounds were determined in the multiple ion detection (MID) mode at the two most intense ions of the molecular ion cluster. PCDD/Fs were quantified using ^{13}C-labeled internal PCDD/F standards (EDF 8999 and EDF 957, Promochem, Wesel, Germany). For the quantification of PCBs and PCNs, ^{13}C-labeled internal PCB standards were used (EC1418, EC1419, EC4064, Promochem). Detection limits for PCBs, PCNs and PCDD/Fs were about 0.1 ng/ml.

3. Results and discussion

3.1. Normal-phase HPLC on nitrophenylpropyl silica

The retention of 49 chlorinated and nonchlorinated aromatic hydrocarbons on the CN phase as well as on NO with HX as mobile phase was investigated aiming at a group specific separation of potentially Cyp1A-inducing compounds. The capacity factors (k) of the analyzed compounds range from about 0.2 to 1.3 for CN and 0.3 to 14.4 for NO (Table 1). There is a correlation between the capacities on CN and NO, but only the resolution of the NO column is sufficient for PAH separation. The application of HX–DCM (95:5, v/v) rather than HX reduced retention times by about a factor of 2. There was linear correlation between the capacity factors with HX (k_{NO100}) and HX–DCM (95:5, v/v) ($k_{NO95/5}$) as mobile phase according to the equation:

$k_{NO95/5} = 0.454k_{NO100} + 0.2527; \quad r^2 = 0.997$

Therefore, the experimental determination of $k_{NO95/5}$ values could be limited to a smaller set of compounds (14 compounds, Table 1). Cut-off points for the fractionation determined for HX could be easily transferred to HX–DCM (95:5, v/v) separation. The separation capacity achieved with HX–DCM (95:5) was still sufficient for group specific PAH fractionation and was applied in order to keep the fractionation time short. However, if a higher resolution is required, HX is recommended as mobile phase.

The capacity factors of the analytes seem to increase with increasing molecular mass. However, this holds true only for nonsubstituted aromatic compounds without C–C single bonds. A comparison of the capacity factors of alkylated, chlorinated or partly saturated compounds with parent PAHs indicates increasing size and π-electron density of the aromatic system as the decisive parameter for the separation.

The capacity factor for acenaphthene is only about half as high as for acenaphthylene. Both compounds have an identical number of carbon atoms and a similar ring structure. The replacement of the single bond in acenaphthene by a double bond in acenaphthylene resulted in almost a doubling of the capacity factor due to the increased delocalized π-electron cloud.

CN66 is eluted significantly earlier than the nonchlorinated naphthalene suggesting a reduction in retention by electron-drawing substituents such as chlorine. This is in agreement with the concept of the formation of charge-transfer complexes between the π-electron systems of the PAHs and the electron-deficient π-electron system of the nitrophenyl group of the stationary phase [12,13].

Methyl substituents in BaA, CHR, and benzo(a)pyrene (BaP) may effect capacity factors isomer-specifically with positive (e.g. 2-methylchrysene (2-MeCHR)) or negative (e.g. 10-MeBaP) deviations from the parent PAH of up to 15%. This indicates steric factors as an additional decisive parameter for the interaction of the analytes with the NO column. This is also supported by the significant differences in retention behavior of other isomers, e.g. naphthalenylbenzothiophenes, binaphthalenes, and benzonaphthofurans.

Despite the modification of retention by steric factors fractionation cut-off points can be established, which allow a group-specific separation with parent PAHs and alkylated compounds eluting in the same fraction. Horizontal lines in Table 1 indicate the suggested cut-off points for fractionation resulting in the collection of eight fractions.

A mixture of 21 compounds was used for the determination of recoveries (Table 1). All components of the standard mixture were detectable exclusively in one fraction, respectively. The recovery in these fractions was 60–86% for most compounds (Table 1). Relatively low recoveries (40–60%) were found for volatile compounds such as naphthalene, acenaphthene, acenaphthylene and fluorene probably due to evaporation losses during concentrating and for high-molecular-mass PAHs (M_w 276, 50–60%).

NP fractionation of cleaned-up Sp and Fo extracts on the NO column with HX–DCM (95:5, v/v) yielded qualitative fraction compositions in good agreement with the expected composition from standard mixture on the basis of twenty-three major components (Table 1). All compounds were present exclusively in the fraction suspected on the basis standard mixture results, respectively, indicating a high reproducibility of the method.

The presented results suggest NP-LC on NO as a suitable tool for the fractionation of mixtures of nonpolar aromatic compounds. It allows a group specific separation according to the size and charge density of the aromatic systems with sufficient resolution and recovery. Dioxin-like acting halogenated compounds with two aromatic rings such as PCBs, PCDD/Fs [14] and PCNs [15] can be properly separated from nonhalogenated PAHs. CYP1A-inducing four to six ring PAHs [16–18] can be group specifically separated from each other and from smaller noninducing PAHs.

3.2. Reversed-phase HPLC

A group of 25 PAHs including TRP, CHR, BaA, binaphthyls, dinaphthofurans and methylated CHRs and BaAs, which coeluted in fraction F5 in NP-LC on the NO phase and has been found to include potent CYP1A inducers (unpublished results) was separated with RP-LC.

The capacity factors in reversed-phase chromatography (Table 2) cover a wide range from 3.50 to

Table 2
Capacity factors (k) of selected polycyclic aromatic compounds in reversed-phase chromatography on C_{18} with acetonitrile–water (75:25) as mobile phase, fractionation course, recovery in the respective fractions and concentrations in fractions of the Forellenbach (C_{Fo}) and Spittelwasser (C_{Sp}) extract

	M_w	k	Recovery (%)	C_{Fo} (μg/kg)	C_{Sp} (μg/kg)
F5.1 (5.0–33.8 min)					
Triphenylene	228	4.39	81	680	750
F5.2 (33.8–38.2 min)					
Cyclopenta(*cd*)pyrene	226	–	–	13	220
Benzo(*ghi*)fluoranthene	226	–	–	930	5000
Benz(*a*)anthracene	228	5.98	72	4000	1700
F5.3 (38.2–43.8 min)					
Chrysene	228	6.84	81	2600	3300
2,2′-Binaphthyl	254	6.95	–	50	110
F5.4 (43.8–46.0 min)					
4/6-Methylbenz(*a*)anthracene	242	7.33	–	41	<2
2-Methylbenz(*a*)anthracene	242	7.38	70	140	<2
5/6-Methylchrysene	242	7.68	–	43	<2
F5.5 (46.0–48.2 min)					
2-Methylbenz(*a*)anthracene	242	7.38	70	2.4	<2
5/6-Methylchrysene	242	7.68	–	13	29
10-Methylbenz(*a*)anthracene	242	7.84	–	3.1	15
1-Methylbenz(*a*)anthracene	242	7.98	70	<2	<2
F5.6 (48.2–53.4 min)					
4-Methylchrysene	242	8.27	–	48	12
Dinaphtho[2,1-*b*;1′,2′-*d*]furan	268	8.62	97	<2	<2
F5.7 (53.4–62.0 min)					
7-Methylbenz(*a*)anthracene	242	9.46	–	<2	<2
3/5-Methylbenz(*a*)anthracene	242	9.54	76	<2	<2
3-Methylchrysene	242	9.78	–	160	150
Dinaphtho[2,1-*b*;1′,2′-*d*]furan	268	–	–	<2	8.3
F5.8 (62.0–72.7 min)					
Dinaphtho[2,1-*b*;2′,3′-*d*]furan	268	11.53	84	<2	1600
9-Methylbenz(*a*)anthracene	242	11.78	81	340	92
1-Methylchrysene	242	12.02	–	150	160
F5.9 (72.7–91.0 min)					
2-Methylchrysene	242	14.77	99	230	310
Dinaphtho[1,2-*b*;2′,1′-*d*]furan	268	16.04	93	<2	<2
F5.10 (91.0–160 min)					
Dinaphtho[1,2-*b*;2′,3′-*d*]furan	268	25.63	81	<2	<2
Dinaphtho[2,3-*b*;2′,3′-*d*]furan	268	27.17	84	<2	<2

–, not determined.

27.17 allowing a resolution of all compounds except 4- and 6-MeBaA, 5- and 6-MeCHR, and 3- and 5-MeBaA, which coelute, respectively. This suggests that the presented method based on RP-LC can be employed as a compound-specific fractionation step following group-specific NP fractionation on NO.

The fractionation course established here was applied to a standard mixture of 13 compounds and to the fractions F5 of Sp and Fo and provided 10 subfractions characterized by one or more individual compounds (Table 2). All components of the standard mixture were detectable exclusively in one fraction, respectively, with a recovery of 70–99% except some overlap between F5.4 and F5.5. The

fractions contain the PAHs TRP, CHR and BaA, methylated and dimethylated derivatives thereof as well as binaphthyls and dinaphthofurans (Table 2). TRP and CHR, which coeluted in GC–MSD analyses, could be separated by RP-LC and differentiated by recording UV-spectra. Despite identical mass and UV spectra and quite similar GC retention data of many isomers, an isomer-specific quantification of MeCHRs and MeBaAs was enabled by the application of the RP fractionation prior to chemical analysis. The isomer-specific analysis of alkylated PAHs is crucial for hazard assessment of complex environmental samples because of the strong dependence of biological activity on the substitution pattern [19].

3.3. Electron-donor–acceptor chromatography

Bulk diaromatic compounds without CYP1A-inducing potency, e.g. naphthalene, biphenyl, and diphenylether were well separated by EDA-LC from PCBs, PCNs, and PCDD/Fs. PCBs eluted according to the general degree of chlorination and planarity, as affected by the number of chlorine substituents in the *ortho* position (Table 3) in agreement with previous investigations [20]. Tri- and tetrachloronaphthalenes eluted together with mono- and di-*ortho* PCBs. Higher chlorinated PCNs as well as PCDD/Fs eluted after a mobile phase gradient to DCM according to the overall degree of chlorination. OCDD and OCDF were retained on the PYE guard column and eluted by backflushing the guard column after 24 h. The retention behavior is caused by the formation of charge-transfer complexes between the electron deficient π-electron clouds of the halogenated aromatic hydrocarbons (HAHs) (electron acceptor) and the π electrons of PYE (electron donor) [20–24].

Based on the retention times of individual compounds the cut-off points for fractionation were established (Table 3). Most of the compounds could be recovered predominantly or exclusively in one

Table 3
Retention times (t_R) of nonchlorinated aromatic compounds and AHHs in electron-donor–acceptor chromatography on 2-(1-pyrenyl)ethyldimethyl silica (PYE), cut-off points of the respective fractions and recovery in the respective fractions

	t_R (min)	Recovery (%)		t_R (min)	Recovery (%)
F1.1 (2.0–2.9 min)			F1.5 (6.0–7.5 min)		
Naphthalene	2.76	–	CB77	6.49	74
Biphenyl	2.76	–	CB81	6.49	–
Diphenylether	2.76	–	F1.6 (7.5–9.0 min)		
F1.2 (2.9–4 min)			CB126	8.23	100
CB209	3.00	–	F1.7 (9.0–11.5 min)		
CB28	3.24	–	CB169	9.91	–
CB101	3.58	–	F1.8 (11.5–16.0 min)		
F1.3 (4–5.1 min)			CN48	–	52
CB118	4.42	71.2	2,3,7,8-TCDD	14.20	48
CN17	4.57	–	2,3,7,8-TCDF	–	48
CB194	4.96	–	F1.9 (16.0–18.0 min)		
CB 123	–	76	CN54	–	78
CB114	–	73	CN67	16.80	82
CB180	–	63	1,2,3,7,8-PeCDD	–	38
CB170	–	110	1,2,3,7,8-PeCDF	–	47
CB105	–	53	F1.10 (18.0–23.5 min)		
CB167	–	14	CN73	19.57	–
F1.4 (5.1–6 min)			1,2,3,6,7,8-HxCDD	–	35
CB105	–	20	1,2,3,6,7,8-HxCDF	–	38
CB167	–	59	F1.11 (23.5–30.0 min)		
CB156	5.44	87	1,2,3,4,6,7,8-HpCDD	–	43
CB157	–	44	1,2,3,4,6,7,8-HpCDF	–	45
CN27	5.49	–	OCDD	24.4	36
			OCDF	24.4	103

fraction. Only the CBs 105, 167, and 157 were distributed between two adjacent fractions with recoveries in the same order of magnitude in both fractions. The total recoveries of all compounds ranged between 35 and 104% with typical recoveries of 70–80% for PCBs and PCNs, and 35–50% for PCDD/Fs. The lower recoveries of PCDD/Fs suggest a selective adsorption behavior on materials used in fractionation, solvent exchange or chemical analysis.

The validation of EDA-LC fractionation for complex environmental mixtures with F1 of the Sp sediment extract provided good agreement with standard mixtures for fractions F1.3 to F1.11 even if PeCDD/Fs and PeCNs were shifted from F1.9 in the standard fractionation to F1.8 for the sediment extract, and HxCDDs from F1.10 to F2.1.9 (Fig. 1). TCNs and HxCNs were isomer specifically recovered in the fractions F1.3–F1.5 and F1.8–F1.9, respectively. The concentrations of non-*ortho*-substituted CBs 77, 81, 126 and 169 as expected in F1.5–F1.7 in the Sp extract were below the detection limits.

HAH analysis of the first fractions F1.1 and F1.2 was renounced because of the high load of bulk compounds such as naphthalene, biphenyl, and diphenylether interfering with HRGC–MS analysis. The HAHs expected in F1.2 include PCBs with less than four chlorine substituents as well as PCBs with two and more ortho chlorine substituents. These compounds are known to not induce CYP1A.

To summarize, EDA-LC proved to be a suitable tool to remove CYP1A noninducing bulk compounds and to fractionate PCBs, PCDD/Fs and PCNs according to the properties relevant for CYP1A induction, namely planarity and the degree of chlorination.

3.4. Size-exclusion chromatography

SE-LC provided a selective separation of HAHs with PCBs and PCDDs eluting in one fraction followed by PCDFs and PCNs (Fig. 2). This is in agreement with former approaches to separate PCBs and PCNs by SE-LC [25]. Technical mixtures of PCBs (Aroclor 1232) and PCNs (Halowax 1013) consisting of numerous compounds elute as one peak, respectively, indicating group specific rather than compound specific separation. However, the hexachlorinated congener CB 169 elutes significantly earlier than Aroclor 1232, which is a technical mixture with a relatively low degree of chlorination containing predominantly mono- and di-chlorinated

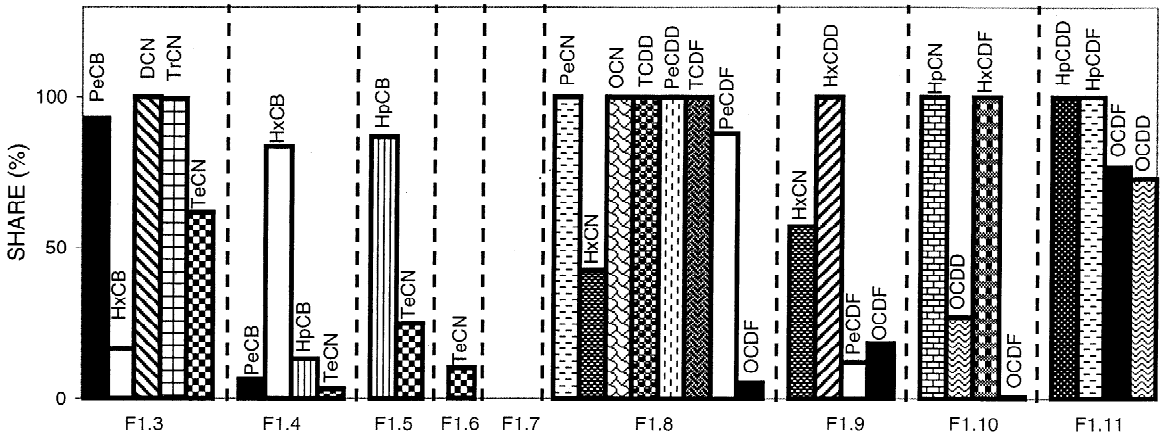

Fig. 1. Distribution of groups of halogenated aromatic hydrocarbons extracted from Sp sediment between EDA-LC fractions F1.3–F1.11. In abbreviations of compound groups prefixes D, Tr, T, Pe, Hx, Hp, and O signify the degree of chlorination (di, tri, tetra, penta, hexa, hepta, and octa) of naphthalenes (CNs), biphenyls (CBs), dibenzo-*p*-dioxins (CDDs) and dibenzofurans (CDFs), respectively. PeCBs, HxCBs, and HpCBs represent mono-*ortho*-chlorinated penta-, hexa-, and heptachlorobiphenyls, respectively, including CBs 105, 118, 156, 157, 167 and 189. TCNs and HxCNs were isomer-specifically recovered in F1.3 to F1.6 and F1.8 to F1.9, respectively with CNs 30, 32, 38, 42, 46, 47, and 48 in F1.3, CNs 27, 29, 39 and 44 in F1.5, CNs 63, 64, 65, 68, 69, 71 and 72 in F1.8, and CNs 66 and 67 in F1.9. Analytically unresolved CNs 36/45 were recovered in F1.3 and F1.5, CNs 28/43 in F1.3 and F1.6, and CN 33/34/37 in F1.3 and F1.4, respectively.

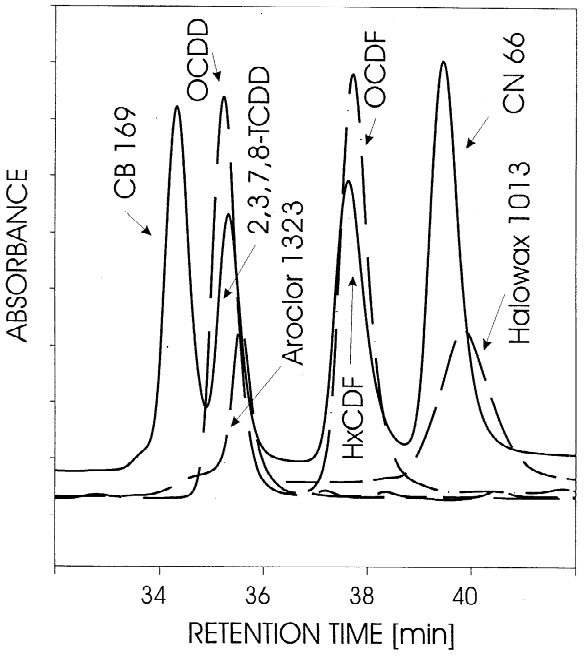

Fig. 2. Overlay of SE-LC chromatograms of standard compounds. For abbreviations see text and Fig. 1.

PCBs. This suggests an influence of chlorine substituents on retention in size-exclusion chromatography.

The results are in agreement with molecular maximum extensions as calculated with SYBYL Molecular Modeling Software 6.5 (Tripos, St. Louis, MO, USA). Depending on the chlorination pattern the molecular maximum extensions of PCDDs, PCBs, PCDFs, and PCNs range from 10.10 to 10.75 Å, from 9.92 to 10.57 Å, from 9.52 to 10.17 Å, and from 7.84 to 8.53 Å, respectively. This explains the good separation of PCNs from the other compounds, while overlap between PCBs, PCDDs and PCDFs can be expected.

The influence of the degree of chlorination suggests a fractionation of PCNs and PCDD/Fs by electron-donor–acceptor chromatography according to the degree of chlorination prior to size-exclusion chromatography.

The recovery analysis was based on four compounds. CB 126 and 2,3,7,8-TCDD were detected only in the first fraction with recoveries of 84 and 80%, respectively. 1,2,3,6,7,8-HxCDF was recovered in the second fraction (79%), and 1,2,3,5,6,7-HxCN in the third fraction (83%).

Size-exclusion chromatography of the Sp sediment extract fractions F1.8, F1.9 and F1.10 provided three fractions containing more than 80% of the PCDDs, PCDFs and PCNs, respectively, as illustrated for F1.8 (Fig. 3). The overall recovery was 60–80%. However, it should be kept in mind, that the pattern for specific congeners can be different. This was true for 2,3,7,8-TCDF, the most toxic TCDF congener but also the one with the greatest maximum interatomic distance. This compound eluted completely in fraction F1.8.1 together with the dioxins. However, this did not apply to other PCDFs with the same maximum extension such as 1,2,3,7,8-PCDF and 2,3,4,7,8 PCDF. Thus, the overlap of PCDDs and PCDFs in size-exclusion chromatography is in principal agreement with the overlap of molecular size expressed as maximum interatomic distance even if additional sorption processes seem to modify the retention behavior on the SE-LC column. Coelution of PCDD/Fs on the one hand and PCNs on the other from the SE-LC column is negligible allowing an excellent group-specific separation of these compounds.

3.5. Fractionation procedure

The separation processes including NP-LC, RP-

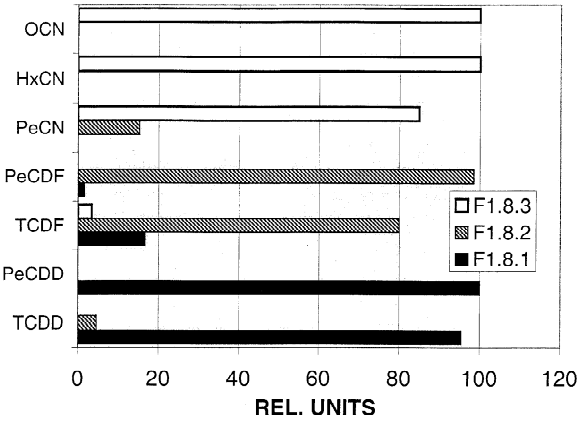

Fig. 3. Normalized distribution patterns of the total amounts of the constituents of the Spittelwasser (Sp) sediment extract fraction F1.8 in the SE-LC subfractions F1.8.1, F1.8.2, and F1.8.3. For abbreviations see Fig. 1.

LC, EDA-LC, and SE-LC are combined to a highly selective fractionation procedure (Fig. 4) for nonpolar aromatic compounds, which include most of the known CYP1A-inducing compounds. The procedure starts with a group-specific fractionation of aromatic compounds according to the number of aromatic carbon atoms using NP-LC on NO.

For the compound-specific subfractionation of the PAH fractions obtained by NP-LC, RP-LC is suggested, which allows a highly selective separation of

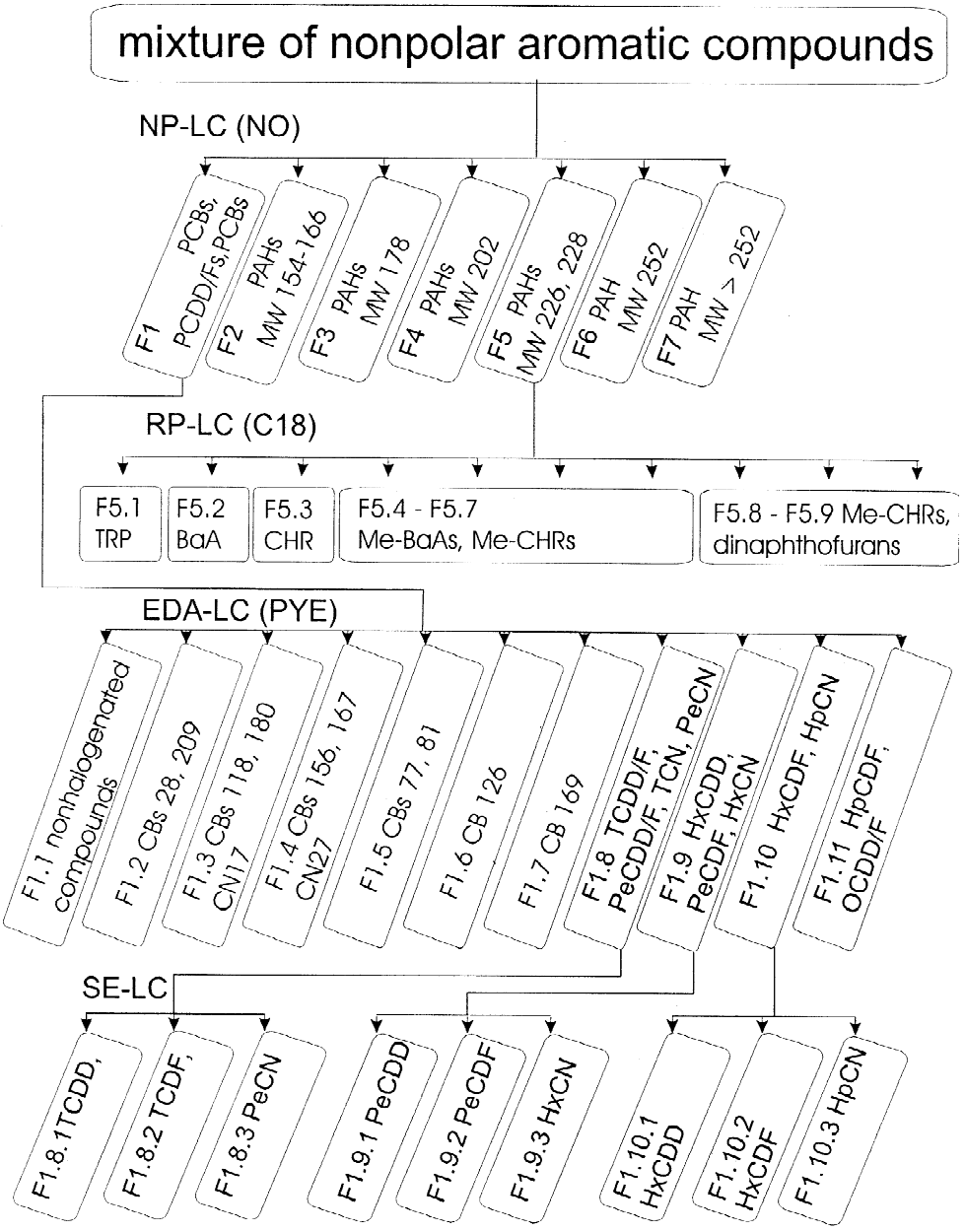

Fig. 4. Fractionation scheme including typical constituents of the fractions. For abbreviations see text and Fig. 1.

isomeric parent PAHs as well as their alkylated derivatives and heterocyclic aromatic compounds as shown for the CHR-like fraction (Fig. 4, F5).

The first NP fraction (Fig. 4, F1) that contains PCBs, PCNs, PCDD/Fs as well as nonhalogenated two-ring compounds is separated by means of EDA-LC on PYE.

PCNs, PCDFs, and PCDDs coelute on the PYE column. In order to enable a separate assessment of the effects of these compound classes, they are separated by SE-LC. This method was shown to be a powerful method for the separation of PCNs from PCBs [25] and was transferred to the fractionation of PCNs, PCDFs and PCDDs.

4. Conclusions

The fractionation procedure for nonpolar aromatic compounds was designed to provide a separation of CYP1A-inducing compounds from noninducers which may mask CYP1A-induction by cytotoxicity or enzyme inhibition and aggravate chemical analysis. The extensive fractionation allows an isomer-specific separation and individual toxicological assessment of CYP1A inducers in complex environmental samples. An identification of unexpected CYP1A inducers is facilitated. The presented procedure proved to be applicable to complex environmental samples such as contaminated sediment extracts.

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