Development of a gas chromatography-ion trap mass spectrometry based method for the quantification of polybrominated diphenyl ethers and polychlorinated biphenyls in adipose tissue.

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ABSTRACT

A combined gas chromatographic mass spectrometric (GC-MS/MS) method for the determination of 7 polybrominated diphenyl ethers (PBDEs) and 7 marker polychlorinated biphenyls (PCBs) in adipose tissue has been developed. Adipose tissue was melted and filtrated through anhydrous sodium sulphate to obtain pure fat. Clean-up was performed using a glass column containing acidified silica, deactivated alumina and anhydrous sodium sulphate. Polybrominated biphenyl (PBB) 155 and mirex were added as internal standards for PBDEs and PCBs respectively. Injection standards, PBB 103 and PCB 143, for PBDEs and PCBs respectively, were added before analysis with GC-MS/MS. The developed GC-MS/MS method has the advantage of being more selective than single MS methods because matrix effects are largely eliminated. Validation of this method was done according to Commission Decision 2002/657/EC. Decision limits for PBDEs and PCBs ranged from 0.06 ng g^{-1} to 0.15 ng g^{-1} and from 0.35 ng g^{-1} to 1.22 ng g^{-1} respectively. Detection capabilities were all between 0.23 ng g^{-1} and 0.55 ng g^{-1} for PBDEs and between 0.98 ng g^{-1} and 2.29 ng g^{-1} for PCBs. Precision, recovery, bias and selectivity were tested with satisfactory results.

Running title: GC-MS/MS analysis of PBDEs and PCBs in adipose tissue

1. INTRODUCTION

The annual production of brominated flame retardants is estimated at 200,000 metric tons of which 70,000 are polybrominated diphenyl ethers (PBDEs)[1]. The reason for using brominated compounds as flame retardants is based on the ability of the organobromine compound to capture free radicals in the gas flame phase and retard both ignition and rate of combustion[2]. PBDEs are a group of additive flame retardants used in plastics, electronic equipment (TV-sets, computers and household appliances), textiles and printed circuit boards. Additive flame retardants are mixed with or dissolved in polymers and are therefore able to leach from the products and into the environment.

Polychlorinated biphenyls (PCBs) have been widely used as complex mixtures in heat transfer fluids, dielectric fluids in capacitors and transformers and as additives in paints, pesticides, copy paper, adhesives and plastics[2]. PCBs are introduced in the environment by escaping from ‘closed’ systems (e.g. capacitors or transformers) or by accidental loss from ‘open’ systems during incineration of industrial or municipal waste.

PBDEs and PCBs are commonly very similar and differ structurally only by the presence of an ether linkage. They each consist of 209 congeners with different numbers and positions of the bromine atoms on the diphenyl ether part and chlorine atoms on the biphenyl part respectively.

Three technical PBDE mixtures are produced, known commercially as the Penta-mix, Octa-mix and Deca-mix. These three mixtures contain less congeners than commercial PCB mixtures. As a consequence the number of PBDE congeners found in environmental samples is much lower than the number of PCB congeners[3]. PBDEs and

PCBs which are persistent and lipophilic, have high binding affinity to particles and a tendency to bioaccumulate through the food chain.

They have been detected in sediments[4-6], air[7,8], biota[9-14] and human blood[15-18] , adipose tissue[19-23] and breast milk[24,25]. The main human exposure route is through the diet, via the aquatic food chain and food products rich in lipids. Highest concentrations have been found in oils, fats, fish and shellfish, meat and eggs[26,27]. After exposure the PBDEs and PCBs accumulate in the adipose tissue and are transported via the blood throughout the body compartments and different organs[20].

Several animal studies indicate that PBDEs are potentially toxic substances. Certain congeners have been shown to cause neurotoxic effects, to interfere with the brain development, to bind to the thyroid and aryl hydrocarbon (Ah) receptor and to have endocrine disrupting properties[28,29]. But overall toxicity is lower than for PCBs.

The Belgian legislation has set maximum limits for the total sum of seven marker PCB congeners (PCBs 28, 52, 101, 118, 138, 153, 180) in different food matrices. For beef, pork, chicken, eggs, animal fat or oil and products thereof a limit of 200 ng g^{-1} fat has been set for products with a fat content above 2 %. According to Council Directive 2003/11/EC[30], the use of penta- and octa-BDE and the placing on the market of articles containing one or both of these substances will be banned within the European Union by the 15th of August 2004. A risk reduction strategy will be established for deca-BDE. Although deca-BDE has been found in a large number of environmental samples, very low levels were detected in biological samples. This is probably due to the very poor bioavailability and fast metabolism of this highly brominated compound[31].

The aim of this study was to develop a gas chromatography-ion trap mass spectrometry method for the determination of 7 PBDE congeners and 7 marker PCB congeners by means of a single clean-up step.

2. EXPERIMENTAL

2.1. Reagents and solvents

All reagents and solvents were of analytical reagent grade. Isooctane, n-hexane Suprasolv® and anhydrous sodium sulphate were obtained from Merck (Darmstadt, Germany). Nonane was purchased from Sigma Aldrich nv/sa (Bornem, Belgium).

Acidified silica was prepared by adding 35.5 ml concentrated sulphuric acid p.a. (Merck) to 100 g silica gel (0.063 - 0.200 mm, Merck) and mixing thoroughly. Water was obtained by a Milli Q gradient system (Millipore, Brussels, Belgium). Preparation of deactivated alumina was done by adding 5 ml water to 45 g alumina B activity I (ICN Biomedicals, Eschwege, Germany). Silane treated glass wool was obtained from Alltech Associates (Deerfield, IL, USA).

2.2. Standard solutions

Individual PBDE standards, IUPAC Nos. 28, 47, 99, 100, 153, 154 and 183 (*see* Table 1), were purchased from Wellington Laboratories (Ontario, Canada). Individual PCB standards, IUPAC Nos. 28, 52, 101, 118, 138, 153, and 180 (*see* Table 1), together with PCB-Mix 3 (10 ng µl $^{-1}$ of each congener), internal standards (mirex and polybrominated biphenyl (PBB) 155) and injection standards (PCB 143 and PBB 103) were purchased from Dr. Ehrenstorfer (Augsburg, Germany).

A standard stock solution containing all 7 PBDE congeners was prepared at a concentration of 1 ng µl $^{-1}$. Standard stock solutions of PBB 155, PBB 103, PCB 143 and mirex were prepared at 10 ng µl $^{-1}$. All stock solutions were stored between 2 °C and 8 °C, protected from light. Suitable working PBDE and PCB solutions were prepared daily by dilution in nonane and isooctane respectively.

2.3. Apparatus

Analysis of the adipose tissue samples was done using a Finnigan GCQ gas chromatograph coupled to a Finnigan GCQ mass spectrometer (Austin, Texas, USA). The autosampler was a CTC 200 series injector (Zwingen, Switzerland). Separation of the PBDE and PCB congeners was performed using a HT8 capillary column (25 m x 0.22 mm x 0.25 µm, SGE, Achrom, Zulte, Belgium). Carrier and collision gas was Alphagaz 2 helium (Air Liquide, Liege, Belgium). Instrument set points and data acquisition were under control of GCQ software.

2.4 Sample collection and storage

Fat tissue samples taken within the frame of the Belgian national monitoring programme for PCBs were collected and stored at –20°C. Only fat samples that contained PCB levels lower than the decision limit (CCa) were used to prepare a pool of blank extracted fat for the validation procedure. Out of this pool a sample was analysed and a new pool was prepared if PBDE levels were above the decision limit (CCa).

Five human adipose tissue samples from the abdominal fat region were obtained from autopsy and stored at -20°C.

2.5. Extraction and clean-up

In order to prevent contamination, glassware was washed extensively and rinsed twice with hexane (Acros, Geel, Belgium). Plastic materials were not used in order to avoid contamination. Approximately 10 g of adipose tissue sample was brought onto a folded Ederol filter paper (VWR, Leuven, Belgium) in a glass funnel together with anhydrous sodium sulphate (10 g). The fat was melted in a microwave oven (600 W – 2 min) and received in a glass recipient. Internal standards PBB 155 (40 µl, 0.1 ng µl $^{-1}$), mirex (20 µl, 1 ng µl $^{-1}$) and n-hexane (5 ml) were added to the melted fat (2 g). A glass column was used containing a wad of silane treated glass wool at the bottom. This column was subsequently filled with n-hexane (25 ml), acidified silica (12 g), deactivated alumina (3 g) and anhydrous sodium sulphate (3 g). After removal of any excess n-hexane from the column, the fat solution was brought onto the column and elution was done with n-hexane (40 ml). The eluate was evaporated in a rotavapor at 40 °C to ca. 4 ml. This solution was transferred to a graduated glass vial (Egilabo, Kontich, Belgium). Injection standards, PBB 103 (40 µl, 0.1 ng µl $^{-1}$) and PCB 143 (20 µl, 1 ng µl $^{-1}$) and keeper solvent isooctane were added. This mixture was concentrated under nitrogen at 40 °C to 100 µl. This condensed isooctane solution was split over 2 GC-MS vials.

2.6. Gas chromatography- mass spectrometry

A 2 µl aliquot of the final sample extract was injected in the splitless mode and the splitless period following injection was 1 min. Injection temperature was set at 300 °C and transferline temperature was 275 °C. The oven was programmed from 70 °C for 1 min to 170 °C at a rate of 30 °C min $^{-1}$, then to 300 °C (15 min) at a rate of 8 °C min $^{-1}$.

Optimisation of GC-MS/MS parameters for PCB analysis was previously done by De Saeger *et al.*[32] In this study optimisation of GC-MS/MS parameters was limited to PBDEs. In order to achieve optimal mass spectrometric conditions ionisation energy was varied between 35 eV and 70 eV. The mass spectrometer was operated in MS/MS mode. Ideal fragmentation conditions were also determined by varying q-value and excitation voltage.

2.7. Validation

Validation of the method was done according to Commission Decision 2002/657/EC[33]. This document gives a description of the performance characteristics that have to be verified for a specific method. Specificity, decision limit (CCa), detection capability (CCß), recovery and precision need to be determined for a quantitative confirmatory method. All validation parameters, except for specificity were determined on the most abundant ion. This ion was also used for quantification purposes. CCa or decision limit was established by the calibration curve procedure and was repeated 5 times. Blank fat samples were fortified with a mixture of 7 PBDE congeners at a level of 0, 0.5, 1, 2, 5 ng g^{-1} per congener and 7 PCB congeners at 0, 1, 5, 10, 20 ng g^{-1} per congener. After identification, the signal was plotted against the added concentration. The corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept is defined as the decision limit (a = 1 %). CCß or detection capability was determined by using the same calibration curves. CCß equals the corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured at the decision limit (ß = 5 %). Specificity was checked by analysing 20 different blank fat

samples for interferences. Recovery is defined as that fraction of mass of the analyte added to the sample which is present in the final extract. For the determination of the recovery 18 blank fat samples were fortified before clean-up at 1, 2 and 4 ng g^{-1} per congener for PBDEs and 5, 10 and 20 ng g^{-1} per congener for PCBs. The responses of these fortified samples were compared with samples fortified after clean-up. Within day and between day precision were calculated as a coefficient of variation (CV %) by fortifying blank fat samples at 3 nominal levels (1, 2 and 4 ng g^{-1} per congener for PBDEs and 5, 10 and 20 ng g^{-1} per congener for PCBs). Bias was determined as an approximation for trueness and was assessed by fortifying 18 blank fat samples at 1, 2 and 4 ng g^{-1} per congener for PBDEs and 5, 10 and 20 ng g^{-1} per congener for PCBs. Measurement uncertainty was determined according to the EURACHEM/CITAC guide (2000)[34]. The global uncertainty was calculated as a combination of the uncertainty associated with precision and the uncertainty associated with accuracy. Although this is the combined uncertainty, the results are expressed as an expanded uncertainty, which corresponds to twice this value.

3. RESULTS AND DISCUSSION

3.1. Extraction and clean-up

Because this method only applies to adipose tissue samples, no particular fat isolation step was necessary except melting. Sodium sulphate extracted any excess water during the melting process. To obtain enough sensitivity it was necessary to extract a minimum of 2 g of fat. Clean-up by sulphuric acid treatment was used to remove potential chromatographic interferences e.g. fatty acids and organic macromolecules.

Chromatographic columns were filled with various quantities of acidified silica, alumina and sodium sulphate. Best results were obtained with 12 g acidified silica, 3 g alumina and 3 g anhydrous sodium sulphate. The columns were eluted with various volumes of n-hexane. To obtain sufficient recoveries 40 ml of n-hexane was necessary. After addition of the injection standard, the eluate was evaporated to 100 µl. It was tested if it could be possible to evaporate all of the eluate and redissolve in isooctane. This approach was discarded because of volatilization of BDE 28 and irreversible adsorption of PCBs to the glassware.

3.2. Optimisation of GC-MS/MS parameters

The HT-8 column, characterised by a carborane phase, was used to separate the 7 marker PCB congeners and 7 PBDE congeners. This type of column was chosen because less co-elutions of target analytes occur. The above mentioned PBDEs were selected because they are the main reported congeners in the literature. It was decided not to include BDE 209 in this study, which is commercially the most important congener, due to significant difficulties in its analysis. BDE 209 is light and heat sensitive and requires a specific GC column owing to its very long retention time.

PBDEs and PCBs had to be determined in two consecutive runs because PCB 180, mirex and BDE 47 could not be separated in a single run. The chromatograms in Figures 1 – 2 show that PBDEs, PCBs, internal standards and injection standards are well separated.

The use of isotopically labelled internal standards was considered but due to software related problems this approach was discarded and mirex and PBB 155 were chosen as alternatives.

Detection of all analytes was done in electron impact (EI) MS/MS. This combination was favoured because less interferences occur and it achieves a higher degree of selectivity. In most studies using EI mass spectrometry the electronisation energy was either 70 eV or between 30 - 40 eV[31]. In this study electronisation energy was varied between 30 eV and 70 eV and no difference was noticed. Therefore the electronisation energy was set at its default value of 70 eV. As previously reported by Pirard *et al.*[31] EI spectra were dominated by M^+ and $[M\text{-}Br_2]^+$ for the lower brominated and higher brominated PBDEs respectively. The molecular ion was chosen as precursor ion because it offers more structural information. Precursor ions were then isolated in the ion-trap and fragmented by collision induced dissociation (CID) resulting into fragment ions. Excitation voltage, excitation time and q-value were optimised and fragment ions were detected in full scan. Fragmentation of the molecular ion only generated a loss of two bromine atoms for all PBDE congeners. As stipulated by Commission Decision 2002/657/EC the two most abundant fragment ions were chosen as diagnostic ions. An overview of the ions monitored and the spectrometric conditions is given in Table 2. Quantification was done with the most abundant fragment ion except for BDE 183 where the sum of m/z 561 to 566 was used. This was necessary because quantification of BDE 183 with a single fragment ion did not generate reproducible results.

3.3. Validation of the method

Because no interfering peaks could be detected when analyzing 20 blank fat samples, it could be decided that the method was specific. Results of the different validation parameters obtained at 2 ng g^{-1} for PBDEs and 10 ng g^{-1} for PCBs are presented in Table 3. Since PBDEs occur in lower concentrations in biological samples compared to

PCBs, calibration curves were determined in a lower linear range resulting in lower decision limits (CCa) and detection capabilities (CCß). Decision limits for PBDEs and PCBs ranged from 0.06 to 0.15 ng g^{-1} and from 0.35 ng g^{-1} to 1.22 ng g^{-1} respectively. Detection capabilities of PBDEs and PCBs varied between 0.23 – 0.55 ng g^{-1} and 0.98 - 2.29 ng g^{-1} respectively. Precision, expressed as the coefficients of variation, did not exceed 7.89 % accounting for the good repeatability of the developed method. In the concentration range 0.5 to 5 ng g^{-1} and 1 to 20 ng g^{-1} for PBDEs and PCBs respectively, the correlation coefficients (R^2) were all higher than 0.995. Recoveries ranged between 88.9 and 98.1 % for PCBs and between 81.5 and 95.3 % for PBDEs. As shown in Table 3 the percent bias was within the -30 % till + 10 % range and -20 % till +10 % range, as prescribed by Commission Decision 2002/657/EC, for concentrations ranging from 1-10 ng g^{-1} and >1-10 ng g^{-1} respectively. Expanded measurement uncertainty varied between 23.5 and 42.8 % for PCBs and 16.5 and 28.2 % for PBDEs.

All identification criteria mentioned by Commission Decision 2002/657/EC were fulfilled. These criteria include that the signal-to-noise ratio for each diagnostic ion is larger or equal then three. Relative retention times (RRT) of the analyte did not differ more than +/- 0.5 % of the RRT of the calibration standard. The relative intensities of the detected ions, expressed as a percentage of the intensity of the most abundant ion, corresponded to those of the samples fortified at comparable concentrations within the tolerances mentioned by Commission Decision 2002/657/EC.

3.4. Analysis of human adipose tissue samples

In order to evaluate the applicability of the developed method, five human adipose tissue samples from the abdominal fat region were analysed. Concentrations of the 7 PBDE and 7 marker PCB congeners are presented in Table 4.

4. CONCLUSIONS

The GC-MS/MS method described in this paper provides a reliable procedure for the determination of above mentioned PCBs and PBDEs. All validation criteria mentioned by Commission Decision 2002/657/EC were fulfilled. The analysis of five human adipose tissue samples demonstrates the applicability of the described method to real samples.

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Table 1 IUPAC numbering of the investigated PBDE and PCB congeners

IUPAC No.	Structure	IUPAC No.	Structure
PCB 28	2,4,4’	BDE 28	2,4,4’
PCB 52	2,2’,5,5’	BDE 47	2,2’,4,4’
PCB 101	2,2’,4,5,5’	BDE 99	2,2’,4,4’,5
PCB 118	2,3’,4,4’,5	BDE 100	2,2’,4,4’,6
PCB 138	2,2’,3,4,4’,5’	BDE 153	2,2’,4,4’,5,5’
PCB 153	2,2’,4,4’,5,5’	BDE 154	2,2’,4,4’,5,6’
PCB 180	2,2’,3,4,4’,5,5’	BDE 183	2,2’,3,4,4’,5’,6

Table 2. Spectrometric conditions and ions monitored

			Mass Spectrometry Ionisation Mode EI		
Compound	Excitation voltage (V)	q	Precursor Ion (m/z)	Product Ions (m/z)	Retention time
PCB 28	1.80	0.450	256	**186** 151	11:50
PCB 52	1.80	0.450	292	**257** 222	12:29
PCB 101	2.00	0.450	326	**256** 291	14:45
PCB 118	2.00	0.450	326	**256** 254	16:35
PCB 138	1.80	0.450	360	**290** 325	17:41
PCB 153	1.80	0.450	360	**290** 325	16:57
PCB 180	1.80	0.450	394	**324** 359	19:21
mirex	1.50	0.450	272	**237** 235	19:32
PCB 143	2.00	0.450	360	**325** 290	16:23
BDE 28	1.50	0.300	406	**246** 248	16:33
BDE 47	1.50	0.300	486	**326** 328	19:34
BDE 99	1.50	0.300	566	**406** 408	22:23
BDE 100	1.50	0.300	566	**406** 408	21:44
BDE 153	1.50	0.300	644	**484** 482	26:19
BDE 154	1.50	0.300	644	**484** 482	24:29
BDE 183	2.00	0.450	722	**564** 562	32:38
PBB 155	1.50	0.300	628	**470** 549	21:13
PBB 103	1.50	0.300	548	**469** 390	19:17

Table 3. Results of the different validation parameters

Compound	CCα (ng g^{-1})	CCβ (ng g^{-1})	Repeatability (CV %)		Bias[a] (%) (n = 6)	Recovery[a] (n = 6) (%)	Expanded measurement uncertainty[a] (%)
			Within day[a] (n = 6)	Between day[a] (n = 6)			
PCB 28	0.56	1.75	5.07	2.69	+ 3.2	98.1	32.4
PCB 52	0.55	1.70	5.12	3.98	+ 2.9	91.7	42.8
PCB 101	0.38	1.25	2.22	2.43	- 2.4	91.7	23.5
PCB 118	0.35	0.98	3.33	4.30	- 0.9	90.5	27.4
PCB 138	0.35	1.28	5.45	0.68	- 3.0	89.4	27.7
PCB 153	0.95	2.21	2.70	4.79	- 7.6	93.2	32.9
PCB 180	1.22	2.29	4.34	2.52	- 3.0	88.9	28.4
BDE 28	0.06	0.23	2.53	3.60	+ 0.50	86.9	23.1
BDE 47	0.06	0.31	3.75	4.98	-1.50	88.4	28.2
BDE 99	0.09	0.30	2.10	5.03	0	95.3	17.6
BDE 100	0.09	0.40	2.39	4.37	+ 1.0	86.5	16.5
BDE 153	0.10	0.55	5.20	3.88	- 3.0	83.0	19.8
BDE 154	0.08	0.46	3.13	4.14	- 1.0	81.5	18.8
BDE 183	0.15	0.53	5.70	7.89	- 6.0	85.4	24.7

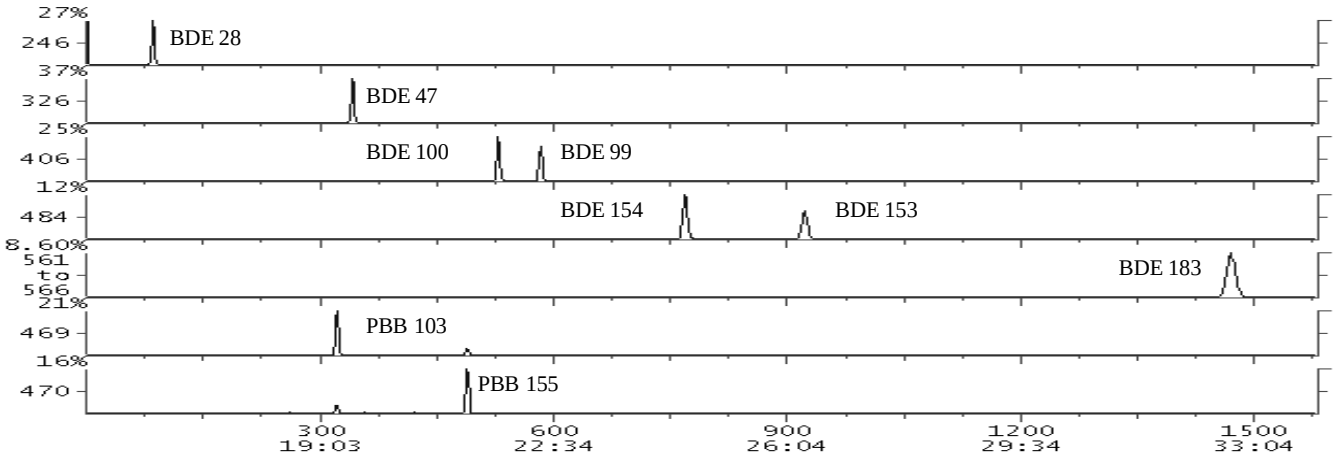
[a] Data were obtained at 2 ng g^{-1} level for PBDEs and 10 ng g^{-1} level for PCBs

Table 4. Concentrations of the different PCB and PBDE congeners in human adipose tissue samples

Sample	PCB (ng g^{-1})								PBDE (ng g^{-1})							
Number	28	52	101	118	138	153	180	SUM	28	47	99	100	153	154	183	SUM
2000/219	<CCß	<CCß	<CCß	4.3	47	208	294	553	<CCß	0.32	<CCß	<CCß	3.5	<CCß	<CCß	3.8
2001/158	3.1	2.4	2.5	15	80	122	118	343	<CCß	1.05	0.40	<CCß	4.8	<CCß	0.72	7.0
2002/129	5.8	<CCß	<CCß	11	30	147	67	261	<CCß	<CCß	<CCß	<CCß	2.7	<CCß	0.78	3.5
2002/130	3.1	2.0	<CCß	32	97	183	222	539	1.7	12	2.1	1.6	1.3	<CCß	0.58	19
2002/3	<CCß	<CCß	<CCß	6.5	67	125	83	282	<CCß	0.77	<CCß	<CCß	3.9	<CCß	0.64	5.3

Figure 1. MS/MS chromatogram of a blank sample fortified at 2 ng/g per PBDE congener.

Figure 2. MS/MS chromatogram of a blank sample fortified at 10 ng/g per PCB congener.


27%
246
BDE 28
37%
326
BDE 47
25%
406
BDE 100
BDE 99
12%
484
BDE 154
BDE 153
8.60%
561
to
566
BDE 183
21%
469
PBB 103
16%
470
PBB 155
300
19:03
600
22:34
900
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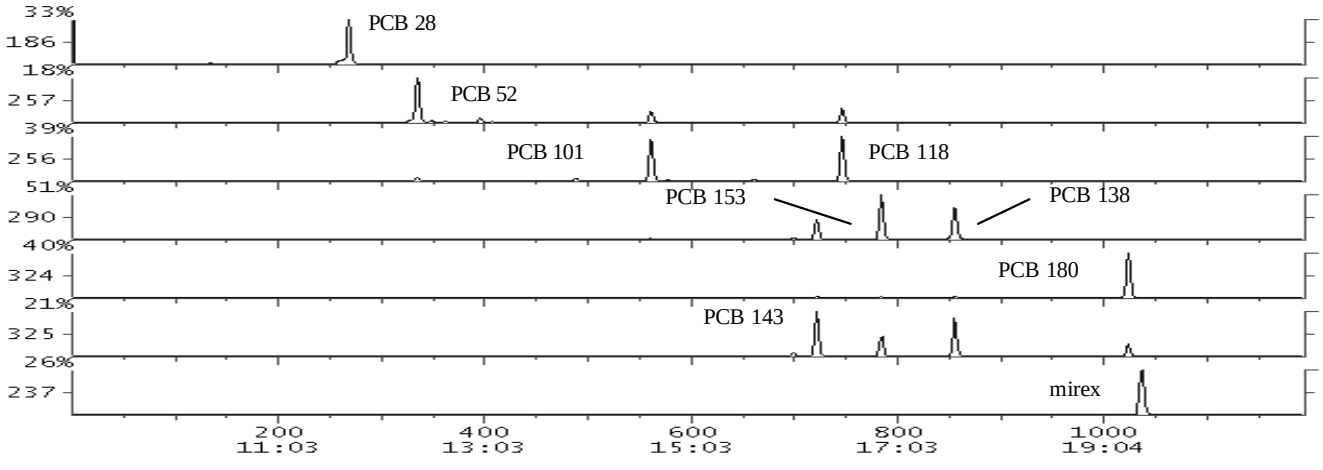
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