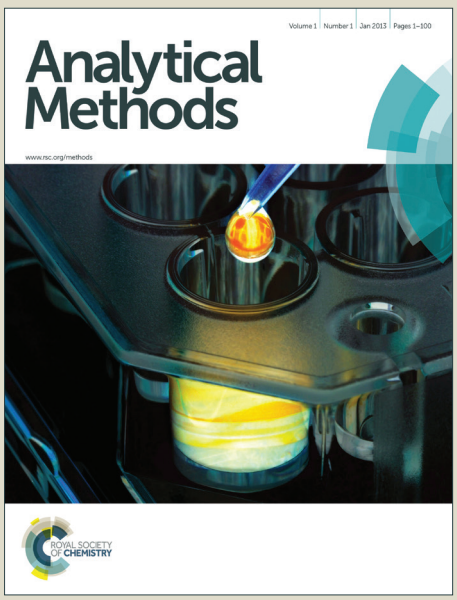
Analytical Methods

Accepted Manuscript



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RAPID SEPARATION 0F HEXABROMOCYCLODODECANE DIASTEREOMERS USING A NOVEL METHOD COMBINING CONVERGENCE CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

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ABSTRACT

Analysis of the brominated flame retardant hexabromocyclododecane (HBCDD) is characterized by the separation of its three predominant diastereomers. This analysis is typically performed using reversed phase liquid chromatography (RPLC) coupled with mass spectrometric (MS) detection with analysis times in the order of 10 minutes or greater. Here we describe a rapid method using supercritical CO_2 and methanol to baseline separate the three most abundant HBCDD diastereomers within a three minute run time using a High Strength Silica (HSS) C18 1.8 µm particle size column. A unique elution order of the α–,β– and γ–HBCDD diastereomers using supercritical CO_2 was observed, and can be used as an orthogonal separation for further confidence in diastereomer identification when used in conjuction with RPLC. A tandem quadrupole mass spectrometer with negative mode electrospray ionization was used for detection, operating in multiple reaction monitoring (MRM) mode. Ionization was enhanced by the addition of a make-up flow, which was introduced to the post-column effluent. Method limit of detection (LOD) and limit of quantification (LOQ) for α–, β–, and γ–HBCDD were based on peak-to-peak signal to noise ratios of greater than 3 or 10, respectively. The LOD for all HBCDD diastereomers as solvent standards was 100 fg on-column, and LOQs 500 fg on-column for α– and γ–HBCDD and 250 fg on-column for β–HBCDD. In order to test the efficiency of this method, small subsets of complex human serum and whale blubber extracts were analyzed using this method, resulting in positive detections in samples of α–HBCDD.

Introduction

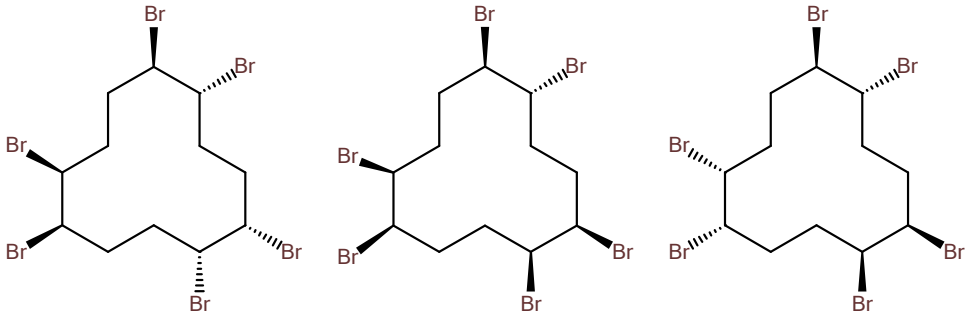
The brominated flame retardant (BFR) hexabromocyclododecane (HBCDD) is commonly monitored for its presence in humans, food samples and the environment [1-10]. In the past, HBCDD has been used as an additive to polystyrene foam, in concentrations ranging from 0.8-4% of total polymer product[11] and is likely to leach out of products as they are not covalently bound with the material [11, 12]. Investigations into the impact of HBCDD on health is ongoing, but initial studies have revealed toxic effects. In the case of HBCDD exposure, liver and thyroid hormone abnormalities have been observed in animal studies [13]. Due to observations of accumulation in the environment and biota and possible health effects, HBCDD has been classified as a persistent, bioaccumulative and toxic (PBT) compound [14]. As of 2011, HBCDD was placed under review for addition to the Stockholm Convention list of Persistent Organic Pollutants, and in 2013 its use was officially restricted [15, 16]. For these reasons HBCDD will continue to be monitored in the same way as other PBT compounds.

Showing a complex stereochemistry, HBCDD has six stereogenic centers [17] and is theoretically composed of 16 possible stereoisomers [18]. The three most common forms of HBCDD as found in technical blends, environmental and biotic samples are the α–,β– and γ–diastereomers (Figure 1), each comprised of a (+/-) enantiomer pair [17]. The three diasteromers have different physico-chemical properties, with melting points ranging from 179-209°C in increasing order of β–,α– and γ– diastereomers; water/octanol coeffcients ranging from 5.07 to 5.47 in increasing order of α–,β– and γ–diastereomers; and water solubility ranging from 48.8 to 2.1 µg/L in decreasing order of α–,β– and γ–diastereomers [19]. As a result of these different properties, the diastereomers exhibit unique interactions. Technical blends contain largely the γ– form, followed by smaller proportions of α– and β– forms [11, 20]. Biotic samples are found to contain largely the α–diastereomer [6, 10, 21]. Zegers et al.[10] propose that this is caused by a stereoselective biotransformation by the cytochrome P450 system, based on a study with marine mammals. In the case of environmental samples there is more diversity in this diastereomer distribution, with findings suggesting a higher proportion of γ– form in sediment and soil [21], mixed proportions of the α– and γ– diastereomers predominate in the air in one study [21], but γ– dominated in another

study [22]. Clearly, the ability to resolve the various HBCDD isomers from one another is an important facet in the analysis of this compound, due to these differences in isomeric distribution in biota, abiotic systems and technical formulations. The need for clear and accurate measurements of the isomer distributions are required for further risk assessment.

Separation of the three α–, β–, and γ– diastereomers of HBCDD can be achieved using reversed phase liquid chromatography (RPLC) and this is currently the method of choice [22, 23]. Originally employed GC separations were unable to resolve the three diastereomers from one another [20], as well as HBCDD being thermally labile resulting in interconversion [5]. Typically, RPLC methods for these compounds are coupled to MS detection [23], and MS/MS detection for low-ng/g level analysis in complex environmental matrices [23].

Convergence chromatography (CC) is a chromatographic technique based upon the use of supercritical fluid CO_2 and has shown to have enhanced efficiency and resolution due to the higher molecular diffusivity and lower viscosity of supercritical fluid compared to liquids [24]. This makes CO_2 well-suited to isomeric separation [25], as well as possessing a reduced column equilibration time [26, 27]. The development of a CC method that analyzes HBCDD also offers the advantage of lower solvent usage, as well as the ability to inject a variety of solvents that are not typically compatible with RPLC analysis. The latter feature has the potential to remove the time consuming solvent exchange step that accompanies many sample preparation procedures. In addition, the coupling of CC with highly sensitive MS/MS detection allows for the detection of low levels of the HBCDD diastereomers. The purpose of this study is to highlight the novelty and potential advantages of using a supercritical fluid based chromatography technique for the separation of HBCDD diastereomers.



α–HBCDD β–HBCDD γ–HBCDD

Figure 1: Structures of the three predominant hexabromocyclododecane diastereomers.

Experiment Details

Chemicals

Individual α–,β– and γ– HBCDD standards were purchased from AccuStandard (New Haven, CT, USA). All standards were stored at 4°C. HPLC grade toluene (Fisher Scientific, USA) and tetradecane 99% (Acros Organics, New Jersey, USA) solvents were used for dilution of standards and samples. Methanol and 2-propanol were Fisher brand Optima grade (Fisher Scientific, USA) and TraceMetal grade ammonium hydroxide was used (Fisher Scientific, USA). Hexane and dichloromethane used in sample preparation were HPLC grade and supplied by Fluka (Steinheim, Germany). Medical grade CO_2 tanks were provided by AirGas (Worcester, MA, USA).

Sample Preparation

Whale blubber and human serum extracts were prepared for a previous analysis as described in Salihovic et. al. [28] for the human serum samples, and Rotander et. al. [29] for the whale blubber samples. Both sample sets were obtained according to legal requirements and ethical practices in their countries of origin and analysis [28,29]. In summary, human serum samples were treated by first protein denaturation using formic acid and sonication,

followed by solid phase extraction using Oasis® HLB cartridges (Waters Corporation, Milford, MA, USA). Target analytes were removed from the cartridge in 1:1 hexane/dichloromethane and 6mL were combined with 25µL tetradecane. Following dry-down using N_2, samples were reconstituted in 500µL hexane. A final multilayer silica gel column clean-up was performed, with analytes eluted in hexane. The samples were then dried-down and reconstituted to 25µL tetradecane for analysis [28]. Whale blubber extracts were pooled by species and homogenized with anhydrous sodium sulfate. Lipid portions were isolated by extraction in 1:1 hexane/dichloromethane in glass columns, and following rotary evaporation lipid content was determined gravimetrically. Clean-up of samples was then performed using multilayer silica gel, and analytes were eluted in hexane [29]. Samples were then exchanged to tetradecane solvent. Prior to analysis described here, the whale blubber extracts were diluted 1:10 in tetradecane.

Convergence Chromatography Conditions

Method optimization and analysis of samples was performed on an Ultra Performance Convergence Chromatography System (UPC^2, Waters Corp., Milford, MA, USA). Upon finalization of method development, a High Strength Silica (HSS) C18 SB 1.8 µm particle size 3.0 x 100 mm column (Waters Corp., Milford, MA, USA) at 40 °C was used, with a total run time of 3 minutes. During method development, an Ethylene Bridged Hybrid (BEH) 1.7µm 2.1x100mm UPC^2 column (Waters Corp., Milford, MA, USA) and Ethylene Bridged Hybrid 2-ethyl pyridine (BEH 2-EP) 1.7µm 2.1x100mm UPC^2 column (Waters Corp., Milford, MA, USA) were also investigated. For all standard and sample injections, a 1µL injection volume was used; during method development. Standards were diluted in toluene for method development steps, and tetradecane was used for the sample analyses both in the case of samples and solvent standards used for calibration curves. This was to account for the samples being in tetradecane, though the diluent choice had no apparent impact on peak response. Initial method development utilized a generic screening gradient, displayed in Table S1of Supplementary Information with CO_2 as mobile phase A and methanol as mobile phase B. The final gradient conditions are displayed in Table S2 of Supplementary Information. A Waters 515 HPLC pump was used to introduce a 0.2 mL/minute make-up flow of 0.1% ammonium hydroxide solution in 2-propanol. This flow was introduced to the post-column effluent prior to introduction into the MS. Several make-up flow compositions were investigated for optimum MS signal enhancement, and the pump was fully purged and primed prior to infusing. For these experiments, five replicate injections of 100 pg solvent standard were used for each make-up flow composition, and peak areas used for comparison.

RPLC Conditions

RPLC separations were performed on an ACQUITY UPLC I-Class (Waters Corp., Milford, MA, USA) using a method described by Shi et al. [7] A BEH C18 1.7µm particle size 50 x 2.1 mm column (Waters Corp., Milford, USA) at 40 °C was used. The gradient is described in Table S3 of Supplementary Information with water as mobile phase A and 1:1 methanol and acetonitrile mix as mobile phase B. A 10µL injection volume was used for all RPLC injections, and standards were diluted in methanol.

MS Conditions

Detection was performed on a Waters Xevo TQ-S operating in MRM mode. Electrospray ionization (ESI) in the negative mode was used. The most abundant transitions were determined and conditions optimized by direct infusion of HBCDD standards, and are displayed in Table S4 of Supplementary Information. The optimized capillary voltage was found to be 2.0 kV. The source temperature was 150 °C, desolvation temperature 500°C, cone gas 150 L/hr and desolvation gas 1000 L/hr. The most abundant transitions were determined and conditions optimized by direct infusion of HBCDD standards. Additional tests were also performed using atmospheric pressure chemical ionization (APCI) and electrospray/chemical ionization switching (ESCi™). Conditions for the APCI and ESCI experiments used the same source temperature, cone gas and cone voltage settings as for ESI. The APCI experiment used a probe temperature of 400°C and desolvation gas flow of 800 L/hr. The ESCI experiment utilized several corona voltage settings, which are described in Figure 5, and the same desolvation temperature and gas flow as the ESI conditions. For column screening experiments and to determine the appropriate ionization conditions, we used a Waters single quadrupole detector (SQD) MS, operating in SIR mode in ESI negative. The operating capillary voltage was set to 3.5 kV. The source temperature was 150 °C,

desolvation temperature 450°C, cone gas 50 L/hr and desolvation gas 800 L/hr. The monitored mass was 640.6, with a dwell time of 0.1 seconds. Full scan MS spectra from m/z 150 to 800 were also acquired on the same instrument.

Results and discussion

Chromatographic Optimization

Three different column chemistries were screened during method optimization under both isocratic and gradient conditions. An HSS C18 column, BEH and BEH 2-EP columns were investigated for their ability to effectively resolve the three HBCDD isomers. Under a 5.6 minute screening gradient (Table S1 of Supplementary Information), only the HSS C18 column and BEH 2-EP column resulted in baseline resolution of the HBCDD diastereomers. Based on these findings, the HSS C18 and BEH 2-EP columns were then assessed for their ability to resolve the HBCDD peaks using isocratic conditions of 10% methanol co-solvent and 90% CO_2. It was found that for both columns the α– and γ– diastereomers were not baseline resolved, and gradient elution on both columns was required for separation of all three diastereomers. Chromatographic peak tailing of standards was also assessed. Peak symmetry at 5% of peak height was measured for the HBCDD diastereomers on the HSS C18 and BEH 2-EP column. The HSS C18 column under gradient conditions was found to be the optimum column for this method based on quality of resolution and minimum peak tailing of HBCDD diastereomers. As mentioned previously, the use of a C18 column chemistry is also widely implemented in current RPLC methods [3-7].

Comparison of CC and RPLC separation

Rapid chromatographic separation of α–, β–, and γ– HBCDD was obtained using CC with a final run time achieving full separation within three minutes (Table S2). Typical RPLC run times for HBCDD separations require at least twice that time [3, 7, 9, 20]. Figure 2 shows a comparison of the chromatograms of the HBCDD isomers at 100 pg/µL using RPLC separation versus CC separation. These results were achieved using HSS C18 column described previously. Peak resolution (R_s) for both chromatographic methods was calculated using the peak width at 50% peak height, and is displayed in Figure 2. Resolution of the HBCDD diastereomers is improved using the CC method. This improved resolution can potentially be attributed to the properties of supercritical fluid CO_2 (increased diffusivity and lower fluid viscosities) which result in an improvement of analyte mass transfer as compared to LC [24]. Additionally, CO_2 has been found to have a solvent strength similar to hexane, which is higher than typical RPLC solvents[30].

As can be seen in Figure 2, the CC separation results in a different elution order than that seen using a C18 column for RPLC conditions. The same elution order of α–,γ– and β–HBCDD has also been observed on C30 column chemistries for RPLC methods [20, 31], as well as on a C18 column where 90:10 methanol/water was used as the mobile phase and the column was held at low temperatures [20]. Enhanced interaction between the stationary phases and the analytes occurs when the viscosity of the mobile phase is increased (e.g. at lower temperatures) and also when a more retentive chemistry such as C30 is used[20]. The findings of Dodder et al.[20] and Stapleton et al.[30] suggest that this enhanced interaction results in the different α–,γ– and β– diastereomer elution order. With respect to the observed elution order achieved when using supercritical CO_2, elution order rearrangements have been found in previous comparisons of RPLC and supercritical fluid chromatography methods [32-34]. These rearrangements are not simply a reversal of the RPLC elution order, and therefore are not exclusively due to analyte polarity [33]. The divergent selectivity observed with this method could be used as an additional confirmation of isomeric identification when used in conjunction with RPLC analysis, as the majority of RPLC methods utilize C18 column chemistries and conditions which result in the α–, β–, and γ– diastereomer elution order.

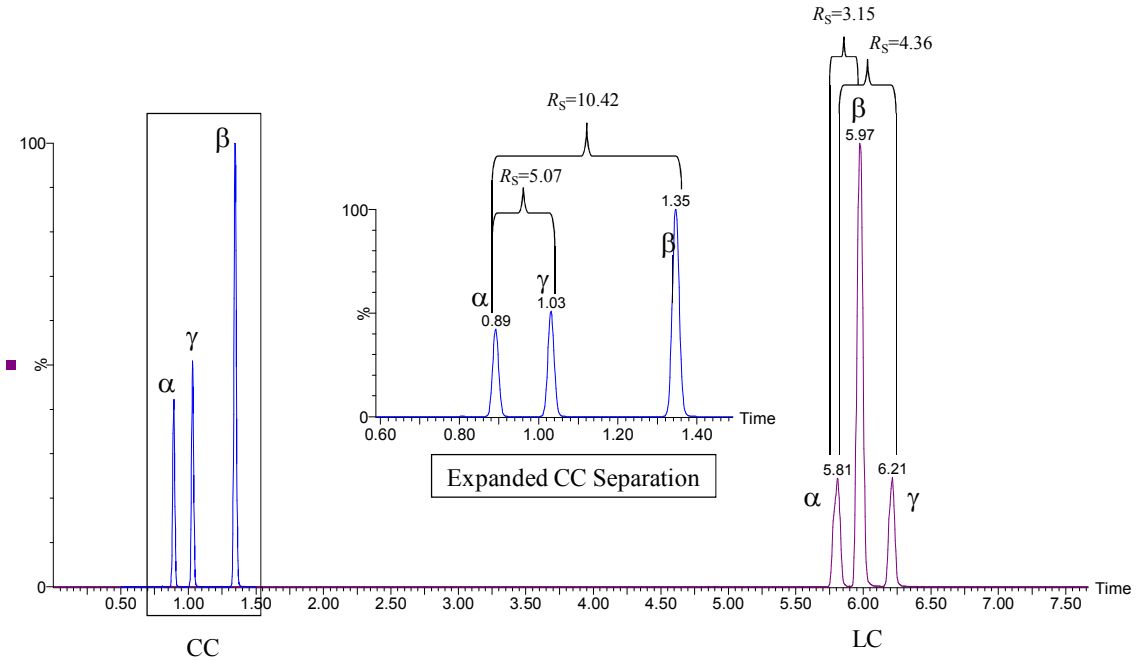


Figure 2: Overlaid chromatograms of CC and LC separations of α–, β–, and γ– HBCDD illustrating the enhanced chromatographic resolution for CC at faster elution rates. In the case of the CC separation, 1 µL of a 100 pg/µL toluene solvent standard was injected, while the LC injection used a 10 µL injection of a100 pg/µL methanol solvent standard.

MS/MS Optimization

Figure 3 is a schematic showing the flow splitter, which enables the mixture of post-column effluent with a supplemental make-up flow introduced using a binary (make-up) pump. Make-up flow solvent composition is combined with the post-column effluent in the first tee-union. A second tee-union connects the automated back pressure regulator (ABPR). This provides an active measurement of system pressure, and the ABPR adjusts as necessary to minimize pressure drop. The combined flow passes through this union into the MS (Figure 3). The make-up flow composition of 0.1% ammonium hydroxide solution in 2-propanol was found to be optimal for HBCDD, with regards to analyte signal intensity following trials of several organic solvent and additive combinations (Figure 4). The use of make-up flow is important for the increase in analyte ionization for the CC method. The HBCDD diastereomers elute at approximately 4-6% methanol co-solvent, hence there is less than 0.2mL/minute organic flow available for protonation when no supplemental make-up flow is added. Additionally, usage of a make-up flow is necessary as the majority of CO_2 present after exiting the MS splitter is in the gas phase, and additional solvent is required to transfer the analyte to the MS ionization source. The MS detection of the HBCDD isomers was enhanced by the use of a post-column make-up flow, which added additional solvent and basic additive in the form of 0.1% ammonium hydroxide solution in 2-propanol.

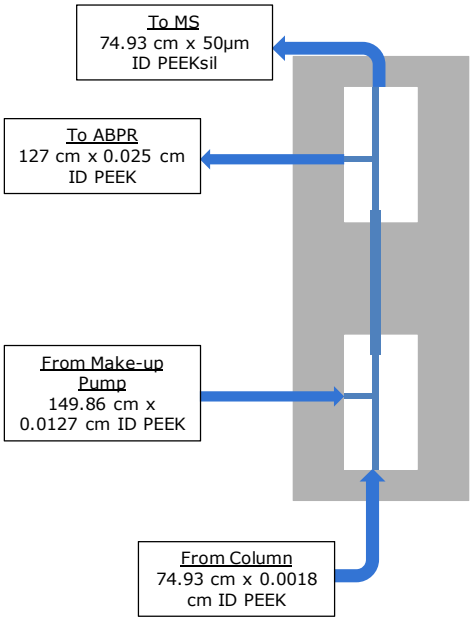


Figure 3: Schematic of flow splitter, with double tee-unions. This configuration is affixed to the CC system.

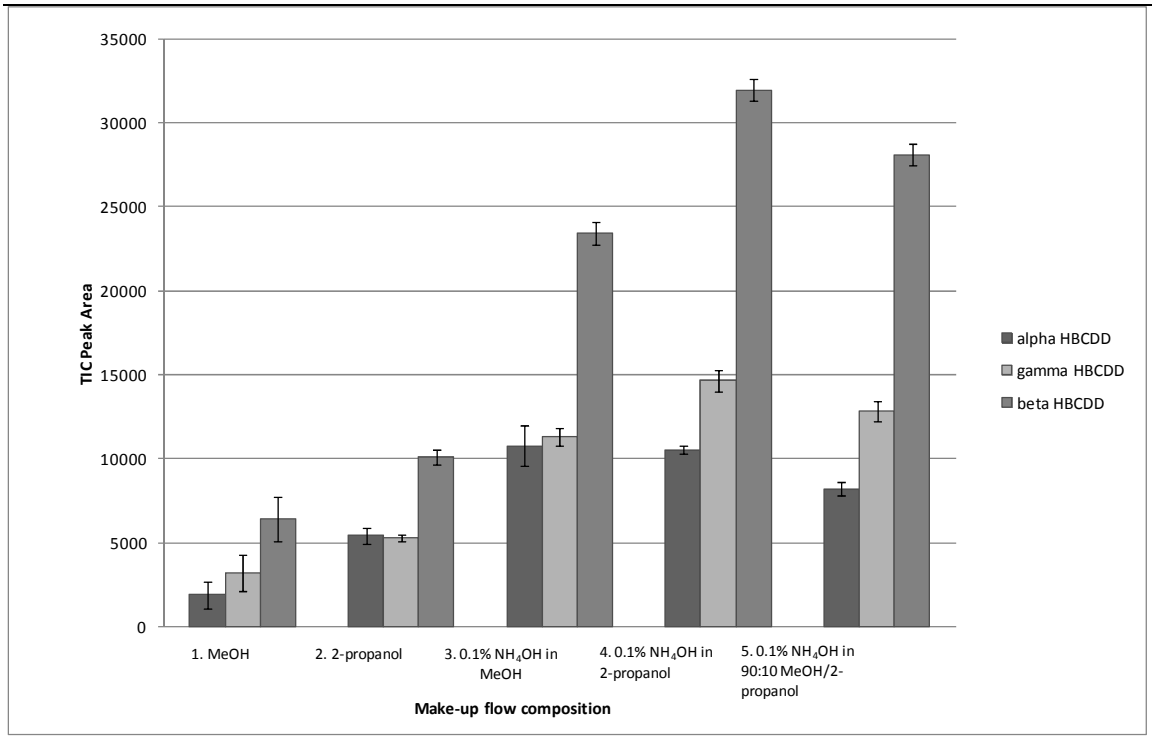


Figure 4: Comparison of peak areas using different make-up flow solvents. Results represent the average peak

area for each diastereomer for five injections, with respective standard deviations. The use of a basic additive in the form of ammonium hydroxide (NH_4OH) notably enhances the MS signal, with the optimum solvent being 2-propanol. Though not shown here, formic acid additive was found to greatly diminish the MS signal.

In addition to ESI, two other ionization techniques were also investigated: atmospheric pressure chemical ionization (APCI) and electrospray/chemical ionization switching (ESCi™), both operating in the negative mode. Depending on the technique, selected parameters which would likely impact the ionization efficiency were assessed. In the case of APCI the make-up flow rate was set at three different values, while for ESCi three different corona voltages were investigated. Corona voltage had previously been optimized for APCI. For the comparison of ESI, APCI and ESCi modes, the $[M-H]^-$ parent ions were monitored, with the most intense signal for HBCDD diastereomers achieved using ESI (Figure 5). Previous RPLC methods have also generally observed a better HBCDD signal when using ESI versus APCI [20, 23].

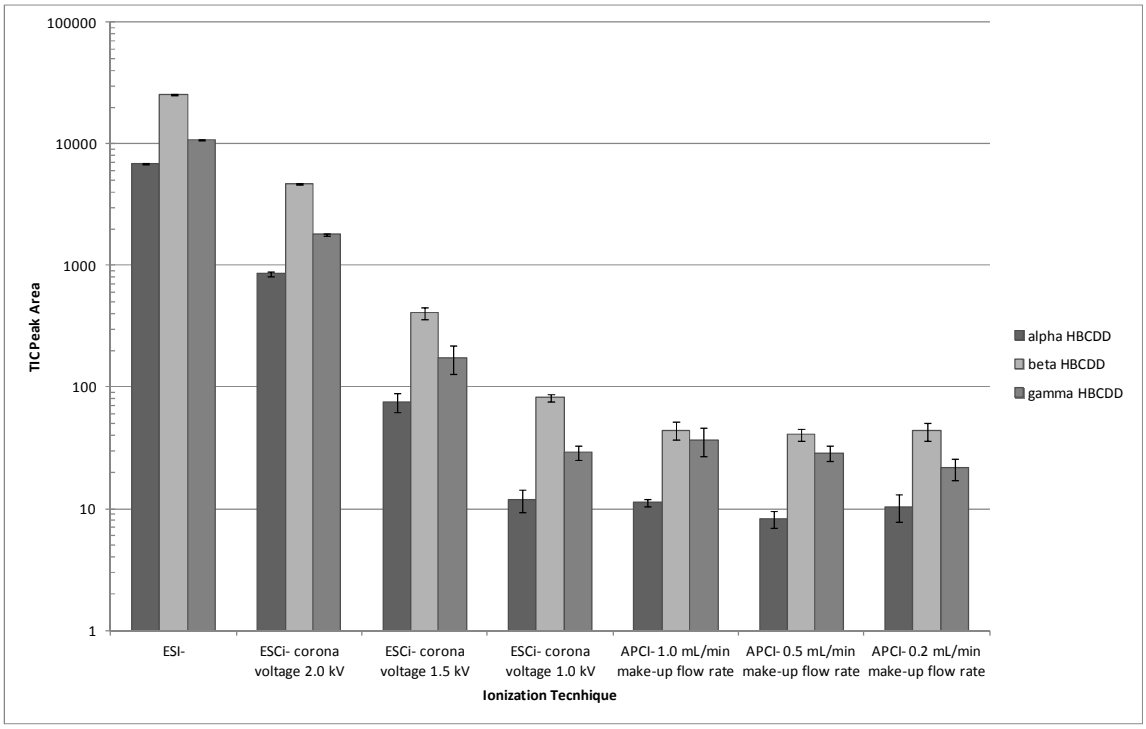


Figure 5: Comparison of ESI$^-$ ionization with APCI$^-$ and ESCi$^-$ methods, showing the average area counts of 5 injections for each HBCDD diastereomer and respective standard deviations.

Limits-of-detection (LODs) and limits-of-quantification (LOQs) were determined using a peak-to-peak signal to noise ratio of 3 and 10, respectively. MRMs of the quantitative transition at the LODs and LOQs are displayed in Figure S1 of Supplementary Information. Measurements were performed using solvent standards, and on the quantification trace (640.6>80.9). The LOD for all HBCDD diastereomers was 100 fg on-column, and LOQs 500 fg on-column for α– and γ– HBCDD, and 250 fg on-column for β–HBCDD. Linearity of the HBCDD calibration curves were calculated using solvent standards across 8 points for β–HBCDD, and 7 for α– and γ–HBCDD. The results showed R^2 values > 0.998 for standards ranging from 0.5 to 100 pg on-column for α–HBCDD and γ–HBCDD, and 0.25 to 100 pg on-column for β–HBCDD. Concentrations of points are described in Tables 1a-b. Repeatability of calculated concentration across five solvent standard injections at

each calibration concentration was also assessed and is displayed in Table 1a. Reproducibility of peak area across the solvent standards for each calibration concentrations in two separate analyses, performed three months apart, is also displayed in Table 5b. No internal standards were used in these analyses, but could be applied in future studies to account for potential matrix effects. Retention time reproducibility was also assessed in both matrix identifications (for α–HBCDD) and solvent standards across the two analyses three months apart. The %RSDs for α–,β– and γ– diastereomers' retention times were 0.77, 0.75 and 0.77 respectively.

Table 1: %RSDs of calculated concentrations for solvent calibration standards for the two analyses three months apart (a) and peak area for solvent calibration standards across both analyses (b). An average of the calculated concentration as pg on-column is displayed for each analyte in parenthesis in (a).

a. Solvent Calibration Curve 1	%RSDs of calculated concentration (n=5) *(Average Calculated Concentration pg on-column)*			Solvent Calibration Curve 2	%RSDs of calculated concentration (n=5) *(Average Calculated Concentration pg on-column)*		
Concentration (pg on-column)	**α-HBCDD**	**β-HBCDD**	**γ-HBCDD**	**Concentration (pg on-column)**	**α-HBCDD**	**β-HBCDD**	**γ-HBCDD**
0.25	<LOQ	7.9 *(0.22)*	<LOQ	0.25	<LOQ	11.7 *(0.26)*	<LOQ
0.5	5.6 *(0.50)*	11.6 *(0.48)*	16.7 *(0.48)*	0.5	9.8 *(0.50)*	6.4 *(0.48)*	15.1 *(0.48)*
1	12.6 *(0.96)*	4.0 *(1.02)*	13.1 *(1.04)*	1	16.8 *(1.06)*	9.4 *(0.94)*	5.7 *(1.06)*
5*	6.8 *(5.05)*	2.3 *(5.15)*	5.3 *(5.25)*	5	4.3 *(4.86)*	1.0 *(4.94)*	2.3 *(5.14)*
10	3.0 *(10.38)*	2.2 *(10.18)*	3.7 *(9.90)*	10	5.4 *(10.08)*	1.0 *(9.88)*	4.4 *(10.04)*
25	5.5 *(24.82)*	2.0 *(25.60)*	1.6 *(25.54)*	25	3.4 *(24.50)*	2.2 *(24.62)*	3.4 *(24.40)*
50	2.3 *(49.94)*	2.2 *(50.52)*	1.8 *(50.06)*	50	1.6 *(48.72)*	0.6 *(49.52)*	1.3 *(49.28)*
100	3.7 *(99.82)*	1.3 *(98.6)*	1.0 *(98.6)*	100	3.4 *(101.8)*	2.6 *(101.12)*	3.5 *(101.12)*
Fit	Linear	Linear	Linear	Fit	Linear	Linear	Linear
Weighting	1/x	1/x	1/x	Weighting	1/x	1/x	1/x
R^2	0.998	0.999	0.999	R^2	0.998	0.999	0.999
*n=4							

b.	%RSDs of peak area for solvent standards (n=10)		
Concentration (pg on-column)	**α-HBCDD**	**β-HBCDD**	**γ-HBCDD**
0.25	<LOQ	11.8	<LOQ
0.5	15.6	11.1	17.7
1	23.5	9.4	11.3
5*	9.9	3.2	5.4
10	10.0	2.8	6.6
25	10.6	3.3	3.6
50	9.2	2.2	4.2
100	11.8	2.2	6.2
*n=9			

Sample analysis

To demonstrate the utility of the method for the analysis of biological samples, a small subset of human serum and whale blubber extracts were screened. Whale blubber extracts were analyzed in this work due to the aforementioned lipophilic properties of HBCDD. Previous studies on the occurrence of persistent organic pollutants, including HBCDD, have focused on marine mammals and found notable levels in their tissue[1, 6, 29, 35]. These analyses were performed using the final gradient method on an HSS C18 column, previously mentioned. These extracts were prepared originally for GC-MS analysis of polybrominated diphenyl ethers (PBDEs) and other persistent organic pollutants (not including HBCDD), as described in the Materials and Methods section.

The chemical properties of HBCDD are similar to PBDEs and other persistent organic pollutants [23], therefore sample preparation techniques used for PBDEs can be applied for HBCDD analysis [6], for semi-quantitative purposes.

The human serum extracts contained one sample found to have α–HBCDD above the LOQ (Figure 6). The presence of this analyte was supported by the conservation of the expected ratio between the two MRM transitions (Table 6); β– and γ–HBCDD were below the LOD. The MRMs of the sample and a solvent standard are displayed in Figure 7. The distribution of the diastereomers is in agreement with that commonly observed in biotic samples [5, 21, 22].

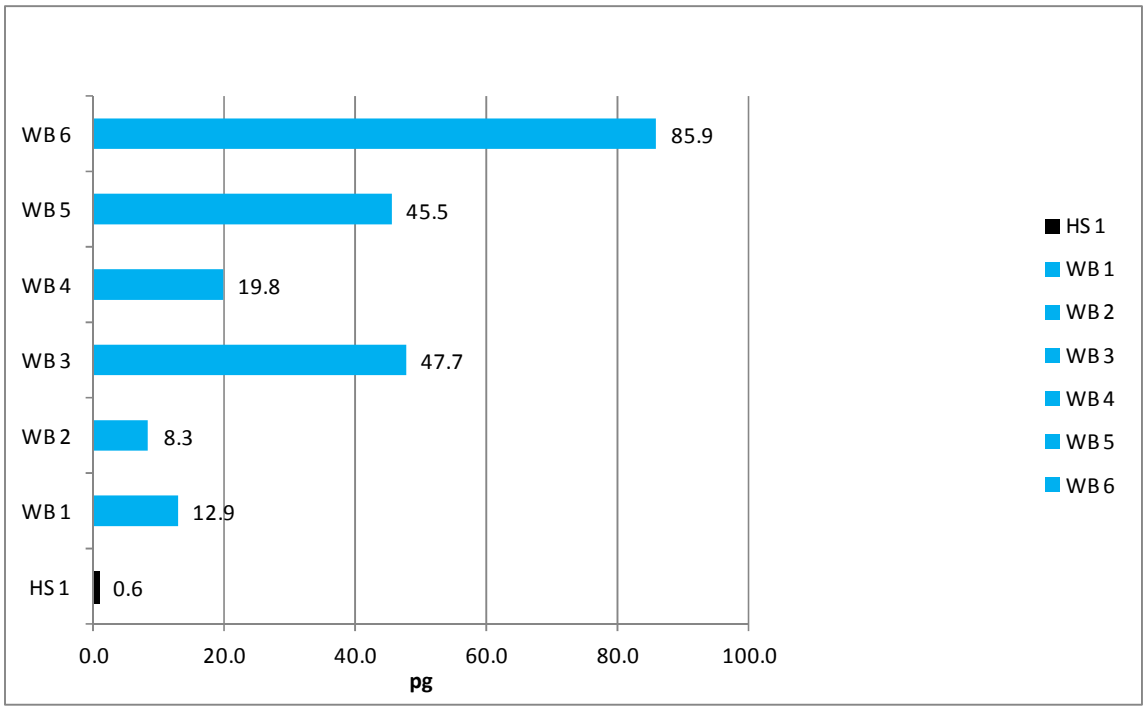


Figure 6: Calculated concentrations (in pg on-column) of α–HBCDD for human serum (HS 1) and whale blubber (WB 1-6) extracts as detected against solvent standards.

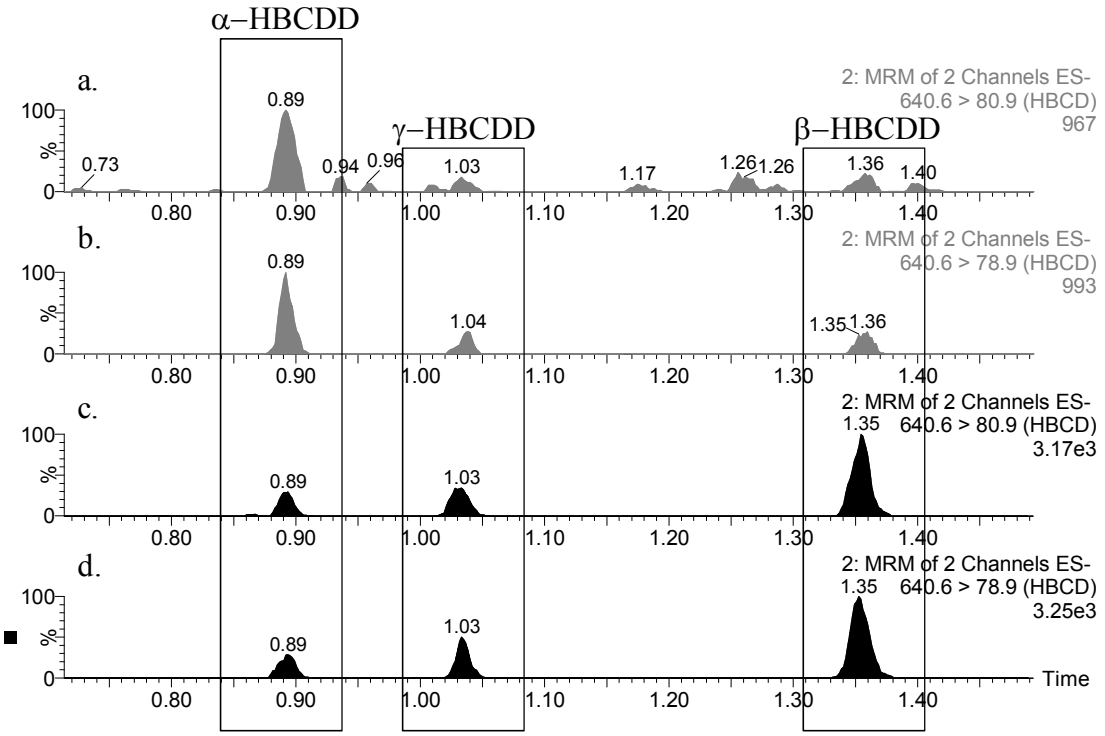


Figure 7: MRM chromatograms of HBCDD in human serum extracts and a solvent standard of similar detected concentration. Quantifier ion (a) and qualifier ion (b) in human serum. Quantifier ion (c) and qualifier ion (d) in solvent standard.

All six whale blubber extracts analyzed had quantifiable levels of the α–HBCDD diastereomer (Figure 6). The whale blubber samples were deduced to be at a sufficiently high concentration to allow for a 1:10 dilution in tetradecane prior to analysis, which is taken into account in the calculated concentrations. The MRMs of HBCDD in the sample and a solvent standard of closely corresponding concentration from the calibration curve are displayed in Figure 8. Again, the predominance of the α–HBCDD diastereomer was as expected for biological samples. With regards to concentrations in the whale blubber samples being generally higher than that of the human serum extract, a previous study looking at adipose tissue in humans and marine predators found lower levels of HBCDD in the human samples [35]. There is evidence to suggest biomagnification of HBCDD [14, 21], and these marine species were top predators [35]. Furthermore, HBCDD is lipophilic (log K_{OW}=5.6 in technical product [14] and therefore more likely to accumulate in lipid rich blubber than serum. Although recovery and matrix effects have not been determined in the scope of this work, the comparative amounts seen between whale blubber and human serum agree with previously published studies[21]. It is recommended that for any future analyses where quantitative results are required, an assessment of the sample preparation procedure for that matrix be performed.

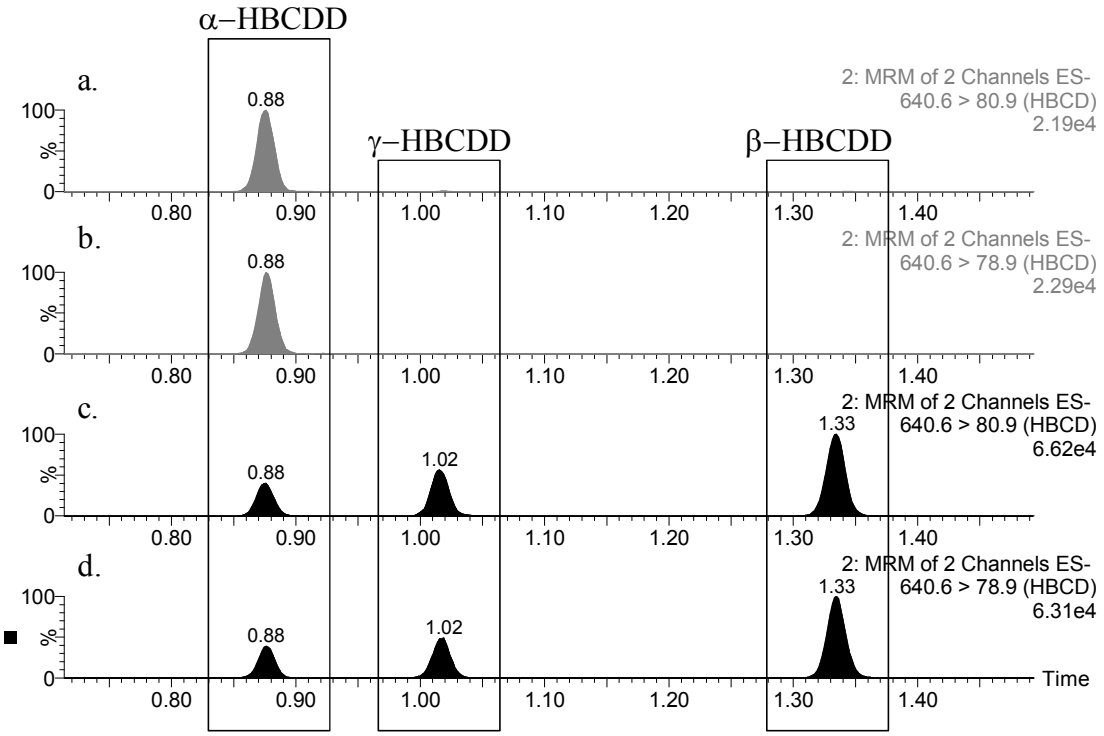


Figure 8: MRM chromatograms of HBCDD in human serum extracts and a solvent standard of similar detected concentration. Quantifier ion (a) and qualifier ion (b) in human serum. Quantifier ion (c) and qualifier ion (d) in solvent standard.

For both sample sets, positive identifications of α–HBCDD were additionally supported by the conservation of ion ratios (640.6>80.9:640.6>78.9). An expected ion ratio for each analysis was calculated by averaging all of the ion ratios in the calibration curve. Table 6 summarizes the calculated ratios in comparison with the expected ratio for all identifications where a peak was observed in both transitions. For all identifications, the observed ion ratio fit within +/- 20% of the expected ratio [36]. In each experiment, expected ion ratios were determined experimentally from those observed for the solvent standards used in each analysis[36].

	α-HBCDD
Expected Ion Ratio Human Serum Analysis	**[1.123]**
HS 1	1.342
Expected Ion Ratio Whale Blubber Analysis	**[1.112]**
WB 1	1.108
WB 2	1.109
WB 3	1.117
WB 4	1.160
WB 5	1.088
WB 6	1.003

Table 6: Ion ratios of 640.6>80.9:640.6>78.9 transitions. The expected ion ratios for the two sample sets are displayed in brackets for each diastereomer, and observed for each samples in the proceeding rows. All ratios are within +/- 20% of these expected values.

Conclusions and Outlook

The use of supercritical fluid CO_2 with methanol co-solvent results in a highly efficient chromatographic separation of the α–,β– and γ–HBCDD diastereomers. The total run time of 3 minutes greatly increases the throughput potential for sample analyses when compared to a typical RPLC based analysis and also offers lower solvent consumption. The use of this method also results in a unique elution order, which can be used alone for identification or in conjunction with RPLC separations to support the identification of a specific diastereomer. Similarities exist with current RPLC methods, namely the use of a C18 column chemistry and ESI negative ion MS providing optimum results. When this method was used to analyze two complex biological matrices, human serum and whale blubber extracts, identifications of the three diastereomers was possible. Confirmation of identifications was afforded by the conservation of ion ratios. Based on these preliminary results, the developed method has been shown to be effective in the analysis of complex samples for HBCDD diastereomers semi-quantitative detection at pg on-column levels.

Acknowledgements

The authors would like to acknowledge Samira Salihovic and Anna Rotander of the MTM Research Centre at Örebro University for sample extracts, Chris Hudalla of Waters Corporation/ProVerde for system schematic details and Baiba Čabovska of Waters Corporation for UPC^2 information and guidance.

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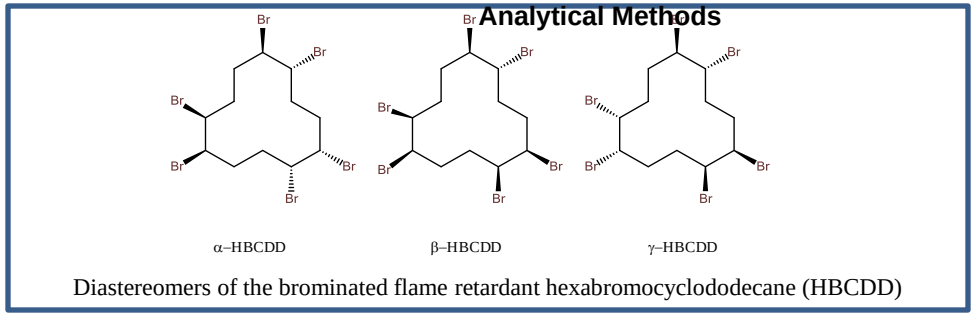
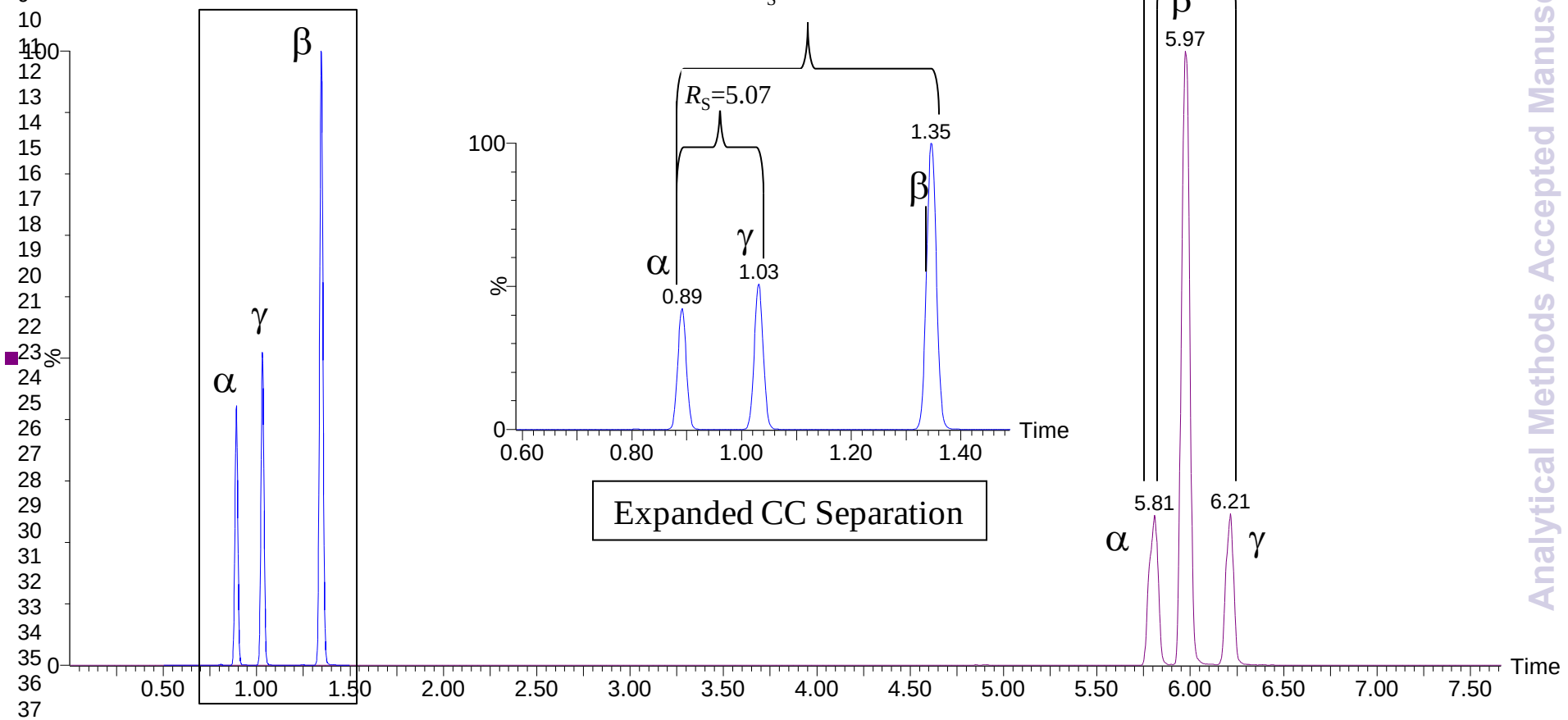


Diastereomers of the brominated flame retardant hexabromocyclododecane (HBCDD)



Expanded CC Separation

Novel Convergence Chromatography (CC) Separation

Typical Reversed Phase Liquid Chromatography (RPLC) Separation