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ORIGINAL RESEARCH

Isomer separation of polybrominated diphenyl ether metabolites using nanoESI-TIMS-MS

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Abstract In this paper, high-resolution nano-electrospray ionization-trapped ion mobility spectrometry coupled to mass spectrometry (nESI-TIMS-MS) is used for the study of hydroxylated polybrominated diphenyl ether (OH-PBDE) metabolites. In particular, experimental ion-neutral collision cross sections (CCS) were measured for five structural OH-PBDE isomers using TIMS-MS. Candidate structures were proposed for each IMS band observed in good agreement with the experimental CCS measurements (5 % error). The analytical power of TIMS-MS to baseline and partially separate structural isomers of OH-BDE in binary and ternary mixtures is shown for single charge species with a mobility resolving power of $R_{IMS} \sim 400$. This work provides the proof of concept for the analysis of low concentration OH-PBDE in environmental samples based on accurate collision cross section and mass measurements without the need for derivatization and pre-fractionation protocols, thus significantly reducing the cost and analysis time.

Keywords Trapped ion mobility spectometry · Mass spectrometry · PBDE · OH-BDE · Collision cross section

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Introduction

Polybrominated diphenyl ethers (PBDEs) are members of the brominated flame retardant family (BFR), which have been frequently added since the 1970s to commercial products (e.g., plastics and textiles) [8]. PBDEs are not chemically bound to the products and are easily released and accumulated in the environment, wildlife and humans [8, 9, 56]. There are three major classes of PBDEs; PBDEs, methoxylated PBDEs (MeO-PBDEs) and hydroxylated PBDEs (OH-PBDEs). The former class is anthropogenically created and released into the environment from commercial production. Previous research has shown that both MeO- and OH-PBDEs are formed from two sources: they are naturally occurring and produced by alga or they are metabolites from commercially produced PBDEs that have been released into the environment [39–41]. Studies have shown that PBDE metabolites such as OH-PBDEs are more toxic than their PBDE counterparts [10, 11, 62, 63]. Differences in toxicity/activity of PBDEs and their metabolites have been noted based on the location of hydroxyl group and bromine atoms on the diphenyl rings [29, 45, 50, 61–63]. The variation in toxicity makes separation and identification of isomeric OH-PBDEs important for exposome profiling in the environment, humans and wildlife.

Traditional methods such as gas chromatography–mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) have allowed for separation and identification of PBDEs and their metabolites. For example, previous studies have utilized GC-MS for profiling of PBDEs, OH-PBDEs and MeO-PBDEs in human breast milk and serum [5, 35, 49]. In addition, GC-MS has been utilized for PBDE analysis in wildlife and environmental samples (e.g., different species of fish and various river sediment samples) [35, 38].

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The use of GC-MS for analysis of OH-PBDEs requires derivatiziation of the molecules to more volatile metabolites such as MeO-BDEs [5, 35, 59]. Previously, Simpson et al. analyzed hydroxylated PBDE metabolites via GC-MS and COnductor-like Screening MOdel for Realistic Solvents (COSMO-RS) to determine experimental retention times and theoretical boiling points of the compounds [59]. In COSMO-RS chemical potentials are calculated in liquids using the screening charge density [30, 31, 36]. Thermodynamic properties of molecules, such as boiling points, were predicted using COSMO-RS, converted to relative retention times and compared to experimental retention time values from GC-MS studies [31, 36, 59] This research proved that OH-PBDE isomers could be separated via retention on a GC-MS column and unknown metabolites could be identified by the combination of theoretically calculated boiling points and experimentally determined retention times after derivatization of the hydroxylated compounds [59]. In addition, four out of the five OH-PBDE metabolites analyzed in this research have previously been extracted and identified from human serum based on GC-MS analysis [5].

Liquid chromatography-mass spectrometry (LC-MS) has also been used for both identification and quantification of PBDE metabolites within various matrices. Lacorte et al. developed an LC-ISP-MS/MS methodology in which eight different OH-PBDEs were identified and quantified from soil, fish and sludge [42]. In these analyses, no sample derivatization was required, saving time and resources as well as allowing for the simultaneous analysis of both OH-PBDEs and MeO-PBDEs metabolites [27, 37, 42]. LC-MS techniques have also been used to successfully analyze for similar metabolites such as the chromatographic separation of 3-OH-BDE-47, 5-OH-BDE-47, and 6-OH-BDE-47 and the subsequent identification and confirmation via tandem mass spectrometry [37]. Although both GC-MS and LC-MS have proven valid methods for analysis and quantification of OH-PBDEs as standards and within biological matrices, the analyses still requires a significant amount of time (i.e., chromatographic separations typically lasting 40 min) and derivatizing agents for GC-MS.

Recent progress in gas-phase, post-ionization separations has been focused on the development of hyphenated techniques in order to achieve higher sensitivity, better separation and reduction of the chemical noise. Different variants of ion mobility spectrometry have been successfully coupled to mass spectrometry (e.g., periodic focusing DC ion guide [20, 21, 57], segmented quadrupole drift cell [25], multistage IMS [23, 32, 34], field asymmetric waveform IMS [33, 48], travelling wave IMS [55], trapped ion mobility spectrometry [12, 15, 26], and cyclic drift tube mobility spectrometry [22, 46]). In particular, TIMS-MS has proved to provide high mobility resolution separations (R ~ 150–300) [26, 58] and the measurement of accurate mobility values using first principles [26]. TIMS-MS provides complementary information separating samples in two dimensions: size-to-charge and mass-to-charge separation on a very limited time scale of analysis (tens to hundreds of milliseconds) [12, 26]. We have previously used TIMS-MS for detection of small molecules within complex matrices [44], the separation of polyaromatic hydrocarbons [1, 7], targeted analysis of endocrine disruptors [2] and the analysis of the conformational dynamics of small molecules and biomolecules [19, 24, 47, 51–54, 58].

In this paper, we explore the potential of TIMS-MS for the analysis of isomeric metabolites of PBDEs. Five OH-tetra-brominated diphenyl ethers are studied: 3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47, 4′-OH-BDE-49 and 2′-OH-BDE-68. Accurate mobility and ion-neutral CCSs were measured using nESI-TIMS-MS. Candidate structures were proposed for each IMS band observed of the individual OH-BDE. The capability of TIMS to separate structural isomers was evaluated for binary and ternary mixtures of OH-BDE. This is the first time resolving powers of ~350–400 are reported for single charge species using TIMS-MS.

Experimental methods

Material and reagents

Hydroxylated tetra-brominated diphenyl ether standards were purchased from Accustandard Inc. (New Haven, CT, USA) and used as received. Five OH-PBDEs were analyzed in this study: 3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47, 4′-OH-BDE-49 and 2′-OH-BDE-68. Binary and ternary mixtures were created by mixing equal volumes of individual standards and diluted to a final concentration of 200 nM. An aliquot (15 μL) of each sample was deposited into a pulled glass capillary tip for nESI-TIMS-MS analysis. All solvents used in these studies were analytical grade or better and purchased from Fisher Scientific (Pittsburg, PA).

TIMS-MS analysis

TIMS-MS is a technique that combines ion mobility separation based on size with ion identification allowed by mass-to-charge ratios [12, 15, 26]. Briefly, this separation technique is built on the utilization of an electric field to hold ions against a flow of moving gas; allowing for the differentiation of ions based on their size-to-charge ratio [12, 15, 26]. The mobility separation in a TIMS cell depends on the velocity of the bath gas (N_2), ion confinement and ion elution parameters [12, 15, 26]. A voltage gradient along the TIMS tunnel provides parameters to trap geometric isomers simultaneously; the isomers are successively eluted from the tunnel by decreasing the electric field in stepwise increments (referred as the "ramp"). The eluted ions are further separated by mass in a

time of flight (TOF) mass spectrometer. The results provide both a mass spectrum of the sample and mobility values which are correlated to collisional cross sections to determine the size of the molecules [4, 12–16, 26, 43]. The mobility in a TIMS analyzer can be described as:

$$K_i = \upsilon_g / E_x(i) = A\left(1/(V_{out} - V_{elu}(i)\right) \quad (1)$$

where υ_g is the velocity of the bath gas in the mobility cell and $E_x(i)$ is the electric field at which the specific packet of ions elute. These parameters can be related to the voltage the ions elute at ($V_{elu}(i)$) and the voltage of the mobility region exit. The A value is a calibration constant that is experimentally determined using a standard of known mobility. From the K or mobility value, the collisional cross section (CCS) can be related by the following equation:

$$CCS = \frac{(18\pi)^{\frac{1}{2}}}{16} \frac{z}{(k_b T)^{\frac{1}{2}}} \left[\frac{1}{m_1} + \frac{1}{m_b}\right]^{\frac{1}{2}} \frac{1}{K} \frac{760}{P} \frac{T}{273.15} \frac{1}{N^*} \quad (2)$$

The charge of the ion is represented by z, k_b represents the Boltzman constant, m_1 and m_b are the masses of molecular ion and the bath gas and N^* is the number density.

The mobility resolving power for the analysis considered was calculated as

$$R = CCS/\Delta CCS \quad (3)$$

Collisional cross sections were calculated using TuneMix as a calibration standard. Details on the Tunemix structures (e.g., $m/z = 322$ $K_0 = 1.376$ cm^2 V^{-1} s^{-1} and $m/z = 622$ $K_0 = 1.013$ cm^2 V^{-1} s^{-1}) can be found elsewhere [17, 26].

TIMS-MS operation parameters

The instrument was operated in wide and narrow mobility selection modes, depending on the analytical challenge. For a wide range mobility analysis, a wide voltage ramp of ΔVramp = 200 V with respect to V_{out} = 60 V was used with a typical ramp step of up to 500 ms. For a narrow mobility selection (higher resolving power), a narrow voltage ramp of ΔVramp = 10 V with respect to V_{out} = 60 V was used with a typical ramp step of up to 500 ms. Notice that, TIMS mobility resolution depends on the ramp speed; the lower the speed the higher the mobility resolution [12, 15, 26]. In addition to the ramp speed, the velocity of the gas also defines the mobility resolution and trapping efficiency of the TIMS analyzer. Increasing the velocity of the gas, by changing the pressure difference between the front (P_1 = 1.6–3.2 mbar) and the end (P_2 = 0.6–1.4 mbar) of the analyzer region also increases the mobility resolution; the higher the velocity of the gas, the higher the drift force and the higher the the electric field required to trap the ions. A constant radiofrequency is applied to the entrance, analyzer and exit region of the TIMS analyzer. Each funnel electrode is divided into four electrically insulated segments, which are used to create a dipole field in the entrance and exit section to focus the ions downstream and a quadrupolar field in the separation region to radially confine the ions during the ion trapping and analsis. That is, in the entrance and exit region the RF between adjacent plates are 180° out phase, while in the analyzer region the RF phase only alternates between adjacent segments. Only the inner diameter and electrode spacing varies between the three sections from 20 to 8 to 1 mm in the entrance, analyzer, and exit region respectively. The adiofrequency was kept constant for all the experiments (frequency of 880 kHz with 300 V peak-to-peak).

A custom-built, pulled capillary nanoESI source was utilized for all the experiments. Quartz glass capillaries (O.D.: 1.0 mm and I.D.: 0.70 mm) were pulled utilizing a P-2000 micropipette laser puller (Sutter Instruments, Novato, CA) and loaded with 10 μL aliquot of the sample solution. Sample solutions consisted single, binary and ternary mixtures of OH-BDEs. A typical nESI source voltage of −600–1200 V was applied between the pulled capillary tips and the TIMS-MS instrument inlet. Ions were introduced via a stainless steel tube (1/16 × 0.020″, IDEX Health Science, Oak Harbor, WA) held at room temperature into the TIMS cell.

Theoretical calculations

A pool of candidate structures were proposed for each OH-BDE standard analyzed in the TIMS- MS experiments. Final structures were optimized at the DFT/B3LYP/6-311G(d,p) level using Gaussian software [18]. Vibrational frequencies were calculated to guarantee that the optimized structures correspond to actual minima in the energy space and zero-point energy corrections were applied to calculate the relative stability between the structures. Theoretical ion-neutral collision cross sections were calculated using MOBCAL [6, 28] software for nitrogen as a bath gas at ca. 300 K. Partial atomic charges were calculated using the Merz-Singh-Kollman scheme constrained to the molecular dipole moment [3, 60].

Results and discussion

TIMS-MS analyses of individual OH-BDE standards including 3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47, 4′-OH-BDE-49 and 2′-OH-BDE-68 resulted in a single IMS band for each structural isomer. Analysis of the mass spectra revealed the expected isotopic pattern of a tetra-brominated compound for the deprotonated molecular ion $[M\text{-}H]^-$ (Fig. 1). Analysis of the IMS projections showed that all individual compounds have very similar experimental CCS values, ranging from 194.5–197.3 $Å^2$ (Fig. 1 and Table 1). In particular, 2′-OH-BDE-68 has the smallest CCS

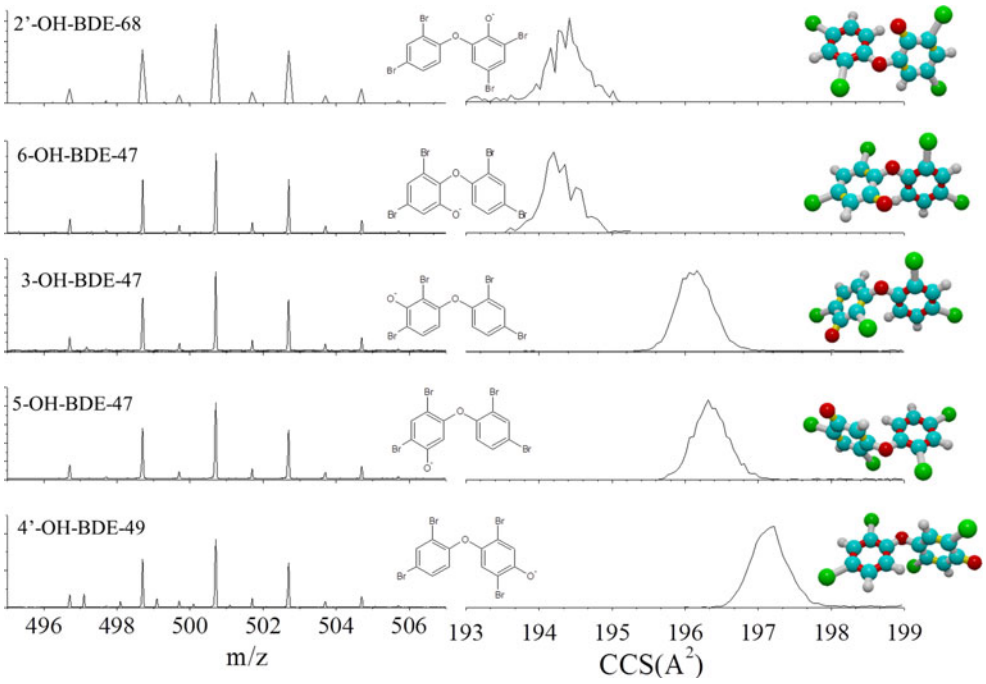

Fig. 1 Typical mass spectra (*left*), IMS projections (*middle*) and candidate structures (*right*) of 2′-OH-BDE-68, 6-OH-BDE-47, 3-OH-BDE-47, 5-OH-BDE-47 and 4′-OH-BDE-49

(194.5 Å^2) while 4′-OH-BDE-49 has the largest CCS (197.3 Å^2). The 6′-OH-BDE-47 and 2′-OH-BDE-68 have very similar CCSs (194.7 Å^2 and 194.5 Å^2, respectively). In addition, 3-OH-BDE-47 and 5-OH-BDE-47 also have very similar experimental CCS (196.0 and 196.6 Å^2, respectively).

Candidate structures were proposed for each IMS band observed (see Fig. 1). Inspection of the optimized structures at the DFT/B3LYP/6-311G(d,p) shows that OH-BDE are near planar conformations, with a slight twist around the central oxygen atom, depending on the position of the Br atoms (ortho *vs* para positions) (Fig. 1). A small error was observed between the theoretical and experimental CCS values (less than ±5 %). This small error can be attributed to the fact that the MOBCAL program does not properly describe bromine atoms for the CCS calculations [6, 28].

Following analysis of individual OH-PBDE compounds, binary and ternary mixtures of OH-PBDE standards were analyzed in order to evaluate the potential of nESI-TIMS-MS for the separation of isomeric mixtures of these compounds. The

Table 1 Experimental and theoretical CCSs for of 2′-OH-BDE-68, 6-OH-BDE-47, 3-OH-BDE-47, 5-OH-BDE-47 and 4′-OH-BDE-4 isomers

	$[M-H]^-$ m/z	Experimental TIMS (Å^2)	Theoretical TM (Å^2)
2′-OH-BDE-68	500.7834	194.5	192
6-OH-BDE-47	500.7834	194.7	186
3-OH-BDE-47	500.7834	196.0	188
5-OH-BDE-47	500.7834	196.6	190
4′-OH-BDE-49	500.7834	197.3	193

analysis of a binary mixture containing 2′-OH-BDE-68 and 3-OH-BDE-47 shows that these two isomers can be baseline resolved with a mobility resolving power of ~350 (Fig. 2). Closer inspection shows that 3-OH-BDE-47 elutes from the mobility cell at a voltage of 143 V and 2′-OH-BDE-68 elutes at a voltage of 141 V while the same isotopic pattern was observed in the *m/z* domain (Fig. 2a). Conversion of the trapping voltage to CCS shows that the 3-OH-BDE-47 peak is centered at 196.0 Å^2 while 2′-OH-BDE-68 is centered at 194.7 Å^2 (Fig. 2b), which are in good agreement with the analysis of individual compounds (Fig. 1). A binary mixture of 4′-OH-BDE-49 and 6-OH-BDE-47 was analyzed using the same instrumental conditions. The 2D-IMS-MS contour plot shows two mobility bands eluting from the cell at trapping voltages of 144 V and 142 V respectively with the same *m/z* profile corresponding to the isotopic distribution of tetrabrominated OH-BDEs (Fig. 2c). Conversion of the trapping voltage to the CCS shows baseline separation of 4′-OH-BDE-49 centered at 197.3 Å^2 and 6-OH-BDE-47 centered at 194.7 Å^2 with a resolving power of ~320–350 (Fig. 2d). It should be noted that in both analyses the narrow range of the ramp voltage (10 V), the higher gas flow velocity, and the slow ramp speed permitted the achievement of high resolving power. In addition, the rigidity of the OH-PBDE molecules in contrast with previously studied systems (e.g., peptides and proteins) utilizing TIMS allows for the observation of narrower IMS bands.

Ternary mixtures of OH-BDE compounds were also created and subsequently analyzed via nESI-TIMS-MS. A mixture of 3-OH-BDE-47, 5-OH-BDE-47 and 2′-OH-BDE-68 showed baseline separation between 2′-OH-BDE-68 and 3-

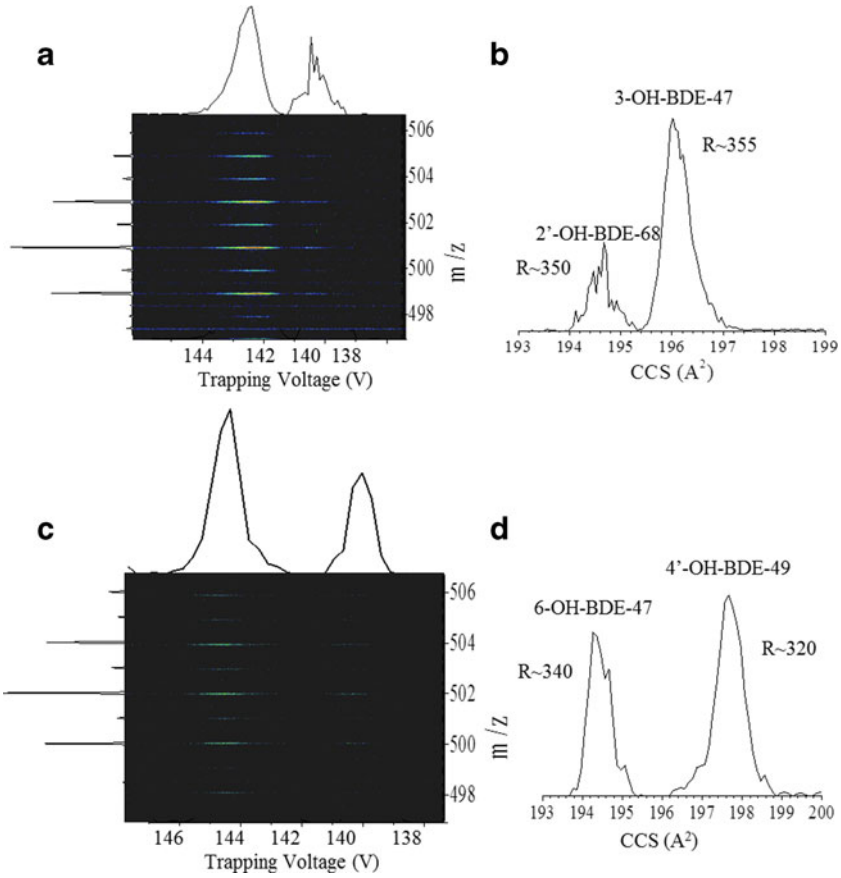

Fig. 2 Typical 2D-IMS-MS contour and IMS projection plots for the binary mixture of 3-OH-BDE-47 and 2′-OH-BDE-68 (**a**) and **b**)) and the mixture of 6-OH-BDE-47 and 4′-OH-BDE-47 (**c**) and **d**))

OH-BDE-47 (CCS = 194.5 Å^2 and 196.0 Å^2) with a resolving power of 320–400. The two BDE-47 compounds in the mixture (3-OH-BDE-47 and 5-OH-BDE47) were only partially resolved; however, the IMS peak clearly shows a bimodal peak with two well defined centers that correlate to the CCS of the single standards (Fig. 3a). A second ternary mixture containing 6-OH-BDE-47, 5-OH-BDE-47 and 4′-OH-BDE-49 was analyzed to further evaluate TIMS-MS as a method to separate isomeric mixtures (Fig. 3b). In this mixture the two metabolites from BDE-47 were baseline separated with a resolving power in the order of 350–400; however, the 4′-OH-BDE-49 and 5-OH-BDE-47 were only partially resolved with centers that correlate to the CCS of the single standards (197.4 Å^2 and 196.6 Å^2, respectively).

Conclusions

In this work we provide the framework for rapid isomer separation of hydroxylated polybrominated diphenyl ethers using nESI-TIMS-MS. During the analysis of five OH-BDE standards, experimental CCS values were calculated using TuneMix as a mobility standard. Candidate structures were proposed for all the IMS bands observed in good agreement with the experimental CCS (within ±5 %). Analysis of binary mixtures showed that baseline separation is possible between 2′-OH-BDE-68 and 3-OH-BDE-47, 6-OH-BDE-47 and 4′-OH-BDE-49, and 6- OH-BDE-47 and 5-OH-BDE-47 with a resolving power of 350–400. Moreover, 3-OH-BDE-47 and 5-OH-BDE-47, and 5-OH-BDE-47 and 4′-OH-BDE-49

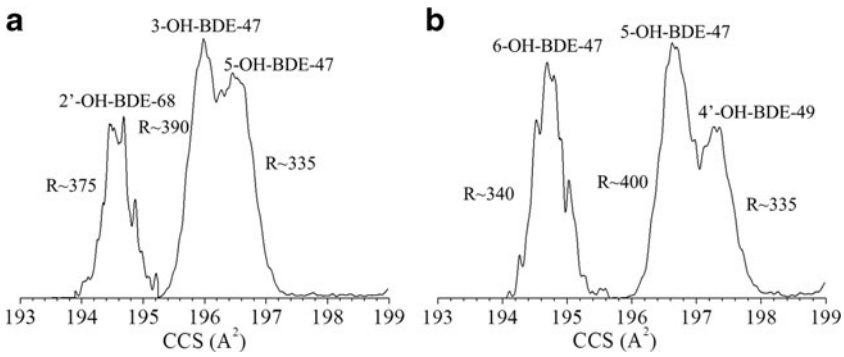

Fig. 3 Typical IMS projection plots from the ternary mixture of 3-OH-BDE-47, 5-OH-BDE-47 and 2′-OH-BDE-68 (**a**) and the mixture of 4′-OH-BDE-49, 5-OH-BDE-47 and 6-OH-BDE-47 (**b**)

isomers were only partially resolved. This work provides the foundation for the analysis of OH-BDE from complex mixtures utilizing small volumes (15 μL), significantly decreasing the amount of material necessary for the analysis without the need for derivatization or chromatographic separation. In addition, separation in the TIMS cell occurred on the millisecond range and experiments typically take less than 5 min per sample significantly decreasing the amount of analysis time when compared to GC-MS and LC-MS analyses. It should be noted that the TIMS-MS operation (as low as 50 ms analysis time [26]) can be easily coupled to GC and LC pre-separation as a way to diminish the matrix effects during nESI for the analysis of complex mixtures.

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Compliance with ethical standards

Competing interest The authors declare no competing financial interest.

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