Development of a liquid chromatography/atmospheric pressurephoto-ionization high-resolution mass spectrometry analytical method for the simultaneous determination of polybrominateddiphenyl ethers and their metabolites: application to BDE-47metabolism in human hepatocytes

Charlotte Marteau, Sylvie Chevolleau-Mege, Isabelle Jouanin, Elisabeth Perdu, Georges De Sousa, Roger Rahmani, Jean-Philippe Antignac, Bruno Le Bizec, Daniel Zalko, Laurent Debrauwer

Research Article

Rapid Communications in Mass Spectrometry

Received: 29 October 2011 Revised: 16 December 2011 Accepted: 16 December 2011 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2012**, *26*, 599–610
(wileyonlinelibrary.com) DOI: 10.1002/rcm.6136

Development of a liquid chromatography/atmospheric pressure photo-ionization high-resolution mass spectrometry analytical method for the simultaneous determination of polybrominated diphenyl ethers and their metabolites: application to BDE-47 metabolism in human hepatocytes

Charlotte Marteau[1], Sylvie Chevolleau[1], Isabelle Jouanin[1], Elisabeth Perdu[1], Georges De Sousa[3], Roger Rahmani[3], Jean-Philippe Antignac[2], Bruno LeBizec[2], Daniel Zalko[1] and Laurent Debrauwer[1]*

[1]INRA, UMR 1331 Toxalim INRA-INP, 180 chemin de Tournefeuille, BP 93173, F31027, Toulouse cedex 3, France
[2]LUNAM, Oniris, USC 1329 INRA, LABERCA, Atlanpôle-La Chantrerie, BP 50707, F44307, Nantes, France
[3]INRA, UMR 1331 Toxalim INRA-INP, 400 route des Chappes, BP 167, F06903, Sophia Antipolis cedex, France

Polybrominated diphenyl ethers (PBDEs) are flame retardants widely used in electronic and domestic goods. These persistent pollutants are present in the environment and in humans, and their toxicological properties are of growing concern. PBDEs can be metabolised into compounds suspected to be responsible for their toxicity. These metabolites have been characterised quite well in rodents and fish, but available information in humans remains scarce. For their identification, an efficient method for the simultaneous analysis of PBDEs, hydroxylated PBDEs (OH-PBDEs), and other PBDE metabolites in a single run was needed and has been developed in this work. Atmospheric pressure ionisation modes were compared, and Atmospheric Pressure Photo-Ionization (APPI) was selected. After careful setting of APPI parameters such as dopant and operating temperature, the optimised method was based on APPI ionization coupled to High-Resolution Mass Spectrometry operating in the full scan mode at a resolution of 60 000. This provided excellent sensitivity and specificity, allowing the discrimination of signals which could not be resolved on a triple quadrupole used as a reference. The full-scan high-resolution acquisition mode allowed monitoring of both parent PBDEs and their metabolites, including hydroxylated PBDEs, with detection limits ranging from 0.1 pg to 4.5 pg injected on-column based on the investigated standard compounds. The method was applied to the study of BDE-47 metabolism after incubation with human primary cultures of hepatocytes, and proved to be efficient not only for monitoring the parent compound and expected hydroxylated metabolites, but also for the identification of other non-targeted metabolites. In addition to hydroxy-BDE-47, several conjugated metabolites could be located, and the formation of a dihydrodiol derivative was evidenced for the first time in the case of PBDEs in this work.

Polybrominated diphenyl ethers (PBDEs) are additive brominated flame retardants (BFR) used to fireproof plastics and textiles. They are commonly used in the manufacture of electric and electronic appliances, as well as transportation and construction foams, with the aim of decreasing fire risk. Three commercial mixtures of PBDEs were originally manufactured. The 'deca-mix', which mainly contains deca-BDE (BDE-209), is now by far the most commonly used, and the most efficient mixture. The production and/or the use of the two other mixtures, namely 'penta-mix' and 'octa-mix', have been phased out in several countries. PBDEs were classified as Persistent Organic Pollutants (POPs) in 2009 by the Stockholm Convention.[1–3]

* *Correspondence to:* L. Debrauwer, INRA, UMR 1331 Toxalim INRA-INP, 180 chemin de Tournefeuille, BP 93173, F31027 Toulouse cedex 3, France.
E-mail: laurent.debrauwer@toulouse.inra.fr

The presence of PBDEs has been demonstrated in all environmental compartments, including sediments and dust. PBDEs are both persistent and bioaccumulative.[4–6] The occurrence of PBDEs in humans has been reported in several studies, with the predominance of congeners 47, 99, 100, 153, 154 and 183. A study focused on both indicators and highly brominated PBDEs has indicated that tri- to deca-BDEs are present in samples collected from the French population, including adipose tissue, maternal milk, serum and cord serum, with higher amounts of highly brominated PBDEs (especially BDE-207 and -209) compared to the more commonly monitored indicator PBDEs (i.e. BDE-28, -47, -99, -100, -153, -154, -183) in serum samples.[7,8] The toxic effects of PBDEs have only partly been characterised so far. They appear to rely both on direct mechanisms and on the capability of PBDEs to behave as endocrine disruptors, as highlighted by recent studies.[9,10] Among others, it was shown that a prenatal exposure of rodents to PBDEs during critical

periods of the brain development can result in decreased learning and memory capacities, as well as modified behaviour, which persist in adulthood.[11,12] Those neurotoxic effects might be linked with the capability of PBDEs to disrupt thyroid hormones homeostasis.[13,14] Some studies also established that PBDEs can alter some sexual characteristics in male and female rats, and modify the metabolism of steroid hormones.[15,16] The biological effects of PBDEs could result, at least in part, from the formation of bioactive metabolites. As first demonstrated by Meerts *et al.*,[17] some PBDE metabolites, especially hydroxylated metabolites (OH-PBDEs) of tetra-BDE such as OH-BDE-47, express a strong affinity for thyroid hormone transporters TTR and TBG.[18] They can also activate estrogen receptor-α.[19] Moreover, a few studies have already established the presence of several PBDE metabolites in human serum and maternal milk.[20–22] Though BDE-209 is by far the main PBDE produced and used nowadays, it is well known that PBDEs can readily debrominate in the environment through photolytic mechanisms, producing a variety of lower brominated analogues.[23,24] In addition, PBDEs have been shown to be metabolised into lower brominated analogues and/or oxidated metabolites, in fish as well as in rats.[25–28] Human cells as well as liver microsomes are also able to metabolise PBDEs through oxidation, debromination, methoxylation, and through the cleavage of the ether bond of the molecule leading to bromophenols.[29–31] However, knowledge on the metabolism of PBDEs by human remains sparse and efficient tools allowing a deep investigation of both those apolar compounds and their polar metabolites are still needed.

Currently, the analysis of PBDEs and OH-PBDEs is most often carried out separately using gas chromatography/mass spectrometry (GC/MS), although derivatisation may be necessary for the analysis of OH-PBDEs. The main advantages of GC/MS are the fine chromatographic resolution of PBDEs congeners, and its great sensitivity, especially when using Electron Capture Negative Ionisation (ECNI). However, GC/ECNI-MS is poorly specific, since the monitored ions are solely bromine ions. Electron ionisation provides more specificity, with the formation of $[M]^{+\cdot}$ and $[M–Br_2]^{+\cdot}$ ions, but this technique is less sensitive (especially for highly brominated PBDEs).[32] Another problem is the thermal degradation of highly brominated PBDEs. To avoid such problems, the analysis of PBDEs by liquid chromatography/mass spectrometry (LC/MS) using Atmospheric Pressure Photo-Ionisation (APPI) has been proposed.[33] The behaviour of PBDEs towards APPI processes has been studied in detail,[34] and several specific methods have been developed for the study of PBDEs in house dust,[35,36] water,[37] and fish samples.[38] Two studies have compared the performances of Atmospheric Pressure Chemical Ionisation (APCI) and APPI for the analysis of PBDEs in sewage sludge[39] and in fish and water.[38,40] Unlike PBDEs, OH-PBDEs can be analysed by Electrospray Ionisation (ESI)[41–43] or APCI.[44,45]

In the present study, we developed a LC/MS method allowing for the first time the simultaneous detection and identification of PBDEs, and their metabolites (with special emphasis on the bioactive OH-PBDEs), with the aim of applying this method to PBDE metabolism studies. The development was carried out on a set of commercially available PBDEs and OH-PBDEs, whose structures and estimated logP values[46] are presented in Fig. 1 and Table 1, respectively. We first compared ESI, APCI and APPI as ionisation means for PBDEs and OH-PBDEs. Based on our previous work,[33,34] APPI of PBDEs and OH-PBDEs was then studied, in order to set up the optimal MS acquisition parameters. A highly sensitive and specific method based on high-resolution MS (HRMS) on a hybrid LTQ-Orbitrap was developed and its performance compared to a targeted multiple reaction monitoring (MRM) approach on a triple quadrupole taken as a reference. Particular attention was also paid to the LC separation of PBDEs and OH-PBDEs. Finally, the method was applied to biological samples in the framework of metabolic studies, through the analysis of culture media extracts obtained after incubation of BDE-47 with human primary cultures of hepatocytes.

EXPERIMENTAL

Chemicals

Standards of OH-PBDEs (3-OH-BDE-47, 4′-OH-BDE-49, 5-OH-BDE-47, 6-OH-BDE-47, 5′-OH-BDE-99, 3-OH-BDE-100, 3′-OH-BDE-154, 6-OH-BDE-180, 4-OH-BDE-187, 4-OH-BDE-188 and 4′-OH-BDE-201) were from Accustandard (New Haven, CT, USA), and standards of PBDEs (BDE-47, -99, -100, -153, -154, -183, -184, -201, -202, -204, -206, -207, -208, -209) from Wellington Laboratories (Guelph, ON, Canada). All solvents used for HPLC were HPLC grade solvents from Scharlau (Barcelona, Spain). Methoxylated tetra-BDEs and bromophenols were synthesised at the laboratory using previously published procedures.[47] Radio-labelled BDE-47 was synthesised from ring-[^{14}C]-diphenyl ether (Izotop, Institute of Isotopes Co. Budapest, Hungary; specific activity: 5.28 MBq.mg^{-1}). Partial bromination of [^{14}C]-diphenyl ether was achieved with iron dibromide. BDE-47 was then purified by HPLC, using a Jasco HPLC system composed of a HPLC pump series PU-980 and a UV detector series UV-975 (Merck,

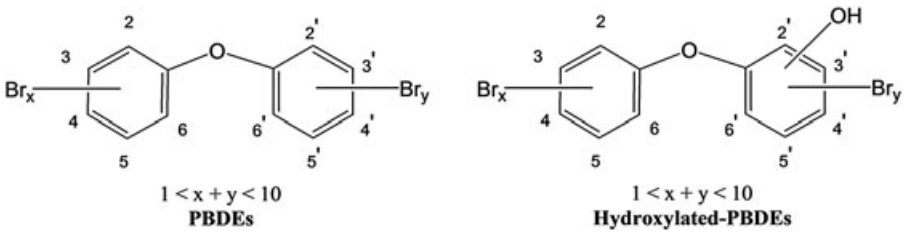

Figure 1. Generic structure of PBDEs and hydroxylated PBDEs.

Rapid Communications in Mass Spectrometry

Table 1. Studied PBDEs and OH-PBDEs and estimation of their logP values

Compounds	IUPAC name	Bromine substitution	logP
Tetra-BDE	BDE-47	2,2′,4,4′	6,40 ± 0,43
Penta-BDEs	BDE-99	2,2′,4,4′,5	7,05 ± 0,57
	BDE-100	2,2′,4,4′,6	7,07 ± 0,59
Hexa-BDEs	BDE-153	2,2′,4,4′,5,5′	7,69 ± 0,75
	BDE-154	2,2′,4,4′,5,6′	7,71 ± 0,76
Hepta-BDEs	BDE-183	2,2′,3,4,4′,5′,6	8,30 ± 0,93
	BDE-184	2,2′,3,4,4′,6,6′	8,32 ± 0,94
Octa-BDEs	BDE-201	2,2′,3,3′,4,5′,6,6′	8,87 ± 1,12
	BDE-202	2,2′,3,3′,5,5′,6,6′	8,85 ± 1,12
	BDE-204	2,2′,3,4,4′,5,6,6′	8,89 ± 1,13
Nona-BDEs	BDE-206	2,2′,3,3′,4,4′,5,5′,6	8,41 ± 0,96
	BDE-207	2,2′,3,3′,4,4′,5,6,6′	8,19 ± 0,84
	BDE-208	2,2′,3,3′,4,5,5′,6,6′	8,17 ± 0,84
Deca-BDE	BDE-209	2,2′,3,3′,4,4′,5,5′,6,6′	8,77 ± 1,04
Hydroxylated tetra-BDEs	3-OH-BDE-47	2,2′,4,4′	5,99 ± 0,57
	5-OH-BDE-47	2,2′,4,4′	6,02 ± 0,59
	6-OH-BDE-47	2,2′,4,4′	5,98 ± 0,56
	4′-OH-BDE-49	2,2′,4,5′	5,96 ± 0,56
Hydroxylated penta-BDEs	5′-OH-BDE-99	2,2′,4,4′,5	6,68 ± 0,71
	3-OH-BDE-100	2,2′,4,4′,6	6,70 ± 0,74
Hydroxylated hexa-BDEs	3′-OH-BDE-154	2,2′,4,4′,5,6′	7,34 ± 0,89
Hydroxylated hepta-BDEs	6-OH-BDE-180	2,2′,3,4,4′,5,5′	7,98 ± 1,05
	4-OH-BDE-187	2,2′,3,4′,5,5′,6	7,91 ± 1,03
	4-OH-BDE-188	2,2′,3,4′,5,6,6′	7,93 ± 1,04
Hydroxylated octa-BDEs	4′-OH-BDE-201	2,2′,3,3′,4,5′,6,6′	8,51 ± 1,21

Nogent-sur-Marne, France) coupled to a model 202 (Gilson, Roissy, France) fraction collector. The structure and purity (>99%) of synthesised BDE-47 (specific activity: 1.78 Mbq. mg^{-1}) were checked by MS and NMR.

Metabolism studies

Hepatocytes were obtained from three human donors (two women: 82 and 31 years old and one man: 64 years old) and were treated separately. Human liver biopsies (resected from secondary tumors and cryopreserved after procurement) were isolated, cryopreserved and cultivated as previously described.[48] After a specific and actually confidential freeze-thaw protocol in Leibowitz L-15 medium supplemented with FBS, insulin and DMSO (progressively decreased in further steps), around 400 000 human hepatocytes were seeded in 12-well plates in L-15 supplemented with FBS culture medium. They were allowed to grow during 24 h at 37°C and under 5% CO_2. Then, medium was removed and replaced by 1 mL of William's E medium with Glutamax supplemented with 0.5% penicillin/streptomycin and 0.1 UI/mL insulin, and incubations were carried out for 24 h at 37°C under 5% CO_2 in the presence of 1 µM or 5 µM radiolabelled BDE-47 (diluted in 5 µL DMSO). At the end of the incubation period, culture media were collected, and cell contents were extracted using acetonitrile/water (50:50, v/v). Both fractions were stored at −20°C until analysis.

Sample preparation for MS analysis

Culture media were treated using methanol (1:3, v/v) for protein precipitation, and were then evaporated to dryness under a nitrogen stream. Each extract was individually retaken in 100 µL methanol and submitted to HPLC separation and fraction collection (1 min fractions) used as a cleanup step with the aim of eliminating potential culture media interfering compounds. Radioactive fractions were pooled, evaporated under a nitrogen stream and redissolved in 25 µL methanol for LC/APPI-HRMS injection.

LC/MS

LC/HRMS experiments were carried out on a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Les Ulis, France) coupled to an Ultimate 3000 LC System (Dionex, Voisins le Bretonneux, France). The Orbitrap cell was operated in the full-scan mode at a resolution power of 60 000. A TSQ Quantum triple quadrupole mass spectrometer (Thermo Fisher Scientific, Les Ulis, France) coupled to an Accela LC system was used to carry out MRM LC/MS/MS experiments. Both devices were operated in the negative APPI, APCI or ESI ionisation modes. APCI was operated with a discharge current of 8 µA. A Syagen APPI source (Thermo Fisher Scientific, Les Ulis, France) operating with a krypton discharge lamp was used for APPI. APPI operating parameters were as follows: sheath gas (N_2), 70 arb. units; and auxiliary gas (N_2), 20 arb. units.

Development steps were completed on the triple quadrupole instrument and later confirmed on the Orbitrap mass spectrometer. The influence of APPI key operating parameters on the ionisation was studied by carrying out five injections of a mixture of PBDEs/OH-PBDEs directly into the source by FIA for each condition tested. Acetone, anisole and toluene were tested as dopants, in 5, 10 and 15% proportions. The dopant was introduced either by post-column addition or directly as LC mobile phase component. The impact of the heated transfer capillary and the nebuliser temperatures on the intensity of

diagnostic ions for PBDEs and OH-PBDEs was studied from 200°C to 350°C and 200°C to 400°C, respectively (50°C steps in both cases).

U-HPLC columns used were an Hypersil Gold C18 column (100 × 2.1 mm, 1.9 µm) and an Hypersil Gold PFP column (100 × 2.1 mm, 1.9 µm) from Thermo Fisher Scientific (Les Ulis, France). Optimised LC separation was achieved on the PFP column at 40°C, at a flow rate of 250 µL.min^{-1} with a multistep gradient of mobile phase A: water/methanol/acetone (80:10:10, v/v/v) and mobile phase B: methanol/acetone (90:10, v/v). Initial conditions were A:B 70:30 v/v held for 1 min followed by a 12 min gradient from A:B 70:30 v/v to 30:70 v/v, then a 13 min gradient from A:B 30:70 v/v to 25:75 v/v and a final 1 min gradient from A:B 25:75 v/v/ to 100% B, held for 5 min.

RESULTS AND DISCUSSION

Ionisation mode

Comparison of APPI, APCI and ESI modes

The performance of the three ionisation modes for LC/MS, i.e. ESI, APCI and APPI, was first compared. As expected, ESI was only suitable for OH-PBDEs, and presented sensitivities in the low pg range in HRMS, comparable to the values generally reported for analysis of OH-PBDEs by LC/ESI-MS/MS.[41–43] This good sensitivity can be attributed, at least in part, to the soft ionisation process of ESI, generating almost only quasimolecular ions. APCI is suitable for analysis of PBDEs and OH-PBDEs. They were ionised in a similar way compared to APPI, and in agreement with other works.[39,40] For PBDEs, the main ions formed were $[C_6Br_xH_{5-x}O]^-$ ions, whereas $[M–Br]^-$ or $[M–Br–xHBr]^-$ ions were produced in a lesser extent (data not shown). OH-PBDEs were ionised as $[M–H]^-$ ions and their subsequent debromination ions. Concerning sensitivity considerations, APCI gave slightly less sensitive detection limits than APPI for both families of compounds with detection limits ranging from 30 to 60 pg injected on the LC column (data not shown). This is in agreement with the instrument detection limits reported by Zhou *et al.*[38,40] who developed both LC/APCI and LC/APPI methods for BFR analysis on a triple quadrupole instrument. Mascolo *et al.* also compared ESI, APCI and APPI as ionisation sources for LC/MS PBDE analysis, and found that APPI gave the best signal intensities although APCI was less sensitive to ion suppression effects when analysing complex matrix samples.[39]

Therefore, APPI ionisation mode was chosen for its sensitivity and informative ionisation pattern which will be discussed later. Both the positive and negative APPI modes were tested. As expected, the positive mode yielded weak current ions, only for lowly brominated PBDEs, in good agreement with previous reports.[33,39] The negative ionisation mode showed a much better sensitivity and was thus selected.

APPI key parameters

APPI operating parameters were set up with emphasis on the dopant and the source operating temperatures known to represent key parameters for efficient and sensitive APPI-based methods. Three dopants (i.e. acetone, anisole and toluene) were tested and led to similar ionisation patterns and signal intensities. By contrast, the proportion of dopant added to the mobile phase was found to influence the signal intensity and should be kept above 5% (data not shown). These results are consistent with previously published works where 10 to 20% of toluene[35,36,39] or acetone[37,38] were used. The adjunction of the dopant directly into the LC mobile phases rather than by post-column addition provided better signal-to-noise (S/N) ratios, likely due to a more homogeneous distribution of the dopant when entering the atmospheric pressure ionisation chamber, in agreement with previous observations.[37,49]

Operating temperatures also greatly influenced the ionisation of PBDEs. After careful optimisation of both the nebulizer and capillary temperatures, results indicate that the capillary temperature should not be set above 250°C to achieve the best intensity for all diagnostic ions. Nebuliser temperature was found to have a qualitative influence on ionisation. High temperatures (from 300 to 350°C) promoted the formation of cleaved $[C_6Br_xH_{5-x}O]^-$ ions over $[M–Br]^-$ ions for PBDEs and to a lesser extent for OH-PBDEs (data not shown). As expected this trend was stronger for nona- and deca-BDEs, which are known to be thermolabile.[32] In the present work, heated transfer capillary and nebulizer temperatures were set at 250°C and 300°C, respectively.

APPI ionisation pattern

In order to select appropriate signals to work on, the ionisation patterns of the studied PBDEs and OH-PBDEs (Table 1) were examined. In previous works reporting the use of APPI-MS for the analysis of PBDEs, phenoxyde $[M–Br+O]^-$ ions were always observed, sometimes with additional debromination products.[33–39] In this study, using the IonMax APPI source and acetone as the doping agent, tetra- and penta-BDEs yielded $[M–Br+O]^-$ ions (data not shown) whereas $[M–Br]^-$ ions were predominantly observed for hepta- to nona-brominated congeners, as shown on the mass spectra presented in Fig. 2. For almost all compounds, the formation of $[C_6Br_xH_{5-x}O]^-$ bromophenoxide ions, resulting from the breakdown of the PBDE ethereal bond, was observed. Usually, such fragment ions are only observed for high molecular weight PBDEs, and can even represent the predominant ions in the case of deca-BDE[33,35,38,39] and nona-BDEs.[35] The differences observed in the present study may be attributed to the use of a different ionisation source, as it is now well known that the source configuration can play on the ionisation pattern of PBDEs.[36,49]

The main APPI-produced ions from OH-PBDEs are listed in Table 2. $[M–Br–HBr]^-$ were the major APPI-produced ions, except in the case of the two congeners hydroxylated at position 6 (namely 6-OH-BDE-47 and 6-OH-BDE-180) which yielded $[M–Br]^-$ ions as the base peak. However, in the case of hydroxylated tetra-BDEs, the intensities of signals arising from the deprotonated $[M–H]^-$ ions were more significant. Figure 3 represents the APPI-MS spectra of the four hydroxylated tetra-BDEs studied in this work. Each isomer shows a different and thus specific ionisation pattern. As already mentioned, 6-OH-BDE-47 was mainly ionised into the corresponding $[M–Br]^-$ ion, whereas 4'-OH-BDE-49 was mainly ionised by deprotonation, giving the corresponding $[M–H]^-$ ion. In the case of 5-OH-BDE-47, the $[M–H]^-$ ion intensity was ca. 60% and the $[M–H–HBr]^-$ ion represented the base peak, and 3-OH-BDE-47 predominantly led to the $[M–Br–HBr]^-$ ion.

Rapid Communications in Mass Spectrometry

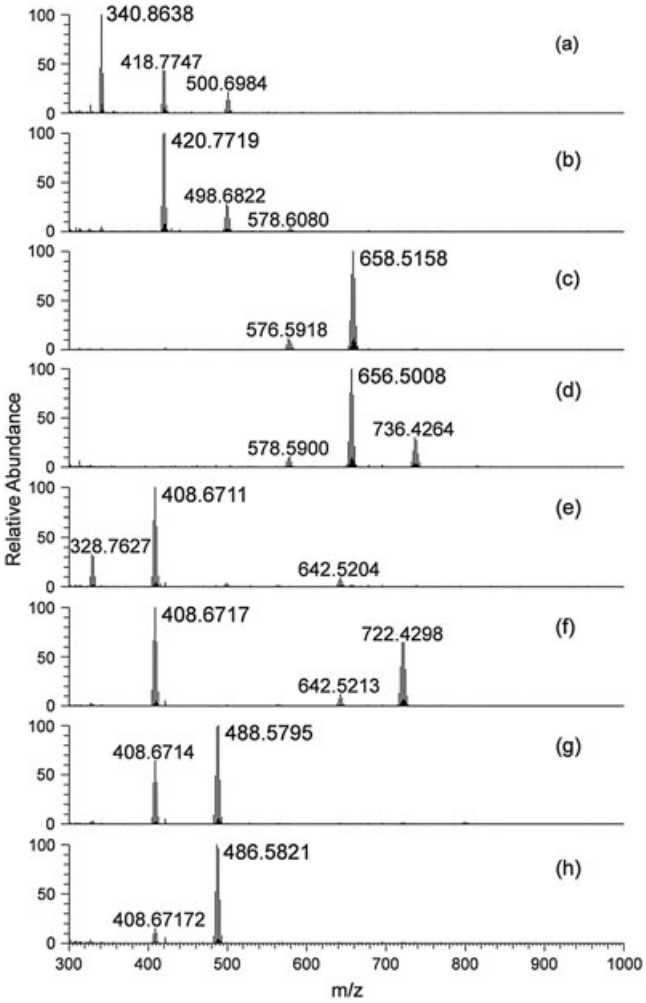

Figure 2. Negative APPI mass spectra of (a) 3-OH-BDE-47, (b) 3-OH-BDE-100, (c) 6-OH-BDE-180, (d) 4′OH-BDE-201, (e) BDE-184, (f) BDE-202, (g) BDE-207, and (h) BDE-209 obtained for the injection of 1 ng PBDE on-column.

In anticipation of our application to a metabolism study, the ionisation of other potential metabolites of PBDEs was also assessed, with particular emphasis on bromophenols and methoxylated PBDEs derived from BDE-47. Those compounds were ionisable by APPI, and yielded specific mass spectra. Bromophenols were ionised as $[M–H]^-$ ions, corresponding to the $[C_6Br_xH_{5-x}O]^-$ ions of PBDEs. As for PBDEs, APPI mass spectra of methoxylated tetra-BDEs derived from BDE-47 displayed $[M–Br]^-$ as major ions together with $[M–Br+O]^-$, $[M–Br–CH_3]^-$ ions and other fragment ions arising from subsequent losses of HBr as minor ions (data not shown).

Optimisation of LC separation

The LC separation of congeners of PBDEs and OH-PBDEs represents a real analytical challenge since PBDE mixtures usually contain several positional isomers. As indicated in Table 1, the logP values estimated for these compounds show that they are highly lipophilic and that, despite the adjunction of a hydroxyl group, the hydroxylated PBDE metabolites are only slightly less lipophilic than the parent compounds. It can be deduced from these observations, as well as from

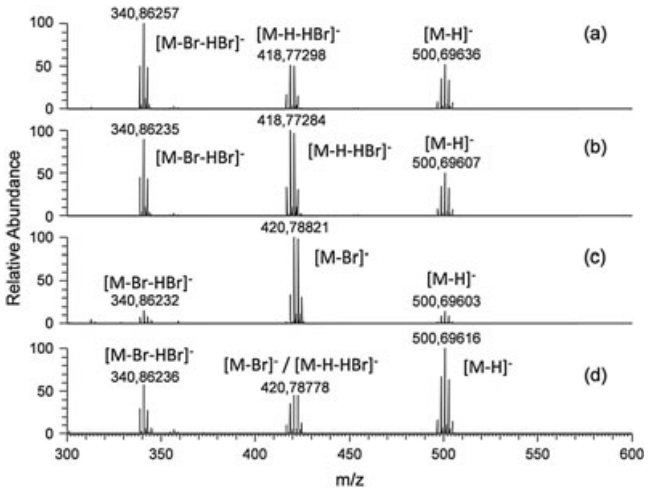

Figure 3. Negative APPI mass spectra of (a) 3-OH-BDE-47, (b) 5-OH-BDE-47, (c) 6-OH-BDE-47, and (d) 4′-OH-BDE-49.

Table 2. Main ions observed from APPI ionisation of OH-PBDEs

	$[M–H]^-$		$[M–Br]^-$		$[M–H–HBr]^-$		$[M–Br–HBr]^-$	
Congeners	calc. *m/z*,	Rel. Int %.	calc. *m/z*,	Rel. Int %.	calc. *m/z*,	Rel. Int %.	calc. *m/z*,	Rel. Int %.
3-OH-BDE-47	496.70285	55	418.79234	5	416.77669	50	338.86618	100
5-OH-BDE-47	496.70285	60	418.79234	3	416.77669	100	338.86618	90
6-OH-BDE-47	496.70285	20	418.79234	100	416.77669	15	338.86618	15
4′-OH-BDE-49	496.70285	100	418.79234	45	416.77669	45	338.86618	60
5′-OH-BDE-99	574.61337	10	496.70285	2	494.68720	40	416.77669	100
3-OH-BDE-100	574.61337	15	496.70285	4	494.68720	40	416.77669	100
3′-OH-BDE-154	652.52388	15	574.61337	10	572.59772	2	494.68720	100
6-OH-BDE-180	730.43439	5	652.52388	100	650.50823	0	572.59772	30
4-OH-BDE-187	730.43439	15	652.52388	70	650.50823	0	572.59772	100
4-OH-BDE-188	730.43439	5	652.52388	10	650.50823	0	572.59772	100
4′-OH-BDE-201	808.34490	2	730.43439	25	728.41874	0	650.50823	100

previously published works, that mixtures of PBDE and OH-PBDE congeners can be hardly separated within an acceptable run time using conventional C18-based reversed-phase chromatographic systems. This is particularly true for the separation of the three nona-BDE isomers and for the separation of BDE-209 and BDE-206.[25,35,38] Other selectivity factors such as π–π or dipole–dipole interactions have thus been considered to achieve the separation of halogenated aromatic compounds like PBDEs and OH-PBDEs. The use of ~2 µm particle size packing is known to provide narrower peaks, which is beneficial for both sensitivity and peak capacity. Besides, water/methanol mobile phases have been found to give better results than water/acetonitrile phases, for the LC/APPI-MS analysis of BFRs by several authors.[33,35–39] For all these reasons, a 1.9 µm particle size Hypersil Gold C18 column was compared to a 1.9 µm particle size Hypersil Gold pentafluorophenyl (PFP) column using water/methanol mobile phases containing 10% of acetone.

Based on the model mixture used in this work (see Table 1), the PFP column proved to be more efficient than the C18 column. In particular, the PFP column allowed a greater retention of the OH-PBDEs which were insufficiently resolved on the C18 column in the presence of acetone in the mobile phase. Besides, the PFP column also allowed a separation of BDE-206 to BDE-209 congeners within a shorter time than the C18 column. A typical chromatogram obtained from a model PBDE/OH-PBDE mixture using the PFP column and the optimised methanol/water/acetone gradient is shown in Fig. 4. The corresponding retention times of each compound are reported in Table 3. Although some of the brominated derivatives were not chromatographically separated (e.g. 5′-OH-BDE-99 and 6-OH-BDE-180), the detection of all the studied compounds was possible within ca. 20 min taking advantage of the specific detection channels of each compound. The presence of acetone in the LC mobile phase likely provided an increased selectivity and constituted a decisive parameter for the separation of BDE-206 and -209 (Fig. 4). Some trials carried out on both C18 and PFP columns without acetone supported this assumption. In a similar way, Abdallah *et al.* have developed a LC gradient providing a complete resolution of those two compounds, likely thanks to the presence of toluene in their mobile phase.[35]

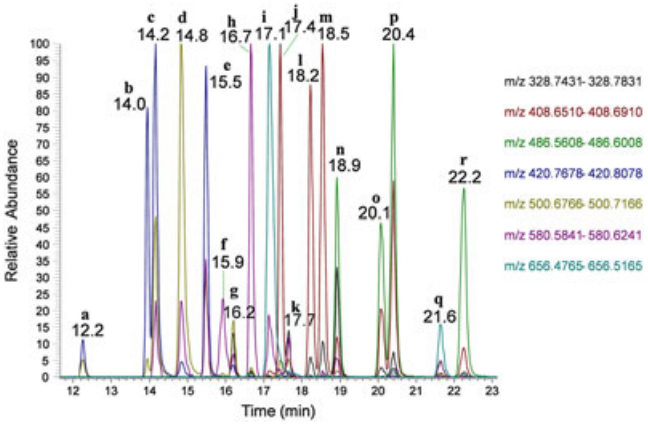

Figure 4. Separation of a PBDE/OH-PBDE mixture (50 pg on-column each) on a PFP column using optimised conditions: (a) 3-OH-BDE-47, (b) 6-OH-BDE-47, (c) 4′-OH-BDE-49 + 3-OH-BDE-100, (d) 3′-OH-BDE-154 + BDE-47, (e) 5′-OH-BDE-99 + 6-OH-BDE-180, (f) BDE-100, (g) BDE-154, (h) BDE-99, (i) 4′-OH-BDE-201, (j) BDE-184, (k) BDE-153, (l) BDE-202, (m) BDE-201, (n) BDE-204, (o) BDE-208, (p) BDE-207, (q) BDE-206, and (r) BDE-209.

HRMS method for the identification of PBDEs and their metabolites

Taking into account both the specific isotopic pattern and mass defects of polybrominated compounds, we took advantage of the high-resolution mass spectrometry capacities to develop a method allowing the tracking of both targeted known metabolites as well as unknown metabolites of PBDEs. In this context, the use of a high resolution power (i.e. R = 60 000) was found to be of particular interest in the case of hydroxylated PBDEs since it allowed discrimination of $[M–H]^-$ ions generated from a given OH-BDE and $[M–H–HBr]^-$ ions generated from an OH-BDE bearing one more bromine atom. An illustration is given in Fig. 5 representing the isotopic clusters of the $[M–H]^-$ ions of 3-OH-BDE-47 (hydroxylated tetra-BDE, Fig. 5(a)) and the $[M–H–HBr]^-$ ions of 5′-OH-BDE-99 (hydroxylated penta-BDE, Fig. 5(b)). Figures 5(c), 5(d) and 5(e) represent expanded views of the *m/z* 498.6972 ion ($^{79}Br_3{}^{81}Br$ isotopomer, theoretical *m/z*: 498.6997) and the *m/z* 498.6803 ion ($^{79}Br_2{}^{81}Br_2$ isotopomer, theoretical *m/z*: 498.6820) ions observed for partially coeluting 6-OH-BDE-47 and 3-OH-BDE-100, for resolution powers of 15 000, 30 000 and 60 000, respectively. They indicate that a minimum resolution of 60 000 must be used to allow the complete resolution of these two signals. However, it is important to note that OH-PBDEs ionised as $[M–Br]^-$ ions still display isotopic clusters rigorously identical to that of $[M–H]^-$ ions produced from OH-PBDEs bearing one less bromine atom.

In the case of non-hydroxylated PBDEs, the diagnostic ions monitored were $[M–Br+O]^-$ or $[M–Br+O–HBr]^-$ ions for both tetra- and penta-BDEs. For congeners ranging from hexa- to deca-BDEs, the bromophenoxide ions were monitored as indicated in Table 3. However, the use of the HRMS full mass spectra acquisition mode also allowed to check for the presence of $[M–Br]^-$ ions, which are more indicative of the number of bromine atoms of a given analyte. Moreover, this acquisition mode enabled to take advantage of the specific behaviour of each hydroxylated tetra-PBDE, illustrated in Fig. 3 and Table 2, and provided an access to additional signals for unambiguous confirmation of the identities of the compounds and for the characterization of untargeted PBDE metabolites (isotopic cluster, mass defect).

Although not developed for quantification purposes, the performances of our LC/APPI-HRMS method (selectivity, sensitivity) were compared to a reference method, i.e. an MRM method elaborated on a triple quadrupole instrument on the same set of reference PBDEs and OH-PBDEs. For the setup of the MRM method, each transition was selected taking into account both sensitivity and specificity. Transitions used for MRM detection of PBDEs and OH-PBDEs are listed in Table 3. Owing to their structure, PBDEs and OH-PBDEs hardly decompose when submitted to collision-induced dissociation (CID) processes. The main decomposition pathway leads to the non-specific Br^- ions as reported in several studies dealing with analysis of PBDEs by LC/MS/MS.[36–39] Due to the lack of other specific fragmentations, pseudo-transitions using the same ion as both precursor and product ion were

Table 3. MRM transitions and exact masses used for identification of PBDEs and OH-PBDEs

	Precursor ion (M′)	Exact mass (*m/z*)	Precursor (*m/z*)	Fragment ions	Fragment (*m/z*)	Compounds monitored	Retention time (min)	HRMS LOD (pg)	MS/MS LOD (pg)
Tetra-BDEs	$[M–H–Br_2+O]^-$	340.8623 / 338.8568	340.9	$[Br]^-/M$	80.9/340.9	BDE-47	14.8	3.87	
	$[M–H–Br_2+O_2]^-$	356.8570 / 354.8591							
Penta-BDEs	$[M–Br+O]^-$	500.6964 / 498.6987	498.8	$[M'–HBr]^-/M$	418.8/498.8	BDE-99	16.7	4.58	
	$[M–H–Br_2+O_2]^-$	436.7655 / 434.7675				BDE-100	15.9	1.41	
Hexa-BDEs	$[C_6Br_3H_2O]^-$	330.7601 / 328.7624	328.8	$[Br]^-/M$	80.9/328.8	BDE-153	17.7	0.81	3.80
						BDE-154	16.2	1.93	3.27
Hepta- and octa-BDEs	$[C_6Br_4H_1O]^-$	408.6705 / 406.6726	408.7	$[Br]^-/M$	80.9 408.7	BDE-183	17.6	0.64	
						BDE-184	17.4	0.09	0.39
						BDE-201	18.5	0.18	0.42
						BDE-202	18.2	0.65	0.70
Octa-, nona-, and deca-BDEs	$[C_6Br_5HO]^-$	488.5785 / 486.5804	488.7	$[Br]^-/M$	80.9/488.7	BDE-204	18.9	0.34	0.40
						BDE-206	21.6	2.30	5.95
						BDE-207	20.4	1.21	0.19
						BDE-208	20.1	1.09	0.78
						BDE-209	22.2	0.25	0.18
OH-Tetra-BDEs	$[M–HBr–Br]^-$	340.8623 / 338.8568	340.9	$[Br]^-/M$	80.9/340.9	3-OH-BDE-47	12.2	1.03	25.10
	$[M–HBr]^-$	418.7730 / 420.7708	420.9	$[Br]^-/M$	80.9/420.9	5-OH-BDE-47	14.1		
	$[M–Br]^-$	420.7877 / 422.7858	420.9	$[Br]^-/M$	80.9/420.9	6-OH-BDE-47	14.0	1.78	20.90
	$[M–H]^-$	500.6964 / 498.6987	498.8	$[M'–HBr]^-/M$	418.8/498.8	4′-OH-BDE-49	14.2	1.52	24.36
OH-Penta-BDEs	$[M–H–Br_2]^-$	418.7730 / 420.7708	420.9	$[Br]^-/M$	80.9/420.9	5′-OH-BDE-99	15.5	0.67	15.89
	$[M–H–Br_2]^-$	418.7730 / 420.7708	420.9	$[Br]^-/M$	80.9/420.9	3-OH-BDE-100	14.2	0.87	18.46
OH-Hexa-BDE	$[M–Br_3]^-$	418.7730 / 420.7708	420.9	$[Br]^-/M$	80.9/420.9	3′-OH-BDE-154	14.8	0.92	18.71
OH-Hepta-BDE	$[M–Br]^-$	658.5146 / 656.5170	658.7	$[M'–HBr]^-/M$	578.8/658.7	6-OH-BDE-180	15.5	0.89	18.61
OH-Octa-BDE	$[M–Br_2]^-$	658.5146 / 656.5170	658.7	$[M'–HBr]^-/M$	578.8/658.7	4′-OH-BDE-201	16.7	2.43	26.29

Rapid Communications in Mass Spectrometry

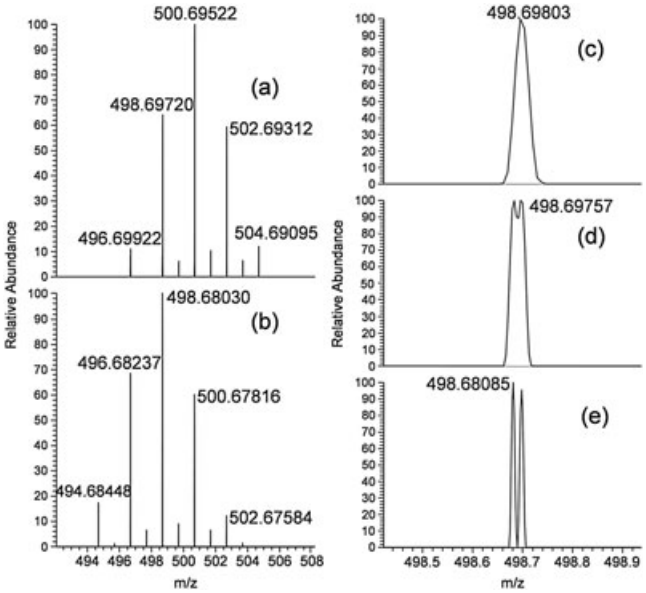

Figure 5. Isotopic clusters of (a) the $[M–H]^-$ ions of 3-OH-BDE-47 and (b) the $[M–H–HBr]^-$ ions of 5′-OH-BDE-99. Expanded view of the $m/z = 498.7$ ions of coeluting 6-OH-BDE-47 and 3-OH-BDE-100 for resolution powers of (c) 15 000, (d) 30 000, and (e) 60 000.

also scheduled. This approach has already been used for the analysis of compounds without abundant fragmentation and has proved to provide cleaner chromatograms.[50,51]

The sensitivities of the two methods were then estimated in terms of limits of detection (LODs, as defined by the injected amount giving a S/N ratio of 3). Results presented in Table 3 indicate that the two methods showed similar performances for PBDEs, with LODs ranging from 0.09 to 4.58 pg injected on-column for LC/HRMS and from 0.18 to 5.95 pg for LC/MS/MS. These values were in the same range as or better than those reported for other published methods based on MRM acquisition on triple quadrupole instruments, with LODs ranging from 0.11 to 100 pg.[38,39] Surprisingly, due to higher chemical noise, detection limits for OH-PBDEs were higher when using MRM compared to HRMS, whereas they were in the same range for PBDEs (see Table 3). It should also be noted that the low resolution of quadrupole mass analyzers did not allow the differentiation of $[M–H]^-$ ions from OH-PBDEs bearing x bromine atoms and $[M–H–HBr]^-$ ions from OH-PBDEs bearing $(x+1)$ bromine atoms.

To our knowledge, this is the first time that OH-PBDEs have been analysed by LC/APPI-MS. This ionisation mode proved to be well adapted for those compounds, providing detection limits in the same range as previously published works reporting LODs in the pg range using ESI or APCI,[41,43,44,52] with the ability to track not only targeted PBDE metabolites (especially OH-PBDEs), but also untargeted metabolites by tracking specific signals of brominated species.

Application to the identification of BDE-47 metabolites produced by primary cultures of human hepatocytes

The developed method was applied to the identification of metabolites produced *in vitro* with BDE-47, one of the major PBDE congeners detected in human samples.[7,8] [^{14}C]-BDE-47 was incubated for 24 h with human primary cultures of hepatocytes. From the three separate experiments, the average rate of metabolism of BDE-47 was 37 ± 6 and $29 \pm 8\%$ for incubations carried out with 1 and 5 μM of BDE-47, respectively. After a cleanup step, culture media were analysed using our developed LC/APPI-HRMS method. Despite an important inter-individual variability, the obtained metabolic profiles were qualitatively similar. A typical chromatogram resulting from these analyses is presented in Fig. 6. It exhibits several peaks detected in particular on the *m/z* 418.775 (Fig. 6(A)), *m/z* 420.790 (Fig. 6(B)) and *m/z* 500.699 (Fig. 6(C)) channels used (precision window width: ±10 ppm) for APPI detection of hydroxylated PBDEs as previously discussed. The whole set of metabolites identified in this study is listed in Table 4, presenting the diagnostic ions used for metabolite characterisation, their proposed molecular formula, and the mass deviation (Δ ppm) with the corresponding calculated accurate mass. The presence of BDE-47 (14.9 min) and hydroxylated metabolites could be evidenced on the basis of their respective

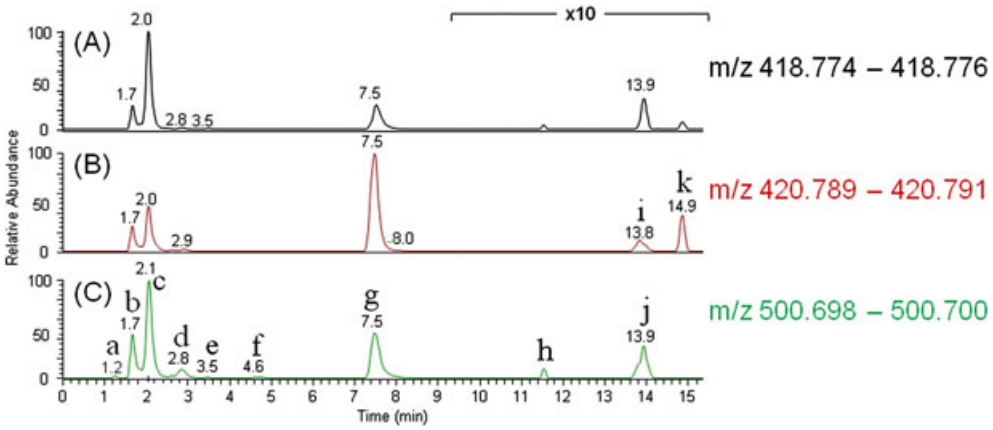

Figure 6. LC/MS chromatogram of a sample culture media analysed on a PFP column, using high-resolution acquisition channels (A) *m/z* 418.774–418.776, (B) *m/z* 420.789–420.791, and (C) *m/z* 500.698–500.700. Identified metabolites are marked as follows: (a) tetra-BDE-dihydrodiol sulfate, (b, c) tetra-BDE-dihydrodiol glucuronide, (d) dihydroxy-tetra-BDE sulfate, (e) monohydroxy-tetra-BDE glucuronide, (f) dihydroxy-tetra-BDE glucuronide, (g) tetra-BDE-dihydrodiol, (h) OH-tetra-BDE, (i) 6-OH-BDE-47, (j) 5-OH-BDE-47, and (k) BDE-47.

Rapid Communications in Mass Spectrometry

Table 4. BDE-47 metabolites identified from the incubation of BDE-47 with human hepatocytes. (* correspond to minor metabolites; n.d.: not detected)

Metabolite	Diagnostic ion molecular formula	Retention time (min)	Ionisation technique	Diagnostic ions (*m/z*) (most abundant isotopomer) Observed *m/z*	Calculated *m/z*	Δ ppm
Tetra-BDE-dihydrodiol glucuronide	$C_{18}H_{15}O_9{}^{79}Br_2{}^{81}Br_2$	1.7	ESI	694.7397	694.7414	−2.45
	$C_{12}H_5O_2{}^{79}Br_2{}^{81}Br_2$		APPI	500.6993	500.6988	1.00
	$C_{18}H_{15}O_9{}^{79}Br_2{}^{81}Br_2$	2.1	ESI	694.7396	694.7414	−2.59
	$C_{12}H_5O_2{}^{79}Br_2{}^{81}Br_2$		APPI	500.6989	500.6988	0.20
Mono-hydroxy-tetra-BDE glucuronide	$C_{18}H_{13}O_8{}^{79}Br_2{}^{81}Br_2$	2.6	ESI	676.7292	676.7308	−2.36
	$C_{12}H_5O_2{}^{79}Br_2{}^{81}Br_2$		APPI	500.6992	500.6988	0.80
	$C_{18}H_{13}O_8{}^{79}Br_2{}^{81}Br_2$	3.3*	ESI	676.7288	676.7308	−2.96
			APPI	n.d.		
	$C_{18}H_{13}O_8{}^{79}Br_2{}^{81}Br_2$	3.5	ESI	676.7287	676.7308	−3.04
	$C_{12}H_5O_2{}^{79}Br_2{}^{81}Br_2$		APPI	500.6988	500.6988	0.04
Dihydroxy-tetra-BDE glucuronide	$C_{18}H_{13}O_9{}^{79}Br_2{}^{81}Br_2$	3.9*	ESI	692.7234	692.7258	−3.37
			APPI	n.d.		
	$C_{18}H_{13}O_9{}^{79}Br_2{}^{81}Br_2$	4.6	ESI	692.7238	692.7258	−2.79
	$C_{12}H_5O_3{}^{79}Br_2{}^{81}Br_2$		APPI	516.6942	516.6937	0.97
Tetra-BDE-dihydrodiol sulfate	$C_{12}H_7O_6S{}^{79}Br_2{}^{81}Br_2$	1.2	ESI	598.6643	598.6661	−2.61
	$C_{12}H_5O_2{}^{79}Br_2{}^{81}Br_2$		APPI	500.6993	500.6988	1.00
	$C_{12}H_7O_6S{}^{79}Br_2{}^{81}Br_2$	4.1*	ESI	598.6667	598.6661	0.78
			APPI	n.d.		
	$C_{12}H_7O_6S{}^{79}Br_2{}^{81}Br_2$	4.3*	ESI	598.6667	598.6661	0.78
			APPI	n.d.		
Mono-hydroxy-tetra-BDE sulfate	$C_{12}H_5O_5S{}^{79}Br_2{}^{81}Br_2$	2.4	ESI	580.6541	580.6556	−2.17
	$C_{12}H_4O_2{}^{79}Br_2{}^{81}Br$		APPI	418.7742	418.7746	−0.95
Di-hydroxy-tetra-BDE sulfate	$C_{12}H_5O_6S{}^{79}Br_2{}^{81}Br_2$	2.8	ESI	596.6492	596.6505	−1.88
	$C_{12}H_5O_3{}^{79}Br_2{}^{81}Br_2$		APPI	516.6942	516.6937	0.97
Tetra-BDE-dihydrodiol	$C_{12}H_7O_3{}^{79}Br_2{}^{81}Br_2$	7.5	APPI	518.7079	518.7093	−2.70
	$C_{12}H_5O_2{}^{79}Br_2{}^{81}Br_2$			500.6974	500.6988	−2.80
OH-tetra-BDE	$C_{12}H_5O_2{}^{79}Br_2{}^{81}Br_2$	11.5	APPI	500.6993	500.6988	0.80
	$C_{12}H_4O_2{}^{79}Br_2{}^{81}Br$			418.7751	418.7746	1.19
6-OH-BDE-47	$C_{12}H_6O_2{}^{79}Br_2{}^{81}Br$	13.8	APPI	420.7903	420.7892	2.61
5-OH-BDE-47	$C_{12}H_4O_2{}^{79}Br_2{}^{81}Br$	13.9	APPI	418.7746	418.7746	−0.01
BDE-47	$C_{12}H_5O_2{}^{79}Br^{81}Br$	14.9	APPI	340.8639	340.8630	2.63

mass spectra and retention times, by comparison with the authentic standards. Two hydroxylated metabolites were eluted respectively at 13.8 and 13.9 min. The hydroxylated metabolite that eluted at 13.8 min (Fig. 6) displayed the same mass spectrum and retention time as standard 6-OH-BDE-47, and was mainly evidenced by the occurrence of its corresponding $[M–Br]^-$ fragment ion, detected at *m/z* 420.7903 (most abundant isotopomer). Therefore, this metabolite was identified as 6-OH-BDE-47. The retention time and the ionisation pattern of the hydroxylated metabolite eluted at 13.9 min (Fig. 6) fitted the chromatographic and mass spectral data of 5-OH-BDE-47, based on the comparison with the authentic standard. A third hydroxylated tetra-BDE was observed with a shorter retention time (11.5 min) than that of 3-OH-BDE-47 (12.2 min). Both 6-OH-BDE-47 and 5-OH-BDE-47 result from the direct hydroxylation of BDE-47. Conversely, the third hydroxylated tetra-BDE could not correspond to a hydroxy-BDE-47 since it did not fit LC and MS characteristics of any of the three possible hydroxylated forms of BDE-47. We assumed that the formation of this metabolite resulted from a cytochrome P-450 dependent oxidation, followed by a 1,2-shift involving the migration of a bromine atom on one of the proximal carbon atoms of the aromatic ring, as described by Marsh *et al.*[53]

The monitoring of specific masses of OH-PBDEs also indicated the presence of another metabolite, more polar than the hydroxylated metabolites, which eluted at 7.5 min (Fig. 6) and actually represented the major metabolite. The highly informative APPI mass spectrum of this metabolite is shown in Fig. 7. This spectrum displays an $[M–H]^-$ ion centred on *m/z* 518.708, together with $[M–Cl]^-$ chloride adduct ions (Fig. 7). Although the occurrence of chloride adduct ions is commonly observed on atmospheric pressure ionisation mass spectra of compounds such as amines,[54] their presence on the APPI-MS spectrum of the BDE-47 metabolites was unexpected since they have not been observed on any APPI spectrum of PBDE derivatives previously. On the spectrum presented in Fig. 7, minor fragment ions are observed at *m/z* 500.697 and

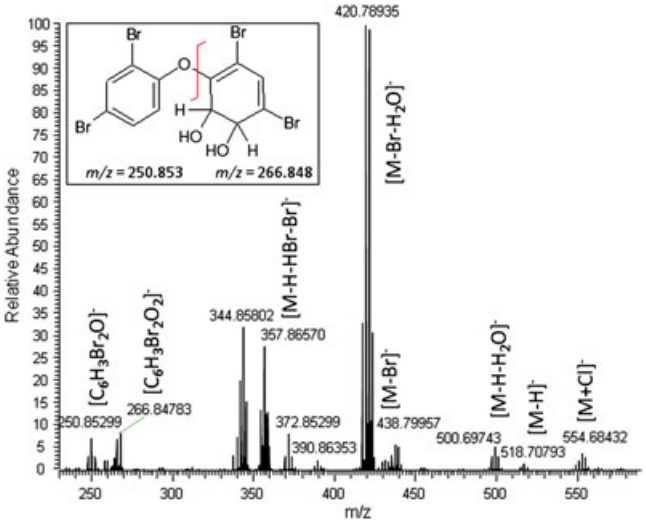

Figure 7. Negative APPI mass spectrum and proposed structure for the BDE-47 metabolite eluted at retention time 7.6 min obtained from the injection of a culture media sample in LC/APPI-HRMS.

438.800, resulting from the loss of H_2O and Br, respectively, whereas the major fragment ion at *m/z* 420.789 can be attributed to a $[M–Br–H_2O]^-$ species. This spectrum also exhibits characteristic complementary fragment ions bearing two bromine atoms each, detected at *m/z* 250.853 and 266.848, whose origin is attributed to the breakage of the ether bond of the metabolite according to the scheme proposed in Fig. 7. These data allowed the identification of this metabolite as dihydrodiol-BDE-47. Taking into account the structure of BDE-47 (2,2′,4,4′-tetrabromodiphenylether), the only possible structure should correspond to 5,6-dihydrodiol-BDE-47. To our knowledge, this is the first time that this kind of metabolite has been observed for PBDEs, despite the known formation of hydroxylated PBDEs,[30,31] dihydroxylated PBDEs,[30] bromophenols,[30,31] and debromination products.[25,53] The formation of such dihydrodiol metabolites has already been described for other aromatic contaminants such as polycyclic aromatic hydrocarbons.[55] Taking into account the enzymatic capability of human hepatocytes, the formation of the dihydrodiol metabolite can be explained considering an initial oxidation of BDE-47 by P450 cytochromes to give an epoxide intermediate (not identified), which is then hydrolysed into a dihydrodiol derivative by epoxide hydrolases.

In addition to those BDE-47 hydroxylated derivatives, other metabolites, displaying very short retention times (Fig. 6), yielded mass spectra which also exhibited the specific ions of monohydroxylated or dihydroxylated tetra-BDEs. The complete characterisation of these metabolites needed an additional LC/HRMS analysis carried out using electrospray in the negative mode for ionisation. This analysis demonstrated that these polar compounds were conjugated metabolites of hydroxylated forms of BDE-47, which are listed in Table 4. Due to the very low amounts available, MS/MS experiments could not be carried out on these metabolites. However, these metabolites could be identified based the characteristic isotopic mass clusters of their $[M–H]^-$ ions, their exact mass measurement giving access to their elemental composition, and the occurrence of some characteristic in-source fragment ions. In particular, in-source fragment ions corresponding to the characteristic loss of 176.0318 a.m.u. for glucuronide conjugates were observed for the metabolites eluted at 2.1, 2.6, 3.5 and 4.6 min (see Table 4), respectively. Similarly, diagnostic in-source fragment ions corresponding to the loss of SO_3 (79.9568 a.m.u.) were also observed for metabolites eluted at 2.4 and 2.8 min (Table 4). All together, twelve conjugated metabolites were evidenced, out of which eight were also detected using APPI (see Table 4). Glucuronide and sulfate conjugates of dibromophenol have already been observed in the urine of rats exposed to BDE-47,[56] as well as glucuronide and glutathionyl conjugates of BDE-99 in rats.[57] However, to our knowledge, this is the first time that phase II metabolites of intact BDE-47 have been characterised. Furthermore, despite the known formation of hydroxylated[30,31] and debrominated[53] PBDEs, this study provides for the first time evidence for the formation of a dihydrodiol metabolite for BDE-47, a structure never characterised before for any PBDE.

In the absence of any standard compound available for dihydrodiol-BDE-47 as well as for conjugated metabolites, no precise quantitative information could be used from the LC/HRMS analyses. However, radio-HPLC analyses allowed to estimate the relative amounts of the different metabolites. The major dihydrodiol metabolite accounted for $47 \pm 11\%$ of the total metabolites, while hydroxylated BDE-47 forms accounted for only $1 \pm 2\%$ of the total metabolites in 5 μM incubations. All together, conjugated metabolites represented $52 \pm 10\%$ of the metabolites, based on radioactivity measurements, showing that BDE-47 undergoes both phase I and phase II metabolism in human hepatocytes.

CONCLUSIONS

This work aimed at developing a LC/MS method for the simultaneous analysis of PBDEs and OH-PBDEs in the framework of metabolism studies. Three atmospheric pressure ionisation modes were compared and APPI was selected for its sensitivity. In addition, APPI was shown to provide key information for the identification of these compounds, including specific ionisation patterns for different isomeric hydroxylated PBDEs. The optimisation of APPI parameters showed that the dopant is not the only important parameter to take into account for PBDEs ionisation, and the operating temperatures should also be carefully adjusted to maximise the sensitivity. The use of an Orbitrap mass spectrometer highlighted the great usefulness of high resolution power (60 000) for the differentiation of diagnostic ions which may overlap, due to the isotopic patterns of these polybrominated compounds. The high-resolution full-scan acquisition mode was an additional critical advantage of the method, since it provided access not only to targeted compounds, but also to non-targeted metabolites, without any loss of sensitivity.

The method was applied to the identification of BDE-47 metabolites produced by human hepatocytes, and proved to be efficient for the monitoring of the parent compounds as well as for the identification of the main known PBDEs metabolites, namely hydroxylated PBDEs. A novel metabolite, namely BDE-47 dihydrodiol, was characterised in this work. The APPI method can also be used to monitor conjugated metabolites, even though ESI was required to give access to their quasi-molecular ions for unambiguous

Rapid Communications in Mass Spectrometry

identification purposes. The developed method is suitable for the simultaneous identification and/or monitoring of PBDEs and their metabolites. Applications to other metabolism studies, and to the analysis of biological samples such as serum or maternal milk, can now be foreseen.

Acknowledgements

The authors would like to thank the Agence Nationale de la Sécurité Environementale et Sanitaire (ANSES) for grant AFSSA EST-046 and for CM PhD fellowship (AFSSET-2007-CRD-33). Anne Riu is thanked for helpful assistance and discussions on *in vitro* experiments. Kind assistance provided by Emilien Jamin on the Orbitrap mass spectrometer is also gratefully acknowledged.

REFERENCES

[1] UNEP. Stockholm Convention on Persistent Organic Pollutants, **2001**.
[2] UNEP, UNEP/POPS/COP.4/SC-4/14 – Listing of hexabromodiphenyl ether and heptabromodiphenyl ether, **2009**.
[3] UNEP, UNEP/POPS/COP.4/SC-4/18 – Listing of tetrabromodiphenyl ether and pentabromodiphenyl ether, **2009**.
[4] R. J. Law, D. Herzke, S. Harrad, S. Morris, P. Bersuder, C. R. Allchin. Levels and trends of HBCD and BDEs in the European and Asian environments, with some information for other BFRs. *Chemosphere* **2008**, *73*, 223.
[5] R. C. Hale, M. J. La Guardia, E. Harvey, M. O. Gaylor, T. M. Mainor. Brominated flame retardant concentrations and trends in abiotic media. *Chemosphere* **2006**, *64*, 181.
[6] C. A. de Wit, D. Herzke, K. Vorkamp. Brominated flame retardants in the Arctic environment - trends and new candidates. *Sci. Total Environ.* **2010**, *408*, 2885.
[7] J. P. Antignac, R. Cariou, D. Zalko, A. Berrebi, J. P. Cravedi, D. Maume, P. Marchand, F. Monteau, A. Riu, F. Andre, B. Le Bizec. Exposure assessment of French women and their newborn to brominated flame retardants: Determination of tri- to deca- polybromodiphenylethers (PBDE) in maternal adipose tissue, serum, breast milk and cord serum. *Environ. Pollut.* **2009**, *157*, 164.
[8] M. Frederiksen, K. Vorkamp, M. Thomsen, L. E. Knudsen. Human internal and external exposure to PBDEs – A review of levels and sources. *Int. J. Hyg. Environ. Health* **2009**, *212*, 109.
[9] I. Meerts, R. J. Letcher, S. Hoving, G. Marsh, A. Bergman, J. G. Lemmen, B. van der Burg, A. Brouwer. In vitro estrogenicity of polybrominated diphenyl ethers, hydroxylated PBDEs, and polybrominated bisphenol A compounds. *Environ. Health Perspect.* **2001**, *109*, 399.
[10] R. Canton, J. Sanderson, R. Letcher, A. Bergman, M. van den Berg. Inhibition and induction of aromatase (CYP19) activity by brominated flame retardants in H295R human adrenocortical carcinoma cells. *Toxicol. Sci.* **2005**, *88*, 447.
[11] P. Eriksson, E. Jakobsson, A. Fredriksson. Brominated flame retardants: A novel class of developmental neurotoxicants in our environment? *Environ. Health Perspect.* **2001**, *109*, 903.
[12] D. C. Rice, E. A. Reeve, A. Herlihy, R. Thomas Zoeller, W. Douglas Thompson, V. P. Markowski. Developmental delays and locomotor activity in the C57BL6/J mouse following neonatal exposure to the fully-brominated PBDE, decabromodiphenyl ether. *Neurotoxicol. Teratol.* **2007**, *29*, 511.
[13] T. Zhou, D. G. Ross, M. J. DeVito, K. M. Crofton. Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol. Sci.* **2001**, *61*, 76.
[14] S. N. Kuriyama, A. Wanner, A. A. Fidalgo-Neto, C. E. Talsness, W. Koerner, I. Chahoud. Developmental exposure to low-dose PBDE-99: Tissue distribution and thyroid hormone levels. *Toxicology* **2007**, *242*, 80.
[15] L. T. M. Van der Ven, T. van de Kuil, A. Verhoef, P. E. G. Leonards, W. Slob, R. F. Canton, S. Germer, T. Hamers, T. J. Visser, S. Litens, H. Hakansson, Y. Fery, D. Schrenk, M. van den Berg, A. H. Piersma, J. G. Vos. A 28-day oral dose toxicity study enhanced to detect endocrine effects of a purified technical pentabromodiphenyl ether (pentaBDE) mixture in Wistar rats. *Toxicology* **2008**, *245*, 109.
[16] T. E. Stoker, S. C. Laws, K. M. Crofton, J. M. Hedge, J. M. Ferrell, R. L. Cooper. Assessment of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture, in the EDSP male and female pubertal protocols. *Toxicol. Sci.* **2004**, *78*, 144.
[17] I. Meerts, J. J. van Zanden, E. A. C. Luijks, I. van Leeuwen-Bol, G. Marsh, E. Jakobsson, A. Bergman, A. Brouwer. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro. *Toxicol. Sci.* **2000**, *56*, 95.
[18] G. R. Marchesini, A. Meimaridou, W. Haasnoot, E. Meulenberg, F. Albertus, M. Mizuguchi, M. Takeuchi, H. Irth, A. J. Murk. Biosensor discovery of thyroxine transport disrupting chemicals. *Toxicol. Appl. Pharmacol.* **2008**, *232*, 150.
[19] H. Kojima, S. Takeuchi, N. Uramaru, K. Sugihara, T. Yoshida, S. Kitamura. Nuclear hormone receptor activity of polybrominated diphenyl ethers and their hydroxylated and methoxylated metabolites in transactivation assays using Chinese hamster ovary cells. *Environ. Health Perspect.* **2009**, *117*, 1210.
[20] S. Lacorte, M. G. Ikonomou. Occurrence and congener specific profiles of polybrominated diphenyl ethers and their hydroxylated and methoxylated derivatives in breast milk from Catalonia. *Chemosphere* **2009**, *74*, 412.
[21] X. H. Qiu, R. M. Bigsby, R. A. Hites. Hydroxylated metabolites of polybrominated diphenyl ethers in human blood samples from the United States. *Environ. Health Perspect.* **2009**, *117*, 93.
[22] M. Athanasiadou, S. N. Cuadra, G. Marsh, A. Bergman, K. Jakobsson. Polybrominated diphenyl ethers (PBDEs) and bioaccumulative hydroxylated PBDE metabolites in young humans from Managua, Nicaragua. *Environ. Health Perspect.* **2008**, *116*, 400.
[23] J. Eriksson, N. Green, G. Marsh, A. Bergman. Photochemical decomposition of 15 polybrominated diphenyl ether congeners in methanol/water. *Environ. Sci. Technol.* **2004**, *38*, 3119.
[24] I. Watanabe, R. Tatsukawa. Formation of brominated dibenzofurans from the photolysis of flame-retardant decabromobiphenyl ether in hexane solution by uv and sun light. *Bull. Environ. Contam. Toxicol.* **1987**, *39*, 953.
[25] A. Riu, J. P. Cravedi, L. Debrauwer, A. Garcia, C. Canlet, I. Jouanin, D. Zalko. Disposition and metabolic profiling of [C-14]-decabromodiphenyl ether in pregnant Wistar rats. *Environ. Int.* **2008**, *34*, 318.
[26] D. F. Staskal, H. Hakk, D. Bauer, J. J. Diliberto, L. S. Birnbaum. Toxicokinetics of polybrominated diphenyl ether congeners 47, 99, 100, and 153 in mice. *Toxicol. Sci.* **2006**, *94*, 28.
[27] C. Munschy, K. Heas-Moisan, C. Tixier, N. Olivier, O. Gastineau, N. Le Bayon, V. Buchet. Dietary exposure of juvenile common sole (*Solea solea* L.) to polybrominated diphenyl ethers (PBDEs): Part 1. Bioaccumulation and elimination kinetics of individual congeners and their debrominated metabolites. *Environ. Pollut.* **2011**, *159*, 229.
[28] H. Stapleton, B. Brazil, R. Holbrook, C. Mitchelmore, R. Benedict, A. Konstantinov, D. Potter. *In vivo* and *in vitro* debromination of decabromodiphenyl ether (BDE 209) by juvenile rainbow trout and common carp. *Environ. Sci. Technol.* **2006**, *40*, 4653.

[29] S. W. Cheng, K. Randall, A. T. Kotchevar. *In vitro* metabolism studies of polybrominated diphenyl ethers using rat and human liver microsomes. *Am. J. Biochem. Biotechol.* **2008**, *4*, 295.
[30] S. J. Lupton, B. P. McGarrigle, J. R. Olson, T. D. Wood, D. S. Aga. Human liver microsome-mediated metabolism of brominated diphenyl ethers 47, 99, and 153 and identification of their major metabolites. *Chem. Res. Toxicol.* **2009**, *22*, 1802.
[31] H. M. Stapleton, S. M. Kelly, R. Pei, R. J. Letcher, C. Gunsch. Metabolism of polybrominated diphenyl ethers (PBDEs) by human hepatocytes *in vitro*. *Environ. Health Perspect.* **2009**, *117*, 197.
[32] A. P. Vonderheide. A review of the challenges in the chemical analysis of the polybrominated diphenyl ethers. *Microchem. J.* **2009**, *92*, 49.
[33] L. Debrauwer, A. Riu, M. Jouahri, E. Rathahao, I. Jouanin, J. P. Antignac, R. Cariou, B. Le Bizec, D. Zalko. Probing new approaches using atmospheric pressure photo ionization for the analysis of brominated flame retardants and their related degradation products by liquid chromatography-mass spectrometry. *J. Chromatogr. A* **2005**, *1082*, 98.
[34] A. Riu, D. Zalko, L. Debrauwer. Study of polybrominated diphenyl ethers using both positive and negative atmospheric pressure photoionization and tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 2133.
[35] M. A. Abdallah, S. Harrad, A. Covaci. Isotope dilution method for determination of polybrominated diphenyl ethers using liquid chromatography coupled to negative ionization atmospheric pressure photoionization tandem mass spectrometry: validation and application to house dust. *Anal. Chem.* **2009**, *81*, 7460.
[36] A. F. Lagalante, T. D. Oswald. Analysis of polybrominated diphenyl ethers (PBDEs) by liquid chromatography with negative-ion atmospheric pressure photoionization tandem mass spectrometry (LC/NI-APPI/MS/MS): application to house dust. *Anal. Bioanal. Chem.* **2008**, *391*, 2249.
[37] A. Bacaloni, L. Callipo, E. Corradini, P. Giansanti, R. Gubbiotti, R. Samperi, A. Lagana. Liquid chromatography-negative ion atmospheric pressure photoionization tandem mass spectrometry for the determination of brominated flame retardants in environmental water and industrial effluents. *J. Chromatogr. A* **2009**, *1216*, 6400.
[38] N. Zhou, E. J. Reiner, C. Marvin, T. Kolic, N. Riddell, P. Helm, F. Dorman, M. Misselwitz, I. D. Brindle. Liquid chromatography-atmospheric pressure photoionization tandem mass spectrometry for analysis of 36 halogenated flame retardants in fish. *J. Chromatogr. A* **2010**, *1217*, 633.
[39] G. Mascolo, V. Locaputo, G. Mininni. New perspective on the determination of flame retardants in sewage sludge by using ultrahigh pressure liquid chromatography-tandem mass spectrometry with different ion sources. *J. Chromatogr. A* **2010**, *1217*, 4601.
[40] N. Zhou, E.J. Reiner, C. Marvin, P. Helm, N. Riddell, F. Dorman, M. Misselwitz, L. Shen, P. Crozier, K. MacPherson, I.D. Brindle. Development of liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry for analysis of halogenated flame retardants in wastewater. *Anal. Bioanal. Chem.* **2010**, *396*, 1311.
[41] S. Mas, O. Jauregui, F. Rubio, A. de Juan, R. Tauler, S. Lacorte. Comprehensive liquid chromatography-ion-spray tandem mass spectrometry method for the identification and quantification of eight hydroxylated brominated diphenyl ethers in environmental matrices. *J. Mass Spectrom.* **2007**, *42*, 890.
[42] C. A. Erratico, A. Szeitz, S. M. Bandiera. Validation of a novel *in vitro* assay using ultra performance liquid chromatography-mass spectrometry (UPLC/MS) to detect and quantify hydroxylated metabolites of BDE-99 in rat liver microsomes. *J. Chromatogr. B* **2010**, *878*, 1562.
[43] H. Chang, Y. Wan, J. Naile, X. W. Zhang, S. Wiseman, M. Hecker, M. H. W. Lam, J. P. Giesy, P. D. Jones. Simultaneous quantification of multiple classes of phenolic compounds in blood plasma by liquid chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. A* **2010**, *1217*, 506.
[44] Y. Kato, S. Okada, K. Atobe, T. Endo, F. Matsubara, T. Oguma, K. Haraguchi. Simultaneous determination by APCI-LC/MS/MS of hydroxylated and methoxylated polybrominated diphenyl ethers found in marine biota. *Anal. Chem.* **2009**, *81*, 5942.
[45] S. J. Lupton, B. P. McGarrigle, J. R. Olson, T. D. Wood, D. S. Aga. Analysis of hydroxylated polybrominated diphenyl ether metabolites by liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 2227.
[46] VCCLAB. ALOGPS 2.1 program: On-line lipophilicity (logP), water solubility (logS) & pKa prediction, **2005**.
[47] G. Marsh, R. Stenutz, A. Bergman. Synthesis of hydroxylated and methoxylated polybrominated diphenyl ethers – Natural products and potential polybrominated diphenyl ether metabolites. *Eur. J. Org. Chem.* **2003**, *14*, 2566.
[48] G. De Sousa, S. Langouët, F. Nicolas, G. Lorenzon, M. Placidi, R. Rahmani, A. Guillouzo. Increase of cytochrome P-450 1A and glutathione transferase transcripts in cultured hepatocytes from dogs, monkeys, and humans after cryopreservation. *Cell Biol. Toxicol.* **1996**, *12*, 351.
[49] I. Marchi, S. Rudaz, J.-L. Veuthey. Atmospheric pressure photoionization for coupling liquid-chromatography to mass spectrometry: A review. *Talanta* **2009**, *78*, 1.
[50] L. S. Haug, C. Thomsen, G. Becher. A sensitive method for determination of a broad range of perfluorinated compounds in serum suitable for large-scale human biomonitoring. *J. Chromatogr. A* **2009**, *1216*, 385.
[51] R. Rodil, J. B. Quintana, T. Reemtsma. Liquid chromatography−tandem mass spectrometry determination of nonionic organophosphorus flame retardants and plasticizers in wastewater samples. *Anal. Chem.* **2005**, *77*, 3083.
[52] C. Marteau, J.-P. Antignac, R. Cariou, L. Debrauwer, P. Marchand, A. Berrebi, D. Zalko, B. Le Bizec. Development and application of a liquid chromatography coupled to high resolution mass spectrometry analytical strategy for measuring BFRs metabolites in human matrices. *Organohalogen Comp.* **2010**, *72*, 1350.
[53] G. Marsh, M. Athanasiadou, I. Athanassiadis, A. Sandholm. Identification of hydroxylated metabolites in 2,2 ′,4,4 ′-tetrabromodiphenyl ether exposed rats. *Chemosphere* **2006**, *63*, 690.
[54] M. Holcapek, R. Jirasko, M. Lisa. Basic rules for the interpretation of atmospheric pressure ionization mass spectra of small molecules. *J. Chromatogr. A* **2010**, *1217*, 3908.
[55] W. Xue, D. Warshawsky. Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: a review. *Toxicol. Appl. Pharmacol.* **2005**, *206*, 73.
[56] J. M. Sanders, L. J. Chen, E. H. Lebetkin, L. T. Burka. Metabolism and disposition of 2,2 ′,4,4 ′-tetrabromodiphenyl ether following administration of single or multiple doses to rats and mice. *Xenobiotica* **2006**, *36*, 103.
[57] L. J. Chen, E. H. Lebetkin, J. M. Sanders, L. T. Burka. Metabolism and disposition of 2,2′,4,4′,5-pentabromodiphenyl ether (BDE99) following a single or repeated administration to rats or mice. *Xenobiotica* **2006**, *36*, 515.

